MELATONIN AND ANTICANCER THERAPY: INTERACTIONS WITH 5-FLUOROURACIL

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ABSTRACT

On the basis of clinical studies, some researchers have advocated that the neurohormone and antioxidant melatonin, shown to possess intrinsic anticancer properties, be used as co-therapy in cancer patients being treated with the antineoplastic agent 5-fluorouracil, as increased patient survival times and enhanced quality of life have been observed. The focus of this research was thus to investigate the mechanisms of this seemingly beneficial drug interaction between 5-fluorouracil and melatonin. Metabolism studies were undertaken, in which it was established that there is no hepatic metabolic drug interaction between these agents by cytochrome P450, and that neither agent alters the activity of this enzyme system. Co-therapy with melatonin is thus unlikely to alter plasma levels of 5-fluorouracil by this mechanism. Novel mechanisms by which 5-fluorouracil is toxic were elucidated, such as the induction of lipid peroxidation, due to the formation of reactive oxygen species; decreases in brain serotonin, dopamine and norepinephrine levels, possibly leading to depression; hippocampal shrinkage and morphological alterations and lysis of hippocampal cells, which may underlie cognitive impairment; and a reduction in the nociceptive threshold when administered acutely. All these deleterious effects are attenuated by the co-administration of melatonin, suggesting that the agent exhibits antidepressive and analgesic properties, in addition to its known antioxidative and free radical-scavenging abilities. This suggests that melatonin co-therapy can significantly decrease 5-fluorouracil-induced toxicity, but this may also exert a protective effect on cancer cells and thus compromise the anticancer efficacy of 5-fluorouracil. It was, furthermore, found that stimulation of indoleamine 2,3-dioxygenase activity, mediated by increases in superoxide anion and interferon-γ levels, may underlie resistance to 5-fluorouracil therapy. Melatonin was shown to increase superoxide anion levels *in vivo*, and this is believed to be by conversion to the metabolite and known oxidant 6-hydroxymelatonin. This highlights that the possible deleterious effects of melatonin metabolites should be studied further. Serum corticosterone levels and cytokine profiles are unaltered by both 5-FU and melatonin, suggesting that these agents may be used by HIV-

infected individuals without promoting the progression to AIDS. It can thus be concluded that melatonin co-therapy is potentially useful in countering 5-fluorouracil toxicity.

This thesis is dedicated to my parents, Kudsiya and Hassan… for all the choices that you have both made to educate my brothers and I… for instilling in us the importance of living with integrity and for always encouraging us to excel academically….

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LIST OF ABBREVIATIONS USED

% RSD:	Percentage relative standard deviation
[^{14}C]:	Radiolabelled carbon isotope 14
µ:	Micro
µm:	Micrometre
µmol:	Micoromoles
13-HODE:	13-Hydroxyoctadecadienoic acid
3-OHK:	3-Hydroxykynurenine
3TC:	Lamivudine
5-FU:	5-Fluorouracil
5-HT:	5-Hydroxytryptamine, serotonin
5-HTP decarboxylase	5-Hydroxytryptophan decarboxylase
5-MT:	5-Methoxytryptamine
6-OHM:	6-Hydroxymelatonin
Ach:	Acetylcholine
ACTH:	Adrenocorticotrophic hormone
AFMK:	N^1-Acetyl-N^2-formyl-5-methoxykynurenine
aHT:	*N*-Acetylserotonin
AIDS:	Acquired immune deficiency syndrome
ALA:	Aminolaevulinic acid
AMPA:	α-Amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
AMPT:	α-Methyl-p-tyrosine
aMT:	Melatonin
ANOVA:	Analysis of variance
ATP:	Adenosine triphosphate
ATPase:	Adenosine triphosphatase
AUFS:	Absorbance units full scale
AVP:	Arginine-vasopressin
AZT:	3'-Azido-3'-deoxythymidine
AZTTP:	AZT-5'-triphosphate
Bd:	Twice a day
BDNF:	Brain-derived neurotrophic factor
BGJb:	Biggers, Gwatkin and Judah
BHT:	Butylated hydroxytoluene
BIS:	Behaviour inhibition system

BP:	British Pharmacopoiea
BROI:	Brain region of Interest
BSA:	Bovine serum albumin
CA:	Cornu ammonis
Ca^{2+}:	Calcium ion
$CaCl_2$:	Calcium chloride
CaMKII:	Ca^{2+}/ calmodulin-dependent protein kinase II
cAMP:	Cyclic adenosine monophosphate
CDHP:	5-Chloro-2, 4-dihydroxypyridine
CDKs:	Cyclin-dependent kinases
cDNA:	Proviral DNA
CEEG:	Computer-analysed electroencephalograms
cGMP:	Cyclic guanosine monophosphate
CKIs:	Cyclin-dependent kinase inhibitors
CMF:	Cyclophosphamide, methotrexate and 5-FU
CMI:	Cell-mediated immunity
CMOS:	CYP450-dependent monooxygenase system
CNS:	Central nervous system
CO:	Carbon monoxide
CO_2:	Carbon dioxide
COMT:	Catechol-*O*-methyl transferase
COX:	Cyclooxygenase
cps:	Centipoise
CREB:	cAMP-responsive element binding protein
CRF:	Corticotrophin-releasing factor
CSF:	Cerebrospinal fluid
CTLA-4:	T lymphocyte-associated antigen-4
CYP:	Cytochrome P
CYP450:	Cytochrome P450
D4T:	Stavudine
DA:	Dopamine
dATP:	Deoxyadenosine triphosphate
ddN:	2', 3'-Dideoxynucleoside
ddNTP:	Dideoxynucleoside-5'-triphosphate
DG:	Dentate gyrus
DMBA:	7,12-Dimethylbenz[a]anthracene
DMSO:	Dimethylsulphoxide

DNA:	Deoxyribonucleic acid
dNTP:	2'-Deoxynucleoside-5'-triphosphate
DOPAC:	3,4-Dihydroxyphenylacetic acid
DPD:	Dihydropyrimidine dehydrogenase
DPM:	Disintegrations per minute
DPX:	1, 3-Diethyl-8-phenylxanthine
dTMP:	Deoxythymidine-5'-monophosphate
dTTP:	Deoxythymidine-5'-triphosphate
dUMP:	Deoxyuridine-5'-monophosphate
dUTP:	Deoxyuridine-5'-triphosphate
ECD:	Electrochemical detection
ECG:	Electrocardiogram
ECT:	Electroconvulsive therapy
EDTA:	Ethylenediamine tetraacetic acid
EGFR:	Epidermal growth factor receptor
EIA:	Enzyme immunoassay
E-LTP:	Early long-term potentiation
EN:	Epinephrine
EOP:	Endogenous opioid peptide
EPSP:	Excitatory post-synaptic potential
f:	Distance from leading edge of peak to peak maximum, at 5% of the peak height
FA:	Monofluoroacetic acid
FABL:	α-Fluoro-β-guanido-propionic acid
FATP:	Fatty acid transport protein
FBAL:	α-Fluoro-β-alanine
FdUMP:	5-Fluorodeoxyuridine-5'-monophosphate
FdUTP:	5-Fluorodeoxyuridine-5'-triphosphate
Fe:	Iron
Fe^{2+}:	Ferrous ion
FUdR:	2'-Deoxy-5-fluorouridine
FUH_2:	5, 6-Dihydro-5-FU
FUMP:	5-Fluorouridine-5'-monophosphate
FUPA:	α-Fluoro-β-ureido-propionic acid
G6PD:	Glucose-6-phosphate dehydrogenase
GH:	Growth hormone
GM-CSF:	Granulocyte-macrophage colony-stimulating factor

gp120:	Glycoprotein 120
GSH:	Reduced glutathione
GSSH:	Oxidised glutathione
H:	Haematin
H·:	Hydrogen radical
H_2O_2:	Hydrogen peroxide
HA:	5-Hydroxyindole acetic acid
HAART:	Highly active antiretroviral treatment
HCl:	Hydrochloric acid
HDL:	High density lipoprotein
Hg:	Mercury
HI:	Humoral immunity
HIOMT:	Hydroxyindole-*O*-methyl transferase
HIV:	Human immunodeficiency virus
HL:	5-Hydroxytryptophol
HO^-:	Hydroxyl ion
HO·:	Hydroxyl radical
HPA:	Hypothalamic-pitutary-adrenal
HPG:	Hypothalamic-pituitary-growth hormone
HPLC:	High-performance liquid chromatography
HPT:	Hypothalamic-pituitary-thyroid
HS:	Haeme saturation
Hsps:	Heat shock proteins
I/ p:	Intraperitoneally
IASP:	International Association for the Study of Pain
ICH:	International Conference on Harmonisation
IDO:	Indoleamine 2,3-dioxygenase
IgE:	Immunoglobulin E
IGF-1:	Insulin-like growth factor 1
I-κB:	Inhibitor of κB
IL:	Interleukin
INFα:	Interferon α
INFγ:	Interferon γ
IQ:	Intelligence quota
IRS:	Inflammatory response system
iu:	International units
K^+:	Potassium ion

KCl:	Potassium chloride
kDa:	KiloDalton
KH_2PO_4:	Potassium dihydrogen phosphate
kyn:	Kynurenine
LDL:	Low density lipoprotein
L-DOPA:	L-Dihydroxyphenylalanine
LHRH:	Luteinising-hormone releasing hormone
L-LTP:	Late long-term potentiation
LOD:	Limit of detection
L-OHP:	Oxalato-platinum
LOQ:	Limit of quantitation
LTP:	Long-term potentiation
LTR:	Long terminal repeat
MA:	5-Methoxyindole acetic acid
MAO:	Monoamine oxidase
MAP:	Mitogen-activated protein
MAPK:	Mitogen-activated protein kinase
mcg:	Microgram
MDA:	Malondialdehyde
Mg^{2+}:	Magnesium ion
MHPG:	3-Methoxy-4-hydroxyphenylglycol
min:	Minute
MIP:	Microsomal iron protoporphyrin
ml:	Millilitre
ML:	5-Methoxytryptophol
mM:	Millimolar
MPO:	Myeloperoxidase
mRNA:	Messenger RNA
MSH:	Melanocyte-stimulating hormone
MT:	5-Methoxytryptamine
mV:	Millivolt
N:	Number of theoretical plates
N_2:	Molecular nitrogen
nA:	NanoAmpheres
Na^+:	Sodium ion
NaCl:	Sodium chloride, saline
NAD:	Nicotinamide adenine dinucleotide

NADPH:	Nicotinamide adenosine dinucleotide phosphate
NaH_2PO_4:	Sodium dihydrogen phosphate
NaOH:	Sodium hydroxide
NAT:	*N*-Acetyl-transferase
NBD:	Nitro-blue diformazan
NBT:	Nitro-blue tetrazolium
NE:	Norepinephrine
NF-kB:	Nuclear factor kB
ng:	Nanogram
nm:	Nanometre
NMDA:	*N*-Methyl-D-aspartate
NMR:	Nuclear magnetic resonance spectroscopy
NO:	Nitric oxide
NSAID:	Non-steroidal anti-inflammatory drug
O_2:	Molecular oxygen
O_2^-:	Singlet oxyen
$O_2^{-}\cdot$:	Superoxide anion
ONOO:	Peroxynitrite
PBS:	Phosphate buffered saline
PCD:	Programmed cell death
pg:	Picogram
PGE:	Prostaglandin E
pM:	PicoMolar
pmol:	Picomoles
p-Npp:	p-Nitrophenyl phosphate
PPC:	Phosphoenolpyruvate carboxykinase
ppm:	Parts per million
psi:	Pounds per square inch
PTHrP:	Parathyroid hormone-related protein
PTSD:	Post-traumatic stress disorder
QA:	Quinolinic acid
R:	Resolution factor
RNA:	Ribonucleic acid
ROS:	Reactive oxygen species
RT:	Reverse transcriptase
s:	Second
SAM:	*S*-Adenosyl methionine

SAMF:	South African Medicines Formulary
SCN:	Suprachiasmatic nucleus
SEM:	Standard error of the mean
SERM:	Selective oestrogen receptor modulators
SOD:	Superoxide dismutase
SSRI:	Selective serotonin reuptake-inhibitor
T:	Tailing factor
t:	Retention time of the respective compound
T_3:	L-Triiodothyronine
TBA:	Thiobarbaturic acid
TCA:	Trichloroacetic acid
TCAD:	Tricyclic antidepressants
TDO:	Tryptophan 2,3-dioxygenase
Tds:	Three times a day
TGFβ:	Transforming growth factor β
TLC:	Thin-layer chromatography
TNFα:	Tumour necrosis factor α
TPEST:	Total pineal endocrine substitution therapy
TRH:	Thyroid-releasing hormone
TSH:	Thyroid-stimulating hormone
TTP:	Thymidine-5'-triphosphate
USP:	United States Pharmacopoiea
USP DI:	United States Pharmacopoiea Drug Information for the Health Care Professional
UV:	Ultraviolet
v/ v:	Volume per volume
VEGF:	Vascular endothelial growth factor
w/ v:	Weight per volume
W:	Width of peak
$W_{0.05}$:	Width of peak at 5% of its height
WHO:	World Health Organisation
B-D-D4FU:	β-D-2', 3'-dideoxy-5-FU
B-L-D4FU:	β-L-2', 3'-dideoxy-5-FU
δ:	Chemical shift

CHAPTER 1

LITERATURE REVIEW AND RESEARCH CONTEXTUALISATION

1.1 Introduction and brief outline of research

Cancer remains a devastating disease whose incidence is steadily and rapidly increasing (Proctor, 1995). Some argue that it is a "disease of civilisation", the inevitable cost of medical, technological and societal advances that have prolonged the population's lifespan and have thus resulted in a greater proportion of people succumbing to age-related diseases, such as cancer and neurodegenerative disorders (Proctor, 1995).

In his book, *Cancer Wars: How Politics Shapes What We Know and Don't Know About Cancer*, Proctor (1995) contextualises public and medical perceptions about cancer against a larger political backdrop. This author exposes how industrialists, politicians and other role players have manipulated the information that the general public receives about cancer, to suit vested interests. These industrialists and politicians have propounded the theory that cancer, or an individual's susceptibility to it, is genetically predetermined. Another school of thought, which believes that cancer is the result of human exposure to environmental carcinogens, believes that the abovementioned genetic theory has been advocated to absolve industries, such as the tobacco industry, from the responsibility they bear in selling or marketing carcinogens, or exposing their workforce to environmental carcinogens, for example asbestos, in the quest for greater profits at the price of worker safety.

The above two theories of the cause of cancer have been manipulated, according to Proctor (1995), so that most people would believe that they are in opposition to one another, and that it is an "either-or" situation. However, these theories have the potential to be well integrated, as they highlight complementary aspects of the pathophysiology of cancer. It is well-established that exposure to certain chemicals or irradiation can cause cancer. The mechanism of this, though, would have to involve interactions on a genetic level, in which the carcinogen disrupts or modifies genetic processes or biochemical reactions in the host, resulting in the formation of cancer cells. The latter are characterised by the ability to rapidly proliferate; are invasive, undifferentiated and can metastasise (Calabresi and Chabner, 2001).

What is evident is that there is no simple cause of cancer, as this disease involves many complex interactions between the body and our environment. Those advocating environmental responsibility for the causation of cancer perceive the body as a passive victim of environmental onslaughts, whereas those who believe in genetic susceptibility regard the

body as a fighting machine, and the ability to fend off cancer as a Darwinian means of achieving genetic superiority (Proctor, 1995). Clearly, the latter perspective lends itself to racism while the former underestimates the body's evolutionary ability to defend itself to some degree.

Due to the multiplicity of "causes" or interactions that eventually give rise to the phenomenon of cancer, there can be no simple "cure" for the disease. This is compounded by the fact that "cancer" itself is manifested in many different ways; there are numerous, sometimes very different, types of cancer. It is thus more appropriate to use the term "cancers". In addition to this, cancers can interact very intimately with the host's internal environment, thus giving rise to a variety of paraneoplastic syndromes. The latter can include almost any imaginable abnormality in the body, from hypercalcaemia to taste disturbances (Hall, 1974).

A humbling consideration for the pharmaceutical industry is that the majority of drugs used to treat cancers are, themselves, toxic to normal host cells, especially to the rapidly-proliferating cells of the bone, gastrointestinal tract, hair and reproductive organs. One may be forgiven for wondering if there is any hope for the effective and safe treatment of cancers, and the best answer at this point in time would be that we, as health-care professionals, have to make use of an holistic approach to cancer treatment: the influences of lifestyle choices and non-pharmacological therapy, such as exercise, rest and meditation, should not be underestimated. Our goal should be to ensure that cancer patients have an improved quality of life, that the efficacy of chemo- or radiation-therapy should be enhanced and the toxicity of these reduced. Instead of focusing scarce resources on developing new drugs, with perhaps new side-effects, we should be focusing our attention towards ensuring the rational and safe use of existing drugs and therapies. The challenges facing the health-care sector are enormous, especially with the current pandemic of HIV/ AIDS. The number of patients who suffer from HIV and cancer is likely to be enormous, and the number of possible interactions between the two disease states, as well as the drugs used to treat them, are very thought-provoking and of great concern indeed.

This research focuses on the interface of oncology and neuroscience, with the aim being to investigate the pharmacological interactions between a commonly-used antineoplastic agent, 5-fluorouracil (5-FU), and the pineal antioxidant melatonin. 5-FU is well-known for its toxicity and, in an encouraging move that perhaps portends the new direction in which cancer research is moving, this drug is the subject of many international studies to ascertain whether its toxicity is reduced, and its efficacy enhanced, when melatonin is administered

concurrently to the patient (Lissoni *et al*, 1999a). This research will thus critically assess the use of such combination therapy, and the implications thereof.

As a brief overview, this thesis starts by determining whether there is an hepatic metabolic drug interaction between 5-FU and melatonin at the level of cytochrome P450. This is important as it will indicate whether melatonin co-administration is likely to alter plasma levels of 5-FU, and thus the possible efficacy or toxicity of this agent. A suitable high-performance liquid chromatography (HPLC) method will be developed and validated to quantitatively analyse concentrations of 5-FU and melatonin subsequent to metabolism. The effects of these drugs on the activities of the enzymes tryptophan 2,3-dioxygenase (TDO) and indoleamine 2, 3-dioxygenase (IDO), key enzymes in the kynurenine pathway, will then be investigated. The implications of these results will subsequently be determined in terms of a neurotransmitter analysis, in which the levels of serotonin, dopamine and norepinephrine will be measured. This will offer insight into the possible roles of 5-FU and melatonin in exacerbating or attenuating the development of depression. The effects of 5-FU and melatonin on pineal indole metabolism will subsequently be determined, in order to ascertain whether these agents alter pineal serotonin metabolism. The effects of 5-FU on hepatic lipid peroxidation, mediated by superoxide anions, are unknown, and it would be useful to determine if this is an additional mechanism of cytotoxicity. It would also be significant to determine if melatonin, a known antioxidant, can counter any 5-FU-induced lipid peroxidation. In Chapter 10, the effects of 5-FU and melatonin on the volume and morphology of the hippocampus will be investigated. This structure is of key importance in cognition and mood regulation. It has recently been reported that 5-FU may result in the phenomenon of "chemobrain" (Winocur *et al*, 2006), which is characterised by cognitive deficits. An investigation into the effects of this agent on the hippocampus may thus offer a possible mechanism by which this occurs, and the extent to which melatonin offers any protection. A behavioural study will be performed to assess the effects of 5-FU and melatonin on locomotor activity, as the effects of the former drug on this are not well documented in the literature. Locomotor activity is often used as an animal model of depression and anxiety. The glucocorticoid hypothesis of the development of Acquired Immune Deficiency Syndrome (AIDS) shall be investigated by determining the effects of 5-FU and melatonin on serum corticosterone and various cytokine levels. Moreover, the use of both drugs in combination with antiretrovirals, which is currently being advocated by some researchers, is critically evaluated. This thesis will end by determining the effects of 5-FU and melatonin on pain perception, a common symptom in cancer.

1.1.1 General hypothesis

Melatonin counteracts 5-FU-induced toxicity and combination therapy is thus recommended in the clinical setting.

1.2 5-Fluorouracil

1.2.1 Chemistry

Originally described by Heidelberger (1957), 5-FU, an essential drug in South Africa (SAMF, 2003), is a fluorinated analogue of the pyrimidine bases uracil and thymine, occurring in RNA and DNA respectively (Chabner *et al*, 2001). 5-FU has the following structural formula, which can undergo keto-enol tautomerism to give the compound on the right, which is less stable (Bayomi and Al-Badr, 1989):

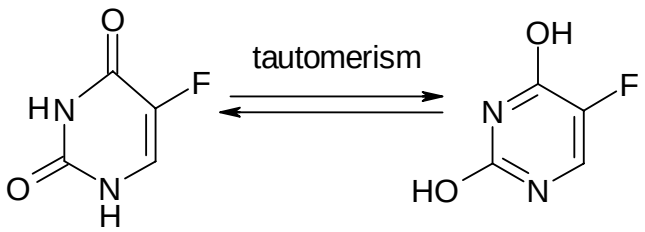


Figure 1: The chemical structure of 5-FU (Bayomi and Al-Badr, 1989)

It contains between 98.5-101.0% of 5-fluoropyrimidine-2,4(1*H*,3*H*)-dione (BP, 2002).

1.2.2 Basic physicochemical data

5-FU has the following physicochemical properties (Dollery, 1991):

- pKa: 8.15, 13.0
- Octanol/ water partition coefficient: 0.1
- Solubility: 1 in 170 (in ethanol) and 1 in 80 (in water)
- Molecular weight: 130.08 g/ mol

5-FU is thus poorly lipid-soluble, due to its low partition coefficient. It is more soluble in aqueous than organic media and has a higher degree of dissociation in the alkaline pH range. The modest lipophilicity that the molecule exhibits is due to the fluorine atom attached to an aromatic system (Park *et al*, 2001); it is suggested that a fluoro group in the 5-position is necessary for penetration into cells (Bryskier and Chantot, 1995) across the lipophilic cell membrane.

1.2.3 Rationale for the development of 5-FU as a chemotherapeutic agent

The carbon-fluoride bond in 5-FU is stronger than the corresponding carbon-hydrogen bond in its natural analogue uracil, or the carbon-methyl bond at the same position in thymine (Chabner *et al*, 2001). This results in the antineoplastic being less susceptible to enzymatic degradation and exhibiting increased chemical stability (Chabner *et al*, 2001; Bayomi and Al-Badr, 1989; Park *et al*, 2001). Such a replacement of a hydrogen with a fluorine is a strategy that is often employed in drug design and development (Alkorta *et al*, 2000; Park *et al*, 2001), and which may well impact on both the pharmacokinetics and pharmacodynamics of a drug (Wakselman, 1999; Park *et al*, 2001).

This drug is used to treat cancer of the skin; head and neck; breast and gastrointestinal tract, in particular colorectal cancer (see 1.2.8). Certain tumours utilise more uracil than normal tissue cells for nucleic acid synthesis and thus growth of the tumour (Rutman *et al*, 1954). 5-FU acts as a fraudulent nucleotide because it has similar physicochemical properties to uracil. Fluorine has a Van der Walt's radius (1.35 Å) very close in value to that of hydrogen (1.2 Å), thereby enabling 5-FU to closely resemble the molecular conformation of uracil (Diasio and Harris, 1989; Park *et al*, 2001). There is, furthermore, increased likelihood of 5-FU fitting into the active sites of the enzymes that metabolise uracil. In fact, the enzyme thymidylate synthase has 1000 times more affinity for 5-FU than for uracil, its endogenous substrate (Park *et al*, 2001). The substitution of hydrogen with fluorine at position 5 gives the best structure-activity relationship, as it simulates the natural situation in which cells replace the hydrogen at position 5 with a methyl group to form dTMP, which is then phosphorylated to its active triphosphate form (Diasio and Harris, 1989). The C-F bond is also much stronger (bond energy = 116 kcal/ mol) than the corresponding C-H bond (bond energy = 99 kcal/ mol), thereby preventing methylation at the 5 position by thymidylate synthase (Chabner *et al*, 2001; Park *et al*, 2001). Substitution of a H with a F thus produces a molecule that closely resembles a natural pyrimidine and can interact with the ezymes involved in the metabolism of this class of compounds (Chabner *et al*, 2001) (see 1.2.6).

1.2.4 Administration and dosage forms

5-FU can be administered orally in capsule form, or parenterally as a sodium salt that is yellowish in solution (Dollery, 1991).

The drug is usually administered intravenously, as oral dosing results in erratic and unpredictable absorption levels and seems to be less effective (Hahn *et al*, 1975), for the reasons outlined below. If it is given orally, fruit juice or any other non-alcoholic drink may be taken as well (Dollery, 1991) in order to reduce gastric irritation.

In order to bypass the difficulties associated with administering 5-FU orally, such as an unpredictable extent of absorption, a prodrug of 5-FU, tegafur, has been formulated. This prodrug is activated by conversion to 5-FU by thymidine phosphorylase and, to a larger extent, by the cytochrome P450 drug-metabolising system, specifically by the isoenzyme CYP 2A6 (Kajita *et al*, 2003). Inhibitors of this isoenzyme, such as 8-methoxypsoralen, thus dramatically reduce the activation of the prodrug (Kajita *et al*, 2003). Tegafur has been compounded into an oral formulation with potassium oxonate and 5-chloro-2, 4-dihydroxypyridine (CDHP), in a ratio of 1: 1: 0.4, called S-1 (Shirasaka *et al*, 1996a, 1996b). CDHP is potent reversible inhibitor of dihydropyrimidine dehydrogenase (DPD), the enzyme that catabolises 5-FU (see 1.2.6.1) (Tatsumi *et al*, 1987; Fujitani *et al*, 2005). This leads to a prolonged elevation in plasma 5-FU levels and increased antitumour activity (Yamashita *et al*, 2006; Ueda *et al*, 2004). Potassium oxonate, a reversible competitive inhibitor of orotate phosphoribosyltransferase, decreases the level of 5-FU and FUMP that is incorporated into RNA (Shirasaka *et al*, 1993; Fujitani *et al*, 2005). It has, furthermore, been found to counter 5-FU-induced gastrotoxicity (Yoshisue *et al*, 2000) and significantly inhibit 5-FU-induced immunosuppression (see 1.2.10.3), by preventing a decrease in natural killer (NK) and interleukin-2 (IL-2) levels (Yamashita *et al*, 2006). The use of S-1 thus enhances the quality of life of cancer patients being treated with 5-FU (Yamashita *et al*, 2006). A phase I and pharmacokinetic study of S-1 combined with the antineoplastic agent paclitaxel has found that such combination therapy exhibits potent anticancer efficacy in the treatment of advanced gastric carcinomas, as well as a low toxicity profile (Fujitani *et al*, 2005). Three phase II studies of S-1, conducted in Japan, have demonstrated a patient median survival time of 8-10 months, which is comparable to other standard treatment options for advanced gastric cancer (Sakata *et al*, 1998; Sugimachi *et al*, 1999; Koizumi *et al*, 2000).

Topically, 5-FU can be administered as a cream and is effective for the treatment of multiple superficial basal cell carcinomas, solar keratoses and uterine neoplasms of the cervix (Dollery, 1991). 5-FU is selectively absorbed into the tumour and is minimally absorbed into the systemic circulation. However, 5-FU is not recommended if the carcinomas are of the invasive type, as delayed hypersensitivity reactions can occur (Dollery, 1991).

A novel drug delivery system of 5-FU is a therapeutic implant. This contains the drug in a protein matrix, consisting of collagen gel, which provides controlled release of the medicament. Epinephrine (EN) is included in the formulation for its vasoconstrictor effects, which result in the retention of 5-FU at a localised area. Such a formulation is administered by intralesional intradermal injection, and is used for the treatment of genital warts (Anonymous, 1996).

When used to treat genital warts (see 1.2.8.8), 5-FU is formulated most commonly as a 5% cream or solution. One percent preparations are also available (Adler and Mindel, 1985; Anonymous, 1988; Davis and Emans, 1989; Pride, 1990).

1.2.5 Biopharmaceutics

5-FU enters cells by simple diffusion and a concentration equilibrium is soon reached between extra- and intracellular compartments (Jacquez, 1962; Kessel and Hall, 1967; Diasio and Harris, 1989). Alternatively, 5-FU can enter cells by a carrier transport system that could be saturated (Wohlhueter *et al*, 1980).

In the ileum, 5-FU is absorbed by a sodium (Na)-dependent transport system. High-performance liquid chromatography (HPLC) shows that the lower gastrointestinal tract, namely the distal colon and ileum, converts 5-FU to metabolites with increased polarity. Metabolism occurs simultaneously with transport, since a greater concentration of metabolites was found on the side to which transport was occurring (Smith *et al*, 1988). Active absorption of 5-FU occurs in the small intestine. This transport system is saturated with concentrations of 5-FU that are much lower than those given in the oral administration of the drug (Lahiri *et al*, 1971; Bateman *et al*, 1971). The passive absorption process thus predominates, resulting in the formation of more polar metabolites, which are more likely to be excreted. This reduces the oral activity of 5-FU since a minimal amount is absorbed intact, thereby reducing the oral bioavailability of the agent (Smith *et al*, 1988).

The extent of first-pass metabolism depends on the dose of the drug, and after saturation of the hepatic microsomal enzyme system has occurred, increased bioavailability results. There is presystemic metabolism in the gastric mucosa (Dollery, 1991), which contains DPD and the high activity of this enzyme results in presystemic hydrogenation of the drug (see 1.2.6).

When administered orally, the bioavailability of 5-FU is erratic. Administration of capsules or oral solution forms gives an equivalent extent of absorption (Dollery, 1991). Portal blood concentrations of the drug are higher when it is administered orally as opposed to parenterally, and this is regarded by some as a motivation to use 5-FU orally to treat cancers in the portal regions (Dollery, 1991).

The following pharmacokinetic information is available (Dollery, 1991):

- Oral absorption: 28%
- Plasma half-life: 11 ± 4 minutes
- Volume of distribution: 0.25 ± 0.12 L/ kg

- Plasma protein-binding: 8-12%
- Presystemic metabolism (i.e. in the gastric mucosal wall): saturable with an increase in dosage

The half-life of 5-FU increases substantially with a reduction in pH. Acidification thus promotes the local retention of 5-FU at a particular site, and this has the potential to impact on the therapeutic efficacy of the drug (Guerquin-Kern *et al*, 1991).

After intravenous administration, plasma levels of 10^{-4} to 10^{-7} mol/ L are rapidly attained and 5-FU is not detectable after 2-3 hours, with plasma levels being less than 10^{-9} mol/ L (Dollery, 1991). Penetration through the blood-brain barrier does occur, even though 5-FU has a low octanol/ water partition coefficient. A slow intravenous injection increases the effect of the drug by prolonging its exposure to dividing cells. A rapid intravenous bolus dose is more convenient, however, and regarded as being equally efficacious (Dollery, 1991). 5-FU can be given interperitoneally, which results in peritoneal levels that are 2-3 times the plasma levels of 5-FU. These peritoneal levels are equivalent to having administered the patient with a 24-hour infusion (Dollery, 1991).

1.2.6 Metabolism and mechanism of action

5-FU undergoes extensive hepatic metabolism, with almost undetectable concentrations of the parent drug being present two hours after administration. Both catabolism and anabolism occur; more than 80% of a dose of 5-FU is inactivated through catabolism and 5-20% of unmetabolised drug is excreted in the urine (Diasio and Harris, 1989). Since catabolism is the predominant metabolic process that occurs, it significantly influences the amount of 5-FU that can be activated and therefore affects the pharmacokinetic profile and potency of the drug.

1.2.6.1 Catabolism of 5-FU

5-FU is catabolised to a number of metabolites, the major one being 5,6-dihydro-5-FU (FUH_2). This metabolite is subsequently converted in the liver to α-fluoro-β-guanido-propionic acid (FABL), which is the major urinary metabolite (Diasio and Harris, 1989). FUH_2 is also converted to α-fluoro-β-ureido-propionic acid (FUPA). In the catabolism of 5-FU, the rate-limiting enzyme (Shiotani and Weber, 1981) is DPD, which forms FUH_2 by reducing the carbon-carbon double bond in the pyrimidine ring. FUH_2 has been found to display some anti-tumour activity (Diasio *et al*, 1985) and so it might not be as inactive as previously believed; it is suggested that FUH_2 could be anabolised to 5-FU *in vivo* and thus prolong and enhance the latter's action (Diasio *et al*, 1985). In the same article, it was noted that the metabolite has a longer half-life than hitherto known, and follows Michaelis-Menten kinetics (Mentre *et al*, 1984). FABL can also be conjugated with bile acids to form biliary

metabolites of 5-FU (Sweeney *et al*, 1987). A newly discovered metabolite of 5-FU is α-fluoro-β-alanine (FBAL), which can also bind to bile acids (Sweeney *et al*, 1987). FBAL is the major urinary catabolite, occurring at more than 95% (Diasio and Harris, 1989). Glucuronide conjugation of 5-FU can also occur (Sommadossi *et al*, 1985) by phase II drug metabolism in the liver, thus forming inactive compounds that can be more readily excreted. The neurotoxicity of FBAL is discussed in 10.4.

DPD is responsible for the inactivation of 5-FU. Some cells may have increased levels of this enzyme and may consequently be able to catalyse the drug more quickly, resulting in resistance to 5-FU (Diasio and Harris, 1989). DPD is present in gastrointestinal, lung and pancreatic tissue in addition to hepatic tissue (Naguib *et al*, 1985). Patients who have genetic abnormalities in DPD expression may experience severe toxicity (Innocenti *et al*, 2000), such as neurological, gastrointestinal and haematological disorders. It has been found that in Indian populations, there is a genetic deficiency of DPD, thus rendering these individuals susceptible to 5-FU toxicity (Saif *et al*, 2006). Patients with breast cancer have reduced DPD activity (Innocenti *et al*, 2000) and this may pose a problem as 5-FU is standard treatment for breast cancer, along with methotrexate and cyclophosphamide (Kajdaniuk *et al*, 2000) (see 1.2.8.7).

There is a 3-25 fold circadian variation (see 1.3.7) in plasma concentrations of 5-FU (Petit *et al*, 1988), which may be attributable to a circadian variation in concentrations of DPD. In patients with genetic DPD deficiency, the pharmacokinetics of 5-FU can be dramatically altered, with almost 90% of the drug being subjected to urinary excretion (Diasio *et al*, 1988; Diasio and Harris, 1989). The circadian variation in DPD activity in peripheral blood mononucleocytes shows a peak at 01h00 and a trough at 13h00 (Milano *et al*, 1996). In addition, a circadian rhythm in plasma 5-FU concentration exists, with a peak at 11h00 and a trough at 23h00 (Milano *et al*, 1996). In patients with advanced gastrointestinal cancer, there is a loss of circadian rhythm in DPD mRNA expression (Raida *et al*, 2002).

DPD activity is lower in women than in men, and this difference is of the same order of magnitude as the difference in the clearance of 5-FU by the sexes (Milano *et al*, 1992). Thymidylate synthase and DPD significantly influence 5-FU activity: a reduction in enzyme concentration or activity correlates with increased cytotoxicity (Milano *et al*, 1996).

Figure 2 below portrays the catabolic metabolism of 5-FU (Dollery, 1991). Eighty percent of 5-FU is eventually converted to carbon dioxide and exhaled within twelve hours of the drug having been administered. Urinary excretion accounts for 15% of drug elimination (Dollery, 1991).

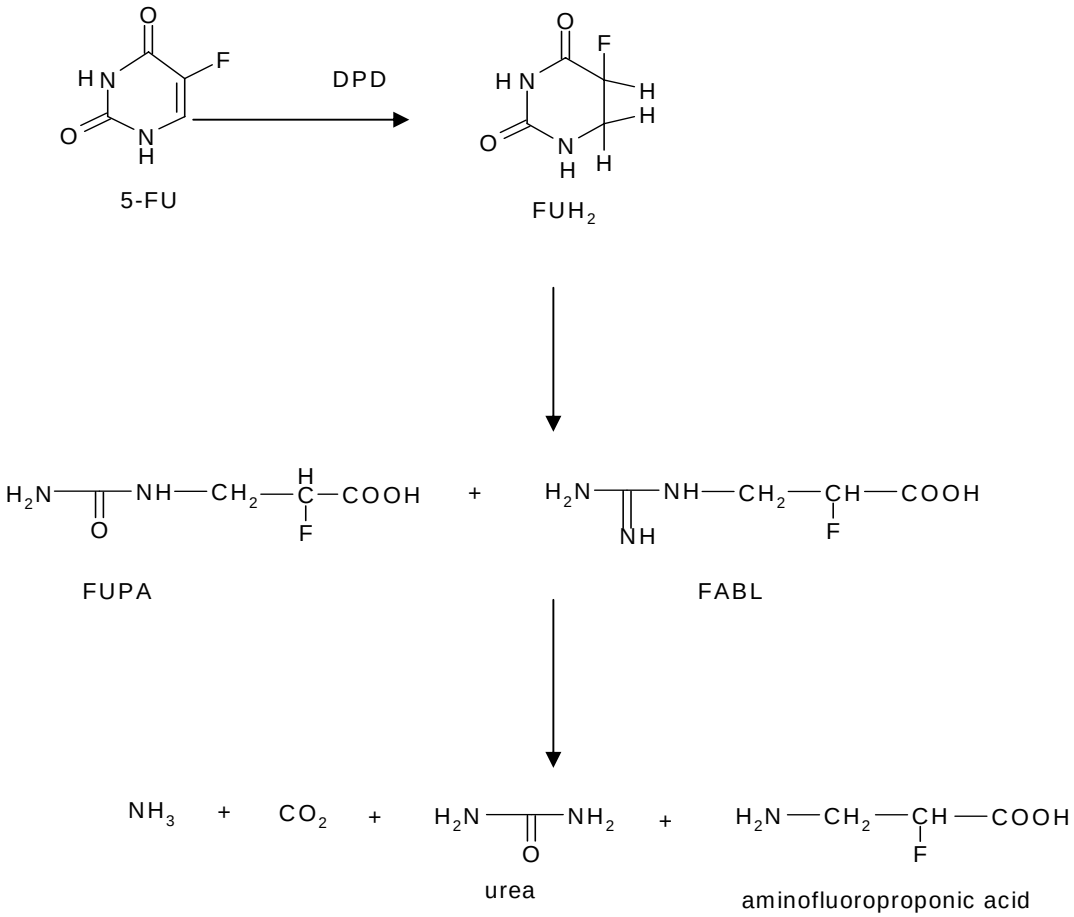


Figure 2: The catabolic metabolism of 5-FU (Dollery, 1991)

1.2.6.2 Anabolism of 5-FU

5-FU undergoes anabolic reactions, which result in its activation within cells. There are three anabolic pathways: (i) uridine phosphorylase and uridine kinase catalyse the formation of 5-fluorouridine-5'-monophosphate (FUMP); (ii) 5-FU is directly converted to FUMP by orotidine-5'-monophosphate phosphoribosyltransferase; and (iii) to a lesser extent, the conversion of 5-FU to 5-fluorodeoxyuridine-5'-monophosphate (FdUMP) by thymidine phosphorylase and thymidine kinase (Diasio and Harris, 1989). The presence of a charged phosphate group in the nucleotide results in it being retained in the cell, while the nucleosides and base can diffuse across the cell membrane, and undergoing further interconversions to a number of other nucleotides. It is not clear which nucleotide actually causes cytotoxicity (Diasio and Harris, 1989). These "fraudulent" nucleotides, which are then phosphorylated into active forms, can be integrated into a cell's RNA and thus result in the production of defunct RNA (Bayomi and Al-Badr, 1989). The antineoplastic activity and toxicity of 5-FU depend on it being converted to nucleotides (Heidelberger, 1975).

Thymidylate synthase converts deoxyuridine-5'-monophosphate (dUMP) into deoxythymidine-5'-monophosphate (dTMP) in mammalian cells and this dTMP is necessary for DNA synthesis. FdUMP, a fraudulent nucleotide resulting from the metabolic activation of 5-FU, has more affinity than the natural dUMP for the enzyme and thus prevents the formation of dTMP, which results in the inhibition of DNA synthesis (Diasio and Harris, 1989). The presence of a stable carbon-fluoride bond at position 5 prevents the formation of an intermediate by thymidylate synthase and results in the enzyme being reversibly inhibited through the formation of a stable ternary complex (Santi *et al*, 1974). Increased concentrations of reduced folates inhibit the dissociation of this ternary complex (Danenberg and Danenberg, 1978), thereby increasing the antitumour potency of 5-FU. This is the rationale behind the co-administration of reduced folates, such as folinic acid, with 5-FU (Diasio and Harris, 1989; Evans *et al*, 1981).

5-FU can also be incorporated into DNA (Ingraham *et al*, 1982): it can be anabolised to FdUTP (Caradonna and Cheng, 1980), which can act as a substrate for DNA polymerase (Cheng and Nakayama, 1983). 5-Fluorodeoxyuridine-5'-triphosphate (FdUTP) is then introduced into DNA instead of deoxythymidine-5'-triphosphate (dTTP), the normal substrate. In fact, when thymidylate synthase is inhibited by FdUMP, the cell is more prone to incorporate FdUTP into DNA (Myers *et al*, 1975). Once 5-FU has been incorporated into DNA, a DNA repair enzyme, uracil glycosylase, may remove the false nucleotide (Caradonna and Cheng, 1980). The sugar-phosphate backbone of the relevant DNA portion is subsequently broken by an apyrimidine endonuclease (Lindahl, 1979), and the correct nucleotide inserted (Diasio and Harris, 1989). With increased concentrations of 5-FU, this repair mechanism is ineffective and DNA fragmentation occurs (Lonn and Lonn, 1984, 1986). It is thus apparent that 5-FU disrupts DNA and RNA synthesis through several mechanisms (Diasio and Harris, 1989). The following scheme illustrates the activation pathway for 5-FU (Chabner *et al*, 2001).

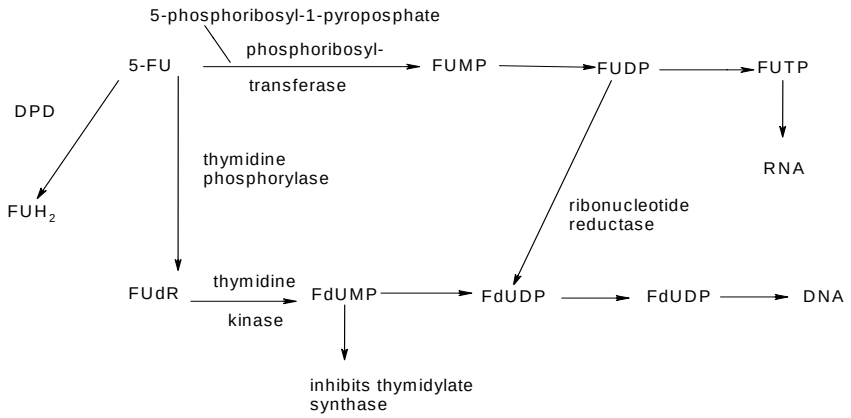


Figure 3: The anabolic metabolism of 5-FU (Chabner *et al*, 2001)

The above interaction between FdUMP and thymidylate synthase, which leads to the depletion of thymidine-5'-triphosphate (TTP), a nucleotide essential in DNA synthesis, requires the vitamin folic acid, in the form of its coenzyme 5, 10-methylenetetrahydofolate. FdUMP and this folate cofactor covalently bind to thymidylate synthase, thereby forming a ternary complex. Due to the presence of the stable C-F bond in FdUMP, the normal methylation reaction that the holoenzyme catalyses cannot occur, thus disrupting DNA synthesis (Chabner *et al*, 2001). The following diagram emphasises the role that the methylated folate cofactor plays in the mechanism of action of thymidylate synthase and hence of 5-FU (Chabner *et al*, 2001).

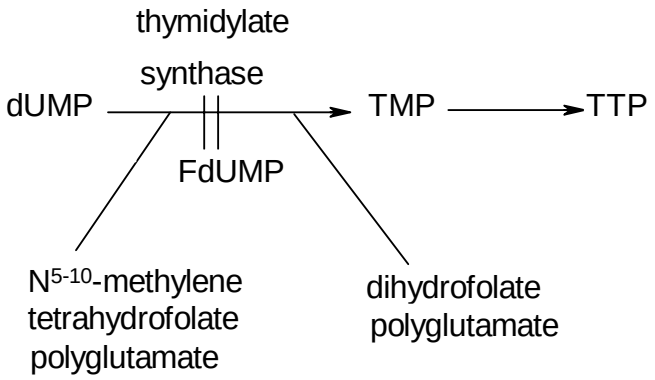


Figure 4: The involvement of folic acid in 5-FU metabolism (Chabner *et al*, 2001)
| | signifies inhibition.

The abovementioned ternary complex has the following structure (Diasio and Harris, 1989).

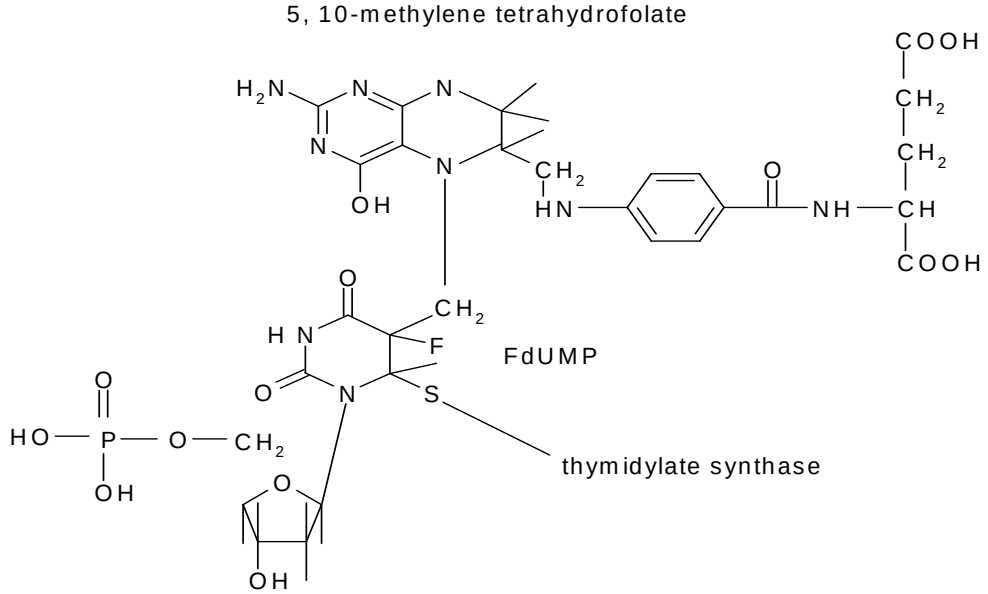


Figure 5: The ternary complex comprising FdUMP, thymidylate synthase and N^{5-10}-methylene tetrahydrofolate (Diasio and Harris, 1989)

1.2.6.3 Membrane alterations

5-FU has another mechanism of action in that it can alter transmembrane potential and charges (Walliser and Redman, 1978) as well as membrane structure, and impair glycoprotein synthesis. The latter increases the volume of the cell, thereby increasing the likelihood of cell lysis occurring (Kessel, 1980).

1.2.6.4 Nitric oxide levels

Nitric oxide (NO), formed by the action of the enzyme NO synthase, is an important mediator of cell proliferation and is believed to contribute to tumour growth, as mentioned in 1.2.9.2. NO synthase exists in three different forms: neuronal, endothelial and inducible. The first two isoenzymes are constitutive forms (Nathan, 1997) and are mainly involved in the normal functioning of the nervous and circulatory systems (Fischmann *et al*, 1999), while the last is induced upon cytokine activation (Nathan, 1997) and exerts immunomodulatory effects that are believed to underlie the adverse effects of NO (Fischmann *et al*, 1999). There are many conflicting reports in the literature on the effects of 5-FU on NO production; some investigators have found that the antineoplastic agent inhibits both inducible NO synthase (Jung *et al*, 2002; Jin *et al*, 1996) and endothelial NO synthase (Casado-Echarren *et al*, 2004) by reducing enzyme expression (Casado-Echarren *et al*, 2004) and blocking the INFγ- and IL-1β-induced increases in NO production (Jin *et al*, 1996; Jung *et al*, 2002). It is believed that this inhibition of NO production thus contributes to the antineoplastic efficacy of 5-FU, by suppressing the proliferation of cancer cells (Jin *et al*, 1996). Other investigators have, however, shown that 5-FU enhances NO synthesis (Jiang *et al*, 2002; Oshima *et al*, 2001) by increasing the expression of inducible NO synthase (Leitão *et al*, 2007) and stimulating this isoenzyme (Jiang *et al*, 2002; Oshima *et al*, 2001). The resulting increase in NO levels is believed to promote the apoptosis of cancer cells (Jiang *et al*, 2002; Oshima *et al*, 2001) through the production of tumour necrosis factor α (TNFα) (Oshima *et al*, 2001). It is thus evident, from these conflicting findings, that both an increase and a decrease in NO levels, induced by 5-FU, can result in anticancer effects, through different mechanisms.

1.2.7 Mechanisms of resistance

There are several biochemical mechanisms of resistance to 5-FU. These include (Chabner *et al*, 2001):

(i) decreased activity of pyrimidine monophosphate kinase, and thus reduced incorporation of the cytotoxic into RNA;

(ii) reduced activity of other enzymes necessary for the activation of 5-FU;

(iii) increased activity and synthesis of thymidylate synthase. The excess enzyme thus saturates the antineoplastic and bypasses its influence;

(iv) the synthesis of a modified thymidyate synthase that is not inhibited by FdUMP (Chabner *et al*, 2001). The activity of 5-FU is inversely related to the levels of degradative enzymes, namely thymidine phosphorylase and DPD, as well as a low level of thymidylate synthase. The levels of the latter enzyme are regulated by an auto-feedback mechanism, alterations of which may be a significant reason for the rapid development of insensitivity of tumour cells to 5-FU (Chabner *et al*, 2001);

(v) certain tumour cells may have reduced levels of the folate cofactor, 5,10-methylene tetrahydrofolate. The subsequent formation with thymidylate synthase of the inhibited ternary complex does not occur to as great an extent and the efficacy of 5-FU is consequently decreased. This can be overcome by giving the patient 5-formyltetrahydrofolate, such as leucovorin, supplementation (Chabner *et al*, 2001). A further advantage to leucovorin supplementation is that the dosage of 5-FU can be reduced (Chabner *et al*, 2001). Besides reducing 5-FU-induced toxicity and enhancing the efficacy of this drug, folate supplementation may, however, be linked to the development of anticancer drug resistance, by modulating the activity and expression of multidrug resistance proteins and transporters (Hooijberg *et al*, 2006). This latter finding points to caution with the use of folate supplements; and

(vi) tumour cells exposed to 5-FU mount the heat shock response, in which heat shock proteins, which protect cells against various types of stresses, are induced. This is known to be associated with resistance towards 5-FU (Gallos *et al*, 2001) (see 1.2.8.1 and 8.4).

1.2.8 Therapeutic indications

5-FU is used to treat a wide variety of cancers, including carcinomas of the liver, colon/ rectum, head/ neck, stomach, breast, bladder, exocrine pancreas, cervix and ovaries (Dollery, 1991). DNA synthesis is inhibited, and thus an effective antineoplastic response attained, with a concentration of 10^{-3} mol/ L. The minimum effective concentration appears to be 10^{-6} mol/ L (Dollery, 1991), but this varies for different tumours situated in different organs. At a concentration of 10^{-5} mol/ L, there is 93% inhibition of gastric cancers and an 81-100% inhibition of breast cancers (Dollery, 1991). More details about the therapeutic indications of 5-FU are given in the next pages:

1.2.8.1 Carcinomas of the rectum and colon

5-FU is the single most effective chemotherapeutic agent for the first-line treatment of colorectal cancer, and is especially effective for drug-resistant tumours (Dollery, 1991). The response rate, though, is less than 20% (Dollery, 1991); this may be due to upregulation of the protective heat shock proteins, which protect cells from stresses (see 8.4). 5-FU can also be used as adjuvant therapy for colorectal carcinomas; in this regard, it is most commonly

administered intravenously into the systemic circulation between two weeks and eighteen months after a surgical procedure (Dollery, 1991; Ansfield, 1977; Ansfield and Greenspan, 1979; Ecanow *et al*, 1977). However, 5-FU is not effective in treating colorectal cancer patients who have a genetic defect termed "high-frequency microsatellite instability", which occurs in approximately 15% of patients with colorectal cancer (http://www.cpaaindia.org/infocentre/clipping_co.htm#top, Adjuvant 5-FU for colorectal cancer does not benefit patients with high-frequency microsatellite instability, *ET* 12/ 08/ 2003). 5-FU can also be administered as a suppository dosage form, which has been shown to successfully treat a third of patients with rectal carcinomas (Takahashi *et al*, 1982).

1.2.8.2 Carcinomas of the head and neck

Of all patients, 50.67% respond to 5-FU therapy, with a low incidence of toxicity and a general increase in health status and survival rates. 5-FU can be advantageously combined with other antineoplastics, such as cyclophosphamide, methotrexate, bleomycin, vincristine and cisplatin. The latter agent, when used with 5-FU, is the most powerful inducer of tumour regression (Dollery, 1991).

1.2.8.3 Carcinomas of the lung

Carcinomas of this type, most notably non-small-cell types, do not respond well to any chemotherapy. The usual response rate is 10-18% (Dollery, 1991). 5-FU is thus not particularly effective, and if it is used in combination with another antineoplastic, such as doxorubicin, the antitumour activity is usually attributed to the other agent (Dollery, 1991).

1.2.8.4 Carcinomas of the pancreas and stomach

The effectiveness of 5-FU in inducing objective tumour regression is associated with its degree of toxicity, with 40.67% of patients responding and developing moderate myelotoxicity (Moertel *et al*, 1964). If the toxicity is severe, there is only a 13% response rate and it can thus be concluded that serious toxicity does not necessarily indicate an adequate chemotherapeutic response. There does not seem to be any added benefit to using 5-FU as monotherapy (Moertel *et al*, 1964; Dollery, 1991). Combination chemotherapy, for example, with mitomycin C and doxorubicin, is more efficacious, especially for tumours of the upper gastrointestinal tract. It is more difficult to assess the effectiveness of 5-FU in treating pancreatic cancers due to the challenges of accessing and analysing the relevant tissues (Dollery, 1991).

1.2.8.5 Carcinomas of the genitourinary tract

5-FU is less effective than other agents, such as platinum, methotrexate and doxorubicin, in treating bladder cancer (Dollery, 1991). It can be used in combination with platinum and doxorubicin and produces a 46% response rate in this regard (Smalley *et al*, 1978). Prostate cancer is typically a more difficult cancer to treat, but it does show up to a 50% response to a combination of 5-FU, doxorubicin and mitomycin C. In the treatment of ovarian cancer, 5-FU is equally as effective as conventional alkylating agents, producing a 33% response rate (Carter, 1981). There is no added advantage of using combination 5-FU therapy instead of single alkylating agents (Miller *et al*, 1980). An objective tumour regression rate of approximately 20% has been recorded with 5-FU in the treatment of cervical carcinomas (Deppe *et al*, 1981). 5-FU is also being used increasingly in the treatment of AIDS-related neoplasias in HIV-positive individuals (see 12.5.1); an example is the use of this agent, in combination therapy with cisplatin (Rich *et al*, 1993), mitomycin (Ajani *et al*, 2006; Flam *et al*, 1996) or radiation therapy (Rich *et al*, 1993; Flam *et al*, 1996; Ajani *et al*, 2006) in the treatment of anal cancer. The use of a 1% 5-FU gel has, furthermore, proven effective in the treatment of intravaginal warts in HIV-positive women already on antiretroviral treatment (Sved *et al*, 1999).

1.2.8.6 Carcinomas of the liver

5-FU is used to treat these hepatocellular carcinomas by being administered via an intra-arterial perfusion. The usual dose is 20-30 mg/ kg/ day for 4-18 days. A combination of 5-FU, vinca alkaloids, doxorubicin and mitomycin C can be used, but there is no added advantage of using combination therapy over monotherapy with 5-FU (Dollery, 1991).

1.2.8.7 Carcinomas of the breast

This is the main type of cancer to have been treated with 5-FU since the 1960s. When used alone, there is a 26% response to 5-FU (Dollery, 1991). Recently it is more common to use combination therapy to treat breast carcinomas, and the most usual combination is 5-FU with cyclophosphamide and methotrexate (CMF). This particular combination has a tumour response rate of 47%; this increases to 73% if doxorubicin is added to the regimen (Dollery, 1991). Another agent that can be added is mitomycin C. It is now standard therapy to treat young women at risk of having a relapse with CMF (Falkson *et al*, 1979). The combination of 5-FU and selective oestrogen receptor modulators (SERM), such as tamoxifen derivatives (Licun and Tannock, 2003) and raloxifene (Fryar-Tita *et al*, 2007), has also proven efficacious against breast cancer cell proliferation. Toxicity is very common, though, in 5-FU-containing regimens, with nausea and vomiting being prominent effects; these side-effects have not detracted from the widespread use of CMF as first-line therapy for breast cancers

(Dollery, 1991). In 10.4, the induction of "chemobrain", a phenomenon of cognitive impairment, in breast cancer survivors treated with 5-FU, is discussed.

1.2.8.8 Other indications

5-FU has, additionally, been used as adjuvant therapy in patients who have undergone surgery to treat glaucoma (Ophir and Ticho, 1992; Goldenfield *et al*, 1994; The Fluorouracil Filtering Surgery Study Group, 1996). It has been suggested that 5-FU increases the success rate of trabeculectomy in patients at risk for failed surgery (Wormald *et al*, 2001), but it was noted that the use of 5-FU in patients with glaucoma is questionable (Wormald *et al*, 2001; Wong *et al*, 1994; O'Grady *et al*, 1993), especially due to the toxic effects of 5-FU on the eye (see 1.2.10.5).

Genital warts, condylomata acuminate, can be treated with 5-FU (Adler and Mindel, 1985; Anonymous, 1988; Davis and Emans, 1989; Pride, 1990), and this drug has also been used with surgery to treat human papilloma virus (HPV)-induced vulvar disease (Reid *et al*, 1990). Although Reid *et al* (1990) have found the efficacy of 5-FU in the case of the latter to be variable, it has been shown that a cream containing 5% 5-FU is effective in decreasing the amount of HPV DNA (Davila *et al*, 1996).

5-FU can also be used topically to treat a variety of skin carcinomas (Dollery, 1991) and actinic keratoses (Epstein, 1998). The excited state structural dynamics of 5-FU have recently been investigated, and it has been found that 5-FU exhibits a profile very similar to that obtained for thymine, with most of the vibrational energy of the molecule localised in the carbon-carbon double bond at position 5 (Billinghurst *et al*, 2006). The photoproducts of thymine induce DNA damage, and it is likely that 5-FU has the same effect when in contact with ultraviolet radiation (Billinghurst *et al*, 2006), thus accounting for its cytotoxic effect. The topical use of 5-FU can induce painful erosions (Epstein, 1998) (see 14.2.2)

1.2.9 Cancer chronotherapy

1.2.9.1 Circadian rhythms

The term "circadian rhythm", as coined by Halberg and Stephens (1959), describes the biological clock. The "circadian clock" consists of an oscillator that produces the rhythmic signal, an input mechanism that links this oscillator to time stimuli from the environment, and an output mechanism that allows for the integration of behaviour and physiology (Canaple *et al*, 2003). The biological clock is represented by the suprachiasmatic nucleus (SCN) of the hypothalamus, which is responsible for generating biological rhythms controlled by the so-

called clock genes (Eriguchi *et al*, 2003), which include period, clock and cytochrome genes (Morse and Sassone-Corsi, 2002; Dunlap, 1999; King and Takahashi, 2000). These biological rhythms regulate various endocrine; immune; nervous and other functions (Eriguchi *et al*, 2003), including locomotor activity (see 11.2).

Different processes, such as the maintenance of blood pressure and the activity of the digestive system, are subjected to circadian control (Mormont *et al*, 2000). The SCN also regulates cellular metabolism and cell proliferation, which are subject to fluctuations. The length of cycles created at the SCN is calibrated by the transition from lightness to darkness, through the secretion of melatonin from the pineal gland (Eriguchi *et al*, 2003). Period genes create circadian rhythms, and these circadian fluctuations are subjected to an auto-negative-feedback mechanism (Eriguchi *et al*, 2003).

Although the SCN is the main pacemaker, there are also oscillators in peripheral tissues, for example the kidney (Kita *et al*, 2002; Canaple *et al*, 2003). This indicates that every cell in the body has its own oscillator (Canaple *et al*, 2003), evidenced by the fact that altering external stimuli, such as breast-feeding time, can reset the oscillators in peripheral tissues, without involving the SCN (Stokkan *et al*, 2001; Damiola *et al*, 2000). However, newer evidence seems to suggest that these peripheral clocks are not completely autonomous of the influence of the SCN, and require input from the latter to produce their rhythms (Pando *et al*, 2002). Although the mechanisms are poorly understood, hormonal influences and neural signals are known to be important in the relationship between the SCN and peripheral clocks (Balsalobre *et al*, 2000). Glucose is effective at resetting the timing signals for peripheral circadian clocks, since it down-regulates period genes and thus generates a circadian rhythm (Hirota *et al*, 2002). Glucose also up- and down-regulates other genes involved in the cell cycle; notably, it up-regulates thymidylate synthase, whose substrate is 5-FU (Canaple *et al*, 2003). The influence of glucose thus highlights the role that fasting and food intake have on circadian patterns (Bjarnason *et al*, 2002), and the cell cycle. The value of circadian rhythms is in assisting the body in being able to adapt to various external stimuli, such as temperature, and to integrate different physiological, molecular, behavioural and biochemical processes (Canaple *et al*, 2003; Grundschober *et al*, 2001; Panda *et al*, 2002; Storch *et al*, 2002; Kita *et al*, 2002; Delaunay and Laudet, 2002).

Support for the role of the pineal gland in controlling the activity of the SCN through a feedback mechanism comes from several findings (Isobe *et al*, 2002): (i) there are high-affinity sites in the SCN to which melatonin binds (Dubocovich, 1995); (ii) melatonin, the chief pineal secretory product (see 1.3) induces dose- and phase-dependent decreases in the

activity of the SCN (McArthur *et al*, 1991; Gillette and McArthur, 1996); (iii) melatonin alters the electrical activity of the SCN *in vitro* (McArthur *et al*, 1991); and (iv) melatonin inhibits arginine-vasopressin (AVP), stored in neurons in the SCN (Morin, 1993; Isobe *et al*, 2001a), whilst AVP, in turn inhibits the pineal secretion of melatonin (Isobe *et al*, 2001b). Furthermore, disturbances in the melatonin circadian rhythm are known to underlie pathological depression, such as seasonal affective disorder (Srinivasan, 1997). Serotonin (5-HT), the precursor of melatonin, is also present in the SCN and here, there appears to be an interaction between the two. For example, it has been shown that oral melatonin administration at night results in an increase in extracellular 5-HT, an effect not observed during the daytime (Yoshioka *et al*, 2000). 5-HT release in the SCN is, moreover, increased by sleep deprivation and wheel running in mice (Mistlberger *et al*, 2000). Since 5-HT is also known for its important role in depression (see 6.2.2.2), the interaction between 5-HT and melatonin in the SCN could play a role in depression resulting from disturbances in such a balance.

1.2.9.2 The cell cycle

The cell cycle, which is a sequence of phases that chart the development of cells, is also an oscillator (Canaple *et al*, 2003). The diagram on the next page illustrates the cell cycle (Calabresi and Chabner, 2001), and indicates the points at which various anticancer drugs function. G_1 is a presynthetic stage; from here, cells move into the S phase, where DNA synthesis occurs. There is then a pre-mitotic phase (G_2) which follows the completion of DNA synthesis. After G_2, cells move into the M phase, in which mitosis occurs. The resultant daughter cells may either re-enter G_1 and undergo DNA synthesis and mitosis themselves, or they may enter a resting phase (G_0) in which they do not undergo proliferation. From the G_0 phase, cells may re-enter the cell cycle at a later point in time (Calabresi and Chabner, 2001).

The major proteins that regulate events in the cell cycle are cyclin-dependent kinase inhibitors (CKIs), cyclin-dependent kinases (CDKs) and the proteins associated with these, and cyclins (Sherr and Roberts, 1999; Obaya and Sedivy, 2002). p53 and pRb genes, which have a tumour-suppressive effect, are also important in this regard (Taya, 1997; Levine, 1997). Not only is the expression of p53 genes subjected to circadian rhythms, so is the cellular apoptosis induced by these genes (Bjarnason *et al*, 1999; Canaple *et al*, 2003). The cell cycle is subject to strict regulation, and defects in these regulatory mechanisms may result in uncontrolled cellular proliferation (Hanahan and Weinberg, 2000; Melo and Toczyski, 2002).

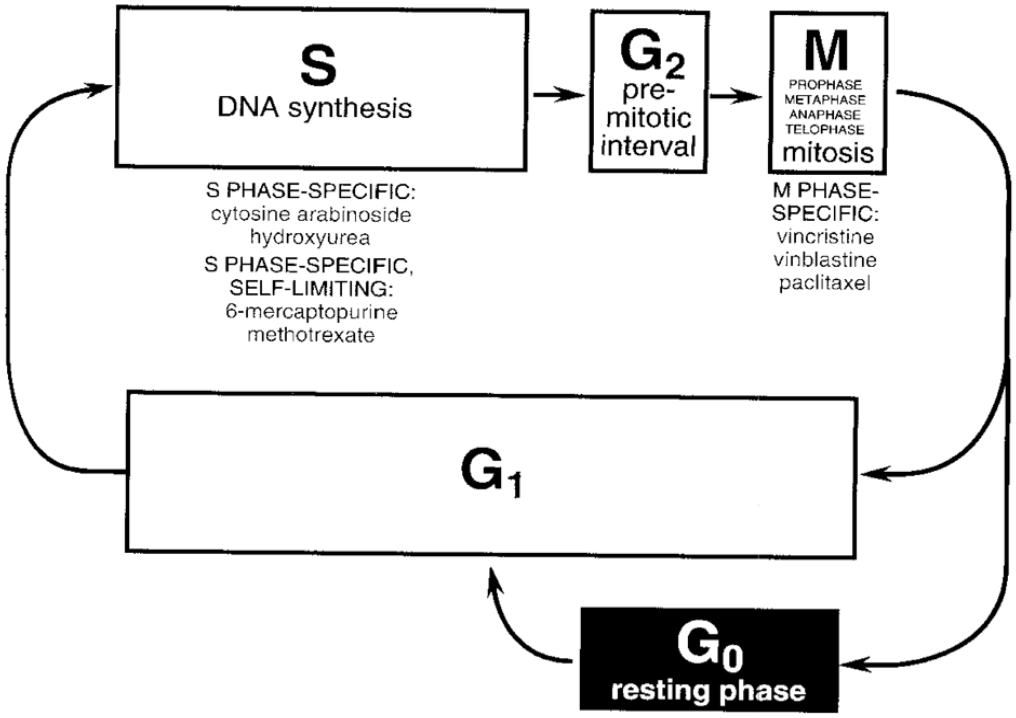


Figure 6: The cell cycle (Calabresi and Chabner, 2001; modified from Pratt *et al*, 1994)

Various studies have confirmed the hypothesis that the cell cycle is regulated by the circadian clock (Canaple *et al*, 2003). For example, the precursors of bone marrow cells were found to undergo circadian fluctuations in proliferation (Smaaland *et al*, 1992), and the same is true for epithelial cells along the gastrointestinal tract (Scheving *et al*, 1978) and neuronal cells (Goergen *et al*, 2002). The circadian clock may, furthermore, influence the sequence of phases in the cell cycle, as the timing of the expression of the clock genes has been found to be correlated with the timing of the different phases in the cell cycle; different clock genes are thus maximally expressed during different stages of the cell cycle (Canaple *et al*, 2003; Bjarnason *et al*, 1999; Bjarnason *et al*, 2001). The examples given above are generally of a proliferative nature, but even some of the proteins in tissues that do not undergo rapid proliferation, such as kidney and muscle tissue, have a circadian rhythm (Storch *et al*, 2002; Kita *et al*, 2002). Canaple *et al* (2003) suggest that cell rhythms and cell division are controlled by different mechanisms, since molecular rhythms still occur in certain rat cells whose division had been arrested (Balsalobre *et al*, 1998). In such non-mitotic cells, clock genes may be involved in controlling various cellular processes that are not proliferative (Canaple *et al*, 2003).

During the growth of tumours, circadian fluctuations are still apparent at all stages of cell division (Granda and Levi, 2002). These proliferating tumour cells, however, have circadian

fluctuations that are out of phase with the rhythms in non-tumour cells (Barbason *et al*, 1995). DNA synthesis in the former, for example, undergoes a distinct diurnal variation (Lakatua *et al*, 1983; Smaaland *et al*, 1993; Klevecz *et al*, 1987). Malignant tumours grow at an even more rapid rate when there are interferences with the circadian rhythm, and one may thus conclude that the circadian clock is probably quite important in endogenously regulating the progression of tumour growth (Filipski *et al*, 2002).

By regulating apoptosis and cell proliferation, some of the circadian clock genes prevent DNA damage and suppress the growth of tumours. This was demonstrated by increased incidences of lymphoma and hyperplasia, as well as a decreased tendency to apoptosis, in mice that had mutated period 2 genes (Fu *et al*, 2002). Mice that had a deficiency of period 2 genes, which are vital clock genes (Canaple *et al*, 2003), were found to have abnormal levels of some of the other genes that have a role to play in apoptsis and cell proliferation, such as mdm 2 (Fu *et al*, 2002). The latter gene functions in the regulation of p53 at a post-transcriptional level, and might thus control the response mechanisms to DNA damage, thereby suppressing tumours (Canaple *et al*, 2003). Furthermore, if the period 2 gene is deactivated, there is overexpression of c-myc genes (Fu *et al*, 2002), which are essential genes in the processes of apoptosis and cell proliferation (Evan and Vousden, 2001). C-myc overexpression consequently results in the development of tumours (Fu *et al*, 2002).

This interrelatedness in biochemical processes is manifested on a relatively larger scale in the common features between the cell cycle and circadian clocks. These two pathways share various molecules, such as casein kinases. Casein kinase I is important in ensuring the phoshorylation of proteins, for example, PER proteins, that are necessary for the functioning of circadian clocks (Eide *et al*, 2002; Lee *et al*, 2001; Lowrey *et al*, 2000; Toh *et al*, 2001); it is also involved in cell division and controlling the repair of damaged DNA (Ho *et al*, 1997). Casein kinase II ensures the stability of proteins involved in the circadian clock (Akten *et al*, 2003; Blau, 2003; Yang *et al*, 2002), is an essential regulator of c-myc proteins and thus has an important role to play in the cell cycle (Channavajhala and Seldin, 2002). It is also responsible for phosphorylating p53 to ensure DNA repair (Kapoor and Lozano, 1998). In cancer, there is often up-regulation of casein kinase II (Ahmed *et al*, 2002).

Another common feature between the two pathways is the involvement of cyclic GMP (cGMP) and cyclic AMP (cAMP). These compounds regulate kinases (Canaple *et al*, 2003). cAMP can enhance the proliferation of some types of cells, and is also able to inibit the proliferation of other cell types. The latter is achieved by influencing mitogen-activated protein (MAP)-kinase pathways (Stork and Schmitt, 2002). Circadian fluctuations in levels of

cGMP and cAMP are vital in maintaining the circadian clock's regulation over the cell cycle (Carre and Edmunds, 1993; Scheving and Gardner, 1998). cAMP interacts with a protein called the cAMP-responsive element binding protein (CREB), and this is involved in resetting the circadian clock (Akashi and Nishida, 2000; Obrietan *et al*, 1999). Synchronisation of the diurnal/ nocturnal cycle with the circadian clock is achieved by a mechanism involving CREB phosphorylation (Travnickova-Bendova *et al*, 2002). The promoter for period 1 genes is induced by CREB pathways, using cGMP and cAMP (Travnickova-Bendova *et al*, 2002). CREB is also involved in DNA repair (Gu *et al*, 1997; Lill *et al*, 1997) and in the processes that alter cells so that these become cancerous (Houglam *et al*, 1997). Phosphorylation is important in ensuring the stability of proteins involved in the cell cycle and in circadian clocks; it also determines the localisation of these proteins within cells (Canaple *et al*, 2003). Proteins are phosphorylated prior to degradation (Lee *et al*, 2001; Naidoo *et al*, 1999), and this degradation, at set stages, is essential in ensuring the progression of the cell cycle (Udvardy, 1996; Yew, 2001; Canaple *et al*, 2003). It is thus evident that the casein kinases, cAMP and cGMP have diverse and far-reaching influences on both circadian clocks and the cell cycle.

Another similarity between these two pathways, on a genetic level, is the E-box, and various transcription factors. The E-box controls the expression of genes under the influence of the circadian clock (Reppert and Weaver, 2002; Dunlap, 1999; Ripperger *et al*, 2000), and also influences cell death, differentiation and proliferation (Canaple *et al*, 2003). For example, the E-box can enhance the activity of c-myc, which can result in cells becoming less susceptible to regulation by growth factors, undergoing apoptosis or progressing through certain phases of the cell cycle, such as the G_1 phase, at a much faster rate (Fuhrmann *et al*, 1999; Littlewood and Evan, 1990).

Other factors that are common between circadian clocks and the cell cycle are: (i) the influence of growth factors, such as glucose; (ii) hormones, in particular, melatonin (see 1.3.7); and (iii) the role that light plays in resetting these systems (Canaple *et al*, 2003). With regard to the latter, many of the genes in the SCN that respond to light stimuli have multiple roles: these genes play a part in resetting circadian rhythms, as well as regulating the cell cycle (Kornhauser *et al*, 1990). Some proteins, such as the photolyase/ cryptochrome subset, can reset circadian rhythms as well as repair DNA that is damaged, in response to visible light stimulation (Thompson and Sancar, 2002).

Other molecules, besides melatonin, can also reset peripheral circadian clocks. This includes retinoids (McNamara *et al*, 2001), which also have a regulating effect on the expression of

myc genes (Thiele and Israel, 1988; Thiele *et al*, 1985). NO, which is also involved in maintaining circadian rhythms (Clément *et al*, 2003; Artinian *et al*, 2001), is believed to contribute to the development of tumours (Lala and Chakraborty, 2001). The activity of the enzyme NO synthase, which is responsible for the formation of NO, is enhanced in tumour cells (Ambs *et al*, 1997) and is subjected to circadian fluctuations (Tunctan *et al*, 2002). This further highlights the link between cancer and circadian rhythms. Moreover, the survival time of patients with some types of cancer, such as breast cancer, can be reasonably well predicted using their circadian rhythm (Sephton *et al*, 2000).

It is suggested that dysfuntioning of circadian clocks can cause the transformation of normal cells into malignant ones (Canaple *et al*, 2003). Circadian rhythms can regulate cell repair, the intracellular generation of toxic substances and the adverse effects that the latter could induce by interacting with cellular components (Tampellini *et al*, 1998; Boughattas *et al*, 1989; Zhang *et al*, 1993). Most tumours, in turn, release growth factors and cytokines, which can interfere with the functioning of circadian systems (Born *et al*, 1997).

The aforementioned findings highlight the links between the circadian and cell cycle systems, which have evolved through independent mechanisms but are still fundamentally related and integrated (Canaple *et al*, 2003), and can thus significantly affect many aspects of physiology. Due to the abovementioned relationships between circadian clocks and cancer from molecular and systemic points of view, it is suggested by Lynch (2004) that cancer is not a localised disease and should not be treated as such.

"Cancer chronotherapy" is "a novel and logical therapy in which anti-cancer drugs are administered with optimal timing according to circadian rhythms of anti-cancer action and those of adverse effects on normal cells" (Eriguchi *et al*, 2003). This approach to cancer treatment is important, because many conventional drug treatments cause considerable toxicity to non-tumor cells, and are not as effective as they could be (Canaple *et al*, 2003). Enhancing the efficacy of drug treatment, while simultaneously reducing toxicity to the host, thus remain desirable goals of cancer treatment. There are many potential ways of achieving this, including using biological modulators, such as melatonin, as adjunctive therapy (see 1.3.9), and the employment of cancer chronotherapy (Canaple *et al*, 2003). Children suffering from acute lymphoblastic leukaemia have a prolonged survival time when subjected to chronotherapy (Fu and Lee, 2003).

Cancer chronotherapy is effective for several reasons. It maximises on the asynchronies between the growth rates of normal and cancer cells, thereby enhancing the efficacy of

anticancer drugs while also reducing the side-effects of these (Lynch, 2004). It it has been found that the effectiveness of anticancer drugs, as well as toxicity, depends on the cell cycle phase and the circadian rhythm (Granda and Levi, 2002; Mormont and Levi, 1997; Mormont and Levi, 2003). This is especially true for drugs with the fluoropyrimidine structure, for example, 5-FU (Mormont and Levi, 2003; Levi, 2001; Levi *et al*, 1997). This may be because drugs, such as 5-FU, whose mechanism of action involves inhibition of DNA synthesis, tend to more selectively target cells undergoing DNA synthesis and because this DNA synthesis in tumour cells occurs in the opposite rhythm to DNA synthesis in non-tumour cells (Barbason *et al*, 1995; Lakatua *et al*, 1983; Smaaland *et al*, 1993; Klevecz *et al*, 1987). The majority of tumour cells are in the transition between the G_2 and M phases in the morning, and inducing apoptosis at this time would thus result in a greater proportion of cancer cells being killed (Canaple *et al*, 2003). Cancer chronotherapy has the potential to also target tumour cells at the time when these are most proliferative (Canaple *et al*, 2003).

1.2.9.3 Chronopharmacokinetics of 5-FU

The circadian dosing time influences the toxic effects of 5-FU by up to 50% in rats (Levi *et al*, 1995). The peak delivery of 5-FU was found to be at 04h00 and, in chronomodulatory studies conducted by Levi *et al* (2000), the most active dosing schedule of 5-FU was also the one with 2-10 times less toxicity. In addition, Levi *et al* have found that chronochemotherapy with 5-FU, folates and L-OHP (oxalato-platinum), the latter being a complex with antiproliferative activity (Mathé *et al*, 1989), produces up to a 50% objective tumour response rate (Levi *et al*, 1997), and that this combination is a good first-line treatment option for patients with colorectal cancer who have unresectable hepatic metastases (Eriguchi *et al*, 2003). Administering 5-FU according to its circadian rhythm was thus found to more than double the efficacy of chemotherapy in patients with metastatic colorectal cancer. This has resulted in improved prognosis, evidenced by the ability to surgically remove metastases that were previously untreatable (Levi *et al*, 1995).

Levi has extensively studied the chronopharmacokinetics of 5-FU and, in an article cited by Eriguchi *et al* (2003), has recommended that 5-FU be administered between 22h00 and 10h00, with a peak at 04h00. This was because, during this time period:

(i) there are fewest cells in the S-phase of the cell cycle; DNA synthesis occurs in the S phase, and so administration at this time would reduce gastrointestinal side-effects and myelosuppression;

(ii) the activity of DPD is greatest, thus inactivating 5-FU and reducing its toxicity;

(iii) the activity of thymidylate synthase is lowest and there is thus less formation of false nucleotides and consequently less incorporation of these into non-tumour cells; and

(iv) the expression of the bcl-2 gene is greatest. This gene suppresses the apoptosis of cells (Eriguchi *et al*, 2003). The above factors reduce the toxicity of 5-FU and will, by the same mechanisms, also reduce the cytotoxicity and hence the effectiveness of the drug.

If administered by continuous intravenous infusion, the plasma concentration of 5-FU has been found to vary in a circadian fashion by as much as 50%; peak concentrations occur at midnight, according to one author (Petit *et al*, 1988). This variation may be attributed to the activity of DPD undergoing circadian fluctuations in the blood (Harris *et al*, 1990), and there is known to be marked interpatient variability in this regard. This emphasises the need for individualised infusion times (Harris *et al*, 1990) in order to reduce the toxicity of 5-FU and increase its efficacy (Young *et al*, 1999).

Thymidylate synthase, the key enzyme that is inhibited by 5-FU and which is used as a marker of S-phase activity, is subjected to significant circadian fluctuation, with maximal levels occurring in the early afternoon (Bjarnason *et al*, 2001). The gene that encodes for thymidylate synthase is controlled by the circadian clock, and interference with the functioning of this gene could potentially represent an effective means of treating cancers that are subjected to circadian influences (Canaple *et al*, 2003).

1.2.10 Toxicity

Fluorine, the most commonly-occurring halogen, was not involved in the evolution of living organisms (O'Hagan *et al*, 2002) and is known to be poisonous due to its microenvironmental effects on various key enzymes (Strunecká *et al*, 2004). In the presence of aluminium ions, fluoride has a synergistic effect and stimulates G-proteins by acting as an analogue of a phosphate group (Sternweis and Gilman, 1981; Strunecká *et al*, 2004), and thereby potentially affecting numerous cellular metabolic processes (see 10.3).

Strunecká *et al* (2004) note the irony that the Nobel prize-winner, Albert Gilman, said in his acceptance speech, that, "The ultimate dream is to design drugs that will prevent aberrant G protein action", while fluoride-aluminium ion complexes induce such aberrations. The issue of overexposure to fluoride by the use of fluorine-containing drugs is of concern to some (Bryson, 2004), especially since there are already substantial fluoride levels in the environment, and there are many who are particularly vulnerable to the effects of fluoride, such as patients with renal impairment (Bryson, 2004).

1.2.10.1 Gastrointestinal toxicity

5-FU has a low therapeutic index and clinical manifestations of its toxicity are delayed, thus complicating treatment and monitoring. The earliest adverse drug reactions are nausea and anorexia, followed by diarrhoea and stomatitis (Chabner *et al*, 2001). The latter is evidenced by the formation of necrotic and ulcerative membranes in the stomach and other parts of the gastrointestinal tract and resulting in gastrointestinal side-effects, such as bleeding. These symptoms are adequate warning that the patient has received a sufficient dose of 5-FU (Bayomi and Al-Badr, 1989; Chabner *et al*, 2001). These gastrointestinal manifestations of toxicity are due to the effect of 5-FU on rapidly-dividing epithelial cells. Pharyngitis and/ or oesophagitis may also occur and, in severe cases, haemorrhagic lesions can develop (Dollery, 1991). Ulcers that develop in the throat or mouth are believed to be due to fungal or viral superinfections and are thus treated with nystatin or acyclovir, respectively (Dollery, 1991).

Nausea, accompanied by severe vomiting, can occur two hours after the administration of 5-FU. A suitable antiemetic, such as a corticosteroid, can be used to reduce the occurrence and/ or severity of this. Diarrhoea occurs within the first week of treatment and is usually mild; haemorrhagic discharge and dehydration may occur in more serious cases. Pulse therapy, which is the intermittent administration of high doses of 5-FU, is associated with fewer gastrointestinal disturbances (Dollery, 1991).

1.2.10.2 Haematological toxicity

The most toxic adverse effect of the drug is myelosuppression; leucopaenia occurs most often and is worst 9-14 days after intravenous administration of 5-FU. Anaemia, thrombocytopaenia, severe alopecia, dermatitis, nail changes and atrophy and pigmentation of the skin may also occur (Chabner *et al*, 2001). These potentially severe and possibly life-threatening adverse haematological toxicities highlight the importance of careful monitoring of the patient by the doctor and pharmacist, as well as emphasise the toxicity of 5-FU (Chabner *et al*, 2001).

Potentially fatal haematological toxicity can develop within ten days of initiating 5-FU therapy, but tends to resolve slowly over three weeks (Stevens *et al*, 1980; Falkson and Schulz, 1962; Floyd *et al*, 1982; Gottlieb and Luce, 1971; Heidelberger, 1957). If patients have a platelet count of less than 20 X 10^9/ L, there is a risk of death from bleeding caused by thrombocytopaenia. In addition, potentially fatal infections can arise due to neutropaenia, if the granulocyte count is less than 500 X 10^9/ L. These cases call for immediate platelet transfusions and antibiotic therapy, respectively (Obrecht *et al*, 1981). The haematological

toxicity of 5-FU is exacerbated if any other drug that causes a suppression of bone marrow cells, or irradiation therapy, is used as well (Dollery, 1991).

Usually toxic effects occur more often if the dosage of 5-FU is greater than 1 g/ day (Dollery, 1991). During the induction phase of the dosing regimen, a full blood count of the drug should be performed weekly, and thereafter every three weeks (Dollery, 1991). If pulse therapy is used, a pre-treatment full blood count is essential, whereas weekly blood counts are required if the drug is administered on a weekly basis. Haemoglobin and platelet levels need to be especially monitored (Dollery, 1991), since one of the major toxic effects of 5-FU is thrombocytopaenia. The systemic toxicity of 5-FU can be decreased by hepatic perfusion of the drug, since more than half of the dose will be subjected to first-pass hepatic metabolism (Dollery, 1991).

1.2.10.3 Immunosuppression

5-FU induces severe immunosuppression (Van Zant, 1984; Větvička *et al*, 1995), with significant inhibition of both primary and secondary immune responses (Ribova *et al*, 1988). The agent exerts toxic effects on central and peripheral lymphoid cells immediately (Větvička *et al*, 1986). A single intravenous dose of 5-FU has been shown to ablate 90% of B lymphocytes, 98% of committed myeloid progenitors, 90% of thymocytes and 50% of total cells in the spleen (Větvička *et al*, 1986). The immunosuppressive effects of 5-FU are attributed to the ability of the agent to selectively decrease B and T lymphocytes; macrophages have been found to exhibit resistance to 5-FU treatment (Větvička *et al*, 1986, 1990). In addition to the abovementioned direct toxic effects on different cells mediating immunity, 5-FU exerts a persistent inhibition on the growth and maturation of cells involved in humoral immunity (Ribova *et al*, 1988). It has been shown that the addition of recombinant cytokines can counteract this 5-FU-induced immunosuppression, by enhancing certain immunoglobulin antibody responses (Větvička *et al*, 1995).

1.2.10.4 "Hand-foot" syndrome

Due to new methods of administration, such as continuous infusions administered by indwelling catheters, new forms of toxicity are being manifested (Dollery, 1991). A "hand-foot" syndrome is common with total daily doses of 500 mg; this is manifested as hyperaemia of the skin on the soles and palms, which is accompanied by burning sensations. This syndrome occurs rapidly if the dosage is reduced or the infusion is stopped (Dollery, 1991). The syndrome, which has been termed a "palmar-plantar erythrodysesthesia syndrome" (Sweetman, 2002) also includes desquamation of skin on the relevant body regions (Lokich and Moore, 1984; Feldman and Ajani, 1985; Atkins, 1985; Curran and Luce, 1989) and may

be attributed to the fact that 5-FU is a photoreactive drug and can thus give rise to photosensitised toxic effects (Dollery, 1991) (see 1.2.8.8). Cessation of drug therapy can terminate phototoxic side-effects, as can oral pyridoxine supplementation (Vukelja *et al*, 1989).

1.2.10.5 Cardiotoxicity

Abnormalities on electrocardiograms (ECG) and cardiac arrhythmias are less common, and these may have very serious effects (Dollery, 1991). Cardiac ischaemia is ten times more common when a continuous infusion of 5-FU has been administered, as compared to an intermittent bolus injection (Douglass and Mitteloran, 1974; Dollery, 1991; Freeman and Costanza, 1988; Collins and Weiden, 1987). Ventricular tachycardia, arrhythmias and cardiac arrest occur in 0.55% of patients on the drug (Keefe *et al*, 1993); the incidence of cardiac events due to coronary artery spasm and angina may be even higher than this (Keefe *et al*, 1993; McLachlan *et al*, 1994; Anand, 1994). Patients who may be at a greater risk of developing cardiotoxic effects are those with a history of heart disease, but cardiotoxicity can occur in patients who do not have this risk factor (McLachlan *et al*, 1994; Anand, 1994; Hannaford, 1994; Kleiman *et al*, 1987; Canale *et al*, 2006). Although uncommon, 5-FU has been known to cause coronary vasospasm as well (Shoemaker *et al*, 2004) (see 14.2.2). These coronary artery spasms may result in S-T segment elevation in ECG profiles (Kleiman *et al*, 1987; Mafrici *et al*, 2003) and thus variant angina (Kleiman *et al*, 1987; Bastion *et al*, 1988) and myocardial infarction (Canale *et al*, 2006). Prophylaxis with drugs used to treat angina, such as nitrovasodilators and calcium-channel antagonists, has been shown to prevent anginal attacks from recurring (Kleiman *et al*, 1987). Haemodynamic impairment, leading to ECG changes mimicking acute myocardial infarction, has also been reported (Mafrici *et al*, 2003).

It has been found that 5-FU-induced cardiotoxicity is not due to the formation of free radicals or lipid peroxidation (Cwikiel, 1996). The agent is also metabolised, in the rat, into two hitherto unidentified compounds, namely monofluoroacetic acid (FA), via cleavage of the pyrimidine ring, and α-fluoro-β-hydroxypropionic acid (Arellano *et al*, 1998). These metabolites are formed in very low concentrations (0.0.67%), but are known to be cardiotoxic (Park *et al*, 2001). This provides an additional mechanism of action for the cardiotoxic effects induced by 5-FU (Arellano *et al*, 1998). The question of whether FA is indeed formed is contentious, and has been refuted by some (Diasio and Harris, 1989). FA undergoes mitochondrial conversion to fluorocitrate via condensation of oxaloacetate and fluoroacetyl coenzyme A in the presence of citrate synthase; this latter enzyme normally provides molecules of acetyl coenzyme A into the citric acid cycle, which is responsible for energy

production in aerobic organisms. Fluorocitrate significantly inhibits the enzyme aconitase, and is thus toxic because it blocks energy production (Strunecká *et al*, 2004; Peters, 1957).

1.2.10.6 Ocular toxicity

More unusual adverse drug reactions include conjunctivitis, which can be chronic or acute, and which can develop into stenosis of the tear ducts (Dollery, 1991). Ocular toxicity due to 5-FU includes severe lachrymation and fibrosis of the tear ducts (Haidak *et al*, 1978). The presence of 5-FU in lacrimal fluid can cause ocular irritation (Christophidis *et al*, 1978), while the most serious effect is bilateral total corneal epithelial erosion (Hirsh *et al*, 1990). Combination therapy has been reported to cause optic neuropathy, which can progress to almost blindness (Adams *et al*, 1984). Diabetic patients with underlying pathology of the eye can develop corneal abscesses and ulcers (Hickey-Dwyer and Wishart, 1993). These toxic ocular effects of 5-FU may be due to the agent's photoreactivity and accumulation in ocular tissues, which are exposed to ambient light.

1.2.10.7 Neurotoxicity

Acute cerebellar effects have been reported after the intrathecal administration of 5-FU (Dollery, 1991). Other symptoms of central neurotoxicity, including emotional lability, confusion, ataxia and disorientation, occur rarely. Patients at a greater risk of developing these side-effects are those receiving high doses of 5-FU (Sweetman, 2002). It has been suggested that patients who have disorders in the metabolism of pyrimidines may have a propensity to developing neurotoxicity (Tuchman *et al*, 1985; Stéphan *et al*, 1995; Takimoto *et al*, 1996). The neurotoxicity induced by 5-FU may be due to the drug inducing a deficiency of the vitamin thiamine, and supplementation with this vitamin may thus be useful in treating these effects (Pirzada *et al*, 2000). The phenomenon of "chemobrain" has recently been described, in which women treated for breast cancer with 5-FU develop cognitive impairments (see 10.4).

1.2.10.8 Hepatotoxicity

Bilirubin levels can also be increased (Dollery, 1991). It has been suggested that a complex which the metabolite FBAL (see 1.2.6.1) forms with bile acids, namely, an FBAL-chenodeoxycholate conjugate, may account for the biliary toxicity associated with the long-term hepatic arterial infusion of 5-FU (Sweeney *et al*, 1988; Diasio and Harris, 1989).

1.2.10.9 Secondary carcinomas

Caution has been raised in the United States Pharmacopoeia Drug Information for the Health Care Professional (USP DI, 1997). Very high concentrations of 5-FU were found to induce

mutagenesis in culture cells, and to cause breaks in chromosomes. Although 5-FU was not found to be carcinogenic, the USP DI (1997) warns that antimetabolites do have the potential to induce secondary carcinomas, although the likelihood of this is less than with alkylating agents used in the treatment of cancer.

1.2.10.10 Algesia

Although there is, to our knowledge, no literature on the effects of this agent on nociception and the mechanisms involved in this process, 5-FU is known to induce a variety of different pains, from the "hand-foot syndrome" (see 1.2.10.4) to chest pains and erythemas when applied topically. This is discussed in more detail in 14.2.2.

1.2.10.11 Metabolic alterations

Tayek *et al* (1995) and Tayek and Chlebowski (1992) have investigated the effects of 5-FU therapy on various metabolic processes, such as amino acid and glucose metabolism. Increased plasma levels of amino acids are frequently observed in patients suffering from colon cancer (Carmichael *et al*, 1980; Eden *et al*, 1984) and abnormalities in glucose metabolism are also common in cancer patients (Tayek, 1992). It has been shown that five days of treatment with 5-FU is sufficient to decrease the oxidation of the amino acid leucine, thus increasing plasma levels of the amino acid, and increase hepatic glucose production, by 10%. Isoleucine levels also fall after 5-FU treatment and the total body protein production decreases by 10% (Tayek and Chlebowski, 1992). It is believed that elevated plasma levels of leucine and enhanced hepatic glucose production are indicative of a poor survival rate for cancer patients, as these markers reflect enhanced tumour burden and metabolism (Tayek *et al*, 1995). This suggests that 5-FU therapy induces metabolic abnormalities that may reflect a poor survival time for patients. After twenty-five days of 5-FU treatment, however, there are no alterations in protein synthesis, leucine oxidation or hepatic glucose production; the latter may be due to the absence of severely malnourished patients in the study, as hepatic glucose production is related to the degree of malnutrition (Tayek *et al*, 1995).

1.2.10.12 Other toxicities

In a study comparing the use of sequential or combined radiation therapy and 5-FU-based chemotherapy in breast cancer survivors, it was shown that the use of both therapies concurrently results in a significant increase in the incidence of skin pigmentation, subcutaneous fibrosis and breast atrophy after a median time of 6.7 years (Toledano *et al*, 2006). Although it was not demonstrated that 5-FU is specifically responsible for these toxicities, these findings are important as 5-FU is very often used in combination therapy with other antineoplastic agents in the treatment of breast cancer (see 1.2.8.7).

1.2.10.13 Overdosage

There is no antidote to 5-FU overdosage, and such a situation is managed supportively by addressing issues such as thrombocytopaenia (Dollery, 1991).

1.2.11 Drug interactions

These include both potentially beneficial and potentially hazardous drug interactions.

1.2.11.1 Beneficial drug interactions

An example is that involving methotrexate and folinic acid. This is due to the added drugs increasing the affinity that thymidylate synthase has for FdUMP. Another potentially useful drug that can be administered with 5-FU is thymidine, which significantly increases the half-life of 5-FU (Dollery, 1991). The potential benefit of combining 5-FU with the pineal hormone melatonin is the cornerstone of this research (see 1.3.9).

Interferon α (INFα) has also been found to modulate the activity of 5-FU by altering its pharmacokinetic profile (Czejka *et al*, 1993). Furthermore, it enhances the anabolism of 5-FU into its active nucleotide forms (Chabner *et al*, 2001).

1.2.11.2 Hazardous drug interactions

Potentially negative drug interactions include combination with other cytotoxics and irradiation, as this can cause an additive myelosuppressive effect (Dollery, 1991). This does not rule out such a combination of cytotoxics, provided that dosages of the cytotoxics are carefully adjusted and the patient is subjected to therapeutic drug monitoring. Aminoglycoside antibiotics can delay the gastrointestinal absorption of 5-FU, and vaccines are not recommended as potentially fatal infections can occur due to the immunosuppressant effects of 5-FU (Dollery, 1991). The antiprotozoal metronidazole has been suggested to reduce the clearance of 5-FU, thereby increasing the toxicity of the latter (Bardakji *et al*, 1986). A metabolite of the antiviral drug sorivudine, which is used to treat herpes zoster infections, has been shown to inhibit DPD, and consequently lead to potentially fatal leucopaenia (Diasio, 1998).

Cimetidine, an antagonist of histamine$_2$ receptors in the stomach, has been found to alter the pharmacokinetic profile of 5-FU. Pretreatment with the former for a period of one month increases the plasma concentration of the antineoplastic following oral or intravenous administration. This increase in concentration may be attributed to cimetidine reducing hepatic blood flow and inhibiting hepatic enzymes (Harvey, 1984).

Metabolic drug interactions occur in the small intestine. 5-FU appears to decrease the enzyme contents of the intestinal mucosa, thereby significantly increasing the plasma levels of nifedipine, a calcium-channel blocker (Yoshisue *et al*, 2001). The sensitivity of enzymes in the small intestine to inhibition by 5-FU is thus much greater than hepatic enzymes. This inhibition, as well as much of the gastrointestinal toxicity of 5-FU, can be reduced by the concomitant administration of potassium oxonate (Yoshisue *et al*, 2000, 2001) (see 1.2.4).

1.2.11.3 CYP450 interactions

5-FU is metabolised by the cytochrome P450 (CYP450) system, but has been found not to inhibit this enzyme system. It has been specifically found not to inhibit the isoenzymes CYP 1A2, 2C9, 2C19, 2C8, 2E1, 2D6, and 3A4. Any drug interactions that occur with 5-FU are thus postulated to occur by other mechanisms, and not by 5-FU directly inhibiting the metabolism of the co-administered drug through the CYP450 system (Park *et al*, 2003). When metabolised by CYP450, positions that are adjacent to the carbon-fluorine bond are targeted, as the presence of a fluorine atom blocks oxidation at that position (Cnubben *et al*, 1994; Park *et al*, 2001). This property has been exploited to design drugs that are not activated by CYP450 to form toxic metabolites (Miller and Miller, 1960; Park *et al*, 2001; Barnard *et al*, 1993), such as fluorination of the antimalarial agent amodiaquine (Ruscoe *et al*, 1995; O'Neill *et al*, 1998).

1.2.12 Contraindications and precautions

5-FU is contraindicated in pregnancy, as it induces multiple congenital abnormalities (Dollery, 1991). The neurotoxicity of 5-FU to the developing foetus is discussed in 10.4. In some cases, however, combination therapy involving 5-FU has been successfully used in the second and third trimesters of pregnancy (Dollery, 1991). 5-FU should be used with caution in malnourished or weak patients, as well as those with impaired hepatic, renal or cardiac function. Topically, 5-FU should not be applied to mucous membranes, and there is excessive absorption if the drug is used on inflamed skin. Occlusive dressings may enhance inflammatory reactions, and the patient should avoid direct sunlight (Sweetman, 2002). Urine produced for 48 hours after an oral dose of the drug, and faeces likewise produced for up to five days should be handled with protective clothing (Harris and Dodds, 1985).

1.2.13 Storage and stability

5-FU is photoreactive and is stored away from moisture and light (BP, 2002). 5-FU preparations are alkaline and are thus incompatible with acidic drugs or those that are unstable at an alkaline pH. The latter includes doxorubicin, an antineoplastic often used in combination chemotherapy with 5-FU (Dorr, 1979). Chemical instability has also been

reported with calcium folinate (Trissel *et al*. 1995) and methotrexate (McRae and King, 1976), which are also often used with 5-FU.

1.2.14 Analysis

It is possible to resolve both the anabolites and catabolites of 5-FU using ion-pair reverse-phase high performance liquid chromatography (Diasio and Wilburn, 1979; Sommadossi *et al*, 1982). Ussing chambers are useful for measuring the metabolism of 5-FU *in vitro* (Smith *et al*, 1988). More details about analysis are given in Chapter 2.

1.2.15 Summary

The mechanisms of action by which 5-FU exerts antineoplastic activity are summarised below:

- It is phosphorylated and converted to a variety of fraudulent nucleotides which inhibit thymidylate synthase, thereby disrupting DNA and RNA synthesis
- High concentrations overwhelm the DNA repair enzyme, uracil glycosylase
- 5-FU promotes cell lysis by altering transmembrane potentials and interfering with glycoprotein synthesis
- The agent has been reported to both increase and decrease NO levels; both changes result in anticancer effects, through different mechanisms

The above mechanisms are illustrated in a model proposed by this author, Figure 7.

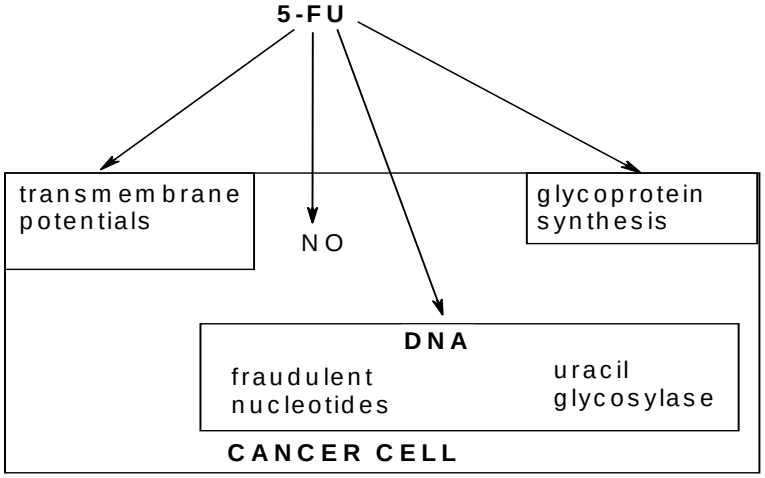


Figure 7: Proposed model of 5-FU's mechanisms of action

1.3 Melatonin (*N*-acetyl-5-methoxy-tryptamine)

1.3.1 Neuroimmunomodulation

Melatonin, an indoleamine hormone, is released from the pineal gland. It is produced by other organs as well, evidenced by the fact that melatonin levels in the gastrointestinal system are 400 times greater than the amounts present in the blood or released by the pineal gland (Bubenik, 2001). The pineal gland plays a pivotal role in regulating biological rhythms (Arendt, 1998; Brzezinski, 1997). The relationship between melatonin secretion and the circadian rhythms of locomotor activity and nociception are explored in Chapters 11 and 14, respectively. Another very important function is in mediating the effect that the psychoneuroendocrine system has on the growth of cancerous cells (Arendt, 1998; Brzezinski, 1997; Cos *et al*, 1998; Jankovic, 1994). When this endocrine role of the pineal gland is suppressed, the organism is more susceptible to developing immunosuppression and the internal environment is more conducive to the development of cancer cells (Regelson *et al*, 1987). Pineal function can be interfered with by various external forces as well, such as electromagnetic fields, resulting in the development of certain cancers (Ronco and Halberg, 1996), particularly breast cancer (Stevens and Davis, 1996). This is because breast cells are, to a large extent, dependent on the influence of hormones and are very sensitive to the effects of magnetic fields. Exposure to the latter inhibits the ability of T cells to proliferate, and reduces melatonin levels (Mevissen *et al*, 1996a, 1996b; Harland and Liburdy, 1997), which together result in a microenvironment conducive to the development of 7,12-dimethylbenz[a]anthracene (DMBA)-induced breast tumours (Mevissen *et al*, 1996a, 1996b). This field of research, which considers the interactions between the endocrine, nervous and immune systems, is termed neuroimmunomodulation, and is believed to be the fastest-growing area of research in the biomedical sciences (Conti, 2000).

Although melatonin has been the most researched pineal hormone, there are other indoles that are secreted and that have been found to exert anticancer activity, namely, 5-methoxytryptamine (MT), 5-methoxytryptophol (ML) and 5-methoxyindole acetic acid (MA), the metabolites of melatonin, evidenced by the finding that total pineal endocrine substitution therapy (TPEST) is also effective in the palliative treatment of patients with metastatic solid tumours (Lissoni *et al*, 2003a) (see 1.3.6).

1.3.2 Uses

The uses of melatonin are varied, and there is much potential for further clinical and diagnostic use of the agent. Its value in oncology will be discussed below. Besides this, melatonin can be used in psychiatry by, for example, assessing the impact that antidepressant

drugs have on the functioning of β receptors in the central nervous system (Miles and Philbrick, 1987), since β stimulation enhances melatonin production (see 7.1). Melatonin can also potentially be used in amniocentesis, to determine whether a foetus is likely to develop spina bifida, since this condition is associated with increased levels of melatonin (Miles and Philbrick, 1987). Pineal tumours secrete excess melatonin, and assays of melatonin concentrations can thus be performed to monitor the progress of neoplastic diseases and to determine whether metastasis has occurred (Miles and Philbrick, 1987). Melatonin can also be used as a marker of prostate cancer, which is associated with reduced levels of the hormone. Melatonin may, furthermore, be of value in the prognosis of breast cancer, since the hormone may be synthesised by epithelial cells in the mammary glands (Maestroni and Conti, 1996). Levels of melatonin are up to six times less in patients with endometrial cancer, and so melatonin can be used as an indicator of disease presence and progression (Grin and Grunberger, 1998).

Melatonin has a distinct function in the cycle between sleep and wakefulness (Cajochen *et al*, 2003). The circadian fluctuations that occur in the secretion of melatonin (see 1.3.7) rationalise the use of exogenous melatonin to treat sleep-pattern and other chronobiological disturbances in various individuals, for example, those experiencing jet lag, the blind (Wirz, 1996; Suhner and Steffen, 1997), those with advanced cancer (Mormont and Waterhouse, 2002; Mormont *et al*, 2002) and the elderly, who often have sleep disorders (Olde Rikkert and Rigaud, 2001). As a chronobiotic, melatonin can better synchronise the circadian patterns in the abovementioned patients with their environments, thus enhancing quality of life and increasing the effectiveness of cancer therapy (Canaple *et al*, 2003). People with visual impairment have been found to have higher endogenous melatonin levels, and a reduced incidence of cancer (Feychting *et al*, 1998).

Others who could benefit from supplemental melatonin include those with gastrointestinal disorders, such as ulcerative colitis (Bubenik, 2001); those who fast regularly, for example Ramadhan adherents (Bogdan *et al*, 2001); those who take total parenteral nutrition (Volodina *et al*, 2001); women who suffer from uterine contractions during menstruation (Schlabritz-Loutsevitch *et al*, 2003), since melatonin has anti-inflammatory action and thus inhibits the synthesis of the prostaglandins responsible for such cramping (Gimeno *et al*, 1980) (see 14.9); and also patients taking certain medication that decreases serum levels of melatonin, such as calcium-channel or β-antagonists (Cagnacci *et al*, 1998) and statins, whose free radical-mediated toxic effects could be reduced by melatonin (Karasek *et al*, 2002; Cagnacci *et al*, 1998). Patients being weaned off benzodiazepine (BZD) therapy can be administered melatonin to facilitate this process (Wagner *et al*, 1998; Rohr and Herold, 2002).

Due to the widespread effects of melatonin, which will be discussed in 1.3.6, 1.3.8 and 1.3.9, this agent can be used to treat numerous and diverse conditions, such as sepsis and diarrhoea (Gitto *et al*, 2001) as well as asphyxia (Bubenik *et al*, 1998; Fulia *et al*, 2001), in children. Melatonin can also potentially be used as a contraceptive, since the hormone has anti-gonadotropic action when administered in high doses (Suhner and Steffen, 1997).

1.3.3 Sources of melatonin

Melatonin is naturally-occurring in all living organisms (Lynch, 2004), including sea animals and micro-organisms, as well as algae (Hardeland and Poeggeler, 2003) and St John's Wort (Karasek and Reiter, 2002; Murch *et al*, 1997). Its occurrence in high concentrations in the latter (Reiter *et al*, 2001) could contribute to the latter's effectiveness in treating depression (Lynch, 2004). High levels of melatonin are also found in natural products, such as some fruits, notably tomatoes and bananas (Burkhardt *et al*, 2001; Manchester *et al*, 2000), sweetcorn and ginger (Lynch, 2004). Smoking marijuana increases endogenous levels of melatonin (Lissoni *et al*, 1986b), as does the ingestion of melatonin from the abovementioned natural products (Tan *et al*, 2003).

1.3.4 Biosynthesis

Melatonin is a metabolite of the neurotransmitter serotonin (5-hydroxytryptamine, or 5-HT), which in turn has been converted from the amino acid tryptophan. The scheme on the next page outlines the biosynthetic pathway of melatonin (Vijayalaxmi *et al*, 2002; Midlane, 1978; Mefford and Barchas, 1980). The role of acetyltransferases in the development of tumours will be discussed in 1.3.12. As mentioned, tryptophan is essential in the endogenous synthesis of melatonin. This essential amino acid occurs in high levels in various foods, such as watermelon and pumpkin seeds, nuts, tofu and beans (Lynch, 2004). Several vitamins are also important in the biosynthesis of melatonin; of note, are vitamins B_3 and B_6 (Lynch, 2004). Low levels of these vitamins would thus be associated with low levels of melatonin.

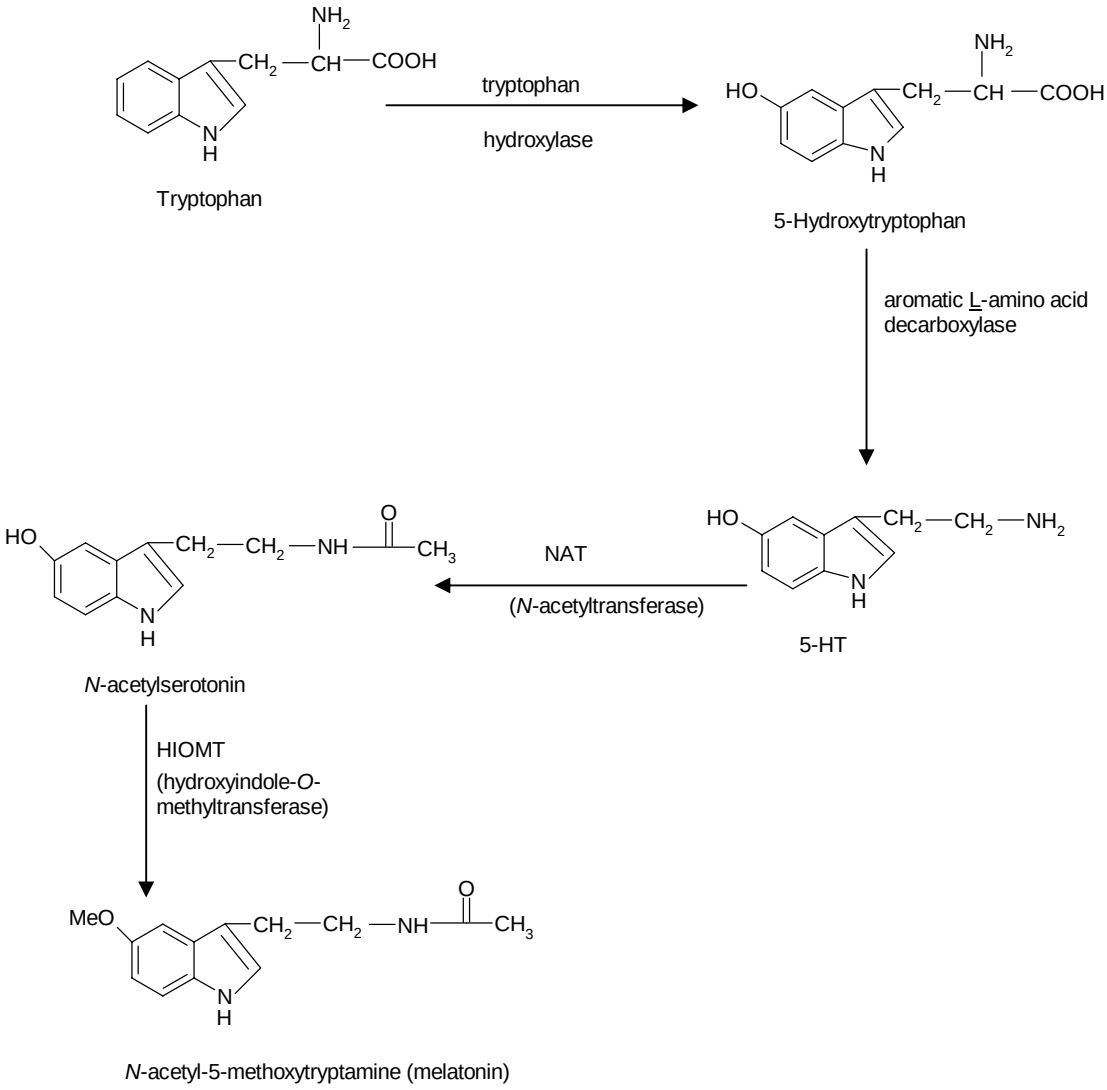


Figure 8: The biosynthetic pathway of melatonin (Vijayalaxmi *et al*, 2002; Midlane, 1978; Mefford and Barchas, 1980)

1.3.5 Metabolism

Melatonin has a short half-life of less than an hour (Reiter and Robinson, 1995) and remains in the bloodstream for only 20-90 minutes (Simonneaux and Ribelayga, 2003; Dawson *et al*, 1996), due to its high lipophilicity and partial hydophilicity, which allow the molecule to cross cell membranes readily and distribute extensively in the body (Kumar, 1996). Melatonin is subjected to extensive first-pass hepatic metabolism and, if exogenous melatonin is ingested orally, there is a reduction in its bioavailability (Lane and Moss, 1985). The normal level of melatonin produced over 24 hours is 28.8 µg, and this is reduced to 12.3 µg/ day in the case of cirrhosis (Lane and Moss, 1985). Melatonin is converted to 6-hydroxymelatonin (6-OHM), its main metabolite, by the cytochrome P450 enzyme system, specifically by the isoenzyme CYP 1A2 (Skene *et al*, 2001). Hepatic microsomes contain the enzyme melatonin

6-hydoxylase, which is NADPH-dependent and which also catalyses the formation of 6-hydroxymelatonin (Skene *et al*, 2001). This metabolite is subsequently subjected to phase II drug metabolism, in which it undergoes sulphate conjugation to form sulphatoxymelatonin (Skene *et al*, 2001); this is more readily eliminated from the body.

It has been found that fluvoxamine, a selective-serotonin reuptake inhibitor (SSRI) used as an antidepressant, increases the serum concentration of melatonin (von Bahr *et al*, 2000). Fluvoxamine is a potent inhibitor of CYP 1A2 and also inhibits CYP 2C19. This indicates that CYP 2C19 is also involved in the hepatic hydroxylation of melatonin (von Bahr *et al*, 2000). The activity of CYP 1A2 is usually measured by determining the clearance of caffeine, which is an inhibitor of the isoenzyme (Ursing *et al*, 2002). High levels of caffeine clearance would thus correspond to low levels of melatonin. However, in this study (Ursing *et al*, 2002), this inverse relationship was not demonstrated. It has been suggested that endogenous levels of melatonin reflect both the amount metabolised by CYP 1A2, as well as the amount secreted by the pineal gland. An increased secretion of the hormone could counteract its increased hepatic metabolism, thus accounting for the lack of an inverse relationship (Ursing *et al*, 2002).

In patients with colorectal cancer, polymorphisms, in particular, linkage disequilibrium, exist in the CYP 1A2 gene (Sachse *et al*, 2003). This has been found to lower the activity of CYP 1A2 (Sachse *et al*, 2003). This has implications for the metabolism of melatonin; since the activity of the main isoenzyme that metabolises it is reduced, the level of melatonin increases. This could account for the favourable effects that are noticed in patients who are being treated with 5-FU for colorectal cancer: there is more melatonin to decrease the toxicity and enhance the efficacy of the antineoplastic.

Melatonin has a local neuromodulatory function in the eye; it is both synthesised (Kvetnoy, 2002) and metabolised in the eye of *Xenopus laevis*. Retinal metabolism proceeds initially by the deacetylation of melatonin, which is catalysed by the enzyme aryl acylamidase and results in the formation of MT (Cahill and Besharse, 1989). This compound is subsequently subjected to deamination by monoamine oxidase (MAO), thereby producing ML and MA (Cahill and Besharse, 1989). The retinal metabolism of melatonin is potentially significant, because 5-FU is a photoreactive drug. If 5-FU accumulates in the eye, the presence of melatonin has the potential to alleviate the phototoxicity induced by the antineoplastic.

It has been established that small amounts (0.3-0.8%) of MT are formed from melatonin in rat liver tissue (Beck and Jonsson, 1981). The enzyme aryl acylamidase is involved, as

mentioned above. MT has a very short half-life of 1 hour, and is rapidly metabolised by MAO. It was noted that the deacetylation of melatonin to 5-MT does not occur in brain tissue, possibly due to a lack of aryl acylamidase in this tissue (Beck and Jonsson, 1981).

The following scheme summarises the abovementioned aspects of melatonin's metabolism:

Figure 9: The metabolism of melatonin (Cahill and Besharse, 1989; Dollery, 1991)

1.3.6 Mechanisms of action in oncology

Whereas melatonin is cytotoxic at pharmacological concentrations, it is cytostatic at physiological concentrations and prevents the proliferation of cancer cells by affecting the cell cycle (Blask *et al*, 2002a). Natarajan *et al* (2001) have found that melatonin inhibits nuclear factor-κB (NF-kB), a regulator found in the nucleus and which is important in transcription. Stimulation of NF-kB controls the expression of various genes involved in the processes of cellular differentiation, cell growth and maintaining the cell cycle. The above authors investigated the effects that melatonin exerts on three functions, namely: (i) the distribution of cell cycle phases, (ii) metabolic activity, and (iii) cell viability. These authors found that, up to a concentration of 2 mM, melatonin had no significant effect on the above functions, although, after 48 hours of melatonin exposure, the proportion of cells in the S phase of the cell cycle was slightly changed. Ninety-eight percent of cells were still viable after continuous exposure to 2 mM of melatonin for 72 hours (Natarajan *et al*, 2001).

1.3.6.1 Oncostatic effects

These are also exerted by melatonin altering protein kinase C activity, oestrogen receptor activity and expression, intracellular structure, calmodulin/ calcium activity and reduction-oxidation reactions (Blask *et al*, 2002a). Melatonin also inhibits various hormones and factors that promote the growth of cancer cells, such as prolactin (Lemus-Wilson *et al*, 1995), oestradiol (Molis *et al*, 1994, 1995) and epidermal growth factor (see later; Cos and Blask, 1994), thereby controlling the proliferation of cells (Blask *et al*, 1999, Tamarkin *et al*, 1981).

Melatonin is a lipohilic and small molecule. It interacts with melatonin receptors, of which there is a diversity: mt1, MT2 and MT3 receptors are found in cell membranes, whereas RZR and ROR receptors are found in the nucleus (Abrial *et al*, 2004). Melatonin receptors are coupled to G-proteins (Maestroni, 2001) and when these are stimulated, various T-helper cell 1 cytokines are released, notably interleukin-2 (IL-2) and interferon γ (INFγ); these undergo immunological reactions with dynorphin B and IL-4 (Maestroni, 1999). INFγ enhances the synthesis and action of NK cells (Abrial *et al*, 2004). The synthesis of IL-1, IL-12 and IL-6 is also increased; IL-1, IL-2 and IL-6 stimulate the development of B and T lymphocytes (Abrial *et al*, 2004), which are important components of the immune system. The production of all these mediators combats immunodeficiency states that result from stress, microbial infection (Maestroni, 2001) or drug therapy (Conti and Maestroni, 1995). These mediators also exert anti-cancer activity through a synergistic relationship with IL-2 (Maestroni, 1999; Conti and Maestroni, 1995).

The neuroendocrine regulation of immunity is influenced by opioid peptides and the secretion of neurohormones by the pineal gland (Barni *et al*, 1988). In a study to determine the effect that altered secretions of neurohormones may have in compromising the immune system's ability to combat cancer, Barni *et al* (1988) have found that the secretion of both the endogenous opioid β-endorphin and melatonin could not be used to predict impaired functioning of the immune system, since no significant relationship was found between the ratio of T-helper and –suppressor cells and melatonin levels.

Melatonin is reported to palliate cancer-related fatigue and cachexia in patients with advanced cancer (Lissoni *et al*, 1996a; Puccio and Nathanson, 1997). The paraneoplastic syndrome of cachexia is manifested by weakness, weight loss, hypofunctioning of the immune system, wasting, fatigue, anorexia and impaired performance. These symptoms do not improve with increased food intake (Puccio and Nathanson, 1997). It was found that while melatonin increases body weight, it does not alter appetite, reduce fatigue or increase physical strength (Anisimov *et al*, 2001). Levels of melatonin in the body increase after eating (Bubenik, 2002), whereas patients with advanced cancer, who suffer from wasting or a loss of appetite, have lower melatonin levels (Grin and Grunberger, 1998; Khoory and Stemme, 1988; Barni *et al*, 1988). This is because melatonin is released in response to eating by the enterochromaffin cells found in the gastrointestinal tract (Bubenik, 2001). Melatonin also stimulates the sympathetic nervous system and may influence feeding habits, thereby altering the rate of metabolism and indirectly regulating body mass (Bartness *et al*, 2002). There are melatonin receptors in adipose tissue cells (Brydon *et al*, 2000) and the activation of these decreases the activity of adenyl cyclase, which results in a decrease in the levels of 3',5'-cAMP (Zwirska-

Korczala *et al*, 2005). There is inhibition of glycogenesis and an increase in blood glucose levels (Kopecky *et al*, 2004). Melatonin also enhances the uptake of glucose by adipocytes (Brydon *et al*, 2000).

Nutritional oncology, whose goals it is "to prevent or reverse the progressive weight loss [observed] in 80 to 90 percent of the oncology patient population at some point in their disease", is becoming increasingly important as clinicians seek a more holistic approach to combating cancer and improving patients' quality of life (Ottery *et al*, 2002). Adequate nutrition is essential for numerous reasons, including the prevention of cachexia, maintaining the competence of the host's immune system and the finding that patients who do not have an optimal nutritional status are more vulnerable to the adverse effects of drug treatment (Ottery *et al*, 2002). 5-FU seems to have been the model chemotherapeutic whose toxicity has been investigated in studies to determine the effects of nutrition on oncotherapy (Bounous *et al*, 1978; Torosian *et al*, 1990; Christensen, 1995; Davis *et al*, 1993).

Melatonin also promotes the stabilisation of metastatic solid neoplasms that are untreatable (Lissoni *et al*, 1996a; Bartsch *et al*, 1995). It is, moreover, oncostatic by promoting the apoptosis of cancer cells, controlling the expression of oncogenes, modulating the immune system mainly by activating IL-2 and inhibiting the generation of free radicals (Bartsch *et al*, 1992; Molis *et al*, 1995; Reiter *et al*, 1997a, 1997b; Lissoni *et al*, 1994a). Melatonin thus enhances the quality of life of cancer patients, particularly those who have advanced cancer and are not responsive to standard chemotherapy (Lissoni *et al*, 1991). It has been found to increase the life span of female mice (Anisimov *et al*, 2001) and slow down various ageing processes, such as the inhibition of oestrous activity. Panzer and Viljoen (1997) have confirmed the oncostatic activity of melatonin, due to its indirect stimulation of the immune system and antiproliferative and antioxidant properties.

Patients with advanced cancer often have endocrine hypofunction of the pineal gland, and this is evidenced in a gradual decrease in the normal physiological increase in melatonin levels during the night (Lissoni *et al*, 1986a; Di Bella *et al*, 1979; Lissoni *et al*, 1989; Sze *et al*, 1993; Lissoni *et al*, 1996).

Insulin-like growth factor I (IGF-I) has been found, *in vitro*, to protect breast cancer cells from apoptosis induced by antineoplastic drugs (Dunn *et al*, 1997) and is thus regarded as a pathogenic factor in this form of cancer. It has been suggested that melatonin could regulate the activity of IGF-I (Cos and Blask, 1994; Lissoni *et al*, 1995c; Kajdaniuk *et al*, 1999), by inhibiting its release or action (Kajdaniuk *et al*, 2000).

Another mechanism of action of melatonin is its interaction with vascular endothelial growth factor (VEGF), a powerfully angiogenic substance. Melatonin has been shown to decrease the secretion of VEGF, thereby controlling the growth of tumours and reducing cell proliferation (Lissoni *et al*, 2001a). Melatonin is consequently anti-angiogenic.

1.3.6.2 13-HODE

An important mechanism by which melatonin prevents tumour cell growth is by blocking the uptake of linoleic acid into the tumour cell; neither melatonin's precursor, *N*-acetylserotonin, nor its metabolite, 6-OHM, demonstrate this ability (Blask *et al*, 1999). Linoleic acid is an essential fatty acid to the tumour cell and is metabolised to 13-hydroxyoctadecadienoic acid (13-HODE). This latter molecule promotes signalling between mitogen-activated protein kinase (MAPK) and the epidermal growth factor receptor (EGFR), as well as the autophosphorylation of EGFR, which result in tumour growth (Blask *et al*, 2002a; Blask *et al*, 1999). The stimulation of G-protein-coupled melatonin receptors results in the inhibition of linoleic acid uptake by blocking the synthesis of cAMP (Blask *et al*, 2002b; Blask *et al*, 1999) through signal transduction processes (Reppert and Weaver, 1995). Inhibition of cAMP synthesis decreases the activity of fatty acid transport protein (FATP) by phosophorylation mediated by a protein kinase. FATP is found on the membrane of tumour cells and facilitates the movement of linoleic acid into the cell (Blask *et al*, 1999). Another mechanism of action of linoleic acid in carcinogenesis is that it promotes cancer cell proliferation by blocking communication across the gap junctions of cells (Hii *et al*, 1995; Hayashi *et al*, 1997); in particular, 13-HODE induces free radical-mediated damage to the connexin molecules that are important in intercellular communication (Hii *et al*, 1995). Melatonin promotes such intercellular communication (Kojima *et al*, 1997; Ubeda *et al*, 1995). This is because the antioxidant, as a very effective scavenger of free radicals (Reiter, 1994, 1997a, 1997b), protects the integrity of the membranes found in gap junctions from free radical damage (Blask *et al*, 1999) induced by various products of the lipid peroxidation of linoleic acid, such as 13-HODE (Glasgow *et al*, 1996; Glasgow *et al*, 1997).

Experiments by Blask *et al* (2002b) show that nocturnal light exposure prevents melatonin secretion, thereby preventing the inhibition of linoleic acid uptake and metabolism to 13-HODE by cancer cells and promoting the progression of cancer. 13-HODE increases the DNA content of tumour cells (Blask *et al*, 1999), and inhibition of this by melatonin would consequently be of great value. Rapidly-proliferating hepatomas overexpress the messenger RNA (mRNA) transcripts necessary for FATP synthesis, thus enabling tumour cells to maintain the uptake of fatty acids, and reducing the efficacy of melatonin (Blask *et al*, 1999). Melatonin has been found to not exert any long-term effects on the genes responsible for the

expression of FATP (Blask *et al*, 1999). The uptake of linoleic acid into tumour cells is greatest during the day, when melatonin levels are at a trough. Similarly, linoleic acid uptake is least at midnight (Blask *et al*, 1999); the uptake of fatty acids has been found to be 60-70% lower and 13-HODE has not been detected at midnight. The latter may also have been due to melatonin inhibiting a lipoxygenase necessary for the synthesis of 13-HODE (Blask *et al*, 1999). These rhythms are not driven by the tumour cells themselves, but regulated passively by the circadian fluctuation of melatonin, evidenced by the fact that removal of the pineal gland experimentally was found to eliminate the variations in tumour linoleic acid uptake and increase the uptake level by a factor of 10-16 times (Blask *et al*, 1999). Pinealectomy doubled the growth rate of tumours in rodents, reduced the latency period that precedes the detection of palpable tumours (Blask *et al*, 1999) and simulated the effect of continuous light exposure on the growth of tumours (Dauchy *et al*, 1997). An antagonist to melatonin receptors (Guardiola-Lemaitre and Delagrange, 1997) reversed the inhibition of fatty acid uptake and metabolism to the mitogen 13-HODE in tumour cells (Blask *et al*, 1999). It has been found that melatonin's oncostatic activity is greater in rodents placed on calorie-restricted diets, as opposed to those who ate freely, thus highlighting the link between melatonin activity and nutrition (Blask *et al*, 1986). A reduction in the energy available to the animal results in a significant decrease in the concentration of linoleic acid in plasma (Sauer *et al*, 1997). The model below illustrates the abovementioned mechanisms (Blask *et al*, 1999).

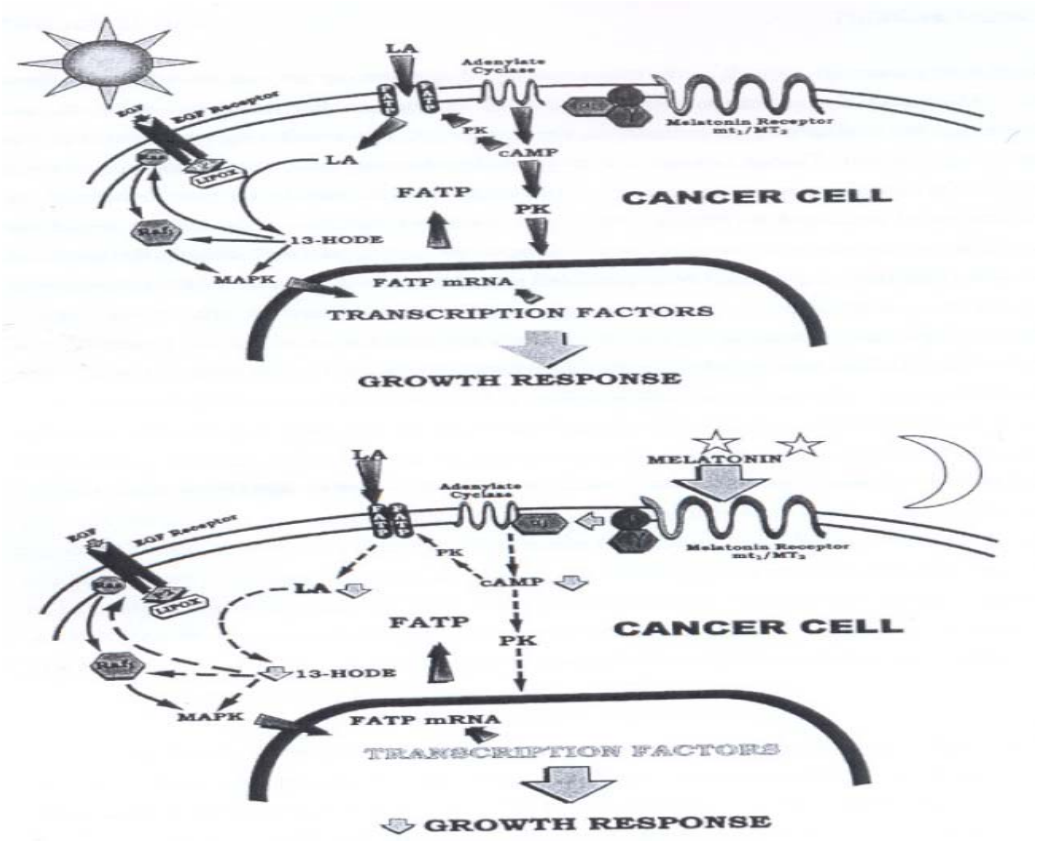


Figure 10: Model of melatonin's inhibition of linoleic acid uptake (Blask *et al*, 1999)

1.3.6.3 Other mechanisms

Melatonin enhances the differentiation of some types of cancer cells and, by altering adhesion molecules, can reduce the potential of these cancer cells to metastasise and be invasive (Blask *et al*, 2002a; Cos *et al*, 1998). Anismov *et al* (1997) studied the effectiveness of melatonin in inhibiting the development of intestinal tumours in rats injected with a known carcinogen, 1,2-dimethylhydrazine. It was found that the rats treated with melatonin do not develop any tumours of the small intestine, and that the incidence of colon tumours, including those that are solely mucosal, as well as the mean number of tumours per rat is much lower in these rats. Melatonin also decreases the incidence of tumours in the ileum and jejunum, and decreases the multiplicity of tumours in the duodenum. The number of small tumours increases, but that of larger tumours decreases in the presence of melatonin (Anisimov *et al*, 1997b). Furthermore, long-term administration of melatonin to the rats decreases the infiltration of lymphoid molecules into colonic mucosa (Anisimov *et al*, 2000a). Melatonin was thus concluded to be an effective inhibitor of the carcinogen 1, 2-dimethylhydrazine in rats; this inhibitory activity has been attributed to melatonin's suppression of the mitotic index of tumour cells, and its promotion of apoptosis (Anisimov *et al*, 2000a). 1, 2-Dimethylhydrazine decreases the number of melatonin-containing cells in the gastrointestinal tract, and this effect is inhibited by the administration of exogenous melatonin (Anisimov *et al*, 1999).

In a subsequent study (Kossoy *et al*, 2000), the spleens of these rats were histologically examined; the carcinogenesis induced by 1, 2-dimethylhydrazine results in significant changes to splenic tissue: the zone of red pulp increases and that of white pulp decreases. This indicates an inadequate immune response to the carcinogen. The administration of melatonin reverses these splenic changes, rapidly increases the density of CD8+ lymphocytes and expands the aforementioned zones by over 100%. Melatonin thus activates components of the lymphatic system, and this results in anti-carcinogenic activity (Kossoy *et al*, 2000). This is in contrast to an earlier study by Lissoni *et al* (1988), which concludes that melatonin does not alter the functioning of lymphocytes in cancer, since the mean percentages of various lymphocytes as well as the mean ratio of T-helper and –suppressor cells (i.e. the T4/ T8 ratio) are not significantly altered, and the cancer progressively worsens. There was, however, a perceived improvement in patients' quality of life (Lissoni *et al*, 1988).

Bartsch and Bartsch (1997) have suggested that melatonin is not the only pineal hormone with antineoplastic action, because when a pineal extract with unknown compounds is administered to tumour cells resistant to melatonin, inhibition of tumour growth occurs.

1.3.7 Circadian fluctuations

Melatonin also undergoes circadian fluctuations, and is thus a chronoimmunomodulator (Ronco and Halberg, 1996). The hormone transmits the circadian rhythm to other organs in the body in order to ensure that physiological processes continue and are regulated (Stehle *et al*, 2003). The effects of this melatonin rhythm on the circadian rhythm of locomotor activity are discussed in 11.1 and 11.2. Endogenous melatonin levels increase in the late evening, and a peak occurs at around midnight; melatonin levels decrease towards morning and are at a trough during the day (Wincor, 1998). Exposure to light at night results in an immediate and significant decrease in physiological levels of melatonin, in all species (Redlin, 2001). It has been suggested that the fluctuating activity of melatonin over time might be due to circadian variations in the activity of melatonin$_1$ and melatonin$_2$ receptors (Akagi *et al*, 2004) (see 1.2.9.1). These circadian fluctuations of melatonin may imply that the pharmacological activity of melatonin varies between the seasons (Maestroni, 2001). Certain drug treatments may have the potential to alter the circadian rhythms of melatonin, thereby affecting its activity (Ronco and Halberg, 1996). Melatonin's circadian variations are very significant and the compound is the only known chronobiological hormone that regulates the growth of neoplastic cells (Blask *et al*, 2002a). Supraphysiological doses of the hormone are necessary to alter the circadian rhythm of tumours (Deacon and Arendt, 1995). Ronco and Halberg (1996) note that the timing of melatonin administration determines whether it enhances or inhibits oncogenesis. In cancer patients, the circadian rhythms of melatonin are disrupted (Abrial *et al*, 2004). The converse is also true, as depressed melatonin levels are associated with the development of spontaneous tumours in the mammary gland, in humans and rats (Anderson *et al*, 2000; Cos *et al*, 1998; Blask *et al*, 1999).

Women working night shifts, for example nurses, are at an increased risk of developing a variety of cancers, in particular rectal, colon or breast carcinomas (http://www.cpaaindia.org/infocentre/clipping_co.htm#top, Nurses' night shifts linked with colon cancer, *Reuters* 03/ 06/ 2003; Schernhammer *et al*, 2003). This is because melatonin levels are depressed due to the presence of artificial light and their not being asleep at night. To a lesser extent, the decreased melatonin levels also tilt the hormonal balance in favour of oestrogen, which has the potential to exert carcinogenic effects (http://www.cpaaindia.org/infocentre/clipping_co.htm#top, Nurses' night shifts linked with colon cancer, *Reuters* 03/ 06/ 2003; Canaple *et al*, 2003).

Lissoni *et al* (1996c) investigated the effects of ML on the serum levels of IL-2, which stimulates the immune system, and IL-6, which is an immunosuppressive cytokine; and showed conclusively that the levels of the former are increased and the latter decrease in the

presence of melatonin. Since ML has an opposite circadian rhythm to melatonin; i.e. its levels peak during the day and reach a nadir at midnight; the authors suggest that the compound should be used together with melatonin, since both agents would be able to both exert immunostimulatory effects at different times of the day, thus potentiating the host's anticancer mechanisms (Lissoni *et al*, 1996c).

In the holistic management of cancer, certain lifestyle or spiritual decisions are important and may impact on physiological processes. It has been found that patients who meditate on a regular basis, for example, have increased melatonin levels (Massion *et al*, 1995; Coker, 1999). These increased melatonin levels are associated with cancer regression (Meares, 1976, 1979, 1980), giving credence to the belief of the power of "mind over matter"!

1.3.8 Antioxidant and free radical-scavenging properties

Melatonin is the most potent natural antioxidant (Reiter *et al*, 1997a, 1997b) and a direct free radical scavenger. It enhances the efficacy and reduces the toxicity of many drugs (Reiter *et al*, 2002). It also stimulates the immune system (Guerrero and Reiter, 1992, 2002), strengthens circadian rhythms (Arendt, 1988) and inhibits cancer initiation (Reiter, 1999) and the growth of tumours (Black *et al*, 1991) by promoting the apoptosis of tumour cells (Lissoni *et al*, 1999a). The hydroxyl radical (HO·) is the most destructive free radical; melatonin is amphiphilic and can thus enter both aqueous and lipid compartments to reduce the damage caused by this free radical (Reiter *et al*, 2002). It has been shown that melatonin is very effective at preventing the DNA damage that results from the HO·, which is generated by ultraviolet light exposure, the Fenton reaction (Yamamoto and Mohanan, 2001a) (see 8.3) as well as by glutathione and toxins, such as cyanide (Yamamoto and Mohanan, 2001b). Melatonin is a very potent scavenger of peroxyl and HO· radicals (Reiter *et al*, 1997a, 1997b). It can defend the chaotic microenvironment within cells from oxidative damage (Karbownik and Reiter, 2002), due to it being an ampiphile, antioxidant (Fowler *et al*, 2003), scavenger of free radicals (Anisimov, 2002) and chelator of metal ions (Antunes *et al*, 1999) (see 8.3).

Melatonin is metabolised in the liver to 6-OHM (Reiter, 1991) (see 1.3.5), which also scavenges free radicals (Pierrefiche *et al*, 1993). Melatonin and some of its metabolites thus exert a cascade-like antioxidant effect (Tan *et al*, 2000b). Melatonin has various mechanisms by which it protects cells against drug toxicity. One of these is its ability to preserve the fluidity of cell membranes (Garcia *et al*, 1997, 1998) by reducing the peroxidation of polyunsaturated fatty acids in the membrane. Melatonin also positions itself within membranes, thus limiting damage to the polyunsaturated fatty acids by toxic substances (Tesoriere *et al*, 1999) and increasing the rigidity of the membrane (Garcia *et al*, 1998).

Melatonin may have intrinsic tumour-inhibiting action, which may be due to its inhibition of cell proliferation (Reiter *et al*, 2002).

There is a school of thought which holds that antioxidants should not be given in drug regimens that make use of a free radical mechanism to destroy cancer cells (Prasad *et al*, 1999), since the efficacy of the latter against cancer cells might be expected to be decreased in the presence of antioxidants (Lamson and Brignall, 1999). It has, however, been found that much damage inflicted on cancer cells is actually by mechanisms other than those induced by free radicals; these other mechanisms result in apoptosis (Schmitt and Lowe, 1999), and antioxidants increase the number of cancer cells thus destroyed (Chinery *et al*, 1997; Mediavilla *et al*, 1999). Lamson and Brignall (1999) consequently conclude that while this may be a rational theoretical concern, it is "too simplistic" to assume that antioxidants interfere negatively with the efficacy of anticancer drug therapy, given the complexity and multiplicity of mechanisms of tumour cell damage (see 1.3.9).

Many cancer cells have impaired defence mechanisms, which makes them less able to utilise antioxidants for the latter's repair properties. There may be a deficiency of catalase in most cancer cells (Oberley and Oberley, 1997). This renders the cells unable to degrade the harmful hydrogen peroxide (H_2O_2) that accumulates in the presence of vitamin C (Benade *et al*, 1969).

It has been suggested that combinations of antioxidants might be particularly effective in cancer treatment, especially since many have intrinsic anticancer activity (Lamson and Brignall, 1999). There is a synergistic relationship between α-tocopherol and β-carotene (Shklar *et al*, 1989). Combinations of melatonin and other antioxidants or vitamins might well be useful. An especially pertinent consideration, in the context of this research, is the finding by Teicher *et al* (1994) that there is a drug-drug interaction between 5-FU and β-carotene. Although the mechanism of interaction is yet to be elucidated, it appears that the antioxidant decreases the efficacy of 5-FU.

1.3.9 Melatonin as adjuvant therapy

Lissoni *et al* (1999a) have extensively studied the effect of melatonin as adjuvant therapy in patients with various forms of advanced cancer, and who were already receiving chemotherapy. These authors found that when melatonin is added to a regimen of 5-FU and folinic acid in patients with gastrointestinal tumours, "the objective tumour regression rate, as well as the one-year survival rate, was improved […] and, moreover, melatonin significantly reduced the frequency of thrombocytopenia, neurotoxicity, cardiotoxicity, stomatitis and

asthenia" (Lissoni *et al*, 1999a). In another study by Lissoni *et al* (1999b), melatonin was furthermore found to counter thrombocytopaenia in cancer patients by increasing the number of circulating platelets. This was suggested to be through altering cytokine functioning, in particular, by enhancing the production of IL-6, which promotes the synthesis of platelets (Abrial *et al*, 2004), and increasing the budding off of platelets from megakaryocytes (Lissoni *et al*, 1999c). Melatonin increased the number of platelets significantly, and 72% of patients achieved normal levels, with no adverse effects to melatonin (Lissoni *et al*, 1999c). Melatonin's effects on platelets may contribute to its ability to enhance the healing of wounds (Maestroni, 1999). Thrombocytopaenia due to disseminated intravascular coagulation is the most difficult to treat (Lissoni *et al*, 1999c). Lissoni *et al*. (1999c) thus concluded that melatonin is a safe and effective means of treating thrombocytopaenia caused by various pathologies.

The differentiation and proliferation of haematopoietic cells is under the control of the neuroendocrine system, and has been shown to vary in a circadian fashion. Melatonin mediates the influence of lighting conditions and the neuroendocrine system on haematopoiesis (Lissoni *et al*, 1996b), and influences the growth of red blood cells by affecting apoptosis-related processes. Melatonin therapy is thus very useful in patients who have various disorders related to blood cells (Lissoni *et al*, 1996b). It is postulated that melatonin possesses various mechanisms of action in enhancing haematopoiesis, such as influencing monocytes, pre-B cells and NK cells (Maestroni, 2001). These are progenitors that eventually result in the formation of erythrocytes. Another suggested mechanism of action is by increasing the activity of cytokines, such as melatonin-induced opioids, that have a specific haematopoietic function by stimulating the κ_1 opioid receptors found on the macrophages in bone marrow (Maestroni, 1999, 2001). Maestroni (2001) suggests that melatonin's role in increasing haematopoiesis may be used advantageously to reduce the haematological toxicity of various chemotherapeutics.

Besides being thrombopoietic (Maestroni *et al*, 1994; Lissoni *et al*, 1995), melatonin is neurotrophic (Mayo *et al*, 1998). The increased survival time in patients with poor clinical status could be due to melatonin preventing 5-FU-induced immunosuppression (Maestroni, 1993); the antioxidant property of melatonin may also decrease the cytotoxicity of the chemotherapy. Melatonin's potent free radical-scavenging ability is believed to account for it attenuating 5-FU's toxicity to non-tumour cells (Lissoni *et al*, 1997a); the heart is especially vulnerable to damage by free radicals and melatonin's scavenging properties might account for the reduced incidence of antineoplastic-induced cardiotoxicity mentioned above. It has been found that melatonin reduces the lipid peroxidation induced by the cardiotoxic agent

doxorubicin and improves cardiac function (Xu *et al*, 2001). As mentioned in 1.2.10.5, however, Cwikiel (1996) reports that 5-FU-induced cardiotoxicity is not due to the formation of free radicals or lipid peroxidation. The mechanisms underlying this cardiotoxicity, and melatonin's ability to counter this, thus remain unclear. Melatonin also exhibits anti-inflammatory action (Lissoni, 2002); it could thus prevent or minimise the inflammatory processes that accompany cytotoxicity. These anti-inflammatory properties could contribute to melatonin's ability to heal wounds (Lissoni *et al*, 1995d; Lissoni *et al*, 1997c). By being a free-radical scavenger, down-regulating inflammatory mediators such as TNFα and certain cytokines (Reiter *et al*, 2000; Perianayagam *et al*, 2005) and decreasing the cellular damage that results from a lack of oxygen (Reiter *et al*, 2003), melatonin limits the damage that is caused in inflammatory processes (Reiter *et al*, 2000). The anti-inflammatory effects of melatonin allow it to inhibit surgery-induced immunosuppression (Lissoni *et al*, 1995d). Lissoni *et al* (1999a) have suggested that further studies are necessary to determine whether the hormone's circadian rhythm influences its beneficial effects in cancer. The following diagram illustrates the effect of melatonin, in combination with conventional cytotoxic therapy, on the survival rate of patients with advanced cancer (Lissoni *et al*, 1999a): As shown, melatonin significantly increases survival time.

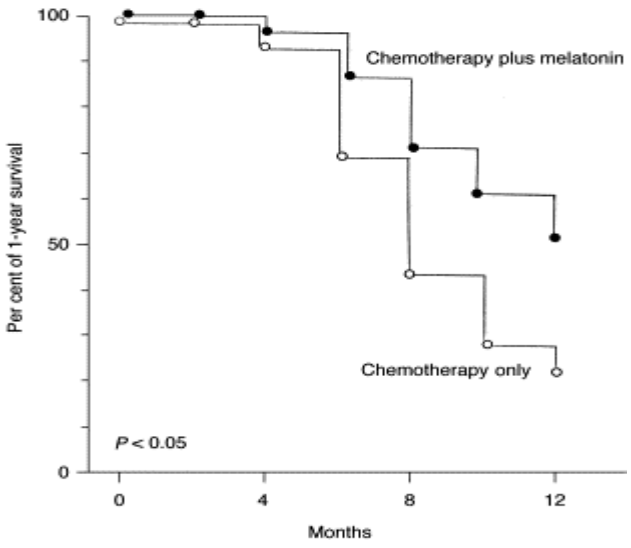


Figure 11: The effect of melatonin as adjuvant therapy to patients treated with 5-FU (Lissoni *et al*, 1999a)

Vijayalaxmi *et al* (2002) have concluded that melatonin's ability to decrease the anxiety and enhance the performance status of patients is a very useful finding in the clinical setting.

Theoretically, since melatonin reduces the toxicity of 5-FU, the dose of the latter could perhaps be increased, thereby enhancing its efficacy (Reiter *et al*, 2002). This point is

contentious, though, due to the low therapeutic index of 5-FU. Alternatively, the increased potency of 5-FU when administered with melatonin could call for a dose reduction of 5-FU, and thus limit adverse effects even further. It should be noted that co-administration of melatonin does not decrease the incidence of alopecia, vomiting, nausea and diarrhoea (Lissoni *et al*, 1999a). Such symptoms may decrease melatonin levels in the body, by simulating a fasting state (Lynch, 2004), which is known to reduce melatonin levels within two days (Bogdan *et al*, 2001).

There may be altered circadian rhythms in the healthy and tumour tissues of cancer patients. After these patients were administered 5-FU, plasma melatonin concentrations showed significant 24 hour rhythms (Mormont *et al*, 2002). The researchers concluded that the rest/ activity status of the patient, and the melatonin rhythms, reflect different aspects of the circadian system and that these may be modified in the presence of cancer. It has been found, for example, that patients with breast cancer show a reduced nocturnal secretion of melatonin (Bartsch *et al*, 1997).

In patients with metastatic colorectal cancer resistant to 5-FU and folates, co-treatment with melatonin was found to improve the patients' quality of life, even though no antitumour activity was demonstrated by melatonin (Barni *et al*, 1990). These positive effects of melatonin may be attributed to its ability to enhance the activity of IL-2 (Barni *et al*, 1995). IL-2 has important anti-cancer activity, usually at high doses that are toxic. Melatonin has been found to increase the ability of IL-2 to induce tumour regression at much lower doses, and thereby increase patient survival rates (Barni *et al*, 1995). It also protects the patient against any adverse effects of IL-2 (Conti and Maestroni, 1995). IL-2, in turn, by enhancing the activity of the immune system, creates an optimum environment for the functioning of melatonin (Maestroni, 1999). The blood concentrations of IL-2 vary in a circadian fashion, with a peak secretion occurring at night; this may, in part, be due to the presence of increased concentrations of melatonin, as the pineal gland has been found to stimulate the endogenous synthesis of IL-2 (Lissoni *et al*, 1998). Advanced neoplasms that do not respond to IL-2 monotherapy have been found to become responsive to IL-2 if melatonin is also administered to the patient; melatonin thus enhances the susceptibility of cancer cells to the effects of IL-2 (Lissoni *et al*, 1994b). This synergistic relationship is emphasised by the suggestion that the simultaneous presence of IL-2 is required in order for melatonin to exert stimulatory activity on the immune system (Lissoni *et al*, 1993).

Although IL-2 is generally immunostimulatory, it does induce immunosuppression as well, through effects mediated by T-helper 2 lymphocytes and macrophages. The main cytokine

that T-helper 2 lymphocytes release that has an immunosuppressive function is IL-10; IL-2 thus enhances the release of this factor (Lissoni *et al*, 1995c). It was found that the administration of IL-12 significantly decreases the IL-2-mediated release of IL-10, and that this inhibition is enhanced in the presence of melatonin. It can thus be concluded that combining IL-12 with melatonin and IL-2 adjunctive therapy will reduce IL-2-induced immunosuppression (Lissoni *et al*, 1995c). The immunosuppressive effect of IL-2 may also be due to this cytokine inducing cortisol secretion (see 12.3.2), thus showing the interaction between the neuroendocrine and immune systems (Lissoni *et al*, 2005a).

It has been suggested that the aforementioned positive effects of IL-2 and melatonin would allow this combination to become effective second-line therapy to metastatic colorectal tumours resistant to 5-FU plus folates; this combination is currently the first-line treatment option (Barni *et al*, 1995). Moreover, it has been found that IL-2 administration increases 5-FU efficacy in patients with colorectal cancer who also exhibit lymphocytopaenia (Lissoni *et al*, 2005b). An in-depth study by Barni *et al* (1992) established that IL-2 plus melatonin achieves tumour stabilisation, and not objective tumour regression. These authors state that whilst the combination neuroimmunotherapy expands the number and type of immune cells activated to mount an antitumour response, by increasing the titre of TNF, eosinophils and lymphocytes, there is no significant tumour regression in patients exposed to treatment with 5-FU (Barni *et al*, 1992).

This implies that neuroimmunotherapy is effective solely as an adjunctive and palliative measure and does not supercede 5-FU therapy. Another advantage of using melatonin with IL-2 is that the combination counters the lymphocytopaenia that results from surgery (Lissoni *et al*, 1995d). It is known that IL-2, as well as TNFα, used in cancer palliation treatment, induce hypotension through stimulating the synthesis of NO, a vasodilator. Melatonin significantly reduces the incidence of cytokine-induced hypotension and thus decreases adverse effects on the cardiovascular system (Lissoni *et al*, 1996e). In an early study, Barni *et al* (1987), investigating the effect that cytotoxic therapy has on the endocrine system, found that CMF (see 1.2.8.7) slightly enhances melatonin levels. More research is needed to assess the clinical significance and extent of this interaction.

In another study that did not involve 5-FU, but nevertheless emphasised the role of melatonin in adjunctive therapy with chemotherapeutics, Lissoni *et al* (1997b) found that melatonin is useful in overcoming the resistance to luteinising-hormone releasing hormone (LHRH) analogues that some prostate cancers develop. Melatonin was found to enhance the efficacy of triptorelin, evidenced by reduced serum levels of prostate-specific antigen, high levels of

which are associated with prostate cancer (Lissoni *et al*, 1997b). Long-term oral administration of 5 mg of melatonin per day resulted in a six-week stabilisation of metastatic prostate cancer resistant to hormones (Shiu *et al*, 2003). A study into the effectiveness of using melatonin with tamoxifen in treating patients with metastatic solid tumours has found that the hormone improves the performance levels of the patients, as well as controls the proliferation of cancer cells (Lissoni *et al*, 1996f). In addition, Panzer (1997) suggests that decreased melatonin levels, together with the increased proliferation of bone marrow cells during puberty, predispose to the development of osteosarcoma, and that the administration of exogenous melatonin might be of value in the adjunctive treatment of this disease (Panzer, 1997).

In patients with malignant melanoma and relapsed lymph nodes, melatonin increases the number of patients who have a disease-free survival time, and also prolongs this period significantly (Lissoni *et al*, 1996d). The authors suggest that this could be a basis for using melatonin as adjunctive therapy in melanoma. This may be particularly relevant with regard to 5-FU, since the latter is often used to treat various forms of skin cancer (see 1.2.8.8). In another study, it was found that patients who were taking melatonin live for longer than patients not on this adjunctive therapy, and experience no toxicity (Lissoni *et al*, 1992).

The effect of melatonin as adjuvant therapy with radiation was also explored in a small study: after one year, a significantly higher proportion of patients who had been taking melatonin as well as radiotherapy were still alive (Lissoni *et al*, 1996g). A suggested reason for melatonin's synergistic effect with radiation therapy (Lamson and Brignall, 1999), and which also applies to drug treatment, is that chemotherapy and radiation therapy cause relatively little damage to tumour cell DNA, and thus, instead of precipitating necrosis, cause apoptosis (Schmitt and Lowe, 1999). Antioxidants, such as melatonin, enhance apoptosis (Chinery *et al*, 1997; Mediavilla *et al*, 1999) and thus increase the efficacy of radiation and chemotherapy. Most tumours are hypoxic due to poor perfusion (Vaupel *et al*, 2001) as well as inconsistency of blood flow through the tumour (Hill *et al*, 1996), and there is anaemia, and thus low oxygen levels, in many cancer patients (Auclerc *et al*, 2003; Thomas, 2002). The effectiveness of radiation therapy is reduced because this requires the presence of oxygen, which generates cytotoxic free radicals (Vaupel *et al*, 2001; Lynch. 2004). In addition, radiation therapy results in anaemia (Harrison *et al*, 2002).

By enhancing thrombopoiesis (Lissoni *et al*, 2001b), melatonin is of value in the treatment of cancer patients who have anaemia and a deficiency of blood platelets (Lissoni *et al*, 1997b). The neurotransmitter 5-HT, which is also a precursor of melatonin, reduces blood flow and

melatonin, by exhibiting anti-serotonergic properties, counters this (Bubenik, 2002), thereby improving blood flow to and within tumours (Vaupel and Hockel, 2000) as well as decreasing the likelihood of cerebral ischaemia resulting from hypoperfusion of the brain (Delagrange *et al*, 2003). The increased blood flow results in an increased delivery of oxygen to the tumour cells and thus the increased efficacy of radiation therapy (Hockel *et al*, 1996). It is suggested that melatonin's lipophilicity allows it to cross into the tumour cell membrane from the blood (Bubenik *et al*, 1998), thereby further enhancing the effectiveness of both chemo- and radiation therapy (Lynch, 2004).

Melatonin thus has numerous benefits in radiation therapy. Besides increasing the life-span of the patient (Blask *et al*, 2002), melatonin also treats many of the adverse effects of radiation therapy (Jatoi and Thomas, 2002), such as neurotoxicity, cardiotoxicity, algesia, sleep disorders, immunosuppression and anaemia (Lissoni *et al*, 1996g). Melatonin furthermore promotes the repair of tissues that have been damaged by radiation (Sorenson, 2002). It has been mentioned that melatonin levels in the gastrointestinal tract are 400 times greater than the quantity released by the pineal gland (Bubenik, 2002) (see 1.3.1); radiation therapy often causes mucositis in the gastrointestinal tract, which significantly decreases the level of melatonin present (Karbownik and Reiter, 2000). The use of exogenous melatonin thus counters this decrease.

The general conclusion with regard to the use of melatonin as adjunctive therapy is that chronobiology improves the outcomes that patients on cancer chemotherapy achieve (Mormont and Levi, 2003; Levi, 2001). There may also be additional positive outcomes that may result from melatonin use and which may greatly enhance the patient's quality of life, such as the alleviation of depression or anxiety (Lissoni *et al*, 1995b). Melatonin also has intrinsic analgesic activity (Shaji and Kulkarni, 1998) and thus reduces the pain from which cancer patients suffer (Raghavendra *et al*, 2000). The hormone has the ability to counter the hyperalgesia that occurs with the inflammation that is present when wounds heal (Raghavendra *et al*, 2000). Since an estimated half of the number of cancer patients die due to poor symptom management, the abovementioned positive outcomes should not be underestimated (Bruera and Neumann, 1998). The use of melatonin is especially recommended when very toxic chemotherapeutics, such as 5-FU, are used (Lissoni *et al*, 1997a; Kajdaniuk *et al*, 2001).

1.3.10 Doses and administration

A dose of 10-50 mg of melatonin, administered at night, is beneficial to patients suffering from cancer, especially if the cancer is untreatable or advanced (Lynch, 2004). If a cancer is

at an early stage of development, or if it has a slow growth rate, it may be necessary to give nocturnal melatonin supplementation of 3-6 mg; the higher dose may be more appropriate for patients who also have disruptions in sleeping patterns (Lynch, 2004).

Melatonin should be administered 30-60 minutes prior to sleeping at night (Lynch, 2004). Since the oral bioavailability of melatonin is low (approximately 15%), sustained-release formulations may be useful for the treatment of insomnia (Hartter *et al*, 2001). It should be remembered by the patient that any nocturnal exposure to light may completely or partially decrease endogenous serum concentrations of melatonin (Brainard *et al*, 1997).

1.3.11 Geroprotection

In an in-depth review article by Anisimov (2001), melatonin has been identified as a geroprotector. Due to the advances of modern medicine and the health-care benefits to which numerous people in developed countries have access, the proportion of elderly people in the population of many countries is increasing. There has thus been an increase in the incidence of diseases known to be associated with advanced age, such as neurodegenerative diseases and cancer (Anisimov, 2001). Various geroprotectors are reviewed in this article, and fall into three categories: (i) those that do not alter the maximum life-span, but increase short-lived peoples' survival rate; (ii) those that reduce the ageing and mortality rates in long-lived people and prolong their life-span; and (iii) those that increase all members of the population's life-span by delaying the commencement of the ageing process (Anisimov, 2001). Antioxidants, including melatonin, fall into the third category (Anisimov, 2001). Geroprotectors are very important in preventing or delaying the development of cancer, and reducing its associated morbidity and mortality.

Melatonin therapy is known to be beneficial in treating patients with Alzheimer's disease (Brusco *et al*, 2000; Asayama *et al*, 2003; Wu and Swaab, 2005), a neurodegenerative disease associated with old age, but there are conflicting reports about melatonin's effects on life-extension. Much of the contention is because many of the reported studies are invalid and it is thus difficult to make credible conclusions (Anisimov *et al*, 2000b).

Anisimov (2001) argues that there is no essential disagreement on whether melatonin has carcinogenic or anticarcinogenic properties. He cites the example of α-tocopherol, an antioxidant that can cause tumours and yet has powerful anticancer and geroprotective properties as well. It is evident that more research is needed before melatonin can be recommended with certainty for use as a geroprotector. It has been found that the pineal gland releases certain peptides (Anisimov, 1998; Yuwiler and Brammer, 1993), such as in the

preparation Epithalamin®, that prolong life-span, inhibit the development of tumours (Anisimov *et al*, 1982, 1989, 1994, 1997a, 1998a) and are useful for treating cancer (Khavinson *et al*, 1998, 1999; Morozov and Khavinson, 1997a, 1997b). Epithalamin® stimulates the pineal gland to synthesise and release melatonin at night (Anisimov *et al*, 1992).

There is less melatonin secreted with advancing age (Reiter, 1995; Touitou *et al*, 1997; Waldhauzer *et al*, 1998) and, due to the oncostatic and other anti-cancer properties of melatonin, this would contribute to the development of an environment conducive to the growth of cancer cells. Furthermore, reduced melatonin secretion would imply a reduced life-span, since pinealectomy was found to decrease the latter (Malm *et al*, 1959; Reiter *et al*, 1999).

A study by Anisimov *et al* (2000b) has found, however, that prolonged nocturnal administration of melatonin increases the incidence of malignant tumours in mice, as well as increasing survival rates. The latter is postulated to occur through a temperature-related mechanism (Anisimov, 2001): melatonin reduces body temperature (Lane *et al*, 1996; Dawson *et al*, 1996), which reduces the rates of various metabolic reactions, thus prolonging life (Lane *et al*, 1996). Melatonin has been found, however, to not be mutagenic by the famous Ames Test (Musatov *et al*, 1998) and prevents the mutagenesis that results from exposure to ionising radiation and substances that exert a cytostatic effect (Vijalaxmi *et al*, 1999; Musatov *et al*, 1997). It is also suggested that the melatonin metabolite, 6-OHM, may be responsible for any possible carcinogenesis, due to its ability to generate HO· and induce DNA damage (see 8.3) (Sakano *et al*, 2004).

Ageing itself results in variations of rhythms within the body (Yamazaki *et al*, 2002). Since ageing is also associated with decreased melatonin levels, this could explain why elderly people are more likely to develop cancers, thus emphasising the importance of geroprotectors. It has been found that elderly men have lower melatonin levels than elderly women, and it is suggested (Lynch, 2004) that this may be one of the reasons why women tend to live longer than men.

1.3.12 Caution

1.3.12.1 Effects on cell proliferation

It is important to be cautious when administering melatonin with 5-FU, and not to use the combination indiscriminately for all types of cancer. This note of caution was sounded by

Furuya *et al* (1994). These authors found that in oestrogen-sensitive human breast cancer cells (MCF7), melatonin exerts a maximum inhibition of tumour growth at 02h00. Blask *et al* (1999) have postulated that's melatonin's ability to inhibit the growth of MCF7 cells is due to it blocking the tumour uptake of linoleic acid, and its subsequent metabolism to 13-HODE. The latter compound promotes mitogenesis (Glasgow *et al*, 1996, 1997; Cos and Blask, 1994). Melatonin inhibits the proliferation of MCF7 cancer cells by up to 60-78% (Hill and Blask, 1988). The antiproliferative effects of melatonin are also attributed to its ability to inhibit DNA synthesis in MCF7 breast cancer cells by inhibiting the incorporation of deoxythymidine nucleotides into the growing DNA strands (Cos *et al*, 1996a). Melatonin affects the cell cycle, by delaying the time that most of the cells enter into the mitotic phase (Cos *et al*, 1996b).

Melatonin induces significant structural changes in MCF7 cells, such as swelling of the nucleus, decreasing the number of microvilli on cell membranes, promoting the shedding of ribosomes and cytoplasm, the formation of vacuoles that are autophages, interference with mitochondrial function and the formation of vesicles from smooth endoplasmic reticulum. These morphological changes are reversible (Hill and Blask, 1988).

Blask *et al* (1997) have demonstrated that the mechanism of melatonin's action in inhibiting the proliferation of MCF7 breast cancer cells involves glutathione as a key component, since the oncostatic effects of melatonin are blocked by inhibiting the rate-limiting enzyme in glutathione production, namely, γ-glutamylcysteine synthetase. Inhibiting this enzyme with L-buthionine-[S,R]-sulphoximine decreases the availability of glutathione to mediate the effects of melatonin (Blask *et al*, 1997). Similarly, inhibiting the metabolism of glutathione by using ethacrynic acid, which inhibits glutathione S-transferase, has been shown to be effective, as adjuvant therapy with melatonin, to inhibit tumour cell growth. Blask *et al* (1997) have further found that MCF7 cells, which are oestrogen-receptor positive cells, metabolise glutathione in a different manner to non-tumour cells.

Melatonin differs from the conventional anti-oestrogens, for example tamoxifen, because it does not prevent oestradiol interacting with its receptor, or bind to this receptor itself (Sanchez-Barcelo *et al*, 2003). Its mechanism of action is postulated to not involve cAMP mediation (Torres-Farfan *et al*, 2003). Melatonin has been found to slow down the rate of breast cancer development (Subramanian and Kothari, 1991) and effectively decreases the development of mammary tumours already present (Rao *et al*, 2000). However, the abovementioned oncostatic effect of melatonin is actually diminished by the co-administration of any amount of 5-FU (Furuya *et al*, 1994). This negative interaction between

5-FU and melatonin is perhaps due to the particular type of tumour cells involved. In this case, further research is needed to elucidate the relative potency and toxicity of the 5-FU-melatonin combination against the many different forms of cancer. Miles and Philbrick (1987) have noted that the administration of chemotherapy to cancer patients reduces melatonin levels significantly.

Barni *et al* (1989) investigated the significance of blood concentrations of melatonin in breast cancer, the normal blood concentration of melatonin being 0.1-10 nmol/ L (Bubenik, 2001), and found that serum concentrations of melatonin are highest in those patients who had the best prognosis. These were patients with node-negative or positive oestrogen receptor breast cancers. The authors thus concluded that melatonin influences the hormone-responsiveness of breast cancer (Barni *et al*, 1989). Falkson *et al* (1990) conclude that increased plasma melatonin concentrations are significantly linked to reduced survival times for patients, and that patients with more advanced breast cancer have higher plasma melatonin concentrations. During the primary tumour growth of various cancers, it has been shown that melatonin levels are reduced, and that these levels only normalise or increase during relapses or in the early stages of tumour growth (Bartsch *et al*, 2002).

The concentration of melatonin and the type of cells to which it is administered appear to be the main determinants of whether the hormone enhances or inhibits cell proliferation (Sainz *et al*, 2003). While melatonin inhibits the proliferation of various types of cancer cells both *in vitro* and *in vivo* (Blask *et al*, 1999; Roth *et al*, 2001), it has been found to promote the proliferation of splenocytes (Nakade *et al*, 1999) and human bone cells (Drazen *et al*, 2001; Zwirska-Korczala *et al*, 2005).

A novel suggestion as to the variations in melatonin levels in tumours of different ages is reviewed by Lynch (2004), who mentions that since humans who age have lower melatonin levels (Schulman and Lunenfeld, 2002; Karasek and Reiter, 2002), the same might be true for ageing tumour cells (Grin and Grunberger, 1998; Mazzoccoli *et al*, 2003), especially since tumour cells grow at a rapid rate (Lynch, 2004). If exogenous melatonin is administered, the levels of the hormone in these ageing tumour cells would increase, and its anticancer activity would be enhanced (Lynch, 2004). This may explain why tumours undergoing the primary phase of growth in which cells grow, and thus age, more rapidly, have lower levels of melatonin (Sephton *et al*, 2000) than tumours that are regressing (Bartsch *et al*, 1989).

Lissoni *et al* (1990) have suggested that those breast cancers that are less proliferative are the ones associated with increased secretion of melatonin, and that this is indicative of an

improved prognosis. It has been suggested that melatonin enhances the affinity that oestrogen receptors in breast carcinomas exhibit for oestrogen (Miles and Philbrick, 1987). Panzer *et al* (1998) have studied the effects of melatonin on MCF7 breast cancer, lymphoblastoid TK6, osteosarcoma MG63 and cervical cancer HeLa cells and conclude that melatonin has no effects on the cell cycle, morphology or growth of these cancer cells, and that melatonin's antiproliferative action is due to it interacting only with a specific, responsive subclone of MCF7 cells, and not with most cancer cells. This may explain why melatonin only inhibits the growth of certain cancer cells, and why the majority of cancer cell types are not responsive to melatonin, or only respond to supraphysiological doses of the hormone (Bartsch and Bartsch, 1997).

Another finding that encourages caution and further research into melatonin is that at concentrations above 200 μM, melatonin has been observed to promote the proliferation of ovarian cancer cells (Kikuchi *et al*, 1989). This was confirmed by Anisimov *et al* (2001), who found that melatonin concentrations of 20 mg/ L induce spontaneous tumours in mice, and thus caution that it was premature to recommend the use of melatonin for protracted periods of time. Anisimov *et al* (2000b) have found that the nocturnal administration of melatonin in water to rodents induces carcinogenesis. It was suggested that this is due to melatonin causing damage to and breakage of DNA in pharmacological doses (Musatov *et al*, 1998).

Holdaway *et al* (1997) have found that the normal seasonal variation of melatonin secretion is reversed in patients with breast cancer; it is this disruption of normal rhythms that may account for the involvement of melatonin in the proliferation of breast cancer cells (Holdaway *et al*, 1997).

An important factor to consider is that the decreased melatonin levels in prostate and breast cancers are associated with mechanisms that differ between species, and it might thus not be appropriate or accurate to simply extrapolate conclusions from experiments performed on rodents (Bartsch and Bartsch, 1997).

1.3.12.2 Carcinogenesis

The acetyltransferases (see 1.3.5) that metabolise 5-HT to melatonin, and are involved in the metabolism of other arylalkylamine hormones produced in the body, have been shown to have the potential to activate various exogenous amines that can induce carcinogenesis (Debiec-Rychter *et al*, 1996). The acetyltransferase enzymes 1 and 2 have good affinities for tryptamine, MT and 5-HT, and acetylate the nitrogen atoms of these compounds. The resultant *N*-acetoxyarylamines are genotoxic and are associated with the induction of various

tumours, such as hepatic, gastrointestinal and breast tumours (King *et al*, 1997). This may explain the 'spontaneous' development of tumours without the host having been exposed to an external carcinogen, and the occasional development of tumours in organs generally not under the influence of aromatic amine compounds (King *et al*, 1997).

1.3.12.3 Autoimmune diseases

Melatonin has been shown to increase cell migration from the bone marrow to bronchiolar fluid, thereby playing a role in the pathogenesis of asthma (Martins *et al*, 2001). It has been found that patients suffering from nocturnal asthma have increased melatonin levels (Sutherland *et al*, 2002). Other than this, excess endogenous synthesis of melatonin is very rarely observed in conditions other than polycystic ovary disease (Luboshitzky *et al*, 2001).

By enhancing the production and release of various inflammatory mediators such as interleukins and cytokines, melatonin may promote the progression or increase the severity of autoimmune diseases, such as rheumatoid arthritis (Maestroni, 2001). It has thus been recommended that melatonin be used with much caution in patients with autoimmune diseases, allergies, asthma or cancer of the immune system (Lynch, 2004). It has been found, almost paradoxically, that the use of melatonin with IL-2 in some cancers of the immune system, such as lymphoma and leukaemia, can prolong the patients' life and stabilise cancer progression (Lissoni *et al*, 2000).

In the case of the abovementioned patients with autoimmune diseases or asthma, melatonin antagonists may have therapeutic value (Blask *et al*, 1999). Further research is needed, however, into whether melatonin also stimulates T-helper 2 cells, since the balance in activity between T-helper 1 and 2 cells is a very significant factor in maintaining homeostasis of the immune system (Maestroni, 2001) (see 5.2.2 and 12.3.2). Lissoni *et al* (1993) suggest that, besides macrophages, the T-helper type 2 cells are the main target of melatonin, and that these cells play a role in the release of IL-5, which promotes the eosinophilia induced by IL-2.

1.3.12.4 General adverse effects

It has been noted that melatonin can cause fatigue and tolerance, if used for a prolonged period and on consecutive nights (Kendler, 1997; Suhner and Steffen, 1997). Melatonin has also been found to be associated with the occurrence of vivid dreams, abdominal pain, headaches and nausea (Karasek *et al*, 2002). It is thus recommended that only the lowest effective dose should be used, and that caution should be exercised since not much is known about the mechanisms of melatonin's involvement in the pathogenesis, palliation or aetiology of many disease states (Kendler, 1997; Suhner and Steffen, 1997). More research is needed in

developing a toxicological profile of melatonin, because even though no deaths have been reported of patients who have taken the agent, very little information is known about its side-effects (Guardiola-Lemaitre, 1997). It is advantageous that melatonin has a short half-life (Simonneaux and Ribelayga, 2003; Dawson *et al*, 1996; Lynch, 2004), as this facilitates the treatment of any adverse effects. Caution is also necessary since many of the melatonin products available are not of acceptable pharmaceutical quality (Suhner and Steffen, 1997). The concentrations of melatonin in natural products, such as St John's Wort, have not been standardised (Lynch, 2004) and these natural products should thus not be used indiscriminately.

1.3.12.5 Metabolic effects

A further note of caution is sounded by Bojková *et al* (2006), who have investigated the effects of prolonged melatonin administration, together with fasting, on various metabolic parameters. These authors have found that melatonin has adverse effects on lipid and carbohydrate metabolism, as the agent promotes hyperlipaemia and gluconeogenesis, respectively (see 9.4). There are conflicting reports on the effects of melatonin on glucose levels (Rodrriguez *et al*, 1989; Milcu *et al*, 1971; Bizot-Espiard *et al*, 1998; Alonso-Vale *et al*, 2004; Fabis *et al*, 2002; Mahata *et al*, 1998). Bojková *et al* (2006) suggest that this inconsistency in results may be attributed to differences in dosages, the time of administration and the duration of melatonin treatment. It is known that melatonin receptors exist in the Islet cells of the pancreas (Peschke *et al*, 2000); thereby suggesting that melatonin plays a role in regulating the synthesis and/ or secretion of pancreatic hormones, such as insulin and glucagon (Bojková *et al*, 2006; Rodriguez *et al*, 1989).

Although melatonin decreases serum cholesterol levels and inhibits the movement of fat into the liver in rats that are hypercholesterolaemic (Ayoma *et al*, 1988) or fed a high-cholesterol diet (Mori *et al*, 1989), as well as decreases low density lipoprotein (LDL) and prevents a fall in high density lipoprotein (HDL) levels, a scenario favourable in countering hypercholesterolaemia, Bojková *et al* (2006) have found that prolonged melatonin treatment and short-term fasting results in an increase in cholesterol and triacylglycerol levels. Body mass also decreases (Bojková *et al*, 2006). Starvation can be a problem in cancer patients (see 1.3.6.1), and may enhance the development of carcinomas (Bojková *et al*, 2006); the combination of melatonin and fasting may thus lead to deleterious metabolic effects.

1.3.12.6 Receptor desensitisation

Indiscriminate or uninformed use of melatonin can result in excessive and prolonged levels of the agent being present in the body; this may result in the desensitisation of melatonin

receptors, an "artificial darkness" state in the body, which may be detrimental to other physiological processes or reactions that are dependent on daylight conditions, the accumulation of metabolites of melatonin that may have adverse or toxic effects, and changes in reproductive functioning, due to alterations in melatonin's circannual fluctuations (Guardiola-Lemaitre, 1997).

1.3.12.7 Use in pregnancy and lactation

Although it has been reported that there are no contraindications to the use of melatonin (Karasek and Reiter, 2002), it does cross the placenta (Klein, 1972) and should thus be used with extreme caution, if at all, by pregnant and breastfeeding women (Karasek *et al*, 2002).

1.3.13 Summary

To summarise the actions of melatonin, this compound has the following mechanisms of action that, when considered in entirety, may well explain its beneficial use as adjuvant therapy in the treatment of cancer in terms of reducing the toxicity and enhancing the efficacy of antineoplastics:

- Melatonin inhibits NF-kB, which controls the expression of genes involved in the cell cycle
- Melatonin is a very potent antioxidant
- It is oncostatic by affecting protein kinase C activity, oestrogen receptor activity and expression, intracellular structure, calmodulin/ calcium activity and reduction-oxidation reactions
- Melatonin inhibits various hormones and factors that promote the growth of cancer cells, such as prolactin, oestradiol and epidermal growth factor
- It promotes the apoptosis and suppresses the mitotic index of cancer cells
- It controls the expression of oncogenes
- It modulates the immune system, particularly by enhancing the activity and reducing the adverse effects of IL-2 and increasing the synthesis of IL-1, IL-6 and IL-12
- Stimulation of melatonin receptors results in the release of INFγ, which enhances the synthesis and activity of NK cells
- It palliates cancer-related fatigue and cachexia
- Melatonin is a direct free radical-scavenger, as is its metabolite 6-OHM
- It regulates the activity of IGF-1, which has a protective influence over cancer cells
- Melatonin reduces the secretion of VEGF and is thus anti-angiogenic

- It blocks the uptake of linoleic acid and its subsequent conversion to 13-HODE in tumour cells, thus preventing the growth of these cells
- It strengthens circadian rhythms, which enhance natural anti-tumour mechanisms
- Melatonin enhances cancer cell differentiation and decreases the potential of these to metastasise and be invasive
- It induces reversible morphological changes in cancer cells
- Melatonin has oncostatic activity through glutathione-dependent mechanisms
- The hormone activates components of the lymphatic system
- It chelates metal ions
- Melatonin reduces the peroxidation of lipid membranes and limits damage to the polyunsaturated fatty acids in membranes
- It inhibits cell proliferation
- It treats thrombocytopaenia by increasing the number of circulating platelets
- Melatonin increases haematopoiesis and thus reduces the haematological toxicity of antineoplastics
- It enhances radiation and chemotherapy by enhancing the blood flow to and within tumours
- Melatonin alleviates depression and anxiety
- It has intrinsic analgesic activity
- It is neurotrophic and thus protects against antineoplastic-induced neuropathy
- It has anti-inflammatory activity and thus minimises the inflammation that accompanies cytotoxicity
- It is a geroprotector

It can thus be gleaned from the above that melatonin has very significant oncostatic, antioxidant and neuroimmunomodulatory functions. The model proposed by this author on the next page summarises the abovementioned actions of melatonin in oncology, and has incorporated the mechanisms of action of 5-FU.

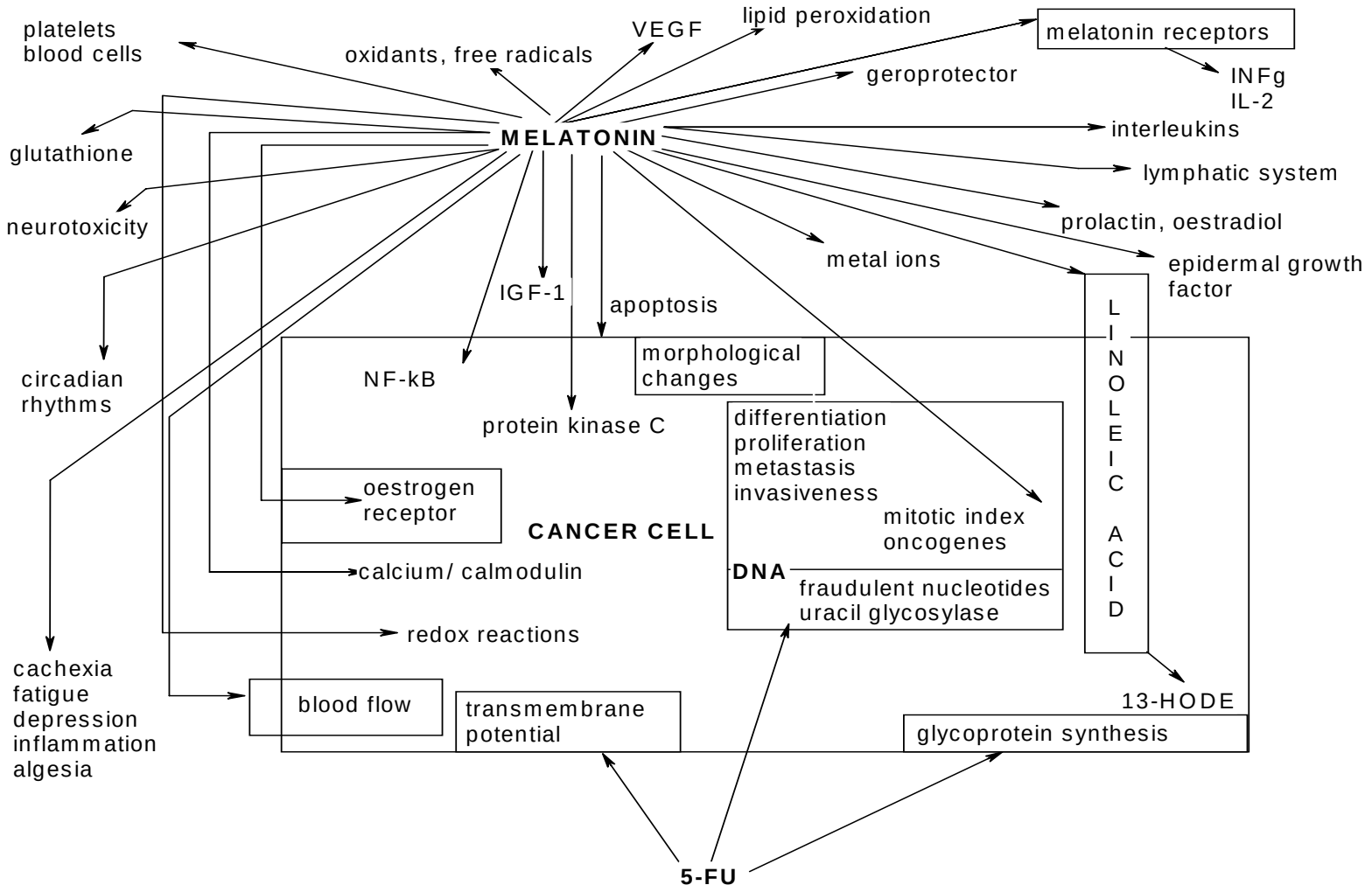


Figure 12: Proposed model of melatonin's anticancer actions

CHAPTER 2

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY: METHOD DEVELOPMENT AND VALIDATION

2.1 Rationale for the use of HPLC

It was decided to choose a suitable method that would allow for the detection and separation of both 5-FU and melatonin, in order for concentrations of each to be accurately measured, to determine whether a metabolic drug interaction occurs between these drugs in the liver. Isolation of the CYP450 system in hepatic microsomes, according to the method of Cinti *et al* (1972), as will be described in detail in chapter 3, is the intended biological application of the chosen method of quantitative analysis. It was initially decided to test whether ultraviolet (UV) spectrophotometry is a suitable method of analysis and whether it adequately allows for the separation and detection of 5-FU and melatonin. A Spectra System UV 2000® UV spectrophotometer was used. A 3 mM stock solution of melatonin was prepared, and serially diluted to 0.0625 µM. Likewise, a 0.0625 µM solution of 5-FU was prepared. When subjected to running wavelength scans by UV spectrophotometry, the following chromatograms, Figures 13 and 14, were obtained, for 5-FU and melatonin respectively. It is apparent from these scans that the lambda maximum, λ_{max}, the wavelength at which maximum absorption of the drug occurs, was 267 nm for 5-FU and 279 nm for melatonin. This is in agreement with the values of 267 nm and 278 nm found by Bayomi and Al-Badr (1989) and Maharaj (2003), respectively, for 5-FU and melatonin.

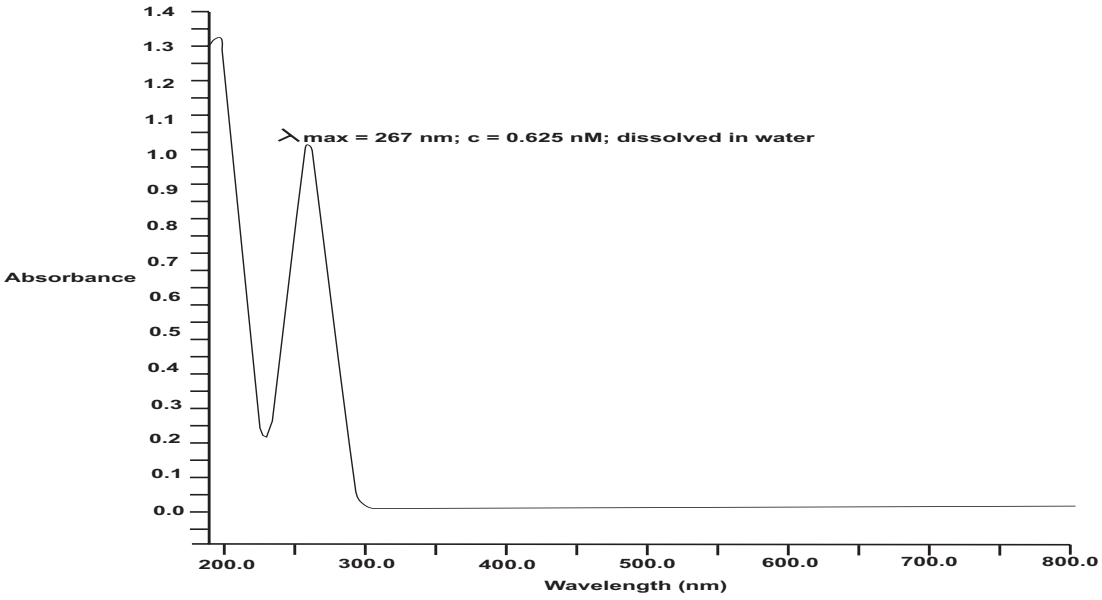


Figure 13: Wavelength scan of a solution of 5-FU by UV spectrophotometry

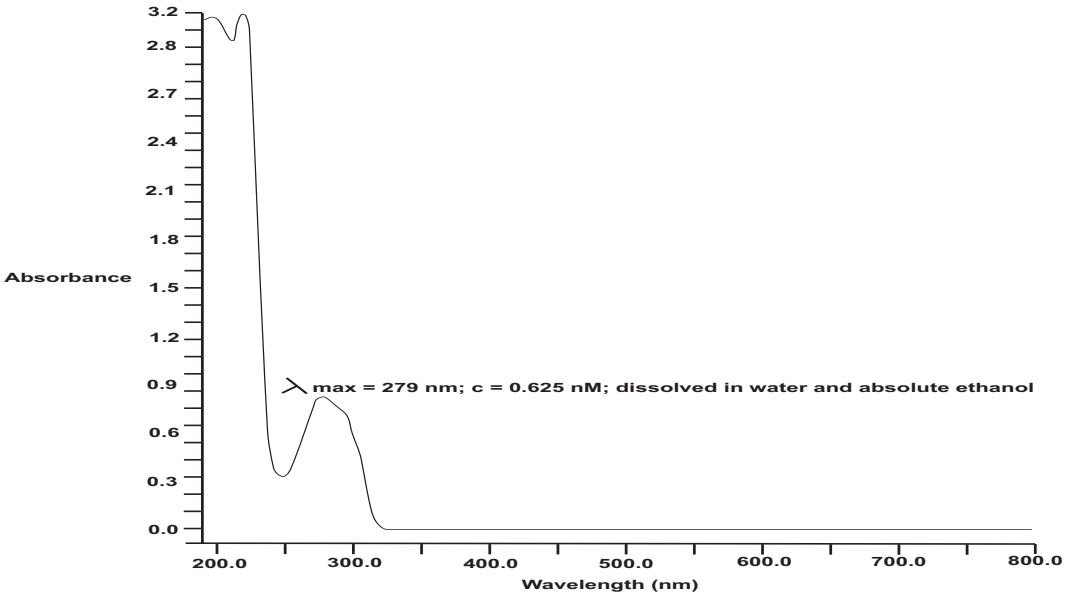


Figure 14: Wavelength scan of a solution of melatonin by UV spectrophotometry

A solution containing equimolar concentrations of both drugs was then subjected to a running wavelength scan, in order to determine whether the drugs can be adequately separated by UV spectrophotometry. Figure 15 shows that both melatonin and 5-FU absorb UV light in the same wavelength spectrum; this is due to both drugs having very electron-dense structures. Since their peaks occur at approximately the same wavelength (250-270 nm), it was concluded that UV spectrophotometry is not sensitive enough a method to allow for the separation of the compounds.

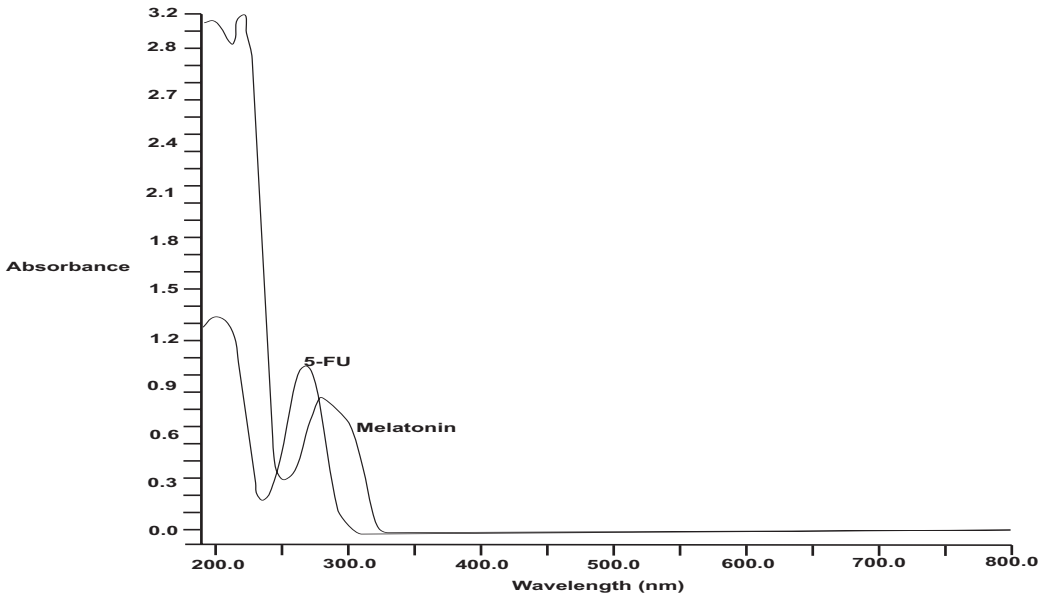


Figure 15: Chromatogram obtained upon UV spectrophotometric analysis of 5-FU and melatonin in solution

Evidence in the literature shows that high performance liquid chromatography (HPLC) is the best method of quantitatively analysing 5-FU in plasma (Christophidis *et al*, 1979), and it was thus decided to develop an HPLC method that would allow for the separation and detection of 5-FU and melatonin.

2.2 Introduction and background to HPLC

HPLC is a method of quantitative pharmaceutical analysis in which drug concentrations can be accurately determined. It is a method of separating compounds in solution using a liquid mobile phase and a stationary phase that is solid (USP, 2005). This separation occurs by high pressure-induced partitioning of the drugs, or analytes, between the aforementioned two phases in a column (USP, 2005). The drugs are dissolved in a suitable mobile phase, which carries these through the column, and the drugs and other components of the solution are eluted at different times, depending on their physicochemical properties. The time until which elution occurs is termed the "retention time" of the particular compound under the given analytical conditions (USP, 2005).

A chromatogram is generated, which shows a peak occurring when the respective compound is eluted. The retention time is measured in terms of the distance from the base of a perpendicular dropped from the peak maximum to an event marker that records the time of injection. Peak height varies according to the concentration of drug in the solution that has been injected into the HPLC machine, and there is a directly proportional relationship between peak height, or alternatively the area under the peak, and analyte concentration. Determination of the tailing factor (T), and a value of this close to unity, is an indicator that the peak obtained is symmetrical, while determination of the resolution factor (R) is necessary to confirm that two peaks, which may correspond to two different drugs, are adequately separated. The resolution factor and number of theoretical plates (N) are indicators of the column's ability to or efficiency in resolving peaks or separating compounds that may elute at times close to each other, and R is a function of N. The value of N is also a function of the chromatographic conditions, and the properties of the compound being analysed; a higher value indicates increased column efficiency (USP, 2005). The equations used to determine the abovementioned factors, and the abbreviations used, are given below (USP, 2005):

$T = W_{0.05} / 2f$

$R = [2(t_2 - t_1)]/ (W_2 - W_1)$

$N = 16(t/ W)^2$

Where: T = tailing factor

$W_{0.05}$ = the width of the peak at 5% of its height as measured from the baseline

f = the distance from the leading edge of the peak to the peak maximum, at 5% of the peak height

R = resolution factor

t = retention time of the respective compound, measured from the time of injection to the time at which the peak maximum occurs

W = the width of the peak, measured by extending the straight edges of the peak to the baseline

N = the number of theoretical plates in the column

2.3 Method development

This process involves determining the exact analytical conditions optimal for the quantitative analysis of the particular drugs. Method development using HPLC is a multistep process in which variables are assessed and changed in a systematic manner in order to ascertain the final chromatographic conditions. These variables will be discussed in more detail below.

2.3.1 Column selection

Drug separation in quantitative analysis occurs in the column. "Normal phase" systems consist of a mobile phase that is nonpolar and a stationary phase that is polar, while the opposite is termed "reversed phase chromatography". The analyte's affinity for the stationary phase, and thus an indication of its retention time, can be altered by varying the polarity of the mobile phase. The stationary phase consists of an organic phase that is bound chemically to silica particles; the latter have a diameter of 3-10 μm (USP, 2005). These small particles, coated with a thin layer of organic phase, allow for the rapid movement of analyte particles between the mobile and stationary phases. The nature of the functional groups in the organic phase determines the degree of polarity of the column. The diameter of the column is usually between 2-5 mm (USP, 2005).

2.3.2 Mobile phase composition

The mobile phase is crucial in allowing the process of drug separation to occur. The mobile phase is usually a combination of solvents and its composition and physicochemical properties are thus very important (Lindsay, 1987). Interactions between the stationary phase packing the column and the mobile phase could result in damage to the column, and the mobile phase should not contain constituents that facilitate such interactions (Maharaj, 2003). The mobile phase should be filtered and degassed prior to use, in order to prevent air bubbles or particulate matter from accumulating in components of the HPLC machine and causing damage (Lindsay, 1987). The solvents selected for use in the mobile phase should not absorb UV radiation to any appreciable extent at the wavelength that has been chosen for analysis, as

this will interfere with the results obtained (Hadden *et al*, 1971). The viscosity of the mobile phase should be below 0.5 cps (Lindsay, 1987), since high viscocity is associated with reduced efficiency of the column (Hadden *et al*, 1971). If a buffer is used as a constituent of the mobile phase, its pH should be varied in the method development stage so that the pH that allows for the optimal separation of the drug is chosen.

2.3.3 Flow rate

The rate at which the mobile phase flows through the column is an important determinant of the retention time of the analyte. Flow rates can vary between 1-5 cm^2/ min (Lindsay, 1987); slower rates preserve the life of the column and pump (Maharaj, 2003).

2.3.4 Chart speed

If a chart recorder is used, instead of an integrator, to record the response of the drug to UV radiation absorption, the chart speed needs to be optimised to give the most symmetrical peaks that show minimal tailing. A slower chart speed gives peaks that are less broad.

2.3.5 Detector and wavelength

Spectrophotometric detectors are used in many HPLC systems. These emit radiation, which is absorbed by the analyte as it is eluted, thereby resulting in energy changes that can be measured. There are various types of spectrophotometric detectors available, and can emit radiation at either variable or fixed wavelengths (USP, 2005).

2.3.6 Sensitivity

The voltage and absorbance units full scale (AUFS) need to be varied to find values that allow sufficient sensitivity to adequately detect the analyte in the required concentration range.

2.4 Method validation

The process of validation is necessary to show that the method of analysis developed is suitable for the purpose for which it will be employed. The USP (2005) defines validation as, "The process that establishes, by laboratory studies, that the performance characteristics of the method meet the requirements for the intended analytical application." It is thus of extreme importance in making the optimal use of available resources, such as money and time, since the results of this process may necessitate changes to the chosen method of quantitative analysis (Green, 1996). The validation process consists of a number of studies; these parameters have been outlined by the International Conference on Harmonisation (ICH Guidelines, 1996). This gathering of regulatory body and industry representatives from Japan, Europe and the USA attempted to standardise validation requirements

(www.labcompliance.com/methods/meth_val.htm). These parameters are outlined below, in the order suggested by Green (1996) to be the most efficient and effective. The ICH Guidelines (1996) concede that it is possible to determine some of these parameters simultaneously.

2.4.1 Establishment of minimum criteria

These are the minimum acceptable criteria for the intended method of analysis, and may vary between different laboratories and research settings. In our Neuroscience Research Group laboratory in the Faculty of Pharmacy at Rhodes University, for example, the limit for the percentage relative standard deviation (% RSD) was set at ± 4% (Maharaj, 2003). This is in contrast to the USP (2005), which states that the % RSD should be ± 2%. Other criteria used were in accordance with the ICH Guidelines (1996).

2.4.2 Specificity

Specificity is the ability to specifically and accurately measure the analyte in the presence of other compounds, such as degradants, endogenous substances that may be present in the sample, impurities (USP, 2005) or excipients (Dantus and Wells, 2004).

2.4.3 Linearity

This study is to demonstrate a directly proportional relationship between the concentration of drug in various solutions over a given concentration range, and the magnitude of its response or ability to provide results (Edwardson *et al*, 1990; ICH Guidelines, 1996; www.labcompliance.com/methods/meth_val.htm; USP, 2005). The data should be visually inspected and a least squares regression line plotted. Even though the USP (2005) states that a regression coefficient of greater than 0.997 for five points over 80-120% of the analyte test concentration (ICH Guidelines, 1996) is sufficient to demonstrate linearity, Green (1996) suggests that it be greater than 0.999. The drug solutions to be used can be prepared by diluting a stock solution (ICH Guidelines, 1996), should cover a wide concentration range (Edwardson *et al*, 1990) and each solution should be analysed a minimum of three times (Green, 1996).

2.4.4 Accuracy and recovery

Accuracy, also termed "trueness" (Dantus and Wells, 2004), is the closeness between the results that have been obtained by the selected method of analysis and true, or reference, values, and should be between 98-102% (USP, 2005). It is determined by using a calibration curve of the analyte; this calibration should consist of at least nine determinations over a range of at least three concentrations (ICH Guidelines, 1996). Once linearity, precision and

specificity have been determined, the accuracy of the method can be inferred (ICH Guidelines, 1996). Included here with accuracy is the parameter of recovery, which involves quantitating the amount of analyte that can be retrieved, for example subsequent to a biological assay, and analysed (Edwardson *et al*, 1990). Indeed, it is suggested that recovery studies be used to determine accuracy (Dantus and Wells, 2004). A sample with no analyte present is first analysed, and then spiked with a known amount of analyte and analysed. The ratio of the difference between these two results and the mass of added analyte is termed the "surrogate" or "marginal" recovery; values significantly different from one are considered as reflecting inaccuracy (ICH Guidelines, 1996; Dantus and Wells, 2004). Accuracy can thus be determined or expressed in a variety of ways, for example as a percentage relative error or as a correlation coefficient (Swartz and Krull, 2005).

2.4.5 Range

The range is the interval between and including the lower and upper concentration values of the analyte to be used (USP, 2005), and depends on the proposed application of the chosen analytical method (ICH Guidelines, 1996). The range must be determined with acceptable levels of accuracy and precision and demonstrate linearity (USP, 2005). The range should cover at least 80-120% of the test concentration of the analyte (ICH Guidelines, 1996).

2.4.6 Method precision

Precision is a measure of the reproducibility of the chosen method (Edwardson *et al*, 1990) and indicates the extent of variability in the results that occurs when the same method is applied to many identical samples. It is given as the % RSD (USP, 2005), the coefficient of variation expressed as a percentage (ICH Guidelines, 1996). A larger % RSD corresponds to less precision in the method used (Dantus and Wells, 2004). Precision is thus an indicator of the degree of consistency achieved in results obtained by the chosen method of analysis (Dantus and Wells, 2004). There are various types of precision, namely: (i) method precision or repeatability, (ii) intermediate precision, and (iii) reproducibility. Method precision is assessed by repeatedly analysing the same sample over a short duration of time (USP, 2005); it is assessed by performing at least nine determinations over the selected concentration range, for example, measuring the concentration of three points in triplicate (ICH Guidelines, 1996). The short period of time is necessary to ensure that the analytical conditions are the same for each determination, and is an indicator of instrument precision (Dantus and Wells, 2004). Reproducibility is the variability that results when another laboratory uses the same analytical method (USP, 2005) to analyse multiple samples (Dantus and Wells, 2004). Intermediate precision will be discussed in 2.4.10.

2.4.7 Limit of detection (LOD)

The LOD is the lowest concentration of analyte that can be reliably detected (Edwardson *et al*, 1990), but not quantitated (USP, 2005). Although there are various methods of calculating the LOD, it is generally best described as being two to three times the signal-to-noise ratio. The latter ratio is the ratio in peak heights between low concentrations of the analyte and baseline noise (ICH Guidelines, 1996). The LOD should be validated by repeated analysis (USP, 2005).

2.4.8 Limit of quantitation (LOQ)

This is the lowest concentration of analyte that can be quantitated with an acceptable level of accuracy and precision (Edwardson *et al*, 1990; USP, 2005). It can also be defined as the lowest concentration of analyte for which a % RSD of up to 2% is obtained from duplicate injections (Edwardson *et al*, 1990). The LOQ is at least ten times greater than the signal-to-noise ratio, and should also be validated (USP, 2005).

2.4.9 Stability studies

During the period of method development and early validation studies, some information can be gleaned as to the stability of the analytes and other reagents, such as those constituting the mobile phase. At this stage, the stability of the analytes should be determined in a systematic way, subjected to various stresses, such as heat, light, and acid- and base-catalysis. Solutions of the analytes should be exposed to these stresses for a period of at least 48 hours (Green, 1996; USP, 2005). These should then be analysed by the chosen method and evaluated in terms of the possible degradation products that are formed and the concentration of analyte that remains (Dantus and Wells, 2004). This is significant because degradation of an analyte decreases the precision of the method of analysis (Szepesi, 1990). If the analytes are not suitably stable for the purposes of the analysis, potential additives or storage conditions should be identified that would enhance stability. It is important that solutions of the analyte are stable enough not to be affected by delays, such as problems with instrumentation (Green, 1996).

2.4.10 Intermediate precision

This refers to the variability that results when the same method is run on different days and at different times (USP, 2005) in the same laboratory (Dantus and Wells, 2004). It thus refers to inter- and intra-day variability; the former is assessed by performing multiple analyses over several days and the latter by performing the same analysis in the morning and evening of the same day; an extended period of time is thus a key feature of intermediate precision (Dantus and Wells, 2004).

2.4.11 Parameters not validated

There are several other parameters that may be determined in the cases where a more advanced and comprehensive validation procedure is required, such as in the efficacy, or phase III stage of drug product development (Green, 1996). These parameters serve to ensure that the validity of the chosen method of analysis is maintained whenever the method is used (ICH Guidelines, 1996), and include: (i) reproducibility (see 2.4.6); (ii) system suitability, which includes an assessment of the type of equipment to be used and any potential impact thereof on the conditions under which analysis is performed (USP, 2005); (iii) robustness, or the ability of the method to not be affected by slight, deliberate variations, such as column temperature, the pH of the mobile phase (USP, 2005) and the volume of injection (Dantus and Wells, 2004); and (iv) ruggedness, which is the degree of variation that results when the same method is performed on different days by different laboratories, using different reagents, instruments, times and temperatures; ruggedness is thus the absence of influence of such variables on the results obtained (USP, 2005; Dantus and Wells, 2004).

2.5 Results and discussion

The results of the method development process and validation studies are given below.

2.5.1 Materials, reagents and equipment

All the chemicals and reagents that were used were of at least analytical reagent quality. Melatonin and 5-FU were purchased from the Sigma Chemical Corporation, St Louis, MO, USA. The HPLC-grade solvents used were obtained locally, while the HPLC-grade water used was purified using a Milli-Ro®-15 water purification system (Millipore, Bedford, MA, USA). The latter consists of an Organex-Q® cartridge, two Ion-X® ion-exchange cartridges and a Super-C® carbon cartridge. Prior to use, the water was double filtered and degassed through a 0.45 µm Millipak® stack filter. The isocratic HPLC system consisted of a Spectraseries UV100 detector with a deuterium lamp, a Spectraphysics Isochrom LC pump (Spectraphysics, San Jose, California, USA) and a Rikadenki chart recorder. Samples were injected from a 100 µL Hamilton syringe (Hamilton Bonaduz AG, Switzerland) into a 20 µL fixed loop Rheodyne injector (Rheodyne, Cotati, California, USA). The column used was a C_{18}, 5 µm Waters Spherisorb analytical column (Waters Corporation, Milford, Massachusetts, USA), with dimensions of 250mm X 4.6mm (internal diameter).

2.5.2 Method development

This process was carried out in a total of twenty stages, as detailed below, in which parameters were systematically altered such that the final optimal chromatographic conditions could be selected. Stock solutions of the drugs, melatonin and 5-FU, separately and together,

were prepared in concentrations of 1 mg/ ml, and dilutions made. 5-FU was dissolved in water, while melatonin was dissolved in water using absolute ethanol as a co-solvent at a final concentration of 0.67% v/ v.

2.5.2.1 Step 1: Selection of mobile phase

A method of detecting 5-FU in the liver has been described in the literature by Del Nozal *et al* (1994), in which the mobile phase consists of a 50 mM phosphate buffer, adjusted to pH 3. It was thus decided to start the method development process using a potassium dihydrogen phosphate (KH_2PO_4) buffer as the mobile phase, at a flow rate of 0.8 ml/ min. The chart speed was set at 30 cm/ hr, the AUFS at 0.1, and voltage at 10 mV. The wavelength selected was 267 nm, as this is the λ_{max} of 5-FU (Bayomi and Al-Badr, 1989). The run time was 10 mins. An average retention time of 3.07 mins for 5-FU was obtained, but the peak was too broad and showed tailing.

2.5.2.2 Step 2: Selection of mobile phase

It was then decided to combine the KH_2PO_4 buffer with 50% methanol, in a ratio of 50: 50. Methanol has been used as a mobile phase to detect 5-FU by several researchers (Yamaguchi *et al*, 1987; Yoshida *et al*, 1988; Jung *et al*, 1997; Kindberg *et al*, 1989) and this particular combination of solvents has been described as an effective gradient mobile phase (Guerrieri *et al*, 1993). The other parameters were kept constant. The average retention time for 5-FU was 3.68 mins, but there was still peak tailing.

2.5.2.3 Step 3: Selection of mobile phase

Methanol only, at a concentration of 50% in water, was tested as a mobile phase. However this proved undesirable as there was peak tailing.

2.5.2.4 Steps 4-6: Selection of mobile phase

The following concentrations of methanol were each used as the mobile phase: 60%, 40% and 30%. The average retention times of 5-FU were, respectively, 2.97 mins, 3.08 mins and 3.19 mins. Each concentration still gave some peak tailing, but the extent of this was least with 60% methanol. Increasing concentrations of the mobile phase resulted in an increase in column pressure and it was decided to not use a concentration greater than 60% as this would result in even greater column pressure and this might be detrimental to the lifespan of the column. The following graph shows the relationship between average retention times of 5-FU and the concentration of methanol constituting the mobile phase. From the graph, it can be observed that increasing concentrations of methanol are associated with the drug eluting at a more rapid rate and the retention time thus decreasing.

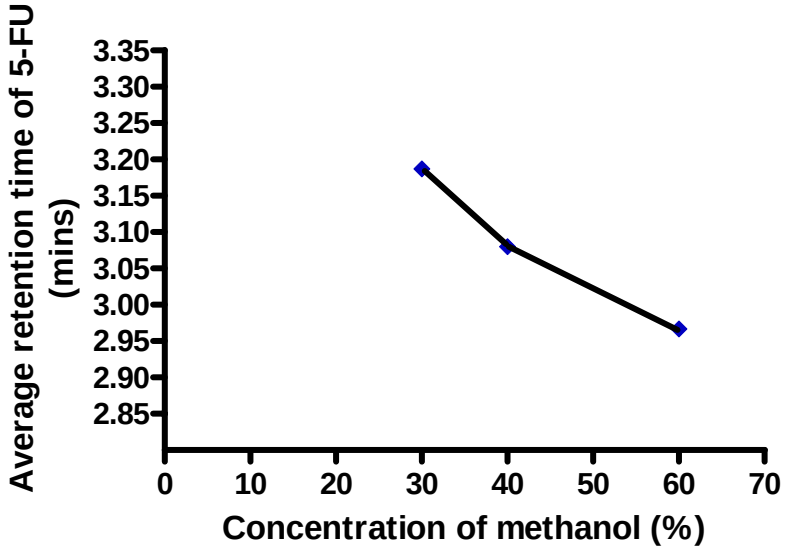


Figure 16: A graph showing the relationship between the retention time of 5-FU and the concentration of methanol in the mobile phase

Melatonin was injected separately using 60% methanol as the mobile phase, in order to determine its peak characteristics and average retention time. The latter was 4.93 mins and the peak tailing was similar to that displayed by 5-FU. The difference in retention times between the two compounds suggests that adequate separation of the two can be achieved. This will be confirmed in Step 7.

2.5.2.5 Step 7: Selection of chart speed and confirmation of drug separation

The chart speed was decreased from 30 cm/ hr to 15 cm/ hr. The peaks were thinner, there was less tailing and it was thus decided that the new chart speed resulted in optimal peaks. The tailing factor, T, was determined and found to be 0.93. Since a value of T close to unity indicates symmetry, the chromatrographic conditions that have been used yield acceptably symmetrical peaks. A solution containing both 5-FU and melatonin was analysed and the average retention times of the drugs were 3.20 mins and 5.08 mins respectively, thus indicating that adequate separation of the two drugs was achieved. The resolution factor, R, was calculated and found to be 96. The number of theoretical plates, N, were found to be 11837.44 with regard to melatonin, and 7744 with respect to 5-FU. The higher N value for melatonin indicates that the column exhibits greater efficiency in separating this compound from the mobile phase (see 2.2).

2.5.2.6 Steps 8-9: Selection of AUFS

The AUFS, which was hitherto 0.1, was varied to 0.5 and 0.05. The former sensitivity resulted in very small peaks, while the latter gave more desirable, larger peaks and was thus the AUFS deemed to be most optimal.

2.5.2.7 Steps 10-11: Selection of voltage

As with the AUFS, smaller values are associated with larger peaks. Voltages of 5 mV and 2 mV were assessed. A voltage of 5 mV was selected as offering optimal sensitivity, as 2 mV resulted in peaks that were deemed too large.

2.5.2.8 Steps 12-13: Selection of flow rate

The flow rate of the mobile phase was hitherto 0.8 ml/ min, and gave retention times of 3.05 mins and 4.73 mins, for 5-FU and melatonin, respectively. This retention time for 5-FU was considered too fast as there may be possible (photo)degradation products of both drugs that could elute before or at that time, thus making detection of 5-FU and melatonin difficult. This consideration stems from the finding that Maharaj *et al* (2002) have made, identifying photoproducts of melatonin that elute from 2-4 mins before the more non-polar parent drug does. It was thus decided to vary the flow rate to 0.6 ml/ min and 1 ml/ min. The former flow rate gave an average retention time of 4.08 mins for 5-FU and 6.81 mins for melatonin, while a flow rate of 1 ml/ min gave an average retention time of 2.40 mins for 5-FU and 3.97 mins for melatonin. The latter retention times were too rapid, and it was decided to select 0.6 ml/ min as the optimal flow rate. The run time for each analysis remained the same at 10 mins. The following graph shows the relationship between the average retention times of both drugs and the flow rate. It can be observed that an increased flow rate is associated with a more rapid elution of the drugs.

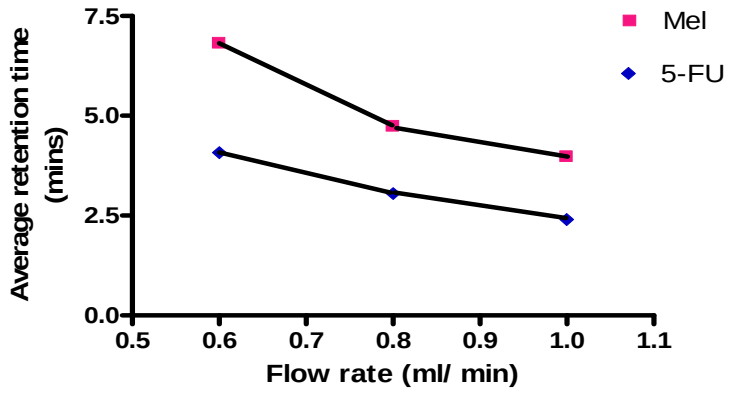


Figure 17: A graph showing the relationship between the average retention times of 5-FU and melatonin, and the flow rate of the 60% methanol mobile phase

2.5.2.9 Steps 14-16: Selection of wavelength

The above analyses were performed at a wavelength of 267 nm, which had been found to be the λ_{max} of 5-FU (see 2.1). The λ_{max} of melatonin was found to be 279 nm (see 2.1). It was decided to vary the wavelength at which the HPLC analysis was performed to 271 nm, 275 nm and 279 nm. The optimal wavelength that was selected was 275 nm, as it gave sufficiently large peaks for both 5-FU and melatonin, and fell between their individual λ_{max} values. Both drugs showed adequate absorption of UV radiation at 275 nm.

2.5.2.10 Steps 17-19: Selection of AUFS II

During the method development process it may be necessary to change certain parameters, if it is found that the selected variables do not provide optimal results, or to re-confirm that these are still the most appropriate, given changes in other parameters. An AUFS of 0.05 was deemed, in 2.5.2.6, to be optimal. Since the wavelength at which analysis was performed was subsequently altered to 275 nm, it was decided to re-confirm that an AUFS of 0.05 was still optimal. Analyses using an AUFS of 0.05, 0.1 and 0.02, were performed. It was found that 0.1 gave peaks that are too small, while 0.02 gave peaks that are too large. An AUFS of 0.05 was thus found to offer adequate sensitivity.

2.5.2.11 Step 20: Selection of voltage II and summary of the selected parameters

It was decided to similarly re-confirm that a voltage of 5 mV, as selected in 2.5.2.7, was still optimal. The peaks that were obtained from 2.5.2.10, of the most dilute solutions, were deemed to be too large, considering the final concentration range to be used and the biological application of this HPLC method. It was thus decided to increase the voltage, and thus reduce the sensitivity with which the drugs were detected. A voltage of 50 mV was used and resulted in optimal peaks being achieved for both dilute and more concentrated solutions.

The results of this method development process are given in the table on the next page.

Table 1: The optimal chromatographic conditions selected for the HPLC analysis of 5-FU and melatonin

Parameter	
Mobile phase	60% methanol in water
Flow rate	0.6 ml/ min
Pressure	1443 psi
Chart speed	15 cm/ hr
AUFS	0.05
Voltage	50 mV
Wavelength	275 nm
Injection volume	20 µL
Run time	10 mins

These parameters result in symmetrical, resolved peaks and adequate separation of 5-FU and melatonin, as shown in the chromatogram below. The average retention times of 5-FU and melatonin are 4.6 mins and 6.8 mins, respectively.

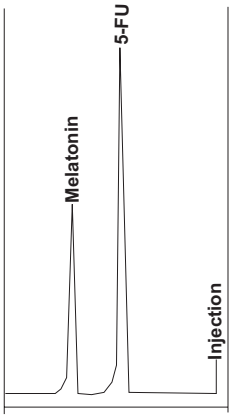


Figure 18: A typical chromatogram showing the separation of 5-FU and melatonin under the final chromatographic conditions selected

2.5.3 Method validation

The HPLC method developed was subjected to various validation studies over the course of one week, and the results of these are given below.

2.5.3.1 Specificity

Solutions of 5-FU and melatonin, separately and together, were analysed and, as previously mentioned, there was adequate separation of the drugs and resolution of their peaks. Various stability studies were conducted (see 2.5.3.8), in which the drugs were subjected to stresses, namely light, heat, time and acid- and base-hydrolysis. The results of these stability studies indicate that the method accurately and adequately detects both 5-FU and melatonin from the degradation products that may be formed.

The biological application of this HPLC method will be the detection of the drugs in microsomes containing CYP450 (see 3.1). It was necessary to thus determine whether the HPLC method developed is sufficiently specific to detect 5-FU and melatonin in the biological matrix that will ultimately be used, and to ensure that other endogenous substances that may be present in the microsomes do not interfere with the analysis. Figure 19 below shows typical chromatograms obtained upon the injection of various samples onto the HPLC system. The control does not contain any drugs, whereas the samples marked 5-FU and melatonin have had the respective drug added to the microsomes prior to a 60 min incubation period (see Chapter 3). The last group received both 5-FU and melatonin.

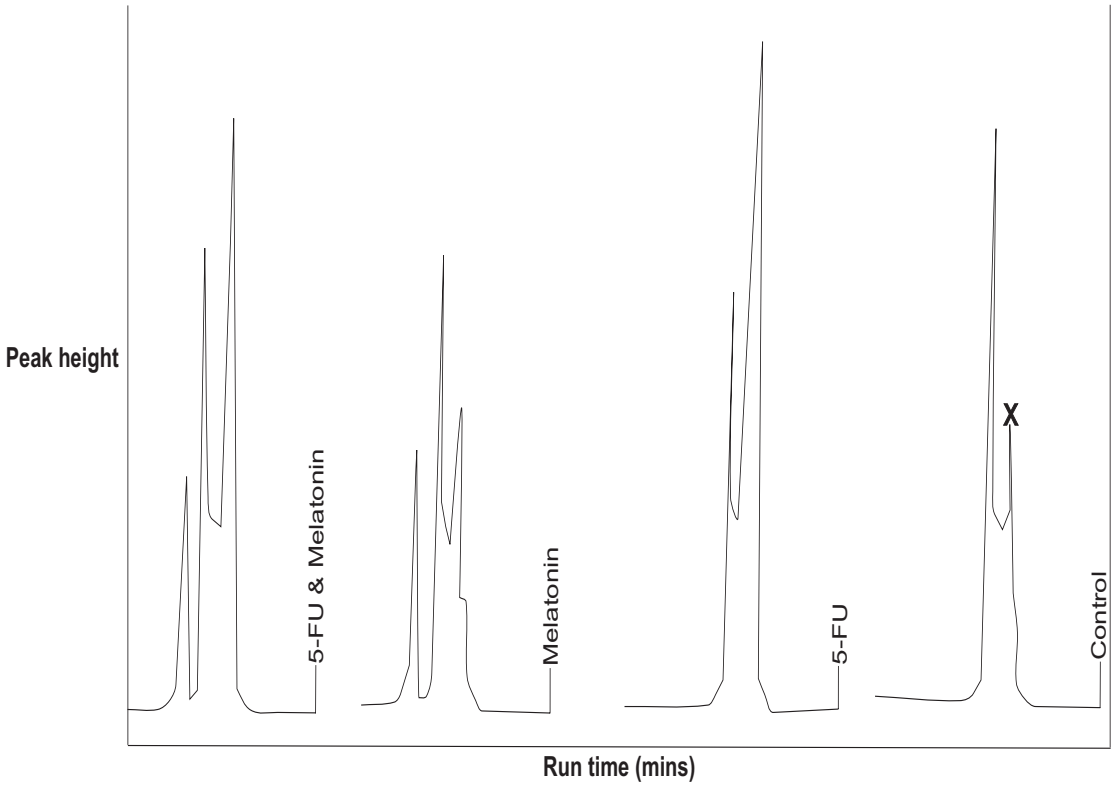


Figure 19: Typical chromatograms obtained upon the HPLC analysis of microsomes incubated with 5-FU and melatonin

As shown in the chromatogram obtained for the control sample, there are endogenous compounds in the microsomal supernatant that elute at average retention times of 4.7 mins and 5.7 mins. 5-FU itself elutes at an average of 4.7 mins. It is evident from the chromatograms of the control and the sample that had had 5-FU added to it, that the latter eluted at the same time as the first endogenous compound(/s), marked X in the chromatogram of the control. The resultant peak is a combination of the peaks obtained for 5-FU and the control compound X alone. It can be concluded that the unknown endogenous compound that elutes at the same time as 5-FU has similar physicochemical properties and interactions with the mobile phase that 5-FU has. It is possible to quantify the amount of 5-FU detected, by subtracting the average control height of compound X from the total peak height in the 5-FU samples. The difference is equal to the peak height obtained for 5-FU alone. Melatonin was found to elute at an average time of 6.8 mins, and there was no interference from endogenous compounds with regard to its detection or separation.

It can thus be concluded that the HPLC method developed is specific for the detection of melatonin, and that even though endogenous compounds in the biological matrix are detected, it is still possible to quantify the amount of 5-FU present.

The concentrations of 5-FU and melatonin that were detected will be discussed in 2.5.3.3.

2.5.3.2 Linearity

A stock solution containing 0.11 mg/ ml of both 5-FU and melatonin was prepared, and serially diluted to give six calibration solutions of 0.0275 mg/ ml, 0.01375 mg/ ml, 0.006875 mg/ ml, 0.0034375 mg/ ml, 0.0017188 mg/ ml and 0.0008594 mg/ ml. Each of these concentrations was analysed thrice. A calibration curve, showing the relationship between concentration and peak height for both 5-FU and melatonin, was plotted and a regression line for each drug was fitted. The correlation coefficients of these were determined and assessed as an indicator of linearity. Typical calibration curves that were obtained are shown on the next page.

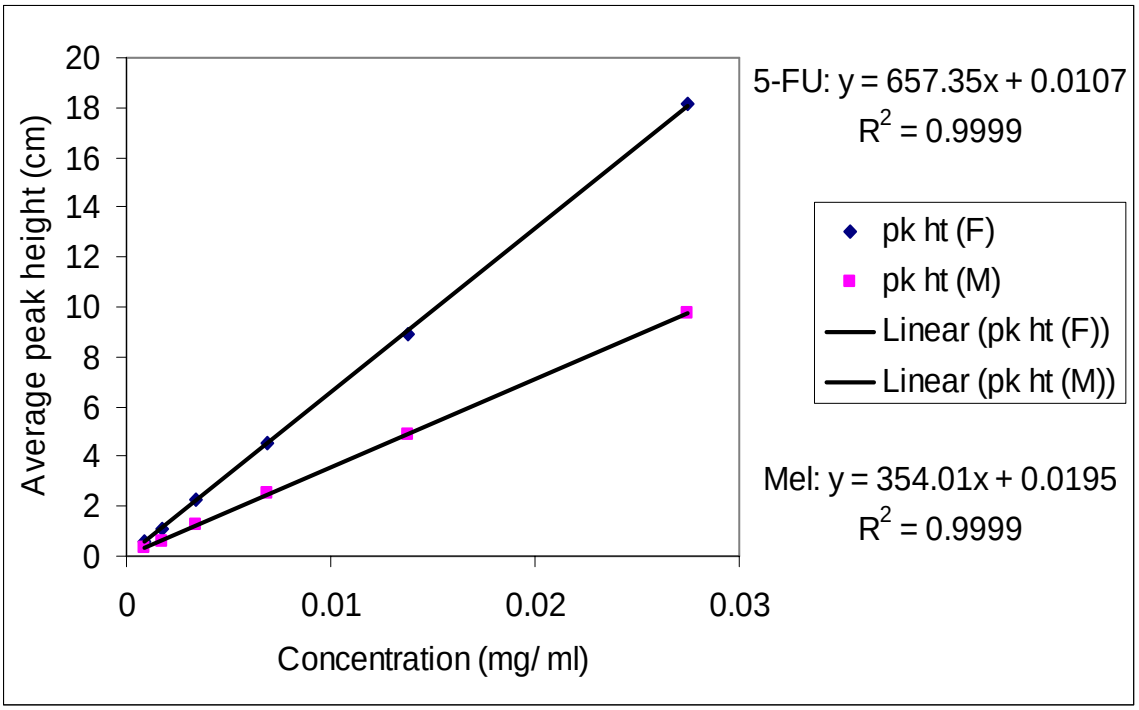


Figure 20: Typical calibration curves of 5-FU and melatonin when analysed by HPLC

F = 5-FU; M = Melatonin; pk ht = peak height

The r^2 values of 0.9999 for both drugs are excellent, considering the required value of 0.997 to demonstrate acceptable linearity (USP, 2005). This indicates that the HPLC method that has been developed provides results that are sufficiently linear.

2.5.3.3 Accuracy and recovery

Accuracy was determined by analysing three different concentrations of the drugs, namely 0.0275 mg/ ml, 0.01375 mg/ ml and 0.006875 mg/ ml, in triplicate. The peak heights obtained were used to calculate the actual concentrations of the drugs, based on the equations of the calibration curves generated in 2.5.3.2. These actual concentrations were expressed as a percentage of the theoretical concentrations. The results are given in the following table.

Table 2: A comparison between the actual and theoretical concentrations of 5-FU and melatonin obtained during accuracy testing

Theoretical concentration (mg/ml)	Average actual concentration of 5-FU (mg/ml)	% of theoretical concentration	Average actual concentration of melatonin (mg/ml)	% of theoretical concentration
0.0275	0.027569	100.25175	0.027534	100.1222
0.01375	0.013574	98.717394	0.013598	98.89466
0.006875	0.006931	100.81175	0.007007	101.918

As shown in the above table, all the actual concentrations obtained fall within 98-102% of the theoretical values, and the method is thus deemed to be accurate, according to USP criteria (USP, 2005) (see 2.4.4).

A recovery study was important to undertake since the biological application of the HPLC method will be to measure the concentration of 5-FU and melatonin remaining after 60 mins of incubation with CYP450 (see Chapter 3). As yet, the extent of metabolism is unknown. It is thus necessary to determine a suitable concentration of the drugs that will be injected into the microsomes; whilst not being overly supraphysiological, this concentration should still be sufficiently high to enable the drugs to be detected subsequent to metabolism and to fall within the calibration range that has been hitherto used. If the drug concentrations after metabolism do not fall within this range, the calibration range and/ or the concentrations of the drugs that are injected, needs to be altered.

A 1 mM solution of both 5-FU and melatonin was prepared and added to the microsomes isolated (see Chapter 3). When analysed in triplicate by HPLC, chromatograms such as those shown in Figure 19 (see 2.5.3.1) were obtained. Immediately after the study, calibration solutions of the same concentrations as described in 2.5.3.2 were analysed and a calibration curve plotted. The concentrations of 5-FU and melatonin were calculated and are reflected in the following table.

Table 3: A recovery study showing the average concentrations of 5-FU and melatonin remaining subsequent to metabolism by CYP450

Drug	Average concentration detected (mg/ ml)
5-FU	0.005517
Melatonin	0.007772
5-FU + Melatonin	0.004381 (5-FU) 0.007493 (Melatonin)

The above concentrations all fall within the calibration range used. It can thus be concluded that the HPLC method developed allows for adequate recovery of 5-FU and melatonin after the intended biological application has been performed. The parameter of recovery will be quantitated in terms of the extent of hepatic metabolism, in the next Chapter.

2.5.3.4 Range

The calibration solutions prepared in 2.5.3.2 have been found, in the above section, to be appropriate for the intended biological assay to be performed. The concentrations of these solutions were: 0.0275, 0.01375, 0.006875, 0.0034375, 0.0017188 and 0.0008594 mg/ ml. The range to be used in HPLC analysis is thus 0.0008594 mg/ ml – 0.0275 mg/ ml.

2.5.3.5 Method precision

Three calibration solutions were each analysed three times within a short period, as described in 2.4.6, and the % RSD calculated for each concentration. The results are presented in the next table.

Table 4: The % RSD obtained for both 5-FU and melatonin during a method precision study

Concentration (mg/ ml)	% RSD (5-FU)	% RSD (Melatonin)
0.0275	1.147978	1.182287
0.01375	1.709916	1.194518
0.006875	1.264271	0

As shown in Table 4, all the % RSD values that were obtained are below the 2% stipulated by the USP (2005) and well below the limit of 4% as set in our laboratory (see 2.4.1). This indicates that acceptable method precision was achieved and that the method shows excellent reproducibility.

2.5.3.6 LOD

Solutions containing both 5-FU and melatonin were successively diluted and analysed in triplicate. The LOD was found to be 0.25 µg/ ml; this concentration yielded signal-to-noise ratios of between 2:1 and 3:1 for both drugs.

2.5.3.7 LOQ

The LOQ was similarly determined and found to be 0.85 µg/ ml. This concentration yielded signal-to-noise ratios of 10:1 for both 5-FU and melatonin.

2.5.3.8 Stability-indicating studies

Solutions of 5-FU and melatonin together were subjected to five stability-indicating tests, namely, light, heat, acid- and base-hydrolysis, and time, as described below. The peak heights of the drugs remaining after each test were measured and compared to control values. The control, of the same concentration of 0.0275 mg/ ml, was kept in the fridge for the duration of the study. Figure 21 shows typical chromatograms obtained for each study.

2.5.3.8.1 Light-induced stress

A 1 ml solution of 5-FU and melatonin was left in a test-tube on a window sill for a period of 48 hours, exposed to ambient light in late summer. As shown in Figure 22, it was found that the average peak height of 5-FU decreased to 16.03 cm, which is 84.98% of the control value of 18.87 cm. The average melatonin peak height decreased by 17.10% from 10.23 cm to 8.48 cm. These values indicate that both drugs are subjected to photolysis, which confirms the product packaging information (Sigma®) that stipulates that storage should be away from light. No photodegradation products were formed and both 5-FU and melatonin were sensitively and accurately detected.

2.5.3.8.2 Heat-induced stress

A 1 ml solution of both drugs was kept in the laboratory oven at 60º for 48 hours. The average peak height of 5-FU decreased substantially to 13.05 cm, which is 69.17% of the control value. The average peak height of melatonin decreased by 31.76% to 6.98 cm. This considerable loss of concentration of both drugs confirms the product packaging information

(Sigma®) which states that both drugs should be stored in the fridge. No degradation products or other compounds were formed.

2.5.3.8.3 Acid-induced stress

An aliquot of 0.5 ml hydrochloric acid (HCl), at a concentration of 32%, was added to a 1 ml solution of both drugs and the test-tube kept in a dark cupboard for 48 hours. Both drugs were subjected to considerable degradation. As shown in Figure 21, a split peak was obtained for 5-FU. This indicates that two compounds with very similar chemical structures were detected. It is likely that 5-FU underwent keto-enol tautomerism (see 1.2.1) by reacting with the hydrogen ions present in the acidic medium. Since the HPLC system that was used was not coupled to mass spectrometry, it was not possible to identify which peaks corresponded to the two tautomers of 5-FU and the peak heights were thus not measured. As shown in Figure 21 the average peak heights for both 5-FU and melatonin are significantly decreased, with melatonin falling to 22.96% of the control value. No degradation products or complexes were formed.

2.5.3.8.4 Base-induced stress

An aliquot of 0.5 ml absolute ethanol was added to a 1 ml solution of 5-FU and melatonin, and the test-tube kept in a dark cupboard for 48 hours. Figure 21 shows that both drugs could still be detected during HPLC analysis, with no other products or compounds being formed. The average peak height of 5-FU decreased to 68.24% of the control value, while that of melatonin fell to 64.82%.

2.5.3.8.5 Time study

A 1 ml solution of the drugs was kept in a cupboard for a period of two weeks and did not show any appreciable loss of either drug when subjected to HPLC analysis.

Figure 21 on the next page shows typical chromatograms obtained for the aforementioned stress studies.

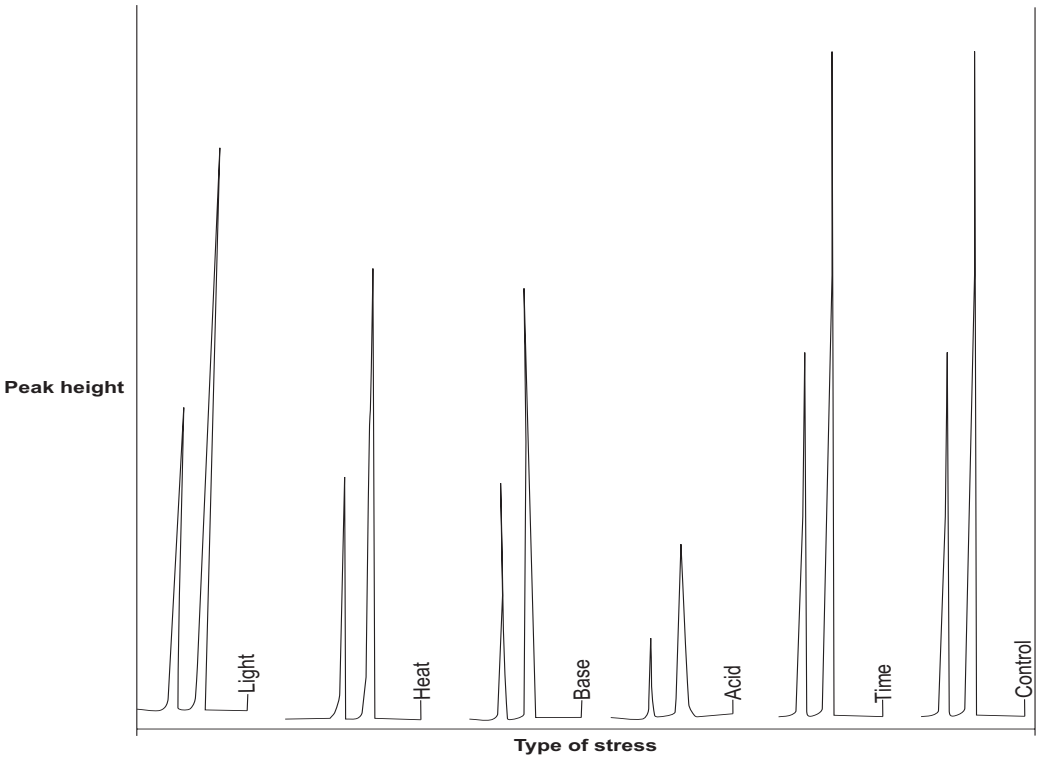


Figure 21: Typical chromatograms obtained after subjecting 5-FU and melatonin to various stability studies

The aforementioned results have been captured in the following graph, to facilitate comparison of the results of the various stability studies.

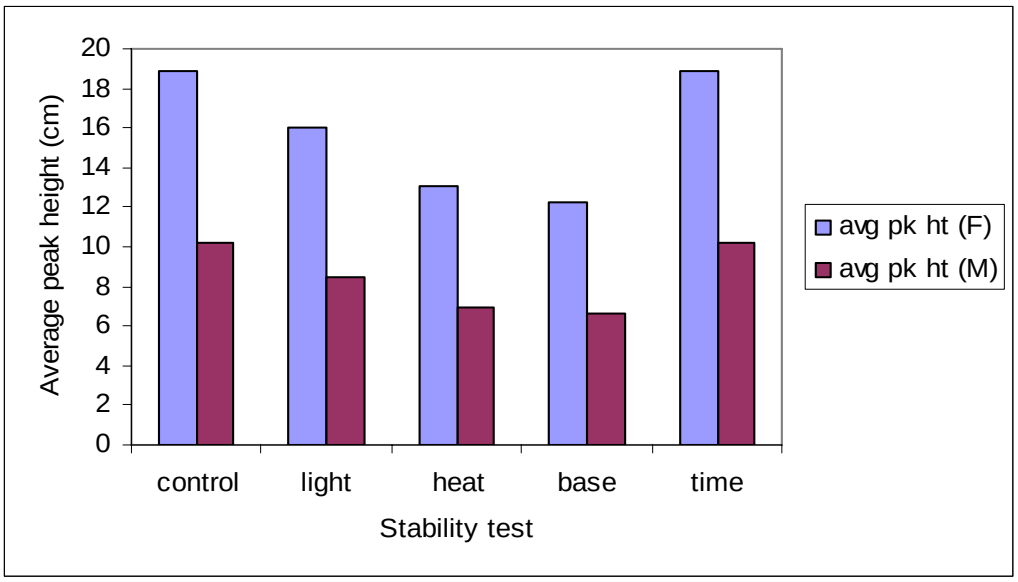


Figure 22: A graph showing the average peak heights obtained when 5-FU and melatonin are subjected to various stability tests

F = 5-FU; M = Melatonin; avg pk ht = average peak height

It can thus be observed that both 5-FU and melatonin are thermo- and photolabile, with the former stress resulting in more drug loss. The concentrations detected after 48 hour exposure to these stresses were relatively high. In contrast, drug loss in the presence of acid and base is very significant, with the former exerting a greater effect. In an acidic medium, 5-FU is believed to undergo keto-enol tautomerism, which may account for the split peak observed. Except for this split peak, 5-FU and melatonin were sensitively and accurately detected after each stability test. The lack of any appreciable drug loss after a period of two weeks suggests that, if stored adequately and with minimal exposure to heat and light, the 5-FU and melatonin solutions that will be used experimentally will have an adequately long shelf life.

2.5.3.9 Intermediate precision

Both intra- and inter-day analyses were run, in order to determine this parameter. The % RSD values were determined and assessed.

2.5.3.9.1 Intra-day precision

Six calibration solutions were analysed as described in 2.5.3.2 and 2.5.3.5. The first assay was run in the morning and the second in the evening. The % RSD values are given in Table 5.

Table 5: Intra-day precision results for solutions of 5-FU and melatonin when analysed by HPLC

	Morning assay		**Evening assay**	
Concentration (mg/ ml)	**% RSD (5-FU)**	**% RSD (Melatonin)**	**% RSD (5-FU)**	**% RSD (Melatonin)**
0.0275	1.477463	1.098901	1.147978	1.182287
0.01375	1.790069	1.264271	1.709916	1.194518
0.006875	2.58515	3.160397	1.264271	0
0.0034375	2.474358	2.249417	0	2.249417
0.00171875	0	0	0	0
0.00085938	0	0	0	0

As shown in the above table, all the % RSD values obtained fall below the limit of 4% as set in our laboratory, and most meet the USP's criterion of being less than 2% (USP, 2005). The intra-day results thus show acceptable precision.

2.5.3.9.2 Inter-day precision

These assays were run over the course of three days. The results are provided in the next table.

Table 6: Inter-day precision results for solutions of 5-FU and melatonin when analysed by HPLC

	Day 1		Day 2		Day 3	
Concentration (mg/ ml)	**%RSD (5-FU)**	**% RSD (Melatonin)**	**%RSD (5-FU)**	**% RSD (Melatonin)**	**%RSD (5-FU)**	**%RSD (Melatonin)**
0.0275	1.477463	1.098901	1.322176	1.2642707	1.137369	0.6711409
0.006875	2.58515	3.160397	1.24608	0	0.643885	0
0.0034375	2.474358	2.249417	2.439508	2.2494166	0	0
0.00171875	0	0	0	0	2.547134	0

These results also all fall within the limit of 4%, and many of the results show no relative deviation at all. This reflects that the HPLC method developed shows acceptable inter-day precision and excellent repeatability.

2.6 Nuclear magnetic resonance spectroscopy study

Nuclear magnetic resonance spectroscopy (NMR) was used to confirm the results of the stability studies (see 2.5.3.8) that no chemical interaction exists between 5-FU and melatonin, such as complexation or the formation of reaction products. The principle behind NMR is that excitation of atoms, usually carbon-13 atoms or protons, is achieved by radiation in the radiofrequency range. This results in the spins of these atoms switching from being aligned to an external magnetic field, to being aligned against this (Watson, 2005). The frequency ranges necessary to produce excitation and the resulting splitting patterns are characteristic of certain functional groups. It is thus possible to elucidate the chemical structure of the molecule, the molecular fingerprint. This ability to obtain detailed information about the molecular structure of compounds is the major advantage of NMR, compared to other methods of quantitative analysis (Watson, 2005). Limitations of this method of analysis are: (i) the use of specialised, expensive equipment; and (ii) insensitivity, as very concentrated solutions of test solution are required (Watson, 2005).

Saturated solutions of 5-FU, melatonin, and a combination of both, were made up using dimethylsulphoxide (DMSO) as the solvent. These solutions were left to stand for 24 hours and subsequently subjected to ^{1}H-NMR analysis. The following spectra were obtained, which shall then be analysed in the ensuing tables.

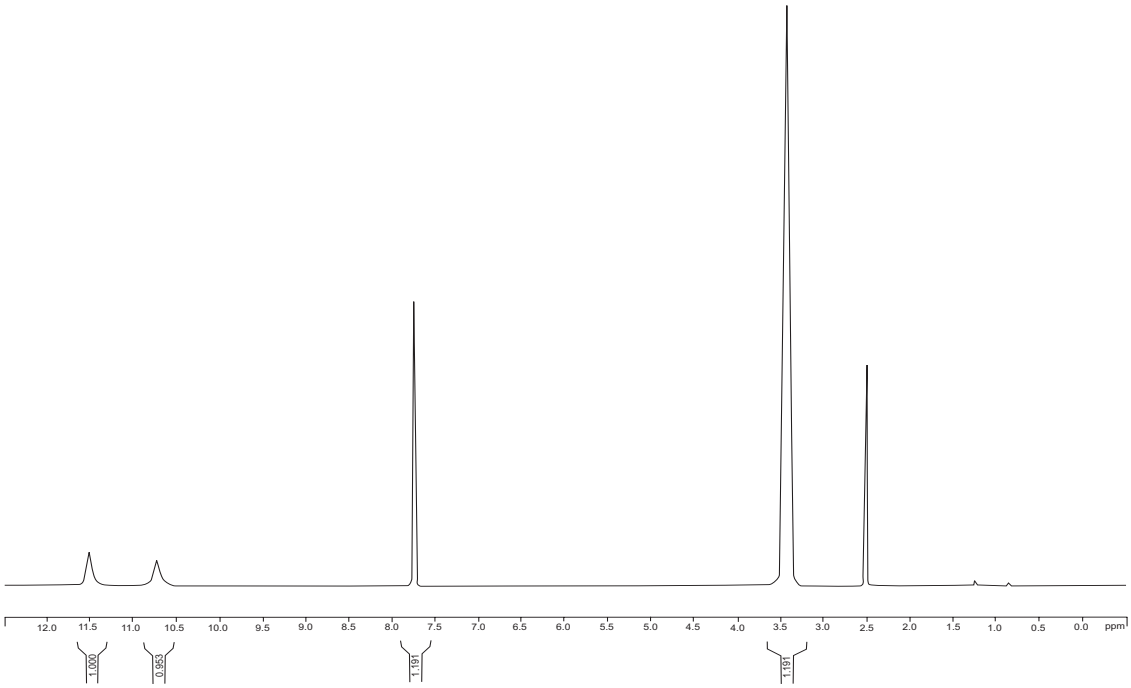


Figure 23: ^{1}H-NMR spectrum obtained for 5-FU, dissolved in DMSO

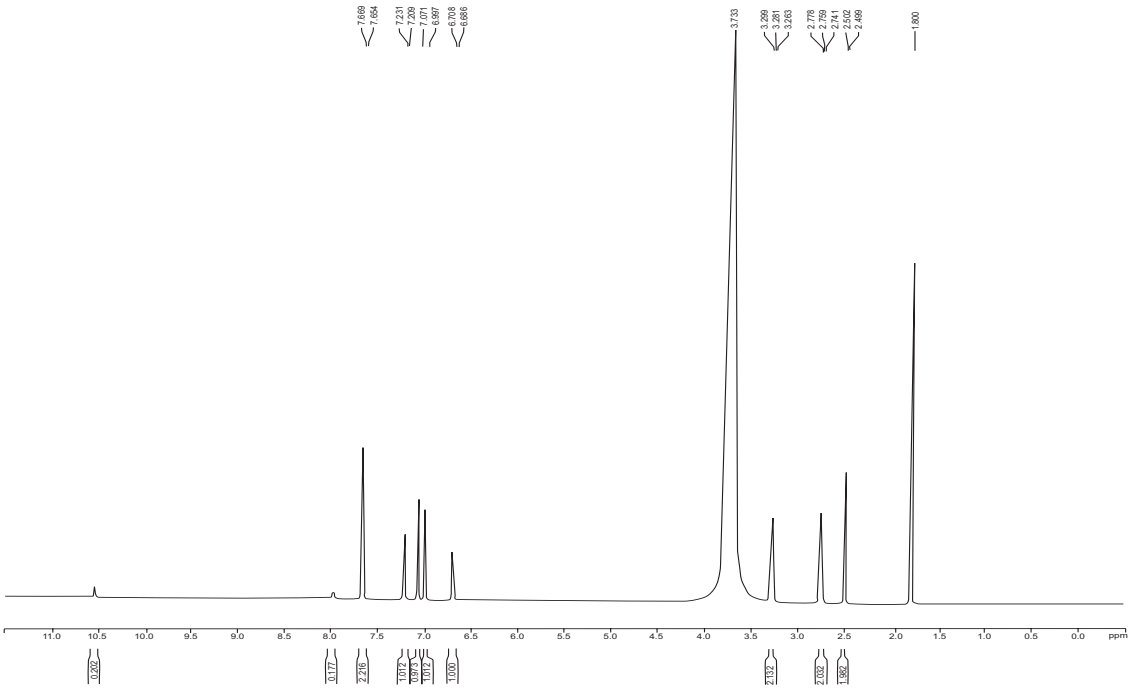


Figure 24: ^{1}H-NMR spectrum obtained for melatonin, dissolved in DMSO

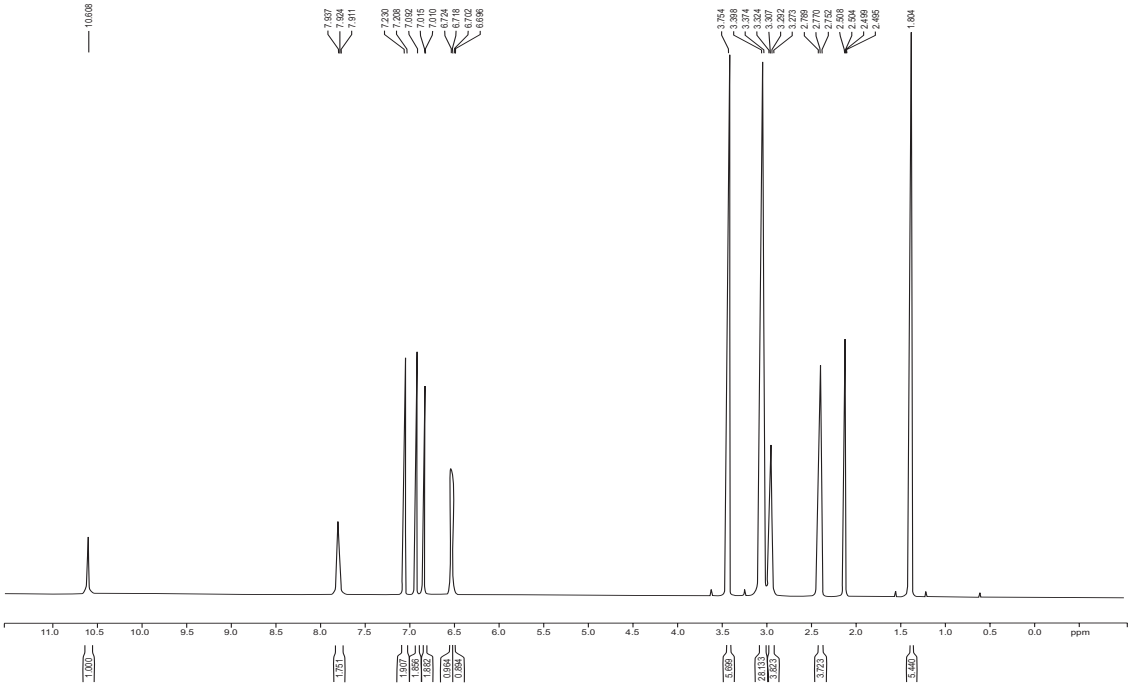


Figure 25: ^{1}H-NMR spectrum obtained for a combination of 5-FU and melatonin, dissolved in DMSO

The following tables give the approximate chemical shift values (δ ppm) in each spectrum, as shown in Figures 23-25, comparative literature reference values (http://www.cem.msu.edu/~reusch/OrgPage/nmr.htm; Watson, 2005; Evans, 1995), and the proposed functional group corresponding to that particular chemical shift. It is necessary to bear in mind the chemical structures of 5-FU and melatonin, which are reproduced below and on the next page. Due to the presence of π electrons in the pyrimidine ring of 5-FU and in the indole ring of melatonin, both compounds are considered aromatic. Melatonin moreover has protons attached to non-aromatic substituent groups. This is important as protons attached to aromatic and non-aromatic groups have different δ ppm. The number of protons appararent from each chemical shift are necessary in order to correctly assign a functional group to a particular shift (Watson, 2005).

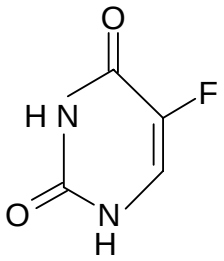


Figure 26: The chemical structure of 5-FU

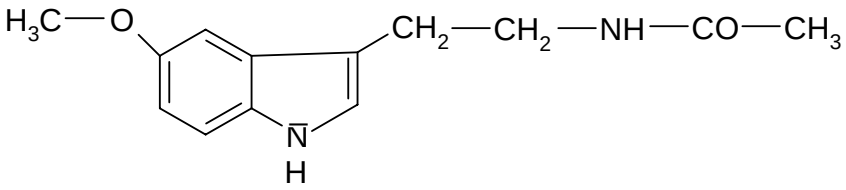


Figure 27: Chemical structure of melatonin

Table 7: Chemical shift analysis of 5-FU, in DMSO, subjected to ^{1}H-NMR

Approximate chemical shift (δ ppm)	Reference value	Number of protons	Proposed functional group
2.5	2.58		(DMSO)
3.4			(Trace of water)
7.75	6.3-8.5	Doublet	-CH
10.7	End of range	Singlet	-NH
11.5	End of range	Singlet	-NH

Table 8: Chemical shift analysis of melatonin, in DMSO, subjected to ^{1}H-NMR

Approximate chemical shift (δ ppm)	Reference value	Number of protons	Proposed functional group
1.8	2.0	Singlet	CH_3-CO
2.5	2.58		(DMSO)
2.8	2.9	Triplet	Ring-CH_2-
3.3	3.3	Triplet	-CH_2-N-
3.4			(Trace of water)
3.75	3.3	Singlet	-OCH_3
6.7		Doublet	
7.0	6-9	Singlet	Indole ring (4H)
7.1		Singlet	
7.3		Doublet	
7.9	End of range	Singlet	-NH in ring
10.6	End of range	Singlet	-NH in side-chain

Table 9: Chemical shift analysis of 5-FU and melatonin, in DMSO, subjected to ^{1}H-NMR

Approximate chemical shift (δ ppm)	Reference value	Number of protons	Proposed functional group
1.7	2.0	Singlet	CH_3-CO (melatonin)
2.5	2.58		(DMSO)
2.8	2.9	Triplet	Ring-CH_2- (melatonin)
3.3	3.3	Triplet	-CH_2-N (melatonin)
3.75	3.3	Singlet	-OCH_3 (melatonin)
6.7		Doublet	
7.0	6-9	Singlet	Indole ring (4H)
7.1		Singlet	
7.2		Doublet	
7.65	6.3-8.5	Doublet	-CH (5-FU)
8.0	End of range	Singlet	-NH in ring (melatonin)
10.55	End of range	Singlet	-NH (5-FU/ melatonin)

From the above NMR analysis, it is evident that no chemical interaction occurs between 5-FU and melatonin. Figure 24 and Table 9 show that in the solution containing both drugs no new chemical structure is formed, as all the chemical shifts have been accounted for as being part of the structure of either 5-FU or melatonin.

2.7 Conclusions

A simple, inexpensive and sensitive isocratic HPLC method has been developed that allows for the separation and detection of 5-FU and melatonin. The resulting peaks are sharp, symmetrical and well-resolved. This method was validated according to ICH Guidelines (1996) and the USP (2005). It meets the criteria for specificity, linearity, accuracy and precision. A preliminary biological assay, similar to the one for which this HPLC method is intended for use, was performed. The drug concentrations obtained after this biological

application fall within the calibration range selected and adequate recovery of the drugs was demonstrated. It was found that an unknown endogenous compound in the biological matrix elutes at the same time as 5-FU, but the simple subtraction of peak heights allows for the measurement of 5-FU concentration to still be possible and accurate.

Various stability-indicating studies were performed, and it was found that solutions of 5-FU and melatonin are thermo- and photolabile, and that 5-FU undergoes a possible tautomerisation reaction in an acidic medium. These findings have implications for the optimal storage of both drugs. Importantly, there is no direct chemical interaction between the drugs, as determined by NMR analysis. The intended biological application of this HPLC method is the focus of the next Chapter.

CHAPTER 3

THE EFFECTS OF 5-FLUOROURACIL AND MELATONIN ON THE ACTIVITY OF HEPATIC CYTOCHROME P450 *IN VITRO* AND *IN VIVO*

3.1 Introduction

The cytochrome P450-dependent monooxygenase system (CMOS) is a drug-metabolising enzyme system found in the smooth endoplasmic reticulum (Kane and Kumar, 2005). It has at least three components (Lu and Coon, 1968; Coon and Lu, 1969; cited in Van der Hoeven and Coon, 1974); namely: (i) the phospholipid phosphatidylcholine (Strobel *et al*, 1970), (ii) the haeme protein CYP450, and (iii) NADPH-CYP450-reductase (Van der Hoeven and Coon, 1974; Kane and Kumar, 2005). The latter is responsible for transferring electrons from NADPH to CYP450, thereby reducing the latter (Kane and Kumar, 2005).

This system is also termed the "mixed-function oxidase system", and has an important role to play in the phase I metabolism of both xenobiotics and endogenous compounds (Kane and Kumar, 2005). It consists of many tightly-bound enzymes (Hashimoto *et al*, 1962), of which CYP450 is one. CYP450 is a terminal oxidase (Mailman *et al*, 1975) and activates oxygen (Imai and Mason, 1971); it is involved in numerous monooxygenase reactions and has unusual spectral properties (see 3.2) (Imai and Sato, 1967). Phase I metabolism involves the oxidation, hydrolysis or reduction of lipophilic compounds in order to increase their hydrophilicity (Kane and Kumar, 2005). It has been found that NADPH-CYP450-reductase, in particular, hydroxylates a wide range of substrates, including alkanes and fatty acids (Van der Hoeven and Coon, 1974). The similarity between these otherwise very different substrates is the presence of an hydrophobic moiety (Jefcoate *et al*, 1969). It has been concluded by Van der Hoeven and Coon (1974) that neither phosphatidylcholine nor NADPH-CYP450-reductase is responsible for the substrate specificity that CMOS demonstrates.

Toxic compounds subjected to metabolism may be rendered non-toxic and unreactive compounds may be metabolised to products that are reactive or carcinogenic. An example of the latter is the activation of benzo[a]pyrene, a chemical present in cigarette smoke, to a secondary metabolite that is carcinogenic. The oxidative reactions in phase I metabolism may give rise to reactive oxygen species (ROS) which contribute to oxidative stress and which have deleterious effects on cells (see Chapter 8). CMOS is most active in the liver, but also occurs in the gastrointestinal mucosa, lung and skin (Kane and Kumar, 2005). CMOS can metabolise an extremely wide variety of substrates and can be induced by numerous

compounds (Haugen *et al*, 1975). CYP450 in the adrenal cortex is involved in the hydroxylation of steroids (Cooper *et al*, 1965; Estabrook *et al*, 1963; Remmer *et al*, 1966), for example. The enzyme is known to occur even in some bacteria (Leibman *et al*, 1969; Cardini and Jurtshuk, 1968).

Other enzymes and enzyme systems, besides CMOS, involved in phase I metabolism and which are both also situated in the smooth endoplasmic reticulum, are the flavin-containing monooxygenase system and prostaglandin-H synthase. The former oxidises nicotine and other amines, while the latter, besides its involvement in the arachidonic acid pathway, metabolises 2-naphthylamine, a carcinogenic ingredient of dyes (Kane and Kumar, 2005).

The primary metabolites obtained from phase I metabolism are subsequently subjected to phase II metabolism, in which these are converted into hydrophilic secondary metabolites that can be excreted from the body via bile, urine or faeces. Phase II metabolism involves glucuronidation, methylation, sulphation or glutathione conjugation reactions (Kane and Kumar, 2005).

It is well known that many substrates of CMOS either inhibit or enhance the metabolism of other substrates or of each other by the enzyme system (Leibman *et al*, 1969; Rubin *et al*, 1964; Anders, 1968; Imai and Sato, 1968). For example, if two barbiturates, such as amobarbital and hexobarbital, are added to CYP450, competitive inhibition occurs. If the second compound is from a different class of drugs, for example isooctane, the inhibition that occurs due to the barbiturate is noncompetitive (Leibman *et al*, 1969).

It has been shown that the food fed to laboratory animals can influence the activity of certain hepatic enzymes (Brown *et al*, 1954). Seasonal differences may also exert an effect on microsomal composition (Garfinkel, 1958).

3.2 Spectral properties of CYP450

CYP450 is a pigment named by Omura and Sato (1964a, b). It was discovered due to the significant peak that occurs at 450 nm, in the blue portion of the UV spectrum (Schenkman and Sato, 1968), when carbon monoxide (CO) is added to liver microsomes in the reduced state (Garfinkel, 1958; Klingenberg, 1958; Mailman *et al*, 1975). In the oxidised state, CYP450 binds to various types of ligands, resulting in two characteristic types of spectra. Type I spectra result from lipophilic interactions that are non-specific in nature and which do not involve the haeme present in CYP450; while type II spectra are due to interactions with some nitrogen-containing bases, which result in the formation of ferrihaemochrome moieties

(Mailman *et al*, 1974, 1975; Schenkman *et al*, 1967). Type I spectral changes have a characteristic peak at 390 nm and a trough at 420 nm (Schenkman *et al*, 1967; Schenkman and Sato, 1968). An example of a type II spectral change is that which occurs as a result of amines displacing CO from its binding site on CYP450 (Schenkman *et al*, 1967). Type II spectra have been further subdivided, into type (a), which shows a maximum absorption between 392-427 nm; and type (b), which peaks between 410-432 nm (Jefcoate *et al*, 1969).

These different spectral changes have led to the classification of ligands based on the spectral profile these induce upon binding to CYP450 (Jefcoate *et al*, 1969); an example of a type I ligand is hexobarbital, and a type II ligand is nicotine (Schenkman *et al*, 1967; Schenkman and Sato, 1968). Type I ligands are all substrates of CMOS, whereas the only known substrate in the type II group is aniline (Schenkman, 1970). There is a proportional relationship between the activity of CYP450 and the existence of type I spectral changes (Schenkman, 1970). Type II compounds can, in addition to inducing type II spectral changes, induce type I changes (Schenkman, 1970). Cytochrome P420 (CYP420) exhibits spectral profiles that, unlike those obtained for CYP450, are normal for haemoproteins (Imai and Sato, 1967; Omura and Sato, 1964a); only one Soret band, for example, is obtained for CYP420 whereas two occur for CYP450. When membrane-bound CYP450 is converted to CYP420, its anomalous spectral properties thus disappear. The electron spin states of the haemoprotein in CYP420, however, retain their unusual properties (Imai and Sato, 1967a; Mason *et al*, 1965).

Small quantities of CYP420 are also present in preparations of CYP450; the former is the reduced form of CYP450 and is so named as it shows an absorption peak at around 420 nm. CYP420 is oxidised to CYP450 in the presence of CO (Van der Hoeven and Coon, 1974; Omura and Sato, 1962) and the size of the subsequent peak at 450 nm, upon spectrophotometric analysis, is thus an indication of the extent to which the pigment is in its reduced form (Omura and Sato, 1962). Protein synthesis is required for the subsequent change in absorption maximum from 420 nm to 450 nm, as well as for any spectral changes that occur due to the addition of inducing agents (Kuntzman *et al*, 1967; Levin and Kuntzman, 1969). CYP450 has also been termed "CO-binding pigment" (Omura and Sato, 1964a; Garfinkel, 1958; Levin and Kuntzman, 1969) and it has been determined that CYP450 binds reversibly (Hashimoto *et al*, 1962) to 1 mole of CO for each mole of haeme (Mason *et al*, 1965). Exposing the CO-CYP450 complex to light at temperatures less than 0ºC causes CO to rapidly dissociate; reassociation occurs at a very fast rate in the dark (Imai and Mason, 1971).

The intensity of the spectral changes observed upon CYP450 binding to substrates is a function of protein concentration, as well as the type and concentration of substrate

(Schenkman *et al*, 1967). NADPH, required for CMOS activity, does not alter the affinity of substrates for CYP450 but does modify both types of the resultant spectral profiles (Schenkman *et al*, 1967).

It has been suggested that the term "CYP450" refer to the form of the pigment that is bound to microsomes, while "CYP420" is its solubilised form (Omura and Sato, 1962). CYP450 undergoes rapid solubilisation in the presence of oxygen and reduction with CO or dithionite should thus be performed under anaerobic conditions. UV absorption studies have shown that there is remarkable similarity between the absorption profiles of both the oxidised and reduced forms of CYP450, and that CYP420 belongs to the cytochrome *b* subclass (Omura and Sato, 1962).

Spectral and kinetic studies on the binding between different ligands and CYP450 have led to the suggestion that CYP450 is a dimer (Schenkman and Sato, 1968) and that its haeme moieties thus occur in pairs and interact (Jefcoate *et al*, 1969; Appleby, 1967). The binding to one by a ligand results in a weakening of the binding force to the other (Jefcoate *et al*, 1969). The nature of ligand binding to CYP450 sheds light on the structure of the latter. The ability of amines with long chains to bind to CYP450 suggests that there is, close to the porphyrin ring, a cavity of sufficient size to accommodate large hydrophobic moieties (Jefcoate *et al*, 1969). It is thought that both type I and II spectral changes occur as a result of changes to the electronegativity of the sixth ligand found in the haeme present in CYP450; such changes can occur due to changes in pH, and can also cause the wavelength at which maximum absorption occurs, to shift (Schenkman and Sato, 1968).

It is believed that one form of CYP450 is an apoenzyme, which requires the binding of a substrate, and another form, in equal quantity, is that which has not reacted with any substrate (Schenkman and Sato, 1968). Upon binding of substrate to the latter, and the formation of an enzyme-substrate complex with CMOS (Schenkman *et al*, 1967), this form of the enzyme is converted into the apoenzyme, thereby giving rise to a type I spectral change. Spectral studies have shown that, in the presence of substrates, one form of CYP450 disappears with a concomitant increase in the concentration of the other, substrate-bound form (Hildebrandt and Estabrook, 1969). Substrate-induced displacement of the abovementioned sixth ligand, from a lipophilic region of the apoenzyme, which may be the active site, is responsible for spectral changes (Schenkman and Sato, 1968). The abovementioned displacement also facilitates the movement of reducing equivalents, such as NADPH, to the haeme moiety; the freed sixth ligand is able to interact with oxygen or CO (Schenkman and Sato, 1968). This has given rise to the term "oxygen-activating enzyme" when referring to CYP450 (Schenkman, 1970).

The following scheme has been suggested as a model for the steps occurring in drug metabolism. The reaction between the sixth ligand and oxygen or CO, and the subsequent interaction with the substrate, as shown below, occurs at an extremely rapid rate, since it is not possible to isolate an oxygenated intermediate product (Schenkman and Sato, 1968). The reduction of CYP450 occurs at a slow rate, with 2-3 μmol being reduced/ min/ mg protein, at a temperature of 18ºC (Schenkman and Sato, 1968).

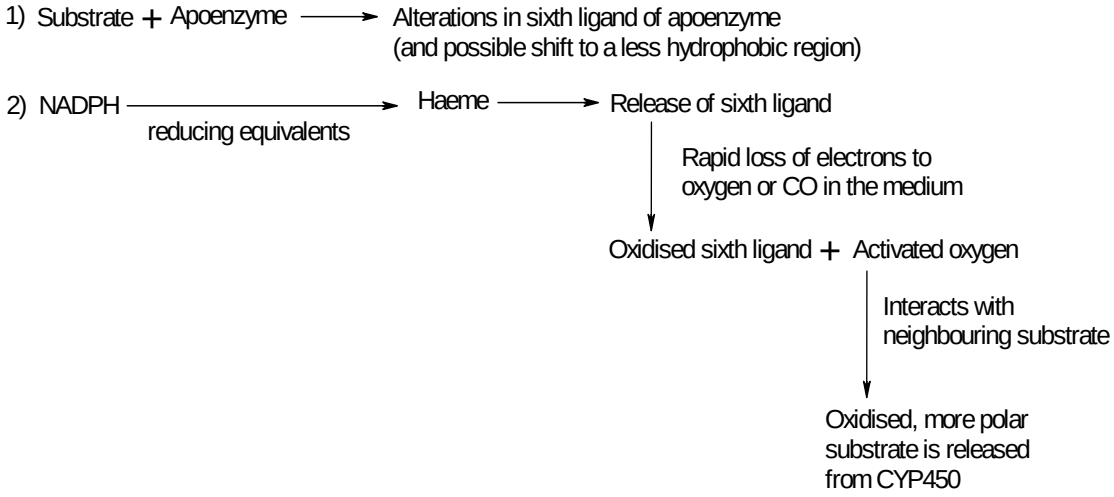


Figure 28: The steps of drug metabolism (Schenkman and Sato, 1968)

It is possible to determine whether drugs have an inductive or inhibitory effect on CYP450 by conducting UV spectrophotometric analyses; interactions between these drugs and CYP450 result in distinct spectral changes of CYP450, such as the heightening of peaks and deepening of troughs, as noted upon the addition of the inducing agent aniline (Remmer *et al*, 1965). Changes in the wavelength at which maximum absorption occurs can also result, with hexobarbital shifting this maximum to 415 nm (Schenkman and Sato, 1968).

There has been much debate as to whether there is a single form of cytochrome in CMOS that is capable of interacting with a wide variety of compounds and thereby exhibiting different spectral profiles, or at least two distinct forms of the enzyme that have greater substrate specificity (Hildebrandt *et al*, 1968; Leibman *et al*, 1969). It has been concluded that there are two forms of CYP450 haemoprotein, with absorption maxima close to each other, and that one of the difficulties of spectral studies with inducing agents is the inability to ascertain which form is being preferentially induced (Hildebrandt and Estabrook, 1969). Interconversion between the different forms of CYP450 is known to occur (Hildebrandt and Estabrook, 1969; Schenkman, 1970) and be dependent on pH (Imai and Sato, 1967). The binding affinities of the different forms for CO is also dependent on pH (Imai and Sato,

1967). These forms exhibit different reduction-oxidation potentials (Imai and Sato, 1966, 1967).

It is believed that the haeme moiety of reduced CYP450 occurs in a hydrophobic region of the enzyme, since binding by lipophilic ligands such as isocyanide result in the formation of the two forms of CYP450, which are spectrally different. This does not occur upon binding by hydrophilic compounds, such as CO (Imai and Sato, 1967). Further evidence for the hydrophobic nature of the haemoprotein location is the fact that only microsomal extraction procedures that destroy the lipid-rich membranes of the endoplasmic reticulum allow for the isolation and solubilisation of the haemoprotein (Imai and Sato, 1967; Omura and Sato, 1964a). It is this hydrophobic region surrounding the haemoprotein, or alternatively a particular conformation of proteins that this maintains, that may be responsible for the anomalous spectral properties of CYP450 (Imai and Sato, 1967a, 1967b).

Figure 29, below, is based on a scheme developed by Leibman *et al* (1969), which summarises the type I and type II interactions with the different forms of the haemoprotein of CYP450, which have been designated CYP420 and CYP395. The latter, when interacting with type II substrates, forms complexes that exhibit spectral profiles similar to CYP420. A type II spectrum thus occurs, with an increase in the absorption peak obtained at 425 nm, and a decrease in the absorption at 395 nm. Likewise, CYP420 can interact with type I substrates, thereby resulting in the formation of complexes that have similar spectral characteristics to CYP395. The enzyme-substrate complexes of both forms of CYP450 can interconvert. Once a threshold quantity of CYP420 has been converted to a CYP395-substrate complex, all the haemoprotein present is able to readily interact with type II substrates (Leibman *et al*, 1969). This scheme illustrates the complexity of CYP450 functioning.

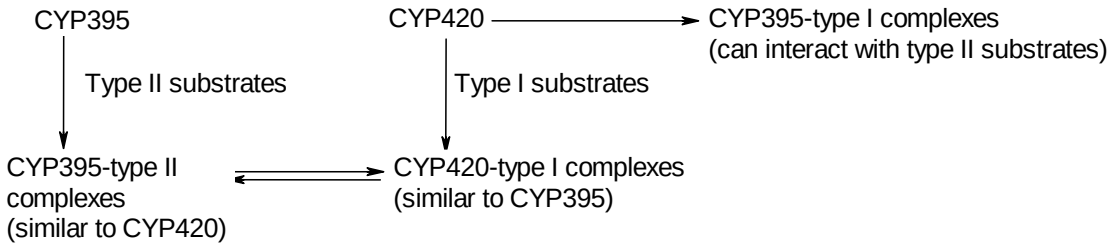


Figure 29: The interconversions and reactions of the different forms of CYP450, giving rise to Type I and II spectral changes (Leibman *et al*, 1969)

3.3 The nature and functions of hepatic microsomes

Microsomes, which contain CYP450 (Van der Hoeven and Coon, 1974), constitute 10-13% of the total liver and are a major component of the endoplasmic reticulum (Claude, 1941; Kamath *et al*, 1971). The endoplasmic reticulum consists of smooth vesicles, which do not have ribosomes, as well as rough vesicles, which contain ribosomes (Palade and Siekevitz, 1956). The smooth component can be further subdivided into two types (Glaumann, 1970). All three kinds of membranes (i.e. smooth I, smooth II and rough) have certain similarities, such as the same composition of phospholipids. An important difference is in microsomal enzyme content; smooth II membranes have high CYP450 and cytochrome b_5 levels but low quantities of other microsomal enzymes (Dallner, 1963; Glaumann, 1970). This highlights the heterogeneous composition of membranes of the endoplasmic reticulum (Glaumann, 1970).

Besides their role in the metabolism of xenobiotics and endogenous compounds by containing and regulating the levels of various enzymes, microsomes also have important roles in the synthesis of proteins and lipids; and in the functioning of electron transport chains, which leads to the generation of energy in the form of adenosine triphosphate (Kamath *et al*, 1971). It has been suggested (Glaumann, 1970) that in certain pathological conditions, there could be an alteration in the velocity at which certain microsomal membranes sediment, when in the presence of cations (see 3.4), and that this would alter the relative distribution of the different types of membranes in the endoplasmic reticulum. Treatment with the CYP450-inuducing agent 3-methylcholanthrene has been found to have the potential to induce widespread microsomal aggregation (Glaumann, 1970).

3.4 Isolation of microsomes

During the process of homogenisation, the endoplasmic reticulum is subjected to fragmentation (Palade and Siekevitz, 1956), thereby liberating microsomes. The average size of microsomes is between 50-300 nm (Palade, 1958) and centrifugation at high velocities for a prolonged period of time is thus required in order to separate them from mitochondria and nuclei (Kamath *et al*, 1971). These authors have developed a simple, less time-consuming, more convenient method for the isolation of microsomes, which is based on the ability of the latter to bind to calcium ions (Ca^{2+}) and results in a greater yield of microsomes being isolated (Kamath *et al*, 1971). This method, modified by Schenkman and Cinti (1972), allows for the isolation of microsomes within 1.5 hours; double this time is required if the conventional, calcium-free methods are used (Schenkman and Cinti, 1972).

3.4.1 Isolation of CYP450

The CYP450 that has been isolated from hepatic microsomes appears to be bound to the microsomal membrane (Coon and Lu, 1969; Lu *et al*, 1969; Van der Hoeven and Coon, 1974) and has been purified up to a concentration of 17.5 nmoles/ mg protein (Van der Hoeven and Coon, 1974). Numerous procedures to isolate CYP450 have been published (Van der Hoeven and Coon, 1974; Mailman *et al*, 1975). These are based on the initial work conducted by Dallner (1963) and Rothschild (1963), which used ultracentrifugation as a method of separation; Dallner (1963) included the use of cesium ions to enhance the process of separation (Mailman *et al*, 1975).

Common features of the abovementioned various methods of isolating CYP450 include: (i) several steps of centrifugation at different velocities, as opposed to the traditional method of ultracentrifugation at 105 000g (Kamath *et al*, 1971), in order to sediment other cellular components. Most modern methods of differential centrifugation are based on the procedure developed by Schneider and Hogeboom (1950) (Cinti *et al*, 1972); and (ii) the addition of Ca^{2+}, in the form of calcium chloride ($CaCl_2$), to result in the final precipitation of CYP450 (Van der Hoeven and Coon, 1974; Kamath *et al*, 1971). This precipitation of microsomes has been found to be irreversible (Schenkman and Cinti, 1972). The presence of other cations, such as K^+ and Mg^{2+}, does not alter the ability of microsomes to bind to Ca^{2+} and thus aggregate (Kamath *et al*, In Preparation; cited in Kamath *et al*, 1971). The presence of these other cations is, in fact, necessary in obtaining a yield of microsomes and may contribute to maintaining the structural integrity of the latter (Korner, 1961; Hultin *et al*, 1961; Kamath *et al*, 1971). At concentrations above 2 mM, Ca^{2+} and Mg^{2+} saturate the sites in the endoplasmic reticulum membrane to which cations bind; it has been suggested that there is a competitive relationship between these ions for binding to secondary phosphate and immidizolium functional groups in microsomes (Carvalho *et al*, 1963; Schenkman and Cinti, 1972).

Binding studies have shown that Ca^{2+} binds to the membranous, lipoprotein-containing part of microsomes, rather than to the portion containing ribosomes (Kamath *et al*, 1971), and also that this binding occurs more to microsomal particles derived from the smooth, rather than the rough, endoplasmic reticulum (Kamath *et al*, 1971; Dallner, 1963). Varying the concentration of cations present influences the purity of the microsomal fraction that is eventually isolated (Mailman *et al*, 1975).

Cinti *et al* (1972) have studied the enzyme kinetics of the CYP450 isolated as described above, in order to determine whether the addition of Ca^{2+} has any effect on the activity of mixed function oxidases. These authors studied the major types of oxidative reactions,

namely, aromatic hydroxylation, *O*-dealkylation, side-chain oxidation and *N*-dealkylation. It was shown that the addition of Ca^{2+} does not alter the activity of CYP450 or any of the other components of CMOS. The result of Ca^{2+} addition is the induction of microsomal sedimentation (Cinti *et al*, 1972), which allows for lower centrifugation speeds (Schenkman and Cinti, 1972). The only difference in these microsomes is the lack of ribosomes present; these occur freely, instead of being bound to membranes of the endoplasmic reticulum (Schenkman and Cinti, 1972). This is in contrast to the effects of Ca^{2+} on other types of cell membranes, which are wide-spread, including a disruption of ion-channel transport and inhibition of the activity of Na-K-ATPase (Skou, 1965; Schenkman and Cinti, 1972). Compared to the aggregation of microsomes, Ca^{2+} addition has the opposite effect on nuclei, allowing for their de-aggregation and isolation in liver homogenate (Cinti *et al*, 1972; Schneider and Peterman, 1950). The presence of Ca^{2+} does not alter the (haemo)protein content of the microsomes (Schenkman and Cinti, 1972).

An important site of protein synthesis is in microsomes (Zamecnik and Keller, 1954; Allfrey *et al*, 1953). It was shown that the microsomes isolated by Ca^{2+}-induced sedimentation do not display any significant difference in this process (Kamath *et al*, 1971).

Van der Hoeven and Coon (1974) have shown that perfusion of livers with pyrophosphate prior to homogenisation enhances the purity as well as increases the recovery of the isolation process by removing interfering haemoglobin. The use of polyethylene glycol separates CYP450 and NADPH-CYP450-reductase from phosphatidylcholine (Van der Hoeven and Coon, 1974).

Stanton and Khan (1976) have developed a higher-yield procedure for the isolation of CYP450, in which the specific activity of CYP450 from uninduced livers is increased from 0.4 nmoles/ mg protein to 4.0 nmoles/ mg protein (Stanton and Khan, 1976). Isolation of microsomes was achieved using Ca^{2+}-induced microsomal sedimentation (Schenkman and Cinti, 1972), differential centrifugation at different speeds for varying periods of time, and final resuspension of the microsomal pellets in phosphate buffer at pH 7.4. More than 90% of the NADPH-CYP450-reductase and 60% of the cytochrome b_5 present were removed by treatment of the microsomal pellets with Subtilisn VII, a protease (Stanton and Khan, 1976), in a time-dependent manner (Comai and Gaylor, 1973). Cytochrome b_5, also a haemoprotein (Levin and Kuntzman, 1969), is known to be present in preparations of liver homogenate (Chance and Williams, 1954) and exhibits an absorption maximum at 560 nm (Keilin and Hartree, 1940). Its presence, as well as the presence of residual haemoglobin, can interfere with the spectral analysis of CYP450 and can result in the removal of CYP450 (Stanton and

Khan, 1976; Comai and Gaylor, 1973). Treatment with phospholipase A removes the phospholipid component of CMOS (Comai and Gaylor, 1973). Titration with polyethylene glycol further purifies CYP450 and column chromatography is used for the final separation (Stanton and Khan, 1976).

Van der Hoeven and Coon (1974) used gel permeation chromatography to determine the apparent molecular weight of CYP450, which was found to be 280 000. The two major polypeptide components of CYP450 have molecular weights of 47 000-49 000 and 52 000-53 000 respectively (Van der Hoeven and Coon, 1974).

It has been found by these authors that, experimentally, in comparison to the intact CMOS, (partially-) purified CYP450 is more active, in terms of having increased substrate turnover. Van der Hoeven and Coon (1974) speculate that this increased turnover may be due to the purification of an additional factor necessary for optimal enzyme activity, or the removal in the purification process of compounds that inhibit CYP450 activity. There is no reported difference in substrate specificity (Van der Hoeven and Coon, 1974).

3.4.2 Properties of the different forms of CYP450

The authors at that time remained uncertain of the existence of different forms of CYP450 (Van der Hoeven and Coon, 1974). These authors have subsequently concluded (Haugen *et al*, 1975) that multiple forms of CYP450 exist (Comai and Gaylor, 1973), since animals treated with different CYP450-inducing drugs demonstrate different microsomal enzyme activities (Gillette, 1966, 1969; Gelboin, 1967; Conney, 1967). Haugen *et al* (1975) have separated and purified the different forms of CYP450 from rats treated with either phenobarbital or β-naphthoflavone, which induce CYP450. Using electrophoresis, four different forms of CYP450 were detected, with the different inducers selectively inducing different forms. These authors have shown that the different forms of the enzyme have different physical properties, such as molecular weight, as well as catalytic abilities (Haugen *et al*, 1975). The various forms of CYP450 may all bind to the same substrates, but the efficiency in which these are hydroxylated differs (Haugen *et al*, 1975).

The different types of CYP450 exist in equilibrium and their relative proportions can be altered by any changes in the environment, such as pH (Imai and Mason, 1971; Jefcoate and Gaylor, 1969) and temperature (Imai and Mason, 1971). The induction of CYP450 by polycyclic aromatic hydrocarbons results in a type of CYP450 that is termed "CYP_I-450" or "CYP448", and its spectra demonstrate less type I binding than CYP450 (Mailman *et al*, 1975). It has been noted that separation of CYP450 from the CMOS structure and its

solubilisation results in significant changes to the spectral profile of CYP450 (Omura and Sato, 1962).

Comai and Gaylor (1973) have concluded, based on the findings of paramagnetic potentiometric studies (Waterman and Mason, 1972), that two forms of CYP450 have haemoproteins that exhibit low spins, and the third form exhibits a high spin state.

3.4.3 Use of CYP450-inducing agents

Stanton and Khan (1976) purified CYP450 from uninduced livers, pointing out that treatment of test animals with drugs that induce CYP450 besides increasing the concentration of CYP450 that is isolated (Conney, 1967), alters the properties of CYP450 (Comai and Gaylor, 1973; Sladek and Mannering, 1966; Thomas *et al*, 1976) and increases the size of the liver (Conney, 1967). The use of inducing agents, such as ethanol, phenobarbital and 3-methylcholanthrene, has also been found to alter the relative proportions (Comai and Gaylor, 1973), spin states (Peisach *et al*, 1972; Nebert and Kon, 1973) and extinction coefficients of the different forms of CYP450 (Omura and Sato, 1964a; Hildebrandt and Estabrook, 1969). There are also effects on other microsomal constituents. The administration of the barbiturate phenobarbital results in an increase in the concentrations of certain peptide components of CYP450 (Van der Hoeven and Coon, 1974). Phenobarbital also preferentially increases phospholipid, RNA and cholesterol levels in rough and smooth I endoplasmic reticulum, while 3-methylcholanthrene has no effect on these levels but affects benzpyrene hydroxylase, a component of the NADPH-dependent electron transport chain (Glaumann, 1970). The use of different types of inducing agents also results in different spectral changes (Remmer *et al*, 1966; Hildebrandt and Estabrook, 1969).

Although it might be more accurate to use uninduced livers, the difficulties associated with this include a low yield of CYP450 and the subsequent use of large amounts of material in order to obtain an acceptable yield (Stanton and Khan, 1976). Another challenge that induction presents is the finding that different inducing agents increase the heterogeneity of CYP450 even further, and that this is exacerbated by the method of CYP450 isolation and even the species and strain of animal used (Mailman *et al*, 1975). These authors have shown that the CYP450 isolated from smooth endoplasmic reticulum has different properties from that found in rough endoplasmic reticulum (Mailman *et al*, 1975). Using uninduced livers is associated with the presence of a greater variety of CYP450 forms, and it has been suggested that the different forms of the enzyme are responsible for preferentially metabolising different groups of substrates (Mailman *et al*, 1975).

Further support for the existence of different forms of CYP450 is the finding that various subfractions display different spectral characteristics and wavelength maxima (Mailman *et al*, 1975), as well as different binding affinities for CO and cyanide. The latter property has been used as a means of separation of the different forms of CYP450 (Comai and Gaylor, 1973). It has been noted that "cross-contamination" of the different forms of CYP450 in various subfractions may occur during the extraction processes (Comai and Gaylor, 1973).

3.5 Differential centrifugation as a method of separation

It has long been established that centrifuging tissue extracts at a series of different velocities has the ability to separate various cellular components that are submicroscopic in nature (Claude, 1938a, 1938b, 1941; Stern and Duran-Reynals, 1939; Henle and Chambers, 1940) and which have been found to be mitochondria (Claude, 1940, 1941).

Centrifugation is a mechanical method of separation, and one of the challenges it faces is the resistance to disintegration offered by membranes, whether these are nuclear or cellular. Hepatic cell membranes are particularly weak, being easily broken by the slightest injury, while nuclear membranes demonstrate a much greater strength (Claude, 1941). Once membranes lyse, cellular contents are released and aggregates of granules that have the same chemical composition can form. The use of isotonic sodium chloride (NaCl) solutions in the formation of these aggregates is unsuitable, as the granules are unstable in this medium and rapidly break down (Claude, 1941). The use of sodium hydroxide (NaOH) or a phosphate buffer results in homogenous granular suspensions that are stable at 4ºC for a prolonged period of time (Claude, 1941).

It is necessary for centrifuging to be performed at 0-6ºC, using refrigerated solvents and equipment, in order to prevent the spontaneous degradation of the tissues being used (Claude, 1941). The initial centrifugation step is performed on tissue homogenate at a low velocity, such as 2400g. This results in the removal of cellular debris, nuclei, red blood corpuscles and coarse tissue fragments greater than 1-2 μm in diameter. The supernatant from this first step of centrifuging is termed the "tissue extract" and is subjected to further centrifuging. Centrifugation results in the sedimentation of particles based on particle size; larger particles and organelles sediment out before finer particles (Claude, 1941). The small granules that eventually sediment out are composed mainly of lipids and proteins. The lipid content is between 40-50%, and phospholipids constitute approximately three quarters of this (Claude, 1941). The major protein component of these particles is a ribose nucleic acid, which constitutes 10-15% of the total protein content. This ribonucleoprotein-phospholipid backbone of these granular particles is present in both tumour and non-tumour cells, and

occupies as much as 12.4% of the dry weight of cells (Claude, 1941). Claude (1941) concludes that the small isolated granular particles are mitochondria, or fragments of these. The larger granular particles are believed to be secretory granules, having a lipid content of 22-24% but being otherwise similar in content to the smaller particles (Claude, 1941). These larger particles have been identified as specialised mitochondria (Claude, 1941; Noël, 1923). It has been found that mitochondrial fractions of liver homogenate contain a major portion of cytochrome *c* (Schneider *et al*, 1948; Schneider and Hogeboom, 1950).

Further differential centrifugation of the initial tissue extract, at 18 000g for 2 hours, results in the isolation of three types of granules (Claude, 1941):
(i) the specialised mitochondria mentioned above, which sediment after 5 mins of centrifugation; these granules are yellow in colour, have a diameter of 0.5-3 μm and form 3.0% of the dry weight of liver tissue;
(ii) the small granules or mitochondria, having a diameter of 60-200 μm and sedimenting after 45-60 mins; these granules are transparent, form milky suspensions and constitute 4.6% of liver tissue; and
(iii) smaller particles, 40-60 μm in diameter, which sediment at a significantly slower rate after 2 hours, are red in colour and account for 6.8% of the mass of liver tissue. All three kinds of granules contain varying amounts of copper, which may function in cellular respiration (Claude, 1941).

Cinti *et al* (1972) have noted that the addition of 0.05 M sucrose and 8 mM $CaCl_2$, and the subsequent standing of the mitochondrion-free homogenate in ice for a period of 10 mins is sufficient to induce the microsomes to sediment, but that further centrifugation is required for the formation of a microsomal pellet.

The following diagram shows the appearance of a microsomal pellet after the process of differential centrifugation.

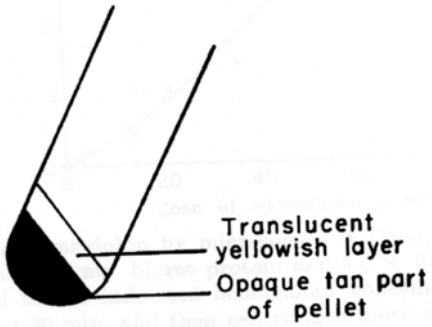


Figure 30: A diagram of the isolated microsomal pellet containing CYP450, obtained by Ca^{2+}-induced sedimentation and differential centrifugation (Garfinkel, 1958)

3.6 Experimentation

Hepatic microsomes containing CYP450 were isolated based on the method developed by Cinti *et al* (1972). The procedure used for the *in vivo* studies was modified slightly from that used *in vitro*, since it was found during the latter that the microsomal pellet obtained per rat was very small. The method was thus optimised to ensure a better yield. Using a larger volume of 8 mM Ca^{2+} achieved this. Protein determination was performed according to Lowry *et al* (1951). For the *in vitro* studies, the protein content of the initial homogenate was determined, whereas for the *in vivo* studies, that of the final isolated microsomal pellet was used.

It was decided not to use inducing agents, for the reasons mentioned in 3.4.3, and also to reduce the possibility of further drug-drug interactions between the inducing agent and either 5-FU or melatonin.

Spectral studies were performed to determine the absorption profile of CYP450, and to ascertain whether the addition of 5-FU or melatonin resulted in any significant changes to this. These studies were based on the experiments conducted by Remmer *et al* (1965).

3.6.1 *In vitro*

3.6.1.1 Materials, animals, reagents and equipment

Melatonin and 5-FU were purchased from the Sigma Chemical Corporation, St Louis, MO, USA. All the other chemicals used were obtained from local suppliers and were of the highest purity available. All solutions were prepared using deionised water (Milli R/Q System, Millipore®), and absolute ethanol at a final concentration of 0.67% v/ v was used as a co-solvent to dissolve melatonin.

Four male Wistar rats, each weighing between 250g and 300g, were used. Male rats were used since it has been noted that the extent to which substrates interact with hepatic microsomes varies between female and male rats (Schenkman *et al*, 1966). The animals were cared for and subjected to the conditions described in Appendix A.

An MSE18 high-speed centrifuge and a Julabo PC water bath (Labotec, South Africa) were used. The isocratic HPLC system used consisted of a Spectraseries UV100 detector with a deuterium lamp, a Spectraphysics Isochrom LC pump (Spectraphysics, San Jose, California, USA) and a Rikadenki chart recorder. Samples were injected from a 100 µL Hamilton syringe (Hamilton Bonaduz AG, Switzerland) into a 20 µL fixed loop Rheodyne injector (Rheodyne,

Cotati, California, USA). The column used was a C_{18}, 5 µm Waters Spherisorb analytical column (Waters Corporation, Milford, Massachusetts, USA), with dimensions of 250 mm X 4.6 mm (see Chapter 2).

3.6.1.2 Method

Four male Wistar rats were killed by cervical dislocation, the livers removed immediately, and perfused with ice-cold 0.9% NaCl to remove blood (Schenkman and Cinti, 1972). The livers were stored in liquid nitrogen at -70ºC until required.

Each liver was chopped on ice and a 10% w/ v mixture prepared using 0.25 M sucrose. The liver was mechanically homogenised and the protein content of this initial homogenate was determined using the method of Lowry *et al* (1951) (see Appendix B). The resulting mixture was centrifuged at 600g for 6 mins. This induces sedimentation of nuclei, erythrocytes, cellular debris and whole cells (Schenkman and Cinti, 1972). The precipitate was discarded and the supernatant centrifuged at 12 000g for 20 mins in a refrigerated MSE 18 high-speed centrifuge at 4ºC. This removes the mitochondria (Schenkman and Cinti, 1972). The precipitate was discarded and the supernatant treated with 1 ml of 8 mM $CaCl_2$; this was centrifuged at 27000g for 30 mins. The resulting pellet was re-suspended using 0.15 M KCl and vortexed. KCl was used to remove any remaining haemoglobin (Cinti *et al*, 1972) or proteins that may be present (Schenkman and Cinti, 1972). Haemoglobin is the most common compound to contaminate microsomal preparations, and is removed by the addition of ionic solutions (Garfinkel, 1958). The resulting suspension was centrifuged at 27 000g for 30 mins and the clear supernatant discarded. A pinkish microsomal pellet remained, which covered a transparent glycogen-containing pellet. Separation from the latter was achieved by gentle tapping of the centrifugation tube (Cinti *et al*, 1972). The presence of glycogen imparts turbidity to the suspension, which presents a problem if UV spectrophotometric analyses are to be performed (Schenkman and Cinti, 1972). The pellets were combined (Schenkman and Cinti, 1972) and sufficient 0.2 M sodium dihydrogen phosphate (NaH_2PO_4) buffer (Imai and Sato, 1967), at a pH of 7.8, was added to cover the pellets. The mixture was vortexed.

An aliquot of 50 µL of either 1.4 mM 5-FU, 1.2 mM melatonin, or both, were added to 100 µL of microsomal suspension, with controls receiving saline. The homogenate from each liver was divided into four groups: (i) 5-FU, (ii) melatonin, (iii) a combination of these, and (iv) a control. The assay was performed in triplicate for each group. These concentrations were used since it was found during the HPLC method validation procedure (see Chapter 2) that these result in convenient and acceptable peak heights. The resulting mixtures were vortexed, the tubes stoppered and wrapped in aluminium foil to prevent degradation of CYP450 and

incubated for 60 mins in a water bath at 37ºC. At the end of the incubation, an aliquot of 1 ml trichloroacetic acid (TCA) was added and the mixture centrifuged at 1200g for 20 mins. An aliquot of 20 µL of the supernatant was then analysed by HPLC, according to the method developed and validated in Chapter 2. A calibration curve was constructed and the concentration of drug metabolised per µg protein of initial homogenate per minute was determined.

3.6.2 *In vivo*

3.6.2.1 Materials, animals, reagents and equipment

Four groups, each containing five male Wistar rats, each weighing between 250g and 300g, were used. The animals were housed as described in Appendix A. All reagents and equipment were as described in 3.6.1.1.

3.6.2.2 Method

The four groups of rats were subjected to a five-day treatment protocol, as outlined in the table below. The injection volumes of saline, 5-FU and melatonin were 0.5 ml. All injections were given intraperitoneally. The control group received 0.9% NaCl at 09h00, 13h00 and 17h00. The 5-FU group received 1 mg/ kg 5-FU and the melatonin group received 1 mg/ kg melatonin at the same times. The third group of rats received both 1 mg/ kg of 5-FU and 1 mg/ kg melatonin at the same times. 5-Fluorouracil is routinely administered at a dosage of 10-30 mg/ kg/ day (D'Souza, 2003), and melatonin at a dosage of at least 5 mg/ kg/ kg (Tan *et al*, 1998a). The dosages used in the present study are sufficiently low as to result in minimal toxicity and are more representative of pharmacological doses.

Table 10: Five-day treatment protocol for rats

Group	**Dose of drug**	**Times of i/p administration**
Control	0.9% NaCl tds	09h00, 13h00, 17h00
5-FU	1 mg/ kg tds	09h00, 13h00, 17h00
Melatonin	1 mg/ kg tds	09h00, 13h00, 17h00
5-FU + Melatonin	1 mg/ kg 5-FU tds + 1 mg/ kg Melatonin tds	09h00, 13h00, 17h00

On the sixth day, the rats were killed and the livers perfused with ice-cold 0.9% NaCl. The livers were stored in liquid nitrogen at -70ºC until required. Microsomes were subsequently isolated as described in 3.6.1.2, except that 3 ml of $CaCl_2$ and a slightly greater volume of 0.2M NaH_2PO_4 buffer were used. The protein concentration of this final microsomal suspension was determined (Lowry *et al*, 1951) (see Appendix B). An aliquot of 50 µL of each drug, as described in 3.6.1.2, was now added to 200 µL of microsomal suspension. The drug concentrations and the rest of the procedure were as detailed in 3.6.1.2.

3.6.3 Spectral study

3.6.3.1 Materials, animals, reagents and equipment

All reagents used were as described in 3.6.1.1. A Spectra System UV 2000® UV spectrophotometer was used.

3.6.3.2 Method

Microsomes were isolated and 100 µL aliquots were incubated with 50 µL aliquots of 5-FU, melatonin, both, or water, as outlined in 3.6.1.2. An aliquot of 2850 µL water was added to each tube after incubation and the contents subjected to a running wavelength scan of between 200-800 nm. A blank of 3 ml HPLC-grade water was used. Oxidation of CYP420 to CYP450 in the presence of CO was not performed.

3.7 Results

The results of the *in vitro* and *in vivo* studies were analysed statistically using a one-way ANOVA, followed by Student-Newman-Keuls multiple-range comparison test (Zar, 1974). The Student's t-test was used to analyse the difference in protein content between the control and 5-FU groups *in vivo*.

3.7.1 *In vitro*

Table 11 on the next page reflects the average concentration of drug metabolised per µg protein of homogenate per minute.

Statistical analysis reveals no differences in the concentration of either drug that was metabolised when in the presence of the other.

Table 11: The average concentration of 5-FU and melatonin metabolised per µg protein of initial homogenate per minute

Group (n = 4)	**Concentration of drug metabolised (µg/ml)/ µg protein/ minute** (mean ±SEM)
5-FU	2.86 ± 0.48
Melatonin	4.47 ± 0.74 *
5-FU (when in combination with melatonin)	2.85 ± 0.47
Melatonin (when in combination with 5-FU)	4.47 ± 0.74 @

* $p<0.01$ compared to 5-FU; @ $p<0.01$ compared to 5-FU (when in combination with melatonin), after analysis by ANOVA followed by the Student-Newman-Keuls multiple-range test

The following Table shows the percentage of each drug subjected to metabolism. As shown, both drugs are metabolised in the 60 mins of incubation, almost to completion, and there are no significant differences in the extent of metabolism across the groups.

Table 12: The average percentage of 5-FU and melatonin metabolised in 60 mins of incubation with liver microsomes

Percentage of drug metabolised (%) (mean ± SEM) (n = 4)			
5-FU	**Melatonin**	**5-FU (when in combination with melatonin)**	**Melatonin (when in combination with 5-FU)**
99.33 ± 0.10	99.86 ± 0.01	99.28 ± 0.02	99.86 ± 0.01

3.7.2 *In vivo*

The following Table shows the average concentration of drug metabolised per µg protein per minute for each of the four groups of rats.

Table 13: The average concentration of 5-FU and melatonin metabolised per µg protein per minute in liver microsomes of rats treated for five days with either or both drugs

	Concentration of drug metabolised (ng/ml)/ µg protein/ minute (mean ± SEM)			
Five-day treatment group (n = 5)	**5-FU**	**Melatonin**	**5-FU (when in combination with melatonin)**	**Melatonin (when in combination with 5-FU)**
Control	22.30 ± 3.57	34.62 ± 5.54	22.27 ± 3.56	34.63 ± 5.53
5-FU	28.01 ± 6.37	43.55 ± 9.90	28.00 ± 6.38	43.55 ± 9.91
Melatonin	41.99 ± 18.82	65.19± 29.25	41.94 ± 18.83	65.20 ± 29.24
5-FU and melatonin	24.53 ± 14.18	38.01± 21.94	24.54 ± 14.20	38.03 ± 21.96

Statistical analysis shows no significant difference in the concentration of 5-FU metabolised/ µg protein/ minute in the presence of melatonin. Likewise, there is no difference in the concentration of melatonin metabolised/ µg protein/ minute in the presence of 5-FU. Neither 5-FU nor melatonin enhances or inhibits the activity of cytochrome P450.

Table 14 on the next page shows that there are no differences in the percentages of 5-FU and melatonin metabolised, and that almost 100% of each drug is metabolised in the 60 mins of incubation.

Table 14: The average percentage of 5-FU and melatonin metabolised in 60 mins of incubation with liver microsomes of rats treated for five days with either or both drugs

	Percentage of drug metabolised (%) (mean ± SEM)			
Five-day treatment group (n = 5)	**5-FU**	**Melatonin**	**5-FU (when in combination with melatonin)**	**Melatonin (when in combination with 5-FU)**
Control	99.69 ± 0.08	99.53 ± 0.03	99.56 ± 0.07	99.55 ± 0.03
5-FU	99.66 ± 0.01	99.62 ± 0.03	99.64 ± 0.06	99.63 ± 0.02
Melatonin	99.72 ± 0.06	99.51 ± 0.02	99.59 ± 0.13	99.53 ± 0.02
5-FU and melatonin	99.91 ± 0.10	99.55 ± 0.03	99.92 ± 0.24	99.59 ± 0.04

The average protein masses in the isolated microsomal pellets from the control and 5-FU-treated groups were compared. As shown in Table 15 below, the 5-FU-treated group exhibit significantly lower protein content.

Table 15: A comparison of the average protein mass in microsomes obtained from the control and 5-FU-treated groups

Group (n = 5)	**Average protein mass** (mg)
Control	1.37
5-FU	1.11 *

*p<0.1 compared to control, after analysis by the Student's t-test

3.7.3 Spectral study

The following figure shows the absorption profile of CYP450 when subjected to a wavelength scan, showing a peak at approximately 420 nm.

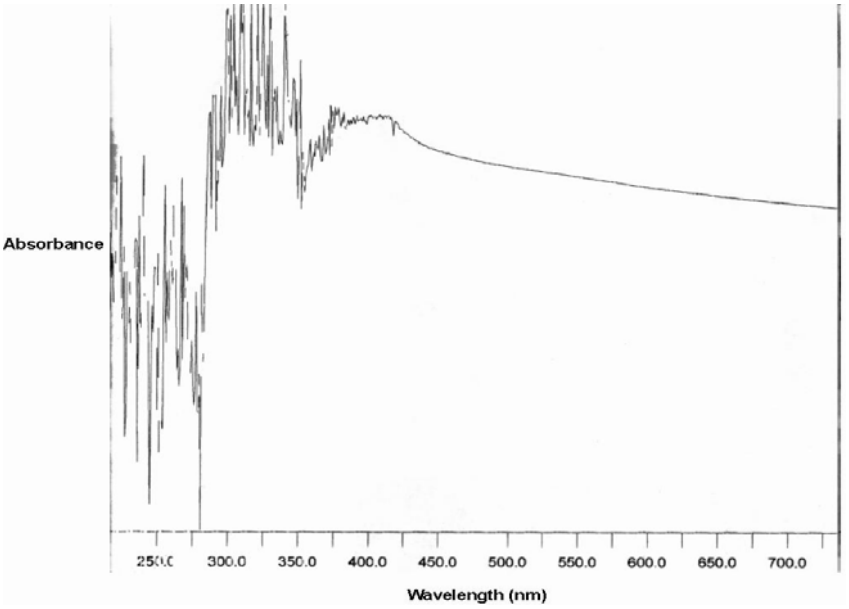


Figure 31: The absorption profile of CYP450 isolated in liver microsomes, when subjected to a wavelength scan by UV spectrophotometry

The absorption profiles obtained for microsomal samples injected with 5-FU or melatonin are given below and on the next page. These do not show any alteration in the position of the peak at 420 nm. The only difference between these profiles is that the one obtained upon the addition of melatonin does not show a small trough that occurs in the 420 nm peak; this trough is present in both the control and 5-FU-injected samples. The sample containing both 5-FU and melatonin also does not show any differences in the absorption profile.

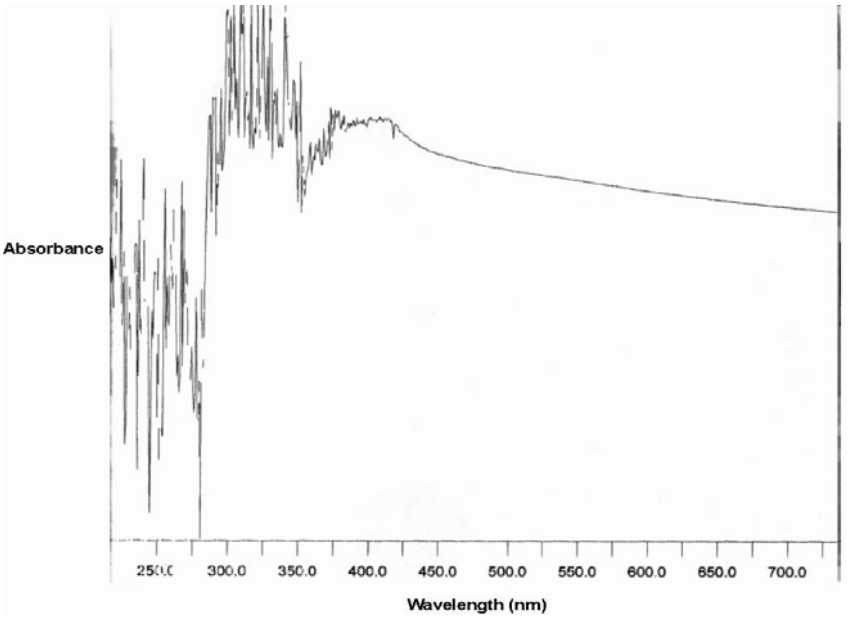


Figure 32: The absorption profile of CYP450 incubated with 1.4 mM 5-FU for 60 mins upon UV analysis

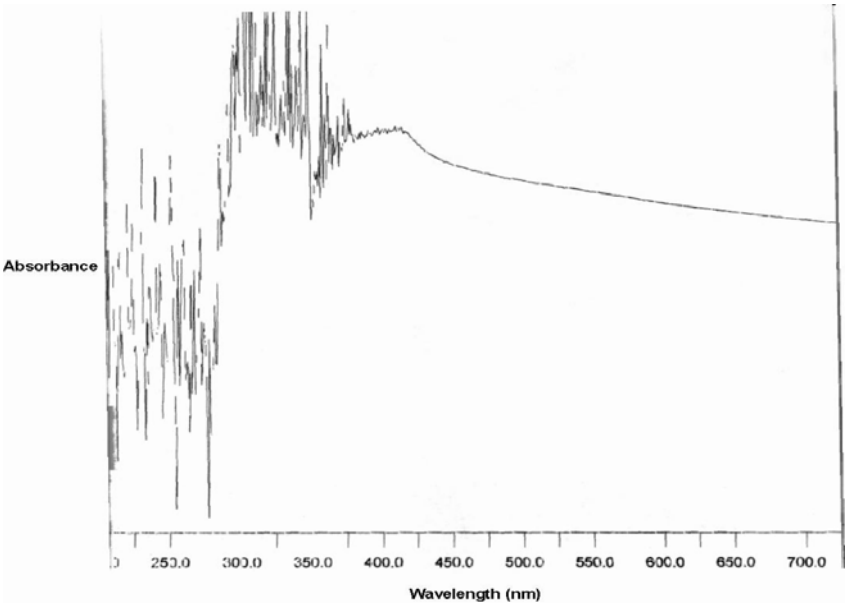


Figure 33: The absorption profile of CYP450 incubated with 1.2 mM melatonin for 60 mins upon UV analysis

3.8 Discussion

The results show that, in both the *in vitro* and *in vivo* studies, the rate of metabolism of 5-FU by CYP450 is not altered in the presence of melatonin, and likewise for melatonin in the presence of 5-FU. This is of significance as it suggests that there is no hepatic metabolic drug interaction between the two. The administration of melatonin thus does not enhance the metabolism of 5-FU, which may result in subtherapeutic concentrations of the latter, nor does it inhibit metabolism, which could potentially result in 5-FU toxicity. Tables 12 and 14 show that almost 100% of each drug is metabolised during the incubation, both *in vitro* and *in vivo*. This confirms that both 5-FU and melatonin are subjected to extensive first-pass hepatic metabolism. The co-administration of melatonin does not alter the percentage of 5-FU metabolised; and likewise 5-FU does not alter the extent to which melatonin is metabolised by CYP450.

As shown in Table 15, the average protein concentration in the microsomes from the rats treated with 5-FU is significantly lower than the average control value. As mentioned in 1.2.10.11, it has been shown by Tayek and Chlebowski (1992) that treatment with 5-FU for five days decreases total body protein production by 10%; the results in Table 15 thus confirm that the antimetabolite has a significant effect on protein levels. Since enzymes are proteins, it might be expected that this lower protein content would lead to decreased metabolic activity. It was found, however, that such treatment does not result in any significant difference in the

concentration of 5-FU or melatonin metabolised per μg protein. The rate of CYP450 activity has thus not been altered.

The results of the spectral study have two important implications:
(i) the presence of a peak at 420 nm confirms that CYP420, the reduced form of CYP450, was indeed isolated. This validates the microsomal-, and CYP450-, extraction procedure used and optimised. The absence of a peak at 560 nm indicates that no contaminating cytochrome b_5 is present (Keilin and Hartree, 1940) and implies that the isolation procedure is relatively specific; and
(ii) the inability of either 5-FU or melatonin to shift the 420 nm peak or result in any significant changes to the spectral profile of CYP450 indicates that neither drug has an inductive nor inhibitory effect on the latter (see 3.2). This finding that neither drug alters the activity of CYP450 is significant, as it reduces the likelihood of drug interactions with other drugs that patients may be receiving and which are metabolised by CYP450. Furthermore, the finding that 5-FU does not alter the activity of CYP450 is in agreement with previous studies (see 1.2.11.3).

The lack of a pharmacokinetic drug interaction between 5-FU and melatonin in terms of metabolism by CYP450 supports the recommendation that melatonin be administered as co-therapy to cancer patients receiving 5-FU treatment.

Further biopharmaceutics studies need to be undertaken, to investigate whether co-therapy with melatonin alters other pharmacokinetic parameters of 5-FU, such as distribution and elimination. Possible metabolism by enzyme systems other than CYP450 should also be investigated, as should the effects of 5-FU on metabolic activity after a period of more than five days.

3.9 Conclusions

A simple, specific method for isolating liver microsomes containing CYP450 was used, based on the principles of Ca^{2+}-induced sedimentation and differential centrifugation. Although 5-FU decreases the protein content of microsomes, it has been shown that no hepatic metabolic drug interaction exists between 5-FU and melatonin, and that neither drug exerts an inhibitory nor inductive effect on CYP450. Co-incubation with melatonin does not alter the rate or extent of 5-FU metabolism and is thus unlikely to result in alterations in plasma levels of 5-FU in the clinical situation.

CHAPTER 4

THE EFFECTS OF 5-FLUOROURACIL AND MELATONIN ON TRYPTOPHAN 2,3-DIOXYGENASE ACTIVITY *IN VITRO* AND *IN VIVO*

4.1 Introduction

Tryptophan 2,3-dioxygenase (TDO), otherwise known as L-tryptophan-oxygen 2,3-oxidoreductase or tryptophan pyrrolase, is an enzyme found predominantly in hepatic cytosol (Badawy, 1977a). Its activity in rat liver has been studied widely. The enzyme consists of both an apoenzyme and a holoenzyme (Feigelson and Greengard, 1961). TDO has a variety of functions, including the detoxification of its substrate, the essential amino acid tryptophan (Badawy, 1977a). TDO is, in particular, responsible for the conversion of L-tryptophan into *N*'-formylkynurenine (Badawy and Evans, 1975b) by irreversibly inserting oxygen into the pyrrole portion of tryptophan (Mehler and Knox, 1954; Kotaka and Massayama, 1936; Hayaishi *et al*, 1957; Walsh *et al*, 1994). TDO activity thus decreases the amount of circulating tryptophan available to the brain and which is subsequently converted into the neurotransmitter 5-HT, thereby potentially contributing to the development of mental disorders (Badawy, 1977a), such as depression (see 6.2.2.5). The TDO apoenzyme utilises haeme as a cofacor, and thus might also function in regulating haeme metabolism. Other hepatic functions of TDO include possibly regulating both gluconeogenesis and the synthesis of NADP and NADPH (Badawy, 1977a) (see 4.3).

The scheme on the next page outlines the metabolism of tryptophan in both the brain (Mefford and Barchas, 1980) and liver (Cho-Chung and Pitot, 1967). The enzyme IDO is also involved in the first step of tryptophan metabolism; IDO catalyses the formation of a compound that is the substrate for TDO (Sun, 1989). The effects of 5-FU and melatonin on this enzyme are investigated in Chapter 5.

4.2 Mechanisms of TDO regulation

The enzyme exists in two forms: an apoenzyme, which requires the presence of haematin for activation, and an active holoenzyme (Feigelson and Greengard, 1961). There are four mechanisms that regulate TDO activity (Schimke *et al*, 1965; Schimke, 1969; Knox, 1966; Badawy, 1977a; Cho-Chung and Pitot, 1967). The first is an hormonal type, in which glucocorticoids induce the synthesis of more apoenzyme. The second mechanism involves tryptophan, which acts as a substrate for TDO and reduces the degradation of apoenzyme that is already present (Badawy, 1977a). The latter mechanism has been termed a cofactor- or

substrate-type mechanism (Greengard and Feigelson, 1961) because tryptophan enhances the saturation of the apoenzyme with haeme, thereby enhancing the activity of the enzyme.

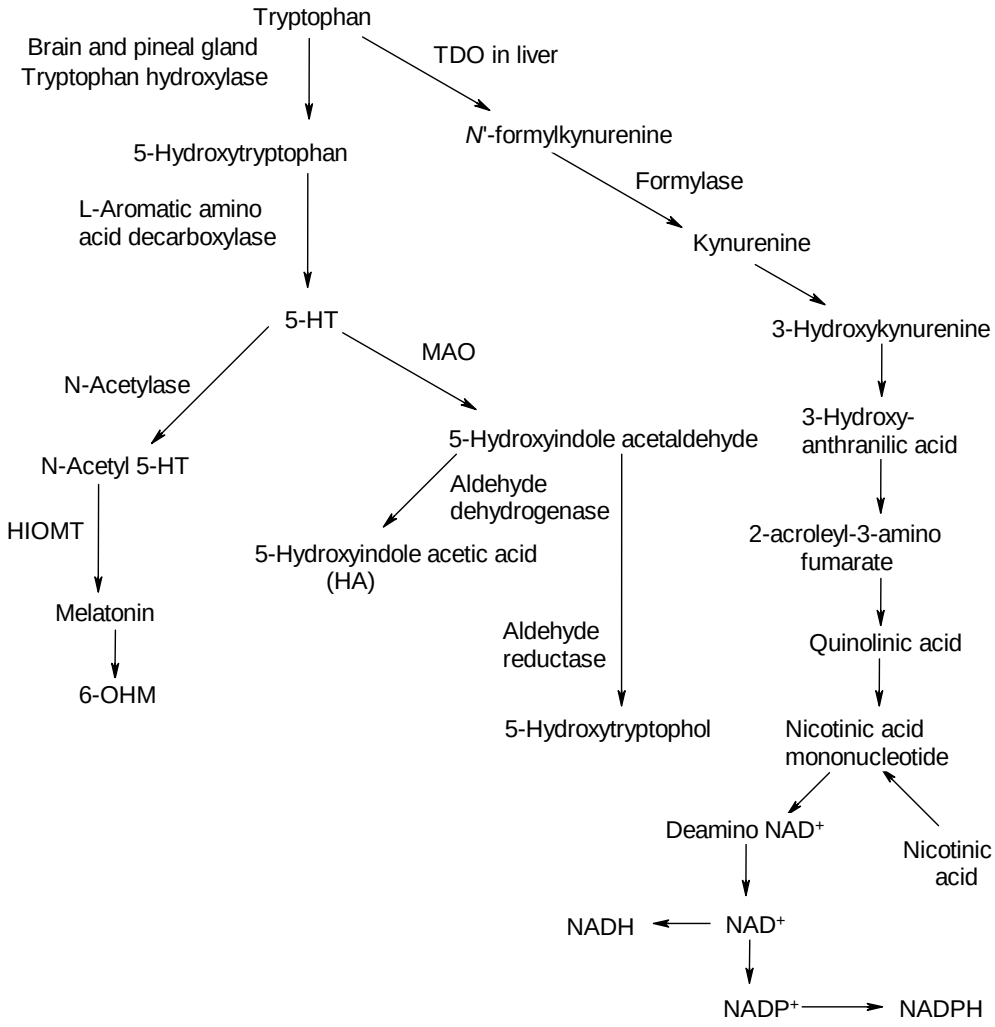


Figure 34: The metabolism of tryptophan in the brain and liver (Mefford and Barchas, 1980; Cho-Chung and Pitot, 1967)

Further work (Badawy, 1977a) has suggested that this cofactor-type of mechanism involving haeme is actually a third mechanism in its own right and has certain differences from the substrate-type mechanism. The fourth mechanism is feedback inhibition of the enzyme by NAD(P)H (Cho-Chung and Pitot, 1967). These mechanisms will be discussed in more detail below (Badawy, 1977a).

4.2.1 Substrate and hormonal mechanisms

TDO activity is enhanced by tryptophan and corticosteroids, as well as compounds that induce the release of the latter (Knox, 1951; Knox and Auerbach, 1955). The administration of tryptophan parenterally increases the activity of TDO significantly (Knox, 1951). This

phenomenon has been termed “adaptation” or “induction”, since it resembles the manner in which micro-organisms develop enzymes in response to the presence of substrates. Not many enzymes in mammals respond in such a manner to the presence of substrates (Greengard and Feigelson, 1961). After the parenteral administration of tryptophan, TDO present in the cytosol of hepatocytes becomes increasingly saturated with its activator, a metalloporphyrin mentioned below. TDO is an enzyme that contains an iron porphyrin group (Auerbach *et al*, 1959; Knox, 1952). The concentration of iron protoporphyrin in microsomes also increases by approximately 20% (Greengard and Feigelson, 1961) and it is this hepatic microsomal iron protoporphyrin (MIP) that is also responsible for stimulating TDO (Feigelson and Greengard, 1961). The MIP in cytosol is lost at 37ºC through interactions with the cytosol. Tryptophan, by preventing this loss of MIP *in vivo*, results in the increased saturation of TDO with its activator. An increase in the activation of TDO accounts for the increased activity of this enzyme in the first hour after the administration of tryptophan. In the next four hours, the increase in the activity of TDO may be due to more protein being present (Greengard and Feigelson, 1961). The MIP is also believed to act as TDO’s prosthetic group (Tanaka and Knox, 1958). The extent of TDO saturation induced by the presence of substrate is equivalent to a decrease in the concentration of available apoenzyme, and this in turn may trigger enhanced TDO synthesis and the rapid increase in the concentration of the enzyme (Greengard and Feigleson, 1961).

Badawy (1977a) suggests a mechanism for substrate activation of TDO: compounds, such as ethanol, which induce the release of catecholamines, increase the concentration of non-esterified fatty acids present in serum. These fatty acids displace tryptophan from binding sites on serum proteins, thereby increasing the amount of tryptophan available for entry into tissues. Tryptophan is the only amino acid that binds to protein (McMenamy *et al*, 1957). The tryptophan concentration in the liver is thus increased and activation of TDO ensues (Badawy and Evans, 1975; 1976a).

The ability to differentiate between the substrate and hormonal mechanisms of TDO regulation is based on the fact that the apoenzyme requires haematin as a cofactor (Feigelson and Greengard, 1961; Greengard and Feigelson, 1961). New apoenzyme is synthesised in the hormonal mechanism, as a result of an increased amount of TDO mRNA (DeLap and Feigelson, 1978; Badawy, 1979), while the substrate mechanism relies on pre-existing apoenzyme that is subjected to less degradation (Badawy, 1979). Glucocorticoids and other steroid hormones stimulate protein synthesis by enhancing transcription. This is due to the formation of a steroid-receptor complex that migrates from the cytoplasm into the nucleus of sensitive cells, where it interacts with chromatin (Ganong, 1993; Haynes and Larner, 1975)

and ultimately results in the synthesis of new apoenzyme (Young, 1981). TDO is subjected to a diurnal circadian rhythm that mirrors that of plasma concentrations of corticosteroids (Salter and Pogson, 1985; Wurtman, 1974) and is opposite to that of melatonin (Van Wyk, 1993; Curzon, 1979). It has been found experimentally that the administration of haematin causes a marked increased in the activity of TDO in the cytosol of hepatocytes, as well as completely reverses the inhibition of TDO induced by the administration of globin (Feigelson and Greengard, 1961). These authors thus suggest that TDO exists with a partially unsaturated prosthetic group and that globin can remove the haeme group present in the holoenzyme, because globin has a strong affinity for metalloporphyrins (Feigelson and Greengard, 1961). Apoenzymes bind haeme to varying extents (Lewis, 1954). The MIP readily dissociates from TDO, thus allowing the haeme affinity of the apoenzyme to resemble that of a coenzyme undergoing dissociation, rather than a prosthetic group that is very tightly bound to TDO (Feigelson and Greengard, 1961).

Saturation of the apoenzyme is enhanced by tryptophan, but not by cortisol (Badawy and Evans, 1975b). The haeme saturation (HS) ratio, which is the ratio of holoenzyme to apoenzyme activity, thus reflects the extent of apoenzyme saturation with haeme and is increased in the presence of tryptophan (Badawy, 1977a, 1979). Cortisol does not have an effect on the formation of haeme (Marver *et al*, 1966a). The substrate mechanism of regulating TDO activity involves increasing the synthesis of the apoenzyme, whereas this is not necessary in the hormonal mechanism (Schimke *et al*, 1965; Knox, 1966; Schimke, 1969). Tryptophan stabilises the apoenzyme (Schimke *et al*, 1965; Shore *et al*, 1971; Garren *et al*, 1964; Badawy and Evans, 1975b) through a poorly-understood mechanism (Badawy, 1977a). Activation with haeme does not in itself enhance the stability of TDO (Badawy and Evans, 1975b), although such activation of the apoenzyme is a requirement for the stabilisation of TDO (Knox, 1966). It has been suggested (Knox, 1966) that, subsequent to activation of the apoenzyme, the enzyme is sequestered by tryptophan and thus protected against degradation. A contentious point is that while it has been found that tryptophan does not promote the synthesis of TDO (Schimke *et al*, 1965), substances that inhibit protein synthesis are able to prevent the effect that tryptophan has on TDO (Badawy, 1977a). Badawy (1977a) explained this by noting that tryptophan could activate TDO through an unknown mechanism, or perhaps the substances that inhibit protein synthesis could have other unrecognised effects. Evidence suggests that tryptophan may also enhance the synthesis of hepatic haeme, thereby regulating TDO (Badawy, 1977a; Greengard and Fiegelson, 1961; Badawy and Evans, 1975b; Badawy and Evans, 1973a; Marver *et al*, 1966b).

Tryptophan may further act by decreasing the activity of 5-aminolaevulinic acid (5-ALA) synthase, the enzyme that catalyses the formation of 5-ALA, the precursor of haeme. This may activate the conversion of 5-ALA into haeme by a mechanism that requires the synthesis of protein, and ultimately enhance the haeme saturation of TDO (Badawy *et al*, 1981). It is noted that there is thus an inverse relationship between the haeme saturation of TDO and the activity of 5-ALA synthase (Badawy *et al*, 1981). This relationship is not always an inverse one (Welch and Badawy, 1980; Badawy *et al*, 1981), but the alteration in haeme saturation of TDO will determine the manner in which 5-ALA synthase activity is altered (Badawy *et al*, 1981). Allopurinol, by preventing the conjugation of haeme with the apoenzyme, thus allows more haeme to be available to further inhibit the activity of 5-ALA synthase (Welch and Badawy, 1980). Compounds, such as metal cations, which decrease haeme levels, either by decreasing the formation of haeme or enhancing its metabolism, thus decrease the haeme saturation of TDO and increase 5-ALA synthase activity (Welch and Badawy, 1980) by impairing the positive-feedback system that regulates 5-ALA synthase activity (De Matteis, 1975; Welch and Badawy, 1980). The activity of the enzyme is inhibited when there is haematin- or endotoxin-induced saturation of TDO with haeme (Welch and Badawy, 1980; Bissell and Hammaker, 1977); the increase in hepatic haeme levels exerts a negative-feedback regulatory effect on 5-ALA synthase activity (Welch and Badawy, 1980). It should be noted that the activity of 5-ALA synthase can be altered without any variation in the extent of the haeme saturation of TDO; this occurs in starvation (Welch and Badawy, 1980). In this case, there is an increase in holoenzyme activity, which may deplete the haeme pool, thus allowing less haeme to be present to regulate 5-ALA synthase levels, thereby increasing the activity of this enzyme (Morgan and Badawy, 1980). This highlights that the utilisation of haeme, as well as haeme saturation, is important in regulating the activity of 5-ALA synthase (Welch and Badawy, 1980).

The following scheme, from Badawy (1979), outlines the activation of TDO *in vitro*.

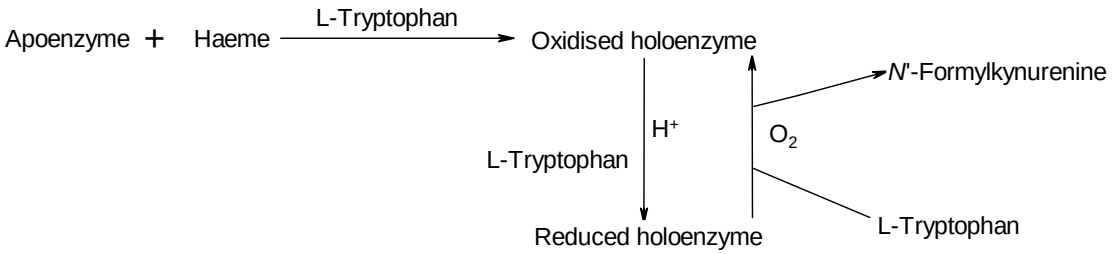


Figure 35: The activation of TDO by tryptophan and haeme (Badawy, 1979)

4.2.2 Cofactor mechanism

The administration of 5-ALA promotes the saturation of the apoenzyme with its activator, haeme (Wetterberg *et al*, 1969; Druyan and Kelly, 1972). This saturation can be altered by the metabolism of haeme and porphyrins (Badawy and Evans, 1973a). Substances that inhibit the synthesis of or degrade haeme thus decrease the extent of haeme saturation of the TDO apoenzyme, while substances that enhance haeme synthesis promote its saturation with the apoenzyme (Badawy, 1977a). The activity of TDO is also enhanced by the administration of haematin (Badawy and Evans, 1975b). 5-ALA or haematin initially increases the saturation of haeme with apoenzyme already present (Badawy, 1977a); at subsequent time intervals, there is an increase in apoenzyme activity, and most of this apoenzyme is saturated with haeme. The effects of haeme, acting as a cofactor, are thus similar to the effects of the TDO substrate tryptophan. The difference is that, unlike tryptophan, haeme saturation does not stabilise TDO (Badawy, 1977a). This may be the reason for tryptophan administration increasing the activity of the total enzyme (Badawy and Evans, 1975b). The effects of 5-ALA are prevented by substances that inhibit protein and/ or RNA synthesis, such synthesis being necessary for TDO activity. The effects of haematin, on the other hand, are blocked by porphyrogens, which degrade haeme (Badawy, 1977a).

4.2.3 Feedback inhibition

Both the nucleotides NADH and NADPH have been found (Cho-Chung and Pitot, 1967) to allosterically inhibit the activity of the TDO apoenzyme, although the extent of inhibition is greater with the latter. Since both NADH and NADPH are the products of tryptophan degradation through the kynurenine pathway (see Figure 34), it has been suggested by these authors that TDO activity could be regulated through a feedback pathway. This has been supported experimentally by the finding that the chronic administration of drugs of dependence, such as ethanol, increases hepatic levels of both reduced nucleotides as well as inhibits the apoenzyme form of TDO (Badawy and Evans, 1975a, c). Furthermore, increased levels of NAD^+ and $NADP^+$, the oxidised forms of the nucleotides, have been reported to reverse the inhibition of TDO (Badawy, 1977a). The relationship between the aforementioned nucleotides and TDO has been investigated further (Badawy and Evans, 1976b). Glucose, which is important in the utilisation of the nucleotides, as well as nicotinamide, the precursor of these, has been found to inhibit the TDO apoenzyme (Yamaguchi *et al*, 1967). Administration of ammonium chloride reverses the aforementioned effect of glucose, thereby indicating that increased hepatic concentrations of the ammonium ion could prevent the inhibition of TDO by altering the concentrations of the reactants required in the glutamate dehydrogenase and β-hydroxybutyrate dehydrogenase systems (Krebs, 1967). Badawy

(1977a) thus concludes that feedback regulation of TDO is likely, and that any alterations in the metabolism of nucleotides or carbohydrates could well affect TDO activity.

4.3 Functions of TDO

The enzyme has numerous functions, ranging from involvement in the detoxification of tryptophan to regulating gluconeogenesis. These will be discussed in the next pages (Badawy, 1977a).

4.3.1 Tryptophan detoxification

Rats that have been adrenalectomised are unable to undergo adrenocorticosteroid-mediated TDO apoenzyme synthesis. The hepatic TDO in these animals is unable to metabolise the surplus of tryptophan; the latter is thus degraded along other pathways to form toxic products, which cause death (Knox, 1966). Administering even a single dose of cortisol has been found to protect these rats against tryptophan-induced mortality (Knox, 1966). It is suggested (Badawy, 1977a; Horita and Carino, 1970) that 5-HT is the specific metabolite of tryptophan responsible for its toxicity, because pretreatment of rats with p-chlorophenylalanine, which inhibits tryptophan hydroxylase, the enzyme which converts tryptophan through an hydrolysis reaction to 5-HT, reduces the toxicity of exogenously administered tryptophan. Indole derivatives other than 5-HT may also contribute to the toxicity of tryptophan (Yang and Carlson, 1972).

The repeated administration of tryptophan has been found to result in poisoning and death in numerous animal species (Hvitfelt and Santti, 1972; Brown and Dodgen, 1968; Johnson and Dyer, 1966; Badawy and Evans, 1976c; Baughman and Franz, 1971; Leklem *et al*, 1969; Spiegel, 1961). All these animal species lack an hormonal mechanism of inducing TDO (Badawy, 1977a). The TDO present in guinea pig liver exists only in the holoenzyme, and not the apoenzyme, form (Badawy and Evans, 1974; Hvitfelt and Santti, 1972). It has been found in many animal species (Badawy and Evans, 1976c) that those not possessing the mechanism of hormonal induction also have an absence of the apoenzyme form of TDO and are susceptible to tryptophan toxicity. Some of these species have a different response to the rat, which possesses the apoenzyme form, in terms of receiving drug treatments that alter the metabolism of tryptophan in both the brain and the liver (Badawy, 1977a); these animals might also have an inadequate kynurenine pathway (Leklem *et al*, 1969; Yang and Carlson, 1972; Green *et al*, 1975) and have lower levels of tryptophan in the liver and circulating in the serum (Badawy and Evans, 1976c). Species that do not possess the hormonal mechanism of TDO induction or the apoenzyme metabolise tryptophan in different ways to species that do possess these (Badawy and Evans, 1976c). In man, for example, the kynurenine pathway is

the main route of metabolism (Hankes *et al*, 1967) while cattle largely utilise the indolylamine pathway (Yang and Carlson, 1972).

Neonates are particularly susceptible to tryptophan toxicity (Badawy, 1977a) and have very low hepatic TDO activity (Auricchio *et al*, 1959). The rate of development of human TDO has not been investigated, but since humans resemble rats in that both species have the hormonal mechanism of induction as well as the apoenzyme form of TDO (Altman and Greengard, 1966), it is likely that the development of human hepatic TDO is similar to that in the rat. The enzyme develops in the latter on the twelfth day after birth (Auerbach and Waisman, 1959). In species that lack the apoenzyme, such as the rabbit, TDO is fully developed within the first day after birth (Nemeth, 1959; Badawy, 1977a). Human neonates are thus unable to adequately detoxify tryptophan, rendering them particularly sensitive to the latter's toxicity (Badawy, 1977a).

4.3.2 Regulation of 5-HT synthesis in the brain

It has been suggested that low levels of the tryptophan metabolite 5-HT, and high levels of TDO, are associated with depression (Curzon, 1969; Lapin and Oxenkrug, 1969) (see 6.2.2.2). Green and Curzon (1968) have shown that the cortisol-induced increase in TDO activity results in a decrease in 5-HT levels in the brain, as expressed by the amount of 5-hydroxyindole acetic acid (HA), the major metabolite of 5-HT. These authors thus suggest that there is an inverse relationship between 5-HT concentrations in the brain and TDO activity (Green and Curzon, 1968; Badawy, 1977a). The specific areas of the brain affected by the aforementioned increase in TDO activity due to cortisol are the pontine and thalamic-hypothalamic areas (Yuwiler and Geller, 1973). It has been found that oestrogens decrease brain levels of 5-HT (Nistico and Preziosi, 1970) as well as induce TDO (Rose and Braidman, 1970). The opposite effects are achieved with repeated administration of the antidepressant imipramine, which increases brain levels of 5-HT (Kivalo *et al*, 1961) and inhibits the activity of TDO (Paracchi, 1967).

There are various mechanisms by which TDO could regulate 5-HT synthesis and/ or metabolism, as discussed below (Badawy, 1977a): (i) the concentration of the metabolites of tryptophan, in particular kynurenine, are increased after TDO induction (Curzon, 1969), and these metabolites could directly inhibit the uptake of tryptophan into the brain; (ii) a decreased amount of non-protein-bound tryptophan freely circulating to the brain; and (iii) TDO activation results in a greater demand in the liver for the coenzyme pyridoxal phosphate, to catalyse reactions in the kynurenine pathway. This results in less of the coenzyme being available in the brain to react with the enzyme 5-hydroxytryptophan decarboxylase (Badawy,

1977a); the latter is a rate-limiting enzyme in humans (Robins *et al*, 1967), although there is no evidence to indicate that a deficiency of pyridoxal phosphate necessarily inhibits the activity of 5-HT decarboxylase in the brain (Davis, 1963; Badawy, 1977a).

The availability of free, circulating tryptophan is ultimately of utmost importance in ascertaining the effects that hepatic TDO exerts on the metabolism of 5-HT in the brain (Badawy, 1977a). It has been found (Green *et al*, 1975) that two hours after TDO has been maximally induced, which is six hours after the administration of cortisol, there is a reduction in the concentration of free tryptophan in serum, the brain and liver. This suggests that increased TDO activity could result in a greater amount of tryptophan entering the liver and thus being subjected to catabolism, thereby reducing the amount of the amino acid that enters the brain (Badawy, 1977a). In species that possess neither the apoenzyme form of TDO nor the hormonal mechanism of inducing the enzyme, such as the gerbil (Badawy and Evans, 1976c; Baughman and Franz, 1971), the administration of cortisol has no influence on cerebral concentrations of 5-HT (Auricchio *et al*, 1959). Ethanol activates TDO in the liver (Badawy and Evans, 1973b, 1973c) and has also been shown to decrease the concentrations of 5-HT and HA in the brain (Badawy and Evans, 1976a). The ethanol-induced increase in TDO activity can be blocked by the administration of allopurinol, and this results in a decrease in the cerebral and serum concentrations of 5-HT (Badawy and Evans, 1976a).

Experiments that investigate the effects of cortisol at earlier periods of time have challenged the importance of TDO in the synthesis of 5-HT in the brain (Badawy, 1977a). The metabolism of tryptophan in the brain is increased three hours after the administration of cortisol (Badawy, 1977a). 2-Allyl-2-isopropylacetamide, a porphyrogen, has been found to increase both 5-HT levels and TDO activity after a similar period of time (Yuwiler *et al*, 1971). The latter enhancement is through the hormonal-type mechanism (Badawy and Evans, 1973a), with 2-allyl-2-isopropylacetamide simultaneously reducing the extent that the apoenzyme is saturated with haeme (Badawy and Evans, 1973a; Badawy, 1977a). Badawy (1977a) thus postulates that corticosterone may mediate the aforementioned effect of 2-allyl-2-isopropylacetamide on 5-HT, which is maximal after four hours. Furthermore, corticosterone promotes the activity of tryptophan hydroxylase (Azmitia and Mc Ewen, 1969, 1974) in the midbrain and/ or the forebrain one and four hours after administration (Azmitia and McEwen, 1976). The effect after four hours requires the synthesis of new proteins, whereas the earlier effect does not and Badawy (1977a) concludes that this mechanism may account for the initial effects of 2-allyl-2-isopropylacetamide and cortisol on 5-HT concentrations in the brain (Hillier *et al*, 1975; Yuwiler *et al*, 1971). The effect of time on the effects of TDO on the synthesis of 5-HT is thus clearly significant (Badawy, 1977a).

As discussed in 6.2.2.2 and 6.2.2.5, low 5-HT levels are correlated with depression, and the kynurenine hypothesis of depression postulates that increased cortisol levels enhance the activity of TDO, and the resulting formation of kynurenine and its metabolites may cause some of the symptoms of depression.

4.3.3 Regulation of the hepatic metabolism of haeme

The synthetic pathway leading to the formation of haeme is usually controlled so that intermediates in this pathway do not accumulate; loss of this type of control results in the development of porphyrias, a group of disorders that can affect almost any bodily system. These are characterised by neurological disorders and abdominal pains, and patients show an increased excretion of porphyrins (Badawy, 1978). The association between porphyrins and depression is discussed in 6.2.2.5.

It has been found that patients suffering from porphyria possess an increased concentration of the metabolites of tryptophan, resulting from the kynurenine pathway, in their urine (Price, 1961), thereby suggesting a possible connection between porphyria and the hepatic metabolism of tryptophan (Badawy, 1977a). These metabolites are the result of an increase in TDO activity that also leads to a deficiency of vitamin B_6; the latter is manifested by the excretion of unusually large amounts of metabolites that have been formed through hepatic reactions requiring the presence of pyridoxal phosphate (Badawy, 1977a). Supplementation with pyridoxine does not alter the abnormal excretion mentioned above, thereby leading Badawy (1977a) to suggest that there may be deficiencies present other than that of vitamin B_6. Price (1961) has found that the chelator ethylinediamine tetra-acetic acid (EDTA) corrects the excess excretion of the tryptophan metabolites exhibited by porphyrics. Badawy (1977a) has found that, *in vitro*, EDTA inhibits the activity of the holoenzyme form of TDO and suggests that this may be due to the chelator inducing the inactivation of the haeme component of the enzyme, ultimately inhibiting TDO in porphyrics.

CYP450 and catalase are haeme proteins that together utilise 82% of hepatic haeme (Marver and Schmid, 1972); these are often used as a test system when investigating the metabolism of haeme and the "free haem pool" (Badawy, 1977a), which is believed to be in the cytosol (Badawy, 1979). Badawy (1977a) believes that TDO should rather be the haeme protein that is employed in this regard, as it offers several advantages over the traditional test system. These advantages are:

(i) both the apoenzyme and the holoenzyme can be measured at the same time, and the level of one can be altered without affecting the other (Badawy and Evans, 1973a, 1975b). On the

other hand, with the CYP450 or catalase test systems, only the holoenzyme activity can be measured (Badawy, 1979);
(ii) TDO responds without a significant lag phase to various stimuli and has a shorter half-life of 2.3 hours compared to catalase and CYP450 (Badawy and Evans, 1975b); and
(iii) activation of the apoenzyme only requires a small quantity of haematin (Badawy and Smith, 1971); in fact, although optimum activation of TDO is achieved using 1 μM haematin (see 4.5.1.2), only one-tenth of this concentration is required to activate TDO in the cytosol (Feigelson and Greengard, 1961; Badawy, 1978). If the activity of TDO is increased in patients with mental diseases, a greater proportion of tryptophan undergoes hepatic metabolism and less of the amino acid is subsequently available to be converted into 5-HT in the brain (Badawy, 1977a). It has been noted that the administraton of haematin can cause CYP450 to release haeme (Bissell and Hammaker, 1976a, 1976b).

TDO responds both to slight and great alterations in hepatic haeme concentrations, whereas catalase and CYP450 respond only to large decreases in haeme concentrations. This highlights the important role that TDO plays in regulating the synthesis of haeme (Badawy, 1979). This sensitivity of TDO to haeme concentrations can be used to detect the haeme depletion that occurs in porphyria (Badawy, 1978). When porphyria is potentiated experimentally, it has been found that the utilisation of haeme by the apoenzyme of TDO is increased, leading to further haeme depletion and the so-called potentiation phenomenon (Badawy, 1978).

The HS ratio (see 4.2.1) is increased if the holoenzyme activity is increased to a greater extent than the total enzyme, which occurs after treatment with drugs that increase haeme availability. This could be achieved by drugs that enhance the concentration or synthesis of haeme, such as 5-ALA, or by increasing haeme utilisation, such as with phenobarbitone (Badawy and Evans, 1973a, 1973e; 1975b; 1977b). The HS ratio is decreased if the holoenzyme activity is decreased, or if only the total enzyme activity is increased. This occurs when the availability of haeme is decreased, by drugs which inhibit its synthesis, such as acetate, or increase its metabolism (Badawy, 1979). Changes in the level of haeme, in turn, alter TDO activity and thus tryptohan metabolism (Badawy *et al*, 1987).

4.3.4 Regulation of gluconeogenesis in the liver

Tryptophan derivatives have an effect on gluconeogenesis and the concentration of glucose in the body. 5-Hydroxytryptophan, for example, has an hypoglycaemic effect (Furman, 1974) while metabolites arising from the hepatic metabolism of tryptophan along the kynurenine pathway have been found to alter gluconeogenesis (Badawy, 1977a). Tryptophan itself

administered exogenously has been shown to result in a marked hypoglycaemia (Mirsky *et al*, 1956) as well as glycogenolysis (Rosen and Nichol, 1964). The activity of phosphoenolpyruvate carboxykinase (PPC), an important enzyme in gluconeogenesis, is altered in an unusual manner by tryptophan (Badawy, 1977a). *In vitro*, there is an increase in the activity of the enzyme (Foster *et al*, 1966), while a decrease is noted *in vivo* (Ray *et al*, 1966). The latter inhibition is evidenced by the increase in the concentration of the intermediates in the gluconeogenesis pathway before phosphoenolpyruvate while there is a decrease in the concentration of the latter and subsequent compounds up to glycogen (Ray *et al*, 1966). It has been shown in the same study that a dose of at least 12.5 mg tryptophan per 100g of body weight is necessary to decrease the concentration of phosphoenolpyruvate after three hours; doses of greater than 5 mg/ 100 g effectively increase the activity of TDO. It has been suggested that quinolinic acid (QA), a metabolite of tryptophan, is responsible for this enhancement of TDO activity (Veneziale *et al*, 1967). Snoke *et al* (1971) suggest that, *in vitro*, QA acts by chelating metal ions whereas its mechanism of action *in vivo* subsequent to the administration of tryptophan is to form a complex with ferrous iron, thereby inducing inhibition of PPC and ultimately of TDO (Lardy, 1971; Snoke *et al*, 1971).

It has been found that TDO is not present in the kidney (Rose, 1972) and that this may be a reason for the activity of PPC in the kidney being significantly less influenced by exogenous tryptophan (Pogson and Smith, 1975; Rose, 1972). It is unknown whether TDO is present in adipose tissue (Badawy, 1977), which has been found to respond to tryptophan administration (Pogson and Smith, 1975).

Much confusion exists in the literature as to the effects of glucocorticoids on PPC (Gunn *et al*, 1975). These authors have studied this relationship in the kidney and liver, using cortisol and triamcinolone, which mimic the effect of exogenous tryptophan (Gunn *et al*, 1975; Pogson and Smith, 1975). Badawy (1977a) thus concludes that the inhibition of the synthesis of PPC that occurs in response to glucocorticoids may be attributed to the presence of TDO, the fact that glucorticoids induce the latter and thereby generate QA.

Another mechanism by which TDO could influence gluconeogenesis is by altering the redox potential of NAD^+; the latter is a crucial component of the process of gluconeogenesis (Badawy, 1977a; Krebs, 1968). Badawy (1977a) questions whether the absence of the TDO apoenzyme in certain species, such as the guinea pig, may be responsible for certain differences in glucogeneic systems; these differences can, for example, result in an unusual redox state for the NAD^+ couple, and this in turn has far-reaching effects, such as causing a 15 minute lag phase in the initial steps of gluconeogenesis (Arinze and Rowley, 1975), due to the

presence of an increased concentration of reduced compounds formed by dehydrogenases, which are NAD^+-dependent (Arinze and Rowley, 1975; Badawy, 1977a). Badawy (1977a) concludes by stating that the rate of the hepatic catabolism of tryptophan, as well as the synthesis of NAD^+, may well be regulated by TDO, thus influencing gluconeogenesis.

4.3.5 Regulation of hepatic NAD(P)H synthesis

TDO is crucial in the synthesis of the cofactors $NADP^+$ and NAD^+ and their reduced equivalents, since TDO is the rate-limiting enzyme in the synthesis of the precursor of these nucleotides, nicotinamide (Badawy, 1977a) (see Figure 34). It was claimed by Powanda and Wannemacher (1971) that it is the availability of tryptophan, as opposed to the activity of TDO, that regulates NAD^+ synthesis, since there was found to be a directly proportional relationship between the amount of tryptophan consumed in the diets of mice and rats and the latters' hepatic NAD^+ levels (Powanda and Wannemacher, 1970). It was, furthermore, found that although allopurinol inhibits the increase in TDO activity that occurs 30 minutes after tryptophan administration, it does not prevent the increase in the concentration of NAD^+ that results 2.5 hours after tryptophan administration (Powanda and Wannemacher, 1971). Allopurinol both inhibits the TDO apoenzyme (Badawy and Evans, 1973d) as well as prevents the tryptophan-induced increase in TDO activity (Badawy, 1977). Badawy (1977a) finds numerous flaws in the aforementioned claim propounded by Powanda and Wannemacher (1971), which shall be mentioned briefly:

(i) a large dose of tryptophan was used, which would activate TDO through the substrate mechanism and activate the kynurenine pathway;

(ii) the concentration was determined 2.5 hours after tryptophan administration, whereas the allopurinol-induced inhibition of TDO activity was measured after 30 minutes;

(iii) the existence of species differences in TDO activity between the mice and rats that were used (Powanda and Wannemacher, 1971; Badawy and Evans, 1976c, 1974); and

(iv) although the administration of tryptophan also increases the concentrations of the other nucleotides, NAD(P)H and $NADP^+$ (Powanda and Wannemacher, 1971), the effect that allopurinol exerts on these nucleotides was not determined. The latter may be of significance because alterations in the concentrations of the nucleotides appear to be independent of each other (Badawy, 1973d, 1976b, 1977a). Considering the claims by Powanda and Wannemacher (1971), as well as others (Greengard *et al*, 1968), Badawy (1977a) calls for further research to differentiate the different roles of TDO and the availability of tryptophan in the synthesis of NAD^+.

Further evidence for the role of TDO in the synthesis of NAD^+ comes from experiments determining the effect of starvation on TDO activity (Badawy and Evans, 1973a). Starvation

for a period of 24 hours was found to increase the activity of TDO. This could be due to starvation inducing an increase in the plasma concentration of non-esterified fatty acids (Hales and Kennedy, 1964); these cause the release of tryptophan, which is bound to plasma proteins (Curzon and Knott, 1974). Starvation, being a stress state, also results in the secretion of corticosterone (Fromweiler *et al*, 1968; Chowers *et al*, 1969), and this may further increase the activity of TDO (Badawy, 1977a). The glucocorticoid-mediated explanation appears to be more significant than the effect of the non-esterified fatty acids for two reasons: (i) in starving rats, the ratio of activities between the holo- and apoenzyme approximates that in cases where TDO has been activated by cortisol (Badawy and Evans, 1973a; Badawy, 1977a); and (ii) actinomycin D blocks the effects of starvation on TDO. The antibiotic does not inhibit the activation of TDO by tryptophan, but does block the induction of the enzyme by cortisol (Badawy, 1977a; Badawy and Evans, 1975b). Starvation furthermore increases the concentrations of the NAD(P)H couples, in particular $NADP^+$ and its reduced form, which is expected since the couple is responsible for the synthesis of various compounds required by the body during starvation (Badawy, 1977a). Actinomycin D also blocks the increase in the concentration of the $NADP^+$ couple, and this may be due to its ability to inhibit TDO (Badawy, 1977a).

4.3.6 Other functions

TDO may have numerous other functions, and further research is needed to elucidate these, in particular with regard to tryptophan and its effects. TDO is the most active of the enzymes responsible for the degradation of tryptophan and, as such, can be a key determinant of the amount of tryptophan available to tissues as well as the concentration of the amino acid in hepatocytes (Badawy, 1977a).

4.4 Effects of TDO regulation on neurotransmitter levels

This will be discussed in Chapter 6, where results of the effects of 5-FU and melatonin administration on various neurotransmitter levels in rat forebrain will be presented.

4.5 Experimentation

The TDO assay used is based on that described by Badawy and Evans (1975b), with some modifications made by Walsh (1997), Anoopkumar-Dukie (2000) and Maharaj (2004).

4.5.1 *In vitro*

4.5.1.1 Materials, animals, reagents and equipment

Male Wistar rats weighing between 200g and 300g were used, and cared for as described in Appendix A. L-Tryptophan, 5-FU, melatonin and haematin were purchased from the Sigma Chemical Corporation, St Louis, MO, USA. All the other chemicals used were obtained from local suppliers and were of the highest purity available. All solutions were prepared using deionised water (Milli R/Q System, Millipore®). Absolute ethanol, at a final concentration of 0.67% v/ v, was used as a co-solvent to dissolve melatonin. A Julabo PC water bath (Labotec, South Africa) was used. The UV spectrophotometer used was a Spectra System UV 2000®.

4.5.1.2 Method

This assay is based on determining the specific activity of TDO, by measuring the concentration of kynurenine formed by the apoenzyme and holoenzyme (Badawy and Evans, 1975b). Kynurenine is a degradation product of L-tryptophan; TDO catalyses the formation of *N*'-formylkynurenine from tryptophan, and the enzyme formylase hydrolyses *N*'-formylkynurenine to form kynurenine (Feigelson and Greengard, 1961). Formylase is present in large quantities in the liver and thus does not alter the results of the assay (Feigelson and Greengard, 1961). In the assay, haematin is added to part of the homogenate (see below); this allows for the determination of total TDO activity whilst the remaining homogenate is used for the determination of the holoenzyme activity, since the holoenzyme is not dependent on haeme for activation. The difference between the total and holoenzyme activities is the activity of the apoenzyme, which does require haeme for activation (Badawy and Evans, 1975b). The HS ratio is obtained by calculating the ratio of holoenzyme to apoenzyme activity (Badawy and Evans, 1975b).

The rats were killed by cervical dislocation and the livers were removed immediately, perfused with 0.9% NaCl and frozen at -70ºC using liquid nitrogen until used in the assay. It has been previously thought (Stowell and Mørland, 1983) that the livers had to be completely perfused, in order to prevent endogenous haeme from interfering with the assay by activating the apoenzyme. Badawy *et al* (1983) have found, however, that the haeme saturation of TDO is not significantly different between perfused and unperfused livers. Each liver was weighed and homogenised manually with an aliquot of 60 ml 0.14 M KCl/ 2.5 mM NaOH; care was taken during this process to ensure that excessive foaming did not occur, as this would result in protein denaturation. A sufficient volume of 0.2 M NaH_2PO_4, pH 7.0, was used to make up a volume of 20% w/v of homogenate and the resulting suspension was stirred gently on ice. An aliquot of 15 ml of the resulting homogenate was added separately to six beakers, each of

which contained 12.5 ml of water, and stirred. An aliquot of 27.6 µL of haematin, made up as haematin chloride in 0.1 M NaOH and freshly prepared prior to the commencement of the assay, were added to three of the six beakers, to provide a final concentration of 2 µM; the mixture was stirred and left for 1 min in order to ensure actvation of TDO. Even though 1 µM of haematin results in optimum activation of TDO, a final concentration of 2 µM was chosen because this would override any minor alterations in the availability of haematin to the apoenzyme *in vitro* (Badawy, 1978). An aliquot of 2.5 ml of 0.03 M L-tryptophan/ 4 mM NaOH was added to each of the six beakers and gently stirred. An aliquot of 3 ml of the above was added immediately to test-tubes kept on ice. 5-FU and melatonin were added according to the table below. The contents of each test-tube were carried out in triplicate in order to ensure reproducibility. Thus a total of sixty test-tubes were used, as shown below. All concentrations are in the units of µM.

Table 16: Template used in the TDO assay <u>in vitro</u>

Test-tube	**Sample A**	**Sample B**	**Sample C**
No H			
No H F (10)			
No H F (50)			
No H F (100)			
No H M (10)			
No H M (50)			
No H M (100)			
No H F(10) + M(10)			
No H F(50) + M(50)			
No H F(100)+M(100)			

H	**Sample A**	**Sample B**	**Sample C**
H F (10)			
H F(50)			
H F(100)			
H M(10)			
H M(50)			
H M(100)			
H F(10) + M(10)			
H F(50) + M(50)			
H F(100)+M(100)			

H = Haematin; F = 5-FU; M = Melatonin

The test-tubes were stoppered and incubated at 37ºC in an oscillating water bath for 60 mins. This period of time was chosen as the most appropriate since it is longer than the lag phase of 30-45 mins in which TDO is activated (Greengard and Feigelson, 1961). Badawy and Evans (1975b) found that TDO activity is at a maximum between 4 and 7 hours of incubation. Aliquots of 2 ml of 0.9 M TCA were added to each test-tube, to stop the reaction. The above was vortexed and the resulting suspension centrifuged at 4000 rpm for 20 mins. An aliquot of 1.5 ml of 0.6 M NaOH was added to 2.5 ml of the filtrate and the mixture vortexed. The absorbance of kynurenine was read spectrophotometrically at 365 nm. The blank consisted of 1.5 ml NaOH and 2 ml TCA. The concentration of kynurenine was determined by using the Beer-Lambert law, with an extinction coefficient ε of 4540 $L.mol^{-1}.cm^{-1}$. The protein concentration of each liver was determined according to the method described by Lowry *et al*

(1951) (see Appendix B). The specific activity of TDO was reported as nanomoles of kynurenine formed per μg of protein per minute.

Stowell and Mørland (1983) have criticised the above assay, pointing out that it could bear three types of errors, which could affect the results obtained. These are:
(i) the activation of TDO by endogenous haeme that is present if the livers are not fully perfused;
(ii) the detection of kynurenine at 365 nm could be disturbed by another reaction that does not involve tryptophan and the products of which are also detected at this wavelength; and
(iii) stimulation of TDO at room temperature after the deaths of the animals used in the assay.

Badawy *et al* (1983) have responded to these criticisms as follows:
(i) these authors have found that perfusion had no effect on TDO activity or haeme saturation. These authors also conducted experiments using allopurinol, which prevents the conjugation between haeme and the apoenzyme (Badawy and Evans, 1973d). The results show that blood present in unperfused or incompletely perfused livers does not provide haeme to be used by TDO in the assay (Badawy *et al*, 1983). These authors suggest that the increase in holoenzyme activity observed by Stowell and Mørland (1983) might be attributed to the latter authors having frozen the unperfused livers; this freezing could have altered the structure of the haemoglobin released from erythrocytes, thereby increasing the availability of haeme for saturation with TDO. Stowell and Mørland (1983) as well as Badawy *et al* (1983) found that freezing perfused livers does not alter TDO activity;
(ii) Badawy *et al* (1983) did not observe any other reaction occurring that was detected at 365 nm; these authors suggest that perhaps dietary factors, in particular the carbohydrate content of the food administered to the animals used, may have accounted for the decrease in absorbance mentioned by Stowell and Mørland (1983); and
(iii) stimulation of TDO cannot occur post-mortem according to the method of Badawy and Evans (1975b) because this method specifies that homogenisation must occur in an ice-cold solution (Badawy *et al*, 1983).

4.5.2 *In vivo*

4.5.2.1 Materials, animals, reagents and equipment

As described in 4.5.1.1. Twenty male Wistar rats, weighing between 250g and 300g, and cared for as described in Appendix A, were used.

4.5.2.2 Method

The abovementioned TDO assay was also conducted after the *in vivo* administration of 5-FU and melatonin to rats, according to the five-day treatment protocol described below and depicted in Table 17. Four groups, each group containing five rats, were used. The injection volumes of saline, 5-FU and melatonin were 0.5 ml, while the injection volume of haematin was 0.1 ml. All injections were given intraperitoneally. Haematin was administered as well because it has been found, in the *in vivo* studies conducted by Badawy and Evans (1975b), to signficantly enhance TDO activity, especially that of the holoenzyme, which it increases by 200%. Haematin is taken up rapidly by the liver and, once there, it saturates the apoenzyme (Welch and Badawy, 1980). It has been noted by Welch and Badawy (1980) that the different forms of haematin and different doses of the compound may result in varying effects on TDO. As mentioned by Daya *et al* (1989), activating TDO enhances the degradation of tryptophan, thereby decreasing the availability of tryptophan for cerebral uptake (Badawy *et al*, 1987).

Table 17: Five-day treatment protocol for TDO assay <u>in vivo</u>

Group	**Dose of drug**	**Times of i/p administration**
Control	0.9% NaCl tds	08h00, 12h00, 16h00
5-FU	1 mg/ kg tds	08h00, 12h00, 16h00
Melatonin	1 mg/ kg tds	08h00, 12h00, 16h00
5-FU + Melatonin	1 mg/ kg 5-FU tds + 1 mg/ kg Melatonin tds	08h00, 12h00, 16h00
ALL – haematin	1 mg/ kg bd	09h30, 17h30

On the sixth day, the rats were sacrificed, the livers perfused and assayed for TDO activity, as described in 4.5.1.2, except that 5-FU and melatonin were not added. The concentration of kynurenine in each liver was determined in triplicate.

4.6 Results

The results of the mean specific activities of the total enzyme, holo- and apoenzyme are shown in the following Figures. All the results were analysed by ANOVA, followed by the Student-Neuman-Keuls multiple-range test.

4.6.1 *In vitro*

The following graphs illustrate the mean specific activities determined from the *in vitro* assay. As shown in Figure 36, a combination of 100 μM of both 5-FU and melatonin results in a significant decrease in total TDO specific activity, from a control value of 80.37 nmol/ μg/ min to 44.97 nmol/ μg/ min. This combination of 5-FU and melatonin also significantly inhibits the specific activity of the apoenzyme, as shown in Figure 37, from a control value of 44.35 nmol/ μg/ min to 12.72 nmol/ μg/ min. Neither drug, alone or in combination, has a significant effect on the activity of the holoenzyme *in vitro*, as shown in Figure 38. There are also no significant differences in the HS ratios.

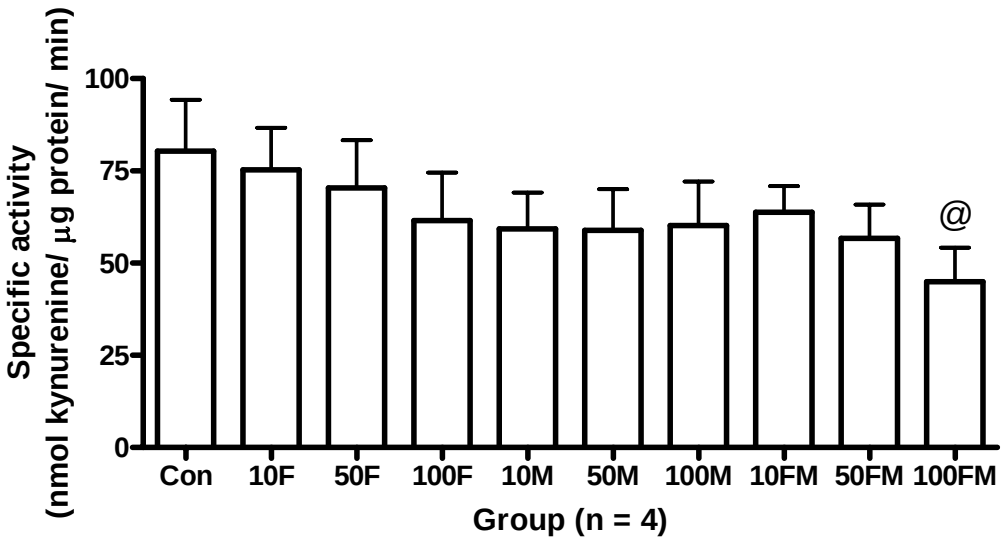


Figure 36: The effects of varying concentrations of 5-FU and melatonin on the total specific activity of TDO <u>in vitro</u>

[@] $p<0.05$ compared to control values, after analysis by ANOVA followed by the Student-Newman-Keuls multiple range test. All concentrations are in μM. Con = Control; F = 5-FU; M = Melatonin; FM = 5-FU + Melatonin. Each bar represents the mean ± SEM

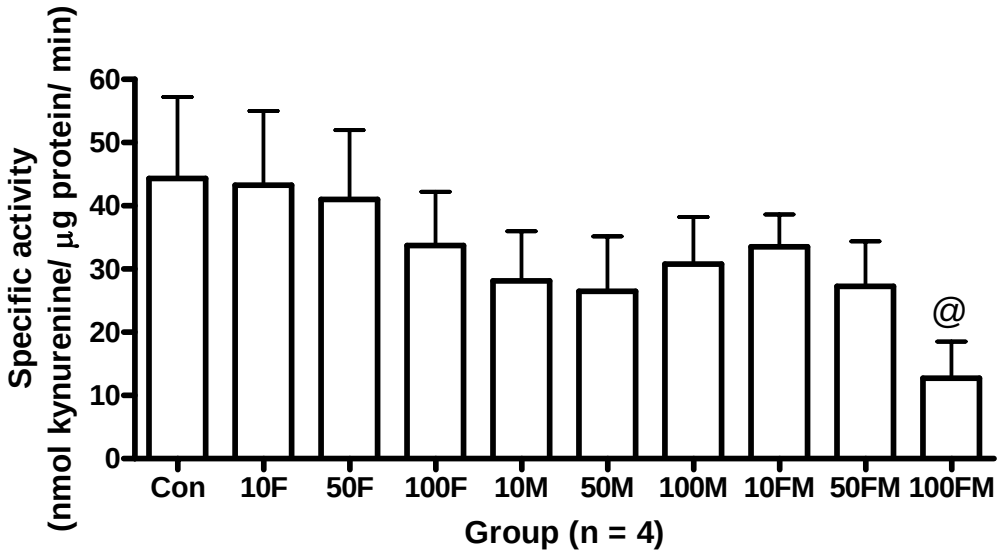


Figure 37: The effects of varying concentrations of 5-FU and melatonin on the specific activity of TDO apoenzyme <u>in vitro</u>

@ $p<0.05$ compared to control values, after analysis by ANOVA followed by the Student-Newman-Keuls multiple-range test. All concentrations are in µM. Con = Control; F = 5-FU; M = Melatonin; FM = 5-FU + Melatonin. Each bar represents the mean ± SEM

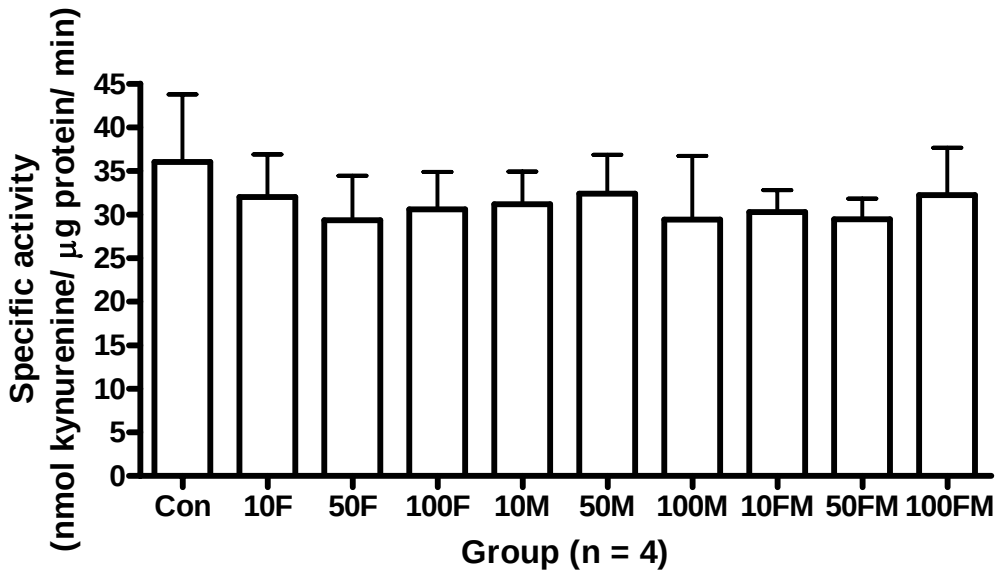


Figure 38: The effects of varying concentrations of 5-FU and melatonin on the specific activity of TDO holoenzyme <u>in vitro</u>

All concentrations are in µM. Con = Control; F = 5-FU; M = Melatonin; FM = 5-FU + Melatonin. Each bar represents the mean ± SEM

4.6.2. *In vivo*

The following graphs illustrate the results obtained from the *in vivo* study.

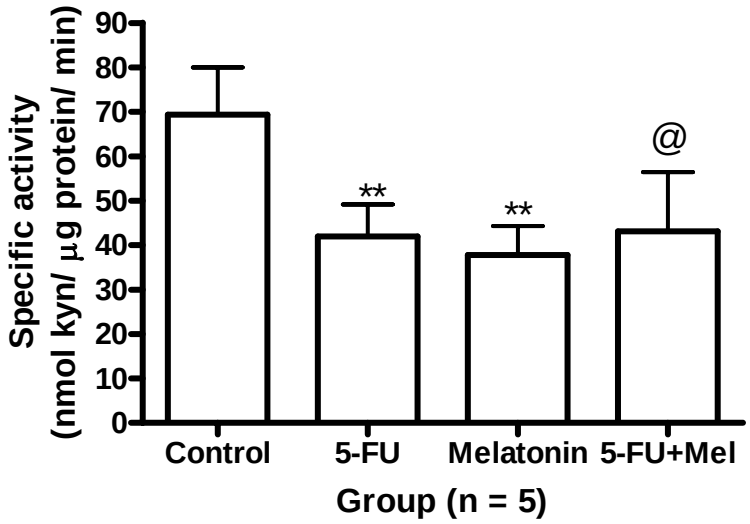


Figure 39: The effects of 5-FU and melatonin on the total specific activity of TDO <u>in vivo</u>

** $p<0.05$, @ $p<0.1$ when compared to control values, after analysis by ANOVA followed by the Student-Newman-Keuls multiple-range test. Each bar represents the mean ± SEM

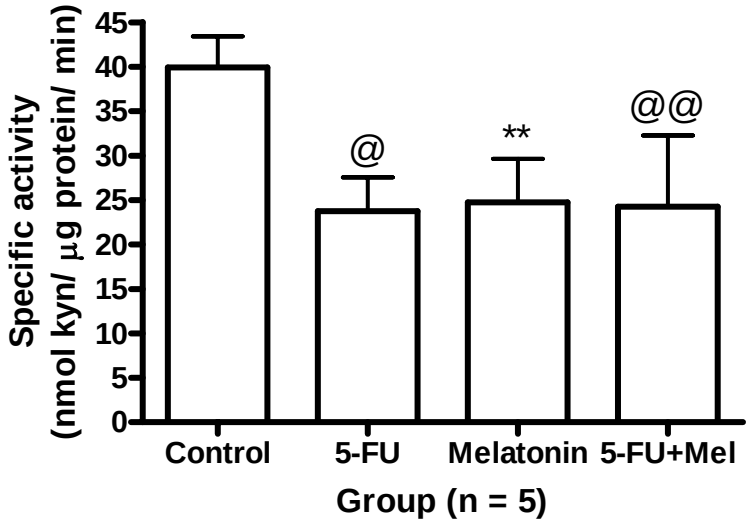


Figure 40: The effects of 5-FU and melatonin on the specific activity of TDO apoenzyme <u>in vivo</u>

** $p<0.05$, @ $p<0.01$, @@ $p<0.1$ when compared to control values, after analysis by ANOVA followed by the Student-Newman-Keuls multiple-range test. Each bar represents the mean ± SEM

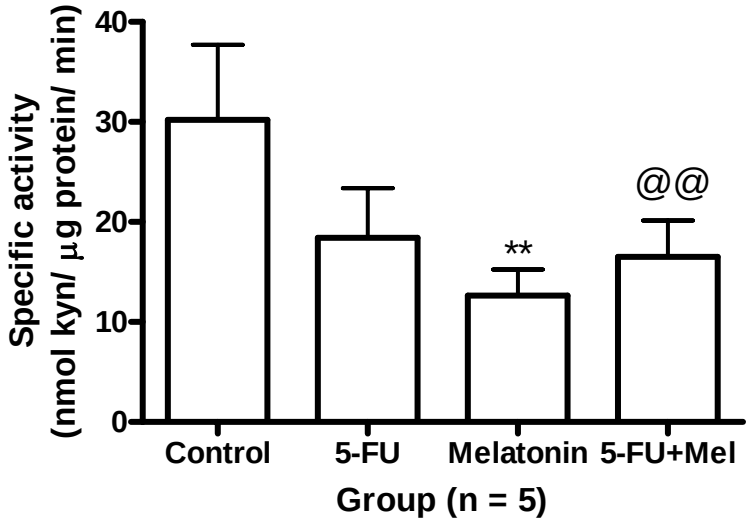


Figure 41: The effects of 5-FU and melatonin on the specific activity of TDO holoenzyme in vivo

** $p<0.05$, $^{@@}$ $p<0.1$ when compared to control values, after analysis by ANOVA followed by the Student-Newman-Keuls multiple-range test. Each bar represents the mean ± SEM

There are no statistically significant differences in the HS ratios.

In vivo, Figure 39 shows that both 5-FU and melatonin inhibit total enzyme activity, with the mean specific activity decreasing from 69.36 nmol/ µg/ min to 41 nmol/ µg/ min and 37.80 nmol/ µg/ min, respectively. When both drugs are administered together, the total enzyme activity is reduced to 43.12 nmol/ µg/ min.

Figure 41 shows that 5-FU does not significantly alter the activity of the holoenzyme, but melatonin results in a marked decrease from 30.21 nmol/ µg/ min to 12.63 nmol/ µg/ min. This is in contrast to the *in vitro* study, which shows that melatonin does not exert a significant effect on holoenzyme activity. Furthermore, for the group receiving both 5-FU and melatonin, the holoenzyme activity decreased to 16.53 nmol/ µg/ min.

In contrast to the *in vitro* study, 5-FU significantly inhibits apoenzyme activity *in vivo*, causing this to decrease from 39.91 nmol/ µg/ min to 23.75 nmol/ µg/ min (see Figure 40). This inhibition due to 5-FU is greater than that resulting from melatonin, with the latter causing apoenzyme activity to fall to 24.74 nmol/ µg/ min. The group exposed to both drugs showed a mean specific apoenzyme activity of 24.24 nmol/ µg/ min; this is an intermediate value to those obtained due to each drug individually. Thus, *in vivo*, 5-FU significantly

inhibits the total enzyme and the apoenzyme. Melatonin significantly inhibits the holoenzyme in addition to its inhibition of the apoenzyme and total enzyme.

4.7 Discussion

It has already been established that melatonin inhibits TDO in a dose-dependent manner at concentrations of between 50 μM and 200 μM (Walsh *et al*, 1991) and that this inhibition is reversible in the presence of excess melatonin (Walsh, 1997). Melatonin is a structural analogue of L-tryptophan and competitively inhibits TDO through a substrate mechanism (Walsh *et al*, 1994), by displacing tryptophan (Walsh and Daya, 1991). It has also been suggested that melatonin, through its free radical-scavenging properties (Hardeland *et al*, 1993; Poeggeler *et al*, 1993), could prevent *N*'-formylkynurenine from being formed (Walsh *et al*, 1994) by reacting with the superoxide anion generated in its formation (Feigelson *et al*, 1964; Walsh *et al*, 1994). This study confirms that melatonin inhibits TDO *in vivo*. *In vitro*, the concentrations of 5-FU and melatonin used individually were insufficient to significantly alter the activity of the total, holo- or apoenzymes; a maximal concentration of the drugs in combination, however, inhibited both the total and apoenzymes. No studies are reported in the literature on the effects exerted by 5-FU on TDO. This study shows that 5-FU inhibits TDO, but to a lesser extent than melatonin. Its mechanism of inhibition has not been established. It is known that the presence of a –NH functional group is necessary for an agent to bind to the catalytic site of TDO (Uchida *et al*, 1992), and since both 5-FU and melatonin possess this functional group, it is suggested that this is a mechanism by which both drugs bind to and inhibit TDO.

5-FU does not alter holoenzyme activity, but counteracts the melatonin-induced inhibition of holoenzyme activity *in vivo*. This has two possible implications:

(i) the fact that 5-FU significantly inhibits the apoenzyme form of TDO but not the holoenzyme suggests that the drug requires the presence of haematin in its mechanism of inhibition; the same may also be true of melatonin, which was found not to inhibit the holoenzyme *in vitro*, and

(ii) there could be a competitive relationship between 5-FU and melatonin, possibly with regard to binding sites on TDO. Further evidence for the latter suggestion arises from the finding that, in rats receiving both drugs *in vivo*, the extent of apoenzyme inhibition is intermediate compared to when the drugs are administered separately. This is in contrast to the *in vitro* study, which shows that the drugs potentiate each other's inhibition of apoenzyme activity.

Neither drug alters the HS ratio significantly, which, according to 4.3.3, suggests that neither drug alters the synthesis or metabolism of haeme. The mechanism of inhibition of the apoenzyme does therefore not involve decreasing the saturation of the aponezyme with its cofactor haeme.

4.8 Conclusions

Both 5-FU and melatonin inhibit TDO. 5-Fluorouracil inhibits only the apoenzyme, whereas melatonin also inhibits the holoenzyme. Melatonin competitively inhibits the enzyme through a substrate mechanism (Walsh *et al*, 1994). The mechanism of 5-FU-induced inhibition is at present unclear, but is thought to be due to the –NH group of the agent blocking the catalytic site of TDO. Enzyme inhibition may require the presence of the cofactor haeme, but does not alter the HS ratio of TDO. The consequences of TDO inhibition on brain neurotransmitter, particularly 5-HT, levels, will be discussed in Chapter 6. The effects of 5-FU and melatonin on IDO, another key enzyme involved in the first step of tryptophan metabolism, are investigated in the next Chapter.

CHAPTER 5

AN INVESTIGATION INTO THE EFFECTS OF 5-FLUOROURACIL AND MELATONIN ON INTESTINAL INDOLEAMINE 2, 3-DIOXYGENASE ACTIVITY *IN VIVO*

5.1 Introduction

In Chapter 4, the effects of 5-FU and melatonin on hepatic TDO activity were determined. As shown in Figure 34, this enzyme plays a key role in the degradation of tryptophan into *N*'-formylkynurenine, a metabolic pathway that accounts for 99% of tryptophan that is not utilised for protein synthesis (Grohmann *et al*, 2003). Another enzyme involved in this first step of tryptophan degradation is IDO.

IDO oxidatively cleaves the indole ring of tryptophan in a rate-limiting step (Grohmann *et al*, 2003), and the resulting anthraniloylalkylamine is subsequently a substrate for TDO (Sun, 1989). Due to its monomeric nature, the enzyme has a broad substrate specificity (Shimizu *et al*, 1978), also metabolising melatonin, 5-HT and tryptamine (Sun, 1989). As shown in Figure 42, melatonin is metabolised to form a kynurenine-type product, N^1-acetyl-N^2-formyl-5-methoxykynuramine (AFMK), which exhibits antioxidant properties (see 8.3). AFMK can also be generated from melatonin in macrophages by an IDO-independent mechanism, in the presence of myeloperoxidase (MPO) (Rodrigues *et al*, 2003). IDO is a haeme-containing enzyme and athough it was originally isolated from the small intestine, it occurs ubiquitously in mammalian organs (Shimizu *et al*, 1978). IDO activity is greatly inhibited in the absence of Mg^{2+}, adenosine triphosphate (ATP), catalase and methylene blue. The latter functions as a H_2O_2-generating system (Higuchi and Hayaishi, 1967). Superoxide anions ($O_2^{-}\cdot$) are essential cofactors in IDO activity (Hirata and Hayaishi, 1971, 1975; Taniguchi *et al*, 1977). If both D- and L- isomers of tryptophan are present, the enzyme preferentially oxidizes L-tryptophan (Higuchi and Hayaishi, 1967). IDO activity also fluctuates significantly according to the season, with activity in winter being 10-20 fold greater than in summer (Shimizu *et al*, 1978).

5.2 Effects of IDO

IDO plays important roles in the immune system, and it facilitates mechanisms that are both cytoprotective and cytotoxic, as described in 5.2.1 and 5.2.2. This has implications for drug efficacy and toxicity, as well as the survival and further growth of tumours. Figure 43 on the next page reflects these dual roles.

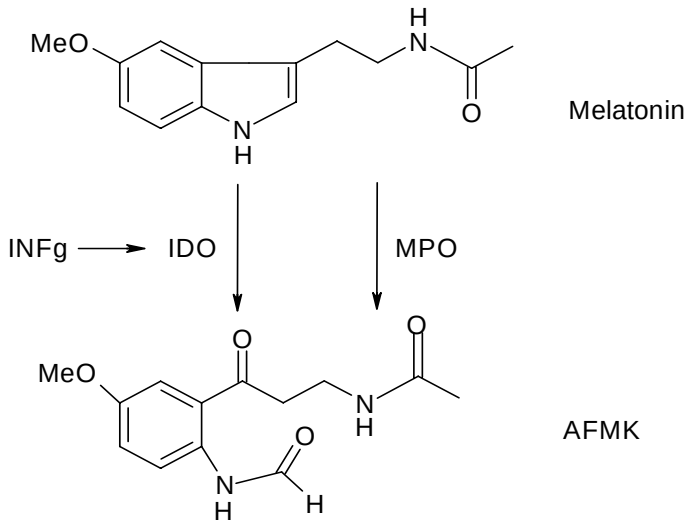


Figure 42: The metabolism of melatonin to AFMK via activation of IDO (Rodrigues *et al*, 2003)

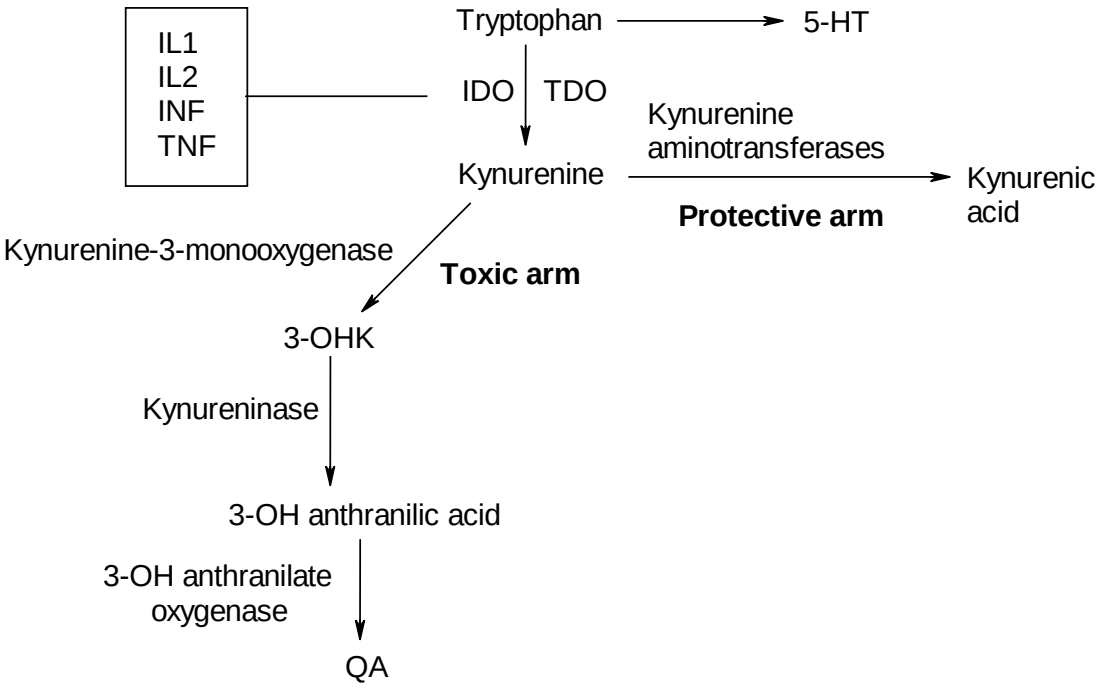


Figure 43: The IDO-catalysed metabolism of tryptophan, which can form both toxic and antioxidative kynurenines (modified from Myint and Kim, 2003)

5.2.1 Protective functions

Upon infection by micro-organisms, lymphocytes and leukocytes accumulate at the region of infection and release INFs. The latter, in particular INF-CE-B_3, stimulate IDO expression and the resultant tryptophan depletion inhibits microbial growth, as this amino acid is an essential requirement for growth to occur (Grohmann *et al*, 2003). Another example of this protective function is the finding that mammalian placental cells synthesis IDO in order to protect the

foetus against attack from maternal T cells (Munn *et al*, 1998). Some catabolites of trytoptophan, such as kynurenic acid (see Figure 43), are cytoprotective, by antagonising the effects of the neurotoxin quinolinic acid (QA) and other *N*-methyl-D-aspartate (NMDA)-receptor agonists (Grohmann *et al*, 2003) (see 6.2.2.16 and 8.2).

5.2.2 Toxic effects

IDO results in the eventual formation of kyurenine and related metabolites, many of which are neuroactive and have deleterious effects, such as QA and 3-OHK (see 4.1 and 8.2). In addition, many of the toxic and antiproliferative effects of INFγ are attributed to the ability of this cytokine to enhance the expression of IDO (Rodrigues *et al*, 2003; Carlin *et al*, 1989; Ozaki *et al*, 1988; Dai and Gupta, 1990), as increased IDO activity stimulates the metabolism of tryptophan. A deficiency of this amino acid results in numerous effects, such as 5-HT depletion in the brain (see 4.1).

IDO-catalysed metabolism of tryptophan occurs predominantly in the periphery, with QA and 3-OHK being able to cross the blood-brain barrier and induce neurotoxicity upon transporter-mediated entry into microglial cells and astrocytes (Grohmann *et al*, 2003). The cerebral catabolism of tryptophan to kynurenines can, furthermore, be induced upon injury to a certain region of the brain. The activity of IDO in the periphery can thus greatly influence the cerebral metabolism of tryptophan. Chronic activation of the immune system, for example, which enhances IDO activity, results in the influx of neurotoxins into the brain as well as depleting 5-HT levels (Grohmann *et al*, 2003). There is also enhancement of the inflammatory, neurodegenerative processes that underlie depression (see 6.2.2.16).

Kynurenines, such as QA and 3-OHK, induce the apoptosis of thymocytes and type 1 T lymphocytes through ROS-mediated mechanisms. This shifts the type 1/ type 2 T cell ratio towards type 2 cells (Grohmann *et al*, 2003) and, as discussed in 12.3.2, this induces immunosuppression and susceptibility to various infections. It is known, for example, that enhanced IDO activity occurs in HIV-positive patients (Schroecksnadel *et al*, 2007; Boasso and Shearer, 2007) and may shorten the progression time to AIDS, by the shift in T cell profile (see 12.3.2). In AIDS patients, increased IDO activity is associated with cachexia, diarrhoea and dementia (Brown *et al*, 1991). Furthermore, by catabolising melatonin, IDO decreases the antioxidative and antitumour effects of this compound (Grohmann *et al*, 2003; Bartsch and Bartsch, 1999).

Some tumours are able to escape immune rejection by upregulating IDO (Friberg *et al*, 2002) and the resultant decrease in the number of T cells. It has been shown that this mechanism of

immune escape is a potentially significant means by which tumour cells survive (Malachowski *et al*, 2005). By enhancing immune tolerance in the periphery, IDO creates a more hospitable host environment for tumour cells. This cytoprotective effect on cancer cells undermines the body's immune response against these cells and results in the maintenance and growth of tumours (Malachowski *et al*, 2005). These findings have prompted research into the development of IDO inhibitors as a novel class of anticancer agents (Malachowski *et al*, 2005; Muller and Prendergast, 2005).

5.2.3 Dual effects

In reconciling the abovementioned protective and toxic effects of IDO, Grohmann *et al* (2003) have suggested that the key role of this enzyme is to maintain immunologic self-tolerance and the homeostasis of T cells. This may be through the synthesis of kynurenine and related products in dendritic cells, a subset of antigen-presenting cells, and the effects that these compounds exert on the survival and proliferation of T cells (Grohmann *et al*, 2003). A benefit of kynurenine-mediated T-cell apoptosis is that lymphocytes possessing the potential to autoreact are targeted. This is important in the maintainance of peripheral tolerance, especially in chronic infections (Grohmann *et al*, 2003). It has been shown that a subset of T cells, Tr cells, can induce IDO-mediated apoptosis by interacting directly with dendritic cells (Grohmann *et al*, 2002). These Tr cells express T lymphocyte-associated antigen-4 (CTLA-4), which is cytotoxic (Grohmann *et al*, 2002; Salomon and Bluestone, 2001). In addition, Tr cells secrete IL-10, which exerts a major regulatory role in the expression of IDO by dendritic cells (Munn *et al*, 2002). Another advantage of IDO activation is that the use of $O_2^{-}\cdot$ decreases the amount of this ROS available to mediate cellular oxidative damage (Grohmann *et al*, 2003), albeit further toxins such as QA can be produced.

It is thus apparent that the immunoregulatory role of kynurenines, and the shift between cytotoxicity and cytoprotection, for both cancer and non-cancer cells, is complex and depends on a variety of pathologic and physiologic conditions and processes.

5.3 Experimentation

The effects of 5-FU and melatonin on IDO activity were determined *in vivo*, according to the method described by Higuchi and Hayaishi (1967) and Shimizu *et al* (1978).

5.3.1 Materials, animals, reagents and equipment

5-FU, melatonin and L-tryptophan were purchased from the Sigma Chemical Corporation, St Louis, MO, USA. NaH_2PO_4, methylene blue, catalase, ascorbate and TCA were purchased from UniLAB (South Africa). Diethyl ether was obtained from Saarchem® (Wadeville, South

Africa). Twenty male Wistar rats, each weighing between 200g and 300g, and cared for as outlined in Appendix A, were used. All solutions were prepared using deionised water (Milli R/Q System, Millipore®). Melatonin was dissolved using absolute ethanol as a co-solvent, at a final concentration of 0.67% v/ v. The Reaction Mixture consisted of: 100 mM NaH_2PO_4 buffer (pH 6.5), 0.4 mM L-tryptophan, 50 mM methylene blue, 50 mM ascorbate and 10 µg catalase. A Julabo PC water bath (Labotec, South Africa) was used. The UV spectrophotometer used was a Spectra System UV 2000®.

5.3.2 Method

Twenty rats, divided into four groups of five, were treated for a period of five days with either: (i) 5-FU, (ii) melatonin, (iii) a combination of these, or (iv) 0.9% saline, as described in Table 10 (see 3.6.2.2). On the sixth day, the rats were killed by inhalation of diethyl ether (see 12.8.2). A section of the ileum was cut and stored at -70ºC until assayed for IDO activity. The contents of each intestinal section was removed, and the section homogenised in 50 mM NaH_2PO_4 buffer, pH 7.0, at a final concentration of 10% w/ v. This was performed at 4ºC, to prevent the thermal degradation of IDO. The homogenate was centrifuged at 10 000g for 20 mins and the supernatant removed. An aliquot of 1.5 ml Reaction Mixture (see 5.3.1) was added to 1.5 ml of supernatant, in triplicate. All the compounds in the Reaction Mixture are required for maximal IDO activity (Higuchi and Hayaishi, 1967). The test tubes were incubated in an oscillating water bath at 37ºC for 60 mins, and the reaction subsequently terminated by the addition of 2 ml of 0.9 M TCA. The mixture was further incubated in an oscillating water bath at 50ºC for 30 mins. This was followed by centrifugation at 12 000g for 10 mins. The absorbance of the turquoise-coloured supernatant was read spectrophotmetrically at 335 nm, the absorption maximum of kynurenine (Higuchi and Hayaishi, 1967), with water as a blank. A protein assay was performed on the intestinal homogenate, as described by Lowry *et al* (1951) (see Appendix B). The concentration of kynurenine was calculated, as described in 4.5.1.2, and the results expressed as the specific activity of IDO (namely, the moles of kynurenine metabolised/ µg protein/ minute).

5.4 Results

Figure 44, on the next page, illustrates the effects of 5-FU and melatonin on the specific activity of rat intestinal IDO *in vivo*. Statistical analysis was performed using ANOVA, followed by the Student-Newman-Keuls multiple-range test.

As shown, both 5-FU and melatonin significantly enhance the specific activity of IDO, both individually and in combination. 5-FU doubles the specific activity from a mean control value of 24.99 mol kyn/ µg/ min to 50.31 mol kyn/ µg/ min, while the administration of melatonin

increases enzymatic activity to 46.75 mol kyn/ μg/ min. The administration of both these agents results in a further marginal increase, to 52.60 mol kyn/ μg/ min.

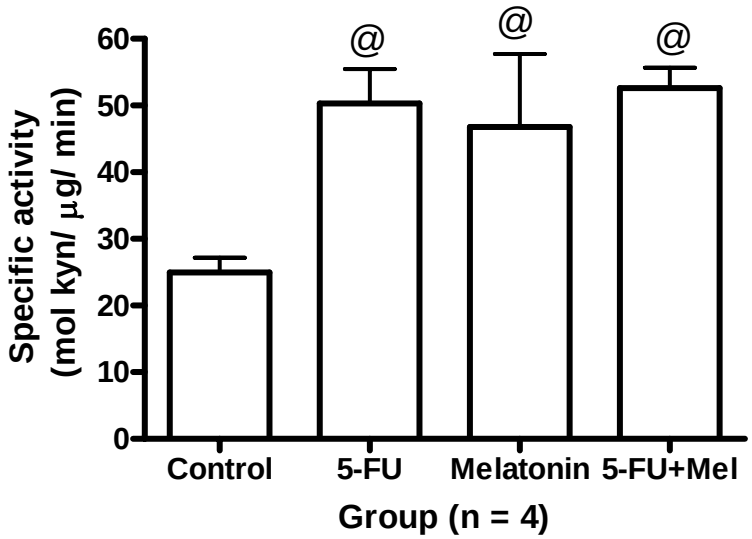


Figure 44: The effects of 5-FU and melatonin on intestinal IDO activity <u>in vivo</u>

[@] $p<0.05$ compared to control value, after analysis by ANOVA followed by the Student-Newman-Keuls multiple-range test. Each bar represents the mean ± SEM

5.5 Discussion

It has been shown that both 5-FU and melatonin increase IDO activity. This could be by possibly increasing the levels of $O_2^{-}\cdot$, essential cofactors for IDO activity, and/ or the pro-inflammatory cytokines shown in Figure 43, which can stimulate IDO. Melatonin is, furthermore, a natural substrate of IDO, and thus the exogenous administration of this agent may stimulate enzymatic activity. The effects of 5-FU and melatonin on $O_2^{-}\cdot$ and cytokine levels will be determined in Chapters 9 and 13, respectively.

There are several significant implications of enhanced IDO activity:

(i) a cytoprotective effect on cancer cells. This results in a decrease in the anticancer efficacy of 5-FU, as tumour cells utilise IDO activity as an immune escape mechanism (Malachowsi *et al*, 2005; Friberg *et al*, 2002). In addition, it has been noted in 1.3.12.1 that melatonin may enhance the proliferation of some cancer cells. The ability of prolonged melatonin administration to enhance IDO activity, and thus make the host environment more hospitable to cancer cells, may well be a mechanism by which this occurs;

(ii) a 5-FU-induced increase in QA and 3-OHK levels may be an additional mechanism of antineoplastic action of this drug;

(iii) a shift from type 1 to type 2 T cells is associated with immunosuppression and renders the patient increasingly susceptible to the development of infections. In HIV-positive patients, these changes promote the progression to AIDS (see 12.3.2). The use of 5-FU and melatonin in HIV infection is investigated in Chapter 12;
(iv) toxic kynurenines, such as QA and 3-OHK, produced as a result of IDO activity, may also cross the blood-brain barrier and worsen 5-FU-induced cerebral neurotoxicity;
(v) the generation of antioxidative kynurenines, such as kynurenic acid, counters some of the above toxic effects on non-cancer cells, to a certain extent. Conversely, these antioxidative kynurenines could exert a protective effect over cancer cells and inhibit QA- or 3-OHK-induced apoptosis of these cells. Similarly, the induction of IDO by 5-FU and the resulting formation of kynurenic acid could exert a protective effect on non-cancer cells, thereby limiting the toxicity and adverse effects of 5-FU; and
(vi) enhanced IDO activity results in a decrease in the amount of tryptophan available for cerebral uptake, thereby decreasing 5-HT levels in the brain (Myint and Kim, 2003) and leading to the development of depression. Furthermore, the toxic kynurenines formed as a result of IDO activity induce neurodegeneration, which is believed to result in depression (Myint and Kim, 2003) (see 6.2.2.16). The effects of 5-FU and melatonin on 5-HT levels will be investigated in the next Chapter.

It is thus apparent that the multiple effects of IDO activity give rise to complex interactions. The relative availability and activity of kynurenine aminotransferases and kynurenine-3-monooxygenase are of key importance in determining whether kynurenine is preferentially metabolised to kynurenic acid or 3-OH and QA, respectively (see Figure 43). The effects of these metabolites on cancer, non-cancer and immune cells can have contrasting implications, as described before.

5.6 Conclusions

The prolonged administration of 5-FU and melatonin both enhance the activity of IDO. Whether this is due to increases in the levels of $O_2^{-}\cdot$ and/ or pro-inflammatory cytokines shall be investigated further in Chapters 9 and 13. Enhanced IDO activity promotes immunosuppression and immune escape by cancer cells, as well as changes in the T lymphocyte profile that favours the progression to AIDS. The 5-FU-induced increase in QA and 3-OHK levels may be an additional mechanism of cytotoxicity, but this is limited by the generation of kynurenic acid. Furthermore, the IDO-induced immune escape mechanism allows the survival and growth of tumours, thereby limiting the anticancer efficacy of 5-FU treatment. IDO stimulation also results in the development of depression, and the effects of both 5-FU and melatonin on neurotransmitter levels will be investigated in the next Chapter.

CHAPTER 6

THE EFFECTS OF 5-FU AND MELATONIN ADMINISTRATION *IN VIVO* ON RAT FOREBRAIN NEUROTRANSMITTER LEVELS

6.1 Introduction

The effects of 5-FU and melatonin on two enzymes of key significance in the kynurenine pathway have been investigated in the preceding two Chapters. In Chapter 4, 5-FU and melatonin were administered to rats according to a five-day treatment protocol and the TDO assay performed on the livers. An increase in the activity of TDO in the liver results in a decrease in the availability of circulating tryptophan that can enter the brain (Maharaj *et al*, 2004). Once in the brain, tryptophan is converted to 5-HT (Fernstrom and Wurtman, 1971; Carlsson and Lindqvist, 1978; Maharaj *et al*, 2004). Daya *et al* (1989) thus conclude that there is an inverse relationship between the levels of 5-HT in the brain and hepatic TDO. TDO activity is a key peripheral indicator of the cerebral availability of tryptophan (Badawy *et al*, 1981). 5-HT, in turn, has been found to have an effect on the levels of other neurotransmitters in the brain, such as norepinephrine (NE) (Xi-Ming *et al*, 2002). NE levels are also influenced by the level of its reduced precursor, the neurotransmitter dopamine (DA) (Mohanakumar, 2004). Low levels of all these neurotrasnmitters are associated with depression (see 6.2.2.1, 6.2.2.2 and 6.2.2.3). In Chapter 5, it was found that the five-day administration of 5-FU and melatonin to rats enhances the activity of intestinal IDO, thereby potentially decreasing the amount of tryptophan available for cerebral uptake and decreasing 5-HT levels. This 5-HT depletion, as well as neurodegeneration induced by the toxic kynurenines formed as a result of IDO stimulation, can lead to the development of depression (Myint and Kim, 2003) (see 6.2.2.16).

In the light of the above findings, and the opposing effects of 5-FU and melatonin on different enzymes involved in tryptophan metabolism, it is of key importance to perform a neurotransmitter analysis and to determine the effects of 5-FU and melatonin on various neurotransmitters. Instead of measuring the levels of individual neurotransmitters, insight into the interdependency and interactions between different monoaminergic systems can be gleaned from measuring the levels of several neurotransmitters and their chief metabolites (Maharaj, 2004).

6.2 Depression

There are various theories about the cause of depression. The word "depression" is an umbrella term that describes numerous conditions and feelings, and is poorly defined since there has been much controversy over its root cause being psychological, physiological or genetic (Skene, 1979). It is now believed that depression is an interactive combination of many factors and influences, some of which have been mentioned above. The main symptom of all depressions is an alteration of mood; the individual experiences intense feelings of sadness, apathy, hopelessness and low self-esteem. The commonality of these symptoms suggests that all depressions have similar underlying neurophysiological pathways. Additional symptoms such as insomnia and changes in appetite may be present (Skene, 1979).

6.2.1 Aetiology of depression

Depression is an interaction of various endogenous and reactive factors; it is a complex convergence of psychological and physiological dysfunction. Some predisposing factors are (Skene, 1979):

(i) neuropharmacological and biochemical dysfunction;

(ii) personality traits (Abraham, 1960; Laughlin, 1956);

(iii) genetic predisposition (Mendlewicz *et al*, 1975; Winokur and Clayton, 1967; Winokur, 1969; Perris, 1966; Angst, 1966; Leonard, 1968; Mendels and Frazer, 1973); and

(iv) adversity in the developmental years (Bowlby, 1973; Granville-Grossman, 1968; Gregory, 1966a, 1966b; Abraham, 1960; Seay *et al*, 1962; Young *et al*, 1973; McKinney *et al*, 1971, 1972).

Factors that may precipitate depression in both predisposed and non-predisposed individuals include:

(i) physiological stresses, such as drug therapy (Goodwin and Bunney, 1971; Simonson, 1964);

(ii) certain disease states (Carpenter *et al*, 1972; Michael and Gibbons, 1963; Whybrow and Hurwitz, 1976); and

(iii) environmental stressors (Feldman, 1976; Gershon *et al*, 1971; Perris, 1966; Lewinsohn, 1974).

An holistic model of depression, that attempts to unify the biological, environmental and psychological facets of this disorder, was proposed by Akiskal and McKinney (1975) and is outlined in Figure 45. This model suggests that various factors could induce biochemical changes, which disrupt normal re-inforcement mechanisms, thereby leading to the development of depression (Akiskal and McKinney, 1973, 1975).

This model is useful as it shows the psychological and physiological reasons for depression, thus highlighting the importance of using a combination of psychotherapy and antidepressant drug therapy in order to successfully treat depressed patients.

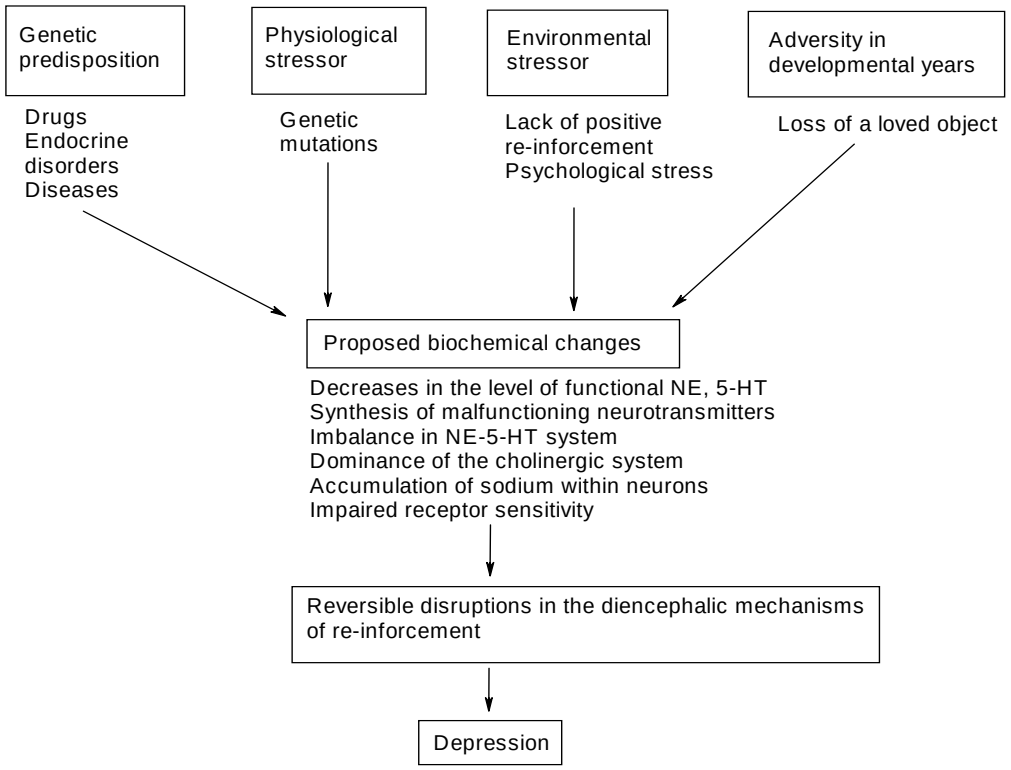


Figure 45: The Akiskal-McKinney unified model of the aetiology of depression (modified from Akiskal and McKinney, 1973; cited in Skene, 1979)

6.2.2 Biological hypotheses of depression

Sixteen possible biological hypotheses of depression are presented below, from the earliest classical biological hypotheses of amine deficiency, to the latest ones of macrophage involvement and neurodegeneration. It is not clear whether these hypotheses are responsible for the development of depression, or a consequence of this condition (Skene, 1979).

6.2.2.1 Catecholamine hypothesis

This hypothesis, presented by Schildkraut (1965), states that depression is due to a deficiency of catecholamines, in particular NE. This deficiency could be relative or absolute (Walsh, 1997). A deficiency of NE leads to fewer interactions with noradrenergic or dopaminergic receptor sites. Very high levels of neurotransmitters are associated with mania, which clinically has the opposite manifestations to depression (Court, 1968; Walsh, 1997). The catecholamine hypothesis is based on the finding that reserpine, which can induce depression (Harris, 1957), induces a deficiency of catecholamines in the brain (Shore, 1962).

Furthermore, an antidepressant, such as iproniazid (Cole *et al*, 1961; Crane, 1957), is thought to function by inhibiting MAO (Spector *et al*, 1960, 1963), thereby increasing the level of catecholamines.

A major shortcoming of the catecholamine hypothesis is that many antidepressants, such as reserpine and MAO inhibitors, also alter 5-HT levels. This, as opposed to the increase in catecholamine levels, may account for the antidepressant effect (Skene, 1979) (see 6.2.2.2).

The catecholamine precursor L-DOPA has been found by several investigators to be ineffective in the treatment of depression (Pare and Sandler, 1959; Schildkraut *et al*, 1963; Davis, 1970). This could be due to the drug's hydrophilicity, which renders it unable to cross the blood-brain barrier, thereby resulting in subtherapeutic brain levels (Bertler *et al*, 1966). L-DOPA is decarboxylated to form DA in the brain, thereby increasing DA levels, but only a small percentage of this DA is converted to NE (Glowinski and Iversen, 1966). There may be an increase in the turnover of NE (Dairman and Udenfriend, 1971), but the general cerebral levels of NE remain the same or decrease (Everett and Borcherding, 1970; Butcher and Engel, 1967).

L-DOPA is, however, found to be effective in treating refractive depression (Bunney *et al*, 1971), if administered on alternate days (Ingvarsson, 1965). High doses of L-DOPA have been shown to be effective in approximately 33% of patients, and this is unaltered in the presence of a decarboxylase inhibitor (Matussek *et al*, 1970; Goodwin *et al*, 1970), which prevents the peripheral conversion of L-DOPA to the hydrophilic DA.

The effects of L-DOPA are not limited to catecholamines; the drug also decreases 5-HT levels (Everett and Borcherding, 1970; Murphy, 1972) by inhibiting the synthesis of this indoleamine neurotransmitter (Dunner and Goodwin, 172; Goodwin *et al*, 1971). This once again highlights the role of the serotonergic system in the aetiology of depression, which is not encompassed in the catecholamine hypothesis of depression.

6.2.2.2 Indoleamine/ biogenic amine hypothesis

This is a modification of the catecholamine hypothesis, and postulates that depression is primarily due to low levels of monoamine neurotransmitters, such as 5-HT, in the brain (Lapin and Oxenkrug, 1969; Bunney and Davis, 1965; Coppen, 1967). This deficiency of 5-HT in the brain decreases the extent of occupation of serotonergic receptors and a decrease in the effects mediated by 5-HT.

Clinical evidence supports the biogenic amine hypothesis, as antidepressants have been found to act by increasing neurotransmitter levels at synaptic clefts. This is done either by inhibiting MAO, the enzyme that degrades these biogenic amines, or by preventing the re-uptake of the neurotransmitters. Tricyclic antidepressants (TCAD), the prototype antidepressants, function according to the last mechanism; re-uptake of neurotransmitters results in less being secreted into the synaptic cleft, and TCAD thus prevent this, thereby maintaining or increasing neurotransmitter levels (Baldessarini, 2001). The turnover of 5-HT in the brain is thus decreased with TCAD treatment (Goodwin and Post, 1974). Electroconvulsive therapy (ECT), which was extensively used to treat depresion prior to the advent of TCAD, is known to increase the levels of HA, the main metabolite of 5-HT, in cerebrospinal fluid (Skene, 1979; Van Praag, 1977). Increased concentrations of HA are indicative of enhanced 5-HT synthesis in the brain (Skene, 1979; Chase, 1972).

The indoleamine hypothesis of depression highlights the importance of 5-HT and states that alterations in the neuronal functioning of 5-HT are a key factor in depression, as evidenced by the success of selective serotonin re-uptake inhibitors (SSRI) as antidepressants (Walsh, 1997; Blier and de Montigny, 1994; Delgado *et al*, 1990). The indoleamine hypothesis is strengthened by the finding that drugs which lower the level of 5-HT in the brain, such as the tryptophan hydroxylase inhibitor PCPA, precipitate depression (Bunney *et al*, 1969). The administration of PCPA to patients on TCAD therapy reverses the efficacy of these (Shopsin *et al*, 1974, 1976).

There are many criticisms of the biogenic amine hypothesis of depression, such as:
(i) the contention that there is no clear relationship between antidepressant drug therapy and biogenic amine levels clinically (Charney *et al*, 1981);
(ii) the fact that there is a long lag phase of several weeks before antidepressants exert an effect, yet MAO inhibition and the prevention of amine uptake are known to occur in minutes (Blier and de Montigny, 1994);
(iii) the finding that compounds such as cocaine, which do not have antidepressant action (Hansson, 1967), increase biogenic amine levels at synaptic clefts (Walsh, 1997); and
(iv) depletion of the 5-HT precursor tryptophan, while inducing a relapse in patients with a history of depression (Moreno *et al*, 1996; Smith *et al*, 1997), does not provoke clinical symptoms of depression in individuals with no history of the disorder (Cowen, 1998).

The effectiveness of L-tryptophan in treating depression, remains equivocal (Cocheme, 1970; Van Praag and Korf, 1970; Bowers, 1970; Murphy *et al*, 1973). It is more positively associated with enhancing the effect of an MAO inhibitor (Coppen *et al*, 1963; Pare, 1963;

Van Praag, 1970) or a TCAD (Wålinder *et al*, 1975) in a subgroup of depressives (Van Praag and Korf, 1971). Some types of cancer can cause a deficiency of 5-HT, and the resultant depression is attenuated with L-tryptophan (Johansson *et al*, 1973; Lehmann, 1972). Patients suffering from Parkinson's disease and who are on L-DOPA therapy may develop depression, due to competition between L-DOPA and tryptophan for uptake into the brain (Lehmann, 1973); less tryptophan enters the brain and is subsequently converted into 5-HT. Alternatively, the increased levels of DA could displace 5-HT from vesicular storage sites (Birkmayer and Neumayer, 1972).

Even after apparent clinical recovery, depressives retain impaired 5-HT metabolism (Mendels *et al*, 1972; Coppen *et al*, 1972). This suggests that while 5-HT deficiency is an important factor in the aetiology of depression, other factors are involved in the full clinical development of this condition (Coppen, 1972; Prange *et al*, 1974; Cowen, 1998).

6.2.2.3 DA hypothesis

Another hypothesis of the cause of depression is the DA hypothesis (Randrup and Braestrup, 1977). DA plays a vital role in the central nervous system (CNS), where it is the most ubiquitous catecholamine neurotransmitter (Pivonello *et al*, 2004). It has been observed that the central nervous system levels of homovanillic acid, a metabolite of DA, are very low (Syvälahti, 1994) and this could suggest that levels of DA could be important in the pathogenesis of depression (Blier and de Montigny, 1994; Syvälahti, 1994). It has been found, furthermore, that TCAD significantly inhibit the re-uptake of DA at synaptic clefts (Randrup and Braestrup, 1977). A DA agonist, piribedil, also reverses the symptoms of depression (Post *et al*, 1978). As mentioned in 6.2.2.1, the use of the DA precursor L-DOPA, seems to have disputed efficacy in the treatment of depression. It is suggested that DA may play a role specifically in the motor dysfunction that is a component of depression (Skene, 1979).

6.2.2.4 Permissive amine hypothesis

The permissive amine hypothesis is a more holistic one than the previous hypotheses. It postulates that although a catecholamine deficiency leads to the clinical development of depression, and a biogenic amine deficiency predisposes an individual to depression, these two deficiencies have to occur in combination in order to cause depression (Kety, 1971; Prange *et al*, 1974). Depression and mania are, furthermore, believed to be on a continuum, rather than being disorders with opposite manifestations (Court, 1968). This is evidenced by the finding that lithium, a traditional anti-manic agent, has some efficacy in the treatment of depression (Goodwin *et al*, 1969). Support for the neurophysiological similarities between mania and depression (Mendels, 1969) comes from the finding that L-tryptophan is useful in

treating mania (Prange *et al*, 1974), by correcting a deficiency of 5-HT (Prange *et al*, 1974; Sheard and Aghajanian, 1970; Knapp and Mandell, 1973). Furthermore, by decreasing 5-HT levels (see 6.2.2.1), L-DOPA is known to induce mania (Murphy *et al*, 1971). It thus seems that while mania is associated with an increase, and depression with a decrease, of catecholamine levels, both these disorders have a 5-HT deficiency in common (Skene, 1979).

6.2.2.5 Kynurenine hypothesis

This states that there are significantly increased levels of plasma cortisol in depression, and that this enhances the synthesis of TDO, which is responsible for the hepatic metabolism of tryptophan (see 4.1). The resultant increase in the metabolism of tryptophan decreases the amount of tryptophan available to enter the brain and be converted to the neurotransmitter 5-HT (Curzon and Bridges, 1970; Curzon, 1969; Lapin and Oxenkrug, 1969) (see 4.1). Metabolites of tryptophan, such as 3-OHK and kynurenine, could induce some of the symptoms of depression (Walsh, 1997). Increased levels of the former have been found in people suffering from depression (Curzon and Bridges, 1970). Hydrocortisone, which stimulates TDO, has also been found to indirectly decrease the levels of 5-HT and HA in the brain (Curzon and Green, 1968).

Low levels of 5-HT in the brain are believed to weaken the inhibitory pathways that regulate the amygdaloid complex (Lapin and Oxenkrug, 1969). The resultant activation of the latter may stimulate the synthesis of adrenocorticotrophic hormone (ACTH) in the anterior pituitary; ACTH, in turn, induces an elevation in cortisol levels (see 6.2.2.9.1.1). As mentioned, increased cortisol production enhances the activity of TDO and thus cerebral 5-HT levels are further depleted (Lapin and Oxenkrug, 1969). This cyclical process is illustrated below (modified from Lapin and Oxenkrug, 1969).

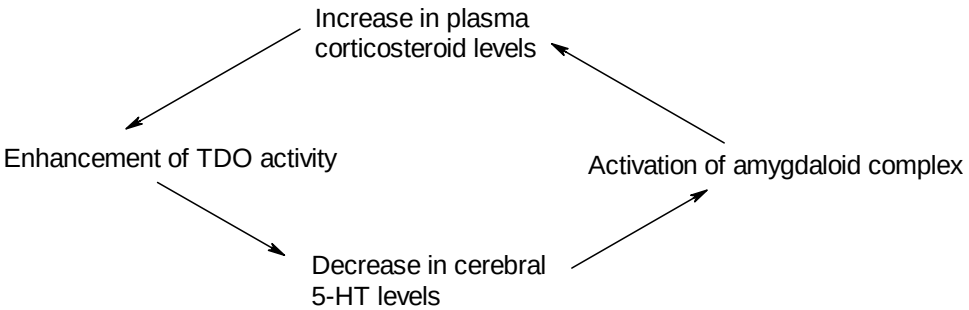


Figure 46: The kynurenine hypothesis of depression (modified from Lapin and Oxenkrug, 1969)

Inhibitors of TDO, such as nicotinamide, have been found to be beneficial in the treatment of depression (Chouinard *et al*, 1977; Curzon, 1969), as the inhibition of this enzyme allows more tryptophan to enter the brain and subsequently be converted to 5-HT, thereby increasing the levels of this neurotransmitter. It has also been found that some TCAD, such as imipramine, also inhibit TDO (Paracchi, 1967) (see 4.3.2), and this could be an additional mechanism of antidepressant action. Furthermore, tryptophan has been found to decrease plasma cortisol levels (Guzik *et al*, 2006) and, as discussed in 6.2.2.9.1.1, this may counter depression. A weakness of the kynurenine hypothesis is that it is as yet unknown whether TDO stimulation significantly alters the amount of the tryptophan that can be converted into 5-HT in depressed humans (Skene, 1979).

It should be noted that some types of porphyria are associated with depression. Badawy and Evans (1973a) suggest that, in patients with hepatic porphyrias, there may be an enhancement of TDO activity due to haeme, the biosynthesis of which is increased due to the stimulation of the enzyme 5-ALA synthase (see 4.3.3). This increased TDO activity might account for the impaired synthesis of 5-HT in these patients, leading to depression (Badawy and Evans, 1973a; Badawy *et al*, 1987). The kynurenine hypothesis is extended in 6.2.2.16.

6.2.2.6 Tryptamine hypothesis

This hypothesis, postulated by Dewhurst (1968a, 1968b), focuses on the influence of 5-HT and NE on receptors in the CNS, and thus diverges from the aforementioned hypotheses of depression. Dewhurst (1968a) classifies monoamines into the following two groups:
(i) Type C, or depressant, amines. These have a general chemical formula of $R'CH(OH)CH_2NHCH_3$; R' is a polar group, such as catechol. An example of a Type C amine is epinephrine (EN), which induces sedation in immature chicks; and
(ii) Type A, or excitant, amines, having the formula $RCH_2CH_2NH_2$; the R' group is a planar, lipophilic hydrocarbon moiety, such as a phenyl group. An example of a Type A amine is tryptamine, which induces both physiological and biological excitation in chicks (Dewhurst, 1968a).

According to this hypothesis, supersensitivity of Type A receptors, or enhanced levels of Type A amines, is responsible for the development of mania. Depression is attributed to subsensitivity of these receptors or a deficiency in Type A amines . The antidepressant effect of tryptophan may be due to it being a precursor of tryptamine; tryptamine levels are also increased by MAO inhibitors (Dewhurst, 1968a)..

6.2.2.7 False neurotransmitter hypothesis

This attributes the development of depression to endogenously-synthesised "false neurotransmitters", which inhibit the activity of the adrenergic system in the brain (Murphy, 1972). This is in contrast to the catecholamine and biogenic amine deficiency hypotheses (Schildkraut, 1965).

It is not known for certain what the roles of false neurotransmitters are, but the involvement of these in the pathogenesis of depression is supported by the fact that α-methyldopa, itself a false neurotransmitter, induces depression, particularly in individuals who have experienced this disorder before (Fullerton and Morton-Jenkins, 1963; McKinney and Kane, 1967).

6.2.2.8 Adrenergic-cholinergic hypothesis

The involvement of the cholinergic system, and in particular the balance between the cholinergic and adrenergic systems, is believed to play an important role in the aetiology of depressive disorders (Janowsky *et al*, 1973a, 1973b, 1972a, 1972b; Fritze, 1993; Janowsky *et al*, 1994; Fritze *et al*, 1995; Overstreet and Janowsky, 1991). These authors have suggested that mania is due to over-enhancement of the adrenergic system, reflected in elevated levels of biogenic amines and a deficiency of acetylcholine (Ach). Depression, on the other hand, is attributed to the domination of the cholinergic system, which results in high levels of Ach and low biogenic amine levels.

Support for this hypothesis comes from the finding that the known depressant, reserpine, has cholinomimetic effects (Baldessarini, 2001; Bogdanski *et al*, 1961). It thus enhances the effects of the cholinergic system, in addition to its known ability to deplete biogenic amine stores (Baldessarini, 2001), thereby precipitating depression through a dual mechanism. The agent α-methyl-p-tyrosine (AMPT) inhibits the synthesis of catecholamines, but yet does not cause depression; this is believed to be due to its lack of effect on the cholinergic system in the brain (Charalampous and Brown, 1967). The TCAD also have anticholinergic effects (Janowsky *et al*, 1972b) and this may be an additional mechanism of their antidepressant action. Furthermore, anticholinergic agents enhance motor activity as well as self-stimulation in the brain (Stein, 1964), functions that are decreased in depression.

Compelling support for the role of the cholinergic system in the aetiology of depression comes from studies on the effects of cholinomimetic drugs, such as physostigmine, in depressed humans. A worsening of depression has been reported in these subjects (Modestin *et al*, 1973; Tamminga *et al*, 1976; Janowsky, 1972b). Cholinesterase inhibitors, which

enhance Ach levels by inhibiting its metabolism, result in numerous effects, one of which is depression (Bowers *et al*, 1964; Rowntree *et al*, 1959).

6.2.2.9 Biochemical abnormalities

The biochemical abnormalties that may play a role in the aetiology of depression fall into two broad categories (Skene, 1979): (i) neuroendocrine, and (ii) electrolyte disturbances.

6.2.2.9.1 Neuroendocrine abnormalities

There are three major neuroendocrine systems that could be disturbed, namely the (i) hypothalamic-pituitary-adrenal (HPA), (ii) hypothalamic-pituitary-thyroid (HPT), and (iii) hypothalamic-pituitary-growth hormone (HPG) systems (Skene, 1979). In each of these systems, the hypothalamus stimulates the pituitary gland to release a hormone that targets a specific end organ, such as the adrenal gland, which can then release further factors or hormones.

6.2.2.9.1.1 HPA axis dysfunction

The HPA axis is illustrated in Figure 47. Physiological stressors stimulate the release of corticotropin-regulating hormone (CRH) from the hypothalamus, into the hypophyseal-portal blood vessels. These blood vessels transport CRH to the adenohypophysis, where it stimulates the secretion of ACTH. The latter hormone acts on the adrenal glands, the end organs of the HPA axis, to enhance the production and release of corticosteroids. Through protein kinase- and adenylate cyclase- mediated mechanisms, ACTH enhances the rate-limiting step in the biosynthesis of steroid hormones, namely the amount of cholesterol that enters mitochondria and is subsequently converted to pregnenolone (Haynes and Larner, 1975). The glucocortoicoids released upon stimulation of the HPA axis interact with glucocorticoid receptors in the pituitary gland and suprahypothalamic regions, in particular the hypothalamus, which is the brain structure with the highest number of corticosterone receptors (McEwen *et al*, 1968). This inhibits further release of CRH and ACTH (Keller-Wood and Dallman, 1984; Haynes and Larner, 1975) through negative feedback mechanisms. Chronic stimulation of the HPA axis results in a depletion of hippocampal glucocorticoid receptors, thus resulting in a loss of sensitivity in the negative feedback mechanism (Sapolsky *et al*, 1986) and a failure to inhibit the adrenal response to stimulation of the HPA axis. The subsequent sustained increase in the levels of glucocorticoids is a feature of chronic stress (see 10.7).

The pineal gland is believed to play a regulatory role in the output of the adrenal glands (Van Wyk, 1993). Pinealectomy has been shown to result in hypertrophy of the adrenal glands

(Wurtman *et al*, 1959; Vaughan *et al*, 1972), which is reversed upon the administration of melatonin (Vaughan *et al*, 1972). Compounds secreted from the pineal gland inhibit the production or release of ACTH from the adenohypophysis (Van Wyk, 1993). Although there are conflicting reports in the literature on the nature of the relationship between the pineal and adrenal glands, it appears that melatonin, secreted by the pineal gland and acting via a central adrengergic pathway, inhibits the secretion of ACTH and thus decreases corticosteroid levels (Van Wyk, 1993; Motta *et al*, 1971; Kinson *et al*, 1967; Armstrong *et al*, 1982). Alternatively, there is evidence that melatonin enhances the activity of adrenal 5α-reductase in the adrenal cortex, to decrease the secretion of glucocorticoids (Johnson, 1982; Ogle and Kitay, 1976). The effects of the pineal gland on adrenal function may also be mediated by the antigonadal effects of the former. Pinealectomy, which removes pineal-induced ovarian inhibition, results in an increase in the levels of oestrogen. This hormone significantly promotes the release of ACTH and inhibits adrenal reductase, thereby altering the synthesis and metabolism of adrenal steroids (Ogle and Kitay, 1976; Van Wyk, 1993). The pineal gland, by regulating the HPA axis, thus functions in promoting physiological adaptation to stress. Although the main pineal derivative that exerts an effect on adrenal function is melatonin, there is increasing evidence that other pineal compounds, such as the pineal peptide hormone arginine vasotocin, also inhibit the release of ACTH and thus decrease adrenal corticosteroid secretion (Porter and Heiman, 1977; Johnson, 1982).

It has long been known that dysfunctioning of the HPA axis is associated with affective disorders (Engel and Margolin, 1941; Michael and Gibbons, 1963; Whybrow and Hurwitz, 1976). Addison's disease, for example, a disorder of hypoactivity of the HPA axis, is associated with symptoms of mental depression (Cleghorn, 1951; Engel and Margolin, 1941). Disorders of HPA overactivity, such as Cushing's syndrome, as well as long-term corticosteroid treatment, are known to induce affective changes, including depression (Borman and Schmallenberg, 1951; Cobb, 1960; Fawcett and Bunney, 1967). Both hypo- and hyper-activity of the HPA axis may result in depression as in both conditions there may be elevated levels of ACTH secreted from the pituitary gland (Skene, 1979). This suggests that glucocorticoids may play a role in the development of depression.

There is also an intimate relationship between HPA functioning and the level of excitatory amino acids, such as glutamate. These amino acids stimulate ACTH secretion (Jezova, 2005; Makatsori *et al*, 2004), thereby increasing cortisol levels. In addition to elevated cortisol levels being associated with depression, cortisol does, in turn enhance cellular susceptibility to excitotoxins (McEwen, 1999; McEwen and Magarinos, 1997) (see 10.7) as well as promote the release of glutamate in the hippocampus (Iqbal *et al*, 2006). This results in excitotoxicity

and neurodegeneration, which are believed to be further mechanisms of the development of depression (Myint and Kim, 2003) (see 6.2.2.16).

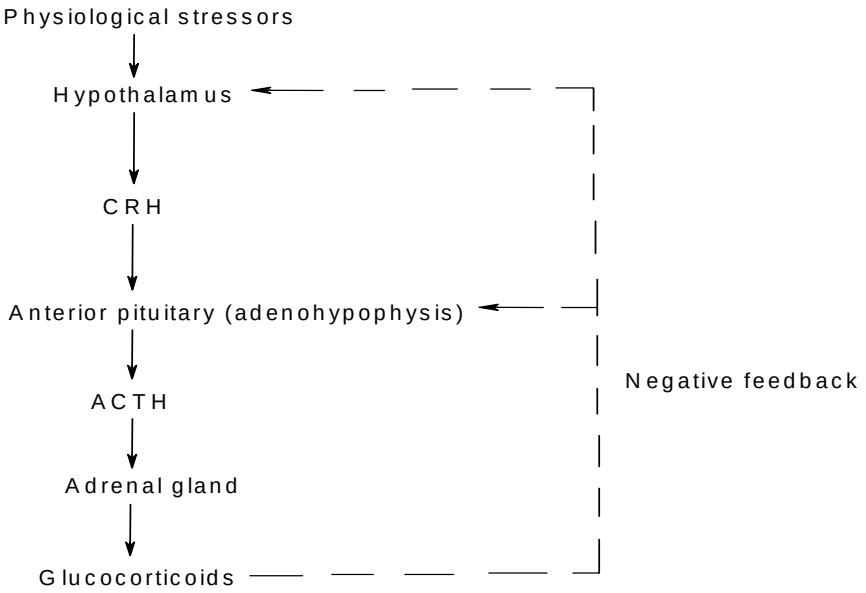


Figure 47: The hypothalamic-pituitary-adrenal (HPA) axis (Haynes and Larner, 1975)

The rate of cortisol secretion is significantly raised in depressives (Carpenter and Bunney, 1971; Gibbons, 1964, 1966; Carroll, 1972; Sachar *et al*, 1973). Plasma levels of cortisol are an indirect reflection of the rate at which ACTH is released from the anterior pituitary (Skene, 1979). It is believed that the aforementioned increase in the rate of cortisol secretion is due to an increase in the rate of ACTH released and is not as a result of adrenal supersensitivity to ACTH (Carroll and Mendels, 1976). There is also an increase in brain cortisol levels (Carroll and Mendels, 1976; Carroll, 1972), although some investigators have reported these levels to be lowered in depressives (Carpenter and Bunney, 1971; McClure and Cleghorn, 1968).

Increased cortisol levels are known to occur in conditions of stress, and it was thus initially believed that the high levels of cortisol in depressed individuals is secondary to an underlying psychological stress (Sachar, 1967; Brooksbank and Coppen, 1967). Curzon (1969), however, suggests that, in depression, hypersecretion of cortisol may also be indicative of dysfunction in the limbic system, which controls emotions and mood (Skene, 1979). This latter hypothesis has been expanded by others (Sachar *et al*, 1973; Carroll and Mendels, 1976), who have found that depressed individuals exhibit disturbances in circadian HPA rhythms. In normal subjects, cortisol levels peak in the morning and trough in the late evening, as the latter is the period when the brain exerts its greatest inhibitory effect on the HPA axis (Carroll, 1972). It has been found, however, that in depressives this trough is significantly less pronounced (Sachar *et al*, 1973; Doig *et al*, 1966; Fullerton *et al*, 1968), and this has been hypothesised to

reflect a failure on the part of the brain to inhibit the HPA axis (Sachar *et al*, 1973; Carroll and Mendels, 1976). This has been corroborated by numerous clinical determinants of HPA functioning (Carroll, 1969, 1972; Jakobson *et al*, 1969; Perez-Reyes, 1972), the most recognised of which is the dexamethasone suppression test. In normal individuals, a dose of dexamethasone administered at midnight results in maximal suppression of plasma levels of cortisol (McHardy-Young *et al*, 1967). It is widely shown that this suppression of cortisol levels either does not occur in depressed individuals (Platman and Fieve, 1968; Butler and Besser, 1968; Stokes, 1972; Endo *et al*, 1974; Carroll and Mendels, 1976), or may occur initially and is then followed by an "escape" from cortisol suppression (Carroll, 1972; Carroll and Mendels, 1976). It has been found that an increase in the severity of depression enables the individual to "escape" abnormally early on the day after dexamthasone administration (Carroll, 1972; Carroll and Mendels, 1976; Skene, 1979).

Steroids other than cortisol may also play a role in the mediating the effects of the HPA axis on the CNS (Goodyer *et al*, 1998). A physiological, age-related decrease in the levels of dehydroepiandrosterone (DHEA), for example, which antagonises the effects of cortisol, and a subsequent increase in the cortisol/ DHEA ratio, may contribute to the development of depression (Cowen, 1998).

Excess cortisol has been shown to lower 5-HT functioning in the brain, in a similar manner to the technique of tryptophan depletion, by decreasing the expression of 5-HT receptors and enhancing the metabolism of the 5-HT precursor tryptophan (Chaouloff, 1995; Maes *et al*, 1990; Cowen, 1998). As low 5-HT levels are known to be associated with depression (see 6.2.2.2), this finding points to an interaction between cortisol and 5-HT that could provide another mechanism by which the complex process of depression occurs. Excess levels of glucocorticoids, furthermore, result in the following serotonergic alterations (Maes *et al*, 1995):

(i) an increase the metabolism of 5-HT in the brain (De Kloet and Reul, 1987; Singh *et al*, 1990, 1991);

(ii) downregulation of 5-HT_{1A} receptors (Bagdy *et al*, 1989; Young *et al*, 1992); and

(iii) enhancement of the sensitivity or number of postsynaptic 5-HT_2 receptors (Kuroda *et al*, 1992).

5-HT itself stimulates HPA functioning via the following mechanisms (Maes *et al*, 1995):

(i) 5-HT_{1C} and 5-HT_2 receptor agonism by arginine vasopressin promotes the secretion of ACTH; and

(ii) the aforementioned receptors, as well as 5-HT_{1A} receptors, stimulate the secretion of CRH (Calogero *et al*, 1993; Van de Kar *et al*, 1989; Rivier and Plotsky, 1986).

A deficiency of the neurotransmitter 5-HT may either (Maes *et al*, 1995):
(i) enhance glucocorticoid-induced negative feedback effects on CRH (Feldman and Weidenfeld, 1992); or
(ii) downregulate mineralocorticoid and glucocorticoid receptors, thereby countering negative feedback effects on the HPA axis (Seckl and Fink, 1991; Brady *et al*, 1992). It is thus evident that the HPA axis and serotonergic mechanisms interact intimately, and malfunctioning in either or both of these systems may result in the development of depression.

6.2.2.9.1.2 HPT axis dysfunction

Hypothyroidism is known to be associated with depression (Whybrow *et al*, 1969; Whybrow and Hurwitz, 1976; Whybrow and Ferrell, 1974; Fountoulakis *et al*, 2004; Staner *et al*, 1992). The hormone L-triiodothyronine (T_3), normally secreted from the thyroid gland, enhances the antidepressant effect of TCAD in women, by enhancing the sensitivity of receptor sites (Prange *et al*, 1969, 1976). Approximately 50% of depressives exhibit a decreased thyroid-stimulating hormone (TSH) response to thyrotropin-releasing hormone (TRH) (Prange, 1975), which regulates TSH release; this substantiates the involvement of HPT dysfunction in depression. This decreased response in TSH levels may be linked to high levels of cortisol (Fountoulakis *et al*, 2004; Musselman and Nemeroff, 1996). It has also been found that often depression is accompanied by subclinical thyroiditis, thus highlighting a possible autoimmune component to the disorder (Schindler, 1985; Fountoulakis *et al*, 2004; Carta *et al*, 2002).

6.2.2.9.1.3 Hypothalamic-pituitary-growth hormone (HPG) dysfunction

The release of growth hormone (GH) can be induced by many stimuli, such as L-DOPA, but a common test used clinically is insulin-stimulated hypoglycaemia (Skene, 1979). NE is believed to play a role in regulating GH release, since blockade of the noradrenergic system results in a diminished release of GH in response to insulin (Martin, 1976). Although depressed individuals exhibit normal basal levels of GH (Carroll and Mendels, 1976), the response in GH levels subsequent to the administration of amphetamine (Langer *et al*, 1976) and insulin-stimulated hypoglycaemia is diminished (Carroll, 1972; Sachar *et al*, 1971; Mueller *et al*, 1969). These low levels of GH are associated with a decrease in urinary levels of 3-methoxy-4-hydroxyphenylglycol (MHPG) (Garver *et al*, 1975, 1976), the major metabolite of NE.

Both DA and NE are released in response to amphetamine administration. Since the response in GH levels to L-DOPA in depressives remains unaltered (Frazer, 1975), the impaired GH response is most likely attributed to a deficiency of NE (Garver, 1979). This is in agreement with the catecholamine hypothesis of depression (see 6.2.2.1). There are other substances, such as somatostatin (Besser, 1974) and 5-HT (Bivens *et al*, 1973), that may also be involved in regulating GH release.

6.2.2.9.2 Electrolyte abnormalities

Electrolytes are essential in the functioning of the CNS. The action potential of cell membranes, for example, is determined by the conentration ratio of extracellular to intracellular sodium ions (Na^+), while the resting potential is similarly dependent on potassium ions (K^+). The action and resting potentials are important in the transmission of nerve signals (Ganong, 1993). The production, storage, release and metabolism of neurotransmitters also requires electrolytes. Abnormalities in electrolyte levels can thus be expected to impair nervous functioning, which may lead to the development of depression. The three electrolytes that shall be considered are: (i) Ca^{2+}, (ii) magnesium (Mg^{2+}), and (iii) Na^+ (Skene, 1979).

6.2.2.9.2.1 Ca^{2+} abnormalities

Ca^{2+} abnormalities may be involved in the aetiology of depression, for several reasons. This cation is involved in regulating K^+ and Na^+ movement across cell membranes and abnormalities may thus influence the normal transmission of nervous impulses (Skene, 1979). Disorders of hypercalcaemia, furthermore, such as vitamin D toxicity (Andersen *et al*, 1968) and hyperparathyroidism (Hockaday *et al*, 1966; Petersen, 1968), are associated with the development of severe stupor and depression.

Depressed patients whose condition has clinically improved have been found to have decreased urinary excretion of Ca^{2+} (Flach, 1966), thus leading this author to link the retention of Ca^{2+} with successful antidepressant treatment. This may be because a fall in levels of extracellular Ca^{2+} inhibits the activity of the enzymes tryptophan hydroxylase and tyrosine hydroxylase (Carman and Wyatt, 1977). These enzymes are integrally involved in the synthesis of the neurotransmitters 5-HT and DA, respectively (Baldessarini, 2001). A decrease in Ca^{2+} levels also enhances the activity of COMT and acetylcholinesterase (Carman and Wyatt, 1977). COMT metabolises biogenic amines and thus levels of these are decreased even further, precipiating depression, according to the biogenic amine hypothesis (see 6.2.2.2). However, in some animal studies, a decrease in cerebrospinal fluid (CSF) levels of Ca^{2+} results in excitation (Pappenheimer *et al*, 1962; Veale and Myers, 1971). A fall in CSF

levels of Ca^{2+} has furthermore been linked to the antidepressant effect of ECT (Carman *et al*, 1974).

Importantly, the association between Ca^{2+} levels and the development of depression has recently been highlighted in the neurodegenerative hypothesis of depression. Excess levels of Ca^{2+} are known to cause overstimulation of the NMDA receptor, thus resulting in excitotoxicity, which may lead to depression (see 6.2.2.16).

6.2.2.9.2.2 Mg^{2+} abnormalities

It is well-known that the total levels of magnesium in plasma are significantly decreased in depressed individuals (Frizel *et al*, 1969; Kirov and Tsachev, 1990; Barragan-Rodríguez L *et al*, 2007; Eby and Eby, 2006) and increase with recovery (Frizel *et al*, 1969; Eby and Eby, 2006). However, the level of Mg^{2+} ions does not differ between depressed and non-depressed individuals, and it is this ionised proportion of magnesium that is assumed to exert physiological effects (Frizel *et al*, 1969).

6.2.2.9.2.3 Na^{+} abnormalities

Several studies have found that depressed individuals exhibit Na^{+} retention (Schottstaedt *et al*, 1956; Russell, 1960; Anderson and Dawson, 1963). The use of radioisotopic techniques initially led to the hypothesis that total Na^{+} accumulation occurs in depression until recovery; by this time, excretion of the excess Na^{+} has occurred (Gibbons, 1960). Later studies, however, have shown that instead of changes in the levels of total Na^{+} in the body of depressives, there is re-distribution of this electrolyte (Coppen and Shaw, 1963; Coppen *et al*, 1965, 1966; Shaw, 1966). The proportion of residual Na^{+}, which comprises Na^{+} in the bone and within cells, is siginificantly increased by 200% in manic (Coppen *et al*, 1966) and 50% in depressed (Coppen and Shaw, 1963) individuals. These levels decrease to normal upon recovery. K^{+} levels were found to be unaltered upon recovery (Coppen and Shaw, 1963; Coppen *et al*, 1966). Severely depressed and schizophrenic patients show a decrease in the entry rate of Na^{+} into the CSF from the blood (Coppen, 1960). Furthermore, there is a decrease in Na^{+}, and an increase in water, content in the brains of depressed individuals who commit suicide (Shaw *et al*, 1969).

There are various mechanisms by which Na^{+} levels could be altered as described above. The increase in cortisol levels that occurs in depression (see 6.2.2.9.1.1) could precipitate an increase in Na^{+} accumulation. The distribution of Na^{+} in the body is additionally influenced by antidiuretic hormone (ADH), aldosterone and progesterone. Shaw (1966) mentions the

importance of ascertaining whether the abnormalities in Na^+ levels are due to depression, or a secondary effect of altered corticosteroid functioning.

It is hypothesised that in depression there is CNS hyperexcitability, due to a decrease in the resting potential of cell membranes, as a result of the accumulation of Na^+ within neurons (Whybrow and Mendels, 1969). The mode of action of lithium ions (Li^+) in treating affective disorders may be due to this cation re-establishing the electrolyte balance across cell membranes by replacing Na^+ within the cell (Coppen *et al*, 1962, 1965; Bunney *et al*, 1972). Since the distribution of electrolytes across the cell membrane has a significant role to play in the transport and ultimate release of neurotransmitters from the presynaptic neuron, the deficiency of biogenic amines, leading to depression (see 6.2.2.2), could be as a result of the accumulation of Na^+ within neurons, which impairs the transport and release of biogenic amine neurotransmitters (Maas, 1972; White and Paton, 1972); Bogdanski *et al*, 1968).

A carrier protein as well as adenosine triphosphatase (ATPase), the latter being Na^+/ K^+ dependent, are necessary for the movement of electrolytes across the presynaptic membrane (Skene, 1979). A deficiency of either ATPase or the carrier protein, which may be due to a genetic abnormality, would inhibit the transport of electrolytes and thereby ultimately interfere with the transport of neurotransmitters (Skene, 1979). It has been found that depressives have reduced ATPase activity (Naylor *et al*, 1973; Hesketh *et al*, 1977), as well as a lower Na^+ content and impaired Na^+ transport (Naylor *et al*, 1970a, 1970b, 1971) in erythrocytes. The increase in intraneuronal Na^+ levels may also inhibit the release of NE from its carrier protein, thus inhibiting the transport of NE (Bunney *et al*, 1972). Further evidence for impaired electrolyte transport in individuals suffering from affective disorders is the finding that in mania there is an impaired ability to transport Li^+ across red blood cells (Pandey *et al*, 1976; Mendels *et al*, 1977). There is also a decrease in 5-HT uptake and retention within platelets in depression (Tuomisto and Tukiainen, 1976).

This hypothesis of abnormalities in Na^+, and other electrolyte, transport, integrates well with the biogenic amine hypothesis of depression, as it provides a mechanism for the deficiency of biogenic amines in the synaptic cleft (Skene, 1979).

6.2.2.10 Adrenergic receptor abnormalities

This hypothesis postulates that abnormalities in postsynaptic adrenergic receptors may account for the dysfunction in neuronal transmission that occurs in depression (Skene, 1979).

Some investigators believe these receptors to be supersensitive to NE (Vetulani *et al*, 1975; Vetulani and Sulser, 1975). This is based on the finding that chronic TCAD or MAO inhibitor treatment decreases the activity of limbic adenylate cyclase, which is NE-dependent. This effect seems to closely follow the antidepressant response in terms of time (Vetulani *et al*, 1975; Vetulani and Sulser, 1975). The enhanced sensitivity of adrenergic receptors may, through negative feedback, inhibit further synthesis of NE. This may account for the low levels of the main NE metabolite MHPG noted in the urine of depressives (Jones *et al*, 1973; Schildkraut *et al*, 1977).

Other researchers have suggested that there is subsensitivity of postsynaptic adrenergic receptors (Ashcroft, 1972; Prange *et al*, 1972). This is based on the finding that the antidepressive effect of TCAD is enhanced by thyroid hormone, which alters the sensitivity of receptors (Prange *et al*, 1969; Wheatley, 1972; Coppen *et al*, 1972b). Some depressives also exhibit decreased sensitivity of α-adrenergic receptors, evidenced by a diminished GH response upon the administration of clonidine (Matussek *et al*, 1977), an α_2-receptor agonist. Although it is yet to be established which school of thought is correct, it is apparent from the above that the altered sensitivity of postsynaptic receptors may well play a role in the aetiology of depression. There may moreover be altered sensitivity of 5-HT autoreceptors in depression (Myint and Kim, 2003); autoreceptors on presynaptic neurons regulate the amount of neurotransmitters available in the synaptic cleft, through a negative feedback mechanism (Baldessarini, 2001).

6.2.2.11 Influence of sex steroids

Depression occurs two to three times more often in women than men (Weissman and Paykel, 1974), although it has been pointed out that this increased incidence is not necessarily due to hormonal factors. Societally, women are more expected to express depressive symptoms than men (Weissman and Paykel, 1974), and the higher incidence of alcoholism among males may indicate a preference among depressed males to use alcohol rather than antidepressant therapy (Winokur *et al*, 1970). Mild depression can occur in the premenstrual, postpartum and menopausal periods in women (Weissman and Paykel, 1974).

The use of oestrogens and progesterones as oral contraceptives is reported to alter the uptake as well as turnover of catecholamines (Fuxe *et al*, 1973). These sex steroids are known to have significant effects on DA metabolism in the brain (Greengrass and Tonge, 1973; Jori and Dolfini, 1976). The use of oral contraceptives is believed to be linked to the development of depression, by inducing vitamin deficiencies (see 6.2.2.13.1). Steroids synthesised

endogenously by the ovaries are also known to alter central monoamine metabolism (Stefano *et al*, 1965; Greengrass and Tonge, 1974; Wirz-Justice *et al*, 1974).

Testosterone exerts effects similar to those induced centrally by catecholamines (Stenn *et al*, 1972; Brovermann *et al*, 1968); this may be due to its ability to directly inhibit the enzyme MAO (Klaiber *et al*, 1967). Although testosterone is successfully used to treat climateric depression in men (Bleuler, 1954), mean plasma levels of this hormone are not altered significantly upon recovery from depression (Sachar *et al*, 1973b).

The CEEG profiles of patients treated with two different sex steroids, the androgen mesterolone and the antiandrogen cyproterone, were analysed (Itil *et al*, 1974). Patients receiving low doses of the latter demonstrated CEEG profiles similar to that obtained for "sedative TCAD", while high doses of cyproterone gave profiles similar to anxiolytics. Low doses of mesterolone gave CEEG profiles similar to the stimulant amphetamine, whereas high doses were similar to the "stimulant" TCAD (Itil, 1974; Skene, 1979). When tested in a clinical trial, mesterolone was found to either have no effect in depressed male individuals, or to be significantly effective. Its efficacy is believed to be due to its inhibition of MAO (Itil *et al*, 1974).

6.2.2.12 Abnormalities in glucose utilisation

Since 1965 it has been known that depressives exhibit a decrease in glucose tolerance, which reflects impairment in the body's utilisation of glucose (Van Praag and Leijnse, 1965). This decrease in glucose tolerance remains for a period of time after recovery (Pryce, 1958). Patients on MAO inhibitor treatment show an improvement in glucose utilisation, which may be due to MAO inhibition potentiating the effects of insulin (Weil-Malherbe, 1970). In recent years, it has been shown that insulin resistance is positively associated with depression (Timonen *et al*, 2005; Lawlor *et al*, 2003) and suicidal risk (Golomb *et al*, 2002; Lawlor *et al*, 2003). Insulin resistance is also a characteristic of metabolic syndrome, which has a prevalence rate of over 21% in the United States of America (Ford *et al*, 2002) and is also characterised by obesity and hypercholesterolaemia (Kinder *et al*, 2004). Metabolic syndrome is associated with depression (Kinder *et al*, 2002, 2004; Chrousos, 2000; Heiskanen *et al*, 2006) and may induce the cardiovascular disease that often accompanies depression (Wulsin *et al*, 1999; Musselman *et al*, 1998).

6.2.2.13 Vitamin abnormalities

Abnormalities in the levels of two vitamins, namely: (i) pyridoxine and (ii) folate, will be discussed.

6.2.2.13.1 Pyridoxine deficiency

Several key enzymes are dependent on pyridoxal phosphate, such as kynureninase aminotransferase and kynureninase. These convert 3-OHK into xanthurenic acid and 3-hydroxyanthranilic acid, respectively (Skene, 1979). A deficiency of pyridoxine thus results in a build-up of kynurenine and 3-OHK, which are associated with inducing depression (see 6.2.2.5).

One of the adverse effects of oral contraceptive use is the development of depression (Nilsson and Sölvell, 1967) (see 6.2.2.11). Oral contraceptive use results in a deficiency of pyridoxine (Salkeld *et al*, 1973) and there are several possible mechanisms by which this can result in depression:

(i) as mentioned above, pyridoxal deficiency increases the levels of 3-OHK and kynurenine (Rose, 1969; Briggs, 1976);

(ii) the oestrogen component of oral contraceptives may induce TDO (Rose, 1969; Salkeld *et al*, 1973) and, according to the kynurenine hypothesis of depression (see 6.2.2.5), this results in low central levels of 5-HT which induce depression; and

(iii) low levels of pyridoxine may decrease the activity of 5-HTP decarboxylase, which results in low levels of 5-HT being synthesised (Skene, 1979). Support for the involvement of TDO in oral contraceptive-induced depression is a finding that the administration of an oral contraceptive to rats for a period of ten days results in enhanced TDO activity and a significantly low level of 5-HT in the brain (Nistico and Preziosi, 1970).

The use of pyridoxine supplements has been found to alleviate depression (McCarty, 2000; Williams *et al*, 2005) in women using oral contraceptives (Leeton, 1974; Winston, 1973) and to normalise the levels of tryptophan metabolites (Salkeld *et al*, 1973; Briggs, 1976).

6.2.2.13.2 Folate deficiency

Low levels of folic acid were reported in the serum of patients afflicted with various affective disorders (Hunter *et al*, 1967), but many patients participating in this study were taking other drugs that also deplete folate, such as anticonvulsants and alcohol. Low serum folate levels in depressives have also been reported by other investigators (Reynolds *et al*, 1970; Carney, 1967). The pteridine cofactor is required in the hydroxylation of tryptophan (Lovenberg *et al*, 1968) and tyrosine (Udenfriend, 1966). A deficiency of folate would thus inhibit this rate-limiting step in the synthesis of 5-HT and catecholamines, respectively (Skene, 1979). There is thus a possible association between folate deficiency and the biogenic amine and catecholamine hypotheses of depression (Reynolds *et al*, 1970).

Low levels of folate, furthermore, result in accumulation of homocysteine, an amino acid which, in the presence of folate, is converted to methionine. Hyperhomocysteinaemia, besides being associated with cardio- and cerebrovascular disease, has been linked to the development of depression (Tiemeier *et al*, 2002; Bottiglieri *et al*, 2000; Folstein *et al*, 2007), possibly by depleting neurotransmitter levels (Folstein *et al*, 2007).

6.2.2.14 Macrophage hypothesis

This is the first hypothesis that links the immune system and the effects of inflammation to the aetiology of depression (Smith, 1991). It postulates that the secretion of the pro-inflammatory cytokine IL-1β from macrophages induces overstimulation of the HPA axis (see 6.2.2.9.1.1), due to an increase in the secretion of CRF from the hippocampus. IL-1β levels are physiologically raised as a response mechanism to infections and stress (Myint, 2007). This hypothesis is extended in the neurodegeneration hypothesis in 6.2.2.16.

6.2.2.15 Neurogenesis and neuroplasticity hypothesis

This hypothesis postulates that in depression there is impaired neurogenesis (Jacobs, 2002), which induces structural alterations in the hippocampus (Sheline, 2000) (see 10.7). Antidepressants have been found to promote the proliferation of progenitor cells in the hippocampus (Malberg *et al*, 2000; Czeh *et al*, 2001) by increasing 5-HT levels (Gould, 1999), and this may be an additional mechanism of antidepressant action. Besides degeneration of the hippocampus, there are alterations in the number (Rajkowska, 2000) and density (Bowley *et al*, 2002) of glial cells in the amygdala in depression. Depression is also associated with disturbances in cellular and neuronal plasticity (Kempermann and Kronenberg, 2003), (see 10.2.1.2 and 10.7) due to alterations in various neurotrophic factors, for example brain-derived neurotrophic factor (BDNF) (Myint, 2007).

6.2.2.16 Neurodegeneration hypothesis

Proposed by Myint and Kim (2003), this hypothesis postulates that the immune system stimulation that occurs in depression results in the production of pro-inflammatory cytokines, which enhance the activity of IDO. IDO stimulation shifts the metabolism of tryptophan away from the production of 5-HT, towards the generation of 3-OHK and ultimately QA, which is neurotoxic (see 8.2).

It is reported that in depression there is stimulation of the inflammatory response system (IRS), with a subsequent increase in the level of pro-inflammatory cytokines, such as IL-1, IL-2, IL-6, IL-12, INFγ and TNFα, as well as PGE (Maes, 1999; Kim *et al*, 2002; Leonard, 2001; Licinio and Wong, 1999). Cytokines, "the hormones of the immune system" (Myint

and Kim, 2003), are produced and released in the nervous system, including the brain (Kronfol and Remick, 2000; Freidin *et al*, 1992), and are subdivided into pro- and anti-inflammatory types. It has been found that pro-inflammatory cytokines can significantly alter the metabolism of NE, DA and 5-HT (Dunn *et al*, 1999). There is a dual mechanism by which cytokines can lower tryptophan levels (Myint and Kim, 2003):

(i) cytokines can decrease the intake of food (Reichenberg *et al*, 2001; Plata-Salaman and Ilyin, 1998), and thus significantly reduce the dietary intake of this amino acid (Smith *et al*, 1997); and

(ii) stimulation of IDO (Guillemin *et al*, 2001; Sakash *et al*, 2002), as illustrated in Figure 48 below. Kynurenine is ultimately converted (Dang *et al*, 2000) into kynurenic acid, an antagonist of excitatory amino acid receptors (Perkins and Stone, 1982); and QA, an agonist at NMDA receptors, which induces excitotoxicity (Schwarcz *et al*, 1983) (see 8.2). Enhanced IDO activity thus decreases the amount of tryptophan that is converted into 5-HT and 5-HT depletion occurs (Myint and Kim, 2003), which leads to depression (see 6.2.2.2). According to this hypothesis, depression occurs via two mechanisms: (i) 5-HT depletion, as mentioned above; and (ii) neurodegeneration induced by the excitotoxic QA.

The neurodegeneration hypothesis of depression may account for the recurrency of major depression, the shortening of time intervals between episodes, and the increased probability of suffering a subsequent episode (Kasper, 1993). It is also supported by the finding that hippocampal atrophy increases with an increase in the duration of a major depressive episode (Bremner *et al*, 2000; Sheline *et al*, 1999; Sheline, 1996).

Another key feature of this hypothesis of depression is the challenge between the neuroprotective ability of kynurenic acid and the neurotoxicity of QA (Myint and Kim, 2003), both formed from kynurenine as a result of IDO stimulation (see Figure 48). If this challenge shifts to the neuroprotective side, it is suggested that some individuals may recover from depression and cope with stress, whereas if the neurodegeration pathway is favoured, depression can be worsened or recur (Myint and Kim, 2003).

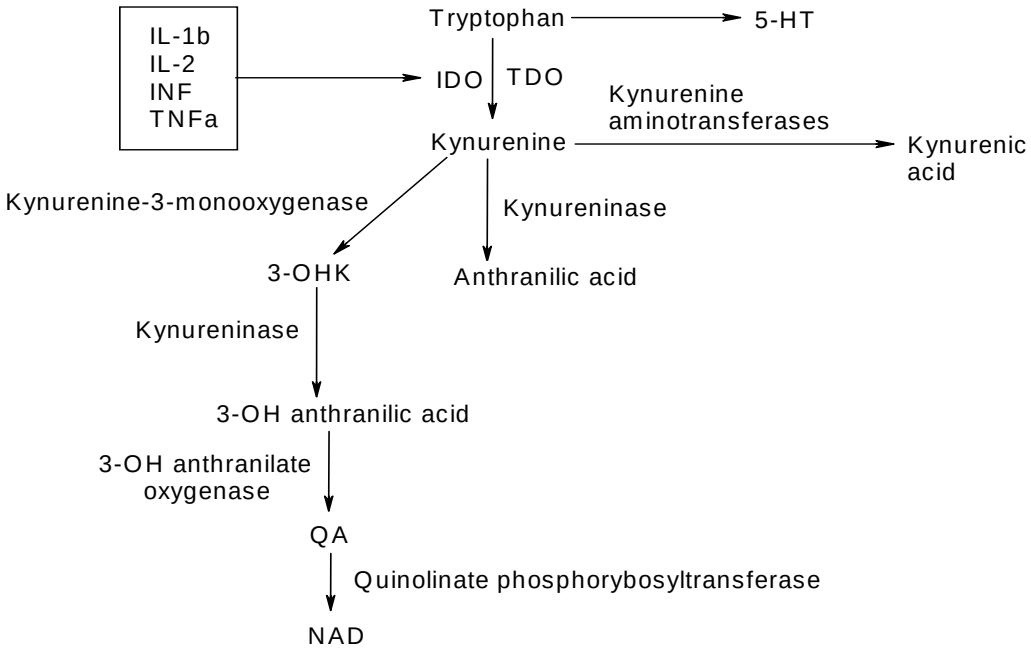


Figure 48: The metabolism of tryptophan to form QA (Myint and Kim, 2003)

The following diagram summarises the neurodegeneration hypothesis of depression (Myint and Kim, 2003). As can be seen, the pro-inflammatory cytokines may also activate the HPA axis, which is another mechanism of the development of depression (see 6.2.2.9.1.1). In the preceding Chapter, it was shown that 5-FU and melatonin both stimulate IDO activity *in vivo*. The effects of these agents on certain cytokine and corticosterone levels are investigated in Chapters 12 and 13, respectively.

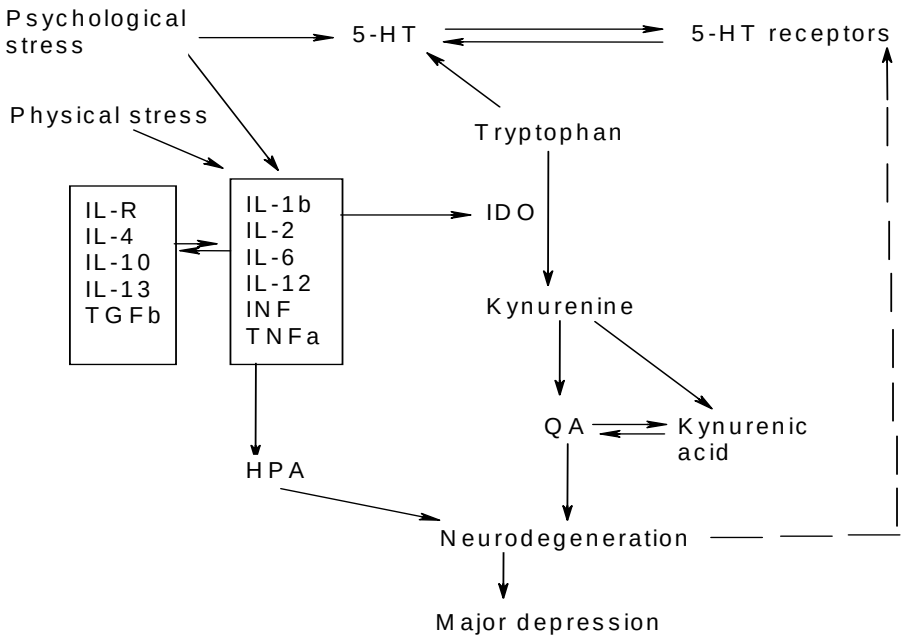


Figure 49: The neurodegeneration hypothesis of depression (Myint and Kim, 2003)

6.3 Experimentation

The forebrains of the rats used in the *in vivo* TDO study were dissected out at the time of sacrifice and stored in liquid nitrogen at -70ºC until the neurotransmitter levels were determined. This was done as described by Muralikrishnan and Mohanakumar (1998) and Maharaj *et al* (2004). The levels of NE, DA, 3,4-dihydroxyphenylacetic acid (DOPAC), 5-HT and HA were determined. It was not possible to determine the levels of the NE metabolite MHPG, as this requires a different mobile phase (Raggi *et al*, 2001; Sastre *et al*, 2004).

6.3.1 Materials, reagents and equipment

Standards of NE, DA, DOPAC, 5-HT and HA were purchased from the Sigma Chemical Corporation, St Louis, MO, USA. EDTA, heptane sulphonic acid, perchlorate, triethylamine, acetonitrile and phosphoric acid were purchased from Merck, Darmstadt, Germany. All other reagents used were obtained locally and of the highest chemical purity. The analysis was performed using HPLC coupled to electrochemical detection (ECD). Separation was achieved using a C_{18} column (4.6 mm X 250 mm), and column pressure was maintained at 1498 ± 20 psi. The mobile phase used, at a flow rate of 0.7 ml/ min, was a combination of: 8.65 M heptane sulphonic acid, 0.27 mM EDTA, 13% acetonitrile, 0.43% triethylamine and 0.22% o-phosphoric acid (v/ v) and was degassed prior to use, using a 0.45 µm membrane filter. ECD was performed at a potential of +0.74 V; the range was 5 nA and the total baseline was +0.43 nA. The oxidation potential was positive.

6.3.2 Method

The forebrains were deproteinised, following sonication at 50 Hz for 60 s in ice-cold 0.1 M $HClO_4$/ 0.00.67% EDTA. The concentration of forebrain was 1 mg tissue in 10 µL of the above solvent. The samples were stored in ice for a period of 15 mins and subsequently centrifuged at 10 000g for 10 mins. The supernatant was injected into the HPLC system for neurotransmitter analysis (Muralikrishnan and Mohanakumar, 1998; Maharaj *et al*, 2004). Standards of the neurotransmitters were similarly injected into the HPLC system at a concentration of 4 pM. Forebrain samples were analysed in triplicate, the concentrations of neurotransmitters were determined and the results expressed as pmol of neurotransmitter per mg tissue.

6.4 Results

A typical chromatogram obtained for the standards is given in Figure 50. The order of elution is as follows: NE, DOPAC, DA, HA and lastly 5-HT. The peaks obtained are well-resolved, sharp and symmetrical. Table 18 summarises the mean peak height obtained for each standard.

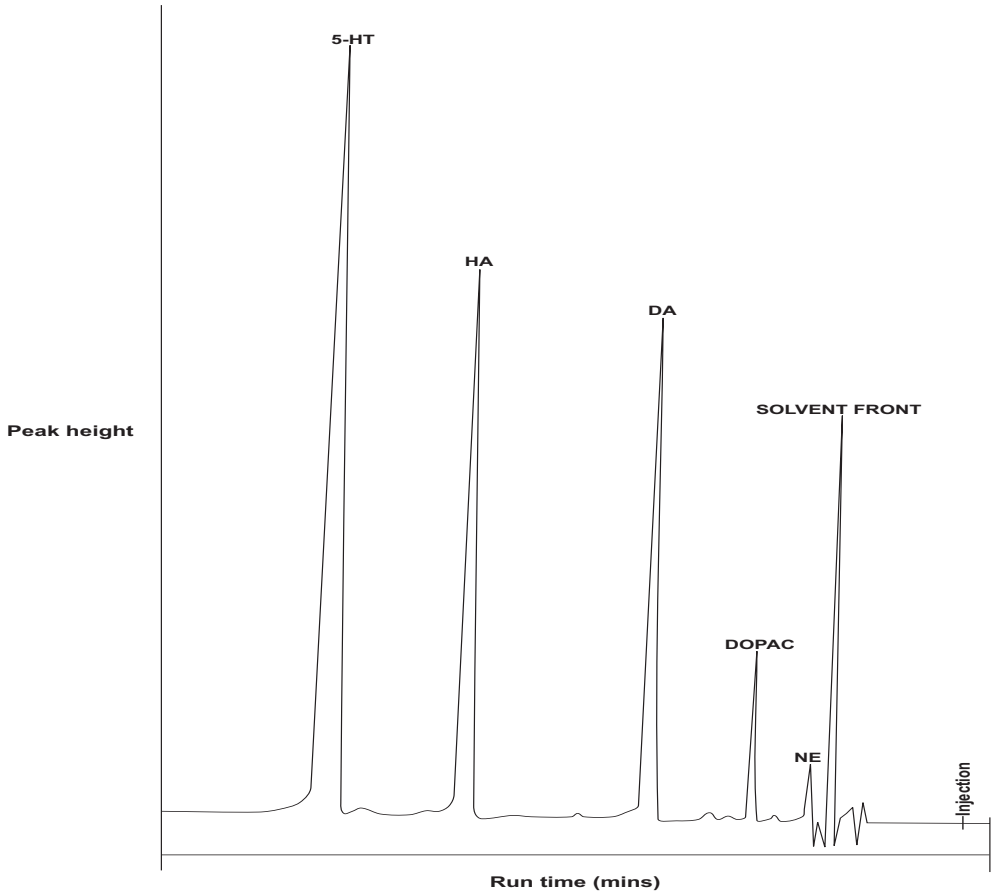


Figure 50: A typical chromatogram showing the separation of standards used in the forebrain analysis of neurotransmitters in rats subjected to the in vivo administration of 5-FU and melatonin

Table 18: The mean peak heights obtained for standards used in neurotransmitter analysis of rats subjected to the in vivo administration of 5-FU and melatonin

Standard	**Peak height (mm) (mean ± SEM)** (n = 3)
NE	15.7 ± 2.52
DOPAC	40.7 ± 1.15
DA	155.7 ± 56.09
HA	127.3 ± 0.58
5-HT	203.3 ± 1.53

The effects of 5-FU and melatonin on various neurotransmitter levels in rat forebrains are reflected in the tables below. Statistical analysis was performed using ANOVA, followed by the Student-Newman-Keuls multiple-range test.

Table 19: The effect of the in vivo administration of 5-FU and melatonin on rat forebrain NE, HA and 5-HT levels

Group	**Concentration (pmol/ mg tissue)** (mean ± SEM) (n = 5)		
	NE	**HA**	**5-HT**
Control	8.58 ± 0.5	0.93 ± 0.064	0.43 ± 0.031
Melatonin	8.36 ± 0.12	0.97 ± 0.018	0.54 ± 0.028 *
5-FU	5.87 ± 0.16 *	0.93 ± 0.032	0.28 ± 0.023 *
5-FU + Melatonin	7.72 ± 0.47 *	0.94 ± 0.078	0.57 ± 0.062 @

* $p<0.05$ compared to control; @ $p<0.01$ compared to 5-FU, after statistical analysis by ANOVA followed by the Student-Newman-Keuls multiple-range test

Table 20: The effect of the in vivo administration of 5-FU and melatonin on rat forebrain DA and DOPAC levels

Group	**Concentration (pmol/ mg tissue)** (mean ± SEM) (n = 5)	
	DOPAC	**DA**
Control	0.94 ± 0.05	1.13 ± 0.074
Melatonin	0.86 ± 0.047	3.83 ± 0.21 @@
5-FU	0.52 ± 0.047	0.59 ± 0.12 @
5-FU + Melatonin	0.84 ± 0.21	1.23 ± 0.036 **

@ $p<0.01$, @@ $p<0.001$ compared to control; ** $p<0.001$ compared to 5-FU, after statistical analysis by ANOVA followed by the Student-Newman-Keuls multiple-range test

As sshown in Tables 19 and 20, 5-FU has marked effects on the levels of neurotransmitters. The antineoplastic decreases NE levels significantly from 8.58 pmol/ mg to 5.87 pmol/ mg, while melatonin induces a negligible decrease. When both drugs are administered together,

NE levels are significantly higher compared to 5-FU alone, but lower than the control levels. Neither drug alters HA levels, but both alter 5-HT levels in opposite ways. While melatonin increases 5-HT levels from 0.43 pmol/ mg to 0.54 pmol/ mg, 5-FU causes a marked decrease to 0.28 pmol/ mg. The administration of both drugs together cause 5-HT levels to increase to levels greater than with melatonin alone.

5-Fluorouracil induces a decrease in DOPAC levels, but this is statistically insignificant; melatonin does not markedly alter DOPAC levels either. Both have significant effects on DA levels. Melatonin increases DA levels from 1.13 pmol/ mg to 3.83 pmol/ mg, while 5-FU causes a decrease to 0.59 pmol/ mg. The group of rats which received both drugs show DA levels of 1.23 pmol/ mg; the concomitant administration of melatonin thus counteracts the effects of 5-FU and increases DA levels to greater than the control values.

6.5 Discussion

As mentioned previously (see 6.1), Daya *et al* (1989) have commented on the inverse relationship that exists between cerebral 5-HT and hepatic TDO levels. Since both 5-FU and melatonin have been found to inhibit TDO, it would be expected that the neurotransmitter analysis would reflect that 5-HT levels would be increased. NE levels are expected to be increased as well, since 5-HT is known to cause the release of this neurotransmitter (Xi-Ming *et al*, 2002). It was found that melatonin increases both 5-HT and NE levels. This is in accordance with the findings of Anton-Tay *et al* (1968) and Wendel *et al* (1974). These increased neurotransmitter levels could be due to the finding that melatonin has been found to inhibit MAO, the enzyme that degrades monoamines (Urry and Ellis, 1975; cited in Walsh and Daya, 1998). 5-FU, unexpectedly, induces a significant decrease in these neurotransmitter levels. Melatonin blunts the 5-FU-induced decrease in NE and 5-HT levels. HA levels are not altered by either drug, thereby suggesting that neither 5-FU nor melatonin alter the main route of 5-HT metabolism.

A possible explanation for 5-FU inhibiting TDO and yet still causing a decrease in 5-HT levels is in considering 5-FU's action as an antimetabolite, disrupting DNA synthesis. The biosynthesis of 5-HT requires enzymes and is thus ultimately controlled at the level of gene expression and protein synthesis. 5-FU is known to enter the brain (see 1.2.5) and can thus disrupt 5-HT synthesis in the brain by inhibiting gene expression of the enzymes necessary to synthesise the neurotransmiter. The same could be true for NE and DA as well. Another possibility is that 5-FU could alter MAO activity; activation of this enzyme enhances the degradation of biogenic amines and thus decreases neurotransmitter levels. It is thus insufficient to assume that merely because 5-FU inhibits TDO it would result in increased

brain levels of 5-HT. It is necessary to have an holistic approach and to bear in mind the other mechanisms of action and effects of 5-FU on other systems in the body.

It has also been shown, in Chapter 5, that 5-FU stimulates IDO activity. This increases the catabolism of tryptophan and results in less tryptophan available for cerebral uptake and conversion to 5-HT, thereby inducing 5-HT depletion (see 6.2.2.16). Melatonin, however, was also shown to enhance IDO activity in Chapter 5, yet increases 5-HT levels. This could possibly indicate that the effects of melatonin on TDO activity outweigh the effects of this agent on IDO activity, whereas the effects of 5-FU on IDO activity outweigh TDO inhibition.

DA levels are increased by the administration of melatonin and decreased by 5-FU. The enzyme dopamine β-hydroxylase catalyses the formation of NE from the catecholamine DA (Menniti and Diliberto, 1989); increased levels of DA would thus be expected to be associated with increased levels of NE. Melatonin, which significantly increases DA levels, has no significant effect on NE levels. 5-FU, however, markedly reduces NE levels. One of the mechanisms for this may be the decrease in DA levels due to 5-FU. 5-FU also decreases 5-HT levels, thereby further reducing NE levels, as 5-HT mediates the release of this neurotransmitter (Xi-Ming *et al*, 2002). Neither drug alters DOPAC levels; DOPAC is a metabolite of DA (Zetterstrom *et al*, 1988) and this finding indicates that neither 5-FU nor melatonin influence the main route of DA metabolism.

These findings have certain implications in the clinical setting. By decreasing 5-HT, DA and NE levels, 5-FU is expected to exacerbate depression, according to the biogenic amine and dopamine hypotheses of depression (see 6.2.2.2 and 6.2.2.3). Patients suffering from cancer might well suffer from depression due to their condition; that this could be worsened by their antineoplastic therapy, besides the other adverse drug reactions of 5-FU (see 1.2.10), indicates that these patients' quality of life could be further adversely affected. Depression, in turn, is known to worsen the progression of cancer (Spiegel *et al*, 1989; Ramirez *et al*, 1989; Faller *et al*, 1999; Fawzy *et al*, 1993).

The call by Lissoni *et al* (1999a) for melatonin to be used as adjunctive therapy with 5-FU, to enhance the efficacy and reduce the toxicity of the latter to improve patients' quality of life, has been extensivley discussed (see 1.3.9). The findings of this study support this, in that melatonin co-therapy with 5-FU increases the levels of NE, 5-HT and DA. Melatonin has been found to have antidepressant properties (Lissoni *et al*, 1995b), and its ability to increase the levels of these neurotransmitters may be one of the mechanisms by which this is achieved.

6.6 Conclusions

It has been found in Chapters 4 and 5 that both melatonin and 5-FU inhibit TDO activity and enhance IDO activity, respectively. By inhibiting TDO, melatonin increases brain 5-HT levels. Melatonin also increases DA levels but has no effect on NE levels. 5-FU has been found to decrease brain levels of all three neurotransmitters. This could be due to a triple mechanism:

(i) the antineoplastic inhibiting the DNA synthesis and gene expression required for the biosynthesis of the enzymes required in the synthesis of these neurotransmitters;

(ii) IDO stimulation, which induces cerebral 5-HT depletion; and

(iii) the decrease in NE levels induced by 5-FU could furthermore be due to decreases in DA and 5-HT levels, since 5-HT is known to cause the release of NE (Xi-Ming *et al*, 2002) and DA is converted into NE (Menniti and Diliberto, 1989).

Even though 5-FU and melatonin alter brain 5-HT levels, neither drug alters the main route of metabolism of 5-HT, evidenced by neither drug affecting brain levels of the metabolite HA. The same is true for the DA metabolite DOPAC. By decreasing the levels of NE, 5-HT and DA, 5-FU could contribute to the development or exacerbation of depression in cancer patients. Melatonin, if administered with 5-FU, could counter this 5-FU-induced depression by significantly enhancing the levels of all these neurotransmitters. Thus the use of melatonin as adjunctive therapy with 5-FU to improve patients' quality of life is recommended.

CHAPTER 7

THE EFFECTS OF 5-FLUOROURACIL AND MELATONIN ON PINEAL INDOLE METABOLISM *IN VITRO* AND *EX-VIVO*

7.1 Introduction

As discussed in 1.3.1, the pineal gland is primarily responsible for the synthesis of indoleamines, the most important of which is the neurohormone melatonin. The mechanisms by which melatonin is synthesised, as well as its metabolism, are detailed below.

The essential amino acid, tryptophan, is taken up into parenchymal cells in the pineal gland (Daya, 1982). The enzyme tryptophan hydroxylase hydroxylates most of this tryptophan in the 5-position, forming 5-hydroxytryptophan (Jequier *et al*, 1969; Lovenberg *et al*, 1967). This undergoes decarboxylation by aromatic-*L*-amino acid decarboxylase (Snyder and Axelrod, 1964), to form 5-HT (Snyder and Axelrod, 1964; Lovenberg *et al*, 1962). The concentration of 5-HT in the pineal gland is higher than in any other gland or region in the body, and is at a maximum during the day. At night, 5-HT levels decrease by at least 80%, as it is converted to melatonin, as detailed below (http://66.249.93.104/search?q=cache:-uw5vJ2m6ZIJ:www.ch.ic.ac.uk/local/projects/s_thipayang/synth.html+serotonin+%225-hydroxyindole+acetic+acid %22&hl=en&gl=2a&ct=clnk&cd=1&dr=lang_en).

5-HT is acetylated by *N*-acetyltransferase (NAT), forming *N*-acetylserotonin (aHT). This step is initiated by the stimulation of β receptors on pinealocytes by NE, which results in the activation of NAT (Olivieri *et al*, 1990; Welman and Daya, 1990) by an increase in the synthesis of a protein involved in its induction (Ebadi *et al*, 1986). The donor of the acetyl group is acetyl coenzyme A (Weissbach *et al*, 1960). As shown in Figure 59, this pathway has been designated pathway I, and is a minor pathway in the metabolism of 5-HT. The aHT thus formed is a substrate for the enzyme HIOMT, which converts it to melatonin (aMT) (Olivieri *et al*, 1990) by *o*-methylating it at position 5 (Daya, 1982; Axelrod and Weissbach, 1960, 1961). HIOMT is one of the most abundant pineal proteins (Jackson and Lovenberg, 1971). The activities of HIOMT and NAT both increase during the night, as the release of NE from sympathetic neurons to parenchymal cells is enhanced during this period. An aryl acylamidase then converts aMT into MT by breaking the amide linkage. MT can either be deaminated by MAO to form ML or oxidised further to yield MA (see 1.3.4 and 1.3.5) (http://66.249.93.104/search?q=cache:-uw5vJ2m6ZIJ:www.ch.ic.ac.uk/local/

projects/s_thipayang/synth.html+serotonin+%225-hydroxyindole+acetic+acid%22&hl=en&gl=2a&ct=clnk&cd=1&dr=lang_en).

Pathway II is the major route of 5-HT metabolism (Maharaj, 2004). MAO oxidises 5-HT, through deamination, to form 5-hydroxyindole acetaldehyde (Axelrod *et al*, 1969), which is unstable and can subsequently undergo two reactions: (i) reduction by aldehyde reductase to form 5-hydroxytryptophol (HL) (McIsaac and Page, 1959); or (ii) oxidation by aldehyde dehydrogenase, forming HA (Lerner and Case, 1960; Wurtman and Larin, 1968), the major product of 5-HT metabolism. HL can undergo methoxylation, catalysed by HIOMT and with *S*-adenosyl-methionine (SAM) being the methyl group donor, forming ML. HA can similarly be converted to MA, as can HL to aMT (Wurtman and Axelrod, 1967). HIOMT is also able to methoxylate 5-HT directly, forming MT (Daya, 1982). This may be considered a third metabolic pathway. HIOMT activity thus determines the rate at which the 5-hydroxyindoles are methylated to form 5-methoxyindoles (http://66.249.93.104/search?q=cache:-uw5vJ2m6ZIJ:www.ch.ic.ac.uk/local/projects/s_thipayang/synth.html+serotonin+%225-hydroxyindole+acetic+acid%22&hl=en&gl=2a&ct=clnk&cd=1&dr=lang_en).
These methoxyindoles have been shown to exhibit anticancer activity (see 1.3.1). While it is known that aMT is a potent antioxidant (see 1.3.1), it has been found that its related indoleamines, 5-HT and aHT, exhibit pro-oxidant effects by reducing Fe^{3+} to Fe^{2+}; the latter is toxic and induces lipid peroxidation (see 8.3) (Chan and Tang, 1996). However, other investigators have reported aHT has antioxidant properties, and is as potent as melatonin in decreasing the production of $O_2^{-}\cdot$ (Perianayagam *et al*, 2005).

The release of 5-HT stored in vesicles in the pineal gland, and its subsequent metabolism by NAT and MAO, is the rate-controlling step in the synthesis of the methoxyindoles and results in elevated nocturnal levels of ML and aMT (http://66.249.93.104/search?q=cache:-uw5vJ2m6ZIJ:www.ch.ic.ac.uk/local/projects/s_thipayang/synth.html+serotonin+%225-hydroxyindole+acetic+acid_%22&hl=en&gl=2a&ct=clnk&cd=1&dr=lang_en). Patients with advanced cancer often exhibit a decrease in the normal physiological increase in aMT levels at night (see 1.3.6). The nocturnal increase in NAT activity is thus not responsible for increased ML levels at night, as the synthesis of the latter does not involve the acetylation of 5-HT. Although pathway II is the major route of 5-HT metabolism, the proportion of 5-HT that is acetylated at any point in time is dependent on the relative activities of MAO and NAT (http://66.249.93.104/search?q=cache:-uw5vJ2m6ZIJ:www.ch.ic.ac.uk/local/projects/s_thipayang/synth.html+serotonin+%225-hydroxyindole+acetic+acid%22&hl=en&gl=2a&ct=clnk&cd=1&dr=lang_en).

The use of β-receptor agonists, such as isoprenaline, have been found to enhance the metabolism of 5-HT and increase levels of aHT and aMT (Axelrod *et al*, 1969; Banoo *et al*, 1987). This is due to activation of NAT, and the stimulation of pathway II described before. It has been shown that the stimulation of α receptors is also involved in aMT synthesis (Klein *et al*, 1983; Alphs *et al*, 1980). NE possesses weak affinity for α receptors and this may also contribute to its ability to stimulate NAT activity.

Figure 51 below summarises the synthesis and metabolism of 5-HT.

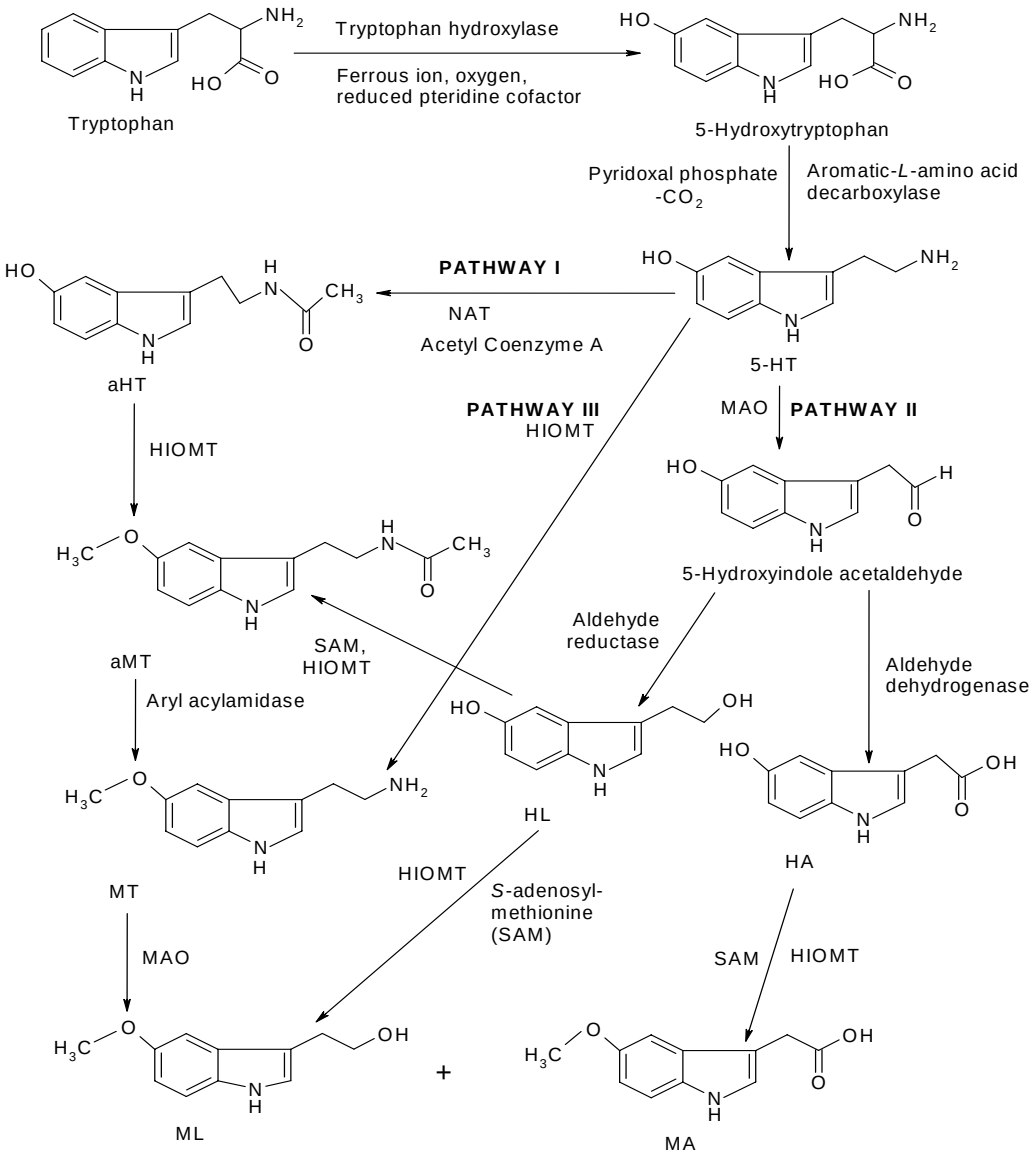


Figure 51: The biosynthesis of 5-HT from tryptophan and its metabolism in the pineal gland

(http://66.249.93.104/search?q=cache:-uw5vJ2m6ZIJ:www.ch.ic.ac.uk/local/projects/s_thipayang/synth.html+serotonin+%225-hydroxyindole+acetic+acid%22&hl=en&gl=2a&ct=clnk&cd=1&dr=lang_en; Daya, 1982)

7.2 Pineal organ culture

Pineal organ culture is the most direct method of investigating the biosynthesis of indoleamines, and is a more selective technique than using homogenates (Klein and Notides, 1969). It is a simple technique, and advantageous in that the pineal gland is small and easily accessible. This eliminates the complications that may arise in *in vivo* studies, such as neuronal, hormonal and other environmental influences (Daya *et al*, 1989; Maharaj, 2004). The sensitivity of the pineal organ culture technique is very good in that it is possible to quantify the amount of radiolabelled products up to the picomole range (Boyd, 1999).

The assay is a radiometric monitoring of the biosynthesis of radiolabelled indoleamine products from a radioactive precursor, such as [^{14}C]-5-HT or [^{14}C]- tryptophan, *in situ* in the pineal gland (Wurtman *et al*, 1968; Klein and Notides, 1969; Klein and Rowe, 1970; Daya and Potgieter, 1982; Morton, 1990; Boyd, 1999). When placed in a culture medium, the pineal gland takes up the radioactive precursor compound and synthesises radiolabelled products during an incubation period, which is normally for 24 hours at 37ºC (Boyd, 1999). Daya and Fata (1986) have shown that maximal levels of indole metabolites are achieved after 16 hours of incubation. The enzymes required for the biosynthesis of indoles are active in culture medium for a period of at least 48 hours (Boyd, 1999). As much as 98% of the radiolabelled indoleamine products thus formed are passively secreted into the culture medium from the pineal gland (Morton, 1990; Klein and Rowe, 1970). The culture medium can be deemed a relatively accurate representation of the pineal gland, as the relative proportions of the indoleamine products are similar (Klein and Notides, 1969; Morton, 1990; Boyd, 1999). Thin-layer chromatography (TLC) can be used to directly analyse the culture medium and separate the different radiolabelled products. These can be quantified using liquid scintillation spectrophotometry (Klein and Notides, 1969).

This technique is specific only for radiolabelled indoleamines synthesised from the specific radiolabelled precursor exogenously added to the biophase; it thus does not reflect endogenous indoleamine synthesis (Boyd, 1999). It is believed that the concentration of endogenously-synthesised indoleamines is negligible compared to those synthesised from the exogenously-added precursor and that the concentration of radiolabelled products thus approximates the true concentration of indoleamines formed (Morton, 1990).

It is perhaps noteworthy that the culture medium used contains tryptophan, at a concentration of 40 mg/ ml. This may act as a source for some of the non-radiolabelled indoleamines produced. It is possible that radiolabelled and non-radiolabelled, “hot” and “cold” substrates respectively, may compete for the same biosynthetic or metabolic pathways, thus influencing

the profile of radiolabelled products formed. In addition, the uptake and compartmentalisation of radiolabelled substrates may differ from that of non-radiolabelled compounds (Boyd, 1999). It is possible to determine the concentrations of the indoleamines that are present endogenously by using HPLC, coupled to electrochemical and fluorometric detection (Boyd, 1999).

The type of TLC system used depends on the number and profile of radiolabelled indoleamines present in the culture medium. This, in turn, depends on the choice of radiolabelled precursor (Morton, 1990; Olivieri *et al*, 1990). In these studies, [^{14}C]-5-HT was used as the precursor, and the following indoleamine products can be isolated and quantified: aHT, aMT, ML, MA, HA and HA. 5-HT and MT cannot be separated and remain together at the origin, as shown in the representative TLC plate in Figure 52 below. A bi-dimensional TLC system, employing two different mobile phases, is used (Klein and Notides, 1969; Morton, 1990). It has been found, by analysis of the remainder of the TLC plate, that no residual radioactivity remains and that there are thus no unidentified radiolabelled products (Morton, 1990).

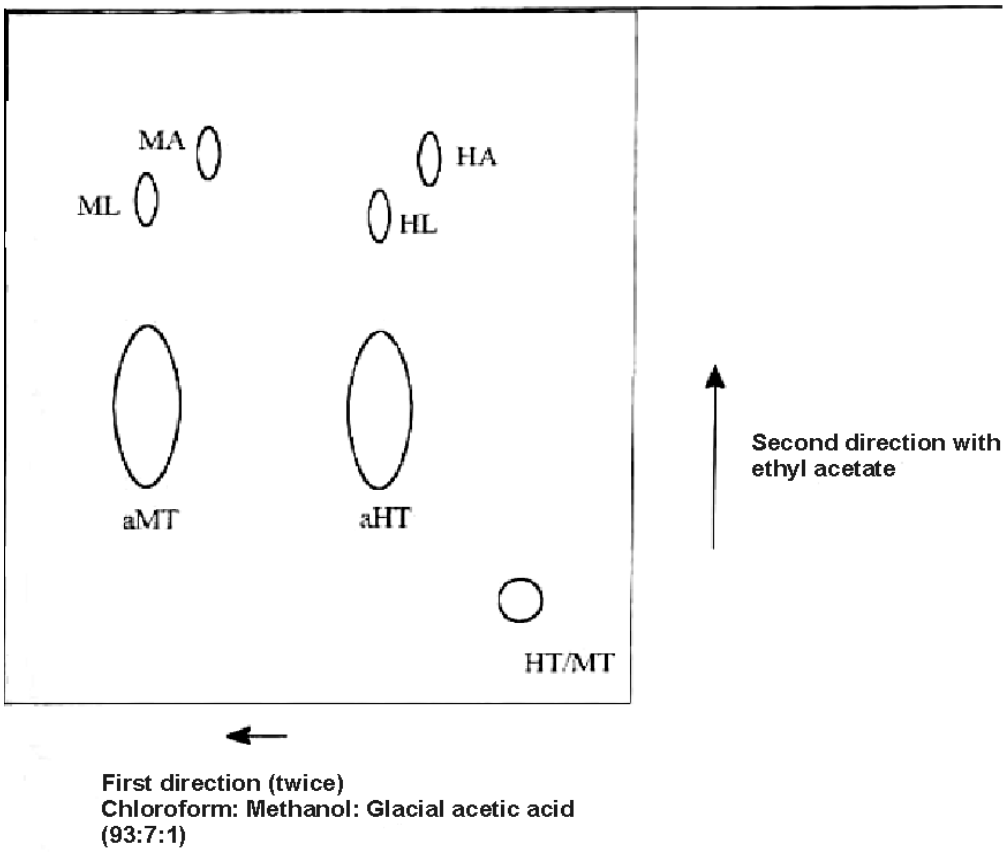


Figure 52: A representative TLC plate showing the separation of [^{14}C]-indoleamine products synthesised from [^{14}C]-5-HT by the pineal gland (Klein and Notides, 1969)

7.3 Experimentation

Pineal organ culture was performed based on the methods developed by Wurtman *et al* (1968), Klein and Notides (1969), Klein and Rowe (1970), Daya and Potgieter (1982) and Morton (1990). The effects of 5-FU and melatonin on the pineal biosynthesis of indoleamines from [^{14}C]-5-HT was investigated.

7.3.1 *In vitro*

7.3.1.1 Materials, animals, reagents and equipment

5-FU, aMT, 5-HT, MT, HA, HL, ML and MA were purchased from the Sigma Chemical Corporation, St Louis, MO, USA. The radiolabelled 5-hydroxy[*side-chain*-2-^{14}C]tryptamine creatinine sulphate, with a specific activity of 58.0 mCi/ mmol, was obtained from Amersham International (Amersham, United Kingdom). All the other chemicals used were obtained from local suppliers and were of the highest purity available. All solutions were prepared using deionised water (Milli R/Q System, Millipore®), and absolute ethanol at a final concentration of 0.67% v/ v was used as a co-solvent to dissolve melatonin. The BGJb culture medium (Fitton-Jackson Modification) was purchased from GibcoBRL Life Technologies (Paisley, Scotland) and antibiotics were aseptically added to this for fortification. Other components of the culture medium include various other amino acids, inorganic salts, vitamins, calcium and glucose (Maharaj, 2004). Emulsifier Scintillator Plus® scintillation liquid was purchased from Packard Bioscience Company (Groningen, The Netherlands). Male Wistar rats, each weighing between 250g and 300g, and cared for as outlined in Appendix A, were used. Kimble tubes were obtained from Vivid Air (Port Elizabeth, South Africa). The aluminium TLC plates, coated with 0.2 mm silica gel 60 F_{254}, were obtained from Merck (Darmstadt, Germany). A Syngene UV lamp (Synoptics Ltd, USA) and Beckman LS 2800 scintillation counter (California, USA) were used.

7.3.1.2 Method

Twenty rats were sacrificed by cervical dislocation and the pineal glands removed. These were immediately placed individually, using a 1 ml syringe needle, into sterile Kimble tubes containing BGJb culture medium. In the control group (n = 5), each pineal gland was placed in 62 µL of culture medium, whereas in the other groups, each pineal gland was placed in 52 µL of culture medium. In the 5-FU group (n = 5), 10 µL of 5-FU, at a final concentration of 10 µM, was added to each tube. Likewise, the melatonin group (n = 5) received 10 µL of melatonin, and the combination group (n = 5) received both drugs, at a final concentration of 10 µM. One of the advantages of performing this assay *in vitro* is the increased certainty that all the exogenously-added drug is available in the culture medium and taken up by the pineal

gland, whereas *ex-vivo* this likelihood could potentially be decreased by the occurrence of pharmacokinetic processes, such as hepatic metabolism.

An aliquot of 8 µL of radioactive [^{14}C]-5-HT was added to each tube, the tubes saturated with Carbogen® gas (95% O_2, 5% CO_2 v/ v) and sealed immediately. The test tubes were placed in the dark in an incubator at 37ºC for 24 hours, after which the pineal glands were flicked out of the culture medium onto the side of the test tubes. These tubes were stored at -20ºC until TLC analysis was performed.

An aliquot of 10 µL of the above culture medium was spotted on a 10 cm X 10 cm TLC plate, at a designated origin. An aliquot of 10 µL of a solution containing all the standard non-radiolabelled indoleamines and 5-HT, dissolved in absolute ethanol, was spotted on top of the culture medium spot. The TLC plates were developed in a saturated tank, with a mobile phase consisting of chloroform: methanol: glacial acetic acid (93: 7: 1 v/ v), until the solvent front was approximately 1 cm away from the top of the plate. The plates were run twice in this system, and gently dried with N_2 gas between and after runs; the use of N_2 prevented indole oxidation (Maharaj, 2004). The plates were subsequently run at 90º to the initial direction, in a mobile phase of ethyl acetate, and dried as before. The primary solvent system allows for the separation of the methoxyindoles from the hydroxyindoles, and aHT and aMT. Glacial acetic acid is effective in separating HA and HL from aHT, and ML and MA from aMT (Maharaj, 2004). UV visualisation and marking of the spots of indoles followed. These spots were carefully cut out and placed into scintillation vials. An aliquot of 3 ml of scintillation liquid was added to each vial, the vials tightly sealed and shaken. The radioactivity in each vial was determined, as counts per minute, using a Beckman LS 2800 scintillation counter. The results were expressed as disintegrations per minute (DPM) per 10 µL of culture medium, which is an indication of the amount of metabolite present.

7.3.2 *Ex-vivo*

7.3.2.1 Materials, animals, reagents and equipment

As described in 7.3.1.1. Twenty-five male Wistar rats were used.

7.3.2.2 Method

Fifteen rats, divided into three groups of five, were treated for five days with either: (i) 5-FU, (ii) melatonin, or (iii) 0.9% saline, as described in Table 10 (see 3.6.2.2). The rats were sacrificed on the sixth day, the pineal glands removed, and the procedure outlined in 7.3.1.2 was followed. The following week, ten rats, divided into two groups of five: (i) a control and

(ii) a 5-FU/ melatonin combination group, were treated for five days (see 3.6.2.2), sacrificed on the sixth day and similarly analysed (7.3.1.2). It was necessary to compare the combination group to this second set of controls, in order to eliminate inter-day variability in experimental conditions. The results for all groups were expressed as DPM/ 10 µL medium.

7.4 Results

Statistical analysis was performed using ANOVA, followed by the Student-Newman-Keuls multiple-range test.

7.4.1 *In vitro*

Figures 53-59 show the results obtained for each radiolabelled metabolite of [^{14}C]-5-HT, as well as the amount of precursor remaining.

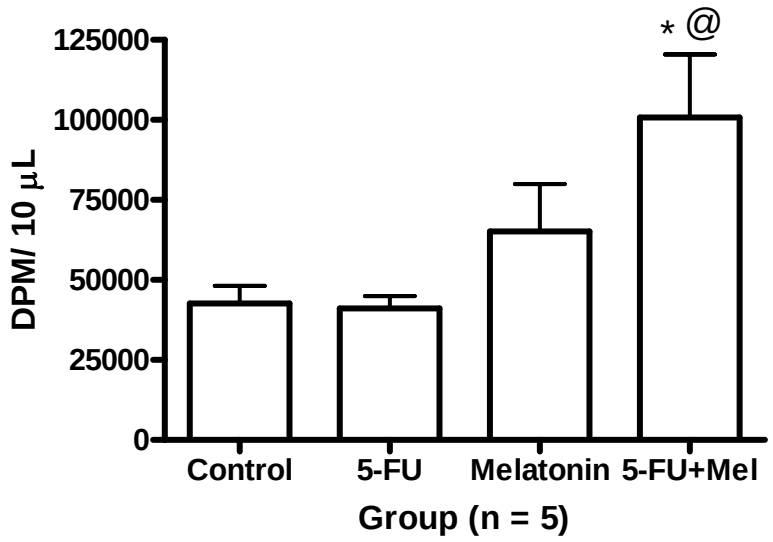


Figure 53: The amount of [^{14}C]-5-HT remaining/ [^{14}C]-MT formed after pineal organ culture <u>in vitro</u>

* $p<0.05$ compared to 5-FU, @ $p<0.05$ compared to control, after analysis by ANOVA followed by the Student-Newman-Keuls multiple-range test. Each bar represents the mean ± SEM

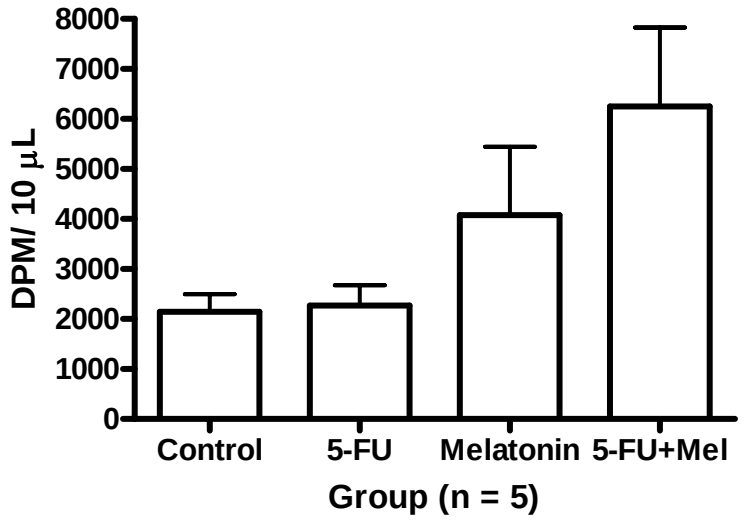


Figure 54: Formation of [^{14}C]-aHT by pineal organ culture <u>in vitro</u>

Each bar represents the mean ± SEM

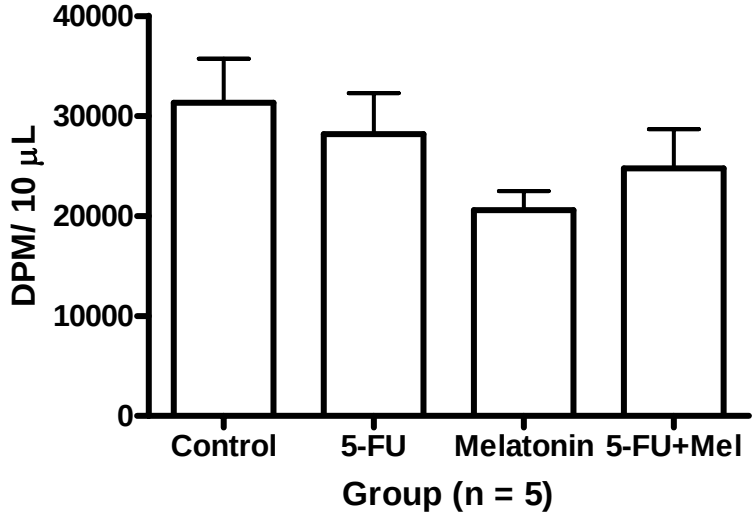


Figure 55: Formatio of [^{14}C]-aMT by pineal organ culture <u>in vitro</u>

Each bar represents the mean ± SEM

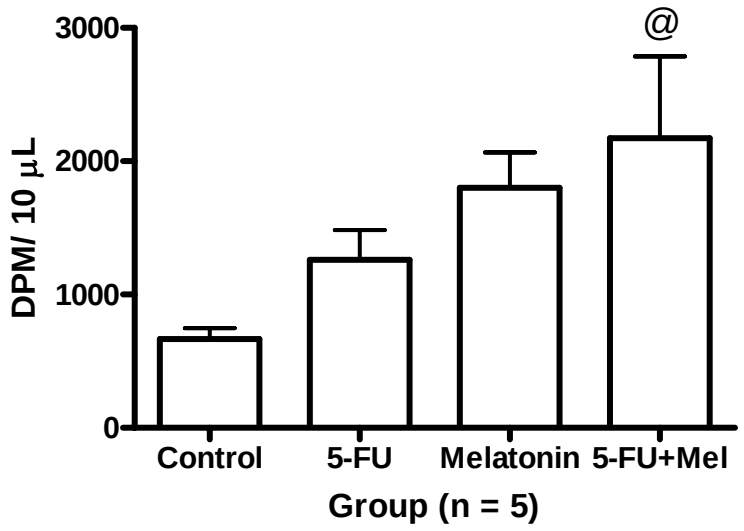


Figure 56: Formation of [^{14}C]-HA by pineal organ culture <u>in vitro</u>

@ $p<0.05$ compared to control, after analysis by ANOVA followed by the Student-Newman-Keuls multiple-range test. Each bar represents the mean ± SEM

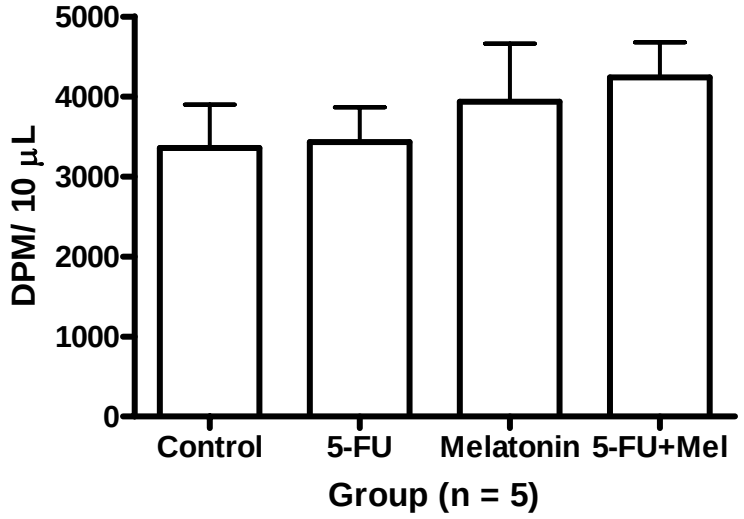


Figure 57: Formation of [^{14}C]-HL formed by pineal organ culture <u>in vitro</u>

Each bar represents the mean ± SEM

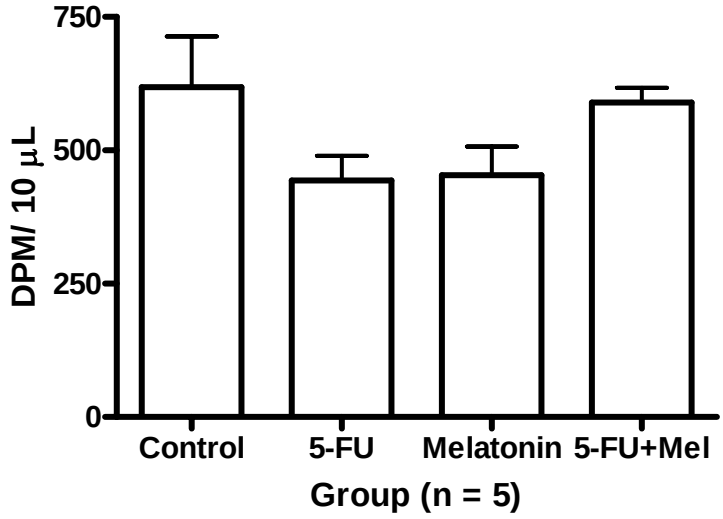


Figure 58: Formation of [^{14}C]-MA by pineal organ culture <u>in vitro</u>

Each bar represents the mean ± SEM

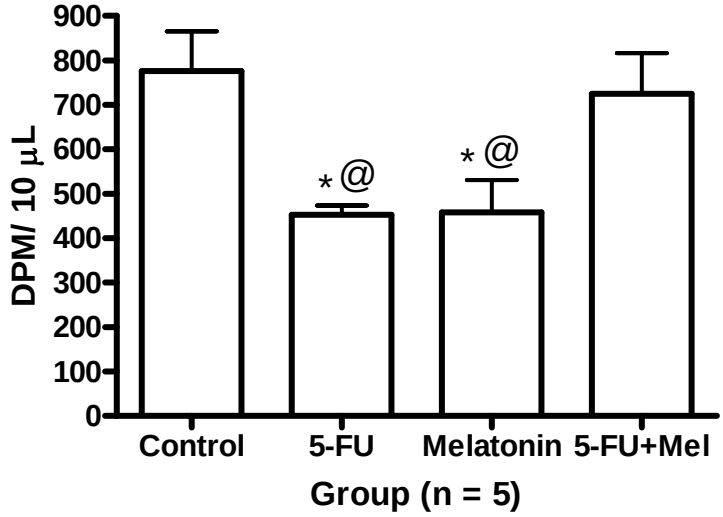


Figure 59: Formation of [^{14}C]-ML by pineal organ culture <u>in vitro</u>

* $p<0.05$ compared to control, $^{@}$ $p<0.05$ compared to 5-FU+Mel, after analysis by ANOVA followed by the Student-Newman-Keuls multiple-range test. Each bar represents the mean ± SEM

As shown in the above graphs, 5-FU and melatonin do not alter the levels of [^{14}C]-aMT, [^{14}C]-aHT, [^{14}C]-HL and [^{14}C]-MA. There are significant alterations in the levels of the following three compounds:

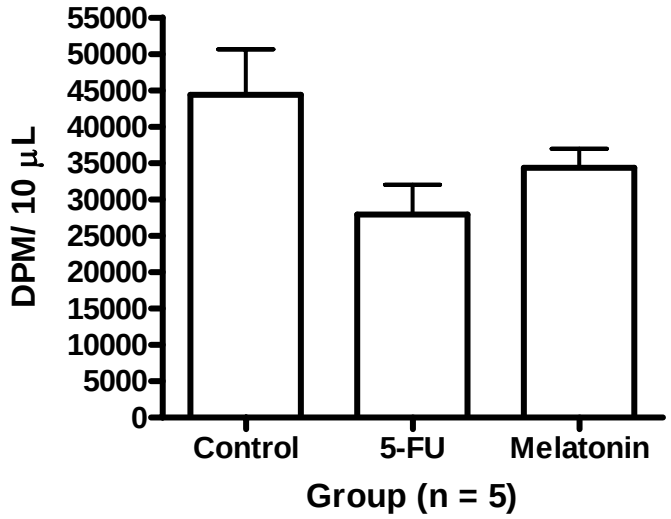
(i) the precursor [^{14}C]-5-HT/ [^{14}C]-MT increased following the addition of both 5-FU and melatonin to the culture medium;

(ii) [^{14}C]-HA levels also increased in the combination group; and

(iii) [^{14}C]-ML levels decreased in both the 5-FU and melatonin groups.

7.4.2 *Ex-vivo*

Figures 60-66, as shown on the next few pages, were obtained for the *ex-vivo* assay.



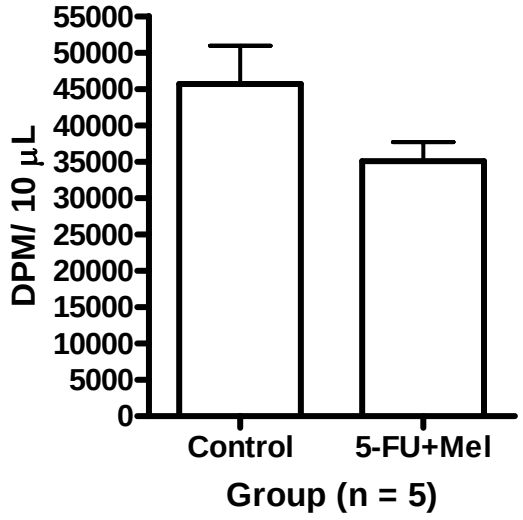
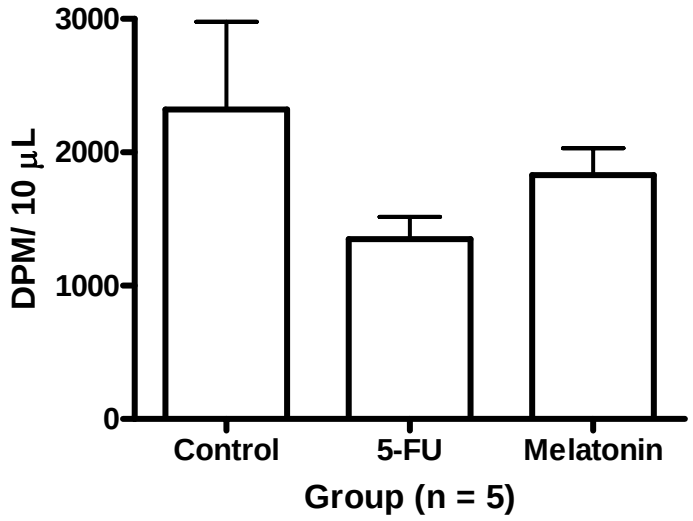


Figure 60: The amount of [^{14}C]-5-HT remaining/ [^{14}C]-MT formed after pineal organ culture ex-vivo

Each bar represents the mean ± SEM



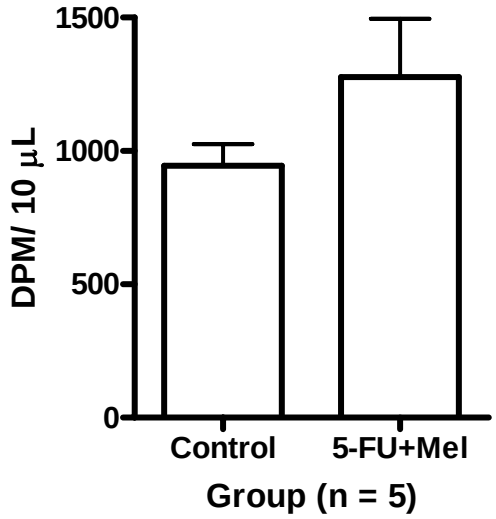
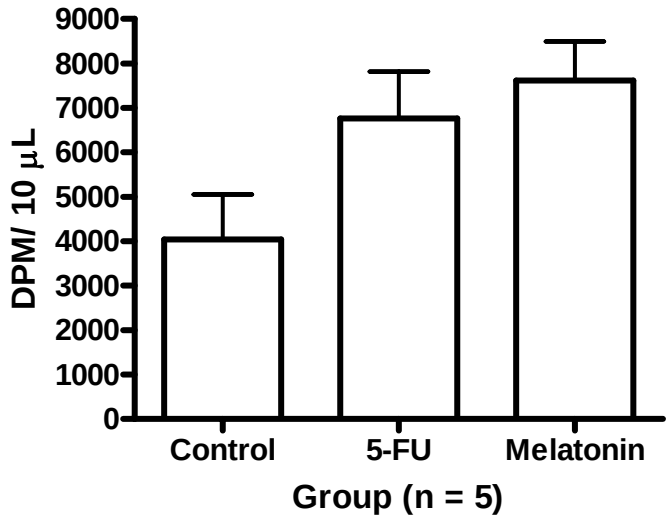


Figure 61: Formation of [^{14}C]-aHT by pineal organ culture <u>ex-vivo</u>

Each bar represents the mean ± SEM



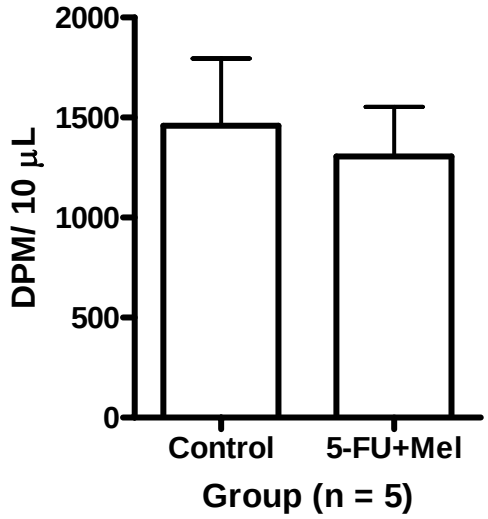
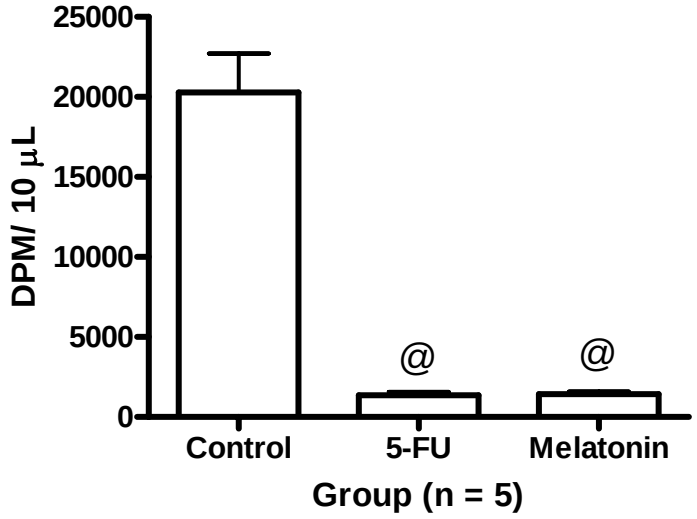


Figure 62: Formation of [^{14}C]-aMT by pineal organ culture ex-vivo

Each bar represents the mean ± SEM



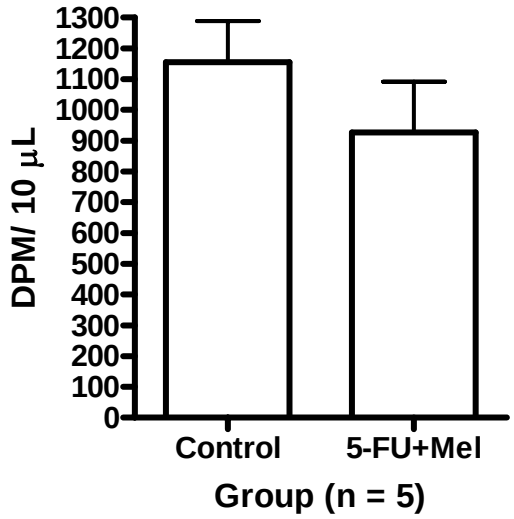
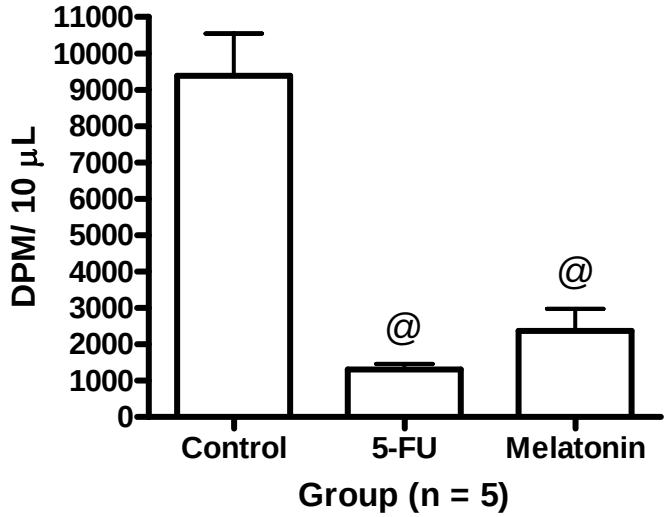


Figure 63: Formation of [^{14}C]-HA by pineal organ culture <u>ex-vivo</u>

[@] $p<0.001$ compared to control, after analysis by ANOVA followed by the Student-Newman-Keuls multiple-range test. Each bar represents the mean ± SEM



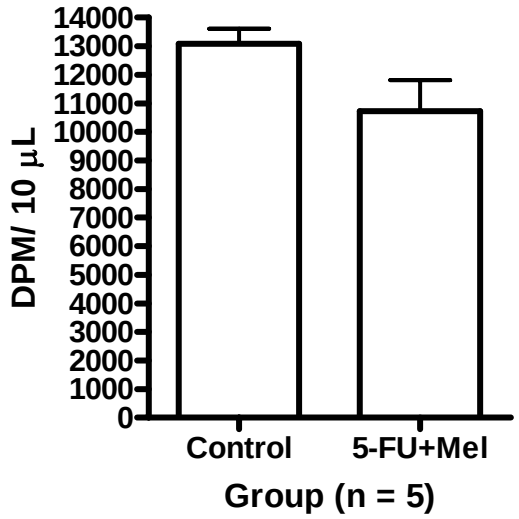
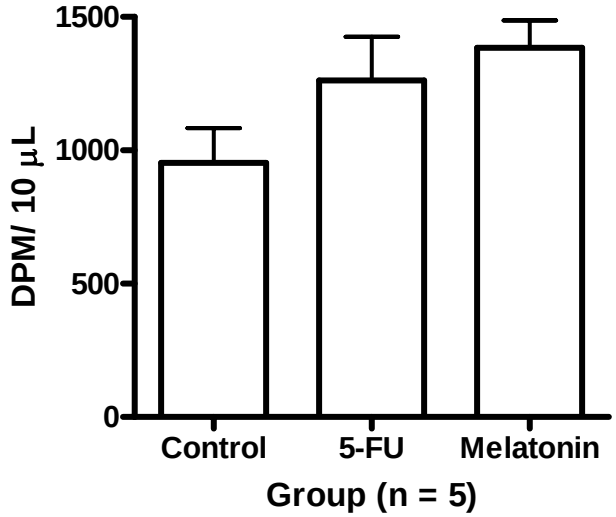


Figure 64: Formation of [^{14}C]-HL by pineal organ culture <u>ex-vivo</u>

@ $p<0.001$ compared to control, after analysis by ANOVA followed by the Student-Newman-Keuls multiple-range test. Each bar represents the mean ± SEM



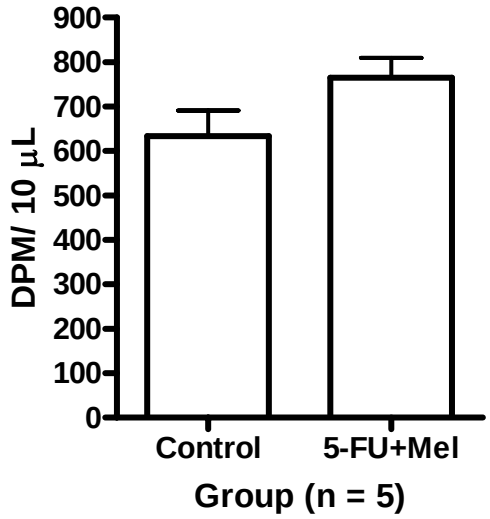
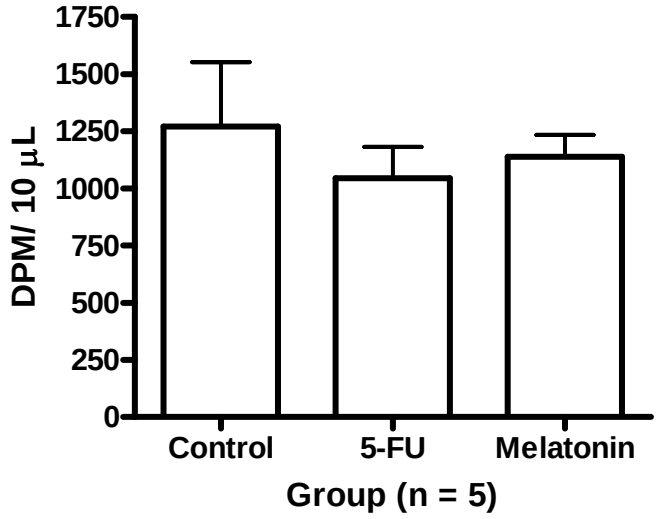


Figure 65: Formation of [^{14}C]-MA by pineal organ culture <u>ex-vivo</u>

Each bar represents the mean ± SEM



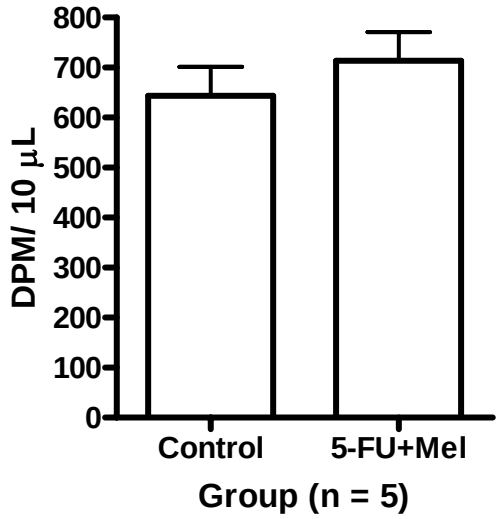


Figure 66: Formation of [^{14}C]-ML by pineal organ culture <u>ex-vivo</u>
Each bar represents the mean ± SEM

As shown in the above graphs, the *ex-vivo* administration of 5-FU and melatonin does not alter the levels of [^{14}C]-aMT, [^{14}C]-aHT, [^{14}C]-HL and [^{14}C]-MA subsequent to pineal metabolism. Furthermore, levels of the precursor [^{14}C]-5-HT/ [^{14}C]-MT are not altered. The two significant changes that do occur were in the levels of the hydroxyindoles; both [^{14}C]-HA and [^{14}C]-HL levels decrease in the 5-FU and melatonin groups, but not in the group of rats receiving both drugs.

7.5 Discussion

It is noticeable that there are some discrepancies between the *in vitro* and *ex-vivo* results. *In vitro*, the group receiving both 5-FU and melatonin show significantly elevated levels of the precursor [^{14}C]-5-HT/ [^{14}C]-MT remaining after metabolism (see Figure 53), while *ex-vivo* there is no change at all (see Figure 60). The levels of both [^{14}C]-hydroxyindole metabolites decrease significantly *ex-vivo* in the presence of either 5-FU or melatonin; there is no change in the combination group, though, as shown in Figures 63 and 64. In the *in vitro* study, [^{14}C]-HA levels increase only in the combination group (Figure 56), whilst [^{14}C]-HL levels remain unaltered (see Figure 57). The final discrepancy is that *in vitro* [^{14}C]-ML levels decrease in both the 5-FU and melatonin groups (see Figure 59), but are unchanged *ex-vivo* (Figure 68). The levels of all other metabolites are unaltered. It is most likely that the abovementioned altered levels observed *in vitro* are artefacts of the experimental conditions, false positives, as these result are not confirmed *ex-vivo*.

It is important to note that [^{14}C]-aMT levels are unchanged both *in vitro* and *ex-vivo*. This indicates that neither 5-FU nor melatonin alters Pathway I metabolism of 5-HT in the pineal gland (see Figure 51) and thus do not alter the activity of NAT (see 7.1). This is supported by the finding that the level of [^{14}C]-aHT, the product of NAT activity, is unchanged. Since the noradrenergic system is involved in stimulating NAT, it is unlikely that either 5-FU or melatonin has β agonist or antagonist activity (see 7.1). In the preceding Chapter (see 6.4), it was found that 5-FU significantly decreases NE levels in the forebrain, and that this is countered by the administration of melatonin. The use of melatonin alone, however, does not alter NE levels. It might thus be expected that since 5-FU decreases NE levels, this might lead to NAT inhibition, with a resultant decrease in the amount of aHT and aMT produced. That this does not occur suggests that there are other compensatory mechanisms, besides the involvement of NE, that maintain NAT activity. An example of such a mechanism is the possible involvement of α receptors (see 7.1).

It is significant that the levels of endogenously-produced melatonin, represented by [^{14}C]-aMT in the assay, are not altered by the exogenous administration of melatonin *ex-vivo*. This suggests that the use of melatonin co-therapy will simple increase the levels of 5-HT that have been lowered due to 5-FU treatment (see 6.4), and will not activate any possible feedback mechanism that will alter endogenous melatonin production.

As shown in Figure 51, Pathway II is the major route of 5-HT metabolism and HA is the primary metabolite of this neurotransmitter (see 7.1). The finding that both 5-FU and melatonin decrease [^{14}C]-hydroxyindole levels *ex-vivo* may be due to a possible inhibition of

the enzyme MAO. It is known that melatonin inhibits MAO (Urry and Ellis, 1975; cited in Walsh and Daya, 1998), but the effect of 5-FU on MAO activity is as yet unknown. MAO inhibition would also result in a decrease in methoxyindole levels (see Figure 51), which does not occur *ex-vivo*. In Chapter 6 it was found that 5-FU does not alter HA levels in the forebrain. This discrepancy in results may be due to an increase in HA turnover, which is masked in the forebrain analyses. The technique of pineal organ culture, by being more selective and reducing the relative likelihood of organ interactions and compensatory mechanisms, allows for the singular decrease in HA levels to be apparent.

Although the mechanisms by which 5-FU and melatonin individually decrease [^{14}C]-hydroxyindole levels is unknown, it is perhaps more significant that this is not found to be the case in the group of rats that received both drugs in combination. In this group, there are no changes in pineal indole metabolism as a result of combination therapy. This suggests that patients who have 5-FU-induced 5-HT deficiency, as shown in Chapter 6, may only have a deficiency of this neurotransmitter and no other underlying disorder in its metabolism. Co-therapy with melatonin would, moreover, correct this deficiency without altering 5-HT metabolism.

7.6 Conclusions

It has been established that neither 5-FU nor melatonin alters the metabolism of 5-HT via Pathway I, which is the minor route of metabolism. This results in the endogenous production of melatonin being unaltered by these drugs. This finding also suggests that neither drug alters the activity of NAT. Although it was found that individually 5-FU and melatonin appear to inhibit the major route of 5-HT metabolism, Pathway II, through possible MAO inhibition, thereby resulting in lowered HA levels, this result does not occur upon combination therapy. This implies that the use of both drugs together only alters the level of 5-HT in the brain, as shown in the preceding Chapter, and is unlikely to alter 5-HT metabolism.

CHAPTER 8

THE EFFECTS OF 5-FLUOROURACIL AND MELATONIN ON HEPATIC LIPID PEROXIDATION *IN VITRO* AND *IN VIVO*

8.1 Introduction

Oxygen is used by cells in numerous biochemical reactions and, while having benefits, these aerobic reactions also result in free radicals being produced (Packer, 1991; Di Mascio *et al*, 1991). These products are chemical entities that possess an unpaired electron, which confers reactivity to the products (Cheeseman and Slater, 1993). Free radicals can induce damage to various important cellular components, such as cell membranes, proteins and DNA (Dean *et al*, 1993; Gey, 1993). Free radicals may also cause the formation of lipid aldehydes and peroxides, resulting in the peroxidation of polyunsaturated fatty acids (Curtis *et al*, 1984; Murphy *et al*, 1989; Dixit *et al*, 1983; Burton and Ingold, 1989; Cheeseman and Slater, 1993). This has many far-reaching effects, such as alterations in the endoplasmic reticulum in the liver (Archakov, 1975) and the uncoupling of mitochondrial oxidative phosphorylation (Hunter *et al*, 1964; Vladimirov and Cheremisina, 1975).

These lipid aldehydes and peroxides can also disrupt the structure of, as well as cause physicochemical alterations in, cell membranes (Galeotti *et al*, 1984; Dixit *et al*, 1983; Dobretsov *et al*, 1977; Curtis *et al*, 1984; Galeotti *et al*, 1986). These cell membranes have high lipid content, in the form of phospholipids. The unsaturated portions of the phospholipid bilayer in cell membranes are especially susceptible to damage by reactive oxygen species (ROS) (Vijayalaxmi *et al*, 2002; Cheeseman and Slater, 1993). An example of the physicochemical changes mentioned above is an alteration in membrane fluidity and electric potential, due to altered interactions between the hydrophobic ends of adjacent phospholipid chains, which facilitate the entry of water molecules into the membrane; this can have serious sequelae (Vijayalaxmi *et al*, 2002; Parasassi *et al*, 1992, 1994). Dobretsov *et al* (1977) have shown that lipid peroxidation decreases membrane fluidity and thus increases its viscosity. This is due to the transfer of fatty acid chains, which have been oxidised, from the interior to the exterior of cell membranes (Witting, 1965; Dobretsov *et al*, 1977). During this transfer, there is a decrease in the proportion of fatty acid chains that are unsaturated, thereby increasing the viscosity of the cell membrane. This alteration of the physical state of the membrane is associated with a loss of function, such as a decrease in respiratory control in mitochondria (Dobretsov *et al*, 1977; Vladimirov and Cheremisina, 1975). Lipid peroxidation, by altering the biophysical properties of cell membranes, interferes with the membrane's

barrier function, thereby potentially having far-reaching sequelae (Cheeseman, 1993). It also disrupts the homeostatic control of cell metabolism, resulting in, for example, malfunctioning of calcium pumps (Albano *et al*, 1991; Cheeseman, 1993).

Lipid peroxidation is one manifestation of the phenomenon of oxidative stress (Santamaría *et al*, 2001a). Oxidative stress can lead to short-term disorders in the brain, such as ischaemia, stroke, trauma and long-term neurodegeneration (Braughler and Hall, 1989; Traystman *et al*, 1991; Jesberger, 1991; Sanatamaría *et al*, 2001b). The ROS formed may, besides oxidising proteins and lipids in cell membranes, result in the breakage of DNA strands, thereby inducing neuronal malfunction (LeBel and Bondy, 1991).

An important endogenous source of ROS is the mitochondria, which can, in turn, be damaged by these ROS (Cadenas and Davies, 2000). MAO, occurring in the outer membrane of this structure, deaminates biogenic amines (see 6.2.2.2), a process that results in the generation of a large quantity of H_2O_2 (Cadenas and Davies, 2000). The electron transport chain, in the inner membrane of the mitochondria, is vital in the production of energy in the form of ATP. Side reactions, however, result in the direct formation of superoxide anions ($O_2^{-}\cdot$) which undergo dismutation to form H_2O_2. This can be converted to the toxic hydroxyl radical ($HO\cdot$) (Cadenas and Davies, 2000). Alternatively, $O_2^{-}\cdot$ may induce the release of Fe^{2+} from dehydratases, and this ion reduces H_2O_2 to $HO\cdot$ (Liochev and Fridovich, 1994).

Cheeseman (1993) classifies lipid peroxidation as occurring in two phases: initiation and propagation. In the initiation phase, the loss of a hydrogen atom from an unsaturated fatty acid results in the formation of a lipid radical (Buege and Aust, 1978; Sevanian *et al*, 1983; Tien and Aust, 1982; Smith *et al*, 1982; Curtis *et al*, 1984; Cheeseman, 1993). This lipid radical reacts with oxygen, forming a variety of ROS and alkyl radicals. These can react with each other to form covalently-bonded oxygen-containing chains (Curtis *et al*, 1984). The propagation phase of lipid peroxidation is this lipid peroxyl radical-mediated initiation of another chain and the degradation of lipid hydroperoxides to free radical intermediates (Cheeseman, 1993). This degradation, besides generating radicals that perpetuate the process of lipid peroxidation, also results in the formation of scission products, such as aldehydes, which may have biological activity (Cheeseman, 1993). It is believed that various products of lipid peroxidation, in particular 4-hydroxynonenal, may alter gene expression (Cheeseman, 1993).

The following scheme (Farmer *et al*, 1943; Holman, 1954; Dahle *et al*, 1962) shows the formation of a lipid radical and peroxide radicals from the peroxidation of linoleate.

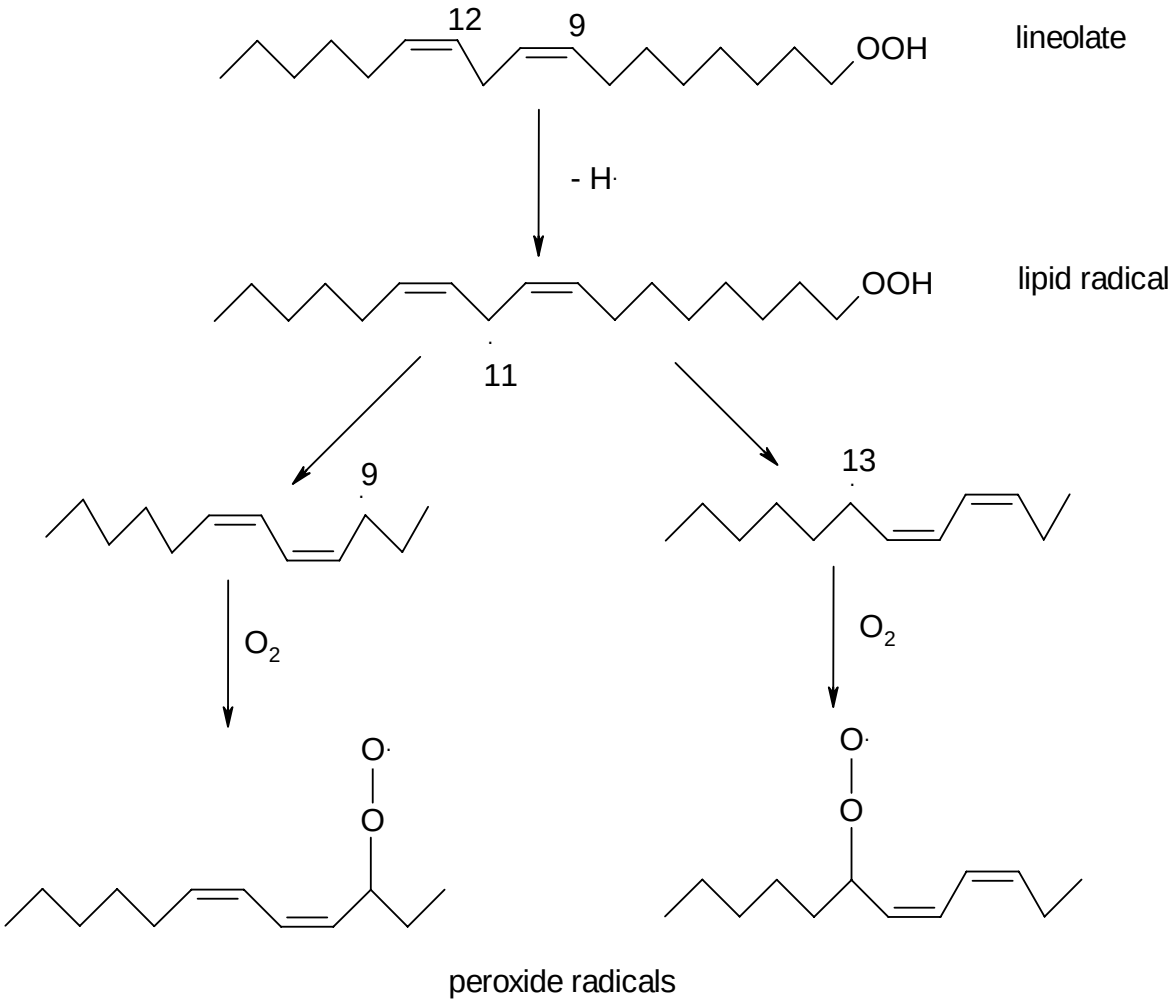


Figure 67: The peroxidation of linoleate to form peroxide radicals (Farmer *et al*, 1943; Holman, 1954; Dahle *et al*, 1962)

Lipid peroxidation, if unchecked, can thus be extremely dangerous as it induces both direct and indirect damage to cells. This is evidenced by destruction of cell membranes and the generation of ROS, respectively (Cheeseman, 1993).

Ottino and Duncan (1997) have surveyed the literature with regard to the effects that free radicals and their products have on the pathogenesis of cancer (Gutteridge, 1993; Gey, 1993). It has been found that free radicals have a role to play in initiating tumour development (Kensler and Taffe, 1986; Sun, 1990). The ongoing production of free radicals may thus contribute to the maintenance of tumours (Ottino and Duncan, 1997). In addition, free radicals may alter antioxidative mechanisms in cells and enhance the activity of procarcinogens (Sun,

1990), such as benzo[*a*]pyrene (Marnett, 1987). There is increasing evidence that free radicals, and especially ROS, have a significant role in the multistep carcinogenesis model (Sun, 1990; Oberley and Oberley, 1986; Fischer *et al*, 1988; Cerutti, 1985; Copeland, 1983; Troll *et al*, 1984). This model divides carcinogenesis into various stages (Friedewald and Rous, 1944; Farber, 1982; Pitot *et al*, 1981, Sun, 1990):

(i) initiation, in which the cell undergoes an irreversible alteration, in a short period of time, resulting from exposure to a carcinogen;

(ii) promotion, in which initiated cells are selected and proliferate, in a process that takes a significant amount of time and that can be partially reversed (Ruddon, 1987; Hennings *et al*, 1983). The promotion phase itself consists of two stages: (a) conversion, in which the initiated cell is altered and (b) propagation, in which the aforementioned cell proliferates to a greater extent than normal cells.

The final step in this model, (iii) progression, is one in which a neoplasm, characterised by malignancy, a rapid growth rate, and an inability to be arrested by host defenses, results (Sun, 1990). Lipid peroxidation is involved in the initiation and promotion stages of carcinogenesis (Cheeseman, 1993).

There are three main causes of cancer: (i) viral, for example oncogenes; (ii) chemical, induced by carcinogens such as cigarette smoke (Pryor, 1986); and (iii) physical causes, such as radiation (Sun, 1990). Free radicals are believed to mediate both radiation- and carcinogen-induced carcinogenesis, as well as play a role in initiating and promoting this process (Sun, 1990). DNA damage induced by free radicals occurs during the initation stage and is characterised by strand breakage, damage to bases, the formation of cross-linkages and alterations to chromosomes (Sun, 1990). In order for such DNA damage to occur, the offending species would have to be generated in the vicinity of the DNA, since reactive species cannot diffuse for any great distance without reacting with compounds (Cheeseman, 1993). It has been suggested, alternatively, that the so-called clastogenic factors, which are stable and cause serious DNA damage, may be linked to lipid peroxidation (Cheeseman, 1993).

Many cancer cells release ROS, such as $O_2^{-}\cdot$ and H_2O_2, which can, in turn, act as "messengers" that promote tumour growth (Burdon, 1995). This may be by (Burdon, 1995):

(i) oxidising growth signal transductor molecules, such as inhibitors of transcription and protein kinases;

(ii) directly interacting with receptors on cancer cells; and

(iii) altering the glutathione oxidation-reduction balance, which impacts on the activity of various proteins involved in signal transduction.

Antioxidants, such as vitamin E (Packer, 1991; Di Mascio *et al*, 1991), may thus be of use in preventing the growth of tumours (Ottino and Duncan, 1997) by reacting with free radicals and thereby preventing lipid peroxidation from occurring (Burton and Ingold, 1989; Murphy *et al*, 1989). Vitamin E has been found to exert antiproliferative effects on some tumour cells (Ottino and Duncan, 1997), which may be due to its antioxidant properties (Rama and Prasad, 1983). Increased concentrations of the vitamin, however, may result in pro-oxidant effects (Cillard *et al*, 1980a, 1980b), which may account for the significant increase in the extent of lipid peroxidation in some tumour cells (Ottino and Duncan, 1997). Cheeseman (1993) summarises the evidence linking lipid peroxidation, and its resultant generation of free radicals, to the development of cancer (Cerutti, 1985; Trush and Kensler, 1991):
(i) various compounds that result in the formation of tumours, such as PMA, produce free radicals;
(ii) organic peroxides, for example benzoyl peroxide, promote the development of tumours;
(iii) the development of tumours is inhibited by antioxidants, such as butylated hydroxytoluene;
(iv) the possible involvement of clastogenic factors in DNA damage; and
(v) the protection that vitamin C and other antioxidants available in the diet can afford against the development of cancer (Doll, 1992; Block, 1991).

Free radicals have also been implicated in the pathogenesis of cardiovascular diseases (Gey, 1993). In order to conclude that lipid peroxidation is the mechanism of toxicity in a particular disease state or disorder, it must be shown that the administration of an antioxidant counters injury to the affected tissue as well as the extent of lipid peroxidation, and that lipid peroxidation can be detected in the period between the administration of a toxin and the death of the cell (Cheeseman, 1993).

Galeotti *et al* (1986) have mentioned the particular importance of lipid peroxidation in cancer. Lipid peroxidation inhibits the growth of tumours (Liepkalns *et al*, 1982) and cell division (Wilbur *et al*, 1957; Cornwell *et al*, 1979; Huttner *et al*, 1977, 1978; Wolfson *et al*, 1956) by its inhibitory effect on DNA synthesis (Slater *et al*, 1984; Galeotti *et al*, 1986). Furthermore, *cis*-unsaturated fatty acids have been found to exert tumoricidal action through free radical-mediated and lipid peroxidation mechanisms (Sagar *et al*, 1992; Begin *et al*, 1985, 1986; Das, 1990, 1990b, 1991; Das *et al*, 1987, 1990). TNFα, which is toxic to cancer cells, exerts its effect through free radical-induced lipid peroxidation; the generation of free radicals occurs via a calmodulin-dependent mechanism (Das *et al*, 1990).

An increase in the growth rate of hepatomas is correlated with a decrease in the susceptibility of membranes to lipid peroxidation, as well as an increase in susceptibility to attack by oxy radicals due to less protection afforded by antioxidative enzymes (Galeotti *et al*, 1986). These antioxidative enzymes, such as superoxide dismutase (SOD) and catalase, are less active in tumour cells (Galeotti *et al*, 1986; Dionisi *et al*, 1975; Mochizuki *et al*, 1971; Bize *et al*, 1980; Bozzi *et al*, 1976; Oberley and Buettner, 1979; Peskin *et al*, 1977; Sun, 1990). It has been shown that the physicochemical composition of cell membranes, in particular the double-bond index, is an important determinant of the extent of lipid peroxidation that occurs (Galeotti *et al*, 1986). These authors have concluded that oxy radical-induced oxidative stress, manifested by lipid peroxidation, alters both the structure and functioning of tumour cell membranes, and that tumours with high rates of proliferation and growth are particularly vulnerable.

Antioxidative enzymes in the body include catalase, glutathione reductase, zinc- and manganese-containing forms of SOD, glutathione peroxidase and glucose-6-phosphate dehydrogenase (G6PD). These enzymes are altered in cancer cells, although it is not known whether such alterations give rise to, or are a result of, cancer (Sun, 1990). Figure 68 illustrates the mechanism of action of antioxidative enzymes in protecting cells from the oxidative stress that results both from insult and metabolism (Sun, 1990); an example of the latter is the formation of H_2O_2 and singlet oxygen (O_2^-) by the xanthine oxidase system through the Haber-Weiss reaction (Haber and Weiss, 1934; Dixit *et al*, 1983). This reaction, occurring in two steps and resulting in the formation of numerous ROS, is given below (Haber and Weiss, 1934):

(1) $H_2O_2 + HO\cdot \rightarrow H_2O + O_2^-$

(2) $H_2O_2 + O_2^{-}\cdot \rightarrow HO\cdot + HO^- + HO\cdot$

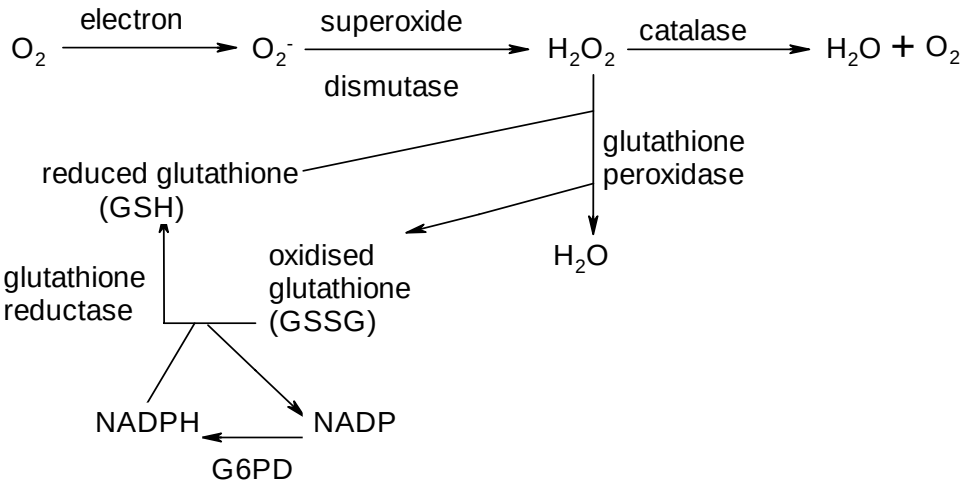


Figure 68: The mechanisms of action of antioxidative enzymes (Sun, 1990)

The abovementioned resistance exhibited by cancer cells towards lipid peroxidation has been widely noted (Cheeseman, 1993; Bartoli and Galeotti, 1979; Cheeseman *et al*, 1986, 1988; Minotti *et al*, 1986). Cheeseman (1993) has summarised the work that has been done on hepatomas as follows:

(i) the extent of lipid peroxidation is much lower than that which occurs in the liver;

(ii) the membranes of hepatomas have a high proportion of cholesterol and low proportions of phospholipids and polyunsaturated fatty acids;

(iii) tumours that have a rapid growth rate and low degree of differentiation show the most resistance to lipid peroxidation;

(iv) CYP450 is present in negligible amounts in hepatomas; and

(v) there are increased levels of some antioxidants in these hepatomas.

This author suggests that, through the generation of hydroxyalkenals and other products that possess biological activity, lipid peroxidation on a long-term basis inhibits the division of cells. This may explain why cells that are rapidly dividing show less susceptibility to lipid peroxidation, as they have developed resistance to this process that curtails their proliferation (Cheeseman, 1993). The finding that there is a close, time-dependent relationship between thymidine kinase and the cyclical activity of antioxidant enzymes (Slater *et al*, 1990) supports the suggestion that cell division and lipid peroxidation are related (Cheeseman, 1993).

The cyclooxygenase (COX) enzyme is a key enzyme in the arachidonic acid pathway that leads to the biosynthesis of eicosanoids, prostaglandins and various inflammatory mediators (Ramwell *et al*, 1980; Dupont, 1987; Slater, 1991; Panganamala and Cornwell, 1982). It has been found that many lipid peroxides, inlcuding prostaglandin G_2 (PGG_2), itself a product of the COX pathway, stimulate this pathway (Lands and Hanel, 1983; Hemler *et al*, 1979; Yamamoto, 1983). High levels of COX activity would thus indirectly imply that there are high levels of lipid peroxides present (Ottino and Duncan, 1997). Malondialdehyde (MDA), a product of lipid peroxidation (see 8.5.1.2), may be formed by the conversion of lipid endoperoxides to prostaglandins (Pryor *et al*, 1975). The thiobarbituric acid (TBA) assay, which determines the level of MDA equivalents in a system (see 8.5.1.2), can thus be used to gauge an increase in COX activity (Ramwell *et al*, 1980), due to the increase in lipid peroxidation (Ottino and Duncan, 1997). The formation of prostaglandin H_2 (PGH_2) from PGG_2, in which COX is also involved through its effects on prostaglandin-hydroperoxidase, results in the formation of many free radicals (Yamamoto, 1983; Egan *et al*, 1976; De Vries and Van Noorden, 1992; Ramwell *et al*, 1980). An increase in COX activity would thus indirectly increase free radical levels and associated damage to cellular components (Ottino and Duncan, 1997).

It has been found that 5-FU enhances COX-2 expression in solid tumours (Mercer *et al*, 2005); the resulting increase in COX-2 levels, which promotes the synthesis of inflammatory mediators and lipid peroxides, may account for the drug's cytotoxicity.

8.2 QA-induced lipid peroxidation

Excitotoxicity in the brain may be induced through several mechanisms, one of which is the formation of free radicals (Lancelot *et al*, 1998). QA, a metabolite of tryptophan (see Figure 34) (see 4.1), induces neurotoxicity through several mechanisms; one of these is the overstimulation of NMDA receptors, which are located on the membranes of nerve terminals (Monahan and Michel, 1987; Sut'astny *et al*, 1999). This results in oxidative damage, increased levels of calcium, a disruption of cellular metabolism and eventually neuronal death (Stone, 1993; Santamaría *et al*, 2001a) (see below).

It also induces neurotoxicity through its ability to generate free radicals, which subsequently cause lipid peroxidation (Santamaría and Ríos, 1993; Ríos and Santamaría, 1991). *In vitro*, QA forms a complex with ferrous ion (Fe^{2+}) (Goda *et al*, 1996); this complex generates $HO\cdot$, the most potent ROS (Sanatamaría *et al*, 2001a), by promoting the Fenton reaction (Iwahashi *et al*, 1999) (see 8.3). These radicals disrupt DNA structure and cause lipid peroxidation (Goda *et al*, 1996; Stípek *et al*, 1997) during the initial stages of neurotoxicity (Santamaría *et al*, 2001a). $HO\cdot$ may further contribute to oxidative stress by altering the activity or decreasing the levels of compounds that have antioxidative and free radical-scavenging functions, such as glutathione and SOD (Santamaría *et al*, 2001a). Other ROS generated as a result of QA are $O_2^{-\cdot}$ and H_2O_2 (Santamaría *et al*, 2001b). QA also causes oxidation by preventing ferrous ion from undergoing auto-oxidation (Murakami *et al*, 1998) (see 8.3). A subsequent effect of QA in inducing oxidative stress may be the development of inflammation and gliosis (Behan *et al*, 1999; Santamaría *et al*, 2001a).

Glutamate is an excitatory amino acid that mediates oxidative stress through two mechanisms (Santamaría *et al*, 2001b; Coyle and Puttfarcken, 1993):
(i) the iron-induced formation of free radicals, which promote lipid peroxidation and subsequent alterations in receptor functioning and the fluidity of neuronal membranes; and
(ii) interactions with glutamate receptors, such as NMDA receptors, which bring about changes in Ca^{2+} levels and the subsequent generation of ROS. Overstimulation of NMDA receptors thus results in an increase in the cytosolic concentration of Ca^{2+}; this enhances the activity of enzymes that are dependent on Ca^{2+}, such as phospholipase A_2 (Choi, 1988), the latter being an important enzyme in the arachidonic acid pathway that leads to the formation of inflammatory mediators. Xanthine dehydrogenase is also irreversibly converted to xanthine

oxidase (Choi, 1988), which results in the formation of NO. The latter is converted to peroxynitrite (ONOO), which is more stable and is responsible for the death of nerve cells (Lafón-Cazal *et al*, 1993).

Santamaría *et al* (2001b) have thus concluded that QA induces significant lipid peroxidation in the CNS (Ríos and Santamaría, 1991; Sutipek *et al*, 1997; Santamaría and Ríos, 1993), and that this can be countered by antagonists of glutamate receptors.

8.3 Effects of melatonin on lipid peroxidation

Melatonin, by virtue of its antioxidant properties, has been shown to protect neurons against QA-induced toxicity (Goda *et al*, 1996; Cabrera *et al*, 2000). Melatonin's antioxidant and free-radical scavenging properties, as well as its mechanisms of inhibiting lipid peroxidation, have been mentioned in 1.3.8. This agent directly scavenges free radicals, stimulates enzymes with antioxidative effects, such as γ-glutamylcysteine synthase (Urata *et al*, 1999), thereby preventing free radical-induced cellular damage (Reiter, 2000); and inhibits the activity of NO synthase (Baydas *et al*, 2001; Rodriguez *et al*, 2004). Its effects on these enzymes occur during periods of oxidative stress, such as after the administration of 6-hydroxydopamine, which induces free radical-mediated damage (Rodriguez *et al*, 2004). Very high levels of free radicals prevent the synthesis of these antioxidant enzymes, thereby contributing to further cellular damage (Brydon *et al*, 2000). Melatonin also has a cascade antioxidant effect (Vijayalaxmi *et al*, 2002), in that, by reacting with the precursor of H_2O_2, it forms N^1-acetyl-N^2-formyl-5-methoxykynuramine (AFMK) (see Figures 42 and 69). AFMK possesses significant antioxidant and free radical-scavenging effects (Tan *et al*, 2000a, 2001). It has been found by Pierrefiche *et al* (1993) that melatonin itself has weak antioxidant effects; it undergoes hepatic metabolism to form 6-OHM, and this main metabolite has been found by Pierrefiche *et al* (1993) to be a potent inhibitor of lipid peroxidation. However, other investigators have found that 6-OHM generates HO· (Matuszak *et al*, 1997; Sakano *et al*, 2004), thus resulting in lipid peroxidation (Bojková *et al*, 2006). The ability of 6-OHM to both generate and scavenge HO· is discussed on page 207. The ability to generate this ROS is supported by the finding that 6-OHM increases heat shock protein levels, a marker of cellular stress (see 8.4).

Melatonin significantly decreases the level of MDA (see 8.5.1.2) and thus inhibits lipid peroxidation (Zwirska-Korczala *et al*, 2005; Baydas *et al*, 2002; Rodriguez *et al*, 2004). Zwirska-Korczala *et al* (2005) have studied the effects of melatonin on the proliferation of, and extent of lipid peroxidation in, 3T3-L1 pre-adipocytes. Besides establishing that melatonin enhances the proliferation of these cells, these authors have found that the

indoleamine decreases MDA levels by increasing the activity of enzymes with antioxidative properties; this activity is apparent after 24 hours of incubation with melatonin, but increases greatly after 48 hours. In a study by Melchiorri *et al* (1995), melatonin's inhibition of MDA formation was found to be dose-dependent. The peroxyl radical generated during lipid peroxidation is also neutralised by melatonin, which is even more potent that vitamin E in this regard (Vijayalaxmi *et al*, 2002; Pieri *et al*, 1994, 1995). It has been found that melatonin's antioxidant mechanisms do not involve breaking chains to any great extent (Antunes *et al*, 1999), unlike vitamin E (Cheeseman and Slater, 1993). The latter functions by scavenging the free radicals that bear the lipid peroxidation reaction chains (Cheeseman, 1993). Melatonin is thus termed a preventive antioxidant, preventing the degradation of lipid hydroperoxides and thus inhibiting the formation of free radicals that propagate lipid peroxidation (Cheeseman, 1993). Its direct antioxidant ability is thus limited (Marshall *et al*, 1996).

Lipid peroxidation occurring in both the liver and brain, due to the toxins lipopolysaccharide and H_2O_2, is decreased by melatonin (Sewerynek *et al*, 1995a, 1995b, 1995c). Its ability to reduce lipid peroxidation both *in vivo* and *in vitro* is thus well-established (Reiter *et al*, 1997a, 2000b; Reiter, 1996), as is its potency compared to traditional antioxidants such as vitamins C and E (Guttekin *et al*, 2001; Montilla *et al*, 2001). In addition, melatonin decreases MDA levels and thus the extent of lipid peroxidation due to ionising radiation (Kaya *et al*, 1999; Ahlers *et al*, 1997). Another mechanism by which melatonin may inhibit lipid peroxidation is through its inhibition of MAO (Urry and Ellis, 1975). This prevents the generation of H_2O_2 that occurs in normal MAO functioning (see 8.1). The following scheme (Vijayalaxmi *et al*, 2002; Reiter, 1996; Reiter *et al*, 2000c, 2001b) summarises the various mechanisms by which melatonin exerts its free radical-scavenging effects.

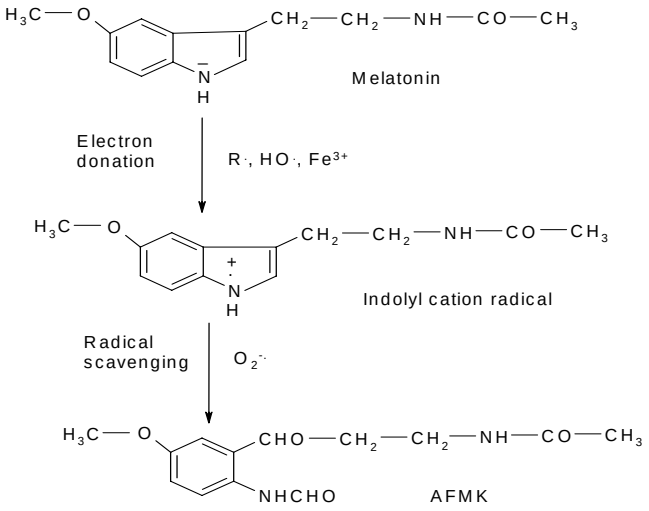


Figure 69: The free radical-scavenging mechanisms of melatonin (Vijayalaxmi *et al*, 2002; Reiter, 1996; Reiter *et al*, 2000c, 2001b)

ROS have a deficiency of electrons and thus react chemically with melatonin, which has a very high electron density (Hardeland *et al*, 1993; Poeggeler *et al*, 1994, 1995, 1996; Mahal *et al*, 1999). Melatonin becomes a melatonyl cation radical, due to the presence of an unpaired electron (Vijayalaxmi *et al*, 2002). This cation radical is less reactive, thereby contributing to a decrease in cellular toxicity (Poeggeler *et al*, 1994; Hardeland *et al*, 1993). The cation radical may either form AFMK or be re-converted to melatonin (Mahal *et al*, 1999); its ability to react with O_2^{-}, another ROS, is significant since this prevents the formation of peroxynitrite anions and HO· and thus the damaging effects of these species (Vijayalaxmi *et al*, 2002).

Melatonin is a potent HO· scavenger, with a rate constant of 2.7 X 10^{10} $M^{-1}s^{-1}$ (Matuszak *et al*, 1997), while the hydroxyindoles 5-HT and 6-OHM may either scavenge or generate this ROS (Matuszak *et al*, 1997). Generation of HO· and O_2^{-}· occurs via the following sequential chemical reactions (Matuszak *et al*, 1997), and may result in iron-induced lipid peroxidation, DNA damage and carcinogenesis (Sakano *et al*, 2004):

(1) $Fe^{3+} + \text{Indole-OH} \rightarrow Fe^{2+} + \text{Indole-O}^{\cdot} + H^{+}$

(2) $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO\cdot + HO^{-}$ (Fenton reaction)

(3) $Fe^{2+} + O_2 \rightarrow Fe^{3+} + O_2^{-}\cdot$

(4) $2O_2^{-}\cdot + 2H^{+} \rightarrow H_2O_2 + O_2$

The H_2O_2 generated in Step (4) may feed into the Fenton reaction, which is an Fe^{2+}-catalysed version of the second step of the Haber-Weiss reaction (see 8.1), and thus result in the formation of further HO· and ultimately $O_2^{-\cdot}$. 6-OHM is able to produce HO· in the absence of H_2O_2, in aerated media containing Fe^{3+} (Matuszak *et al*, 1997). Melatonin interacts with two HO·, thus producing cyclic-3-hydroxymelatonin, which is excreted in the urine and can be used as an indication of the extent of melatonin's HO· scavenging *in vivo* (Tan *et al*, 1998b). Melatonin is known to bind to metal cations (Limson *et al*, 1998; Gulcin *et al*, 2003). Its chelation to Fe^{3+} prevents this ion from producing Fe^{2+}, and the subsequent generation of the above free radicals is inhibited (Limson *et al*, 1998).

The probability of melatonin protecting cells *in vivo* from attack by HO· is low, due to very low physiological concentrations of melatonin in the nanomolar range. It is thus more probable that melatonin exerts its protective effect by scavenging other ROS, including those formed by HO· (Matuszak *et al*, 1997).

It must, however, be noted that melatonin, whilst protecting against oxidative stress, has been reported to also induce this at concentrations in the millimolar range (Clapp-Lilly *et al*, 2001). Ianas *et al* (1991) have shown that at concentrations less than 250 μM, melatonin has pro-oxidant effects while it is an antioxidant at concentrations higher than this. There thus seems

to be confusion as to the possible pro-oxidant effect of melatonin, and at which concentration range this could occur. In a study of melatonin and other indoleamines *in vitro* it was found that melatonin exhibits only antioxidant effects and lacks a pro-oxidant effect on substrates that are non-lipid in nature (Chan and Tang, 1996).

8.4 Effects of 5-FU and melatonin on heat shock protein 70

One of the cellular sequelae of stress, be it heat or otherwise, is protein denaturation, in particular disrupted folding, which the cell counters by mounting the heat shock response. This involves cessation of protein synthesis and a significant increase in the production of heat shock proteins (Hsps) (Smith *et al*, 1998; Schlesinger *et al*, 1982; Lindquist and Craig, 1988). Hsp, in particular Hsp72, levels are thus a useful marker of the extent of (oxidative) stress in a cell (Gonzalez *et al*, 1989; Lindquist, 1986).

There are many families of Hsps, such as Hsp40, Hsp60, Hsp70, Hsp90 and Hsp100, the number indicating the approximate kiloDalton molecular size of the members within each family. Smaller Hsp families, 20-25 kDa in size, also exist (Smith *et al*, 1998). Hsp70, a molecular chaperone, plays a significant role in protein refolding. This occurs by Hsp70 binding to a particular protein, enabling the appropriate conformational folding and oligometric assembly to occur, and transporting the protein to its target location within the cell (Hendrick and Hartl, 1993). Hsp70 also exerts a protective effect over cells following injury, evidenced by the finding that ischaemic heart tissue recovers upon heat stress (Currie *et al*, 1988), and that this is correlated with an elevation in Hsp70 levels (Yellon and Latchman, 1992; Marber *et al*, 1995). This protective effect is via Hsp70 interaction with apoptotic protease-activating factor, resulting in inhibition of procaspase 9. This prevents apoptosis from occurring (Beere *et al*, 2000).

As mentioned in 1.2.7 and 1.2.8.1, it has been reported that Hsp70 levels in colon cancer cells are significantly elevated in response to 5-FU treatment (Grivicich *et al*, 2007; Gallos *et al*, 2001; Hisatomi *et al*, 2006), and are associated with an exponential increase in resistance to 5-FU (Gallos *et al*, 2001; Grivicich *et al*, 2007). This is due to the Hsp70-mediated anti-apoptotic effect, which exerts a protective influence over cancer cells and allows recovery from 5-FU-induced injury. This may account for the low response rate of less than 20% demonstrated by patients with colon cancer receiving 5-FU therapy (see 1.2.8.1). 5-FU-induced increases in Hsp70 levels are not due to the effect of the agent on the genes encoding for the latter, as the antineoplastic is not able to alter levels of the Hsp70 gene promoter; this suggests that Hsp70 induction is due to proteotoxicity (Ait-Aissa *et al*, 1999) and cellular stress.

Maharaj (2003) shows that melatonin decreases QA-induced Hsp70 production, demonstrating the protective effects of melatonin on QA-induced cellular oxidative damage. The administration of 6-OHM, however, both alone and with QA, results in a significant increase in Hsp70 levels (Maharaj, 2003). This indicates that 6-OHM results in cellular stress, possibly due to the generation of HO· (see 8.3).

8.5 Experimentation

The lipid peroxidation assay was based on those described by Placer *et al* (1966), Sagar *et al* (1992), Esterbauer and Cheeseman (1990), Draper and Hadley (1990) and Ottino and Duncan (1997). The TBA assay is used to measure the amount of lipid peroxidation that occurs in cells (Placer *et al*, 1966). It involves the chemical reaction that takes place between MDA equivalents and TBA (Ottino and Duncan, 1997; Sagar *et al*, 1992; Draper and Hadley, 1990; Placer *et al*, 1966), to form a pink-coloured complex (Kohn and Liversedge, 1944; Bernheim *et al*, 1947; Wilbur *et al*, 1949) that has an absorption maximum at 532-535 nm (Esterbauer and Slater, 1981). MDA is a product of lipid peroxidation (Patton and Kurtz, 1951; Dahle *et al*, 1962) and is often the most abundant aldehyde formed from this event (Esterbauer and Cheeseman, 1990). Its measurement spectrophotometrically thus gives an indication of the extent of lipid peroxidation, and thus of the oxidation of polyunsaurated fatty acids, occurring in a system (Placer *et al*, 1966). The TBA assay is advantageous in determining the extent of lipid peroxidation because MDA is water-soluble and the pink colour is so intense that it allows for very sensitive MDA detection (Dahle *et al*, 1962). The pink complex is formed by the reaction of one mole of MDA with two moles of TBA (Dahle *et al*, 1962; Sinnhuber *et al*, 1958). Unless the profile and amount of unsaturated fatty acids present, as well as the stoichiometry of the formation of MDA from these fatty acids, is known, which is unlikely in biological systems, the TBA assay can only provide an indirect measure of the presence of the products of oxidation in a system; this is in comparison to determining the peroxide value of a system, for example, which is a more absolute test (Dahle *et al*, 1962). The concentration of MDA formed increases with an increase in the degree of unsaturation in the fatty acids present (Dahle *et al*, 1962; Wilbur *et al*, 1949), and the intensity of colour is directly proportional to the extent of diene conjugation (Dahle *et al*, 1962). The scheme on the next page (Dahle *et al*, 1962) proposes a mechanism for the formation of MDA from the cyclic peroxides generated by lipid peroxidation.

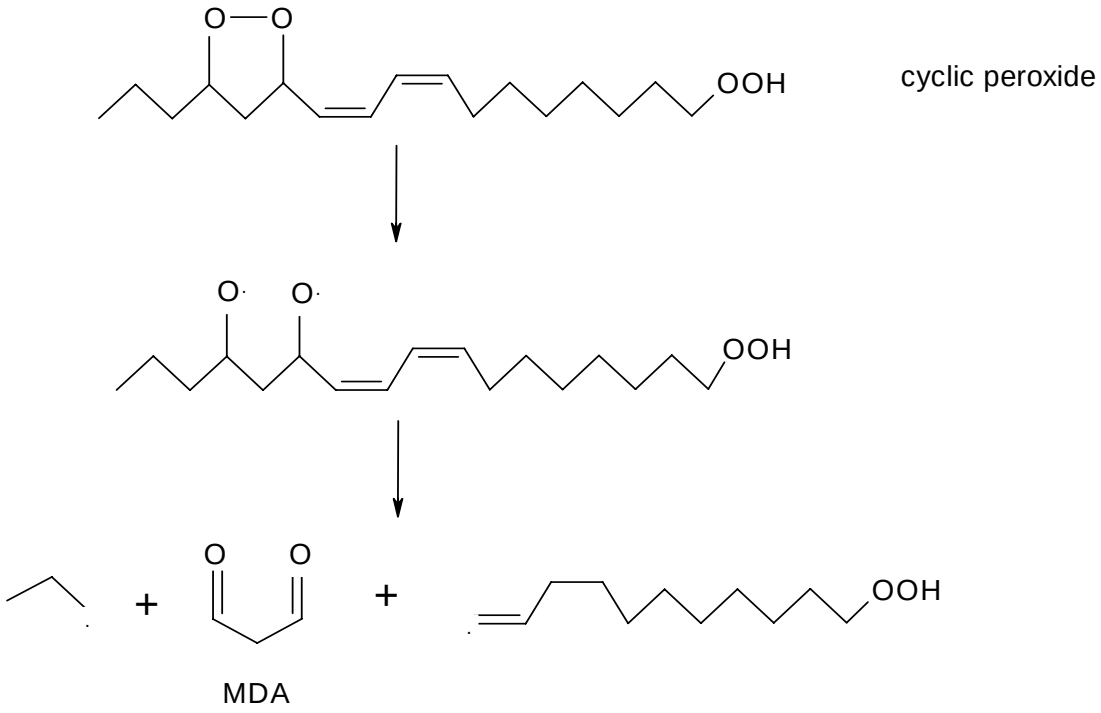


Figure 70: A proposed mechanism for the formation of MDA (Dahle *et al*, 1962)

Another limitation of the TBA assay, besides it being only an empirical, indirect measure of oxidation products, is the possibility of side-reactions occurring (Tarlagdis *et al*, 1962). Esterbauer and Cheeseman (1990) and Draper and Hadley (1990) summarise the arguments against and in favour of using the TBA assay as a marker of lipid peroxidation, and conclude that the assay is an accurate indicator of free MDA levels. MDA forms complexes with various compounds, such as proteins, *in vivo*, evidenced by the determination of these products in urine. This may interfere with the determination of free MDA in tissues (Draper and Hadley, 1990). It can be concluded that the reaction conditions have a very significant role to play in the sensitivity of the TBA assay performed (Esterbauer and Cheeseman, 1990; Draper and Hadley, 1990).

In the present study, the extent of lipid peroxidation was determined in rat livers. It is useful to perform this assay in livers for three main reasons:
(i) the cell membranes of liver and brain cells are similar, differing in the phospholipid content (Kane and Kumar, 2005). It can thus reasonably be expected that an agent that induces, or prevents, lipid peroxidation in the liver is likely to exert a similar effect in the brain;
(ii) one of the consequences of hepatoxicity might be a "leakage" of TDO out of the liver into the bloodstream. This may be as a direct result of lipid peroxidation, as the cell membrane is no longer able to function effectively as a barrier retaining cellular contents. This "leakage" is

known to occur, for example, with the serum aminotransferses (Sturgill and Lambert, 1997; Kaplan, 1993); and
(iii) the liver is particularly vulnerable to xenobiotic-induced toxicity (Thurman *et al*, 1986; Sturgill and Lambert, 1997) due to its role as the primary site of metabolism in the body.

The ability of 5-FU to bind to Fe^{2+} was also investigated, using the method described by Decker and Welch (1990). This is important, as binding to this ion prevents the Fenton reaction from occurring (see 8.3) and thus blocks the formation of HO·, thereby inhibiting lipid peroxidation. As discussed in 8.3, melatonin chelates Fe^{2+} and is thus able to prevent HO·-induced lipid peroxidation from occurring.

8.5.1. *In vitro*

8.5.1.1 Materials, animals, reagents and equipment

Male Wistar rats, each weighing between 200g and 300g, and cared for as described in Appendix A, were used. Melatonin, 5-FU and QA were obtained from the Sigma Chemical Corporation, St Louis, MO, USA. All the other chemicals used were obtained from local suppliers and were of the highest purity available. All solutions were prepared using deionised water (Milli R/Q System, Millipore®), except for butylated hydroxytoluene (BHT), which was made up in methanol. Absolute ethanol, at a final concentration of 0.67% v/ v, was used as a co-solvent to dissolve melatonin. TBA is photolabile and was thus prepared immediately prior to use. A Julabo PC water bath (Labotec, South Africa) was used. The UV spectrophotometer used was a Spectra System UV 2000®.

8.5.1.2 Method

Rats were killed by cervical dislocation and the livers were removed immediately, perfused with 0.9% NaCl and frozen at -70ºC using liquid nitrogen until used in the assay. Each liver was weighed and homogenised manually using a suitable volume of NaH_2PO_4 buffer, pH 7.0, until a 10% w/ v homogenate was achieved. An aliquot of 0.9 ml homogenate was added to each test-tube. An aliquot of 0.1 ml of the relevant drug was subsequently added, according to the table on the next page; the controls received 0.1 ml water. All concentrations were carried out in triplicate; thus, a total of fifty-one test-tubes were used. QA, at a concentration of 100 µM was added to one set of test-tubes, as a positive control, since it is an accepted lipid peroxidation-inducing toxin. Any lipid peroxidation occurring as a result of 5-FU can thus be compared to the standard QA.

Table 21: Template used in lipid peroxidation assay <u>in vitro</u>

Test-tube	**Sample A**	**Sample B**	**Sample C**
Control			
QA (100 μM)			
5-FU (10 μM)			
5-FU (50 μM)			
5-FU (100 μM)			
Melatonin (10 μM)			
Melatonin (50 μM)			
Melatonin (100 μM)			
5-FU (10 μM) + Mel (10 μM)			
5-FU (10 μM) + Mel (50 μM)			
5-FU (10 μM) + Mel (100 μM)			
5-FU (50 μM) + Mel (10 μM)			
5-FU (50 μM) + Mel (50 μM)			
5-FU (50 μM) + Mel (100 μM)			
5-FU (100 μM) + Mel (10 μM)			
5-FU (100 μM) + Mel (50 μM)			
5-FU (100 μM) + Mel (100 μM)			

The test-tubes were vortexed and incubated in an oscillatory water bath at 37ºC for 60 mins. Aliquots of 0.5 ml BHT, an antioxidant, were added to each test-tube, to ensure that minimal lipid peroxidation occurs during the experiment (Esterbauer and Cheeseman, 1990; Draper and Hadley, 1990). Aliquots of 1 ml TCA were then added. The tubes were vortexed and incubated in a water bath at 95ºC for 15 mins. The test-tubes were subsequently centrifuged at 2000g for 20 mins, in order to remove any insoluble proteins present (Ottino and Duncan, 1997) and also to make the assay more specific for MDA (Esterbauer and Cheeseman, 1990). Aliquots of 2 ml of the resulting supernatant were placed in new test-tubes, and 0.5 ml TBA added. Incubation in a water bath at 95ºC for 60 mins followed. Aliquots of 2 ml of butanol were added, after the test-tubes had cooled down. The test-tubes were vortexed and subsequently centrifuged at 2000g for 5 mins. Butanol is used because it allows for spectrophotometric detection by removing turbidity due to the precipitation of proteins and produces clear pink solutions (Placer *et al*, 1966). Theoretically, endogenous compounds, such as desoxysugars, which produce MDA after undergoing oxidation, may result in a pink

colour and thereby possibly interfere with the validity of the assay. Placer *et al* (1966) have, however, concluded that there is not much likelihood of this occurring, and the use of blanks would correct for it. Although the TBA assay is not specific for MDA alone (Esterbauer and Cheeseman, 1990), since compounds similar to MDA, such as amino acids, can result from thermal degradation or acid catalysis that occurs during the assay (Esterbauer and Cheeseman, 1990; Marcuse and Johansson, 1973; Terao and Matsushita, 1981; Witz *et al*, 1986; Gutteridge, 1981), using this protocol is believed to minimise the extent of this (Esterbauer and Cheeseman, 1990; Esterbauer and Slater, 1981; Esterbauer *et al*, 1987; Lang *et al*, 1984). The supernatant was removed and the absorbance of MDA was read spectrophotometrically at 532 nm. Butanol was used as a blank. The concentration of MDA was determined using the Beer-Lambert law and a suitable calibration curve (see Appendix C). The results were expressed as the nanomoles of MDA formed per milligram of tissue. The concentration of MDA can alternatively be determined using HPLC (Esterbauer and Cheeseman, 1990; Bull and Marnett, 1985; Csallany *et al*, 1984), and confirms that the TBA assay is an accurate indication of free MDA concentration (Esterbauer and Cheeseman, 1990). Gas chromatgraphic methods have also been reported (Draper and Hadley, 1990).

8.5.2 *In vivo*

8.5.2.1 Materials, animals, reagents and equipment

As described in 8.4.1.2. Sixteen male Wistar rats were used.

8.5.2.2 Method

Four groups of rats were treated according to the five-day treatment protocol described in Table 10 (see 3.6.2.2). The assay was performed as described in 8.4.1.2, and the results expressed as nanomoles MDA per milligram tissue.

8.5.3 Fe^{2+}-chelating activity of 5-FU

The ability of 5-FU to chelate Fe^{2+} was determined using the method of Decker and Welch (1990). EDTA, a known chelator of this ion (Que *et al*, 2006), was used as a positive control.

8.5.3.1 Materials, reagents and equipment

As described in 8.4.1.1. EDTA and iron (II) sulphate ($FeSO_4$) were purchased from Saarchem® (Wadeville, South Africa) and 3-(2-pyridyl)-5, 6-diphenyl-1, 2, 3-triazine-4', 4''-disulphonic acid sodium (ferrozine) was obtained from the Sigma Chemical Corporation (St Louis, MO, USA).

8.5.3.2 Method

An aliquot of 1 ml, containing 5-FU in increasing concentrations of 6.25 µg/ml, 12.5 µg/ ml, 25 µg/ ml, 50 µg/ ml and 100 µg/ ml was added to 3.7 ml of deionised water (n = 5 for each concentration of 5-FU). Aliquots of 0.1 ml of 2 mM $FeSO_4$ and 0.2 ml of 5 mM ferrozine were added and the mixture was left to react for a period of 20 mins. The controls received 0.1 ml water instead of 5-FU. Upon the addition of ferrozine, the mixture turns pink in colour, due to the formation of a ferrozine-Fe^{2+} complex. The concentration of this complex present is directly proportional to the intensity of colour; a lighter colour indicates that the drug used binds Fe^{2+}, thus leaving less of the ion available to interact with ferrozine. The absorbance was subsequently read spectrophotometrically at 562 nm, with water as the blank. EDTA, the positive control, was used separately at the same concentrations as 5-FU. The chelating activity of 5-FU and EDTA was calculated using the following formula: chelating activity = $(A_1-A_0)/A_1$, where A_1 was the absorbance of the control and A_0 was the absorbance of the mixture in the presence of either 5-FU or EDTA. The results were expressed as the percentage chelating activity of each agent.

8.6 Results

The results were analysed for statistically significant differences using ANOVA, followed by the Student-Newman-Keuls multiple-range test

8.6.1 *In vitro*

As shown in Figure 71 on the next page, QA at a concentration of 100 µM induces lipid peroxidation *in vitro*, evidenced in the increase of MDA levels from a control value of 0.36 nmol/ mg to 0.85 nmol/ mg. 5-Fluorouracil increases MDA at all the concentrations used, with 10 µM 5-FU increasing MDA levels to 0.71 nmol/ mg and 100 µM 5-FU increasing the level to 1.03 nmol/ mg. When compared to MDA levels after the addition of 10 µM 5-FU, the addition of 10 µM melatonin causes a decrease to 0.43 nmol/ mg, while the addition of 100 µM melatonin reduces MDA levels to 0.23 nmol/ mg, a value lower than the control. Lipid peroxidation induced by higher concentrations of 5-FU is not altered by melatonin.

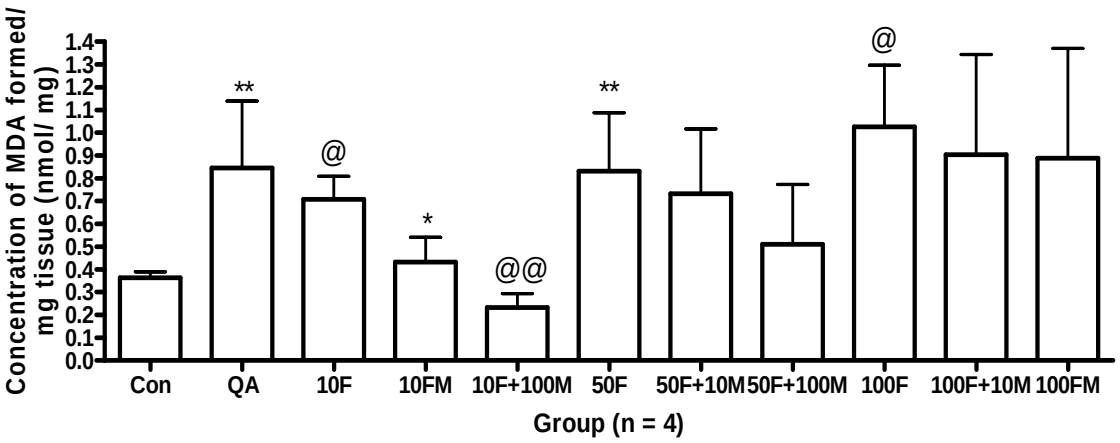


Figure 71: The effects of varying concentrations of 5-FU and melatonin on hepatic lipid peroxidation <u>in vitro</u>

** $p<0.1$, $^{@}$ $p<0.05$ compared to control values; * $p<0.1$, $^{@@}$ $p<0.01$ compared to 5-FU (10 μM), after analysis by ANOVA followed by the Student-Newman-Keuls multiple-range test. All concentrations are in μM. Con = Control; F = 5-FU; M = Melatonin; FM = 5-FU + Melatonin. Each bar represents the mean ± SEM

8.6.2 *In vivo*

Figure 72 shows the *in vivo* lipid peroxidation results.

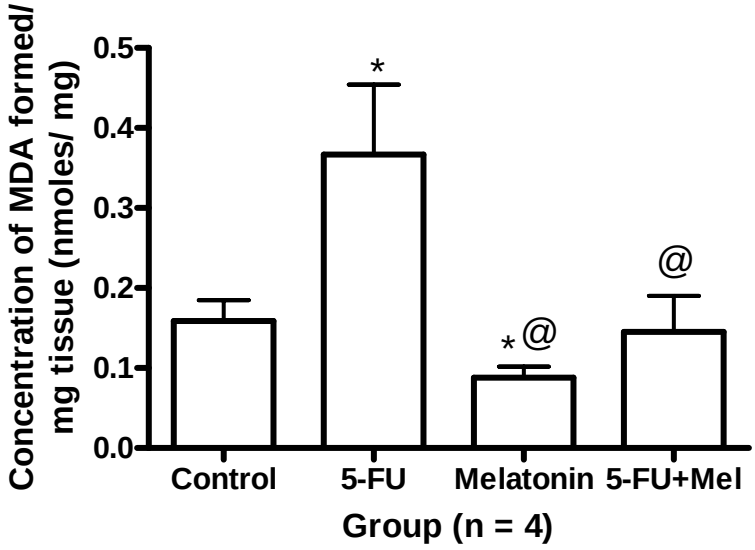


Figure 72: The effects of 5-FU and melatonin on hepatic lipid peroxidation <u>in vivo</u>

* $p<0.05$ compared to control value; $^{@}$ $p<0.05$ compared to 5-FU, after analysis by ANOVA followed by the Student-Newman-Keuls multiple-range test. Each bar represents the mean ± SEM

As shown in Figure 72, the administration of 5-FU increases MDA levels significantly, to 0.37 nmol/ mg. The administration of melatonin results in a reduction of MDA levels to not less than 0.09 nmol/ mg. The co-administration of melatonin to rats also receiving 5-FU results in MDA levels being reduced to 0.15 nmol/ mg, which is significantly less than the MDA levels obtained from rats which only received 5-FU.

8.6.3 Fe^{2+}-chelating activity of 5-FU

Figure 73 below shows that 5-FU demonstrates negligible chelating activity towards Fe^{2+}. The percentage binding to this cation does not exceed 7.16%. This is in contrast to the profile obtained for EDTA, which shows an increase in Fe^{2+}-binding, to a maximum of 93.86%; this increase is initially dose-dependent and then becomes more gradual at concentrations greater than 40 µg/ ml.

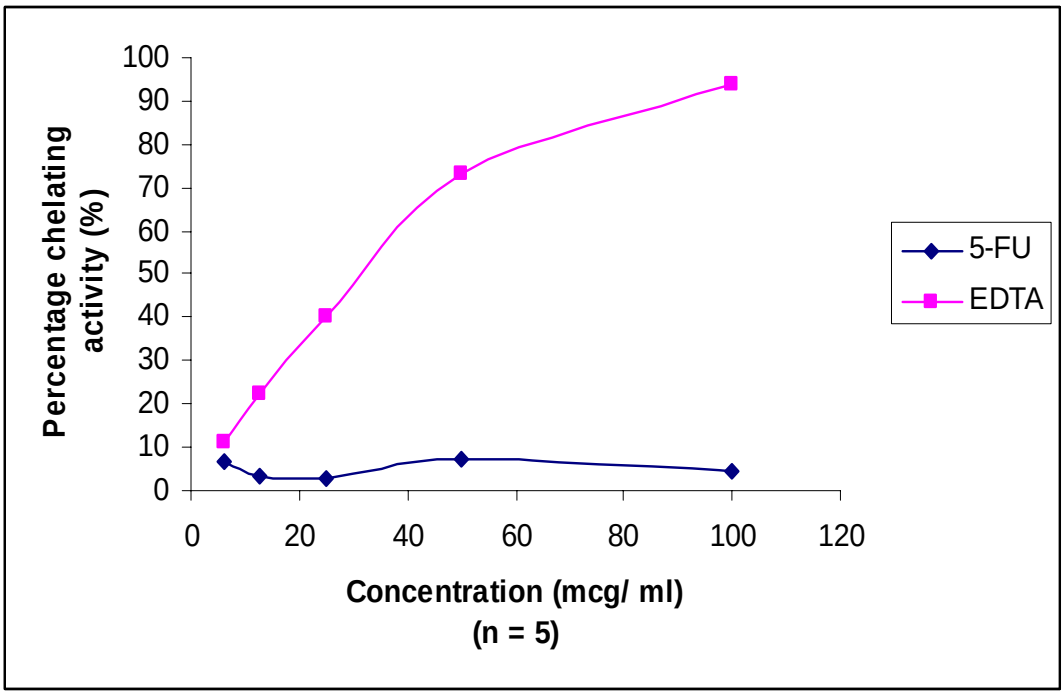


Figure 73: The Fe^{2+}-chelating activity of 5-FU and EDTA

8.7 Discussion

Although it has been shown that fluorides induce lipid peroxidation in the brain (see 10.3), there are no reported cases in the literature, to our knowledge, on the effects of 5-FU on lipid peroxidation. QA was used in the *in vitro* study since it is well-known to induce lipid peroxidation. A comparison with the lipid peroxidation caused by QA would thus be a useful means of ascertaining whether 5-FU itself causes such cellular damage. As a potent

antineoplastic agent, it might be expected that 5-FU could result in lipid peroxidation, especially in the light of the agent elevating Hsp70 levels, a marker of cellular stress (see 8.4).

From Figure 71, it is shown that even a low concentration of 10 μM 5-FU induces a significant increase in MDA levels. A concentration of 100 μM 5-FU increases MDA levels to greater than that induced by an equimolar concentration of QA. It can thus be concluded that 5-FU does indeed result in lipid peroxidation, evidenced by the increased levels of MDA. The addition of melatonin *in vitro* significantly reduces the extent of lipid peroxidation induced by a low concentration of 5-FU. As the concentration of the latter increases, 100 μM of melatonin is no longer sufficient to counteract lipid peroxidation. The *in vivo* study confirms both that melatonin significantly inhibits lipid peroxidation when administered alone, and that the co-administration of melatonin decreases the extent of 5-FU-induced lipid peroxidation at the dosages used, as shown in Figure 72.

5-Fluorouracil-induced lipid peroxidation could occur as a result of 5-FU enhancing the generation of various ROS, such as $O_2^{-\cdot}$ and HO·. The effects of 5-FU and melatonin on $O_2^{-\cdot}$ levels are investigated in Chapter 9. Possible chemical mechanisms by which 5-FU could generate these ROS are postulated by the author in the next pages. The suggested chemical formation of MDA is shown in Figure 70 (see 8.5.1.2), and melatonin's interactions with free radicals is shown in Figure 69 (see 8.3). The 5-FU metabolites FA and FBAL (see 1.2.6.1) are known to decrease cellular resistance to oxidative stress, thus allowing the influx of toxins into cells (see 10.4). 5-FU-induced lipid peroxidation could thus facilitate further cellular toxicity mediated by other toxins.

Figure 74 shows that 5-FU abstracts a H· from a typical lipid peroxide, resulting in a lipid radical. This is stabilised by π electrons in Step (2) and cleavage in Step (3). Any nucleophile in the environment, such as 5-FU itself, could then attack the partially-positive carbonyl carbon atom to form a carbocation in Step (4). The fluoride group in 5-FU is a good leaving group; the resulting fluoride ion is very stable and a strong nucleophile (Park *et al*, 2001). The carbocation from Step (4) is stabilised by the release of $O_2^{-\cdot}$ in Step (5).

As postulated in Figure 75 on the next page, an oxygen in a typical lipid peroxide may abstract a H· from 5-FU, thus leading to the formation of an unstable oxygen ion in Step (1). This is stabilised by cleavage of the dioxygen bond in Step (2). Attack on the carbonyl carbon by any nucleophile in the environment may result in the formation of the carbocation shown in Step (3). This is subsequently stabilised by loss of HO·.

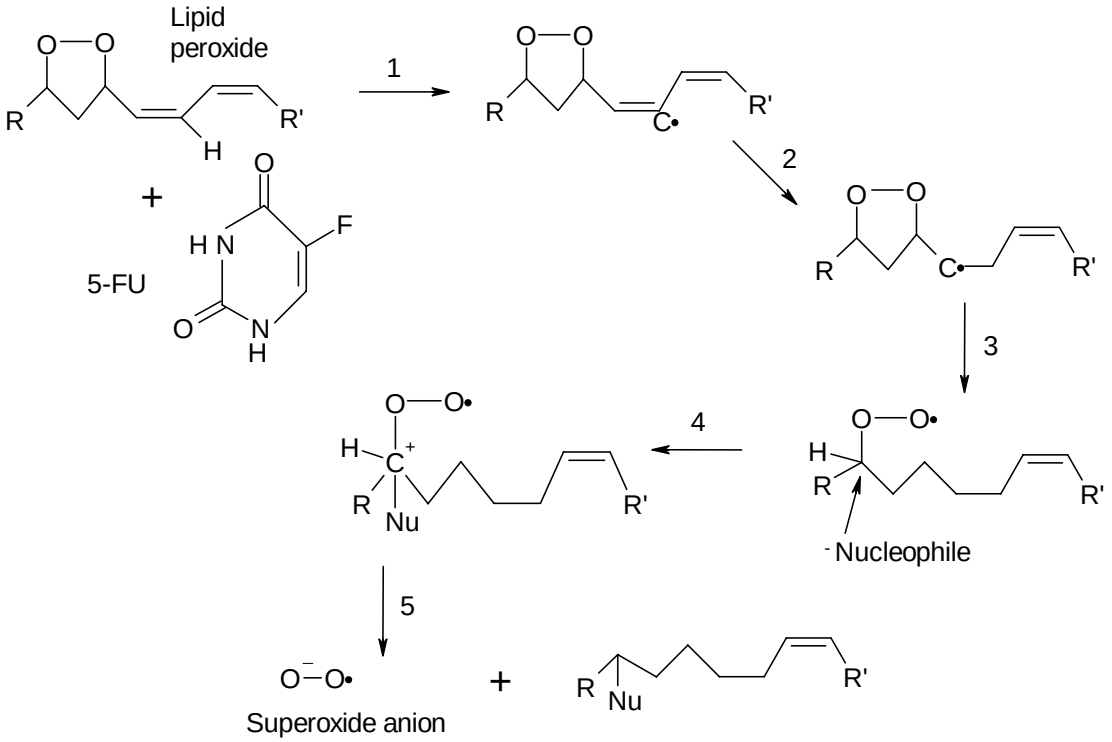


Figure 74: Proposed reaction mechanism for the 5-FU-induced generation of $O_2^{-\cdot}$

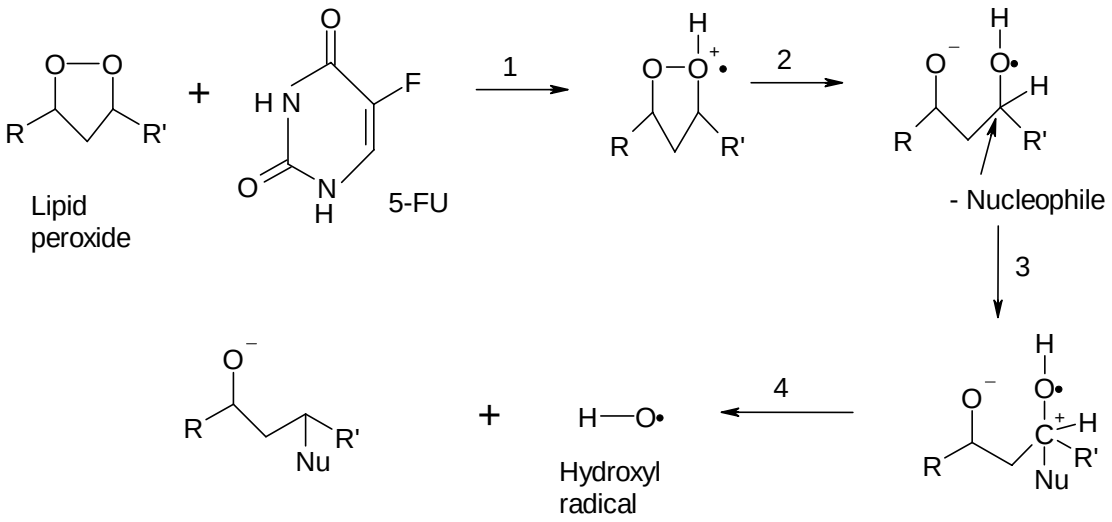


Figure 75: Proposed reaction mechanism for the 5-FU-induced generation of HO·

Alternatively, or perhaps additionally, $O_2^{-\cdot}$ generated by 5-FU can undergo the second step of the Haber-Weiss reaction (see 8.1) to generate HO·. In addition, HO· may itself undergo the first step of the Haber-Weiss reaction (see 8.1), ultimately giving rise to $O_2^{-\cdot}$, giving rise to a cyclical increase in the levels of both ROS.

The ferrozine assay, which demonstrates that 5-FU exhibits negligible binding towards Fe^{2+}, suggests indirectly that 5-FU does indeed generate HO·. A failure to bind to Fe^{2+} implies that

a drug will not prevent Fenton reaction-generated HO· from being formed, in the presence of endogenous iron, thus resulting in lipid peroxidation.

The ability of melatonin to inhibit lipid peroxidation, both alone and in the presence of 5-FU, is due to a variety of effects (see 8.3):
(i) the antioxidant and free radical-scavenging properties of the indoleamine (Reiter, 2000) (see 1.3.8);
(ii) the stimulation of antioxidative enzymes, such as γ-glutamylcysteine synthase (Urata *et al*, 1999);
(iii) inhibition of NO synthase (Baydas *et al*, 2001; Rodriguez *et al*, 2004), thus preventing the formation of NO, which induces oxidative stress;
(iv) the formation of AFMK, which has significant antioxidative ability (Tan *et al*, 2000a, 2001); and
(v) the formation of 6-OHM which, although it can generate HO· (see 8.3), is also known to scavenge this species (Pierrefiche *et al*, 1993).

The findings in this chapter could have two important, seemingly opposing, implications:
(i) lipid peroxidation may be an additional mechanism by which 5-FU exerts its cytotoxic effects, by increasing the oxidative stress that cancer cells are exposed to and disrupting their integrity. 5-Fluorouracil has another mechanisms of action, besides disruption of DNA synthesis, in that it can alter transmembrane potential and charges (Walliser and Redman, 1978) as well as membrane structure, thereby increasing the likelihood of cell lysis occurring (Kessel, 1980) (see 1.2.6.3). Lipid peroxidation may be the mechanism by which 5-FU causes such lysis. As mentioned in 10.3, fluorides decrease the activity of various antioxidative enzymes, such as SOD and catalase (Chinoy and Shah, 2004; Wang *et al*, 2004). 5-FU might thus also exert such an effect, thereby inhibiting cellular mechanisms of countering oxidative stress, in addition to inducing such stress. The co-administration of melatonin would have a protective effect on cancer cells and thus reduce the efficacy of 5-FU. This may explain the decreased efficacy of 5-FU upon the administration of the antioxidant vitamin E (see 1.3.8); and (ii) melatonin protects non-cancer cells against 5-FU-induced lipid peroxidation; this would decrease the toxicity of the antineoplastic and be a further mechanism by which melatonin improves the survival time and quality of life of cancer patients receiving 5-FU treatment (Lissoni *et al*, 1999a).

8.8 Conclusions

It has been shown that 5-FU induces lipid peroxidation in rat liver homogenate, both *in vitro* and *in vivo*, as determined by an increase in the concentration of MDA. The co-administration of melatonin significantly reduces the extent of 5-FU-induced lipid peroxidation. This may well support the recommendation by Lissoni *et al* (1999a) that melatonin be used as adjunctive therapy in cancer patients to decrease the toxicity of 5-FU. The use of combination therapy would, however, have to balance the concerns of antineoplastic toxicity and efficacy, since the latter may be compromised due to melatonin also protecting cancer cells against lipid peroxidation.

CHAPTER 9

THE EFFECTS OF 5-FLUOROURACIL AND MELATONIN ON THE GENERATION OF SUPEROXIDE ANIONS IN RAT LIVER *IN VITRO* AND *IN VIVO*

9.1 Introduction

As shown in the previous Chapter, 5-FU induces lipid peroxidation in the liver and the co-administration of melatonin protects cells from this oxidative stress. We have decided to investigate whether the ROS responsible for 5-FU-induced lipid peroxidation is $O_2^{-}\cdot$. As mentioned in 8.2, these anions contribute to QA-induced lipid peroxidation. It is also important to determine the effects of 5-FU and melatonin on $O_2^{-}\cdot$ levels, as it was shown in Chapter 5 that both agents stimulate IDO activity, and it was suggested that this could be by increasing the levels of $O_2^{-}\cdot$, essential cofactors for IDO activity (Higuchi and Hayaishi, 1967) (see 5.5).

9.2 Experimentation

The assay used was based on that described by Sagar *et al* (1992) and Das *et al* (1990). It involves the reduction of nitro-blue tetrazolium (NBT) into the insoluble purple-coloured diformazan in the presence of $O_2^{-}\cdot$. This assay was conducted *in vitro* and *in vivo*.

9.2.1 *In vitro*

9.2.1.1 Materials, animals, reagents and equipment

Melatonin, 5-FU, NBT and nitro-blue diformazan (NBD) were obtained from the Sigma Chemical Corporation, St Louis, MO, USA. All the other chemicals used were obtained from local suppliers and were of the highest purity available. Male Wistar rats, each weighing between 200g and 300g, and cared for as described in Appendix A, were used. All solutions were prepared using deionised water (Milli R/Q System, Millipore®), except for NBD, which was dissolved in glacial acetic acid. Melatonin and NBT were dissolved using absolute ethanol as a co-solvent, in a final concentration of 0.67% v/ v. A Julabo PC water bath (Labotec, South Africa) was used. The UV spectrophotometer used was a Spectra System UV 2000®.

9.2.1.2 Method

Twenty rats were sacrificed by cervical dislocation. The livers were removed, perfused with ice-cold 0.9% saline and stored at -70ºC until assayed. Each liver was homogenised with 0.2

M NaH_2PO_4 buffer, pH 7.4, to give a final concentration of 10% w/ v. An aliquot of 100 µL of 100 µM 5-FU or melatonin, or both, was added to 1 ml of homogenate from each liver. This was performed in triplicate. The controls received 100 µL of water. It was decided to use a concentration of 100 µM of both 5-FU and melatonin, as this concentration of 5-FU has been previously shown to induce lipid peroxidation *in vitro* (Figure 70) (see 8.5.1). Melatonin, furthermore, is known to be a modest scavenger of $O_2^{\cdot-}$ (Chan and Tang, 1996; Marshall *et al*, 1996) and thus a concentration lower than 100 µM might not be effective in decreasing levels of this species. An aliquot of 0.5 ml of 0.1% NBT was added to each test-tube. The test-tubes were vortexed. The samples were incubated in an oscillating water bath at 37ºC for 60 mins, and subsequently centrifuged at 2000g for 10 mins. The supernatant was discarded and 2 ml glacial acetic acid added to the precipitate. The mixture was vortexed and centrifuged at 2000g for a further 5 mins. The absorbance of the supernatant was read in a UV spectrophotometer at 560 nm, with the blank being glacial acetic acid. The absorbance was converted to concentration of diformazan using an NBD calibration curve (see Appendix D). A protein assay was performed as described by Lowry *et al* (1951) (see Appendix B). The results were expressed as µmol diformazan produced per µg protein.

9.2.2 *In vivo*

9.2.2.1 Materials, animals, reagents and equipment

As described in 9.2.1.1.

9.2.2.2 Method

Twenty rats were subjected to the five-day treatment protocol described in Table 10 (see 3.6.2.2), and cared for as outlined in Appendix A. The rats were sacrificed on the sixth day, the livers removed and the NBT assay performed as described in 9.2.1.2, except that no 5-FU or melatonin was added to the homogenate.

9.3 Results

The following results were obtained for the effects of 5-FU and melatonin on the generation of $O_2^{\cdot-}$. Statistical analysis was performed using ANOVA, followed by the Student-Newman-Keuls multiple-range-test.

9.3.1 *In vitro*

The *in vitro* results are illustrated in Figure 76 on the next page.

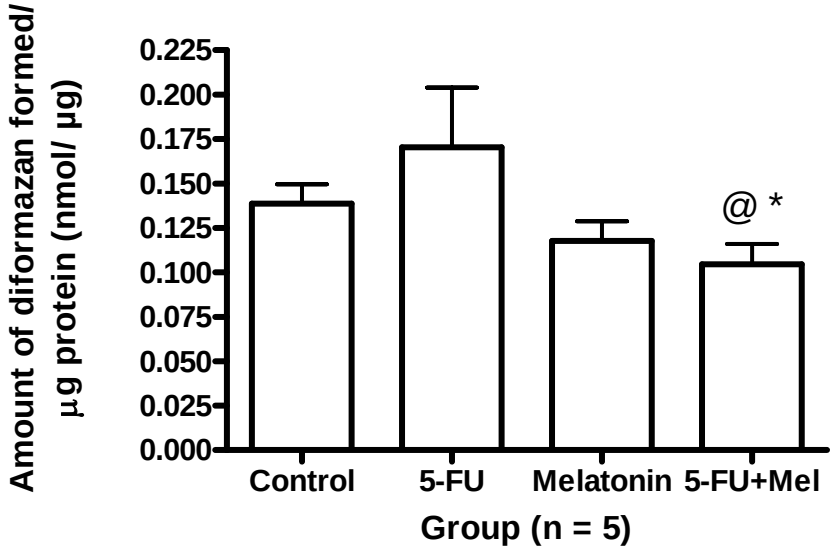


Figure 76: The effects of 5-FU and melatonin on the generation of $O_2^{-}\cdot$ in rat liver <u>in vitro</u>, reflected in the amount of diformazan formed

* $p<0.05$ compared to control value; $^{@}$ $p<0.1$ compared to 5-FU value, after analysis by ANOVA followed by the Student-Newman-Keuls multiple-range test. Each bar represents the mean ± SEM

As shown in Figure 76, although 5-FU does increase diformazan levels, and melatonin does decrease these, these alterations are not statistically significant compared to control values. The addition of melatonin to liver homogenate also incubated with 5-FU, however, results in a significant blunting of diformazan levels, compared to both control values and samples incubated with 5-FU only.

9.3.2 *In vivo*

These results are provided in Figure 77 on the next page. It is evident that 5-FU significantly enhances the generation of O_2^{-}, since the concentration of diformazan produced increases to 0.023 nmol/ µg, from a control value of 0.015 nmol/ µg. Melatonin also significantly increases diformazan levels, to a similar value. The co-administration of melatonin does not alter the 5-FU-induced generation of $O_2^{-}\cdot$ to any significant extent.

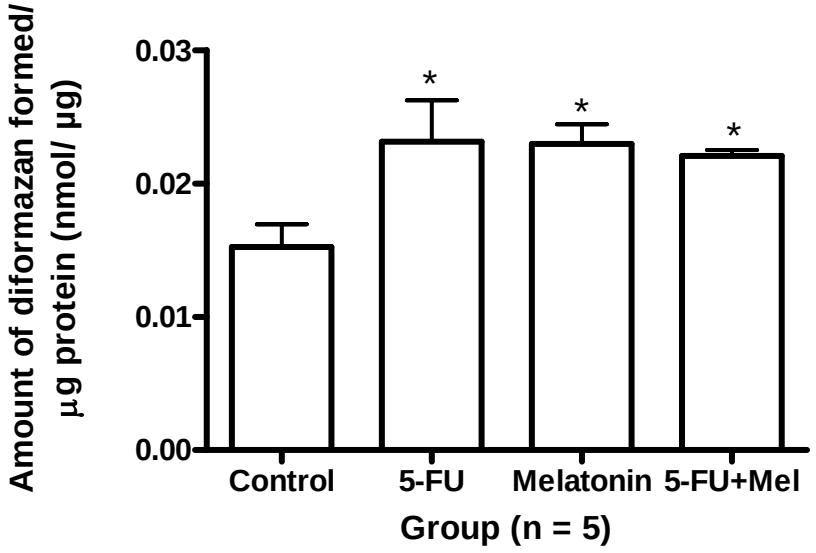


Figure 77: The effects of 5-FU and melatonin on the generation of $O_2^{-}\cdot$ in rat liver <u>in vivo</u>, reflected in the amount of diformazan formed

* $p<0.05$ compared to control value, after analysis by ANOVA followed by the Student-Newman-Keuls multiple-range test. Each bar represents the mean ± SEM

9.4 Discussion

The *in vitro* results show that a concentration of 100 µM of either 5-FU or melatonin is not sufficient to significantly alter $O_2^{-}\cdot$ levels, as reflected in diformazan levels. Since it has been shown in Chapter 8 that a concentration as low as 10 µM 5-FU is sufficient to induce lipid peroxidation in the liver *in vitro*, it is implied that this 5-FU-induced lipid peroxidation *in vitro* is mainly due to ROS other than $O_2^{-}\cdot$, such as possibly $HO\cdot$. Melatonin's antioxidant property (see 8.3) is evidenced by its ability to significantly decrease diformazan levels in homogenate that is concurrently incubated with 5-FU. In this group, the slight increase in diformazan levels induced by 5-FU, and the modest decrease induced by melatonin alone is sufficient to result in a statistically significant difference. It is thus evident that *in vitro*, at a concentration of 100 µM, melatonin has an antioxidant effect and can counteract the modest $O_2^{-}\cdot$-induced oxidative damage induced by 5-FU. It is believed that melatonin may exhibit pro-oxidant effects at higher concentrations (see 8.3).

In vivo, 5-FU significantly enhances the generation of $O_2^{-}\cdot$. It is thus likely that this ROS is involved in 5-FU-induced lipid peroxidation. In Chapter 8, it was suggested that the results of the Fe^{2+}-chelation study of 5-FU indirectly show that 5-FU generates $HO\cdot$, by failing to prevent the Fenton reaction from occurring. As mentioned in 8.7, $HO\cdot$ may result in the formation of $O_2^{-}\cdot$, and *vice versa*, through the Haber-Weiss reaction (see 8.1), thus resulting in

the cumulative production of ROS. The inability of melatonin to counteract the generation of $O_2^{-}\cdot$ shown in Figure 77 is in agreement with the findings of many investigators (Chan and Tang, 1996; Marshall *et al*, 1996; Zang *et al*, 1998; Perianayagam *et al*, 2005), who have found that melatonin is not particularly effective in scavenging this particular ROS. The fact that melatonin is able to decrease the extent of 5-FU-induced lipid peroxidation (Figures 71 and 72) (see 8.5) implies that $HO\cdot$ generated by 5-FU play an important role in the induction of lipid peroxidation, as melatonin is known to be a potent quencher of this radical (see 1.3.8. and 8.3).

In contrast to effecting an expected decrease in $O_2^{-}\cdot$ levels *in vivo*, the administration of melatonin for five days results in a significant increase in the level of this ROS. This oxidant effect could be attributed to the hepatic metabolism of melatonin, which has a half-life of less than 60 minutes (Reiter and Robinson, 1995), to 6-OHM (see 1.3.5). This metabolite has been found to possess pro-oxidant properties and results in the oxidation of DNA through the generation of $HO\cdot$ (Sakano *et al*, 2004) (see 1.3.5 and 8.3). An increase in 6-OHM availability has been suggested to be the underlying reason for an increase in lipid peroxidation noticed in female rats subjected to long-term melatonin treatment (Bojková *et al*, 2006). 6-OHM, furthermore, induces Hsp70 (see 8.4), thus indicating that the metabolite generates cellular stress. The findings of this Chapter suggest that this stress is oxidative in nature, and mediated by an increase in $O_2^{-}\cdot$ levels.

It is known that ethanol induces the formation of ROS, in particular $O_2^{-}\cdot$ (Novitskiy *et al*, 2006; Li *et al*, 2007). The absolute ethanol used as a co-solvent to dissolve melatonin is used at a final concentration of 0.67% v/ v; over the five days of drug treatment to which rats are exposed, this amounts to a total volume of 50 µL of absolute ethanol per rat. The ethanol-induced formation of $O_2^{-}\cdot$ mentioned above (Novitskiy *et al*, 2006) was investigated to determine the effects of alcoholic beverages on the liver; the concentration of ethanol used in this Chapter and throughout this research, is very low compared to that in widely-available alcoholic beverages. It is thus unlikely that the use of absolute ethanol, in the very low concentration used as a co-solvent, contributes to the melatonin-induced increase in $O_2^{-}\cdot$ levels after five days of treatment.

It is important that the co-administration of melatonin with 5-FU does not result in a synergistic or potentiated increase in $O_2^{-}\cdot$ levels, as shown in Figure 75. In fact, there is a decrease, which may indicate a competitive antagonism between 5-FU and melatonin in the generation and/ or activity of $O_2^{-}\cdot$. This indicates that even though accumulation of 6-OHM may occur, the long-term co-administration of melatonin is unlikely to exacerbate 5-FU-

induced toxicity. Further studies examining the effects of melatonin on $O_2^{-}\cdot$ levels after a period of administration longer than five days are thus recommended.

The results of this Chapter point to caution with the possible use of melatonin as co-therapy with 5-FU, as due consideration should be given to the effects of its metabolites in the body. Bojková *et al* (2006) have furthermore cautioned that melatonin be used with caution clinically (see 1.3.12.5). These authors have investigated the metabolic effects of long-term melatonin administration coupled with short-term fasting in male and female rats. The effects of fasting are deemed to be important, as starvation could be a causative factor in the development of cancer (Bojková *et al*, 2006) and an assessment of the effects of melatonin co-therapy to cancer patients is thus necessary. The long-term administration of this agent has been found to result in undesirable metabolic effects, particularly on lipid metabolism (Bojková *et al*, 2006). The concentrations of triacylglycerols and cholesterol are increased, as a result of hyperlipaemia. There is an increase in serum corticosterone and a decrease in body mass (Bojková *et al*, 2006) (see 1.3.6.1); the latter is believed to be due to a melatonin-mediated effect on insulin secretion (Rodriguez *et al*, 1989; Bojková *et al*, 2006). Gluconeogenesis is also promoted (Bojková *et al*, 2006). As discussed in 1.3.12.5, the effects of melatonin on the endocrine system are widespread. If melatonin is used clinically as co-therapy with 5-FU, patients should have regular tests to examine the levels of various pancreatic hormones, blood glucose and cholesterol. The significance of nutritional oncology (see 1.3.6.1) is highlighted, as adequate nutrition is essential in cancer patients and also serves to attenuate some of the adverse metabolic effects of melatonin (Bojková *et al*, 2006).

In addition to its role in the process of lipid peroxidation, $O_2^{-}\cdot$ is a cofactor required by IDO (Higuchi and Hayaishi, 1967). It was shown in Chapter 5 that both 5-FU and melatonin enhance the activity of this enzyme *in vivo*, and the results presented in 9.3 indicate that this is due to both agents increasing $O_2^{-}\cdot$ levels *in vivo*.

9.5 Conclusions

5-FU induces the generation of $O_2^{-}\cdot$ *in vivo*, thereby resulting in lipid peroxidation and IDO activation. The acute, *in vitro* addition of melatonin decreases this generation of $O_2^{-}\cdot$, but the prolonged administration of melatonin *in vivo* has been shown to increase levels of this ROS. This may be due to the hepatic metabolism of melatonin to 6-OHM, which is known to have oxidant properties. By failing to potentiate the 5-FU-induced increase in $O_2^{-}\cdot$ levels, melatonin co-therapy may be non-toxic in cancer patients being treated with 5-FU. It is recommended that adequate nutrition is ensured and regular endocrine tests performed.

CHAPTER 10

THE EFFECTS OF 5-FLUOROURACIL AND MELATONIN ON RAT HIPPOCAMPUS, USING DESIGN-BASED STEREOLOGY

10.1Introduction to stereology

Stereology is deemed the most accurate and reliable method of quantitative histology (Glaser *et al*, 2006; Schmitz and Hof, 2005). Histology, the study of tissue on a microscopic level and the techniques used to prepare such tissues, allows for the visualisation of the cytoarchitecture of either animal or plant tissues (Hodgson and Bernard, 1992). These tissues can be preserved, sectioned and stained, allowing for microscopic analysis. Although histology is undoubtedly useful in visualising structural abnormalities in tissues, it is a subjective, qualitative technique and cannot determine statistically significant differences in cytoarchitecture that occur between physiologic and pathologic states (Schmitz and Hof, 2005).

The origins of stereology date back to 1733, when Buffon discovered relationships between the principles of probability and geometry, and in 1843 the first practical method of determining the three-dimensional volume fraction of an area fraction was invented by Delesse. Originating from the Greek word *stereos*, or "solid", the word "stereology" was introduced in 1961 and defined as "the spacial interpretation of sections" (http://en.wikipedia.org/wiki/Stereology). Stereology is thus the science of determining three-dimensional information from one- or two-dimensional samples (Haug, 1986; Weibel, 1979; 1980; 1992), and it is possible to use this technique to determine some three-dimensional quantities, such as volume, without three-dimensional reconstruction (http://en.wikipedia.org/wiki/Stereology).

Stereology is based on fundamental geometrical and statistical principles, such as Cavalieri's principle and survey sampling, respectively. Extrapolating from a few planar sections into three dimensions requires that the sections be representative of all sections that are in the material. In the earlier days of stereology, this was achieved by the model-based sampling inference approach, which assumes homogeneity of the material. Modern stereology, however, makes use of design-based sampling inference. This approach recognises that material may not be homogeneous in composition, and therefore samples are randomly taken throughout the section (http://en.wikipedia.org/wiki/Stereology). In a review article, Schmitz and Hof (2005) have shown that design-based stereology yields results that are representative

for the whole material, and are not dependent on the spatial distribution or orientation, shape or size of the cells being studied (West, 2002). Furthermore, bias can be minimised and the extent of variability determined, thus establishing design-based stereology as the most optimal and robust method of quantitative neuromorphology (Schmitz and Hof, 2005; West, 1993, 2002).

There are a variety of design-based stereological methods, or probes, that can be used to analyse either the local or global characteristics of tissues. An example of a local characteristic is the mean volume of a certain type of cell in a particular region, whereas an example of a global characteristic is the volume of a certain cell layer (Schmitz and Hof, 2005). Some important characteristics that can be studied include: number, volume, spatial distribution, connectivity and the length of linear structures. These characteristics can either be expressed as relative values, such as the density of a particular type of cell in a specific brain region, or as absolute values, such as the total number of neurons in a particular structure (Schmitz and Hof, 2005). In the medical sciences, stereology is widely used, and is responsible for the uncovering of a century-old misconception of the structure of mammalian liver. Another field in which stereology is useful is metallurgy (http://en.wikipedia.org/wiki/Stereology).

10.2 Structure and function of the hippocampus

The following figure illustrates the structure of the brain, and shows the position of the hippocampus. It is a thin, subcortical structure adjacent to the temporal lobe. The hippocampus is an important component of the limbic system and functions in regulating learning, short-term memory and emotions (Perry, 2007).

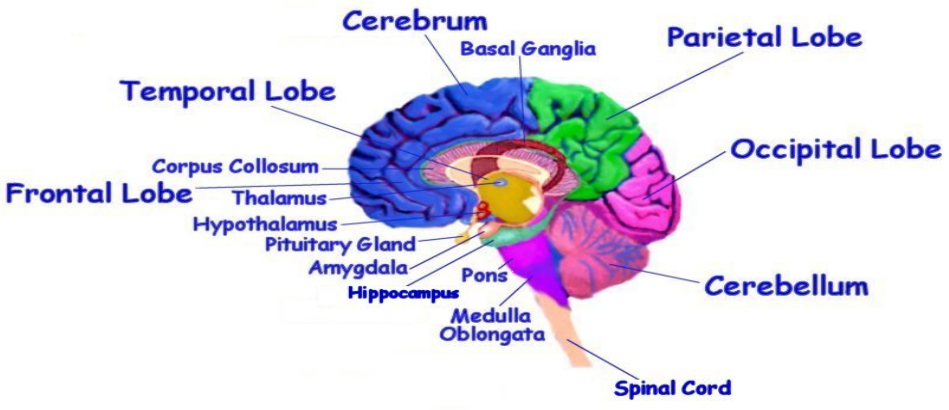


Figure 78: A longitudinal section through the brain (www.rmcybernetics.com/science/cybernetics/ai_vision_perception_brain.htm)

Figure 79 shows a coronal view of both hemispheres of the hippocampus, and the proximity of these to the cerebrum. Named for its seahorse-like structure, the hippocampus consists mainly of the dentate gyrus (DG), subiculum and CA1, CA2, CA3 and CA4 fields, as illustrated in Figure 80.

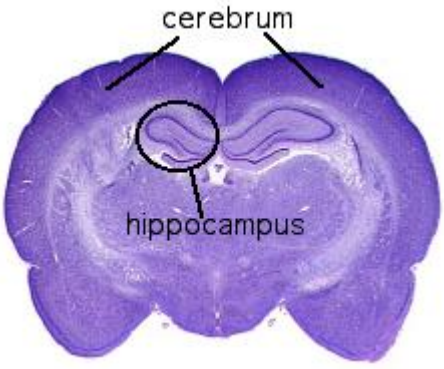


Figure 79: A Nissl-stained coronal section of rat brain, showing the hippocampus (http://instruct1.cit.cornell.edu/courses/bionb424/students/cth5/images/brain_cross_section_showing_hipp.jpg)

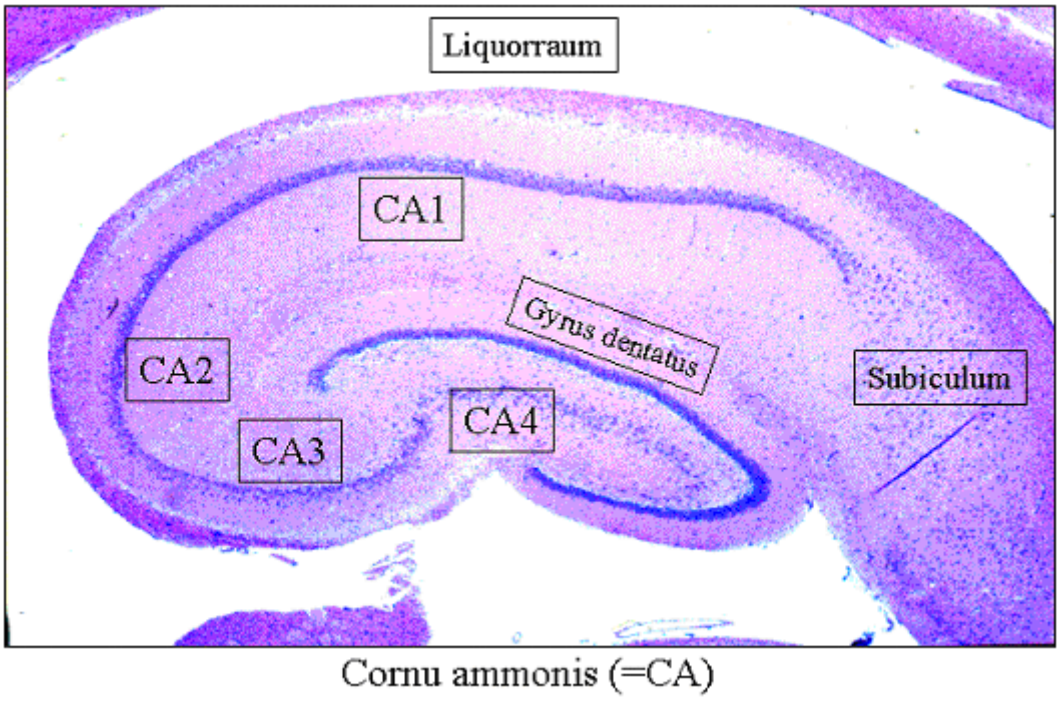


Figure 80: A Nissl-stained coronal section of the hippocampus, showing its major regions
(http://edoc.hu-berlin.de/dissertationen/schubert-stephan-nicolas-2003-09-6/HTML/schubert_html_21d219b1.png)

The brain consists of two major types of cells, namely neurons and supportive glial cells. Neurons are highly specialised, and are readily excited to generate and conduct nerve impulses. As shown in Figure 81, a typical neuron consists of a large circular cell body, with an axon and dendrites. The latter two structures are long cytoplasmic extensions from the cell body. A large nucleus occurs in the centre of the neuron. Tissues containing neurons innervate most organs in the body (Maharaj, 2004; Perry, 2007).

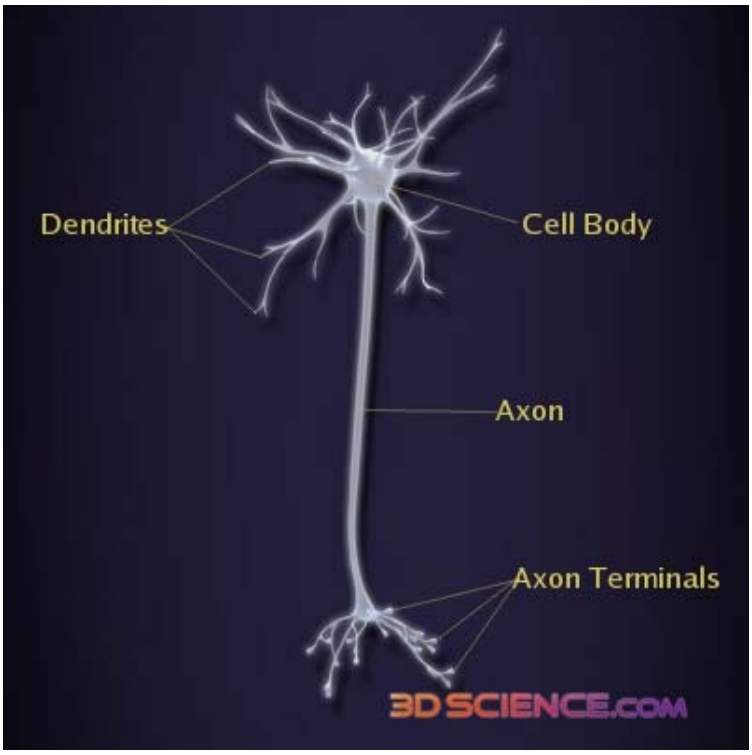


Figure 81: A typical neuron, as shown under high-power magnification (http://www.puffafish.net/ai/images/3d_neuron.jpg)

10.2.1 Functions of the hippocampus

10.2.1.1 Spatial memory

Multimodal sensory stimuli from the olfactory, temporal and parietal cortices are received by the anterior region of the CA1 (Rockland and Hosen, 1999). Cells in the hippocampus respond to both the organism's intention/ position attributes within the environment and the object and spatial attributes of environmental stimuli (Eichenbaum and Otto, 1993). Anterior hippocampal CA1 and CA3 cells interact with surrounding cells that spatially map the relative position and head orientation of the organism, in a complex process that results in the spatial mapping of certain objects (Georges-Francois *et al*, 1999; McHugh *et al*, 1996) and the formation of episodic memories (Georges-Francois *et al*, 1999). These are memories which are independent of observer orientation and contain information about both stimuli and the

organism's response (Eichenbaum and Otto, 1993). Episodic memories are important in enabling the hippocampus to extract novel experiences through a process of sifting against familiar stimuli, so that further memory encoding and consolidation can occur in the parahippocampal, frontal and prefrontal regions of the brain (Brewer *et al*, 1998; Wagner *et al*, 1998). The hippocampus thus continually compares real stimuli against cognitive expectations, thereby maintaining the accuracy of the representations of reality, which in turn contributes to future cognitive expectations (Strange *et al*, 1999).

The right hemisphere of the hippocampus responds to pictorial stimuli or object representations (Brewer *et al*, 1998), whereas the left hemisphere is responsive to linguistic or verbal stimuli (Wagner *et al*, 1998). In addition, the hippocampus responds to the contextual significance and relevance of stimuli, evidenced by the finding that the posterior region of the hippocampus responds to familiar stimuli, whereas the anterior region is stimulated by novel perceptions, defined as mismatches to expected representations (Strange *et al*, 1999). Damage to the posterior region of the hippocampus thus results in retrograde amnesia, while damage to the anterior part induces anterograde amnesia (Strange *et al*, 1999). The abovementioned mismatches between expectation and reality noted by the anterior hippocampus can lead to the development of anxiety; it is an alerting mechanism that is termed "the behaviour inhibition system" (BIS) and is stimulated by unpredictable representations (Gray, 1982a, 1982b, 1988).

10.2.1.2 Synaptic plasticity

Memory consolidation occurs through a process of long-term potentiation (LTP), a form of synaptic plasticity (Bliss and Lomo, 1973) (see 10.2.1.3). Synaptic plasticity, an inherent characteristic of the nervous system, is the ability of a synapse or neuron to adjust its internal characteristics as a response to a past experience (http://en.wikipedia.org/wiki/Plasticity). It enables animals to adjust and survive in changing environmental conditions (Park *et al*, 2006). It is furthermore a fundamental mechanism by which learning and memory occur, as according to the Hebbian theory memories are believed to be represented by numerous interacting synaptic networks (Hebb, 1949). Synaptic plasticity is a form of neuroplasticity, in which an entire region of the brain can adjust to environmental adversity, such as trauma, or to repeated learning. An example of neuroplasticity is the assumption of (some of) the functions of a damaged brain region by another region (http://en.wikipedia.org/wiki/Plasticity).

Synaptic plasticity involves a change in the strength of the synapse, or connection, between two neurons. This is achieved by the interaction of many complex mechanisms, some of

which include alterations in the amount of neurotransmitters released from the presynaptic neuron, and the altered responses of the post-synaptic neuron to these neurotransmitters (Gaiarsa *et al*, 2002). Two fundamental mechanisms underlying synaptic plasticity are:
(i) modifications in existing proteins, such as protein kinases, which result in functional changes of the synapse (Shi *et al*, 1999); and
(ii) changes in the amount of important synaptic proteins, due to neurotransmitter-induced alterations in gene transcription. This occurs over a longer period of time, has a longer duration and provides the basis for the storage of long-term memory (http://en.wikipedia.org/wiki/Plasticity).

Synaptic strength is also a function of the number of ion channels present in the synapse (Debanne *et al*, 2003). It is known that the density of receptors on post-synaptic neuronal membranes can be altered as a mechanism to regulate neuronal excitability to stimuli; glutamatergic receptors, such as NMDA receptors, for example, can be added to or removed from post-synaptic membranes through exocytosis and endocytosis, respectively, and this can be influenced by synaptic activity (Pérez-Otaňo and Ehlers, 2005; Shi *et al*, 1999).

Negative feedback mechanisms are in place to regulate plasticity. These include metaplasticity and scaling (Pérez-Otaňo and Ehlers, 2005). The former decreases the consequences of plasticity over a period of time; for example, if a neuron has undergone extensive plasticity, further plasticity is minimised. Metaplasticity may occur as a result of alterations in NMDA receptors, such as conformational changes that favour a decrease in intracellular Ca^{2+} levels (Pérez-Otaňo and Ehlers, 2005). Scaling, another mechanism of negative feedback, occurs over several hours or days, and involves an alteration in the number of synaptic NMDA receptors. This results in the maintenance of relative synaptic strength, through increasing the amplitudes of small excitatory post-synaptic potentials (EPSP) after continued inhibition, and decreasing the amplitudes of these potentials after prolonged excitation (Pérez-Otaňo and Ehlers, 2005).

10.2.1.3 LTP

LTP plays a leading role in the long-term storage of information for memory and learning (Bliss and Collingridge, 1993; Kandel, 2001; Grimwood *et al*, 2001); and LTP-like processes are involved in mediating a wide range of behavioural responses to experience (Malenka, 2003), in particular inhibitory avoidance behaviour (Whitlock *et al*, 2006). LTP is a prolonged increase in synaptic strength, which lasts from minutes to a period of several days or years, following high-frequency stimulation of the synapse. The response of the post-synaptic

neuron to further single-pulse stimuli is thus enhanced (Lomo, 2003). The counterpart of LTP is long-term depression.

The hippocampus is of importance as not only was LTP discovered in this organ in 1966 (Bliss and Lomo, 1973; Lomo, 2003), and due to the pivotal role that this organ plays in learning and memory, but LTP studies are often performed on hippocampal slices (http://en.wikipedia.org/wiki/Long-term_potentiation). The CA1 region of the hippocampus is most often used to study LTP, due to its predictable composition and the ease with which LTP can be induced (http://en.wikipedia.org/wiki/Long-term_potentiation). LTP occurs in a variety of structures in the brain and different forms of LTP exist, depending on the age of the organism (Yasuda *et al*, 2003), the specific type of glutamatergic receptor involved in the signalling pathway (Malenka and Bear, 2004), and the anatomical location in which LTP occurs (Harris and Cotman, 1986). The most extensively-studied form of LTP is NMDA receptor-dependent LTP that occurs in the CA1 region (Malenka and Bear, 2004).

Although there are various mechanisms by which LTP occurs, there are two major events that occur (http://en.wikipedia.org/wiki/Long-term_potentiation):
(i) induction, in which a high-frequency stimulus is applied to a synapse and results in the release of glutamate from the pre-synaptic neuron. This excitatory neurotransmitter interacts with AMPA receptors in the membrane of the post-synaptic neuron, thereby resulting in the influx of Na^+ into the neuron, which depolarises the membrane and generates an EPSP. The induction of LTP in the post-synaptic neuron depends on the magnitude of the EPSP. Repeated, high-frequency stimuli generate many EPSPs, which have a summative effect and result in progressive depolarisation of the cell membrane. In NMDA receptor-dependent LTP synapses, this results in a lifting of a Mg^{2+}-induced NMDA blockade, which allows the influx of Ca^{2+} into the neuron. The rapid increase in Ca^{2+} in the neuron triggers the early phase of LTP; and
(ii) expression, which is divided into early (E-LTP) and late (L-LTP) phases. E-LTP occurs for two to three hours following LTP induction and does not involve protein synthesis. Various protein kinases (Sweatt, 1999; Otmakhova *et al*, 2000; Huang and Hsu, 1999; English and Sweatt, 1997), such as Ca^{2+}/ calmodulin-dependent protein kinase II (CaMKII) (Malenka and Bear, 2004), phosphorylate AMPA receptors and mediate the insertion of AMPA receptors into the membrane of the post-synpatic neuron (Malenka and Bear, 2004; Malinow, 2003). These receptors are the most abundant glutamatergic receptors in the brain, and the increase in AMPA receptor density during E-LTP facilitates the generation of larger EPSPs upon excitatory stimulation (Malenka and Bear, 2004). L-LTP occurs from hours to weeks after LTP induction (Lynch, 2004) and results in a marked increase in post-synaptic

responses, which occurs through new protein synthesis and synaptic remodelling. The initial phase of L-LTP involves protein synthesis, while the second phase involves transcription in addition to protein synthesis (Lynch, 2004; Frey *et al*, 1996; 1988). The synthesis of structural proteins, glutamate receptors and transcription factors enhances synapses and generates further synaptic connections. Protein synthesis also occurs in the presynaptic neuron and synaptic cleft (Lynch, 2004). L-LTP may be modulated by various compounds, such as the β receptor agonist, NE (Straube and Frey, 2003).

There have been several studies on the effects of melatonin on LTP, as discussed below. There are melatonin receptors in the CA1, CA3, DG and subiculum of the hippocampus (Musshoff *et al*, 2002). Some investigators have found that the nocturnal administration of melatonin markedly increases the firing of neurons in the CA1 region, but that this excitatory effect is diminished during the day (Musshoff *et al*, 2002). Other investigators have found that melatonin either decreases the amplitude of EPSPs (El-Sherif *et al*, 2003), does not alter synaptic transmission (Collins and Davies, 1997; Wang *et al*, 2005), or exhibits a biphasic effect, initially depressing synaptic transmission and subsequently inducing an amplification phase (El-Sherif *et al*, 2002). It is widely reported that melatonin blocks LTP induction in the DG and CA1 regions (Collins and Davies, 1997; Hogan *et al*, 2001; Gonzales and Armstrong, 1995). The finding by Chaudhury *et al* (2005) that melatonin enhances LTP at night, when its own endogenous secretion is maximal, may reflect the influences of other hormones that have circadian rhythms (Ozcan *et al*, 2006). Ozcan *et al* (2006) have studied the effects of melatonin on the mossy fibres of the CA3 region, which exhibit NMDA receptor-independent LTP (Harris and Cotman, 1986), as well as the CA1 region, which has NMDA receptor-dependent LTP. Synaptic transmission and LTP were investigated and it was found that melatonin depresses synaptic transmission in a dose-dependent manner in the CA1 region, by decreasing the amplitude of EPSPs, and also blocked LTP in this region. The effects in the CA3 are less pronounced and not statistically significant (Ozcan *et al*, 2006). Since melatonin is known to not alter NMDA receptor-mediated responses (Collins and Davies, 1997), the mechanism by which the agent depresses LTP in the CA1 region is not through NMDA receptor antagonism (Ozcan *et al*, 2006).

It is thought that the effects of melatonin in the CA1 region are mediated by type-2 melatonin receptors (Ozcan *et al*, 2006), and that the results of these interactions regulate the adenylyl cyclase-protein kinase A pathway, thereby altering synaptic plasticity (Wang *et al*, 2005). Melatonin exerts both inhibitory (Ayar *et al*, 2001; Zeise and Semm, 1985) and stimulatory (Leon *et al*, 1998; Brotto and Gorzalka, 2000) effects on neurons, although the inhibitory effect is greater (Ozcan *et al*, 2006). This interaction with both inhibitory and excitatory

neurotransmitter pathways (Skaper *et al*, 1998; Saenz *et al*, 2004) could imply that melatonin has a regulatory role in the mechanisms that underlie memory formation (Ozcan *et al*, 2006).

10.3 Effects of fluoride on the brain

This has been widely studied, due to the presence of fluorides in the water supplies of many countries (Mullenix *et al*, 1995; Schettler *et al*, 2000), and in many commercial products, such as toothpaste and mouthwashes (Beltran and Szpunar, 1988; Whitford, 1989). Chinese studies linking impaired cognitive ability in children with high levels of fluoride have added momentum to research the neurological effects of this ion (NRC, 2006; Xiang *et al*, 2003a, 2003b; Lu *et al*, 2000; Calvert *et al*, 1998; Wang *et al*, 1996).

Young rats exposed to 100 ppm fluoride in drinking water have been shown to exhibit significant signs of neurodegeneration in the hippocampus, cerebellum, motor cortex and amygdala (Shivarajashankara *et al*, 2002a; Zhavoronkov, 1977). This is evidenced by a decrease in total neuronal number and size, cortical gliosis and a reduction in the number of cerebellar Purkinje cells (Shivarajashankara *et al*, 2002a). Hippocampal toxicity may in part be due to fluoride altering Ca^{2+} current kinetics within cells (Mullenix *et al*, 1995). In addition, fluoride may alter enzyme activity by the formation of strong hydrogen bonds with amides, increase the hydrolysis of phosphoinositide and inhibit the activity of adenylyl cyclase in the cortex (Spittle, 1994).

Long-term fluoride toxicity in animals induces brain lesions (Shashi *et al*, 1994; Shashi, 1992; Vani and Reddy, 2000), altered cerebrovascular and neuronal functioning (Varner *et al*, 1998), and behavioural abnormalities (Mullenix *et al*, 1995). Concentrations as low as 1 ppm have also been found to directly induce neurotoxicity by resulting in the following (Varner *et al*, 1998):

(i) a decrease in the phospholipid content of cells. This alteration in the composition of cell membranes could facilitate the "leakage" of cell contents and cell death (see 8.1) (NRC, 2006; Guan *et al*, 1998);

(ii) a decrease in the number of nicotinic cholinergic receptors, which are important for memory retrieval (NRC, 2006; Chen *et al*, 2003; Long *et al*, 2002);

(iii) malfunctioning in antioxidant mechanisms. In particular, SOD, catalase and glutathione levels are decreased (Chinoy and Shah, 2004; Wang *et al*, 2004) (see 8.1);

(iv) the formation of β-amyloid plaques, which is characteristic of Alzheimer's disease (Wang *et al*, 1997);

(v) an increase in aluminium uptake, which is also implicated in the aetiology of Alzheimer's disease. In addition, fluorides enhance the generation of free radicals, thus contributing to the

development of Alzheimer's disease (NRC, 2006). The generation of free radicals is attributed to alterations in the content of coenzyme Q in the brain (Wang *et al*, 1997);
(vi) fluoride accumulation in the pineal gland (Varner *et al*, 1998); and
(vii) an increase in lesions that result from a deficiency of iodine (Varner *et al*, 1998).

Histologically, fluoride induces hippocampal neurodegeneration, evidenced by mitochondrial swelling, membrane involution and chromatin aggregation in cell bodies of the DG and CA3 and CA4 regions (Bhatnagar *et al*, 2002). Apoptosis, DNA damage (Chen *et al*, 2002, 2000; Lu *et al*, 2000) and altered protein synthesis (Shashi *et al*, 1994) also occur. Besides cellular distortions, vascular inclusions are also apparent (Issacson *et al*, 1997).

The above biochemical abnormalities result in poor performances in maze and motor co-ordination animal tests (Bhatnagar *et al*, 2002; Paul *et al*, 1998); and impaired learning and memory (Zhang *et al*, 1999, 2001; Sun *et al*, 2000), which is associated with structural changes in synapses (Zhang *et al*, 1999) and a fluoride-induced decrease in RNA and DNA levels in the cerebral hemisphere (Shah and Chinoy, 2004). Synaptic alterations include a decrease in post-synaptic density and an increase in the width of the synaptic cleft (Zhang *et al*, 1999).

In humans, chronic fluoride toxicity also results in neurological impairments, including decreased mental acuity, vertigo, spasticity and paralysis (Waldbott *et al*, 1978). It is believed that the behavioural changes noticed in rats also apply to humans (Mullenix *et al*, 1995). Paralysis, seizures and tremors occur as a result of fluorides directly interacting with nerve tissue (Shashi, 2003). The brains of aborted foetuses from areas richly exposed to fluoride were examined stereologically, and it was shown that there is a decrease in mean neuron volume and the volume density of mitochondria. Furthermore, the nucleus: cytoplasm ratio is increased, as is the volume of undifferentiated neuroblasts (Du, 1992). Exposure during foetal development or early childhood also results in a decrease in the intelligence quota (IQ) (Li *et al*, 1995) due to impairment both in memory and the ability to learn (Shivarajashankara *et al*, 2002a). This decrease in IQ is apparent even after corrections for lead and iodine exposure, family income and educational status (Xiang *et al*, 2003a, 2003b). Exposure to high levels of fluoride, besides decreasing mental work capacity, affect zinc metabolism (Li *et al*, 1994), thus altering plasma levels of this essential trace element.

Damage to the hippocampus results in cognitive impairment and hyperactivity (Altman, 1987), as this structure is the integrating centre of stimuli from memory, the environment and motivation, resulting in modifications to memory and decisions regarding behaviour

(DeLong, 1992). Fluoride exposure and toxicity thus can thus result in dysfunctional CNS output (Mullenix *et al*, 1995). The cholinergic system in particular may be affected as fluorides are known to inhibit enzymes in this system (Lin Fa-Fu *et al*, 1991), such as acetylcholinesterase and cholinesterase (Ekambaram and Paul, 2001; Shah and Chinoy, 2004). Learning and memory deficits are postulated to occur as a result of oxidative stress, which is induced by fluorides through decreased SOD activity, enhanced lipid peroxidation (Wang *et al*, 2004; Shen *et al*, 2004) and the protein oxidation of receptors (Shan *et al*, 2004). Increased oxidative stress in the brain is well documented (Shivarajashankara *et al*, 2002b; Shao *et al*, 2000).

It is suggested that the hippocampus might be the brain region most vulnerable to fluoride (Mullenix *et al*, 1995). The extent to which fluoride alters behaviour is dependent on the age at which exposure occurs and the extent of accumulation of fluoride in the brain (Mullenix *et al*, 1995). Fluorides can cross the blood-brain barrier (Zhao and Wu, 1998; Zhai *et al*, 2003) and accumulate in the hippocampus (Zhai *et al*, 2003). From histological analysis, in which an increase in reactive microglia is apparent, as well as the presence of immunoglobulin M and intracellular neurofilaments, it is believed that fluoride-induced dementia may result (NRC, 2006).

In 1.2.10 the ability of fluorides to activate G proteins was mentioned. These proteins mediate neurotransmitter release in the CNS. The effects of fluorides can be deleterious through a dual mechanism (NRC, 2006):
(i) fluorides alter the concentrations of neurotransmitters, such as 5-HT and NE (Li *et al*, 1994), as well as prostaglandins, glucagons and CNS peptides, for example vasopressin; and
(ii) by interacting with ADP and GDP, fluorides prevent the formation of the triphosphate forms of these compounds that are essential in providing energy to brain cells (NRC, 2006). The production of energy is thus impaired and energy-dependent processes, such as the transport of molecules across cell membranes and the transmission of impulses across synapses, are inhibited (Lakshmi Vani and Pratap Reddy, 2000).

10.4 Effects of 5-FU on the brain

As early as 1983, it was reported that 5-FU induces neurotoxicity, in particular lesions, cellular oedema and haemorrhagic necrosis (Neuwelt *et al*, 1983). The administration of 5-FU during late gestation to rats results in the developing brain showing signs of microencephaly, olfactory lobe regression and an absence of dorsal surface fissures (Kumar *et al*, 2005; Kumar *et al*, 2006), besides significant body weight loss and mortality (Kumar *et al*, 2006). Microscopic analysis of the olfactory lobe reveals disruption of the orderly arrangement of

olfactory cortical layers, the absence of the olfactory ventricle and the aggregation of glial cells and neurons that have undergone degeneration. The hippocampus and cerebral cortex also exhibit disrupted cytoarchitecture, with a decrease in nuclear mitotic figures occurring in the latter (Kumar *et al*, 2005). The hippocampus is also significantly shrunken. There is a marked reduction in the number of glial cells and a weakening of the fibrous structure in the subcortical zone of the cerebrum. These histological abnormalities indicate that 5-FU is neurotoxic to the developing brain and should thus not be used in pregnancy (Kumar *et al*, 2005).

It has been found that metabolites of 5-FU, in particular FA and FBAL (see 1.2.6.1), are neurotoxic, with the latter being more potent in this regard (Okeda *et al*, 1990) (see 1.2.10.7). Two major pathological changes are apparent (Okeda *et al*, 1990):
(i) necrosis occurs, especially in animals treated with FBAL. Symmetrical necrosis occurs in the thalamus, oculomotor nuclei and colliculi; and
(ii) both metabolites result in the development of vacuoles with a 20-50 μm diameter, distributed primarily in the white matter, nuclei of the cerebrum and the brain stem. Using electron microscopy, it was found that these vacuoles are caused by splitting of the separation line between the innermost myelin layer and the axon (Okeda *et al*, 1990).

It is furthermore suggested that FBAL may have a deleterious effect on energy metabolism in the brain (Okeda *et al*, 1990), which may be through the G-protein-mediated mechanism described in 10.3. In addition to the above, there is a decrease in cellular resistance to oxidative stress, and this facilitates the influx of neurotoxins through the blood-brain barrier and into brain cells (Subramaniam *et al*, 1994).

It has been found that chemotherapeutic agents used to treat breast cancer, for which 5-FU is standard therapy (see 1.2.8.7), result in cognitive impairment (Van Dam *et al*, 1998; Tchen *et al*, 2003; Phillips and Bernhard, 2003; Ahles and Saykin, 2002; Schagen *et al*, 1999; Anderson-Hanley *et al*, 2003; Jansen *et al*, 2005), particulary concentration and memory problems, and that this impairment is significantly greater at higher doses (Van Dam *et al*, 1998). It is suggested by these authors that CNS toxicity be perceived as a dose-limiting toxicity. In addition to cognitive impairment, women receiving chemotherapy also experience greater fatigue, menopausal symptoms and a poorer quality of life compared to controls (Tchen *et al*, 2003). Yoshikawa *et al* (2005) have, however, found that chemotherapy does not alter cognitive function or the volume of the hippocampus in patients who have been successfully treated for breast cancer. Likewise, there is no alteration in hippocampal volume

in women with breast cancer who have experienced a first major episode of depression (Inagaki *et al*, 2004).

In a more recent study (Winocur *et al*, 2006), the results of Van Dam *et al* (1998) were confirmed. A dosage of 75 mg/ kg of 5-FU was administered every week for three weeks to mice, and evidence of impaired learning and memory was apparent (Winocur *et al*, 2006). The cognitive deficit that patients experience has been termed "chemobrain", and has been attributed to standard doses of 5-FU and methotrexate (Winocur *et al*, 2006). It is suggested that 5-FU specifically induces damage in the hippocampus and frontal lobes, as mice experienced difficulties with spatial memory tasks and strategic/ working tasks, respectively, tasks co-ordinated from these two brain regions (Winocur *et al*, 2006; Sanderson *et al*, 2006; O'Keefe and Nadel, 1978; Save *et al*, 1992), the former through an oestrogen-dependent mechanism (Zurkovsky *et al*, 2006). Spatial memory may furthermore play a role in the creation of episodic memories (Sanderson *et al*, 2006) (see 10.2). The study by Winocur *et al* (2006) focused on the acute effects of 5-FU, and it was noted that chronic administration may involve some degree of cognitive recovery (Winocur *et al*, 2006). This author has found that "chemobrain" is due to clinical causes, and its symptoms are not as a result of anxiety or stress, as had been believed. The extent of this cognitive dysfunction is mild to moderate, and is the equivalent of the normal ageing process. Winocur *et al* (2006) thus recommend that patients continue with 5-FU, and perform cognitive rehabilitation tasks, such as mnemonic and concentration aids; the use of cholinergic drugs used to treat Alzheimer's disease may furthermore be of some value (Winocur *et al*, 2006).

There is one report of an investigation into the effects of long-term 5-FU treatment on cognition in female rats (Lee *et al*, 2006). Complex spatial learning tasks, such as the Stone 14-unit T-maze and the Morris water maze, which are sensitive to hippocampal damage (Ingram, 1988; Gallagher and Nicolle, 1993) were perfomed after a recovery period from chemotherapy. The effects of 5-FU on LTP were not determined. Despite clear clinical signs of toxicity, such as weight loss, nail and dentition damage, poor coat quality, and delayed haematotoxicity, it was found that 5-FU treatment actually results in a transient enhancement of cognitive ability, reflected in better performances in the learning tasks.

These authors (Lee *et al*, 2006) have explained the above unexpected results and limitations of the study by noting that:
(i) in many clinical studies, multiple antineoplastic drugs are used, complicating an assessment of which specific agent is responsible for any cognitive impairment noted;

(ii) the patients used in many clinical studies already suffer from cancer, and this disease state may predispose to neurological impairment. It has been found, for example, that 36% of breast cancer patients in a study, exhibited cognitive impairment prior to antineoplastic treatment (Wefel *et al*, 2004);
(iii) it has been noted that after a recovery period of at least two years, breast cancer patients who had been treated with 5-FU demonstrate enhanced cognitive ability (Schagen *et al*, 2002);
(iv) female rats were used in the study, and fluctuations or modifications in the oestrous cycle may account for the transient enhancement of cognition, as it is known that low levels of oestradiol are associated with improved performances in the Morris water maze (Healy *et al*, 1999; Galea *et al*, 1995). 5-FU itself may have an effect on oestrogen levels;
(v) 5-FU results in hypophagia and subsequent weight loss. Diet restriction is known to induce neuroprotection in rodents (Mattson *et al*, 2002; Prolla and Mattson, 2001);
(vi) the learning tasks used in the study may not necessarily reflect the areas in the brain related to cognition that are damaged by the antineoplastic agent. This is supported by the finding that more patients treated with 5-FU, in combination with cyclophosphamide and methotrexate, reported experiencing problems in concentration, than those reporting memory problems (Schagen *et al*, 1999). Behavioural models of attentional mechanisms should thus be developed (Jongen-Relo *et al*, 2002; Lee *et al*, 2006);
(vii) cognitive impairment noticed in patients treated with 5-FU may be due to co-morbidity with other conditions, such as depression, which affects a large proportion of cancer patients and is very often undiagnosed (Bottomley, 1998). It has been suggested though, by the author, that 5-FU may precipitate depression by decreasing the levels of key neurotransmitters in the brain (Cassim *et al*, 2006) (see Chapter 6). Fatigue, related to a poor mental health score, very often accompanied by pain and not correlated with anaemia, was also found to occur up to three years later in 20% of breast cancer patients treated with 5-FU and other cytotoxics (Nieboer *et al*, 2005).

It thus seems likely, from the above studies by Winocur *et al* (2006) and Lee *et al* (2006), that whilst acute 5-FU treatment may well induce the phenomenon of "chemobrain", which is mild to moderate in nature, on a chronic basis some degree of cognitive recovery occurs, possibly through neuroplasticity mechanisms.

10.5 Experimentation

The effects of 5-FU and melatonin on rat hippocampus are visualised with the Nissl stain, and the effects of these agents on hippocampal volume are quantitated using design-based stereology.

10.5.1 Materials, animals, reagents and equipment

5-Fluorouracil and melatonin were purchased from the Sigma Chemical Corporation, St Louis, MO, USA. Absolute ethanol, isopropanol, xylene, sodium acetate, glacial acetic acid, Triton X-100 and cresyl violet were purchased from Merck, Darmstadt, Germany. 1, 3-Diethyl-8-phenylxanthine (DPX) was obtained from Serva, Heidelberg, Germany. All solutions were made up using deionised water (Milli R/Q System, Millipore®). Melatonin was dissolved using absolute ethanol as a co-solvent at a final concentration of 0.67% v/ v. A 1 M sodium acetate buffer solution was made; 40 ml of this stock solution, with an aliquot of 9.6 ml glacial acetic acid, was made up to 1 L with deionised water and designated Solution I. A 2% Triton X-100 stock solution was prepared and left to stir for 1 hour; 2.5 ml of this was added to 50 ml water and 150 ml absolute ethanol, and designated Solution II. Cresyl violet solution at a concentration of 0.1% w/ v was prepared, consisting of 0.25 g cresyl violet, 250 ml deionised water, 0.0512 g sodium acetate and 0.75 ml glacial acetic acid. The solution was left overnight and the pH adjusted to 3.5.

Twenty-four male Wistar rats, each weighing between 250g and 300g, and cared for as outlined in Appendix A, were used. Superfrost Plus glass slides were obtained from Meuzel, Braunschweig, Germany; and a Leica CM3050 cryostat (Leica, Rijswijk, The Netherlands) was used to cut sections. Stereology was performed on a stereology workstation, consisting of stereology software (StereoInvestigator; MicroBrightField Inc., Williston, VT, USA) and a framegrabber board, a modified light microscope (Olympus BX50; Olympus, Tokyo, Japan), Olympus UplanApo objective lenses (1.25X, 10X), electronic microcator and motorised specimen stage (Multicontrol2000; Märzhäuser, GmbH Wetzlar, Germany), coupled to a Heidenhain ND281 detector (Heidenhain, Germany), as shown below.

Figure 82: The stereology workstation used for analysis

Photomicrographs were taken using an Olympus DP70 digital camera, attached to an Olympus AX70 microscope (Olympus, Tokyo, Japan) and cellP Imaging Software for Life Sciences (version 2.3, Soft Imaging System, Münster, Germany), as shown below.

Figure 83: The workstation used for photomicroscopy

10.5.2 Method

Twenty-four rats, divided into four groups of six, were treated with either: (i) 5-FU, (ii) melatonin, (iii) a combination of both, or (iv) 0.9% saline, for five days as described in Table 10 (see 3.6.2.2). The rats were sacrificed by cervical dislocation on the sixth day, and the brains removed and stored in liquid nitrogen at -80ºC until required. Three series of 30 µm-thick sections at 120 µm intervals through the hippocampus were cut at -17ºC using a cryostat, giving a total of twelve sections per brain. Sections should not be thicker than 30 µm, in order to facilitate optimal diffusion of the cresyl violet stain (see 10.5.2.1) into the tissue, thereby increasing the proportion of stained neurons (Cooper *et al*, 1988); this thickness is also adequate for stereological purposes (Schmitz and Hof, 2005). Each section was placed on a gelatin-coated glass slide. In order for sections to be analysed by stereology, these are required to be adequately prepared (Schmitz and Hof, 2005). The use of paraffin or methacrylate, for example, to embed the sections, may result in a non-uniform compression of the tissue in the z-plane when it is cut, thereby resulting in heterogeneous densities of particles in this plane. The use of a cryostat to cut sections is not associated with this source of error (Hatton and von Bartheld, 1999; Gardella *et al*, 2003). Furthermore, the use of a cryostat minimises the loss of tissue or structure at the upper and lower surfaces of sections, which may result when using a knife to cut tissue (Schmitz and Hof, 2005). The sections were stored at -80ºC until required.

10.5.2.1 Nissl staining

Sections were air-dried for 60 mins, bathed in 96% ethanol for 10 mins and further air-dried for 10 mins. Sections were subsequently placed in Solution I for 20 mins, followed by 20 mins in Solution II and a further 20 mins in a fresh Solution I. The Triton X-100 in Solution II functions as a defatting agent, removing lipids from the tissue. Cresyl violet solution at a concentration of 0.1% was used to stain the sections, as this high concentration has been found to increase the proportion of neurons that are stained (Cooper *et al*, 1988). Termed the Nissl stain, after the neurologist Franz Nissl, this particular stain is widely-used to visualise neuons in different regions of the brain under a light microscope (Maharaj, 2004; Bear *et al*, 2001). The Nissl stain distinguishes neurons from the surrounding glial cells (Maharaj, 2004) and stains the endoplasmic reticulum in cells an intense purple colour. The Nissl stain is a regressive, as opposed to a progressive, stain (Cooper *et al*, 1988). It thus involves over-staining the section and subsequently removing excess stain from non-staining structures by immersion in a solvent. The Nissl stain is long known to effectively stain unfixed, cryostat-cut sections (Sandoz and Meier, 1978; Lindroos and Leinonen, 1983). Various staining times of 15, 20 and 35 mins were attempted, and 35 mins was found to yield optimal results. This is in agreement with the observation made by Cooper *et al* (1988), that optimal staining occurs with an increase in staining time. It is noted that the cresyl violet stain leaves 25% of neurons unstained, even in sections that are less than 30 µm thick (Cooper *et al*, 1988). The sections were thereafter washed thrice, for 1 min each, in fresh Solution I. This was followed by 1 min in absolute ethanol; and bathing twice, each time for 5 mins, in isopropanol. The sections were dehydrated by being bathed twice, each time for 5 mins, in xylene. This staining procedure is summarised in the following table.

Table 22: The Nissl staining procedure for cryostat-cut unfixed brain sections

Step	**Time (mins)**
Air drying	60
96% Ethanol	10
Air drying	10
Solution I	20
Solution II	20
Solution I	20
Cresyl violet solution	35
3 X Solution I	3 X 1
Absolute ethanol	1
2 X Isopropanol	2 X 5
2 X Xylene	2 X 5

A few drops of DPX glue and a coverslip were added, and the slides left to dry in a horizontal position for a few days.

10.5.2.2 Design-based stereology

The first step in stereological assessment is the delineation of the brain region of interest (BROI) (Schmitz and Hof, 2005). The hippocampus is stained with cresyl violet and thus readily visualised. The boundaries of the BROI can be traced on video images that are displayed on computer (Glaser and Glaser, 2000). The DG, CA 1 and 2 fields (combined as CA1-2) and CA3 regions of both hippocampal hemispheres were delineated at a magnification of 360X, using the 10X objective lens. The area of each BROI was determined, and the volume calculated and expressed in mm^3.

10.5.2.3 Photomicroscopy

Photomicrographs were taken of a representative hippocampus from each treatment group, at a final magnification of 20X, 400X and 1000X, using the 2X, 40X and 100X objective lenses respectively.

10.6 Results

The following volume measurements and photomicrographs were obtained. Statistical analysis was performed on the volume measurements, using ANOVA followed by the Student-Newman-Keuls multiple-range test.

10.6.1 Volume measurements

As shown in Figure 84 below, although 5-FU does decrease the volume of the DG, this is not to a statistically significant extent. Melatonin does not alter the volume of the DG either.

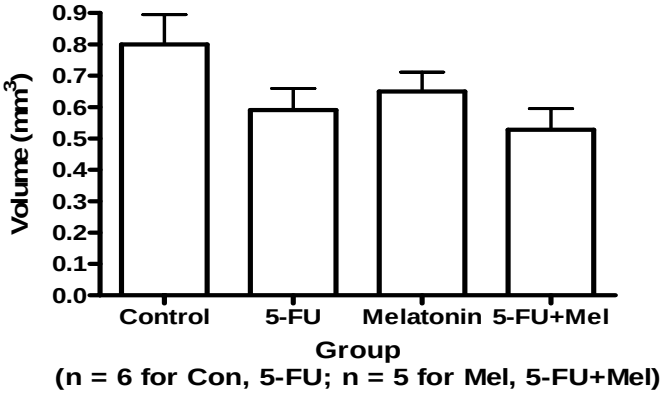


Figure 84: The effects of 5-FU and melatonin treatment <u>in vivo</u> on the volume of the DG in rat hippocampus

Each bar represents the mean ± SEM

The effects of these agents on the CA1-2 and CA3 regions are given in Figures 85 and 86. Figure 87 shows the effects of 5-FU and melatonin on the volumes of all three BROI combined.

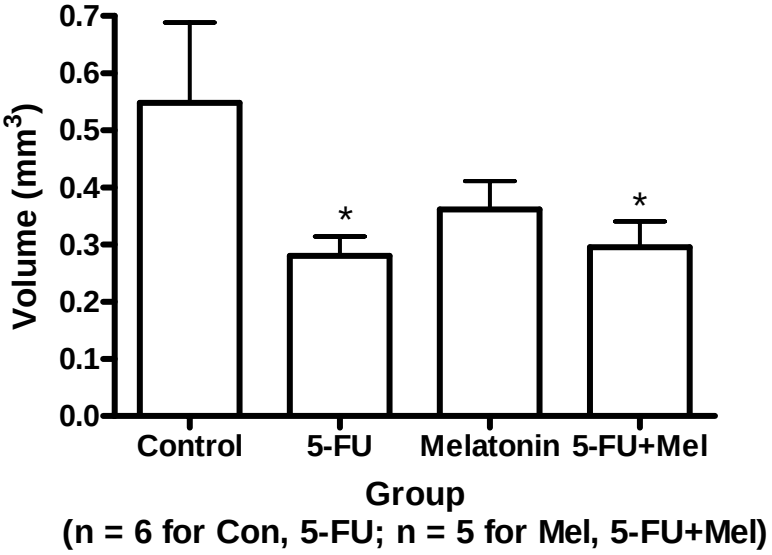


Figure 85: The effects of 5-FU and melatonin treatment <u>in vivo</u> on the volume of the CA1-2 region in rat hippocampus

* $p<0.1$ compared to control values, after analysis by ANOVA followed by the Student-Newman-Keuls multiple-range test. Each bar represents the mean ± SEM

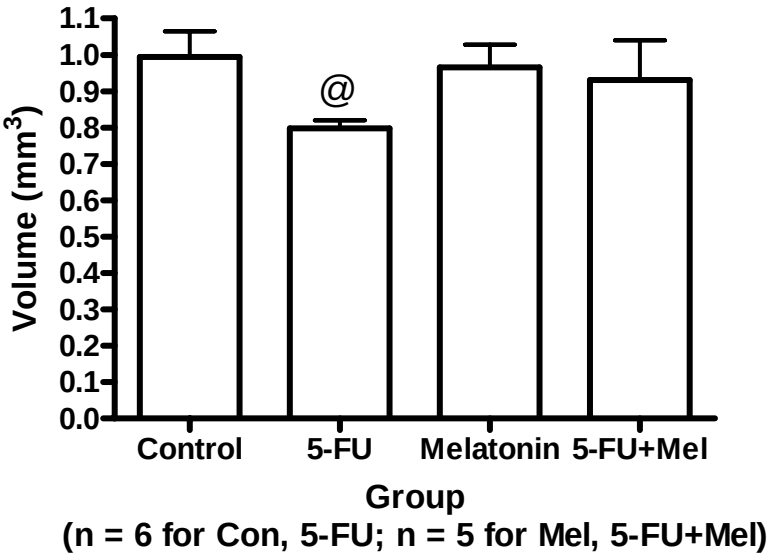


Figure 86: The effects of 5-FU and melatonin treatment <u>in vivo</u> on the volume of the CA3 region in rat hippocampus

@ $p<0.05$ compared to control values, after analysis by ANOVA followed by the Student-Newman-Keuls multiple-range test. Each bar represents the mean ± SEM

Although no statistically significant differences are noted in the volume of the DG, Figures 85 and 86 show that 5-FU significantly decreases the volume of the CA1-2 and CA3 regions of the hippocampus. The co-administration of melatonin slightly counters this decrease in the CA1-2 region, from a mean of 0.280 mm^3 to 0.295 mm^3. The latter value is still well below the mean control volume of 0.548 mm^3. Melatonin co-administration exerts a more marked effect in the volume of the CA3 region, which is increased back to levels that are statistically no different from control values. 5-FU decreases the mean CA3 volume from 0.994 mm^3 to 0.798 mm^3, and the co-administration of melatonin results in an increase to a mean volume of 0.931 mm^3.

The volumes of the DG, CA1-2 and CA3 regions have been combined and the effects of 5-FU and melatonin on this nett value are reflected in Figure 87 below. It is shown that 5-FU significantly decreases this nett hippocampal volume from 2.342 mm^3 to 1.669 mm^3, and that the co-administration of melatonin results in an overall modest increase of hippocampal volume to 1.754 mm^3, which is still significantly lower than control values.

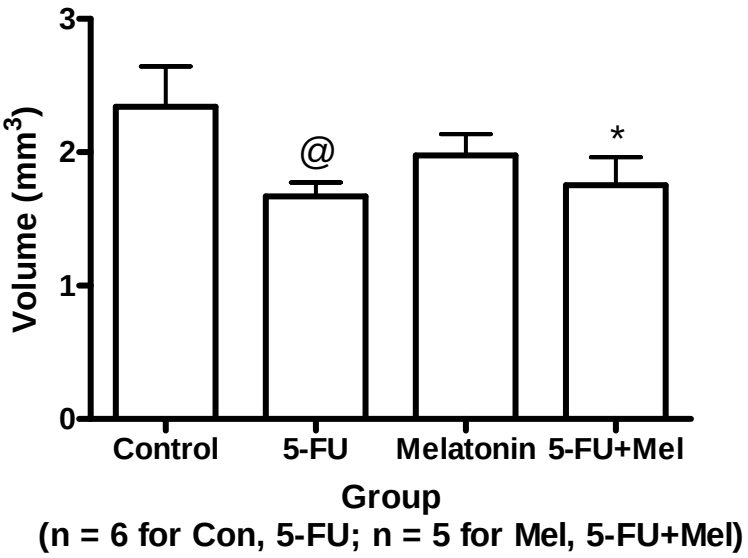


Figure 87: The effects of 5-FU and melatonin treatment <u>in vivo</u> on the combined volumes of the DG, CA1-2 and CA3 regions in rat hippocampus

@ $p<0.05$ and * $p<0.01$ compared to control values, after analysis by ANOVA followed by the Student-Newman-Keuls multiple-range test. Each bar represents the mean ± SEM

10.6.2 Photomicrographs

Figures 88-91 show a typical hippocampal hemisphere from each of the four treatment groups, at a final magnification of 20X. As can be seen, all hippocampi resemble that shown in Figure 80 (see 10.2) and no differences in structure or cytoarchitecture can be observed at this magnification.

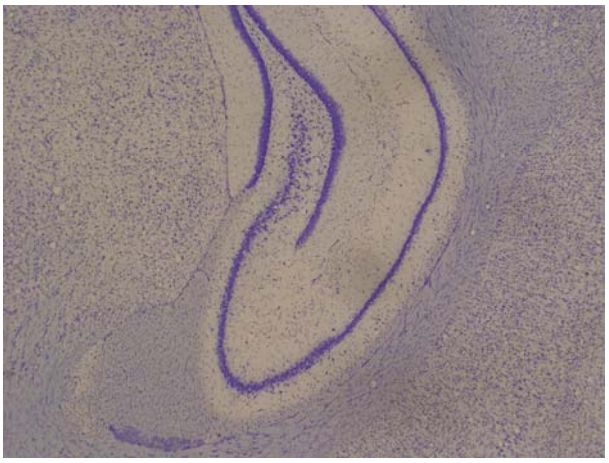


Figure 88: Photomicrograph of a typical control hippocampus, viewed at 20X magnification

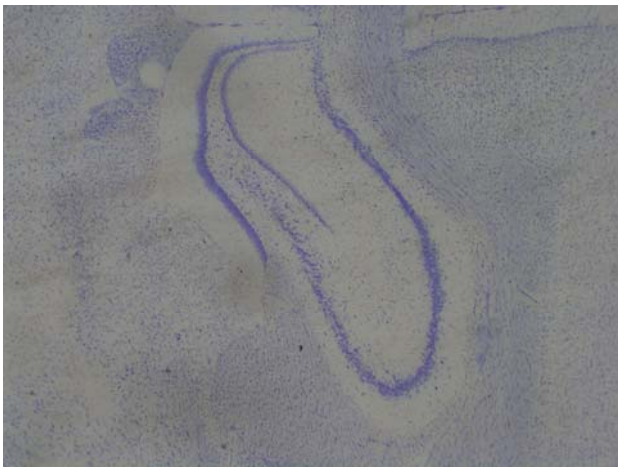


Figure 89: Photomicrograph of a typical hippocampus of a 5-FU-treated rat, viewed at 20X magnification

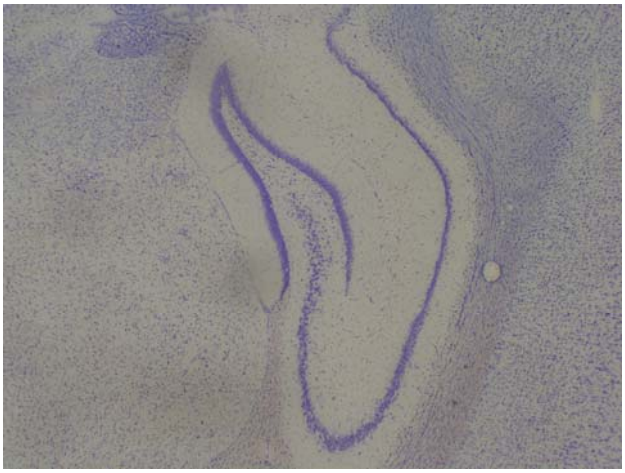


Figure 90: Photomicrograph of a typical hippocampus of a melatonin-treated rat, viewed at 20X magnification

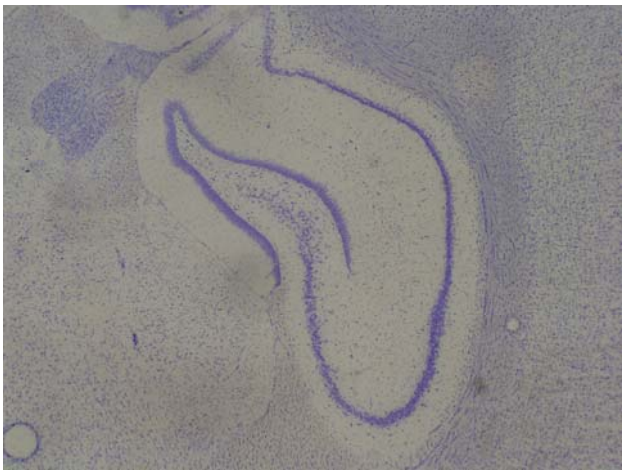


Figure 91: Photomicrograph of a typical hippocampus of a 5-FU- and melatonin-treated rat, viewed at 20X magnification

The next series of photomicrographs show a typical DG from each group, under 400X and 1000X magnification.

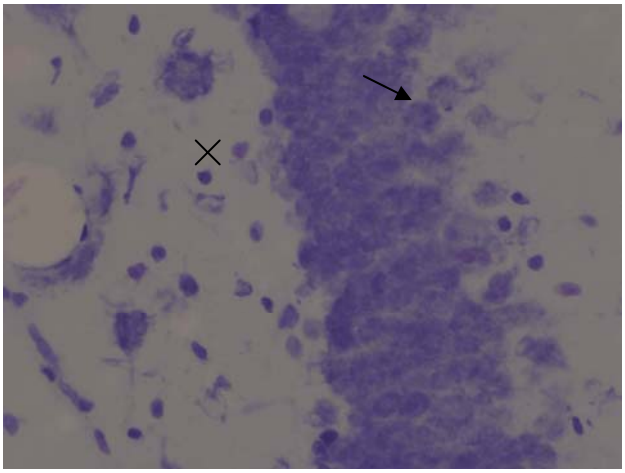


Figure 92: Photomicrograph of a typical DG of a control rat, viewed at 400X magnification. The arrow shows a typical neuron, and X is above a glial cell

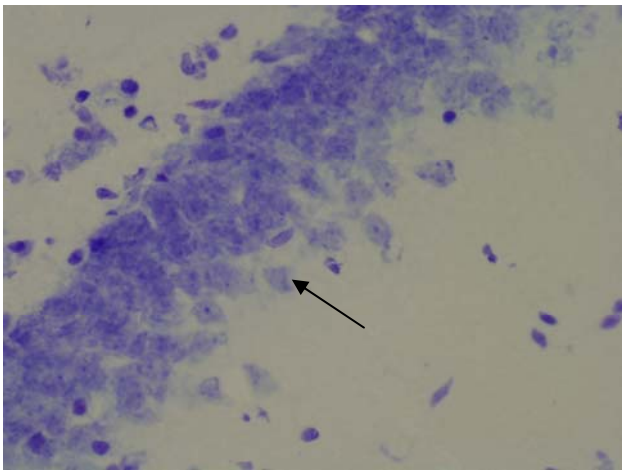


Figure 93: Photomicrograph of a typical DG of a 5-FU-treated rat, viewed at 400X magnification. The arrow shows a typical neuron

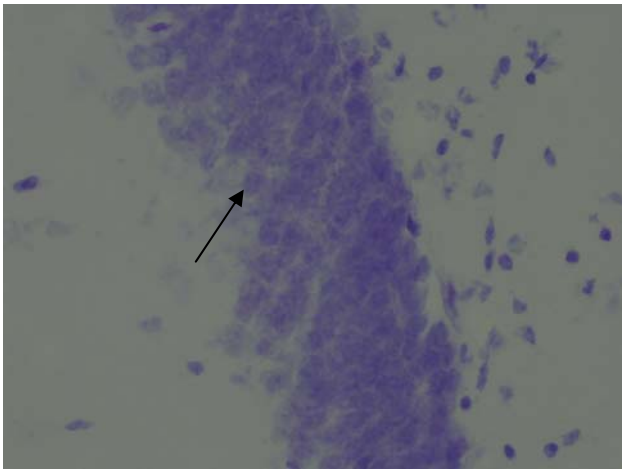


Figure 94: Photomicrograph of a typical DG of a melatonin-treated rat, viewed at 400X magnification. The arrow shows a typical neuron

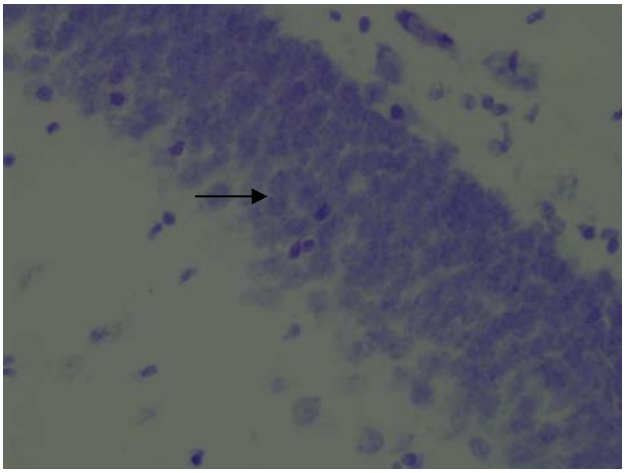


Figure 95: Photomicrograph of a typical DG of a 5-FU- and melatonin-treated rat, viewed at 400X magnification. The arrow shows a typical neuron

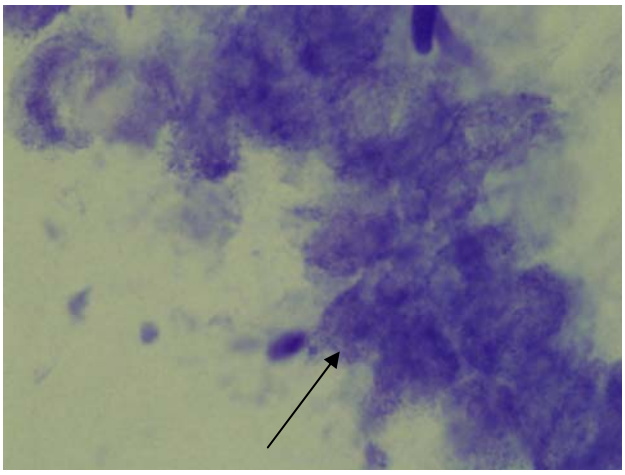


Figure 96: Photomicrograph of a typical DG of a control rat, viewed at 1000X magnification. The arrow shows a typical neuron

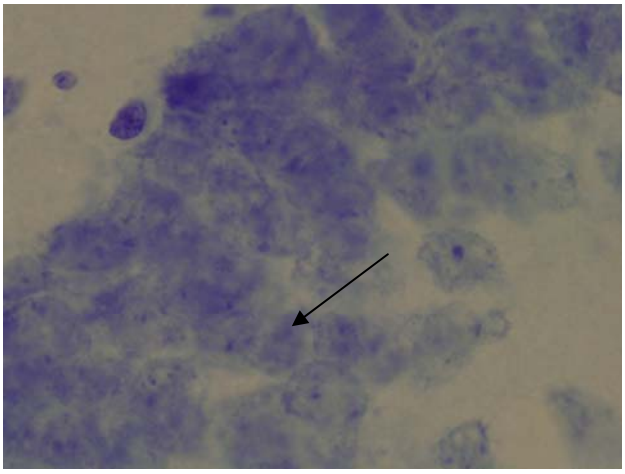


Figure 97: Photomicrograph of a typical DG of a 5-FU-treated rat, viewed at 1000X magnification. The arrow shows a typical neuron

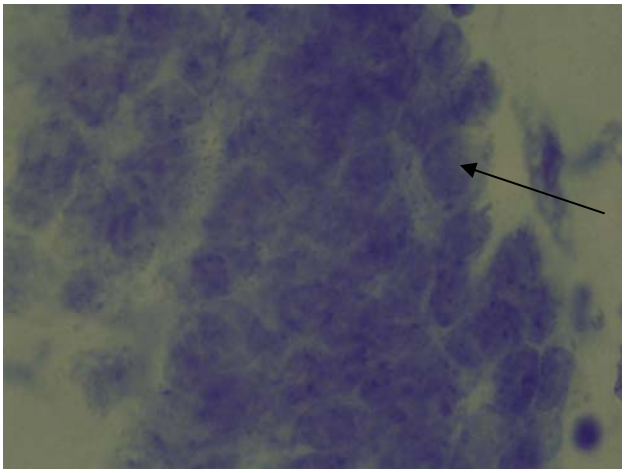


Figure 98: Photomicrograph of a typical DG of a melatonin-treated rat, viewed at 1000X magnification. The arrow shows a typical neuron

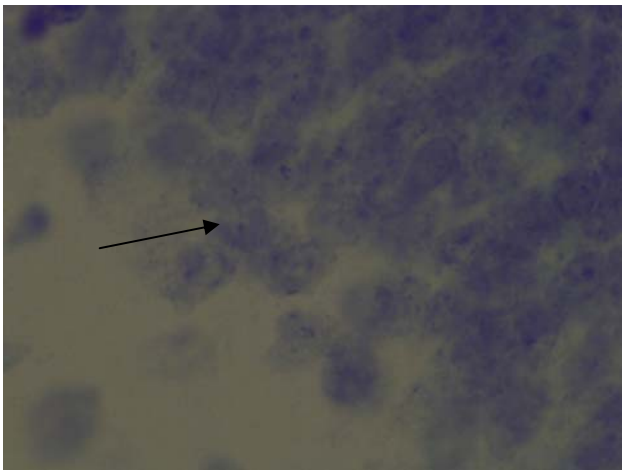


Figure 99: Photomicrograph of a typical DG of a 5-FU- and melatonin-treated rat, viewed at 1000X magnification. The arrow shows a typical neuron

As shown in Figures 91 and 96, typical neurons from the control group are circular, with a distinct dark blue nucleus. It can be observed that the administration of 5-FU results in neurons losing this circular shape, appearing smaller and signs of cell lysis are present, as shown in Figure 93. The DG of 5-FU-treated rats also appears to be narrower in diameter than the DG of control rats. Neurons from the Melatonin group have a similar appearance to those from the control group. The co-administration of melatonin to 5-FU-treated rats results in neurons retaining a circular shape and the structural integrity of the DG being retained, as illustrated in Figures 95 and 99. The small dark blue cells surrounding neurons are glial cells, indicated below the X in Figure 92.

The effects of 5-FU and melatonin on the CA1-2 region of the hippocampus are shown in Figures 100-107.

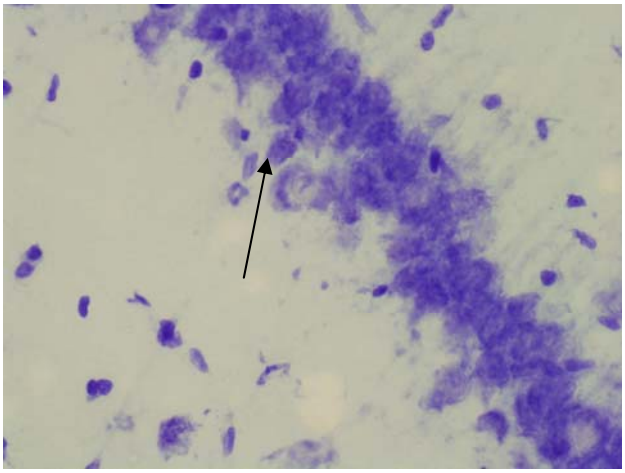


Figure 100: Photomicrograph of a typical CA1-2 region of a control rat, viewed at 400X magnification. The arrow shows a typical neuron

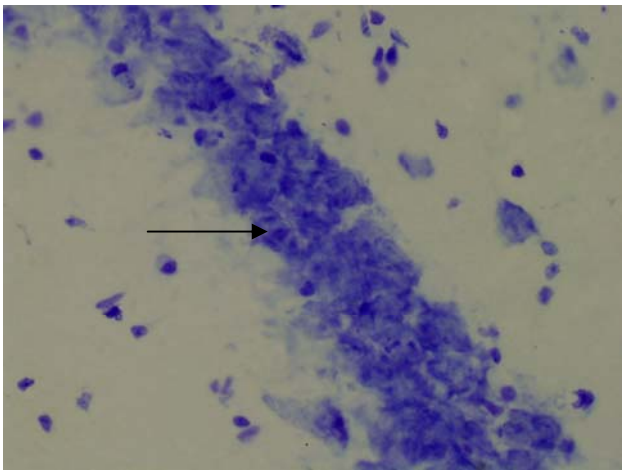


Figure 101: Photomicrograph of a typical CA1-2 region of a 5-FU-treated rat, viewed at 400X magnification. The arrow shows a typical neuron

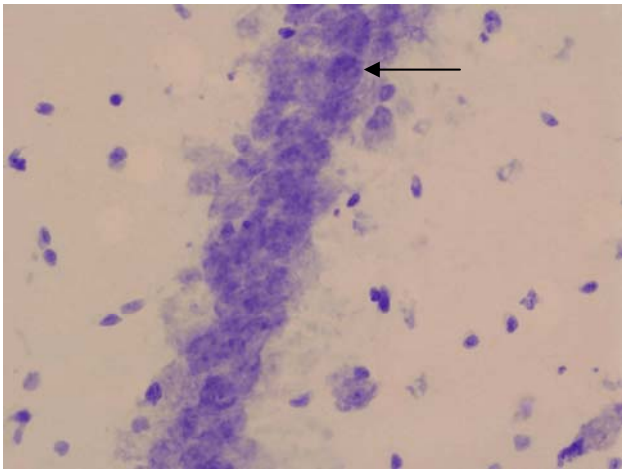


Figure 102: Photomicrograph of a typical CA1-2 region of a melatonin-treated rat, viewed at 400X magnification. The arrow shows a typical neuron

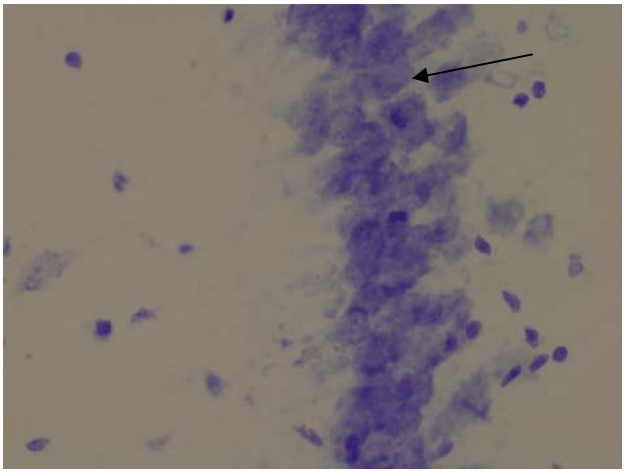


Figure 103: Photomicrograph of a typical CA1-2 region of a 5-FU- and melatonin-treated rat, viewed at 400X magnification. The arrow shows a typical neuron

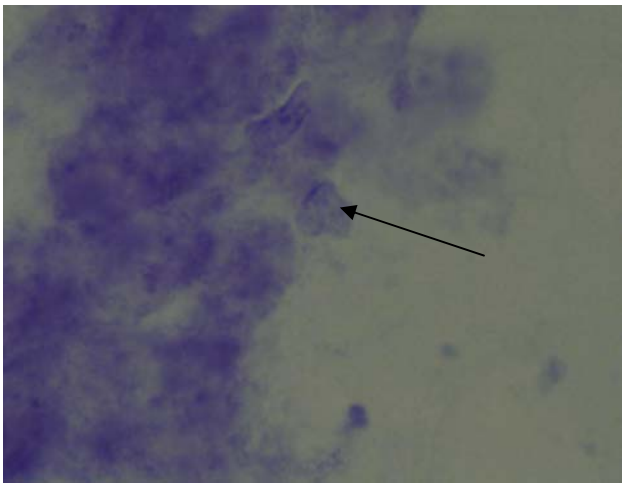


Figure 104: Photomicrograph of a typical CA1-2 region of a control rat, viewed at 1000X magnification. The arrow shows a typical neuron

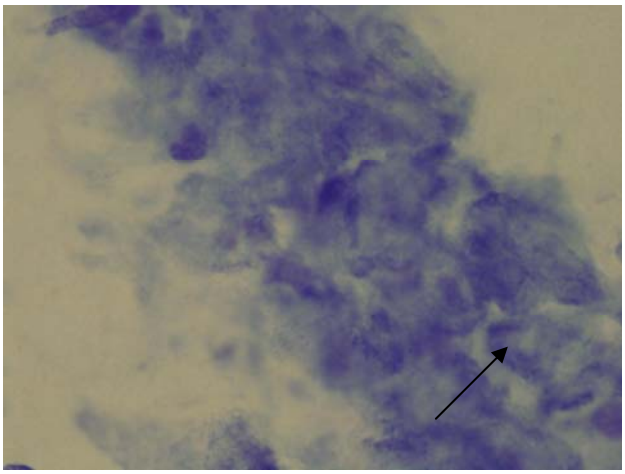


Figure 105: Photomicrograph of a typical CA1-2 region of a 5-FU-treated rat, viewed at 1000X magnification. The arrow shows a typical neuron

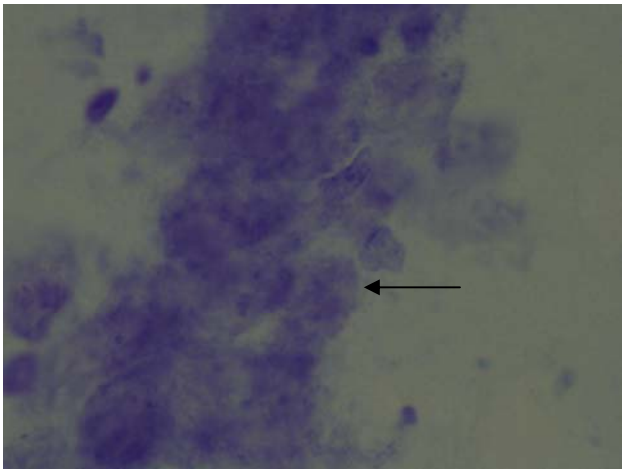


Figure 106: Photomicrograph of a typical CA1-2 region of a melatonin-treated rat, viewed at 1000X magnification. The arrow shows a typical neuron

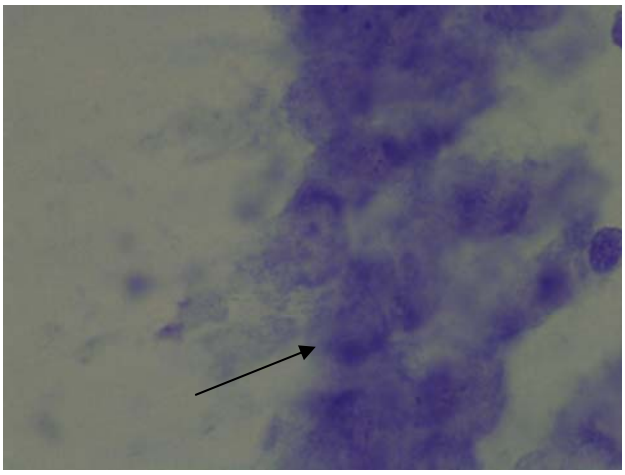


Figure 107: Photomicrograph of a typical CA1-2 region of a 5-FU- and melatonin-treated rat, viewed at 1000X magnification. The arrow shows a typical neuron

The above photomicrographs of the CA1-2 regions show that the administration of 5-FU results in a decrease in neuronal size and a loss of structural integrity, with the possible occurrence of cell lysis. The co-administration of melatonin counters this; neurons retain a more circular shape, and cell packing appears less disrupted, as shown in Figure 107.

Photomicrographs of the effects of 5-FU and melatonin on the CA3 region are provided in the following pages.

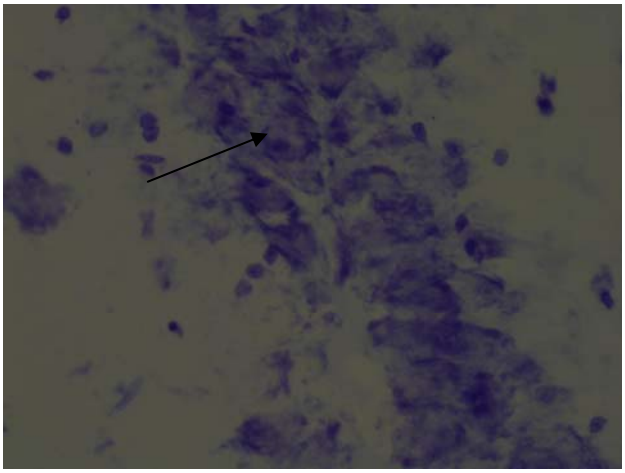


Figure 108: Photomicrograph of a typical CA3 region of a control rat, viewed at 400X magnification. The arrow shows a typical neuron

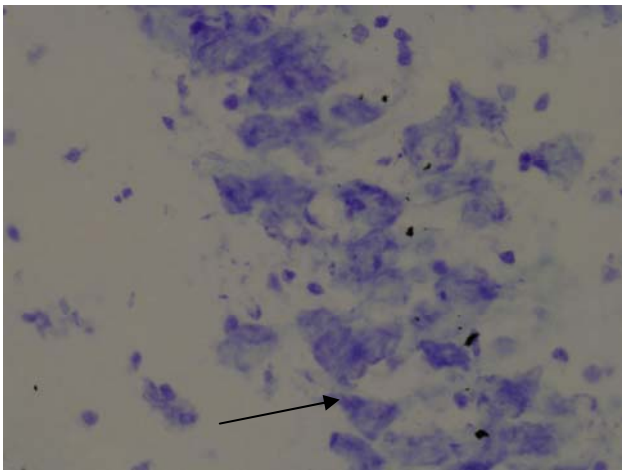


Figure 109: Photomicrograph of a typical CA3 region of a 5-FU-treated rat, viewed at 400X magnification. The arrow shows a typical neuron

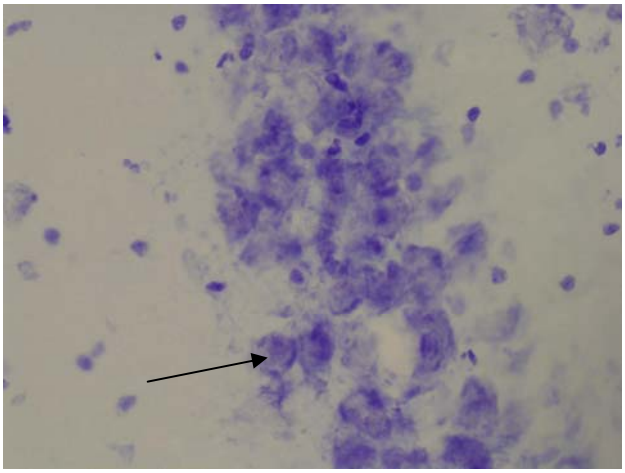


Figure 110: Photomicrograph of a typical CA3 region of a melatonin-treated rat, viewed at 400X magnification. The arrow shows a typical neuron

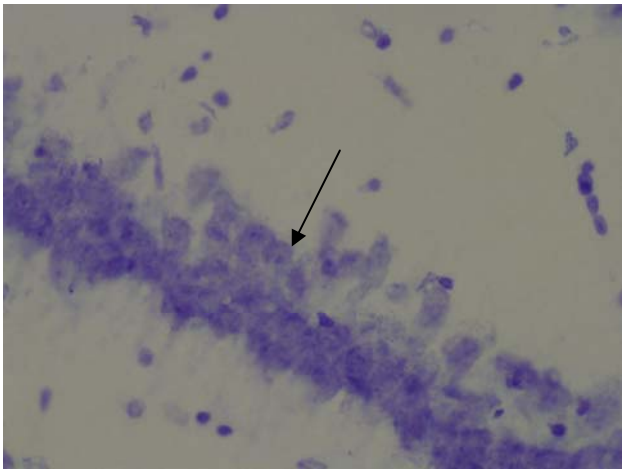


Figure 111: Photomicrograph of a typical CA3 region of a 5-FU- and melatonin-treated rat, viewed at 400X magnification. The arrow shows a typical neuron

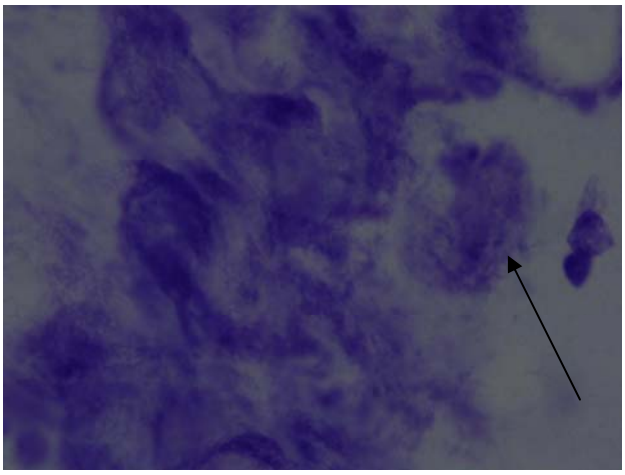


Figure 112: Photomicrograph of a typical CA3 region of a control rat, viewed at 1000X magnification. The arrow shows a typical neuron

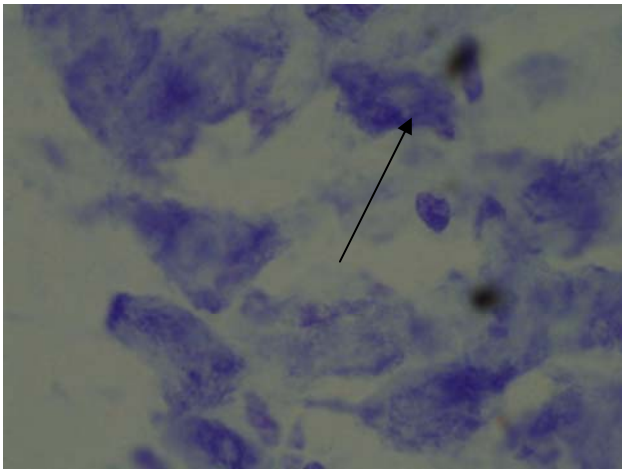


Figure 113: Photomicrograph of a typical CA3 region of a 5-FU-treated rat, viewed at 1000X magnification. The arrow shows a typical neuron

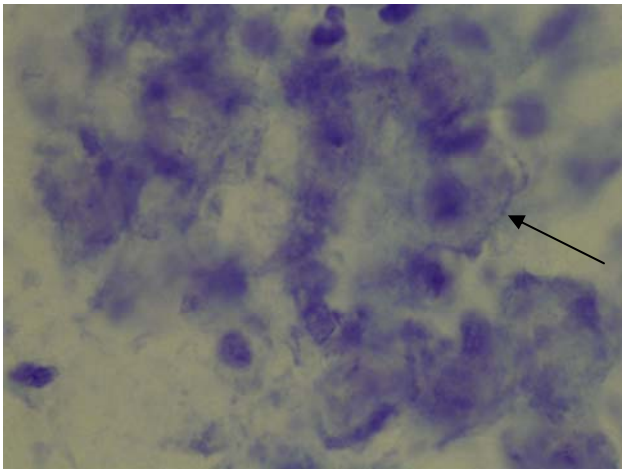


Figure 114: Photomicrograph of a typical CA3 region of a melatonin-treated rat, viewed at 1000X magnification. The arrow shows a typical neuron

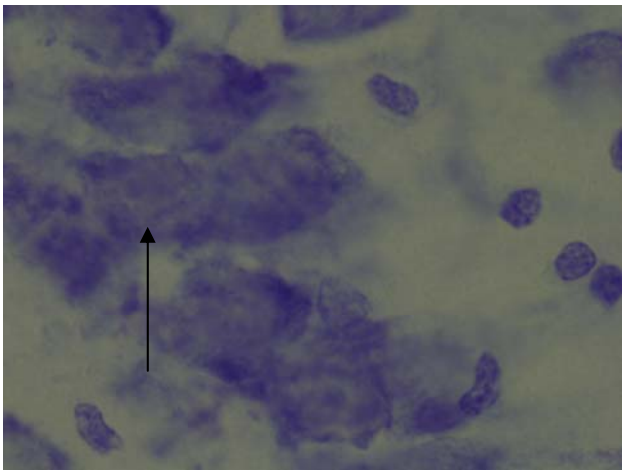


Figure 115: Photomicrograph of a typical CA3 region of a 5-FU- and melatonin-treated rat, viewed at 1000X magnification. The arrow shows a typical neuron

The photomicrographs of the CA3 region show that the administration of 5-FU significantly alters the morphology of this region of the hippocampus. As shown in Figures 109 and 113, the cells of the CA3 region are no longer closely packed; the spatial distribution of neurons in this region has thus been disrupted. Neurons are smaller and irregular in shape. The co-administration of melatonin results in less disruption of the closely-packed, band-like structure of the CA3 region. Neuronal integrity is less impaired, evidenced by less cellular shrinkage and loss of shape, as shown in Figures 111 and 115.

10.7 Discussion

It has been shown stereologically that 5-FU decreases the volume of the CA1-2 and CA3 regions of the hippocampus. The volume of the latter region is more significantly altered by 5-FU. The decrease in the volume of the DG is not statistically significant, but this may be a

statistical type II error as it has been noted by Schmitz and Hof (2005) that stereology may be associated with an increase in the probability of this type of error, which concludes that no significant difference exists between groups when such a difference does indeed occur.

The co-administration of melatonin counters 5-FU-induced decreases in the volumes of the CA1-2 and CA3 regions. The volume of the former region is only slightly increased, to levels still significantly lower than control values, whereas the volume of the CA3 region is similar to that of the control values. The effects of melatonin, particularly on the CA1-2 region, may have been more pronounced if the drug was administered at a higher dose. This agent is routinely administered at a dosage of 5 mg/ kg to attenuate kainic acid-induced hippocampal damage (Tan *et al*, 1998a), compared to the 1 mg/ kg used in this study.

Several possible reasons for 5-FU-induced decreases in CA1-2 and CA3 volume are proposed below:
(i) this agent could decrease the number of neurons and/ or glial cells in these regions. It is not possible to determine cell numbers in this study, as the unfixed nature of the brains hinders individual cell visualisation, which is necessary to count cells using stereology;
(ii) an increase in the lipid peroxidation of cell membranes occurs, resulting in the loss of cell contents and a subsequent decrease in cellular volume. It is shown in Chapter 8 that 5-FU induces lipid peroxidation, and that melatonin counters this;
(iii) 5-FU may impair neuro- and/ or synaptic plasticity, by inhibiting the synthesis of the genes required to express the enzymes and structural proteins required for these phenomena to occur. In addition, by decreasing NE levels (see Chapter 6), 5-FU is likely to inhibit LTP (Straube and Frey, 2003). The implications of impaired plasticity will be discussed below. 5-FU is routinely used in stem cell research to deplete cycling progenitor haemopoietic stem cells and direct the differentiation plasticity of these cells (Randall and Weissman, 1997; Graf, 2002). In neuroscience, 5-FU may also very well result in plastic changes that inhibit or promote the differentiation of certain cell types ;
(iv) neurons in the CA1-2, CA3 and DG regions are susceptible to the influence of stress, and the hippocampus plays a role in terminating the HPA axis (Badowska-Szalewska *et al*, 2006) (see 6.2.2.9.1.1). The CA3 region is very receptive to glucocorticoid stimulation. Enhanced hippocampal sensitivity to glucocorticoids could be a mechanism to ultimately blunt stress-induced cortisol responses (Murburg, 1997). Patients with post-traumatic stress disorder (PTSD) exhibit significantly decreased hippocampal volumes (Bremner *et al*, 1995, 1997; Gurvitis *et al*, 1996), and impaired short-term memory (Bremner *et al*, 1995). It was found that if the stress occurs early in life, neural plasticity could adapt to hippocampal damage and assign the processes involved in verbal memory to other structures, thereby attenuating the

extent of memory deficits (Bremner *et al*, 1997). 5-FU may possibly increase levels of the stress hormone and glucocorticoid, cortisol, and this might result in structural damage to the hippocampus (Badowska-Szalewska *et al*, 2006). The effects of 5-FU and melatonin on corticosterone, the predominant glucocorticoid in rats (Van Wyk, 1993), are investigated in Chapter 12. It has been found that stress decreases hippocampal volume and results in morphological changes and atrophy, due to an increase in glucocorticoid levels, a decrease in BDNF and the inhibition of neurogenesis (Bremner, 2006; Gould *et al*, 1999). Glucocorticoids inhibit metabolism within cells; enhance susceptibility to excitotoxins, such as glutamate (McEwen, 1999; McEwen and Magarinos, 1997; Watanabe, Gould and McEwen, 1992) (see 6.2.2.9.1.1) and impair neuronal ability to survive insults (Sapolsky *et al*, 1986). In a review article (Lucassen *et al*, 2006), however, it is noted that the effects of chronic stress on the volume of the hippocampus and cell number is modest, and that neuronal plasticity can be restored following cessation of the stress. Functional alterations may still be significant (Lucassen *et al*, 2006). It has been shown that exogenous melatonin administration decreases the affinity of corticosterone receptors in the hippocampus for corticosterone, thereby protecting neurons against glucocorticoid-induced neurotoxicity and preventing the loss of glucocorticoid receptors (Marinova *et al*, 1991); and

(v) recurrent depression is associated with a reduction in hippocampal volume, which may be due to increased levels of glucocorticoids, which enhance hippocampal atrophy (Sheline *et al*, 1999; American Scientist, 2000) (see 6.2.2.15). SSRIs, by increasing 5-HT levels, enhance cell division in the DG and this neurogenesis, which occurs over several weeks, may be an additional mechanism of antidepressant action (Jacobs, 1994; Jacobs and Azmitia, 1992). NE is also believed to enhance cell division occurring in the DG (American Scientist, 2000). In Chapter 6, it was found that 5-FU significantly decreases NE and 5-HT levels, thereby possibly leading to the development of depression. These decreases in neurotransmitter levels may thus inhibit neurogenesis in the hippocampus and result in a decrease in hippocampal volume. Melatonin was found to counteract this fall in neurotransmitter levels (see 6.4), and the resultant promotion of cell proliferation may account for melatonin's ability to increase the volume of the CA3 region when co-administered with 5-FU.

The abovementioned alterations in the volumes of hippocampal regions are visually supported by the photomicrographs, in particular Figures 108-111, which show that with the administration of 5-FU, the CA3 region loses its band-like appearance and the region appears smaller in diameter than the control CA3. The structural integrity of the closely-packed CA3 region of a 5-FU-treated rat is also impaired, with an increase in space between neurons; this could result in impaired neuronal signaling and the transmission of impulses. Neurons are furthermore smaller and irregular in shape, and cell lysis may have occurred, possibly due to

the enhanced lipid peroxidation of cell membranes. These deleterious effects are all attenuated by the co-administration of melatonin, and the hippocampal cells of rats that received combination therapy have a similar appearance to control cells.

As discussed in 10.4, it is believed that 5-FU induces the phenomenon of "chemobrain", or cognitive impairment, in breast cancer patients (Winocur *et al*, 2006; Anderson-Hanley *et al*, 2003). The abovementioned histological, morphological alterations induced by a very low dose of 5-FU in the hippocampus may well represent a mechanism by which such cognitive deficits occur. In addition, 5-FU has been previously shown to decrease NE levels, thus possibly precipitating depression (see Chapter 6), which may itself result in cognitive impairments. The co-administration of melatonin not only protects against kainic acid-induced neuronal damage, but also alleviates the neurobehavioural manifestations of this toxicity, such as seizures, due to melatonin's antioxidant and free radical-scavenging properties, and ability to decrease lipid peroxidation (Tan *et al*, 1998b). The effects of combination melatonin therapy with 5-FU may very well be similar.

Chronic 5-FU administration may be associated with a degree of cognitive recovery (Winocur *et al*, 2006) or even enhancement (Lee *et al*, 2006) (see 10.4), and this could be due to the generation of new hippocampal cells, which occurs throughout life in the DG (Piatti *et al*, 2006). Compensatory mechanisms of plasticity could also be developed, in which memory processes are assigned to different structures, as mentioned before.

10.8 Conclusions

The stereological and pictorial results presented in this Chapter confirm that 5-FU induces hippocampal toxicity, in particular morphological alterations and a decrease in hippocampal volume. The CA3 region is most susceptible to 5-FU-induced toxicity, and the co-administration of melatonin is beneficial in alleviating the severity of this damage.

CHAPTER 11

THE EFFECTS OF 5-FLUOROURACIL AND MELATONIN ON LOCOMOTOR ACTIVITY IN RATS

11.1Introduction

The effects of melatonin on locomotor activity are widely documented. Whilst most reports show that melatonin decreases locomotor activity in rats, there are some conflicting findings. This may be due to a variety of experimental conditions being used and limitations in study design, which make comparison of the studies difficult.

Wong and Whiteside (1968) have shown that a dosage of 10 µg/ day of melatonin to food-deprived rats results in a decrease in the extent of running on a wheel. This supports the findings of Reiss *et al* (1963), who have shown that pinealectomy is associated with enhancement of treadwheel activity. Pinealectomy also increases locomotor activity, measured by the open field and activity cage models (Sampson and Bigelow, 1971; Karppanen *et al*, 1973). Male rats designated as "slow runners" have been shown to possess a greater density of pineal cells, and thus higher endogenous levels of melatonin, compared to "fast runners" (Reiss *et al*, 1967). Melatonin also decreases spontaneous locomotor activity in mice, in a dose-dependent manner (Sugden, 1983).

It has been found that bilateral injections of the indoleamine directly into the nucleus accumbens induce inhibition of locomotor activity (Gaffori and Van Ree, 1985). This inhibition is dose-dependent, with 0.1 ng being the lowest effective dose, 10 ng inducing maximal effect and 1000 ng resulting in no effect on locomotor activity. The rats in this study, in addition to displaying a decrease in locomotor activity, demonstrate increased emotivity, evidenced by an increase in the frequency of sniffing and grooming (Gaffori and Van Ree, 1985). The intranigral administration of 0.01 µg and 0.1 µg melatonin has been found to decrease spontaneous locomotor activity by 50% and 80%, respectively (Bradbury *et al*, 1985). This inhibition of activity is partially reversed by the DA_2 antagonist sulpiride, thus indicating that melatonin may modulate dopaminergic activity (Burton, 1989). This is supported by a decrease in dopaminergic functioning in the limbic and striatal regions (Bradbury *et al*, 1985). The finding that the sulpiride-induced reversal of locomotor inhibition occurs only to a certain extent and not totally suggests that the serotonergic system is also affected by melatonin (Bradbury *et al*, 1985), since 5-HT is a key factor in the release of DA from the substantia nigra and may be involved in the nigro-striatal pathway (Williams and

Davies, 1983). Gaffori and Van Ree (1985), in the abovementioned experiment, injected melatonin into the nucleus accumbens and demonstrated that haloperidol and sulpiride do not antagonise the effects of melatonin on locomotor activity. Such antagonism is achieved by the injection of 5-HT and various antidepressants into the nucleus accumbens, thus highlighting the involvement of 5-HT in locomotor changes induced by melatonin (Gaffori and Van Ree, 1985).

Studies on other animal species have also shown that melatonin administration decreases locomotor activity. For example, Hendel and Turek (1978) have found that the perch hopping activity of sparrows significantly decreases, leading the authors to suggest that melatonin can influence the circadian rhythm of locomotor activity, and may be involved in the extent and timing of activity demonstrated in the avian activity-rest cycle. The ability of melatonin to entrain the locomotor activity of lizards is discussed in 11.2. Entrainment in this context refers to the ability of melatonin to align the circadian rhythm of locomotor activity with its own rhythm, particularly with respect to phase and period (http://en.wikipedia.org/wiki/Entrainment_%28chronobiology%29). Pinealectomy in the lizard has been demonstrated to decrease the amount of daily locomotor activity (Underwood, 1981).

Some investigators have, however, found that melatonin administered at a dosage of between 50 µg/ day and 200 µg/ day fails to alter locomotor activity in rats (Kastin *et al*, 1973; Kovács *et al*, 1974; Sampson and Bigelow, 1971). Rats are, furthermore, nocturnal animals, and demonstrate greatest locomotor activity at night, when physiological levels of melatonin are highest (Burton, 1989). Pre-treatment of mice with melatonin, furthermore, counters sleep deprivation-induced decreases in locomotor activity (Kalonia and Kumar, 2007). There are some reports also of pinealectomy either exerting no effect (Quay, 1968, 1970; Kovács *et al*, 1974; Relkin, 1970a, 1970b; Remley *et al*, 1969) or decreasing locomotor activity (Kincl *et al*, 1970). In view of these conflicting findings, it is suggested by Burton (1989) that melatonin exerts an indirect effect on locomotor activity, which may be mediated by serotonergic and dopaminergic mechanisms in the aforementioned regions of the brain.

11.2 Circadian rhythm of locomotor activity

The SCN, or circadian clock, and the relationship between this and the pineal gland, is described in 1.2.10.1. The SCN plays an important role in regulating the sleep-wake cycle, an indicator of which is the occurrence or absence of locomotor activity (Redlin, 2001). Although the circadian rhythm of locomotor activity is not regulated as precisely as melatonin rhythms, the timing of locomotor activity can very accurately indicate the phase of the sleep-

wake cycle in nocturnal animals, such as rats (Redlin, 2001). In diurnal animals, light stimulates activity (Gander and Moore-Ede, 1983; Kas, 1999; Rajaratnam and Redman, 1999), whereas in nocturnal animals a rapid decrease in locomotor activity occurs (Redlin and Mrosovsky, 1999; Borbely, 1978; Borbely and Huston, 1974). Conversely, the application of dark pulses to nocturnal animals during daylight hours can stimulate locomotor activity (Redlin, 2001)

The administration of 1 mg/ kg/ day of melatonin in the morning for two weeks is sufficient to synchronise the circadian rhythm of locomotor activity in rats (Baturin and Arushanyan, 1990). The same treatment at noon does not exert an effect, and the sensitivity of rats to melatonin varies (Baturin and Arushanyan, 1990). In a study examining the relationship between the circadian rhythms of melatonin synthesis and locomotor activity in the pineal gland, Hastings *et al* (1987) have found that a decrease in the duration of the photoperiod increases both melatonin production and locomotor activity, and that during the re-entrainment period, these two rhythms are held in a stable relation. These authors surmise that one signal regulates both melatonin production and locomotor activity through as yet unknown mechanisms (Hastings *et al*, 1987). Laakso *et al* (1995) have also shown that changes in the rhythm of melatonin synthesis during entrainment as a result of a shorter photoperiod mirror the changes in the locomotor activity rhythm. This also occurs when the transition from light to dark is gradual, involving a twilight phase of two hours, instead of abrupt (Laakso *et al*, 1992).

The effects of melatonin on the circadian rhythm of locomotor activity may, however, be limited, as it has been found that the daily administration of melatonin to rats kept in constant darkness does not entrain a free-running rhythm of locomotor activity (Bonnefond *et al*, 1993). In another study, however, Hyde and Underwood (1995) report that the administration of melatonin every alternate day to lizards entrains locomotor activity rhythms to a periodicity of twenty-four hours. In pinealectomised lizards, a dose of 0.1 μg/ day of melatonin results in mid-infusion levels similar to mid-dark levels of melatonin in pineal-intact lizards, thus supporting the hyothesis that the pineal gland, by secreting melatonin according to a circadian rhythm, exerts an important regulatory role on the circadian rhythm of locomotor activity (Hyde and Underwood, 1995). These findings are in agreement with those of Golombek and Cardinali (1993), who have demonstrated that melatonin accelerates the re-entrainment of locomotor activity rhythms of hamsters exposed to phase advancement of the light-dark cycle. Control animals require a day per hour of phase advance in order for resynchronisation of locomotor activity rhythms to occur; the administration of melatonin decreases this resynchronisation time by 34% (Golombek and Cardinali, 1993). These effects of melatonin

are abolished by the benzodiazepine (BZD) antagonist flumazenil, thus suggesting that melatonin may exert its effects on the circadian rhythm of locomotor activity through a BZD-type mechanism (Golombek and Cardinali, 1993). This is supported by a study by Shaji and Kulkarni (1998), in which the CNS depressant effects of melatonin, evidenced by a decrease in locomotor activity and body temperature, and enhancement of phenobarbitone-induced hypnosis, are likened to the effects of BZDs. BZDs function by enhancing binding to $GABA_A$ receptors. γ-Aminobutyric acid (GABA) is an inhibitory amino acid, and binding to its receptors facilitates the entry of Cl^- into the cell, subsequently hyperpolarising the cell membrane potential and decreasing its excitability to impulses. This results in sedation and, at higher doses, hypnosis (McNamara, 2001). It is believed that melatonin similarly enhances $GABA_A$ binding (Shaji and Kulkarni, 1998), due to similarities with the CNS profiles of agents, such as BZDs, that modulate GABA binding (Holmes and Sugden, 1982; Coloma and Niles, 1988).

The timing of melatonin administration and the intensity of light may be important determinants on the occurrence of entrainment of locomotor activity rhythms, as it has been found that synchronisation of these rhythms occurs only in rats exposed to dim light or constant darkness, and not to bright light (Marumoto *et al*, 1996). In a study to determine the conditions under which melatonin entrains locomotor activity in hamsters, it was found that dim lighting is necessary, and that the effects of melatonin may occur via alteration of the functioning of the SCN (Schuhler *et al*, 2002). Furthermore, the entraining ability of melatonin is dose-dependent (Warren *et al*, 1993; Cassone *et al*, 1986), occurs only at pharmacological doses (Armstrong and Redman, 1985; Slotten *et al*, 1999; Cassone *et al*, 1986) and when the onset of melatonin and locomotor activity rhythms coincide (Redman *et al*, 1983; Pitrosky *et al*, 1999). The median effective dose for entraining locomotor activity in rats has been shown to be 5.45 ± 1.33 μg/ kg of melatonin daily for a period of two weeks (Cassone *et al*, 1986). The administration of melatonin prenatally to Syrian hamsters results in synchronisation of the onset of locomotor activity of the new-born pups for three weeks (Davis and Mannion, 1988).

In addition to its effects on locomotor activity rhythms, melatonin also entrains the rhythms of 6-sulphatoxymelatonin levels (see 1.3.5) (Brown *et al*, 1993) and body temperature (Golombek and Cardinali, 1993; Brown *et al*, 1993), the regulation of the latter being very closely associated with locomotor activity (Isobe *et al*, 2002). It is known that melatonin plays a key role in setting body temperature (Haim and Zisapel, 1997; Cagnacci *et al*, 1997). The indoleamine decreases the core body temperature of chickens in a dose-dependent manner (Rozenboim *et al*, 1998), which may represent a key component of melatonin's ability to alter

circadian rhythms (Shaji and Kulkarni, 1998). The temperature of peripheral skin is, meanwhile, increased (Rozenboim *et al*, 1998). In a study to investigate the effects of melatonin on body temperature and locomotor activity of rats at different times of the day, it has been shown that melatonin dose-dependently decreases locomotor activity both in the light and dark phases (Isobe *et al*, 2002), which may be due to the sedative effects of the agent (Isobe *et al*, 2002; Sugden, 1983; Holmes and Sugden, 1982). It was found that melatonin induces hyperthermia during the light phase, and hypothermia during the dark phase (Isobe *et al*, 2002; Cagnacci *et al*, 1997). Melatonin-induced hypothermia may be due to the inhibition of prostaglandin synthesis (see 14.3.1) and NO formation (see 14.9) (Nava *et al*, 1997). The hyperthermia noted during the light phase may be mediated by a melatonin-induced decrease in the neuropeptide AVP (see 1.2.9.1) (Isobe *et al*, 2002, 2001a). Melatonin's effect on body temperature rhythms may be by acting at the thermoregulatory centre, situated in the hypothalamus (Cassone *et al*, 1986), which also houses the SCN (Isobe *et al*, 2002). Melatonin may set the body temperature to be consistent with the metabolic rate (Shaji and Kulkarni, 1998; Dubocovich, 1995).

It is believed that melatonin exerts a specific effect in regulating various types of circadian rhythms (Brown *et al*, 1993). This may be by acting as a messenger between both the pineal gland and eyes, which may function as circadian pacemakers (see 1.2.9.1), and the rest of the multioscillatory circadian system (Underwood, 1981). This suggestion comes from the finding that the long-term administration of melatonin to blinded and/ or pinealectomised lizards increases the length of the activity rhythm and decreases the total amount of daily locomotor activity, which may indicate the interaction of melatonin with extraocular and extrapineal sites (Underwood, 1981).

11.3 Locomotor activity in cancer patients

In a study of 11.3% of all nursing home admissions with cancer in the United States, it was found that 55% of individuals relied on a wheelchair as the main means of locomotion (Buchanan *et al*, 2005). Cancer-associated wasting syndrome is characterised by a decrease in locomotor activity, and a loss of body weight, muscle and fat tissue. This syndrome is associated with the presence of pro-inflammatory cytokines, such as IL-5, IL-6, IL-11 and leukaemia-inducing factor (Onuma *et al*, 2005). It has recently been shown that parathyroid hormone-related protein (PTHrP) inhibits locomotor activity and energy metabolism, leading to the development of cancer-associated wasting syndrome (Onuma *et al*, 2005).

11.4 Effects of 5-FU on locomotor activity

To our knowledge, there is only one report in the literature on the effects of 5-FU on locomotor activity, by Matsuura *et al* (1985). In this study, at doses between 62.5 mg/ kg and 500 mg/ kg, administered orally, 5-FU does not alter methamphetamine-induced locomotor activity, spontaneous activity, gross behaviour or rectal temperature in rats and mice (Matsuura *et al*, 1985).

The phenomenon of "chemotherapy-related malaise" is discussed by Malik *et al* (2006). Cancer patients on anticancer drug therapy often experience fatigue, malaise, symptoms of dyspepsia, decreased gastric emptying and anorexia. Disruptions in circadian rhythms, also experienced, are due to low physiological levels of melatonin, as mentioned in 1.3.2. It is believed that this reponse of the body to cytotoxic drugs is similar to the manner in which the body responds to infections and toxins, described as "illness behaviour" (Andrews and Morrow, 2002). In a study to determine the effects of the antineoplastic agent cisplatin on locomotor activity, which is also a feature of "illness behaviour", it was found that cisplatin significantly decreases locomotor activity in the first eight days of treatment, particularly during the dark phase, and that this activity recovers in the subsequent seven days of the study (Malik *et al*, 2006). These authors were not able to ascertain whether the decrease in locomotor activity was independent of a significant decrease in food intake. It is suggested that the administratin of cisplatin to rats over a two week period results in behavioural changes, for example in locomotor activity, that strongly resemble those reported by patients being treated with antineoplastic drugs.

11.5 Experimentation

Due to the well-documented effects of melatonin on locomotor activity and the circadian rhythms regulating this (see 11.1 and 11.2), the paucity of information on the effects of 5-FU on locomotor activity and the decrease in physical activity demonstrated by cancer patients, it was thus decided to investigate the effects of melatonin on locomotor activity in the absence and presence of 5-FU. The measurement of locomotor activity is a behavioural model frequently employed in the primary stages of drug evaluation and commonly used as an animal model both of depression and movement disorders. Its robustness as a model of depression is limited by the findings that both increases and decreases in locomotor activity can reflect depression (Willner, 1990). As a model of movement disorders, such as Parkinson's disease and tardive dyskinesia, an assessment of locomotor activity offers insight into possible dopaminergic impairment. A novel application of the locomotor activity model is in determining the effects of various stressors on the immune system (Wrona *et al*, 2004). It has been found, for example, that the responses of rats to certain stressful locomotor activity-

measuring tests, such as the open field test, are associated with immunological changes, particularly the suppression of spleen and blood NK cell activity (Wrona *et al*, 2004).

Locomotor activity in this study was measured using the Noldus Ethovision® video-tracking system. The parameters investigated were: (i) total distance moved; (ii) maximum distance moved; (iii) mean velocity; (iv) total duration of movement; and (v) rearing frequency.

11.5.1 Materials, animals, reagents and equipment

5-Fluorouracil and melatonin were purchased from the Sigma Chemical Corporation, St Louis, MO, USA. All solutions were prepared using deionised water (Milli R/Q System, Millipore®), and absolute ethanol at a final concentration of 0.67% (v/ v) was used as a co-solvent to dissolve melatonin.

Thirty-two male Wistar rats, each weighing between 200g and 300g, and cared for as outlined in Appendix A, were used. Male rats were selected to prevent oestrous effects on locomotor activity. Black henna, used as a dye, was purchased locally. The Noldus EthoVision® Video Tracking System for Automation of Behavioral Experiments and software (Noldus Information Technology, Wageningen, The Netherlands, version 3: Pro), with an attached Picolo camera, was used to measure locomotor activity.

11.5.2 Method

Thirty-two male Wistar rats were divided into four groups of eight rats per group: (i) control, (ii) 5-FU, (iii) melatonin, and (iv) a group receiving both drugs. Since the Noldus EthoVision® video-tracking system used can only detect two rats per group, and a maximum of eight rats simultaneously, the animals were monitored and treated in stages, as outlined below. Two rats from each treatment group were subjected to the protocol described below, at the same time; this process was repeated a further three times on the remaining rats. The study thus ran for a total of four weeks, with two rats per treatment group undergoing the following protocol every week. The locomotor activity of two rats from each group was measured prior to drug treatment, for a twelve hour period, from 07h00 to 19h00. Each set of two rats was placed in a separate cage, and all four cages were placed in a black tank, as shown in Figure 116. Since the Noldus EthoVision® video-tracking system differentiates between the two rats in each cage using the monochrome object detection method, based on size, the bottom half of one rat in each cage was dyed black with henna the day prior to video recording. The video tracking system thus detects the undyed animal as “large” and the dyed one as “small”. Using the Noldus EthoVision® software, each cage was delineated as a separate arena, and the area within each cage as a zone, according to the Noldus EthoVision® instruction manual. The

container of food and water in the top left-hand corner of each arena was designated a hidden zone, as rats often nestle underneath it and such designation allows for the rats to be detected even though not visible to the camera. Distance was calibrated using a 30 cm ruler. The trial protocol was set, with the trial duration set for 12 hours, the sample rate at 5/ s and the monochrome object detection method being grey scaling. The background noise, or light, was manually updated prior to each trial. Grey scaling is the fastest method of detecting objects, but is heavily influenced by the intensity of background lighting. Prior to the commencement of the trial, the limits of the noise threshold were set. These values are the minimum and maximum levels between which the video tracking system will detect and track the two biggest objects that show up as red or orange. Other pixels within these limits that show up as green will not be tracked. The noise threshold is manually updated before each trial, in order to eliminate as much background light as possible and to ensure that all rats are detected. The light bulb was left on in the room in which the experiment was run, for the entire duration of the trial, in order to allow the video-tracking system to detect the rats, and the windows covered with black paper to reduce ambient light filtering through. After the trial, data was analysed using the Noldus EthoVision® video tracking system software, and the following parameters calculated for each rat:

(ii) the total distance moved;

(iii) the maximum distance moved;

(iv) the mean velocity;

(v) the total duration of movement, expressed both in minutes and as a percentage; and

(vi) the frequency of rearing, defined by the rat standing up on its hind legs and thus measured by a change in surface area.

On the day following this pre-treatment trial, the two rats from each group were subjected to the five-day treatment protocol described in Table 10 (see 3.6.2.2). On the sixth day, the rats were subjected to another twelve-hour video recording trial as described above. This procedure was repeated on all sets of two rats. ANOVA, followed by the Student-Newman-Keuls mulptiple range test, was used to determine if there were any statistically significant differences in the pre- and post-treatment locomotor activities of rats across the groups.

Figure 116: Picture of the cages, or arenas, each containing two rats, used in the video-tracking of locomotor activity over 12 hours

11.6 Results

Figure 117 below shows the effects of 5-FU and melatonin on the mean total distance moved. 5-FU significantly increases the total distance moved, while melatonin does not change this significantly. The group of rats receiving both drugs shows a marked increase in the mean total distance moved, to levels higher than that induced by 5-FU alone.

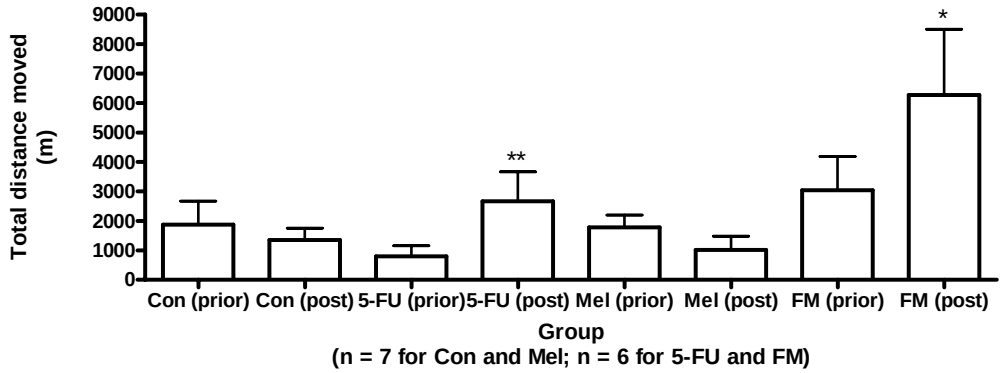


Figure 117: The effects of 5-FU and melatonin on the total distance moved by rats over 12 hours of constant bright light exposure

* $p<0.05$ compared to FM (prior); and ** $p<0.1$ compared to 5-FU (prior), after analysis by ANOVA followed by the Student-Newman-Keuls multiple-range test. Con = Control; FM = 5-FU + Melatonin; prior = total distance moved prior to five days of drug treatment; post = total distance moved post five days of drug treatment. Each bar represents the mean ± SEM

The mean maximum distances moved at a time by rats in each group are shown in the next Figure. There are no significant differences between the groups after drug treatment.

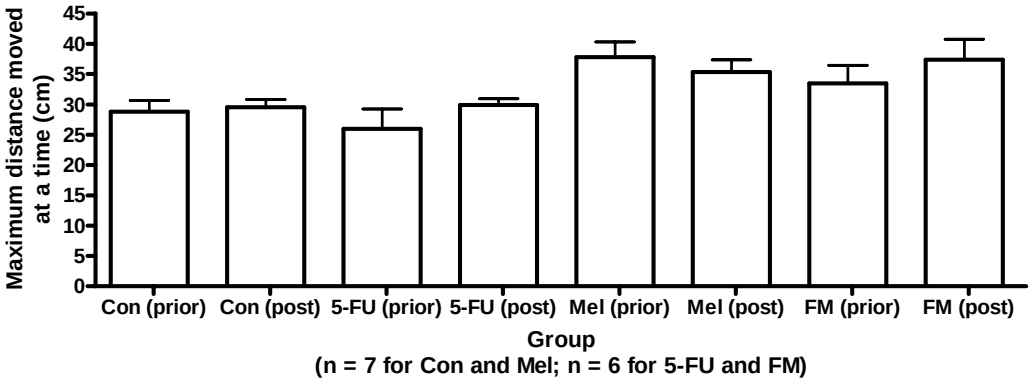


Figure 118: The effects of 5-FU and melatonin on the maximum distance moved at a time by rats over 12 hours of constant bright light exposure

Con = Control; FM = 5-FU + Melatonin; prior = maximum distance moved at a time prior to five days of drug treatment; post = maximum distance moved at a time post five days of drug treatment. Each bar represents the mean ± SEM

The effects of 5-FU and melatonin on the mean velocity of the rats is shown in Figure 119 below. It is evident that 5-FU significantly increases the mean velocity, from a pre-treatment value of 2.08 cm/ s to 6.18 cm/ s. Melatonin has no effect on the velocity of rats, but the group receiving both 5-FU and melatonin show a significant increase in mean velocity, from 7.04 cm/ s to 16.57 cm/ s.

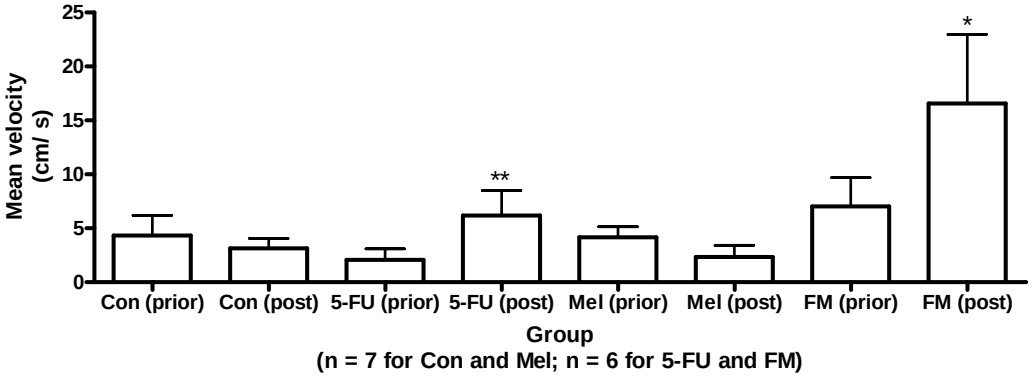


Figure 119: The effects of 5-FU and melatonin on the mean velocity of rats over 12 hours of constant bright light exposure

* $p<0.05$ compared to FM (prior); and ** $p<0.1$ compared to 5-FU (prior), after analysis by ANOVA followed by the Student-Newman-Keuls multiple-range test. Con = Control; FM = 5-FU + Melatonin; prior = mean velocity prior to five days of drug treatment; post = mean velocity post five days of drug treatment. Each bar represents the mean ± SEM

Figures 120 and 121 illustrate the effects of 5-FU and melatonin on the total duration of movement, expressed in minutes and as a percentage of the total duration of the trial, respectively. These Figures show that 5-FU significantly increases the total duration of movement carried out by rats, from 77.54 mins (10.77%) prior to drug treatment, to 198.48 mins (27.57%). Melatonin decreases the total duration of movement, from 126.64 mins (17.59%) to 79.47 mins (11.04%). The group of rats receiving both 5-FU and melatonin do not demonstrate any alterations in levels.

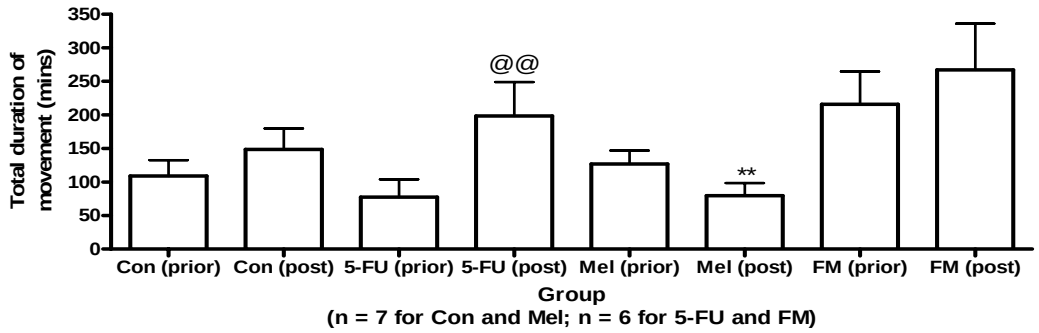


Figure 120: The effects of 5-FU and melatonin on the total duration of movement of rats over 12 hours of constant bright light exposure

$^{@@}$ $p<0.05$ compared to 5-FU (prior); and ** $p<0.1$ compared to Mel (prior), after analysis by ANOVA followed by the Student-Newman-Keuls multiple-range test. Con = Control; FM = 5-FU + Melatonin; prior = total duration of movement prior to five days of drug treatment; post = total duration of movement post five days of drug treatment. Each bar represents the mean ± SEM

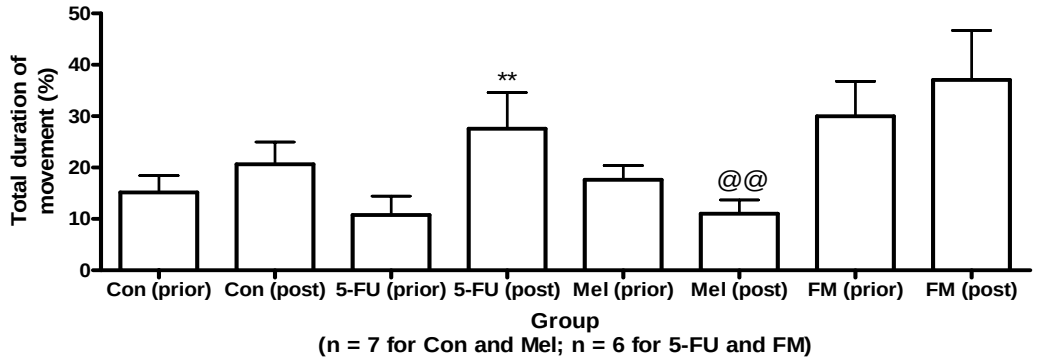


Figure 121: The effects of 5-FU and melatonin on the percentage of total duration of movement of rats over 12 hours of constant bright light exposure

** $p<0.1$ compared to 5-FU (prior); and $^{@@}$ $p<0.1$ compared to Mel (prior), after analysis by ANOVA followed by the Student-Newman-Keuls multiple-range test. Con = Control; FM = 5-FU + Melatonin; prior = total duration of movement prior to five days of drug treatment; post = total duration of movement post five days of drug treatment. Each bar represents the mean ± SEM

Figure 122 shows that 5-FU has no effect on the frequency of rearing demonstrated by rats, but that melatonin significantly decreases the incidence of this from 2027.43 to 870.29. The group of rats receiving both drugs shows no alterations in the occurrence of rearing.

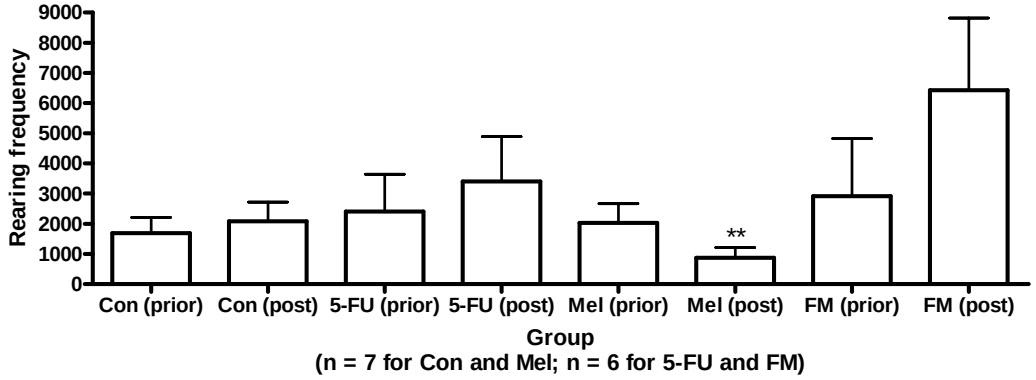


Figure 122: The effects of 5-FU and melatonin on the incidence of rearing demonstrated by rats over 12 hours of constant bright light exposure

** $p<0.1$ compared to Mel (prior) after analysis by ANOVA followed by the Student-Newman-Keuls multiple-range test. Con = Control; FM = 5-FU + Melatonin; prior = rearing frequency prior to five days of drug treatment; post = rearing frequency post five days of drug treatment. Each bar represents the mean ± SEM

11.7 Discussion

The results presented in Figures 117-122 show that 5-FU increases the total distance moved, duration of movement and mean velocity. Melatonin decreases the total duration of movement and the frequency of rearing. The co-administration of 5-FU to rats treated with melatonin significantly increases the total distance moved and the mean velocity, and abolishes the effects of melatonin on rearing frequency.

The melatonin-induced decreases in the total duration of movement and rearing frequency may be due to the CNS depressant effects of this agent (see 11.2). Although in the present study melatonin does not significantly decrease the total distance moved by rats over a twelve hour light period, this may be due to the duration of treatment being short, since melatonin is reported to entrain locomotor activity when administered for a period of two weeks (see 11.2). This study shows that melatonin decreases locomotor activity in rats, evidenced by a decrease in the total duration of movement and rearing, which is in agreement with most reports in the literature (see 11.1 and 11.2). This inhibitory effect of melatonin is abolished with the co-administration of 5-FU, which exerts an excitatory and stimulant effect, evidenced by an increase in the mean velocity and total distance moved by the rats. This may also indicate a

possible anxiogenic effect of 5-FU. In a study of patients receiving both cisplatin and 5-FU, it was found that 42% of patients displayed anxiety, as measured by the Manifest Anxiety Scale (Fujii *et al*, 2001). In another study of breast cancer patients being treated with CMF (see 1.2.8.7), it was found that patients exhibit insomnia, which induces anxiety, during the active phase of drug treatment (Kuo *et al*, 2006). Although neither of these two studies determined which specific antineoplastic agent is responsible for inducing anxiety, it cannot be ruled out that 5-FU exerts such an effect. Significant alterations in locomotor activity may also be an indicator of depression (see 11.5), and it was hypothesised in Chapter 6 that 5-FU may induce depression due to its lowering of 5-HT, DA and NE levels.

The ability of melatonin to decrease the frequency of rearing, an indicator of aggressive behaviour due to elevated arterial blood pressure (McGregor *et al*, 2004; Dielenberg and McGregor, 2001; Velíçek, 2006), suggests that melatonin has anti-aggressive effects. The aetiology of aggression, which is behaviour that is destructive to objects, others or the self, is complex and there are a variety of pathological causes, one of which may be altered levels of key neurotransmitters, some of which are discussed below (Swann, 2003; Eichelman, 1990):
(i) low levels of 5-HT are associated with the development of aggression (Brown *et al*, 1979; Brown and Linnoila, 1990). In Chapter 6 it was found that 5-FU significantly decreases 5-HT levels in the forebrain; it might thus be expected that 5-FU induces aggressive behaviour. This was not found to be the case, in this Chapter. This indicates the involvement of other neurotransmitter systems in the aetiology of aggression, or perhaps that 5-FU induces aggressive behaviour only at higher doses. It was found, however, in Chapter 6 that melatonin increases 5-HT levels, and this may account, in part, for the decrease in rearing frequency noted in the present Chapter;
(ii) high levels of the catecholamines DA and NE (Swann, 2003; Eichelman, 1990). It Chapter 6 it was shown that 5-FU decreases the levels of both these neurotransmitters, thus perhaps accounting for the finding in this Chapter that 5-FU does not alter rearing frequency in rats. Melatonin, however, increases the levels of these neurotransmitters (see 6.4), but in this Chapter was found to exert an anti-aggressive effect. This discrepancy may be due to the hypothesis that altered levels of one neurotransmitter are insufficient to induce aggression; Swann (2003) suggests that fluctuations in NE levels, superimposed on an imbalance between 5-HT and DA levels, are necessary to result in impulsive aggression (Swann, 2003; Oquendo and Mann, 2000); and
(iii) a decrease in the functioning of inhibitory amino acids, notably GABA, and thus a shift to the effects of excitatory amino acids, such as glutamate (Swann, 2003; Lang *et al*, 1995; Eichelman, 1990). The ability of melatonin to enhance GABAergic functioning is discussed

in 11.2; this may be an additional mechanism by which the indoleamine decreases rearing frequency and thus aggression.

The implications of these findings for cancer patients warrant further investigation. As mentioned before, cancer patients demonstrate disrupted circadian rhythms (see 11.4) and those receiving drug cocktails including 5-FU show a likelihood of experiencing insomnia. The findings of this Chapter suggest that 5-FU-induced anxiety and depression may be causative factors that contribute to compromised quality of life. The use of melatonin in cancer patients would strengthen impaired circadian rhythms, induce sleep, and exert an anti-depressant, anti-aggressive and anxiolytic effect. These beneficial effects of melatonin are limited when the agent is co-administered with 5-FU, as it is shown in this Chapter that the co-administration of melatonin does not alter the total distance moved by rats, or the mean velocity, and it is suggested that this is due to the relatively short period of five days of drug treatment.

11.8 Conclusions

It has been found that 5-FU significantly increases the locomotor activity of rats exposed to twelve hours of constant bright light, evidenced by increases in the total distance moved, total duration of movement and mean velocity. It is suggested that this excitatory effect may be due to 5-FU-induced anxiety and/ or depression. The use of melatonin, in the absence of 5-FU, decreases the rearing frequency of rats and the total duration of movement. This is due to the sedative and possibly anti-aggressive effects of the agent. The administration of 5-FU to rats receiving melatonin treatment abolishes the effects of melatonin. It is thus recommended that whilst melatonin co-therapy is useful in cancer patients, to normalise physiological parameters and enhance patients' quality of life, the administration of this agent on a chronic basis is necessary in order to effectively counteract 5-FU-induced adverse effects.

CHAPTER 12

DETERMINATION OF THE EFFECTS OF 5-FLUOROURACIL AND MELATONIN ON SERUM CORTICOSTERONE LEVELS AND IMPLICATIONS IN HIV

12.1 Introduction

The Human Immunodeficiency Virus (HIV) pandemic is undoubtedly one of the most devastating in human history, with 39.5 million people infected worldwide by December 2006, and South Africa having one of the highest infection rates in the world (http://data.unaids.org/pub/EpiReport/2006/20061121_EPI_FS_GlobalFacts_en.pdf).

HIV, which is spread by contact with infected bodily fluids, is a nontransforming retrovirus in the lentivirus category and results in Acquired Immune Deficiency Syndrome (AIDS) (Abbas, 2005). The glucocorticoid hypothesis states that the progression to AIDS occurs primarily due to excess glucocorticoids (Corley, 1996) (see 12.3). Recently, the use of 5-FU in the treatment of HIV infection has been investigated (see 12.5); melatonin is used by some HIV-infected individuals due to its immunostimulatory effects (see 12.6). It is thus important to determine whether these agents have any effect on glucocorticoid levels, as this could indicate potential effects on the progression time to AIDS in HIV-infected patients. It has, furthermore, been shown in Chapter 5 that both 5-FU and melatonin enhance IDO activity; an increase in the activity of this enzyme is associated with the progression to AIDS (see 5.2.2).

There are two genetically different forms of the virus: HIV-1, which occurs predominantly in Europe, the United States and central Africa; and HIV-2, which is more common in India and West Africa (Abbas, 2005). As shown in Figure 123, the structure of the HI virion is spherical, and consists of a cone-shaped, electron-dense core that is covered with a host cell membrane-derived lipid envelope (Abbas, 2005). The core of the virus consists of the following (Abbas, 2005):

(i) p24, a major capsid protein and the most easily detected antigen;
(ii) two copies of the viral genomic RNA;
(iii) the nucleocapsid protein p7/ p9; and
(iv) the enzymes reverse transciptase (RT), protease and integrase.

Between the viral core and the lipid envelope there is a protein matrix, p17. As shown in the following diagram, the glycoproteins gp120 and gp41 stud the lipid envelope; these glycoproteins are essential for the virus to infect cells. Gp41 extrudes from the envelope and

gp120 is non-covalently attached to gp41 (Abbas, 2005). The RNA genome of HIV-1 is shown in Figure 121. The *env*, *pol* and *gag* genes code for viral proteins; the accessory genes *vif*, *rev*, *nef*, *tat*, *vpu* and *vpr* are responsible for the assembly and regulation of infectious particles and viral pathogenicity (Frankel and Young, 1998; Abbas, 2005).

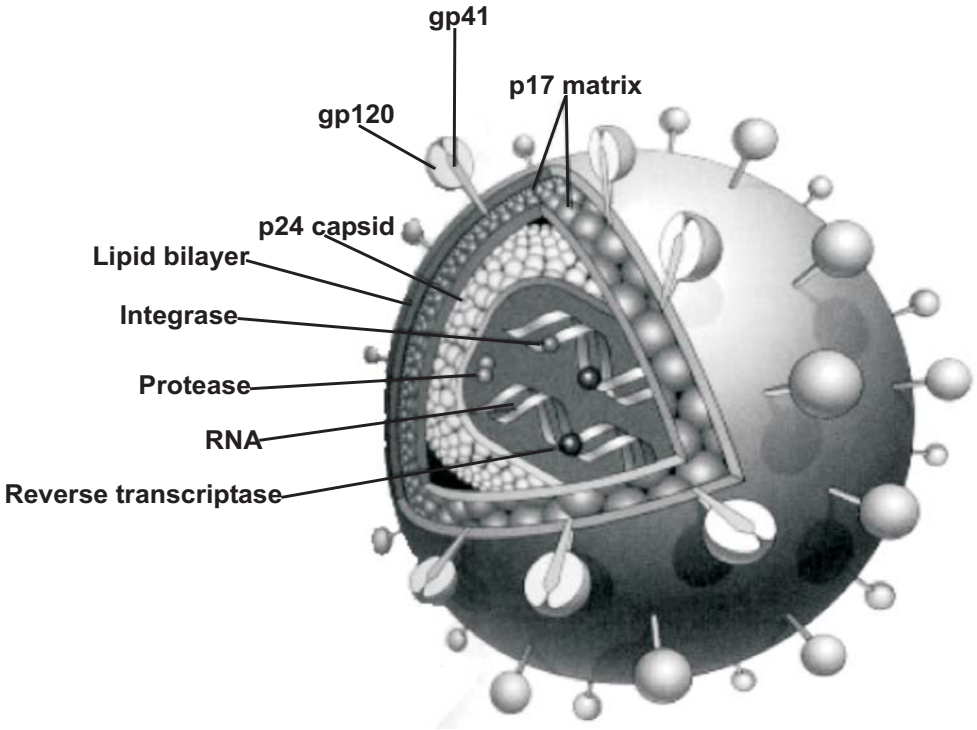


Figure 123: A schematic representation of the structure of HIV (Abbas, 2005)

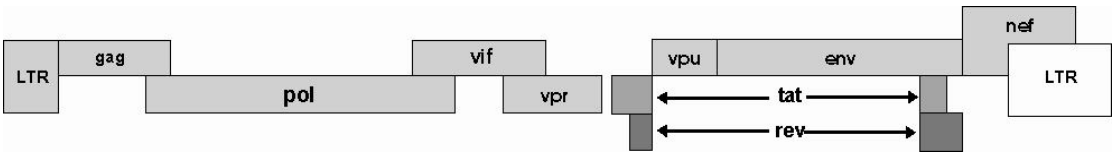


Figure 124: The proviral HIV RNA genome, showing the standard gag, pol and env genes, as well as accessory genes that regulate viral pathogenicity (Abbas, 2005)

CD4 cells are high-affinity receptors for HIV, and the virus exhibits selective tropism for the following types of CD+ cells: CD4+ T cells, macrophages, monocytes and dendritic cells. The primary infection of CD4 cells occurs in mucosa and blood (Abbas, 2005). The gp120 group of HIV binds to the CD4+ cell, thereby inducing a conformational change that reveals a recognition site on gp120 for two chemokine receptors, CCR5 and CXCR4, that are on the surface of the host cell (Littman, 1998; Berger *et al*, 1999; Abbas, 2005). Conformational alterations in gp41 follow, which facilitate the insertion of a fusion peptide, which is found at the tip of gp41, into the membrane of the host cell (LaBranche *et al*, 2001). Fusion of gp41

with the target cell membrane ensues, allowing the viral core to enter the cytoplasm of the host cell. The RNA genome of the virus subsequently undergoes reverse transcription, resulting in the formation of proviral DNA (cDNA) (Abbas, 2005). In cells that are quiescent, this cDNA remains in a linear form; whereas it circularises in dividing cells, enters the nucleus and is incorporated into the genome of the host cell by the enzyme integrase. Subsequent to this integration, the provirus either remains locked in the chromosome for a period of time that could extend to years, resulting in the particular cell being latently infected, or it could be transcribed. The latter process results in the synthesis of various infectious viral particles, which bud from the host cell membrane and induce cell death (Abbas, 2005). The activation of latently-infected host T cells occurs upon interaction with an antigen or certain cytokines. This activates kinases in the cytoplasm, which phosphorylate and induce the degradation of the inhibitor of kB (I-kB), thereby releasing complexed NF-kB (Abbas, 2005). This factor enters the nucleus and binds to kB sites in various genes, including the long terminal repeat (LTR) sequence of the viral genome (see Figure 124) (Greene and Peterlin, 2002; Abbas, 2005). This triggers the transcription of cDNA, viral replication and cell lysis. Pro-inflammatory, or type 1 cytokines (see 12.3.2), also stimulate the activity of NF-kB, thereby enhancing viral replication (Abbas, 2005). Viral replication is thus enhanced when host T cells and macrophages are activated physiologically by cytokines or antigens, such as HIV or other micro-organisms (Abbas, 2005).

The natural history of HIV encompasses the following stages (Abbas, 2005; Christeff *et al*, 1997):

(i) acute retroviral syndrome, which is characterised by a high level of virion production and widespread seeding into lymphoid tissues in the body. The host's immune system adequately controls the initial infection, which clinically presents as flu-like symptoms several weeks after HIV infection. There are distinct changes in humoral immunity (HI) and cell-mediated immunity (CMI) (see 12.2.2). There is a notable increase in the level of CD8+ T lymphocytes (Christeff *et al*, 1997) as well as B lymphocytes (Laurence *et al*, 1993);

(ii) a chronic middle phase of approximately 7-10 years, during which the virus is clinically latent. There are no marked alterations in the immune system, but continual viral replication occurs, especially in lymphoid tissues. Little weight loss, usually less than 10% of the individual's total weight, and minor opportunistic infections may occur;

(iii) the progression to full-blown AIDS, or advanced HIV, which is characterised by severe immunosuppression and a significant increase in plasma levels of HIV. Clinical signs include weight loss of over 10% of the total body weight (Christeff *et al*, 1997), fatigue, a fever that has persisted for over a month, and diarrhoea. The development of neurological disease, secondary neoplasms or serious opportunistic infections occurs, the latter due to depletion of,

and abnormalities in, the remaining CD4+ T lymphocytes (Christeff *et al*, 1997; Abbas, 2005).

HIV replication is enhanced by UV radiation (Breuer-McHam *et al*, 2001; Akaraphanth and Lim, 1999) through the following mechanisms (Termorshuizen *et al*, 2002a, 2002b):

(i) the penetration of UV radiation into the skin induces the isomerisation of dermal *trans*-urocanic acid to the *cis* form; this isomer results in immunosuppression by inhibiting T-cell responses both dermally and systemically; and

(ii) photodamage of DNA also inhibits T-cell-mediated immune responses.

This ability of the virus to be activated by ambient UV radiation is highlighted in the finding that patients with advanced HIV infection exhibit a significantly lower CD4+ T lymphocyte count in mid-summer (Termorshuizen *et al*, 2002a), and may be a factor in the alarming incidence and rapid progression of the infection in individuals living in sub-Saharan Africa.

12.2 Effects of HIV

Major targets of HIV are the CNS and the lymphoid system (Abbas, 2005). HIV-induced neurotoxicity will be discussed below, as well as the effects of the virus on the HPA axis. The latter is significant because the glucocorticoid hypothesis of AIDS (see 12.3) is the major focus of this Chapter.

12.2.1 HIV-induced dementia

HIV results in a wide range of physiological abnormalities, one of which is HIV-induced dementia. HIV infection is the leading cause of dementia in people younger than 60 years of age (Janssen *et al*, 1992), and the average survival time upon diagnosis is six months (Harrison and McArthur, 1995). Antiretroviral treatment, by increasing the survival time of HIV-positive patients, renders patients increasingly susceptible to developing dementia in later years (Lopez *et al*, 1999). Some investigators argue that antiretroviral therapy is decreasing the incidence of this disorder (Sacktor *et al*, 1999; Berger and Arendt, 2000), as certain antiretroviral agents, such as 3'-azido-3'-deoxythymidine (AZT) and stavudine (D4T) penetrate the blood-brain barrier (Klecker *et al*, 1987; Haworth *et al*, 1998) and thus provide clinical improvement (Schmitt *et al*, 1988; Rolinski *et al*, 1997) by decreasing the viral load in the brain.

The early stages of dementia involve a subtle slowing of physical and mental activity (Navia *et al*, 1986). This progresses within months to behavioural changes and forgetfulness, with impairment in performing daily tasks (Koutsilieri *et al*, 2002). This is followed by severe loss of memory, depression and apathy, with patients also exhibiting symptoms of bradykinesia,

tremor and impaired gait (Navia *et al*, 1986). Language disorders develop as the patient experiences a worsening in memory. Features of the final stage of HIV-induced dementia include severe retardation in psychomotor activity and general cognitive malfunctioning. Hallucinations, incontinence, seizures and coma precede death (Navia *et al*, 1986).

HIV, a neurotrophic virus, invades the subcortical regions of the CNS, and concentrates in the basal ganglia, ventral midbrain and thalamus (Wiley *et al*, 1998; Kure *et al*, 1990). The virus targets lymphocytes, microglia and macrophages in the brain (Koutsilieri *et al*, 2002). Although neurons are not targeted (Lopez *et al*, 1999), there is a loss of synapses and neurons in the frontal neocortex (Ketzler *et al*, 1990). Hallmark features of HIV-induced dementia include the influx of mononucleocytes into brain parenchyma; the formation of large multinucleated cells (Masliah *et al*, 1992), microglial nodules; cellular atrophy and myelin pallor (Sharer *et al*, 1997). These hallmark features do not correlate well with the clinical presentation of HIV-induced dementia (Glass *et al*, 1993). The neurodegeneration that occurs in HIV-induced dementia is associated with neurotoxicity induced by excitatory amino acids, ROS and cytokines (Lipton, 1994), as well as the direct neurotoxicity of the HIV proteins gp41, gp120 and Tat (Nath and Geiger, 1998).

Patients with HIV-induced dementia share many clinical symptoms of patients suffering from Parkinson's disease (Koutsilieri *et al*, 2002; Berger and Arendt, 2000), such as the bradykinesia, tremor and abnormal gait described before, rigidity and speech difficulties (Currie *et al*, 1988; Navia *et al*, 1986). Neurochemically, there is a significant decrease in the levels of DA and its metabolite homovanillic acid, in HIV-induced dementia (Sardar *et al*, 1996); a deficiency of DA is the underlying disorder in Parkinson's disease. HIV-positive patients treated with neuroleptics are more likely to develop Parkinsonism, and this sensitivity to the use of DA antagonists further confirms an underlying abnormality in DA levels present in HIV infection (Hriso *et al*, 1991; Koutsilieri *et al*, 2002). DA has been found to regulate the expression of the HIV gene in immune and neuronal cells *in vitro* (Sawaya *et al*, 1996; Koutsilieri *et al*, 1997; Rohr *et al*, 1999). DA furthermore stimulates HIV in T lymphoblasts that are chronically infected (Scheller *et al*, 2000) by enhancing cellular oxidative stress (Koutsilieri *et al*, 2002). It is thus suggested by these authors that antioxidants be used to treat HIV-positive patients being treated with dopaminergic agents (Koutsilieri *et al*, 2002), as glutathione and *N*-acetylcysteine have been found to be effective in inhibiting DA-induced HIV activation (Scheller *et al*, 2000).

In addition to similarities in DA deficits and clinical symptoms between HIV-induced dementia and Parkinson's disease, stereological studies have shown that in AIDS, there is a

25% decrease in the neuron count of the substantia nigra pars compacta (Reyes *et al*, 1991), a region of the brain rich in dopaminergic neurons and severely affected in Parkinson's disease. Lewy bodies, which are a characteristic feature of Parkinson's disease, are, however, not reported to be present in HIV-induced dementia (Reyes *et al*, 1991).

12.2.2 Effects on the HPA axis

HIV infection results in a wide range of endocrine and metabolic abnormalities (Grinspoon and Bilezikian, 1992). The HPA axis is activated and, as discussed in 12.3.1 and 12.3.2, the secretion of cortisol is enhanced (Verges *et al*, 1989; Christeff *et al*, 1992a). One of the deleterious effects of this is the phenomenon of HIV lipodystrophy syndrome, which is characterised by the accumulation of fat in the dorsocervical region, abdominal obesity, elevated trigylcerol levels and insulin resistance (Carr *et al*, 1998).

12.2.3 Depression

It has been shown, in a large cohort longitudinal study, that seropositive HIV women are four times more likely to suffer from depression than seronegative women, and also experience more symptoms of anxiety (Morrison *et al*, 2002). This increased susceptibility to depression is not linked to the use of antiretrovirals (Morrison *et al*, 2002). Many studies have found that HIV-seropositive men are more likely to develop depression than uninfected males, but that this prevalence is not higher than in HIV-seronegative men (Perkins *et al*, 1994; Williams *et al*, 1991; Rabkin *et al*, 1997). Depression could be as a result of complex interactions between biochemical alterations induced by the virus, such as DA depletion (see 12.2.1 and 6.2.2.3), as well as psychosocial factors.

12.3 Glucocorticoid hypothesis of AIDS

This hypothesis shows the interactions between the immune and endocrine systems, and the mechanisms by which excess glucocorticoids can hasten the progression to AIDS in HIV-positive individuals.

12.3.1 HIV-induced increases in cortisol levels

HIV interacts intimately with the humoral system, thereby altering the relationship between the immune system and glucocorticoids (Corley, 1996, 1997). By altering immunological functioning and the metabolism of glucocorticoid hormones, HIV produces an excess of cortisol (Corley, 1995). This is countered by a significant down-regulation in the number of glucocorticoid receptors (Smith *et al*, 1993), which results in cortisol resistance (Corley, 1996). It is believed (Corley, 1996, 1997) that AIDS is an HIV-induced malfunctioning in the metabolism of glucocorticoids.

Under normal conditions, an increase in cortisol levels is a physiological mechanism to depress the initial stress-induced immune response of the body (Haq *et al*, 1993). This increase in cortisol levels results in a range of cellular and immunological effects (Gabriel *et al*, 1992), including a decrease in the number of peripheral CD4+ (Hennig *et al*, 1993), CD3+ and CD8+ T lymphocyte cells (Munschauer *et al*, 1993). It has been found that glucocorticoids influence all the stages in the selection, development and regulation of lymphoid cells (Crompton *et al*, 1992). Furthermore, the ratio of CD4+/ CD8+ cells is positively associated with increased cortisol levels and the development of anxiety and depression (Charles *et al*, 1992).

The gp120 envelope protein of HIV increases cortisol levels through several related mechanisms, which are summarised in Figure 125:
(i) upregulation of mRNA that codes for ACTH (Hashemi *et al*, 1992), thereby stimulating the release of cortisol by activating the HPA axis (see 6.2.2.9.1.1);
(ii) infusion of gp120 into rat brain has been found to induce the pro-inflammatory cytokine IL-1; increase the concentration of plasma steroids; and decrease the activity of NK cells, which maintain cellular immunity, in the spleen and blood (Sundar *et al*, 1991). IL-1 induces the release of CRH, which stimulates the release of ACTH and ultimately cortisol (Biglino *et al*, 1993; Sandi and Guaza, 1995; Kasckow *et al*, 1994). The secretion of IL-1 is stimulated by the amino terminus of gp120 (Clouse *et al*, 1991). The addition of melanocyte-stimulating hormone (MSH) prevents gp120-stimulated IL-1 secretion, and antagonises many of the biological effects of this cytokine (Davidson *et al*, 1992). Melanocytes do, however, synthesise IL-1α and IL-1β (Swope *et al*, 1994), and Corley (1996) believes that this highlights a homeostatic mechanism between IL-1 and MSH, which may be influenced by the presence of gp120;
(iii) gp120 contains an octapeptide sequence, referred to as peptide T, which is a retro-copy fragment of the carboxy terminus of γ-3-MSH (Dominy, 1988). Peptide T may thus, besides interacting with CD4+ cells (Ramsdale *et al*, 1993), also competitively antagonise MSH receptors. This results in inhibition of the MSH-induced blockade of IL-1; the enhanced levels of the latter subsequently stimulate cortisol secretion (Corley, 1996);
(iv) the aforementioned blockade of MSH receptors results in an increase in circulating MSH, which itself is immunosuppressive, and since these levels are already elevated, less ACTH is converted into MSH by granulocyte-associated neutral endopeptidase (Corley, 1996). These elevated ACTH levels stimulate the secretion of cortisol, which further stimulates the glucocorticoid response element of HIV. This enhances the replication of HIV (Justement *et al*, 1990), which thus further increases cortisol levels in the cyclical process depicted in Figure 122 (Corley, 1996). The predecessor of ACTH and MSH is proopiomelanocortin (see

14.2) (Zhou *et al*, 1993), and it is known that the gene for the melanocortin receptor is located on chromosome 18 (Chowdhary *et al*, 1995). This chromosome is associated with a variety of retroviruses (Corley, 1997) and contains an endogenous retroviral sequence (Brack-Werner *et al*, 1989; Leib-Mosch *et al*, 1989), thus further highlighting the genetic mechanisms by which HIV interacts with host cells and ultimately increases cortisol levels (Corley, 1997); and
(v) IL-1 enhances the proliferation of cytotoxic CD8+ T lymphocytes (Nagoya *et al*, 1994). It is thus believed (Corley, 1996, 1997) that the gp120 group of HIV enhances the activity of IL-1 as a mechanism of utilising the genome of a lymphocyte in order to increase glucocorticoid levels, thereby stimulating further viral replication. The synthesis of CD8+ T lymphocytes is also enhanced by the increase in cortisol levels. Immunosuppression, a defining feature of AIDS, thus occurs via a dual mechanism (Corley, 1996), namely: (a) an increase in CD8+ T lymphocytes (Sinicco *et al*, 1994); and (b) lysis of infected CD4+ lymphocytes. These infected CD4+ T lymphocytes, prior to lysis, serve as "factories" for the synthesis of ACTH, IL-1 and glucocorticoids (Corley, 1996).

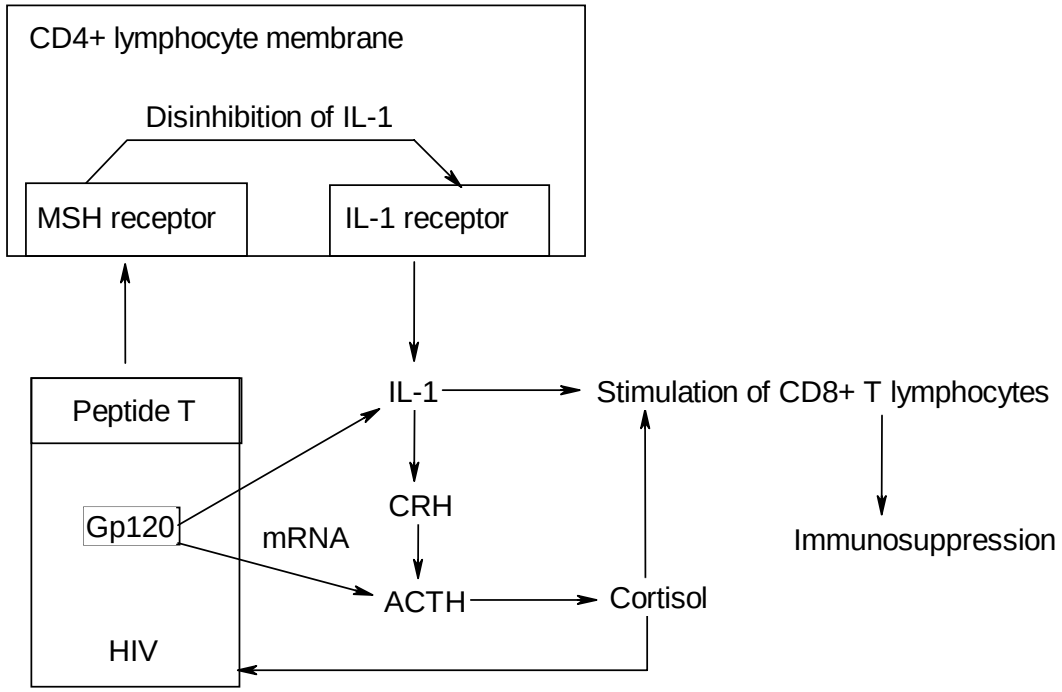


Figure 125: Proposed model of the mechanisms by which HIV increases cortisol levels (Corley, 1996)

Although HIV-positive patients treated with peptide T do exhibit increased levels of IL-1, there is an overall improvement in clinical status, due to a decrease in TNFα and increased activity of the lymphoproliferative system (MacFadden *et al*, 1993). Peptide T administration to HIV-positive patients results in a competitive antagonism with gp120, resulting in a wide array of effects that improve the clinical status of these patients (Doob and MacFadden, 1992;

MacFadden and Doob, 1992). Such positive effects include an inhibition of TNFα-induced HIV replication (Race *et al*, 1994), a decrease in gp120-induced neurotoxicity (Buzy *et al*, 1992), as well as an improvement in peripheral neuropathy (MacFadden and Doob, 1991) and neuropsychiatric symptoms, such as fatigue (Bridge *et al*, 1991; Doob and MacFadden, 1992).

It has also been found that HIV interacts intimately with the human genome. Like chromosome 18, chromosome 2 is known to be associated with a variety of immunodeficiency states (Corley, 1997), Epstein Barr-associated lymphoma (Yoshizawa *et al*, 1993), AIDS-associated Burkitt's leukaemia (Berkowicz *et al*, 1989) and retroviruses (Ohara *et al*, 1993). Chromosome 2 is associated with HIV and ultimately enhances the replication of this virus via three main mechanisms (Corley, 1997), namely:
(i) a factor located on the chromosomal long arm induces viral transactivation by the Tat protein, which is encoded by HIV. This process is facilitated by the transactivation responsive region, located in HIV-LTR (Cupelli, 1992);
(ii) the gene encoding for the IL-1 receptor antagonist, IL-1RA, also resides in the long arm of chromosome 2 (Patterson *et al*, 1993; Steinkasserer *et al*, 1993). It is believed that the integration of HIV into the human genome interferes with the IL-1RA gene, thereby preventing IL-1 antagonism. This may contribute to the elevation in IL-1 levels displayed by cells infected with HIV (Merrill *et al*, 1989; Corley, 1997). The inhibitory effect of HIV on the IL-1RA gene is supported by the finding that antiretroviral agents, besides decreasing IL-1 levels, increase the release of IL-1RA (Sadeghi *et al*, 1995). Zavala *et al* (1995) have found, however, that HIV induces the synthesis of IL-R1A after an initial lag phase; there might thus be an internal homeostatic mechanism in which the HIV-induced increase in IL-1 levels indirectly triggers a feedback mechanism to increase the levels of its antagonist, IL-1RA (Corley, 1997); and
(iii) the gene encoding for CD26, or dipeptidyl peptidase IV, is also located on the long arm of chromosome 2 (Abbott *et al*, 1994). CD26 is a lymphocyte cell-surface antigen; its levels are enhanced upon the activation of T cells; and it is involved in the entry of HIV into cells, possibly by being a coreceptor of gp120 (Hovanessian *et al*, 1994; Corley, 1997). The results of HIV interaction with chromosome 2 are thus an increase in IL-1 levels and enhanced viral infectivity (Corley, 1997).

It has also been found that HIV-induced increases in IL-1 levels may play a significant role in the development of Kaposi's sarcoma (Corley, 1997; Louie *et al*, 1995), a carcinoma characteristic of AIDS, via activation of IL-6, which serves as a growth factor for Kaposi sarcoma-derived cells (Yang and Offermann, 1993). These cells demonstrate high levels both of mRNA for IL-1 (Ensoli *et al*, 1989) and glucocorticoid receptors (Guo *et al*, 1993), thus

emphasising the role of IL-1 and cortisol in the development of Kaposi's sarcoma. Furthermore, HIV directly promotes the growth of Kaposi's sarcoma, as the HIV-1 Tat protein enhances the growth of sarcoma-derived spindle cells (Albini *et al*, 1995). It has been found that IL-1RA completely blocks IL-1-stimulation of the growth of Kaposi's sarcoma cells (Louie *et al*. 1995).

The glucocorticoid hypothesis thus postulates that HIV is essentially a viral-induced disorder in ACTH/ glucocorticoid/ MSH metabolism (Corley, 1996, 1997), and is supported by findings that cells resistant to glucocorticoids exhibit total resistance to infectivity by HIV (Kawa *et al*, 1993). Clinical studies have shown that patients with AIDS have similar clinical features to those with Cushing's disease, a disorder of excess glucocorticoids, but without the effects of excess androgens or mineralocorticoids (Norbiato *et al*, 1994). Based on this hypothesis, a treatment strategy for HIV would involve the use of agents that decrease cortisol levels, and/ or promote glucocorticoid resistance.

12.3.2 Cortisol/ anticortisol imbalances

Besides the abnormal activation of HI described in 12.3.1, the progression from HIV to full-blown AIDS is marked by impaired CMI (Clerici and Shearer, 1993, 1994) (see 12.1). The susceptibility to developing tumours and opportunistic infections results from a failure to develop delayed-type hypersensitivity reactions upon challenge by various antigens (Clerici *et al*, 1997). Impaired CMI results in a decrease in the levels of the type 1 cytokines INFγ, IL-2 and IL-12, while stimulation of HI results in an increase in the levels of the type 2 cytokines IL-4, IL-5, IL-6, IL-10 (Clerici and Shearer, 1993, 1994). It is suggested that this progression from type 1 to type 2 cytokines be considered a marker of HIV progression, as it has been shown to predict the following clinical features and events (Clerici *et al*, 1997; Clerici and Shearer, 1993, 1994):

(i) a decrease in the CD4+ count (Lucey *et al*, 1991);
(ii) the time to which the patient is diagnosed with AIDS; and
(iii) survival time (Dolan *et al*, 1995).

HIV-positive individuals progressing to AIDS thus exhibit a weak type 1 and enhanced type 2 cytokine profile, while paediatric patients and those with absent or delayed HIV progression show the opposite profile (Vigano' *et al*, 1995; Clerici *et al*, 1996). The shift from a type 1 to type 2 cytokine profile renders CD4+ T lymphocytes increasingly susceptible to programmed cell death (PCD) (Ameisen and Capron, 1991; Clerici *et al*, 1994), thus accounting for the significant drop in CD4+ T lymphocyte levels characteristic of HIV progression to AIDS. HIV-positive patients with acquired glucocorticoid resistance exhibit an altered cytokine

profile that favours type 1 cytokines (Clerici *et al*, 1997; Norbiato *et al*, 1992). The following diagram illustrates the alterations in cytokine profiles that occur in HIV.

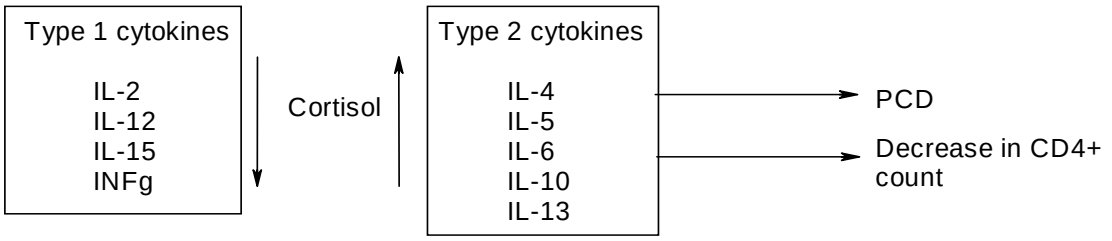


Figure 126: The cortisol-induced alterations in cytokine profiles that occurs in HIV infection (Clerici *et al*, 1997)

HIV progression is, additionally, characterised by an elevation in the level of glucocorticoids, as discussed in 12.3.1, as well a marked decrease in the levels of dehydroepiandrosterone (DHEA) and DHEA sulphate; the latter two are androgen hormones that have opposing actions to cortisol (Grinspoon and Bilezikian, 1992; Christeff *et al*, 1992a; Mayo *et al*, 2002) and a decrease in the cortisol/ DHEA ratio has been implicated in the development of depression (Goodyer *et al*, 1998) (see 6.2.2.9.1.1). Glucocorticoids are known to impair CMI and augment HI (Clerici *et al*, 1997), thereby promoting the shift from type 1 to type 2 cytokines (Clerici *et al*, 1997; Mayo *et al*, 2002) via the following mechanisms:
(i) glucocorticoids inhibit the transcription of genes responsible for the expression of INFγ and IL-2 (Vacca *et al*, 1992);
(ii) inhibition of the synthesis of IL-1, which activates T cells (Clerici *et al*, 1997);
(iii) glucocorticoids enhance the effects of IL-4 in promoting and differentiating B lymphocytes into plasma cells that synthesise immunoglobulin E (IgE) (Wu *et al*, 1991). The concentration of IgE is increased in HIV (Clerici *et al*, 1997); and
(iv) by directly enhancing IL-4 synthesis, glucocorticoids promote the synthesis of type 2 cytokines (Daynes and Araneo, 1989).

Importantly, the *vpr* gene present in HIV, which plays a role in the replication of the virus in monocytes and T lymphocytes as well as in the induction of mitotic arrest and apoptosis (Zhu *et al*, 2001), has been shown to undergo a direct interaction with a cellular protein that forms part of the transcriptional complex associated with glucocorticoids (Rafaeli *et al*, 1995). Agents that display an antiglucocorticoid effect are found to inhibit the *vpr*-induced promotion of HIV replication (Rafaeli *et al*, 1995). The PCD of mature T lymphocytes and a wide range of cells is also promoted by glucocorticoids (Cohen *et al*, 1992; Golstein *et al*, 1991). The imbalance between type 1 and type 2 cytokines is necessary in inducing the susceptibility of CD4+ T lymphocytes to PCD (Clerici *et al*, 1994), as the type 1 cytokines

inhibit PCD while the type 2 cytokines either stimulate or have no effect on this phenomenon (Clerici *et al*, 1997). These type 2 cytokines and glucocorticoids thus exert a synergistic decrease in CD4+ T lymphocyte levels by promoting PCD (Clerici *et al*, 1997).

The abovementioned deleterious effects of glucocorticoids, in particular cortisol, on HIV progression can thus be summarised as follows (Clerici *et al*, 1997):
(i) glucocorticoids enhance the synthesis of type 2 cytokines and inhibit that of type 1 cytokines;
(ii) inhibition of CMI;
(iii) HIV transcription and replication are stimulated through the *vpr* gene; and
(iv) PCD is promoted.

Support for the role of the cortisol/ anticortisol imbalance in the progression of HIV comes from the preliminary finding that HIV-positive patients treated with DHEA demonstrate a significantly decreased viral load compared to those receiving antiretroviral treatment (Salvato, 1996). By promoting the activity of helper T cells and increasing the synthesis of IL-2 by these cells, DHEA counteracts the glucocorticoid-induced inhibition of this cytokine (Daynes *et al*, 1990), thereby strengthening the type 1 cytokine profile and its more inhibitory effects on HIV progression (Clerici *et al*, 1997).

Cortisol and DHEA play an important role in maintaining body weight and nutrition (Homo-Delarche *et al*, 1991; Nunez and Christeff, 1994). HIV progression is characterised by cachexia and severe immunosuppression, especially in the advanced stages (see 12.1). Whilst the immunosuppression is attributed to a loss of CD4+ T lymphocytes, as mentioned earlier, the decrease in body weight is as a result of a decrease in fat-free mass, as opposed to a loss of adipose tissue (Christeff *et al*, 1997). Cortisol and DHEA influence the metabolism of amino acids that occurs in muscle tissue and plays a role in the development of HIV cachexia (Nunez and Christeff, 1994). Cortisol induces muscle loss by enhancing the activity of the enzymes responsible for the catabolism of amino acids, such as TDO and transaminase. DHEA, in contrast, promotes the anabolism of amino acids, especially in bone muscles, and strengthens bones by increasing the levels of bone proteins (Kochakian, 1964).

It has been found clinically that whilst cortisol levels are approximately 30-50% higher in patients at all stages of HIV infection, compared to seronegative individuals, the level of DHEA in serum is closely associated with the stage of infection (Christeff *et al*, 1997). In the early stages of HIV infection, DHEA levels are 60-85% of normal, and decrease to 15-30% below normal in patients with AIDS (Christeff *et al*, 1997). The mechanisms by which

cortisol levels are increased are mentioned in 12.3.1. This increase could also be attributed to the inhibition of cortisol catabolism, which occurs as a result of altered fatty acid levels (Christeff *et al*, 1988), or to modifications in the binding properties or availability of corticosteroid-binding globulin (Martin *et al*, 1992). It is suggested (Christeff *et al*, 1997) that the gradual decrease in DHEA levels that mirrors the progression of HIV could occur in a similar manner to the decrease in androgen levels that occurs in various stressful situations (Woolf *et al*, 1985), such as endotoxin shock (Christeff *et al*, 1987) and septic shock (Christeff *et al*, 1992b). In the same clinical study (Christeff *et al*, 1997), it was found that the increase in cortisol and decrease in DHEA levels is associated with a fall in the CD4+ T lymphocyte count and a decrease in fat-free body mass. These authors suggest that the initial increase in DHEA levels that occurs early in HIV infection could stimulate the synthesis of IL-2 (Daynes *et al*, 1990), the activity of T cells (Padgett and Loria, 1994) and the anabolism of proteins in muscle tissue (Christeff *et al*, 1997), as well as inhibit the proliferation of lymphocytes (Blauer *et al*, 1991). This counters cortisol-induced immunosuppression and protein catabolism. The increase in the cortisol/ DHEA ratio that occurs in the later stages of HIV infection, with a drop in DHEA levels, may worsen immunosuppression and result in imbalances between the catabolism and anabolism of amino acids (Christeff *et al*, 1997). The initial elevated levels of DHEA could thus have an anticortisol effect, which decreases in amplitude as the cortisol/ DHEA ratio increases and the infection progresses (Christeff *et al*, 1997). Cortisol and DHEA may thus be regarded as an agonist-antagonist duo (Nunez and Christeff, 1996), and the relative effects of the two play an important role in the development of hallmark features of AIDS, as illustrated in the Figure below.

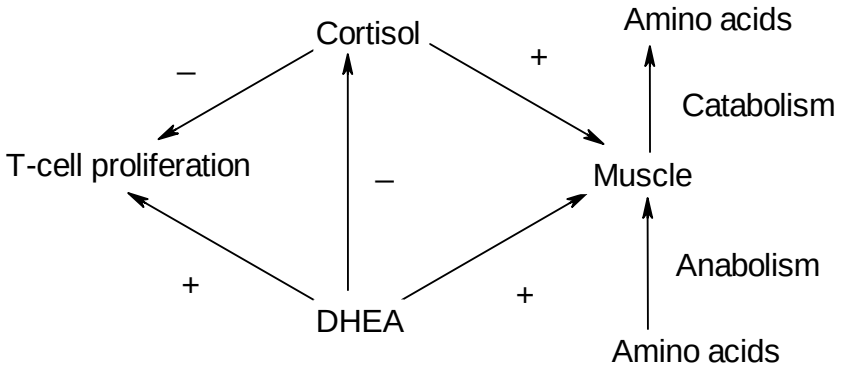


Figure 127: The metabolic and immune changes induced by alterations in the cortisol/ DHEA ratio that accompany the progression from HIV to AIDS (Christeff *et al*, 1997)
The (-) signs signify inhibition and the (+) signs indicate stimulation of the relevant processes

12.4 Effects of antiretrovirals on cortisol levels

It has been found that certain antiretroviral agents, in particular the non-nucleoside reverse transcriptase inhibitors (NNRTIs) and protease inhibitors (PIs), induce hypercortisolemia (Collazos *et al*, 2004). Drugs from each class that were found to exert a profound effect in this regard are efavirenz and ritonavir/ saquinavir, respectively (Collazos *et al*, 2004). The nucleoside reverse transcriptase inhibitors (NRTIs) are not known to alter serum levels of cortisol (Saint-Marc *et al*, 1999; Collazos *et al*, 2003). Ritonavir is an inhibitor of CYP450, and the subsequent inhibition of steroid metabolism (Christeff *et al*, 1999; Inaba *et al*, 1997) may account for the increase in serum cortisol levels (Collazos *et al*, 2004). The mechanism of efavirenz-induced hypercortisolaemia is possibly more complex, however, as this agent induces CYP450 (Clarke *et al*, 2001; Ruiz, 1999; Barry *et al*, 1999). Efavirenz may possibly interact with different isoforms of CYP450 and also undergo extrahepatic metabolism (Collazos *et al*, 2004; Hesse *et al*, 2001; von Moltke *et al*, 2001).

This highlights a potential problem with certain antiretrovirals, as the induction of hypercortisolaemia may worsen the patient's immunosuppression and viral load, according to the glucocorticoid hypothesis of AIDS (see 12.3)

12.5The use of 5-FU in HIV-positive patients

12.5.1 Treatment of carcinomas

5-Fluorouracil has been used in the clinical setting to treat AIDS-related neoplasias (Gilman *et al*, 1990). As HIV infection rates and mortality due to AIDS increases, there is an increase in the occurrence of these AIDS-related neoplasias and malignancies (Sadaie *et al*, 2004). When 5-FU is used as co-therapy with AZT, itself intially developed as an anticancer agent, there is reported to be an enhanced antineoplastic effect in treating chronic myeloid leukaemia associated with HIV (Tosi *et al*, 1993), other malignancies (Posner *et al*, 1992; Danesi *et al*, 1998) and intravaginal warts (Sved *et al*, 1999) (see 1.2.8.5). 5-FU is also used in combination with other antineoplastic agents and radiation therapy in the treatment of anal cancer in HIV-positive individuals (see 1.2.8.5). It is well-established, however, that 5-FU is a toxic immunosuppressive drug, exerting its immunosuppressive effect through a selective decrease and suppression of B and T lymphocytes (Větvička *et al*, 1986) (see 1.2.10.3).

12.5.2 Effects on HIV

Although antiretroviral drugs have a significant effect on the mortality and morbidity of HIV by inhibiting key enzymes that are involved in viral replication, such as protease and RT, there are several limitations associated with the use of antiretrovirals (Sadaie *et al*, 2004):

(i) these agents are non-curative;
(ii) the proviral load is not eradicated;
(iii) severe adverse drug reactions are associated with the use of these drugs;
(iv) complex regimens are necessary, which may discourage patient compliance; and
(v) the development and transmission of antiretroviral resistance, which decreases the efficacy of anti-HIV treatment (Sadaie *et al*, 2004).

These limitations, as well as the continual soar in infection rates across the world, and the existence of subtypes of HIV, have propelled the search for novel means to treat HIV infection (Sadaie *et al*, 2004). One of these novel strategies is the use of low doses of nucleotide-based antineoplastic agents, such as 5-FU, as potential anti-HIV drugs (Sadaie *et al*, 2004) and in combination with the pyrimidine-based NRTIs AZT and D4T (Gao *et al*, 1999; Ahluwalia *et al*, 1996).

12.5.2.1 Anti-HIV activity

The anti-HIV effects of 5-FU can be subdivided into two categories and will be explored below: (i) inherent to the drug; and (ii) in combination therapy with certain existing antiretroviral agents.

12.5.2.1.1 Inherent anti-HIV activity

It has been found by some some investigators that 5-FU exhibits anti-HIV activity, by inhibiting HIV-1 RT (Shi *et al*, 1999). A range of analogues of 5-FU were synthesised, with varying potency against HIV and cytotoxicity compared to the parent 5-FU (Shi *et al*, 1999; Choo *et al*, 2003). A range of sugar moieties are added through glycosidic bonds to the nitrogen in the pyrimidine ring of 5-FU to form various nucleosides, two of which are shown below. β-D-2', 3'-dideoxy-5-fluorouracil (β-D-D4FU) exhibits weaker anti-HIV and cytotoxicity compared to 5-FU, while the β-L anomer is more potent (Shi *et al*, 1999). These agents are competitive inhibitors of HIV-1 RT (Shi *et al*, 1999).

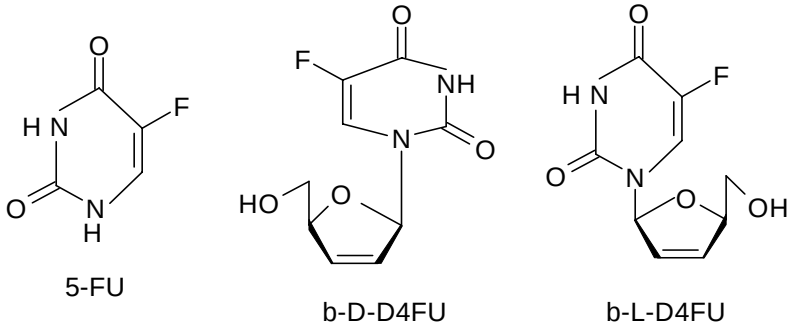


Figure 128: The chemical structures of some analogues of 5-FU that exhibit anti-HIV activity, as well as the structure of 5-FU (Shi *et al*, 1999)

Monotherapy against HIV is now seldom used, due to the rapid development of drug resistance (Gao *et al*, 1999). Viral counts of the p24 antigen, for example, return to baseline values after six months of D4T therapy (Gong *et al*, 1996). High viral replication rates, as well as infidelity in RT activity and the lack of DNA proofreading ability (Roberts *et al*, 1988) results in an increase in the frequency in which uncorrected deoxynucleotides are inserted into the growing DNA chain, and thus the formation of drug-resistant mutants (Gao *et al*, 1999). Highly active antiretroviral therapy (HAART) thus consists of the use of multiple drugs: two 2', 3'-dideoxynucleoside (ddN) drugs with complementary resistance profiles and which inhibit RT, such as AZT and lamivudine (3TC) (Larder *et al*, 1995), as well as a drug that inhibits another stage of viral replication, for example a protease inhibitor (Gao *et al*, 1999). It has been suggested, however, that sensitivity to individual ddNs can be partially restored by combination therapy with inhibitors of thymidylate synthase, such as 5-FU (Gao *et al*, 1999), as discussed below.

As discussed in 1.2.6.2, 5-FU is anabolised to nucleotides that inhibit the enzyme thymidylate synthase. This enzyme plays a key role in the synthesis of the nucleotide dTTP, and inhibition thus depletes dTTP pools and blocks DNA synthesis (Gao *et al*, 1995). It has been found that at clinically-used doses, the 5-FU deoxynucleoside 2'-deoxy-5-fluorouridine (FUdR), through inhibition of thymidylate synthase (Santi *et al*, 1974), inhibits the synthesis of HIV-1 p24 antigen (Gao *et al*, 1995).

12.5.2.1.2 Combination therapy with AZT or D4T

Furthermore, the use of FUdR, at a low concentration of 0.25 μM, significantly decreases the concentration of D4T required to decrease p24 levels (Gao *et al*, 1995). In host cells, this dTTP depletion results in a compensatory increase in the activity of thymidine kinase, which catalyses the initial phosphorylation of D4T. In the absence of FUdR, thymidine kinase is under feedback inhibition by dTTP (Bresnick and Karjala, 1964; Black and Hruby, 1992). The presence of the fraudulent nucleotide thus results in an increase in the activity of thymidine kinase, by decreasing dTTP levels, and this enhances the phosphorylation of D4T (Gao *et al*, 1995; Ahluwalia *et al*, 1996). This further increases the antiretroviral efficacy of the drug, as D4T triphosphate is the active form that inibits RT. HIV does not have the ability to synthesise dTTP, and the use of the FUdR is thus beneficial as it enhances the antiretroviral effect of D4T as well as decreases the concentration of the latter required to exert such an effect (Gao *et al*, 1995), thereby potentially decreasing the adverse effects experienced by the patient.

The ability of ddNs, such as AZT and D4T, to inhibit RT and terminate viral chains of DNA does not depend on the absolute concentration of the active triphosphate form (ddNTP) generated within cells, but rather on the ratio of ddNTP to 2'-deoxynucleoside-5'-triphosphate (dNTP) (Gao *et al*, 1999). An example of this, using AZT, is the AZTTP/ dTTP ratio. The relevant dNTP is generated by the host cell, is an absolutely essential requirement for HIV replication and competes with the ddNTP for insertion into the DNA chain. The levels of dNTP thus exert an important influence over the efficacy of ddN antiretrovirals (Gao *et al*, 1995). Considering the ddNTP/ dNTP ratio, it is evident that an enhanced antiretroviral effect can be achieved by an increase in ddNTP levels, or a decrease in dNTP levels (Gao *et al*, 1999). 5-FU, by depleting dTTP levels through the inhibition of thymidylate synthase, thus increases the ratio and promotes the inhibition of HIV replication by the antiretroviral, thereby enhancing HIV sensitivity to the latter (Gao *et al*, 1999; Johns and Gao, 1998; Lori *et al*, 1997). Co-therapy with a single dose of 5-FU or FUdR has been shown to fully restore HIV sensitivity to AZT (Gao *et al*, 1999; Medina *et al*, 1996). It is necessary for both 5-FU and AZT or D4T to be present simultaneously either before or during the process of reverse transcription, in order to inhibit the activity of RT (Gao *et al*, 1999).

Resistance to ddNs is a result of amino acid substitutions in the structure of RT, which decreases the affinity of the enzyme for ddNTPs. There is also a loss in the substrate affinity of ddNTPs, which indicates an impaired ability to incorporate within and terminate HIV-1 DNA chains (Gao *et al*, 1999; Martin *et al*, 1993; Ueno *et al*, 1995). Resistance thus develops because even though there is little alteration in the catalytic efficiency of RT, which is reflected in the ability to utilise dNTPs for the process of reverse transcription, there is impaired inhibition of the enzyme by ddNTPs and a failure of these nucleotides to compete with dNTPs for insertion into DNA chains (Gao *et al*, 1999). Lowering the level of dNTPs would thus promote binding of ddNTPs to RT (Gao *et al*, 1999). The use of 5-FU, an agent that targets host cells, with agents that selectively target the virus, is thus useful in controlling antiretroviral resistance (Lori *et al*, 1997). The concentration of 5-FU used, 0.5-2.0 μM is significantly lower than doses used clinically for an antineoplastic effect, and this decreases cytotoxicity to host cells (Gao *et al*, 1999). A concentration of 9 μM has been used and found to be non-toxic to peripheral blood mononuclear cells (Gong *et al*, 1996). The 5-FU-induced decrease in dTTP levels is, furthermore, transient, and this attenuates toxicity to host cells. Once dTTP levels return to normal, extension of HIV DNA chains cannot occur, because RT lacks a 3'-5'-exonucleolytic ability and thus cannot correct the insertion of the fraudulent ddNTP instead of the physiological dNTP. Normal host cells do, however, possess DNA polymerases, which are able to differentiate between ddNTPs and dNTPs and thus insert the

correct nucleotide into growing DNA chains, thus further minimising damage to host cells (Gao *et al*, 1999).

The aforementioned ability of 5-FU to enhance the efficacy of the pyrimidine-based ddNs, AZT and D4T, is not as a result of the inherent cytotoxicity of 5-FU, as control experiments demonstrate that 5-FU does not enhance the anti-HIV efficacy of purine-based ddNs (Gong *et al*, 1996), possibly due to a lack of effect on dUTP pools. This strategy can be used, however, to restore sensitivity to other (purine-based) ddNs, provided that depletion of the appropriate dNTP can be induced (Lori *et al*, 1997). An example is the use of hydroxyurea to enhance dideoxyinosine sensitivity by depleting dATP levels (Gao *et al*, 1994)

12.5.2.2 Enhanced HIV expression

Some investigators have shown that 5-FU, as well as some other anticancer agents, enhances the expression of HIV in latently-infected ACH2 (CD4+) cells (O'Brien *et al*, 1995). This is due to a twenty-six-fold increase in the production of p24 Gag protein. 5-FU does not alter the methylation pattern of DNA in the infected cells, as its mechanism of action is not one of alkylation (O'Brien *et al*, 1995). These authors also show that 5-FU stimulates the binding activity of NF-kB to DNA (O'Brien *et al*, 1995). NF-kB elements situated in the LTR region of HIV play an important role in regulating viral expression in a variety of cells (Raziuddin *et al*, 1991; Nabel and Baltimore, 1987).

In a study using human cervical carcinoma HeLa cells (Panozzo *et al*, 1996), clinically-used doses of 5-FU were found to increase the activation HIV-LTR. An increase in the expression of HIV-LTR is associated with cell death (Schreck *et al*, 1995; Libertin *et al*, 1994; Woloschak *et al*, 1994). The 5-FU-induced increase in HIV-LTR expression is modest in comparison to that induced by the antineoplastic doxorubicin (Panozzo *et al*, 1996). 5-FU-induction of HIV-LTR occurs over a longer period of time, namely several days, (Panozzo *et al*, 1996; Zoumpourlis *et al*, 1991), and the magnitude of induction is not more than double (Panozzo *et al*, 1996). The effects of 5-FU are delayed as the antimetabolite requires time in order to disrupt metabolic processes and result in cell death (Panozzo *et al*, 1996). The combination of UV radiation and 5-FU does not result in an increase in HIV-LTR expression (Panozzo *et al*, 1996).

Even though 5-FU induces the expression of HIV, it is suggested that the virions produced are likely to be non-functional, due to the presence of fraudulent nucleotides within HIV DNA chains. A limitation of the aforementioned studies that have been performed on HIV expression (O'Brien *et al*, 1995; Panozzo *et al*, 1996) is that these experiments were

conducted *in vitro* and have not focused on the potency or pathogenicity of the virus that has been induced.

12.6 Use of melatonin by HIV-positive patients

More than one-third of HIV-infected patients resort to alternative, or complementary, medicine, and one of the agents often used for its ability to boost the immune system, is melatonin. The use of such complementary medication is often without the knowledge of the patient's medical practitioner, and drug interactions with antiretrovirals may exist (Manfredi and Chiodo, 2000). It is thus important to critically assess the use of melatonin in HIV infection.

Melatonin has two important effects that impact on its potential use by HIV-positive individuals:
(i) it enhances the immune system (see 1.3.6.1), via mechanisms mediated by lymphokines and T-helper cell-derived opioid peptides (Maestroni, 1993). The hormone is also immunostimulatory by activating monocytes (Morrey *et al*, 1994) and enhancing the signals required for the proliferation of T cells, as well as the presentation of antigens to T cells by macrophages in the spleen (Pioli *et al*, 1993). By enhancing the synthesis of IL-12, melatonin strengthens the type 1 cytokine profile and its immunostimulatory effects. Melatonin administration in HIV is thus of value, as it has been found that during HIV infection, endogenous melatonin and IL-12 levels are progressively decreased (Nunnari *et al*, 2003); and (ii) it induces both the mRNA required for IL-1α and IL-1β synthesis and the secretion of IL-1 (Morrey *et al*, 1994), and thus promotes the replication of HIV by the mechanisms described in 12.3.1 and 12.3.2 (Corley, 1996). This ability to induce HIV replication may counter its usefulness in stimulating the defunct immune system (Morrey *et al*, 1994).

12.7 Effects of 5-FU and melatonin on corticosterone levels

The focus of this Chapter is to investigate the effects of 5-FU and melatonin on levels of corticosterone, the major glucocorticoid in rats. The effects of 5-FU on HIV have been discussed (see 12.5), as well as the use of melatonin by HIV-positive patients (see 12.6). According to the glucocorticoid hypothesis, the progression of HIV to AIDS is stimulated by excess levels of glucocorticoids, the major one in humans being cortisol (see 12.3). If 5-FU and melatonin are to be used by HIV-positive patients, it is thus of paramount importance to determine the effects of these drugs on glucocorticoid levels.

12.8 Experimentation

The effects of 5-FU and melatonin on serum corticosterone levels in rats after treatment for five days were determined by the use of a Correlate-EIA™ Corticosterone Enzyme Immunoassay Kit.

12.8.1 Materials, animals, reagents and equipment

5-Fluorouracil and melatonin were purchased from the Sigma Chemical Corporation, St Louis, MO, USA. Diethyl ether was obtained from Saarchem® (Wadeville, South Africa) and heparin sodium was purchased from Wallace's Pharmacy (Grahamstown, South Africa). All solutions were prepared using deionised water (Milli R/Q System, Millipore®), and absolute ethanol at a final concentration of 0.67% v/ v was used as a co-solvent to dissolve melatonin. The Correlate-EIA™ Kit was purchased from Assay Designs Inc, Ann Arbor MI, USA. This kit contained the following:

(i) a microtitre plate with 96 wells, coated with donkey anti-sheep immunoglobulin G (IgG);
(ii) corticosterone EIA conjugate, a blue solution of corticosterone conjugated with alkaline phosphatase;
(iii) corticosterone EIA antibody, a yellow solution of sheep polyclonal antibody to corticosterone;
(iv) Assay Buffer 15 concentrate, which consists of Tris-buffered saline with proteins and the preservative sodium azide;
(v) Wash Buffer concentrate, consisting of Tris-buffered saline and detergents;
(vi) corticosterone standard solution at a concentration of 200 000 pg/ ml;
(vii) a solution of p-nitrophenyl phosphate in buffer (p-Npp substrate);
(viii) a Stop Solution, of trisodium phosphate in water; and
(ix) a plate sealer.

Prior to the experiment, 10 ml of the supplied Assay Buffer 15 was diluted to 100 ml with deionised water, and 5 ml of the supplied Wash Buffer concentrate was similarly diluted to 100 ml. Standards were prepared as outlined in Appendix E. The kit was stored at 4ºC until required.

Twenty male Wistar rats, each weighing between 200g and 300g, and cared for as outlined in Appendix A, were used. An Ika-Vibrax-Vxr plate shaker (Ika Labortechnik Staufen, Janke and Kunkel GmbH and Co., KG, Germany) was used. Results were read using a PowerWave X 96-well microplate reader (Bio-Tek Instrument Inc, South Africa), with KC Junior Software (Bio-Tek Instruments Inc, South Africa).

12.8.2 Method

Twenty rats, divided into four groups of five, were treated for a period of five days with either: (i) 5-FU, (ii) melatonin, (iii) a combination of these, or (iv) 0.9% saline, as described in Table 10 (see 3.6.2.2). On the sixth day, the rats were anaesthetised with diethyl ether in a sealed glass container. An aliquot of 1 ml of 1000 iu/ ml heparin was injected into the renal artery and blood was drawn from the heart. The blood samples were centrifuged at 4000 rpm for 2 mins, and the serum supernatant was pipetted off and collected. The serum was stored at -70ºC until the assay was performed.

The procedure employed was as specified in the Correlate-EIA™ Kit manual (Assay Designs Inc, Ann Arbor MI, USA). This procedure has been validated by the manufacturers, according to the National Committee for Clinical Laboratory Standards Evaluation Protocols (1989), and exhibits a sensitivity of 26.99 pg/ ml, acceptable linearity and precision, and 112% recovery of corticosterone from equine serum (Correlate-EIA™ Kit manual, 2004). All samples and reagents were left at room temperature (20-25ºC) for at least 30 mins. An aliquot of 100 µL of diluted Assay Buffer 15 was pipetted into the NSB and Bo wells (see Figure 129 for an illustration of the microtitre plate and the labelling of wells). This was followed by the addition of 100 µL of the corticosterone standards and serum samples into the appropriate wells. The serum samples were added to duplicate wells. An aliquot of 50 µL of diluted Assay Buffer 15 was added to the NSB wells. This followed by 50 µL of blue Conjugate into each well, except the TA and Blank wells. An aliquot of 50 µL of yellow Antibody was added to each well, except for the TA, NSB and Blank wells. This antibody binds to corticosterone present in the sample or standard. All wells were green in colour, except for the blue NSB wells, and the TA and Blank wells were empty. The plate was incubated at room temperature on a plate shaker for 2 hours, at 500 rpm. The wells were emptied and washed by the addition of 400 µL of diluted Wash Buffer to each well. The wells were subsequently emptied and washed twice more in the same way. After this, the wells were emptied and the plate firmly tapped onto lint-free paper to remove any remaining Wash Buffer. It was ensured that no Wash Buffer remained, as this would result in variations in the results that are obtained. An aliquot of 5 µL of blue Conjugate was added to the TA wells, followed by 200 µL of p-Npp Substrate solution to each well on the plate. The plate was incubated at room temperature for 1 hour without shaking. An aliquot of 50 µL of Stop Solution was added to each well to stop the enzymatic reaction and the yellow colour generated was read immediately on a microplate reader at 405 nm.

The optical intensity of the yellow colour is inversely proportional to the concentration of corticosterone present. The average net optical density bound for each sample and standard

was calculated by subtracting the average NSB optical density from the average optical density bound. The net optical density bound was subsequently expressed as a percentage of the maximum binding wells (Bo), thereby giving the percentage of corticosterone bound in each well. This was converted to the concentration of corticosterone present by using the calibration curve equation generated by the standards (see Appendix E).

Blank (substrate+stop solution)	Std 1	Std 5	5FU 2A	Mel 5A	Con 3B	Mel 1B	FM 4B
Blank	Std 1	Std 5	5FU 3A	FM 1A	Con 4B	Mel 2B	FM 5B
TA (conjugate, substrate+stop solution)	Std 2	Con 1A	5FU 4A	FM 2A	Con 5B	Mel 3B	
TA	Std 2	Con 2A	5FU 5A	FM 3A	5FU 1B	Mel 4B	
NSB (conjugate, substrate+stop solution)	Std 3	Con 3A	Mel 1A	FM 4A	5FU 2B	Mel 5B	
NSB	Std 3	Con 4A	Mel 2A	FM 5A	5FU 3B	FM 1B	
Bo (conjugate, antibody, substrate+stop solution)	Std 4	Con 5A	Mel 3A	Con 1B	5FU 4B	FM 2B	
Bo	Std 4	5FU 1A	Mel 4A	Con 2B	5FU 5B	FM 3B	

Figure 129: An illustration of the IgG- coated microplate used for the Correlate-EIA™ corticosterone assay

Std = Standard; Con = Control; Mel = Melatonin; FM = 5-FU + Melatonin

12.9 Results

Figure 130 shows the average concentraton of corticosterone obtained for all treatment groups. The results were statistically analysed using ANOVA, followed by the Student-Newman-Keuls multiple-range test.

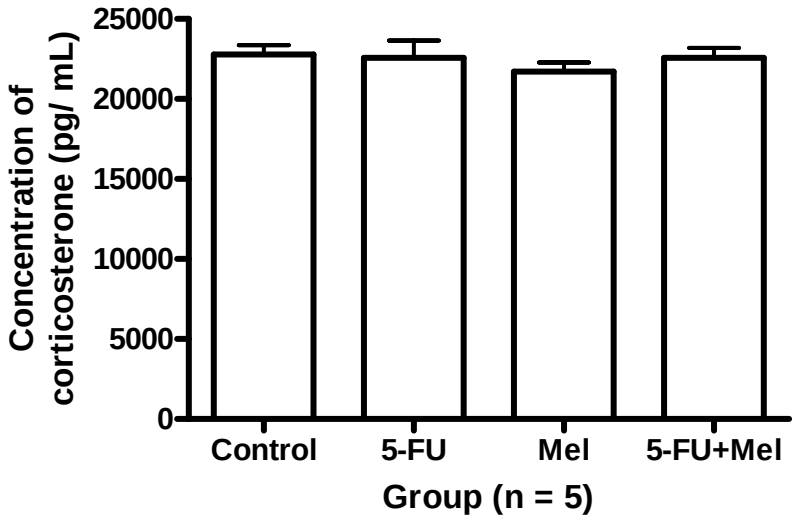


Figure 130: The effects of five days of 5-FU and melatonin administration on serum corticosterone levels

Each bar represents the mean ± SEM

As shown in the above graph, no signifcant differences occur across the groups.

12.10 Discussion

Figure 130 shows that neither 5-FU nor melatonin alters serum corticosterone levels. From the perspective of the glucocortcoid hypothesis of AIDS (see 12.3), and extrapolating the data to reflect cortisol levels in humans, it would thus imply that 5-FU and melatonin can be used in HIV-positive patients without shortening the progression time to AIDS. The use of a low dose of 5-FU in conjunction with either AZT or D4T would seem to be beneficial. The anti-HIV activity of the antretroviral is enhanced due to the inherent ability of 5-FU to inhibt HIV-p24 levels (see 12.5.2.1.1) and inhibit RT, as well as 5-FU-induced depletion of dTTP pools, which also enhances HIV sensitivity to the antiretroviral (see 12.5.2.1.2). The latter effect is achieved *in vitro* at doses of 5-FU that are lower than those used in cancer therapy, thereby minimising host cell toxicity. Further studies are recommended to determine the doses of 5-FU to be used *in vivo* as co-therapy with AZT or D4T, in order to minimise the adverse effects of 5-FU, which include immunosuppression. The dose of 5-FU used is also of importance as it has been shown in Chapter 6 that 5-FU decreses DA levels, and thus the use

of this agent might worsen or enhance the progression of HIV-induced dementia (see 12.2.1) and/ or depression (see 12.2.3). The co-administration of melatonin was found to counter this 5-FU-induced decrease in DA levels (see Chapter 6), and thus the use of melatonin in conjunction with low-dose 5-FU as co-therapy in HIV might be beneficial in attenuating adverse biochemical alterations induced by the latter.

Several investigators have reported that 5-FU induces HIV-LTR expression, thus increasing virion levels *in vitro* (see 12.5.2.2). These studies, however, did not determine the potency of the virions whose expression was enhanced by 5-FU. Due to a lack of a suitable *in vivo* model of HIV, it is thus not known whether these virions, with DNA containing 5-FU-derived fraudulent nucleotides, are as potent and infectious as the wild-type virus. 5-FU exerts its antineoplastic action through the insertion of fraudulent nucleotides into the growing DNA chains of cancer cells, thus inhibiting DNA synthesis. 5-FU also inhibits RNA synthesis through the same mechanism (see 1.2.6.2). It might well be that, in HIV-infected cells, 5-FU-derived fraudulent nucleotides are similarly incorporated into the RNA of the virus. When this RNA is integrated into the host cell DNA, in the process of reverse transcription, defunct DNA is synthesised that cannot code for infectious viral particles and proteins.

Melatonin-induced increases in IL-1 levels are discussed in 12.6, and this increase may theoretically enhance glucocorticoid levels, thereby promoting the progression to AIDS (see 12.3.1). Although an increase in corticosterone levels were found after the long-term administration of melatonin by one author (Bojková *et al*, 2006), this has not been found in the present study, in which melatonin was administered for a period of five days. Based on the results of this Chapter, the co-administration of melatonin to HIV-positive patients is unlikely to promote the progression to AIDS as glucocorticoid levels are not altered. This suggests that melatonin does not significantly increase IL-1 levels, or perhaps that this increase is counteracted by other physiological mechanisms that maintain IL-1 levels in the immunocompetent rats used in this study. The effects of 5-FU and melatonin on IL-1 and other cytokine levels will be investigated in Chapter 13. This is important, as decreases in type 1 and increases in type 2 cytokine levels mirror the progression to AIDS (see 12.3.2). Further studies are required to determine the time point at which any melatonin-induced alterations in glucocorticoid levels occurs. The benefits of melatonin administration would be its inherent immunostimulatory effects, as well as a decrease in 5-FU, and perhaps AZT/ D4T toxicity.

It is known that cortisol secretion undergoes a circadian rhythm, peaking at 08h00 and troughing between 23h00 and 02h00, and that this rhythm is not disturbed in advanced cancer

patients (Raida *et al*, 2002). It would be important to determine is there is an alteration in this periodicity of secretion in HIV/ AIDS.

Despite the lack of alterations in corticosterone levels due to both 5-FU and melatonin, these agents were both shown in Chapter 5 to enhance the activity of IDO. This could worsen the increased IDO activity that occurs in HIV-positive patients (Schroecksnadel *et al*, 2007; Boasso and Shearer, 2007) and result in the formation of kynurenines, such as 3-OHK and QA, which induce the apoptosis of thymocytes and type 1 T lymphocytes through ROS-mediated mechanisms (see 5.2.2). This shifts the type 1/ type 2 T cell ratio towards type 2 cells (Grohmann *et al*, 2003) and this decreases the progression time to AIDS (see 12.3.2). Increased IDO activity in AIDS patients is also associated with cachexia, diarrhoea and dementia (Brown *et al*, 1991). The effects of 5-FU and melatonin on IDO activity, and the deleterious effects of such activity on the immune system could thus potentially offset any beneficial effects of 5-FU and melatonin use in HIV infection. Further studies are required to determine the lowest possible doses of these agents, in order to avoid IDO stimulation and minimise changes in the profile of T lymphocytes.

12.11 Conclusions

Neither 5-FU nor melatonin alters serum corticosterone levels, thus suggesting that the use of these agents by HIV-positive patients is unlikely to hasten the progression to AIDS, according to the glucocorticoid hypothesis. The use of low-dose 5-FU co-therapy is beneficial in enhancing the anti-HIV activity of AZT and D4T as well as decreasing antiretroviral resistance. Melatonin administration both enhances the immune system of these immunocompromised patients as well as decreases the toxicity of the other drugs being used. However, it has been shown previously that both 5-FU and melatonin enhance IDO activity, which may induce changes in the T lymphocyte profile that favour the progression to AIDS. It is thus recommended that the lowest possible doses of 5-FU and melatonin are used, and that further studies are undertaken to determine these dosages. The findings of this Chapter are limited by the lack of a suitable *in vivo* rat model of HIV. Using such a model, further studies are recommended to determine the potency of HIV upon 5-FU treatment and the long-term effects of 5-FU and melatonin treatment on glucocorticoid levels in an immunocompromised host. The effects of 5-FU and melatonin on cytokine levels will be investigated in the next Chapter; this is significant as alterations in the cytokine profile are indicative of the progression to AIDS.

CHAPTER 13

THE EFFECTS OF 5-FLUOROURACIL AND MELATONIN ON CYTOKINE LEVELS

13.1 Introduction

Various references to cytokines, also termed the "hormones of the immune system" (Myint and Kim, 2003) and the "messenger molecules of the immune system" (Abbas, 2005), have been made throughout this thesis. These shall be summarised on the next page.

Cytokines are signalling proteins that mediate interactions between various types of immune cells, are soluble and possess a short duration of action (Abbas, 2005). Most cytokines mediate a diversity of effects, and can be synthesised from different kinds of cells. There are many types of cytokines: interleukins are those which have been defined on a molecular basis and which facilitate communication between leukocytes; monokines are derived from monocytes, lymphokines from lymphocytes; and other polypeptides that play a role in regulating inflammatory, immune and reparative responses by the host (Abbas, 2005).

Cytokines are divided into the following five functional categories, although some may belong to more than one group (Abbas, 2005):
(i) those involved in natural immunity, for example TNFα, IL-1, IL-6, IL-12, INF-1 and INFγ. IL-12 and INFγ mediate both natural immunity as well as adaptive immunity, in response to micro-organisms found intracellularly. IL-1 and TNFα mediate the recruitment of leukocytes and respond to acute inflammation, whereas the interferons mediate immunity against viral infections;
(ii) those regulating the growth, stimulation and differentiation of lymphocytes, such as IL-2, IL-4, IL-10, IL-12, IL-15 and transforming growth factor β (TGFβ). IL-2 promotes the growth of T cells, while IL-4 is responsible for the differentiation of T helper 2 cells and IL-12 enhances the differentiation of T helper 1 cells. IL-15 enhances NK cell activity, and TGFβ and IL-10 play roles in down-regulating immunological responses;
(iii) those influencing the movement of leukocytes. These cytokines are also termed "chemokines" and are divided into two categories, based on the position of cysteine moieties in their structure: C-C chemokines are synthesised predominantly by T cells, while C-X-C chemokines are produced by activated tissue cells and macrophages. These different chemokines mediate the movement of various types of leukocytes to local sites of inflammation. A further function of chemokines is the anatomic distribution of different types of cells, for example the localisation of B and T cells in certain lymphoid regions;

(iv) cytokines that stimulate inflammatory cells and thus result in acute inflammation. This includes IL-5, INFγ, TNFα and TNFβ, which activate eosinophils, macrophages, neutrophils and endothelial cells, respectively; and
(v) those mediating haematopoiesis, by interacting with haematopoietic progenitor cells. This group includes colony-stimulating factors and stem cell factor (Abbas, 2005).

The following references have been made to cytokines in the preceding chapters:
(i) tumours release cytokines, which interfere with circadian functionng (Born *et al*, 1997) (see 1.2.9.2);
(ii) the addition of cytokines counters 5-FU-induced immunosuppression by enhancing certain immunoglobulin antibody responses (Větvička *et al*, 1995) (see 1.2.10.3);
(iii) the stimulation of melatonin receptors results in the release of the following cytokines: IL-2 (Maestroni, 1999); INFα (Maestroni, 1999), which stimulates the synthesis and activity of NK cells (Abrial *et al*, 2004); IL-1; IL-6 and IL-12 (Abrial *et al*, 2004) (see 1.3.6.1);
(iv) melatonin increases the levels of IL-2, which stimulates the immune system by promoting the development of T and B lymphocytes (Abrial *et al*, 2004), and decreases the levels of IL-6, which is immunosuppresive (Lissoni *et al*, 1996c) (see 1.3.7);
(v) another report has shown that melatonin increases IL-6 levels, thus promoting the synthesis of platelets (Abrial *et al*, 2004) and the budding off of platelets from megakaryocytes (Lissoni *et al*, 1999c). This counters thrombocytopaenia by increasing the number of circulating platelets (Lissoni *et al*, 1999b) (see 1.3.9);
(vi) melatonin enhances haematopoiesis by increasing the activity of melatonin-induced opioids, cytokines which stimulate κ_1 opioid receptors on macrophages in bone marrow (Maestroni, 1999, 2001) (see 1.3.9);
(vii) melatonin has anti-inflammatory properties, by down-regulating inflammatory mediators, some of which are cytokines (Reiter *et al*, 2000; Perianayagam *et al*, 2005) (see 1.3.9);
(viii) IL-2 also has immunosuppressive properties, by increasing the release of IL-10 (Lissoni *et al*, 1995c). The administration of melatonin and IL-12 greatly inhibits the IL-2-mediated release of IL-10 (Lissoni *et al*, 1995c) (see 1.3.9);
(ix) IL-2 induces cortisol secretion and this is a further mechanism by which this cytokine is immunosuppressive (Lissoni *et al*, 2005a) (see 1.3.9);
(x) melatonin counters IL-2-induced hypotension (Lissoni *et al*, 1996e) (see 1.3.9);
(xi) by increasing the synthesis of some cytokines, melatonin contributes to the development of autoimmune diseases (Maestroni, 2001) (see 1.3.12.3);
(xii) INFγ enhances the expression of IDO (Rodrigues *et al*, 2003; Carlin *et al*, 1989; Ozaki *et al*, 1988), thus resulting in both antiproliferative and toxic effects (see 5.2.2). IDO is also

stimulated by IL-1, IL-2 and TNF (Myint and Kim, 2003) (see 5.2) and one of the effects of such stimulation is immunosuppression and changes in T cell profiles indicative of AIDS (see 5.2.2);

(xiii) the macrophage hypothesis of depression states that IL-1β (Smith, 1991) and other pro-inflammatory cytokines (Myint and Kim, 2003) induce overstimulation of the HPA axis, due to an increase in CRF secretion from the hippocampus (Smith, 1991). High levels of IL-1β are also noted in response to infections and stress (Myint, 2007) (see 6.2.2.14);

(xiv) the stimulation of the immune system in depression results in the production of pro-inflammatory cytokines, namely IL-1, IL-2, IL-6, IL-12, INFγ and TNFα (Maes, 1999; Kim *et al*, 2002; Leonard, 2001; Licinio and Wong, 1999). These enhance IDO activity and the metabolism of tryptophan is thus shifted towards the generation of the toxic metabolites QA and 3-OHK (Myint and Kim, 2003). The pro-inflammatory cytokines mentioned before also decrease tryptophan levels by reducing food intake (Reichenberg *et al*, 2001; Plata-Salaman and Ilyin, 1998); the metabolism of NE, DA and 5-HT is, furthermore, altered (Dunn *et al*, 1999) (see 6.2.2.16);

(xv) the pro-inflammatory cytokines IL-5, IL-6, IL-11 and leukaemia-inducing factor are associated with cancer-associated wasting (Onuma *et al*, 2005) (see 11.3);

(xvi) type 1, or pro-inflammatory, cytokines interact with host T cells and macrophages latently infected with HIV, and activate these. This results in the release of NF-κB and thus increased viral transcription, replication and host cell lysis (Abbas, 2005) (see 12.1);

(xvii) cytokines induce neurotoxicity, which occurs in HIV-induced dementia (Lipton, 1994) (see 12.2.1);

(xviii) the gp120 moiety of HIV induces the synthesis of IL-1 (Sundar *et al*, 1991; Clouse *et al*, 1991), which stimulates the release of CRH and ultimately ACTH and cortisol (Biglino *et al*, 1993; Sandi and Guaza, 1995). This hastens the progression to AIDS in HIV-infected individuals, according to the glucocorticoid hypothesis (Corley, 1996, 1997) (see 12.3.1);

(xix) HIV disease progression is characterised by impaired CMI and stimulation of HI. The former results in a decrease in the levels of the type 1 cytokines INFγ, IL-2 and IL-12; whereas the latter is associated with an increase in the type 2 cytokines IL-4, IL-5, IL-6, IL-10 (Clerici and Shearer, 1993, 1994). It is suggested that the progression from type 1 to type 2 cytokines be considered as a marker of HIV progression (Clerici *et al*, 1997). This shift towards a strengthened type 2 cytokine profile renders CD4+ T lymphocytes more susceptible to PCD (Ameisen and Capron, 1991; Clerici *et al*, 1994), thus leading to the depletion of CD4+ cells that occurs in AIDS (see 12.3.2);

(xx) HIV-induced increases in IL-1 levels may play a significant role in the development of Kaposi's sarcoma (Corley, 1997; Louie *et al*, 1995), via activation of IL-6, which serves as a growth factor for Kaposi sarcoma-derived cells (Yang and Offermann, 1993) (see 12.3.1);

(xxi) glucocorticoids promote the shift from type 1 to type 2 cytokines (Clerici *et al*, 1997; Mayo *et al*, 2002; Dayneo and Araneo, 1989) (see 12.3.2);
(xxii) DHEA counteracts the glucocorticoid-induced inhibition of IL-2 (Daynes *et al*, 1990) and strengthens the type 1 cytokine profile and its more inhibitory effects on HIV progression (Clerici *et al*, 1997) (see 12.3.2);
(xxiii) melatonin increases IL-12 levels, thus strengthening the type 1 cyokine profile (Nunnari *et al*, 2003) (see 12.6); and
(xxiv) melatonin induces the mRNA required for the synthesis of IL-1α and IL-1β, and also increases the secretion of IL-1 (Morrey *et al*, 1994), thus promoting HIV replication (see 12.6).

As shown in the above, the effects of cytokines can vary widely, and even one type of cytokine, for example IL-2, can exert both immunostimulatory and immunosuppressive effects, via different mechanisms. This emphasises the importance of cytokine balance, and the complex results that could occur as a result of alterations in cytokine levels.

13.2 Properties of cytokines

Cytokines share the following general properties, even though different members may exert a diversity of effects (Abbas, 2005):
(i) cytokines may be produced by many different types of cells. IL-1, for example, can be synthesised by fibroblasts, endothelial cells and leukocytes;
(ii) cytokines exert three kinds of effects, namely: autocrine, paracrine and endocrine effects. Autocrine effects are when a cytokine produced by a specific type of cell acts on that particular cell; for example, IL-2 is produced by antigen-activated T cells and induces the growth of these cells. Paracrine effects involve cytokines affecting other neighbouring cells, such as IL-7, synthesised by bone marrow cells and enhancing B-cell progenitor maturation. Endocrine effects are systemic effects, affecting many cells; an example of such an effect is the acute inflammatory response evoked systemically by TNFα and IL-1;
(iii) cytokines are pleiotropic; one cytokine can thus interact with a wide variety of cell types, and mediate a host of effects. IL-2, for example, mediates the growth and differentiation of T, B and NK cells. Different cytokines may also mediate the same or similar physiological responses; this implies that some cytokines may be redundant; and
(iv) cytokines interact with their target cells by binding to high-affinity receptors on these cells. An illustration of this is that the use of monoclonal antibodies to IL-2 receptors on T cells prevents IL-2 from interacting with these receptors and thus inhibits the activation of T cells which occurs during the rejection of transplanted organs (Abbas, 2005).

Knowledge gained about the properties of cytokines is providing the basis for novel means to treat certain pathologies. The use of certain recombinant cytokines, for example, can boost immunity against infections and cancer, while the use of antagonists to TNFα decreases the inflammation occurring in rheumatoid arthritis (Abbas, 2005).

13.3Experimentation

The effects of 5-FU and melatonin on cytokine levels are of key importance in this research. It has been shown in Chapter 5 that both agents induce IDO activity, and this was attributed to the increase in $O_2^{-}\cdot$ levels *in vivo* noted in Chapter 9. However, as illustrated in Figure 43 (see 5.2), IDO activity can also be stimulated by various pro-inflammatory cytokines, such as IL-1, INFγ and TNFα (see 5.2). It is thus useful to determine whether an increase in cytokine levels underlies this increase in IDO activity, in addition to an increase in the levels of $O_2^{-}\cdot$. Although it was shown in Chapter 12 that neither 5-FU nor melatonin alters serum corticosterone levels, an analysis of the effects of these agents on individual cytokines is useful in ascertaining whether there are opposing effects on corticosteroid levels that counteract each other. Changes in the cytokine profile are also indicative of the progression to AIDS in HIV-infected patients, and would thus impact on the use of 5-FU and melatonin by these patients. Furthermore, as discussed in 13.1, cytokines mediate a wide variety of effects on the immune and endocrine systems, and on cell growth and differentiation. The effects of 5-FU, a known immunosuppressant (see 1.2.10.3), and melatonin, an immunostimulant (see 1.3.6.1), on individual cytokines can thus offer potentially valuable insight into the anticancer efficacy and toxicity of these agents, particularly when used in combination.

The levels of nine cytokines, namely IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-10, granulocyte-macrophage colony-stimulating factor (GM-CSF), INFγ and TNFα, will be analysed simultaneously in rat serum, by the Bio-Plex™ Rat Cytokine 9-Plex A Panel Kit (Catalogue number 171-K11070, Bio-Rad Laboratories Inc., Germany). IL-2 and INFγ are type 1 cytokines; while IL-4, IL-6 and IL-10 are type 2 cytokines (see 13.1). This assay is a multiplex immunoassay; it is thus possible to measure the levels of up to 100 different cytokines in a single well, using as little as 12.5 μL of serum sample, at the same time. The advantages of this multiplex system compared to traditional immunoassays, which measure the levels of cytokine at a time, include (Bio-Plex™ Rat Cytokine 9-Plex A Panel Kit manual, Bio-Rad Laboratories Inc., Germany):

(i) a decrease in experimental time, including less time spent on the preparation of samples and reagents;

(ii) the use of coloured beads, instead of a coated well, as the solid phase allows for up to 100 different cytokines to be detected and quantitated. An entire cytokine profile can thus be built from a limited volume of sample; and
(iii) enhanced throughput.

This Bio-Plex suspension array system consists of three fundamental technologies (Bio-Plex™ Rat Cytokine 9-Plex A Panel Kit manual, Bio-Rad Laboratories Inc., Germany):
(i) a family of microspheres, or polystyrene beads, 5.6 μm in diameter. Each set of beads is colour-coded with a fluorescent dye and conjugated with a specific monoclonal antibody, which exhibits a great affinity for the cytokine to be studied. A mixture of different beads is added to a small volume of sample; examples of the latter include serum and cell lysate. An antigen in the sample, which is the cytokine of interest, binds to the monoclonal antibody, forming a bead-antibody-antigen complex. Unbound protein is removed by multiple washing steps, and a specific complementary biotinylated antibody is added to the beads. This detection antibody binds to the antigen in this complex, forming an antibody sandwich around the target cytokine, as shown in the Figure below. The addition of streptavidin-phycoerythrin to this complex allows for the reaction to be detected;
(ii) the contents of each well are drawn up into a flow cytometer, comprising two lasers. This system identifies the reaction occurring on the surface of each microsphere and quantitates this, based on the colour of the bead and fluorescence. Reporter molecules, marked fluorescently and associated with every different target cytokine, are used to measure the extent of the reaction; and
(iii) the Bio-Plex Manager™ digital processor and software automatically calculates the concentration of each cytokine in the sample.

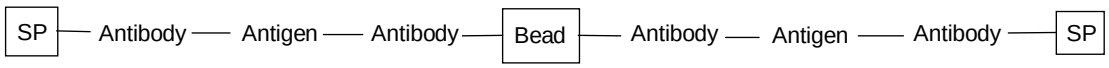


Figure 131: The microsphere complex formed allowing for the detection of different types of cytokines in rat serum (Bio-Plex™ Rat Cytokine 9-Plex A Panel Kit manual, Bio-Rad Laboratories Inc., Germany)
SP = Streptavidin-Phycoerythrin

13.3.1 Materials, animals, reagents and equipment

5-Fluorouracil and melatonin were purchased from the Sigma Chemical Corporation, St Louis, MO, USA. All other reagents were obtained locally and were of the highest chemical purity. All solutions were prepared using deionised water (Milli R/Q System, Millipore®), and absolute ethanol at a final concentration of 0.67% v/ v was used as a co-solvent to dissolve

melatonin. The cytokine kit used was a Bio-Plex™ Rat Cytokine 9-Plex A Panel Kit (Catalogue number 171-K11070), purchased from Bio-Rad Laboratories Inc., Germany. The kit consisted of:

(i) Bio-Plex Assay Buffer;

(ii) Bio-Plex Wash Buffer;

(iii) Bio-Plex Detection Antibody Diluent;

(iv) one vial of Streptavidin-Phycoerythrin;

(v) anti-cytokine conjugated beads;

(vi) one tube of lyophilised recombinant cytokine standards, at a concentration of 32 000 pg/ml;

(vii) Bio-Plex rat serum Sample Diluent;

(viii) a sterile 96-well filter microplate; and

(ix) a plate cover and tray.

The cytokine standards were prepared as outlined in Appendix F. The kit was stored at 4ºC until required, and the Streptavidin-Phycoerythrin was also kept in the dark. Prior to the commencement of the experiment, anti-cytokine conjugated beads were diluted 25-fold with Bio-Plex Assay Buffer, forming the multiplex bead working solution, which was covered with aluminium foil due to the photolability of the beads. The Bio-Plex Detection Antibody was diluted 25-fold with Detection Antibody Diluent and stored at room temperature in the dark. Streptavidin-Phycoerythrin was diluted 100-fold with Bio-Plex Assay Buffer and stored in the dark. Twenty male Wistar rats, each weighing between 200g and 300g, and cared for as outlined in Appendix A, were used. Bio-Plex Manager™ 4.0 computer software (Bio-Rad Laboratories Inc., Germany) was used to read the results and calculate the concentration of cytokines.

13.3.2 Method

Twenty rats, divided into four groups of five, were treated for a period of five days with either: (i) 5-FU, (ii) melatonin, (iii) a combination of these, or (iv) 0.9% saline, as described in Table 10 (see 3.6.2.2). On the sixth day, the rats were sacrificed and the serum collected and stored as described in 12.8.2.

Levels of the nine cytokines were measured according to the procedure specified in the Bio-Plex™ Rat Cytokine 9-Plex A Panel Kit manual (Bio-Rad Laboratories Inc., Germany), which has been tested and validated by the manufacturers. Prior to the commencement of the experiment, all serum samples were left at room temperature and allowed to thaw. Each sample was diluted three-fold with Bio-Plex rat serum Sample Diluent. All other reagents

were also brought to room temperature. The wells in the filter plate were pre-wet with an aliquot of 100 µL of Bio-Plex Assay Buffer per well. The plate was then placed on a calibrated filter plate vacuum and the Bio-Plex Assay Buffer removed by vacuum filtration, at a pressure of 1-2" Hg. The bottom of the filter plate was dried thoroughly with lint-free paper towels. The multiplex bead working solution was vortexed for 15-20 s at medium speed; 50 µL of this was pipetted into each well, and the buffer removed by vacuum filtration. An aliquot of 100 µL of Bio-Plex Wash Buffer was added to each well, and the buffer removed by vacuum filtration as before. The bottom of the plate was dried with lint-free paper. The bottom of each diluted standard and sample tube was gently flicked a few times. An aliquot of 50 µL of diluted standard or sample was added to the appropriate well, as shown in Figure 132. A blank, without any standard or sample, was also used. The plate was sealed, placed on a microplate shaker and covered with aluminium foil. The speed of the shaker was slowly increased to 1100 rpm and maintained at this for 30 s; the speed was subsequently decreased to 300 rpm and the plate incubated at this speed for 30 mins. After this first incubation, the plate was placed on a flat surface and the buffer removed by vacuum filtration. The wells were washed thrice, each time with a volume of 100 µL of Bio-Plex Wash Buffer. After each wash, buffer was removed by vacuum filtration and the bottom of the plate dried with lint-free paper towels. The diluted Bio-Plex Detection Antibody was vortexed gently and 25 µL added to each well. The plate was sealed, covered with aluminium foil and agitated on a plate shaker as before, initially at 1100 rpm for 30 s and then at 300 rpm for 30 mins. After this second incubation, the plate was removed and the buffer removed by vacuum filtration. The wells were washed thrice and the plate dried, as described before. The diluted Streptavidin-Phycoerythrin was vortexed vigorously and 50 µL pipetted into each well. The plate was sealed, covered with aluminium foil and agitated on a plate shaker. The initial speed at which incubation occurred was 1100 rpm for 30 s, followed by 300 rpm for 10 mins at room temperature. The plate was subsequently removed from the shaker and the buffer removed by vacuum filtration. The wells were washed thrice with 100 µL of Bio-Plex Wash Buffer per well, and the plate dried as described before. The beads in each well were resuspended with 125 µL of Bio-Plex Assay Buffer. The plate was then sealed, agitated on a shaker at 1100 rpm for 30 s at room temperature, and the fluorescence intensity of each cytokine read immediately using the Bio-Plex Manager™ digital processor and software. The concentrations of cytokines were calculated using the calibration curves generated in Appendix F, and expressed in pg/ ml.

Blank	Std 4	Std 8	5-FU 2	Mel 5
Blank	Std 4	Std 8	5-FU 3	FM 1
Std 1	Std 5	Con 1	5-FU 4	FM 2
Std 1	Std 5	Con 2	5-FU 5	FM 3
Std 2	Std 6	Con 3	Mel 1	FM 4
Std 2	Std 6	Con 4	Mel 2	FM 5
Std 3	Std 7	Con 5	Mel 3	
Std 3	Std 7	5-FU 1	Mel 4	

Figure 132: An illustration of the microplate used for the Bio-Plex™ Rat Cytokine 9-Plex A Panel assay

Std = Standard; Con = Control; Mel = Melatonin; FM = 5-FU + Melatonin

13.4 Results

The results were analysed by ANOVA, followed by the Student-Newman-Keuls multiple-range test. Figures 133-141 were obtained. As shown in Figure 134, whilst neither 5-FU nor melatonin alone alters IL-1β levels, the co-administration of these agents results in an increase in the levels of this cytokine, from a control value of 30.11 pg/ ml to 43.61 pg/ ml. The administration of 5-FU increases the levels of INFγ, as shown in Figure 140, from a control value of 184.45 pg/ ml to 686.45 pg/ ml. This increase was completely abolished by the co-administration of melatonin. No other differences in cytokine levels occur, and the administration of melatonin does not alter the levels of any of the nine cytokines analysed.

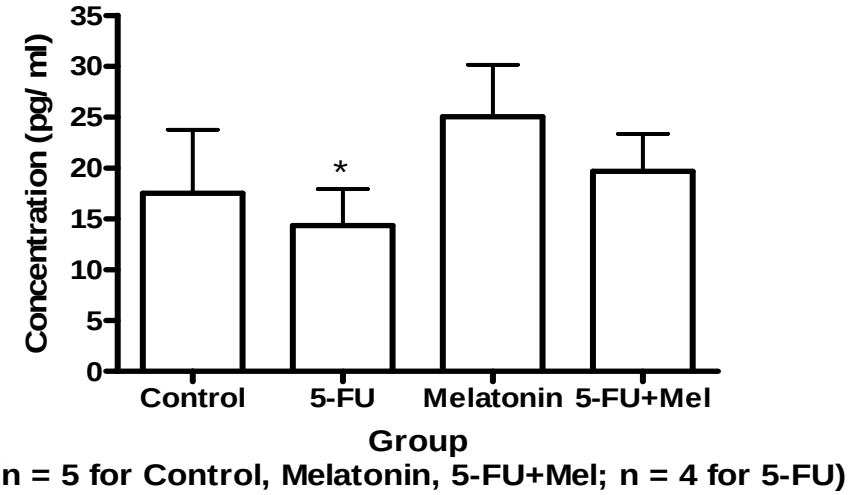


Figure 133: The effects of 5-FU and melatonin on IL-1α levels <u>in vivo</u>

* $p<0.1$ compared to Melatonin, after analysis by ANOVA followed by the Student-Newman-Keuls multiple range test. Each bar represents the mean ± SEM

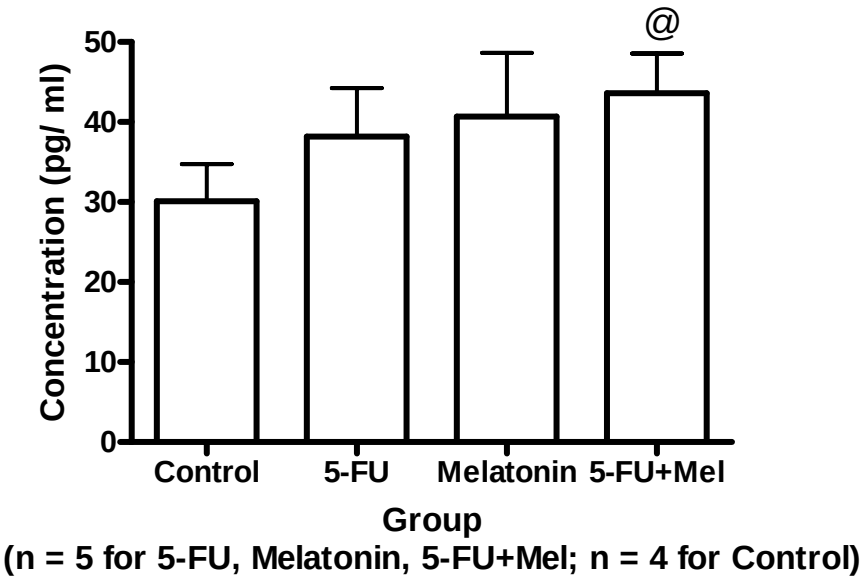


Figure 134: The effects of 5-FU and melatonin on IL-1β levels <u>in vivo</u>

[@] $p<0.05$ compared to control, after analysis by ANOVA followed by the Student-Newman-Keuls multiple range test. Each bar represents the mean ± SEM

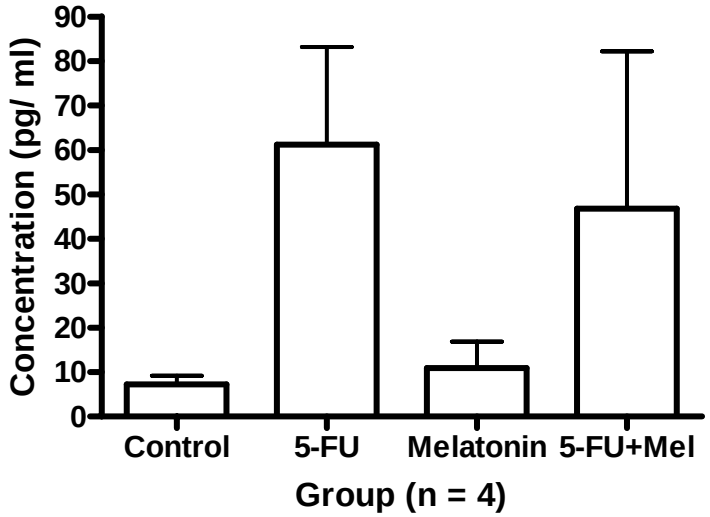


Figure 135: The effects of 5-FU and melatonin on IL-2 levels <u>in vivo</u>

Each bar represents the mean ± SEM

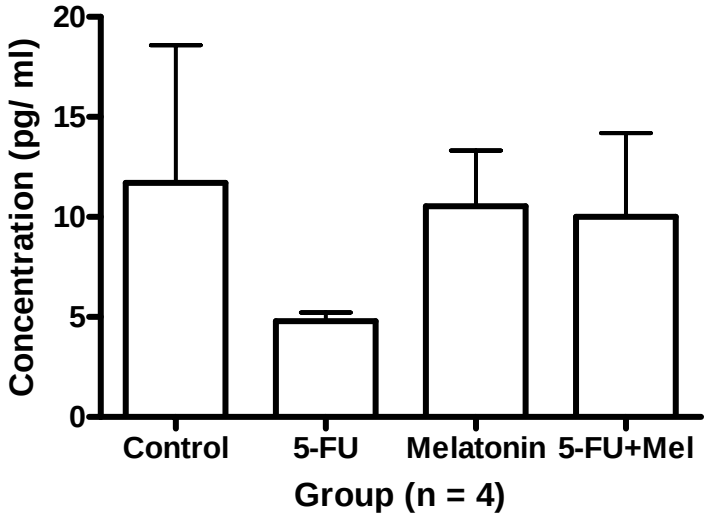


Figure 136: The effects of 5-FU and melatonin on IL-4 levels <u>in vivo</u>

Each bar represents the mean ± SEM

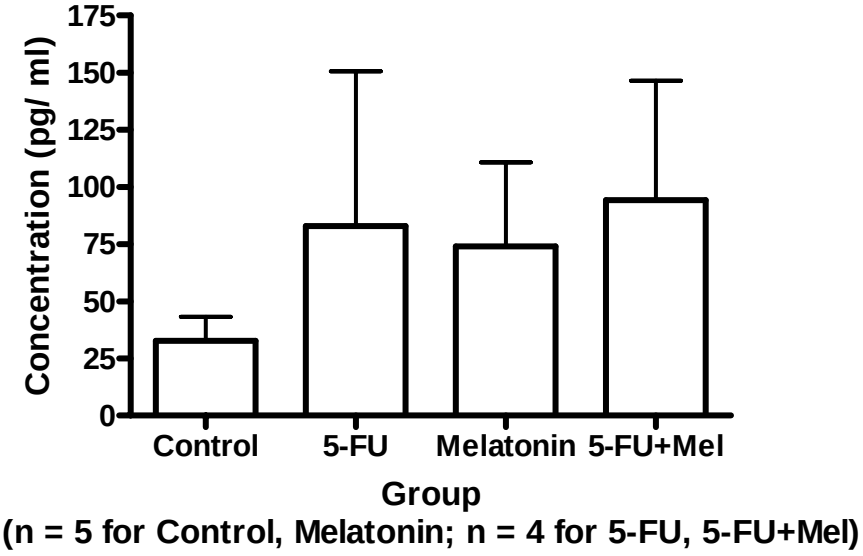


Figure 137: The effects of 5-FU and melatonin on IL-6 levels <u>in vivo</u>

Each bar represents the mean ± SEM

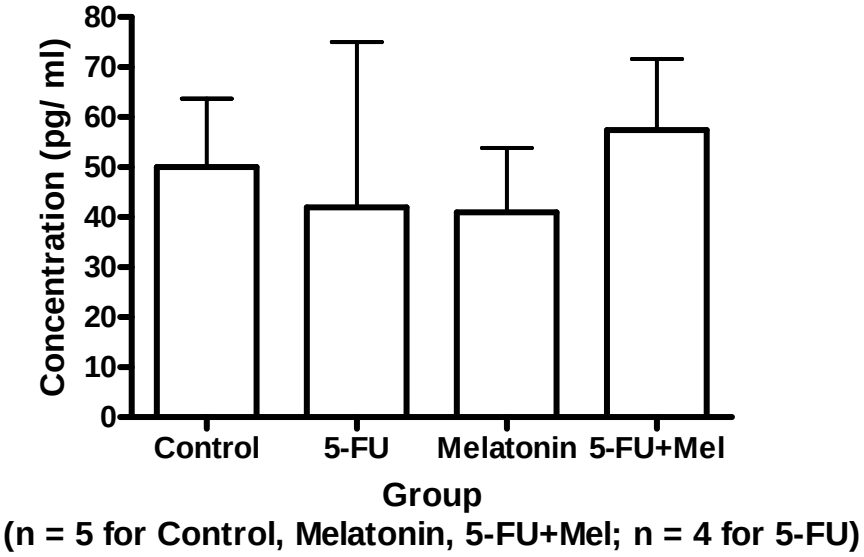


Figure 138: The effects of 5-FU and melatonin on IL-10 levels <u>in vivo</u>

Each bar represents the mean ± SEM

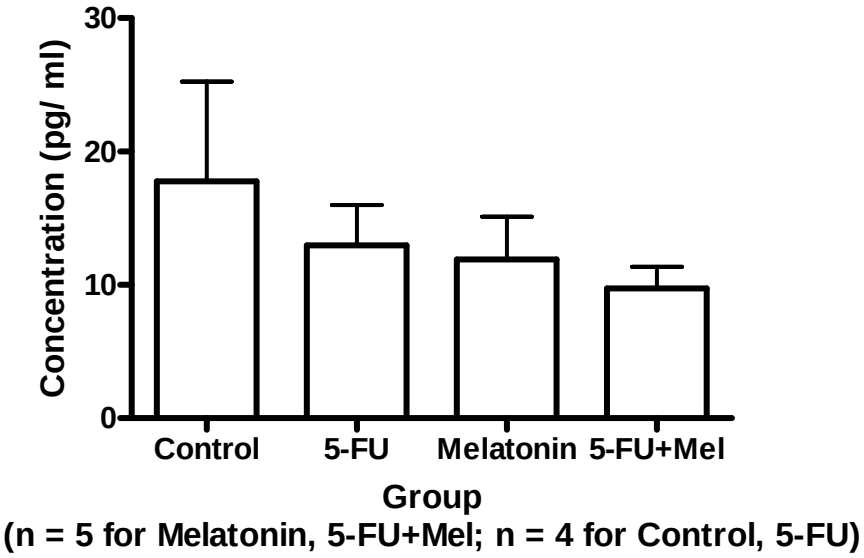


Figure 139: The effects of 5-FU and melatonin on GM-CSF levels <u>in vivo</u>

Each bar represents the mean ± SEM

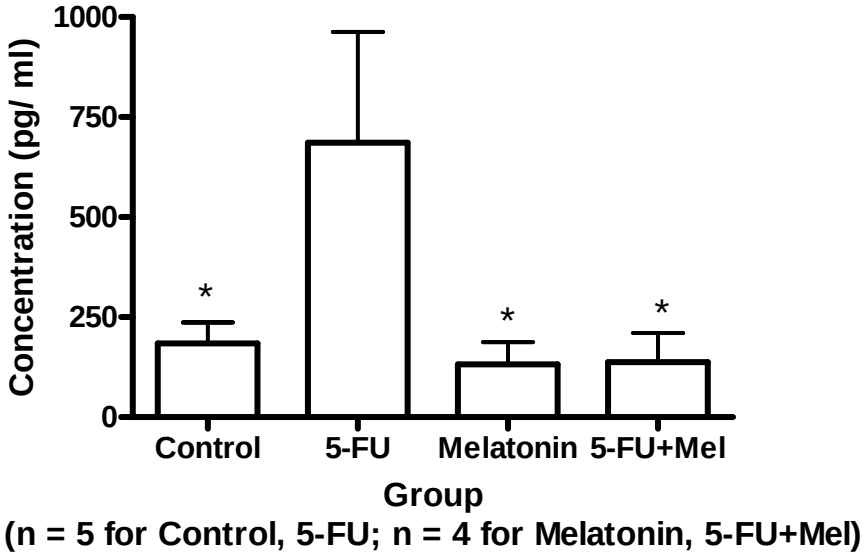


Figure 140: The effects of 5-FU and melatonin on INFγ levels <u>in vivo</u>

* p<0.1 compared to 5-FU, after analysis by ANOVA followed by the Student-Newman-Keuls multiple-range test. Each bar represents the mean ± SEM

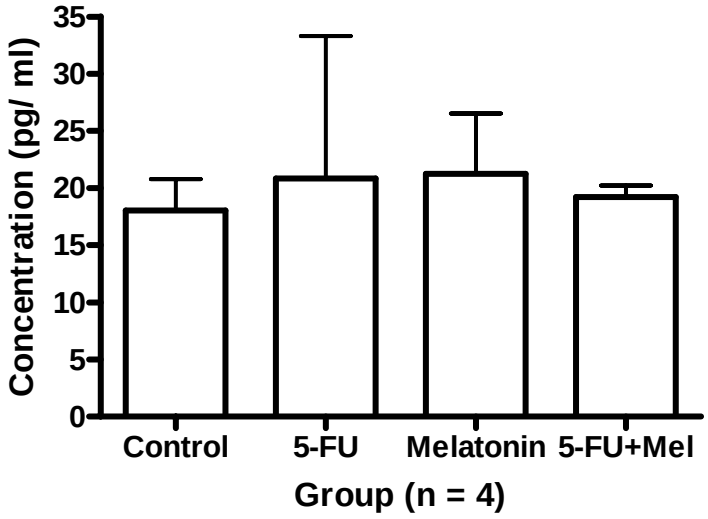


Figure 141: The effects of 5-FU and melatonin on TNFα levels <u>in vivo</u>

Each bar represents the mean ± SEM

13.5 Discussion

The results presented in 13.4 indicate that 5-FU and melatonin exert remarkably few effects on the levels of the nine cytokines studied, at the dosages used. It is noticeable from the error bars in the graphs obtained that there is often considerable intra-group variation in results; this may perhaps mask any real differences between groups. Melatonin is reported to alter the

levels of IL-1, IL-2, IL-6 and IL-10 (Maestroni, 1999; Abrial *et al*, 2004; Lissoni *et al*, 1996c) (see 13.1), yet these differences are not noted in this study. The co-administration of 5-FU and melatonin, however, increases the levels of the pro-inflammatory cytokine IL-1β (see Figure 134). This suggests that when used in combination, these agents exert a summative effect which is sufficient to significantly alter IL-1β levels. The mechanism by which melatonin contributes to the increase in the levels of this cytokine may be by induction of the mRNA required for IL-1β synthesis and an increase the secretion of this cytokine (Morrey *et al*, 1994) (see 12.6); the mechanism by which 5-FU contributes to an increase in IL-1β levels is unknown. The mechanism by which 5-FU increases INFγ levels (see Figure 140) is also at present unknown. The effect of 5-FU on INFγ levels is abolished by the co-administration of melatonin; this suggests that althought melatonin does not exert an effect on endogenous INFγ levels when administered alone, it is able to counter the abnormal increase in INFγ levels induced by 5-FU. This finding is significant, as it shows that combination therapy with melatonin prevents the 5-FU-induced increase in the levels of the pro-inflammatory INFγ. This could represent a mechanism by which melatonin exerts an anti-inflammatory effect, in the presence of an agent that induces inflammatory changes.

The abovementioned increases in IL-1β and INFγ levels are significant, for a number of reasons, discussed below. These pro-inflammatory cytokines activate IDO (Myint and Kim, 2003) (see Figure 43) (see 5.2), with INFγ enhancing the expression of this enzyme (Rodrigues *et al*, 2003; Carlin *et al*, 1989; Ozaki *et al*, 1988) (see 5.2.2). The 5-FU-induced increase in INFγ levels is thus a mechanism by which 5-FU enhances the activity of IDO, as shown in Chapter 5. This is in addition to the ability of the agent to increase the levels of $O_2^{-}\cdot$, cofactors for IDO activity, as found in Chapter 9. The inability of melatonin to alter the levels of the pro-inflammatory cytokines studied in this Chapter suggests that the melatonin-induced increase in IDO activity noted in Chapter 5 is primarily due to an increase in the levels of $O_2^{-}\cdot$ *in vivo* (see Chapter 9), rather than an increase in the levels of pro-inflammatory cytokines.

IDO activation results in both protective and toxic effects, as described in 5.5. Some of these deleterious effects include (see 5.5):

(i) increased tryptophan metabolism, and thus a decrease in the availability of tryptophan for cerebral uptake, leading to depletion of 5-HT in the brain (Myint and Kim, 2003), which is associated with depression (see 6.2.2.2). IDO stimulation thus represents an additional mechanism by which 5-FU decreases forebrain 5-HT levels, as shown in Chapter 6;

(ii) the formation of excitotoxic kynurenine metabolites, such as QA and 3-OHK. Whilst these may be toxic to cancer cells, non-cancer cells are also vulnerable to the effects of these metabolites. Thymocytes and type 1 T lymphocytes, for example, undergo apoptosis; this

shifts the type 1/ type 2 T cell ratio towards type 2 cells (Grohmann *et al*, 2003) and results in immunosuppression and decreases the progression time to AIDS in HIV-infected individuals (see 12.3.2). Furthermore, QA and 3-OHK induce neurodegeneration, leading to depression (see 6.2.2.16); and

(iii) the production of cytoprotective kynurenine metabolites, such as kynurenic acid, whilst protecting non-cancer cells, also represent a means by which cancer cells are protected and allowed to escape detection by the immune system. This creates a more favourable environment for the survival and growth of tumours, and may account for resistance towards 5-FU therapy and/ or decreased antineoplastic efficacy (Malachowsi *et al*, 2005; Friberg *et al*, 2002).

Interleukin-1 has significant effects in HIV infection, by stimulating the release of CRH and ultimately ACTH and cortisol (Biglino *et al*, 1993; Sandi and Guaza, 1995). This hastens the progression to AIDS in HIV-infected individuals, according to the glucocorticoid hypothesis of AIDS (Corley, 1996, 1997) (see 12.3.1). It has been shown in Chapter 12 that neither 5-FU nor melatonin alters serum corticosterone levels, whether administered alone or together. This implies that the increase in IL-1β levels induced by the co-administration of both agents is not sufficient to induce an increase in corticosterone levels. This confirms the suggestion in 12.10 that melatonin does not exert significant effects on IL-1 levels. HIV and the progression to AIDS is marked by changes in the cytokine profile, with a decrease in type 1 and an increase in type 2 cytokines (see 12.3.2). By enhancing the levels of INFγ, a type 1 cytokine, 5-FU thus counteracts the decrease in the level of this cytokine that occurs in HIV infection. Whether this solitary change can slow down the progression time to AIDS, and the extent to which it is offset by the pro-inflammatory and other deleterious effects of INFγ is subject for future study. Co-therapy with melatonin prevents this increase in INFγ. This effect of the two agents in combination, as well as the finding that neither 5-FU nor melatonin alters the levels of the other cytokines studied suggests that neither agent exerts a significant effect in altering the overall cytokine profile and could potentially be safely used by HIV-positive patients without hastening the progression time to AIDS.

The finding that neither agent alters levels of GM-CSF (see Figure 139), a cytokine important in haematopoiesis (see 13.1), suggests that 5-FU exerts its haematological toxicity (see 1.2.10.2) through other mechanisms, such as inhibiting the synthesis of other colony-stimulating factors and progenitor cells. This lack of effect on GM-CSF levels supports the finding by Větvička *et al* (1990) that macrophages exhibit resistance to the effects of 5-FU (see 12.5.1). It is also possible that melatonin exerts its stimulatory effects on haematopoiesis

(see 1.3.9) via other mechanisms, or possibly by enhancing the levels of other colony-stimulating factors.

13.6 Conclusions

The co-administration of 5-FU and melatonin increases the levels of IL-1β. This increase is, however, not sufficient to alter corticosterone levels, as shown in Chapter 12. The administration of 5-FU alone increases the levels of INFγ, which is abolished by the co-administration of melatonin, suggesting an anti-inflammatory role for melatonin. These increases in the levels of the above two pro-inflammatory cytokines represent another mechanism by which 5-FU increases the activity of IDO, as shown in Chapter 5, in addition to increasing the levels of its cofactor $O_2^{-}\cdot$ (see Chapter 9). The inability of melatonin to alter INFγ levels when administered alone, and its lack of effect on other pro-inflammatory cytokines suggest that this agent activates IDO primarily by increasing $O_2^{-}\cdot$ levels *in vivo* (see Chapter 9). Neither 5-FU nor melatonin alters the levels of IL-1α, IL-2, IL-4, IL-6, IL-10, GM-CSF and TNFα at the dosages used. This supports the use of low doses of both agents by HIV-positive patients, the focus of Chapter 12, as neither drug induces alterations in the cytokine profile indicative of the progression to AIDS.

CHAPTER 14

THE EFFECTS OF ACUTE AND LONG-TERM ADMINISTRATION OF 5-FLUOROURACIL AND MELATONIN ON PAIN PERCEPTION IN RATS

14.1 Introduction

Pain, a ubiquitous symptomatic experience, is probably the most common reason for seeking medical attention (Loeser and Melzack, 1999). The health-care costs associated with chronic pain are high, compounded by the costs of subsidies and salary replacements for those who cannot work due to pain (Loeser and Melzack, 1999).

It is now widely recognised that there are various facets of pain, and that psychological and physiological factors have a role to play in the sensation of pain experienced by an individual. The International Association for the Study of Pain (IASP) defines pain as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage" (Merskey and Bogduk, 1994). The Melzack-Wall gate control theory of pain (Melzack and Wall, 1965) postulates that CNS mechanisms regulate the perception of a painful stimulus by afferent nerves, thereby integrating peripheral nervous stimulation and central processes (Loeser and Melzack, 1999). This theory does not, however, take cognisance of the long-term changes induced in the CNS by noxious stimulation, and environmental and social factors that could influence the individual (Loeser and Melzack, 1999). Tissue injury is accompanied by local inflammation and the presence of a variety of inflammatory mediators, which alter the function of local nociceptors (Levine and Taiwo, 1994). This local inflammation, occurring during the healing process, is transient (Loeser and Melzack, 1999). It has been found, though, that intense acute noxious stimulation can alter the functioning of the spinal cord, by amino acid-induced excitotoxicity, and that this can lead to the development of chronic pain (Dubner and Ruda, 1992).

Plasticity (see 10.2.1.2) plays an important role in pain. Repeated noxious stimulation induces synaptic potentiation, and this can alter physiological responses to painful stimuli (Rainville *et al*, 1996; Graceley *et al*, 1992). It is evident clinically, such as in the case of the phantom limb phenomenon, that the brain is able to generate the perception of pain without the peripheral stimulation of nociceptors (Loeser and Melzack, 1999; Hansson *et al*, 2001). This suggests that there is a neuromatrix, or mechanism of pattern formation, in the brain and that this sustains a sensory image of the body (Coderre *et al*, 1992; Melzack, 1990). Sensory inputs into this neuromatrix, as well as information from brain regions involved in cognitive

and affective processes, result in output that determines pain perception (Loeser and Melzack, 1999). The anticipation of pain and/ or previous experiences of certain environmental stimuli may, furthermore, generate or sustain pain behaviours (Loeser and Melzack, 1999). Figure 142 below illustrates the neuromatrix, the various sensory inputs that feed into this and the widespread central processes that generate pain. It integrates the pyschosocial and physiological inputs that result in the perception of pain, and highlights the dynamism of the CNS and its ability to respond to tissue damage in the periphery and stress-regulating systems, such as the HPA axis (Loeser and Melzack, 1999).

In addition to generating pain, injury induces stress. The body seeks to cope with this stress through various hormonal, neural and behavioural mechanisms of homeostasis (Chrousos and Gold, 1992; Loeser and Melzack, 1999). These mechanisms occur locally at the site of injury, such as the release of cytokines; in the immune system; in peripheral organs, for example corticosteroid release from the adrenal cortex; and in the brain (Loeser and Melzack, 1999).

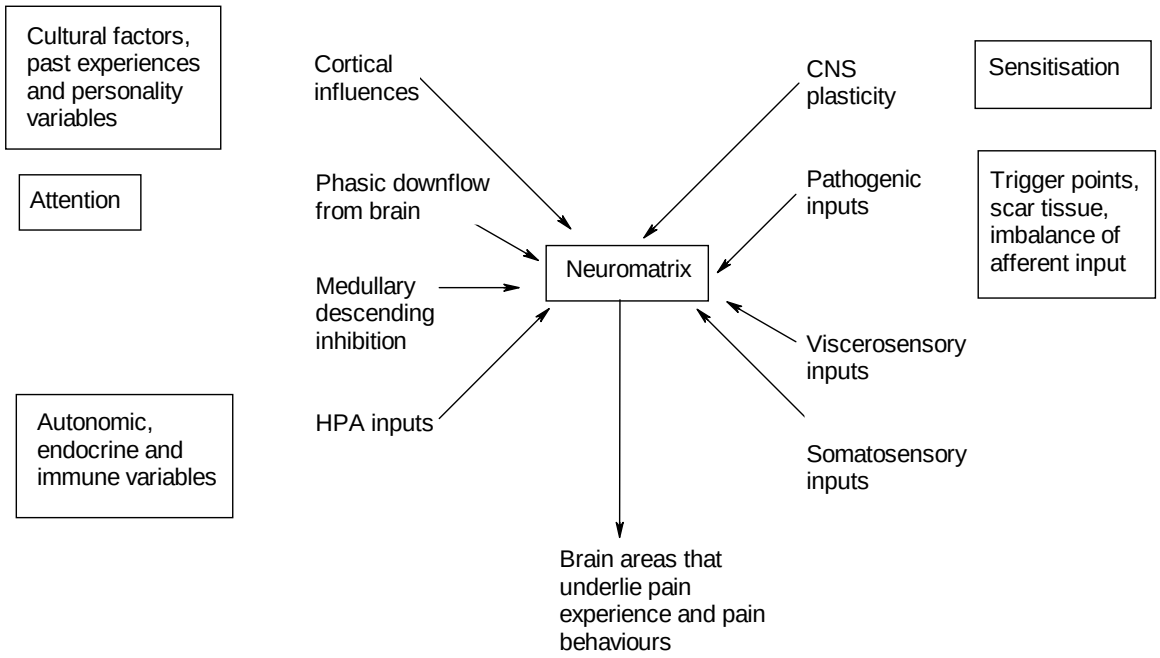


Figure 142: The neuromatrix, receiving a variety of inputs and generating pain (Loeser and Melzack, 1999; adapted from Melzack, 1990)

It is suggested by Loeser (1980) and Loeser and Malzeck (1999) that there are four general categories of pain, as described below, and that each category includes psychological, physiological and anatomical inputs:

(i) nociception. Specialised transducers structurally attached to A delta and C fibres detect tissue damage. Local inflammation may activate these transducers, and non-steroidal anti-

inflammatory drugs (NSAIDs) exert an analgesic effect by decreasing this local inflammation and thus restoring transducer sensitivity to baseline;
(ii) pain perception, which can occur in the absence of nociception and is triggered by various noxious stimuli, for example an injury or disease. Lesions in the nervous system may also induce pain, such as diabetic neuropathy. Analgesics are less effective at treating pain due to nerve injury than pain due to tissue damage (Dellemijn, 1999; Loeser and Melzack, 1999);
(iii) suffering, a negative experience that results from pain as well as various psychological states, such as stress, fear, loss of beloved objects and anxiety; and
(iv) pain behaviours, which occur as a result of suffering and pain. This includes a physical reaction to pain, such as limping, as well as medical consultations and sick leave (Loeser and Malzeck, 1999).

There are three main types of pain, based on duration (Loeser and Malzeck, 1999):
(i) transient pain, which occurs due to the stimulation of nociceptive transducers and which disappears after the impinging stress is removed from the body. No tissue damage is present, and transient pain occurs in everyday life;
(ii) acute pain, which involves tissue injury. The latter alters the responsiveness of the local transducers and autonomic nervous system. Physiological healing mechanisms are stimulated and often pain perception stops before the healing process is complete. Drug and/ or medical treatment is not necessary, but can be useful in decreasing the intensity of pain experienced and hastening the recovery time. Acute pain can last up to a few days or weeks, but certain chronic diseases, such as cancer, can continually induce acute episodes of pain through the malignant invasion of cancer cells into different tissues (Loeser and Malzeck, 1999); and
(iii) chronic pains, which are usually precipitated by a disease or injury, but which may be sustained by other factors. Somatic and autonomic reflexes, which are associated with acute pain, disappear as the pain becomes chronic. Chronic pain can often be of an intensity disproportonal to the amount of tissue damage present (Loeser and Malzeck, 1999), with nerve axons firing spontaneously due to injury-induced lowering of membrane potentials (Paice, 2003). Examples of chronic pains are postherpetic neuralgia and lower back pains (Loeser and Malzeck, 1999). Patients treated for cancer often suffer from neuropathic pain (Paice, 2003). The body's ability to heal itself is inadequate, possibly due to widespread trauma, the loss of limbs or nerve injury. The intensity of pain experienced is dispropotionately greater than the amount of tissue damage, or the pain may occur without any accompanying evidence of injury. Due to the persistence of chronic pain, psychosocial and environmental factors may amplify the original pain (Loeser and Melzack, 1999). Chronic pain differs from acute pain not only due to the longer period of time involved, but also because the body fails to restore homeostatic mechanisms of pain control and healing.

Traditional analgesics that are effective therapy for acute pain are, furthermore, often inadequate in treating chronic pain, do not treat the underlying disorders, and pain continues upon cessation of drug therapy (Loeser and Melzack, 1999). It is believed that down-regulation of N-type Ca^{2+} and Na^{+} channels, as well as an increase in afferent neuron sensitivity to the CNS, are also involved in the development of neuropathic pain (Paice, 2003). A loss of spinal cord GABA receptors, and thus a decrease in pain inhibition pathways, as well as a fall in CSF and blood levels of adenosine may also induce neuropathic pain (Ossipov *et al*, 2001; Paice, 2003). One of the consequences of chronic neuropathic pain is an alteration in cortical functioning (Apkarian *et al*, 2001), thus emphasising the interactions between the various factors, inputs and variables described in Figure 142.

The World Health Organisation (WHO) has recommended a "ladder approach" to treating pain (WHO, 1996). This involves initially using NSAIDs and, if these are inadequate in offering pain relief, to use more potent agents, such as opioids. These may be used alone or with adjuvant therapy, and the dosage titrated upwards, depending on the occurrence of adverse effects (WHO, 1996; Cruciani *et al*, 2003).

14.2 Endogenous analgesics

The efficacy of opioids, such as morphine, as analgesics results from the ability of these compounds to mimic endogenous opioid peptides (EOP), also termed endorphins. The EOP system mediates a wide array of effects, including inhibition of nociception; modulating the functions of the endocrine, gastrointestinal and autonomic systems; promoting reward and addictive behaviour; and influencing cognitive processes (Gutstein and Akil, 2001). Although the EOP system contains only four types of receptors, there are over a dozen endogenous substrates. There are three families of EOP, each derived from a specific polypeptide and showing a distinct distribution in the body. These families are: (i) enkephalins, derived from preproopiomelanocortin; (ii) endorphins, products of preproenkephalin; and (iii) dynorphins, derived from preprodynorphin (Gutstein and Akil, 2001). Proopiomelanocortin gives rise to the main opioid peptide, β-endorphin, as well as to non-opioid peptides, such as ACTH and MSH (see 12.3.1), thus highlighting the relationship between opioid systems and stress (Gutstein and Akil, 2001). This has been supported by evidence of stress-induced algesia (Akil *et al*, 1985).

The families of opioid receptors include the classic μ, κ and δ receptors, as well as the recently-discovered N/OFQ receptor (Gutstein and Akil, 2001). These receptor families have distinct distribution patterns in the CNS and periphery (Mansour *et al*, 1988). The so-called "apparent paradox" is that a wide variety of endogenous substrates activate the relatively few

opioid receptors. This is unlike many neurotransmitter systems, in which a few substrates interact with a variety of structurally-different receptors (Gutstein and Akil, 2001). The μ, κ and δ receptors are coupled to second messenger systems: positively to receptor-operated K^+ channels, and negatively to both voltage-gated Ca^{2+} channels (Duggan and North, 1983) and adenylyl cyclase activity (Herz, 1993). Membrane hyperpolarisation by activating K^+ currents and inhibiting the entry of Ca^{2+} into cells may be a mechanism by which opioids prevent the release of neurotransmitters and the propagation of painful impulses along neuronal routes (Gutstein and Akil, 2001). Other second messenger systems, such as phospholipase C and MAP kinases, may also be coupled to these opioid receptors (Gutstein and Akil, 2001).

The fundamental mechanisms by which opioids induce analgesia are by directly inhibiting the upward transmission of nociceptive impulses from the dorsal horn of the spinal cord and activating pain inhibition pathways extending from the midbrain to this region. Opioids are also released from immune cells peripherally at sites of local inflammation (Gutstein and Akil, 2001), and stimulate opioid receptors located on peripheral nerves (Fields *et al*, 1980), thereby inducing local analgesia.

14.3 Inducers of pain

Both endogenous substances and xenobiotics can induce pain, as described in 14.3.1 and 14.3.2.

14.3.1 Endogenous compounds

There are many endogenous substances that stimulate nociception. Prostaglandins, for example, products of COX activity (see 8.1), play a significant role in the processing of nociceptive stimuli, in the spinal cord and periphery (Scheuren *et al*, 1997). PGE_2 and $PGF_{2\alpha}$, in particular, are powerful inducers of pain. Prostaglandin release is promoted by cytokines, such as IL-1, IL-8 and TNFα, and bradykinin (Roberts and Morrow, 2001). Prostaglandins decrease the threshold of nociceptors attached to C fibres, thereby enhancing nociceptor excitability and responsiveness to chemical and mechanical stimulation, generating pain. Other pain inducers include NO (Eaton, 2003) and various neuropeptides, in particular calcitonin gene-related peptide and substance P (Roberts and Morrow, 2001; Eaton, 2003).

The neurotransmitter acetylcholine is involved in mediating visceral pain (Vogel and Vogel, 1997; Paice, 2003). It is believed that noradrenergic neurons in the brainstem play an important role in regulating the transmission of nociceptive impulses, and that this regulation of nociception is abnormal in hypertensive rats (Taylor *et al*, 2000).

14.3.2 Chemotherapy-induced algesia

Several antineoplastic agents are known to damage sensory neurons in the periphery, thereby inducing peripheral neuropathy in a dose-dependent manner (Paice, 2003). Paclitaxel, for example, induces hyperalgesia and allodynia (Polomano *et al*, 2001). In addition to these adverse effects, vincristine results in peripheral neuropathy by interfering with the transport of impulses in nerve cell axons (Topp *et al*, 2000) and slowing down the velocity at which impulses are conducted in sensory fibres (Tanner *et al*, 1998). CNS alterations also occur, including the sensitisation of nociceptive neurons (Nurmikko, 2000; Dickenson *et al*, 2001). This sensitisation process, which results in hyperalgesia, is induced by neuropeptides and excitatory amino acids, such as glutamate, which stimulate NMDA receptors (Paice, 2003). Primary afferent processes in the dorsal horn of the spinal cord may, furthermore, be rearranged, thus contributing to the development of peripheral neuropathy. The terminals of C fibres within the dorsal horn degenerate due to the damage of peripheral nerves, and this facilitates the sprouting of mechanoreceptors. This provides a means for stimuli, for example touch, to activate nociceptors in the CNS, resulting in tactile allodynia, which is commonly experienced by patients suffering from neuropathic pain (Paice, 2003).

Other antineoplastic agents associated with algesia are ifosfamide and cisplatin, which induce neuropathic pain (Frisk *et al*, 2001), and oxiplatin, which results in acute allodynia (Quasthoff and Hartung, 2002) and peripheral neuropathy (Kern *et al*, 2001). Radiation therapy may also induce pain syndromes, such as radiation myelopathy, as well as the development of nerve tumours (Foley, 1987; Coyle, 2003).

Treatment-induced pain can occur either within weeks of therapy, and is commonly self-limiting, or up to years after cessation of drug therapy (Coyle, 2003). In addition to therapy-induced algesia, cancer patients may also experience pain due to the cancer itself, and the presence of tumours (Paice, 2003), which may infiltrate a variety of tissues, including nerve tissue (Coyle, 2003).

As mentioned in 1.2.10.10, although the effects of 5-FU on nociception are unknown, this agent is associated with the development of various types of pains, many of which are the result of very different pathologies. It has recently been reported that the topical use of 5-FU can exacerbate sensory peripheral neuropathy in a patient with normal levels of DPD (Wasif Saif *et al*, 2006).

5-FU induces cardiotoxicity, for example, possibly due to coronary artery spasms. Variant angina and acute coronary syndrome, the latter evidenced by chest pain and ECG changes, are

uncommon adverse effects but have been reported with the use of 5-FU theray (Sevket *et al*, 2002; Kleiman *et al*, 1987; Mafrici *et al*, 2003) (see 1.2.10.5).

High doses of 5-FU induce painful acral erythema, which can be successfully treated with corticosteroids (Esteve *et al*, 1995). Burning and pain in the skin has been reported with the continual infusion of 5-FU (Jorda *et al*, 1991), and this phenomenon has been described as the "hand-foot syndrome" (see 1.2.10.4). 5-FU is used topically to treat actinic keratoses, and is associated with the development of painful erosions in the skin. The use of pulse therapy topically decreases the intensity of this adverse effect (Epstein, 1998). The use of 5-FU in a suppository dosage form, whilst effective in the treatment of rectal carcinomas, induces tenesmus, anal pain and bleeding, possibly due to the effects of 5-FU on rectal musocal tissue (Takahashi *et al*, 1982). The use of 5-FU topically to treat cervical HPV infection, as mentioned in 1.2.8.8, is often limited by dysuria and vulvar pain that occurs due to leakage of the drug into surrounding vulvar tissue. It has been shown that these pains are eliminated with the use of a cervical cap (Davila *et al*, 1996).

It should be borne in mind that cancers induce pain, and 5-FU, through its anticancer action, may thus decrease the pain experienced by patients. It has been reported, for example, that a patient with an extremely painful carcinoma of the bladder experienced total pain relief within seven days of receiving an infusion of 5-FU (Confer *et al*, 1977). A novel application of the effects of 5-FU on algesia is the differential diagnosis of duodenal/ gastric ulcers and acute pancreatitis (Vinnik *et al*, 2002). If, within 15-20 mins after the start of an intravenous infusion of 250 mg 5-FU, dissolved in a 5% glucose solution to a final volume of 200 ml, a patient experiences significant pain relief, a diagnosis of acute pancreatitis is made. If there is no alteration in pain, the patient is more likely to suffer from a perforated duodenal or gastric ulcer (Vinnik *et al*, 2002).

14.4 Circadian rhythm of nociception

It is well established that there is a circadian rhythm of pain perception in humans, with subjects demonstrating enhanced sensitivity in the afternoon (Procacci, 1972; Glynn and Lloyd, 1967; Davis *et al*, 1978). Somatosensory evoked potentials recorded in the mornings and afternoons demonstrate decreased pain sensitivity, as well as naloxone-induced hyperalgesia, in the morning. This diurnal fluctuation in pain sensitivity may be mediated by endorphins (Davis *et al*, 1978). It it known that plasma levels of the endorphin β-lipotropin peak in the morning and trough in the late afternoon and evening (Krieger *et al*, 1977). Factors that influence this circadian rhythm include gender, with females exhibiting greater sensitivity; and whether the subject stays at home or not (Glynn and Lloyd, 1967). Mice also

exhibit a diurnal rhythm in pain sensitivity, but are more pain tolerant in the afternoon and evening (Frederickson *et al*, 1977; Morris and Lutsch, 1969). The fluctuations in pain sensitivity are blocked by the opioid antagonist naloxone. This suggests that the circadian fluctuations in pain perception are regulated in part by an opioid-mediated mechanism (Frederickson *et al*, 1977). Although the levels of met-enkephalin do not vary during the day, upon noxious stimulation these levels are enhanced in the globus pallidus of the brain in the afternoon only, corresponding to the time of the day during which mice exhibit greatest pain tolerance (Frederickson *et al*, 1977; Wesche and Frederickson, 1981). This study suggests that the globus pallidus may be involved in the neuroanatomical mechanism of pain perception (Wesche and Frederickson, 1981).

El-Slobsky *et al* (1976) have made the surprising observation that naloxone does not counteract nociception induced experimentally in humans, leading these authors to surmise that there is no continuous endogenous release of an opioid-type compound, but that this is released only in certain situations. This may account for some psychological states, such as in accidents and war, when the individual does not experience pain even when severely injured (El-Slobsky *et al*, 1976).

14.5 Effects of melatonin on nociception

There are conflicting reports in the literature on the effects of melatonin on pain perception, and the circadian rhythms which regulate this. Whilst there is a report of melatonin enhancing formaldehyde-induced algesia in mice (Takahashi *et al*, 1987), other investigators have found that the intraperitoneal administration of melatonin during the light phase has an analgesic effect (Ying and Huang, 1990). In the latter study, melatonin was also shown to potentiate the analgesic effects of morphine and meperidine, leading the authors to suggest that melatonin may be an endogenous inhibitor of nocifensors (Ying and Huang, 1990). As mentioned in 1.3.9, melatonin has been reported to counter hyperalgesia during wound healing (Raghavendra *et al*, 2000) and possess intrinsic analgesic activity (Shaji and Kulkarni, 1998), as discussed below. In an investigation of the effects of melatonin on nociception, as determined by the tail-flick test (see 14.6), Shaji and Kulkarni (1998) have found that a dose of 10-400 mg/ kg melatonin produces significant analgesia by increasing the reaction time of rats to noxious thermal stimulation. This was hypothesised to occur due to three possible mechanisms (Shaji and Kulkarni, 1998):

(i) modulation of endogenous circadian rhythms of nociception and age-induced alterations in opioid receptors (Kavalsiers *et al*, 1983);

(ii) opioid-mediated increases in melatonin levels at night (Lowenstein *et al*, 1984); and

(iii) melatonin-induced release of EOP, which interact with opioid receptors (Erlich and Apuzzo, 1985).

El-Shenawy *et al* (2002) have found that melatonin exerts analgesic and anti-inflammatory effects and enhances the effects of the NSAID indomethacin and the COX-2 inhibitor rofecoxib, possibly due to CNS-mediated mechanisms. It has been shown that certain strains of mice, such as CBA/N and BALB/c, which exhibit diurnal melatonin levels in the pineal gland, possess elevated pain thresholds (Takao and Uchida, 1999). Other investigators have reported that melatonin, MT and pinealectomy exert no effect on the circadian rhythm of pain perception, leading these authors to postulate that the pineal gland does not play a role in the regulation of endogenous alterations in pain perception occuring during the diurnal cycle (Brown *et al*, 1986). John *et al* (1994), however, in a study of both thermal and mechanical pain, have concluded that rats subjected to twelve hours of lighting and twelve hours of darkness exhibit a diurnal rhythm in perceiving both types of pain, and that pinealectomy reverses this rhythm of thermal pain perception, and eliminates the rhythm of mechanical pain perception. The administration of melatonin was found, furthermore, to counteract the effects of pinealectomy and restore the diurnal rhythms of pain perception. This suggests that an indoleaminergic-opioid system may exist to regulate pain perception (John *et al*, 1994).

14.6 Animal models of nociception

The use of animal models of pain and spasticity have allowed for insight into the mechanisms leading to the development of these disorders, and have served as a means of evaluating the efficacy of possible analgesic agents. Despite the myriad of animal models available, as outlined below, an important limitation of these models is that few mimic chronic human pain. Behavioural responses to acute thermal, tactile and chemical stimuli are assessed, which simplifies the polymorphic nature of human pain (Eaton, 2003). In addition, transient pain is measured, thus making an accurate measurement of the onset and duration of action of analgesics difficult (Dubuisson and Dennis, 1977).

There are several methods of determining the pain threshold in animals, and the efficacy of analgesic agents. These tests fall into three categories:
(i) thermal tests, such as the foot withdrawal response to noxious heat, the hot-plate test (Woolfe and MacDonald, 1944), and the tail-flick test (D'Amour and Smith, 1941). The latter two are the most frequently-used animal models of pain (Eaton, 2003);
(ii) chemical tests, including formalin-induced nociception (Dubuisson and Dennis, 1977) and the acetic acid-induced writhing test (Turner, 1965; Vogel and Vogel, 1997; Cervero and Larid, 1999). Chemical tests of nociception are more selective for opioid κ receptors, while

thermal tests preferentially target the involvement of opioid μ receptors (Furst *et al*, 1988; Abbott and Young, 1988); and
(iii) mechanical tests, such as the pinch test (Mayer *et al*, 1971; Collier, 1962), the classic von Frey filaments test (1896) and the electronic pressure-meter paw test (Vivancos *et al*, 2004).

14.7 Experimentation

The effects of the acute and long-term administration of 5-FU and melatonin on the reaction time of rats subjected to the modified tail-flick test, were determined. This test requires immersion of the animal's tail in hot water (Ben-Bassat *et al*, 1959). The original tail-flick test used a smaller area of stimulation, and only the tip of the animal's tail was exposed to thermal stimulation (Bass and Vanderbrook, 1952; D'Amour and Smith, 1941). The modified tail-flick method was chosen, as opposed to the hot plate test (see 14.6), to minimise tissue damage and unecessary pain experienced by the animals.

14.7.1 Acute study

The effects of 5-FU and melatonin on thermal pain sensitivity were determined 60 mins after the administration of these agents. This period of 60 mins was designated as the acute administration of 5-FU and melatonin, as both drugs have half-lives of less than 60 mins (see 1.2.5 and 1.3.5). Other researchers have, furthermore, used this time period as an indication of the acute effects of drugs on nociception (Euchenhofer *et al*, 1998).

14.7.1.1 Materials, animals, reagents and equipment

5-FU and melatonin were purchased from the Sigma Chemical Corporation, St Louis, MO, USA. All other reagents were obtained from local suppliers and were of the highest chemical purity. Forty male Wistar rats, each weighing between 200g and 300g, and cared for as described in Appendix A, were used. All solutions were prepared using deionised water (Milli R/Q System, Millipore®). Melatonin was dissolved using absolute ethanol as a co-solvent, at a final concentration of 0.67% v/ v. A Julabo PC water bath (Labotec, South Africa) was used.

14.7.1.2 Method

Forty male Wistar rats were divided into four groups of ten. A sample size of ten rats per group, and not five, as previously used, was employed in order to decrease the inter-animal variability that often occurs in behavioural experiments, and allow for statistical analysis to be more accurate. The reaction time of each rat prior to drug treatment was measured by holding the rat above a water bath maintained at 38 ± 0.5 ºC, immersing the tail entirely in the water and timing until the first flick or movement of the tail, an indication of nocifensive behaviour, occurred. The animal was held and not restrained in a contraption as it has been noted that

restraint may induce pain (Hill *et al*, 1952a, 1952b). An independent observer was responsible for timing the rats and the experiment was conducted in the afternoon. The rats were subsequently injected with either 1 mg/ kg 5-FU, 1 mg/ kg melatonin or a combination of these, intraperitoneally. The control group received 0.9% saline. After 60 mins, the modified tail-flick test was again performed and the reaction time measured.

14.7.2 Long-term study

This study involved the administration of 5-FU and melatonin to rats over a period of five days, and determining if any alteration in reaction times occurred.

14.7.2.1 Materials, animals, reagents and equipment

As described in 14.7.1.1.

14.7.2.2 Method

Four groups, each consisting of ten rats, were subjected to the modified tail-flick test and the reaction times measured by an independent observer, as described in 14.7.1.2. The rats were subsequently left for ten days, to recover from the experience and minimise the occurrence of a learned response when re-exposed to the test at a later stage. After this ten-day period, the rats were subjected to a five-day treatment protocol with either: (i) 5-FU, (ii) melatonin, (iii) a combination of these agents, or (iv) 0.9% saline, as described in Table 10 (see 3.6.2.2.). On the afternoon of the sixth day, the modified tail-flick test was again performed and the reaction times measured.

14.8 Results

The reaction times both pre- and post-treatment for each group of rats were analysed statistically using ANOVA followed by the Student-Newman-Keuls multiple range test. The following results were obtained.

14.8.1 Acute study

Figure 143 on the next page shows the reaction times obtained upon the acute administration of 5-FU and melatonin.

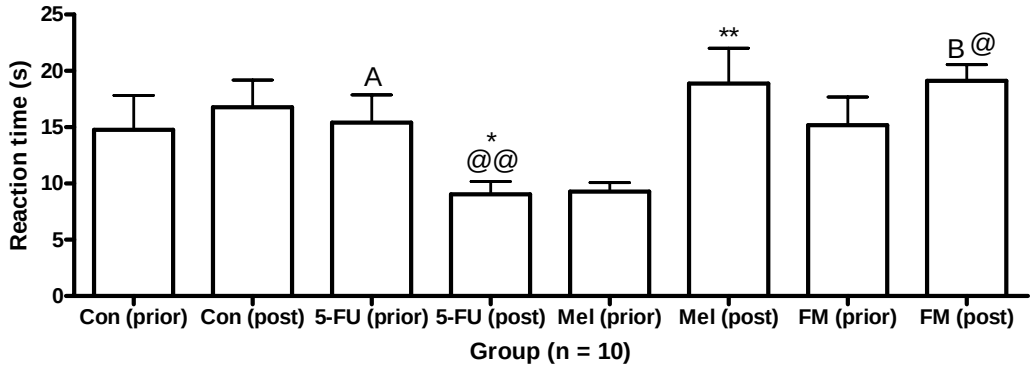


Figure 143: The effects of the acute administration of 5-FU and melatonin on the reaction times of rats subjected to the modified tail-flick test prior to and 60 mins after drug administration

(A) $p<0.05$ compared to 5-FU (post); * $p<0.01$ compared to Mel (post); $^{@@}$ $p<0.01$ compared to Con (post); ** $p<0.01$ compared to Mel (prior); $^{@}$ $p<0.001$ compared to 5-FU (post); and (B) $p<0.1$ compared to FM (prior) after analysis by ANOVA followed by the Student-Newman-Keuls multiple range test. Con = Control; FM = 5-FU + Melatonin; prior = reaction time prior to drug treatment; post = reaction time 60 mins post drug treatment. Each bar represents the mean ± SEM

As shown in the Figure above, the acute administration of 5-FU significantly decreases the reaction time of rats, from an average time of 15.40 s to 9.03 s. This is also less than the average reaction time obtained for the Melatonin group and the group receiving both drugs subsequent to treatment. The administration of melatonin significantly increases the average reaction time in the modified tail-flick test from 9.28 s to 18.86 s. The group of rats treated with both 5-FU and melatonin demonstrates an increase in average reaction time, from 15.19 s to 19.11 s.

14.8.2 Long-term study

Figure 144 on the next page shows that subsequent to the five-day administration of 5-FU and melatonin, there are no significant differences in reaction times across the groups.

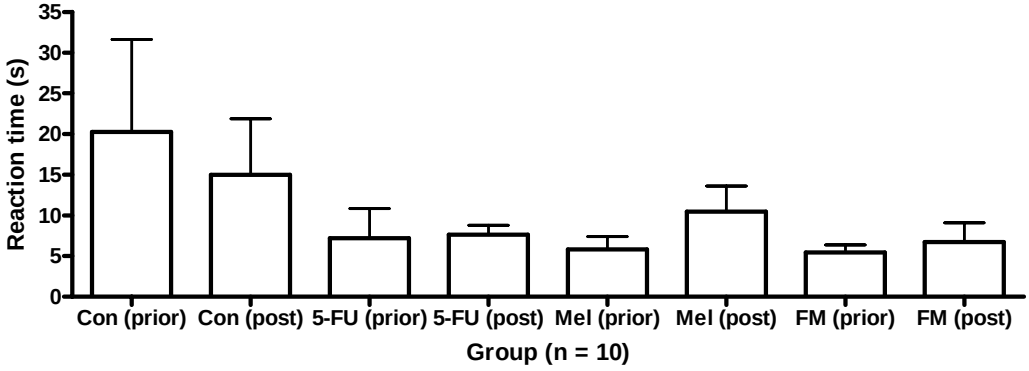


Figure 144: The effects of the long-term administration of 5-FU and melatonin on the reaction times of rats subjected to the modified tail-flick test prior and subsequent to five days of drug treatment

Con = Control; FM = 5-FU + Melatonin; prior = reaction time prior to five days of drug treatment; post = reaction time post five days of drug treatment. Each bar represents the mean ± SEM

14.9 Discussion

Figures 143 and 144 show that whilst 5-FU and melatonin both have significant effects on the reaction time of rats to the modified tail-flick test when administered acutely, these effects are abolished after five days of drug treatment.

The acute administration of 5-FU, by decreasing reaction time, indicates that the antineoplastic increases pain sensitivity and decreases the pain threshold of rats to a thermal stimulus. This is unlikely to be due to inhibition of the synthesis of the genes necessary to express endogenous opioids, as this process requires time to occur, and in this study rats were re-tested 60 mins after drug administration. Possible reasons for the acute effect of 5-FU include:

(i) the stimulation of peripheral nociceptors within the body, thereby decreasing the pain threshold towards subsequent thermal stimulation;

(ii) damage to nerve cells, nociceptors and/ or opioid receptors. Damage to the former two might potentiate pain perception, whilst damage to opioid receptors may prevent EOP-induced analgesia;

(iii) activation of COX-2 (Mercer *et al*, 2005) (see 8.1), thereby leading to the production of various prostaglandins, many of which are powerful inducers of pain (see 14.3.1);

(iv) enhancing the release of other mediators of pain and inflammation. It has been reported that 5-FU does not alter histamine levels (Eschalier *et al*, 1988; Decorti *et al*, 1997). The ability of 5-FU to both increase and decrease NO levels is discussed in 1.2.6.4; a singular

increase in NO production (Jiang *et al*, 2002; Oshima *et al*, 2001; Leitão *et al*, 2007) would result in nociception. The effects of the antineoplastic on bradykinin levels are unknown and a possible increase in these levels could represent another mechanism of 5-FU-induced algesia.

The acute administration of melatonin significantly decreases pain sensitivity and enhances the pain threshold, by increasing the average reaction time to thermal stimulation. This result is in agreement with the findings of Shaji and Kulkarni (1998) (see 14.5). This analgesic effect may be due to:

(i) exogenous melatonin enhancing the phase of pain tolerance in the circadian rhythm of nociception;

(ii) melatonin mediating the release of EOP, which interact with opioid receptors and induce algesia (see 14.5);

(iii) inhibition of endothelial and inducible NO synthase. It is known that melatonin inhibits this enzyme both *in vitro* (Tamura *et al*, 2006) and *in vivo* (Silva *et al*, 2007; Valero *et al*, 2007; Chucharoen *et al*, 2007; Dong *et al*, 2003), thereby preventing the synthesis of NO, which is known to induce pain (see 14.3.1). NO synthase inhibition is believed to be due to blockade of Ca^{2+} movement from intracellular deposits (Silva *et al*, 2007). Melatonin furthermore inhibits the transcriptional activation of inducible NO synthase by inhibiting the acetylation, binding and subsequent transactivation of its p52 moiety (Deng *et al*, 2006);

(iv) inhibition of COX-2 (Deng *et al*, 2006; Dong *et al*, 2003), also via inhibition of p52 acetylation and the transcriptional activation of the enzyme (Deng *et al*, 2006). COX-2 inhibition prevents the formation of pro-inflammatory and algesia-inducing prostaglandins (see 14.3.1); and

(v) in 13.6, it was suggested that melatonin exerts an anti-inflammatory effect by blocking the 5-FU-induced increase in the levels of the pro-inflammatory cytokine INFγ shown in Chapter 13; this anti-inflammatory effect may possibly be associated with analgesia.

The co-administration of melatonin to rats receiving 5-FU increases the pain threshold, possibly through the abovementioned mechanisms, suggesting that the use of melatonin as co-therapy in patients treated with 5-FU may be useful in counteracting 5-FU-induced algesia. This may potentially be of great value in cancer patients, who often also experience pain due to the disease itself.

The abovementioned alterations in pain sensitivity induced by 5-FU and melatonin do not occur upon the long-term administration of these agents. The inability of 5-FU to decrease the pain threshold indicates that the antimetabolite does not inhibit the synthesis of the genes required to express EOP, or that perhaps there are compensatory mechanisms at play to

counteract this. Since patients on 5-FU therapy are usually receiving the drug on a chronic basis, it is pertinent that no enhancement of pain sensitivity occurs. No melatonin-induced increase in pain tolerance occurs, suggesting normalisation of any changes in the circadian rhythm of nociception induced upon the acute administration of melatonin.

14.10 Conclusions

The administration of 5-FU may initially decrease the pain threshold and induce algesia, evidenced by a decrease in the average reaction time of rats subjected to the modified tail-flick test. This increase in pain sensitivity is counteracted by the co-administration of melatonin, which may possibly strengthen the circadian rhythm of pain tolerance, in addition to intrinsic analgesic effects. These alterations in pain sensitivity disappear upon long-term treatment with these agents, possibly due to compensatory physiological mechanisms. The use of chronic 5-FU therapy is thus unlikely to result in, or exacerbate, nociceptive changes in the cancer patient.

CHAPTER 15

SUMMARY CHAPTER

This thesis focuses on the mechanisms of interaction between 5-FU, a commonly-used and toxic antineoplastic agent, and melatonin, a pineal hormone and powerful antioxidant. Chapter 1 discusses the pharmacokinetic and pharmacodynamic properties of each agent, and describes clinical findings that show that the administration of melatonin to cancer patients receiving 5-FU therapy results in a significant increase in patient survival time (Lissoni *et al*, 1999a). Mechanisms by which melatonin enhances the efficacy and decreases the toxicity of 5-FU in the clinical setting are explored, and the effects of melatonin on cancer cells are critically evaluated.

In Chapter 2, a suitable method of quantitative analysis, to allow for the detection and separation of 5-FU and melatonin in solution, was developed. This simple, inexpensive and sensitive HPLC method was validated according to international guidelines (ICH Guidelines, 1996; USP, 2005), and found to meet the criteria for specificity, linearity, accuracy and precision. A preliminary biological assay was performed, and adequate recovery of the drugs was shown from the biological sample. Stability-indicating studies reveal that both 5-FU and melatonin are photo- and thermolabile, and the former undergoes possible tautomerisation in acidic media. The results of these stability-indicating studies offer insight into the optimal storage conditions for both drugs. ^{1}H-NMR analysis reveals that there is no direct chemical interaction between 5-FU and melatonin.

This HPLC method was utilised in Chapter 3, to measure the concentrations of 5-FU and melatonin remaining after hepatic metabolism by CYP450, the primary site of drug metabolism in the body. Microsomes containing this enzyme system were isolated from the liver, through a method of differential centrifugation and Ca^{2+}-induced sedimentation. It was shown that both drugs are extensively metabolised by CYP450 within 60 minutes, almost to completion, and that no hepatic metabolic drug interaction exists between 5-FU and melatonin. Furthermore, although 5-FU decreases the protein content of microsomes, neither drug inhibits nor induces CYP450 activity. This suggests that the co-administration of melatonin is unlikely to alter plasma levels of 5-FU clinically, and that neither drug is likely to alter the metabolism of other agents that may be administered to the patient.

The effects of 5-FU and melatonin on TDO activity were determined in Chapter 4. This enzyme is responsible for the hepatic metabolism of the essential amino acid tryptophan, and its activity in the periphery is an indicator of the amount of tryptophan that will be available for cerebral uptake and conversion into the neurotransmitter 5-HT. It was found that both melatonin and 5-FU inhibit TDO *in vivo*; 5-FU inhibits the apoenzyme form, while melatonin inhibits both the apoenzyme as well as the holoenzyme. Melatonin exerts this effect through a substrate mechanism (Walsh *et al*, 1994), leading to competitive inhibition of TDO. Both agents possess –NH functional groups, which block the catalytic site of TDO, thus leading to enzyme inhibition. It is expected that this inhibition of TDO will result in increased cerebral levels of 5-HT.

In Chapter 5, the effects of 5-FU and melatonin on intestinal IDO activity were investigated. This enzyme is the rate-limiting enzyme involved in tryptophan metabolism; the product of its activity is a substrate for TDO. It was shown that both 5-FU and melatonin stimulate IDO activity *in vivo*. This is postulated to occur by increases in the levels of pro-inflammatory cytokines, which activate IDO, and/ or the IDO cofactor $O_2^{-}\cdot$. An increase in IDO activity is associated with the generation of the toxic kynurenine metabolites 3-OHK and QA, and the cytoprotective metabolite kynurenic acid. The 5-FU-induced production of 3-OHK and QA may be an additional mechanism of cytotoxicity, but this is limited by the synthesis of kynurenic acid, which may protect cancer cells against apoptosis and thus provide an immune escape mechanism that allows for the survival and growth of tumours. This may limit the anticancer efficacy of 5-FU. Enhanced IDO activity is also associated with the development of depression, immunosuppression, and changes in the T lymphocyte profile that favour the progression to AIDS in HIV-positive individuals.

Levels of key neurotransmitters, namely 5-HT, DA and NE, were analysed in rat forebrain in Chapter 6. The levels of the metabolites HA and DOPAC were measured and found to be unaltered. It was found that melatonin increases 5-HT levels, due to TDO inhibition, and also raises DA levels. Despite its ability to inhibit TDO, 5-FU was shown to decrease the levels of all three neurotransmitters. This is suggested to be by the antineoplastic agent's ability to impair DNA synthesis and thus inhibit the expression of the genes required to synthesise the enzymes necessary for the formation of 5-HT, DA and NE. By enhancing IDO, furthermore, 5-FU induces central 5-HT depletion. Low levels of both 5-HT and DA contribute to a decrease in NE levels. By lowering these neurotransmitter levels, the administration of 5-FU could potentially result in the development, or exacerbation, of depression in cancer patients, according to the catecholamine, biogenic amine and dopamine hypotheses. The co-

administration of melatonin counters the 5-FU-induced decrease in 5-HT, DA and NE levels, suggesting that melatonin could alleviate depression resulting from 5-FU therapy.

In Chapter 7, pineal organ culture was used to examine the effects of 5-FU and melatonin on the metabolism of 5-HT by the pineal gland. The results show that neither drug alters the acetylation of [^{14}C]-5-HT by NAT, and the production of [^{14}C]-aMT is not altered. The deamination of [^{14}C]-5-HT by MAO, the major route of [^{14}C]-5-HT metabolism leading to the formation of [^{14}C]-HA, appears, however, to be inhibited by both drugs individually, suggesting MAO inhibition. This lowering in [^{14}C]-HA levels is abolished when 5-FU and melatonin are administered together, implying that the use of combination therapy, whilst lowering 5-HT levels in the brain, is unlikely to alter the metabolism of this neurotransmitter.

Lipid peroxidation is a toxic process, mediated by ROS and resulting in cell lysis. The ability of melatonin, a powerful free radical-scavenger, antioxidant and enhancer of antioxidative enzyme functioning, to protect cells against lipid peroxidation is well documented. There are no reports in the literature, though, on the effects of 5-FU on lipid peroxidation. This was thus the subject of Chapter 8. It was found that 5-FU significantly induces hepatic lipid peroxidation both *in vitro* and *in vivo*, as determined by measuring MDA levels. Fe^{2+}-chelation studies indirectly show that by failing to bind to Fe^{2+}, 5-FU may allow the Fenton reaction-generated production of HO· to occur, which may result in lipid peroxidation. The co-administration of melatonin reduces the extent of this 5-FU-induced lipid peroxidation, providing a role for melatonin in countering 5-FU toxicity. However, the induction of lipid peroxidation may be an additional mechanism by which 5-FU exerts its cytotoxic effects, and the co-administration of melatonin thus protects cancer cells from this and decreases the antineoplastic efficacy of 5-FU.

The effects of 5-FU and melatonin on O_2^{-}· levels were determined in Chapter 9, by the NBT assay. It was found that both agents induce the formation of this ROS *in vivo*; an increase in O_2^{-}· levels is associated with lipid peroxidation as well as IDO stimulation. It is suggested that the increase in the levels of this ROS noted in the group of rats receiving melatonin is due to the melatonin metabolite 6-OHM, which is known to exhibit oxidant properties. The co-administration of melatonin does not potentiate the 5-FU-induced increase in O_2^{-}· levels, implying that the use of melatonin by cancer patients on 5-FU therapy is unlikely to exacerbate the toxicity of the latter.

The hippocampus is a brain structure intimately involved in learning and memory. In Chapter 10, Nissl staining and stereology was used to show that 5-FU significantly decreases the

volumes of the CA1-2 and CA3 regions of the hippocampus and induces morphological alterations and structural damage. Hippocampal cells are smaller, with signs of possible cell lysis due to lipid peroxidation, and the closely-packed structure of hippocampal regions is disrupted. These may represent mechanisms by which 5-FU induces the phenomenon of "chemobrain", or cognitive impairment, in breast cancer survivors who have been treated with this drug, and may also result in depression. The co-administration of melatonin counters the 5-FU-induced decrease in hippocampal volume, particularly in the CA3 region, and cells retain their structural integrity and appear less damaged than those exposed to 5-FU alone. This provides evidence that melatonin prevents 5-FU-induced hippocampal toxicity, and may improve the quality of life of cancer patients being treated with 5-FU.

In Chapter 11, the effects of 5-FU and melatonin on locomotor activity were explored. It was found that melatonin administration decreases rearing frequencing and the total duration of movement, possibly due to the BZD-like sedative effects of the compound. The administration of 5-FU alone significantly increases the locomotor activity of rats exposed to twelve hours of constant bright light, evidenced by increases in the total distance moved, total duration of movement and mean velocity. This excitatory effect suggests that 5-FU may induce anxiety and/ or depression. The co-administration of 5-FU to rats receiving melatonin treatment abolishes the effects of melatonin on locomotor activity, implying that whilst melatonin co-therapy is useful in cancer patients, to normalise physiological parameters and enhance patients' quality of life, it is necessary to administer this agent on a chronic basis in order to effectively counteract the adverse effects of 5-FU.

Chapter 12 discusses the glucorticoid hypothesis of AIDS. This postulates that the progression to AIDS in HIV-positive patients is fundamentally due to excess cortisol, which induces immunosuppression. The effects of 5-FU and melatonin on levels of corticosterone, the predominant glucocorticoid in rats, were investigated. This is because, in addition to being used to treat AIDS-related neoplasias, the use of 5-FU to treat HIV is being advocated, due to the ability of this agent to inhibit RT (Shi *et al*, 1999). Furthermore, 5-FU, by depleting dTTP levels, enhances the activity of AZT and D4T and decreases resistance towards these antiretrovirals (Gao *et al*, 1995, 1999). Although it has been reported that 5-FU enhances HIV expression (O'Brien *et al*, 1995), it is likely that the virions produced are non-functional, due to the presence of fraudulent nucleotides within HIV DNA. Melatonin is often taken by patients due to its immunostimulatory effects. It was shown in this Chapter that neither 5-FU nor melatonin alters serum corticosterone levels, suggesting that the use of these agents by HIV-positive patients is unlikely to promote the progression to AIDS. However, the lowest possible doses of these drugs should be used, since it has been demonstrated in Chapter 5 that

these agents induce IDO, which promotes immunosuppression. It was noted that the findings of Chapter 12 are limited by the lack of a suitable *in vivo* rat model of HIV/ AIDS.

The progression to AIDS is also marked by changes in the cytokine profile, with a decrease in type 1 cytokines, and an increase in type 2 cytokines. It was thus necessary to determine the effects of 5-FU and melatonin on cytokine levels. It was demonstrated in Chapter 13 that the co-administration of 5-FU and melatonin increases IL-1β levels, but this is insufficient to raise glucocorticoid levels, as shown in Chapter 12. It was also found that 5-FU enhances the levels of the pro-inflammatory cytokine INFγ, which may be a further mechanism by which the agent enhances IDO activity, and that this increase is abolished by the co-administration of melatonin. This suggests an anti-inflammatory role for melatonin. Neither drug alters the levels of IL-1α, IL-2, IL-4, IL-6, IL-10, GM-CSF and TNFα at the dosages used. It can thus be concluded that neither 5-FU nor melatonin induces alterations in the cytokine profile indicative of the progression to AIDS.

In Chapter 14, the effects of 5-FU and melatonin on pain perception were investigated. Pain is a common problem, particularly in cancer patients. It was found that the acute administration of 5-FU significantly lowers the pain threshold of rats exposed to the modified tail-flick method, and that the co-administration of melatonin counters this. Melatonin was thus shown to exhibit analgesic properties, and may possibly strengthen the circadian rhythm of pain tolerance. It was also found that these 5-FU- and melatonin-induced alterations in pain sensitivity disappear with the long-term administration of both agents. This suggests that long-term 5-FU therapy is thus unlikely to induce or worsen nociceptive changes in cancer patients.

CHAPTER 16

FINAL CONCLUSIONS

This research focuses on examining the various mechanisms of interaction between 5-FU and melatonin, so that the call made by Lissoni *et al* (1999a) for melatonin to be used as co-therapy to enhance the efficacy and decrease the toxicity of the antineoplastic agent, can be critically evaluated.

This study has, firstly, established that there is no hepatic metabolic drug interaction between 5-FU and melatonin, at the level of CYP450. The use of melatonin co-therapy is thus unlikely to result in subtherapeutic, or toxic, plasma levels of 5-FU in the clinical setting. It is also important tht neither drug was found to alter the activity of CYP450; cancer patients are often treated with a cocktail of drugs, and the use of 5-FU and melatonin is thus unlikely to inhibit or induce the metabolism of these other agents. It was also shown that there is no direct chemical interaction between 5-FU and melatonin, and a sensitive HPLC method was developed and validated to separate and detect these drugs. This technique could possibly assist other researchers in quantitatively analysing biological samples containing 5-FU and melatonin.

Secondly, the toxicity of 5-FU by novel mechanisms, and the ability of melatonin to counter these, has been demonstrated:
(i) The agent induces hepatic lipid peroxidation, through the generation of $O_2^{-}\cdot$ and possibly HO·. The co-administration of melatonin decreases 5-FU-induced lipid peroxidation, indicating a cytoprotective role for melatonin against 5-FU toxicity. However, it was also demonstrated that five days of melatonin administration *in vivo* results in a significant increase in $O_2^{-}\cdot$ levels, and it was suggested that this is due to the formation of the metabolite 6-OHM, which is known to increase ROS levels. This finding suggests caution with the use of melatonin, as it is metabolised to compounds that may have deleterious effects. Co-administration of melatonin does not enhance 5-FU-induced increases in $O_2^{-}\cdot$ levels, implying that melatonin co-therapy is unlikely to worsen 5-FU-induced toxicity;
(ii) 5-FU decreases forebrain levels of 5-HT, NE and DA, thereby possibly leading to the development of depression. These decreases in neurotransmitter levels are believed to be primarily due to 5-FU's ability to inhibit DNA synthesis; this prevents the expression of the genes required to synthesise the enzymes necessary for the biosynthesis of neurotransmitters. 5-FU does not, however, alter the metabolism of 5-HT and DA. The co-administration of

melatonin counters the abovementioned 5-FU-induced decreases in NE, DA and 5-HT levels, suggesting that, in the clinical setting, melatonin co-therapy may alleviate 5-FU-induced depression;
(iii) 5-FU induces hippocampal toxicity, by decreasing the volumes of the CA1-2 and CA3 regions, and causing morphological alterations. This could be a mechanism by which 5-FU results in the phenomenon of "chemobrain". These effects are countered by the administration of melatonin, implying that melatonin may attenuate 5-FU-induced cognitive impairment;
(iv) The administration of 5-FU raises levels of the pro-inflammatory cytokine IL-1β, which is abolished in the presence of melatonin; and
(v) The acute administration of 5-FU decreases the pain threshold, suggesting that 5-FU may induce algesia. This is countered by the co-administration of melatonin, which enhances pain tolerance.

Thirdly, it was shown that both 5-FU and melatonin have significant effects on tryptophan metabolism, by inhibiting TDO activity and enhancing IDO activity. TDO inhibition is likely to be a result of the –NH functional groups in both agents blocking the catalytic site of the enzyme, and in the case of melatonin this inhibition results in an increase in cerebral 5-HT levels. It has been concluded that 5-FU stimulates IDO activity by increasing both $O_2^{-}\cdot$ and INFγ levels, whereas it is believed the melatonin-induced stimulation of IDO is primarily due to an increase in $O_2^{-}\cdot$.

Fourthly, the induction of lipid peroxidation and IDO-induced increases in 3-OHK and QA levels may be additional mechanisms by which 5-FU exerts its antineoplastic effects, and the co-administration of melatonin, by countering the former, may decrease the anticancer efficacy of 5-FU by protecting cancer cells against oxidative stress. IDO stimulation may also be a mechanism of 5-FU-induced immunosuppression, but a consequence of such stimulation is immune escape by cancer cells. The production of kynurenic acid may exert a protective influence over cancer cells, resulting in resistance towards 5-FU therapy.

The fifth finding of this research is that if melatonin is to be used as co-therapy to decrease the adverse effects of 5-FU, in particular possible anxiety and depression manifested by locomotor activity changes, long-term administration of this agent is necessary.

The sixth major finding of this research is that, by not altering serum corticosterone levels or changing the cytokine profile, 5-FU and melatonin can be used by HIV-positive patients, without promoting the progression to AIDS, according to the glucocorticoid hypothesis. 5-FU may be of benefit in these patients due to its intrinsic anti-HIV activity as well as its ability to

decrease antiretroviral resistance, while melatonin is immunostimulatory. This finding is perhaps very relevant, due to soaring HIV infection levels, especially in South Africa, and the mounting problem of antiretroviral resistance. It is recommended, though, that the lowest possible doses of 5-FU and melatonin are used, in order to prevent IDO-induced cytokine changes and immunosuppression.

The findings of this research lend themselves to a plethora of future studies. These include determining whether there are any pharmacokinetic interactions between 5-FU and melatonin, apart from hepatic metabolism by CYP450; detecting 6-OHM levels and investigating the effects of this metabolite on oxidative stress in detail; chronobiology studies to determine the best time at which to administer 5-FU and melatonin therapy, and at which time melatonin administration is likely to have optimal protective effects on non-cancer cells and not interfere with 5-FU-induced toxicity towards cancer cells; the optimal dosage of melatonin and the duration of administration should be investigated; and a suitable *in vivo* rat model of HIV/ AIDS should be developed, the effects of 5-FU and melatonin studied, and optimal dosages of these agents in combination with antiretrovirals ascertained.

It can thus be concluded that prolonged melatonin co-therapy may be useful in cancer patients being treated with 5-FU, and could potentially attenuate many adverse effects of 5-FU and thus enhance the patient's quality of life. Cognisance should, however, be taken of the possibly deleterious effects of melatonin metabolites, and patients should be regularly monitored and adequate nutrition ensured.

APPENDIX A

HOUSING OF ANIMALS

The animals used in this research were male Wistar rats, each weighing between 200g and 300g, obtained from the South African Institute for Medical Research (Johannesburg, South Africa). Unless otherwise stated (see 10.5.2 and 11.5.2), the rats were randomly assigned into groups of five and each group housed in a separate cage. These cages consisted of opaque plastic sides and metal grid covers and floors; each cage held a container of water and food, to which the animals had access *ad libitum*. The cages were cleaned once a day. The animal room in which the cages were housed was windowless, maintained at a temperature of 20-25ºC and ventilated by means of an extractor fan. An automatic lighting control ensured that the rats were subjected to a diurnal lighting cycle of twelve hours of light, commencing at 06h00, and twelve hours of darkness, commencing at 18h00. The intensity of the lighting during the light phase was 300 μWatts/ cm^2. All work involving these animals was performed with the approval of the Rhodes University Ethics Committee.

APPENDIX B

PROTEIN DETERMINATION ASSAY (Lowry *et al*, 1951)

A stock solution of 300 µg/ ml of bovine serum albumin (BSA) was made up. The following table gives the composition of the calibration solutions, each of which was made in duplicate:

Table 23: Volumes required to formulate BSA calibration solutions

Concentration (µg/ ml)	**Volume of BSA stock required (ml)**	**Volume of HPLC-grade water required (ml)**
0	0	3.0
30	0.3	2.7
60	0.6	2.4
90	0.9	2.1
120	1.2	1.8
150	1.5	1.5
180	1.8	1.2

To each of the BSA solutions above, an aliquot of 6 ml of Copper reagent was added. The mixtures were vortexed and left to stand for 10 minutes. The Copper reagent consisted of: 1 ml of 0.67% copper sulphate, 1 ml of 2% sodium tartrate and 98 ml of 2% disodium carbonate; the latter contained 0.1 M NaOH. An aliquot of 0.3 ml of Folin reagent was subsequently added to the above, the mixtures vortexed and left to stand in the dark for 30 mins. Each of the solutions, which were dark blue in colour, was read spectrophotometrically at 500 nm and the absorbances obtained were used to calculate a calibration curve. A calibration curve was obtained with an excellent r^2 of 0.9992 (see Figure 145).

Aliquots of 30 µL of the homogenate preparation, containing an unknown concentration of protein, were added to 2970 µL of deionised water, in triplicate, and the absorbance read. Deionised water was used as the blank. The concentration of protein in the extract was then determined by using the calibration curve obtained and multiplying the concentration so obtained by the dilution factor.

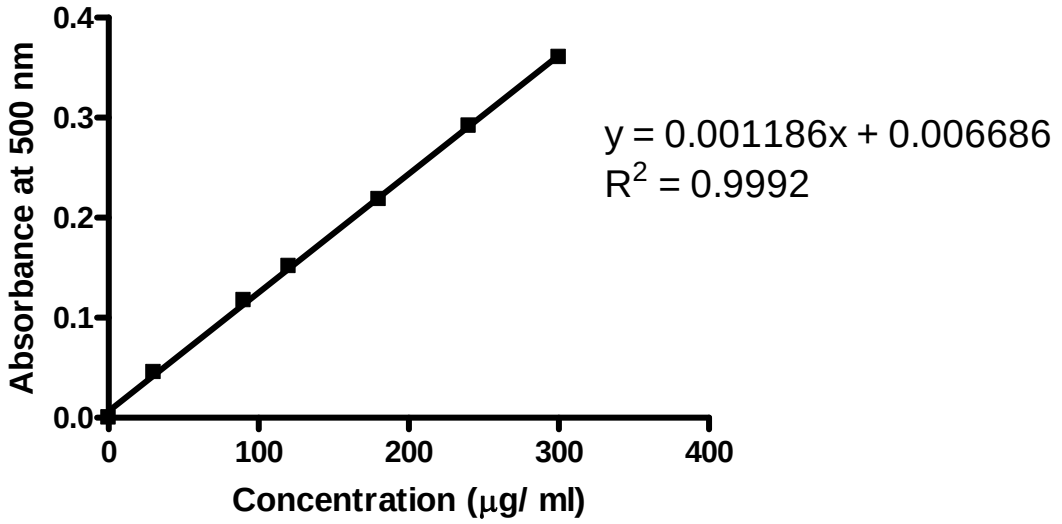


Figure 145: Calibration curve obtained for BSA assay

APPENDIX C

LIPID PEROXIDATION CALIBRATION CURVE (Placer *et al*, 1966)

A stock solution of 50 ng/ ml MDA was made up and serial dilutions of MDA made up according to the table below. An MDA equivalent, 1,1,3,3-tetramethoxypropan, was used. The solutions were made up using KH_2PO_4 buffer, pH 7.4. The procedure was carried out in duplicate.

Table 24: Volumes required to formulate MDA calibtration solutions

Concentration of MDA (nM)	**Volume of MDA stock solution required (ml)**	**Volume of KH_2PO_4 required (ml)**
0	0	3.0
5	0.3	2.7
10	0.6	2.4
15	0.9	2.1
20	1.2	1.8
25	1.5	1.5
30	1.8	1.2
40	2.4	0.6
50	3.0	0

The test-tubes were vortexed. Aliquots of 0.5 ml BHT were added to each, followed by 1 ml TCA and further vortexing. An aliquot of 0.5 ml was removed, 0.5 ml TBA added and the test-tubes vortexed. Incubation in a water bath at 95ºC for 60 mins followed. An aliquot of 2 ml of butanol was added, the test-tubes centrifuged at 2000g for 5 mins, and the absorbance of MDA read spectrophotometrically at 532 nm. Butanol was used as the blank. A calibration curve was constructed, with an acceptable r^2 of 0.9988.

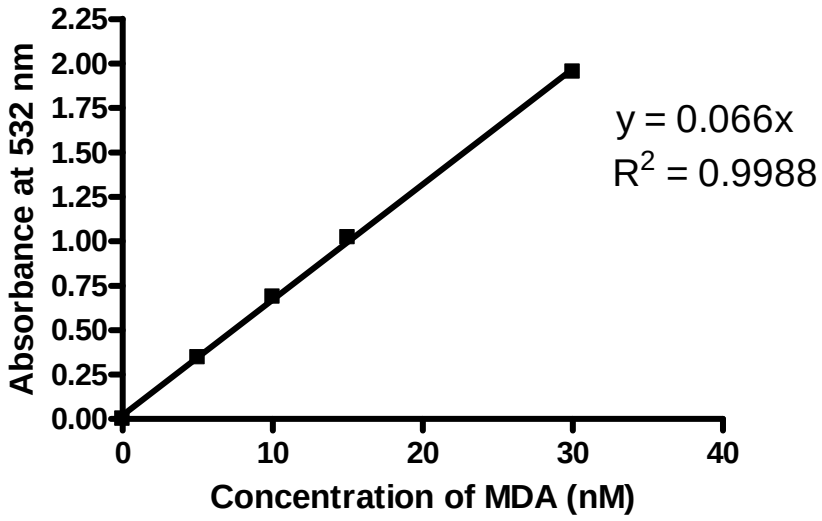


Figure 146: Calibration curve obtained for lipid peroxidation assay

APPENDIX D

NBD CALIBRATION CURVE

A stock solution of 500 µM NBD was made up in glacial acetic acid and serial dilutions were made to give the calibration solutions described below.

Table 25: Volumes required to formulate NBD calibtration solutions

Concentration of NBD (µM)	Volume of NBD stock solution required (ml)	Volume of glacial acetic acid required (ml)
0	0	3.0
100	0.6	2.4
200	1.2	1.8
300	1.8	1.2
400	2.4	0.6
500	3.0	0

The test-tubes were vortexed and the absorbance of each at 560 nm was read spectrophotometrically. The following calibration curve was obtained, with an excellent r^2 of 0.9996.

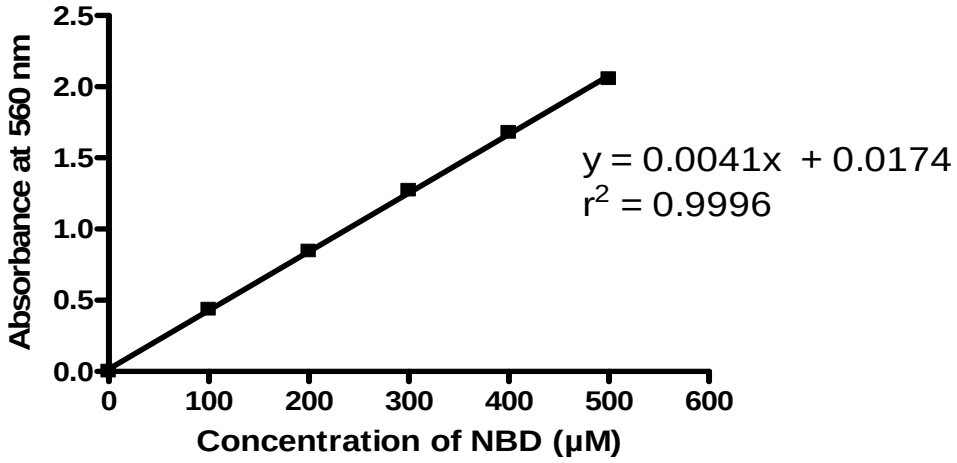


Figure 147: Calibration curve obtained for NBD

APPENDIX E

CORTICOSTERONE CALIBRATION CURVE (Correlate-EIA™ Kit manual, 2004)

A 200 000 pg/ ml supplied stock solution of corticosterone was warmed to room temperature and calibration standards of corticosterone were prepared according to the following Table. The diluent used was Assay Buffer 15 (see 12.8.1)

Table 26: Volumes required to formulate corticosterone calibtration solutions

Standard	Diluent volume (µL)	Volume added (µL)	Concentration of corticosterone (pg/ ml)
1	900	100 of stock	20 000
2	800	200 of [1]	4000
3	800	200 of [2]	800
4	800	200 of [3]	160
5	800	200 of [4]	32

The standards were pipetted into microplate wells and subjected to the protocol described in 12.8.2. The optical density was read in a microplate reader at 405 nm and the concentration of corticosterone present was calculated as outlined in 12.8.2. The calibration curve on the next page has been obtained. A linear regression line with an acceptable r^2 of 0.9223 has been obtained.

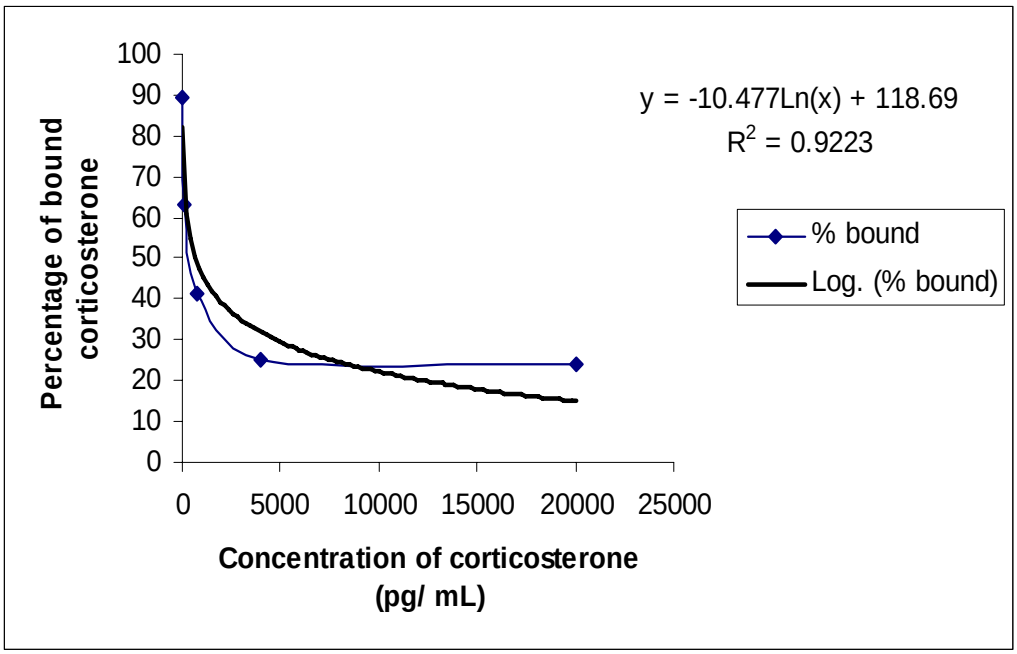


Figure 148: Calibration curve obtained for corticosterone assay

APPENDIX F

CYTOKINE CALIBRATION CURVES (Bio-Plex™ Rat Cytokine 9-Plex A Panel)

One tube of lyophilised recombinant cytokine standard, at a concentration of 32 000 pg/ ml, was warmed to room temperature and reconstituted with 500 µL of Bio-Plex rat serum Standard Diluent. This was gently vortexed for a few seconds and incubated on ice for a period of 30 mins. The following calibration solutions were prepared from a stock solution of 3200 pg/ ml, as described in Table 27. The diluent used was Bio-Plex rat serum Standard Diluent.

Table 27: Volumes required to formulate cytokine calibtration solutions

Standard	**Diluent volume (µL)**	**Volume added (µL)**	**Concentration of cytokine (pg/ ml)**
1	0	150 of stock	3200
2	150	50 of [1]	800
3	150	50 of [2]	200
4	150	50 of [3]	50
5	150	50 of [4]	12.5
6	150	50 of [5]	3.13
7	150	50 of [6]	0.78
8	150	50 of [7]	0.2

The solutions were vortexed gently, and the fluorescence intensity of each cytokine read using the Bio-Plex Manager™ digital processor and software (Bio-Rad Laboratories Inc., Germany) (see 13.3.2). The following calibration curves and equations were obtained.

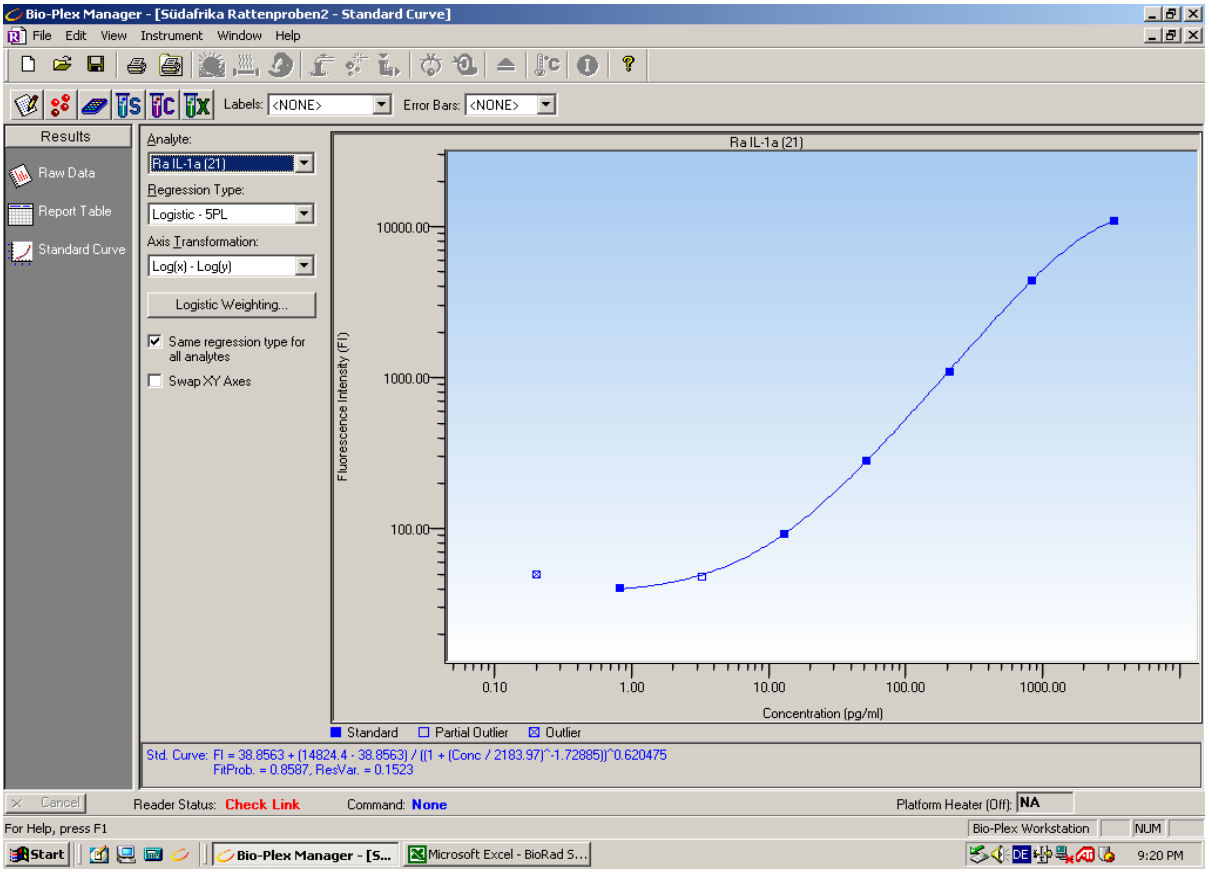


Figure 149: Calibration curve obtained for IL-1α

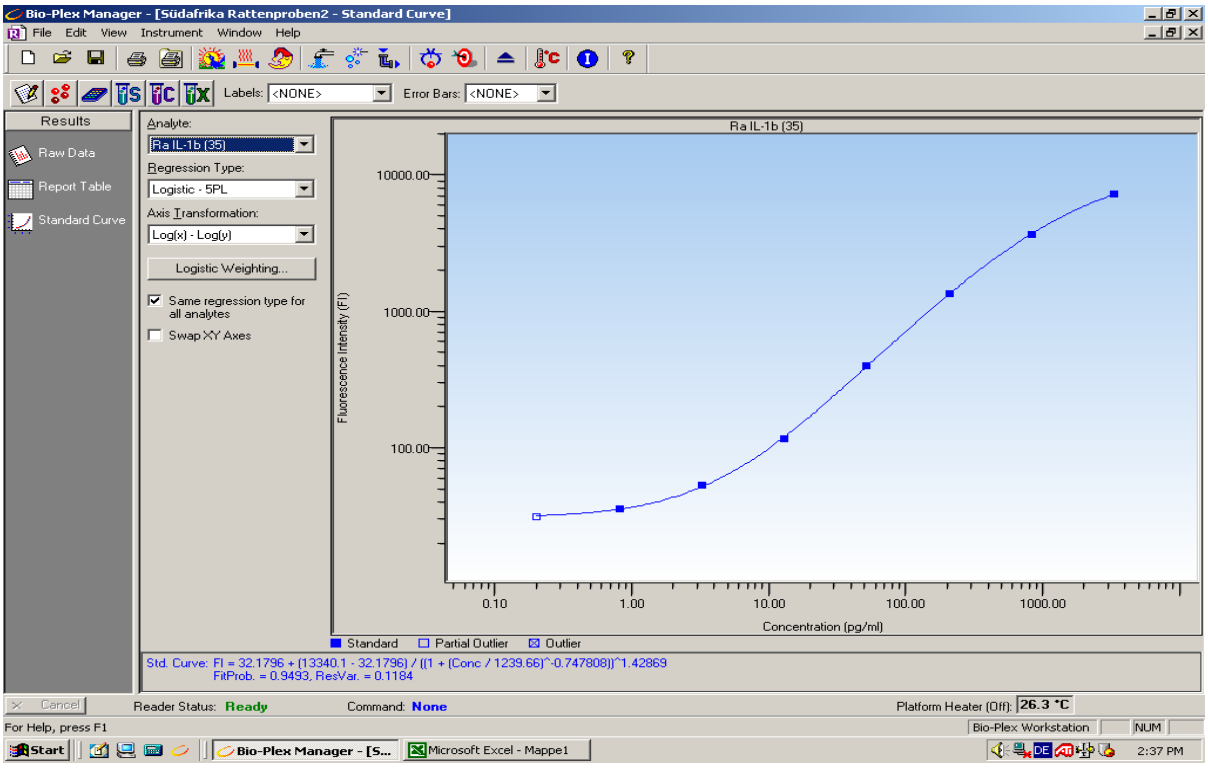


Figure 150: Calibration curve obtained for IL-1β

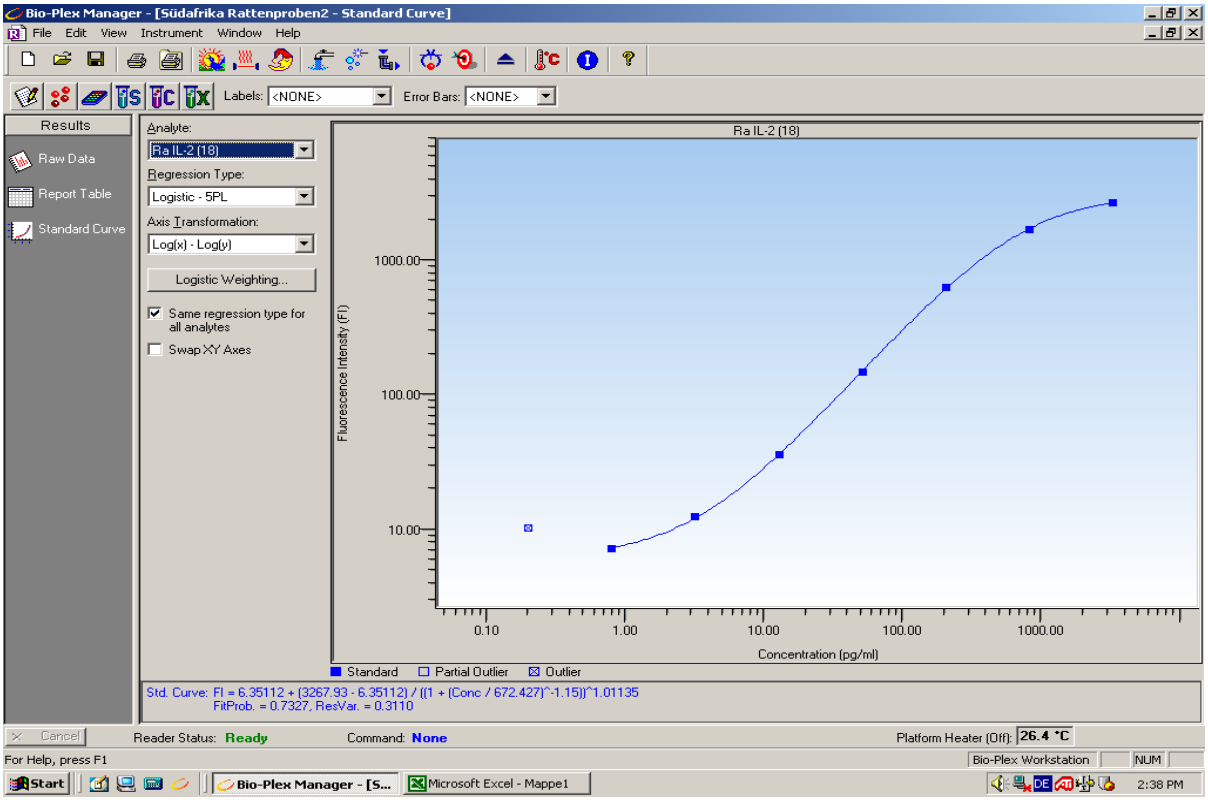


Figure 151: Calibration curve obtained for IL-2

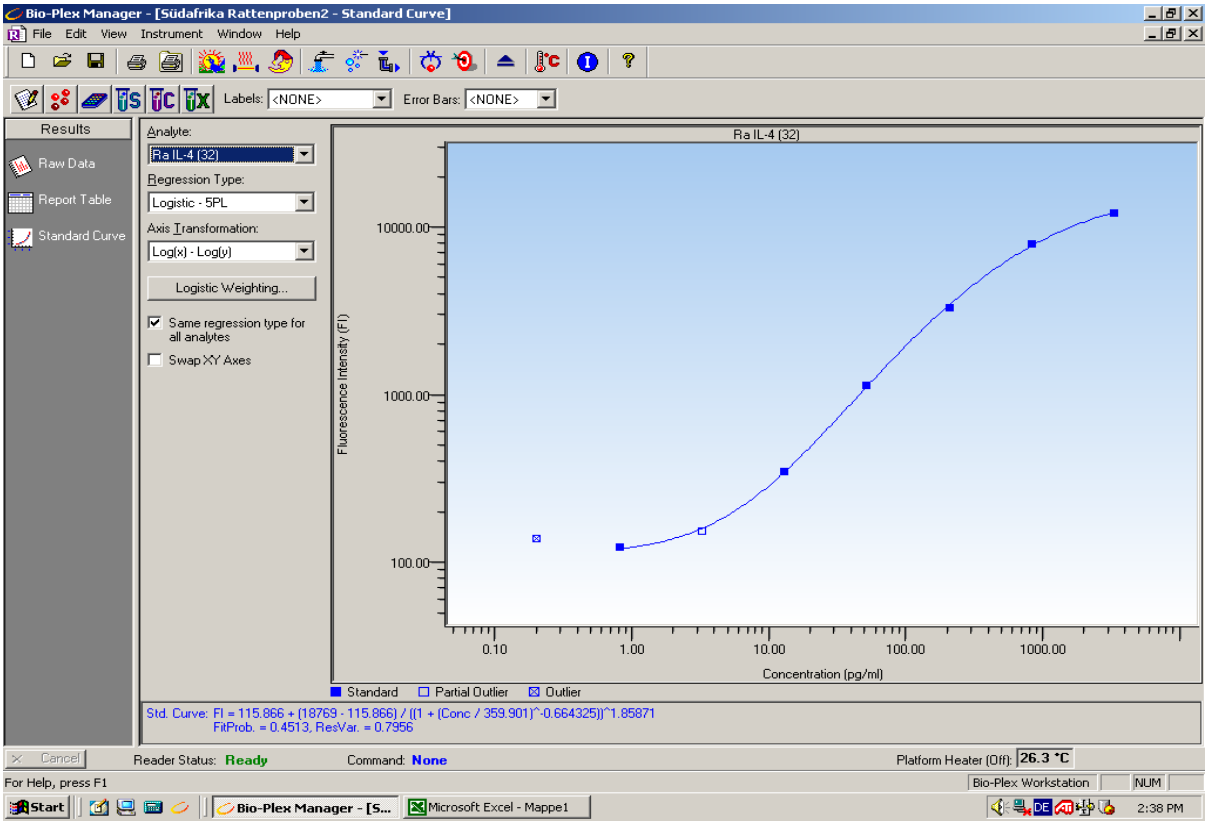


Figure 152: Calibration curve obtained for IL-4

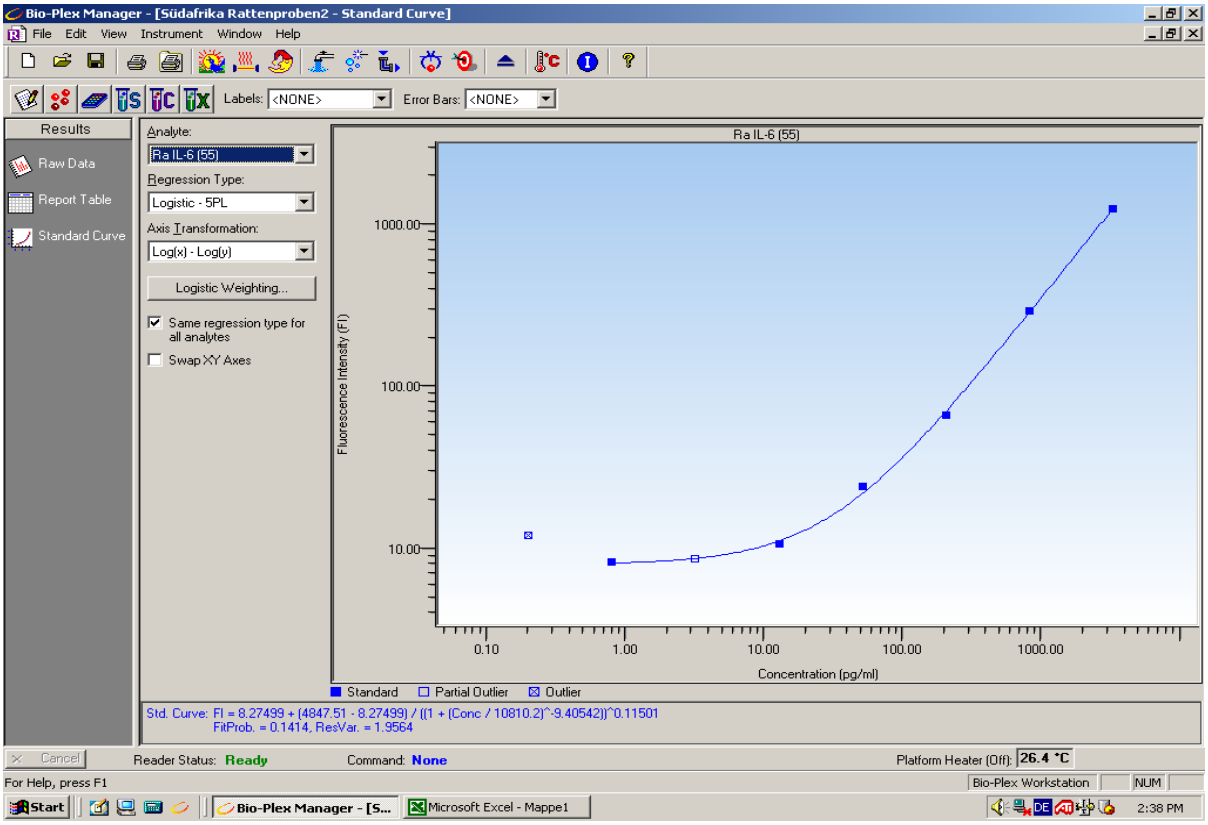


Figure 153: Calibration curve obtained for IL-6

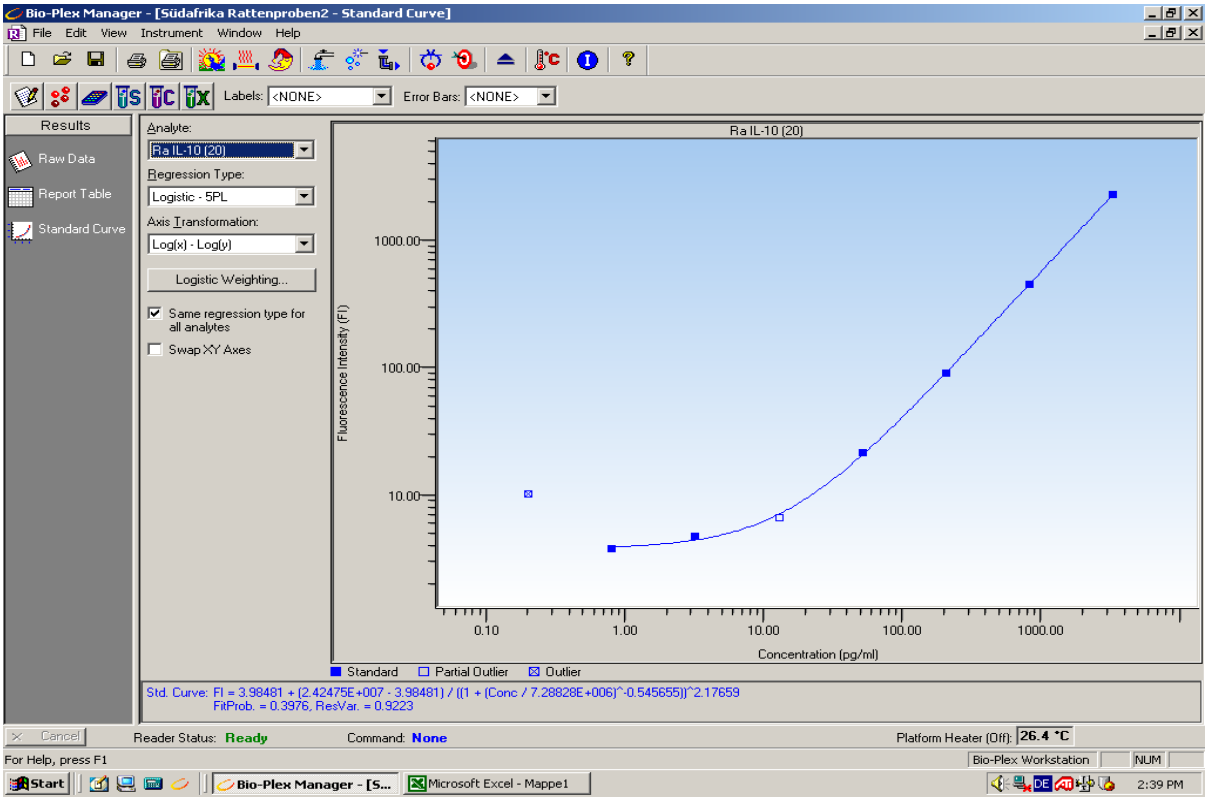


Figure 154: Calibration curve obtained for IL-10

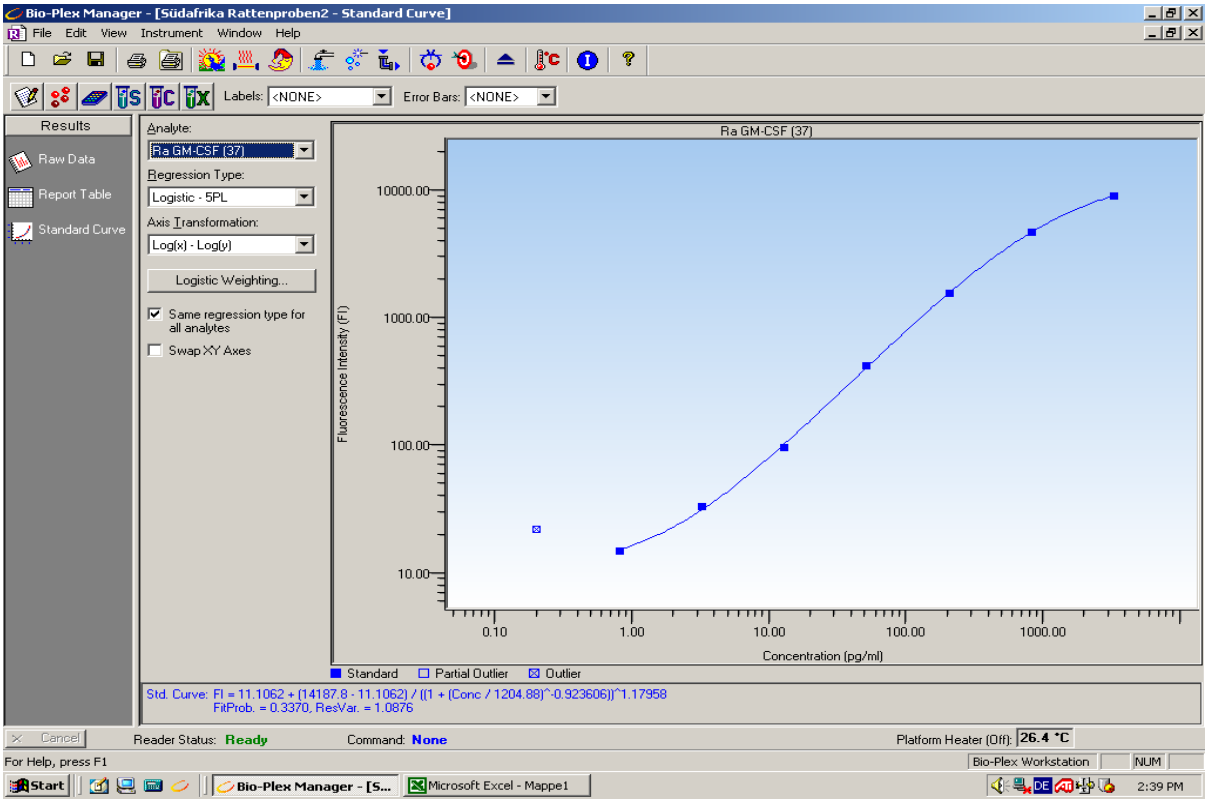


Figure 155: Calibration curve obtained for GM-CSF

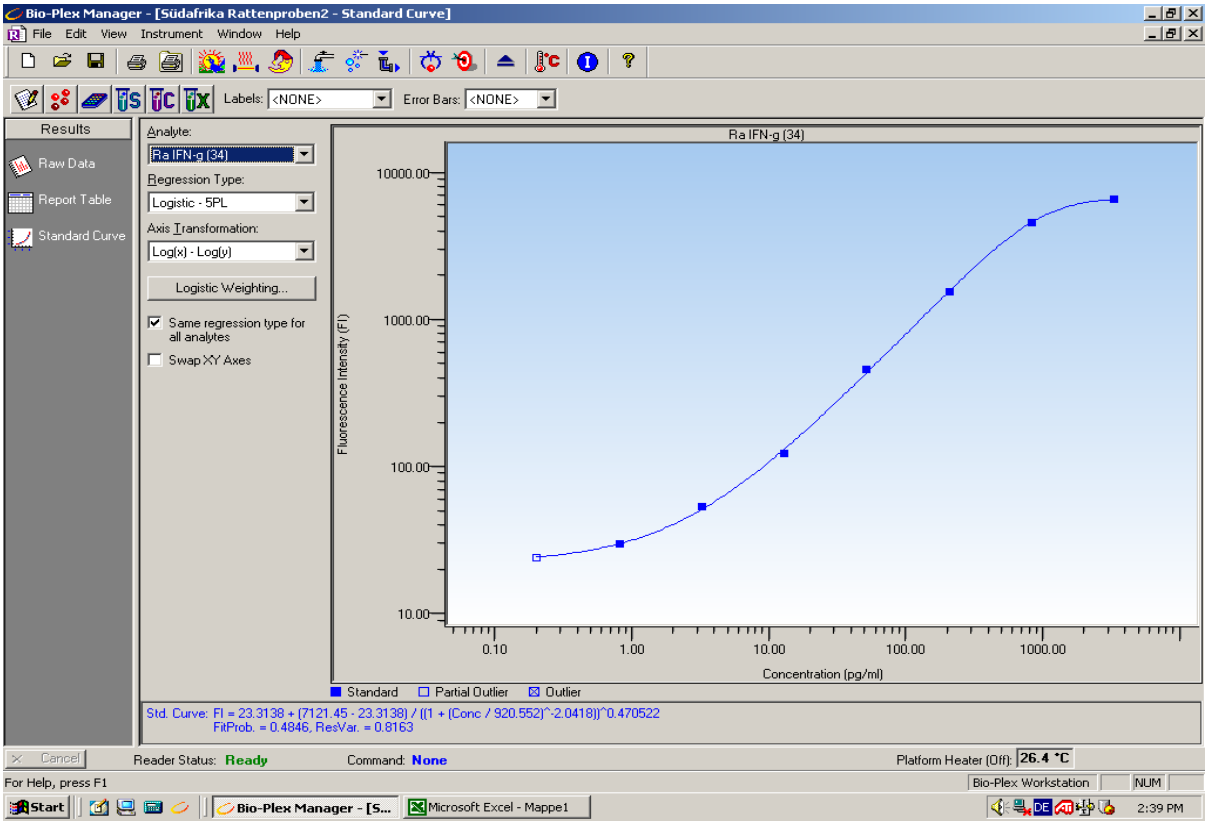


Figure 156: Calibration curve obtained for INFγ

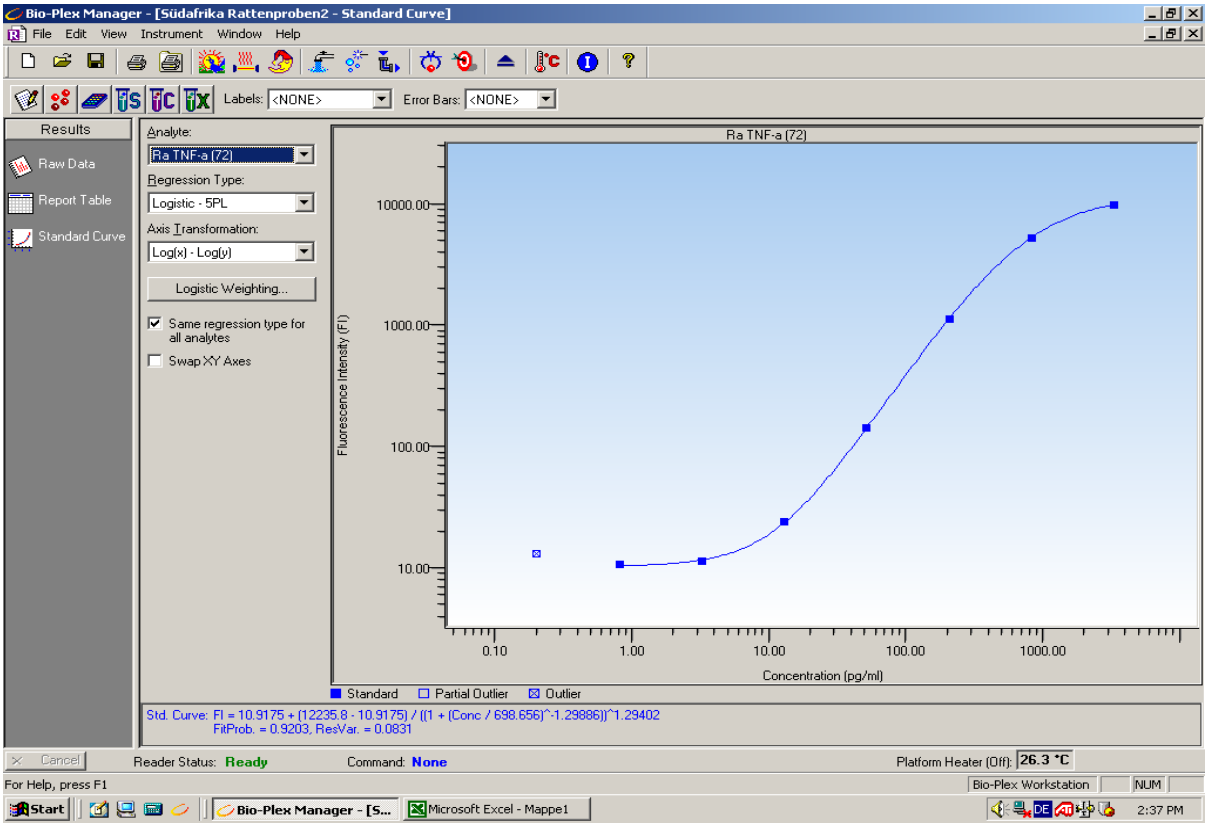


Figure 157: Calibration curve obtained for TNFα

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