NEUROPROTECTIVE MECHANISMS OF NEVIRAPINE AND EFAVIRENZ IN A MODEL OF NEURODEGENERATION.

Thesis submitted in fulfilment of the requirements for the degree of

MASTER OF SCIENCE (PHARMACY)

of

RHODES UNIVERSITY

by

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DECEMBER 2007

ABSTRACT

AIDS Dementia Complex (ADC) is a neurodegenerative disorder implicated in HIV-1 infection that is associated with elevated levels of the neurotoxin, quinolinic acid (QA) which causes a cascade of events to occur, leading to the production of reactive oxygen species (ROS), these being ultimately responsible for oxidative neurotoxicity. In clinical studies, Non-nucleoside reverse transcriptase inhibitors (NNRTIs), efavirenz (EFV) and nevirapine (NVP) have been shown to potentially delay the progressive degeneration of neurons, thus reducing the frequency and neurological deficits associated with ADC. Despite these neuroprotective implications, there is still no biochemical data to demonstrate the mechanisms through which these agents offer neuroprotection. The present study aims to elucidate and further characterize the possible antioxidant and neuroprotective mechanisms of NVP and EFV *in vitro* and *in vivo*, using QA-induced neurotoxicity as a model.

Research has demonstrated that antioxidants and metal chelators have the ability to offer neuroprotection against free radical induced injury and may be beneficial in the prevention or treatment of neurodegeneration. Hence the antioxidant and metal binding properties of these agents were investigated respectively. Inorganic studies, including the 1, 1-diphenyl-2 picrylhydrazyl (DPPH) assay, show that these agents readily scavenge free radicals *in vitro*, thus postulating the antioxidant property of these agents. The enhancement of superoxide radical generation and iron mediated Fenton reaction by QA is related to lipid peroxidation in biological systems, the extent of which was assayed using the nitroblue tetrazolium and thiobarbituric acid method respectively. Both agents significantly curtail QA-induced lipid peroxidation and potentially scavenge superoxide anions generated by cyanide *in vitro*. Furthermore, *in vivo* results demonstrate the ability of NVP and EFV to protect hippocampal neurons against lipid peroxidation induced by QA and superoxide radicals generated as a consequence thereof.

The alleviation of QA-induced oxidative stress *in vitro* possibly occurs through the binding of iron (II) and / or iron (III), and this argument is further strengthened by the ability of EFV and not NVP to reduce iron (II)-induced lipid peroxidation *in vitro*

directly. In addition the ferrozine and electrochemistry assay were used to measure the extent of iron (II) Fe^{2+} and iron (III) Fe^{3+} chelation activity. Both assays demonstrate that these agents bind iron (II) and iron (III), and prevent redox recycling of iron and subsequent complexation of Fe^{2+} with QA which enhances neuronal damage.

Both NNRTIs inhibit the endogenous biosynthesis of QA by inhibiting liver tryptophan 2, 3-dioxygenase activity *in vivo* and subsequently increasing hippocampal serotonin levels. Furthermore, these agents reduce the turnover of hippocampal serotonin to 5-hydroxyindole acetic acid. NVP and not EFV increase 5-hydroxyindole acetic acid and norepinephrine levels in the hippocampus. The results of the pineal indole metabolism study show that NVP increases the synthesis of melatonin, but decreases N-acetylserotonin, 5-hydroxyindole acetic acid and 5-hydroxytryptophol levels. Furthermore, it shows that EFV decreases 5-hydroxyindole acetic acid and melatonin synthesis. Behavioural studies using a Morris water maze show that the post-treatment of rats with NVP and EFV significantly improves QA-induced spatial memory deficits in the hippocampus.

This study therefore provides novel information regarding the neuroprotective mechanisms of NVP and EFV. These findings strengthen the argument that these NNRTIs not only have antiviral effects but possess potential neuroprotective properties, which may contribute to the effectiveness of these drugs in the treatment of ADC.

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%	percent
ΔE_P	change in electrical potential
ΔI_A	change in current
^oC	degrees Celsius
$-CH_2-$	methylene group
µg	microgram
µL	microlitre
µm	micrometers
3-HA	3-hydroxyanthranillic acid
3-HAO	3-hydroxyanthranillic acid oxygenase
3-HK	3-hydroxykynurenine
5-ALA	5-aminolaevulinate
5-HIAA	5-hydroxyindole acetic acid
5-HT	serotonin
5-HTOH	5-hydroxytryptophol
5-MIAA	5-methoxyindole acetic acid
5-MT	5-methoxytryptamine
5-MTOH	5-methoxytryptophol
a	amperes
ACN	acetonitrile
AD	Alzheimer's disease
ADH	aldehyde dehydrogenase
ADC	AIDS Dementia Complex
AIDS	acquired immune deficiency syndrome
AMPA	α-amino-3-hydroxy-5-methylisoxazole-4-proprionate
aMT	melatonin
ANOVA	one-way analysis of the variance
aq	aqueous phase
AR	aldehyde reductase
ART	antiretroviral therapy
ASV	adsorptive stripping voltammetry
ATP	adenosine triphosphate
BHT	butylated hydroxytoluene
BSA	bovine serum albumin
CA	cornu ammonis
Ca^{2+}	calcium
CAT	catalase
Ci	curies
cm	centimetre

CNS	central nervous system
CPM	counts per minute
CR	conditional response
CS	conditional stimulus
CSF	cerebrospinal fluid
Cyt *c*	cytochrome c
DA	dopamine
DG	dentate gyrus
DNA	deoxyribose nucleic acid
DPPH	1, 1-diphenyl-2-picryl-hydrazil
DPP $H^{\bullet}$	1, 1-diphenyl-2-picryl-hydrazil radical
DPM	disintegrations per minute
e^{-}	electron
EC	entorhinal cortex
EDTA	etyhlenediaminetetraacetic acid
E_P	electrical potential
ETC	electron transport chain
EFV	efavirenz
FAD	flavin adenine dinucleotide
$FADH_2$	dihydroflavin adenine dinucleotide
Fe^{2+}	ferrous ion
Fe^{3+}	ferric ion
$FeCl_3$	anhydrous ferric chloride
$FeSO_4.7H_2O$	hydrated ferrous sulphate
g	gram
GCE	glassy carbon electrode
gp120	glycoprotein 120
GPx	glutathione peroxidase
GSH	reduced glutathione
GSSG	oxidized glutathione
H	hydrogen
H^{+}	hydrogen ion
$H^{\bullet}$	hydrogen radical
H_2O	water
H_2O_2	hydrogen peroxide
HAD	HIV-Associated dementia
HCl	hydrochloric acid
$HClO_4$	perchloric acid
HD	Huntington's disease
HIOMT	hydroxyindole-O-methyl transferase
HIV	human immunodeficiency virus
HIV-1	human immunodeficiency virus type-1
HNE	hydroxynonenal
$HO^{\bullet}$	hydroxyl radical

$HOO^{\bullet}$	hydroperoxyl radical
HSA	heptane sulphonic acid
HSE	herpes simplex encephalitis
H-W	Haber-Weiss
HPLC-ECD	high performance liquid chromatography-electrochemical detector
i.h.	intrahippocampal
i.p.	intraperitoneal
I_A	current
IDO	indoleamine 2, 3-dioxygenase
IL	interleukin
KCl	potassium chloride
KCN	potassium cyanide
kg	kilogram
KH_2PO4	potassium dihydrogen phosphate
KP	kynurenine pathway
KYNA	kynurenic acid
LHPA	limbic-hypothalamic-pituitary-adrenal
L-KYN	L-Kynurenine
$LOO^{\bullet}$	lipid peroxyl radical
LP	lipid peroxidation
LTM	long-term memory
LTP	long term potentiation
M	molar
MAO	monoamine oxidase
MDA	malondialdehyde
MEM	Minimum Essential Medium
MeOH	methanol
mg	milligram
Mg^{2+}	magnesium
min	minute
ml	millilitre
mm	millimetre
mM	millimolar (millimoles per litre)
mmoles	millimoles
mV	millivolt
n	sample size
Na^{+}	sodium
Na^{2+}/ K^{+}	sodium/potassium
NaCl	sodium chloride
NAD	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
NaOH	sodium hydroxide
NAS	N-acetylserotonin

NAT	N-acetyltransferase
NBD	nitroblue diformazan
NBT	nitroblue tetrazolium
NE	norepinephrine
NMDA	N-methyl-D-aspartate
nmole	nanomoles
NNRTIs	non-nucleoside reverse transcriptase inhibitors
NO	nitric oxide
NOS	nitric oxide synthase
ns	not significant
NVP	nevirapine
O_2	oxygen
$^1O_2$	singlet oxygen
$O_2^{\bullet -}$	superoxide anion
od	once daily
$ONOO^-$	peroxynitrite
PA	phosphoric acid
PBS	phosphate buffered saline
PD	Parkinson's disease
PIs	protease inhibitors
PLA_2	phospholipase A_2
pmoles	picomoles
PT	permeability transition
PUFAs	polyunsaturated fatty acids
QA	quinolinic acid
redox	reduction-oxidation
RNS	reactive nitrogen species
$RO^{\bullet}$	alkoxyl radical
$ROO^{\bullet}$	peroxyl radical
ROOH	lipid peroxide
ROS	reactive oxygen species
R-SH	thiol-containing compounds
RT	reverse transcriptase
s	seconds
SD	standard deviation
SH	sulfhydryl groups
SOD	superoxide dismutase
S-R	stimulus–response
STM	short-term memory
T5H	tryptophan-5-hydroxylase
TBA	thiobarbituric acid
TCA	trichloroacetic acid
TDO	tryptophan 2, 3-dioxygenase

TEA	triethylamine
TLC	thin layer chromatography
TNF-α	tumour necrosis factor alpha
TRP	tryptophan
TYR	tyrosine
UR	unconditional response
US	unconditional stimulus
UV	ultraviolet
UV/VIS	ultraviolet/visible
V	volts
v/v	volume by volume
Vs^{-1}	Volts per second
w/v	weight by volume
xg	relative centrifugal field
ε	extinction coefficient
λ_{max}	lambda max

In memory of my dear colleague, friend and mentor, Dr Amichand Dairam

(27 February 1980 - 19 May 2007)

who inspired me to never give up and to work whole heartedly in all I do in life.

ACKNOWLEDGEMENTS

First of all I thank Almighty God for his divine intervention and strength over my life and because of his grace and guidance throughout this journey, I was able to complete my research project.

I thank my family for their encouragement, love and support throughout my academic career. I am very grateful for the influence they have over my life. Above all they have taught me to have faith in God and to trust him to bring a breakthrough in my academic career.

I also thank my supervisor, Professor Santy Daya, for his guidance, support and advice in all aspects of my research project. I am grateful for helping me in my applications for different scholarships, I obtained during my studies.

Thank you to my Neuroscience Research Group colleagues, Mark Scheepers, Layla Cassim and Paul Vogt, for being ever ready to lend a helping hand whenever they could.

Many thanks go to Andrew Mellon Foundation of Rhodes University and the National Research Foundation of South Africa for the financial contribution towards my research project.

Words are not enough to express my gratitude and appreciation to my friend and colleague, Adrienne Müller for her input and for proof reading my thesis. Her constructive criticism, gave me confidence to write this thesis. I also thank Professor Afolayan for proof reading my thesis.

I thank my friend, mentor and colleague, the late Dr Amichand Dairam for helping me come up with most of the studies pertaining to my thesis. He helped with the writing of my first scientific paper, to be published soon. Hence I dedicate my thesis in memory of him. May his soul rest in peace.

I thank my river of life cell group members for their spiritual up liftment and prayers. A special thanks to Ps Debbie Sloane, Nelisha, Antonia, Nyaradzo and Happy for lending me a shoulder to cry on whenever the work was overwhelming.

Thanks to the academic staff of the Faculty of Pharmacy, technical staff, particularly Mr and Mrs Morley, Mr L. Purdon and Mr T. Samkange for ensuring that I had all the chemicals and reagents I required for all my experiments. I also thank the service staff, Quinett, Welcome and Prudence for their help throughout my research project.

Thank you to the following: Mike Naidoo, for helping me secure the drugs I used for my project, Aspen Pharmaceutical Company in Port Elizabeth for kindly supplying the drugs I used for my project and Dr Limson and the biosensor research group for helping with my iron binding studies.

I thank the other postgraduate students in the Faculty of Pharmacy and Chemistry department for lending me an ear when I needed them. I also thank all my friends for their support throughout my academic career.

CHAPTER 1

LITERATURE REVIEW

1.1. AIDS DEMENTIA COMPLEX (ADC)

1.1.1. Background and Epidemiology

Human Immunodeficiency virus type-1 (HIV-1) continues to spread globally. Currently, 40 million people worldwide are estimated to be infected, of which 37 million are adults and 3 million are children. More than 3 million die every year because of various complications associated with the illness and its progression to acquired immune deficiency syndrome (AIDS) (Kumar *et al*., 2007). HIV infection of the central nervous system (CNS) leads to severe neurological complications in about 25 % of adults and half of children with AIDS (Navia *et al*., 1986; Price *et al*., 1988).

The mechanisms involved in the pathogenic consequences involve, neurological disorders, neuropsychological dysfunctions, motor disturbances, behavioural changes and neurobehavioural disorders including HIV-Associated dementia (HAD), which progresses to AIDS dementia complex (ADC) (Navia *et al*., 1986; Price *et al*., 1988; Kumar *et al*., 2007). As many as 25-50 % of adult patients and children with AIDS suffer from neurological manifestations, including impaired psychomotor functions and memory which progresses to clinical disorders associated with cognitive motor disorder and dementia, which independently predict shortened survival (Navia *et al*., 1986; Price *et al*., 1988; Lipton, 1994; McGuire and Marder, 2000).

ADC, the syndrome of cognitive and motor dysfunction observed after HIV-1 infection is the most common cause of neurological dysfunction (Elder and Sever, 1988). The

delivery of the virus in the brain through infected macrophages, allows for long term persistence of HIV-1 in the CNS and its preferred localization in the subcortical and striatal circuitaries (Ho *et al*., 1985; Wiley *et al*., 1986).

1.1.2. Pathology and pathogenesis of ADC

HIV infection in the brain is characterized by widespread reactive astrocytosis, myelin pallor, and infiltration predominantly by monocytoid cells, including blood-derived macrophages, resident microglia and multinucleated giant cells. However, numbers of HIV-infected cells, multinucleated giant cells or viral antigen in CNS tissue do not correlate well with measures of cognitive function (Glass *et al*., 1995; Masliah *et al*., 1997; Kaul *et al*., 2001). The pathological features closely associated with the clinical signs of ADC include increased numbers of microglia (Glass *et al*., 1995), evidence of excitotoxins (Heyes *et al*., 1991; Giulian *et al*., 1996), decreased synaptic and dendritic density (Masliah *et al*., 1997; Everall *et al*., 1999) and selective neuronal loss (Masliah *et al*., 1992; Fox *et al*., 1997).

1.1.2.1. Viral Proteins

Extracellular protein shed from virions and infected cells, particularly the envelope glycoprotein, gp120 has profound effects on neuronal and astrocytic cell function with resultant neurotoxicity around infected macrophages producing viral antigens (Brenneman *et al*., 1988; Pulliam *et al*., 1993; Dawson and Dawson, 1994; Toggas *et al*., 1994). gp120 exerts potent toxic effects on hippocampal neurons, which also become depleted in the brains of ADC patients (Masliah *et al*., 1992). The neurotoxicity operates in a nitric oxide (NO) dependent manner that requires calcium (Ca^{2+}) and glutamate, the primary excitatory amino acid in the brain (Dawson and Dawson, 1994). This occurs through activation of voltage sensitive Ca^{2+} channels and glutamate sensitive N-methyl-D-aspartate (NMDA) channels leading to unregulated Ca^{2+} influx and neuronal dysfunction via an excitotoxic mechanism (Dawson and Dawson, 1994; Lipton, 1994). In addition, cell damaging superoxide anions ($O_2^{\bullet -}$) also induced by gp120, play a role in mediating neurotoxicity (Dawson and Dawson, 1994).

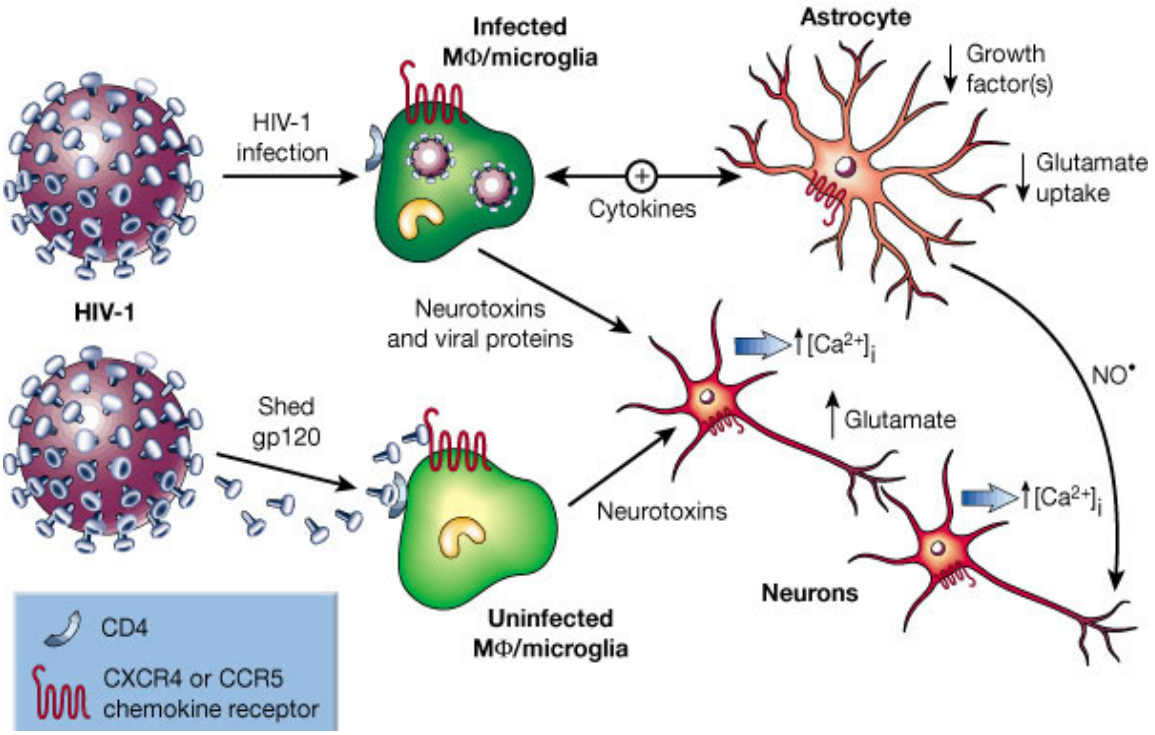

Figure 1.1: Current model of HIV-related neuronal damage involving cell-cell signalling (Kaul *et al*., 2001).

1.1.2.2. HIV-1 infected and activated macrophages

HIV-1-infected macrophages migrate into the brain and constitute the principal route of viral entry into the CNS (Gartner, 2000). Immune-stimulated macrophages/microglia directly damage neurons by releasing excitotoxic substances including eicosanoids, platelet activating factor (PAF), NO and QA (Achim *et al*., 1993; Nottet and Gendelman, 1995; Lipton *et al*., 1994), which engenders excessive Ca^{2+} influx and free radical (NO and $O_2^{\bullet -}$) formation through excessive activation of NMDA receptors. In addition, indirect neurotoxicity is mediated by macrophage- and microglial-derived inflammatory cytokines, such as interleukin (IL) and tumour necrosis factor-alpha (TNF-α), free radicals and viral proteins (Dreyer *et al*., 1990; Giulian *et al*., 1993; Lipton and Gendelman, 1995).

The levels of these potential neurotoxins are elevated in the cerebrospinal fluid (CSF) or brain tissue of patients with the neurologic disorder (Achim *et al*., 1993; Griffin *et al*., 1994; Sei *et al*., 1995). Of the cytokines, TNF-α mediates neurotoxicity by activation of neuronal α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) subtype of glutamate receptor channels (Gelbard *et al*., 1993). Furthermore as shown in Figure 1.1., cytokines inhibit astrocytic glutamate reuptake, compounding the processes leading to Ca^{2+} influx and neuronal damage via voltage dependent Ca^{2+} channels and NMDA operated receptor channels (Fine *et al*., 1996).

1.2. NEURODEGENERATION

1.2.1. Mechanisms of neurodegeneration

1.2.1.1. Introduction

Neuronal cell death can be initiated by three inter-related mechanisms, namely free radicals and oxidative stress, excitotoxicity and mitochondrial dysfunction (Alexi *et al*., 1998; Beal, 2000), resulting in neurodegenerative disorders, which are morphologically characterised by progressive cell loss in specific neuronal populations (Jellinger, 2001). Two types of cell death have been discussed in neurodegeneration: necrosis and apoptosis. Necrosis is a passive pathological process, arising from spontaneous insults such as trauma and stroke and ultimately leads to an inflammatory response (Ankarcrona *et al*., 1995; Clarke, 1999; Levin *et al*., 1999).

Whilst apoptosis, involves gene-directed programmed cell death which is triggered by oxidative stress, toxins and viruses (Wyllie *et al*., 1980; Majno and Joris, 1995; Reed, 2000 and Yuan and Yankner, 2000).

1.2.1.2. Free radicals and Oxidative stress

The interest in the role of oxygen-free radicals generally known as reactive oxygen species (ROS) in experimental and clinical medicine has grown in the last two decades (Halliwell and Gutteridge, 1999). Free radicals can be defined as molecules or molecular fragments containing one or more unpaired electrons in atomic or molecular orbitals. These unpaired electrons confer a considerable degree of reactivity to the free radical (Halliwell and Gutteridge, 1999; Valko *et al*., 2004). In most circumstances, ROS are products of metal-catalyzed reactions, macrophage inflammatory response and mitochondria-catalyzed electron transport reactions (Cadenas, 1989). However, in biological systems levels of these radicals are controlled through deactivation of antioxidant defense systems (Alexi *et al*., 2000). Mediation of damage to cell structures including lipid membranes, proteins and nucleic acids by ROS results in oxidative stress due to an imbalance between free radical production and antioxidant defense mechanisms (Poli *et al*., 2004; Valko *et al*., 2004). Radical-related damage to DNA, proteins and lipids has been proposed to play a key role in the development of cancer (Johnson, 2004), arteriosclerosis (Witztum, 1994) and neurodegenerative disorders such as Parkinson's Disease (PD) (Alexi *et al*., 2000; Koutsilieri *et al*., 2002), Alzheimer's Disease (AD) (De la Monte *et al*., 2000; Ansari *et al*., 2006;), Herpes Simplex Encephalitis (HSE) (Valyi-Nagy and Dermody, 2005) as well as ADC (Lipton and Rosenberg, 1994; Mollace *et al*., 2001).

1.2.1.2.1. Superoxide Radical

The $O_2^{\bullet -}$ is generated through the reduction of molecular O_2 by the mitochondrial respiratory chain (Boveris and Cadenas, 1975), where electrons passing through the electron transport chain (ETC), directly "leak" from complexes I and III onto O_2, the ultimate electron acceptor (Halliwell and Gutteridge, 1990; Turrens, 1997). Intracellular accumulation of $O_2^{\bullet -}$ plays a key role in oxidative chain reactions through excitotoxicity and disturbed Ca^{2+} homeostasis, yielding highly toxic oxidants such as hydroxyl radicals ($HO^{\bullet}$) from H_2O_2 by making ferrous ion (Fe^{2+}) available for the Fenton reaction (Stohs and Bagchi, 1995; Liochev and Fridovich, 2001). Accumulations of such toxic free radicals, increases the susceptibility of brain tissues to oxidative damage leading to either direct injury via membranous lipid peroxidation, protein and DNA oxidation or indirect

damage via inflammation and apoptosis (Traystman *et al*., 1991; Kuroda and SiesjÖ, 1997; Chan, 2001).

1.2.1.2.2. Hydroxyl Radical

The $HO^{\bullet}$ is highly reactive and is known to react close to its site of formation *in vivo* (Valko *et al*., 2006). As mentioned above, the majority of $HO^{\bullet}$ generated come from metal-catalyzed breakdown of H_2O_2. According to the Fenton reaction, $O_2^{\bullet -}$ participates in the Haber-Weiss reaction to form the reactive $HO^{\bullet}$ (Liochev and Fridovich, 2002). The formation of this radical can be accounted for when the Haber-Weiss reaction is catalyzed by traces of transition metal ions, such as Fe^{2+} and ferric ion (Fe^{3+}) in the Fenton reaction (Halliwell and Gutteridge, 1986; Liochev and Fridovich, 2002), as shown in equations 1.1, 1.2, 1.3.

$$Fe^{3+} + O_2^{\bullet -} \longrightarrow Fe^{2+} + O_2 \qquad \text{equation 1.1.}$$

$$Fe^{2+} + H_2O_2 \longrightarrow HO^{\bullet} + OH^{-} + Fe^{3+} \qquad \text{equation 1.2.}$$

Fenton Reaction

$$O_2^{\bullet -} + H_2O_2 \xrightarrow{\text{metal catalyst}} O_2 + HO^{\bullet} + OH^{-} \qquad \text{equation 1.3.}$$

Haber-Weiss Reaction

1.2.1.2.3. Peroxyl Radical ($ROO^{\bullet}$)

The peroxyl radical is typical of additional radicals that are derived from O_2 and can be formed in living systems. The simplest $ROO^{\bullet}$ is the dioxyl (hydroperoxyl) radical $HOO^{\bullet}$, the conjugate acid of $O_2^{\bullet-}$. $HOO^{\bullet}$ formation is as a result of the reaction between $O_2^{\bullet-}$ with any substrate containing acidic protons (Collins *et al*., 2001). Perhaps the most interesting feature of $HOO^{\bullet}$ is the diversity of those biological reactions in which they participate, including DNA cleavage, protein backbone and lipid modification (Halliwell and Gutteridge, 1989) and the synergistic enhancement of $O_2^{\bullet-}$ enhanced DNA damage (Valko *et al*., 2006). The intermittent citation of detection and measurement of lipid peroxidation has been used as evidence to support the involvement of $ROO^{\bullet}$ reactions in toxicology and human diseases (Gutteridge, 1995; Cadenas and Sies, 1998).

1.2.1.2.4. Nitric Oxide (NO)

Due to its extraordinary properties, NO acts as an important oxidative biological signalling molecule in diverse physiological processes, including neuronal transmission, synaptic plasticity in the CNS as well as regulation of the immune system (Archer, 1993; Bergendi *et al*., 1999; Alderton *et al*., 2001). Inflammatory processes cause oxidative burst resulting in the release of both $O_2^{\bullet-}$ and NO by cells of the immune system. Therefore it is under such conditions, that the two react to produce a much more oxidatively active molecule, peroxynitrite anion ($ONOO^-$) which may promote apoptosis (Radi *et al*., 1991; Van der Vliet et *a*l., 1994; Carr *et al*., 2000).

1.2.1.2.5. Peroxynitrite Anion

The wide spectrum of toxicity in biological systems associated with the highly oxidizing and short lived $ONOO^-$ involves, DNA fragmentation, lipid peroxidation, protein oxidation and nitration, inhibition of mitochondrial respiration and a reduction in cellular antioxidant defenses (Misko *et al*., 1998; Carr *et al*., 2000; Szabó, 2003; Viràg *et al*., 2003). Such events lead to apoptotic cell death at lower concentrations and necrosis at higher concentrations of $ONOO^-$ (Viràg *et al*., 2003).

1.2.1.3. Mitochondrial dysfunction

Excitotoxicity (section 1.2.1.4.) and respiratory poisons such as cyanide (section 1.2.4.) lead to mitochondrial dysfunction, which in turn may lead to depression of oxidative phosphorylation, the consequences of which are leakage of electrons and a reduction in ATP production (Zhang *et al*., 1990; Alexi *et al*., 2000). In the presence of O_2, these electrons are involved in the formation of free radicals including $O_2^{\bullet-}$ which ultimately leads to oxidative stress (Beal, 2000). Oxidative stress is also associated with the opening of pores in the inner mitochondrial membrane which causes changes in ion homeostasis, breakdown of mitochondrial membrane potential and ultimately necrotic cell death (Hengartner, 1998; Beal, 2000).

The brain uses the most energy in the human body, accounting for 20 % of the total oxygen consumption despite accounting for only for 2 % of the total body mass (Papa, 1996). Neurons, like muscle cells and especially cardiac muscle cells, are particularly vulnerable to the effects of mitochondrial damage due to their high dependence on energy (Thyagarajan and Byrne, 2002; Schon and Manfredi, 2003). Consequently, many well-recognized mitochondrial diseases have CNS consequences (Thyagarajan and Byrne, 2002), including Parkinson's disease (PD), Alzheimer's disease (AD), Huntington's disease (HD) (Beal, 2000; Orth and Shapira, 2001; Moro *et al*., 2002; Van Houten *et al*., 2006) and ADC (Akaike *et al*., 1990; Schwarz, 1996). They all share the common features of mitochondrial Ca^{2+} and ATP or ROS metabolism disturbances (Brookes *et al*., 2004).

1.2.1.4. Excitotoxicity

Glutamate-mediated excitotoxicity is a mechanism of neuronal cell death mediated by overstimulation of glutamate receptor sub-types, such as NMDA and AMPA (Griffiths *et al*., 2000). Excessive NMDA receptor stimulation allows for an influx of sodium (Na^+) and Ca^{2+} ions (Cotman *et al*., 1989). It is this disturbance in ion homeostasis that induces several detrimental intracellular signals such as mitochondrial Ca^{2+} overload and release of cytochrome *c* (Cyt *c*), free radical generation (NO, $O_2^{\bullet-}$, $HO^{\bullet}$) (Choi 1992; Dykens 1994; White and Reynolds 1996), lipid peroxidation and chromatin condensation

(Bonfoco *et al*., 1995; Tenneti, 1998; Budd *et al*., 2000; Ghatan *et al*., 2000) that contribute to neuronal death by apoptosis or necrosis.

As shown in Figure 1.2., there exist two separate excitotoxic mechanisms:

(1) The influx of Na^+ is accompanied by the influx of chloride ions, creating an osmotic imbalance between the neuron and extracellular environment. As a result water flows into cells, causing swelling, damage and lysis, which constitute necrosis (Olney, 1969; Rothman and Olney, 1987; Choi, 1987, Tilson and Mundy, 1995).

(2) The massive Ca^{2+} ion influx triggers activation of intracellular calcium-dependent enzymes including lipases, proteases and nucleases, causing a delayed toxicity that accounts for most cell deaths due to the hyperstimulation of glutamatergic system (Cotman *et al*., 1989; Choi, 1992;).

Increases in intracellular Ca^{2+} may cause mitochondrial dysfunction (section 1.2.1.3) by depression of oxidative phosphorylation and activation of nitric oxide synthase (NOS), which catalyzes production of the toxic NO (Zhang *et al*., 1990; Kiedrowski *et al*., 1992). The consequences of which are discussed in section 1.2.1.2.4.

Activation of a lipase type of enzyme, phospholipase 2 (PLA_2) by Ca^{2+} ions results in the release of arachidonic acid and polyunsaturated fatty acids (PUFAs) from membranes. These are further metabolized by cyclooxygenase or lipoxygenases, further causing free radical generation (Traystman *et al*., 1991). The free radicals in turn promote elevations in Ca^{2+} ions concentrations, precipitating a vicious cycle, which enhances further cell damage (Mattson and Mark, 1996). In addition the released arachidonic acid inhibits glutamate uptake into neurons and glial cells thus prolonging the excitotoxic action of this amino acid on its receptors (Volterra *et al*., 1992). The metabolic and neurochemical perturbations arising as a result of excitotoxicity occur in many neurodegenerative disorders including depression (Hayley *et al*., 2005; Yao and Reddy, 2005) and AD (Olney *et al*., 1995; Hynd *et al*., 2004).

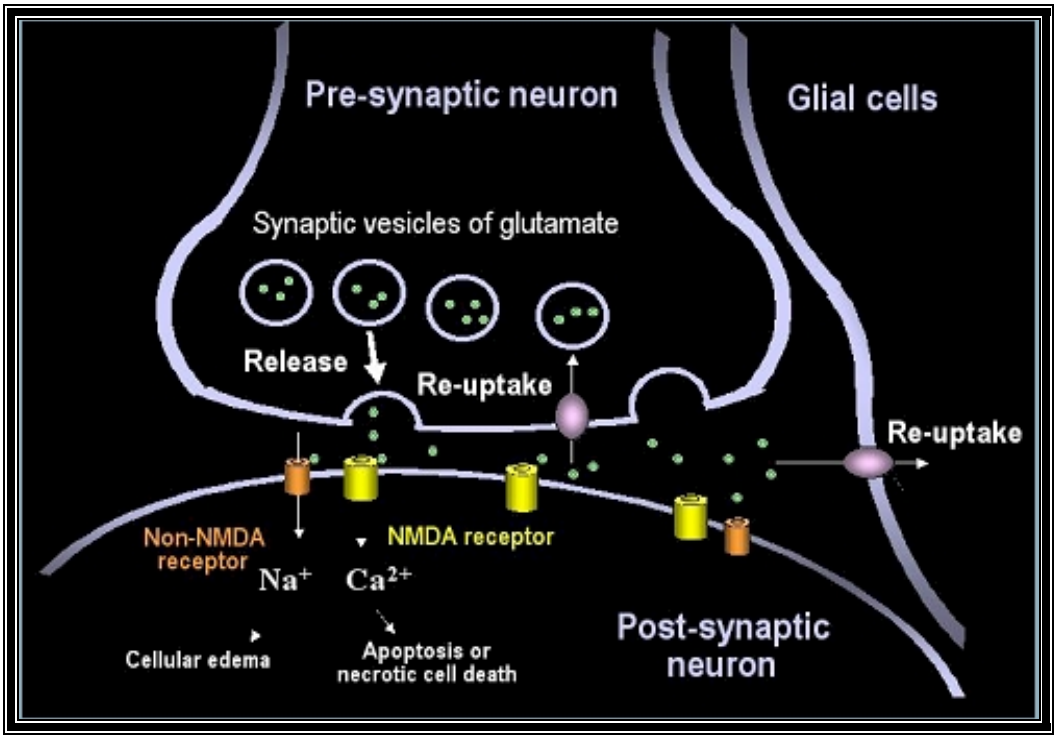

Figure 1.2. A modified diagram showing mechanisms of excitotoxicity. Stimulation of glutamate receptors and cellular pathways ultimately leads to excitotoxicity by increasing oxidative stress and ultimately apoptotic cell death (Epstein and Gendelman, 1993).

1.2.2. Oxidation of biological molecules

1.2.2.1. Introduction

In living organisms, various ROS can form by different ways. Normal aerobic respiration stimulates polymorphonuclear leukocytes and macrophages, and peroxisomes appear to be the main endogenous sources of most of the oxidants produced by cells (Halliwell and Gutteridge, 1989; Davies 1994; Robinson *et al*., 1997). ROS induce some oxidative damage to biomolecules which may lead to ageing, cancer, and other diseases (Kehrer, 1993; Aruoma, 1994). As a result of this, ROS have been implicated in more than 100 diseases, including malaria, AIDS, heart disease, stroke, arteriosclerosis, diabetes, and

cancer (Tanizawa *et al*., 1992; Hertog *et al*., 1993; Duh, 1998; Alho and Leinonen, 1999; Yildirim *et al*., 2000).

1.2.2.2. Oxidation of proteins

Radical-mediated damage to proteins may be initiated by electron leakage, metal-ion-dependent reactions and autoxidation of lipids (Kim *et al*., 1985; Rivet *et al*., 1985; Davies, 1987; Stadtman, 2004). The consequent protein oxidation is O_2- dependent, and involves several propagating radicals, notably alkoxyl radicals (Barron *et al*., 1955; Davies *et al*., 1987; Neuzil and Stocker, 1993; Stadtman, 1997), to produce protein hydroperoxides which further react to form protein carbonyls (Stadtman, 1992; Butterfield and Stadtman, 1997).

Oxidative modification of proteins can lead to diminished specific protein functions, which may ultimately result in cell death (Hensley *et al*., 1995; Butterfield *et al*., 1997; Dean *et al*., 1997). Cells can detoxify some of the reactive species, e.g. by reducing protein hydroperoxides to unreactive hydroxides. However, certain oxidized proteins gradually accumulate with time, and together with possible alterations in the rate of production of oxidized proteins, may contribute to the observed accumulation and damaging actions of oxidized proteins during aging and in pathologies such as diabetes, atherosclerosis and neurodegenerative diseases including AD to produce protein hydroperoxides which further react to form protein carbonyls (Stadtman, 1992; Butterfield *et al*., 1997).

1.2.2.3. Oxidation of nucleic acids

Free radicals can damage DNA through a number of mechanisms including direct alteration of base pairs resulting in miscoding and some pre-mutogenic changes (Olinski *et al*., 2002). Miscoding can result in a decrease in critical proteins within neurons (Jaruga *et al*., 1999). Modification of nucleic acids by ROS can induce chromosomal aberrations with high efficiency (Cerutti, 1985), suggesting that chromosomal damage exhibited in neurons of patients with PD, might be related to abnormally high oxidative stress (Mosley *et al*., 2006). ROS including $HO^{\bullet}$ are known to react with all components

of the DNA molecule, damaging both purine and pyrimidine bases (Figure 1.3.), as well as the deoxyribose backbone (Dizdaroglu *et al*., 2002), causing permanent modification of genetic material, which represents the first steps involved in mutagenesis, carcinogenesis and ageing (Mannet, 2000; Cooke *et* al., 2003). Among the most promising biomarkers of oxidative damage to nucleic acids is nucleoside 8-hydroxyguanine (8-OH-G), the oxidized base produced by free radical attack on DNA by C8-hydroxylation of guanine as shown in Figure 1.3. This is one of the most frequent nucleic acid modifications observed under conditions of oxidative stress (Loft and Polsen., 1996).

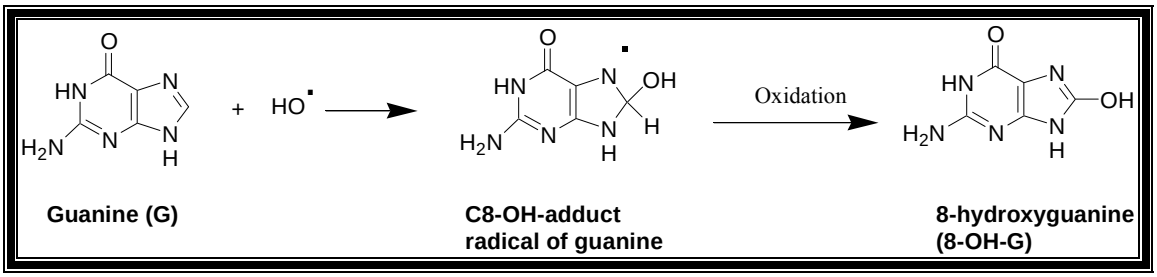

Figure 1.3. A modified reaction of guanine with hydroxyl radical to form a guanine radical which in turn is oxidized to a hydroxyguanine (Shigenaga *et al*., 1989).

This reaction with $HO^{\bullet}$ proceeds via addition to double bonds of the DNA bases, such as guanine to form a guanine radical, as shown in Figure 1.3., which then undergoes oxidation to form a hydroxyguanine, both a mutagenic and carcinogenic species (Shigenaga *et al*., 1989). The oxidized DNA base product formed is often used as a good biomarker for free radical-mediated DNA damage and oxidative stress in organisms (Halliwell and Gutteridge, 1989; Helbock *et al*., 1999). Since guanine bases are particularly sensitive to oxidation, the oxidized DNA bases always exist at some basal level, although cells have numerous repair systems to remove such species (Lindahl and Wood, 1999). In the event that these species occur at critical sites which are not quickly repaired, functional problems can occur (Klaunig *et al*., 1998).

1.2.2.4. Lipid Peroxidation

Neuronal membranes, being the 'site of action' for many essential brain functions have an abundant supply of PUFAs, which makes them highly vulnerable to lipid peroxidation (LP) (Halliwell and Gutteridge, 1989; Calabrese *et al*., 2000). LP is a mode of oxidative injury triggered and promoted by different radical and nonradical ROS or by the catalytic decomposition of preformed lipid hydroperoxides in tissues by several agents including most transition metals and microsomal cytochromes (Slater, 1984; Halliwell and Gutteridge, 1989; Niki *et al*., 2005). The peroxidative injury not only causes structural and functional derangement of phospholipid bilayer of membranes but also produces several deleterious aldehydic end products including malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE), which may inflict secondary damage to proteins and DNA (Halliwell and Gutteridge, 1989; Janero, 1990; Uchida, 2003; Luo and Shi, 2005).

Schematic diagram of the sequence of events in LP

- Cell damage → $HO^{\bullet}$ + CH_2 → $C^{\bullet}H$ →conjugated diene + O_2 → $CHO_2^{\bullet}$ + CH_2→ $C^{\bullet}H$ + lipid peroxide
- Fe^{2+}-complex + lipid peroxide → $CHO^{\bullet}$
- Fe^{3+}-complex + lipid peroxide → $CHO^{\bullet}_2$ + H^{+} + Fe^{2+}-complex

Hydrogen (H) abstraction from a methylene group (-CH_2-) in the side chain of an unsaturated fatty acid by free radicals provides the first step of a peroxidation sequence in membranes. The removal of the H atom leaves behind an unpaired electron on the carbon (-$C^{\bullet}H$-) to which it was originally attached. The presence of a double bond in the fatty acid weakens the C-H bonds on the carbon atom adjacent to the double bond, facilitating the removal of H (Halliwell and Gutteridge, 1989). The resulting carbon-centred radical stabilizes by molecular rearrangement to form a conjugated diene (Figure 1.4.), which reacts with O_2 (which is hydrophobic and concentrates in to the interior of

membranes) to form $CHOO^{\bullet}$. These radicals are capable of abstracting H from adjacent fatty acid side chains to form lipid hydroperoxides (**C** in Figure 1.4.), which marks the propagation step of LP. When combined, the carbon-centered radical (**A** Figure 1.4.), undergoes minor reactions including cross linking of fatty acid side chains and attack of protein membranes. The $CHOO^{\bullet}$ (**B** in Figure 1.4.), may also attack protein membranes and form singlet oxygen ($^1O_2$), a highly reactive species capable of damaging macromolecules within cells, when combined with each other (Halliwell and Gutteridge, 1989).

Extensive LP in biological membranes results in the following: (1) alterations in fluidity and increase in permeability to H^+ and other ions. (2) Inactivation of membrane-bound receptors and enzymes (Gutteridge and Halliwell, 1990). The eventual alterations in membrane fluidity and membrane potential, permits leakage of ions such as Ca^{2+} into cells, causing cells to rupture and release its contents including lysosomal hydrolytic enzymes (Halliwell, 1994).

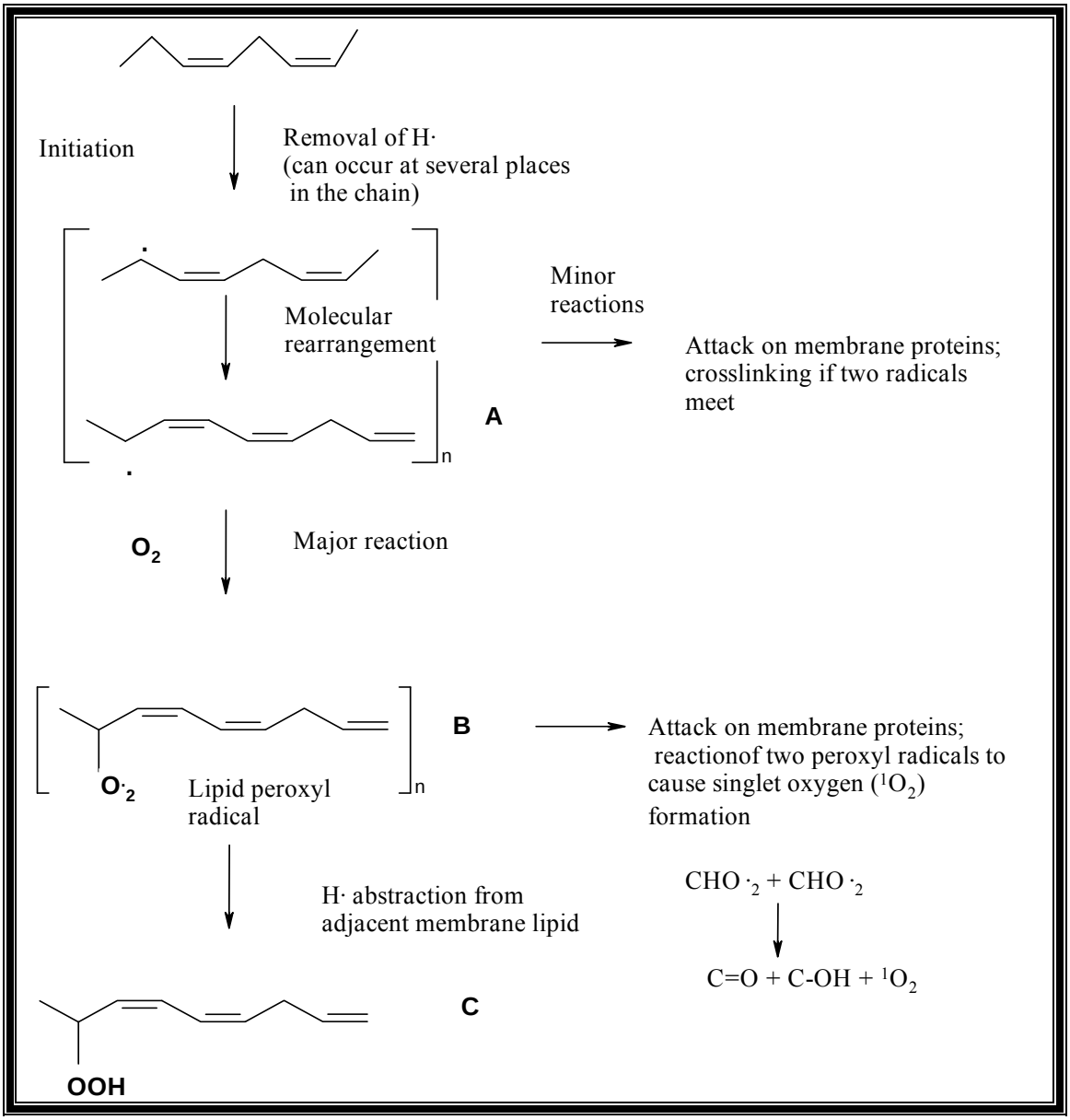

Figure 1.4. An outline of the mechanism of LP (Gutteridge and Halliwell, 1990).

1.2.3. Metal ions

The ions of transition metals including copper (Cu) and iron (Fe) are involved in many free radical reactions leading to generation of ROS (Halliwell and Gutteridge, 1989) as shown in Figure 1.5. In case of Fe, the Fe^{2+} accelerates lipid oxidation by breaking down

hydrogen and lipid peroxides to reactive free radicals via the Fenton Reaction, shown in section 1.2.1.3.3., equation 1.1 and 1.2 (Halliwell and Gutteridge, 1984; Miller, 1996).

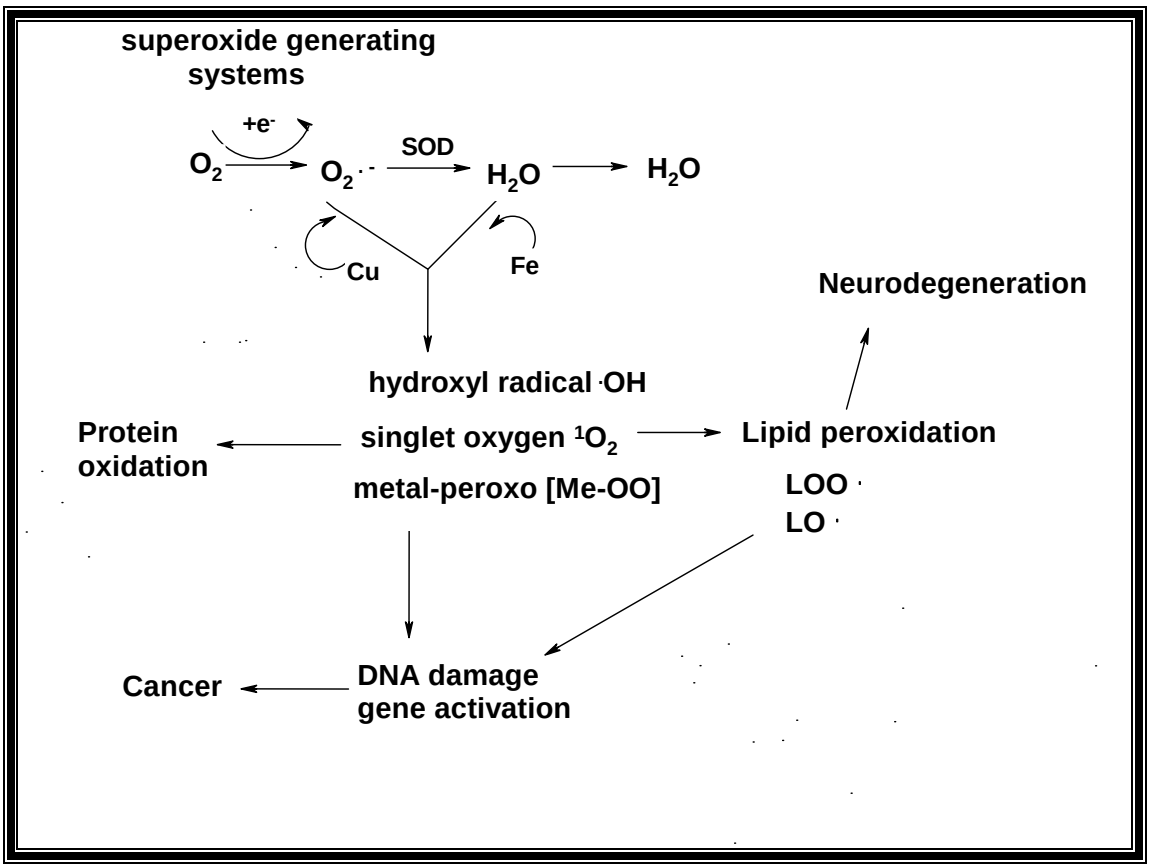

Figure 1.5. A modified pathway of metal-induced oxidative stress (Valko *et al*., 2005).

The reduction of Fe^{3+} by $O_2^{\bullet -}$ augments the Fenton reaction, thus regenerating Fe^{2+} and producing $HO^{\bullet}$ as in the iron-catalyzed Haber-Weiss type of reaction (section 1.2.1.3.3., equation 1.3.).

As shown in Figure 1.5., Fe dependent formation of $HO^{\bullet}$ is detrimental to cells, since this radical can react at diffusion-limited rates with various biomolecules, including lipids, proteins and DNA (Valko *et al*., 2005). Oxidative damage to these biological molecules

consequently results in various disorders including cancer and neurodegeneration. In addition, cells try to prevent its formation and remove H_2O_2 to inactive sites (Fahn and Cohen, 1992). Notably the $HO^{\bullet}$ has a short half life but however H_2O_2 crosses the blood-brain barrier where it is converted to $HO^{\bullet}$ in brain tissue, causing detrimental damages to neurons.

Certain pathological states of the CNS such as ADC are associated with increased Fe accumulation (Savarino *et al*., 1999), thus enhancing the generation of ROS, LP and neurodegeneration. The role of Fe in neurodegeneration has been further discussed in chapter 5, section 5.2.

1.2.4. Cyanide

Cyanide is one of the most potent respiratory poisons not only known to man but in all aerobic forms of life (Yen *et al*., 1995). The nerve cells of the respiratory centre are prone to damage by acute doses of cyanide because of enhanced susceptibility to hypoxia (Greer and Jo, 1995). It exerts its toxicity through histotoxic hypoxia and mitochondrial dysfunction (section 1.2.1.3.) (Bhattacharya and Lakshmana Rao, 2001) causing alterations in ionic homeostasis and elevated Ca^{2+} levels. The elevation of Ca^{2+} in the brain (Johnson *et al*., 1987) and cytosol often leads to increased oxidative stress and excitotoxicity. In addition, Ca^{2+} activates phospholipases and proteases causing surface blebbing and cytoarchitectural defects of neuronal cells (Nicotera *et al*., 1989).

1.2.5. Quinolinic acid

1.2.5.1. Introduction

QA is a neuroactive metabolite of the tryptophan-kynurenine pathway that is normally present in nanomolar concentrations in human brain and cerebrospinal fluid (CSF) and is often implicated in the pathogenesis of a variety of human neurological diseases (Chao *et al*., 1996; Lipton, 1998). Substantial increases in QA have been found in the brain and CSF of patients with inflammatory neurological disorders because it is produced by

immune activated macrophages and microglia (Heyes *et al*., 1998; Huengsberg *et al*., 1998).

1.2.5.2. Biosynthesis of quinolinic acid

1.2.5.2.1. Introduction

Tryptophan (TRP) is an essential amino acid having various important biological functions. In mammals, about 90 % of dietary TRP is metabolized along the kynurenine pathway (KP) (Musajo and Benassi, 1964; Price *et al*., 1965; Wolf, 1974), which represents the major catabolic route of the essential amino acid TRP over multiple metabolic steps (Figure 1.6.).

Among the metabolites, some are neurotoxic while others can be neuroprotective. The neurotoxic mechanism of 3-hydroxykynurenine (3-HK) and 3-hydroxyanthranilic acid involves the generation of $O_2^{\bullet -}$ and H_2O_2, which contribute to oxidative processes that are implicated in the production of reactive free radicals (Eastman *et al*., 1992; Goldstein *et al*., 2000; Vazquez *et al*., 2000) and potentiation of QA- induced neurotoxicity (Guidetti and Schwarcz, 1999). In contrast, kynurenic acid (KYNA) is an antagonist of the excitotoxic NMDA receptors which preferentially prevents QA-induced neurodegeneration (Foster *et al*., 1984). Hence KP is mostly implicated in the pathophysiology of various diseases associated with inflammation leading to brain injury, such as multiple sclerosis, ADC and cerebral malaria (Stone, 2001; Stone *et al*., 2003; Nemeth *et al*., 2005).

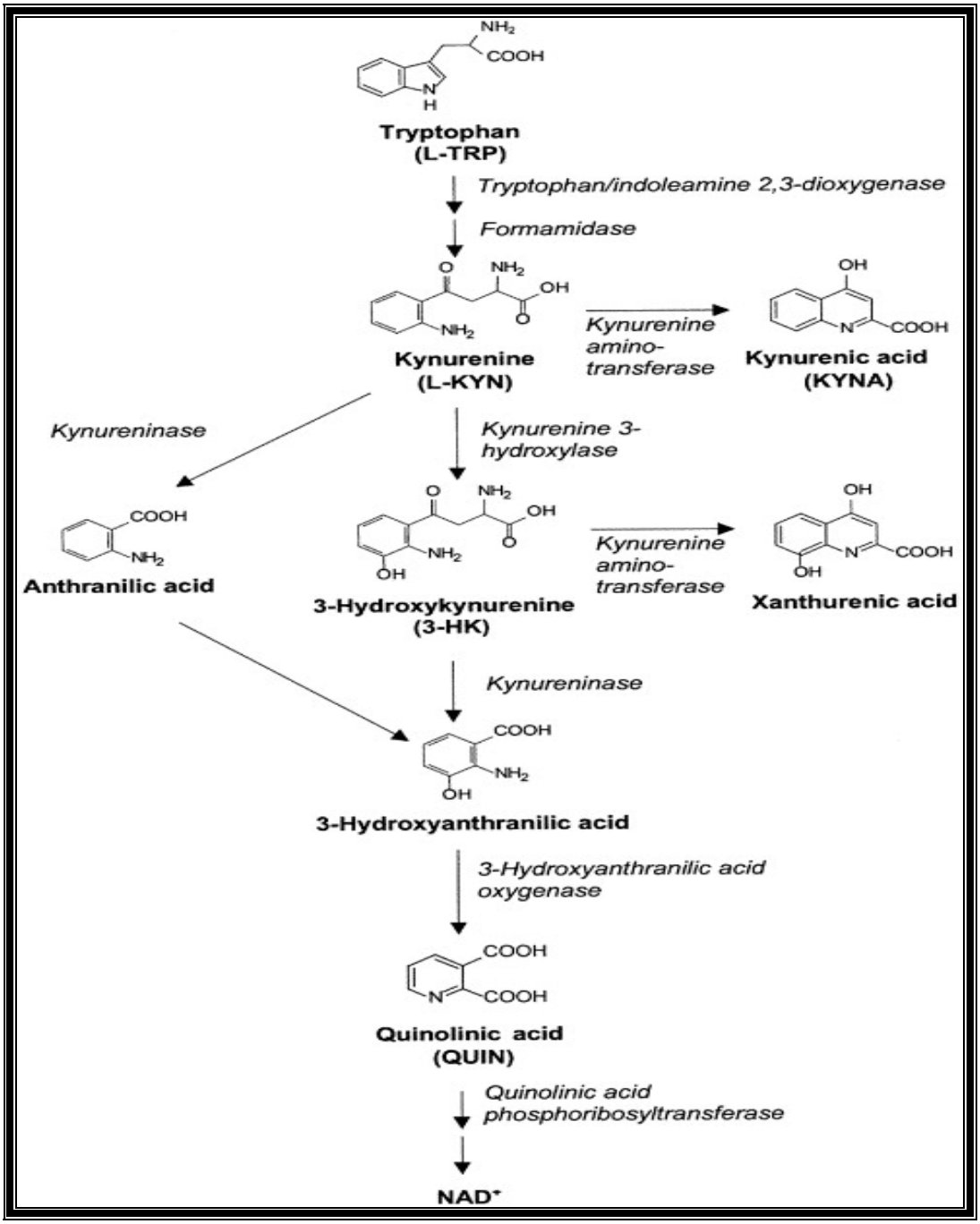

Figure 1.6. Graphical representation of the KP. Tryptophan (2, 3)-dioxygenase (TDO), indoleamine (2, 3)-dioxygenase (IDO) catalyze the formation of kynurenine. Formamidase, Kynurenine-3-hydroxylase, Kynureninase and 3-Hydroxyanthranilic acid oxygenase (3-HAO) regulate the QA/Kynurenic Acid balance (Sas *et al*., 2007).

1.2.5.2.2. Enzymes regulating the Kynurenine Pathway

1.2.5.2.2.1. Tryptophan-2-3-dioxygenase

Tryptophan-2-3-dioxygenase (TDO) is a haeme-containing dioxygenase, present in the liver of mammals, including humans and rats. This enzyme is involved in catalyzing the addition of molecular O_2 across the 2, 3-double bond of the indole ring of TRP, leading to the cleavage of the indole ring to form *N*-formylkynurenine (Tanaka and Knox, 1959; Hayaishi and Nozaki, 1969; Leeds *et al*., 1993; Dick *et al*., 2001). There is a considerable proportion of the enzyme that is present as the apoenzyme form, and is therefore inactive until the additional haeme is made available, either by the administration of haeme precursors *in vivo* or by the addition of haematin during incubation *in vitro* (Bender, 1982).

1.2.5.2.2.1.1. TDO regulation

1.2.5.2.2.1.2. TDO induction

The activity of TDO determines the relative TRP flux into the serotogenic and KP. Hepatic TDO is specific for TRP as the substrate (Hayaishi, 1980) and is subject to hormonal as well as substrate induction (Knox and Auerback, 1955). Its activity is enhanced by TRP concentration (Satyanarayana and Rao, 1980; Smith *et al.*, 1980; Saito *et al.*, 1990), high cortisol (Salter and Pogson, 1985) and haeme or its precursor 5-aminolaevulinate (5-ALA) (Badawy *et al*., 1987).

Haeme is required for activity and reversible binding of the inactive apoenzyme (Figure 1.7.) to form the oxidized and inactive holoenzyme (Knox and Piras, 1966). TRP then binds to the inactive holoenzyme reducing it to the fully active form. The binding of L-TRP to the enzyme induces a fundamental change in the ligand binding affinity of the catalyst in addition to increasing the reactivity of the haeme iron towards the substrate (Makino *et al*., 1980). The sequential events in enzymatic catalysis due to substrate induction include; (1) Enzyme saturation by activator haeme, and (2) Increased levels of total enzyme concentrations (Frieden *et al*., 1961).

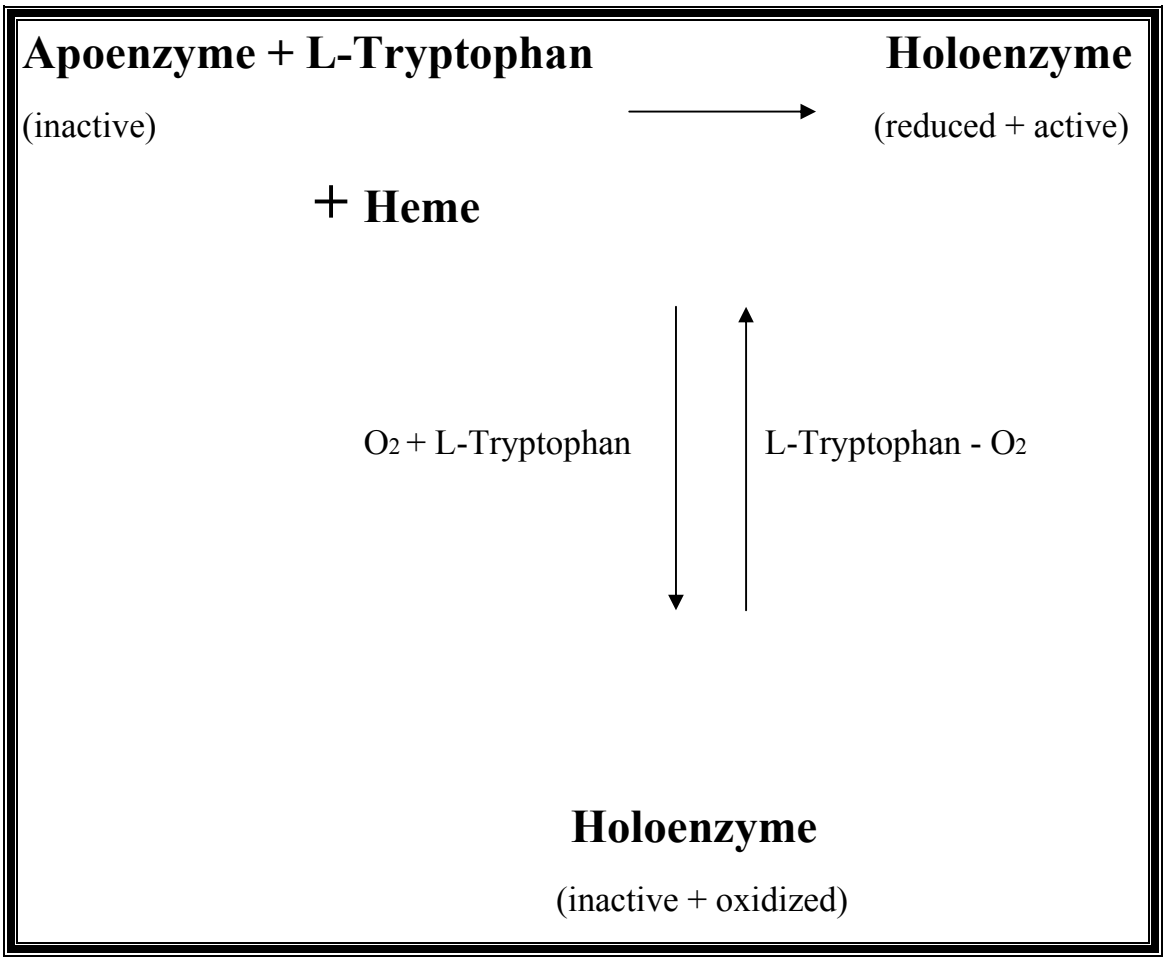

Figure 1.7. The activation of TDO (modified from Walsh, 1996).

1.2.5.2.2.1.3. TDO inhibition

The administration of novel indoles with inhibitory effects on TDO and serotonin (5-HT) reuptake (Madge *et al*., 1996), ultimately lead to increased concentrations of L-TRP and 5-HT in CSF, which could be vitally important for antidepressant therapy (Hardeland and Rensing, 1968; Walsh and Daya, 1998). In addition Walsh and Daya (1997) demonstrated that melatonin (aMT) and 5-HT can differentially regulate TDO activity, with the former and latter exhibiting competitive and allosteric inhibition.

1.2.5.2.2.2. Indoleamine-2-3-dioxygenase

Indoleamine-2-3-dioxygenase (IDO) is the second enzyme that catalyzes oxidative cleavage of the indole ring of TRP through incorporation of molecular O_2 or $O_2^{\bullet-}$ (Hirata and Hayaishi, 1971; Tanigushi *et al*., 1977). It is an interferon-induced protein (Yoshida *et al*., 1981), widely distributed in mammalian extrahepatic tissues including brain, lung, gastric and intestinal mucosa, kidney, heart and adrenal gland. Besides interferon, IDO is also induced by bacteria (Yoshida and Hayaishi, 1978; Urade *et al*., 1983), virus infections (Yoshida *et al*., 1979) and tumour cells (Yoshida *et al*., 1984).

IDO has a broader specificity than TDO (Figure 1.6.), as it catalyses oxidative cleavage of various indoleamines, including 5-hydroxytryptophan, tryptamine, 5-HT as well as aMT (Hirata and Hayaishi, 1972). The induction of IDO causes a marked increase in TRP catabolism in the body with the production of kynurenine and total depletion of TRP in the cells as shown in Figure 1.6 (Bertazzo *et al*., 2001).

1.2.5.2.2.3. Formamidase

The immediate product of TDO and IDO, formylkynurenine (Figure 1.6.) is rapidly hydrolyzed into kynurenine by the tissues formamidase. This enzyme has low substrate specificity and is able to release formate from a variety of aryl-formylkynurenines although its greatest activity is towards N-formylkynurenine (Bender, 1975; Stone, 1993).

1.2.5.2.2.4. Kynurenine-3-hydroxylase

Kynurenine-3-hydroxylase is a flavin adenine dinucleotide (FAD)-dependent monoxygenase which governs the conversion of L-KYN to 3-hydroxykynurenine (3-HK) (Schwarcz and Pellicciari, 2002). It is localized in the outer mitochondrial membrane and present in the brain at low activity (Antunes, 1998). In the presence of nicotinamide adenine dinucleotide hydrogen (NADPH) an electron donor, the prosthetic group FAD is reduced to dihydroflavin adenine dinucleotide ($FADH_2$) which is subsequently oxidized to FAD by molecular O_2 (Breton et *al*., 2000).

1.2.5.2.2.5. Kynureninase

Kynureninase is a cytosolic and pyridoxal phosphate-dependent enzyme that catalyses the hydrolysis of both L-KYN and 3-HK into anthranillic acid and 3-hydroxyanthranillic acid respectively as shown in Figure 1.6. (Braunstein *et al*., 1949; Schwarcz and Pellicciari, 2002). Inhibition of this enzyme results in an increase in urinary and plasma KYN and 3-HK with the resultant modification of the cerebral concentration of the metabolites and delayed QA metabolism (Stone, 1993).

1.2.5.2.2.6. 3-Hydroxyanthranilic acid oxygenase

3-Hydroxyanthranilic acid oxygenase (3-HAO) is an anabolic enzyme that is responsible for the synthesis of QA via an unstable intermediate 2- acroleylaminofumurate QA (Schwarcz, 1993). The enzyme is present in the mitochondrial membrane (Stone, 1993) and at excitatory synapses (Antunes, 1998), thus allowing QA to act on NMDA receptors (Schwarcz, 1993; Stone, 1993). Increase in QA production causes lesions, which in turn increase the activity of 3-HAO. Several excitatory amino acids, TRP and KYNA have no influence on the enzyme with regard to its activity (Stone, 1993).

1.2.5.2.3. The Effect of QA biosynthesis on Brain Indoleamine Metabolism

TRP is hydroxylated to 5-hydroxytryptophan by tryptophan hydroxylase (T5H), which is present in high concentrations in the pineal gland, and is the rate-limiting step in the synthesis of 5-HT (Lovenberg *et al*., 1968). Thus enhanced TRP catabolism by the TDO enzyme (Hayaishi, 1980) and IDO (Heyes *et al*., 1993; Mellor and Munn, 1999) during QA biosynthesis in the KP, reduces the amount of TRP available in the brain for conversion to 5-HT. A negative correlation exists between free plasma TRP levels and depression during acute TRP depletion (McDougle *et al*., 1993), where a lack of the amino acid has a pronounced effect on the depressive symptoms (Curzon and Bridges, 1970) in pathological conditions such as ADC. Hence the indoleamine hypothesis, asserts that modifications in the 5-HT neuronal function are a core feature of depression (van

Praag, 1982). Post-mortem studies have shown reduced levels of plasma TRP (Delgado *et al*., 1990), concentrations of 5-HT and its metabolite 5-hydroxyindole acetic acid (5-HIAA) (Gibbons and Davis, 1986) in brains of depressed or suicidal patients.

5-HT is an important regulator of various physiological and biochemical processes and it acts as a neurotransmitter at central and peripheral regions (Kema *et al*., 2000). As shown in Figure 1.8., it can be *N*-acetylated to form *N*-acetyl serotonin (NAS), in a reaction catalyzed by the enzyme *N*-acetyltransferase (NAT) or oxidized by monoamine oxidase (MAO) to 5-hydroxyindole acetaldehyde, which is further oxidized to 5-HIAA by acetaldehyde dehydrogenase (ADH) (Lerner and Case, 1960; Weissbach *et al*., 1960). However, a significant portion of the aldehyde may also be reduced to 5-hydroxytryptophol, when the redox potential of brain shifts to a more reducing state than normal (Bender, 1983).

All 5-hydroxyindoles namely, 5-hydroxytryptophan, 5-hydroxytryptamine, 5-HIAA, 5-hydroxytryptophol and NAS can be methylated by hydroxyindole-O-methyl transferase (HIOMT) (Axelrod and Weissbach, 1960; Axelrod and Weissbach, 1961) to form 5-methoxytrptophan, 5-methoxytryptamine, 5-methoxyindole acetic acid, 5-methoxytryptophol and aMT (Klein and Notides, 1969).

In the last several years, aMT, the chief secretory product of the pineal gland (Reiter, 1991), has been found to be both a direct free radical scavenger and a potent antioxidant (Tan *et al*., 1993; Hardeland *et a*l., 1995; Reiter *et al*., 1997), in addition to its function as a neurohormone. It has been demonstrated to reduce oxidative damage in the CNS (Reiter, 1998). Hence patients with a reduction in aMT secretion are more prone to depression and susceptible to neurodegeneration.

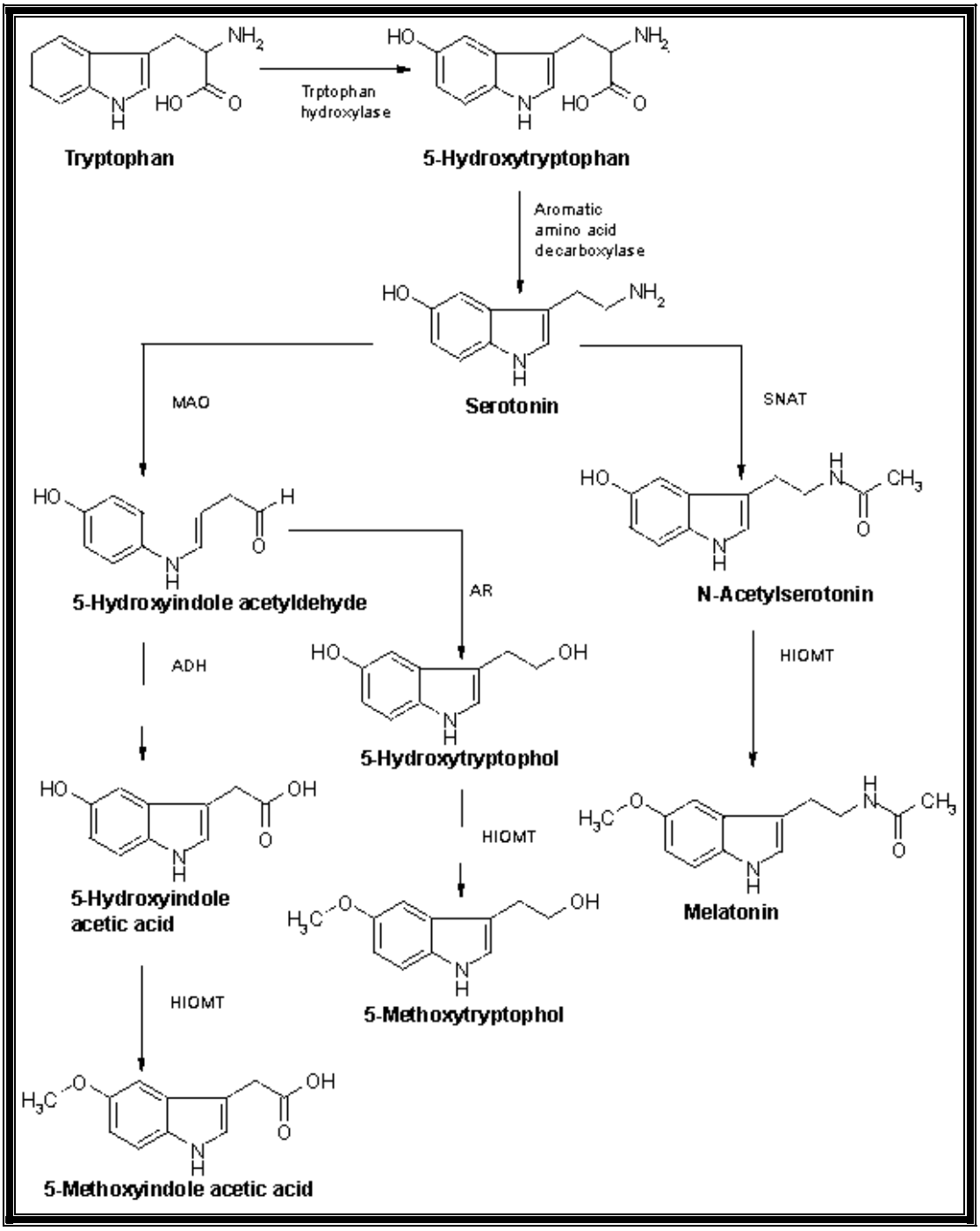

Figure 1.8. Schematic representation of pineal indole metabolism (modified from Young and Silman, 1982).

1.2.5.3. The Neurotoxicity of QA

QA is an established agonist at receptors for the glutamate analogue NMDA that has become a widely used tool for the study of neuronal damage resulting from activation of these receptors (Stone and Perkins, 1981; Perkins and Stone, 1983; Stone, 1993; Behan *et al*., 1999). Excessive activation of glutamate receptors in mammalian brain represents a cytotoxic mechanism that is potentially involved in neurodegenerative processes (Coyle and Puttfarcken, 1993). Since QA is not readily metabolized in the synaptic cleft, it stimulates NMDA receptors for prolonged periods resulting in Ca^{2+} influx into neurons entraining a destructive sequence of events within cells which enhances generation of ROS and often molecular damage and cell death (Choi, 1987; Hartley *et al*., 1993; Atlante *et al*., 1997; Perez Velazquez *et al*., 1997). Studies by Stipêk *et al*, (1997) showed that QA was able to modulate LP through its interaction with Fe^{2+} to form QA- Fe^{2+} complexes that mediate generation of ROS which are implicated in alterations of profiles of some endogenous antioxidants (Rodríguez- Martinez *et al*., 2000).

1.2.5.3.1. Impairment of Learning and Memory

1.2.5.3.1.1. Introduction

Memory formation and excitotoxic cell death in the hippocampus involves excitatory glutamatergic neurons, especially of the NMDA type (Haberny *et. al*., 2002; Silva, 2003), which has three basic features including high permeability to Ca^{2+} ions, voltage dependent blockage by magnesium (Mg^{2+}) ions and slow gating kinetics. These features make the NMDA receptor ideally suitable for mediating plastic changes in the brain, such as learning and memory (Dairam, 2005).

1.2.5.3.1.2. The Hippocampus

This is the most medial portion of the cerebral cortex developed from the stalk, of the original cerebral vesicle (Smythies, 1970). The hippocampus is a bi-lateral limbic structure which plays a role in emotion, motivation and memory (Amaral and Witter, 1989; Butler, 1993). Each hippocampus consists of two thin sheets of neurons,

resembling two C's: the dentate gyrus (DG) and the Ammon's horn or *Cornu Ammonis* (CA), leaning together at the top and spread apart at the base (Figure 1.9.).

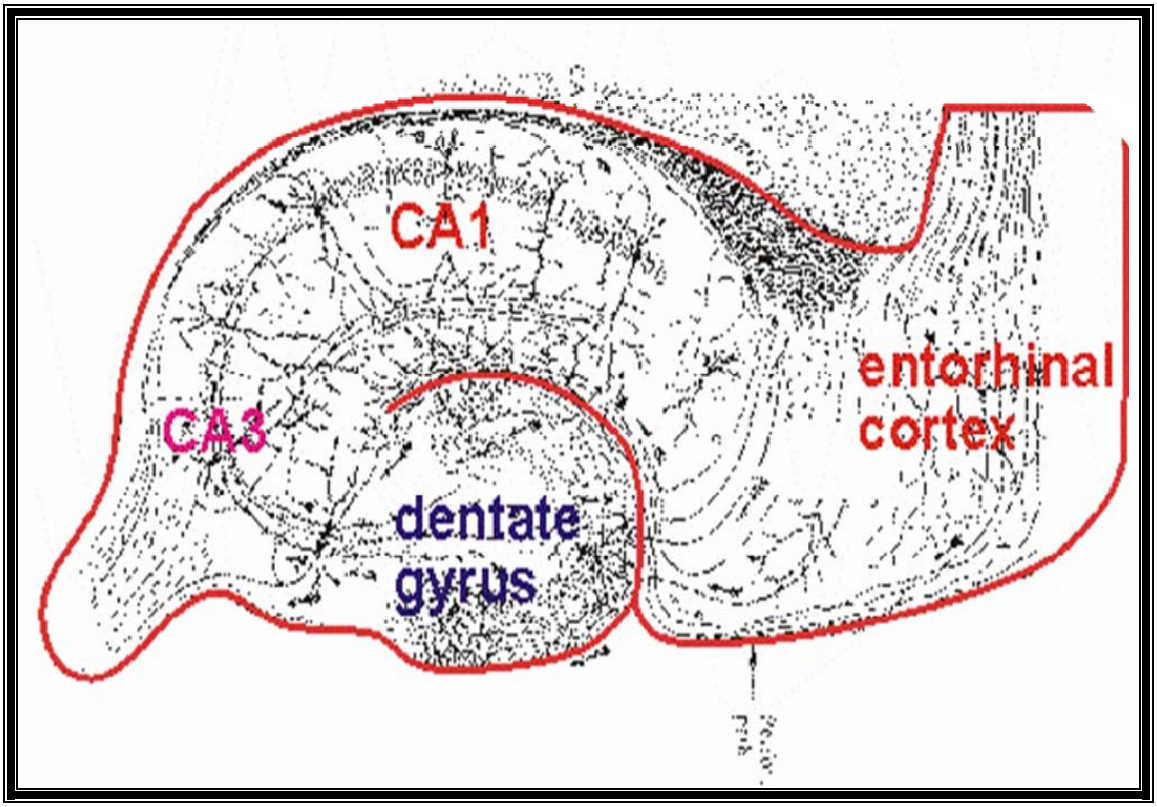

Figure 1.9. Cross sections through the major regions of the hippocampus. (www.neuroscience.bham.ac.uk/.../hippocampus.png).

The Ammon's horn, also known as the hippocampus proper has four divisions (CA1-CA3), of which the most important are the CA1 and CA3. The *stratum pyramidale* is the principle cell layer of the Ammon's horn that contains cells bodies of pyramidal cells, whilst the *stratum granulosm* consisting of cell bodies of granule cells, constitutes the main cell layer of the DG. Although the DG is commonly included as part of the hippocampus, it is cytoarchitectonically distinct from the hippocampus proper (Amaral, 1978; Bayer, 1985; Amaral and Witter, 1989). The hippocampal region (the CA field, the

dentate gyrus and subicular complex) is important for mammalian memory (Squire 1992), and damage to these regions impairs performance on a variety of tasks of learning and memory (Eichenbaum and Cohen, 2001; Broadbent *et. al*., 2004)

1.2.5.3.1.3. Long Term Potentiation

The stimulation of glutamatergic fibre pathways in the hippocampus enhances synaptic neurotransmission between stimulated axons and post-synaptic cells that initiates long term potentiation (LTP) (Bliss and Lomo, 1973; Danysz and Parsons, 2003). LTP is an example of plastic changes in the brain and is believed to model basic mechanisms of memory formation.

As shown in Figure 1.10., the initiation of LTP results in massive release of glutamate, which binds to both NMDA and AMPA receptors. The binding of glutamate alone on NMDA receptors is insufficient to bring about ion channel opening. Thus it is the continuous activation of the AMPA receptors that causes massive influx of Na^+ ions across the postsynaptic membrane leading to depolarization required to remove the positively charged Mg^{2+} from the narrow region in the NMDA receptor. Once the Mg^{2+} ion has dissociated from the receptor, the ion channel opens to allow the passage of Na^+ and Mg^{2+} ions (Bliss and Collingridge, 1993; Lodish *et al*., 1995). It is only at this stage where Ca^{2+} ions can freely enter the cell via the NMDA receptor channel and initiate a number of enzymatic processes that enhance synaptic strength (Dairam, 2005). Thus loss of hippocampal NMDA receptors, which have a pivotal role to play in LTP, will result in learning and memory impairment.

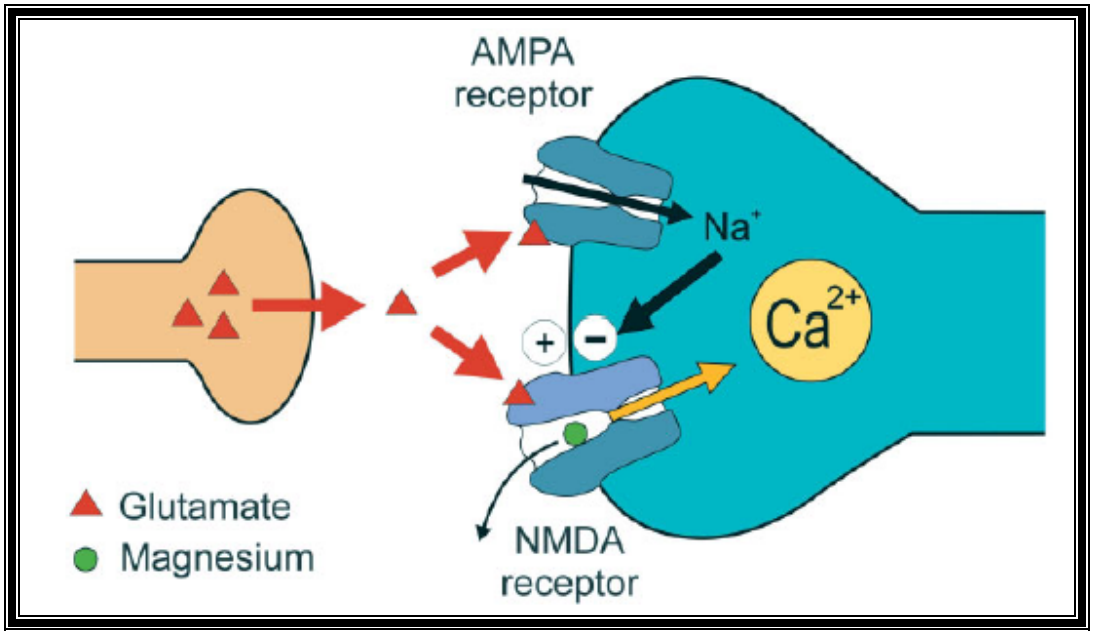

Figure 1.10. Diagram showing the involvement of NMDA and AMPA receptors in the induction of LTP (Danysz and Parsons 2003).

1.3. NEUROPROTECTION

1.3.1. Introduction

The phenomenon of neuroprotection involves both mechanisms and strategies used to protect against neuronal injury and degeneration of the CNS, following acute disorders including, stroke, nervous system injury/trauma or chronic neurodegeneration (Slikker *et al*., 1999, Fahn and Sulzer, 2004).

1.3.2. Antioxidant therapy

The overproduction of ROS can be detoxified by endogenous antioxidants, causing the cellular stores to be depleted (Candelario-Jalil *et al*., 2001). According to Halliwell and

Gutteridge (1989), "an antioxidant is any substance that when present at low concentrations compared to those of an oxidizable substrate significantly delays or inhibits oxidation of that substrate". In addition, the antioxidants can act at different levels in the oxidative sequence.

1.3.2.1. Enzymatic antioxidant systems

The principal cellular defence systems against oxygen free radicals are superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) (a haeme enzyme) (Figure 1.11.). These enzymes scavenge reactive chemical species and help to maintain cells in a reduced state (Fahn and Cohen, 1992).

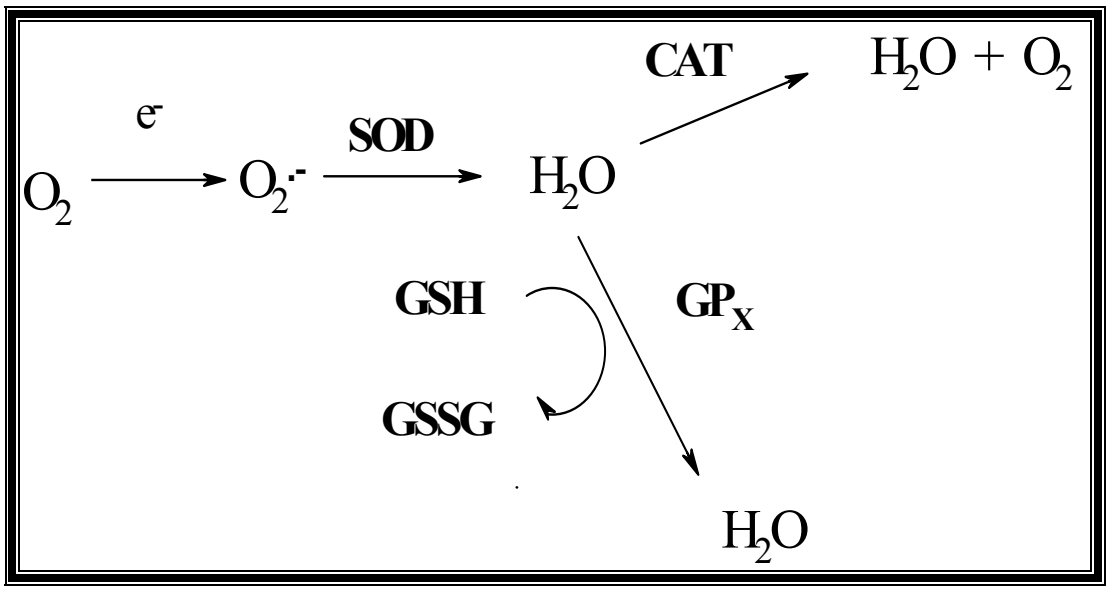

Figure 1.11. An outline of the three main antioxidant enzymes involved in preventing formation of ROS. Abbreviations: Superoxide Dismutase **(SOD)**, Catalase **(CAT)** and Glutathione Peroxidase **(GPX)**, Oxidized Glutathione (**GSH**) and Reduced Glutathione (**GSSG**), modified from Reiter, 1995.

The breakdown of $O_2^{\bullet -}$ by SOD yields H_2O_2 and O_2. There are two distinct SOD's in eukaryotes; the manganese-containing SOD localized in the mitochondrial matrix and the copper- zinc-containing SOD found in the cytoplasm. H_2O_2 is converted to water (H_2O) in the presence of CAT and GPx, thus delaying the generation of the potent $HO^{\bullet}$ and the progression of the Fenton reaction (section 1.2.1.3.2.) (Fahn and Cohen, 1992). Besides metabolizing H_2O_2 to H_2O, GPx also functions as a peroxynitrite reductase which removes the highly damaging $ONOO^-$ (section 1.2.1.3.5.) (Sies *et al*., 1997).

Due to low CAT activity in the brain, GPx acts as the major enzyme for the detoxification of H_2O_2 in the brain (Bharath *et al*., 2002). Glutathione (GSH) is the major cellular thiol participating in the maintenance of cellular redox status of the neuron and neuronal mitochondria. A decreased level of GSH may severely impair normal cellular functions (Butterfield *et al*., 2002).

1.3.2.2. Metal Chelators

Fe cations are known to generate free radicals through the Fenton and Haber-Weiss reaction as described in section 1.2.1.2.2 (Halliwell and Gutteridge, 1990). Hence through metal ion-chelation activity, the antioxidant molecule reduces the concentration of the catalyzing transition metal in LP, thus preventing oxyradical generation and the consequent oxidative damage (Kehrer, 1993; Duh *et al*., 1999). It is reported that chelating agents reduce the redox potential by forming σ-bonds with a metal, thereby stabilising the oxidised form of the metal ion (Gordon, 1990; Srivastava *et al*., 2006).

Evidence shows that neurotoxicity resulting from increased Fe content in the substantia niagra of post-mortem parkinsonian brains (Gerlach *et al*., 1994) and in AD patients (Perry *et al*., 2003), is mediated by H_2O_2 (Behl *et al*., 1994). Hence agents with the ability to chelate Fe and thus reduce its content in the brain could be of therapeutic importance in oxidative stress-induced CNS disorders. Such agents include desferrioxamine, an Fe chelator, usually used to reduce brain Fe content in models of Fe-loaded rats (Ward *et al.*, 1995).

1.3.2.3. Free radical scavengers

1.3.2.3.1. Introduction

Free radical scavengers are antioxidants, which are oxidized in place of biomolecules (Halliwell and Gutteridge, 1989) and undergo radical terminating reactions with other free radicals, thus reducing further initiation of oxidative damage. The scavenging of free radicals by antioxidants, either involves hydrogen abstraction or addition to form an antioxidant radical (Halliwell and Gutteridge, 1989). In addition, this radical is insufficiently reactive to cause hydrogen abstraction or addition of biomolecules because of the delocalization of the unpaired electron in the aromatic ring (Halliwell and Gutteridge, 1989).

1.3.2.3.2. Endogenous scavengers of free radicals

Vitamin E and Vitamin C are both important free radical scavengers that work synergistically to prevent oxidation of membranes (Buechter, 1988; Fang *et al*., 2002). Vitamin E, an important natural antioxidant in living cells is known as a "chain-breaking antioxidant". It has the ability to transfer its phenolic hydrogen to lipid peroxyl radicals ($LOO^{\bullet}$), thereby terminating LP chain reactions and preventing further peroxidation of PUFAs in membrane phospholipids with the formation of a relatively unreactive vitamin E radical (Palozza and Krinsky, 1991; Fang *et al*., 2002).

Vitamin C is another compound which acts as an important free radical scavenger in the aqueous phase within cells and plasma. As a reducing agent, it reacts with a vitamin E radical to yield a vitamin C radical while generating vitamin E. In addition, the vitamin C radical is converted back to Vitamin by GSH (Buechter, 1988; Fang *et al*., 2002).

1.3.2.3. Melatonin

Melatonin (*N*-acetyl-5-methoxytryptamine) is a naturally occurring chemical mediator that is predominantly synthesized in the pineal gland (Shida *et al*., 1994; Costa *et al*.,

1995). The synthesis of the hormone is under rhythmic control (Reiter, 1987) with peak levels produced at night in darkness. aMT, a lipophilic molecule, is able to traverse almost every organ in the body (Reiter *et al*., 1995). It has been demonstrated (Morgan and Williams, 1989) that it can bind to specific membrane receptors as well as receptors in the nuclei (Acuña-Castroviejo *et al*., 1993).

Although the exact mechanism of its action on receptors is yet to be determined, aMT has been demonstrated to be a powerful antioxidant and free radical scavenger (Tan *et al*., 1993). It chelates Fe (Limson *et al*., 1998), and scavenges the $ONOO^-$ (Cuzzocrea *et al*., 1997; Gilad *et al*., 1997) and the $HO^\bullet$ which is generated from H_2O_2 via the Fenton reaction (Tan *et al*., 1993; Stasica *et a*l., 1998). In addition, aMT not only stabilises cell membranes allowing them to resist free radical processes more effectively (Garcia *et al*., 1998) but it also stimulates antioxidative enzymes including SOD and GPx (Antolin *et al*., 1996; Barlow-Walden *et al*., 1995; Reiter, 1998; Albarran *et al*., 2001). Hence stimulation of these enzymes reduces oxidative damage by converting toxic radicals and reactive oxygen intermediates to non-toxic products.

1.3.3. Anti-Excitotoxin therapy

One of the consequences of oxidative stress and ROS, as mentioned earlier in section 1.2.1.4., is an increase in extracellular glutamate associated with subsequent excitotoxic neuronal cell death (Mattson, 2003). Hence prevention of excitotoxicity may have therapeutic potential in both PD (Alexi *et al*., 2000) and HD (Hynd *et al*., 2004), where the pathology involves this phenomenon.

Memantine, an uncompetitive NMDA receptor antagonist (Grossberg *et al*., 2006) and riluzole are believed to inhibit glutamate release and so reduce excitotoxicity (Bensimon *et al*., 1994; Kriz *et al*., 2003; Traynor *et al*., 2003) in AD. Memantine has been shown to reduce clinical deterioration (Reisberg *et al*., 2003) without interfering with the physiological actions of glutamate required for memory and learning whilst riluzole shows neuroprotective properties against hypoxic brain damage and cerebral ischemia (Mufson *et al*., 1999).

1.3.4. Non-nucleoside reverse transcriptase analogues

1.3.4.1. Introduction

The Non-nucleoside reverse transcriptase (RT) inhibitors (NNRTIs) of HIV-1 have been studied extensively in recent years in both laboratory and clinical settings. The NNRTIs are a structurally and chemically dissimilar group of antiretrovirals that are potent and highly selective inhibitors of HIV-1 RT. The NNRTIs comprise a structurally diverse series of compounds that are highly specific for inhibition of the HIV-1 RT (Young, 1993). The compounds bind to a common site on the RT heterodimer that is distinct from the enzyme's active site (Wu *et al*., 1991; Dueweke *et al*., 1992; Smerdon *et al*., 1994). Kinetically, the NNRTIs' inhibitory activities are generally non-competitive with respect to the template-primer and nucleotide substrate (Frank *et al*., 1991; Koop *et al*., 1991; Althaus *et al*., 1993; Carroll *et al*., 1993). The compounds appear to function by mediating a noted decrease in the enzyme's polymerizing activity (Spence *et al*., 1995). The compounds are active in their native state, requiring no phosphorylation or other activity-dependent alteration. They are extensively metabolized in the liver; very little drug is excreted unchanged (Murphy and Montaner, 1996).

1.3.4.2. Efavirenz

1.3.4.2.1. Introduction

Efavirenz (EFV, DMP 266) (Figure 1.12.) belongs to the NNRTIs class of drugs, and has gained a definitive and important place in the treatment of HIV-1/AIDS as part of combination therapy (Ward *et al*., 2003).

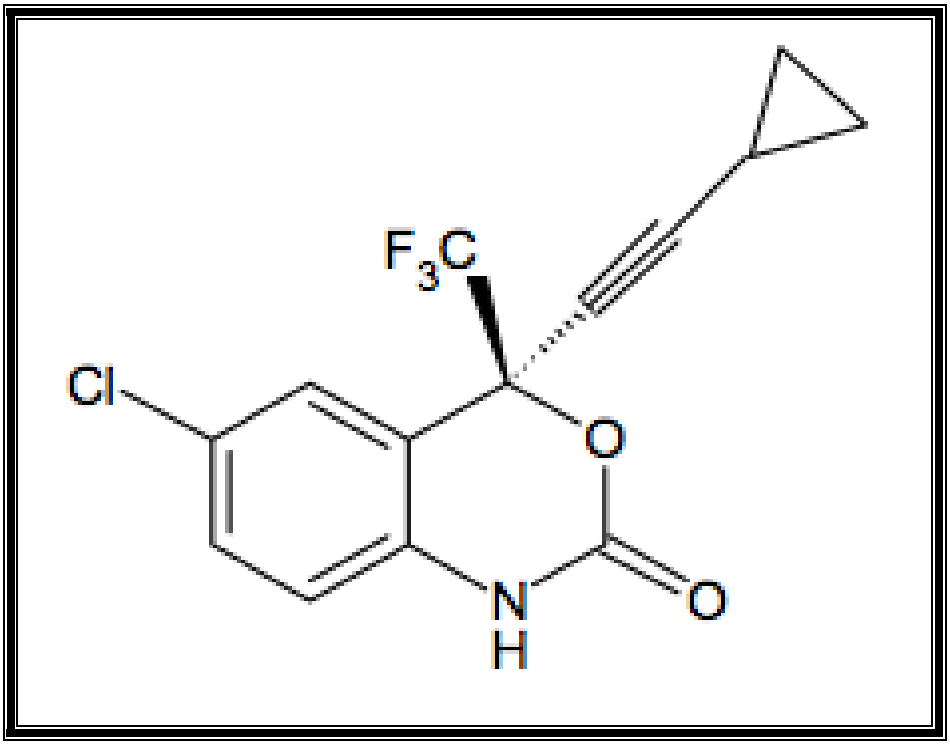

Figure 1.12. Structure of EFV (modified from Wang *et al*., 2001).

It alters the function of the reverse transcriptase enzyme, rendering it incapable of converting viral RNA to DNA (Young *et al*., 1995). EFV has a sustained antiretroviral efficacy when used in combination with either a protease inhibitor (PI) or/and nucleoside reverse transcriptase inhibitors (NRTIs) and exhibits clinical activity in reducing plasma levels of HIV-1 RNA (Benedek *et al*., 1999).

1.3.4.2.2. Pharmacology

1.3.4.2.2.1. Dosing

The usual adult dose of EFV is 600 mg per day (usually given at bedtime) (DuPont Pharmaceuticals, 1998), and is usually taken on an empty stomach at bedtime to reduce neurological and psychiatric adverse effects.

1.3.4.2.2.2. Adverse Effects

The principal adverse effects of EFV involve the central nervous symptoms which include: dizziness, drowsiness, insomnia, depression and euphoria. These tend to occur in about 50 % of patients and during the first days of therapy and may resolve while medication is continued. Occasionally, EFV causes skin rash early in therapy in up to 28 % of patients (Katzung, 2004).

1.3.4.2.2.3. Drug interactions

EFV is extensively metabolized by the cytochrome P450 enzymes, primarily CYP3A4 (Eagling *et al*., 1995; Adkins and Noble, 1998; Barry *et al*., 1999). All currently available PIs are inhibitors of CYP3A4, therefore, drug-drug interactions should be expected when EFV is coadministered with them. It has been reported that ritonavir produces a 21 % increase in EFV concentrations (Pfister *et al*., 2003). Therefore, appropriate dose adjustments may be necessary for these drugs. Drugs including phenobarbital, rifampin and rifabutin, induce CYP3A4 activity thereby increasing the clearance of EFV resulting in lowered plasma concentrations in patients (Katzung, 2004).

1.3.4.2.3. Pharmacokinetics

1.3.4.2.3.1. Absorption

Animal studies suggest the oral bioavailability of this drug is about 40 %, and studies in humans have shown that bioavailability is increased by 50 % following a high-fat meal. Time-to-peak plasma concentrations were 3–5 hours and steady-state plasma concentrations were reached in 6 to 10 days (DuPont Pharmaceuticals, 1998). EFV is highly protein-bound (> 99.5 %), predominantly to albumin. In one small study, doses of 200–600 mg once daily produced mean CSF concentrations of 0.69 % of the corresponding plasma concentration. This proportion is about 3-fold higher than the non-protein-bound (free) fraction in plasma (Balani *et al*., 1996).

1.3.4.2.3.2. Distribution

EFV is widely distributed in body compartments and is likely to be effective in protected tissues such as the CNS (Taylor *et al*., 2001; Wynn *et al*., 2002).

1.3.4.2.3.3. Metabolism

Studies in humans and *in vitro* studies using human liver microsomes have demonstrated that EFV, being a substrate (Mutlib *et al*., 1999; Smith *et al*., 2001), an inhibitor (Von Moltke *et al*., 2001), and an inducer of cytochrome P450 (Mouly *et al*., 2002), exhibits multiple interactions with the P450 system. It is principally metabolized by the cytochrome P450 system to hydroxylated metabolites with subsequent glucuronidation of these hydroxylated metabolites that include 8- and 7-hydroxyEFV, which are essentially inactive against HIV-1 (Mutlib *et al*., 1999).

1.3.4.2.3.4. Excretion

In vitro experiments with hepatocytes in culture and pharmacokinetic studies of EFV disposition in healthy volunteers and HIV-infected patients suggest that hepatic metabolism is the major route of EFV elimination (Fiske *et al*., 1999).

1.3.4.3. Nevirapine

1.3.4.3.1. Introduction

Nevirapine (NVP) (Figure 1.13.), formerly known as BI-RG-587 (NVP), is a potent NNRTI specific for HIV-1 which shows good characteristics for development as a potential therapeutic agent (Merluzzi *et al*., 1990). It belongs to the dipyridodiazepinone analogues, which bind directly to RT and blocks the RNA- and DNA-dependent DNA polymerase activities by causing a disruption of the catalytic site of the enzyme (Merluzzi *et al*., 1990; Richman *et al*., 1991).

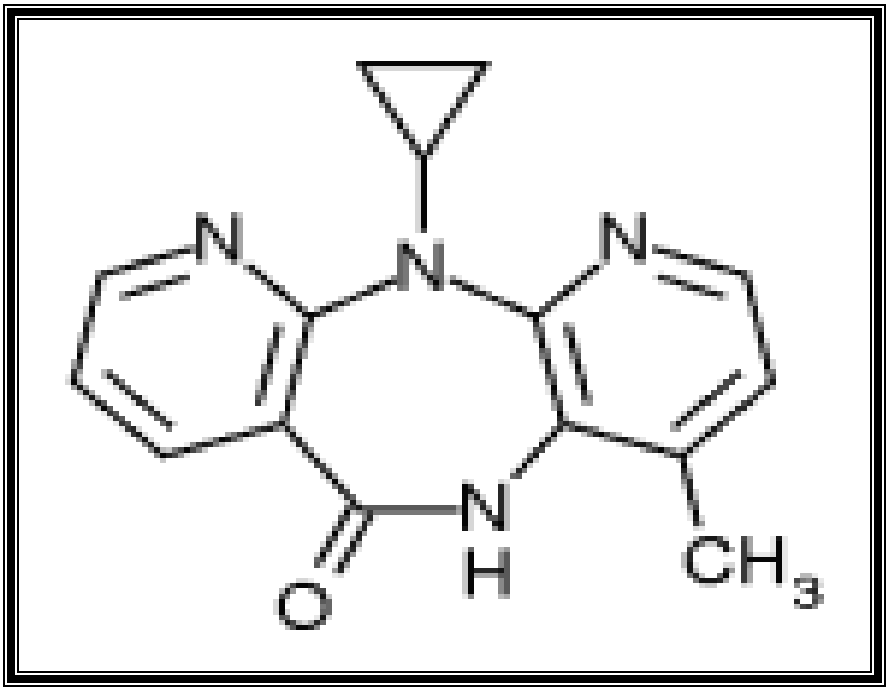

Figure 1.13. Modified structure of NVP (Riska *et al*., 1999).

NVP was the first NNRTI widely introduced in clinical practice and is regarded to be a potent (Murphy *et al*., 1999) and an effective component of highly active antiretroviral therapy (HAART) used in the treatment of HIV - 1 (Carr *et al*., 1996; D'Aquila *et al*., 1996; Harris *et al*., 1998; Montaner *et al*., 1998). In addition, it has good penetration into the CNS, making it an attractive option for patients with HIV-associated CNS disease (Yazdanian *et al*., 1999).

1.3.4.3.2. Pharmacology

1.3.4.3.2.1. Dosing

Due to its long half-life, NVP can be given as part of a twice-daily dosing regimen (Cheeseman *et al*., 1995; Miller *et al*., 1997)http://www.journals.uchicago.edu/CID/journal/issues/v32n1/000421/000421.text.ht

ml - rf7#rf7http://www.journals.uchicago.edu/CID/journal/issues/v32n1/000421/000421.text.html - rf8#rf8.

Adults: The recommended dose of NVP is 200 mg once a day for the first 14 days, then 200 mg twice a day in combination with other antiretroviral agents (Mirochnik *et al*., 2000).

Paediatrics: The recommended dose of NVP for children is based on age and weight. The oral dose of patients from 2 months up to 8 years of age is 4 mg/kg once daily for the first 14 days followed by 7 mg/kg twice daily thereafter. For patients 8 years and older the recommended dose is 4 mg/kg once daily for two weeks followed by 4 mg/kg twice daily thereafter. The total daily dose should not exceed 400 mg for any patient (Mirochnik *et al*., 2000).

1.3.4.3.2.2. Adverse Effects

The most common adverse effect of NVP is the development of mild or moderate rash in 32-48 % patients (Havlir *et al*., 1995; Carr *et al*., 1996). Severe or life-threatening skin reactions have been observed in 0.5 % of patients, including Stevens-Johnson syndrome, which is a toxic epidermal necrolysis and hypersensitivity reaction (Murphy and Montaner, 1996). NVP may cause severe or life-threatening liver toxicity, usually emerging in the first six weeks of treatment (González de Requena *et al*., 2001).

1.3.4.3.2.3. Drug interactions

In vivo studies in humans and *in vitro* studies with human liver microsomes have shown that NVP induces the cytochrome P450 enzymatic system (Sahai *et al*., 1997; Murphy *et al*., 1999). Evidence has shown that rifampicin decreases serum NVP concentrations at a level similar to those produced by EFV (Lopez-Cortes *et al*., 2001) and ritonavir (Burman *et al*., 1999; Pozniak *et al*., 1999). It seems reasonable to assume that overall; cytochrome enzyme system induction by rifampicin is the main mechanism responsible for the decrease in serum concentrations of NVP produced in patients receiving anti-

tuberculosis therapy that contains rifampicin (Esteban *et al*., 2001). Due to inductive effects of this drug, caution must be exercised when using with methadone, oral contraceptives, as well as PIs including ritonavir (Back *et al*., 2003).

1.3.4.3.3. Pharmacokinetics

1.3.4.3.3.1. Absorption

NVP is readily absorbed (> 90 %) after oral administration in healthy volunteers and in adults with HIV-1 infection. Absolute bioavailability in 12 healthy adults following single-dose administration was 93 ± 9 % (mean ± SD) for a 50 mg tablet and 91 ± 8 % for an oral solution. NVP tablets and suspension have been shown to be comparably bioavailable and interchangeable at doses up to 200 mg (Murphy *et al*., 1999).

1.3.4.3.3.2. Distribution

NVP is well absorbed orally with > 90 % bioavailability, distributes well to all tissues, and is approximately 60 % bound to plasma proteins (Murphy and Montaner, 1996).

1.3.4.3.3.3. Metabolism

NVP is extensively biotransformed via cytochrome P450 through oxidative metabolism to several hydroxylated metabolites namely 2- and 3-hydroxyNVP glucuronide (Riska *et al*., 1996; Riska *et al*., 1999).

1.3.4.3.3.4. Excretion

Renal excretion is the primary mode of elimination, accounting for 81.3 ± 11.1 % of the radiolabeled dose compared with faeces (10.1 ± 1.5 %). Excretion of the NVP parent compound in urine represented approximately 2.7 % of the dose (Riska *et al*., 1999).

1.4. RESEARCH OBJECTIVES

Immune activated macrophages and microglia release neurotoxins such as oxidative free radicals and QA, which are implicated in pathogenesis of different disorders such as AD (De la Monte *et al*., 2000), PD (Koutsilieri *et al*., 2002) as well as ADC (Mollace *et al*., 2001). Recent studies suggest that NNRTIS are of therapeutic importance in patients with ADC (Enting *et al*., 1998; Tashima *et al*., 1998).

The first objective of this study was conducted to determine the potential neuroprotective effects of the NNRTIs, EFV and NVP under a number of neuropathological conditions and to attempt to elucidate the mechanism of neuroprotection, should it be occurring, using various inorganic studies and biological assays. These experiments include:

1) Measuring the antioxidant and free radical scavenging potential of these agents in the presence of various neurotoxins in rat brain homogenates as well as in hippocampal neurons.
2) Metal binding studies involving Fe^{2+} and Fe^{3+} were conducted by electrochemical analysis.

The second objective was to investigate the effect of NVP and EFV on the enzyme responsible for QA production in the KP. Due to the implication of QA in spatial memory deficits and progressive degeneration of neurons, a decrease in the synthesis of QA could be beneficial. Hence the effect of EFV and NVP on the enzyme responsible for QA production will be assessed. Furthermore, due to the inverse relationship between TDO activity and brain 5-HT, any effect exhibited by these on TDO activity will be investigated by measuring levels of brain 5-HT and related neurotransmitter levels in the hippocampus. High brain 5-HT levels are of importance in alleviating or preventing depression, hence the effect of NVP and EFV on 5-HT metabolism was also determined.

These studies mentioned above would provide important information on the possible antidepressant properties of these drugs as well as any effects that they may possess in inhibiting the enzyme responsible for the synthesis of endogenous, QA, a potent neurotoxin.

It is hoped that this study enhances further understanding of the beneficial use of NVP and EFV in ADC. In addition, the knowledge gained could ultimately lead to further research on the use of these NNRTIs in the treatment of other neurodegenerative diseases associated with depression.

CHAPTER 2

FREE RADICAL SCAVENGING ACTIVITY

2.1. INTRODUCTION

ROS are free radicals generated in many redox processes that induce oxidative damage to biomolecules such as proteins, lipids and DNA (Figure 2.1) (Farber, 1994). The most commonly formed ROS are $O_2^{\bullet -}$ and $HO^{\bullet}$, and non-free radicals, such as H_2O_2 and $^1O_2$. Biomolecule degeneration, followed by initiation and propagation driven oxidative chain reactions, cause accelerated aging, inflammation and neurodegenerative diseases (Castro and Freeman, 2001), such as AD (De la Monte *et al*., 2000), PD (Koutsilieri *et al*., 2002) as well as ADC (Mollace *et al*., 2001).

Cells have several antioxidant defence mechanisms that play an important role in the elimination of ROS and lipid peroxides, and therefore, protect the cells against such toxic effects of ROS and lipid peroxides (Halliwell, 1991; El-Habit *et al*., 2000). These defence mechanisms include antioxidative enzymes, such as SOD, CAT, and GPx and of small molecules such as GSH and vitamins C and E (Fridovich, 1999). The efficiency of the antioxidant defence system is altered under pathological conditions (Aruoma, 1994; Halliwell, 1994), as the detoxifying mechanisms are often inadequate to remove the continuously produced ROS (Halliwell *et* al., 1992). The imbalance between ROS and antioxidant defence mechanisms leads to oxidative stress and LP (El-Habit *et* al., 2000).

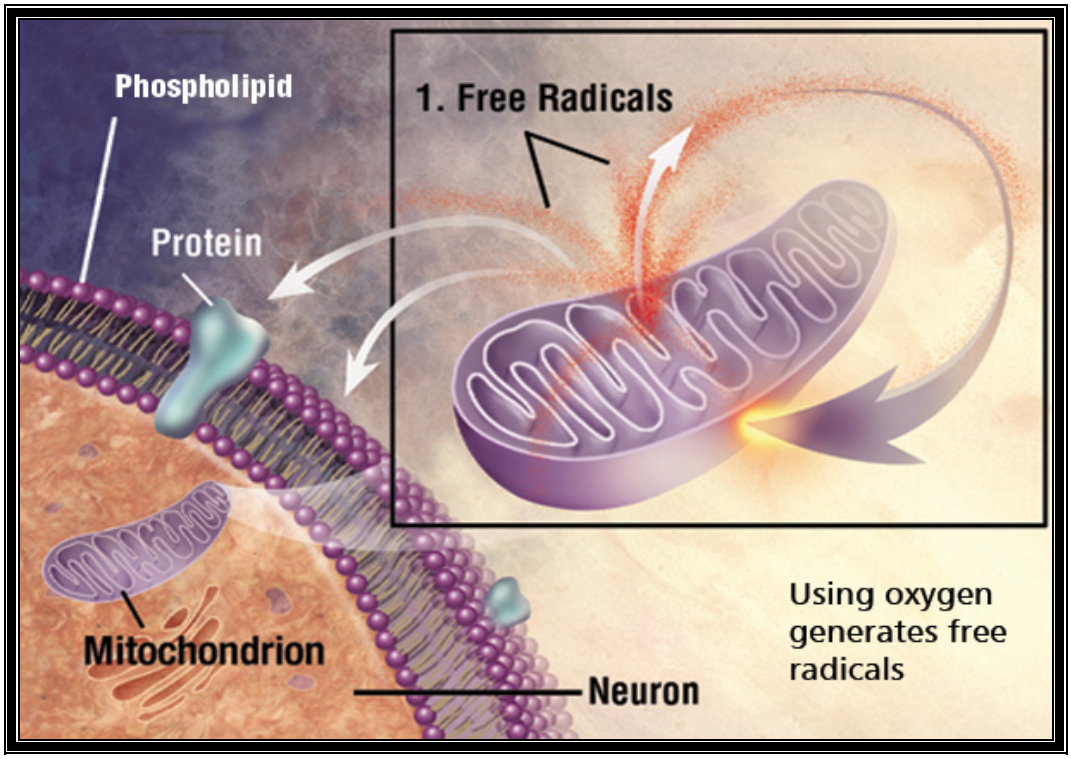

Figure 2.1. Source: Alzheimer's Disease Unraveling the Mystery. US Department of Health and Human Services, National Institute of Health, NIH Publication Number: 02-3782, 2002; 36. (*www.nia.nih.gov/Alzheimers/Resources/HighRes.htm*).

Based on the growing interest in free radical biology and the lack of effective therapies for most degenerative diseases, the search for antioxidants in protection against these diseases is warranted (Robak and Marcinkiewicz, 1995). Antioxidants play an important role in preventing or alleviating neurodegeneration, by reducing the oxidative damage to cellular components caused by ROS (Ceriello, 2003). Hence, compounds exhibiting antioxidant activity have the potential to reduce and/ or prevent neurodegenerative disorders via direct radical scavenging (Figure 2.2.), chelation of catalytic metal ions and delayed oxidation of biomolecules through inhibition of chain initiation reactions and continued hydrogen abstraction (Ames *et al*., 1993; Robak and Marcinkiewicz, 1995; Jackson *et al*., 2002).

In vitro model systems to investigate antioxidant activity against various ROS are a valuable tool in classifying and screening for potential antioxidants to be used *in vivo* (Halliwell *et al*., 1995). Hence the evaluation of existing *in vitro* mechanisms in predicting a specific aspect of antioxidant activity behaviour *in vivo* is of major importance (Bartasiute *et al*., 2007).

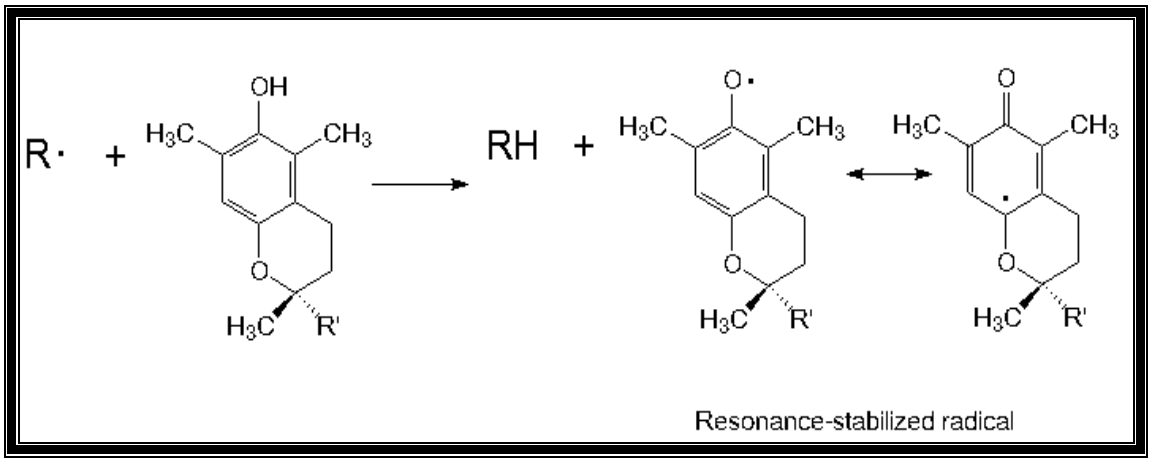

Figure 2.2. Mechanism of action of vitamin E by binding to the free radical and neutralising its unpaired electron (www.uic.edu/.../Vitamin%20E%20Chemistry.htm).

Pharmacological efforts to attenuate oxidative injury in degenerative diseases have typically focused on drugs with antioxidant properties. Such approaches provide a 'first line of defence' against free radicals. The free radical scavenging activity of antioxidants can be based on the ability to scavenge the stable radical 1, 1-diphenyl-2-picryl-hydrazil (DPPH) (Oyaizu, 1986). The DPPH free radical ($DPPH^{\bullet}$) reacts with hydrogen donors to form the corresponding colourless, hydrazine analogue (DPPH: H) (Figure 2.3), by either accepting an electron or hydrogen radical to become a stable diamagnetic molecule (Soares *et al*., 1997). In this assay, the nitrogen-centred DPPH free radical acts as both an oxidizable substrate and as the reaction indicator molecule (Dorman *et al*., 2003)

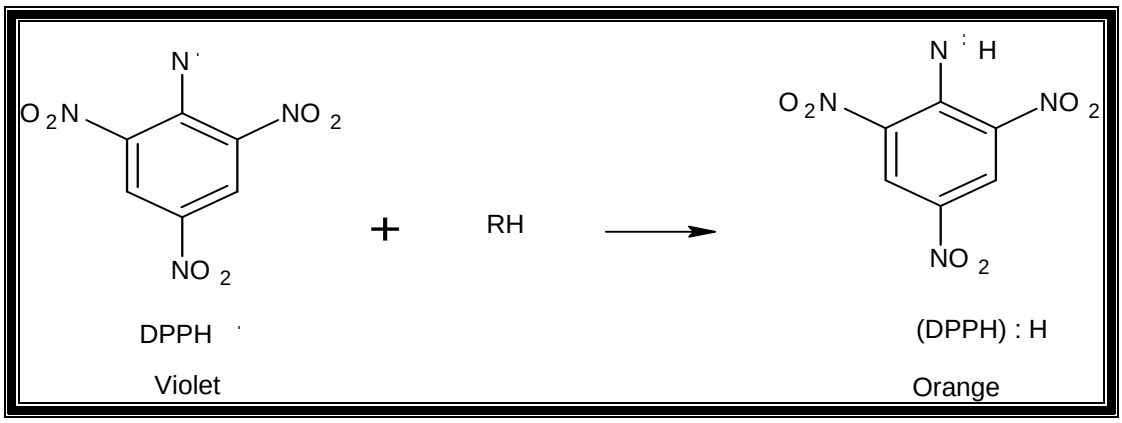

Figure 2.3. A modified reaction of DPPH• with an antioxidant (RH) to form the stable diamagnetic molecule (DPPH): H. (Prakash, 2001).

This assay provides information on the scavenging ability of antioxidants towards the stable free radical. Because of the odd electron, DPPH shows a strong absorption band at 517 nm in visible spectroscopy. As this electron becomes paired off in the presence of free radical scavenger, the absorption band vanishes, and the resulting decolorisation is stochiometric with respect to the number of electrons taken up (Badmis *et al*., 2003). The degree of discoloration indicates the scavenging potential of the antioxidant.

There exist two mechanisms for antioxidants to scavenge DPPH (Brand-Williams *et al*., 1995; Litwinienko and Ingold, 2003). The first is a direct H atom- abstraction process (Equation 2.1.), and the second is a proton concerted electron-transfer process (Equation 2.2.).

$$DPPH^{\bullet} + RXH \rightarrow DPPHH + RX^{\bullet} \quad (1)$$ **Equation 2.1.**

$$DPPH^{\bullet} + RXH \rightarrow DPPH^{-} + RXH^{\bullet +} \rightarrow DPPHH + RX^{\bullet} \quad (2)$$ **Equation 2.2.**

Where X represents O, N, S or C

2.2. THE COMPARATIVE FREE RADICAL SCAVENGING REACTIONS AND ANTIOXIDANT ACTIVITIES OF NVP AND EFV.

2.2.1. Introduction

The brain is particularly susceptible to free radical damage because of its high utilization of O_2 and its relatively low concentration of antioxidant enzymes and free radical scavengers (Brannan *et al*., 1981; Reiter, 1995; Reiter, 1998). The mechanism by which free radicals induce tissue injury is attributed to the ability to initiate the apoptosis of brain cells (Lipton and Nicotera, 1998; Raha and Robinson, 2001), hence agents that reduce free radical generation could potentially delay the progression of neurodegeneration. This experiment was conducted to determine the free radical scavenging effects of increasing concentrations of NVP or EFV (100-200 μg/ml) on the DPPH radical *in vitro*.

2.2.2. Materials and Methods

2.2.2.1. Chemicals and Reagents

EFV and NVP were kindly supplied by Aspen Pharmaceutical Company, South Africa. 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) was purchased from the Sigma Chemical Corporation, St. Louis, MO, U.S.A. DPPH, efavirenz and nevirapine were dissolved in absolute ethanol before use.

2.2.2.2. DPPH Assay

The free radical scavenging activity of EFV and NVP was measured by 1, 1-diphenyl-2-picryl-hydrazil (DPPH) using a modified method of Brand-Williams *et al*., (1995).

Briefly, 1.2 ml of DPPH (0.1 mM) dissolved in absolute ethanol was incubated for a period of 5 hours with 0.3 ml of NVP or EFV at final concentrations of 100 μg/ml and

200 µg/ml. The absorbance was measured at 517 nm against absolute ethanol as the blank at different time intervals for 5 hours. In the presence of the compounds capable of donating H-atom or an electron there is a decrease in absorbance at 517 nm. The lower absorbance of the reaction mixture indicates a higher free radical scavenging capacity.

The DPPH radical scavenging activity was calculated using the following equation:

Scavenging activity (%) = $\{(A_o - A_1)/A_o\}$ x 100

Where A_o is the absorbance of the control reactions which consisted of DPPH and were free of EFV and NVP. A_1 is the absorbance of DPPH in the presence of the test compound.

2.2.2.3. Statistical Analysis

All results were analyzed using a one-way analysis of the variance (ANOVA) followed by the Student-Newman-Keuls Multiple Range Test. The level of significance was accepted at $p < 0.05$ (Zar, 1974).

2.2.3. Results

Fig. 2.4. shows that both NNRTIs have free radical scavenging properties with NVP being more effective than EFV. At 200 µg/ml NVP scavenges approximately 21 % of the DPPH free radicals while EFV scavenges approximately 13 %.

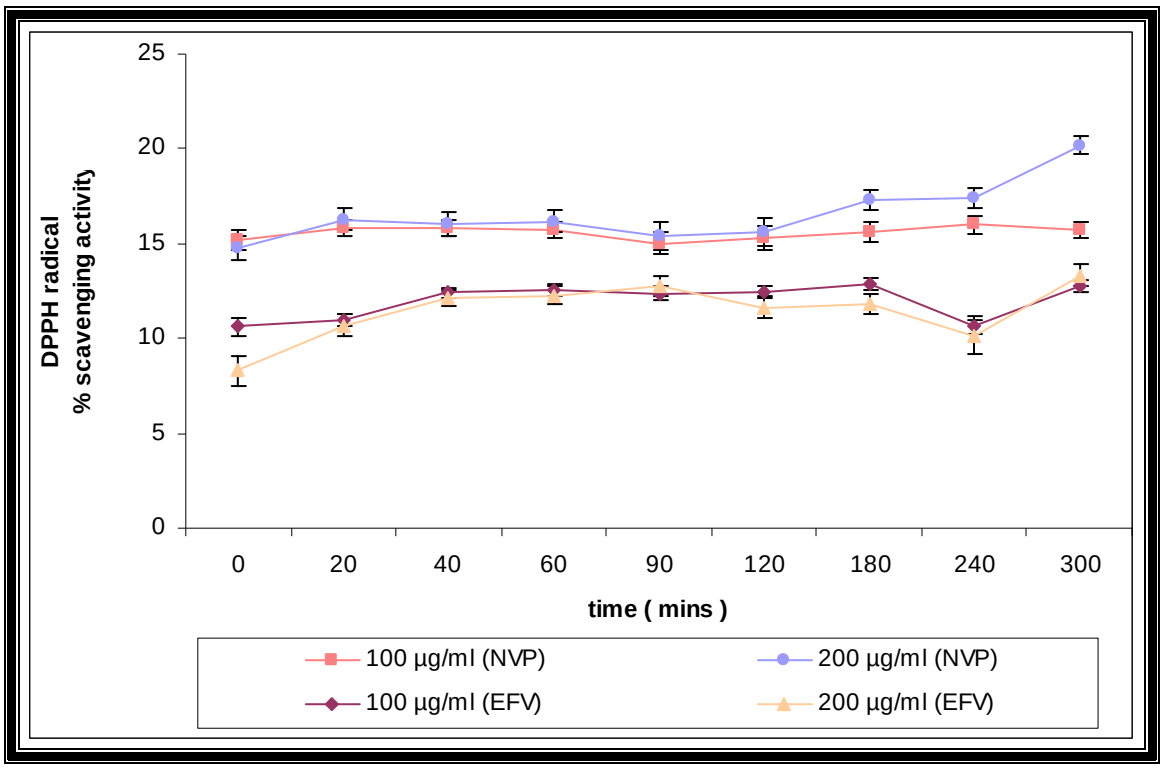

Figure 2.4: Percentage scavenging activity of DPPH radical by NVP and EFV. Each point represents the mean ± S.D. (n=3).

2.2.4. Discussion

The pivotal role of radical damage in various neurological conditions has stimulated research on the potential use of antioxidants to slow or prevent the progression of these processes (Bartasiute *et al*., 2007). Research has demonstrated that antioxidants have the ability to offer neuroprotection against free radical induced injury and may be beneficial in the prevention or treatment of neurodegeneration (Anoopkumar-Dukie *et al*., 2003; Santamaria *et al*., 2003).

Oxidative degradation of susceptible biomolecules and cellular systems is a multi-step process involving chain initiation and propagation steps (Halliwell and Gutteridge, 1989). The prevention of the chain initiation step by scavenging various ROS is

considered to be an important antioxidant mode of action. Therefore, it was considered important to characterize the ability of these NNRTIs to scavenge the DPPH radical.

The model of scavenging the stable DPPH radical is a widely used method to evaluate antioxidant activities. This study shows that both agents effectively scavenge the DPPH radical (Figure 2.4.). The effect of antioxidants on DPPH radical scavenging is due to their hydrogen donating ability. Based on the structure of EFV and NVP, it is possible that the pyridine moieties as well as the ketone group on EFV are responsible for free radical scavenging activities as these have delocalized electrons to donate.

Through hydrogen donation, these NNRTIs prevent the abstraction of hydrogen from susceptible PUFAs and therefore the initiation of free radical-mediated chain reactions in cells. Hence, these results suggest that the antioxidant mechanism displayed by both agents, are possibly due to oxidative chain termination by radical scavenging capacity.

2.3. Conclusion

The antioxidant activity of NVP and EFV can be correlated to the radical -scavenging potency exhibited by these agents in this study.

CHAPTER 3

LIPID PEROXIDATION

3.1. INTRODUCTION

The PUFAs composition of membrane phospholipids plays a direct role in a variety of multicellular processes, including inflammation (Grimble, 1998) and immunity (Yehuda and Mostofsky, 1997) with implications for neurodegenerative diseases. While several biomolecules may be affected, oxidative damage to lipids is of particular significance especially in the CNS because of its enrichment in PUFAs (Bassett and Montine, 2003) such as docosahexanoic acid (22:6) (Moriguchi *et al*., 2000 ; Crawford *et al*., 2003), linoleic acid (18:2) and arachidonic acid (20:4) (Gamor *et al*., 1999). PUFAs composition of neural membranes is a key factor for brain structural development and biologic functions (Crawford *et al*., 2003) and enhanced peroxidation of PUFAs, leads to cellular oxidative stress and generation of reactive LP products such as MDA (Esterbauer, 1982). The amount of MDA present is measured as an index of LP and marker of oxidative stress (Janero, 1990).

Oxidation of lipids in biological systems proceeds via a chain reaction consisting of three phases: initiation, propagation, and termination as shown in Figure 3.1. (Halliwell and Gutteridge, 1990). In the former case, peroxidation occurs by abstraction of a hydrogen atom from the lipid substrate (LH) to generate a highly reactive lipid radical ($L^{\bullet}$). The lipid peroxyl radical ($LOO^{\bullet}$) formed in the propagation phase, can abstract a hydrogen atom from a number of *in vivo* sources, such as DNA and proteins, to generate free radicals and lipid hydroperoxides (LOOH) (Bentinger *et al*., 2007). These in turn propagate the radical chain which once initiated, is extremely difficult to terminate (Krinsky, 1992). The use of antioxidants such as α-tocopherol (α-TOH) is another mechanism essential to limit radical-induced LP (Palozza and Krinsky, 1991).

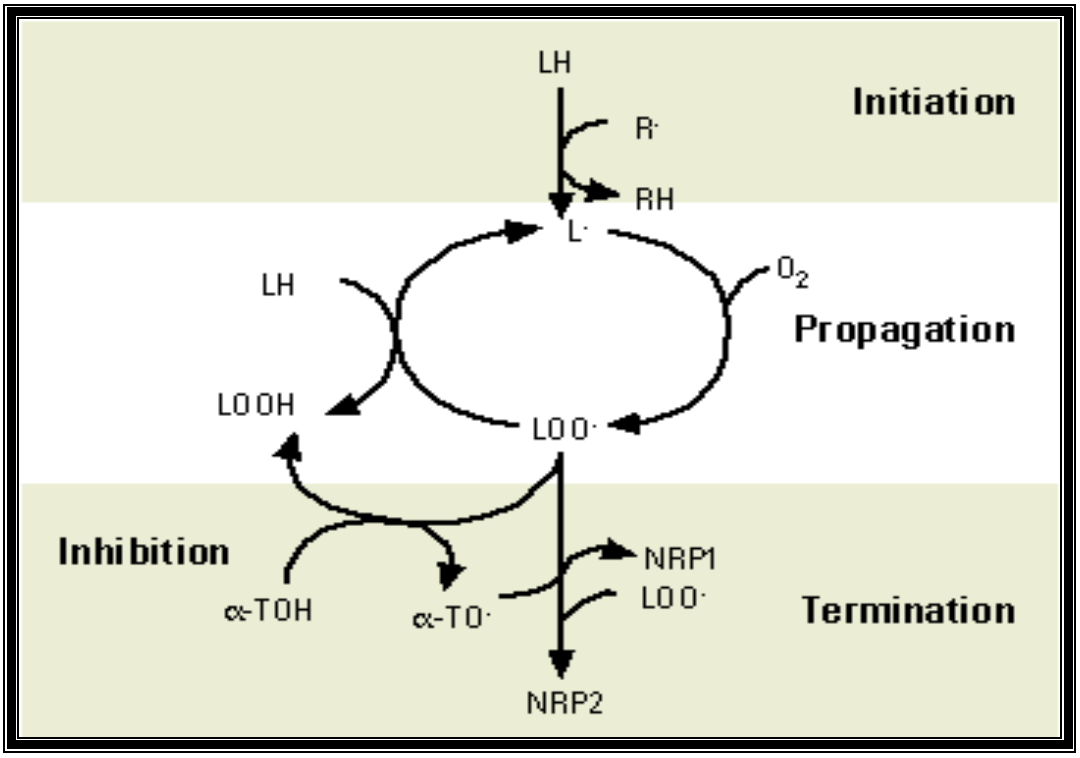

Figure 3.1. Overview of LP. Abbreviations: NRP, non radical product; LOOH, lipid hydroperoxide; α-TOH, α-tocopherol; α-TO•,α-tocopherol radical; LH, lipid substrate; LOO•, lipid peroxyl radical. (Waldeck and Stocker, 1996).

LP products modify the physical characteristics of biological membranes (Servanian and Ursini, 2000), and because direct analysis of endogenous primary LP products is complicated, the extent of LP is typically assessed by measuring levels of secondary oxidation products (Janero, 1990). The primary lipid oxidation products, LOOH, are unstable and decompose to form secondary products such as aldehydes and ketones through a multitude of reaction pathways (Esterbauer and Schaur, 1990). The resulting diverse array of breakdown products, coupled with the small *in vivo* concentration of these products, presents a challenge for accurate quantification of LP (Janero, 1990).

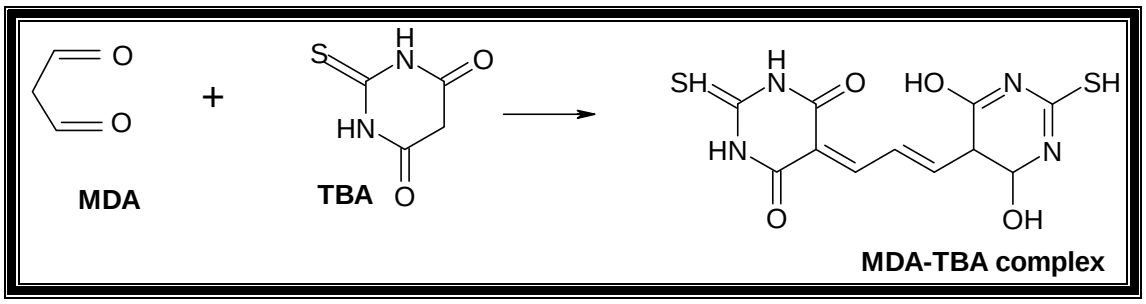

Figure 3.2. Reaction between 1 molecule of MDA and 2 molecules of TBA to produce a pink MDA-TBA complex (modified from Mead *et. al*., 1986).

The most used assay for LP is the thiobarbituric acid (TBA) test, which relies on the production of a coloured adduct from the reaction of LP products and TBA (Janero, 1990). The test material is heated at low pH with TBA resulting in a pink chromogen, formed by a MDA-TBA complex (Figure 3.2.) which is extracted into butanol and measured by absorbance at 532 nm or by fluorescence at 553 nm (Gutteridge and Halliwell, 1990). Some of the MDA detected in the TBA test result from the breakdown of LOOH during the peroxidation process itself (Janero, 1990), but most is generated during the acid-heating stage of the test (Gutteridge and Halliwell, 1990).

However, because TBA reacts with a number of other oxidation products including other unsaturated aldehydes (Kosugi *et al*., 1987) and endoperoxides from enzymatic routes (Shimizu *et al*., 1981), this test is non-specific (Janero, 1990). Therefore, the TBA test can only give a crude measure of LP by quantifying MDA levels formed.

3.2. THE COMPARATIVE EFFECTS OF NVP AND EFV ON QA-INDUCED LIPID PEROXIDATION IN RAT BRAIN HOMOGENATE *IN VITRO*.

3.2.1. Introduction

QA is involved in the neurocytotoxicity associated with several major inflammatory brain diseases (Stone, 2001) such as ADC (Heyes *et al*., 1991) and other viral brain infections (Heyes *et al*., 1992). The massive elevation of QA concentration within the brain produced by immune activated macrophages and activated microglia in pathological states such as ADC and HSE (Heyes *et al*., 1998; Huengsberg *et al*., 1998; Pláteník *et al*., 2001), forms an integral part of the inflammatory response in the CNS (Heyes *et al*., 1992; Stone, 1993) and exerts neurotoxicity through excitotoxic (Schwarcz *et al*., 1983; Stone 1993) and oxidative (Santamaría *et al*., 2001) mechanisms.

Since QA-induced neurotoxicity is mediated at least in part by generation of excitotoxins and free radicals in the brain, the following study was aimed at investigating the effect of QA on LP in rat brain homogenate *in vitro*, in the absence and presence of NVP or EFV.

3.2.2. Materials and Methods

3.2.2.1. Chemical Reagents

QA, butylated hydroxytoluene (BHT), 2-thiobarbituric acid (TBA) and 1, 1, 3, 3-tetramethoxypropane (99 %) were purchased from Sigma Chemical Corporation, St Louis, MO, U.S.A. EFV and NVP were kindly supplied by Aspen Pharmaceutical Company, Port Elizabeth, South Africa. Trichloroacetic acid (TCA) ethanol and butanol were purchased from Saarchem, Johannesburg, South Africa. All other reagents used were of the highest quality available. QA, TBA and TCA were prepared by dissolving in Milli-Q water while NVP, EFV and BHT were prepared by dissolving in absolute ethanol.

3.2.2.2. Animals

Adult male Wistar rats, purchased from the South African Vaccine Producers (Johannesburg, South Africa) were used throughout the study. The animals were housed under artificial illumination with a daily photoperiod of 12 hours (lights on at 06h00). The animal-house temperature was maintained at a constant 20 ^{o}C to 24 ^{o}C, while an extractor fan ensured the constant removal of stale air. The rats (200-250 g) were housed five per cage with food and water provided *ad libitum*. The Rhodes University Animal Ethics Committee approved all protocols for the experiments.

3.2.2.3. Brain Removal

Rats were sacrificed by neck fracture and decapitated. The brain was exposed by making an incision through the bone on either side of the parietal structure, from the foramen magnum to near the orbit. The calvarium was removed, exposing the brain, which was easily removed for use in experiments.

3.2.2.4. Preparation of Tissue

Once the brain was removed, it was weighed and rapidly homogenized in a glass-teflon hand held homogenizer in iced cold 0.1 M phosphate buffered saline (PBS), at pH 7.4 to yield a 10 % w/v homogenate. This was used immediately for assay. PBS buffer was used as it has been shown not to scavenge free radicals (Anoopkumar-Dukie *et al*., 2001).

3.2.2.5. Preparation of the MDA Standard Curve

1, 1, 3, 3-Tetramethoxypropane was used as a standard. Varying concentrations of the standard were prepared in reaction tubes using PBS as the diluent. To these tubes BHT (0.5 ml) and TCA (1 ml) was added. An aliquot of 2 ml of this solution was added to TBA (0.5 ml) and incubated for 1 hour. A calibration curve was generated by measuring the absorbance at 5 nmole intervals. The absorbance was read at 532

nm using a GBC UV/VIS 916 spectrophotometer and plotted against the concentration (Appendix I).

3.2.2.6. Lipid Peroxidation Assay

A modification of the TBA Assay as described by Placer *et al*. (1966) was used.

Triplicate samples of rat brain homogenate (1 ml) contained 1mM QA (100 µl) in the absence and presence of increasing concentrations (0-1mM) of NVP AND EFV (100 µl). The samples were incubated at 37 ^{o}C for an hour in an oscillating water bath. Control samples did not contain QA or test compounds. The reaction was terminated at the end of the incubation period by the addition of 1 ml TCA (15 % in milli-Q water) and 0.5 ml BHT (0.5 % in ethanol) to each sample. The samples were heated at 95 ^{o}C for 15 minutes in a water bath to release protein-bound MDA. Following this, samples were cooled and centrifuged at 2000 x g for 20 minutes to yield a protein-free supernatant. This supernatant (2 ml) was then transferred to a clean set of test tubes and 0.5 ml TBA (0.33 % in milli-Q water) was added. All samples were heated at 95 ^{o}C for an hour in a water bath to allow for the formation of the MDA-TBA complex. After rapidly cooling the test tubes on ice, 2 ml butanol was added to extract the pink complex. The samples were then centrifuged at 2000 x g for 15 minutes. An aliquot of the extracted complex in butanol (the top layer) was read at 532 nm using a GBC UV/VIS spectrophotometer. MDA levels were then determined from a standard curve generated from 1, 1, 3, 3-tetramethoxypropane as described in section 3.2.2.5. Final results were expressed as MDA (nmoles /mg tissue).

3.2.2.7. Statistical Analysis

This was performed as described in section 2.2.2.3.

3.2.3. Results

Figure 3.3. illustrates that 1 mM QA significantly increases the amount of MDA in comparison to control ($p < 0.001$), and both agents blunt the 1 mM QA-induced LP. The latter response from EFV proceeds in a concentration-dependent manner and

suppression of MDA is highly significant for each concentration of the drugs used in comparison to samples with QA alone. The drugs alone have no significant effect on LP ($p > 0.05$).

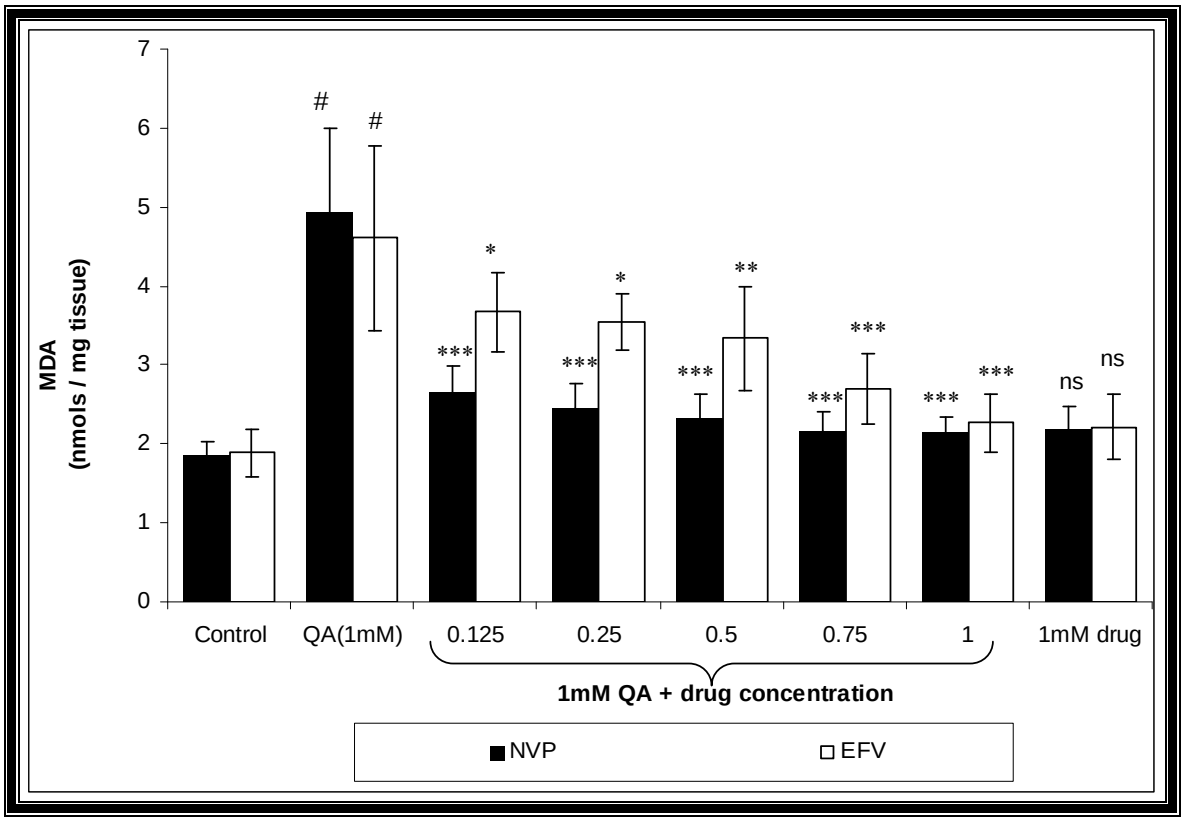

Figure 3.3. Effect of 1 mM QA alone and in combination with either EFV or NVP on LP in rat brain homogenate *in vitro*. Each bar represents the mean ± SD (n = 5). # ($p < 0.001$) and ns ($p > 0.05$) in comparison to control and * * * ($p < 0.001$); * * ($p < 0.01$) and * ($p < 0.05$) in comparison to 1 mM QA (ANOVA and Student–Newman–Keuls Multiple Range Test).

3.2.4. Discussion

Rios and Santamaria (1991) reported that the effect of QA on LP involves increased free radical generation and therefore QA-induced neurotoxicity could be partially attenuated by free radical scavengers (Stone *et al*., 1993; Tan *et al*., 1993; Behan *et al*., 1999). The ability of these agents to prevent the initiation of free radical-mediated

chain reactions associated with PUFAs and consequently LP possibly emanates from the possible free radical scavenging properties demonstrated in chapter 2.

The oxidising activity of QA on LP *in vitro* requires the obligatory presence of Fe^{2+} ions and this mechanism involves Fe^{2+}- chelation by QA (Stipek *et al*., 1997). Tissue homogenization releases metal ions especially Fe from storage sites within cells (Barber, 1963; Gutteridge and Stocks, 1976). Ultraviolet-visible absorption spectra have shown the ability of 2-pyridinecarboxylic moiety containing compounds such as QA to chelate Fe^{2+} ions (Figure 3.4) (Iwahashi *et al*., 1999).

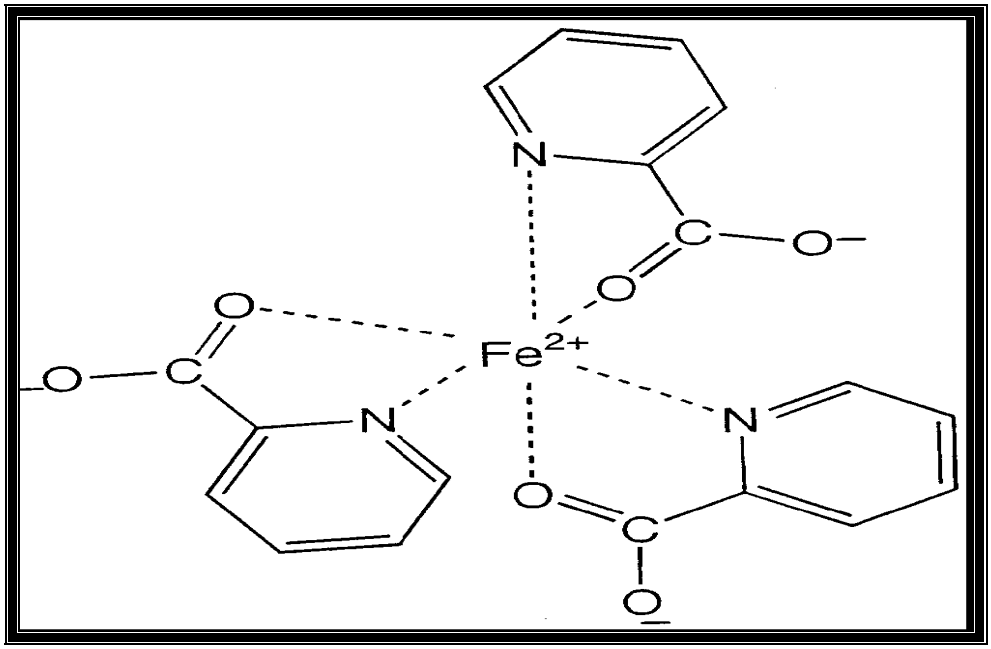

Figure 3.4. Structure of 2-pyridinecarboxylic acid moiety/ Fe^{2+} ion complex (Iwahashi *et al*., 1999).

Upon addition of H_2O_2 the visible band disappeared, indicating the oxidation of Fe^{2+} ions in the Fe^{2+} ion / 2, 6-pyridinecarboxylic complex (Iwahashi *et al*., 1999) to Fe^{3+} ions with the subsequent formation of the potent $HO^{\bullet}$ radical (Equation 3.1.). This radical is potent enough to initiate LP.

Fe^{2+} / 2, 6-pyridinecarboxylic + H_2O_2

$\rightarrow Fe^{3+} + OH^- + HO^{\bullet}$ + 2, 6-pyridinecarboxylic. **Equation 3.1.**

The ability of the QA-Fe^{2+} complex to inhibit the auto-oxidation of Fe^{2+} (Murakami *et al*., 1998), allows Fe to be kept in the ferrous form which participates in the Fenton reaction, hence weak chelation of Fe^{2+} by QA could enhance the Fenton reaction and subsequently, LP. Furthermore the potential of Fe^{3+} to undergo redox recycling (Gutteridge, 1994) in the presence of $O_2^{\bullet -}$ provides a constant supply of Fe^{2+} for the Fenton reaction.

Stipek and his co-workers in 1997 postulated that in the presence of desferoxamine, an Fe chelator, the increase in QA-induced LP is abolished. Therefore it is evident that the ability of these NNRTIs to ameliorate QA-induced LP is possibly related to an ability to strongly bind Fe^{2+} and/ or Fe^{3+}. If these agents strongly bind endogenous Fe^{2+} in rat brain homogenate, then fewer of these ions will be available to participate in the Fenton reaction and bind QA to form the QA-Fe^{2+} complex. Similarly strong Fe^{3+} binding by these NNRTIs would prevent the redox recycling of this ion and the subsequent formation of Fe^{2+}. Therefore the possibility of NVP and EFV binding Fe^{2+} and Fe^{3+} ions shall be investigated in Chapter 5.

3.3. THE EFFECT OF QA ALONE AND IN COMBINATION WITH NVP OR EFV IN THE RAT HIPPOCAMPUS *IN VIVO*.

3.3.1. Introduction

The intrahippocampal (i.h) injection of QA, induces neuronal lesions (Schwarcz *et al*., 1983) causing neurodegeneration (Southgate and Daya, 1999) through excitotoxicity and oxidative stress. Although both agents significantly ($p < 0.001$) protect against QA-induced LP *in vitro* (section 3.2.3.), this experiment aims to determine if NVP and EFV could alter the deleterious effects initiated by QA *in vivo* on lipid biomolecules.

3.3.2. Materials and Methods

3.3.2.1. Chemicals and Reagents

As described in section 3.2.2.1. Sodium phenobarbitone was purchased from Merck, Germany and was used at a concentration of 60 mg/ml.

3.3.2.2. Animals

Adult male Wistar rats were purchased from the South African Vaccine Producers (Johannesburg, South Africa) and cared for as described in section 3.2.2.1.

3.3.2.3. Drug Treatment

The rats were divided into four groups of five each (Table 1).The control and QA groups received PBS while the remaining two groups received daily doses of 5 mg kg^{-1} (0.25 mL) of drug (NVP or EFV) injected intraperitoneally (i.p), five days prior to i.h QA injection. On the 6^{th} day, after dosing the animals with the respective drug or vehicle, the animals were bilaterally injected with QA directly into the hippocampal region. QA was dissolved in PBS made up to pH 7.4. A dose of QA (120 nmol) was used to induce neurotoxicity as this concentration of QA is known to cause severe

behavioural disturbances and total loss of hippocampal neurons (Schwarcz *et al*., 1984). Following the i.h injections of QA, the animals in group 3 and 4 received subsequent daily doses of the drugs respectively, each day for five days, while as before; the animals in groups 1 and 2 received daily doses of drug vehicle (PBS) for five days.

Table 3.1. Treatment regime for each group of animals

Treatment group	**Daily i.p injection for 5 days prior to i.h injection.**	**i.h injection**	**Daily i.p injection for 5 days after stereotaxic surgery**
1 (control)	drug vehicle	2µL PBS	drug vehicle
2 (drug (-))	drug vehicle	120 nmol QA	drug vehicle
3 (drug (+))	5 mg kg^{-1} EFV	120 nmol QA	5 mg kg^{-1} EFV
4 (drug (+))	5 mg kg^{-1} NVP	120 nmol QA	5 mg kg^{-1} NVP

3.3.2.4. Surgical Procedures

3.3.2.4.1. Anesthesia

Diethyl ether anesthesia was employed for all surgical procedures carried out. Animals were placed, one at a time, in a desiccator containing cotton wool soaked in diethyl ether. Once the animals were sedated, and were removed and placed on the operating surface. A small conical flask containing cotton wool soaked in ether was placed approximately 3cm from the rats' nose. This flask remained in this position throughout surgery, except in cases where respiration became too weak. A good indication of the depth of anesthesia was monitored by the colour of the limbs and tail, which displayed a faint, almost pale pinkness. This was indicative of the optimum level of anesthesia, meaning a satisfactory rate and depth of respiration with good narcosis. A purple colour of the limbs was an indication of cyanosis.

3.3.2.4.2. Bilateral Intrahippocampal QA Injection

QA was injected intrahippocampally using stereotaxic surgical techniques. Each animal was anaesthetized as described above in section 3.3.2.4.1. QA was dissolved in phosphate buffered saline (PBS), pH= 7.4, and (120 nmol in 2 μL) was infused bilaterally into the hippocampii employing rat brain stereotaxic apparatus (Stoelting, IL, USA). The skull was orientated according to the König and Klippel stereotaxic atlas (1963). After a saggital cut in the skin of the skull, the bregma suture was located and bilateral holes were manually formed with a 21 gauge needle at the following coordinates; 4.0 mm anterior, 2.5 mm lateral to the saggital suture. Care was taken not to lesion the meninges. A Hamilton syringe, with a 26s gauge cannula with an outer diameter of 0.47 mm was used to inject 120 nmol of QA in 2 μL of PBS, pH= 7.4., 3 mm ventral of the dura. The rats in the control group were sham-lesioned with injections containing PBS only. Each injection was administered at a rate of 1 μL per minute and the cannula was left *in situ* for a further 2 minutes to allow for passive diffusion away from the cannula tip and to minimize spread into the injection tract. The cannula was then slowly removed and the scalp was closed with sutures. Animals were kept warm until recovery from the anesthesia.

3.3.2.4.4. Sham Lesioned Rats

The rats used as controls were subjected to the same surgical procedures described in section 3.3.2.3. However, stereotaxic injections into the hippocampus were free of QA and comprised solely of PBS.

3.3.2.4.5. Dissection of the Hippocampus

On the 6^{th} day following the i.h injection of QA, the brains were removed as described in section 3.2.2.3 and the hippocampii rapidly dissected according to a modified method of Glowinski and Iversen, (1966).

3.3.2.5. Preparation of Tissue

The preparation for each portion of the hippocampal tissue was performed as described for each whole brain in section 3.2.2.4. However, the homogenate concentration was 5 % w/v instead of 10 % w/v.

3.3.2.6. Lipid Peroxidation Assay

The assay was performed as described in section 3.2.2.6. However, no exogenous QA or test compound was added and the first incubation at 37 ^{o}C for an hour was omitted. The assay resumed with the termination of LP by addition of TCA and BHT to the triplicate samples of rat brain homogenate.

3.3.2.7. Statistical Analysis

This was performed as described in section 2.2.2.3.

3.3.3. Results

As shown in Figure 3.5., a two-fold increase ($p < 0.001$) in MDA levels in comparison to the control treated rats is observed following i.h. injection of QA (120 nmol). However, single daily i.p doses of either NVP or EFV (5 mg kg^{-1}) for five days are able to significantly reduce QA-induced MDA production ($p < 0.001$). Furthermore NVP significantly reduces LP to below basal control levels.

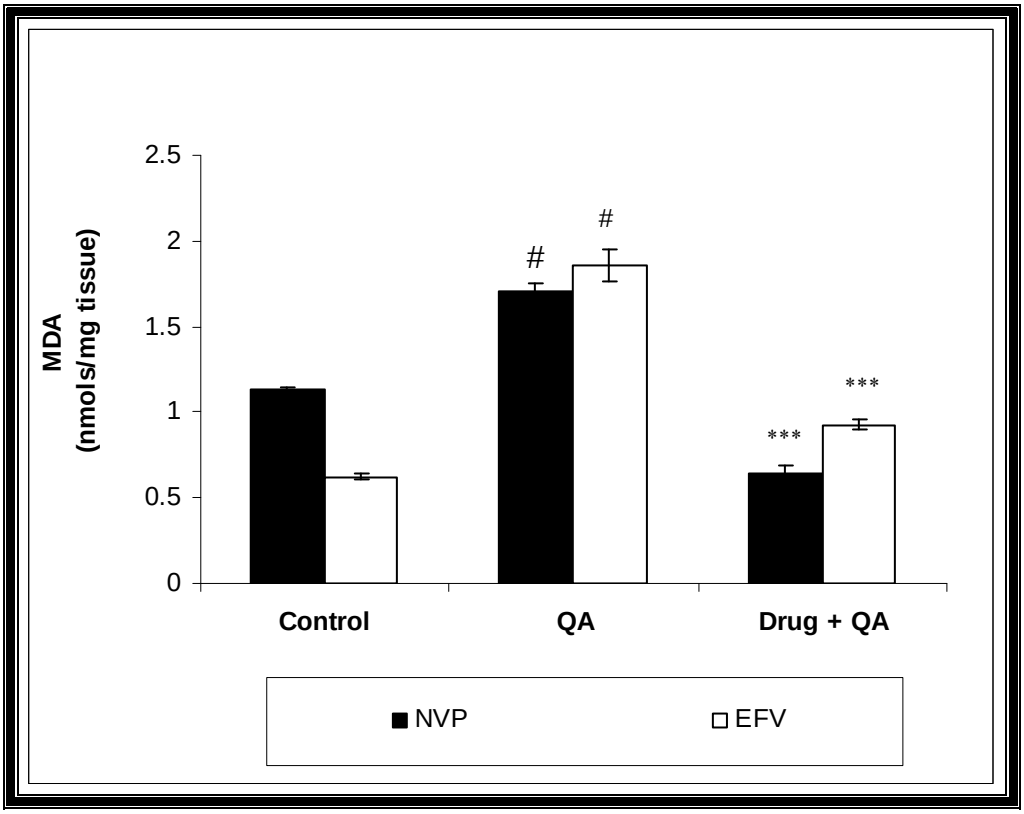

Figure 3.5. Effect of QA alone and in combination with NVP or EFV in rat hippocampal homogenate *in vivo*. Each bar represents the mean ± S.D; n = 5. # ($p < 0.001$) in comparison to control; * * * ($p < 0.001$) in comparison to QA (ANOVA and Student–Newman–Keuls Multiple Range Test).

3.3.4. Discussion

The potent *in vivo* induction of LP by QA is in agreement with the *in vitro* study discussed in section 3.2.3, which shows severe QA-induced LP in rat brain homogenate. The intrahippocampal administration of QA results in an increase in MDA level production (Figure 3.5.). These results are parallel to those of Santamaria and Rios (2003), who postulated that QA induces LP in the CNS by overstimulating NMDA receptors resulting in excessive intracellular Ca^{2+} influx, which sets off a cascade of events that culminate in free radical generation, subsequently causing neuronal damage. Free radical formation therefore significantly contributes to QA-induced damage. During LP, sulfhydryl groups on NMDA receptors of the

glutamatergic system in the hippocampus are damaged (Van der Vliet and Bast, 1992). Hence QA-induced LP *in vivo* may inactivate these receptors, consequently reducing the incidence of LTP at the glutamatergic synapses which have survived the excitotoxic insults (Müller, 2006). Although the amelioration of QA-induced LP by these agents could improve memory function, this possibility will be investigated in Chapter 8.

Antioxidants are necessary for preventing the formation of free radicals and they inhibit some of the deleterious actions of free radicals on lipids, DNA and proteins (Halliwell, 1996). The observed reduction in MDA levels in rats following administration of NVP and EFV is one indicator of the antioxidant and free radical scavenging activity of these agents.

Since high Fe levels accumulate in the basal ganglia following single unilateral injections of QA into rat ventral-striatal regions (Shoham *et al*., 1992), it is possible that intrahippocampal injections of QA cause a similar effect, consequently forming complexes with Fe which may enhance $HO^{\bullet}$ production and further initiate LP in this manner (Müller, 2006). The decrease in QA-induced MDA production demonstrated by these agents indicates a possibility of Fe binding and free radical scavenging activity. This further strengthens the argument that these NNRTIs have free radical scavenging activities.

Although NVP has been shown to effectively reduce LP associated with tissue injury (Strzelecki *et al*., 2001), there is no evidence of its antioxidant activity and biochemical effect in an *in vivo* animal study. It may be argued that binding of MDA, the product of QA-induced LP by NVP, produces MDA levels that are below those of the control. However, instead, NVP could possibly be competing with QA to bind NMDA receptors. This points to the possibility that NVP could act directly on these receptors or indirectly via second messenger processes that in turn influence NMDA receptor processes. Further studies have to be implemented in order to augment this possibility.

3.3.5. Conclusion

Although, QA significantly induces LP in rat brain homogenates, NVP and EFV have the ability to blunt this effect. Hence this study provides substantial and novel information regarding the potential of these agents as free radical scavengers in QA-induced neurodegenerative disorders.

CHAPTER 4

SUPEROXIDE ANION ASSAY

4.1. INTRODUCTION

The brain uses the most energy in the human body, accounting for 20 % of the total oxygen consumption despite accounting for only 2 % of the total body mass (Papa, 1996). The mitochondria consume nearly 85 % to 90 % of the cell's O_2 to support oxidative phosphorylation and subsequent synthesis of ATP.

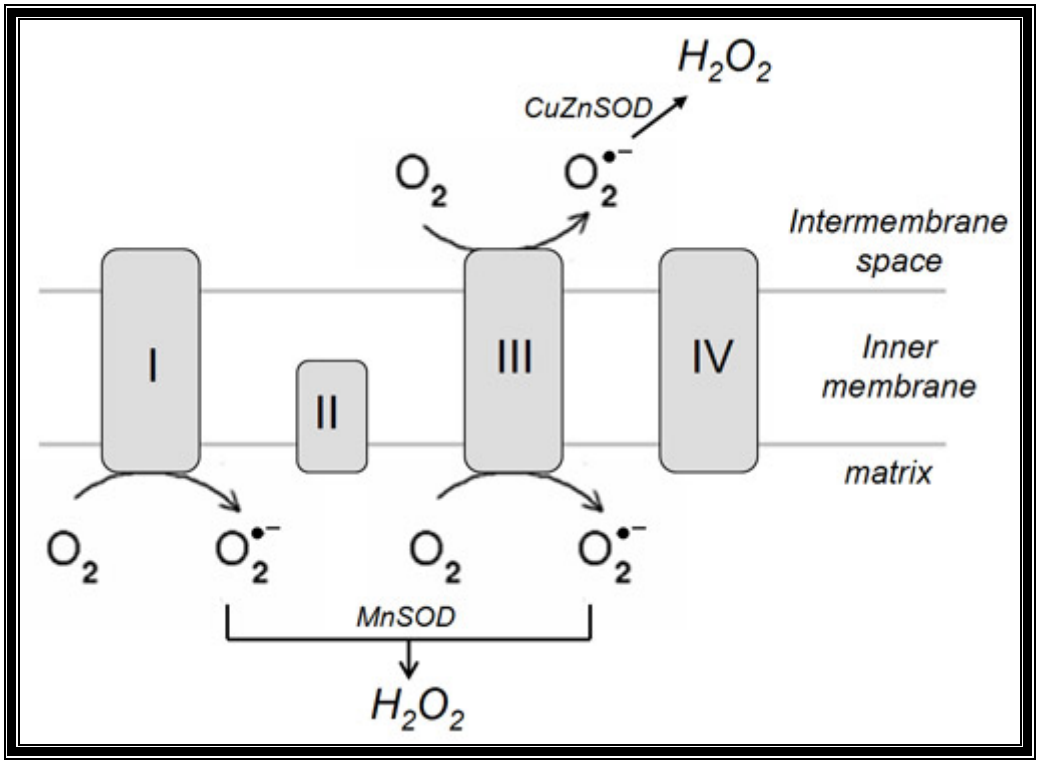

Figure 4.1. Mitochondrial superoxide production and disposition. (Szeto, 2006).

O_2 normally serves as the ultimate electron acceptor and is reduced to water. However, electron leakages through complexes I and III to O_2 generates ROS such as $O_2^{\bullet-}$ (Turrens, 1997) (Figure 4.1).

SODs such as MnSOD and CuZnSOD, catalyze the dismutation of $O_2^{\bullet-}$ to H_2O_2 and molecular O_2, hence maintaining intracellular concentrations of $O_2^{\bullet-}$ under control (Equation 4.1).

$$2O_2^{\bullet-} + 2H^+ \xrightarrow{\text{SOD}} H_2O_2 + O_2 \qquad \textbf{Equation 4.1}$$

ROS concentrations are governed by the balance between the production of ROS and their elimination by antioxidants. An appropriate balance is crucial to normal cell and tissue function. Under pathological conditions, enzyme activity is reduced, hence there exists an imbalance between the amount of $O_2^{\bullet-}$ formed and the ability of the enzymes to remove it and consequently leading to oxidative stress (Muscoli *et al*., 2003). Oxidative stress, leading to the formation of free radicals, has been implicated in a final common pathway for neurotoxicity in a wide variety of acute and chronic neurologic diseases (Lipton and Rosenberg, 1994). Excessive stimulation of excitatory amino acid receptors in these disorders may trigger the production of free radicals. In particular, neurotoxicity associated with overstimulation of NMDA receptors is thought to be mediated by an excessive Ca^{2+} influx, leading to a series of potentially neurotoxic events (Lipton and Rosenberg, 1994). Such events include NOS activation and Ca^{2+} overload of mitochondria, leading to the generation of more $O_2^{\bullet-}$ and NO (Lafon-Cazal *et al*., 1993).

An increasing amount of $O_2^{\bullet-}$ interacts with NO to yield the highly toxic ONOO− (Equation 4.2), which causes oxidative and nitrative damage to the mitochondria and ultimately leads to cell death pathways (Figure 4.2) (Radi *et al*., 1991; Van der Vliet et *a*l., 1994).

$$O_2^{\bullet -} + NO^{\bullet} \longrightarrow ONOO^{-} \qquad \textbf{Equation 4.2}$$

Excess $O_2^{\bullet -}$ leads to the formation of many other ROS, including $OH^{\bullet}$ and perhydroxyl radicals ($OH_2^{\bullet}$). At physiological pH, $O_2^{\bullet -}$ chemically dismutates to H_2O_2, which further reacts with $O_2^{\bullet -}$ in the Haber-Weiss reaction to form the highly destructive $OH^{\bullet}$ (Cheeseman and Slater, 2003). Accumulation of toxic free radicals increases the susceptibility of brain tissues to oxidative damage leading to either direct injury via membranous LP, protein and DNA oxidation or indirect damage through inflammation and apoptosis (Figure 4.2) (Traystman *et al*., 1991; Kuroda *et al*., 1997; Chan, 2001).

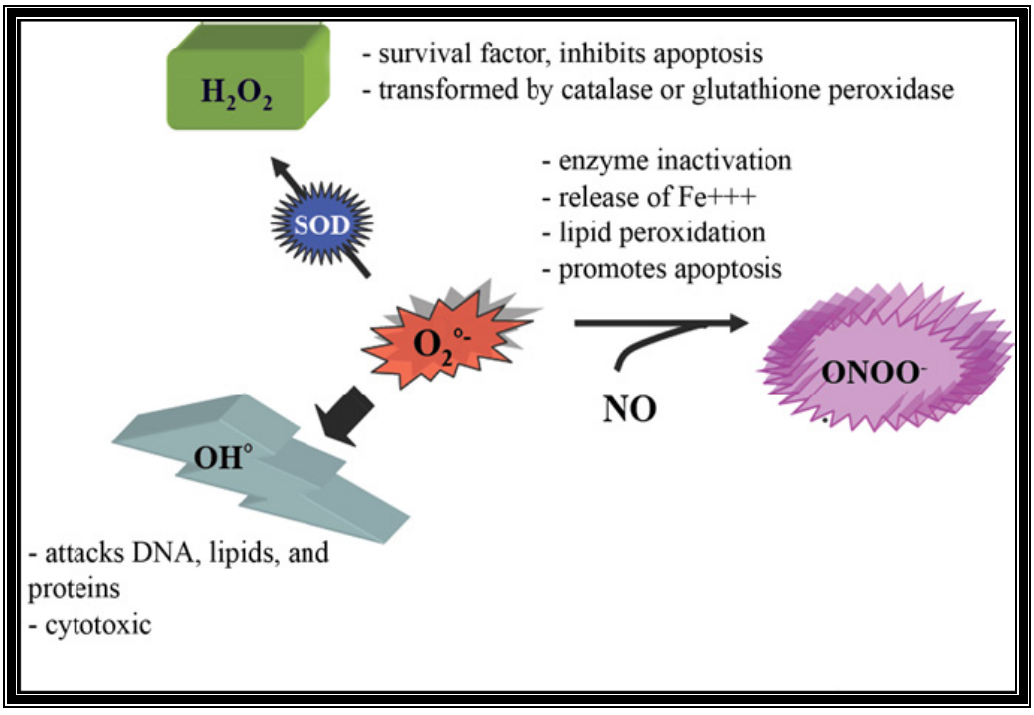

Figure 4.2. A diagram showing the effects of superoxide anion ($O_2^{\bullet -}$) and its derivates on biological molecules (Afonso *et al*., 2007).

One strategy to protect the brain is to decrease oxidative damage by scavenging the excessively produced toxic free radicals. The nitroblue tetrazolium (NBT) assay was used as a reliable method to assay for $O_2^{\bullet -}$ generated in the presence of the drugs in combination with toxic agents such as potassium cyanide (KCN) and QA. The principle behind the assay involves the reduction of NBT to water insoluble nitroblue diformazan (NBD), which can be extracted using glacial acetic acid and quantified by spectrophotometric analysis.

4.2. THE COMPARATIVE EFFECTS OF EFV AND NVP ON CYANIDE-INDUCED SUPEROXIDE ANION GENERATION IN THE RAT HIPPOCAMPUS *IN VITRO*.

4.2.1. Introduction

Cyanide, a well established respiratory poison, exerts its toxic effects by inhibiting cytochrome oxidase a_1a_3, the terminal electron acceptor enzyme of the mitochondrial electron transport chain (ETC) (Albaum *et. al*., 1946; Isom and Way 1984). These electrons have the capacity to leak out of the mitochondria resulting in the generation of free radicals, which exert destructive effects on cellular components with neurodegeneration being one such consequence. Other studies have shown that cyanide produces elevated levels of brain Ca^{2+} (Johnson *et. al*., 1987), which is associated with free radical-induced damage of neuronal membranes (Braughler *et. al*., 1985).

Antioxidant enzymes such as SODs, as well as antioxidant vitamins A, C and E protect biological membranes from the destructive effects of free radicals (Halliwell and Guteridge, 1989; Fahn and Cohen, 1992; Fang *et al*., 2002). Oxidative stress is believed to play an important role in cyanide induced neurotoxicity due to the inhibition of antioxidant enzymes by cyanide (Ardelt *et. al*., 1989). Such neurotoxicity has been recognized to cause cellular anoxia in the brain (Ballantyne, 1987; Yamamato and Tang, 1996) and dopaminergic toxicity accompanied by impaired motor function (Gunasekar *et. al*., 1996). Hence, the administration of antioxidants to cyanide toxified animals should have ameliorating influences on the severity of the resultant tissue damage.

The objective of this study was to therefore ascertain the protective effects of NVP and EFV against cyanide induced $O_2^{\bullet -}$ in rat brain homogenate.

4.2.2. Materials and Methods

4.2.2.1. Chemicals and Reagents

KCN, nitroblue diformazan (NBD), nitroblue tetrazolium (NBT) and QA were purchased from Sigma Chemical Corporation, St Louis, MO, U.S.A. Glacial acetic acid and ethanol was purchased from Saarchem, Johannesburg, South Africa. All other chemicals used were of the highest quality available from commercial sources.

4.2.2.2. Animals

Adult male rats of the Wistar strain, weighing between 250-300 g were purchased from the South African Vaccine Producers (Johannesburg, South Africa). The animals were housed in a controlled environment with a 12-hour light: dark cycle, and were given access to food and water ad libitum. The Rhodes University animal ethics committee approved protocols for the experiments

4.2.2.3. Brain Removal

Rats were sacrificed and the brains removed s described in section 3.2.2.3.

4.2.2.4. Preparation of Tissue

Each brain was weighed and rapidly homogenized in a glass-teflon hand held homogenizer in ice cold 0.1 M PBS at pH 7.4 to obtain a homogenate concentration of 10 % w/v. This is necessary to prevent lysosomal damage of the tissue. PBS buffer was used as it has been shown not to scavenge free radicals (Anoopkumar- Dukie *et al*., 2001).

4.2.2.5. Preparation of the NBD Standard Curve

Nitroblue diformazan (NBD) was used as a standard. A series of NBD standards (0-400 nmoles/ml) were prepared in triplicate using glacial acetic acid as a diluent to give a final volume of 1 ml. A calibration curve was generated by measuring the

absorbance at 10 μM intervals at 560 nm using a GBC UV/VIS 916 Spectrophotometer and plotting these against the molar equivalent weight of NBD (Appendix II).

4.2.2.6. Nitroblue Tetrazolium Assay

The assay procedure was a modification of Das *et al*., 1990; Ottino and Duncan, 1997. Samples of rat brain homogenate (1 ml), in triplicate, contained 1 mM KCN (250 μL) in the absence and presence of increasing concentrations of either NVP or EFV (250 μL) at final concentrations of 0.125, 0.25, 0.5, 0.75 and 1 mM and 0.4 ml NBT (0.1 % dissolved in ethanol and then made up to the required volume with milli-Q water). The samples were incubated in an oscillating water bath for 1 hour at 37 ^{o}C. Control samples did not contain KCN, NVP or EFV.

The reaction was terminated and the NBD formed extracted by centrifuging the samples for 10 minutes at 2000 x g followed by resuspension of the pellets with 2 ml of glacial acetic acid. To remove insoluble debris, the samples were centrifuged for 5 minutes at 2000 x g. An aliquot of the supernatant (extracted NBD in glacial acetic acid) was read at 560 nm using a GBC UV/VIS Spectrophotometer. NBD levels were then determined from a standard curve generated as described in section 4.2.2.5. Final results were expressed as diformazan (nmoles /mg tissue).

4.2.2.7. Statistical Analysis

This was performed as described in section 3.2.2.7.

4.2.3. Results

Figure 4.3. illustrates that 1 mM KCN has a significant effect ($p < 0.001$) on $O_2^{\bullet-}$generation *in vitro*, in comparison to the control. Co-incubation of rat brain homogenate with EFV and NVP significantly ($p < 0.001$) reduces this effect. These drugs alone have no effect on $O_2^{\bullet-}$ generation.

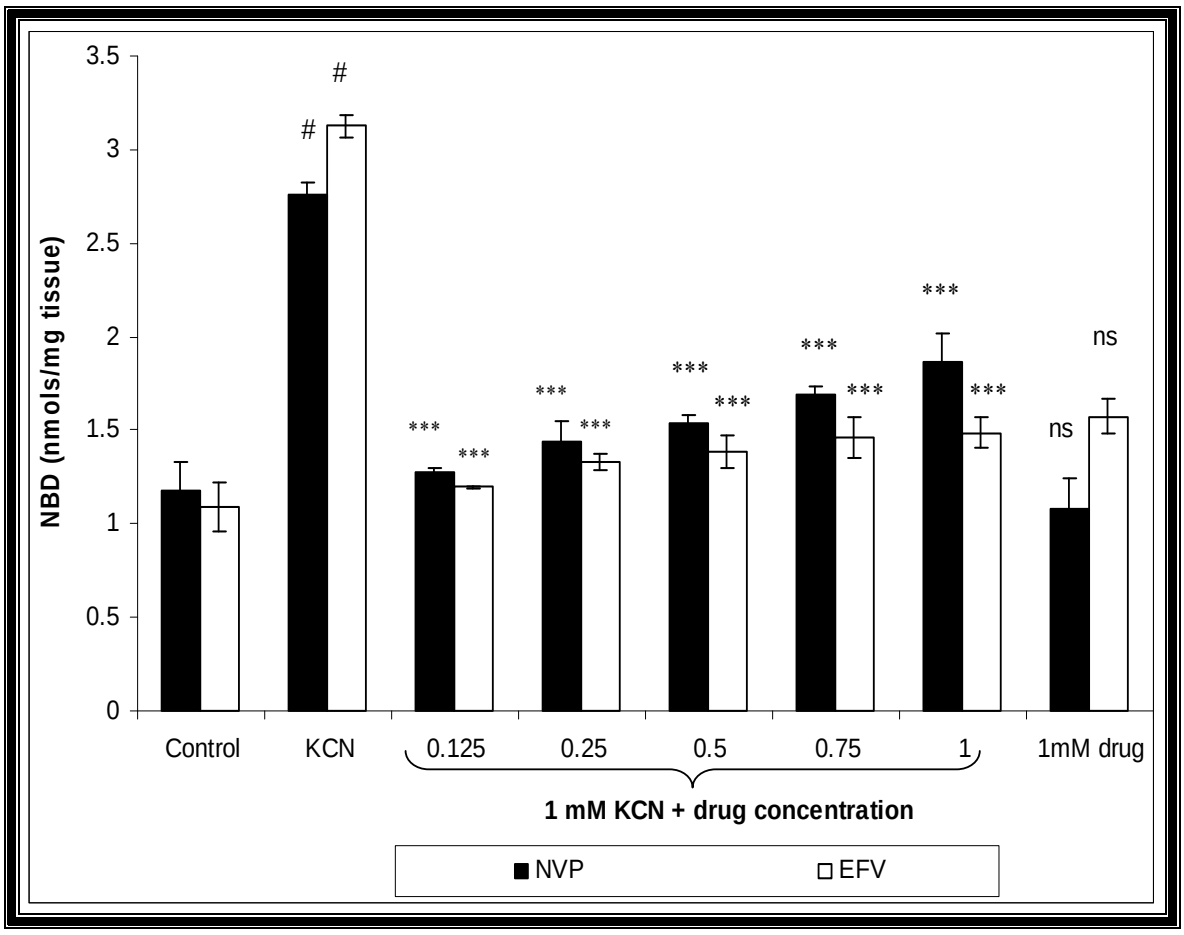

Figure 4.3. The effect of NVP and EFV on 1 mM KCN-induced $O_2^{\bullet-}$ generation in whole rat brain homogenate *in vitro*. Each bar represents the mean ± S.D; (n=5). # ($p < 0.001$) in comparison to control; *** ($p < 0.001$), ** ($p < 0.01$), * ($p < 0.05$) and ns ($p > 0.05$) in comparison to KCN (ANOVA and Student-Newman-Keuls Multiple Range Test).

4.2.4. Discussion

The results from this chapter demonstrate that KCN is a potent neurotoxin which results in the generation of $O_2^{\bullet-}$ *in vitro* as evident from Figure 4.3. Cyanide inhibits antioxidant enzymes and disrupts the mitochondrial ETC (Way, 1984), and consequently the homeostatic ATP-dependent Na^{2+}/ K^{+} and Ca^{2+} pumps. This leads to increased levels of intracellular Ca^{2+}, which activate numerous neuronal Ca^{2+}-

dependent events and ultimately resulting in oxidative stress and free radical generation that will cause cellular damage (Southgate and Daya, 1999; Andreyev *et al*., 2005).

As stated earlier, the destructive effects exerted on cellular components by ROS, culminate in neurodegenerative diseases. Free radical scavengers, therefore become increasingly important as a means of reducing or preventing the effects of ROS and their respective inducers. Results from Figure 4.3, clearly indicate that both agents significantly reduce the conversion of NBT to NBD by the $O_2^{\bullet -}$. These agents reduce the levels of $O_2^{\bullet -}$either by possibly reducing and / or preventing the influx of Ca^{2+} or scavenging these free radicals. The possibility that NVP and EFV may prevent the influx of Ca^{2+} into the mitochondria and reduce $O_2^{\bullet -}$ generation needs to be further investigated.

This study confirms that these agents possess antioxidant properties in the presence of the neurotoxin, KCN, in rat brain homogenate *in vitro*. However these properties were further investigated to ascertain antioxidant effects *in vivo*. Since it is not possible to use KCN *in vivo*, it was decided to investigate the antioxidant effects *in vivo* using QA.

4.3. THE COMPARATIVE EFFECTS OF EFV AND NVP ON QA-INDUCED SUPEROXIDE RADICAL GENERATION IN THE RAT HIPPOCAMPUS *IN VIVO*.

4.3.1. Introduction

QA induced neurotoxicity results from the activation of ion channels, leading to the influx of Ca^{2+} into cells (Stone, 1993) and consequently free radical generation (Southgate and Daya, 1999). Among the ROS produced by these cells, $O_2^{\bullet -}$ plays a key role in oxidative chain reactions, yielding highly reactive oxidants such as $OH^{\bullet}$ that are extremely toxic for neurons and endothelial cells (Cadenas and Davies, 2000; Chan, 2001). Since NVP and EFV significantly reduce QA-induced LP in experiments 3.2 and 3.3, it was decided to determine whether these agents could reduce or prevent the QA-induced $O_2^{\bullet -}$ generation in rat hippocampal tissue. The concentration of QA, NVP and EFV used in this experiment was similar to that used in experiment 3.3.

4.3.2. Materials and Methods

4.3.2.1. Chemicals and Reagents

As per section 4.2.2.1. In addition, Quinolinic acid (2, 3-pyridinedicarboxylic acid) and diethyl ether were purchased from Sigma Chemical Corporation, St Louis, MO, U.S.A. All other chemicals used were of the highest quality available from commercial sources. QA was prepared by dissolving in Milli-Q water.

4.3.2.2. Animals

Adult male Wistar rats were purchased from the South African Vaccine Producers (Johannesburg, South Africa) and cared for as described in section 4.2.2.2.

4.3.2.3. Dosing of Animals

As described in section 3.3.2.2.

4.3.2.4. Surgical Procedures

These procedures were conducted as described in section 3.3.2.3

4.3.2.5. Nitroblue Tetrazolium Assay

The assay procedure was a modification of Das *et al*., 1990; Ottino and Duncan, 1997. Homogenate (1 ml) was incubated with 0.4ml of 0.1 % NBT in a shaking water bath for 1hour at 37 ^{o}C. The remainder of the experiment was conducted as described in section 4.2.2.6.

4.3.2.6. Statistical Analysis

Final results were expressed as nmoles of Diformazan/ mg tissue and analyzed for statistical significance as described in section 2.2.2.3.

4.3.3. Results

The intrahippocampal infusion of QA (120 nmols) significantly ($p < 0.001$) increases $O_2^{\bullet -}$ generation (Figure 4.4). The treatment of the animals with either NVP or EFV markedly ($p < 0.001$) reduces the QA-induced $O_2^{\bullet -}$ generation.

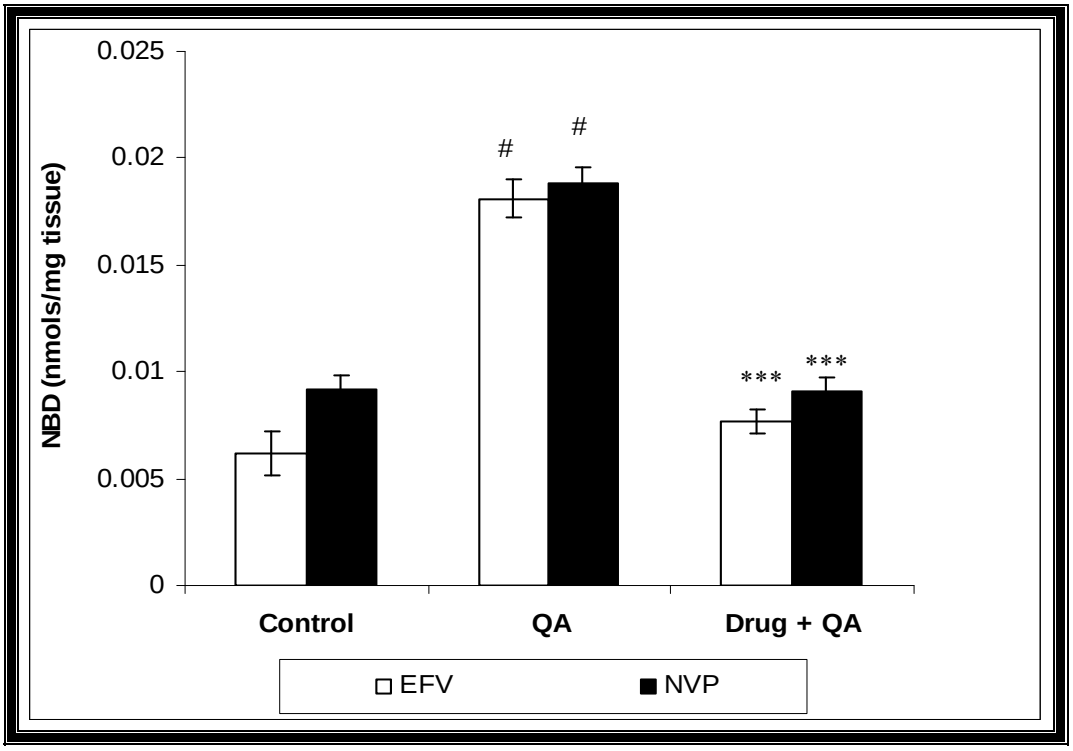

Figure 4.4. The effect of NVP and EFV on QA-induced $O_2^{\bullet -}$ generation in rat hippocampus. Each bar represents the mean ± S.D. (n=5). # ($p < 0.001$) in comparison to control; *** ($p < 0.001$) in comparison to QA (ANOVA and Student-Newman-Keuls Multiple Range Test).

4.3.4. Discussion

Free radical destruction of neurons has been linked to a number of neurological diseases and to the normal aging processes of the CNS (Bonilla *et al*., 1999; Gilad *et al*., 1997). The results show that intra-hippocampal injections of QA cause a significant induction of $O_2^{\bullet -}$generation and LP in the rat hippocampus. The significant *in vivo* pro-oxidant effects of QA are attributed to sustained stimulation of NMDA receptors (Stone and Perkins, 1981; Stone, 1993; Santamaría *et al*., 2003; Pérez-Severiano *et al*., 2004,) which results in Ca^{2+} dependent increase in oxidative stress (Hartley *et al*., 1993; Atlante *et al*., 1997; Perez Velazquez *et al*., 1997) and mitochondrial dysfunction (Hengartner, 1998; Beal, 2000) which induces $O_2^{\bullet -}$ production (Choi 1992). It has previously been shown that QA induces $O_2^{\bullet -}$

generation in rat hippocampus (Schwarcz *et al*., 1983; Santamaria *et al*., 2001b) and treatment of the animals with antioxidants reduces or prevents the QA-induced neurotoxicity (Sies, 1997; Pérez-Severiano *et al*., 2004). The results of the present study show that both agents significantly reduce QA-induced $O_2^{\bullet-}$ generation (Figure 4.4). The alteration in Ca^{2+} levels by QA, which results in the generation of ROS, could therefore be attenuated by these NNRTIs, which would also explain the potent action exhibited by these agents observed in Figure 4.4. Possible mechanisms by which NVP and EFV exhibit neuroprotective properties include the reduction of Ca^{2+} dependent oxidative stress by either preventing the generation of the $O_2^{\bullet-}$ or scavenging the free radicals generated. These findings demonstrate an ability of both agents to mitigate radical-induced brain injury. The present study reports the antioxidant and protective role of these agents against QA-induced neurotoxicity in rat brain, thus supporting the hypothesis that NNRTIs have the ability to protect against HIV-induced neurodegeneration.

4.3.5. Conclusion

NVP and EFV have been shown, in the present study, to possess potent antioxidant activity at inhibiting both KCN and QA-induced $O_2^{\bullet-}$generation. The results of this study not only show that NVP and EFV limit the undesirable effects of QA, but provide confirmation, of the antioxidant properties previously shown by these agents in chapter 2, section 2.2.3. This study therefore, suggests that the antioxidant properties of these agents could be exploited as a possible therapeutic approach against HIV-induced neurodegenerative disorders.

CHAPTER 5

IRON CHELATION STUDIES

5.1. INTRODUCTION

In biological systems, Fe usually exists in the form of Fe^{2+} and Fe^{3+}, with Fe^{3+} being relatively biologically inactive. However, it can be reduced to Fe^{2+}, (Strlic *et al*., 2002), and oxidized back through Fenton type reactions, with production of $HO^{\bullet}$, or Haber–Weiss Cycle reactions with $O_2^{\bullet -}$ (Kehrer, 2000).

Fe is required in the CNS to facilitate important cellular functions including electron transport, myelination of axons, and synthesis of neurotransmitters. Fe deficiency, as well as Fe overload, can be deleterious to the CNS, and balancing the beneficial and harmful effects is an essential aspect of cell survival. Fe, being the most abundant transition metal in the brain, is considered the most potent potential toxin. The increased level of brain tissue Fe has been implicated as a major generator of ROS which are capable of damaging biological molecules such as lipids, carbohydrates, proteins, and nucleic acids (Riederer *et al*., 1989; Youdim *et al.*, 1993). Oxidative stress resulting from the increased Fe levels, and possibly also from defects in antioxidant defence mechanisms, is widely believed to be one of the causes responsible for neuronal death in neurodegenerative diseases (Riederer and Lange, 1992; Dawson and Dawson, 1996).

Redox-dependent reactions have proven to be important in regulating numerous processes that determine the physiological and pathophysiological function of cells and tissues. Consistently, strategies to modulate intracellular redox status by antioxidants and other redox enhancing agents show remarkable therapeutic potential (Sen, 1998).

Chelating agents may inactivate metal ions and potentially inhibit and/or decrease the metal-dependent processes and incidences associated with neurodegeneration (Finefrock *et al*., 2003).

In 1997, Stipek and co-workers postulated that *in* vitro, QA induces LP by complexing with Fe in brain homogenates. The direct interaction and possible complexation of Fe with NVP and EFV as a possible mechanism for reducing LP was investigated using the TBA assay, ferrozine assay and electrochemical analysis.

5.2. THE EFFECT OF IRON (II) ALONE AND IN COMBINATION WITH EFV OR NVP ON LIPID PEROXIDATION IN RAT BRAIN HOMOGENATE *IN VITRO*.

5.2.1. Introduction

The brain in addition to having an abundant supply of PUFAs also contains a high content of transition metals such as copper and Fe in several regions (Halliwell and Gutteridge, 1989; Calabrese *et al*., 2000). In biological systems Fe acts as the LP pro-oxidant via the Fenton reaction through the formation of the LP initiating $OH^{\bullet}$ (Halliwell and Gutteridge, 1984; Miller, 1996) as shown below in equation 5.1.

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\bullet} + OH^{-} \qquad \textbf{Equation 5.1.}$$

The Fenton reaction occurs in the presence of ethylenediamine tetraacetate (EDTA) which complexes Fe^{3+} to form a soluble complex which generates $OH^{\bullet}$ and initiates LP in the presence of reducing agents such as ascorbate, which promote the redox recycling of soluble Fe complexes (Rauhala *et al*., 1998).

The Fenton reaction has been implicated in the lipoperoxidative effect of QA *in vitro*, and in section 3.2.3., NVP and EFV significantly reduce QA-induced LP *in vitro*. Therefore the direct effects of these NNRTIs on the Fenton reaction in LP were investigated.

5.2.2. Materials and Methods

5.2.2.1. Chemicals and Reagents

Chemicals and reagents used were as described in section 3.2.2.2., with the exception of QA. In addition, ferrous sulphate ($FeSO_4.7H_2O$) was obtained from Merck, Darmstadt, Germany. EDTA was obtained from Holpro Chemical Corp., Johannesburg, South Africa. H_2O_2 was obtained from BDH Laboratory Supplies, Pool, England and ascorbic acid was obtained from Saarchem, Krugersdorp, South Africa.

5.2.2.2. Animals

As described in section 3.2.2.1.

5.2.2.3. Brain Removal

This was performed as described in section 3.2.2.3.

5.2.2.4. Preparation of Tissue

As described in section 3.2.2.4.

5.2.2.5. Preparation of the MDA Standard Curve

As described in section 3.2.2.5.

5.2.2.6. Lipid Peroxidation Assay

The assay was performed as described in section 3.2.2.6. However, triplicate samples of rat brain homogenate (1 ml) containing 1mM Fe^{2+} (100 µL) in the absence and presence of increasing concentrations (0-1 mM) of NVP and EFV (100 µL), together with 0.2 mM H_2O_2 (100 µL), 100 µM EDTA (100 µL) and 1 mM ascorbic acid (100

μL) incubated in a shaking water bath for an hour at 37 ^{o}C. Control samples had no Fe^{2+} or the test compound.

5.2.2.7. Statistical Analysis

This was performed as described in section 2.2.2.3.

5.2.3. Results

Figure 3.5. illustrates that 1 mM Fe^{2+} in the presence of H_2O_2 and ascorbate, increases the amount of MDA produced in comparison to the control. EFV has the ability to significantly ($p < 0.001$) reduce 1 mM Fe^{2+}-induced LP in a concentration dependent manner. In the presence of 1 mM Fe^{2+}, NVP has no significant effect on LP in comparison to samples containing 1 mM Fe^{2+} alone.

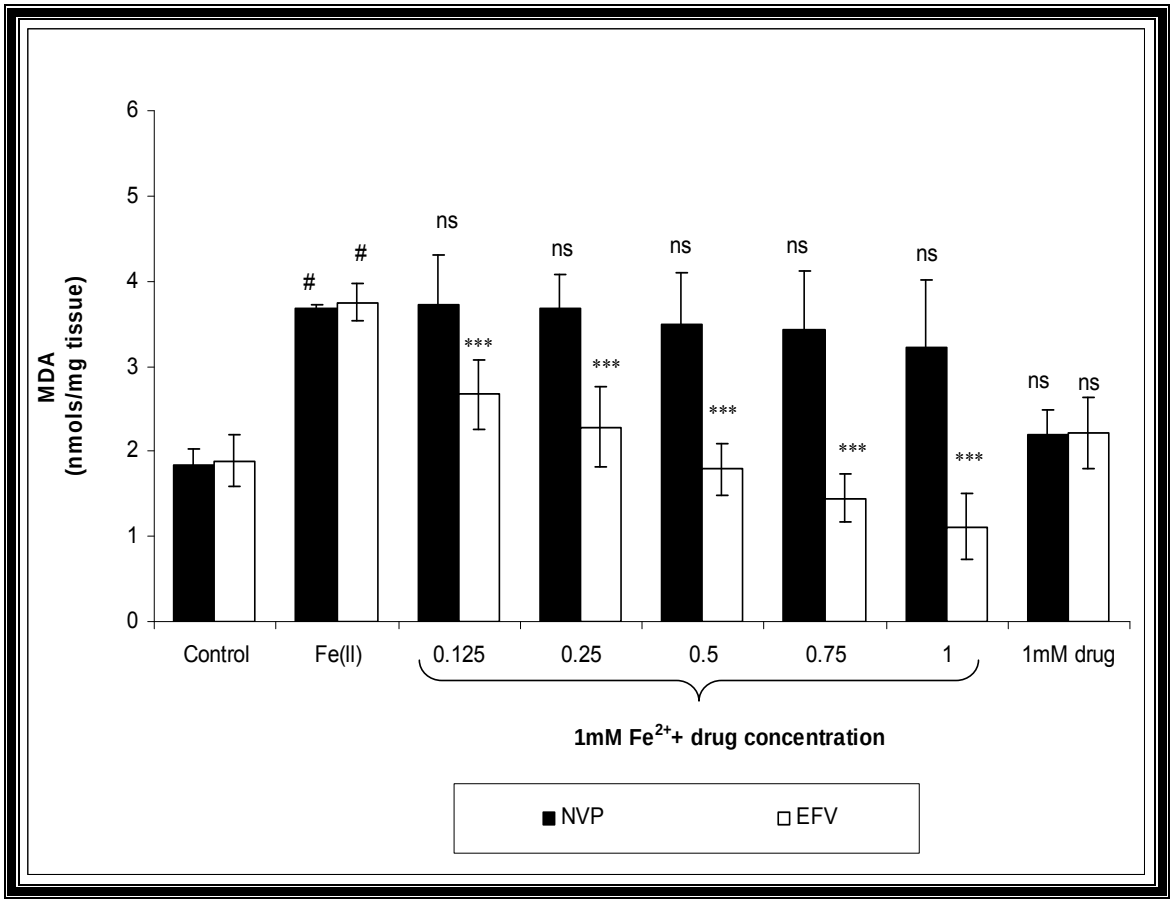

Figure 5.1. Effect of 1 mM Fe^{2+} alone and in combination with NVP or EFV on LP in rat brain homogenate *in vitro*. Each bar represents the mean ± S.D; n = 5. # ($p < 0.001$) and ns ($p > 0.05$) in comparison to control; * * * ($p < 0.001$) and ns ($p > 0.05$) in comparison to 1 mM Fe^{2+} alone (ANOVA and Student–Newman–Keuls Multiple Range Test).

5.2.4. Discussion

A combination of Fe and ascorbate represents a physiologically relevant pro-oxidant system in the brain (Sen *et al*., 2006), and the ability of 1 mM Fe^{2+} to produce

significant amounts of MDA in comparison to control indicates the potential role demonstrated by the Fenton reaction on LP.

By-products of LP such as LOOH (lipid hydroperoxide) react with EDTA-Fe^{3+} / QA-Fe^{2+} complexes and decompose to alkoxyl and peroxyl radicals (Halliwell and Gutteridge, 1989) as shown in equations 5.2.and 5.3. These radicals together with $OH^{\bullet}$ produced during the Fenton reaction and in the presence of Fe^{2+} and Fe^{3+} ions can abstract $H^{\bullet}$ from PUFAs in membranes, further stimulating LP.

$$LOOH + Fe^{2+}\text{-complex} \rightarrow Fe^{3+}\text{-complex} + OH^{-} + \underset{\text{alkoxyl radical}}{L\text{-}O^{\bullet}} \qquad \textbf{Equation 5.2.}$$

$$LOOH + Fe^{3+}\text{-complex} \rightarrow \underset{\text{peroxyl radical}}{L\text{-}O\text{-}O^{\bullet}} + H^{+} + \underset{\text{further reaction to alkoxyl radical}}{Fe^{2+}\text{-complex}} \qquad \textbf{Equation 5.3.}$$

The finding that NVP shows no significant effect ($p > 0.05$) on Fe^{2+}-induced LP in comparison to QA-induced LP (section 3.2.3.), indicates that NVP reduces LP only via free radical scavenging and not by interference with the Fenton reaction. The ability of NVP to scavenge free radicals was demonstrated in section 2.2.3, and in chapter 4.

Since EFV significantly reduces Fe^{2+}-induced LP ($p > 0.001$), the argument that EFV may be binding Fe^{2+} and/ or Fe^{3+} ions is strengthened. The concentration dependent decrease that occurs could also be attributed to scavenging of free radicals, particularly those associated with Fe^{2+}-induced LP such as alkoxyl radicals, $HO^{\bullet}$ and $O_2^{\bullet -}$, as shown in Chapter 4.

5.3. THE COMPARATIVE EFFECTS OF EFV AND NVP ON THE INHIBITION OF THE FERROZINE-IRON (II) COMPLEX.

5.3.1. Introduction

Ferrozine (Figure 5.2.) forms purple complexes with Fe^{2+} ions (Figure 5.3.) in a quantitative manner (Decker and Welch, 1990; Dinis *et al*., 1994) with a metal to ligand chelation ratio of 1 to 3 (Figure 5.3.). In the presence of chelating agents, the formation of the Fe^{2+}- ferrozine complex is reduced or inhibited resulting in a decrease in the colour of the complex.

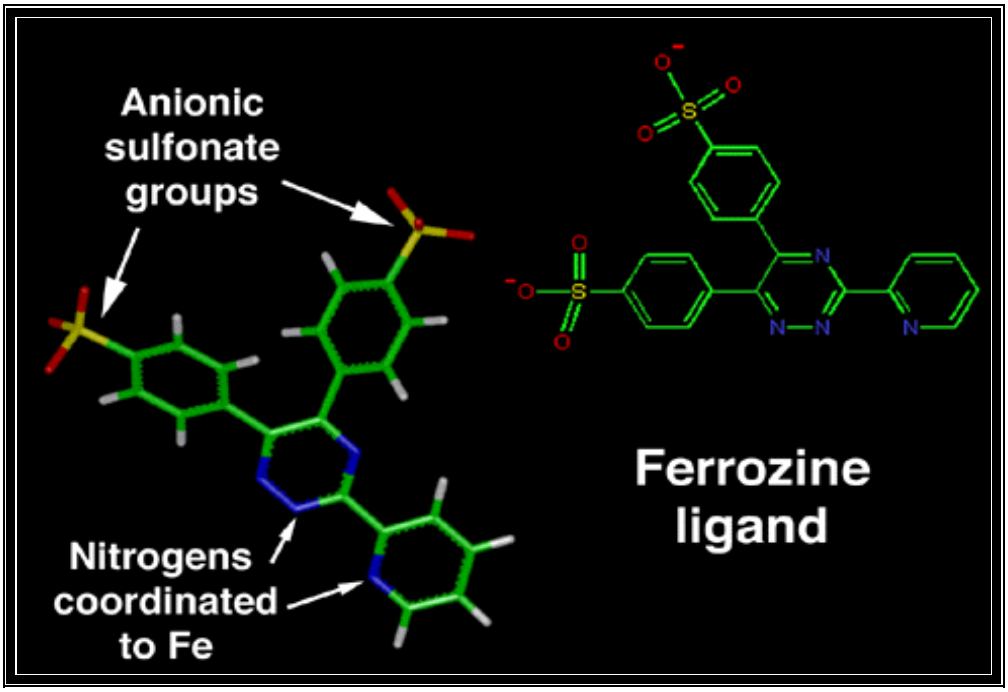

Figure 5.2. Representation of the ferrozine ligand. The left figure is a stick representation whereas the right figure is a ChemDraw® representation of the ligand. The carbon atoms are green, the hydrogens are white, the nitrogen atoms are blue, the oxygen atoms are red, and the sulphur atoms are yellow. (http://www.chemistry.wustl.edu/~edudev/Ferritin/FerritinGraphics/ferrozine_net.gif)

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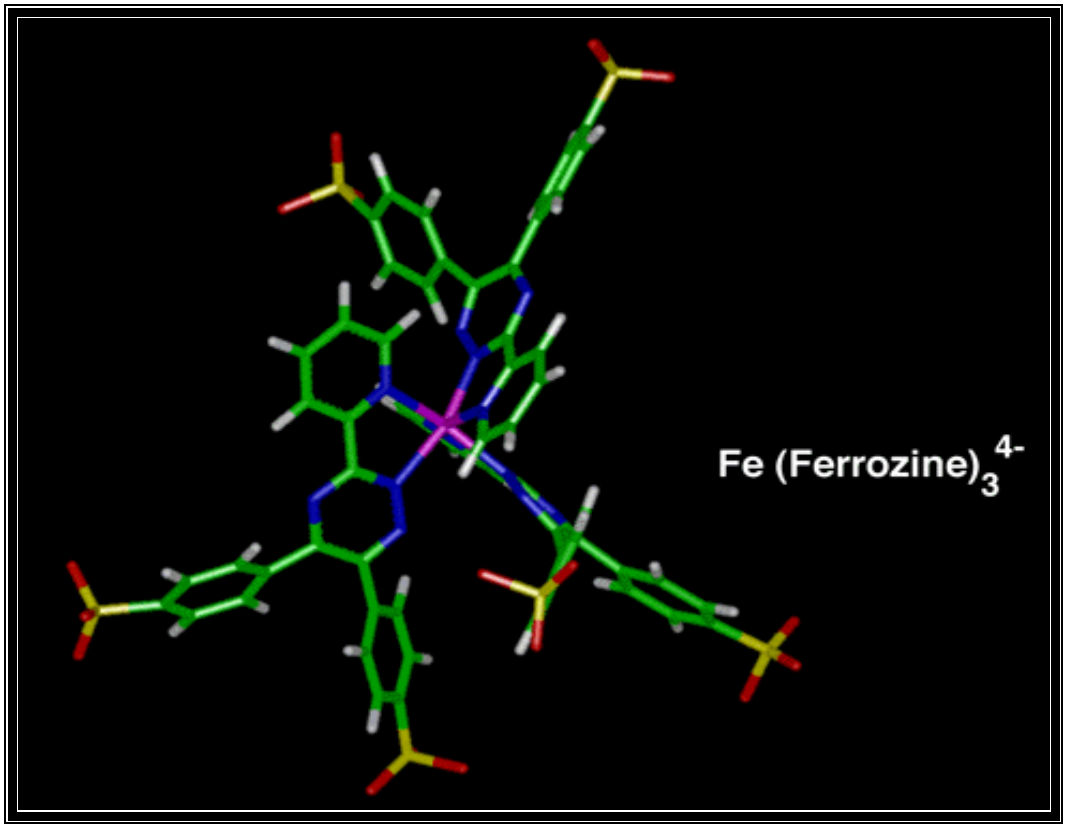


Figure 5.3. Molecular stick representation of the Fe^{2+}-ferrozine complex where Fe^{2+} is complexed with three (3) ferrozine ligands. The carbon atoms are green, the hydrogens are white, the Fe^{2+} is purple, the nitrogen atoms are blue, the oxygen atoms are red, and the sulphur atoms are yellow. (http://www.chemistry.wustl.edu/~edudev/LabTutorials/Ferritin/FerritinGraphics/fefe rr_net.gif).

Measurement of colour reduction allows estimation of the chelating activity of the coexisting chelator (Yamaguchi *et al*., 2000). The chelation of Fe^{2+} ions, which are required for free radical production by the Fenton reaction (Equation 5.1.), could be of significant relevance in the clinical therapeutics of progressive neurodegenerative disorders (Cuajungco *et al*, 2000). Therefore, the ability of NVP and EFV to chelate Fe^{2+} was investigated using the ferrozine assay.

5.3.2. Materials and Methods

5.3.2.1. Chemicals and Reagents

NVP and EFV were kindly supplied by Aspen Pharmaceutical Company, South Africa. Ferrous chloride ($FeCl_2$) and 3-(2-Pyridyl)-5, 6-bis (4-phenyl-sulfonic acid)-1, 2, 4-triazine (Ferrozine) were purchased from the Sigma Chemical Corporation, St. Louis, MO, U.S.A.

5.3.2.2. Chelating Activity of NVP and EFV

The chelating activity on Fe^{2+} ions by NVP and EFV was measured by the method of Decker and Welch, (1990). Briefly, 1 ml solution containing either 100 or 200 μM EFV or NVP was added to 3.7 ml of H_2O. This mixture was then reacted with 0.1 ml of 2 mM $FeCl_2$ and 0.2 ml of 5 mM ferrozine. The absorbance of the solutions was measured spectrophotometrically at 562 nm at different time intervals for 90 minutes. The lower absorbance of the mixture indicates higher chelating activity.

The percentage chelation activity on the Fe^{2+}- ferrozine complex with time, was calculated using the following equation: Chelating activity (%) = $\{(A_o - A_1)/A_o\}$ x 100, where A_o is the absorbance of the control reactions and A_1 is the absorbance in the presence of the test compounds.

5.3.3. Results

Figure 5.4. shows that both agents have the capacity to sequester Fe^{2+} and hence inhibit the formation of the Fe^{2+} - ferrozine complex. EFV has a higher chelating potency than NVP since at 200 μg/ml EFV chelates approximately 10 % of the Fe^{2+} ions while NVP chelates approximately 7 %.

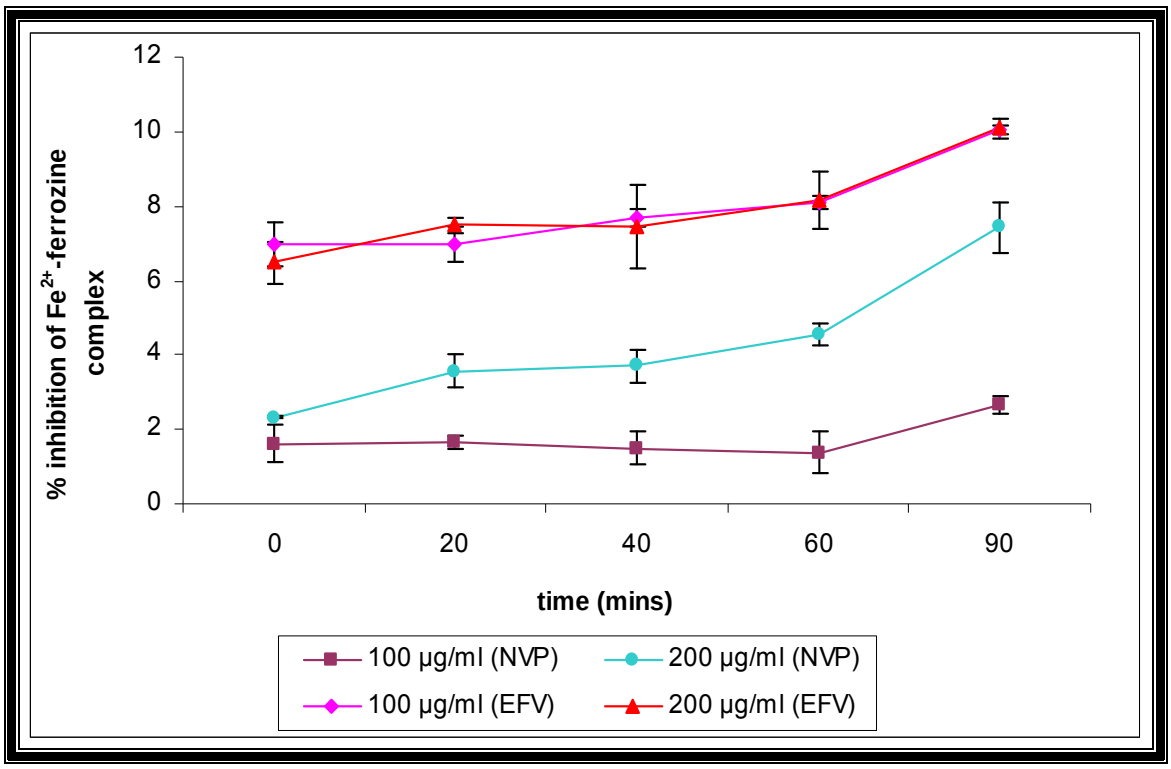


Figure 5.4. Percentage inhibition of Fe^{2+}-ferrozine complex by NVP and EFV. Each point represents the mean ± S.D. (n=3).

5.3.4. Discussion

Antioxidants prevent or delay oxidative damage of biomolecules through scavenging radicals and binding to the metal ions. In this assay both agents reduce the formation of the Fe^{2+}-ferrozine complex (Figure. 5.4.), suggesting the chelation of Fe^{2+} ions. At both concentrations (100-200 µg/ml) EFV shows a greater ability to inhibit the Fe^{2+}-ferrozine complex and therefore a greater affinity and chelating activity of Fe^{2+} than NVP. This could be attributed to the multidentate character and increased steric hindrance associated with the molecular structure of NVP.

It may therefore be concluded that Fe^{2+} is more likely to be bound by EFV than NVP, hence fewer of these ions will be available to participate in the Fenton reaction and bind QA to form the QA-Fe^{2+} complex. This provides an adequate explanation for the

inhibition of Fe^{2+}-induced LP by EFV and not NVP, and another mechanism through which EFV reduces QA-induced LP (section 3.2.3).

The relatively high Fe^{2+} chelating activity of these agents is of great significance, because it has been proposed that the transition metal ions contribute to the oxidative damage in neurodegenerative disorders, and one of the lines of treatments entails binding of transition metals (Vardarajan *et al*., 2000; Bush, 2003).

5.4. AN ELECTROCHEMICAL ANALYSIS OF IRON (III) ALONE AND IN THE PRESENCE OF NVP OR EFV.

5.4.1. Introduction

The transfer of electrons during a chemical reaction is the basis for electrochemical analysis (Pecsok *et al*., 1968). Electrochemistry has been used successfully in metal-ligand interactions (Limson *et al*., 1998; Matlaba *et al*., 2000). The ability of metal ions in solution to accept and donate electrons, allows for both qualitative and quantitative analysis. For trace metal ions in solution it is necessary to employ a preconcentration step, for isolation at the working electrode where the reactions take place (Limson, 1998). The introduction of a ligand to a metal solution increases the preconcentration of the metal at the electrode resulting in the increase in current. In the present study, adsorptive stripping voltammetry (ASV) was employed to determine the interaction between NVP and EFV with Fe^{3+}.

The sequence of steps that occur in ASV is given by equations 5.4 -5.6. The formation of a metal-ligand complex (Equation 5.4) is followed by its controlled interfacial accumulation (deposition) onto a working electrode at a fixed deposition potential during the deposition step (Equation 5.5) (Limson *et al*., 1998). The reduction of the adsorbed metal complex involves the application of a potential in the negative direction, during which reduction of the adsorbed metal complex occurs, releasing the metal and ligand back into solution (Equation 5.6) (Limson *et al*., 1998).

$$M^{n+} + nL_{aq} \rightarrow ML_n^{n+}, _{aq} \qquad \textbf{Equation 5.4.}$$

$$MLn^{n+}, _{aq} \rightarrow MLn^{n+}, _{ads} \qquad \textbf{Equation 5.5.}$$

$$MLn^{n+}, _{ads} + xe^{-} \rightarrow M^{(n-x)+} + nL \qquad \textbf{Equation 5.6.}$$

where M is the metal and L the ligand.

This technique relies on the natural tendencies of analytes in solution to adsorb at the surface of the electrode in solution (Limson, 1998). Theoretically, when a suitable ligand is added to a metal solution, the reduction wave observed for the metal ligand complex should exhibit a significant change in current strength as well as a potential shift over the reduction potential of the metal alone in solution.

The affinity of the ligand for the metal is expressed by the extent of an increase in current response of the metal on addition of the ligand, whilst the stability of the metal complex and the strength of the metal-ligand bond are determined by the extent of the shift in the reduction potential (Limson *et al*., 1998). A more negative potential shift may occur due to strong metal-ligand interactions, hence indicating a lower tendency for the metal-ligand complex to become reduced and a positive shift is associated with weaker metal-ligand interactions (Limson, 1998). At relatively high ligand concentrations, a decrease in current indicates a possible competition between the free ligand and the metal –ligand complex for the binding sites at the electrode, while at low ligand concentrations, it is more likely to be due to the formation of strong metal-ligand bonds where the metal is not easily reduced (Limson, 1998).

5.4.2. Materials and Methods

5.4.2.1. Chemicals and Reagents

As described in section 5.2.2.1. Tris-HCl was purchased from the Sigma Chemical Corporation, St. Louis, MO, U.S.A and anhydrous ferric chloride ($FeCl_3$) was purchased from BDH Laboratory Supplies, Poole, England.

5.4.2.2. Apparatus

Adsorptive stripping voltammograms (ASVs) were recorded on the Autolab PGSTAT 30 (Netherlands) voltammeter equipped with a Metrohm (Sweden) VGA cell stand. A 3 mm diameter glassy carbon electrode (GCE) was employed as a working electrode for voltammetric experiments.

A silver/silver chloride (KCl=3 M) and a platinum wire were employed as reference and auxiliary electrodes, respectively, for all electrochemical work. Prior to use and between scans, the GCE was cleaned by polishing with alumina on a Buehler pad, followed by washing in nitric acid and rinsing in Milli-Q water.

5.4.2.3. Adsorptive Stripping Voltammetry

All solutions were degassed with nitrogen. For all experiments 0.01 mM of Fe^{3+} was introduced into an electrochemical cell containing the electrolyte, 0.2 M Tris–KCl buffer, pH 7.4 which was then deareated for 5 minutes. Thereafter, an optimum deposition potential for Fe^{3+} was identified and applied for 60 s to effect the formation and adsorption of the metal ion onto the GCE. A potential scan in the negative direction from the deposition potential to at least 0.50 V beyond the reduction of the metal was applied, at a scan rate of 0.10 V s^{-1}, to strip the adsorbed metal species from the electrode.

During the stripping step, current responses due to the reduction of the metal species were measured as a function of potential to generate voltammograms. The procedure was repeated between successive additions of an appropriate concentration of the ligand (0-0.06 mM) to the electrolyte containing the metal ion in the electrochemical cell. All potential values quoted are referenced against the silver/silver chloride reference electrode. Current vs concentration and potential vs concentration plots were constructed to measure the extent of shifts in current response and reduction potential of the metal species with increasing concentrations of the ligand.

5.4.3. Results

In Figure 5.5., the ASV for 0.01 mM Fe^{3+} alone in 0.2 M tris-HCL, pH 7.4 buffer shows a peak reduction potential of 5.14 x 10^{-6} A at -0.41 V. Upon addition of 0.01 mM NVP, there is an increase in current response with a slight shift in the reduction potential of 0.03 V to a more negative potential. The addition of increasing concentrations of NVP causes a significant concentration-dependent decrease in the current response in comparison to 0.01 mM NVP.

In Figure 5.6., the ASV for 0.01 mM Fe^{3+} alone in the electrolyte shows a peak reduction potential of 5.52 X 10^{-6} A at -0.41 V. In the presence of increasing concentrations of EFV, there is a significant concentration-dependent decrease in the current response and a slight shift in the reduction potential to a more negative potential.

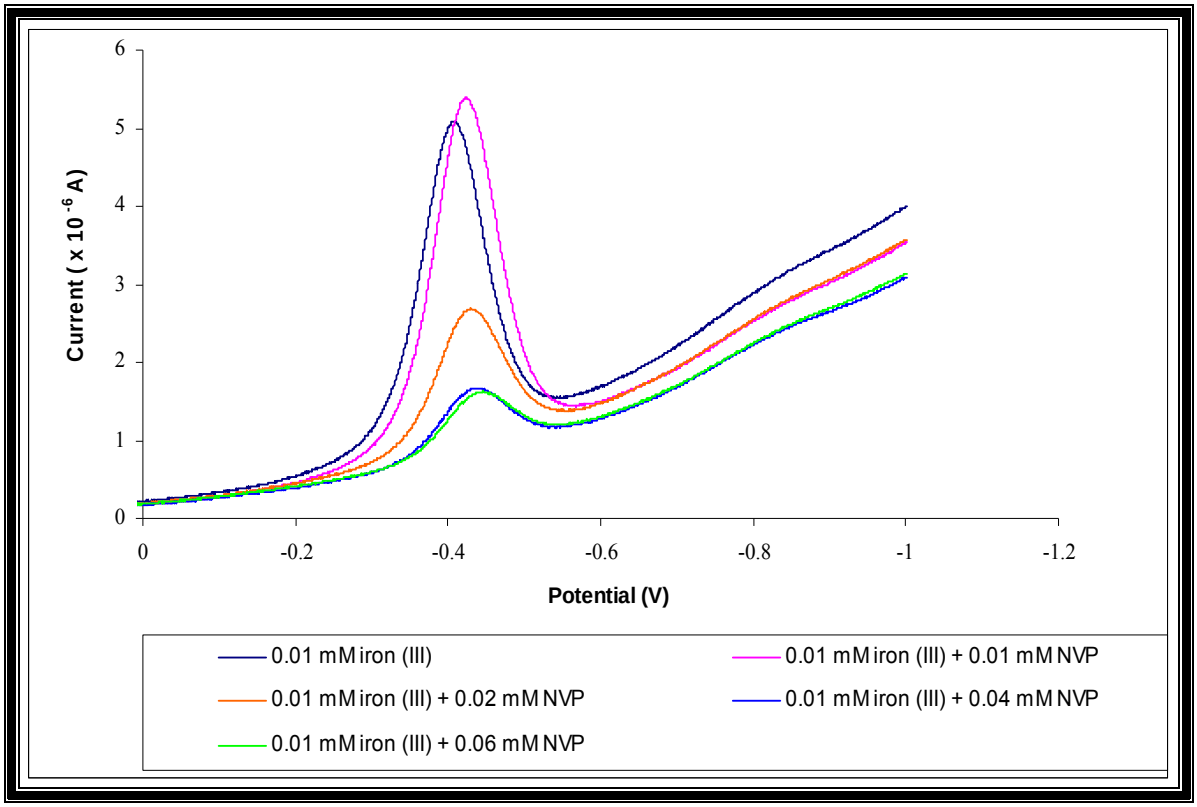


Figure 5.5. ASVs of Fe^{3+} (0.01 mM) alone and in the presence of increasing (0.01-0.06 mM) concentrations of NVP.

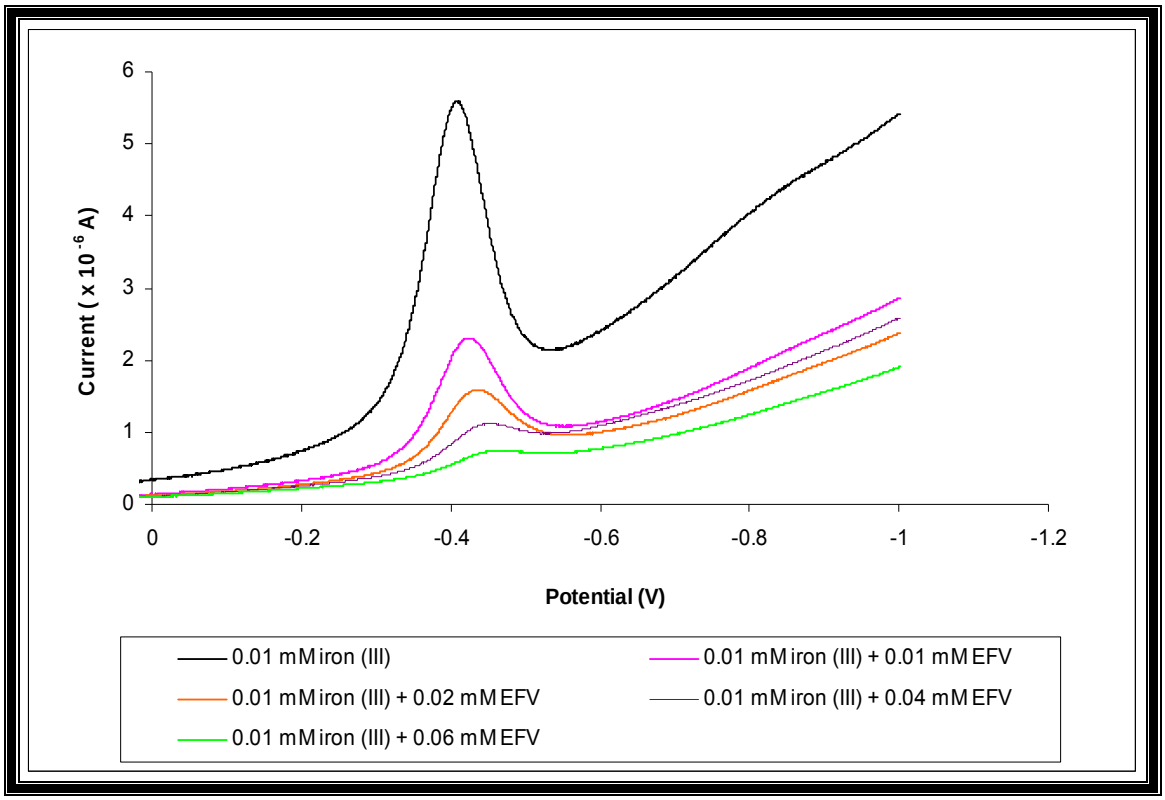


Figure 5.6. ASVs of Fe^{3+} (0.01 mM) alone and in the presence of increasing (0.01-0.06 mM) concentrations of EFV.

Figure 5.7. illustrates the overall concentration-dependent decrease in reduction potential of Fe^{3+}, exhibited by NVP and EFV. This effect is greater for efavirenz (ΔE_P = -9.03), than for NVP (ΔE_P = -5.18).

Figure 5.8. depicts an overall reduction in peak current response of Fe^{3+} with concomitant increases in ligand concentration. This effect is greater for NVP (ΔI_A = -0.68), than for EFV (ΔI_A = -0.62).

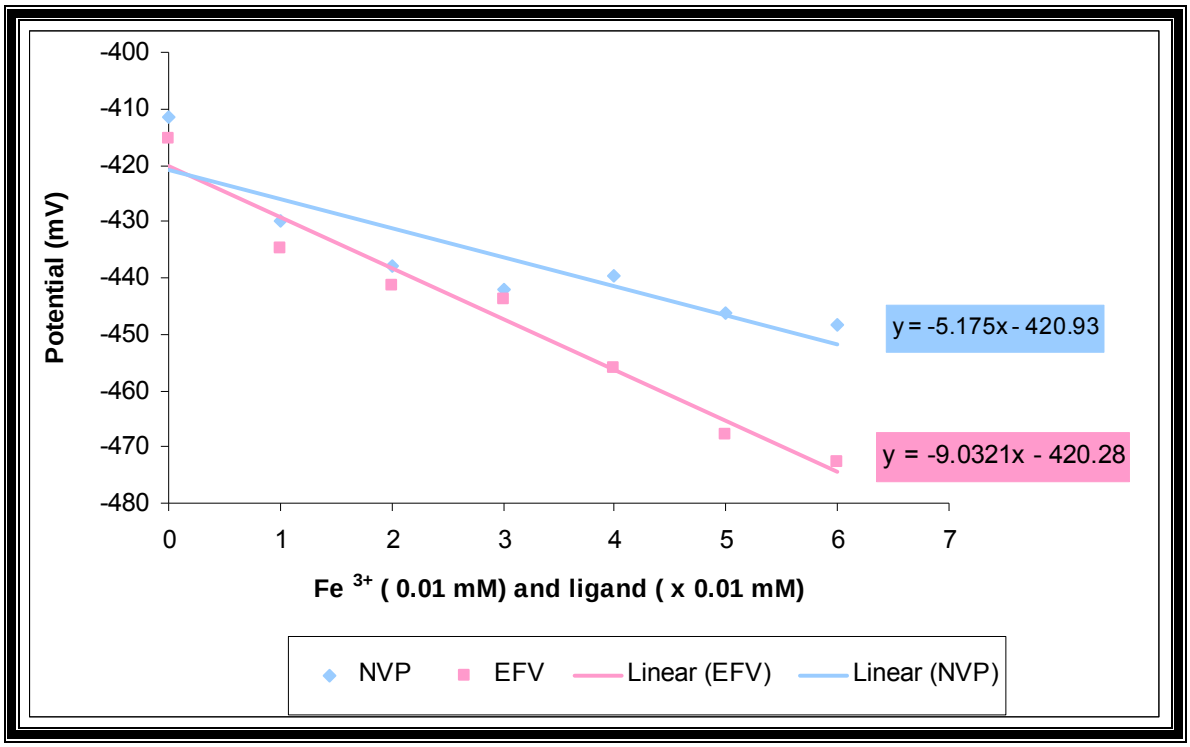


Figure 5.7. Effect of increasing concentrations of NVP and EFV on the reduction potential of 0.01 mM Fe^{3+}.

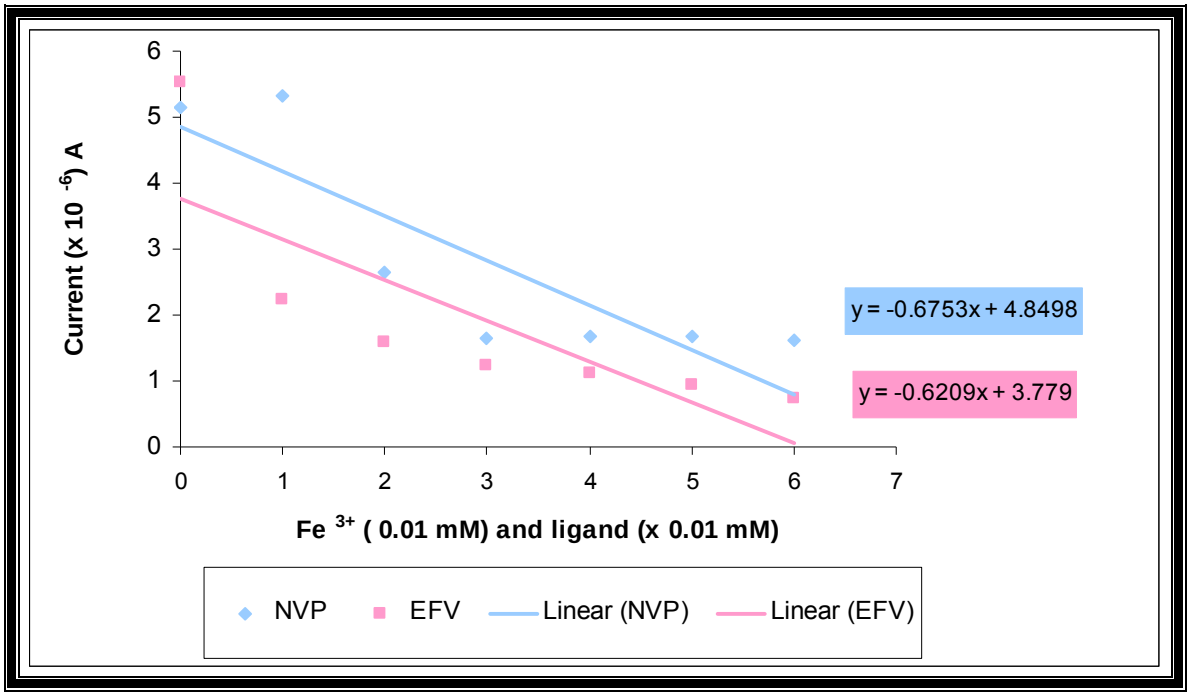


Figure 5.8. Effect of increasing concentrations of NVP and EFV on the peak current response of 0.01 mM Fe^{3+}.

5.4.4. Discussion

During the Fenton reaction (Equation 5.1.), Fe^{3+} is reduced to Fe^{2+}, therefore in the presence of chelators, the reduction potential of Fe^{2+} and/ or Fe^{3+} is altered (Winterbourn, 1995). The reduction potential is an indication of the stability and strength of complexes formed with Fe^{3+} and the ease with which Fe^{3+} is reduced in the complex (Limson *et al*., 1998). Both agents have the ability to form complexes with Fe^{3+} which have more negative reduction potentials than Fe^{3+} alone as shown in Figures 5.5. and 5.6.

The gradient of Figure 5.7. allows for an estimation of the magnitude of the reduction potential shift at increasing concentrations of NVP and EFV, which in turn measures the stability of the complexes formed. The gradient for EFV (ΔE_P = -9.03) is relatively larger in comparison to NVP (ΔE_P = -5.18), thus indicating the formation of a more stable complex with Fe^{3+}.

This in turn reflects the difficulty in reducing the complex as compared to the reduction of Fe^{3+} alone. Through this strong binding of Fe^{3+}, EFV prevents the reduction of Fe^{3+} to Fe^{2+}, thus less Fe^{2+} will be available to participate in the Fenton reaction. Therefore this could be one of the neuroprotective mechanisms through which EFV reduces Fe^{2+} -induced LP (section 5.2.3).

As previously mentioned in Chapter 3, the oxidizing effects of QA on LP *in vitro*, requires the presence of Fe^{2+} and this mechanism involves Fe^{2+} –chelation by QA (Stipek et al., 1997). By binding Fe^{3+}, these agents prevent the reduction of Fe^{3+} to Fe^{2+} and consequently reduce the amount of Fe^{2+} available to participate in the formation of the QA- Fe^{2+} complex and the subsequent LP associated therewith. Thus, this could be another mechanism through which these agents reduce QA-induced LP (section 3.2.3).

The decrease in peak current response which occurs in a concentration-dependent manner (Figure 5.8.) after the formation of metal-ligand complexes observed in Figures 5.5. and 5.6., most likely indicates that the reduction rate of the complexes is lower than that of the metal species. This implies that the complexes formed are strong enough to prevent the reduction of Fe^{3+} to Fe^{2+} thus reducing current flow.

5.4.5. Conclusion

The binding of Fe^{2+} and Fe^{3+} ions by these agents, is of great relevance to the Fenton reaction, where Fe^{2+} generates the toxic $HO^{\bullet}$ and is oxidized to Fe^{3+}, the stable oxidation state of Fe which reacts to form insoluble polymers which are toxic to cells. The fact that both agents bind Fe^{3+}, may thus suggest that these NNRTIs remove unbound Fe^{3+}, preventing it from recycling back to Fe^{2+}, the form responsible for free radical formation. The neuroprotective activities of NVP and EFV could be ascribed to their known free radical scavenging properties (Chapter 2) and Fe-chelating effectiveness (Chapter 5). Due to the lipophilic nature and ability to penetrate the blood-brain barrier, these agents are able to facilitate the removal of toxic metals from the CNS. These studies therefore suggest an important role for these agents and a further increase in the prospects for the clinical application of these potent antioxidants in ADC.

CHAPTER 6

THE BIOSYNTHESIS OF QUINOLINIC ACID

6.1. INTRODUCTION

Tryptophan (TRP) is an essential amino acid which is required in several physiological processes. In addition to protein synthesis (Sidransky, 1976), more than 90 % of the total TRP is degraded in the liver through the kynurenine pathway (KP) as shown in Figure 6.1. (Musajo and Benassi, 1964; Price *et. al*., 1965; Wolf, 1974; Young *et al*., 1978). The KP not only occurs in the CNS, but in the periphery and such dynamics of this pathway involves interplay between KP metabolism in the periphery and brain (Schwarcz and Pellicciari, 2002). Along this pathway, TRP is first oxidized to N-formylkynurenine, which is quickly catabolised to kynurenine. In the liver this step is catalyzed primarily by tryptophan 2, 3-dioxygenase (TDO) (Schimke *et al*., 1965; Knox, 1966).

TDO has high substrate specificity for L-TRP and can be regulated by glucocorticoids (Knox and Mehler, 1951; Schimke *et al*., 1965; Voigt and Sekeris, 1980) and TRP itself (Knox, 1951, 1966), although possibly by different mechanisms (Civen and Knox, 1959). Saturation of this enzyme with haeme or with haeme precursor, 5-ALA results in an increased activity of this enzyme and enhanced L-TRP catabolism (Badawy and Evans, 1975). The untoward effect of enhanced TDO activity is not only an increase in blood-borne kynurenines which are responsible for the biosynthesis of the neurotoxin QA, but also a reduction in the availability of TRP for uptake into the brain (Müller, 2006). Thus changes in TRP concentrations in peripheral blood may be significant as a determinant of the concentration of 5-hydroxytryptamine (5-HT) in the brain, in which case acute depletion of TRP consequently affects serotonergic functions (Sidransky, 1976; Young and Leyton, 2002).

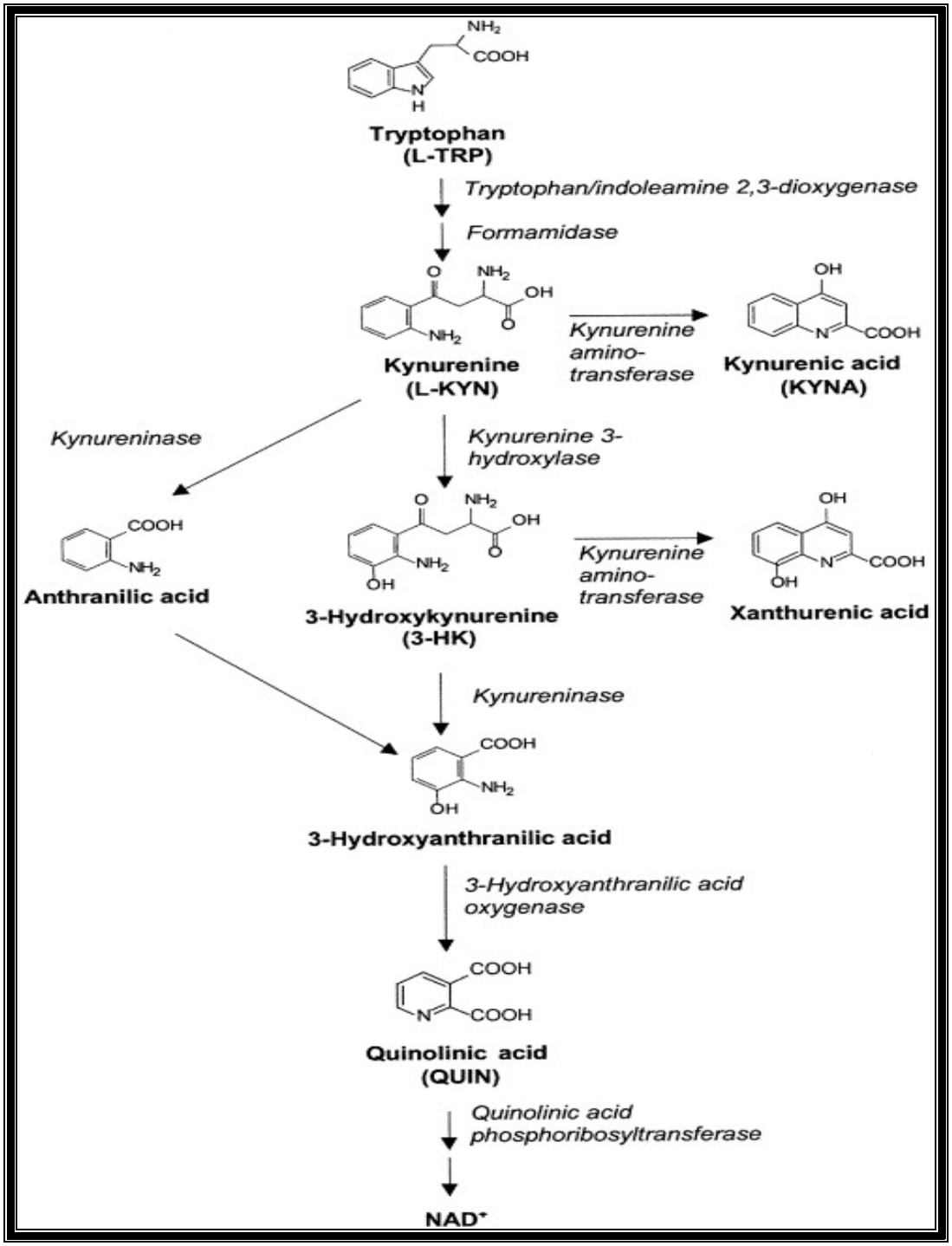


Figure 6.1. Graphical representation of the oxidative tryptophan metabolism along the KP showing the metabolites and enzymes of this pathway. Tryptophan (2, 3)-dioxygenase (TDO) and indoleamine (2, 3)-dioxygenase (IDO) catalyze the formation of kynurenine (Sas *et al*., 2007).

The role of TDO in the regulation of 5-HT is of significance considering that the majority of patients suffering from depression, AD and ADC have elevated cortisol levels (Curzon, 1988; Freda and Bilezikian, 1999) and that corticoids are potent inducers of TDO activity (Salter and Pogson, 1985). Such induction of liver TDO enhances blood-borne kynurenines which are converted to the neurotoxin QA in the brain which ultimately leads to progressive hippocampal damage (Sapolsky *et al*., 1986; Seckl and Olsson, 1995). Since the hippocampal formation forms part of the limbic-hypothalamic–pituitary-adrenal (LHPA) axis, any increase in cortisol levels activates receptors which influence neuronal excitability, neurochemistry and structural plasticity (McEwen, 2000), thus impairing neuronal plasticity and possibly contributing to neurodegeneration.

One of the major metabolites formed by the KP is QA, which binds to glutamate receptors activated by NMDA. QA is an agonist at these receptors and may be neuroexcitotoxic at physiological concentrations (Perkins and Stone, 1983; Schwarcz *et al*., 1983). Elevated levels of QA have been found in the CSF of people with dementia due to AIDS and cerebral malaria as well as the lesions associated with AD which have been shown to enhance neuronal damage (Heyes *et al*., 1989; Guillemin *et al*., 2005).

A reduction in the synthesis of QA via TDO inhibition could attenuate the amount of brain damage (Stone, 2003) and impaired neuronal plasticity. Hence the aim of this chapter is to assess the ability of these NNRTIs to ameliorate the activity of TDO, the enzyme which catalyses the first step in the conversion of TRP to QA.

6.2. THE EFFECT OF NVP AND EFV ON TDO ACTIVITY IN RAT LIVER HOMOGENATE *IN VIVO*.

6.2.1. Introduction

Enhanced TRP degradation is observed in diseases concomitant with cellular immune activation. Disturbed metabolism of TRP affects biosynthesis of 5-HT, and it appears to be associated with an increased susceptibility for depression (Widner *et al*., 2002). TRP is required for 5-HT synthesis, the rate-limiting step of which is the conversion of TRP to 5-HT by T5H. This enzyme is thought to be fully saturated with substrate *in vivo* (Knowles and Pogson, 1984).

Since TDO is the rate-limiting enzyme for the conversion of TRP through the KP, inhibition of this enzyme makes more TRP available for conversion to 5-HT. (Schwarcz and Pellicciari, 2002). This will in turn increase the saturation of T5H, which is known to be unsaturated under normal conditions, and increase 5-HT availability in the brain (Pogson *et al.,* 1989).

Previously an association was found between decreased TRP concentrations in patients with HIV infection and progressed cognitive inability (Fuchs *et al*., 1990). Patients with HIV infection present with decreased TRP and increased kynurenine concentration thus indicating accelerated TRP degradation. Antiretroviral Therapy (ART) has been shown to have the ability to significantly reduce TRP degradation: TRP concentration increases whereas kynurenine concentration decreases, and improves cognitive impairment (Judd *et al.*, 2000; Suarez *et al*., 2001). Hence the increase in TRP concentrations during ART could relate to the improved depressive symptoms in patients with HIV infection (Judd *et al*., 2000).

Antiretroviral agents that reduce extrahepatic TRP oxidation have proven effective in reducing QA concentrations clinically, thereby lowering the risk of ADC (Heyes *et al*., 1991). However, such a relationship has to be proven in future studies. Thus, due to the potential reduction in TRP catabolism and the consequential decrease in the

biosynthesis of QA associated with ART in ADC, the effect of NVP and EFV on the activity of the total, holo- and apoenzyme of TDO was determined.

6.2.2. Materials and methods

6.2.2.1. Chemicals and Reagents

EFV and NVP were kindly supplied by Aspen Pharmaceutical Company, Port Elizabeth, South Africa. Haematin, L-TRP, Folin's reagent and bovine serum albumin (BSA) were purchased from Sigma Chemical Corporation, St Louis, MO, U.S.A. All other reagents were purchased from Merck, Darmstadt, Germany and were of the highest chemical purity.

6.2.2.2. Animals

Adult male Wistar rats, purchased from the South African Vaccine Producers (Johannesburg, South Africa) and cared for as described in section 3.2.2.2.

6.2.2.3. Treatment regimes

The animals were treated in a similar manner as described in section 3.3.2.2 except that there were no intrahippocampal injections of QA. Rats were divided into 3 groups, control, EFV and NVP treated. The rats were injected intraperitoneally with drug, 5mg/kg/day for 5 days. On the morning of the 6^{th} day the rats were sacrificed by neck fracture. The livers were rapidly removed, perfused with 0.9 % saline then frozen in liquid nitrogen and stored at -70 ^{o}C until use.

6.2.2.4. Preparation of the Tissue

The livers were thawed, chopped into fine pieces and homogenised with 60 ml 140 mM KCl/2.5 mM NaOH using a glass–teflon hand held homogeniser in 0.01 M PBS pH 7.4, to give a final concentration of 10 % w/v. The homogenate was then sonicated for a period of 2 minutes at 30 s intervals for complete removal of the enzyme from cells. The entire procedure, where possible, was conducted on ice.

6.2.2.5. Determination of TDO Activity

A modification of the method described by Badawy and Evans (1975).

An aliquot of 15 ml homogenate was added to a flask containing 12.5 ml water. An aliquot of haematin (100 µL) at a final concentration of 2 µM (Badawy and Evans, 1975) was added to samples that were used to determine the total activity of the enzyme. This was stirred for 1 min to allow for the activation of the enzyme. Finally, 2.5 ml of 0.03 M L-TRP was added to all flasks and gently stirred. The assay was conducted in triplicate. Aliquots of 3 ml of the assay mixture was transferred to test tubes, stoppered under carbogen and incubated for 1 h at 37 °C in an oscillating water bath. The enzyme activity was determined in the absence and presence of haematin in order to determine the activity of the holo- and apoenzyme of TDO. The apoenzyme in isolation is inactive but in the presence of haematin becomes fully active. The holoenzyme activity was measured in the absence of haematin while the total activity was measured in the presence of added haematin. The reaction was terminated with the addition of 2 ml of 0.9 M TCA to the reaction mixture and incubated for 2–4 min. The mixture was filtered through a Whatman no. 1 filter paper. Filtrate (2.5 ml) was added to 1.5 ml of 0.6 M NaOH and vortexed. The kynurenine present in the solution was measured at 365 nm spectrophotometrically using the molar extinction coefficient of kynurenine: $\varepsilon = 4540\ M^{-1}.cm^{-1}$. The blank consisted of 2 ml TCA and 1.5 ml NaOH. The TDO activity was expressed as nmol/mg protein/min. Protein estimation was performed using the method described by *Lowry et al*., 1951.

6.2.2.6. Protein Assay

Protein estimation was done using the method previously described by Lowry *et al*., (1951). A calibration curve was generated using BSA which was prepared in Milli-Q water (1 ml) at concentration intervals of 60 µg/ml. The aliquots of BSA were incubated with copper reagent (6 ml) for 10 minutes at room temperature.

Folin's reagent (0.3 ml) was added and samples were left to stand for 30 minutes in the dark, at room temperature. The absorbance was then read at 500 nm on a GBC 916 UV/VIS spectrophotometer and the curve generated by plotting concentration against absorbance (Appendix III).

6.2.2.7. Statistical Analysis

The results were analysed as described in section 2.2.2.3.

6.2.3. Results

As shown in Figure 6.2 and 6.3., it is evident that EFV and NVP, administered 5 mg/kg/day for five days induce a significant ($p < 0.001$) decrease in the apoenzyme, holoenzyme and total enzyme activity of TDO.

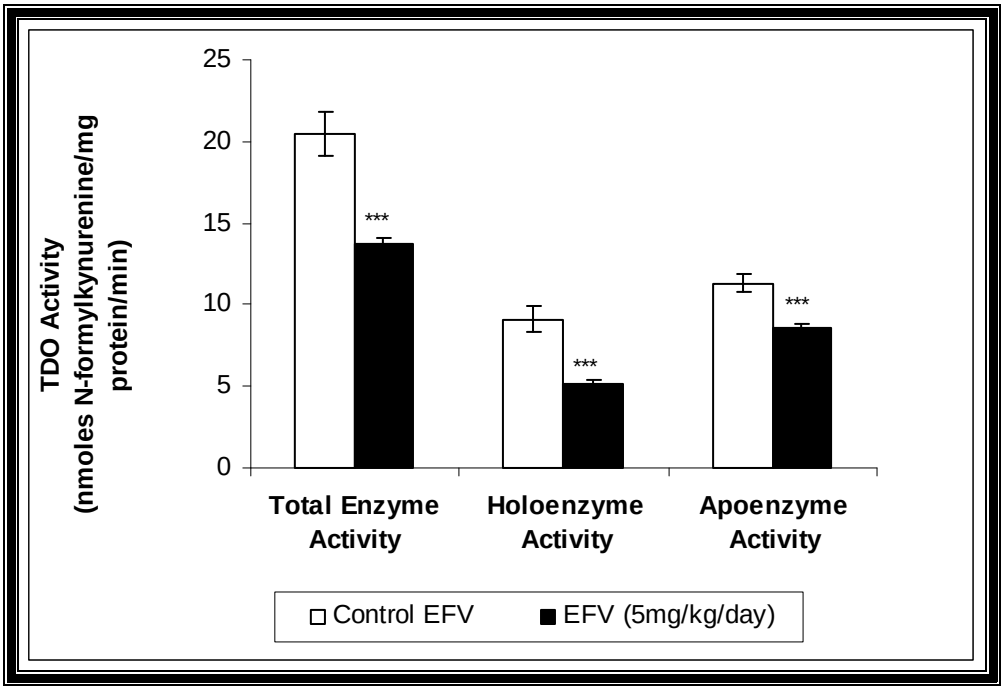


Figure 6.2. Effect of EFV (5mg/kg/day for 5 days) on TDO enzyme activity in rat liver homogenate *in vivo*. Each bar represents the mean ± SD (n = 5). *** ($p < 0.001$) in comparison to control (ANOVA and Student- Newman-Keuls Multiple Range Test).

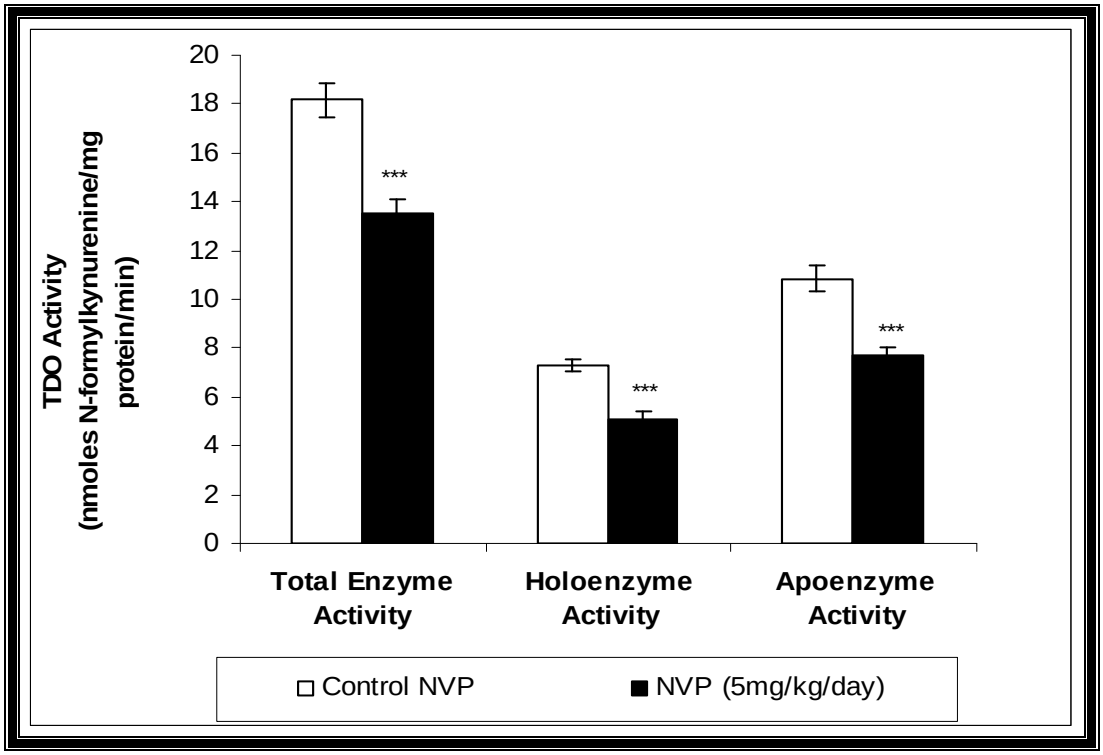


Figure 6.3. Effect of NVP (5 mg/kg/day for 5 days) on TDO enzyme activity in rat liver homogenate *in vivo*. Each bar represents the mean ± SD (n = 5). *** ($p < 0.001$) in comparison to control (ANOVA and Student- Newman-Keuls Multiple Range Test).

6.2.4. Discussion

Liver TDO plays an imperative role in determining the levels of circulating TRP in the blood (Badawy *et. al*., 1981). An increase in the activity of this enzyme enhances the conversion of TRP to N-formylkynurenine, thus reducing the amount of TRP available for uptake into the brain (Badawy, 1979). Thus agents, which inhibit TDO, increase plasma levels of TRP and subsequently induce a rise in brain TRP and 5-HT levels (Daya *et al*., 1989). Tricyclic anti-depressants inhibit TDO and this could be part of their mode of action in attenuating depression (Badawy and Evans, 1981).

The inhibition of TDO occurs via two mechanisms:

(a) Interference with the conjugation of the apoenzyme and its co-factor, haeme by some agents (Badawy *et al*., 1981)

(b) The structure-activity relationship of the test compound at the active site of the co-enzyme. The presence of an indole -NH group has been reported to be essential for substrate binding to the catalytic site of TDO (Uchida *et al*., 1992). Hence test compounds which possess at least an indole-NH group, competitively inhibit the binding of TRP to the inactive holoenzyme, thereby inhibiting holoenzyme activity, whilst those without the indole-NH group demonstrate non-competitive inhibition (Uchida *et al*., 1992). Compounds without the indole-NH such as NVP and EFV, may non-competitively inhibit TDO at this level by interfering with TRP binding through interaction at a site other than that designated for TRP, consequently leading to decreased holoenzyme activity. However, this needs to be further investigated.

The inhibition of TDO by these NNRTIs appears to occur at the apoenzyme level as well (Figure 6.3 and 6.4.), which implies that NVP and EFV probably interfere with the conjugation of haeme to the apoenzyme.

Because T5H is approximately 50 % saturated with TRP *in vivo* (Pogson *et al.,* 1989), 5-HT levels will be more sensitive to decreases in TRP than increases in TRP. Therefore, an increased availability of TRP in the presence of these agents will increase the activity of T5H, and therefore increase the synthesis and vesicular stores of 5-HT (Schaechter and Wurtman, 1989). It has been shown that the inhibition of liver TDO results in the elevation of brain TRP and 5-HT concentrations and a decrease in peripheral kynurenine levels (Curzon and Bridges, 1970; Walsh and Daya, 1998).

Thus the consequential neuronal damage contributed by QA in the CNS (Stone, 1993), particularly as a result of TDO induction by endogenous corticoids associated with ADC and depression is ameliorated. Therefore apart from increased TRP levels, through TDO inhibition, these agents could also reduce QA production and the consequential impaired neuronal plasticity associated therewith. Although the results imply that NVP and EFV could cause a rise in circulating TRP levels resulting in elevated brain 5-HT levels, this possibility is further investigated in Chapter 7.

6.2.5. Conclusion

Liver TDO is an important enzyme in the metabolism of TRP, a precursor of 5-HT synthesis. The treatment of animals with NVP or EFV (5mg/kg/day for 5 days) decreases the total holoenzyme as well as the total apoenzyme activity of TDO. Since this enzyme activity is reduced, it is possible that more TRP is available for 5-HT synthesis. The amount of 5-HT and other biogenic amine levels will be measured in the following chapter. The inhibition of TDO may also reduce the synthesis of the excitatory amino acid, QA.

CHAPTER 7

THE BIOSYNTHESIS AND METABOLISM OF SEROTONIN

7.1. INTRODUCTION

Viral infections have been shown to have substantial effects on neurotransmitters in the brain, especially the catecholamines (Zalcman *et al*., 1994) and the indoleamine, 5-HT (Dunn, 1992). In addition, neuropathology and certain clinical symptoms of HAD also confirm dysfunctions in monoamine neurotransmitters systems, such as dopamine (DA) (Hriso *et al*., 1991), and 5-HT (Kumar *et al*., 2001), which result in neurocognitive deficits and mental health problems (Kumar *et al*., 2007). Subsequent findings of high viral load in the hippocampus and basal ganglia, areas of the brain rich in both dopaminergic and serotonergic activities, led to the concept that deficits in DA and 5-HT might be associated with the neurological disorders, cognitive impairments and behavioural changes (Dursun, 1993; Kumar *et al*., 2001). The neurological problems and behavioural changes observed in patients with ADC were found to be similar to those found in subcortical dementia of PD (Hriso *et al*., 1991; Nath *et al*., 2000).

It has been demonstrated that CSF levels of 5-HT, were severely diminished in patients with ADC (Kramer and Sanger, 1990; Kumar *et al*., 2001). Furthermore, while levels of 5-HT are significantly decreased in patients with ADC, there was no change in the levels of 5-hydroxyindole acetic acid (5-HIAA), the major metabolite of 5-HT (Kumar *et al*., 2007). Depression may involve functional abnormality of one or more of monoamine systems in the brain (Fuxe *et al*., 1970). On the other hand, evidence accumulated over half a century ago has implicated the involvement of another monoamine neurotransmitter, norepinephrine (NE), in depression. Thus

activation rather than suppression of monoaminergic activity seems to be linked to antidepressant activity by enhancing serotonergic (Lapin and Oxenkrug, 1969; Blier and de Montigny, 1990) and noradrenergic systems (Schildkraut, 1965) via reuptake inhibition (van Praag, 1982b) and liver TDO activity inhibition causing elevation of brain TRP and 5-HT concentrations (Badawy and Evans, 1981; Walsh and Daya, 1998).

Since NVP and EFV have been shown to inhibit liver TDO activity in the previous chapter (section 6.2.5), and considering the role of monoamine synthesis and metabolism dysfunction in ADC, PD and depression as illustrated above, an investigation into the effects of these agents on the monoamine levels in the hippocampus was carried out to assess the ability of these NNRTIs to alleviate biochemical consequences of the disorders.

7.2. THE EFFECT OF NVP AND EFV ON 5-HT AND RELATED NEUROTRANSMITTER LEVELS IN RAT HIPOCCAMPUS HOMOGENATE *IN VIVO*.

7.2.1. Introduction

Alterations in brain 5-HT levels are known to result in mood disorders, particularly depression (van Praag, 1982). Brain 5-HT is synthesized from TRP, which is taken up from the peripheral circulation (Badawy *et al*., 1981). In turn, the availability of TRP for 5-HT synthesis in the brain depends on the rate of TDO activity. Thus an inverse relationship exists between the activity of this enzyme and brain 5-HT levels (Daya *et al*., 1989). The inhibition of TDO by NVP and EFV (section 6.2.5), may ultimately lead to a rise in brain 5-HT and its major metabolite, 5-HIAA.

5-HT is known to play a role in NE release in the brain (Xi-Ming Li *et al*., 2002). These neurotransmitters are synthesized within the brain from their precursors, the large neutral amino acids, TRP and tyrosine (TYR) respectively, the latter being also the precursor of DA (Curzon and Sarna, 1984; Lucca *et al*., 1994). Both these molecules may thus compete for transport into the brain via the neutral amino acid transporter (Müller, 2006). Hence an increase in peripheral TRP through TDO inhibition by NVP and EFV may possibly reduce brain uptake of peripheral TYR by increasing 5-HT and reducing NE.

The existence of a distinct inter-reliance between the two monoaminergic systems, led to an investigation into the effects of NVP and EFV on 5-HT, 5-HIAA and NE levels in rat brain. In this study, much attention was focused on the hippocampus as the importance of this brain region lies in brain functions, such as emotion, motivation, learning and memory, which may be related to the expression of depression (Leonard and Kafoe, 1976; Sapolsky *et al*., 2000).

7.2.2. Materials and Methods

7.2.2.1. Chemicals and Reagents

EFV and NVP were kindly supplied by Aspen Pharmaceutical Company, Port Elizabeth, South Africa. Heptane sulphonic (HSA) acid, EDTA, triethylamine (TEA), phosphoric acid (PA), perchloric ($HCLO_4$) acid, NE, 5-HT and 5-HIAA were purchased from Sigma Chemical Corporation, St Louis, MO, U.S.A. HPLC grade acetonitrile (ACN) was purchased from Saarchem, Johannesburg, South Africa.

7.2.2.2. Animals

Adult male Wistar rats, purchased from the South African Vaccine Producers (Johannesburg, South Africa) and cared for as described in section 3.2.2.2.

7.2.2.3. Drug Treatment

Animals were cared for and treated as described in section 3.3.2.2.

7.2.2.4. Brain Removal

On the morning of the 6^{th} day (after receiving EFV or NVP, 5mg/kg/day for 5 days) the animals were killed by neck fracture and the hippocampus of each rat was removed and frozen in liquid nitrogen and stored at -70 ^{o}C.

7.2.2.5. Preparation of Tissue

The brain regions were thawed on ice, weighed and individually homogenised (1 mg/10 μL) in ice-cold $HCLO_4$ (0.1 M) containing 0.01 % EDTA, by sonication at 50 Hertz for 30 seconds using an ultrasonic cell disruptor. The homogenate was kept on ice for 20 minutes before being centrifuged at 10 000 x g for 10 minutes using a bench top centrifuge. The supernatant (10 μL) obtained was directly injected into the

High Performance Liquid Chromatography-Electrochemical Detector (HPLC-ECD) system for analysis (Muralikrishnan and Mohanakumar, 1998).

7.2.2.6. Instrumentation

Samples were analyzed on an isocratic HPLC system coupled to an electrochemical detector. The chromatographic system consisted of a Waters Millipore Model 510 pump, Waters electrochemical detector and a Rikadenki chart recorder. The chart speed was set at 15 cm/h. Samples were introduced into the system using a Rheodyne Model 772Si fixed loop injector, fitted with a 20 μL loop.

7.2.2.7. Chromatographic Conditions

Separation was achieved using a C_{18} (Waters Spherisorb©, 5μm, 250 x 4.6mm n-octadecylsilane column). The mobile phase consisted of 8.32 mM HSA, 0.27 mM, EDTA, 13 % ACN, 0.4-0.45 % TEA and 0.2-0.25 % PA (v/v) and made to 1000 ml using Milli-Q water. Mobile phase was degassed twice using a 0.45 μM membrane filter prior to use. The mobile phase was recycled but was changed every 3 days. The flow rate was set at 0.6 ml/min and the electrochemical detection was performed at +0.74 V. Results were expressed as pmoles/mg tissue.

7.2.2.8. Statistical Analysis

The results were analysed as described in section 2.2.2.3.

7.2.3. Results

Table 7.1. illustrates that the hippocampus of rats treated with NVP and EFV show a significant increase in the levels of 5-HT ($p < 0.001$) and a decrease in 5-HT turnover ($p < 0.001$) in comparison to controls. NVP exhibits significantly higher levels of NE ($p < 0.001$) and 5-HIAA ($p < 0.01$) in comparison to EFV.

Table 7.1. Effect of NVP and EFV on rat hippocampal NE, 5-HT and 5- HIAA levels.

Treatment Groups	**NE (pmoles/mg tissue)**	**5-HT (pmoles/mg tissue)**	**5-HIAA (pmoles/mg tissue)**	**5-HT turnover (5-HIAA : 5-HT)**
Control	2.00 ± 0.63	0.77 ± 0.32	2.50 ± 0.46	0.56 ± 0.1
NVP	3.41 ± 0.34 ***	2.10 ± 0.34 ***	3.73 ± 0.63 **	0.31 ± 0.6 ***
Control	1.58 ± 0.415	0.38 ± 0.07	2.40 ± 0.24	0.30 ± 0.3
EFV	1.80 ± 0.423 ns	0.80 ± 0.39 ***	2.75 ± 0.53 ns	0.19 ± 0.6 ***

Each value represents the mean ± SD (n=4). *** ($p < 0.001$), ** ($p < 0.01$) and ns ($p > 0.05$) in comparison to controls. All results were analyzed using one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls Multiple Range Test.

7.2.4. Discussion

The involvement of 5-HT in the pathogenesis of depression and its mechanistic role in the action of antidepressant drugs has been well established (Delgado *et al.*, 1990; Briley and Moret, 1993). The interest in the correlations between 5-HT and depression was stimulated by the finding that the first generation of antidepressants enhance the availability of 5-HT at central receptors (van Praag, 1982). As shown in Table 7.1, both agents significantly ($p < 0.001$) increase hippocampal 5-HT levels. This increase could possibly through inhibition of the TDO enzyme, as shown in Chapter 6, section 6.2.5.

5-HT is known to play a role in NE release (Xi-Ming Li *et al*., 2002) by stimulating 5-HT_3 receptors on noradrenergic axon terminals (Shachar *et al*., 1997), thus the increased 5-HT levels by NVP, may have triggered the elevated response to NE levels. Although the increase in NE levels by NVP could be another mechanism through which this agent can assist in elevating the mood of depressed patients, further studies are required to substantiate this possibility. Furthermore, inhibition of TDO and thus increased brain TRP levels is confirmed to be one of the most likely mechanisms of increased brain 5-HT levels by these agents.

The majority of 5-HT is oxidized by the enzymes monoamine oxidase (MAO) and aldehyde dehydrogenase (ADH) to 5-HIAA (Wurtman *et al*., 1968). Thus, the rise in hippocampal 5-HIAA levels after treatment with NVP may be a result of increased hippocampal 5-HT levels available as substrate for MAO. NVP may also have the ability to affect the functioning of the enzyme, ADH. This would mean less 5-hydroxyindole acetaldehyde is converted to 5-hydroxytryptophol and more was converted to 5-HIAA. Conversely, the decrease in 5-HT turnover could imply the ability of these agents to slow 5-HT metabolism thus maintaining the increased levels for longer (Müller, 2006) As a result, NVP and EFV could be useful particularly in ADC, where 5-HT turnover is increased (Moeller and Pirke, 1990).

Currently there is much interest in the role played by postsynaptic 5-HT_{1A} receptors in the inhibition of 5-HT cell firing induced by administration of 5-HT_{1A} agonists (Ceci *et al*., 1994; Celada *et al.*, 2001). It is well known that elevated extracellular 5-HT concentrations within the somatodendritic regions will suppress serotonergic neuronal cell firing via its action at the inhibitory presynaptic 5-HT_{1A} receptors (Blier and de Montigny 1985; Dawson and Nguyen, 1998). Electrophysiological data has demonstrated the desensitization of presynaptic 5-HT receptors during chronic treatment with 5-HT specific reuptake inhibitors (Blier *et al*., 1987) allowing an enhancement in synaptic 5-HT, particularly in terminal areas (Bel and Artigas, 1993; Blier and de Montigny, 1994).

The combined action of these agents through TDO inhibition and decreased 5-HT metabolism may produce 5-HT levels greater than either effect alone, which may lead to a faster down regulation of $5\text{-HT}_{1A/1D/1B}$ receptors and hence a faster return to

normal firing and release as well as maintaining enhanced synaptic 5-HT prior to autoreceptor down regulation, thus enhancing the onset of postsynaptic changes in processing and prompting rapid therapeutic onset (Bel and Artigas, 1993; Blier and de Montigny, 1994).

The ability of these NNRTIs to elevate brain 5-HT raises a possibility of increased aMT synthesis in the pineal gland. To assess the extent to which this hypothesis holds true, the effect of these agents on 5-HT metabolism will be investigated in section 7.3.

7.3. THE EFFECT OF NVP AND EFV ON INDOLE METABOLISM IN RAT PINEAL GLANDS *IN VIVO*.

7.3.1. Introduction

The pineal gland, considered to be the "seat of the soul" by Descartes (1596-1650), has received extensive attention in the past two decades. Many studies have established the pineal gland as a fully functional organ that is responsible not only for indoleamine synthesis, and exists as an integral and essential component of the neuroendocrine system (Wainwright, 1977; Reiter, 1989). The elevated 5-HT concentrations in the pineal, exhibit a striking diurnal rhythm which remains at a maximum level during daylight hours and falls by more than 80 % soon after the onset of darkness as the 5-HT is converted to aMT, 5-hydroxytryptophol and other methoxyindoles (Axelrod *et al*., 1965 ; Klein and Weller, 1970).

Indole metabolism in the pineal gland occurs in the pinealocytes. The concentrations of pineal 5-HT may be reduced mainly via two pathways:

i) N-acetylation to form NAS through the action of the enzyme NAT,
ii) Oxidative deamination by MAO (Axelrod *et al*., 1969).

The primary functions of this organ, allows for coordination of effects of light/dark cycles on physiology, through secretion of one of the metabolites of 5-HT, the neurohormone, aMT (Arendt, 1988). In addition, there is evidence which suggests the actions of aMT to be pharmacologically and physiologically relevant (Reiter, 1997), through scavenging free radicals. Collectively, this data is indicative of the antioxidant role displayed by aMT in organisms (Reiter, 1997). Hence this study investigates whether NVP and EFV have the potential to manipulate 5-HT pineal metabolism to increase 5-HT and/ or aMT.

In order to obtain an overall picture of indole metabolism in the pineal gland, the organ culture technique was used to monitor the indole metabolites with

pharmacological and biochemical manipulations. The assay used in this study was previously described by Klein and Notides, 1969 and modified by Daya *et al*., 1989. The organ culture technique used allows room for the manipulation of experimental conditions, to avoid complications of *in vivo* interactions. The pineal gland is ideal for organ culture due to its small size, and its ability to remain viable for as long as six days under optimum conditions. It is able to utilize exogenous radioactive (^{14}C) 5-HT to produce various indoles including aMT (Daya *et al*., 1989), thus the direct effects of NVP and EFV on 5-HT metabolism, are established.

As much as 95 % of the synthesized indoles are secreted into the culture medium which can then be isolated and quantified. Isolation of the pineal indoles is achieved by using the Bi-dimensional Thin Layer Chromatography (TLC) system (Klein and Notides, 1969). This method not only effectively separates trace quantities of the pineal indoles, but it accurately determines quantities of the components of such mixtures (Skoog and West, 1980). TLC separation is achieved on a planar surface to allow for a 2-dimensional operation, and only samples which could be partially separated with one solvent alone, may be completely separated by the combined solvents. Hence two different solvent systems can be utilized in developing a single chromatogram (Ewing, 1960).

7.3.2. Materials and Methods

7.3.2.1. Chemicals and Reagents

EFV and NVP were kindly supplied by Aspen Pharmaceutical Company, Port Elizabeth, South Africa. ^{14}C labelled 5-HT was obtained from Amersham International, England. The culture medium, Minimum Essential Medium (MEM), was purchased from Highveld Biological (PTY) LTD, Lyndhurst, South Africa and fortified with the antibiotics, amphotericin B, streptomycin and benzyl penicillin (Hoechst, South Africa). The aluminium TLC plates coated with silica gel 60, Type F254 (0.25 mm), were purchased from Merck, Darmstadt, Germany. Beckman

Ready-Sol multipurpose liquid scintillation fluid was purchased from Beckman RIIC Ltd, Scotland. The indole standards, 5-HT, N-acetylserotonin (NAS / aHT), aMT, 5-

hydroxytryptophol (5-HTOH/ HL), 5-methoxytryptophol (5-MTOH/ ML), 5-methoxytryptamine (5-MT), 5-hydroxyindole acetic acid (5-HIAA/HA) and 5-methoxyindole acetic acid (5-MIAA/ MA) were purchased from Sigma Chemicals Co, St Louis, USA. Ascorbic acid, ethanol, chloroform and glacial acetic acid were purchased from Saarchem, Krugersdorp, South Africa.

7.3.2.2. Animals

Adult male Wistar rats, purchased from the South African Vaccine Producers (Johannesburg, South Africa) and cared for as described in section 3.2.2.2.

7.3.2.3. Drug Treatment

Animals were cared for and treated as described in section 3.3.2.2.

7.3.2.4. Pineal Gland Removal

On the morning of the 6^{th} day (after receiving NVP or EFV, 5mg/kg/day for 5 days) the animals were killed by neck fracture. The brain was exposed in the manner described in section 3.2.2.2, and the pineal organ was carefully removed. The stalk and any tissue adhering to the gland were also removed.

7.3.2.5. Organ Culture of the Pineal Glands

The pineal glands were removed and individually placed into sterile borosilicate (75 x 10 mm) Kimble containing 52 µL of MEM culture medium, supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml) and amphotericin B (2.5 µg/ml). ^{14}C 5-HT (8 µL) with a specific activity 55mCi/mmoles was added. The tubes, the test compounds were then saturated with carbogen (95 % Oxygen: 5 % Carbon dioxide) and immediately sealed. The tubes were incubated for a period of 24 hours at 37 ^{o}C in the dark in a Forma Scientific model 3028 incubator.

The incubation was terminated after 24 hours by the removal of the pineal glands from the culture medium. The culture medium was then analyzed by TLC.

7.3.2.6. Separation of Indoles by Thin Layer Chromatography

A modification of the technique employed by Klein and Notides (1969) was used to separate the radiolabelled indoles. The TLC plates were activated by placing these in an oven at 100 ^{o}C for 10 minutes. Ten µL of the culture medium was spotted on a 10 x 10 cm TLC plate, to form a spot no larger than 4–5mm. The spotting took place under a gentle stream of nitrogen to aid in the drying of the spotted-media. Drying with nitrogen prevented the atmospheric oxidation of the indoles. The indole standard solutions prepared using 1 mg of each of the pineal metabolites: 5-HT, aMT, 5-MT, NAS, 5-HIAA, 5-MIAA, 5-MTOH and 5-HTOH, were vortexed after addition of 2.5 ml 1 % ascorbic acid (an antioxidant) in 0.1 M HCl. Thereafter, 10 µL of the standard solution containing all the indoles, was spotted on top of the already spotted culture medium, the standard was dried under nitrogen.

The spotted TLC plates were placed in a TLC tank which containing chloroform: methanol: glacial acetic acid (93:7:1, Solvent A). Glacial acetic acid allows for complete separation of 5-MIAA from 5-HIAA. The plate was allowed to develop until the solvent front had reached 9 cm from the starting point. The plate was removed from the tank and dried under a stream of nitrogen. Once the required distance was reached, the plate was dried under a stream of nitrogen and placed in a second solvent system (ethyl acetate, Solvent B) at right angles to the first direction. Ethyl acetate separates 5-HTOH from NAS and it improves separation of aMT from 5-MIAA and 5-MTOH. The small amount of acid which remains on the gel from the first solvent development enhances the effective separation by solvent B. 5-HT and 5-MT remain at the origin (Klein and Notides, 1960).

Once the plate was dried under nitrogen again, this was placed under a UV Visible light to detect the indole spots. The spots were cut out and placed in scintillation vials containing 3 ml Beckman Ready-Sol multipurpose liquid scintillation fluid. The vials were tightly sealed and shaken for a period of 30 minutes.

The radioactivity of each metabolite was quantified by a Beckman LS 2800 scintillation counter. The results were obtained as counts per minute (CPM) and

corrected for the counting efficiency of the scintillation counter to disintegrations per minute (DPM). The results were expressed as DPM / 10 µL /pineal.

7.3.2.7. Statistical Analysis

The results were analysed as described in section 2.2.2.3.

7.3.3. Results

Figure 7.1 shows a typical bi-dimensional TLC of the pineal indole metabolites. Clear separation of the six spots was achieved and the positions of the metabolites were identified by following the schematic representations of the chromatogram in Figure 7.1. As discussed in section 7.3.2.6., 5-HT and 5-MT do not migrate from the origin, therefore the results expressed for the origin are those of 5-HT and 5-MT.

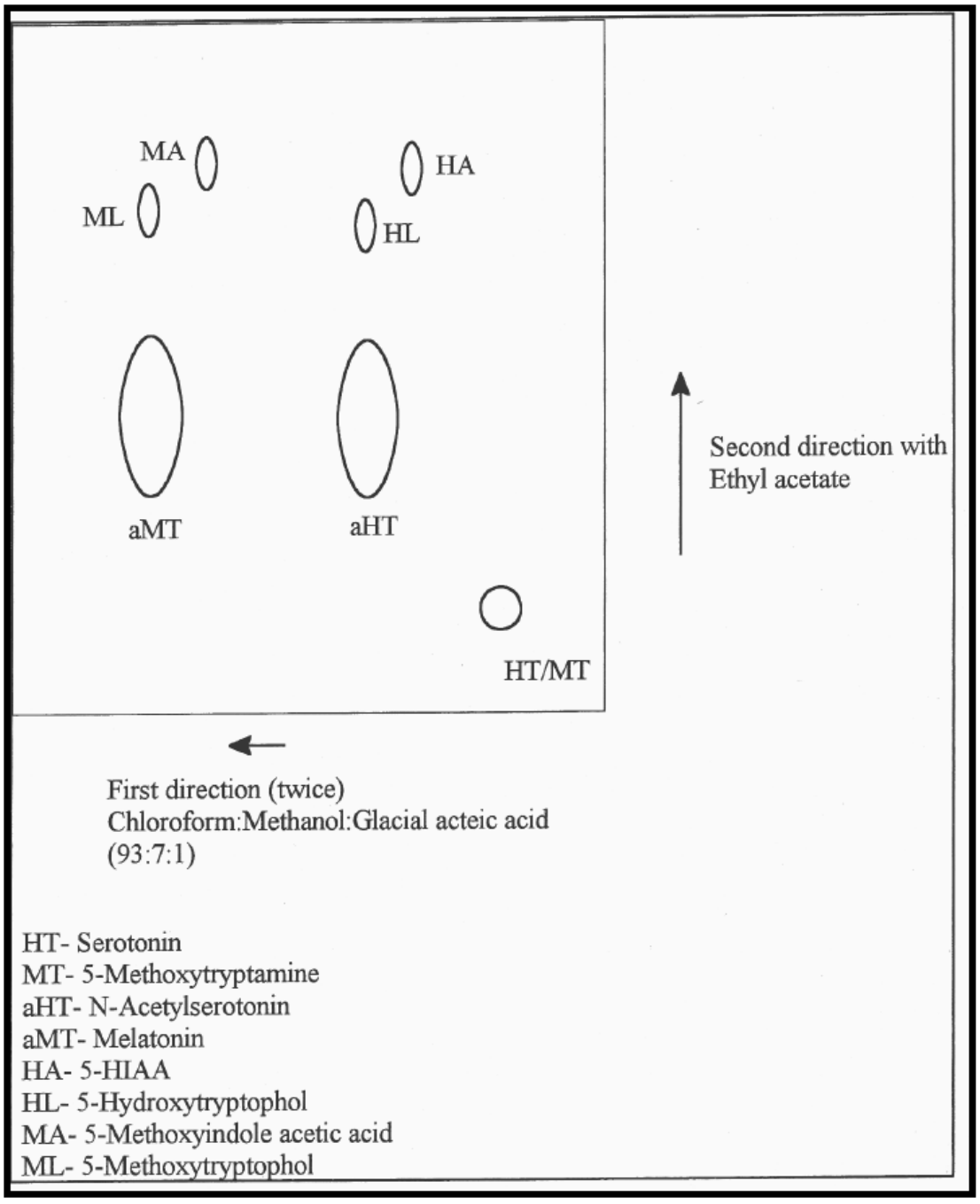


Figure 7.1. A typical bi-dimensional thin layer chromatogram illustrating the location of the pineal indole metabolites (Klein and Notides, 1969).

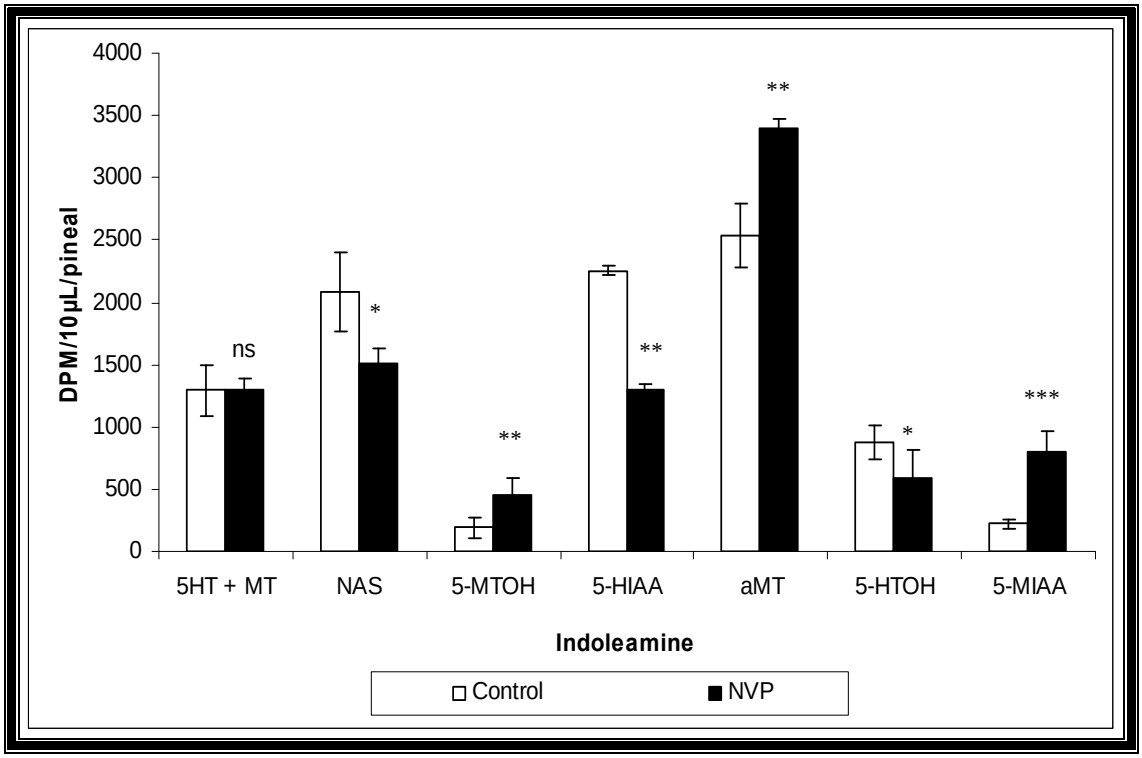


Figure 7.2. Effect of NVP on indole metabolism in rat pineal gland *in vivo*. Each bar represents the mean ± SD; n=4. *** ($p < 0.001$), ** ($p < 0.01$), * ($p < 0.05$) and ns ($p > 0.05$) in comparison to controls (ANOVA and Student-Newman-Keuls Multiple Range Test).

Figure 7.2. shows that there is a significant decrease in the DPM / 10 µL /pineal for NAS ($p < 0.05$), 5-HIAA ($p < 0.001$) and 5-HTOH ($p < 0.05$) of NVP treated group in comparison to the control group, while a significant increase in the DPM / 10 µL /pineal is observed for aMT ($p < 0.01$) and 5-MIAA ($p < 0.001$). The effect of NVP on 5-HT + 5-MT is insignificant.

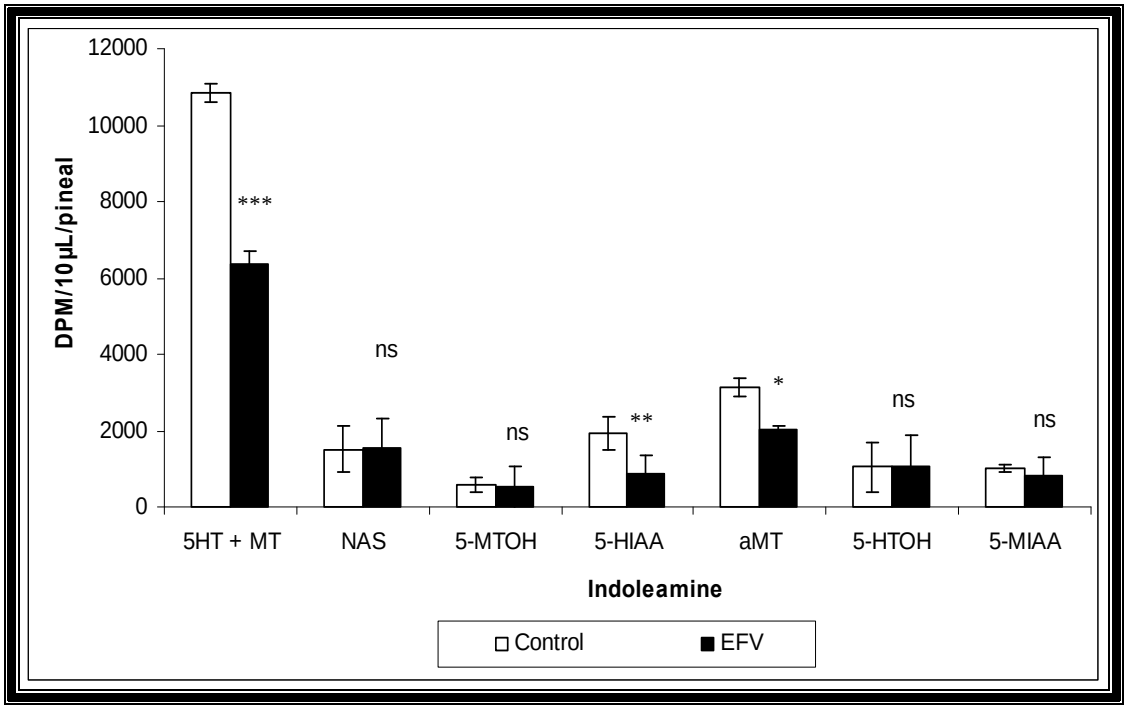


Figure 7.3. Effect of EFV on indole metabolism in rat pineal gland *in vivo*. Each bar represents the mean ± SD; n=4. *** ($p < 0.001$), ** ($p < 0.01$), * ($p < 0.05$) and ns ($p > 0.05$) in comparison to controls (ANOVA and Student-Newman-Keuls Multiple Range Test).

Figure 7.3. shows that there is a significant decrease in the DPM / 10 µL /pineal for 5-HT + 5-MT ($p < 0.001$), 5-HIAA ($p < 0.01$) and aMT ($p < 0.05$) of EFV treated group in comparison to the control group, while the effect of EFV on NAS, 5-MTOH, 5-HTOH and 5-MIAA ($p > 0.05$) is insignificant.

7.3.4. Discussion

The rat pineal gland metabolizes radiolabelled 5-HT to various pineal indoleamines when subjected to organ culture. The effect of exogenous substances on indole metabolism can then be assessed by comparing drug treated pineals to untreated ones. 5-HT can either be taken up by pinealocytes for conversion to NAS and then aMT or

by the sympathetic nerve terminals where it is subjected to MAO degradation to form 5-HIAA and 5-HTOH (Olivieri *et al*., 1990). As shown in Figure 7.3., the decrease in 5-HT + 5-MT after treatment with EFV is thus related to increased uptake and utilization of 5-HT at one or both of these two sites. It is unlikely to be due to a decrease in 5-MT, as this would elevate 5-HT since less of it is converted to 5-MT. Since there is a significant decrease in one of the metabolites of 5-HT formed in the pinealocytes, namely aMT, then the increased uptake and utilization of 5-HT after treatment with EFV most likely occurs at the sympathetic nerve terminals and not in the pinealocytes.

Most 5-HT undergoes deamination by MAO and then oxidised to 5-HIAA by the enzyme ADH, or reduced to 5-HTOH by aldehyde reductase (AR). The significant decrease in 5-HIAA (Figure 7.2. and 7.3.), could be due to the inhibitory effects of these agents on ADH, whilst an inhibitory effect on AR reduces synthesis of 5-HTOH as indicated in Figure 7.2. However, it is also interesting to note that the decrease in 5-HIAA and 5-HTOH synthesis is accompanied by an increase in the production of 5-MIAA and 5-MTOH (Figure 7.2.). Therefore it is possible that this agent has stimulatory effects on HIOMT enzyme which catalyses the conversion of hydroxyl indoles to methoxy metabolites.

NE stimulates T5H to convert TRP to 5-hydroxytryptophan (Wurtman *et al*., 1971) and eventually 5-HT, which is acted upon by NAT to NAS. In the presence of NE and β-adrenergic receptor agonist, NAT activity is increased between 30- and 100-fold (Klein *et al*., 1970), thus enhancing NAS production.

Although NVP significantly enhances NE release in rat hippocampus (section 7.2.3.), which in turn stimulates NAT activity and increases NAS levels in the pineal, Figure 7.2. shows a significant reduction in NAS levels. Thus it could be possible that the amounts of NE produced may not be sufficient to induce NAT activity.

An increase in NAT activity produces a greater concentration of substrate for HIOMT which then synthesizes an increased concentration of aMT. However, it appears that NVP has an inhibitory effect on NAT and a stimulant action on HIOMT, as indicated

by an increase in aMT (Figure 7.2.). On the other hand, EFV shows no significant effects on NAS levels, but reduces aMT synthesis. Therefore this agent may have inhibitory effects on HIOMT activity. It is therefore necessary to determine the effect of these NNRTIs on NAT and HIOMT activity.

Reduced levels of aMT are secreted from the pineal of patients with depression, and whether the increased amounts of aMT produced by the rat pineal gland in the presence of NVP are sufficient to regain the normal physiological concentrations of aMT is debatable. Furthermore, it is unlikely that the rise in aMT induced by NVP is a consequence of inhibition of TDO in the liver as it has been shown (Daya *et. al*., 1989), that such inhibition does not alter aMT levels. A possible reason for the NVP-induced increase in aMT synthesis could be a direct effect of this drug on the aMT synthesis pathway in the pineal gland.

Therefore future studies involving enzyme activity and pineal function are required, in order to establish the effects these agents have on 5-HT metabolism.

7.3.5. Conclusion

These studies provide novel information that these agents not only affect hippocampal neurotransmitter levels but also pineal indole metabolism. Both NNRTIs induce a rise in 5-HT and 5-HIAA levels and a decrease in 5-HT turnover, which implies that these agents may have the potential to slow 5-HT metabolism, allowing for potentiated 5-HT effects in depression associated with ADC. The rise in aMT induced by NVP in rats indicates that this agent could play a significant role in neuroprotection as aMT has been shown to be a potent antioxidant.

CHAPTER 8

LEARNING AND MEMORY

8.1. INTRODUCTION

Learning depends on experience and leads to the acquisition of new behaviour. Many studies have identified conditioning as a universal learning process (Tsitolovsky *et al*., 2004). Classical and operant conditioning are the two different types of conditioning that yield different behavioural patterns (Deporter, 1992; Eldema, 1992; Sylwester, 1993). The typical paradigm for classical conditioning, involves repeatedly pairing a conditioned stimulus (CS) with an unconditioned stimulus (UCS) to produce an unconditioned response (UCR), hence the CS acquires the ability to evoke a conditional response (CR) (http://en.wikipedia.org/wiki/Psychology_of_learning; Guthrie, 1935; Hull, 1943). Pavlov experimentally discovered this type of learning, and found out that when food (UCS) is paired with the sound of a bell (CS), it evokes salivation, the UCR in dogs (Anderson, 2000). On the other hand, operant conditioning occurs when a response to a stimulus is reinforced (Tsilovosky *et al*., 2004) and it deals with the modification of voluntary behaviour. The classic study of operant conditioning was done in 1898 and 1933 by Thorndike who discovered that by placing a hungry cat in a box (stimulus) with food outside (the reinforcer), the cat learns how to press a specific area of the box in order to escape and eat the food. With time the random behaviour of the cat diminished (Anderson, 2000) and when placed in the box, finding the area to press became instinct.

There are several ways to classify memories, based on duration, nature and retrieval of information. A basic and generally accepted classification of memory is based on duration of memory retention, and identifies two distinct types of memory, short-term and long-term. Short-term memory (STM) is supported by transient patterns of

neuronal communication, dependent on regions of the frontal and parietal lobe, and it occurs in the form of either reference or working memory (Baddeley, 1992). Working memory retains rapidly changing information and it is useful for "trial-specific information", while reference memory refers to the memory of information that remains constant over a relatively long period of time and is required for "task-specific information" (Honig, 1978; Olton *et al*., 1979). Long-term memory (LTM), on the other hand, is maintained by more stable and permanent changes in the neural connections widely spread throughout the brain (Eldema, 1992; Sylwester, 1993). More recently it was suggested that LTM, can be divided into declarative and non-declarative memory (Schacter, 1987; Squire *et al*., 1993). While declarative memory involves the ability to store and recall (semantic memory) or recognise events and situations (episodic memory) (Lesch and Pollatsek, 1993), non-declarative memory refers to internal, rather than external information and is reinforced through extensive practice, conditioning, or habits such as brushing teeth or reading a book. Memory processing occurs in three main stages mainly, encoding, storage and retrieval. The encoding stage processes and combines received information for storage, whilst storage requires a physiological change to occur first before memory can be stored. During the retrieval phase, the stored memory is retrieved and the process of encoding is reversed, by using either recognition or recall (van der Veeen *et al*., 2006).

The nervous system and the brain are the physical foundation of the human learning process. Although neuroscience links observations about the biological basis of consciousness, perception, memory, and learning with the actual physical processes that support such behaviour. This theory is still "young" and is undergoing rapid, controversial development (Deporter, 1992; Edelman, 1992; Sylwester, 1993). Throughout the history of research on animal learning there have been conflicting views concerning the fundamental issue of what animals learn (Packard and McGaugh, 1996).

Cognitive theorists such as Tolman proposed that animals acquire knowledge of ''what leads-to-what'' that result in expectations of the consequences of their behaviour (Packard and McGaugh, 1996). In contrast, following the pioneering work of Thorndike and Pavlov, other theorists proposed that animal learning consists of the formation of stimulus–response (S-R) habits (Hull, 1943; Guthrie, 1935) or simply,

the learning of motor responses. In experiments addressing this issue, rats were trained in mazes and subsequently tested in a variety of ways in an attempt to discover whether the learning was based on acquisition of knowledge or learning of responses (Tolman *et al*., 1946; Blodgett *et al*., 1949; Hill and Thune, 1952).

Disturbances in structure and or function of the nervous system emerging from injury, diseases, development of abnormalities and the presence of toxins cause neurological disorders. The relationship between neurobiology and behaviour is important as it is often that abnormal behaviour that first presents as a sign of neurological disorder. Hence such changes bring about clues as to which anatomical or chemical site of the nervous system is attacked (Bondy, 1985) and permit integration of explicit behavioural assessment into a wide variety of ongoing studies and chronic bioassays (Dairam, 2005).

8.2. THE EFFECT OF QA ALONE AND IN COMBINATION WITH EFV AND NVP ON RAT SPATIAL REFERENCE MEMORY *IN VIVO*.

8.2.1. Introduction

The hippocampus is considered as a brain region critical for the acquisition, consolidation and retrieval of several kinds of memory (Squire 1992; Eichenbaum 2000) that are non-specific for the type of information, such as declarative memory, (Cohen and Eichenbaum, 1991 and Eichenbaum *et al*., 1992) working memory or spatial mapping of recent memories (Buzsaki *et al*., 1982 and Olton, 1990), reference memory (Olton *et al.*, 1979) and configural learning. (Sutherlane and Rudy, 1989) The hippocampus has been traditionally linked to cognitive functions, particularly spatial memory, at least in rodents (Morris *et al*., 1982 and Silva *et al*., 1998). Previous studies have shown that hippocampus has an exclusive role in working memory or spatial mapping of recent memories (Buzsaki *et al*., 1982 and Olton, 1990). The hippocampal regions which consist of the CA fields, dentate gyrus and subicular complex, are part of a system that is important for mammalian memory (Squire, 1992). Damage to these regions impairs learning and memory (Eichenbaum and Cohen, 2001). It has been thought that the hippocampus may be particularly essential for tasks that depend on relating or combining information from multiple sources, as in spatial memory tasks (O'Keefe and Nadel, 1978). It has been reported that hippocampal lesions impair recognition memory performance in humans (Manns *et al*., 2003), monkeys (Beason-Held *et al*., 1999; Zola *et al.*, 2000) and rodents (Clark *et al*., 2000; Clark *et al*., 2001). In the rodent, hippocampal lesions encompassing 40 % of the total hippocampal volume markedly impair learning in the water maze (Moser *et al.*, 1993; Moser *et al.*, 1995). A loss of hippocampal neurons had been considered a hallmark of normal aging and, furthermore, to be a substrate of age-related learning and memory deficits. Because the neuron is the basic functional unit of the nervous system, it is of fundamental importance to establish, as a first step in identifying the structural basis of age-related decline in memory, the extent to which neuron numbers correlate with performance on tasks known to involve the

hippocampus, such as the Morris water maze (Morris, 1984). CA1 pyramidal neurons are a subpopulation of hippocampal neurons that are the most sensitive to cognitive deficits (Olsen *et al*., 1994). There was a significant correlation between working memory impairments and the neuronal cell loss in hippocampal CA1 subfield of demented rats (Stepanichev *et al*., 2004).

The hippocampus plays an important role in episodic memory, such that lesioning of this limbic structure after learning a spatial orientated task induces spatial memory deficits (Winocur *et al*., 2005). Memory formation in the hippocampus involves stimulation of excitatory glutamatergic neurons, especially of the NMDA type (Haberny *et al*., 2002; Silva, 2003). Although the NMDA receptor plays an important role in memory formation, excessive stimulation by agonists such as QA can induce neuronal dysfunction, cell damage or even death. NMDA receptor-mediated neuronal damage and impaired hippocampal LTP have been implicated in the neurocognitive deficits associated with ADC (Lipton, 1994, Lipton, 1998). QA is known to cause lesioning in the hippocampus (Schwarcz *et al*., 1984; Speciale *et al*., 1987) and consequently resulting in spatial memory deficits (Moser *et al*., 1993; Clark *et al*., 2000; Zola *et al*., 2000; Clark *et al*., 2001; Manns *et al*., 2003). Furthermore, since QA concentrations are elevated in the brain tissue and CSF of patients with ADC, and there is a direct correlation between the degree of elevation of CSF QA and the degree of neuropsychological deficit observed (Archim *et al*., 1993; Brew *et al*., 1996) due to lesioning of the hippocampus (Price *et al*., 1988; Masliah *et al*., 1992) it was decided to assess whether NVP and EFV, the current treatment of HIV/AIDS, could attenuate QA-induced memory impairment in ADC.

Several authors suggest that the dorsal hippocampus is particularly involved in the acquisition of water-maze spatial memory tasks (Hock and Bunsey 1998; Hampson *et al* 1999; Ferbinteanu and McDonald 2001; Hölscher, 2003; Pothuizen *et al*., 2004). The most frequently used paradigm to evaluate learning and memory abilities in rodents is the Morris water task (D'Hooge and De Deyn, 2001), which is a spatial navigation task in which the animal swims to find a hidden platform, using extra-maze visual and other sensory cues (Lipton *et al*., 1991) to locate it. To assess hippocampal dependent spatial learning and memory, mice were trained in a standard Morris water maze task (Morris *et al*., 1982; Silva et *al*., 1998; Stackman *et al*., 2002).

It was suggested that the short time periods that the rats are allowed to remain on the platform after each swimming trial are used for spatial orientation in the service of acquiring the spatial relationships between the platform and the cues in the environment (Sutherland and Dyck, 1984). Since the platform location remains unchanged throughout the test trials, this task predominantly measures spatial reference memory rather than working memory. The operant type of learning is employed in this task by training the rats to swim and escape from the opaque tank of water onto the hidden platform. The Morris water maze test is generally presented as taxing spatial memory, and thus is sensitive to the ability to consolidate and deal with spatial representations depending on hippocampal functions. The task itself may also be sensitive to non-cognitive biases (Lindner , 1997, van der Staay, 2002), or be solved by strategies that either do not exclusively rely upon the constitution and use of a cognitive map such as, path integration or the ability to integer, represent and use self-movements (Sutherland and Hamilton, 2004). After training, the hippocampi of the rats are lesioned and these animals undergo treatment. Local lesions in the CNS are a common tool to interfere with brain structures and neurotransmitter systems, respectively, to assess their relevance for the behaviour and basal brain functions (File *et al*., 1979). The effects of the water maze task experienced before lesioning on spatial memory, is determined by reintroducing the rats to the same water maze and assessing the latency (time in seconds) to escape to the platform (Morris, 1981).

8.2.3. Materials and Methods

8.2.3.1. Chemicals and Reagents

All chemicals and reagents were as per section 4.2.2.1.

8.2.3.2. Animals

Adult male Wistar rats were housed and cared for as described in section 3.2.2.2.

8.2.3.3. Animal training in the Morris water maze task

The apparatus consisted of a circular water tank (150cm in diameter and 40 cm high). A platform (12.5 cm in diameter and 31cm high) invisible to the rats, was set inside the tank, which was filled with water maintained at approximately 23 ^{o}C to a height of 33 cm. The platform was submerged 2 cm below the surface of the water. The tank was located in a large room where there were several brightly colored cues external to the maze: these were visible from the pool and could be used by the rats for spatial orientation. The position of the cues remained unchanged throughout the study. For each training session each rat was put into the water at one of four starting positions, the sequence of the positions being selected randomly. Training was conducted for 7 consecutive days, twice a day with each session consisting of 4 trials. Animals were trained for 7 days before being subjected to the treatment protocol.

8.2.3.4. Surgical procedures and treatment regimes

For the purpose of these experiments, rats were divided into four groups as described in the table 8.1.

Table 8.1. Treatment regimes for the behavioural studies

Treatment Regime	i.h. injection (2 μL)	Received daily dose for 5 days after i.h. injection
Control	PBS	Vehicle
QA (i.h.) + vehicle	120 nmoles QA	Vehicle
QA + EFV (5 mg/kg/day for 5 days)	120 nmoles QA	EFV 5mg/kg/day
QA + NVP (5 mg/kg/day for 5 days)	120 nmoles QA	NVP 5mg/kg/day

After 7 days of training, animals were divided into the above 4 groups and received intrahippocampal injections as described above. Surgical procedures were conducted as described in section 3.3.2.3. Thereafter the animals were dosed intraperitoneally with either EFV or NVP at a dose of 5mg/kg/day for 5 days. On the morning of the 6th

day, animals were re-introduced to the water maze task and test trials were conducted for 7 days with animals receiving 1 test trial a day.

8.2.3.5. Morris water maze test of spatial reference memory

During test trials, rats were placed into the tank at the same starting point, with their heads facing the wall of the tank. The time taken for each rat to find the hidden platform from the starting point was measured by a person unaware of the experimental conditions.

8.2.3.6. Statistical analysis

All results were analysed using repeated measures ANOVA with group as a factor and latency to escape measured over 7 days. Student-Newman-Keuls multiple range test was used for post hoc analysis. The level of significance was accepted at $p < 0.05$ (Zar, 1974).

8.2.4. Results

In Figure 8.1. and 8.2., the results of the statistical analysis show a significant difference in latency to escape between the EFV ($F = 15.77$, $p < 0.001$) and NVP treated groups ($F= 18.81$, $p < 0.001$). Post hoc analyses (Student-Newman-Keuls' Multiple Range test) show that the latency to escape (over 7 days) of rats that received intrahippocampal injections of QA was significantly longer compared to the control group ($p < 0.001$).

The EFV and NVP treated animals which also received intrahippocampal injections of QA significantly show faster escape times in comparison to the QA alone group ($p < 0.001$) (Figure 8.1. and 9.2.). There is no significant difference between the latencies to escape of the control group in comparison to the drug treated groups ($p > 0.05$).

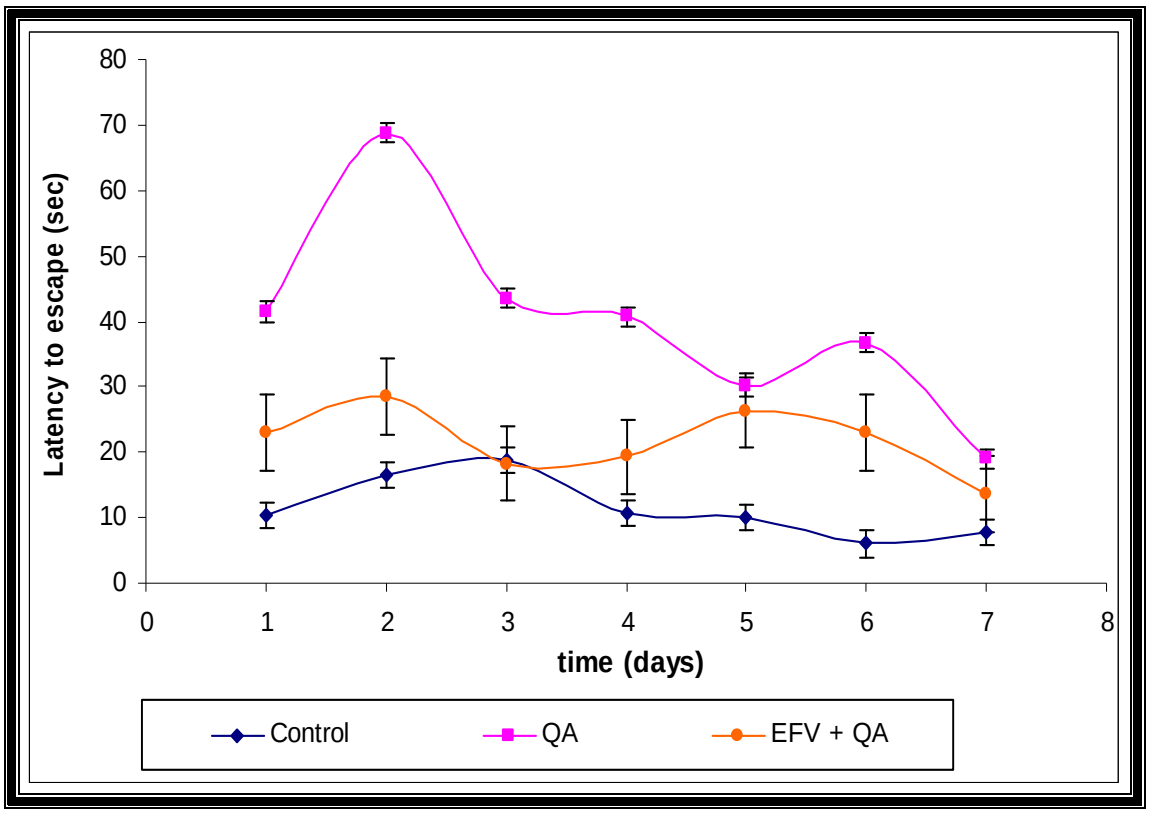


Figure 8.1. Performance of the rats in the water maze task. Each point represents the mean latency ± SD to escape of 5 rats. Trials were conducted once daily, every day for 7 days. Results were analyzed using repeated measures ANOVA with group as a factor and latency measured over 7 days. Student-Newman-Keuls multiple range test for multiple group comparison was used for post hoc analysis.

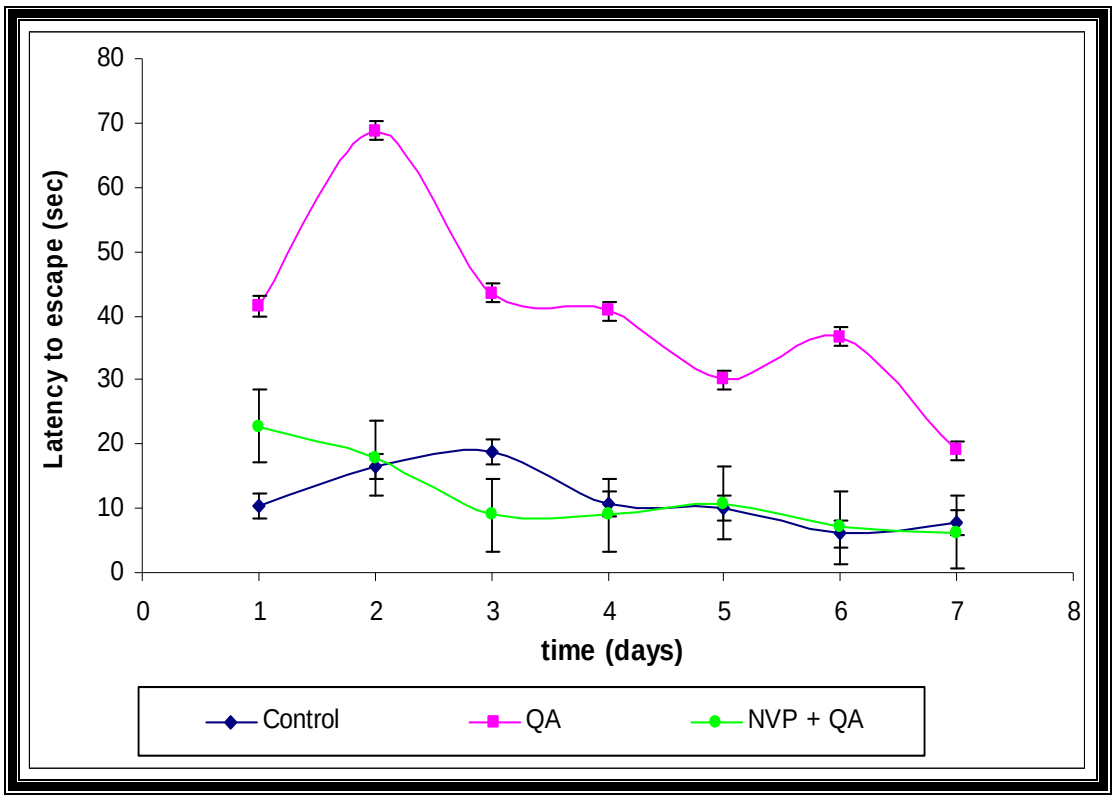


Figure 8.2. Performance of the rats in the water maze task. Each point represents the mean latency ± SD to escape of 5 rats. Trials were conducted once daily, every day for 7 days. Results were analyzed using repeated measures ANOVA with group as a factor and latency measured over 7 days. Student-Newman-Keuls multiple range test for multiple group comparison was used for post hoc analysis.

8.2.3. Discussion

Memory formation in the hippocampus involves stimulation of excitatory glutamatergic neurons, especially of the NMDA type (Haberny *et al*., 2002; Silva, 2003). The NMDA receptors are critically involved in various types of synaptic plasticity, including LTP, a neural process underlying memory and learning in the hippocampus (Collingridge and Bliss, 1987; Bliss and Collingridge, 1993; Asztely and Gustafsson, 1996). The normal physiological stimulation of the NMDA receptors evokes an influx of Ca^{2+}, known as the calcium signal, required during development of LTP (Bliss and Collingridge, 1993; Asztely and Gustafsson, 1996; Danysz and

Parsons, 2003). Although NMDA receptors play an important role in memory formation, excessive stimulation by agonists such as QA can induce neuronal dysfunction, cell damage or even death (Stone, 1993; Nakai *et al*., 1999). QA is an analogue of aspartate, which is a known agonist at NMDA receptors (Stone and Perkins, 1981; Perkins and Stone, 1983; Stone, 1993). The concentrations of QA in the CNS have been shown to rise several hundred-fold in some circumstances, such as infection by HIV, to levels that produce a marked activation of NMDA receptors (Heyes *et al*., 1991).

The presence of QA on the postsynaptic neuron, results in temporal uncoordinated and continuous pathological stimulation of NMDA receptors, producing enhanced noise, thus decreasing the probability of detecting the relevant calcium signal (Danysz and Parsons, 2003), produced in response to the firing of the presynaptic neuron. The firing of hippocampal neurons during the test trails (after lesioning), occurs when partial cues are detected and the retrieval processes of autoassociation are placed in motion (Müller, 2006). The potentiated synapses in all groups of rats are formed by induction and subsequent expression of LTP between CA1 and CA3 subfields of the hippocampus, in the encoding and storage processes during the 7 days of training. However in QA lesioned rats, the Ca^{2+} signal generated in the glutamatergic synapses is not detected, suggesting that CA1 cells are unable to operate as recorders for the recalled information from the CA3 cells, as a result the recalled information is not efficiently represented to enable initiation of recalling (Müller, 2006).

The ability of 120 nmoles of QA to significantly reduce the retrieval of spatial memory as shown in Figure 8.1.and 8.2., could also be due to postsynaptic neuronal degeneration of the potentiated glutamatergic synapses, as a result of overstimulation of ionotropic glutamate receptor sub-types, such as NMDA and AMPA. It is these properties of QA that alter the ability of the rats to retrieve spatial reference memory. However, treatment of the animals with EFV and NVP, improves behavioural response in comparison to the QA treated group. In addition, both agents appear to be effective in improving memory to the extent that there is no significant difference in comparison to the control group ($p > 0.05$).

The ability of NVP and EFV to improve spatial reference memory deficits induced by QA, a product of HIV-induced neurotoxicity, could possibly be due to a reduction in

the noise-to-signal ratio as well as scavenging ROS (section 2.2.3; 3.2.3 and 3.3.4) hence protecting the hippocampus from QA-induced neurotoxicity. Since NMDA receptors are vital for brain function (Forrest *et al*., 1994) memory formation and learning (Bliss and Collingridge 1993), and QA has been demonstrated to reduce the number of NMDA receptors in rat hippocampus (Southgate *et al*., 1998; Nakai *et al*,. 1999), It is possible that these NNRTIs may reduce the loss of receptors and hence prevent the loss of hippocampal neurons thereby improving the QA-induced spatial reference memory deficit. Although these results may suggest this possibility, it is not yet known whether these agents bind to NMDA receptors, hence histological and receptor binding studies have to be implemented in order to augment this possibility.

8.2.4. Conclusion

NVP and EFV treated animals have lower escape latencies compared to the untreated animals that also received intrahippocampal injections of QA. This effect is speculated to be related to the ability of these agents to protect against QA-induced hippocampal neurodegeneration, hence improve retrieval of the spatial information stored in potentiated synapses through LTP and autoassociation at the NMDA receptors.

CHAPTER 9

SUMMARY OF RESULTS AND GENERAL CONCLUSIONS

9.1. SUMMARY OF RESULTS

Oxidative stress-induced neuronal death implicated in ADC is often associated with an immune response, in which microglia are activated to produce and release many neurotoxic products including oxidative free radicals and QA, which have the ability to cause neurodegeneration. Although there are several hypotheses for the involvement of causative agents in neuro-AIDS, there is particularly a strong case for believing that the excitotoxin QA may have special relevance to the development of CNS dysfunction and damage in ADC. Numerous studies point to a neuroprotective potential for NNRTIs, EFV and NVP, which currently are the drugs of choice in the treatment of HIV/AIDS.

Free radicals cause deleterious oxidative damage to biological molecules leading to various neurodegenerative disorders including ADC. Therefore in Chapter 2, the antioxidant effect of these NNRTIs was determined based on the percentage scavenging of DPPH radicals. The results show that, NVP and EFV scavenge the DPPH radical and this is speculated to be through hydrogen donation. In addition this helps to prevent further initiation of free radical-mediated chain reactions in cells by preventing the abstraction of hydrogen from susceptible PUFAs.

Increased ROS formation and oxidative stress are important features involved in QA-induced neurotoxicity, ultimately causing oxidative damage to biological molecules including lipids, proteins and DNA. Chapter 3 illustrates that QA significantly induces LP in rat brain homogenates *in vitro* and that both, NVP and EFV blunt this effect. It is speculated that *in vitro*, QA forms complexes with Fe^{2+} ions that enhance

the Fenton reaction to produce $HO^{\bullet}$ which in turn participates in LP. The possibility of the NNRTIs binding to Fe^{2+} and Fe^{3+} and reducing Fe^{2+}-induced LP in rat brain homogenate was investigated in chapter 5. Both NNRTIs once again, reduce QA-induced LP *in vivo*, which is thought to occur through NMDA-dependent excitotoxicity in addition to the free radicals produced by the QA-Fe^{2+} complex. The results of this chapter speculate that this effect could be related to the ability of these agents to either scavenge the free radicals being produced or binding Fe^{2+} and / or Fe^{3+} ions. Furthermore, NVP reduces QA-induced LP *in vivo*, below control levels, and this may possibly be related to interactions with NMDA receptors. More studies are required to augment this possibility. In addition since QA inactivates NMDA receptors which have an important function in learning and memory, a reduction in QA-induced neurotoxicity by these agents is thought to be of therapeutic value in improving learning and memory function. Hence this possibility was investigated in Chapter 8.

Since Chapters 2 and 3 demonstrate that NVP and EFV have antioxidant and free radical scavenging properties, it was decided to determine if these agents could scavenge, the toxic, $O_2^{\bullet -}$. Initially, *in vitro* experimental results illustrate that KCN acts as a potent inducer of $O_2^{\bullet -}$ production in rat brain homogenate, and that both agents significantly attenuate this effect. Hence the ability of these agents to reduce KCN-induced $O_2^{\bullet -}$ formation was speculated to be possibly through a reduction and / or prevention of Ca^{2+} influx or scavenging of $O_2^{\bullet -}$. Intrahippocampal injections of QA induce a significant increase in $O_2^{\bullet -}$, and the presence of this free radical *in vivo* is influenced by NMDA stimulation associated with QA, which implies that the reduction in $O_2^{\bullet -}$ generation brought about by NVP and EFV is most likely and primarily related to the ability of these agents to prevent $O_2^{\bullet -}$ formation rather than scavenging this free radical.

In view of the possibility that NVP and EFV alleviate QA enhanced Fe^{2+}-induced LP through chelation of Fe^{2+} and Fe^{3+} , the purpose of Chapter 5 was to conduct Fe chelation studies. The possibility of the NNRTIs to directly inhibit the Fenton reaction was assessed by conducting Fe^{2+}-induced LP *in vitro*. Rat brain homogenates exposed to Fe^{2+} exhibited significant LP by a mechanism which may involve Fe-mediated decomposition of lipid hydroperoxides to yield alkoxyl or peroxyl radicals, leading to

the chain reaction of LP. EFV exhibits an inhibitory effect on Fe^{2+}-induced LP, hence its ability to ameliorate QA-induced LP was speculated to be through binding of Fe^{2+} and/ or Fe^{3+}. Furthermore, the decrease in QA-induced LP by NVP was speculated to be through free radical scavenging and not by interference with the Fenton reaction as indicated by the non-significant effect on Fe^{2+}-induced LP *in vitro*. The ferrozine assay was used to determine Fe^{2+} chelating activity by these NNRTIs, and the results demonstrate that EFV has a greater chelating activity than NVP, indicating that it is more likely to bind Fe^{2+} and therefore provides an adequate explanation for its significant inhibition of Fe^{2+}-induced LP. The ASVs from electrochemical analysis confirms the metal binding and further characterized the redox potentials of the complexes in comparison to the free Fe^{3+}. It was concluded that the NVP- Fe^{3+} and EFV- Fe^{3+} complexes are more difficult to reduce than the respective free metal ion. These results also demonstrate stronger Fe^{3+} binding for EFV than NVP. Thus through binding Fe^{2+} and Fe^{3+}, these NNRTIs may prevent redox recycling of Fe^{3+} to Fe^{2+} and consequently reduce the amount of Fe^{2+} available to participate in the formation of the QA- Fe^{2+} complex and the subsequent LP associated therewith. Furthermore, these results provide an adequate explanation for the inhibition of QA-induced LP by these agents (Chapter 3).

The KP is involved in the synthesis of the neurotoxin QA, and Chapter 6 aims to assess the effect these NNRTIs have on the biosynthesis of QA by measurement of the activity of the enzyme TDO, which is involved in catalyzing the conversion of TRP, a precursor of 5-HT synthesis to QA. The untoward effect of enhanced TDO activity is that it induces TRP catabolism, thus increasing blood-borne kynurenines for the biosynthesis of QA, and reducing the availability of TRP for uptake into the brain, where it is utilized for the synthesis of the neurotransmitter 5-HT. *In vivo* studies demonstrate that the treatment of animals with NVP and EFV (5mg/kg/daily for 5 days) decreases the apo-, holo- and total enzyme activity of TDO. It was proposed that both NVP and EFV most likely interfere with the conjugation of haeme to the apoenzyme by competitive inhibition.

The inhibition of TDO may not only reduce the synthesis of the excitatory amino acid, QA but increases amounts of TRP for 5-HT synthesis. 5-HT has emerged as an important neurotransmitter in normal brain function and abnormal synthesis,

utilization and metabolism are appalling. Hence, Chapter 7 investigated the effect of NVP and EFV on these aspects of 5-HT and other biogenic amines.

NVP and EFV (5mg/kg/daily for 5 days), both increase the production of 5-HT in the hippocampus. The study confirms the earlier findings in Chapter 6 that both NNRTIs have profound inhibitory effects on liver TDO activity, which culminates in a rise in central 5-HT levels. It was therefore concluded that TDO inhibition and thus increased brain TRP is confirmed to be the most likely mechanism of increased brain 5-HT levels by these NNRTIs. NVP also induces a rise in hippocampal 5-HIAA levels which was attributed to the fact that there is increased hippocampal 5-HT levels available as substrate for ADH and MAO which oxidize 5-HT to 5-HIAA. However despite this, 5-HT turnover was reduced, which could imply that both NNRTIs may have the potential to slow the metabolism of 5-HT, thus maintaining the increased levels for longer.

The results of the pineal organ culture showed that EFV treatment decreases the pineal level of 5-HT + 5-MT. It was hypothesized that this effect is related to an increased uptake and utilization of 5-HT at the sympathetic nerve terminals and not in the pinealocytes. The significant decrease in 5-HIAA is observed and is thought to occur via ADH inhibition, whilst an inhibitory effect on AR reduces synthesis of 5-HTOH as indicated in NVP treated pineals. However, it is also interesting to note that the decrease in 5-HIAA and 5-HTOH synthesis in NVP treated animals is accompanied by an increase in the production of 5-MIAA and 5-MTOH. Therefore it is possible that this agent has stimulatory effects on HIOMT. In addition, NVP increases aMT synthesis by the pineal gland. This increase was thought to be as a result of NVP's effect on the aMT synthesis pathway in the pineal gland rather than the inhibition of TDO. Although increased NE levels stimulate NAT activity , thus increasing pineal NAS levels, the decrease in pineal NAS levels shown by NVP treated animals is speculated to be as a result of insufficient NE available to induce NAT activity.

Therefore the direct effect of NVP on NAT activity needs to be investigated. These results also indicate that the mechanism of action of these agents is not only confined to the hippocampus but also the pineal gland with regard to alteration of

indoleamines. The resultant increase in pineal aMT levels following NVP administration could have implications in neuroprotection as aMT has been demonstrated to be a potent antioxidant in the brain.

Since the hippocampus plays a major role in learning and memory, lesioning of this limbic structure would induce spatial memory deficit. QA is known to cause lesioning in the hippocampus, hence Chapter 8 demonstrates that i.p. post-treatment of NVP and EFV, improves QA-induced spatial memory deficits in the hippocampus, in a Morris water maze task model of behavioural studies. QA significantly affects the animal's performance in the water maze by virtue of the longer latencies to escape to the platform when compared to the control group. NVP and EFV treated animals have shorter latencies to escape in comparison to the untreated animals that also received intrahippocampal injections of QA. Therefore this experiment speculates that the reduced spatial memory deficits could be related to the ability of these NNRTIs to protect against QA-induced hippocampal neurotoxicity. NVP treated animals had shorter latencies to escape when compared to EFV treated animals. This effect may be related to the ability of NVP to protect against QA-induced hippocampal neuronal damage to below control levels, as shown in Chapter 3.

9.2. CONCLUSIONS

Some of the factors implicated in the pathogenesis of neurodegenerative disorders such as AD, PD and ADC, include free radicals (Gutteridge 1995; Floyd 1999; McCord 2000), excitotoxicity (Choi 1985; Rothman and Olney 1987) as well as an imbalance in metal homeostasis (Bush 2000). Since there is no cure for these debilitating diseases, current therapeutic strategies which aim to delay the onset and/ or slow down the progression of the diseases will be of therapeutic value. This study has demonstrated that the NNRTIs, EFV and NVP may possibly have the potential to be used in the treatment or prevention of other neurological disorders such as AD and HSE.

NVP and EFV have shown to possess antioxidant and metal binding properties, which may also contribute towards the neuroprotection offered by these agents. Both agents reduce LP, scavenge the $O_2^{\bullet -}$, bind Fe^{2+} and Fe^{3+}, protect rat hippocampal neurons against QA-induced neurotoxicity and subsequently reduce QA-induced spatial memory deficits. EFV demonstrated stronger interactions with Fe^{2+} and Fe^{3+}, hence exhibiting significant reduction in Fe^{2+}-induced LP *in vitro*.

Both NVP and EFV inhibit TDO and increase 5-HT levels in the hippocampus. Furthermore, NVP but not EFV increases the pineal synthesis of aMT, a well established neuroprotectant. Hence this could be another mechanism through which NVP may offer neuroprotection although it was stated that this increase is not due to TDO inhibition. TDO inhibition also may be a mechanism through which these agents reduce the synthesis of QA, and consequently decrease excitotoxicity and the subsequent neurodegeneration associated therewith.

Thus from the preceding evidence, the possible mechanisms of neuroprotection, by which these agents function provides a variety of alternatives in attenuating various biochemical processes that are exhibited in neurodegeneration. Furthermore, this study highlights the potential therapeutic benefits that these agents could have in treating the neurodegenerative disorder, ADC.

CHAPTER 10

RECOMMENDATIONS FOR FUTURE STUDIES

In Chapter 3, the NNRTIS reduce QA-induced neurotoxicity *in vivo*. QA has been shown to enhance synaptosomal glutamate release, resulting in indirect excitotoxicity through elevation of glutamate levels in the synaptic cleft (Tavares *et al*., 2005). Hence the effect of these agents on synaptosomal glutamate release needs to be further investigated. Excitotoxicity produced by sustained NMDA receptor activation by QA is associated with elevated cytosolic Ca^{2+} concentrations, followed by Ca^{2+} dependent enhanced free radical production, which leads to molecular damage and often cell death (Stone and Perkins, 1981; Santamaría *et al*., 2003; Pérez-Severiano *et al*., 2004). Thus apart from scavenging the free radicals generated, these NNRTIs may also reduce intracellular Ca^{2+} concentrations and / or interact directly with NMDA receptor or indirectly via second messenger processes that in turn influence NMDA receptor processes. Further studies need to be conducted in order to determine the effect, if any, of these NNRTIs on NMDA receptors and the binding of Ca^{2+}.

KCN may induce LP through disruption of the mitochondrial ETC (Way, 1984), which consequently leads to increased levels of intracellular Ca^{2+}, and ultimately free radical generation and oxidative stress (Southgate and Daya, 1999; Pillay *et al*., 2002). The effect of KCN on LP is reduced by the co-incubation of the homogenate with the NNRTIs, as illustrated in Chapter 4. NVP and EFV may initiate this effect by scavenging the free radicals generated. It was also thought that these agents prevent the influx of Ca^{2+} into the mitochondria or prevent the inhibition of the mitochondrial enzyme a_1a_3. Further research needs to be conducted on the effects of these NNRTIs on the mitochondria.

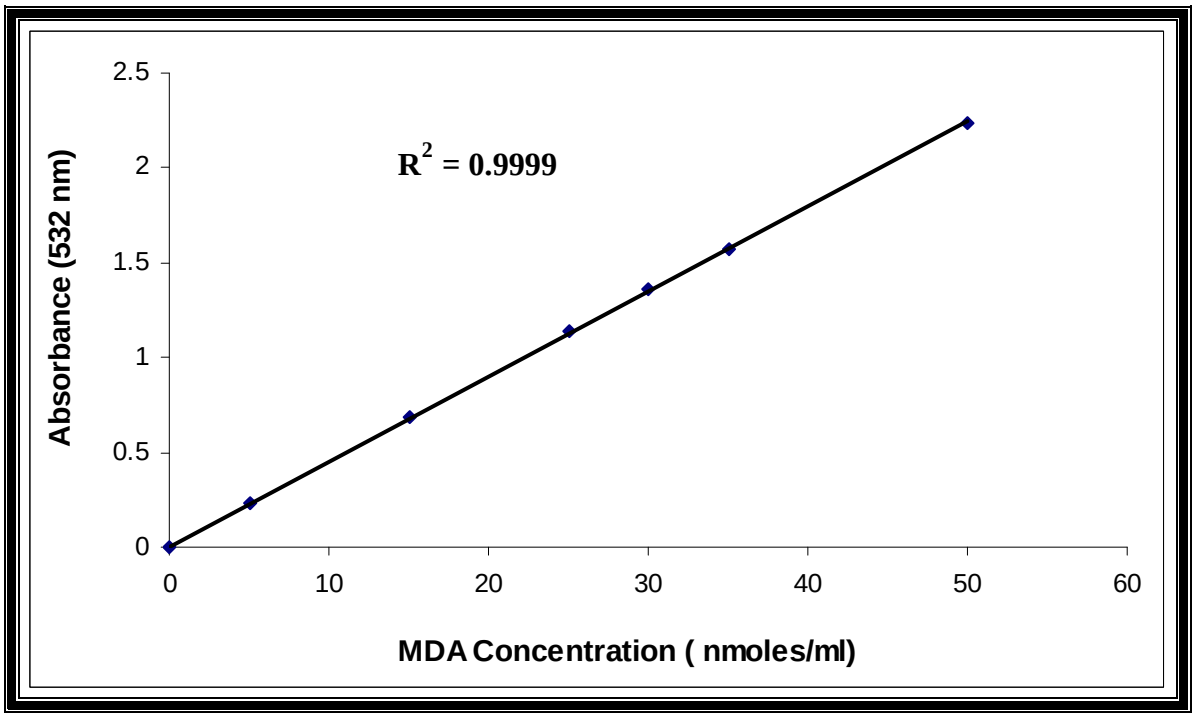
In Chapter 6, the treatment of the animals with NVP and EFV (5 mg/kg/day for 5 days), decreases the apo-, holo- and total enzyme activity of TDO. It was proposed

that these agents may be interfering with conjugation of haeme to the apoenzyme by competitive inhibition. This effect needs to be further explored. Another area of further research would be an investigation into the effect of these NNRTIs on the biosynthesis of QA by measurement of the activities of other enzymes involved in catalyzing the conversion of TRP to QA, namely IDO and 3-HAO. The cofactors, $O_2^{\bullet-}$ and Fe^{2+} are required for the activity of IDO and 3-HAO respectively, and since these NNRTIS have demonstrated the ability to reduce QA-induced $O_2^{\bullet-}$ generation *in vivo*, and bind Fe^{2+} as illustrated in Chapter 4 and 5 respectively. Further studies are necessary to investigate the effect these agents have on the activity of both enzymes.

The enzymes, MAO and ADH are implicated in the oxidation of 5-HT to 5-HIAA, and NVP increases 5-HIAA levels in the hippocampus. Hence more studies involving mechanisms through which this agent increases 5-HIAA needs to be established. It is also suggested that the effect of this agent on the enzyme responsible for 5-HT metabolism, MAO-A, also be studied, as this would provide a further understanding into the effects it has, regarding the role it plays in altering brain 5-HT levels. NVP increases aMT synthesis. It is unlikely that the rise in aMT is a consequence of inhibition of TDO in the liver but rather a direct effect on the pineal gland. More research needs to be conducted on the direct effects of NVP on the aMT synthesis pathway.

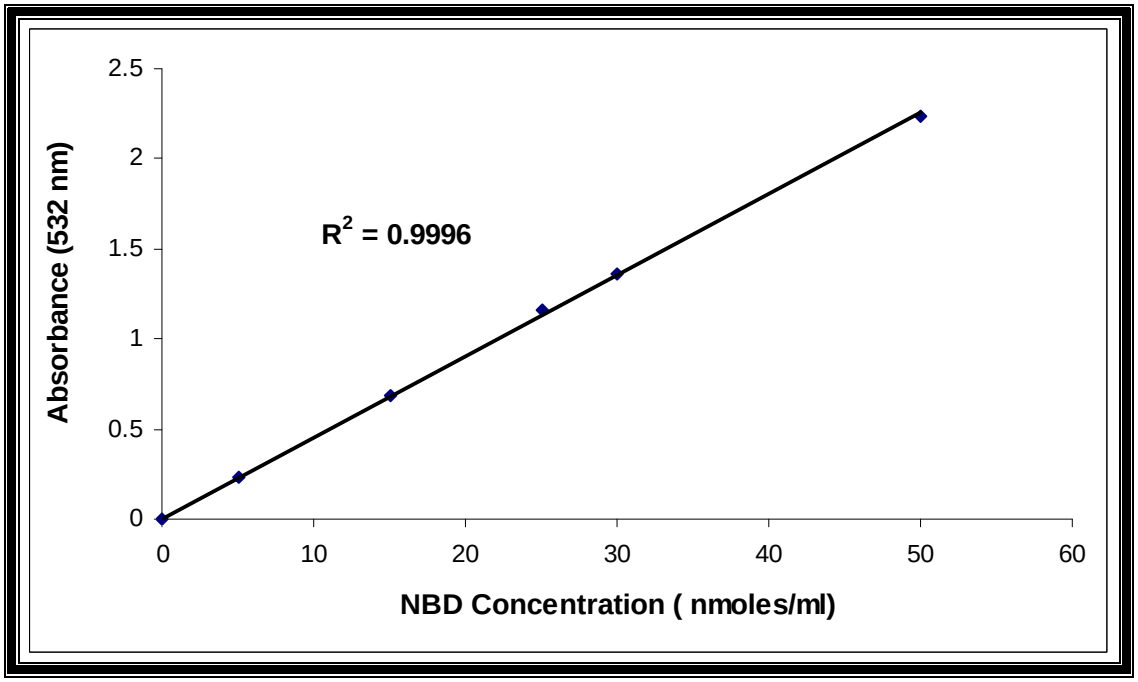
QA has been reported by some authors to cause selective pyramidal cell death in the hippocampal CA1, CA3 and CA4 regions (Behan *et al*., 1999), which are responsible for learning and memory. In Chapter 8, both agents illustrate the ability to significantly improve QA-induced spatial memory deficits. Hence the ability of these agents to protect against pyramidal cell death, if any, needs to be further investigated using both apoptotic and histological analyses. These investigations will provide useful information pertaining to the degree to which the neuroprotection offered by these agents dictates the efficiency of spatial reference memory.

APPENDIX I



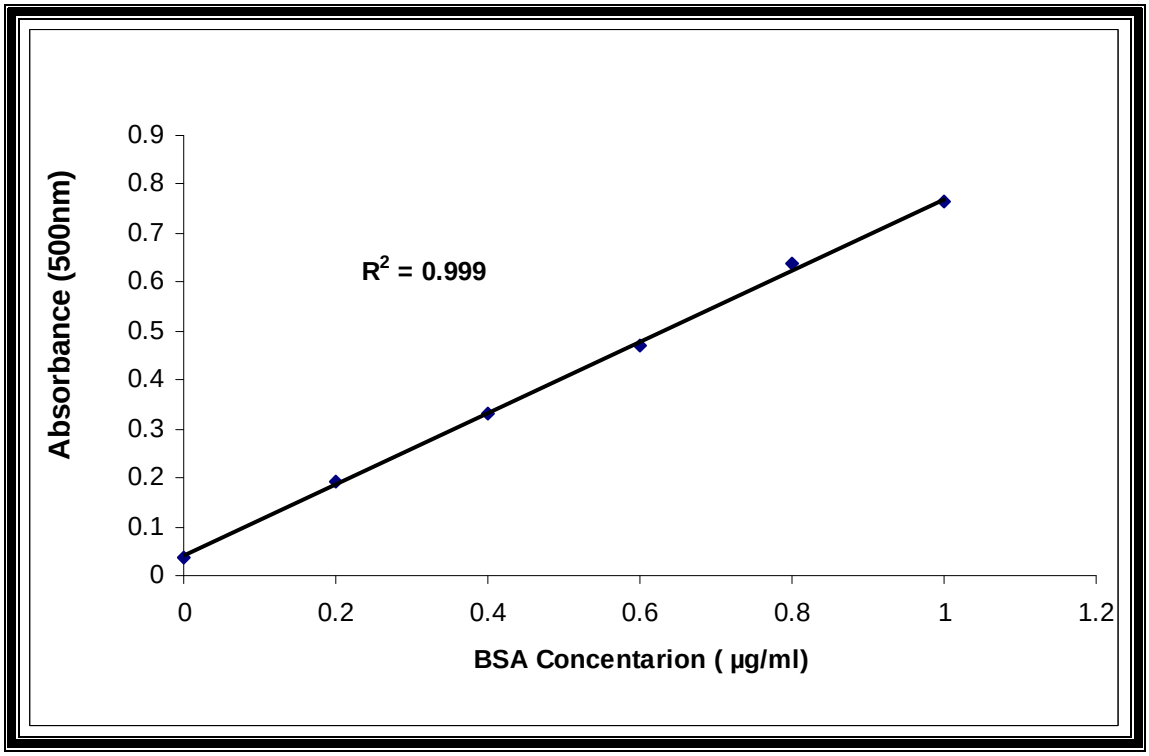
MDA Standard Curve

APPENDIX II



NMD Standard Curve

APPENDIX III



Protein Standard Curve

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