

**Analysis of the anti-cancer activity of
novel indigenous algal compounds in
breast cancer:**

**Towards the development of a model for
screening anti-cancer stem cell activity**

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Abstract

Breast cancer, the most common malignancy diagnosed in women, is one of the leading causes of death in women worldwide. In South Africa only 32% of women diagnosed with advanced breast cancer survive more than five years. The search for new chemotherapeutic agents capable of effectively treating breast cancer is therefore essential. Recent evidence supporting the cancer stem cell theory of cancer development for breast cancer challenges the current theories of cancer development and hence treatment. Cancer stem cells are a small subpopulation of tumour cells that possess properties of both cancer cells and stem cells and are believed to be the tumour-initiating population of many cancers. Cancer stem cells are inherently resistant to many chemotherapeutic agents and in this way have been associated with repopulation of tumours after chemotherapy. This phenomenon is proposed as a possible mechanism for cancer relapse after treatment. Cancer stem cells have also been implicated in metastasis, the major cause of mortality in cancer patients. Therefore, any treatment that is capable of targeting and removing breast cancer stem cells may have the theoretical potential to effectively treat breast cancer. However, there are currently no such treatments available for clinical use. We were provided access to a library of novel indigenous small molecules isolated from red and brown algae found off the Eastern Cape of South Africa. The aim of this project was to analyse the anti-cancer and anti-cancer stem cell properties of the compounds in this library and to identify „hit“ compounds which could form the basis for future development into new anti-cancer drugs. Ten novel compounds of algal origin were tested for cytotoxicity, by determining their ability to inhibit the growth of MCF12A breast epithelial cells and MCF7 breast cancer cells using the colorimetric MTT [(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] cell proliferation assay. All but one of the compounds tested exhibited cytotoxicity towards the MCF7 cancer cell line, with IC_{50} values (the concentration of the compound that leads to a 50% inhibition in cell growth) of between 3 μ M and 90 μ M. The chemotherapeutic drug paclitaxel was used as a positive control. Four of the compounds (RUMB-001, RUMB-002, RUMB-007 and RUMB-010/saragaquinoic acid) were significantly more toxic to the MCF7 cancer cell line, than the „normal“ MCF12A breast cells and were selected as priority compounds for further analyses. In addition, two other compounds were selected as priority compounds, one highly cytotoxic towards both MCF12A and MCF7 cell lines (RUMB-015) and one which was non toxic to either cell line (RUMB-017/018). Preliminary studies into the mechanism of cytotoxicity using Western blot analysis for poly (ADP-ribose) polymerase (PARP) cleavage and Hoechst 33342 immunostaining in MCF-7 cells were largely unsuccessful. The Hoechst 33342 immunostaining assay did provide tentative evidence that selected priority compounds were capable of inducing

apoptosis, although these assays will need to be repeated using a less subjective assay to confirm the results. The priority compounds were subsequently investigated for their cytotoxic effect on the cancer stem cell-enriched side population in MCF7 cells. The ability of the priority compounds to selectively target the cancer stem cell containing side population was assessed using two complementary flow cytometry-based techniques – namely the Hoechst 33342-exclusion assay, and fluorescent immunostaining for the expression of the putative cancer stem cell marker, ABCG2⁺. The ABCG2⁺ staining assay was a novel technique developed during the course of this study. It remains to be fully validated, but it may provide a new and reliable way to identify and analyse cancer stem cell containing side population cells. The MCF7 cells were treated with the compounds and the proportion of putative cancer stem cells compared with the size of the population in untreated cells was assessed. Three compounds (RUMB-010, RUMB-015 and RUMB-017/018) capable of reducing the proportion of side population cells within the MCF7 cell line were identified. Taking these data together, we identified two potential „hit“ compounds which should be prioritised for future research. These are compounds RUMB-010/sargaquinoic acid and RUMB-017/018. RUMB-010 is of interest as it was shown to target the putative cancer stem cell population, in addition to the bulk MCF7 tumour line, but was relatively less toxic to the „normal“ MCF12A cell line. RUMB-017/018 is of interest due to the ability to selectively target the cancer stem cell enriched side population, while having little effect on the normal (MCF12A) or bulk tumour (MCF7) cell lines tested. These compounds will be important as „hit“ compounds for drug development and as tool compounds to study cancer and cancer stem cell biology.

Table of Contents

ABSTRACT	2
TABLE OF CONTENTS	4
LIST OF FIGURES	7
LIST OF TABLES	8
ACKNOWLEDGEMENTS	9
OUTPUTS	10
AUTHOR'S DECLARATION	11
ABBREVIATIONS	12
1 LITERATURE REVIEW	14
1.1 Cancer	15
1.2 Cancer Development	15
1.3 Breast Cancer	16
1.3.1 Breast cancer subtypes	16
1.4 Theories of cancer development	17
1.4.1 Breast Cancer Development and Progression	17
1.4.1.1 STOCHASTIC MODEL OF CANCER DEVELOPMENT AND SPORADIC AND FAMILIAL BREAST CANCER	17
1.4.1.2 CANCER STEM CELL THEORY OF CANCER DEVELOPMENT AND BREAST CANCER STEM CELLS	19
1.5 Breast CSC and metastasis	23
1.6 Current Breast Cancer Treatments and Limitations	26
1.7 Problem Statement and Knowledge Gap	31
1.8 Hypothesis	32
1.9 Objectives	32
2 MATERIALS AND METHODS	33
2.1 Novel Indigenous Algal Compounds Isolated from Indigenous Red and Brown Algae	34
2.2 Cell Culture	34
2.3 Cytotoxicity Assays	36

2.4	Putative cancer stem cell /Side Population Analyses.....	37
2.4.1	Flow Cytometry Based on Exclusion of Hoechst 33342	37
2.4.2	Flow Cytometry Based on ABCG2 Staining	38
2.5	Apoptosis Assays.....	39
2.5.1	PARP detection using Western blot analysis	39
2.5.2	Immunofluorescent detection of apoptosis	39
3	DETERMINATION OF CYTOTOXICITY OF MARINE COMPOUNDS IN MCF12A AND MCF7 CELLS	41
3.1	Introduction	42
3.2	Results and Discussion	43
3.2.1	Optimisation and validation of MTT assay on MCF12A and MCF7 cells using Paclitaxel	44
3.2.2	Determination of the IC ₅₀ values of algal compounds in MCF12A and MCF7 cells	45
3.2.2.1	RUMB-001	46
3.2.2.2	RUMB-002	47
3.2.2.3	RUMB-003	47
3.2.2.4	RUMB-004	48
3.2.2.5	RUMB-005	48
3.2.2.6	RUMB-007	48
3.2.2.7	RUMB-008	49
3.2.2.8	RUMB-010	49
3.2.2.9	RUMB-015	50
3.2.2.10	RUMB-017/018	50
3.3	Comparison of toxicity in normal and cancer cell lines	64
3.4	Conclusion.....	69
4	DETERMINATION OF THE CYTOTOXICITY OF THE PRIORITY COMPOUNDS ON THE SIDE POPULATION (SP) IN THE MCF7 BREAST CANCER CELL LINE 71	
4.1	Introduction	72
4.2	Results and Discussion	75
4.2.1	Identification of the Side Population in MCF7 cell line using Hoechst staining	75
4.2.2	Analysis of the percentage of ABCG2 ⁺ cells present in MCF7 cells after treatment with the priority compounds using flow cytometry.....	79
4.3	Conclusion.....	82
5	DETERMINATION OF THE APOPTOTIC-INDUCING ABILITY OF THE PRIORITY COMPOUNDS.....	84
5.1	Introduction	85
5.2	Results and Discussion	87
5.2.1	Detection of PARP in MCF7 cells after treatment with the priority compounds	87
5.2.2	Detection of apoptosis in MCF7 cells after treatment with the priority compounds using Hoechst 33342 immunostaining.....	89
5.3	Conclusion.....	95
6	DISCUSSION AND CONCLUSIONS.....	96
6.1	Results Summary	97

6.2	Novel algal compounds as potential anti-cancer and anti-CSC agents and identification of potential hit compounds	101
6.3	A hit-to-target approach for screening potential anti-cancer and anti-CSC agents.....	103
6.4	Importance of identifying anti-CSC and anti-breast CSC agents.....	106
7	REFERENCES	108
8	APPENDICES.....	123
8.1	APPENDIX 1	124
8.1.1	Reagents Used	124
8.1.2	Compound Dilution Calculations.....	125
8.2	SDS-PAGE Resolving and Stacking Gel Constituents	126
8.2.1	12% Resolving Gel	126
8.2.2	4% Stacking Gel	126
8.3	APPENDIX 2	127
8.3.1	IC ₅₀ calculations from graphs generated from MTT assays in MCF12A and MCF7 cells	127
8.4	APPENDIX 3	133

List of Figures

Figure 1: Schematic representation of the proposed origins of breast CSC.	25
Figure 2: Reduction of MTT by metabolically active cells.....	37
Figure 3: Optimisation of the MTT assay on MCF7 cells using paclitaxel.	51
Figure 4: Determination of IC ₅₀ values of paclitaxel in MCF12A and MCF7 cells.....	52
Figure 5: Determination of IC ₅₀ values of RUMB-001 in MCF12A and MCF7 cells. ...	53
Figure 6: Determination of IC ₅₀ values of RUMB-002 in MCF12A and MCF7 cells. ...	54
Figure 7: Determination of IC ₅₀ values of RUMB-003 in MCF12A and MCF7 cells. ...	55
Figure 8: Determination of IC ₅₀ values of RUMB-004 in MCF12A and MCF7 cells. ...	56
Figure 9: Determination of IC ₅₀ values of RUMB-005 in MCF12A and MCF7 cells. ...	57
Figure 10: Determination of IC ₅₀ values of RUMB-007 in MCF12A and MCF7 cells. ..	58
Figure 11: Determination of IC ₅₀ values of RUMB-008 in MCF12A and MCF7 cells. ..	59
Figure 12: Determination of IC ₅₀ values of RUMB-010 in MCF12A and MCF7 cells. ..	60
Figure 13: Determination of IC ₅₀ values of RUMB-015 in MCF12A and MCF7 cells. ..	61
Figure 14: Determination of IC ₅₀ values of RUMB-017/018 in MCF12A and MCF7 cells.	62
Figure 15: A comparison of the average cytotoxicity of the marine algal compounds in MCF12A (blue) and MCF7 (red) cells.	63
Figure 16: Different approaches available for the determination of toxicity to CSC	74
Figure 17: Confirmation of the presence of the side population in MCF7 cells using flow cytometry.....	76
Figure 18: Comparison of the average percentage of the Hoechst 33342-excluding cells in MCF7 cells treated with selected marine algal compounds.	77
Figure 19: The effect of the priority compounds on the relative proportions of the ABCG2 ⁺ population in MCF7 cells	81
Figure 20: Simplified schematic diagram showing the convergence of extrinsic and intrinsic apoptotic pathways.	86
Figure 21: Detection of PARP cleavage in MCF7 cells after treatment with the priority compounds using SDS-PAGE and Western blot analysis.	88
Figure 22: Representative of (normal) non-apoptotic (A) and apoptotic (B) nuclei in MCF7 cells using Hoechst-33342 staining.	91
Figure 23: Identification of Apoptotic Nuclei by Hoechst 33342 staining in MCF7 cells treated with novel indigenous compounds	92
Figure 24: Determination of the IC ₅₀ value of RUMB-001 in MCF12A (A) and MCF7 (B) cells	128
Figure 25: Determination of the IC ₅₀ value of paclitaxel (1), RUMB-001 (2) and RUMB-002 (3) in MCF12A (A) and MCF7 (B) cells.	129
Figure 26: Determination of the IC ₅₀ value of RUMB-003 (1), RUMB-004 (2) and RUMB-005 (3) in MCF12A (A) and MCF7 (B) cells.	130
Figure 27: Determination of the IC ₅₀ value of RUMB-007 (1), RUMB-008 (2) and RUMB-010 (3) in MCF12A (A) and MCF7 (B) cells.	131
Figure 28: Determination of the IC ₅₀ value of RUMB-015 (1) and RUMB-017/018 (2) in MCF12A (A) and MCF7 (B) cells.....	132
Figure 29: Comparison of the percentage of ABCG2 ⁺ cells present in MCF7 cells after treatment with the priority compounds.....	133

List of Tables

Table 1: The code, name, structure, molecular mass and source of the compounds used in the anti-cancer and anti-cancer stem cell analyses.....	35
Table 2: Codes, names, structures and IC ₅₀ values in MCF12A and MCF7 cells of selected marine algal priority compounds and paclitaxel	68
Table 3: A summary of the cytotoxicity of the algal compounds in MCF12A and MCF7 cells and the effect of the priority compounds on MCF7 SP cells.....	98
Table 4: Reagents Used.....	124
Table 5: Stock concentrations (mM) of novel marine compounds dissolved in DMSO	125
Table 6: Amount (µl) of DMEM added to 1 µl of stock compound to give a 1 mM ..	125
Table 7: Concentrations of priority compounds used in treatment of MCF7 cells to determine the effect of these compounds on the MCF7 side population ...	133

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Outputs

Publications

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Conference Outputs

Poster and oral presentation: Symposium on Chemico- and Biomedical Research, Thursday 5 November 2009, Rhodes University

Oral presentation: Eastern Cape South African Society of Biochemistry and Molecular Biology Conference, Friday 6 November 2009, Nelson Mandela Metropolitan University

Author's Declaration

I, Jessica Clair Lawson, declare that this thesis, which I now submit for the degree of Master of Science, is my own unaided work and has not been submitted for a degree at any other university. During the course of this study a review pertaining to the work presented in this thesis was published (see Lawson, J. C., Blatch, G. L. and Edkins, A. L. (2009) Cancer stem cells in breast cancer and metastasis. Breast Cancer Research and Treatment, 118, 241-254).

Signed: _____

on this _____ day of _____ 2010

Abbreviations

17-AAG	17-Allylamino-17-demethoxygeldanamycin
17-DMAG	17-Dimethylaminoethylamino-17-demethoxygeldanamycin Hydrochloride
ABCG2	ATP-binding Cassette Transporter sub-family G member 2
ALDH1	Aldehyde dehydrogenase 1
AML	Acute Myeloid Leukaemia
BRCA1	Breast Cancer Susceptibility Gene 1
BRCA2	Breast Cancer Susceptibility Gene 2
CASY	Cell Counter and Analyser System
CO ₂	Carbon Dioxide
COX-2	Cyclooxygenase 2
CSC	Cancer Stem Cells
CXCR4	CXC Chemokine Receptor 4
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
dUTP	Deoxyuracil Triphosphate
ER	Oestrogen Receptor
FACS	Fluorescence Assisted Cell Sorting
FCS	Foetal Calf Serum
FITC	Fluorescein Isothiocyanate
H ₂ O ₂	Hydrogen Peroxide
HER-2	Human Epidermal Growth Factor Receptor 2
HIV/AIDS	Human Immunodeficiency Virus/ Acquired immune deficiency syndrome
HRP	Horseradish Peroxidase
MMTV	Mouse Mammary Tumour Virus
MTT	(3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide
NADH	Nicotinamide Adenine Dinucleotide
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NMR	Nuclear Magnetic Resonance
NOD/SCID	Non-Obese Diabetic/ Severe Combined Immune Deficiency
NSP	Non-Side Population

PARP	Poly(ADP-ribose) Polymerase
PBS	Phosphate Buffered Saline
PR	Progesterone Receptor
RNAi	RNA Interference
SAR	Structure Activity Relationship
SDS-PAGE	Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis
SP	Side Population
TBST	Tris-Buffered Saline with 0.1% Tween-20
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
VEGF	Vascular Endothelial Growth Factor

1 LITERATURE REVIEW

1.1 Cancer

Cancer is a serious disease and is the second most common cause of death worldwide, exceeded only by heart disease, and kills more of the world's population than tuberculosis, malaria and HIV/AIDS combined (Singh et al., 2004). A total of 12.5% of deaths worldwide is due to cancer and it was estimated that there would be 12.1 million new cancer cases worldwide in 2007, 45% in developed countries and the remaining 55% in developing countries (Bao et al., 2006b; Bertram, 2000; Garcia et al., 2007). It was predicted that there would be 7.6 million deaths as a result of cancer in 2007, 38% in developed countries and 62% in developing countries (Garcia et al., 2007). In Southern Africa in particular, there were an estimated 78 000 new cancer cases and over 54 000 deaths as a result of cancer and the top three diagnosed malignancies in Southern Africa are cervical, breast and prostate cancers respectively (Garcia et al., 2007).

1.2 Cancer Development

There are six properties that make cells capable of cancerous growth; they are not under the control of signals that regulate cell proliferation, they are resistant to apoptosis, they overcome limitations on proliferation by avoiding replicative senescence and evading differentiation, they are genetically unstable, they are able to invade surrounding tissues and they are capable of metastasis (Bertram, 2000; Fleming, 2003). In order for a cancerous tumour to develop, cancer cells must overcome replicative cell senescence and become „immortalised“ i.e. continue dividing indefinitely (Hornberg et al., 2006). Tumours are only considered to be cancerous if they are capable of metastasis and colonising surrounding tissue (de Castro Junior et al., 2006; Hornberg et al., 2006). Even though most cancers are thought to be monoclonal, the development of cancer takes place over many years and involves multiple processes (Bertram, 2000; Garcia et al., 2007; Hart, 2004). The original abnormal cell must undergo some change that allowed it to grow much faster than „normal“ cells in the same tissue and this property had to be passed on to subsequent daughter cells (Bertram, 2000; Garcia et al., 2007; Hart, 2004; Singh et al., 2004). For cancer to successfully develop, numerous but independent changes must occur in this one cell (Bertram, 2000; Garcia et al., 2007; Hart, 2004). Most cancers are thought to be a result of genetic mutations in the DNA of the cancerous cells and these mutations can be a result of internal elements, such as inherited mutations, and external elements, including chemical carcinogens and viruses (Bertram, 2000; Garcia et al., 2007; Hart, 2004). The daughter cells of the original aberrant cell must then undergo further mutations to allow them to divide uncontrollably and thrive in the tumour

environment and eventually outnumber the healthy cells in the tissue (Bertram, 2000; Garcia et al., 2007; Hart, 2004).

1.3 Breast Cancer

Breast cancer is the most common form of cancer diagnosed in women worldwide, affecting an estimated 10% of women, with over 1.3 million new cases and over 400 000 deaths predicted for 2007 (Garcia et al., 2007; Imyanitov and Hanson, 2004). There are different factors that contribute to the incidence of breast cancer, including advanced age (Garcia et al., 2007; Imyanitov and Hanson, 2004), high oestrogen levels (Clemons, 2001), extensive chromosomal mutations (Lerebours and Lidereau, 2002), breast cancer genes *BRCA1* and *BRCA2* (especially significant in hereditary breast cancer) (Nathanson et al., 2001), DNA hypermethylation (Widschwendter and Jones, 2002) and mutations in the mitochondrial DNA of epithelial mammary cells (Bianchi et al., 2001). Also being re-considered is the potential viral origin of human breast cancer (Mant and Cason, 2004; Mant et al., 2004). Two of the most commonly reported viruses investigated as potential causal agents in breast cancer are the Epstein Barr virus and mouse mammary tumour virus (MMTV). There is evidence of genetic material from the Epstein-Barr virus present in 21% of breast tumours analysed in 1995 (Labrecque et al., 1995) and in another 100 invasive breast carcinomas, 51% of tumours were positive for the Epstein-Barr viral genome while 90% of healthy tissue adjacent to the tumours tested was negative for the viral genome (Bonnet et al., 1999). More recently, genes encoding the envelope for MMTV, which, as its name suggests, causes mammary tumours in mice, were found in 40% of human breast cancer patients tested but the same genes were present in only 4% of normal breast tissue samples tested (Cotterchio et al., 2002). Another study showed evidence of the MMTV-*env* gene in between 38% and 40% of breast cancer samples tested but the gene was not present in any of the healthy tissue tested (Wang et al., 1998; Wang et al., 1995). It is not known, however, whether these viruses are responsible for the cancer that developed or whether they are there for another reason (Brower, 2004).

1.3.1 Breast cancer subtypes

Normal breast epithelium, the component of the mammary gland that gives rise to breast cancer, contains a basal layer of myoepithelial cells and an inner layer of luminal cells and human breast cancers are either myoepithelial, basal or luminal (Daniel and Smith, 1999). Gene expression profiling has enabled the classification of breast tumours into subtypes,

namely luminal, basal and HER-2 (human epidermal growth factor receptor 2) over-expressing tumours (Cheang et al., 2008; Jumppanen et al., 2007). Luminal breast tumours express progesterone receptor (PR), oestrogen receptor (ER) and HER-2, can often be treated successfully with hormone therapy and in general have a good prognosis (Adelaide et al., 2007; Phipps et al., 2008). Basal breast tumours carry the „triple negative“ phenotype; that is they do not express ER, PR or HER-2 and are considered to be highly aggressive and have a much poorer prognosis than their luminal counterparts (Adelaide et al., 2007; Phipps et al., 2008). Basal tumours account for between 10% and 15% of breast tumours, are associated with younger patient age, have no specific treatment and have high metastatic potential (Adelaide et al., 2007; Jumppanen et al., 2007; Phipps et al., 2008). HER-2 over-expressing breast tumours also have a poorer clinical prognosis than luminal breast tumours but unlike basal tumours, HER-2 positive tumours do have specific and standardized treatments such as trastuzumab (discussed further later) and lapatinib (Adelaide et al., 2007; Tzong-Der et al., 2005). The over-expression of HER-2 is observed in between 25% and 30% of breast cancers (Hortobagyi et al., 1999; Wilson et al., 2005) but the development of resistance to trastuzumab is a problem. Between 66% and 88% of breast cancer patients who initially respond to trastuzumab develop resistance and present with disease progression within one year of being treated with trastuzumab (Nahta and Esteva, 2006; Nahta et al., 2006). Another kind of breast cancer, myoepithelial breast cancers, are rare and aggressive and little is known about their aetiology or the long-term prognosis of patients with myoepithelial tumours following treatment (Behranwala et al., 2004; Woo et al., 2005).

1.4 Theories of cancer development

1.4.1 Breast Cancer Development and Progression

1.4.1.1 Stochastic model of cancer development and sporadic and familial breast cancer

The theory of cancer development that has informed the way cancer has been treated over the last few decades holds that for cells to become malignant they must be subjected to a series of mutations in the cell's DNA. The damage to the genome can be a result of internal or external factors. Internal factors that can contribute to genome damage include faulty DNA replication and unstable DNA base pairs. External factors that may contribute to mutations in the genome

include chemical carcinogens, exposure to UV radiation and, in some cancers, viruses (Bertram, 2000; Fleming, 2003; Hart, 2004).

The mutations that lead to malignancy are those mutations that confer the ability to escape regular homeostatic mechanisms of the cell and are generally those that abolish the function of a certain gene, called a tumour suppressor gene, or those that increase the expression or activation of a gene, called an oncogene (Armitage and Doll, 1961; Bertram, 2000). For a cancer to develop, four to seven independent mutations need to occur within genes crucial to cellular processes (Armitage and Doll, 1961; Fleming, 2003). A cell containing the initial mutation needs to replicate and accumulate multiple mutations that will allow the cell to replicate in an uncontrolled manner and invade surrounding tissues. This depends on clonal expansion, a process by which the number of cells increases exponentially as the production of daughter cells, all arising from a single original cell, progresses, as the probability of the cell carrying the initial mutation acquiring another mutation in the necessary location is almost negligible (Armitage and Doll, 1961; Bertram, 2000). Clonal expansion continues so that the mutations necessary for cancer development can be accumulated (Armitage and Doll, 1961; Bertram, 2000).

Sporadic breast cancer is the result of genetic alterations to the genomic material in the somatic breast cells rather than a result of an inherited, or germline, mutation (Lerebours and Lidereau, 2002; Ma et al., 2003). Little is known about the molecular basis and genetic events involved in the stochastic development of sporadic breast cancer, partly due to the vast clinical, biological and geographical diversity of breast cancers (Lerebours and Lidereau, 2002; Ma et al., 2003). According to the stochastic theory of cancer development the development and progression of breast cancer is a result of a series of accumulated mutations which, due to clonal expansion, result in successively aggressive clones that eventually lead to an invasive carcinoma (Lerebours and Lidereau, 2002). The progression of sporadic breast cancer is believed to be linear, starting with a premalignant atypical ductal hyperplasia which develops into a preinvasive ductal carcinoma *in situ*, which in turn develops into an invasive ductal carcinoma (Ma et al., 2003). The activation of oncogenes and inactivation of tumour suppressor genes is thought to be an early event in breast cancer development, followed by mutations in at least four other genes (Kenemans et al., 2004). Oncogenes commonly activated in sporadic breast cancers include *MYC*, which encodes a transcription factor involved in cell growth, *CCND1*, which codes for cyclin D1, and *HER*. Tumour suppressor

genes commonly inactivated in breast cancers include *TP53*, which codes for a transcription factor involved at many stages in the cell cycle and *RB1*, the retinoblastoma gene (Kenemans et al., 2004; Lerebours and Lidereau, 2002; Ma et al., 2003).

Approximately 4% to 9% of breast cancers are inherited and mutations in two major breast cancer susceptibility genes, *BRCA1* and *BRCA2*, account for the majority of inherited breast cancers (Joseph et al., 1996). *BRCA1* has been mapped to a region on chromosome 17q and *BRCA2* to the q12-13 region on chromosome 13 (Joseph et al., 1996) and mutations in these two genes are rare in sporadic breast cancer (Lerebours and Lidereau, 2002). Both *BRCA1* and *BRCA2* are tumour suppressor genes and are inactivated by their mutations in hereditary breast cancer, although not all hereditary breast cancers carry mutations in these genes (Cheang et al., 2008; Joseph et al., 1996). Hereditary *BRCA1* breast cancers resemble basal breast cancers and are more frequently aneuploid than other hereditary breast cancers (Cheang et al., 2008; Joseph et al., 1996; Phipps et al., 2008). The death rate and tumour recurrence associated with *BRCA1* are also lower than those observed in *BRCA2* breast cancers (Joseph et al., 1996).

1.4.1.2 Cancer stem cell theory of cancer development and breast cancer stem cells

An alternative model of cancer development that has gained popularity over the last few years is the Cancer Stem Cell theory of cancer development. Cancer stem cells (CSC) are tumorigenic cells capable of self-renewal to produce another CSC, as well as differentiation to produce non-tumorigenic cancer cells. The cancer stem cell theory proposes that during cancer development the genes involved in stem cell self-renewal regulation are disturbed (Al-Hajj et al., 2004). The recent discovery of CSC has changed the way scientists think about cancer, which in turn has implications for how the treatment of this disease is approached. The stem cell origin of cancer hypothesizes that CSC are capable of self-renewal, are resistant to chemotherapy and are able to repopulate the tumour mass after chemotherapy (Marotta and Polyak, 2009; Wicha et al., 2006). CSC were first discovered in acute myeloid leukaemia (AML) (Bonnet and Dick, 1997) and have since been shown to exist in B and T acute lymphoblastic leukaemia (Cobaleda et al., 2000; Cox et al., 2004), as well as solid tumours including colon and brain tumors (Bao et al., 2006a; Bao et al., 2006b; Dalerba et al., 2007; Dick, 2003; Piccirillo et al., 2006) and in pancreatic, lung, prostate and breast cancers (Al-Hajj et al., 2003; Kim et al., 2005; Li et al., 2007; Xin et al., 2005).

The origin of CSC remains to be conclusively determined; however two theories have been proposed. One theory proposes that normal stem cells, which are long-lived, undergo some mutation which gives rise to CSC while the other theory poses that a differentiated cell undergoes a „de-differentiation“ process through which it gains stem-like properties (Burns et al., 2005; Sell, 1993). It has been shown that after long-term culture of four to five months, normal human adult mesenchymal stem cells can give rise to stem cells with cancerous properties, supporting the suggestion that CSC are a result of a genetic alteration in normal stem cells (Rosland et al., 2009; Rubio et al., 2005; Shi et al., 2007). There is also experimental evidence supporting the idea that a differentiated cell can be induced to de-differentiate into a pluripotent cell with stem-like properties, where pluripotent stem cells were induced from adult mouse fibroblasts using four factors including Oct-3/4 under embryonic stem cell conditions (Takahashi and Yamanaka, 2006). These cells expressed embryonic stem cell markers and showed the same growth and morphology characteristics as embryonic stem cells (Takahashi and Yamanaka, 2006). Also, upon injection into nude mice, these cells gave rise to tumors (Takahashi and Yamanaka, 2006).

The way in which the human mammary gland changes and develops suggests the presence of a stem cell population within the breast tissue and it has been experimentally shown in mice that a functional mammary gland can be generated in a cleared mammary fat pad from a single cell (Shackleton et al., 2006; Wicha et al., 2003). The cells capable of doing this were found in the $\text{Lin}^- \text{CD}29^{\text{hi}} \text{CD}24^+$ population within breast tissue and were also found to be capable of self-renewal and are multi-potent and have thus been defined as breast stem cells (Shackleton et al., 2006). It has since been suggested that a hierarchical relationship exists between human breast stem cells and their differentiated progeny (Shackleton et al., 2006) and the existence of an adult breast stem cell lends support to the idea that breast CSC may arise from a transformed adult breast stem cell.

There is evidence for the existence of breast CSC within breast tissue in the human mammary gland, first reported by Al-Hajj and colleagues in 2003 using pleural effusions and tumour implantations (Al-Hajj et al., 2003). It was shown that only a small subpopulation of cells, namely those with the phenotype $\text{CD}44^+ \text{CD}24^-$, was able to initiate the formation of a new tumour when a small number of these cells, sometimes as few as 100, was injected into the cleared mammary fat pad of non-obese diabetic severe combined immunodeficient (NOD/SCID) mice. The resultant tumours also contained a subpopulation of $\text{CD}44^+ \text{CD}24^-$ cells (Al-Hajj et al., 2003). Cells with different phenotypes, such as $\text{CD}44^+ \text{CD}24^+$, were

unable to do this, even when tens of thousands of cells were used (Al-Hajj et al., 2003). This is supported by another study in which the stem-like properties of $CD44^+CD24^-$ cells were confirmed and the cells were propagated *in vitro* as anchorage-independent mammospheres (Abraham et al., 2005), a characteristic typical of normal breast stem cells (Dontu et al., 2003). As with other CSC, there is still uncertainty as to the origin of breast CSC; whether they arise from a transformed normal mammary stem cell or whether from a differentiated breast cell that has undergone dedifferentiation and malignant transformation (Figure 1). There is experimental evidence to support the proposal that breast CSC arise from a normal breast stem cell having transformed (Boulanger and Smith, 2001; Taketo et al., 1991). FVB/N mice, an inbred mouse strain favoured for transgenic analyses (Taketo et al., 1991), show decreased susceptibility to MMTV-induced breast cancer if the proliferative capacity of the normal mammary stem cells *in situ* is decreased (Boulanger and Smith, 2001). The mammary gland, which is comprised of an extensive network of ducts, undergoes massive changes and expansion during puberty unlike other organs, which for the most part fully develop during foetal development (Melchor and Smalley, 2008; Sternlicht, 2006).

CSC exist within a small subpopulation of tumour cells distinct from the main tumour cells known as the “side population” (SP), a group of cells defined by the ability to actively exclude the fluorescent dye Hoechst. SP cells are known to express high levels of ABC transporters, especially ABCG2, and these ABC transporters are responsible for the exclusion of Hoechst 33342 from the SP cells (Hirschmann-Jax et al., 2004; Zhou et al., 2001). CSC do not constitute the entire side population and reports differ with respect to the proportion of the side population comprised of $CD44^+CD24^-$ cells. The cancer stem cell population within a tumour has been proposed to comprise only a small percentage of the total tumour, namely between 0.01% and 2% of the tumour (Fillmore and Kuperwasser, 2008), as observed in AML where the proportion of the total tumour cells comprised of alleged CSC was 0.2% (Bonnet and Dick, 1997) however, this is not always seen in experimental data. For example, cells expressing CD133 have been proposed as potential brain CSC and in a study conducted by Singh and colleagues cells expressing CD133 comprised between 3.5% and 45.4% of the total tumour mass from different patients (Singh et al., 2003). In addition, the cancer stem cell population described by Al-Hajj made up between 11% and 35% of the total tumours (Al-Hajj et al., 2003) while in another immunohistochemical study a significant proportion of the breast tumour samples (12.4%) has a $CD44^+CD24^-$ population of greater than 10% (Mylona et al., 2008). Also worth noting is that the $CD44^+CD24^-$ cancer stem cell-representative

phenotype seems to be predominant in *BRCA1*, tumours but the same cells are uncommon in HER2 positive tumours (Honeth et al., 2008). $CD44^{+}CD24^{-}$ cells are present in normal breast epithelium in large numbers in the basal layer and so this may be the origin of cancer in basal and *BRCA1* type cancers although these cells are not present in all types of breast tumours (Honeth et al., 2008). Are $CD44^{+}CD24^{-}$ cells only tumour initiating cells in certain types of cancer and if so, what is the tumour initiator in breast tumours lacking $CD44^{+}CD24^{-}$, and where do they originate from? Another marker, aldehyde dehydrogenase 1 (ALDH1), has been used to separate fractions of breast tumours in tumour initiating and non-tumour initiating cells, although it is only found in 30% of breast tumours (Ginestier et al., 2007).

One of the defining characteristics of CSC is their inherent resistance to chemotherapy (Eyler and Rich, 2008). The chemoresistance of CSC allows for their survival during treatment and as such enables them to initiate tumour repopulation and therefore patient relapse, providing a major challenge in the effective treatment of cancer (Eyler and Rich, 2008). One such reported instance of this is reported in a study on the effect of conventional chemotherapy drugs on the $CD44^{+}CD24^{-}$ population in breast cancer biopsy samples. Patients with advanced breast cancer were subjected to 12 weeks of treatment with docetaxel or doxorubicin and cyclophosphamide. The percentage of $CD44^{+}CD24^{-}$ cells present in the tumour biopsies before treatment commenced was 4.7%. After 12 weeks of treatment the proportion of these cells had increased to 13.6% and these cells were capable of forming anchorage-independent mammospheres, suggesting that enrichment for CSC had occurred (Li et al., 2008). In addition to this Li and colleagues established xenografts of the breast cancer biopsies in SCID mice. In the mice transplanted with pre-chemotherapy breast cancer biopsy samples, 29% of the xenografts formed tumours whereas in the mice transplanted with post-chemotherapy breast cancer biopsy samples 50% of the xenografts formed tumours, suggesting that the cells that survived treatment with the chemotherapy drugs are enriched for tumorigenic cells (Li et al., 2008). The results from the studies by Al-Hajj and colleagues (2003) and Li and colleagues (2008), assuming they are representative of the environment and events in a tumour in a breast cancer patient, show that these cells are tumorigenic and resistant to chemotherapy, both considered to be properties of CSC. Understanding of the molecular mechanisms responsible for the observed chemoresistance in CSC may lead to treatments which either eradicate the CSC themselves or render the CSC susceptible to current chemotherapy treatments (Eyler and Rich, 2008). CSC exist in a small side population within the tumour and these side population cells express a high level of ABC transporter proteins,

especially ABCG2, a multi-drug resistant transporter that may confer some of the observed chemoresistance to the side population cells (Hirschmann-Jax et al., 2004; Zhou et al., 2001). The expression of ABCG2 by the cancer stem cell-containing SP has been exploited as a means of identifying the SP and is currently being used as a marker for the side population (Challen and Little, 2006; Goodell et al., 1996).

The part played by the CD44⁺CD24⁻ phenotype in breast CSC is still controversial but there it has been shown that chemotherapeutic treatment results in an increase in the percentage of CD44⁺CD24⁻ subpopulation cells in breast cancer patients after chemotherapy, suggesting that they are resistant to chemotherapy (Li et al., 2008) in accordance with the stem cell theory of cancer. In addition to CD44⁺CD24⁻, the expression of ALDH1 and CD133 are being used as markers for breast CSC (Ginestier et al., 2007; Wright et al., 2008). As already mentioned, breast CSC display some of the properties that define normal breast stem cells. It is not unreasonable then to believe that both breast stem cells and breast CSC are reliant on the same pathways for their self-renewal and asymmetric division. Integrins, in particular β 1 integrin, have already been suggested to be involved in the same processes in breast CSC. Knockout of the β 1 integrin has been shown to obstruct tumorigenesis in a transgenic mouse model of human breast cancer. Moreover, α -6 integrin was recently shown to be critical for tumorigenic cells isolated from the side population of MCF7 cells to generate tumours in SCID mice (Cariati et al., 2008).

1.5 Breast CSC and metastasis

Metastasis is the most common cause of mortality in breast cancer patients but there is as yet no standardized treatment (Freudenberg et al., 2009; Gralow, 2005). The most common sites of metastasis in breast cancer patients are the bone marrow and lungs (Abraham et al., 2005; Kang et al., 2003; Mastro et al., 2007). If CSC are responsible for the initiation and maintenance of tumours, this raises the question of whether they are involved in metastasis and if so, to what extent.

The expression of CD44 is believed to be a marker of breast CSC (Al-Hajj et al., 2003) and activation of CD44 results in cell motility, promotion of cellular adhesion and activation of cell survival mechanisms (Draffin et al., 2004). A potential role of CD44 expression in metastasis has recently been described by Draffin and colleagues. They detected CD44

expression in a metastatic breast cancer cell line MDA-MB-231 but did not detect it in the weakly invasive MCF7 breast cancer cell line (Draffin et al., 2004). In addition to this, down-regulation of CD44 expression in the metastatic cell line by RNAi resulted in decreased invasiveness and adherence to human bone marrow endothelial cells, while expression of CD44 in the MCF7 cell line after transfection resulted in increased cellular adhesion to human bone marrow endothelial cells (Draffin et al., 2004). This evidence suggests a role for CD44 in breast cancer metastasis, especially to the bone marrow (Draffin et al., 2004), which is one of the main development sites of breast cancer metastasis (Kang et al., 2003).

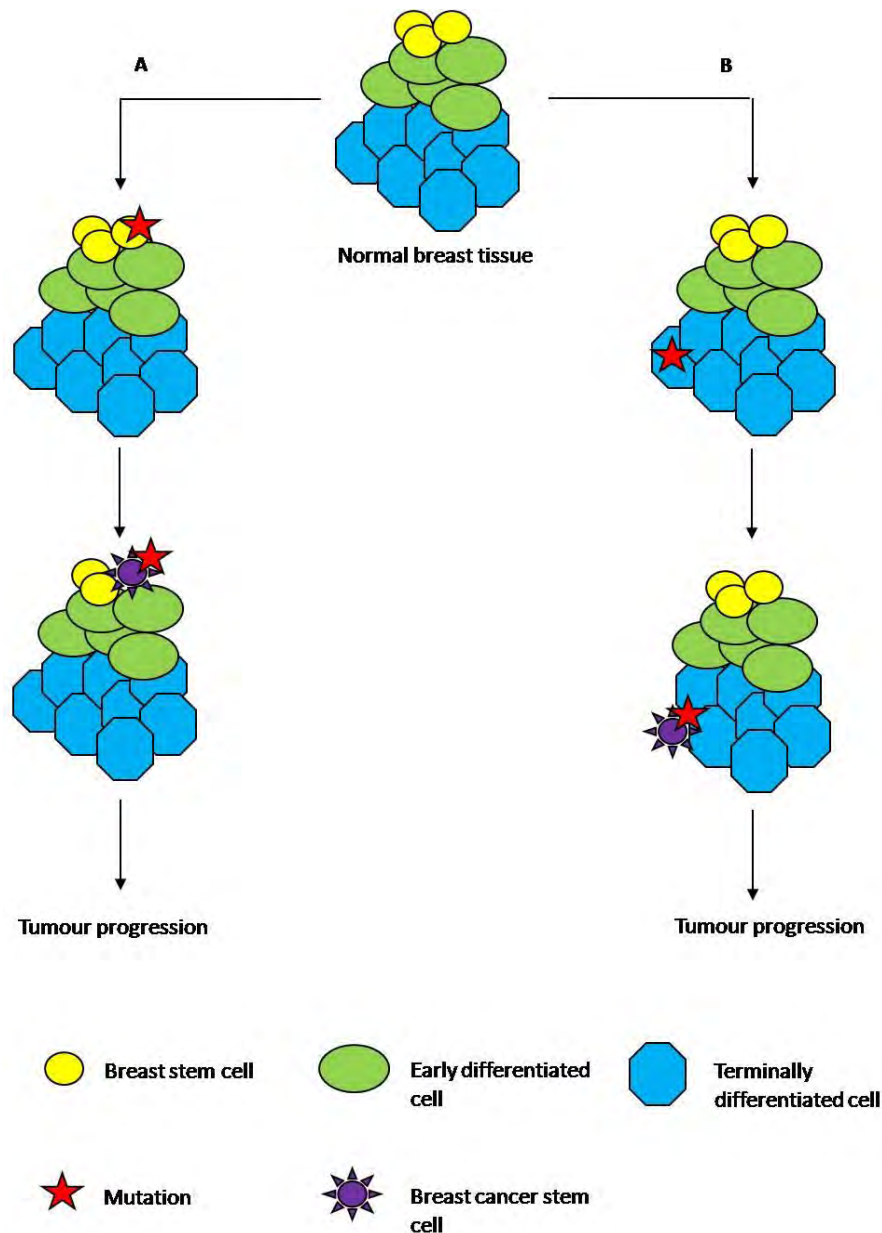


Figure 1: Schematic representation of the proposed origins of breast CSC.

(A) A stem cell within the breast tissue undergoes transformation to become a malignant breast cancer stem cell. (B) A differentiated breast cell is induced to a pluripotent cell with malignant properties, thus a breast cancer stem cell. The breast CSC are capable of self-renewal and asymmetric division such that the cancer stem cell population is maintained and proliferation of invasive cancer cells occurs (Boulanger and Smith, 2001; Ponti et al., 2006; Shackleton et al., 2006; Taketo et al., 1991).

Osteopontin, one of the ligands recognised by CD44, is a sialic rich phosphoglycoprotein and evidence of its involvement in metastasis is growing (Chakraborty et al., 2008; Suzuki et al., 2007; Wai and Kuo, 2004). In breast cancer in particular, osteopontin overexpression triggers the expression of vascular endothelial growth factor (VEGF), thereby inducing tumour invasion, progression, angiogenesis and metastasis (Chakraborty et al., 2008; Tuck et al., 1999; Tuck et al., 1998; Tuck et al., 1997). Osteopontin is able to induce breast epithelial cells to invade and migrate (Tuck et al., 1999; Tuck et al., 1998) while inhibition of osteopontin in the highly metastatic breast cancer cell line MDA-MB-231 has been shown to decrease the metastatic ability of these cells (Adwan et al., 2004). Breast CSC are believed to express CD44, the receptor for osteopontin, a matrix protein often expressed at the sites of breast cancer metastases. The interaction between CD44 and osteopontin may result in the attraction of metastatic cancer cells to certain tissues in the body (Lawson et al., 2009). Osteopontin is expressed in, among other tissues, bone marrow and lung tissue (Brown et al., 1992) and so it is possible that osteopontin facilitates the homing of metastatic breast cancer cells to these tissues. If breast CSC are responsible for metastasis then it is also possible that the CD44 expressed on the surface of the breast CSC interacts with the osteopontin present in the tissues at the sites of metastasis (Lawson et al., 2009). CD24, on the other hand, is believed to decrease the metastatic potential of cells by inhibiting the migration-inducing activity of chemokine receptor CXCR4 (Darash-Yahana et al., 2004). The proposed breast CSC are CD24 deficient, which may positively influence the migration, and thus metastatic potential, of these cells (Schabath et al., 2006). Furthermore, the majority (71%) of cells in early bone marrow metastases of breast cancer patients have been shown to be CD44⁺CD24⁻ cells (Balic et al., 2006). The evidence of the involvement of CD44 and osteopontin in breast cancer metastasis and well as the absence of metastasis-inhibiting CD24 on the putative breast CSC supports the theory that breast CSC are responsible, at least in part, for breast cancer metastases.

1.6 Current Breast Cancer Treatments and Limitations

Even though more is known today about the biology of different tumours, there has been little marked increase in the last two decades in the survival of patients whose cancer was diagnosed at an advanced stage (Etzioni et al., 2003). The decrease observed in the number of deaths as a result of cancer is attributed primarily to prevention and early diagnosis rather than the successful treatment of advanced cancers (Etzioni et al., 2003). Current cancer treatments include surgery, chemotherapy and radiation. Surgeries performed on cancer patients include

preventative surgery, where tissue that has a high chance of becoming cancerous is removed, curative surgery, where it is expected that the entire tumour can be removed by surgery and palliative surgery which aims to treat complications that arise as a result of advanced cancers. There are different chemotherapeutic drugs that target the different stages of cancer and there is still much research into the alteration of the cellular processes involved in each of these stages and potential ways of blocking them. Angiogenesis, for example, can be halted by bevacizumab (Avastin), a compound that blocks vascular endothelial growth factor signals from the tumour (Velcheti et al., 2006). Other drugs that have been found to interfere with the angiogenesis of a tumour are cyclophosphamide (Neosar, Cytosan), paclitaxel (Taxol) and COX-2 inhibitors celecoxib and thalidomide (Celebrex, Thalomid) (Brock, 1996; Fuchs and Johnson, 1978; Solomon et al., 2005). Imatinib, more commonly known as the drug Gleevec, inhibits the tyrosine kinase *Bcr-Abl* in chronic myelogenous leukaemia and is also known to inhibit c-kit and PDGF-R (platelet-derived growth factor receptor) (Deininger and Druker, 2003). Radiation therapy aims to damage cancer cells using ionizing radiation by subjecting the tumour to high-energy particle beams, for example gamma-rays, but unfortunately, as with chemotherapy, normal cells are also destroyed.

Breast cancer is often diagnosed only after it has passed through the early and most treatable stages (Garcia et al., 2007; Imyanitov and Hanson, 2004). Currently, only 32% of women in southern Africa diagnosed with advanced breast cancer survive more than 5 years (Garcia et al., 2007). The majority of chemotherapy drugs currently employed in the treatment of breast cancer such as paclitaxel and doxorubicin are complex organic structures containing ring structures and nitrogen (Sigurdsson et al., 1986; Tian et al., 2004; Wang et al., 2006; Yayoi et al., 2009). There is currently no standardized treatment for metastatic breast cancer, which is the major cause of death in breast cancer patients (Gralow, 2005). Recently however, trastuzumab, a recombinant monoclonal antibody directed against the extracellular domain of HER-2, has been used to treat HER2-positive early and metastatic breast cancer with some success, especially when combined with traditional chemotherapeutic drugs (Emens, 2005; Hall and Cameron, 2009; Neyt et al., 2008). A response rate of up to 84% was seen in clinical trials when trastuzumab was used in conjunction with conventional chemotherapy drugs such as paclitaxel, doxorubicin and docetaxel with improved response duration, time to progression and survival (Hall and Cameron, 2009; Marty et al., 2005; Slamon et al., 2001). Whether trastuzumab acts by down-regulating HER-2 expression or by inhibiting HER-2 cleavage however, remains unknown (Hall and Cameron, 2009). While current cancer treatments often

appear initially successful, relapse occurs often. It has been proposed that this relapse may be a result of the CSC, which are chemoresistant and thus unaffected by chemotherapy and remain behind after treatment and are able to repopulate the tumour. CSC, like other stem cells, exhibit the ability to self-renew and are resistant to chemotherapeutics due to a high level of expression of anti-apoptotic proteins such as Bcl-2 and Bcl-x_L (Domen et al., 1998; Peters et al., 1998) and the presence of multi-drug resistance transporters, for example ABCG2 (Zhou et al., 2001). A further disadvantage of many of the drugs used in chemotherapy is non-specific toxicity and therefore the adverse effect they have on other tissues. Chemotherapy can cause problems in the cardiovascular, respiratory, hepatic and gastrointestinal systems in a patient (Carr et al., 2008) and the ionizing beams used in radiation are also harmful to healthy cells.

Humans have been using plants and other natural resources to treat diseases for centuries (Weldegerima, 2009). Between 1981 and 2002, 62% of novel small molecules approved as chemotherapy drugs were natural products, derivatives thereof or synthetic replicas of natural products (Newman et al., 2003). The investigation of natural products for new drugs is ongoing. Paclitaxel, isolated from the Pacific yew tree *Taxus brevifolia* and docetaxel, isolated from the European yew tree, are both used in the treatment of breast cancer and are both natural products (Newman et al., 2003). In an analysis by Fabricant and Farnsworth, 122 compounds with a defined chemical structure were identified, all of which were isolated from only 94 plant species (Fabricant and Farnsworth, 2001). While plants have historically been the main source of compounds with drug properties, marine organisms in particular are known to be a rich source of chemically and biologically diverse compounds, many of which may well prove to be effective anti-cancer agents (Valeriote et al., 1994; Weldegerima, 2009). Cytosine arabinoside, isolated from *Cyptothecia crypto*, was the first compound of marine origin to be approved as an anti-cancer drug and is used in the treatment of acute myeloid leukaemia and non-Hodgkin's lymphoma (Mayer and Gustafson, 2003; Pigneux et al., 2007; Wang et al., 1997). Two other compounds of marine origin that have proven to have anti-cancer activity are didemnin B from *Trididemnum solidum* which exhibited anti-cancer activity in kidney, ovarian and breast cancer (Mittelman et al., 1999; Rinehart et al., 1988; Taylor et al., 1992), and bryostatin from *Bugula neritina*, which exhibited anti-cancer activity in lung and prostate tumours and non-Hodgkin's lymphoma (Hornung et al., 1992; Kerr et al., 1996). Didemnin B, however, caused allergic reactions in patients and clinical trials were terminated (Nuijen et al., 2000). There are, however, many other compounds isolated from

marine organisms that display anti-cancer activity as reviewed by Mayer and Gustafson (Mayer and Gustafson, 2003), and over the past 15 years several new experimental compounds of marine origin entered preclinical and clinical trials (Schwartsmann et al., 2001). Halogenated monoterpenes are a class of small organic compounds produced only by *Plocamium*, *Porteria* and *Ochtodes* algal species (Naylor et al., 1983). Over 100 of these compounds have been identified, some of which have been shown to have anti-cancer activity even though they are chemically and structurally very different from current chemotherapy drugs (Naylor et al., 1983; Polzin et al., 2003). Species of the *Plocamium* genus produce both linear and cyclic halogenated monoterpenes as secondary metabolites (Afolayan et al., 2009). Of the ten compounds used in the research presented here, nine were halogenated monoterpenes isolated from *Plocamium corallorhiza* and *Plocamium cornutum* collected off the Eastern Cape coast of South Africa (Knott et al., 2005; Mann et al., 2007). These compounds have been demonstrated as having anti- cancer activity in human oesophageal cancer cell line WHCO1 (Knott et al., 2005; Mann et al., 2007). The remaining compound used in this study was sargaquinoic acid, a tetraprenylquinone isolated from the brown algae *Sargassum heterophyllum* that has been shown to have both the ability to induce apoptosis in human keratinocyte cell line HaCaT (Hur et al., 2008) and to induce neural differentiation in cells derived from a pheochromocytoma of rat adrenal medulla (Kamei and Tsang, 2003; Tsang et al., 2001).

Chemotherapy regimens currently in use have adverse effects on healthy tissues in the body (Carr et al., 2008). There are over 200 chemotherapeutic agents in use and unfavourable side effects have been reported for the majority of these drugs, with the long-term side effects of others, such as trastuzumab, being widely unknown (Carr et al., 2008). It is difficult to develop a standardized treatment for the different breast cancer subtypes based on the characteristics of the tumours because breast cancers by nature are geographically, aetiologically, biologically and clinically diverse (Lerebours and Lidereau, 2002). The cancer stem cell theory of cancer development has implications for cancer treatment and any compound with the ability to target CSC and either kill them or force them to differentiate has the theoretical capacity to effectively treat cancer, however there are no such treatments yet in existence. Research into drugs that target CSC has become one of the focuses of cancer research and drug discovery and it is possible to selectively target this population, as has been shown by one study in which the proliferation and colony forming ability of breast cancer cells expressing the CD44⁺CD24⁻ phenotype in the MCF7 cell line are preferentially inhibited

by three NF- κ B pathway inhibitors, namely parthenolide, pyrrolidinedithiocarbamate and its analog diethyldithiocarbamate (Zhou et al., 2008). Another study showed that *BRCA1* breast cancers become more sensitised to current chemotherapy drugs after treatment with 17-DMAG (17-dimethylaminoethylamino-17-demethoxygeldanamycin hydrochloride), an Hsp90 inhibitor (Wright et al., 2008). Another Hsp90 inhibitor, 17-AAG (17-allylamino-17-demethoxygeldanamycin), is able to inhibit the growth of human gliomas stem cells both *in vitro* and *in vivo* (Sauvageot et al., 2009). As with the other properties of different breast cancers, it appears that the alleged cancer stem cell responsible for tumour development and maintenance in one type of breast cancer is not responsible for the same in a different breast cancer subtype (Honeth et al., 2008). The tumorigenic cells responsible for tumour initiation and progression have not been identified for all breast cancer subtypes and it is unknown whether these cells are completely different or whether they have some common characteristic that could be targeted.

In order to design treatments that can effectively target CSC it is crucial to identify and understand the differences between the CSC, normal adult stem cells and normal mature cells and a potential way this could be done is comparing the gene and protein expression profiling of these cells (Zhao et al., 2008). Unfortunately, not enough is currently known about CSC and the mechanisms by which they survive in the tumour environment and whether they have any unique biological characteristics that could be exploited in the development of novel drugs to target the CSC (Zhao et al., 2008). Should an effective treatment targeting CSC be developed it may be best to use this treatment in conjunction with conventional chemotherapy so that both the rapidly proliferating bulk tumour cells and the slow-dividing CSC are both eliminated (Zhao et al., 2008).

1.7 Problem Statement and Knowledge Gap

New therapies that are able to effectively treat cancer are always in demand as the current treatment strategies available often cause damage to healthy tissues, including cardiovascular, intestinal and respiratory tissues, and are not always successful in eradicating the tumour and preventing relapse (Carr et al., 2008). In addition to this, the long-term side effects of many currently used drugs, including trastuzumab, remain unknown (Carr et al., 2008).

Breast cancer is the most commonly diagnosed malignancy in women, affecting an estimated 10% of the world's female population and in southern Africa in particular, only 32% of women diagnosed with advanced breast cancer survive more than five years (Garcia et al., 2007). Numerous cancers, including breast cancers, are thought to arise from tumorigenic CSC which are believed to be responsible for the initiation and maintenance of tumours. These cells are biologically distinct from the bulk tumour cells and are capable of self-renewal and asymmetric differentiation, a property they share with normal stem cells. CSC are also resistant to chemotherapy and therefore may repopulate the tumour after treatment with conventional chemotherapy drugs, leading to relapse. CSC are also believed to be responsible for metastasis, the most common cause of mortality in cancer patients. In the case of breast cancer, CSC are believed to be $CD44^{+}CD24^{-}$ (Al-Hajj et al., 2003), express the ABCG2 drug transporter and have been shown to be chemoresistant (Li et al., 2008). There is also no standardized treatment for breast cancer metastases, the leading cause of death in breast cancer patients (Freudenber g et al., 2009; Gralow, 2005). A therapy able to successfully target CSC, either by forcing them to differentiate or by forcing them to undergo apoptosis, has the theoretical potential to effectively treat cancer if used in conjunction with conventional chemotherapy treatments. If CSC are indeed responsible for metastasis and a treatment targeting CSC is developed, successful standardized treatment of metastases may be achievable. Unfortunately, no treatments that specifically target CSC have yet been developed. This does, however, pave the way for new research into novel compounds that target CSC.

The compounds used in this study are of marine origin and were isolated from the red algae *Plocamium corallorhiza* and *Plocamium cornutum* and the brown alga *Sargassum heterophyllum*, all found off the Eastern coast of South Africa. The compounds isolated from the *Plocamium* species were halogenated monoterpenes, containing either chlorine or bromine or both, but not nitrogen, and are much simpler organic compounds than those of currently

used chemotherapy drugs. These compounds have also been shown to have cytotoxic effects in the human oesophageal cancer cell line WHCO1 (Knott et al., 2005; Mann et al., 2007). The compound isolated from *Sargassum heterophyllum*, a tetraprenylquinone known as sargaquinoic acid, contains a ring structure and is more complex than the halogenated monoterpenes but is less complex than conventional chemotherapy drugs. It was not tested for cytotoxicity in WHCO1 cells but it is known to promote neurite growth and has been shown to induce apoptosis in human keratinocyte cell line HaCaT (Hur et al., 2008; Kamei and Tsang, 2003).

1.8 Hypothesis

Novel indigenous compounds of marine origin isolated from *Plocamium* species and *Sargassum heterophyllum* will have anti-breast cancer activity and anti-breast cancer stem cell activity.

1.9 Objectives

The broad objective of this investigation was to screen novel indigenous algal compounds for toxicity against breast cancer cells and breast CSC. Using novel small molecules, namely marine compounds isolated from red and brown algae, most already shown to have anti-cancer activity in human oesophageal cancer cell line WHCO1, the specific objectives of this study were to:

1. Screen the indigenous algal compounds for cytotoxicity in breast epithelial cell line MCF12A and breast epithelial cancer cell line MCF7 and determine the concentration at which 50% growth inhibition occurs (IC_{50}) values of the compounds in both cell lines
2. Identify the cancer stem cell-containing side population in breast cancer epithelial cell line MCF7 and determine the cytotoxic effect of selected algal compounds on the side population in this cell line
3. Perform a preliminary investigation into the apoptosis-inducing ability of selected indigenous algal compounds in MCF7 epithelial breast cancer cells

2 MATERIALS AND METHODS

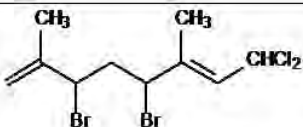
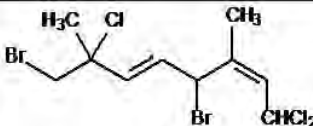
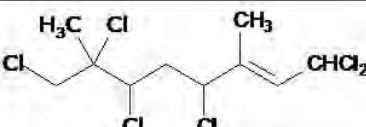
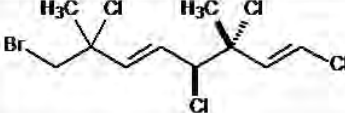
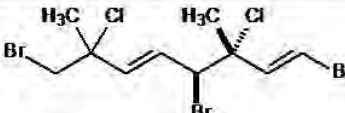
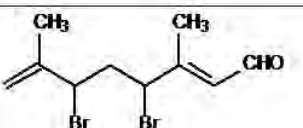
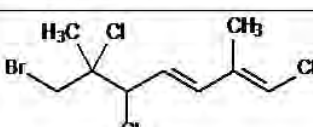
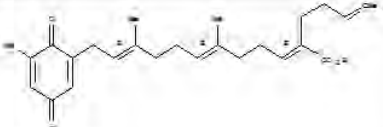
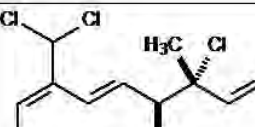
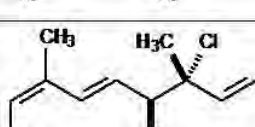
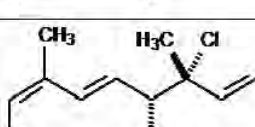
2.1 Novel Indigenous Algal Compounds Isolated from Indigenous Red and Brown Algae

The compounds used for cytotoxicity testing on cell lines are shown in Table 1. The compounds are halogenated monoterpenes isolated from *Plocamium* sea sponges off the Eastern Cape coast in South Africa. The exception is RUMB-010, a tetraprenylquinone isolated from *Sargassum heterophyllum* (Knott et al., 2005; Mann et al., 2007). The structures of all compounds were verified by nuclear magnetic resonance (NMR). All compounds were initially dissolved in dimethyl sulfoxide (DMSO) (Sigma) before dilution to working concentrations in cell culture media.

2.2 Cell Culture

The cell lines used for cytotoxicity testing were the MCF12A and MCF7 cell lines (both from ATCC). The MCF12A cell line is a breast epithelial cell line that underwent spontaneous immortalisation and was derived from a 60 year old Caucasian adult female (Paine et al., 1992; Pauley, 1993). The MCF7 cell line is a breast epithelial adenocarcinoma cell line derived from a metastatic pleural effusion in a 69 year old Caucasian adult female (Soule et al., 1973). MCF12A cells were cultured using a 1:1 ratio of Ham's F10 (Sigma Aldrich) and DMEM supplemented with 5% Foetal Calf Serum (FCS) (PAA Laboratories), 100 U.ml⁻¹ penicillin-streptomycin (BioWhittaker), 20 ng.ml⁻¹ epidermal growth factor, 100 ng.ml⁻¹ cholera toxin, 500 ng.ml⁻¹ hydrocortisone and 10 µg.ml⁻¹ insulin. MCF7 cells were routinely cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco) supplemented with 5% heat inactivated FCS, 100 U.ml⁻¹ penicillin-streptomycin and 2 mM L-glutamine. Cells were subcultured at a ratio of 1:2 every 3 to 4 days by aspirating the media from the flask, washing the cells with sterile 1 × PBS warmed to 37°C (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄·2H₂O, 1.47 mM KH₂PO₄, pH 7.4), adding 1 ml 1 × Trypsin/EDTA (ethylenediaminetetraacetic acid) (porcine trypsin with 0.2 g.l⁻¹ EDTA) and incubating the cells at 37°C and 5% CO₂ (carbon dioxide) until the cells lifted. The trypsinisation was stopped by the addition of serum-containing media, and cells collected by centrifugation at 800g. The media supernatant was discarded and the cells resuspended in 20 ml media warmed to 37°C and 10 ml each transferred to T75 culture flasks (Corning).

Table 1: The code, name, structure, molecular mass and source of the compounds used in the anti-cancer and anti-cancer stem cell analyses

Code	Name	Structure	Mass (g.mol ⁻¹)	Source
RUMB-001	Plocoralide A		364.93	<i>Plocamium corallorhiza</i>
RUMB-002	Plocoralide B		399.38	<i>Plocamium corallorhiza</i>
RUMB-003	Plocoralide C		346.94	<i>Plocamium corallorhiza</i>
RUMB-004	Plocoralide D		354.96	<i>Plocamium corallorhiza</i>
RUMB-005	Plocoralide E		443.83	<i>Plocamium corallorhiza</i>
RUMB-007	Plocoral A		310.03	<i>Plocamium corallorhiza</i>
RUMB-008	Plocoral B		300.02	<i>Plocamium corallorhiza</i>
RUMB-010	Sargaquinoic acid		424.58	<i>Sargassum heterophyllum</i>
RUMB-015	Plocornulide A		308.46	<i>Plocamium cornutum</i>
RUMB-017/018	Plocornulide C - erythro		284.02	<i>Plocamium cornutum</i>
	Plocornulide C - threo			

2.3 Cytotoxicity Assays

The cytotoxicity of the compounds (Table 1) in MCF12A and MCF7 cells was determined using the Cell Proliferation Kit I (MTT) (Roche) according to the manufacturers' instructions. The assay is based on the reduction of the yellow 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to purple formazan crystals in metabolically active cells (Berridge and Tan, 1993). A 100 μl volume of 6×10^3 cells. ml^{-1} cell suspension (6000 cells) was added to each well in 96 well plates (Nunc) and incubated overnight at 37°C and 5% CO_2 . The optimum treatment time was determined by conducting MTT assays on MCF7 cells after treatment with paclitaxel at concentrations ranging from 0.01 μM to 5 μM after 24 hours, 48 hours, 72 hours and 96 hours. Thereafter, MCF7 cells were treated with the differing concentrations of compounds and controls as indicated in figure legends and incubated for 96 hours at 37°C and 5% CO_2 . The assay was validated using MCF12A and MCF7 cells treated with paclitaxel at concentrations ranging from 0.01 μM to 0.15 μM included on each 96-well plate and assayed in the same way as the cells treated with the algal compounds. The cytotoxicity testing for individual compounds was performed in triplicate, with each concentration of that compound tested in quadruplicate. After treatment with the compounds the cells were observed under the microscope to determine qualitatively whether the compounds had any cytotoxic effect on the cells. A 5 μl volume of MTT reagent was added to each well and incubated for 4 hours at 37°C and 5% CO_2 , following which 100 μl of solubilisation solution was added to each well and incubated overnight at 37°C and 5% CO_2 . The colour change from yellow to purple was measured by quantifying the absorbance of each well at 550 nm using a PowerWave spectrophotometer (BioTek). The average absorbance of each compound at each different concentration was calculated and the cells treated with 0.25% DMSO were regarded as having 100% cell survival. The average percentage survival of each compound at each concentration on each 96 well plate was calculated using the 100% survival of the DMSO-treated cells as a reference. The percentage survival of the cell lines at the different concentrations of each compound was plotted on a graph from which the IC_{50} values (the concentration at which 50% growth inhibition is reached) for each compound were calculated. Student's *t*-test (two-tailed, two sample with equal variance) was conducted to determine whether the difference in IC_{50} values of each compound in the MCF7 cell line was statistically significant when compared with the IC_{50} values in the MCF12A cell line. All error bars on the graphs from which the IC_{50} values were calculated represent the standard deviation of the relevant results.

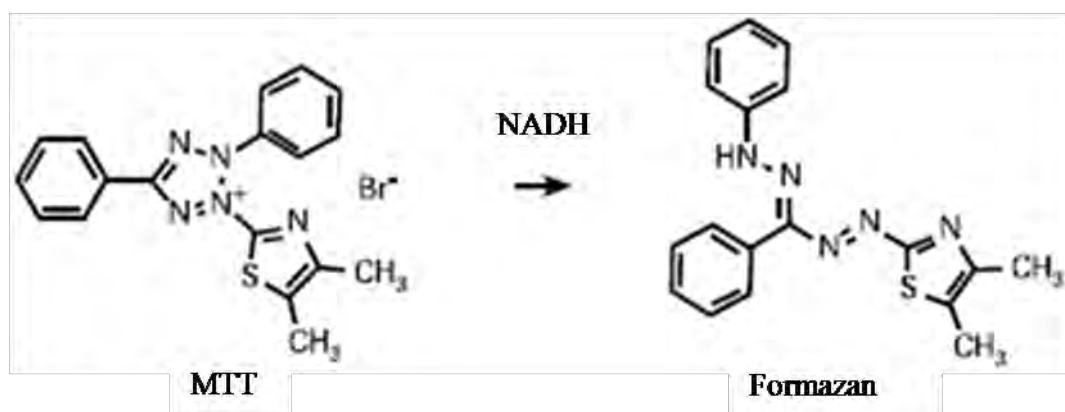


Figure 2: Reduction of MTT by metabolically active cells

Reduction of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) to formazan by NADH, as happens in metabolically active cells (adapted from (Berridge and Tan, 1993).

2.4 Putative cancer stem cell /Side Population Analyses

2.4.1 Flow Cytometry Based on Exclusion of Hoechst 33342

The presence of the side population in MCF7 cells was first confirmed by treating MCF7 cells as follows: MCF7 cells were counted manually using a haemocytometer and resuspended in media to a concentration of 1×10^4 cells.ml⁻¹. A 1 ml volume of the cell suspension was added to each of two T75 culture flasks and a further 9 ml of media was added to each flask. The MCF7 cells were incubated at 37°C and 5% CO₂ for 48 hours, when the cells had formed a confluent layer. One flask was washed with $1 \times$ PBS (pH 7.4) warmed to 37°C and then incubated at 37°C for 10 minutes in serum-containing media with 5 µg.ml⁻¹ reserpine (Sigma), an ABCG2 inhibitor. Both flasks were washed with serum-containing media warmed to 37°C and incubated in at 37°C for 2 hours in serum-containing media with 5 µg.ml⁻¹ Hoechst 33342, with sporadic agitation. The media was removed, the cells washed twice with cold $1 \times$ PBS (pH 7.4) and then harvested by trypsinisation. The cells were collected by centrifugation at 800g and 4°C and kept on ice in the dark until flow cytometric analysis.

To determine the cytotoxic effect of selected algal compounds on the MCF7 side population, MCF7 cells were counted using a haemocytometer and resuspended in media to a concentration of 1×10^5 cells.ml⁻¹. A 1 ml volume of this cell suspension was seeded into each well of a 12-well culture plate and the cells were incubated at 37°C and 5% CO₂ for 24 hours before treatment. The MCF7 cells were then treated for 96 hours with 1 ml solutions of

0.25% DMSO and 100 nM paclitaxel, RUMB-007, RUMB-008, RUMB-010 and RUMB-015 at their IC_{50} values as determined by the cytotoxicity assays. The media was not replenished during this 96 hours (discussed in section 4.2.1). The cells were incubated with $5 \mu\text{g} \cdot \text{ml}^{-1}$ Hoechst 33342 as follows. Cells were incubated in media containing Hoechst 33342 as adherent cultures in the original culture flasks for 2 hours at 37°C with sporadic agitation. The cells were harvested by trypsinisation, washed twice with cold $1 \times \text{PBS}$ (pH 7.4) and collected by centrifugation at $800g$ and 4°C and kept on ice in the dark until flow cytometric analysis. Cells were sorted based on the presence or absence of Hoechst 33342 using a FACSVantage SE flow cytometer (BD Biosciences) equipped with a Coherent Innova 300C water-cooled laser (BD Biosciences). The Hoechst 33342 dye was excited at 350 nm and dual fluorescence emission measured at 450 nm in flow channel 1 (FL-1) (Hoechst Blue) and 675 nm in flow channel 3 (FL-3) (Hoechst Red). All error bars on the graphs depicting the Hoechst 33342-excluding cells represent the standard deviation of the relevant results.

2.4.2 Flow Cytometry Based on ABCG2 Staining

MCF7 cells were counted manually using a haemocytometer and resuspended to give a cell concentration of $1 \times 10^6 \text{ cells} \cdot \text{ml}^{-1}$. A 1 ml volume (1×10^6 cells) of cells was seeded into each well of 3 6-well plates and another 1 ml of culture media was added to each well. The cells were incubated at 37°C and 5% CO_2 for 24 hours before treatment. The cells were then treated with 0.25% DMSO, 10 nM paclitaxel and selected compounds at a concentrations higher than their IC_{50} values determined from the results of the MTT cytotoxicity analyses (Section 2.3 and Chapter 3, Figure 15.). All samples were incubated at 37°C and 5% CO_2 for 96 hours, following which they were harvested and washed with cold $1 \times \text{PBS}$ (pH 7.4). Each sample was then incubated with $1 \mu\text{g}$ biotinylated anti-human CD338 (ABCG2) (BioLegend) in cold $1 \times \text{PBS}$ (pH 7.4) for 1 hour at 4°C following which the cells were washed with ice cold $1 \times \text{PBS}$ (pH 7.4). The cells were then incubated in $2 \mu\text{g}$ streptavidin-FITC (Sigma) at 4°C in the dark for 30 minutes, washed with ice cold $1 \times \text{PBS}$ (pH 7.4), resuspended in $100 \mu\text{l}$ ice cold $1 \times \text{PBS}$ (pH 7.4) with $1 \mu\text{g} \cdot \text{ml}^{-1}$ propidium iodide (Fluka) and analysed by flow cytometry using the Beckman Coulter FC 500 flow cytometer. The FITC was excited at 488 nm and its fluorescence measured at 530 nm in flow channel 1 (FL-1).

2.5 Apoptosis Assays

2.5.1 PARP detection using Western blot analysis

MCF7 cells were counted manually using a haemocytometer and resuspended to give a cell concentration of 2×10^5 cells.ml⁻¹. A 500 µl volume (1×10^5 cells) of cells was seeded into each well of 2 24-well plates and the cells were incubated at 37°C and 5% CO₂ for 24 hours before treatment. The MCF7 cells (1×10^5) were treated with 0.25% DMSO, serum starvation, 100 nM hydrogen peroxide (H₂O₂), 100 nM paclitaxel and the selected novel indigenous algal compounds at the IC₅₀ values as determined by the cytotoxicity assays for 24 hours at 37°C and 5% CO₂. The cells were harvested in 50 µl SDS (sodium dodecyl sulphate) loading buffer (125mM Tris-HCl pH 6.8, 2% SDS, 20% glycerol, 0.2% bromophenol blue) and boiled for 5 minutes. A 20 µl volume of each sample (40 000 cells) was loaded into each well of an SDS gel with a 12% SDS resolving gel and 4% stacking gel (Appendix 1). The proteins were resolved at 150 V and visualized by staining the gels with Coomassie staining solution (40% methanol; 10% acetic acid; 50% water; 0.1 % (w/v) Coomassie Brilliant Blue R250) and destain solution (40% methanol; 10% acetic acid; 50% water). The proteins on the gels were transferred from the SDS gels to Western nitrocellulose membranes (BioRad) in Western transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol) at 100 V for 1 hour. The membranes were incubated at 4°C in 5% (w/v) milk block in Tris-buffered saline-Tween (TBST) (50 mM Tris, 150 mM NaCl, 0.1% Tween-20, pH 7.5) for 1 hour following which they were incubated in a 1 in 500 dilution of purified mouse anti-human PARP (poly (ADP-ribose) polymerase) (BD Pharmingen) in 5% (w/v) milk block in TBST (pH 7.6) at 4°C overnight with shaking. The membranes were washed four times for 15 minutes each with TBST to remove the primary antibody following which they were incubated in a 1 in 2000 dilution of goat anti-mouse HRP in 5% milk block for 1 hour at 4°C with shaking. The membranes were washed with TBST as before. The presence of PARP on the membranes was visualized using the ECL Advance Western Blotting Kit (GE Healthcare). Images were recorded using the Molecular Imager ChemiDoc XRS System (BioRad).

2.5.2 Immunofluorescent detection of apoptosis

To investigate the ability of selected compounds to induce apoptosis, MCF7 cells were counted manually using a haemocytometer and resuspended to give a cell concentration of 1×10^5 cells.ml⁻¹. A 200 µl volume (2×10^4 cells) of cells was seeded into each chamber of an 8-chamber microscope slide and the cells were incubated at 37°C and 5% CO₂ for 24 hours

before treatment. Treatments included 0.25% DMSO, 100 nM paclitaxel and RUMB-001, RUMB-002, RUMB-007, RUMB-010, RUMB-015 and RUMB-017/018 at the IC_{50} values as determined by the cytotoxicity assays (Section 2.3 and Chapter 3, Figure 15.) for 48 hours. The cells were washed with $1\times$ PBS (pH 7.4) and then treated with $10\ \mu\text{g}.\text{ml}^{-1}$ nuclear stain Hoechst 33342 at room temperature in the dark for 10 minutes. The cells were then washed with $1\times$ PBS (pH 7.4) again and the coverslip mounted on the slide using fluorescent mounting medium (Dako). The cells were visualized using a Zeiss LSM META 510 confocal laser scanning microscope. A total of 150 cells from each sample were counted from at least three randomly selected fields of view. The number of apoptotic cells present in each sample of 150 cells was recorded and the percentage of apoptotic cells in each sample calculated.

3 DETERMINATION OF CYTOTOXICITY OF MARINE COMPOUNDS IN MCF12A AND MCF7 CELLS

3.1 Introduction

When searching for novel drugs or compounds to target cancer cells, the level of cytotoxicity of the compounds in both the target cells and other normal cells that may be adversely affected should be determined (Balis, 2002). Should a compound exhibit a high level of cytotoxicity in the target cells but also in other non-target or normal cells, the compound may not be effective as a drug. One of the main problems facing chemotherapy has been the toxicity of chemotherapeutic drugs to normal cells. When investigating potential novel chemotherapeutic drugs, therefore, the difference in dose response between the normal and cancer cells is vital. An ideal chemotherapeutic drug is one which has a high, specific cytotoxicity to the target cells, in this case the cancer cells, while having little or no cytotoxicity in normal cells (DeVita and Chu, 2008).

Monoterpenes have already been used in the treatment of cancer (Gould, 1997; Myers, 1997). With respect to breast cancer, some of these monoterpenes act by interfering with signal transduction pathways, thereby changing gene expression and resulting in tumour regression (Gould, 1997). Some monoterpenes have been patented as potential chemotherapeutic drugs and in addition to having anti-cancer activity these compounds are also capable of sensitising select tumours to radiation (Myers, 1997). Marine organisms are known to be a source of chemically and biologically varied compounds, many of which might prove to be effective anti-cancer agents (Valeriote et al., 1994). Among some compounds isolated from marine organisms that have shown anti-cancer activity are didemnin B from *Trididemnum solidum* (Mittelman et al., 1999; Rinehart et al., 1988; Taylor et al., 1992) and bryostatin from *Bugula neritina* (Hornung et al., 1992; Kerr et al., 1996). Some marine halogenated monoterpenes have been demonstrated as having anti-cancer activity, some as far back as in 1992 (Fuller et al., 1992; Polzin et al., 2003). A halogenated monoterpene, isolated from *Portieria hornemannii*, containing both bromine and chlorine groups was shown to have high levels of cytotoxicity against brain, colon and kidney tumours (Fuller et al., 1992). In another study, four halogenated monoterpenes from *Plocamium cartilagineum* showed anti-cancer activity against colon adenocarcinoma cell line SW480 and cervical adenocarcinoma cell line HeLa but showed no toxic effects against non-cancerous Chinese hamster ovary cell line (de Ines et al., 2004). Nine halogenated monoterpenes isolated from the red alga *Plocamium corallorhiza* and *Plocamium cornutum* and one tetraprenylquinone isolated from the brown algae *Sargassum heterophyllum*, all collected off the eastern coast of South Africa, were investigated for their cytotoxicity towards MCF12A and MCF7 cell lines.

The MCF12A cell line represents „normal“ cells while the MCF7 cell line represents cancer cells. These compounds were selected for cytotoxicity analyses in MCF12A and MCF7 cell lines because some of them have been shown to exhibit moderate cytotoxic activity towards the WHCO1 human oesophageal cancer cell line and have IC_{50} values in these cells of between 10 μ M and 90 μ M (Knott et al., 2005; Mann et al., 2007). The IC_{50} value of a compound, or the concentration at which 50% cell death occurs, is commonly used as a measure of cytotoxicity in cells. The aim of the cytotoxicity assays was to select compounds with a more pronounced cytotoxic effect in the MCF7 cell line than in the MCF12A cell line for further analysis and the difference between the IC_{50} values calculated for the different compounds in each cell line would ideally be statistically significant as determined by Student's *t*-test. Four compounds that had IC_{50} values at least three times higher in MCF12A cells than in MCF7 cells were selected so that treatment at a concentration toxic to the cancer cells would have little effect on normal cells. A further two compounds were selected, one which displayed high cytotoxicity in both cell lines and one which was not cytotoxic to either cell line.

3.2 Results and Discussion

Before determining the effect of the algal compounds in MCF12A and MCF7 cells it was necessary to define what was meant by the term „cytotoxicity“ in the context of this study. The MTT assay is commonly used as a measure of cytotoxicity through the identification of metabolically active cells (Berridge and Tan, 1993). While all dead cells will indeed be metabolically inactive, not all metabolically inactive cells will necessarily be dead. Rather these cells may have entered replicative cell senescence (Matsumura et al., 1979; Shay and Wright, 2000). This is a potential limitation of the MTT assay, which measures cell proliferation on the basis of metabolism and therefore may not discriminate between dead and senescent cells. For the purpose of this study, the term „cytotoxicity“ will be used to describe the lack of proliferation or the inhibition of proliferation of the cells as measured by the MTT assay. The IC_{50} values calculated for the algal compounds in the different cell lines is therefore representative of the concentration at which 50% growth inhibition, regardless of whether it is a result of death or cellular senescence, is reached. The cytotoxicity of each compound in both MCF12A and MCF7 cell lines was determined using the MTT assay method in which MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) is reduced to formazan crystals by NADH and NADPH in metabolically active cells (Figure 2) (Berridge and Tan, 1993). This change was then measured spectrophotometrically at 550 nm.

3.2.1 Optimisation and validation of MTT assay on MCF12A and MCF7 cells using Paclitaxel

The first step in the analysis of the cytotoxicity of the algal compounds was the determination of the optimal time for which cells should be treated with the algal compounds and this was done using MCF7 cells treated with a range of concentrations of the existing chemotherapeutic agent, paclitaxel. The MCF7 cells (6000) were treated with between 0.01 μM and 5 μM paclitaxel for 24 hours, 48 hours, 72 hours and 96 hours before cell survival was measured using the MTT assay (

Figure 3 3). The most pronounced dose response was observed after treatment of the MCF7 cells with paclitaxel for 96 hours. It was decided that all subsequent MTT assays in which the cytotoxic effect of the algal compounds were investigated in MCF12A and MCF7 cells would be conducted after the cells were treated with differing concentrations of the algal compounds for 96 hours, even though a similar dose response was seen after treatment of the MCF7 cells with paclitaxel for 72 hours.

MCF12A and MCF7 cells (6000 cells) were treated with between 0.5 μM and 100 μM of each compound for 96 hours, following which the anti-proliferative activity of the compounds was measured. This concentration range was chosen as it would allow observation of a cytotoxic effect in both the nanomolar range and the micromolar range, following which the concentration range could be narrowed if necessary. The effect of each compound on the cells lines was represented graphically as a function of concentration (on the x-axis) versus percent survival (on the y-axis). The DMSO-treated cells were taken as 100% survival, and the percent survival of the cells after treatment with each compound tested could then be calculated relative to the DMSO control. From these representations the concentration of each compound at which 50% growth inhibition (IC_{50}) is reached can be calculated from a linear trendline fitted to the graph using the straight line equation $y = mx + c$. Paclitaxel was used as a positive control for cytotoxicity and has a known IC_{50} in MCF7s of approximately 100 nM but the IC_{50} in MCF12As is unknown.

The results of the MTT assays carried out on MCF12A and MCF7 cells after treatment with varying concentrations of paclitaxel (between 0.01 μM and 0.15 μM) for 96 hours are shown in Figure 4. After conducting the MTT assay on the MCF7 cells treated with between 0.01 μM and 5 μM to determine the optimum treatment time it was observed that treatment with

0.5 μM and 5 μM elicited the same cytotoxic response at each time point. The concentration range of paclitaxel used in subsequent assays in which the cytotoxicity of the algal compounds was tested was therefore changed to between 0.01 μM and 0.15 μM in order to obtain a linear dose response.

Figure 4 shows the results of the validation of the cytotoxicity tests for MCF12A (Figure 4A) and MCF7 (Figure 4B) cells when treated with different concentrations of paclitaxel, a comparison of the cell survival in MCF12A and MCF7 cells (Figure 4) and the structure of paclitaxel and the calculated IC_{50} values in both cell lines (Figure 4D). The IC_{50} values of paclitaxel in MCF12A and MCF7 cells were calculated to be 5.36 nM and 88.7 nM respectively (Figure 4). No published data could be found regarding the IC_{50} of paclitaxel in MCF12A cells but from these results the IC_{50} was calculated to be 5.36 nM. The published IC_{50} of paclitaxel in MCF7 cells is approximately 100 nM (Dwight et al., 1997), close to the calculated IC_{50} of 88.7 nM obtained from these data, thereby validating the MTT assay being used. The calculated IC_{50} of paclitaxel in MCF12A cells was 5.36 μM but no published value for this could be found. The difference in the IC_{50} values in MCF12A and MCF7 cells was found to be statistically significant using Student's *t*-test ($p < 0.03$). Closer observation of the shape of the curve of Figure 4A suggests that the calculated IC_{50} value for paclitaxel in MCF12A cells may be higher than the actual value. The anti-proliferative effect of the paclitaxel on the MCF12A cells was already at its highest after treatment of the cells at 0.01 μM , the lowest concentration point. As such, calculation of the IC_{50} of paclitaxel in MCF12A cells from the curves shown in Figure 4A may not be accurate. To accurately determine the IC_{50} of paclitaxel in MCF12A cells it would be necessary to repeat the assay using concentrations of paclitaxel within the linear range (0 – 0.01 μM).

3.2.2 Determination of the IC_{50} values of algal compounds in MCF12A and MCF7 cells

The cytotoxic effects of the indigenous algal compounds were investigated in MCF12A and MCF7 cells using the MTT assay and concentrations of between 0.5 μM and 100 μM . This concentration range was chosen as it would allow the observation of a cytotoxic effect over a wide concentration range, from the low (0.5 μM) to the high micromolar range (100 μM). In addition to this the IC_{50} values of these compounds in WHCO1 oesophageal cancer cells ranges from 7.5 μM to 88 μM (Knott et al., 2005; Mann et al., 2007). The concentration range selected for compound testing in MCF12A and MCF7 cells would allow for any effect on the cells in the same range as seen in WHCO1 cells to be observed but would also allow for

effects below and above this range to be seen. The data are presented for individual compounds as dose response curves showing the percent survival of MCF12A and MCF7 cells at each concentration of the different compounds in triplicate. Also shown is a dose response curve showing the average percentage survival both cell lines at the different compound concentrations as well as the structure of each compound and the calculated IC_{50} values for the particular compound. To determine whether the difference in the IC_{50} values calculated for the different compounds in each cell line was statistically significant two-tailed two-sample equal variance Student's *t*-tests were conducted. The cytotoxic effect of each novel compound was investigated using the validated MTT assay by treating the MCF12A and MCF7 cell lines with five different concentrations (including 0 μ M) of each compound. Each concentration was repeated in quadruplicate on a 96 well plate and each plate was repeated in triplicate. Not all the compounds tested resulted in 50% cell death and so the calculation of the IC_{50} values for these compounds relies on the extrapolation of the data beyond the concentration range tested. Unfortunately, data extrapolation may result in inaccurate calculations and the IC_{50} values calculated for all compounds tested in both MCF12A and MCF7 cells in which 50% cell death was not observed may be an underestimation. In order to obtain a more accurate IC_{50} value for those compounds, a further experiment in which the concentration range of the compounds tested is expanded would be required.

3.2.2.1 RUMB-001

RUMB-001, also called Plocoralide A, is a polyhalogenated monoterpene containing both chlorine and bromine isolated from *Plocamium corallorhiza*. The results obtained from the MTT assays on MCF12A and MCF7 cell lines after treatment with RUMB-001 are shown in Figure 5. A dose response was observed in both the MCF12A (Figure 5A) and MCF7 (Figure 5B) cells. The dose response in the MCF7 cells appeared to be more pronounced than that observed in the MCF12A cells, as is shown in Figure 5C. The IC_{50} values of RUMB-001 in MCF12A and MCF7 cell lines were calculated to be 164.3 μ M and 58.1 μ M respectively, a difference was calculated to be statistically significant ($p < 0.05$) using Student's *t*-test. RUMB-001 is almost three times more toxic in MCF7 cells than in MCF12A cells. There was a standard deviation of 69 μ M observed when calculating the IC_{50} of RUMB-001 in MCF12A cells which is a large margin of error considering the calculated IC_{50} value of this compound in the MCF12A cells was 164.3 μ M. In addition, the calculated IC_{50} of RUMB-001 in MCF12A cells is higher than the highest concentration of the compound tested. The IC_{50} of

this compound in MCF12A cells may well be an underestimation and so can be said to be greater than 164.3 μM ($\text{IC}_{50} > 164.3 \mu\text{M}$).

3.2.2.2 RUMB-002

Figure 6 shows the results from the MTT assays after treatment of MCF12A and MCF7 cells with RUMB-002, a chlorinated and brominated monoterpene also called Plocoralide B isolated from *Plocamium corallorhiza*. There is a clear dose response seen in the MCF7 cells treated with RUMB-002 (Figure 6B). A lesser dose response was seen in the MCF12A cells (Figure 6A) than in the MCF7 cells which is shown in Figure 6C. The IC_{50} of RUMB-002 in MCF12A and MCF7 cells was calculated to be 238.3 μM and 65.1 μM respectively and this difference was statistically significant ($p < 0.05$). As with RUMB-001, the IC_{50} of RUMB-002 is approximately three times lower in MCF7 cells than in MCF12A cells, meaning that this molecule is three times more toxic to the MCF7 cancer cells than the MCF12A breast epithelial cells, using this assay. In addition, the IC_{50} of RUMB-002 in MCF12A cells is higher than the highest concentration of the compound tested and so the calculated IC_{50} may be an underestimation. As such, the IC_{50} of RUMB-002 in MCF12A cells can be said to be greater than 238.3 μM ($\text{IC}_{50} > 238.3 \mu\text{M}$).

3.2.2.3 RUMB-003

RUMB-003, also called Plocoralide C, is a polychlorinated monoterpene isolated from *Plocamium corallorhiza*. The results from the cytotoxicity analyses obtained using the MTT assay on MCF12A and MCF7 cells after treatment with this compound are shown in Figure 7. A slight dose response was observed in MCF12A cells (Figure 7A) and a more marked dose response was seen in the MCF7 cells (Figure 7B). The IC_{50} values of RUMB-003 were calculated to be 184.7 μM and 90.0 μM in MCF12A and MCF7 cells respectively. The difference between the IC_{50} values of this compound in the two cell lines was calculated to be significantly different ($p < 0.05$). As with RUMB-001 and RUMB-002, the IC_{50} of RUMB-003 in MCF12A cells is higher than 100 μM , the highest compound concentration tested. The IC_{50} of RUMB-003 may therefore be higher than 184.7 μM ($\text{IC}_{50} > 184.7 \mu\text{M}$).

3.2.2.4 RUMB-004

The results from the MTT assay carried out after treatment of MCF7 and MCF12A cells with RUMB-004 is shown in Figure 8. RUMB-004, or Plocoralide D, is a polyhalogenated monoterpene isolated from *Plocamium corallorhiza* and contains both chlorine and bromine. The IC_{50} of RUMB-004 in MCF12A cells was calculated even though it appears to have little effect on the cells with no distinct dose response observed (Figure 8A). A more distinct dose response was observed in the MCF7 cells treated with RUMB-004 (Figure 8). The concentrations of RUMB-004 which results in 50% growth inhibition in MCF12A and MCF7 cells are 225 μ M and 90.0 μ M respectively, a difference that was confirmed as being significant using Student's *t*-test ($p < 0.05$). Also, in spite of the fact that RUMB-004 is more cytotoxic in the MCF7 cancer cells than the MCF12A cells, the concentration required to achieve 50% cell death in the cancer cells was still observed to be much higher than desired for a potential hit compound as treatment of the MCF12A cells with 90.0 μ M of the compound may induce a dose response. As a result of the IC_{50} of RUMB-004 in MCF12A cells being higher than the highest compound concentration tested and the necessary use of data extrapolation to calculate this IC_{50} , the IC_{50} of this compound in MCF12A cells can be said to be greater than the 225 μ M it was calculated to be ($IC_{50} > 225 \mu$ M).

3.2.2.5 RUMB-005

RUMB-005, also known as Plocoralide E, is a polyhalogenated monoterpene, containing chlorine and bromine, isolated from *Plocamium corallorhiza*. The results from the cytotoxicity determination using the MTT assay after treatment of MCF12A and MCF7 cells with RUMB-005 is shown in Figure 9. The IC_{50} calculated for RUMB-005 in MCF12A cells was 34.8 μ M and in MCF7 cells was 69.7 μ M. RUMB-005 was the only compound tested that exhibited a significantly ($p < 0.05$) higher level of cytotoxicity towards the normal breast epithelial MCF12A cells than the MCF7 breast adenocarcinoma cells and in this case the IC_{50} in MCF12A cells was almost exactly half of that in MCF7 cells.

3.2.2.6 RUMB-007

RUMB-007 is a brominated monoterpene isolated from *Plocamium corallorhiza* and is also known as Plocoral A. The cytotoxicity results obtained from treating MCF12A and MCF7 cells with RUMB-7 are shown in Figure 10. Dose responses were observed in both the MCF12A (Figure 10A) and MCF7 (Figure 10B) cells although the dose response in the

MCF7 cells was significantly higher ($p<0.05$) in the MCF7 cells. The IC_{50} values calculated for RUMB-007 in MCF12A and MCF7 cells were 127.6 μ M and 33.0 μ M respectively, although the standard deviation found when calculating the IC_{50} in MCF12A cells was 30.2 μ M.

3.2.2.7 RUMB-008

RUMB-008, or Plocoral B, is a chlorinated and brominated monoterpene isolated from *Plocamium corallorhiza*. The results obtained from the MTT assays on MCF12A and MCF7 cells after treatment with RUMB-008 are shown in Figure 11. Both the MCF12A (Figure 11A) and MCF7 (Figure 11B) showed marked dose responses as a result of treatment with this compound. The IC_{50} value calculated for RUMB-008 in MCF12A cells was 62.3 μ M and for MCF7 cells was 28.8 μ M. Even though the difference in the IC_{50} values of RUMB-008 in MCF12A and MCF7 cells was not as great as with some other compounds, the difference was still found to be significant ($p<0.05$).

3.2.2.8 RUMB-010

RUMB-010, also known as sargaquinoic acid, is a tetraprenylquinone isolated from *Sargassum heterophyllum*. The results obtained from the MTT assay carried out on MCF12A and MCF7 cells after treatment with RUMB-010 are shown in Figure 12. RUMB-010 was the only marine molecule used in this study that has not been previously tested for anti-cancer activity on WHCO1 oesophageal cancer cells (Knott et al., 2005; Mann et al., 2007). A dose response was seen in the MCF7 cells treated with RUMB-010 (Figure 12B). The IC_{50} of RUMB-010 in MCF7 cancer cells was calculated to be 68.4 μ M, showing that RUMB-010 is cytotoxic towards these cells, albeit not at the same level as, for example, RUMB-007 and RUMB-008. When looking at Figure 12A, no obvious dose response in MCF12A cells treated with RUMB-010 was observed. The lack of dose response seen in the MCF12A cell line treated with RUMB-010 resulted in an inability to properly fit trendlines to the curves. As such the IC_{50} of RUMB-010 in this cell line, as well as whether the difference in the dose response between the two cell lines was significant, could not be calculated. In this case, the IC_{50} has been recorded as indeterminate.

3.2.2.9 RUMB-015

RUMB-015 is a chlorinated monoterpene isolated from *Plocamium cornutum* and is otherwise known as Plocornulide A. The results of the cytotoxicity analyses in the MCF12A cell line treated with RUMB-015 are shown in Figure 13. Distinct and strong dose responses were seen in both the MCF12A and MCF7 cells treated with this compound. After treating the MCF7 cell line with concentrations of RUMB-015 ranging from 0.5 μM to 100 μM the concentration range was adjusted to 0.1 μM to 5 μM (Figure 13B). RUMB-015 showed high levels of cytotoxicity in both cell lines with calculated IC_{50} values of 3.5 μM and 3.0 μM in MCF12A and MCF7 cells respectively. The strong cytotoxic effect of RUMB-015 in both these cell lines can be clearly seen in Figure 13A and B where the percentage of cell survival drops sharply after the addition of low concentrations of RUMB-015. From Figure 13C it seems that the MCF12A cells (blue line) are as susceptible to RUMB-015 as MCF7 cells (red line) as they show decreased cell survival in comparison to the MCF7 cells. The IC_{50} values calculated for RUMB-015 in MCF12A and MCF7 cells, 3.5 μM and 3.0 μM respectively, are similar, as was confirmed by Student's t -test ($p>0.05$).

3.2.2.10 RUMB-017/018

RUMB-017/018, or Plocornulide C, contains both chlorine and bromine and exists as a mixture of enantiomers and was isolated from *Plocamium cornutum*. The results of the cytotoxicity assays carried out on MCF12A and MCF7 cells after treatment with RUMB-017/018 are shown in Figure 14. RUMB-017/018 had little cytotoxic effect in both MCF12A and MCF7 cells and as shown in Figure 14A and B there is no distinct dose response observed after treating both cell lines with this compound. The lack of dose response exhibited in both cell lines is shown in Figure 14C, which shows little difference in cell survival between both cell lines and also shows little cell death. As such, no IC_{50} values for this compound in MCF12A and MCF7 cells could be determined.

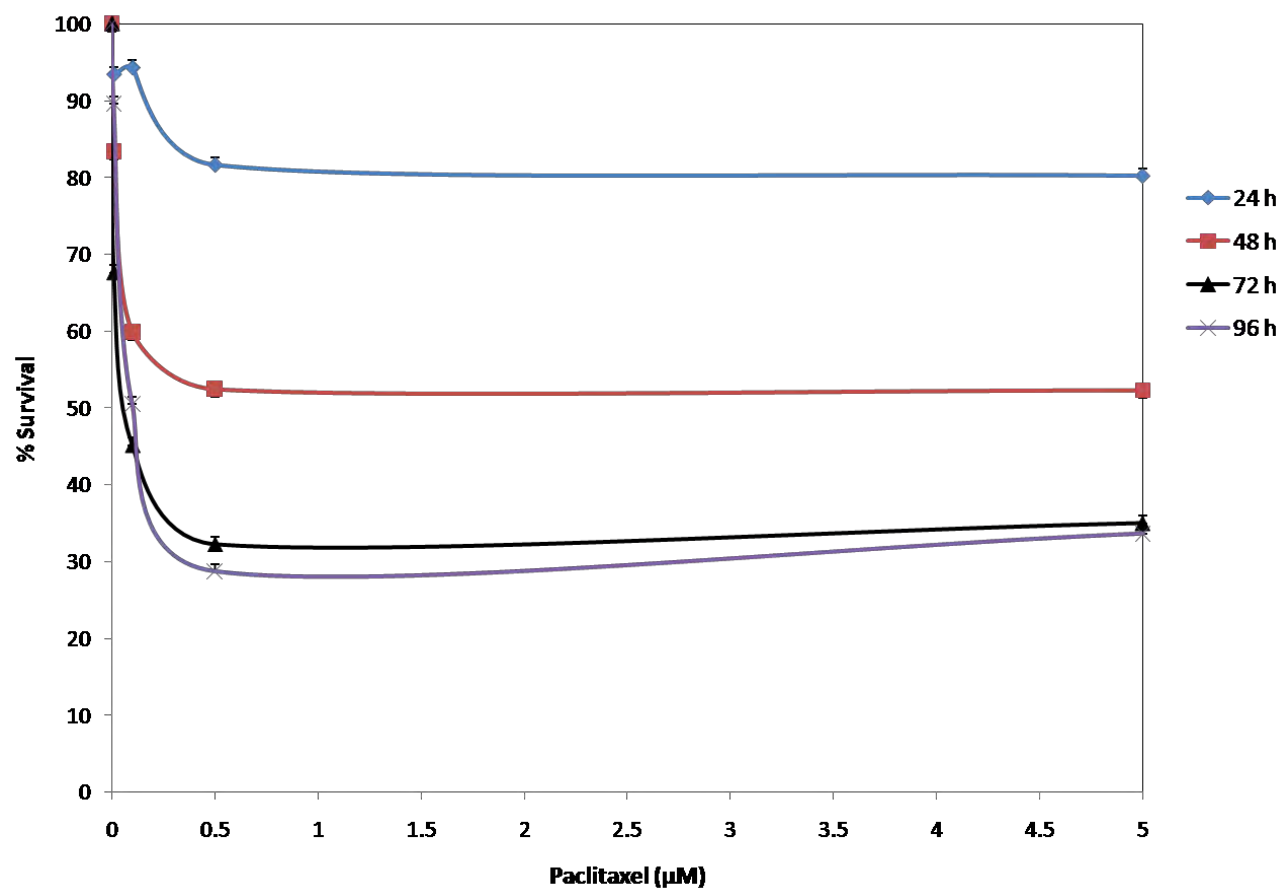


Figure 3: Optimisation of the MTT assay on MCF7 cells using paclitaxel.

Survival curve of MCF7 cells after treatment with paclitaxel at concentrations ranging from 0.01 µM to 5 µM after 24 hours (blue line), 48 hours (red line), 72 hours (black line) and 96 hours (purple line) as measured by the MTT assay. The percentage survival of cells was calculated by normalizing the survival of cells treated only with DMSO and no paclitaxel to be 100% and the survival of the cells at each concentration was calculated relative to this value.

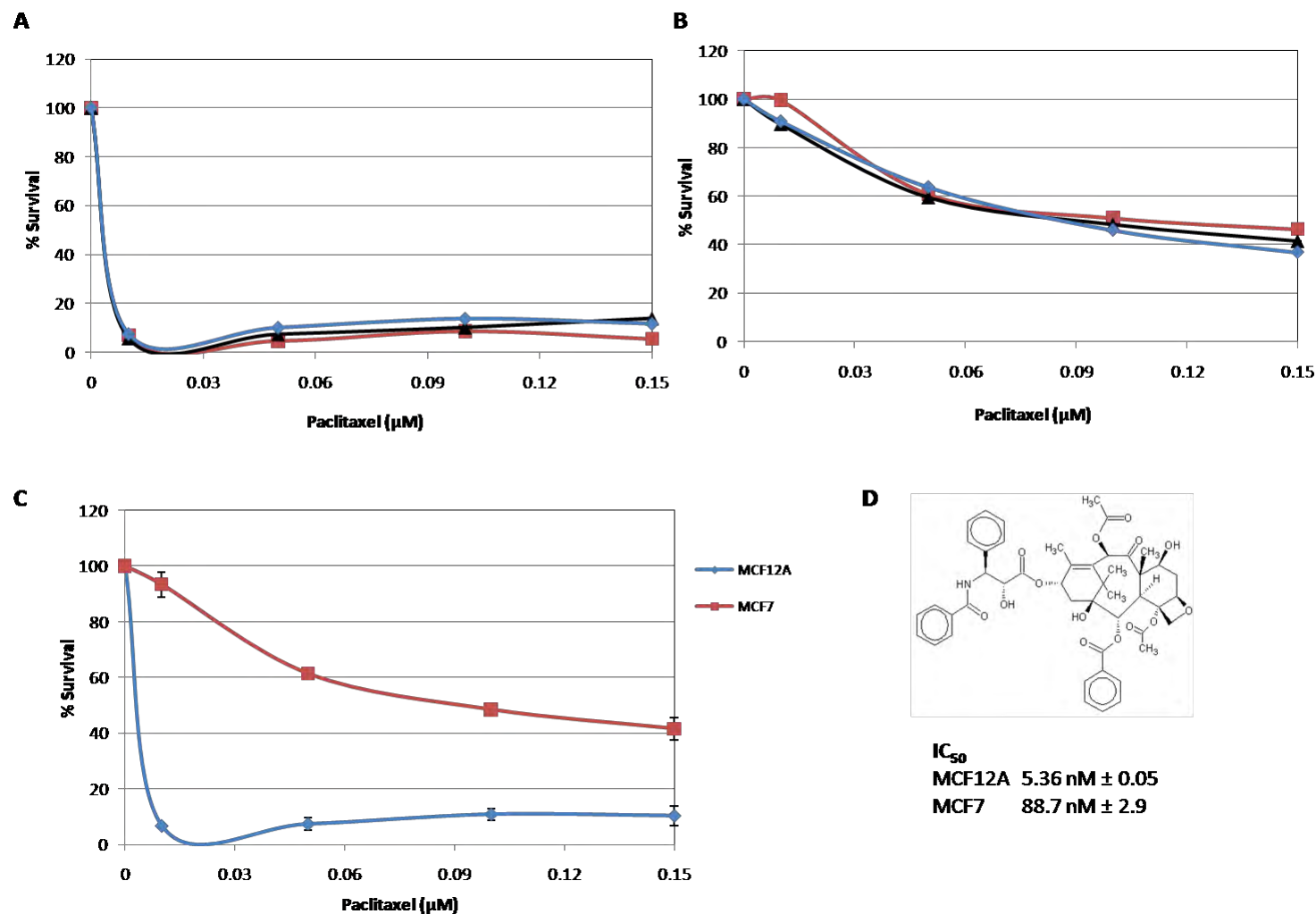


Figure 4: Determination of IC_{50} values of paclitaxel in MCF12A and MCF7 cells.

A: MCF12A and B: MCF7 cells were treated with paclitaxel ranging in concentration from 0.01 μM to 0.15 μM for 96 hours (triplicates shown as black, red and blue lines). C: Comparison of the average percentage cell survival of MCF12A (blue line) and MCF7 (red line) cells after treatment with paclitaxel at concentrations ranging from 0.01 μM to 0.15 μM after 96 hours. Percentage survival was calculated relative to the DMSO control (100% survival). D: Structure of paclitaxel and the IC_{50} value of this compound in MCF12A and MCF7 cells (see Appendix 2 for calculations).

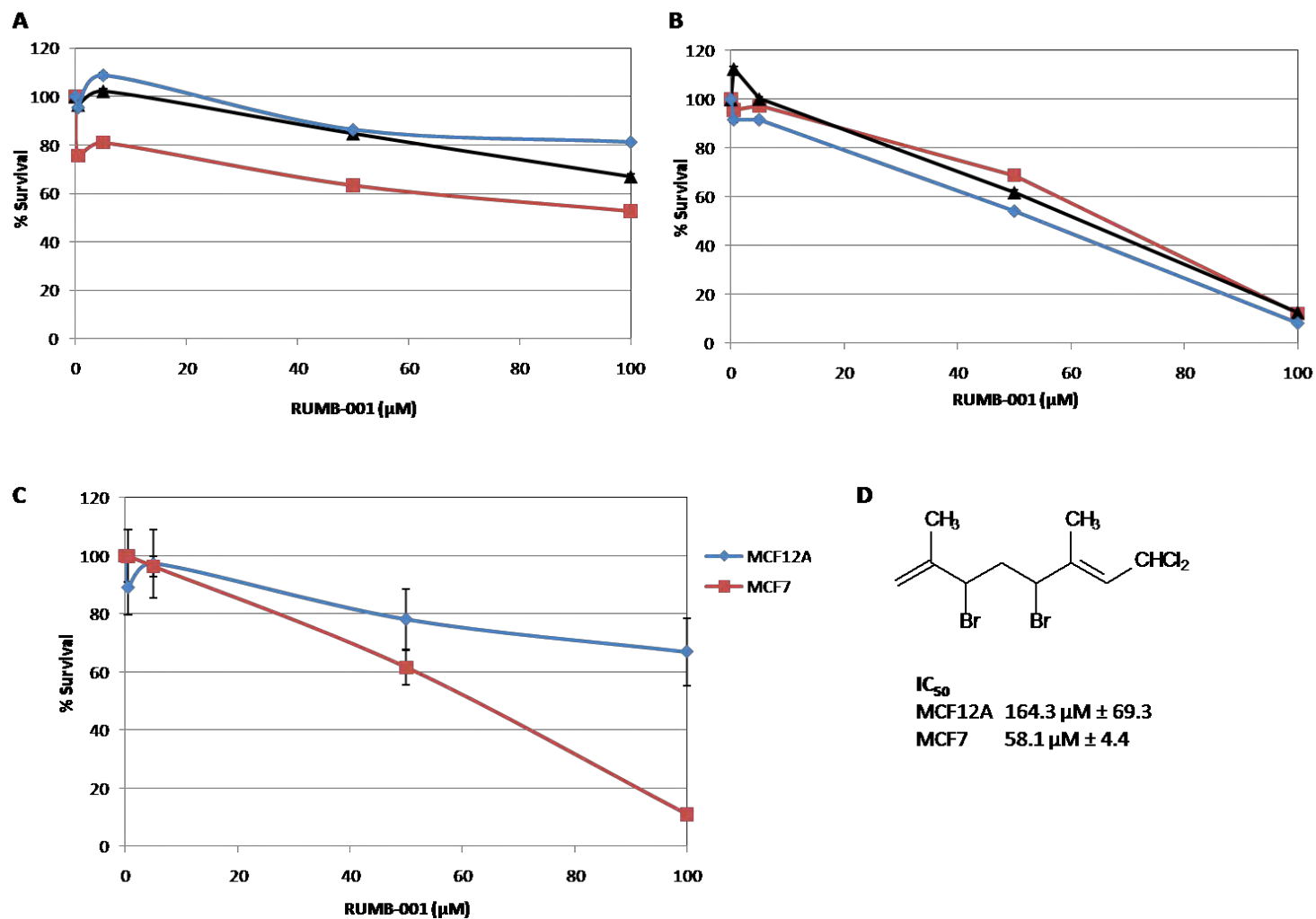


Figure 5: Determination of IC_{50} values of RUMB-001 in MCF12A and MCF7 cells.

A: MCF12A and B: MCF7 cells were treated with RUMB-001 ranging in concentration from 0.5 μM to 100 μM for 96 hours (triplicates shown as black, red and blue lines). C: Comparison of the average percentage cell survival of MCF12A (blue line) and MCF7 (red line) cells after treatment with RUMB-001 at concentrations ranging from 0.5 μM to 100 μM after 96 hours. Percentage survival was calculated relative to the DMSO control (100% survival). D: Structure of RUMB-001 and the IC_{50} value of this compound in MCF12A and MCF7 cells (see Appendix 2 for calculations).

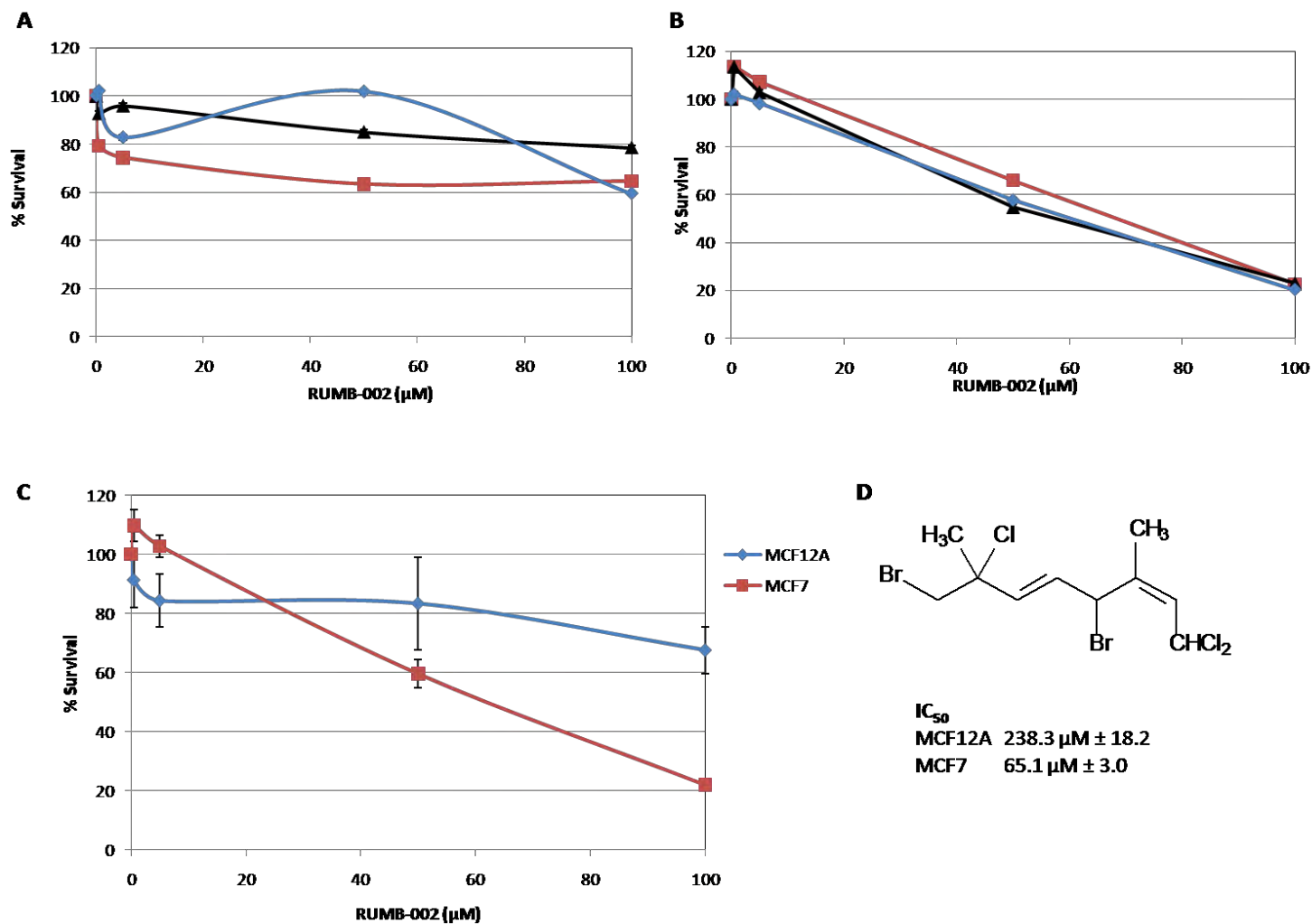


Figure 6: Determination of IC_{50} values of RUMB-002 in MCF12A and MCF7 cells.

A: MCF12A and B: MCF7 cells were treated with RUMB-002 ranging in concentration from 0.5 μM to 100 μM for 96 hours (triplicates shown as black, red and blue lines). C: Comparison of the average percentage cell survival of MCF12A (blue line) and MCF7 (red line) cells after treatment with RUMB-002 at concentrations ranging from 0.5 μM to 100 μM after 96 hours. Percentage survival was calculated relative to the DMSO control (100% survival). D: Structure of RUMB-002 and the IC_{50} value of this compound in MCF12A and MCF7 cells (see Appendix 2 for calculations).

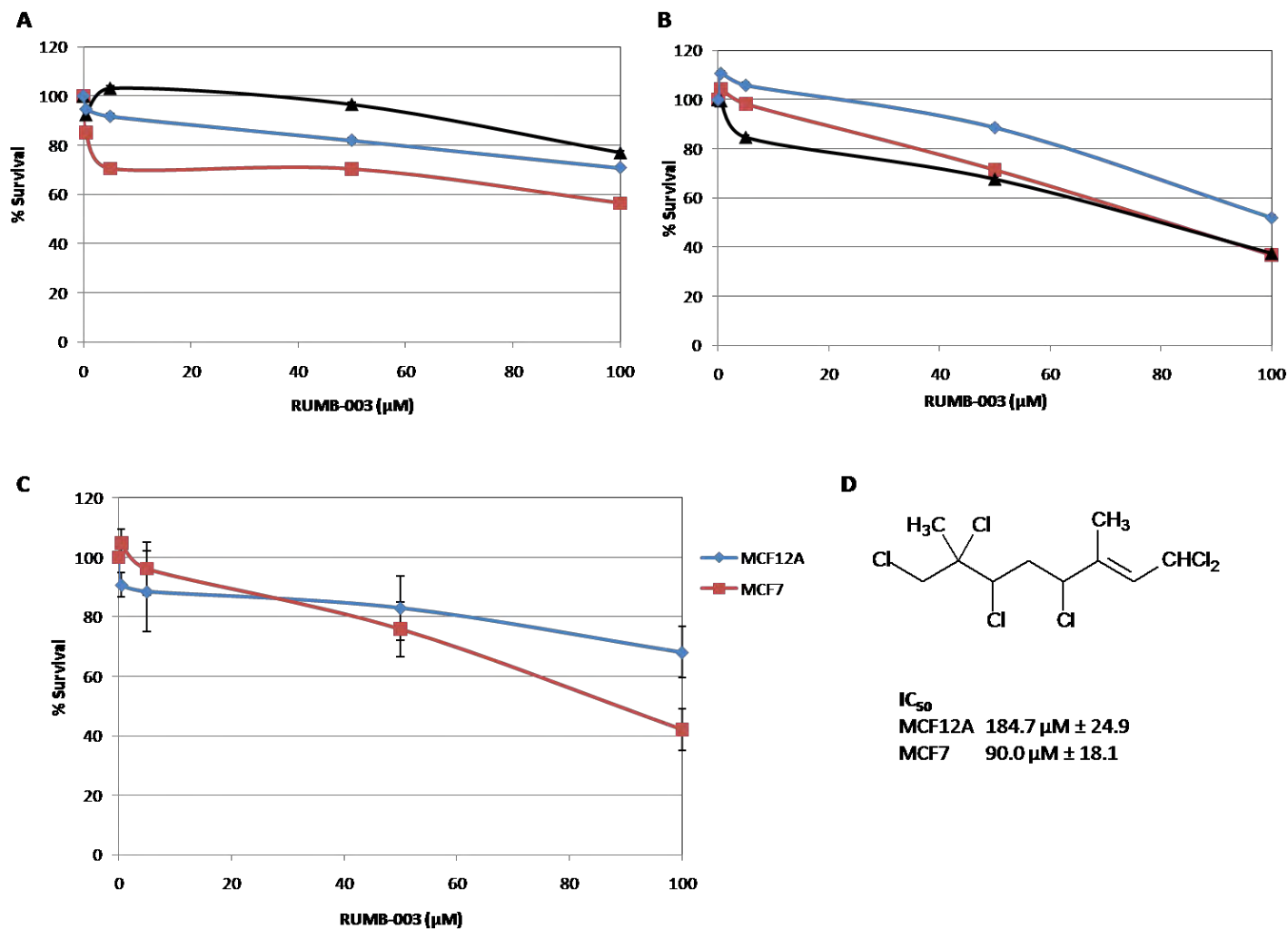


Figure 7: Determination of IC_{50} values of RUMB-003 in MCF12A and MCF7 cells.

A: MCF12A and B: MCF7 cells were treated with RUMB-003 ranging in concentration from 0.5 μM to 100 μM for 96 hours (triplicates shown as black, red and blue lines). C: Comparison of the average percentage cell survival of MCF12A (blue line) and MCF7 (red line) cells after treatment with RUMB-003 at concentrations ranging from 0.5 μM to 100 μM after 96 hours. Percentage survival was calculated relative to the DMSO control (100% survival). D: Structure of RUMB-003 and the IC_{50} value of this compound in MCF12A and MCF7 cells (see Appendix 2 for calculations).

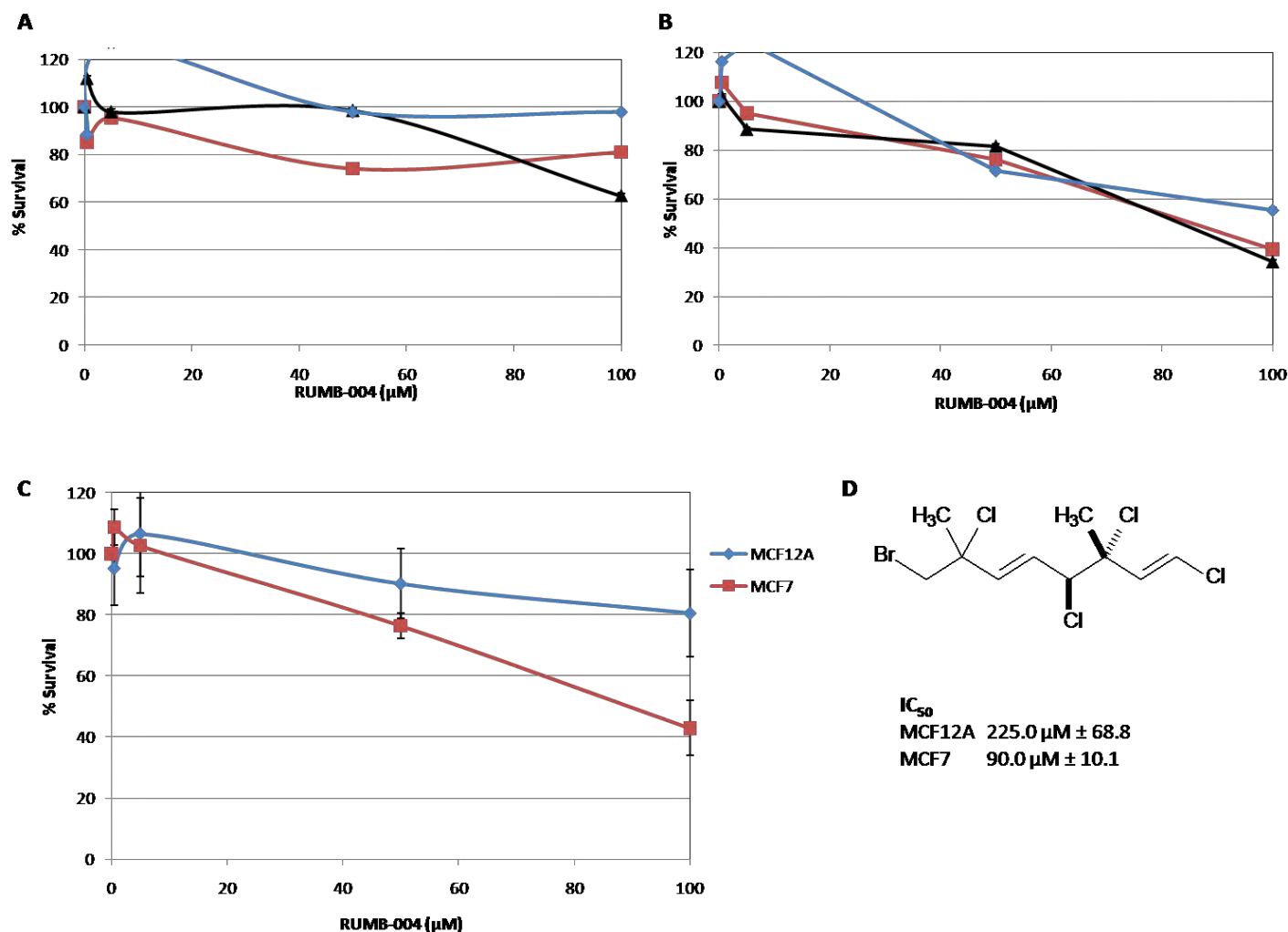


Figure 8: Determination of IC_{50} values of RUMB-004 in MCF12A and MCF7 cells.

A: MCF12A and B: MCF7 cells were treated with RUMB-004 ranging in concentration from 0.5 μM to 100 μM for 96 hours (triplicates shown as black, red and blue lines). C: Comparison of the average percentage cell survival of MCF12A (blue line) and MCF7 (red line) cells after treatment with RUMB-004 at concentrations ranging from 0.5 μM to 100 μM after 96 hours. Percentage survival was calculated relative to the DMSO control (100% survival). D: Structure of RUMB-004 and the IC_{50} value of this compound in MCF12A and MCF7 cells (see Appendix 2 for calculations).

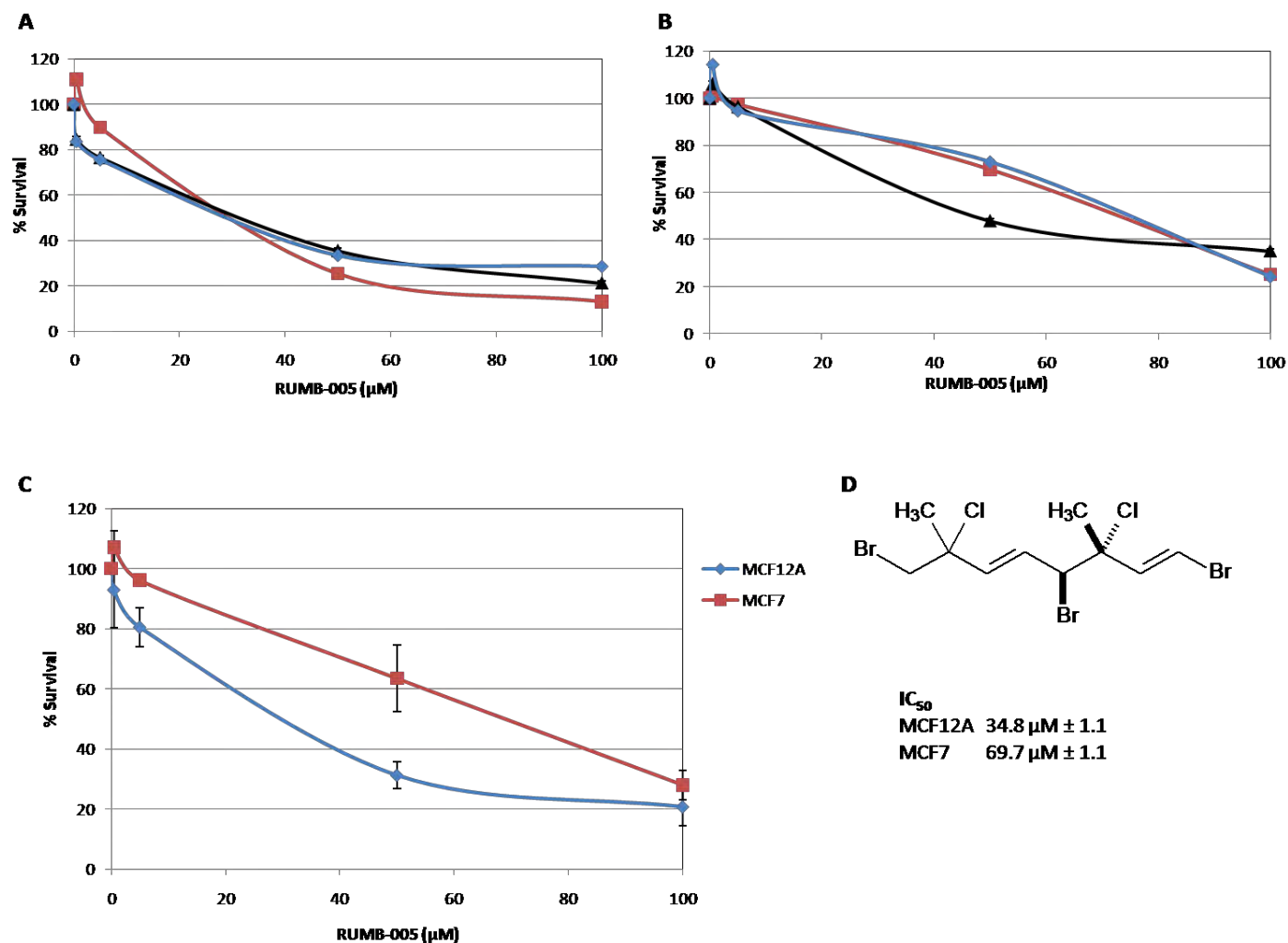


Figure 9: Determination of IC_{50} values of RUMB-005 in MCF12A and MCF7 cells.

A: MCF12A and B: MCF7 cells were treated with RUMB-005 ranging in concentration from 0.5 μM to 100 μM for 96 hours (triplicates shown as black, red and blue lines). C: Comparison of the average percentage cell survival of MCF12A (blue line) and MCF7 (red line) cells after treatment with RUMB-005 at concentrations ranging from 0.5 μM to 100 μM after 96 hours. Percentage survival was calculated relative to the DMSO control (100% survival). D: Structure of RUMB-005 and the IC_{50} value of this compound in MCF12A and MCF7 cells (see Appendix 2 for calculations).

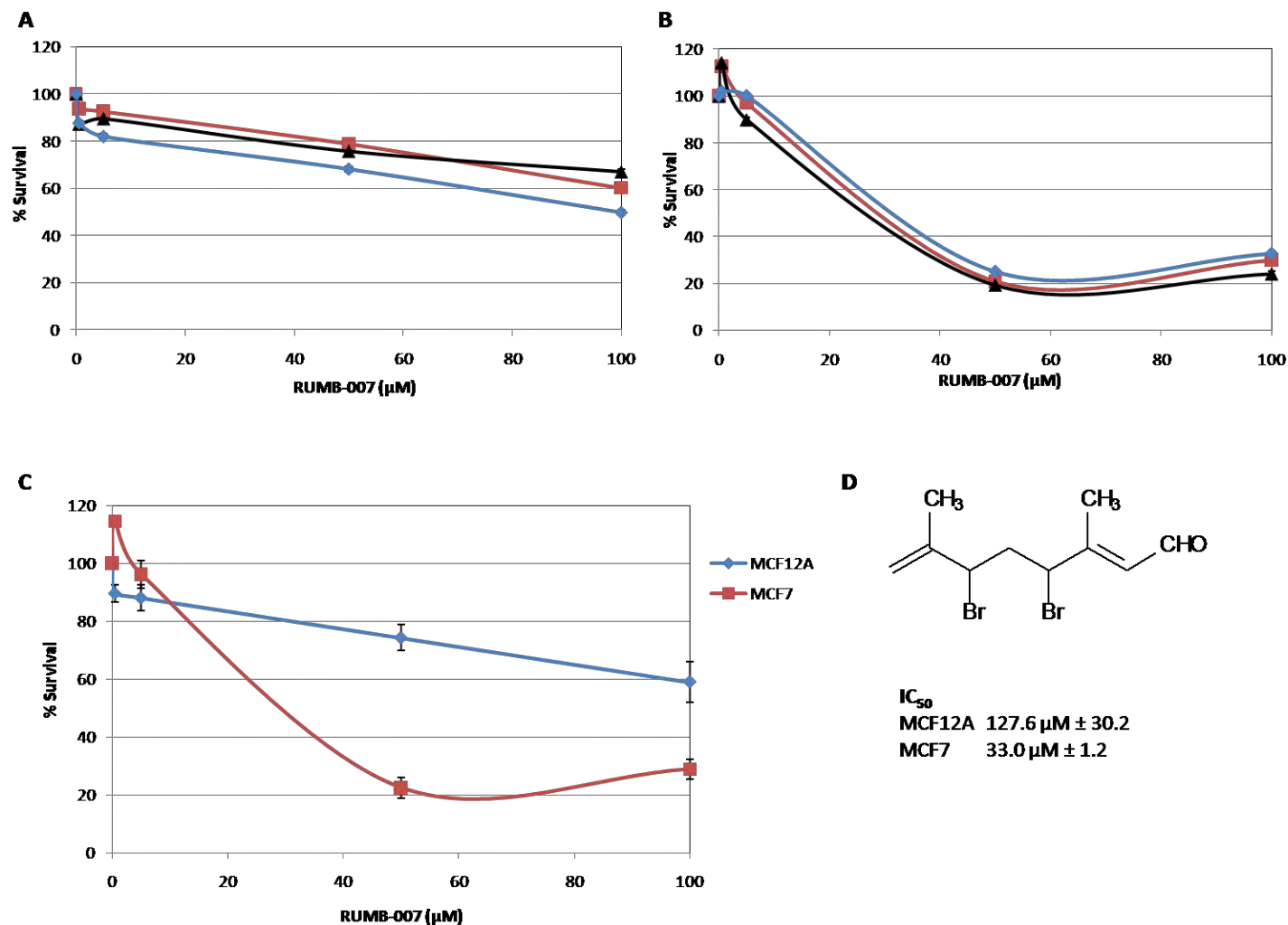


Figure 10: Determination of IC₅₀ values of RUMB-007 in MCF12A and MCF7 cells.

A: MCF12A and B: MCF7 cells were treated with RUMB-007 ranging in concentration from 0.5 μM to 100 μM for 96 hours (triplicates shown as black, red and blue lines). C: Comparison of the average percentage cell survival of MCF12A (blue line) and MCF7 (red line) cells after treatment with RUMB-007 at concentrations ranging from 0.5 μM to 100 μM after 96 hours. Percentage survival was calculated relative to the DMSO control (100% survival). D: Structure of RUMB-007 and the IC₅₀ value of this compound in MCF12A and MCF7 cells (see Appendix 2 for calculations).

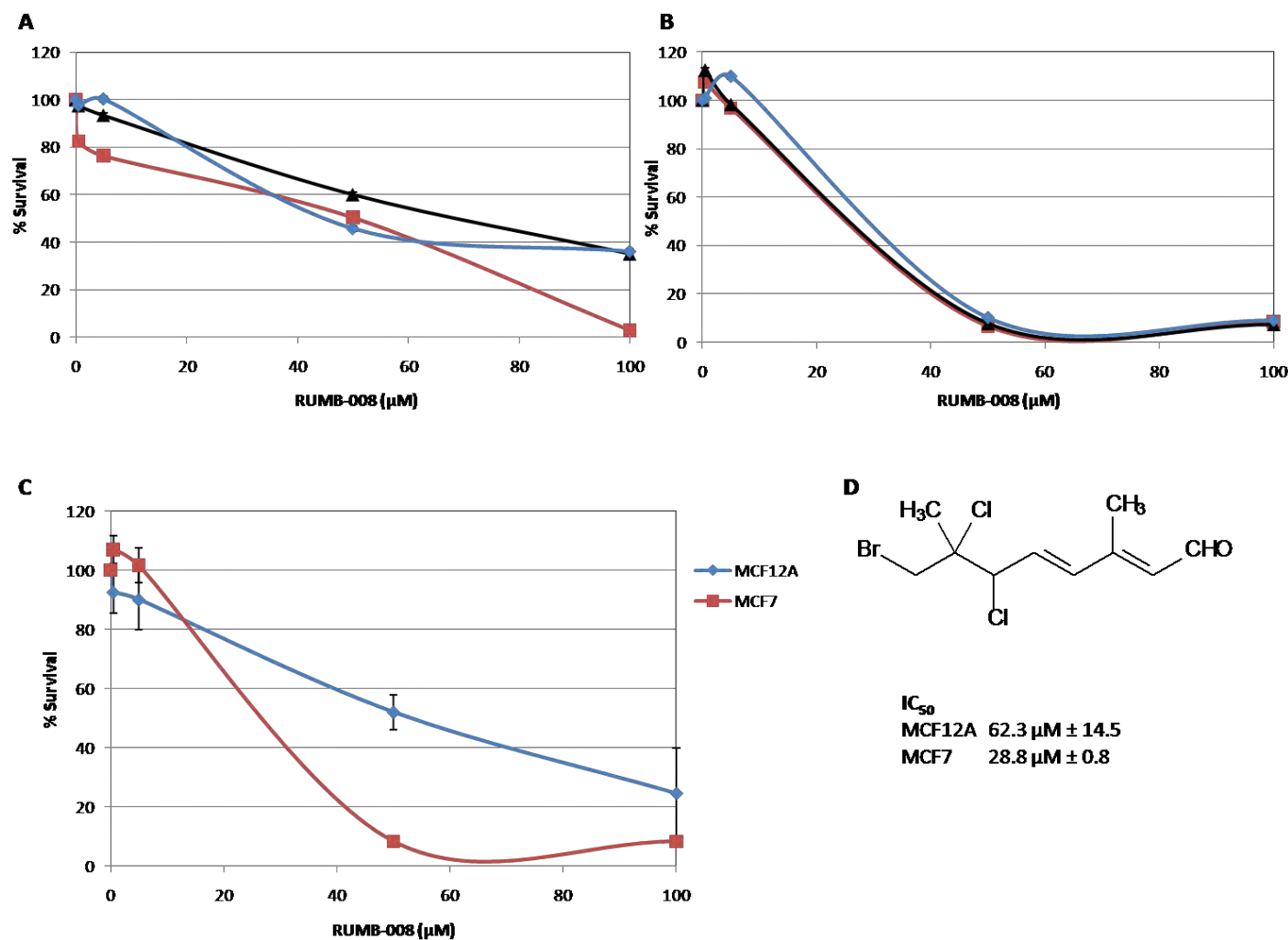


Figure 11: Determination of IC_{50} values of RUMB-008 in MCF12A and MCF7 cells.

A: MCF12A and B: MCF7 cells were treated with RUMB-008 ranging in concentration from 0.5 μM to 100 μM for 96 hours (triplicates shown as black, red and blue lines). C: Comparison of the average percentage cell survival of MCF12A (blue line) and MCF7 (red line) cells after treatment with RUMB-008 at concentrations ranging from 0.5 μM to 100 μM after 96 hours. Percentage survival was calculated relative to the DMSO control (100% survival). D: Structure of RUMB-008 and the IC_{50} value of this compound in MCF12A and MCF7 cells (see Appendix 2 for calculations).

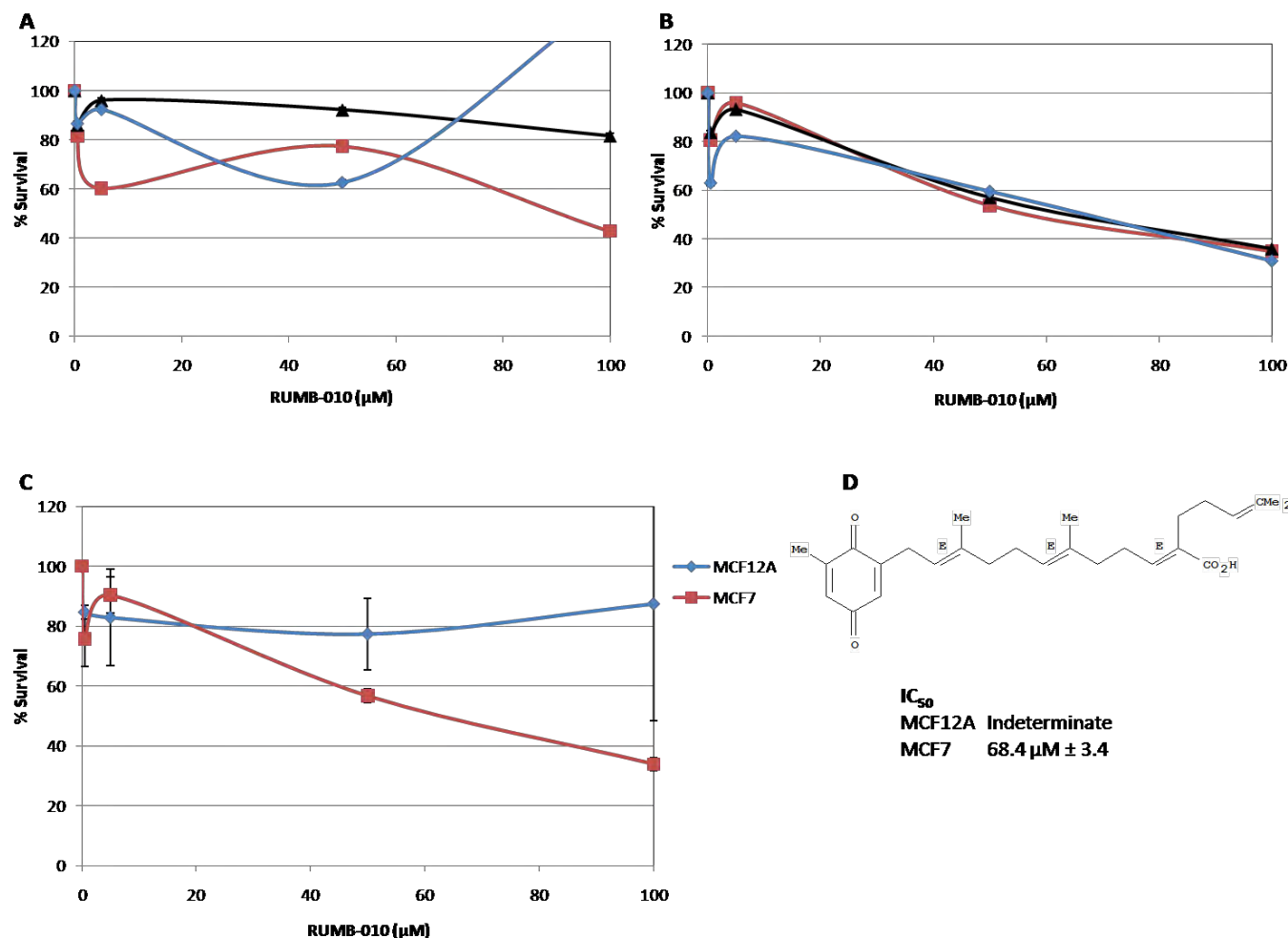


Figure 12: Determination of IC₅₀ values of RUMB-010 in MCF12A and MCF7 cells.

A: MCF12A and B: MCF7 cells were treated with RUMB-010 ranging in concentration from 0.5 μ M to 100 μ M for 96 hours (triplicates shown as black, red and blue lines). C: Comparison of the average percentage cell survival of MCF12A (blue line) and MCF7 (red line) cells after treatment with RUMB-010 at concentrations ranging from 0.5 μ M to 100 μ M after 96 hours. Percentage survival was calculated relative to the DMSO control (100% survival). D: Structure of RUMB-010 and the IC₅₀ value of this compound in MCF12A and MCF7 cells (see Appendix 2 for calculations).

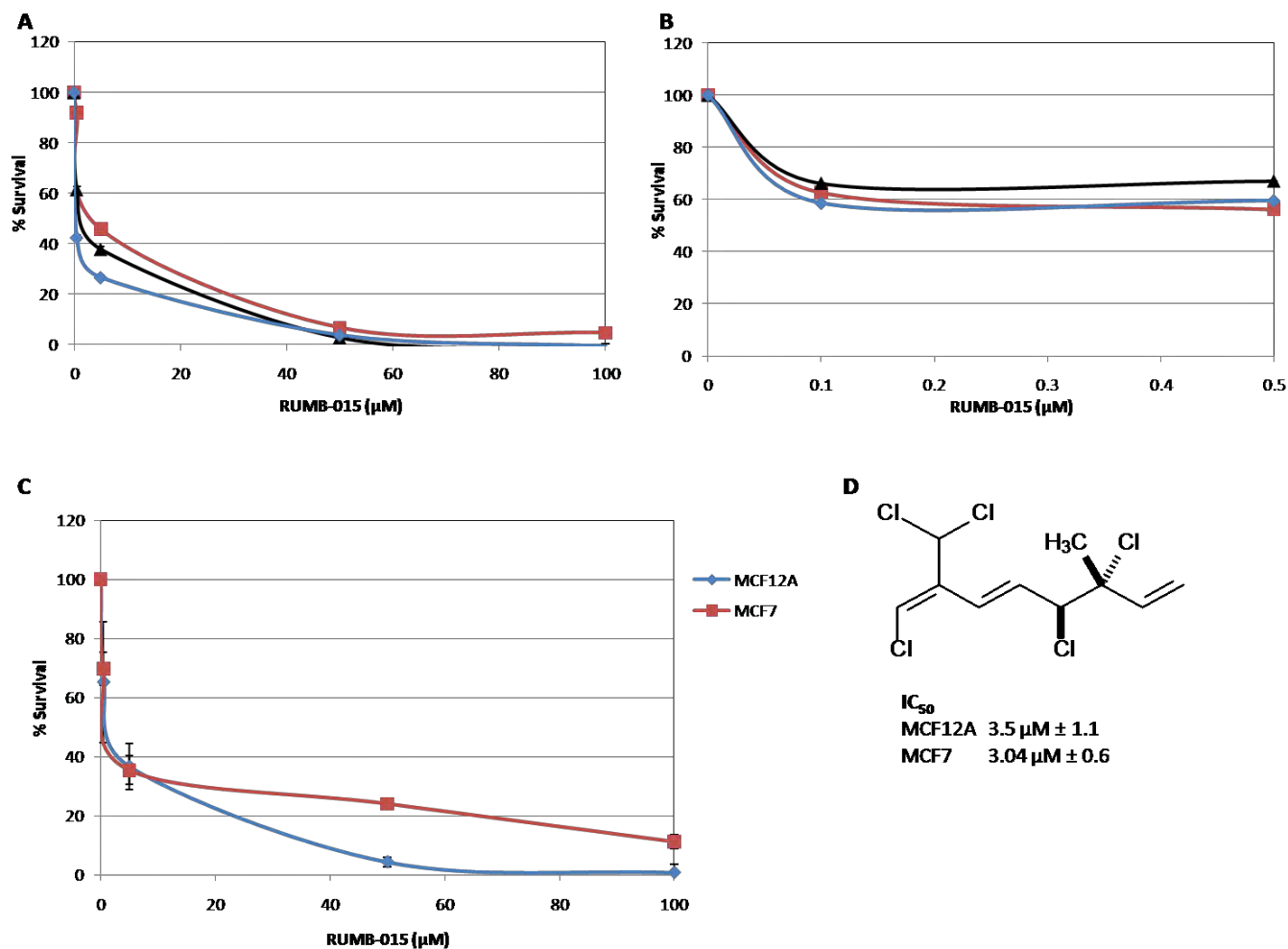


Figure 13: Determination of IC₅₀ values of RUMB-015 in MCF12A and MCF7 cells.

A: MCF12A cells were treated with RUMB-015 ranging in concentration from 0.5 μM to 100 μM for 96 hours and B: MCF7 cells treated with RUMB-015 ranging in concentration from 0.1 μM to 5 μM for 96 hours (triplicates shown as black, red and blue lines). C: Comparison of the average percentage cell survival of MCF12A (blue line) and MCF7 (red line) cells after treatment with RUMB-015 at concentrations ranging from 0.5 μM to 100 μM after 96 hours. Percentage survival was calculated relative to the DMSO control (100% survival). D: Structure of RUMB-015 and the IC₅₀ value of this compound in MCF12A and MCF7 cells (see Appendix 2 for calculations).

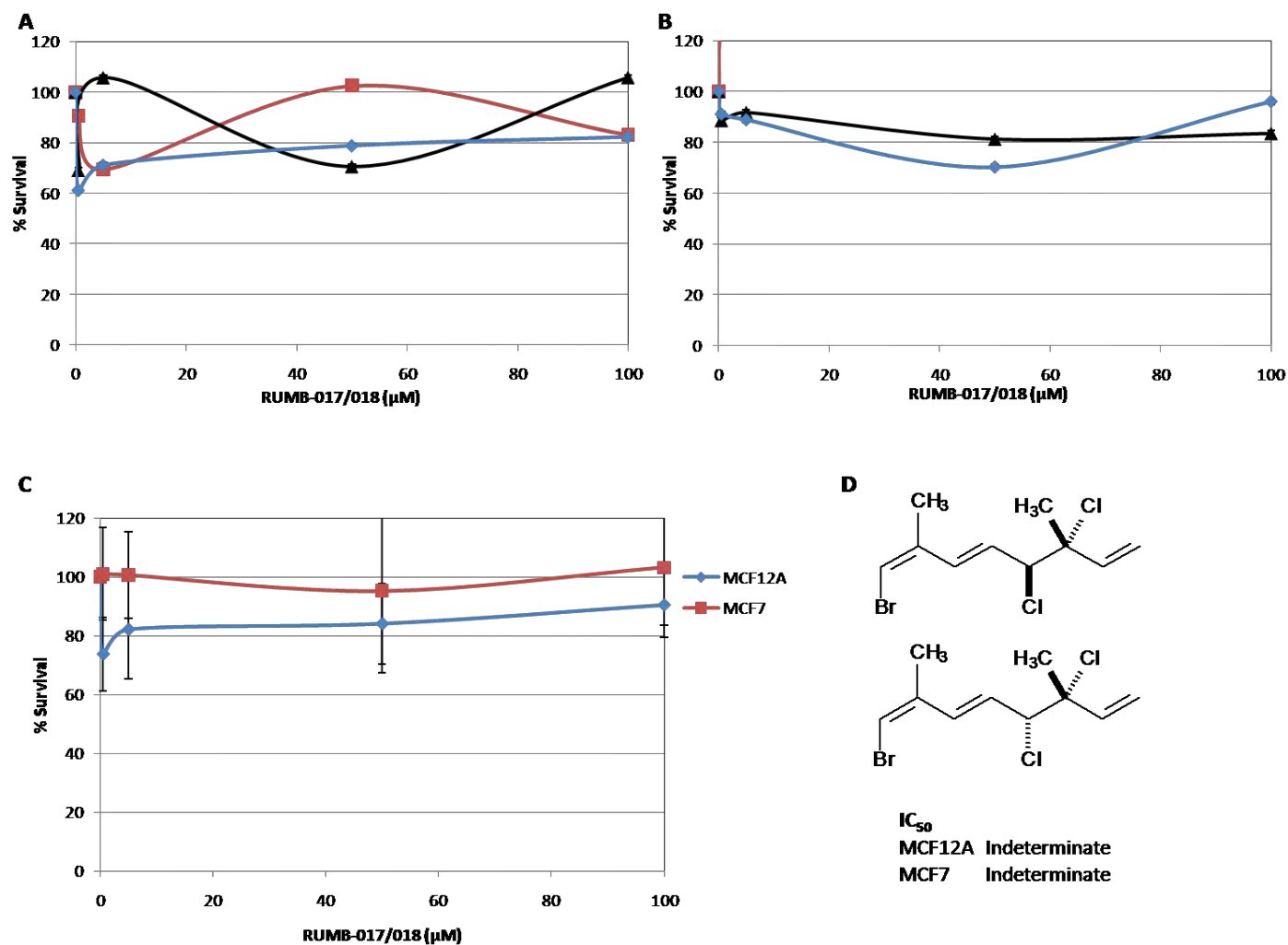


Figure 14: Determination of IC_{50} values of RUMB-017/018 in MCF12A and MCF7 cells.

A: MCF12A and B: MCF7 cells were treated with RUMB-017/018 ranging in concentration from 0.5 μM to 100 μM for 96 hours (triplicates shown as black, red and blue lines). C: Comparison of the average percentage cell survival of MCF12A (blue line) and MCF7 (red line) cells after treatment with RUMB-017/018 at concentrations ranging from 0.5 μM to 100 μM after 96 hours. Percentage survival was calculated relative to the DMSO control (100% survival). D: Structure of RUMB-017/018 and the IC_{50} value of this compound in MCF12A and MCF7 cells (see Appendix 2 for calculations).

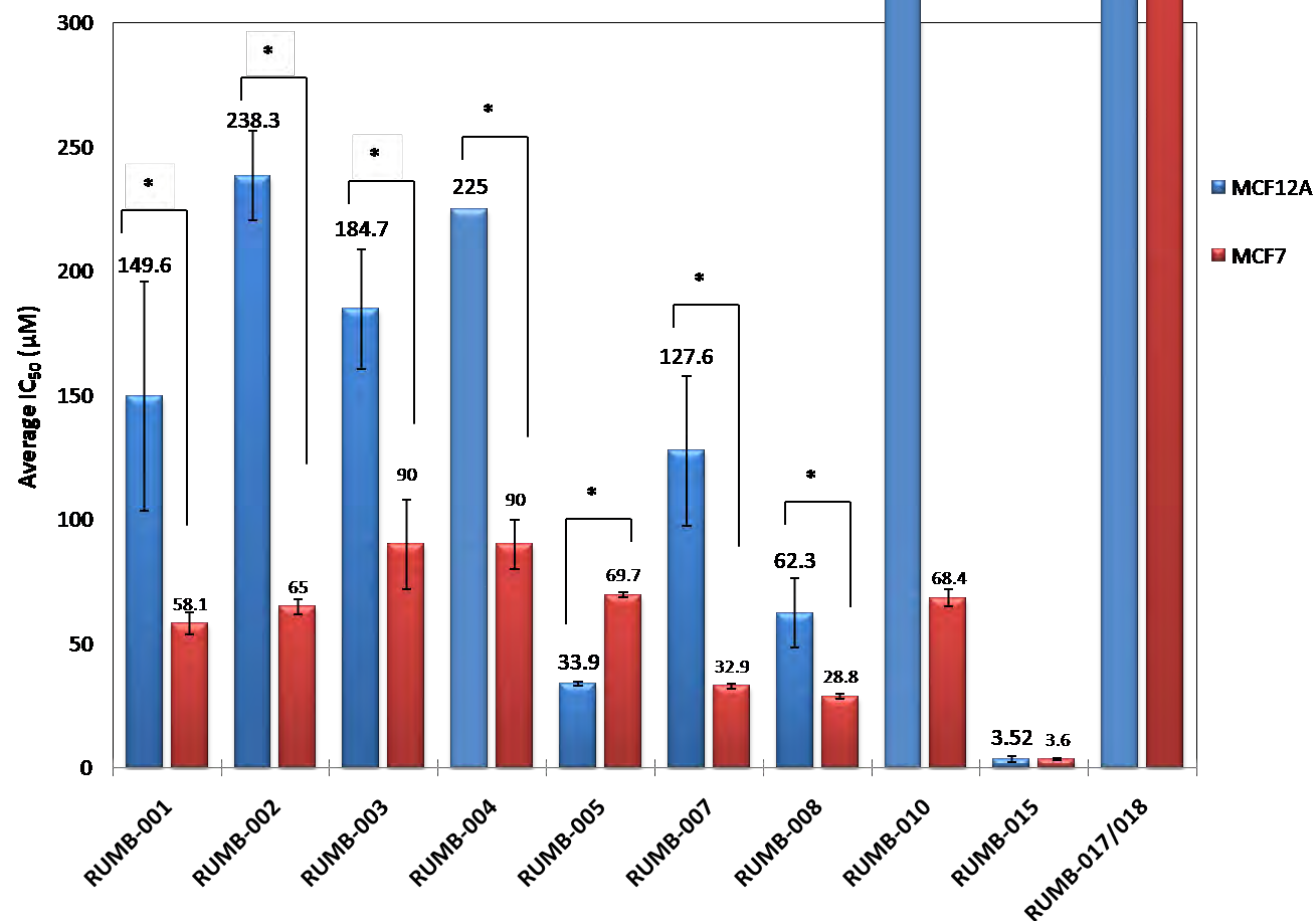


Figure 15: A comparison of the average cytotoxicity of the marine algal compounds in MCF12A (blue) and MCF7 (red) cells.

RUMB-001, RUMB-002, RUMB-007 and RUMB-010 were selected for further studies due to their higher cytotoxicity in MCF7 cells relative to the MCF12A cells. As a result of their respective high and low toxicity in both MCF12A and MCF7 cells RUMB-015 and RUMB-017/018 were also included in subsequent studies. The average IC₅₀ values of RUMB-010 in the MCF12A cell line and RUMB-017/018 in the MCF12A and MCF7 cell lines were indeterminate and are represented as being greater than 300 µM. Each IC₅₀ was calculated (Appendix 2) using the three cytotoxicity curves, each of which represented the average of quadruplicate samples (* $p < 0.05$).

3.3 Comparison of toxicity in normal and cancer cell lines

A comparison of the average IC_{50} values of all the marine small molecules tested on MCF12A and MCF7 cells using the MTT assay is shown in Figure 15. The average IC_{50} of each compound in MCF12A cells is shown in blue and the average IC_{50} of the compounds in MCF7 cells is shown in red and the standard deviations for each value are also shown. Student's *t*-test was conducted for all compounds on both cell lines and a statistically significant dose-dependent decrease in cell survival was observed in MCF12A cells for all compounds except RUMB-001, RUMB-004, RUMB-010 and RUMB-017/018 ($p < 0.05$ for untreated cells as compared with cells treated with 100 μ M of the compound). In the MCF7 cell line statistically significant dose-dependent decreases in cell survival were observed in all compounds except RUMB-017/018 ($p < 0.05$ for untreated cells compared with cells treated with 100 μ M of the compound). Compounds with differential toxicities were identified. For example compounds more toxic to the MCF7 cancer cells than the MCF12A cells (RUMB-001, RUMB-002, RUMB-003, RUMB-004, RUMB-007 and RUMB-010), a compound more toxic to the normal MCF12A cells than the MCF7 cells (RUMB-005), compounds toxic to both the normal and the cancer cells (RUMB-008 and RUMB-015) and a compound non-toxic to both the MCF12A and MCF7 cells (RUMB-017/018). The differences in the IC_{50} values of the compounds in MCF12A cells and MCF7 cells were found to be significant for RUMB-001, RUMB-002, RUMB-003, RUMB-004, RUMB-005, RUMB-007 and RUMB-008 ($p < 0.05$). There was no significant difference in the IC_{50} values in MCF12A and MCF7 cells for RUMB-015 and RUMB-017/018 and the statistical significance of the difference in IC_{50} values in MCF12A and MCF7 cells for RUMB-010 could not be calculated as it was not possible to calculate the IC_{50} of this compound in MCF12A cells. The cytotoxicity assays in the MCF12A cell line were carried out in two consecutive stages and so the passage numbers of the cells used in this analysis differed by only one passage. The cellular alterations that occur during the continuous and extensive sub-culturing of cells, including changes in gene expression, cellular growth rates and morphological changes (Chang-Liu and Woloschak, 1997; Esquenet et al., 1997; Wenger et al., 2004), would therefore not have contributed to the variation observed in the results obtained in the cytotoxicity analyses carried out in the MCF12A cell line. The MCF12A cell line was not an ideal „normal“ control as the *in vitro* growth of these cells required stimulation by a number of agents including cholera toxin, epidermal growth factor, hydrocortisone and insulin. Therefore it was not possible to determine whether the observed effects of the compounds in this cell line were a direct result of the compounds on the cells

themselves or as a result of the compound interfering or interacting with one of the stimulatory factors present in the culture medium. Another concern with using the MCF12A cell line is that it is an immortalised cell line and these cell lines are known to have altered gene expression profiles when compared with the primary cell lines from which they were derived (Olschlager et al., 2009; Sawada et al., 2005; You et al., 2004). Many immortalised cell lines show a loss of p53 expression which often results in the cells exhibiting a higher level of chemoresistance (Bunz et al., 1999; Dean et al., 2005; Donehower et al., 1992; Lowe et al., 1993). The MCF12A cell line used may therefore have exhibited a higher level of resistance to the compounds than would have been observed in a primary cell line, suggesting that the compounds would have greater cytotoxic activity in both a primary cell line and in normal breast cells *in situ*. A better control cell line to use may be the breast epithelial cell line Hs578Bst. This cell line does not require stimulants for growth and is part of a paired cell line, having a tumorigenic partner cell line, Hs578T, derived from the same origin as the Hs578Bst cells (Hackett et al., 1977). Using these two cell lines in place of the MCF12A and MCF7 cells would allow the investigation of the effect of the compounds on a cancerous and normal cell line while eliminating any difference that may be observed due to different genetic backgrounds of the cells.

From these results six compounds (termed priority compounds) were selected for further studies (Table 2). For a compound to be selected as a priority compound the IC_{50} in MCF7 cells had to be at least three times lower than in MCF12A cells and this difference should ideally be statistically significant. The reason for this is that any concentration used at which the compound would have a cytotoxic effect on the MCF7 cancer cell line there should be a minimal cytotoxic effect on the MCF12A normal cell line. Having said this, however, any compound that exhibited either high cytotoxicity or no cytotoxicity to both the MCF12A and MCF7 cell lines could also be of interest in future studies and in the determination of the cause of cytotoxicity or lack thereof in the different cells. These 6 selected compounds, shown in Table 2, will hereafter be referred to as priority compounds. The IC_{50} values calculated for paclitaxel in both MCF12A and MCF7 cells are also shown in Table 2 and are 5.35 nM and 88.7 nM respectively. As mentioned previously, the IC_{50} of paclitaxel in MCF12A cells is unknown but in MCF7 cells has been reported as being 100 nM (Munster et al., 2002), close to the value of 88.7 nM calculated here. The results obtained from the cytotoxicity assays in the MCF12A and MCF7 cell lines show that paclitaxel has a greater cytotoxic effect on the MCF12A normal breast epithelial cells than on the

MCF7 cancer cells. Current chemotherapeutic agents are known to have adverse side effects on normal, healthy tissues (Carr et al., 2008) but a chemotherapy drug that exhibits a vast difference in cytotoxicity towards cancer and normal cells as paclitaxel does in MCF12A and MCF7 cells raises the question as to whether this drug is actually viable as an anti-cancer agent if it is causing so much damage to the normal breast tissue. As discussed previously, there are various limitations present when using the MCF12A cell line, including the numerous stimulants the cells require for growth and the fact that it is an immortalised cell line. The extent to which paclitaxel induces cell death in the MCF12A cells may therefore not be a true reflection of the effect of paclitaxel on normal breast cells *in situ* but rather a result of the nature of the MCF12A cells. The IC_{50} values calculated for paclitaxel in the MCF12A and MCF7 cells lines are in the nanomolar range, whereas those calculated for the algal compounds are all in the micromolar range. While four of the priority compounds were selected as a result of the higher cytotoxicity exhibited in the MCF7 cancer cells, these compounds are not cytotoxic to the same extent. Indeed, the majority of chemotherapy drugs have IC_{50} values in the nanomolar range (Sigurdsson et al., 1986; Tian et al., 2004; Yayoi et al., 2009). Priority compounds were selected based on their differential cytotoxicities in the MCF12A and MCF7 cell lines but in order to be considered as potential hit compounds the IC_{50} values in the cancer cells would have to be lowered, perhaps by alteration of the compounds by chemical modification (discussed in section 6.2).

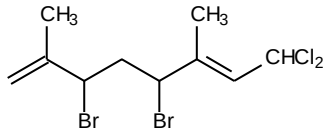
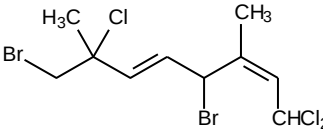
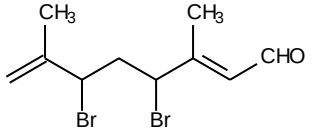
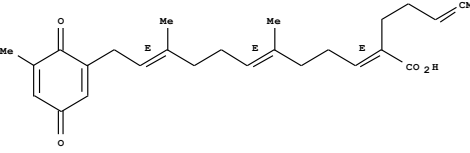
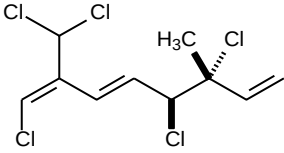
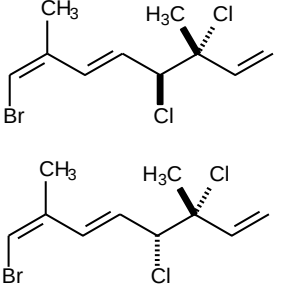
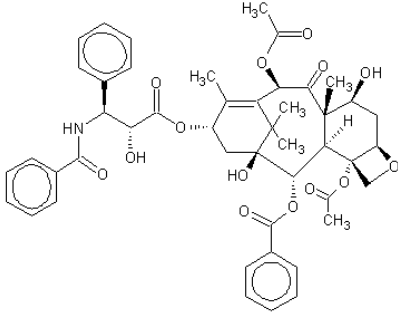
Four of the compounds tested, namely RUMB-001, RUMB-002, RUMB-007 and RUMB-010, showed three times higher cytotoxicity in the MCF7 cancer cells than in the MCF12A cells. The difference in the IC_{50} values of RUMB-001, RUMB-002 and RUMB-007 in MCF12A and MCF7 cells were shown to be statistically significant using Student's *t*-test ($p < 0.05$). No statistical test could be done on the difference in the IC_{50} values of RUMB-010 in MCF12A and MCF7 cells as the IC_{50} of this compound in MCF12A cells could not be calculated and is considered to be indeterminate. While the difference in the IC_{50} values of RUMB-010 in MCF12A and MCF7 cells could not be verified as statistically significant, RUMB-010 was chosen as a priority compound as there is a large difference in the IC_{50} in MCF12A cells ($IC_{50} > 300 \mu M$) and MCF7 cells ($IC_{50} = 68.4 \mu M$). RUMB-010 is also of interest as it has a different structure to the other compounds in this study. Of the remaining two compounds, RUMB-015 showed high cytotoxicity to both the MCF12A and the MCF7 cells while RUMB-017/018 displayed little to no cytotoxicity in both MCF12A and MCF7 cells. The similar effects of these compounds on the

different cell lines were confirmed by Student's *t*-test which showed that the differences in IC₅₀ between MCF12A and MCF7 cells were not statistically significant ($p>0.05$).

The IC₅₀ values of RUMB-003 and RUMB-008 in MCF12A cells are double that in MCF7 cells and so treatment of the cancer cells at the IC₅₀ concentrations may still elicit a cytotoxic response in the normal cells and as such RUMB-003 and RUMB-008 were not selected as priority compounds. RUMB-004, while having little cytotoxic effect in MCF12A cells still requires a relatively high concentration to achieve an effect in MCF7 cells and has therefore also not been chosen for further analyses. RUMB-005, interestingly, is the only compound tested that showed more cytotoxicity towards the normal MCF12A cells than the MCF7 cancer cells. This is not desirable in a cancer treatment, although it was observed in the treatments of MCF12A and MCF7 cells with paclitaxel, and so RUMB-005 was not used in subsequent experiments. This also suggests that the MCF12A „normal“ control cell line used was a sub-optimal control cell line, most likely due to the exogenous stimulation these cells required to grow in culture.

The majority of current chemotherapy drugs, including paclitaxel, doxorubicin, 17-AAG and geldanamycin are complex organic structures that contain multiple ring structures and nitrogen (Sigurdsson et al., 1986; Tian et al., 2004; Wang et al., 2006; Yayoi et al., 2009) but only one of the novel marine compounds tested here, namely RUMB-010, possess a ring structure and none of the compounds contain nitrogen. The concentration at which a drug is administered is of extreme importance because of the potential effects it could have on other tissues in the body as well as the practical issues involved with administering large amounts of a drug (Lipp and Bokemeyer, 1999). All the IC₅₀ values reported here for the marine compounds are in the micromolar range, whereas drugs currently used in chemotherapy have IC₅₀ values mostly in the nanomolar range, for example the IC₅₀ values of paclitaxel, doxorubicin, 17-AAG and geldanamycin in MCF7 cells are 100 nM, 8.44 nM, 72 nM and 25 nM respectively (Munster et al., 2002; Yang et al., 2001; Zheng et al., 2000). The IC₅₀ values found for the marine compounds tested are considerably higher than these values and while those with lower IC₅₀ values in MCF7 cells and higher IC₅₀ values in MCF12A cells were selected as priority compounds (i.e. „hit“ compounds), their cytotoxicity may still be too high for them to be considered lead compounds, though this may be made lower with rational chemical modification (discussed further later) and so may represent a starting point for chemical modification.

Table 2: Codes, names, structures and IC₅₀ values in MCF12A and MCF7 cells of selected marine algal priority compounds and paclitaxel

Code	Name	Structure	IC ₅₀ (μM) in MCF12A cells	IC ₅₀ (μM) in MCF7 cells
RUMB-001	Plocoralide A		>164.3 ± 69.3	58.1 ± 4.4
RUMB-002	Plocoralide B		>238.3 ± 18.2	65.1 ± 3.0
RUMB-007	Plocoral A		>127.6 ± 30.2	33.0 ± 1.2
RUMB-010	Sargaquinoic acid		Indeterminate	68.4 ± 3.4
RUMB-015	Plocornulide A		3.5 ± 1.1	3.0 ± 0.6
RUMB-017/018	Plocornulide C		Indeterminate	Indeterminate
Paclitaxel			5.35 ± 0.05 nM	88.7 ± 2.9 nM

The reason for the difference in cytotoxicity levels between the different novel marine molecules tested is unclear but may be due to the electronegativity of the compounds, one of the properties on which the effectiveness of current chemotherapy is based (Holshouser et al., 2002; Maini et al., 1997; Wu et al., 2000).

When looking at the selected priority compounds for example, RUMB-001 and RUMB-007 are almost identical, differing only in the substitution of the $-\text{CHCl}_2$ group in RUMB-001 for $-\text{CHO}$ in RUMB-007 and their IC_{50} values in MCF12A and MCF7 cell lines are similar. Also, RUMB-015 is highly chlorinated which may confer increased electronegativity to this compound and therefore higher cytotoxicity than, for example, RUMB-017/018, which is halogenated to a lesser extent. RUMB-015 and RUMB-017/018 also have very similar backbones but differ vastly in their cytotoxicities, raising the question as to whether this is due to the difference in electronegativity or some other difference. Successive molecular substitution of the chlorine and bromine groups on these compounds with proton or methyl groups, and subsequent testing of these derivatives for changes in cytotoxicity, may provide information on the structure activity relationship (SAR) of these compounds. It should also be considered whether the existence of RUMB-017/018 as an enantiomer has an effect on the cytotoxic activity of the compound and may explain the high IC_{50} of this compound in both the MCF12A and MCF7 cells. It cannot be determined from the tests carried out here whether both enantiomers are in fact inactive, whether one is more cytotoxic but is masked by the other or even whether any difference in their effect on the cells exists. These suggestions could be determined conclusively by rational chemical modification of selected compounds.

3.4 Conclusion

Ten algal compounds were tested for cytotoxicity in MCF12A „normal“ breast epithelial cells and MCF7 epithelial breast cancer cells from which compounds with differential cytotoxicity were identified. Seven compounds, namely RUMB-001, RUMB-002, RUMB-003, RUMB-004, RUMB-007, RUMB-008 and RUMB-010 exhibited lower cytotoxicity in MCF12A cells than in MCF7 cells with significant differences ($p < 0.05$) in IC_{50} values of these compounds in each cell line except for RUMB-010 for which an IC_{50} in MCF12A cells could not be determined. RUMB-005 had higher cytotoxicity in MCF12A cells when compared with MCF7 cells and the

difference in the IC_{50} values of this compound in the different cell lines was also calculated to be significant ($p < 0.05$). RUMB-015 and RUMB-017/018 exhibited high and low cytotoxicity respectively in both cell lines. The difference in the IC_{50} values of RUMB-015 was not significant ($p > 0.05$) but no significance could be calculated for the effect of RUMB-017/018 on the different cell lines as the IC_{50} value of RUMB-017/018 in both these cell lines was indeterminate. From the ten compounds tested six were selected as „priority“ compounds based on their very different or similar cytotoxicities in the „normal“ MCF12A cells and the cancerous MCF7 cells. In the case of RUMB-001, RUMB-002, RUMB-007 and RUMB-010 their selection as priority compounds was based on their IC_{50} value being at least three times lower in the MCF7 cells than in the MCF12A cells. The high cytotoxicity of RUMB-015 in both MCF12A and MCF7 cells and the apparent lack of cytotoxicity of RUMB-017/018 in MCF12A and MCF7 cells resulted in these two compounds also being selected as priority compounds. There is a lack of obvious SAR of these compounds and as such there is a need to study the cytotoxicities of compounds that are systematic structural derivatives of each priority compound. The priority compounds were then used for further cytotoxic and preliminary mechanistic studies.

4 DETERMINATION OF THE CYTOTOXICITY OF THE PRIORITY COMPOUNDS ON THE SIDE POPULATION (SP) IN THE MCF7 BREAST CANCER CELL LINE

4.1 Introduction

The side population (SP) are small populations of cells that are enriched in undifferentiated cells and are also believed to be enriched for stem cells (Decraene et al., 2005; Hadnagy et al., 2006; Larderet et al., 2006). They have been identified in human normal tissues, including breast tissue (Alvi et al., 2003; Behbod et al., 2006; Dontu et al., 2003), as well as in tumours and cancer cell lines (Grichnik et al., 2006; Hirschmann-Jax et al., 2004; Kondo et al., 2004). These SP cells are known to express high levels of ABC transporters, including multi-drug resistance transporter ABCG2 (Zhou et al., 2001), a property that has been successfully exploited when studying the cancer stem cell containing-side population where Hoechst 33342 is driven out of the side population cells by the ABC transporters but retained in the non-side population cells (Challen and Little, 2006; Goodell et al., 1996). While the SP, which is thought to comprise between 0.1% and 2% of total tumour mass (Chuthapisith et al., 2009) has been shown to be heterogeneous, the characterisation of cells within the SP has shown that they are undifferentiated and tumorigenic with the most poorly differentiated cells being the most tumorigenic (Decraene et al., 2005; Hadnagy et al., 2006), suggesting the SP is the population in which the proposed CSC reside. The presence of the multi-drug resistant transporter on the surface of the cancer stem cell-containing SP cells is believed to contribute to the observed chemoresistance of these cells (Hirschmann-Jax et al., 2004; Li et al., 2008). There is much debate over the existence and importance of CSC, particularly since there is currently no accepted marker profile for identifying breast CSC, although many groups suggest that this population is defined by the CD44⁺CD24⁻ phenotype (Dontu, 2008). Many of the current chemotherapy drugs target the rapidly dividing cells of the bulk of the tumour, leaving the CSC behind in an apparent enrichment for these cells, which are then able to repopulate the tumour which may lead to relapse (Dean et al., 2005). Theoretically, targeting the CSC would remove the source of and the cells responsible for the maintenance of the tumour, after which the bulk of the tumour could disintegrate or be removed using chemotherapy (Figure 16). Various techniques exist that allow for the investigation of toxicity of drugs and compounds to CSC, both in isolation and *in situ*. The MTT assay is commonly used to determine the cytotoxicity of compounds in different cell lines (Berridge and Tan, 1993) and should SP be successfully isolated and propagated *in vitro*, while maintaining their stem cell characteristics, it may be possible to test the toxic effects of novel compounds and potential drugs on CSC-containing SP using this technique. It is also possible to perform toxicity studies on CSC *in situ*. As already discussed SP the exclusion of Hoechst 33342 dye from SP cells has already

been used successfully in identification and isolation of the SP in various cell lines (Challen and Little, 2006; Decraene et al., 2005; Goodell et al., 1996; Larderet et al., 2006) and so the effect of novel compounds and potential drugs on the SP can be determined by treating the entire cell population and then investigating the changes in the proportion of Hoechst-33342 excluding or ABCG2⁺ cells by flow cytometry (Figure 16B). These techniques and others will have to be employed in the search to find compounds that are able to effectively target and remove CSC from the tumour environment. The CSC theory of cancer development proposes that CSC cause cancer and as a result of their inherent chemoresistance may play a large role in relapse after treatment. The only way to completely remove a tumour is to remove the sustaining cell population. The aim of this study was to investigate the cytotoxic effect of the priority compounds on the ABCG2⁺ cancer stem cell-containing side population in the MCF7 breast cancer cell line.

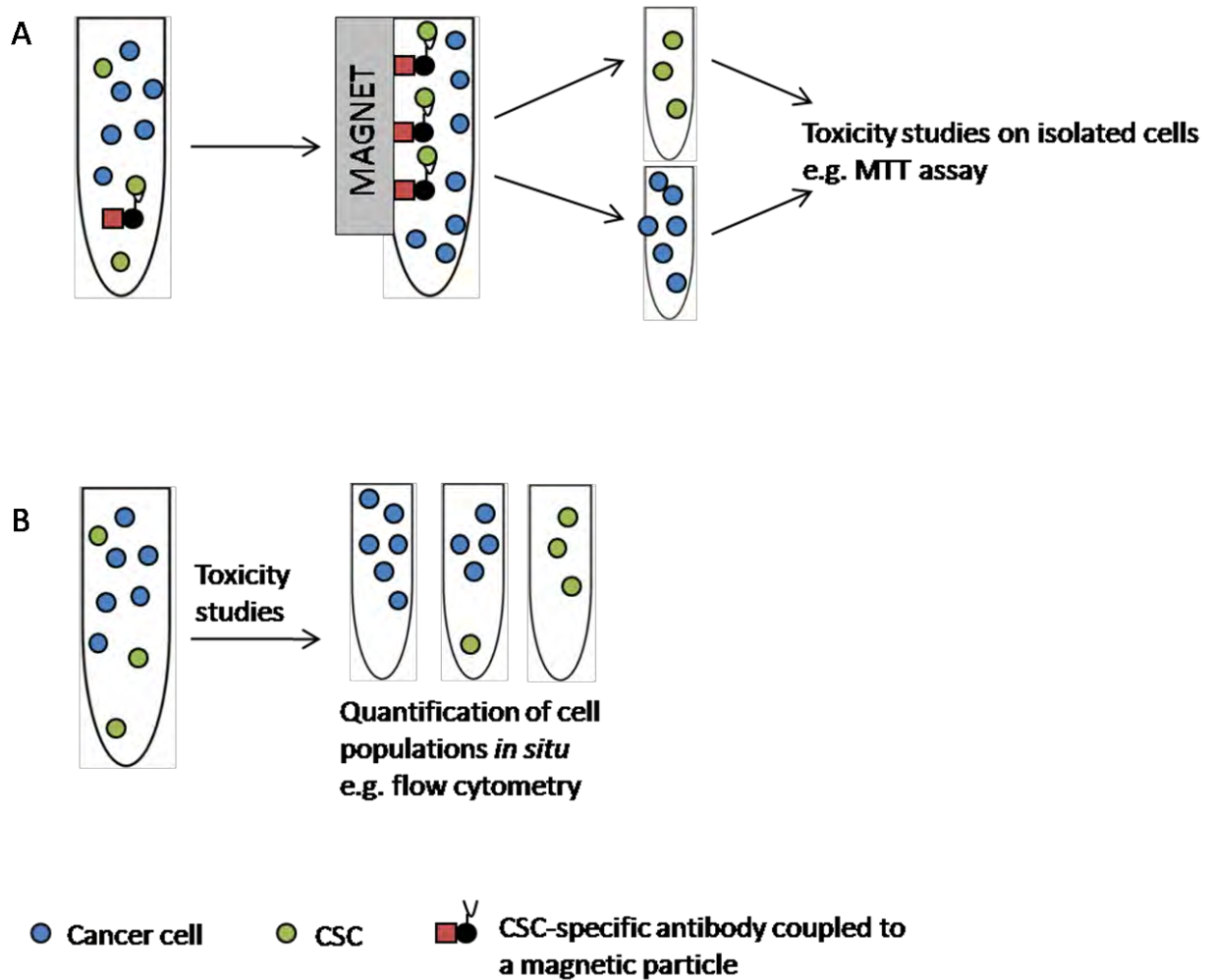


Figure 16: Different approaches available for the determination of toxicity to CSC

Schematic representation of CSC isolation for toxicity studies using magnetic cell separation (A) versus *in situ* toxicity studies (B). Isolation of the SP by magnetic cell separation using a magnetically labelled SP-specific antibody (A) allows for toxicity studies to be carried out on the different subpopulations within a heterogeneous tumour or cell line. Toxicity studies can also be carried out *in situ* by analyzing the different cell populations present in a heterogeneous tumour sample or cell line after treatment (B) using, for example, flow cytometry.

4.2 Results and Discussion

4.2.1 Identification of the Side Population in MCF7 cell line using Hoechst staining

The CSC containing-side population cells express ABC transporter proteins, especially ABCG2, at high levels. As a result of the high expression levels of these transporters, the fluorescent dye Hoechst 33342 is actively excluded from these “side population (SP)” cells while the “non-side population (NSP)” cells retain the dye (Chuthapisith et al., 2009; Goodell et al., 1996). Therefore it is possible to distinguish between the different populations using flow cytometry. In order to confirm the presence of the SP in MCF7 cells one sample of MCF7 cells was incubated with 50µM reserpine [known to inhibit the function of ABCG2 transporter (Shukla et al., 2005)] followed by Hoechst 33342 staining, while another sample of MCF7 cells was incubated only with Hoechst 33342. Both cell sets were analysed by flow cytometry using a FACSVantage SE flow cytometer (BD Biosciences). The Hoechst 33342 was excited at a wavelength of 350 nm and the fluorescence measured at 450 nm (Hoechst Blue) and 675 nm (Hoechst Red). Reserpine is an ABCG2 inhibitor which removes the ability of ABCG2 to exclude Hoechst 33342 thereby causing the cells to retain Hoechst 33342. This was done to confirm that the SP could be identified using the Hoechst 33342-exclusion method and a known inhibitor of ABCG2. This was necessary before attempting to investigate the effect of the selected algal compounds on the SP in MCF7 cells so that when analysing the cells after treatment with the selected algal compounds the SP and any changes in the SP could be accurately identified.

Figure 17A shows the dot plot of MCF7 cells stained with Hoechst 33342 only and Figure 17B shows the dot plot of the MCF7 cells treated with reserpine and stained with Hoechst 33342. Cells that exclude Hoechst 33342 would be expected to appear in the bottom left of the dot plot, an area representing „low“ Hoechst 33342 staining. In a dot plot SP cells appear as a tail (Goodell et al., 1996). This is observed in the box enclosing the SP cells (3037 cells) in Figure 17A. If the SP was correctly identified in Figure 17A, treatment of the MCF7 cells with reserpine would result in an apparent decrease in the SP cells observed. The number of SP cells counted in the MCF7 cells treated with reserpine and Hoechst 33342 was 947 cells, a decrease from the 3037 cells counted in the MCF7 cells stained with Hoechst 33342 alone. This suggests that the SP in MCF7 cells was correctly identified using this technique.

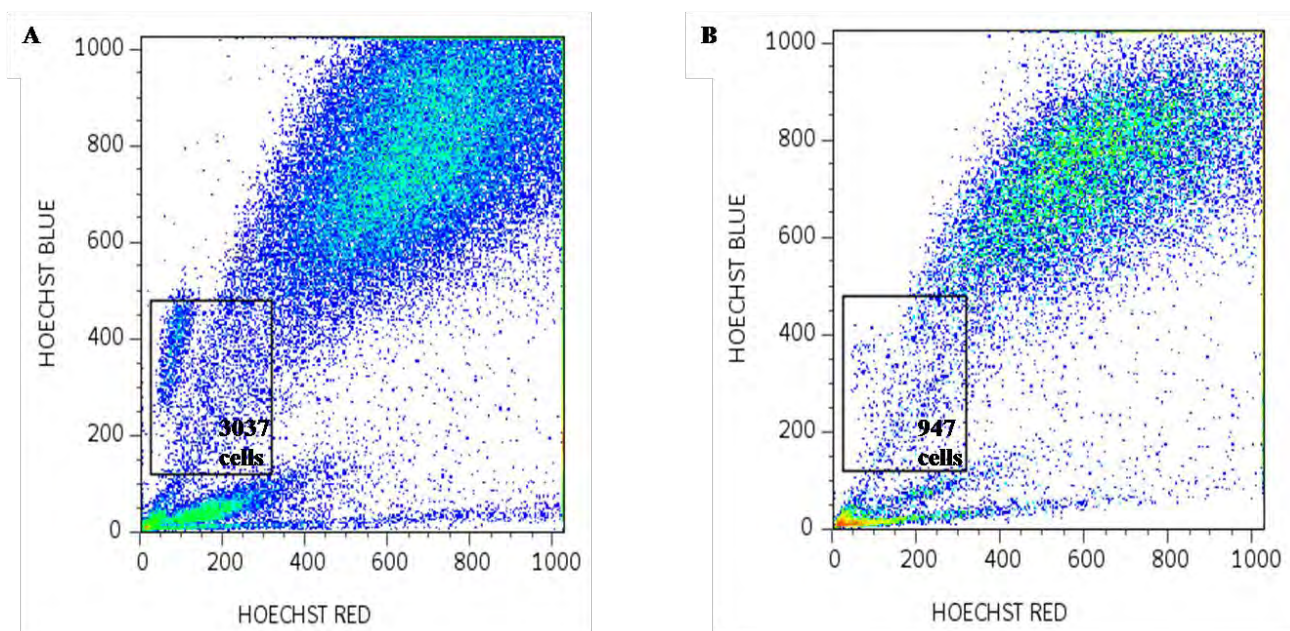


Figure 17: Confirmation of the presence of the side population in MCF7 cells using flow cytometry

Flow cytometry was carried out with a FACSVantage SE flow cytometer. The Hoechst 33342 was excited at 350 nm and the fluorescence measured at 450 nm (Hoechst Blue) and 675 nm (Hoechst Red). A: Untreated MCF7 cells stained with $5 \mu\text{g}.\text{ml}^{-1}$ Hoechst 33342. Cells expressing ABCG2 will be negative for Hoechst 33342 and appear in the bottom left quadrant of the dot plot and are represented here in the rectangular gate. There are 3037 ABCG2⁺ cells present in this gate. B: MCF7 cells treated with 50 μM reserpine and stained with $5 \mu\text{g}.\text{ml}^{-1}$ Hoechst 33342.

At the time of conducting this experiment the cytotoxicity analyses of all the compounds on the MCF12A cell line had not been completed. The compounds chosen for this study, listed below, were selected as preliminary priority compounds based on their cytotoxic effect in MCF7 cells alone and therefore, this analysis was not conducted for all the priority compounds. MCF7 cells were treated with DMSO, paclitaxel, RUMB-007, RUMB-008, RUMB-010 and RUMB-015 for 96 hours before being incubated with $5 \mu\text{g}.\text{ml}^{-1}$ Hoechst 33342 for 2 hours at 37°C. The cells were harvested by trypsinisation, washed with ice-cold PBS buffer (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄.2H₂O, 1.47 mM KH₂PO₄, pH 7.4) and analysed by flow cytometry using a FACSVantage SE flow cytometer. The Hoechst 33342 was excited at 350 nm and its fluorescence measured at 450 nm (Hoechst Blue) and 675 nm (Hoechst Red) and the same gate used to define the SP in the untreated MCF7 cells (Figure 17) was used to calculate the proportion of the SP in the MCF7 cells treated with DMSO, paclitaxel and selected algal compounds. The results are shown in Figure 18.

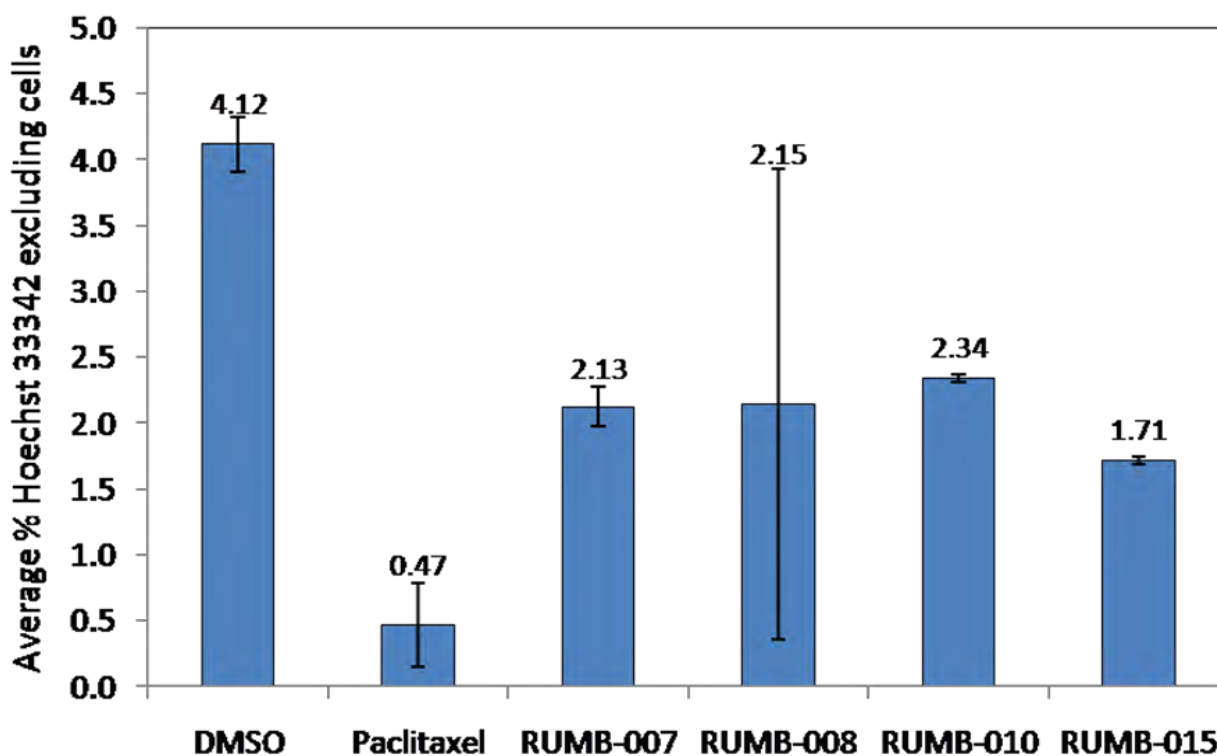


Figure 18: Comparison of the average percentage of the Hoechst 33342-excluding cells in MCF7 cells treated with selected marine algal compounds.

The average percentage of the Hoechst 33342-excluding cells present in the rectangular gate in MCF7 cells treated with 0.25% DMSO, 100 nM paclitaxel, 33 μ M RUMB-007, 29 μ M RUMB-008, 68 μ M RUMB-010 and 3 μ M RUMB-015 for 96 hours and stained with 5 μ g.ml⁻¹ Hoechst 33342 and analysed by flow cytometry. The data presented here are representative of replicate samples.

The percentage of SP cells in the MCF7 cells treated with DMSO was 4.12%, higher than the 0.1% to 2% that is generally accepted as being the proportion of cancer comprised of SP cells (Chuthapisith et al., 2009) but in the expected range for SP cells present in MCF7 cells which has been reported to be between 0.2% and 7.5% (Cariati et al., 2008; Engelmann et al., 2008; Patrawala et al., 2005). It was expected that treatment of MCF7 cells with paclitaxel would result in an apparent enrichment in the SP cells due to the inherent drug resistant nature of this population. However, there was a decrease in the percentage of these cells (0.47%) when compared with the DMSO sample. After treatment with 33 μ M RUMB-007, 29 μ M RUMB-008, 68 μ M RUMB-010 and 3 μ M RUMB-015 for 96 hours, the percentages of Hoechst 33342-excluding cells were 2.13%, 2.15%, 2.34% and 1.71% respectively. These values are lower than that of the DMSO-treated cells suggesting that these compounds may have had some cytotoxic effect on the SP; however this experiment would have to be repeated in order to verify that these compounds do, in fact, have a cytotoxic effect on the SP. It must be kept in mind that the media

used was not changed during the 96-hour treatment of the MCF7 cells with DMSO, paclitaxel and algal compounds. The media depletion that would have occurred during this time may have resulted in more stress upon the cells and therefore increased cell death. The observed cytotoxic effects, or lack thereof, of the compounds may be an overestimation as some of the cell death in both the SP and NSP may be a result of nutrient deficiencies.

Due to its reliance on a biological process and its nature as a biological assay, the Hoechst 33342 assay to investigate side populations is a very sensitive assay and may be affected by any changes in assay conditions and is especially sensitive to temperature (Goodell et al., 1996). Because Hoechst 33342 exclusion is an active and sensitive process falsely positive and negative results may occur. In addition, different numbers of cells were counted for each sample, making comparisons between the samples difficult. The validity of any conclusions drawn from these data is questionable because different cell numbers were counted for each sample, including the duplicate samples of cells treated with the same compound. The results for the different compounds cannot therefore be compared accurately and with confidence. Due to time constraints and equipment limitations this experiment was performed only once and therefore the reproducibility and robustness of this assay when used to determine the effect of the algal compounds on the SP is unknown. Validation of the results from this experiment would require repetition of the assay. The decrease in SP cells after treatment with paclitaxel to 0.47% when compared with the 4.12% in the DMSO-treated cells instead of the expected increase or enrichment for these cells (Li et al., 2008; Marotta and Polyak, 2009; Wicha et al., 2006) brings the accuracy, or the extent to which the results agree with known values, of this assay into question. As a result of the shortcomings encountered in the Hoechst 33342 assay for the side population in MCF7 cells, namely the highly sensitive nature of the assay, the difference in the numbers of cells counted and the fact that the priority compounds had not been selected at the time of doing this experiment, another assay in which the number of cells expressing the ABCG2 transporter was investigated directly using an antibody against ABCG2. This assay was developed by our group and to our knowledge this is the first time this type of assay has been used to investigate the effect of novel compounds on CSC. Using an antibody against ABCG2, the transporter responsible for the efflux of the Hoechst 33342 dye, is specific for SP cells but unlike Hoechst 33342 this assay is not based on a biological procedure and so is less sensitive than the Hoechst 33342-exclusion assay.

4.2.2 Analysis of the percentage of ABCG2⁺ cells present in MCF7 cells after treatment with the priority compounds using flow cytometry

MCF7 cells (1×10^6) cells were treated with DMSO, paclitaxel and RUMB-001, RUMB-002, RUMB-007, RUMB-010, RUMB-015 and RUMB-017/018 for 96 hours at 37°C at the concentrations indicated in the figure legend (Figure 19). The cells were harvested and incubated with 1 µg biotinylated anti-human CD338 (ABCG2) (BioLegend), following which they were stained with 2 µg streptavidin-FITC (Sigma) and analysed by flow cytometry using the Beckman Coulter FC 500 flow cytometer. The FITC was excited at a wavelength of 488 nm and its fluorescence emission measured at 530 nm. Single-colour histograms (Figure 19) were generated from the flow cytometry data and the gates used to define the ABCG2⁺ and ABCG2⁻ populations in the DMSO sample were used to determine the percentage of the ABCG2⁻ NSP and ABCG2⁺ SP populations in the samples treated with paclitaxel and selected indigenous compounds. The percentage of ABCG2⁺ cells present in each sample is shown in Figure 19.

It has been reported that the SP of cancers make up between 0.1% and 2% of the total tumour mass (Chuthapisith et al., 2009; Fillmore and Kuperwasser, 2008) but other groups have found that the SP comprises between 0.2% and 7.5% of MCF7 cells (Engelmann et al., 2008; Patrawala et al., 2005). The DMSO treated sample showed an ABCG2⁺ population of 2.47% which is in the range reported for MCF7 cells (Engelmann et al., 2008; Patrawala et al., 2005). Treatment of the MCF7 cells with paclitaxel was expected to result in an apparent enrichment for ABCG2⁺ cells, as these cells express the ABCG2 drug transporter and are reported to be resistant to chemotherapy drugs. The proportion of side population cells should increase relative to the rest of the tumour cells after treatment with a drug such as paclitaxel that has a high cytotoxicity in the bulk tumour cells but lower cytotoxicity to the CSC population (Dean et al., 2005; Hirschmann-Jax et al., 2004; Zhou et al., 2001).

The results of the cytotoxicity of the novel compounds against the SP are shown in Figure 19. The percentage of SP cells present in the MCF7 cells treated with paclitaxel was 4.8%, indicating an apparent enrichment in the SP cells as compared with the SP of 2.47% in the DMSO-treated cells. This is in accordance with reports in the literature (Li et al., 2008). The percentage of SP cells present in the MCF7 cells treated with RUMB-001, RUMB-002, RUMB-007 and RUMB-

010 were 2.99%, 2.04%, 2.01% and 1.33% respectively. The percentage of SP cells in all of these samples were in the same range as the MCF7 cells treated with DMSO (2.47%), implying that these compounds have little effect on the cancer stem cell-containing side population in MCF7 cells even though they showed cytotoxicity towards the bulk of the MCF7 cells (Chapter 3, Figure 15). After treatment with RUMB-010 and RUMB-017/018, the ABCG2⁺ populations present in the MCF7 cell samples made up 0.67% and 0.9% of the total cell population respectively, indicating a drop in the percentage of SP cells relative to the untreated DMSO control (2.47%). This suggests that these compounds were able to preferentially target and reduce the SP. It would appear therefore, that RUMB-010 has a cytotoxic effect on both the bulk MCF7 cancer cells and the cancer stem cell-containing SP cells but not on the MCF12A normal breast epithelial cells. Interestingly, RUMB-017/018 had very little effect on both the MCF12A normal breast epithelial cells and the whole MCF7 breast cancer cells but did appear to have a cytotoxic effect on the SP.

This assay was developed in our group during this project and therefore due to time constraints and equipment availability this experiment was performed only once. Therefore, it will be essential to repeat this experiment to validate the results. The results from the DMSO and paclitaxel samples do suggest this assay was successful, because they are in accordance with published values. The reproducibility of the assay, which is the degree to which the experiment gives the same results when repeated under the same conditions, however, is as yet untested. For a measurement to be valid it must be both accurate, meaning the measurement but be close to the actual value, and reproducible. The results obtained are accurate when compared with values reported in literature (Chuthapisith et al., 2009; Fillmore and Kuperwasser, 2008) but the results cannot be pronounced valid until the assay has been repeated. The design of the experiment may also be improved by changing the concentrations of priority compounds at which the MCF7 cells were tested. Analysis of the effect of the priority compounds on the SP at a range of different concentrations, from below the IC₅₀ o above the IC₅₀ value, may provide more conclusive information regarding the effect of the compounds on the SP and the minimum concentration required for cytotoxicity to the SP. Another assay that could be included is a kinetic analysis in which the effects of different concentrations of the compounds on the MCF7 SP cells are investigated over a pre-determined time course.

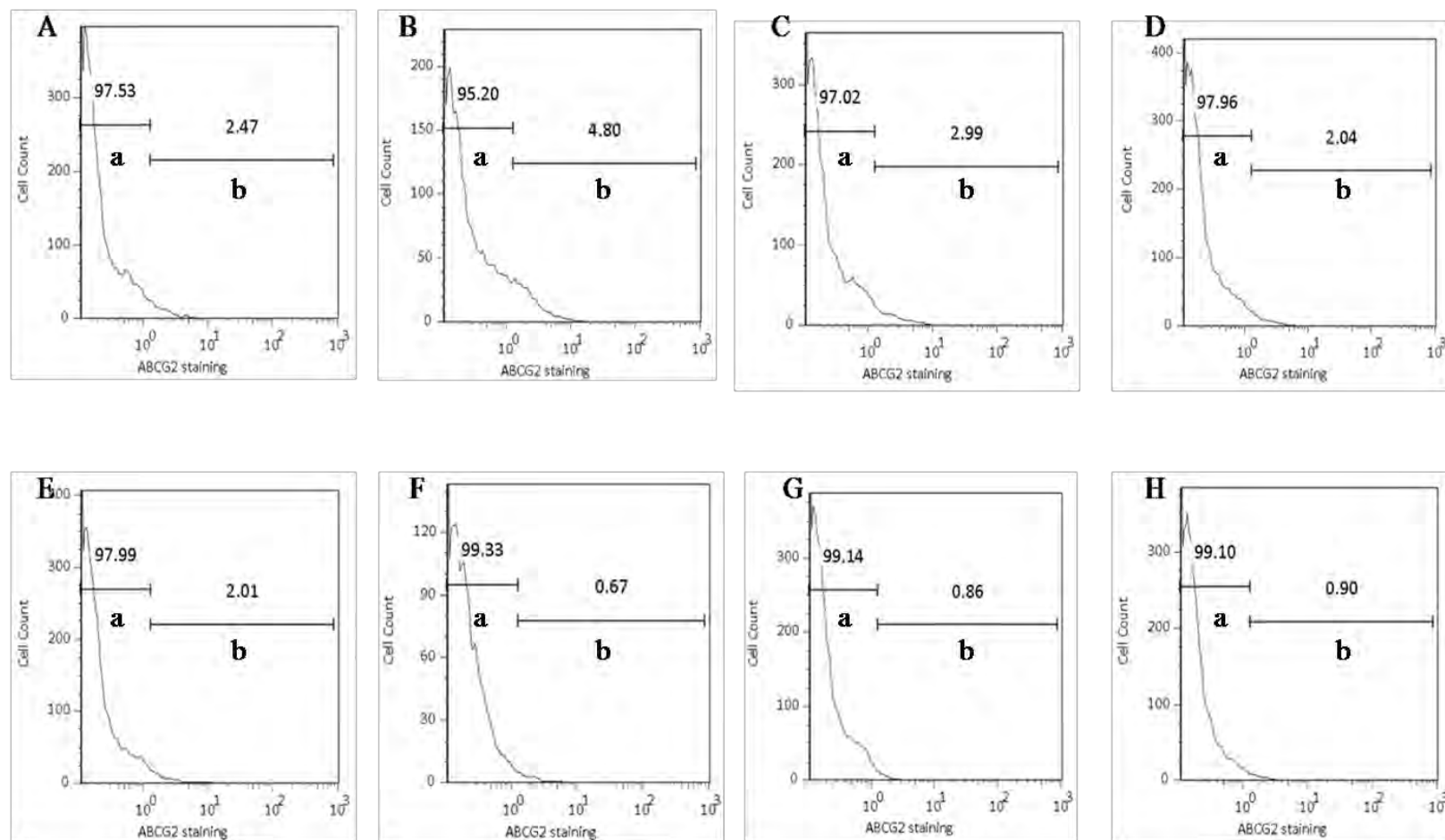


Figure 19: The effect of the priority compounds on the relative proportions of the ABCG2⁺ population in MCF7 cells

Single-colour histograms generated from the flow cytometric analysis of the percentage of ABCG2⁺ cells in 20 000 MCF7 cells after treatment with selected algal compounds. The NSP is designated as „a“ and the SP as „b“. The numbers indicate the percentage of these cells within the total population. Cells were analysed after treatment with 0.25% DMSO (A), 10 nM paclitaxel (B), 80 μ M RUMB-001 (C), 80 μ M RUMB-002 (D), 40 μ M RUMB-007 (E), 80 μ M RUMB-010 (F), 10 μ M RUMB-015 (G) and 300 μ M RUMB-017/018 (H) for 96 hours. The DMSO sample (A) was used to define the gates for the ABCG2⁺ (2.47% in DMSO sample) and ABCG2⁻ (97.53% in DMSO sample) populations and these gates were used for all subsequent samples. Flow cytometry was performed using the Beckman Coulter FC 500 flow cytometer and data was collected in the FL1 channel.

4.3 Conclusion

Two different techniques were used to investigate the effect of selected indigenous compounds on the SP of MCF7 breast cancer cells, namely the Hoechst 33342-exclusion assay (Goodell et al., 1996) and an assay developed during this project in which an antibody against ABCG2 was used to identify and analyse the SP in MCF7 cells by flow cytometry. While both of these assays make use of ABCG2 expression in the SP the antibody staining has some distinct advantages over the Hoechst 33342-exclusion assay. The efflux of Hoechst 33342 from cells is a biological process and as such is extremely sensitive to any changes in assay conditions, especially any fluctuations in temperature (Goodell et al., 1996). The staining of the MCF7 cells with an antibody against ABCG2 is not reliant on a biological process so the staining procedure is much less sensitive to environmental fluctuations and more reliable. This assay still retains the specificity for the SP but is more robust than the Hoechst 33342-exclusion assay making it a desirable alternative for use in the identification of the SP and the results obtained after treatment of the MCF7 cells with DMSO and paclitaxel justify the development of this assay for SP analysis.

Not only is the antibody staining for ABCG2 to identify the SP a novel technique, but the indigenous algal compounds investigated for their cytotoxicity towards the SP are also novel compounds. Three of the priority compounds, namely RUMB-001, RUMB-002 and RUMB-007, had little apparent cytotoxicity towards the SP while RUMB-015 showed moderate cytotoxicity. RUMB-010 and RUMB-017/018 were the most effective at reducing the proportion of ABCG2⁺ MCF7 cells, with the percentage of these cells making up 0.67% and 0.9% respectively after treatment. This value is lower than that of the DMSO sample where the SP makes up 2.47% of the total cell population. It is important to note is that had RUMB-017/018 been excluded from this study based on its lack of cytotoxicity towards MCF7 breast cancer cells, its apparent cytotoxic effect on the cancer stem cell-containing ABCG2⁺ population would have been missed. It is possible; therefore, that other compounds that displayed little cytotoxicity in the bulk MCF7 cell line, and in the MCF12A cell line, may have a cytotoxic effect on the SP in MCF7 cells and testing these compounds may provide further interesting results. These experiments however need to be repeated not only to confirm these results but also to test the reproducibility of the assay.

RUMB-010 and RUMB-017/018 are perhaps the most interesting of the priority compounds and may have the most potential as drug candidates should chemical modification be able to increase the cytotoxicity of these compounds in the NSP and/or SP in a cancer cell line. However further characterisation of the effects of these compounds on both the NSP and SP cells and the mechanisms by which they act would be required before a decision could be made regarding their potential as hit compounds.

Work by other researchers has also indicated that it is possible to target the SP, for example three NF- κ B pathway inhibitors that are capable of inhibiting the proliferation of CD44⁺CD24⁻ MCF7 cells (Zhou et al., 2008), which are thought to reside in the SP (Al-Hajj et al., 2003; Mylona et al., 2008). Interferon-alpha (IFN- α) has also been shown to reduce the percentage of SP cells relative to NSP cells in both ovarian cancer (Moserle et al., 2008) and adult T-cell leukaemia/lymphoma (Kayo et al., 2007). Preliminary studies into the mechanism of toxicity of the priority compounds were then investigated using Western blot analyses for PARP cleavage and Hoechst 33342 immunostaining for apoptotic nuclei.

5 DETERMINATION OF THE APOPTOTIC-INDUCING ABILITY OF THE PRIORITY COMPOUNDS

5.1 Introduction

Apoptosis is a Greek word used to describe the falling or dropping off of a petal or leaf from a flower or tree. It is often mispronounced as the second „p“ is silent. The second part of the word is a direct derivation of „ptosis“, the Greek word for „falling“. Originally called „shrinkage necrosis“, apoptosis is defined as programmed cell death, a controlled process by which cells are lost from tissues in an effort to maintain tissue homeostasis which would otherwise be disrupted as a result of cell proliferation or damage from harmful cells. Apoptosis involves two distinct stages; first the formation of apoptotic bodies, during which nuclear and cytoplasmic condensation occurs, followed by the phagocytosis and degradation of the apoptotic bodies by the surrounding cells. Apoptosis causes no inflammation as is seen with necrosis and also allows for the recycling of cellular constituents (Kerr et al., 1972; Nagata, 1997).

Apoptosis can occur either extrinsically, whereby cell death is induced by the presence of external signals or intrinsically, when cell death occurs as a result of the absence of external factors that inhibit apoptosis and involves the release of cytochrome *c* from the mitochondrion and can occur through a number of different pathways (Figure 20) (Green and Reed, 1998; Kerr et al., 1972; Kroemer et al., 1995). The extrinsic and intrinsic apoptotic pathways converge in the final stages of apoptosis, the execution phase, during which caspases exert their function, cleaving numerous substrates (Elmore, 2007; Slee et al., 2001). A group of proteases known as the caspases are cysteine proteases responsible for apoptosis and once activated, caspases in turn activate an irreversible proteolytic signaling cascade that ultimately results in cell death and over 60 cellular proteins have been identified as caspase substrates (Morgan et al., 2002). It is during these final stages of apoptosis that caspase-3 cleaves poly(ADP-ribose) polymerase (PARP) (Elmore, 2007; Levrand et al., 2006; Regula and Kirshenbaum, 2005; Slee et al., 2001). (PARP), a 113 kDa nuclear zinc-finger DNA binding protein detects DNA strand breaks in cells and is involved in the poly(ADP-ribosyl)ation of nuclear proteins using NAD^+ as a co-enzyme and is activated by binding to DNA ends or strand breaks (Boulares et al., 1999; Oliver et al., 1999). The cleavage of PARP produces two fragments; an 89 kDa fragment containing the active site of the enzyme and a 24 kDa fragment containing the DNA-binding domain of the enzyme (Kaufmann et al., 1993). This cleavage therefore abrogates the ability of PARP to respond to DNA strand breaks and as such is thought to contribute to the depletion of NAD and ATP in the cell, ultimately resulting in cell death (Boulares et al., 1999; Kaufmann et al., 1993; Oliver et al.,

1999). PARP cleavage is therefore used as a marker of the late stages of apoptosis in cells (Kaufmann et al., 1993). The enzyme primarily responsible for the cleavage of PARP during apoptosis is caspase-3, one of the aforementioned proteins involved in the proteolytic cascade (Boulares et al., 1999). The cleavage site in PARP recognised by caspase-3 (DEVD) is highly conserved amongst different species, alluding to the importance of PARP cleavage during apoptosis (Boulares et al., 1999; Lazebnik et al., 1994; Le Rhun et al., 1998).

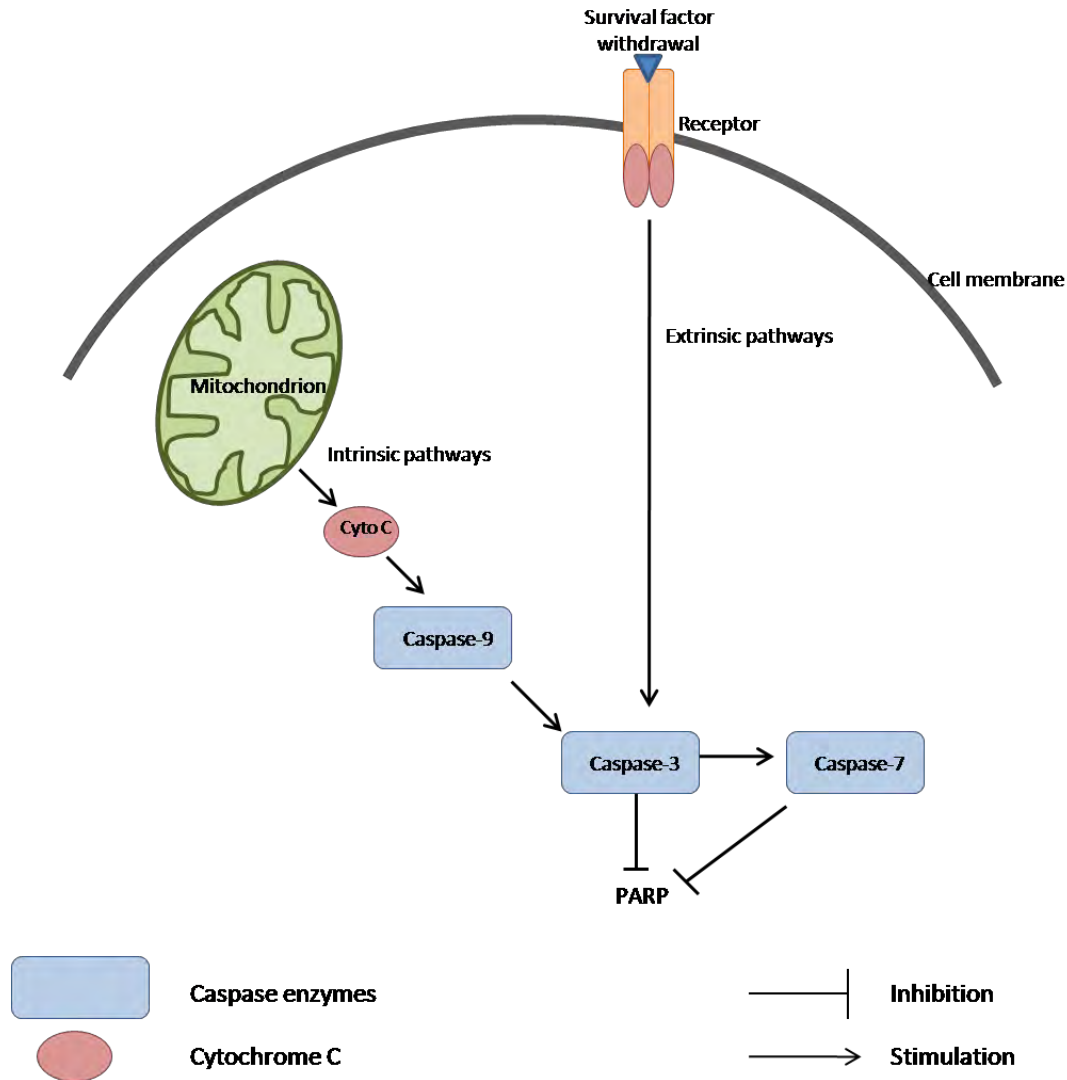


Figure 20: Simplified schematic diagram showing the convergence of extrinsic and intrinsic apoptotic pathways.

This schematic representation shows the convergence of the extrinsic and intrinsic apoptotic pathways on caspase-3 during the final stages of apoptosis and the resultant cleavage action of caspase-3 on PARP [poly(ADP-ribose) polymerase]. In cases where caspase-3 is absent caspase-7 is sometimes capable of compensating for the lack of caspase-3 and of cleaving PARP (Elmore, 2007; Janicke et al., 1998; Slee et al., 2001).

One of the defining characteristics of malignant cells is their ability to evade apoptosis (Bertram, 2000; Johnstone et al., 2002; Shao et al., 2004) and many chemotherapy drugs exert their effect on cancer cells by inducing apoptosis (Saunders et al., 1997). Originally the activation of apoptosis in breast cancer cells proved to be difficult, even when treated with these otherwise cytotoxic chemotherapeutic agents, but paclitaxel was one of few compounds able to successfully induce an apoptotic response in breast cancer cells (Saunders et al., 1997). The principal target of taxanes is thought to be the microtubule system and paclitaxel acts by binding to β -tubulin, thereby stabilizing microtubules and causing the cell cycle to arrest in the G₂/M phase of the cell cycle, eventually resulting in apoptosis (Manfredi and Horwitz, 1984; Marone et al., 2001; Reshkin et al., 2003; Wang et al., 2000). It would make sense, therefore, that the ability of a compound to induce apoptosis in cancer cells would be one of the properties looked for when investigating the potential anti-cancer activity of novel compounds.

The aim of the apoptosis assays was to identify which of the priority compounds, five of which were determined to be cytotoxic in the MCF7 breast cancer cell line (RUMB-001, RUMB-002, RUMB-007, RUMB-010 and RUMB-015), were able to elicit an apoptotic response (as indicated by PARP cleavage and Hoechst analysis) in these cells.

5.2 Results and Discussion

5.2.1 Detection of PARP in MCF7 cells after treatment with the priority compounds

The ability of each of the selected priority compounds to induce apoptosis was investigated by Western blot analysis. MCF7 cells (1×10^5) were treated with serum starvation, 100 nM hydrogen peroxide (H₂O₂), 0.25% DMSO, 100 nM paclitaxel and the priority compounds at their IC₅₀ values as determined by the cytotoxicity assays for 24 hours at 37°C, after which the cleavage of PARP was analysed by Western analysis (Figure 21). Serum starvation, H₂O₂ and paclitaxel were used as positive controls for the cleavage of PARP and DMSO was used as a negative control for PARP cleavage.

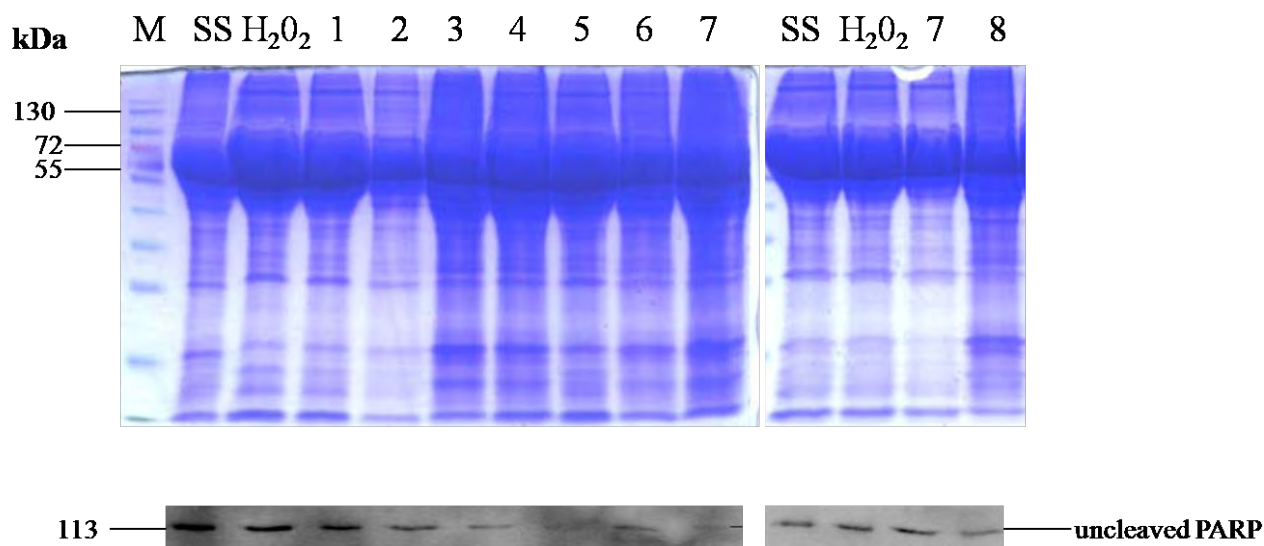


Figure 21: Detection of PARP cleavage in MCF7 cells after treatment with the priority compounds using SDS-PAGE and Western blot analysis.

SDS-PAGE and Western blot analysis of full-length and cleaved PARP in MCF7 cells (1×10^5 /lane) treated with serum starvation (SS), 100 nM H₂O₂ (H₂O₂), 0.25% DMSO (Lane 1), 100 nM paclitaxel (Lane 2), 58 μ M RUMB-001 (Lane 3), 65 μ M RUMB-002 (Lane 4), 33 μ M RUMB-007 (Lane 5), 68 μ M RUMB-010 (Lane 6), 3 μ M RUMB-015 (Lane 7) and 300 μ M RUMB-017/018 (Lane 8) for 24 hours. Lane M shows a molecular weight marker. Full-length PARP (~130kDa) was detected in all samples but no cleaved PARP (~85kDa) was detected.

Full-length PARP was detected but PARP cleavage was not detected in any of the MCF7 samples analysed by Western blot, including in the positive controls of serum starvation, H₂O₂ and paclitaxel, which are known to induce apoptosis in MCF7 cells (Egeblad and Jaattela, 2000; Perillo et al., 2000; Saunders et al., 1997). This result was consistently observed for repeated experiments, despite the reports in literature of apoptosis induction by serum starvation, paclitaxel and H₂O₂ (Egeblad and Jaattela, 2000; Perillo et al., 2000; Saunders et al., 1997). After further investigation into these conflicting results it was found that MCF7 cells are caspase-3 deficient as a result of a 47-bp deletion in the *CASP-3* gene that abolishes the expression of caspase-3 (Jänicke, 2009; Janicke et al., 1998). Caspase-3 is required for PARP cleavage during apoptosis (Slee et al., 2001). Caspases-3, -6 and -7 are known as the executioner caspases and are thought to be responsible for the actual cell damage during apoptosis (Slee et al., 2001). It has been shown that caspase-6 and caspase-7 perform minor roles while caspase-3, the primary executioner caspase, is required for many of the proteolytic events that occur in the final stages of

apoptosis (Slee et al., 2001). In the absence of caspase-3 apoptosis in MCF7 cells occurs through a caspase-7-activated pathway and caspase-7 takes the place of caspase-3 as the primary executioner caspase in MCF7 cells (Twiddy et al., 2006) although it has been established that caspase-3 and caspase-7 perform distinct and different roles during apoptosis (Slee et al., 2001). It appears that in the above samples (Figure 21) caspase-7 was not able to compensate for the loss of PARP cleavage during apoptosis that would otherwise have occurred in the presence of caspase-3, although caspase-7-dependent PARP cleavage has been shown in MCF7 cells treated with staurosporine (Twiddy et al., 2006).

The assays for PARP cleavage in MCF7 cells treated with the priority compounds were unsuccessful in determining the ability of these compounds to induce apoptosis. However, the data are consistent with the major role of caspase-3 in the proteolytic processing of PARP during apoptosis found by others (Jänicke, 2009; Slee et al., 2001). There were two possible alternative options available for determining whether the priority compounds induce apoptosis. These were to change the cell line and use one which does express caspase-3 and in which PARP cleavage is known to occur; or change the assay and use a technique that is independent of caspase-3. It was decided that the assay used would be changed as all the other experiments in this study were carried out using MCF7 cells and the data obtained for the cytotoxicity of the algal compounds in both the MCF7 cells and the MCF7 SP would thus be more comparable. A kinetic analysis incorporating a full range of dose concentrations assayed at different time points may allow for the eventual identification of PARP cleavage, especially if caspase-7 is only able to compensate for caspase-3 activity after a certain amount of time.

5.2.2 Detection of apoptosis in MCF7 cells after treatment with the priority compounds using Hoechst 33342 immunostaining

The morphological changes that allow the identification of a cell undergoing apoptosis include cell shrinkage which results in smaller cells with denser cytoplasm and more tightly packed organelles; chromatin condensation, the phase during which the nuclear material in the cell aggregates and the most characteristic feature of apoptosis; and membrane blebbing, a process in which membrane-bound cell fragments bud off from the cell as apoptotic bodies carrying some organelles and sometimes nuclear material (Elmore, 2007; Kerr, 1971; Kerr et al., 1972). The

ability of the priority compounds to induce apoptosis was determined by investigating whether treatment with the compounds induced chromatin condensation using staining with Hoechst 33342, a nucleic acid stain, and immunofluorescence analysis. For the purposes of this study non-apoptotic cells were defined as those showing intact nuclei and less intense staining. Apoptotic nuclei were defined as those nuclei showing chromatin condensation and therefore more intense staining, disintegration and the formation of potential apoptotic bodies containing nuclear material close to the cell (Figure 22). The difference between a non-apoptotic and an apoptotic nucleus is shown in Figure 22 where cells from the MCF7 cell line are treated with 100 nM paclitaxel for 48 hours. The cell representative of a non-apoptotic nucleus in Figure 22 was intact whereas the cell representative of an apoptotic nucleus shows chromatin condensation and nuclear disintegration and more intense staining.

MCF7 cells (1×10^5) were treated with 0.25% DMSO, 100 nM paclitaxel and the priority compounds at their IC_{50} values for 48 hours at 37°C following which they were stained with using $10 \mu\text{g}.\text{ml}^{-1}$ Hoechst 33342 and visualized using a Zeiss LSM META 510 laser scanning microscope at 405 nm (Figure 23). A total of 150 MCF7 cells from 3 randomly chosen fields were counted for each of the sample treatments, except in the case of RUMB-002 where only 69 cells were counted because only 69 cells were present on the microscope slide. The number of apoptotic cells in each representative sample of 150 cells was counted, from which the percentage of apoptotic cells in each sample after treatment for 48 hours could be calculated (Figure 23).

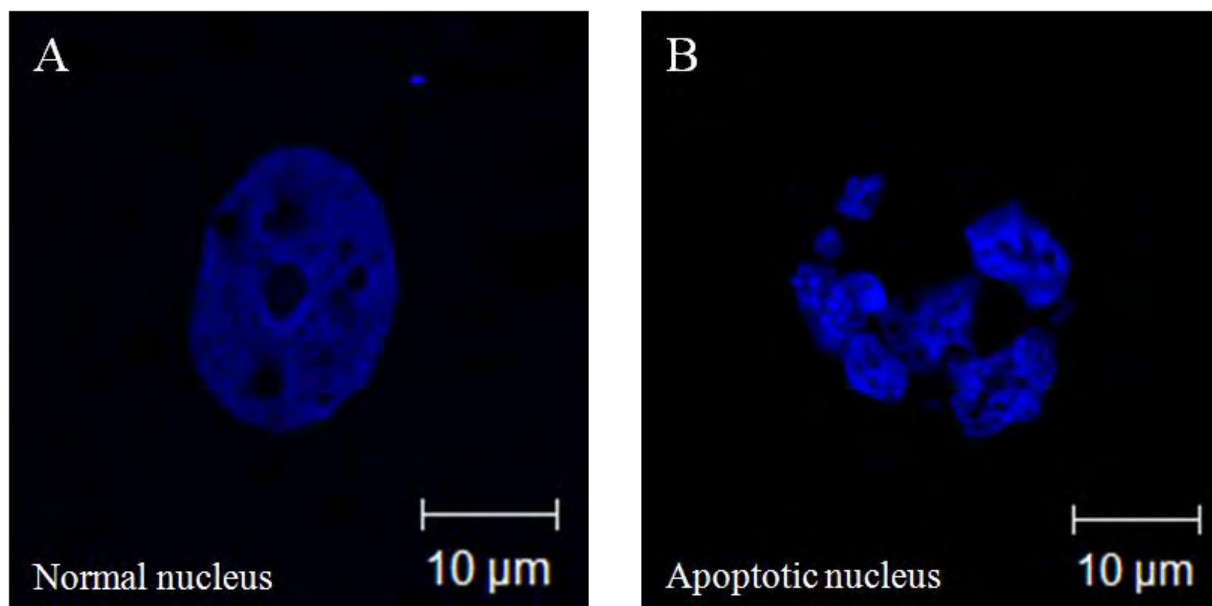


Figure 22: Representative of (normal) non-apoptotic (A) and apoptotic (B) nuclei in MCF7 cells using Hoechst-33342 staining.

(A) Normal (non-apoptotic) and (B) apoptotic nucleus in MCF7 cells after treatment with 100 nM paclitaxel for 48 hours and stained with $10 \mu\text{g}.\text{ml}^{-1}$ Hoechst 33342 and visualized at $60\times$ magnification on a Zeiss LSM META 510 laser scanning microscope at 405 nm. A: The non-apoptotic nucleus is intact and shows no obvious sign of nuclear condensation. B: An apoptotic nucleus showing both nuclear condensation, and therefore more intense nuclear staining, and nuclear disintegration, both characteristics of apoptosis.

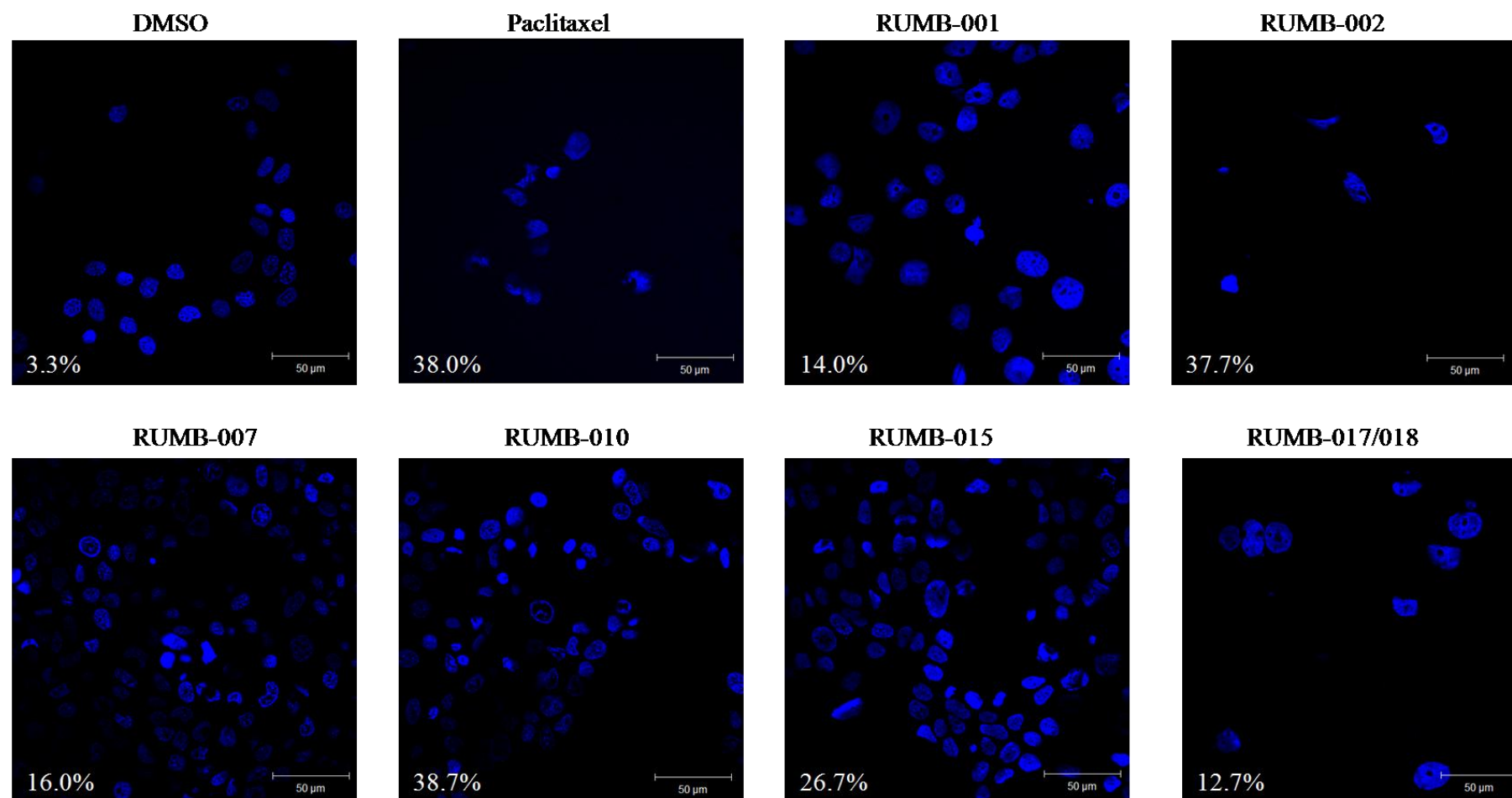


Figure 23: Identification of Apoptotic Nuclei by Hoechst 33342 staining in MCF7 cells treated with novel indigenous compounds

Representative images of apoptotic nuclear staining in MCF7 cells treated with 0.25% DMSO, 100 nM paclitaxel, 58 μM RUMB-001, 65 μM RUMB-002, 33 μM RUMB-007, 68 μM RUMB-010, 3 μM RUMB-015 and 300 μM RUMB-017/018 for 48 hours at 37°C and stained with 10 $\mu\text{g}.\text{ml}^{-1}$ Hoechst 33342. The cells were visualized at 40 $\times$ magnification using a Zeiss LSM META 510 laser scanning microscope. A total of 150 cells from three different fields of view were counted for each sample and the average percentage of apoptotic nuclei for each treatment in triplicate, shown in the bottom right of each image, was calculated. One field of view for each sample is shown here.

A total of 3.3% of the cells present in the DMSO negative control were judged as apoptotic while 38.0% of the cells present in the positive control treated with 100 nM paclitaxel showed signs of apoptosis, namely chromatin condensation (and therefore brighter staining) and disintegration of the cell into apoptotic bodies. All the priority compounds tested appeared able to induce apoptosis in MCF7 cells, albeit at different levels. In the cells treated with the priority compounds, 38.7% and 26.7% of the cells in the RUMB-010 and RUMB-015 treated samples respectively appeared to be apoptotic according to the Hoechst staining pattern. These results imply that these compounds are able to induce apoptosis in MCF7 cells to approximately the same extent as paclitaxel. The percentage of apoptotic cells present in MCF7 cells treated with RUMB-001, RUMB-007 and RUMB-017/018 were calculated to be 14.0%, 16.0% and 12.7% respectively. While these compounds do not appear capable of inducing apoptosis to the same degree as paclitaxel, RUMB-010 and RUMB-015, they do activate apoptosis to a greater extent than seen in the DMSO negative control, which showed 3.3% apoptotic cells. Further experiments and statistical analysis would be required to determine whether these differences are significant. RUMB-002 appeared to be successful in inducing apoptosis with 37.7% of the cell present undergoing apoptosis, however only 69 cells were present in this sample and these few cells may not be representative of the effect of RUMB-002 on a larger population of cells.

The MCF7 cells were treated with the priority compounds at their IC_{50} values. As such, it was expected that, if these compounds induce death solely through apoptosis, 50% of the cells in each sample would be show signs of apoptosis. Cells treated with RUMB-010 and RUMB-015 showed the highest incidences of apoptosis (38.7% and 26.7% respectively), however none of the priority compounds induced apoptosis in as many as 50% of the MCF7 cells as measured by this assay. Therefore, the mechanism by which these compounds bring about cell death may not be solely by apoptosis. It is possible that they induce cell death via other pathways rather than purely apoptotic pathways. In addition, the Hoechst 33342 assay used to identify apoptotic nuclei by immunostaining is a subjective assay and the difference between apoptotic and non-apoptotic cells is determined qualitatively. The Hoechst 33342 stain is non-specific and stains all nuclear material, not only that of cells undergoing apoptosis. In many cases it was difficult to determine whether a cell was undergoing apoptosis based on the features defined for apoptotic and non-apoptotic cells for this assay, highlighting the bias of this assay. The Hoechst 33342 immunostaining for apoptotic nuclei was used for this study as the Hoechst 33342 stain and other

reagents and equipment required were readily available whereas reagents required for more specific analyses were not. Data about the ability of the priority compounds to induce apoptosis in MCF7 cells was obtained and in some cases implied that certain compounds were able to induce apoptosis to a similar degree as paclitaxel, however we now appreciate the challenges presented by this assay as a result of the subjective nature of the interpretation of data. As such, no conclusive statements about the ability of these compounds to induce apoptosis can be made based on the data obtained from the Hoechst staining. Further studies should allow better analysis of the ability of the priority compounds to induce apoptosis.

There are many other assays that are specific for apoptosis that would have yielded less biased and more reliable results. The combination of Hoechst 33342 and propidium iodide, which also stains DNA, are often used for the detection of apoptotic cells versus necrotic cells by fluorescence microscopy. Theoretically, the Hoechst 33342 should allow a distinction to be made between apoptotic and non-apoptotic cells and the propidium iodide, as a result of being membrane impermeable, only stains the DNA of dead cells. This should allow the identification of live cells, cells undergoing apoptosis and dead cells although some of the difficulties encountered when using Hoechst 33342 only will most likely be present when using Hoechst 33342 in conjunction with propidium iodide. There are other techniques more specific for apoptosis. One such technique exploits the translocation of phosphatidylserine from the inner leaflet of the plasma membrane to the cell surface, a universal and early event in apoptosis. Annexin V, for example, binds to phosphatidylserine and if conjugated to a fluorescent label would allow the accurate identification of cells undergoing apoptosis by both fluorescence microscopy and flow cytometry (van Engeland et al., 1998; Zhang et al., 1997). Another technique commonly used to identify apoptosis is the TUNEL assay. The execution phase of apoptosis results in DNA fragmentation and the TUNEL assay is based on the detection of DNA fragmentation by terminal deoxynucleotidyl transferase, which catalyses the addition of dUTPs to any nicks present in DNA (Elmore, 2007; Gavrieli et al., 1992). Apoptotic cells can thus be identified by using fluorescently labeled dUTPs and immunostaining for fluorescence microscopy (Gavrieli et al., 1992). This technique has two main drawbacks. Firstly, it is based on an event that happens late in apoptosis and so cells undergoing early apoptosis may be missed. Also, cells that have been subjected to severe DNA damage will also be labeled with the fluorescent dUTPs, giving false positives.

The Annexin V assay would be preferred should this experiment be repeated as it is specific for apoptosis and is based on an early event during the apoptotic pathway and so cells in the early stages of apoptosis would not be missed. In addition to using a technique specific for apoptosis, the design of the experiment may also be improved upon. The MCF7 cells were treated with the priority compounds at only their IC_{50} value and only after 48 hours. Increasing the range of concentration of treatment may provide more useful information regarding the minimum concentration required to induce apoptosis. A further change that may improve the quality and validity of this assay may come from testing different concentrations of the priority compounds over different time periods for the induction of apoptosis.

5.3 Conclusion

Induction of apoptosis is a way of targeting death in a particular cell without inducing an immune response that will lead to the death of nearby cells. All the priority compounds tested were predicted as capable of inducing apoptosis to some extent in the MCF7 cells, as determined by the immunofluorescent identification of nuclear condensation using Hoechst 33342. RUMB-010 and RUMB-015 appeared to be the most successful at inducing apoptosis when treating MCF7 cells with these compounds at their IC_{50} values with the percentage of apoptotic cells present in cells treated with these compounds being in the same range as the percentage apoptotic cells in the sample treated with 100 nM paclitaxel. RUMB-002 also appeared successful at inducing apoptosis but as a result of the small cell numbers counted for this sample it is not comparable with the other samples and in order to determine the ability of RUMB-002 to induce apoptosis this experiment would need to be repeated. The remaining three priority compounds, RUMB-001, RUMB-007 and RUMB-017/018 were able to induce apoptosis, albeit not to the same extent as the aforementioned compounds however, this technique is limited and so further analysis would be required to determine this conclusively.

6 DISCUSSION AND CONCLUSIONS

6.1 Results Summary

The aim of this study was to screen a group of novel organic molecules isolated from algae off the East Coast of South Africa for their activity in cells representative of normal healthy cells and cancer cells using the MTT cell proliferation assay, as well as in the cancer stem cell containing-SP using flow cytometry. A summary of the characteristics of the novel algal compounds and the cytotoxicity in MCF12A and MCF7 cells and the SP analyses discussed above is presented below in Table 3. Based on these data, we proposed to carry out preliminary investigations into the mechanism by which any cytotoxic compounds acted in the cancer cells. The approach that was developed in this study included the investigation of the effects of the compounds on the bulk tumour and the SP, which is believed to contain the cancer stem cell population responsible for the maintenance of the tumour.

Compounds with differential cytotoxicities in the MCF12A breast epithelial (representing normal cells) and MCF7 breast cancer cell lines were identified using the MTT cell proliferation assay. Most of the novel marine organic molecules tested were more toxic towards MCF7 cells than not the MCF12A cells, although one compound was toxic to the MCF12A cells to a greater extent than to the MCF7 cells (RUMB-005), one compound was highly cytotoxic to both cell lines (RUMB-015) and one compound was apparently non-toxic to either cell line (RUMB-017/018). Six compounds (termed priority compounds) were selected for further analysis (Table 3). Four of these compounds, namely RUMB-001, RUMB-002, RUMB-007 and RUMB-010, exhibited differential toxicity in MCF7 and MCF12A cells with at least three-fold higher cytotoxicity in the MCF7 breast cancer cells than in the MCF12A breast epithelial cells. RUMB-015, which had the lowest IC_{50} value in both cell lines, and RUMB-017/018, which exhibited no apparent cytotoxicity to either cell line, were also selected as priority compounds. It was anticipated that these compounds would be used to validate future assays, as they were from the same class of molecules as the other priority compounds and could therefore represent positive or negative controls for cytotoxicity

Table 3: A summary of the cytotoxicity of the algal compounds in MCF12A and MCF7 cells and the effect of the priority compounds on MCF7 SP cells

Compound	Toxicity in MCF12A cells (MTT assay)	Toxicity in MCF7 cells (MTT assay)	Toxicity to SP cells (Hoechst 33342-exclusion assay)	Toxicity to SP cells (ABCG2 antibody assay)
RUMB-001	Low	High	Not tested	Low
RUMB-002	Low	High	Not tested	Low
RUMB-003	Low	Moderate	Not tested	Not tested
RUMB-004	Low	Moderate	Not tested	Not tested
RUMB-005	High	High	Not tested	Not tested
RUMB-007	Low	High	Low	Low
RUMB-008	Moderate	High	Low	Not tested
RUMB-010	Low	High	Low	High
RUMB-015	High	High	Low	High
RUMB-017/018	Low	Low	Not tested	High

RUMB-005 was more toxic to the normal MCF12A cells than to the MCF7 breast cancer cells. This was considered to be an undesirable property for a potential novel hit compounds and so RUMB-005 was not selected as a priority compound. Interestingly, the data for paclitaxel-treated MCF12A and MCF7 cells, however, indicated that paclitaxel, which has an IC_{50} of approximately 100 nM in MCF7 cells, was more toxic to the MCF12A cell line, in which it was calculated to have an IC_{50} of 5.36 nM, than to the cancer cells. This once again highlights the potential unsuitability of MCF12A cells as a „normal“ control because of the growth stimulants these cells required for proliferation. Paclitaxel is currently used as a chemotherapy drug. One of the features of cancer cells is their ability to evade apoptosis, and so perhaps the anti-apoptotic mechanism in MCF7 cells enables them to tolerate more of a toxic compound than normal cells that lack such mechanisms. There are no reports of the IC_{50} value for paclitaxel in MCF12A cells, despite the fact that MCF12A cells are often used as a „normal“ cell control in cytotoxicity analyses (Lin et al., 2004; Van Zijl et al., 2008).

Determination of the effect of the selected priority compounds against the SP was achieved using an antibody against the SP-specific ABCG2 and flow cytometry. Three of the priority compounds, RUMB-001, RUMB-002 and RUMB-007 had no apparent effect on the SP, as

indicated by the fact that the size of the ABCG2⁺ population in these treated cells was similar to that observed in the untreated cells. Three compounds, namely RUMB-010, RUMB-015 and RUMB-017/018, appeared to potentially target the SP in MCF7 cells, as indicated by a decrease in the percentage of ABCG2⁺ cells in the treated cells (0.67%, 0.86% and 0.9%, respectively) compared with the untreated cells (2.47%). However, the assay in which the effect of the priority compounds on the ABCG2⁺ SP of MCF7 cells was determined was developed during this project and due to time and equipment constraints this study was completed only once. Further analyses would be required to confirm and validate the data obtained as well as to ensure the reproducibility and robustness of the assay.

The summary shown in Table 3 allows for the identification of any compounds that appear to have the most potential as high priority hits for development into lead compounds. The results presented here suggest that RUMB-010 and RUMB-017/018 may be most promising as potential hit compounds; RUMB-010 because it is non-toxic to the MCF12A „normal“ cells but toxic to both the MCF7 cancer cells and the MCF7 SP and RUMB-017/018 because it seems to be capable of specifically targeting the SP. An analysis of the effect of the non-priority compounds on the SP of MCF7 cells using the assay developed during this study may result in the identification of further compounds able to specifically target the SP. Before these compounds can be considered hit compounds and move on to become potential lead compounds further analysis is required. It would also be essential that both RUMB-010 and RUMB-017/018 are tested for toxicity in both embryonic and adult stem cells. These compounds exhibited cytotoxicity towards the CSC-containing SP in MCF7 cells and may therefore have a toxic effect on either embryonic or adult stem cells or both. Since adult stem cells are the source of maintenance of most adult tissues a drug that targets these cells would most likely have extensive and serious detrimental effects on normal tissues (Beltrami et al., 2009; Clarke and Frisén, 2001; Zhao et al., 2008). The mechanism of toxicity and the cellular target of the compound must be identified and chemical modification to enhance the cytotoxic effects on the target cells can be carried out. For example, the role of the halogen groups in the cytotoxicity of the compounds may be determined by successively substituting the bromine and chlorine groups with proton or methyl groups. Any change in the cytotoxicity of the derivative compounds could then be attributed to the substitution, shedding light on the role played by the electronegative halogen groups. The chemical integrity and stability of the compound must be analysed and

optimized and the structure-activity relationship of the compound must be assessed (Bleicher et al., 2003). The SAR of over 800 multidrug resistance reversal agents for the treatment of cancer were reviewed using *in silico* structure-activity analyses (Klopman et al., 1997). The information provided by SAR analyses can lead to the development of more effective drugs. For example after conducting SAR studies on glutamine analogues that were membrane impermeable, it was found that linking the analogues to a peptide rendered them membrane permeable, and thus able to enter the cell and act accordingly (Klopman et al., 1997; Marshall et al., 2003). Similar SAR studies could be conducted these algal compounds *in silico* using molecular modelling software or by the generation of successive derivatives of the test compounds (Bleicher et al., 2003; Klopman et al., 1997; Marshall et al., 2003).

A preliminary investigation into the mechanism by which these priority compounds exert their cytotoxicity by immunostaining using Hoechst 33342 revealed that all the priority compounds seemed capable of inducing apoptosis, albeit to different extents. Two of the compounds, namely RUMB-010 and RUMB-015, appeared to be able to induce apoptosis in the MCF7 cells to the same extent as the chemotherapeutic drug paclitaxel. RUMB-001, RUMB-007 and RUMB-017/018 were able to induce apoptosis to some extent but not to the same degree as paclitaxel or RUMB-010 and RUMB-015. The data obtained from the MCF7 cells treated with RUMB-002 in this study was inconclusive and in order to determine whether RUMB-002 is capable of inducing apoptosis and if so to what extent repetition of the study and further analysis would be required. The Hoechst 33342 immunostaining used for the detection of apoptotic nuclei is a non-specific and biased assay and for the results to be confirmed and validated another more specific assay and repeated experiments would be required. The results from this study were not included in Table 3 as the technique attempted here was flawed and the data obtained was inconclusive. Work into the ability of these compounds to induce apoptosis is ongoing and is currently being investigated in MDA-MB-231 cells, a breast cancer cell line in which the IC_{50} values of these compounds have also been determined.

6.2 Novel algal compounds as potential anti-cancer and anti-CSC agents and identification of potential hit compounds

Halogenated monoterpenes are a class of naturally occurring acyclic organohalogens found only in 3 genera of red algae; *Plocamium*, *Ochtodes* and *Portieria*. Over one hundred halogenated monoterpenes have been isolated and characterised, some of which have been demonstrated to have anti-cancer properties (de Ines et al., 2004; Fuller et al., 1992; Polzin et al., 2003; Wise and Croteau, 1999), making them potential scaffolds for future drug design.

The only compound tested here that was not a halogenated monoterpene was RUMB-010, or sargaquinoic acid, a tetraprenylquinone isolated from the brown alga *Sargassum heterophyllum*. The components of the cell that these algal compounds target and the mechanism by which they act are largely unknown, but are very important in the identification of potential novel drugs (Naylor et al., 1983; Wise and Croteau, 1999). There is some evidence in literature that sargaquinoic acid (RUMB-010) induces apoptosis in certain cells and has been demonstrated to do so in human keratinocyte cell line HaCaT as well as in mice skin cells (Hur et al., 2008). Analysis of major signalling pathways or immunoprecipitation should enable the determination of the mechanism of cytotoxicity of these compounds and the cellular component targeted by the compounds. Three signalling pathways that could be investigated are the Hedgehog, Notch and Wnt signalling pathways, all of which have already been implicated in many cancers, including breast cancer (Evangelista et al., 2006; Kasper et al., 2009; Zardawi, 2009). These three pathways have also been proposed to play important roles in the self-renewal and survival of CSC (Howe and Brown, 2004; Kopper and Hajdu, 2004; Liu et al., 2005; Zardawi, 2009). A simple way of investigating the effect of the priority compounds on these pathways would be to select a protein essential to the pathway, for example β -catenin in the Wnt pathway, and determine whether the protein is affected (expression level or localisation) after treatment with the compounds using Western blot analyses (Prasad et al., 2007; Wang et al., 2009). This could be done for all the major proteins in the pathways. This technique has been used successfully to investigate the disruption of Hsp90 associations with its client proteins in various cancer cell lines by certain compounds (Li et al., 2006; Luo et al., 2007; Niikura et al., 2006; Zhang et al., 2008). Alternatively, the compounds could be conjugated to agarose and used as the bait in pull down assays to identify cellular components to which the compound binds (Golubovskaya et al., 2008; Wang et al., 2009).

The structures of the halogenated monoterpenes and sargaquinoic acid bear little chemical and structural resemblance to those drugs currently being used in chemotherapy, which often contain multiple rings and nitrogen groups and have IC₅₀ values in the nanomolar range (Sigurdsson et al., 1986; Tian et al., 2004; Wang et al., 2006; Yayoi et al., 2009). The algal compounds tested in this body of work had IC₅₀ values in the micromolar range, which would be considered too high for a potential hit compound. Elucidation of the mechanism of cytotoxicity and the specific targets of these compounds within the cell may be able to inform rational chemical modification to enhance the cytotoxicity of these compounds in the cancer cells and potentially even the CSC. Chemical modification has been widely used to alter the properties of certain compounds and to increase the toxic effects of anti-cancer drugs (Gomez-Orellana, 2005; Majumdar and Mitra, 2006). Perhaps one of the most successful chemical modifications is that of 17-AAG, a derivative of geldanamycin and inhibitor of Hsp90, that has shown promise as a potential breast cancer treatment in clinical trials (Nimmanapalli et al., 2001; Schulte and Neckers, 1998; Yayoi et al., 2009). Paclitaxel derivatives have also been investigated for water solubility and increased cytotoxicity and some derivatives have been shown to be successful in this regard (Rose et al., 1997; Safavy et al., 1999). Other derivatives of chemotherapeutic drugs that have shown enhanced cytotoxicity include derivatives of gambogic acid and CPT-11, a derivative of camptothecin (Tsuruo et al., 1988; Wang et al., 2009; Zhang et al., 2004). It is not unreasonable, therefore, to consider the possibility that chemical modification of the novel marine organic compounds tested for cytotoxicity in MCF7 cells could improve the solubility of the compounds in water as well as enhance their anti-tumour activity. Structural comparison of the algal compounds with current chemotherapeutics may also provide a basis for rational chemical modification. The electronegativity of these compounds is one of the features on which chemotherapy relies (Holshouser et al., 2002; Maini et al., 1997; Wu et al., 2000) and so modification of the electronegativity of the algal compounds may enhance their cytotoxic activity. One potential modification is the substitution of one of the end groups on the algal compounds for a group which is highly electronegative and one which is not electronegative and investigating the change in cytotoxicity of these compounds in cancer cells seen as a result of higher or lower electron withdrawing capabilities, enlightening us as to whether electronegativity is the cytotoxic factor. Modification of the algal compounds to include a nitrogen group may also have some effect on the cytotoxicity of the algal compounds in cancer cells.

Sargaquinoic acid (RUMB-010) has been shown to induce apoptosis in HaCaT and mice skin cells (Hur et al., 2008) but in other cell lines it appears to promote neurite outgrowth, especially in PC12D cells, a cell sub-line derived from the pheochromocytoma of rat adrenal medulla (Kamei and Tsang, 2003; Tsang et al., 2001). RUMB-010 appeared capable of specifically targeting and removing the SP in MCF7 cells but this was determined only by investigating the proportions of SP cells to NSP cells using flow cytometry. This technique is limited as it gives no indication as to whether RUMB-010 induced the cells to undergo apoptosis, removing them by killing them, or if it forced the undifferentiated cells in the SP to differentiate, lose their SP markers and appear in the NSP proportion. The isolated SP would have to be treated with RUMB-010 as a purified fraction in order to determine whether the compound induces cell death or forces the cells to differentiate. Unfortunately, SP cells are prone to asymmetric division because of the CSC population that resides within the SP. In order to determine the effect of a compound on the SP alone a culture method that prevents the asymmetric division of the cells would have to be developed. It would also be necessary to validate that the SP is in fact enriched for CSC. This could be achieved by isolating the SP cells using flow cytometry and then investigating the expression of proposed breast CSC surface phenotypes, for example CD44⁺CD24⁻ and ALDH1 (Al-Hajj et al., 2003; Ginestier et al., 2007; Mylona et al., 2008).

6.3 A hit-to-target approach for screening potential anti-cancer and anti-CSC agents

Hit-to-target approaches are commonly used in drug development and are dependent on high-throughput screening (Starkuviene and Pepperkok, 2007). The approach developed in this study is a novel hit-to-target approach that can be used for the screening of potential anti-cancer and anti-CSC compounds. This approach makes use of three distinct cell types for screening experiments – normal non-cancerous cells, cancer cells and the CSC-containing SP to screen novel compounds and has an advantage in that it incorporates components that may have been neglected in the past, in particular the effect of compounds on the SP that may reside within tumours. The results presented in this thesis show that the type of assay used to screen potential drugs may influence the data collected and so it is important when screening novel compounds for potential hit compounds that the most specific, reproducible and robust assays are used. The

shortcomings of certain assays are also highlighted in this body of work and relate to the types of assays and cell models used for the assessment of cytotoxicity.

The results from the MTT cell proliferation assay were used to select priority compounds that were at least three fold more cytotoxic in the MCF7 cancer cells than in the normal MCF12A cells. RUMB-017/018 did not meet the criteria defined for priority compounds but was selected for further studies based on its lack of cytotoxicity in both the MCF12A and MCF7 cells. It was later found that this compound appeared able to specifically target the SP in MCF7 cells. Other compounds that were not selected as priority compounds due to their insufficient cytotoxicity towards the MCF7 cancer cells may also be able to specifically target the SP but based on the results of the MTT cell proliferation assay, were disregarded for further analysis during this study. This raises the question as to how many other compounds have been discarded in the past based on their lack of potent cytotoxic activity in cancer cells without having been tested on the SP or the CSC subpopulations.

There are ways in which this approach can be improved; mostly by the use of different and more specific assays. An alternative to the MTT cell proliferation assay is an adaptation of the MTT assay. The MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay results in the production of a water soluble product (Cory et al., 1991). The MTS assay has two distinct advantages over the MTT assay: MTS is more efficiently reduced than MTT and the water soluble product removes the possibility of any toxicity to the cells of the insoluble formazan produced in the MTT assay (Cory et al., 1991). The MTS assay also eliminates the need for the solubilisation step that is required in the MTT assay. Another technique that could be used is CASY cell counting technology, which is based on the integrity of the plasma membrane of the cell (Lindl et al., 2005). The plasma membrane of viable cells is intact while that of dead cells is broken and contains pores. Application of a low voltage electric field to a cell sample enables the distinction between viable and non-viable cells (Cory et al., 1991; Lindl et al., 2005). An electric current cannot pass through the intact plasma of a viable cell but can pass through the pores present in the membrane of a dead cell (Cory et al., 1991; Lindl et al., 2005). One of the main advantages of the CASY cell counting technique is the speed at which data is obtained and its reproducibility (Cory et al., 1991; Lindl et al., 2005).

The analysis of the SP by flow cytometry and staining with an antibody against ABCG2⁺ is a newly developed assay that has not previously been used for the identification and analysis of the SP of cancer cell lines. It is also the first time that an assay of this nature has been used to investigate the effect of compounds, novel or otherwise, on the SP within a tumour. The main advantage of this antibody-based approach over the Hoechst 33342-exclusion assay is that it is not reliant on a biological process and so is much less sensitive to false negatives and therefore more reliable. Validation of both the development of the assay and the results presented here would require repetition of the assay. In addition to being repeated, the assay requires further validation regarding the non-specific binding of the ABCG2 antibody. Non-specific binding of this antibody would produce false positives, thus skewing the results. The determination of the extent to which this antibody is susceptible to non-specific binding could be achieved by using a SP-deficient cell line or by separating the SP and the NSP prior to incubation with the antibody against ABCG2. Isolation and culture of the CSC-enriched SP would allow further characterisation of the effects of compounds on the SP, as well as confirmation of the effects already seen in SP analyses conducted *in situ*, and calculation of IC₅₀ values for the toxic compounds. This would also allow validation of the SP as being enriched for CSC using CSC-specific markers (such as CD44) and functional assays (anchorage-independent growth) (Al-Hajj et al., 2003; Mylona et al., 2008).

A better indication of the mechanism of action of the compounds could be obtained using a different and more reliable apoptosis detection technique. One such technique is the staining of the cells with Annexin V conjugated to a fluorescent molecule. Annexin V binds to phosphatidylserines which are translocated from the inner plasma membrane to the surface of the cell early in apoptosis and so staining with Annexin V conjugated to a fluorescent molecule allows for the identification of cell undergoing apoptosis (van Engeland et al., 1998; Zhang et al., 1997).

Another way in which this approach could be improved is by using paired cell lines as the model system, as opposed to the MCF12A and MCF7 cell lines. The disadvantages of using MCF12A cells, which need various stimulants in order to grow in culture, have already been discussed (Chapter 3). Using a cell line that is a true normal equivalent of the cancer cell line used is

important. For instance, these normal equivalents should grow in culture and should ideally not require complex external stimulants to ensure that any effect on the cells observed was a result of the test compounds acting on the cells themselves and not a result of the compounds interfering with one of the stimulatory factors. The use of cancerous and non-cancerous paired cell lines will also account for any difference that arise due to a difference in the overall genetic background of the cell. For example, the paired cell lines, Hs578Bst and Hs578T, are a good example of normal and cancerous breast cell lines (respectively) and which have the same origin (Hackett et al., 1977; Smith, 1979).

6.4 Importance of identifying anti-CSC and anti-breast CSC agents

Cancer is one of the leading causes of death worldwide and as the most commonly diagnosed malignancy in women, breast cancer is of global importance (Garcia et al., 2007). In southern Africa, breast cancer comprised 10.% of the 78 100 new cancer cases diagnosed in 2007 and only 32% of women diagnosed with advanced breast cancer in southern Africa survive more than five years (Garcia et al., 2007). The major cause of death in breast cancer patients is metastasis and there is no standardized treatment for metastatic breast cancer yet available, although trastuzumab, a monoclonal antibody directed against HER-2, has been recently used to successfully treat HER-2 positive early and metastatic breast cancer (Emens, 2005; Gralow, 2005; Hall and Cameron, 2009; Neyt et al., 2008).

Recently discovered evidence that supports the cancer stem cell theory of cancer development has implications for the treatment of cancer and rapid advancement in this field has provided hope for the development of an effective and reliable anti-cancer treatment as the only way to completely remove a tumour will be to remove the cell population sustaining that tumour (Al-Hajj et al., 2004; Klonisch et al., 2008). The cancer stem cell theory of cancer proposes that CSC are self-renewing and resistant to chemotherapy and are believed to be responsible for tumour initiation and maintenance (Marotta and Polyak, 2009; Wicha et al., 2006). Because of their resistance to chemotherapy, after treatment of a tumour with conventional chemotherapy drugs the CSC remain behind and are able to repopulate the tumour, leading to relapse (Marotta and Polyak, 2009; Wicha et al., 2006) which has prompted exploration into new drugs that are able to

target and kill CSC (Chuthapisith et al., 2009; Li et al., 2008). Any treatment capable of targeting and killing CSC has the theoretical potential to effectively treat cancer, especially if such a treatment is used in conjunction with conventional chemotherapy. Unfortunately, no such treatments are yet available although research into compounds that are specifically able to target CSC is being undertaken. Two Hsp90 inhibitors, 17-AAG and 17-DMAG, have been demonstrated to be capable of inhibiting the growth of glioma stem cells and sensitising *BRCA1* breast cancers to conventional chemotherapeutics respectively (Sauvageot et al., 2009; Wright et al., 2008) and inhibitors of the NF- κ B pathway have been shown to preferentially inhibit the proliferation and colony forming abilities of the putative CD44⁺CD24⁻ CSC from the MCF7 cell line (Zhou et al., 2008).

The search for effective new anti-cancer treatments is therefore ongoing and natural products have historically been a rich source of treatments for many human diseases (Newman et al., 2003). While the idea of cancer stem cell specific anti-cancer treatments is promising, should compounds capable of effectively and specifically targeting and killing CSC be identified, a variety of other questions will need to be answered. The differences between normal adult stem cells and CSC and the properties thereof need to be defined in order to fully understand CSC, the mechanisms by which they operate and the most effective way in which they can be targeted for anti-cancer therapy (Trosko et al., 2005; Zhao et al., 2008). There is still much more work that needs to be done in order to not only understand CSC and their biology but also in the discovery and development of a specific and effective therapy aimed at removing the cancer stem cell population. This does not, however, exclude the ongoing work into novel therapies aimed at the bulk of the tumour cells. The search for new anti-cancer drugs is ongoing in an effort to find treatments that are more effective in killing the cancer cells but have fewer adverse side effects on healthy cells. The evidence supporting the existence of CSC provides an attractive target for drug development.

7 References

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8 Appendices

8.1 APPENDIX 1

8.1.1 Reagents Used

Table 4: Reagents Used

Dulbecco's Modified Eagle Medium (DMEM)	Gibco, USA
Ham's F10	Sigma Aldrich, USA
Foetal calf serum (FCS)	PAA Laboratories, USA
L-glutamine	Gibco, USA
Penicillin-Streptomycin	BioWhittaker, USA
Epidermal growth factor (EGF)	Sigma Aldrich, USA
Cholera toxin	Sigma Aldrich, USA
Hydrocortisone	Sigma Aldrich, USA
Insulin	Sigma Aldrich, USA
Trypsin/ethylenediaminetetraacetic acid (EDTA)	Sigma Aldrich, USA
Phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄·2H₂O, 1.47 mM KH₂PO₄, pH 7.4)	Not applicable
Tris-buffered saline-Tween (50 mM Tris, 150 mM NaCl, 0.1% Tween-20, pH 7.5)	Not applicable
Purified mouse anti-human PARP [poly (ADP-ribose) polymerase]	BD Pharmingen, USA
ECL Advance Western Blotting Kit	GE Healthcare, USA
Hoechst 33342	Invitrogen, USA
Biotin anti-human CD338 (ABCG2)	BioLegend, USA
Streptavidin-FITC	SigmaAldrich, USA
Propidium iodide	Fluka, USA
Reserpine	Sigma Aldrich, USA
MTT Cell Proliferation Kit I	Roche, USA

8.1.2 Compound Dilution Calculations

All compounds received were diluted in 50 μ l DMSO to give stock concentrations shown in Table 5. A 1 μ l volume of each compound was mixed with different amounts of serum-containing DMEM (Table 6: Amount (μ l) of DMEM added to 1 μ l of stock compound to give a 1 mM) such that the concentration of the mixture was 1 mM.

Table 5: Stock concentrations (mM) of novel marine compounds dissolved in DMSO

RUMB Code	Stock (mM)
RUMB-001	87.688
RUMB-002	125.194
RUMB-003	40.353
RUMB-004	253.571
RUMB-005	193.77
RUMB-007	625.75
RUMB-008	119.99
RUMB-010	18.84
RUMB-015	79.1
RUMB-017/018	507.01

Table 6: Amount (μ l) of DMEM added to 1 μ l of stock compound to give a 1 mM

RUMB Code	DMEM (μ l)	Calculation
RUMB-001	86.688	The amount of 1 mM solution required to make the 100 μ M compound dilution required for the MTT assays was calculated using the equation $c_1v_1=c_2v_2$ where c_1 = initial concentration (1 mM), v_1 = volume required, c_2 = final concentration (0.1 mM) and v_2 = final volume (1.5 ml)
RUMB-002	124.194	
RUMB-003	39.353	
RUMB-004	252.571	
RUMB-005	192.77	
RUMB-007	624.75	
RUMB-008	118.99	
RUMB-010	17.84	
RUMB-015	78.1	Therefore: $c_1v_1=c_2v_2 \rightarrow 1 \text{ mM} \times v_1 = 0.1 \text{ mM} \times 1.5 \text{ ml}$ $v_1 = 0.15 \text{ ml} \rightarrow v_1 = 150 \mu\text{l}$ of 1 mM solution + 1350 μ l DMEM
RUMB-017/018	506.01	

The same equation was used to calculate the amount of 1 mM solution required to make the 50 μ M compound dilutions, the amount of 50 μ M solution required to make the 5 μ M compound dilutions and the amount of 5 μ M solution required to make the 0.5 μ M compound dilutions required for the MTT assays.

8.2 SDS-PAGE Resolving and Stacking Gel Constituents

8.2.1 12% Resolving Gel

3.2 ml dH₂O

2.6 ml 1.5 M Tris (pH 8.8)

4 ml 30% acrylamide

100 µl 10% SDS

100 µl 10% ammonium persulphate (APS)

20 µl TEMED

Final volume = 10ml

8.2.2 4% Stacking Gel

6.1 ml dH₂O

2.5 ml 0.5 M Tris (pH 6.8)

1.3 ml 30% acrylamide

100 µl 10% SDS

50 µl 10% ammonium persulphate (APS)

20 µl TEMED

Final volume = 10ml

8.3 APPENDIX 2

8.3.1 IC₅₀ calculations from graphs generated from MTT assays in MCF12A and MCF7 cells

The average IC₅₀ values of the marine compounds in MCF12A and MCF7 cells were calculated using the straight line equations of the trendlines fitted to the three curves generated from the results of the MTT assays. Shown below are the calculations of the IC₅₀ values for RUMB-001 in MCF12A and MCF7 cells (Figure 24).

MCF12A

$$y = mx + c$$

$$50 = -0.3267x + 100.19$$

$$x = 153.63 \mu\text{M}$$

$$y = mx + c$$

$$50 = -0.3516x + 85.512$$

$$x = 101.00 \mu\text{M}$$

$$y = mx + c$$

$$50 = -0.2138x + 100.95$$

$$x = 238.31 \mu\text{M}$$

The average IC₅₀ of RUMB-001 in MCF12A cells is therefore $164.31 \mu\text{M} \pm 69.3$

This value is greater than the highest compound concentration tested and may be an underestimation. Therefore, the IC₅₀ > 164.31 μM .

MCF7

$$y = mx + c$$

$$50 = -0.9279x + 106.24$$

$$x = 60.61 \mu\text{M}$$

$$y = mx + c$$

$$50 = -0.8371x + 100.78$$

$$x = 60.66 \mu\text{M}$$

$$y = mx + c$$

$$50 = -0.8732x + 96.266$$

$$x = 52.98 \mu\text{M}$$

The average IC₅₀ of RUMB-001 in MCF7 cells is therefore $58.09 \mu\text{M} \pm 4.4$

This approach was used to calculate all of the IC₅₀ values (paclitaxel and the remaining compounds) from the dose response curves shown in Figure 25, Figure 26, Figure 27 and Figure 28.

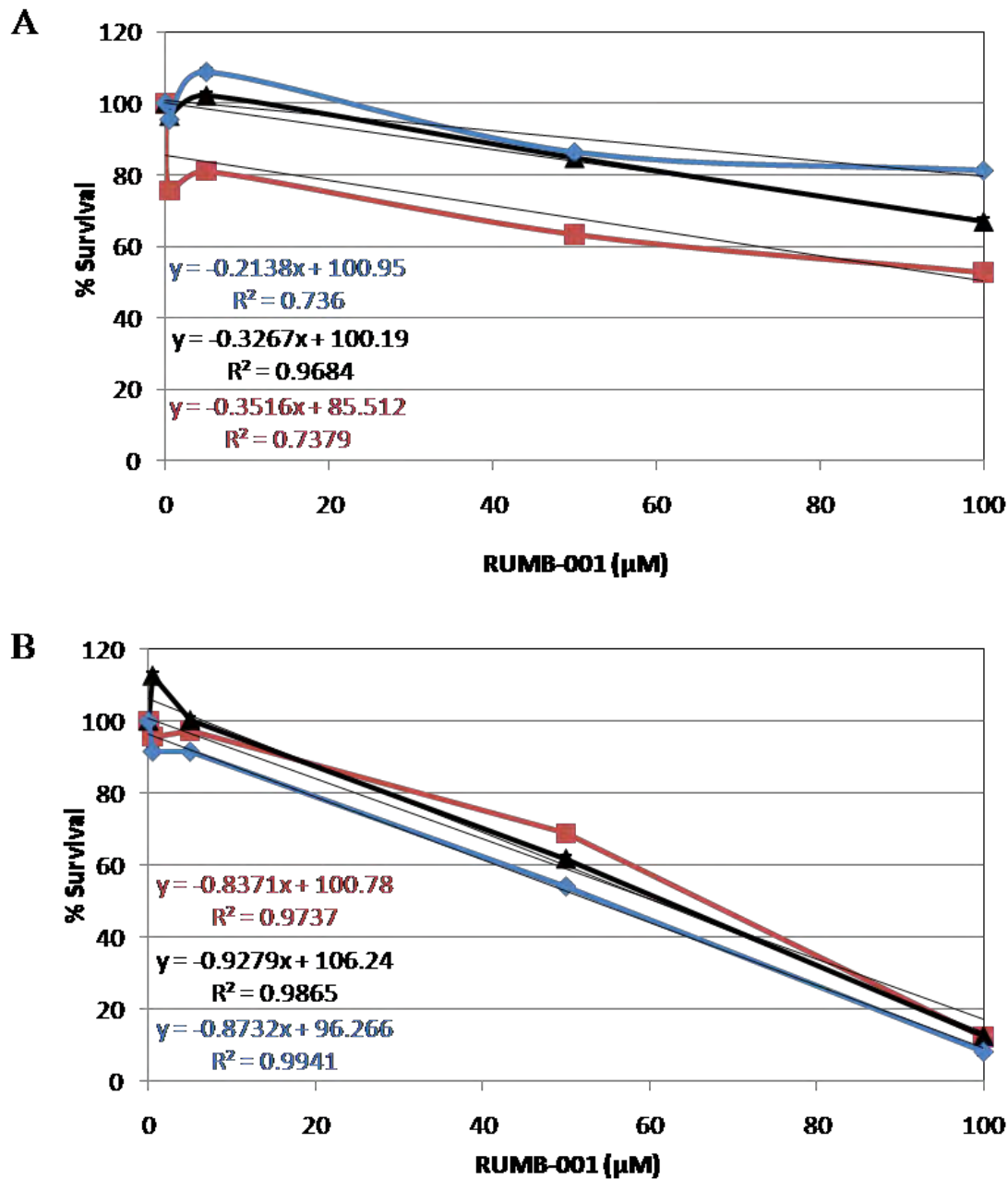


Figure 24: Determination of the IC₅₀ value of RUMB-001 in MCF12A (A) and MCF7 (B) cells

MCF12A (A) and MCF7 (B) cells were treated with between 0.5 μM and 100 μM RUMB-001 for 96 hours (triplicates are shown as black, red and blue lines). The IC₅₀ values of RUMB-001 in the different cell lines was calculated from the trendlines of the triplicate curves and an average of the triplicate IC₅₀ values was calculated to determine the overall average IC₅₀ value of RUMB-001 in each cell line.

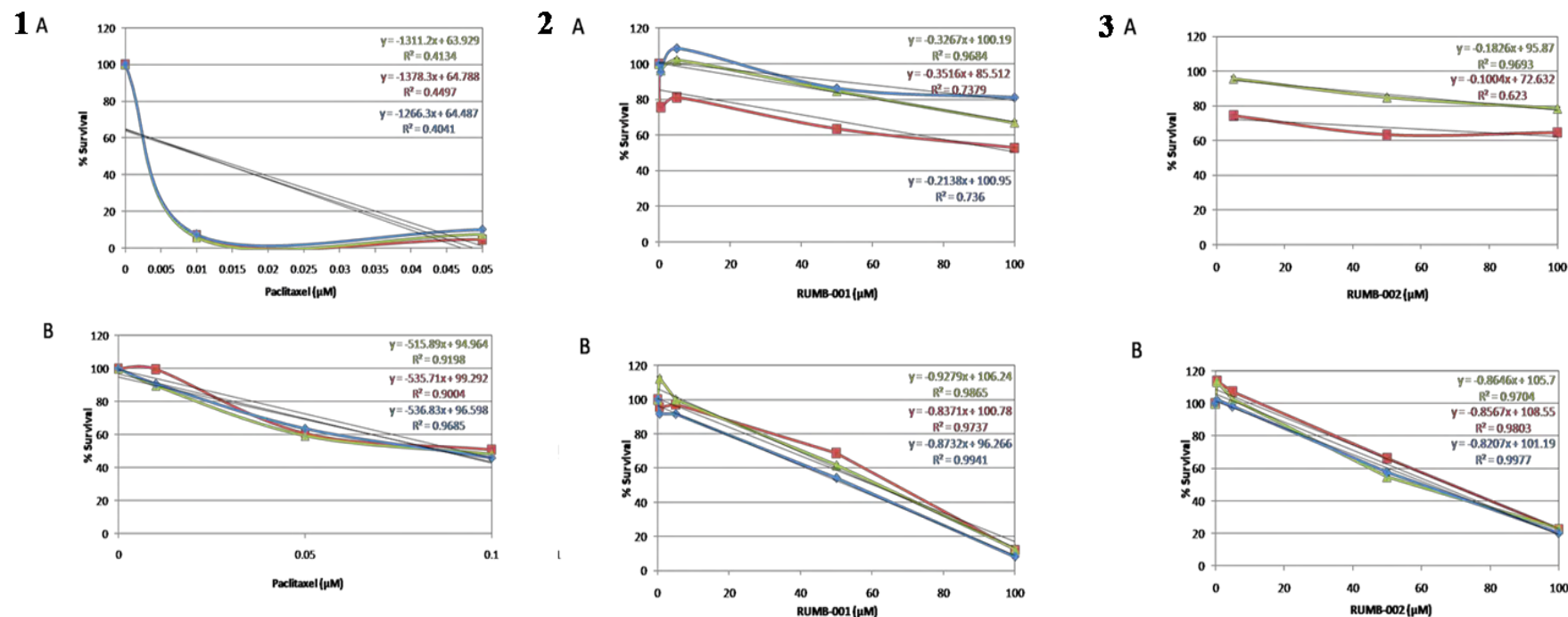


Figure 25: Determination of the IC₅₀ value of paclitaxel (1), RUMB-001 (2) and RUMB-002 (3) in MCF12A (A) and MCF7 (B) cells.

1: MCF12A (A) and MCF7 (B) cells were treated with between 0.01 μ M and 0.15 μ M paclitaxel for 96 hours, 2: MCF12A (A) and MCF7 (B) cells were treated with between 0.5 μ M and 100 μ M RUMB-001 for 96 hours, 3: MCF12A (A) and MCF7 (B) cells were treated with between 0.5 μ M and 100 μ M RUMB-002 for 96 hours (triplicates are shown as green, red and blue lines). The IC₅₀ values of these compounds in the different cell lines was calculated from the trendlines of the triplicate curves and an average of the triplicate IC₅₀ values was calculated to determine the overall average IC₅₀ value of the different compounds in each cell line.

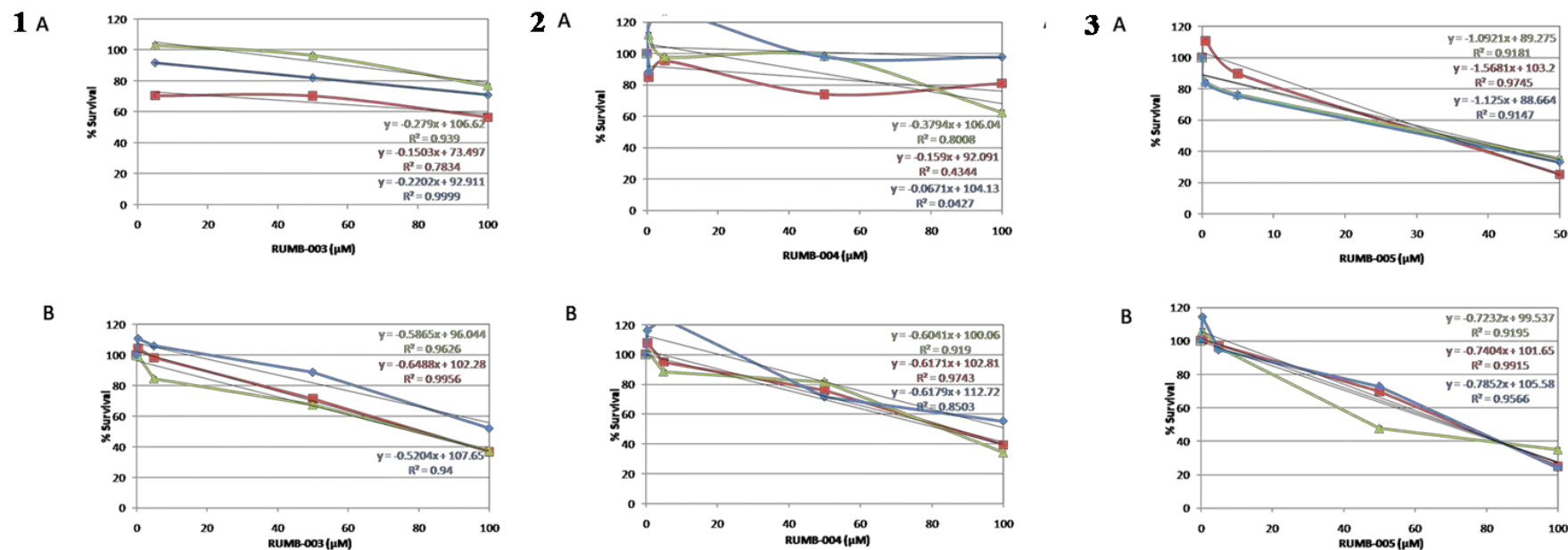


Figure 26: Determination of the IC₅₀ value of RUMB-003 (1), RUMB-004 (2) and RUMB-005 (3) in MCF12A (A) and MCF7 (B) cells.

1: MCF12A (A) and MCF7 (B) cells were treated with between 0.05 μ M and 100 μ M RUMB-003 for 96 hours, 2: MCF12A (A) and MCF7 (B) cells were treated with between 0.5 μ M and 100 μ M RUMB-004 for 96 hours, 3: MCF12A (A) and MCF7 (B) cells were treated with between 0.5 μ M and 100 μ M RUMB-005 for 96 hours (triplicates are shown as green, red and blue lines). The IC₅₀ values of these compounds in the different cell lines was calculated from the trendlines of the triplicate curves and an average of the triplicate IC₅₀ values was calculated to determine the overall average IC₅₀ value of the different compounds in each cell line.

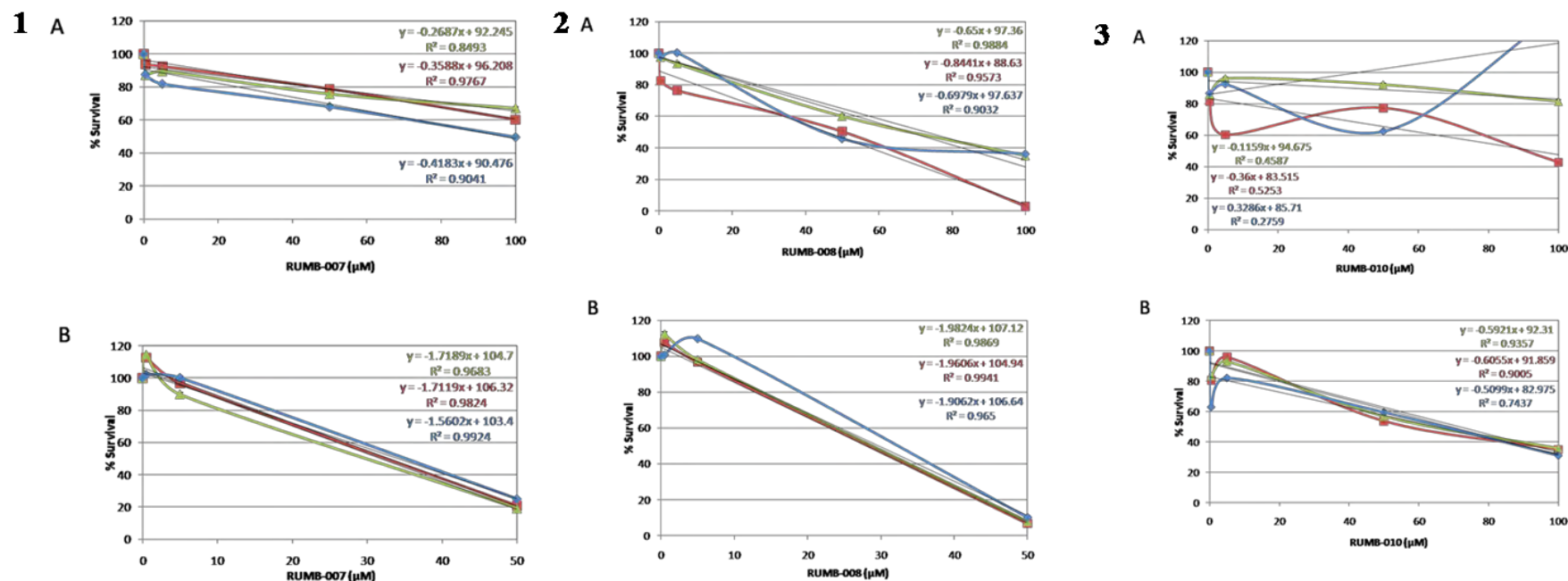


Figure 27: Determination of the IC₅₀ value of RUMB-007 (1), RUMB-008 (2) and RUMB-010 (3) in MCF12A (A) and MCF7 (B) cells.

1: MCF12A (A) and MCF7 (B) cells were treated with between 0.05 μM and 100 μM RUMB-007 for 96 hours, 2: MCF12A (A) and MCF7 (B) cells were treated with between 0.5 μM and 100 μM RUMB-008 for 96 hours, 3: MCF12A (A) and MCF7 (B) cells were treated with between 0.5 μM and 100 μM RUMB-010 for 96 hours (triplicates are shown as green, red and blue lines). The IC₅₀ values of these compounds in the different cell lines was calculated from the trendlines of the triplicate curves and an average of the triplicate IC₅₀ values was calculated to determine the overall average IC₅₀ value of the different compounds in each cell line.

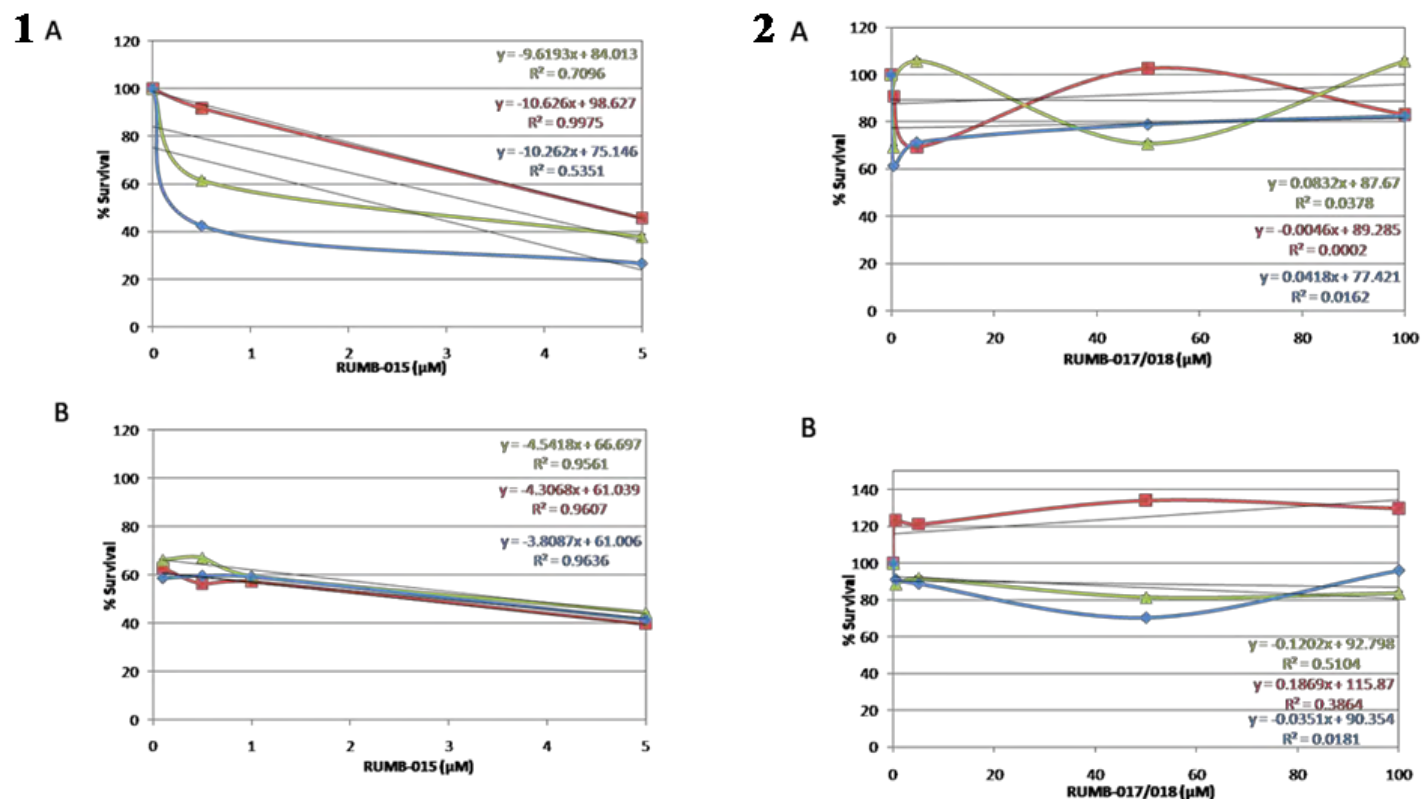


Figure 28: Determination of the IC_{50} value of RUMB-015 (1) and RUMB-017/018 (2) in MCF12A (A) and MCF7 (B) cells.

1: MCF12A (A) cells were treated with between 0.5 μ M and 100 μ M RUMB-015 and MCF7 (B) cells were treated with between 0.01 μ M and 5 μ M RUMB-015 for 96 hours, 2: MCF12A (A) and MCF7 (B) cells were treated with between 0.5 μ M and 100 μ M RUMB-017/018 for 96 hours for 96 hours (triplicates are shown as green, red and blue lines). The IC_{50} values of these compounds in the different cell lines was calculated from the trendlines of the triplicate curves and an average of the triplicate IC_{50} values was calculated to determine the overall average IC_{50} value of the different compounds in each cell line.

8.4 APPENDIX 3

Table 7: Concentrations of priority compounds used in treatment of MCF7 cells to determine the effect of these compounds on the MCF7 side population

Compound	Concentration (μM)
RUMB-001	80
RUMB-002	80
RUMB-007	40
RUMB-010	80
RUMB-015	10
RUMB-017/018	300

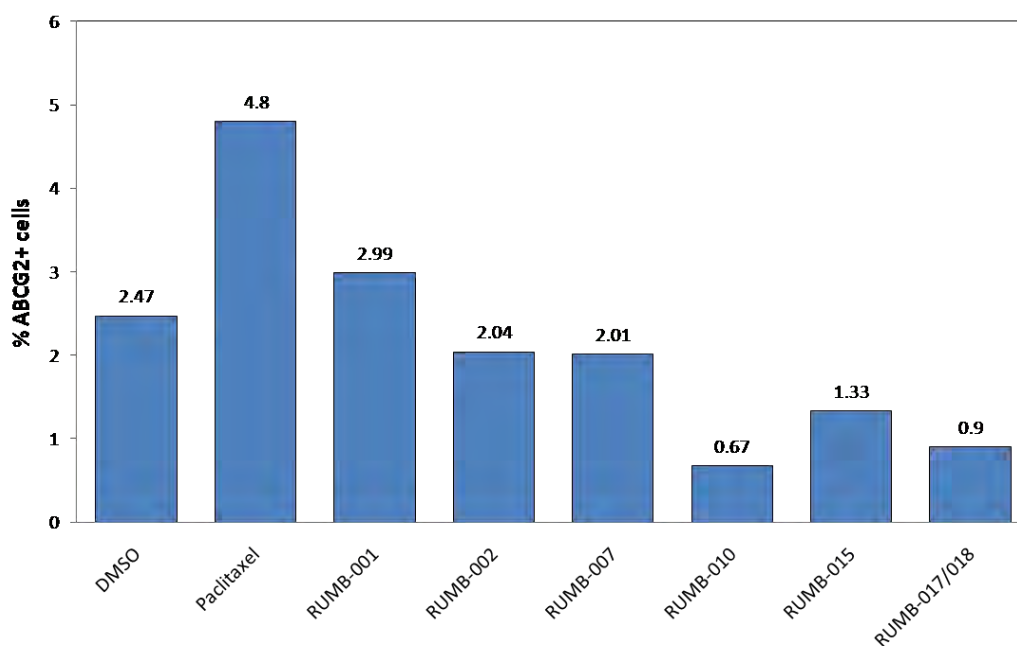


Figure 29: Comparison of the percentage of ABCG2⁺ cells present in MCF7 cells after treatment with the priority compounds.

The percentage of ABCG2⁺ cells present in MCF7 cells (20 000 cells counted) after treatment with 0.25% DMSO, 10 nM paclitaxel, 80 μM RUMB-001, 80 μM RUMB-002, 40 μM RUMB-007, 80 μM RUMB-010, 10 μM RUMB-015 and 300 μM RUMB-017/018 for 96 hours and analysed by flow cytometry after staining with an anti-human ABCG2 antibody.