IDENTIFICATION OF NOVEL MARINE ALGAL COMPOUNDS WITH DIFFERENTIAL ANTI-CANCER ACTIVITY: TOWARDS A CANCER STEM CELL-SPECIFIC CHEMOTHERAPY

Thesis submitted in fulfillment of the requirements for the degree of

Doctor of Philosophy (Biochemistry)

of

Rhodes University

by

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March 2012

ABSTRACT

Breast cancer remains the leading cause of cancer-related death in women worldwide. Furthermore, it has been demonstrated that the treatment-resistant ER^-PR^-HER2/neu^- sub-type is more common among women of African descent, necessitating the search for novel chemotherapies for this form of the disease. The secondary metabolites produced by marine algae represent a rich source of structurally unique compounds with chemotherapeutic potential, particularly in South Africa, whose oceans allegedly host 15 % of the total number of species in the world. Indeed, a recent study reported the isolation of a range of novel compounds from South African red and brown algae of the *Plocamium*, *Portiera* and *Sargassum* genera which displayed cytotoxicity against oesophageal cancer cells *in vitro*. The molecular mechanisms mediating this toxicity were unknown, as was the effect of these and similar compounds on metastatic ER^-PR^-HER2/neu^- breast cancer cell lines or breast cancer stem cells. The current study aimed to address these questions by screening a library of twenty-two novel marine algal compounds for the ability to inhibit MDA-MB-231 and Hs578T breast cancer cells, while having no adverse effects on non-cancerous MCF12A breast epithelial cells. While twelve of these were toxic in the micromolar range against breast cancer cells, only the polyhalogenated monoterpenes RU004 and RU007, and the tetraprenylated quinone sargaquinoic acid (SQA) were identified as hit compounds based on the criteria that their cytotoxicity was specific to breast cancer and not healthy breast cells *in vitro*. On the other hand, the halogenated monoterpene RU015 was found to be highly toxic to both breast cancer and non-cancerous breast cell lines, while the halogenated monoterpene stereoisomers RU017 and RU018 were non-toxic to either of these cell lines.

The mode of action of RU004, RU007, RU015 and SQA, together with the previously characterized carotenoid fucoxanthin (FXN), was assessed in terms of the type of cell death induced and the effect on cell cycle distribution of these compounds. Flow cytometric analysis of the extent of Hoescht 33342 and propidium iodide staining along with PARP cleavage studies suggested that SQA induced apoptosis in MDA-MB-231 cells. On the other hand, the highly toxic compound RU015 appeared to induce necrosis as evidenced by 50 kDa PARP cleavage product in MDA-MB-231 cells. The flow cytometry profiles of MDA-MB-231 and Hst578T cells treated with the hit compounds RU004 and RU007 were suggestive of the induction of apoptosis by these compounds. Cell cycle analysis by flow cytometry with propidium iodide staining revealed that both SQA and FXN induced G_0-G_1 arrest together with an increase in the apoptotic sub-G_0 population, which agreed with previous reports in the literature.

The molecular mechanism of action of SQA and FXN were further investigated by the identification of specific signal transducer molecules involved in mediating their anti-cancer activities. SQA was found to require the activity of numerous caspases, including caspase-3, -6,

-8, -9, -10 and -13, for its cytotoxicity and was demonstrated to decrease the level of the anti-apoptotic protein Bcl-2. On the other hand, FXN was shown to require caspase-1, -2, -3, -9 and -10 for its toxicity. This, together with the ability to decrease the levels of Bcl-2, pointed to the involvement of the intrinsic pathway in particular in mediating the activity of FXN.

The screening of algal compounds against non-cancerous breast epithelial cells carried out in this study, together with the investigation into their mechanisms of action, represent one of the few reports in which characterization of algal metabolites goes beyond the initial cytotoxicity assays.

Finally, in order to assess the potential anti-cancer stem cell activity of the marine algal compounds, a subset of these was screened using a mammosphere assay technique developed in this study. The cancer stem cell (CSC) theory proposes that cancers arise from and are maintained by a specific subpopulation of cells able to undergo asymmetric cell division and termed CSCs. These CSCs are capable of anchorage-independent growth in serum-free culture conditions, such as those in the mammosphere assay. Using this assay, the novel halogenated monoterpene stereoisomers RU017 and RU018 were demonstrated to possess putative anti-CSC activity as evidenced by their ability to completely eliminate mammosphere formation *in vitro*. Furthermore, since RU017 and RU018 were non-toxic to both breast cancer and healthy breast cells, it appeared that the activity of the compounds was potentially specific to the CSCs. The results require further validation, but represent the first report of selective anti-CSC activity.

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17-AAG	17-Allylamino-17-demethoxygeldanamycin
17-DMAG	17-Dimethylaminoethylamino-17-demethoxygeldanamycin hydrochloride
ABC protein	ATP-binding cassette protein
AIF	Apoptosis inducing factor
AML	Acute myeloid leukemia
Apaf-1	Apoptotic protease-activating factor-1
BCRP1	Breast cancer resistance protein 1
BCSC	Breast cancer stem cell
bFGF	Basic fibroblast growth factor
BMP4	Bone morphogenic protein 4
BSA	Bovine serum albumin
CDK	Cyclin-dependent kinase
CRD	Cysteine-rich extracellular domain
CREB	Cyclic adenosine triphosphate-response element binding protein
CSC	Cancer stem cell
DED	Death effector domain
DISC	Death inducing signalling complex
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EDTA	Ethylene diamine tetra-acetic acid
EGF	Epidermal growth factor
EMT	Epethelial-mesenchymal transition
EndoG	Endonuclease G
ER	Estrogen receptor
ERK	Extracellular signal-related kinase
FACS	Fluorescence-activated cell sorting
FADD	Fas-associated death domain
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
FLICE	FADD-like interleukin-1-converting enzyme
FXN	Fucoxanthin
GSK-3	Glycogen synthase kinase-3
H33342	Hoescht 33342 dye
HC	Hydrocortisone
HER-2	Human epidermal growth factor-2
HGF	Hepatocyte growth factor
HIV/AIDS	Human Immunodeficiency Virus/ Acquired immune deficiency Syndrome
HRP	Horseradish peroxidise

Hsp90	Heat shock protein 90
IAPs	Inhibitory apoptotic proteins
ILK	Integrin linked kinase
JNK	c-Jun N-terminal kinase
LY294002	2-(4-morpholinyl)-8-phenyl-chromone
MAPK	Mitogen-activated protein kinase
MAPKK	Mitogen-activated protein kinase kinase
MAPKKK	Mitogen-activated protein kinase kinase kinase
mCSC	Metastatic cancer stem cell
MEK	Mitogen-activated ERK kinase
MOMP	Mitochondrial outer membrane permeabilisation
MPT pore	Mitochondrial permeability transition pore
mRNA	Messenger RNA
mTOR	Mammalian target of Rapamycin
MTT	(3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NMR	Nuclear magnetic resonance
NOD/SCID	non-obese diabetic/severe combined immunodeficient
OPG	Osteoprotegerin
PARP	Poly(ADP- ribose) polymerase
PB	Permeabilisation buffer
PBS	Phosphate buffered saline
PDK	Phosphoinositide-dependent kinase
PH domain	Pleckstrin homology domain
PI	Propidium iodide
PI3K	Phosphatidylinositide-3-kinase
PIP_2	Phosphoinositide-4,5,-bisphosphate
PIP_3	Phosphoinositide-3,4,5-triphosphate
PP2A	Protein phosphatase 2A
PLAD	Preligand assembly domain
PSA	Penicillin/streptomycin/amphotericin
PR	Progesterone receptor
PTEN	Phosphatase and tensin homologue deleted on chromosome 10
PTX	Paclitaxel
PWB	Permeabilisation wash buffer
ROS	Reactive oxygen species
RNA	Ribonucleic acid
SCUBA	Self-contained underwater breathing apparatus
SDS	Sodium dodecyl sulphate
SIMPs	Soluble intermembrane proteins
siRNA	Small interfering RNA
SMAC	Smooth mitochondrial activator of caspases
SP	Side population
SQA	Sargaquinoic acid
SHQA	Sargahydroquinoic acid

TBS	Tris-buffered saline
TNF	Tumour necrosis factor
TNF-R	Tumour necrosis factor receptor
TRADD	TNF-α-associated death domain
TRAIL	TNF-related apoptosis inducing ligand
VEGF	Vascular endothelial growth factor

ACKNOWLEDGEMENTS

First and foremost, I wish to thank my wonderful supervisors, Dr Adrienne Edkins and Prof Greg Blatch. Your passion and dedication has been an inspiration to me and I count myself extremely lucky to have had two such phenomenal mentors.

For supplying the algal compounds, I would like to thank Dr Denzil Beukes and his students from the Faculty of Pharmacy. In particular, my thanks to Maynard Chiwakata for generating the chemical structures.

I would also like to thank my husband Clint for supporting me and for being a single parent for all those week-ends so that I could finish this manuscript! To my baby girl, Rachel: thank you for that magical little smile that always makes everything ok. A big thank you, also, to my very special mom, for being my rock. To my best friend Michelle, thank you for your help with this manuscript and for being there for me when I needed it most.

To my family away from home, BioBRU, thank you for all the shared reagents, advice, laughter and tears. You are an awesome group of people and I am very grateful to have spent the last three years working with you. In particular: Mrs Sally Terry, Dr Petra Gentz and Dr Mugdha Sukhthankar, in their capacity as Laboratory Managers, for running a tight ship; to Ms Buhle Moyo for assistance in tissue culture and Mr Jason Sterrenberg and Dr Mugdha Sukhthankar for establishing the mammosphere culture system. Thank you.

I gratefully acknowledge Rhodes University, The National Research Foundation and The Henderson Foundation for personal funding as well as The Cancer Research Initiative of South Africa (CARISA) for funding the project.

Finally, I would like to dedicate this manuscript to my late uncle, Colin.

RESEARCH OUTPUTS

PUBLICATIONS

Prinsloo, E., Cooper, L.C., Moyo, B., **de la Mare**, **J-A**., Lawson, J.C., Edkins, A.L. and Blatch, G.L. (2010). Heat Shock Proteins in Normal and Cancer Stem Cell Biology: Implications for Regenerative and Chemotherapeutic Medicine IN Stem Cells, Regenerative Medicine and Cancer (©2010 Nova Science Publishers, Inc.). Editor: Shree Ram Singh, Chapter 27, pp.693-713. ISBN: 978-1-61761-342-5.

de la Mare, **J-A**., Lawson, J.C., Chiwakata, M.T., Beukes, D.R., Blatch G.L. and Edkins, A.L. (2011). Quinones and halogenated monoterpenes of algal origin show anti-proliferative effects against breast cancer cells *in vitro*. Revised manuscript under review (Manuscript reference: DRUG 1125).

de la Mare, **J-A**., Sterrenberg, J.N., Sukhthankar, M.G., Beukes, D.R., Blatch, G.L. and Edkins, A.L. Halogenated Monoterpenes from the Red Algae *Plocamium corallorhiza* Display Specific Anti-Cancer Stem Cell Activity in Breast Cancer Cells *in vitro*. Article in preparation.

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B Moyo, **J-A de la Mare**, JC Lawson, D Beukes, AL Edkins, GL Blatch. (2010). Screening of novel compounds of South Africa marine origin that modulate Hsp90 function. The 5th International Meeting on the Hsp90 chaperone machine. Les Diablerets, Switzerland. POSTER PRESENTATION.

J-A de la Mare, JC Lawson, D Beukes, MT Chiwakata, AL Edkins, GL Blatch. (2011). Natural products isolated from the South African *Plocamium corallorhiza* red alga show anti-proliferative effects against breast cancer cells *in vitro.* Signalling in cell death survival, proliferation and degeneration. International Cell Death Society meeting. São Paulo, Brazil. ORAL PRESENTATION.

MG Knott, E Antunes, J Bolton, **J-A de la Mare**, AL Edkins, D Beukes (2011). Harrietones A-B, a new structural class of cyclic halogenated monoterpenes from marine algae. 7th European Conference on Marine Natural Products, Strömstad, Sweden. ORAL PRESENTATION.

CHAPTER 1

LITERATURE REVIEW

1. INTRODUCTION

1.1 Cancer: definition and significance

Cancer, defined as a disease state in which uncontrolled growth and proliferation occurs in one or more cell types (Rhaffagello *et al*., 2008), is currently responsible for more deaths worldwide than HIV/AIDS, malaria and tuberculosis combined (Parkin and Bray, 2006). At the turn of the millennium, Hanahan and Weinberg proposed six "hallmarks" to describe the features of cancer cells, including: 1) independence of exogenous proliferation signals, 2) insensitivity to growth-inhibitory signals, 3) evasion of apoptosis, 4) metastasis and invasion of other tissues, 5) the support of angiogenesis and 6) the development of drug resistance (Hanahan and Weinberg, 2000). Significant progress in the understanding of the etiology of cancer has led to the recent addition of two more hallmarks, namely the deregulation of metabolic processes and the evasion of the immune response (Colotta *et al*., 2009; Hanahan and Weinberg, 2011).

A number of the hallmarks of cancer arise from the deregulation of signal transduction pathways (Hanahan and Weinberg, 2000; Dhillon *et al*., 2007). Cells control and coordinate biochemical activities in response to a range of environmental stimuli via complex signalling systems (Rosen, 1987). These signalling pathways control survival, proliferation, differentiation and apoptosis and allow the cell to cope with changes and stresses in its environment (Weston and Davis, 2007). In human cancers, the tight control of the above cellular processes is lost at least in part through multiple alterations in the signal transduction machinery (Dhillon *et al*., 2007).

What follows is a description of some of the major signal transduction pathways deregulated in cancer cells, their significance in terms of the origin and maintenance of the malignant state, as well as attempts to target cancer cells by the development of pathway specific inhibitors.

1.2 The relationship between apoptosis and cancer

As described above, the evasion of apoptosis is one of the key hallmarks of cancer (Hanahan and Weinberg, 2000). This occurs either by the over-expression of anti-apoptotic molecules or the decreased expression of pro-apoptotic molecules (Speir *et al*., 2011). While conventional cancer treatments such as chemotherapy and radiation aim to induce apoptosis by causing damage to the DNA of the transformed cell, it is these alterations in apoptotic molecules that allows cells to become resistant to these treatments (Debatin and Krammer, 2004; Klener Jr. *et al*., 2006).

1.2.1 The cellular apoptotic machinery

The term apoptosis is derived from the Greek description of falling leaves or petals and is an essential part of life for multicellular organisms (Jin and El-Deiry, 2005; Klener Jr. *et al*., 2006) Apoptosis or programmed cell death is an active process which was first described in 1972 and was found to be vital for development, immune function, tumour suppression and the maintenance of cellular homeostasis (Kerr *et al*., 1972; Jin and El-Deiry, 2005; Iannolo *et al.*, 2008). During development, an organism produces more cells than are required and apoptosis allows for the elimination of these excess cells without eliciting an inflammatory response (Meier *et al*., 2000; Iannolo *et al*., 2008). Characteristic apoptotic morphological features include membrane blebbing, cell shrinkage, DNA fragmentation and chromatin condensation (Savill and Fadok, 2000; Jin and El-Deiry, 2005). Other forms of programmed cell death which do not elicit an immune response include autophagy and anoikis. Autophagy describes the process within a dying cell whereby the cellular components are degraded in autophagosomes and is often associated with infection of cells by viruses and other parasites (Lockshin and Williams, 1965; Lockshin and Zakeri, 2004). On the other hand, anoikis is a form of programmed cell death associated with the detachment of anchorage-dependent cells from the extracellular matrix (Frisch and Francis, 1994; Gilmore, 2005).

In contrast to programmed cell death mechanisms, necrosis is described as a passive, uncontrolled and irreversible process occurring in response to a major environmental insult (Fink and Cookson, 2005; Klener Jr. *et al*., 2006). Here, the cell ruptures and releases its contents into the surrounding tissues, which produces a strong inflammatory response (Leist and Jaattela, 2001; Jin and El-Deiry, 2005). Another form of uncontrolled cell death is mitotic catastrophe where aberrant mitosis produces giant, multinucleate cells containing uncondensed chromosomes (Takai *et al*., 2000).

Caspases or cysteine aspartic acid-specific proteases are central initiators and executioners of apoptosis (Green and Kroemer, 1998; Jin and El-Deiry, 2005). These enzymes are synthesized as inactive precursors called zymogens, which are activated by proteolytic cleavage, resulting in cascades where upstream caspases activate their downstream counterparts at specific aspartic acid residues (Stennicke and Salvesen, 2000; Klener Jr. *et al*., 2006). Caspases are divided into two groups: those that participate in inflammation (caspase-1, -4, -5, -11, -12, -13 and -14) and those involved in mediating apoptosis (caspase-2, -3, -6, -7, -8, -9, 10) (Thornberry *et al*., 1998; Martinon *et al*., 2002; Jin and El-Deiry, 2005; Klener Jr. *et al*., 2006). The apoptotic caspases are further divided into initiator and effector caspases. The initiator caspases (2, 8, 9 and 10) are auto-activated in the presence of certain adaptor proteins by a proximity-induced mechanism and activate downstream effector caspases (Thornberry *et al*., 1997; Boatright *et al*., 2003). The

effector caspases (3, 6 and 7) are more abundant than the initiator variety and cleave various cellular substrates to induce cell death (Jin and El-Deiry, 2005).

Apoptosis is induced by both external and internal signals and occurs via two major pathways: the extrinsic or receptor-mediated pathway and the intrinsic or mitochondrial pathway (Nagata, 1997; Kroemer and Reed, 2000). These pathways converge on a final step, which results in the cleavage of regulatory and structural molecules (Klener Jr. *et al*., 2006).

1.2.1.1 The extrinsic/receptor-mediated apoptotic pathway

The extrinsic apoptotic pathway, shown in Figure 1.1, is activated by external signals and involves the binding of a death-inducing ligand to a cell surface receptor (Nagata, 1997; Klener Jr. *et al*., 2006). Death ligands are type II membrane glycoproteins belonging to the tumour necrosis factor (TNF) superfamily and are arranged in stable homotrimers (Gruss *et al*., 1996; Jin and El-Deiry, 2005). These ligands are either membrane bound or cleaved by metalloproteinase into soluble form (Gruss *et al*., 1996). The TNF ligand family includes TNF-α (TNFSF2/cachectin), Fas ligand (FasL/TNFSF6/CD95L/Apo-1L) and TNF-related apoptosis inducing ligand (TRAIL, TNFSF10) (Ashkenazi and Dixit, 1998; Jin and El-Deiry, 2005). These death ligands exert their effects by binding to their respective receptors on the cell surface (Klener Jr. *et al*., 2006).

The death receptors are type I membrane proteins, which belong to the TNF receptor (TNF-R) superfamily (Ashkenazi and Dixit, 1998). The TNF-R family is divided into death receptors and decoy receptors (DcR) and comprises TNF-R1, which binds TNF-α; Fas (TNFRSF6, Apo-1, CD95), which binds FasL (TNFSF6/CD95L/Apo-1L); TRAIL-R1 (DR4/TNFRSF10A/APO-2/MGC9365) and TRAIL-R2 (DR5/TNFRSF10B /KILLER/TRICK2), which both bind TRAIL; DR3 and DR6 (Pan *et al.*, 1997b; Walczak *et al*., 1997; Wu *et al*., 2000; Klener Jr. *et al*., 2006). Decoy receptors include the Fas decoy, DcR3, and the TRAIL decoys, DcR1 (TNFRSF10C/TRAIL-R3/TRID), DcR2 (TNFRSF10D/TRAIL-R4/TRUNDD) and osteoprotegerin (OPG) (Jin and El-Deiry, 2005). Receptors belonging to the TNF-R family possess a cysteine-rich extracellular domain (CRD), which is responsible for interaction with the death ligand (Baud and Karin, 2001; Jin and El-Deiry, 2005). Whereas death receptors possess a highly conserved intra-cytoplasmic death domain, decoy receptors lack this domain and are unable to transduce the apoptotic signal but compete for binding of the death ligand, thereby abrogating apoptosis via the extrinsic pathway (Pan *et al*., 1997a; Ashkenazi and Dixit, 1998).

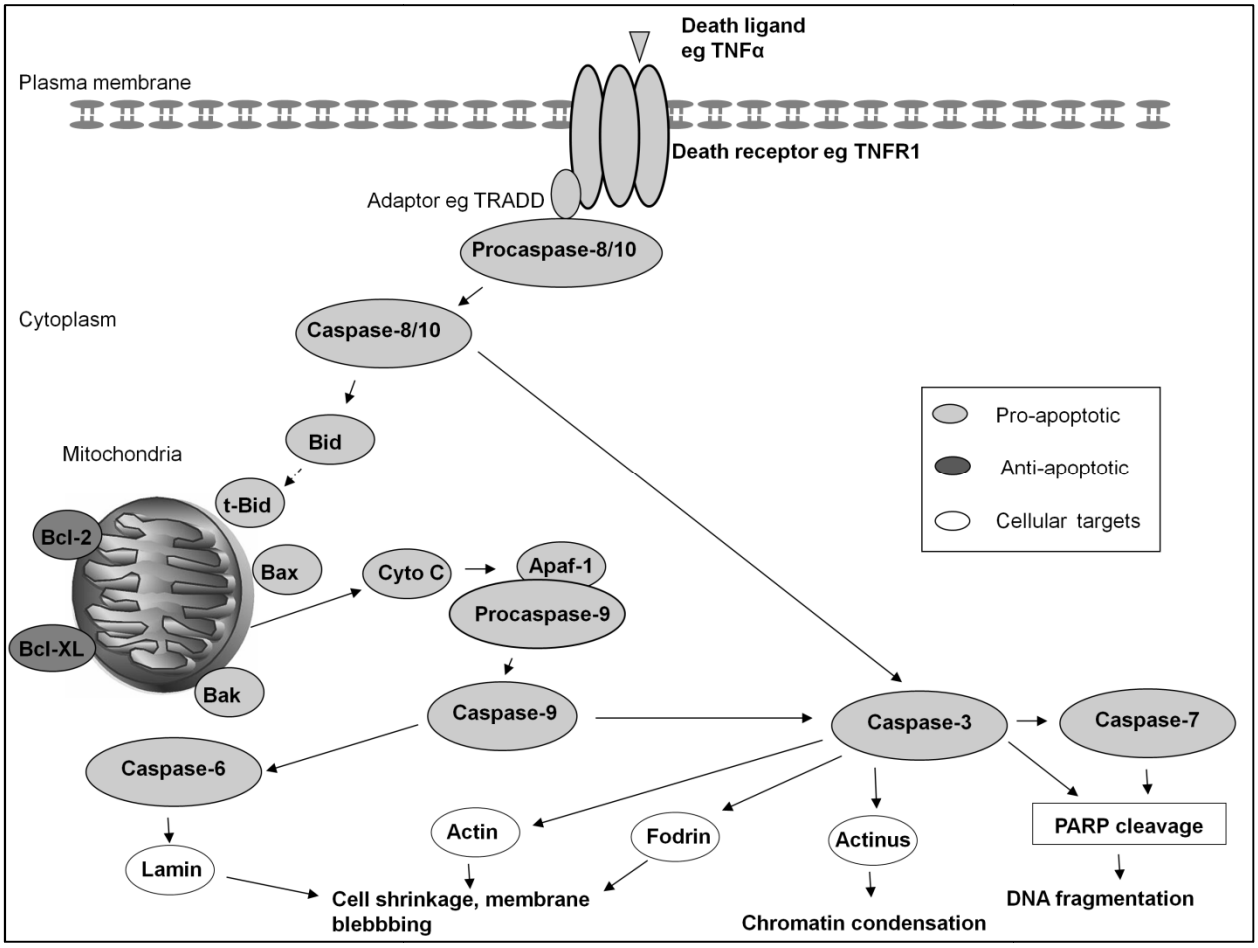

Figure 1.1: Overview of the extrinsic, receptor-mediated apoptotic pathway. The binding of a TNF family ligand to its relevant receptor results in the cleavage of procaspase-8 via the interaction of an adaptor protein in a death-inducing signalling complex (DISC). The subsequent caspase cascade converges on caspase-3 where it is interlinked with the intrinsic mitochondrial pathway. The end results of this pathway are cell shrinkage, membrane blebbing, DNA fragmentation and chromatin condensation leading to apoptosis (adapted from Klener Jr. *et al*., 2006).

Before death ligands can bind their respective receptors, these receptors must associate by means of their extracellular preligand assembly domain (PLAD) to form complexes (Chan *et al*., 2000; Jin and El-Deiry, 2005). Upon binding of the ligand, the receptor either undergoes an activating conformational change or forms higher order complexes in order to become "signal competent" (Jin and El-Deiry; Klener Jr. *et al*., 2006). Thereafter, a homophilic interaction occurs between the death domains of the receptor and an adaptor protein such as TNF-α-associated death domain (TRADD) and Fas-associated death domain (FADD) (Chinnaiyan *et al*., 1996). These adaptor proteins allow for the recruitment of procaspase-8 or FADD-like interleukin-1-convertin enzyme (FLICE) and procaspase-10 to the membrane via their death effector domains (DEDs) to form a death-inducing signalling complex (DISC) at the death

receptor (Kischkel *et al*., 2001; Seol *et al*., 2001). Procaspase-8 is then autocatalytically cleaved to form active caspase-8, which activates numerous downstream targets including caspase-3 and Bid, the cleavage of which represents the convergence point of the extrinsic and intrinsic apoptotic pathways (Chou *et al*., 1999; Klener Jr. *et al*., 2006). The pro-apoptotic regulator Bid is cleaved to a truncated form, t-Bid, which then translocates to the mitochondrial membrane and stimulates the release of pro-apoptotic effectors (Figure 1.2) (Chou *et al*., 1999).

1.2.1.2. The intrinsic/mitochondrial apoptotic pathway

The intrinsic pathway depicted in Figure 1.2 is initiated within cells in response to various cellular stresses including growth factor deprivation, osmotic shock, hypoxia, DNA damage, heat shock, UV radiation, cytotoxic agents, the accumulation of misfolded proteins and the disruption of cytoskeletal structures (Jin and El-Deiry, 2005; Klener Jr. *et al*., 2006; Iannolo *et al*., 2008). All of these stress stimuli converge on pivotal cellular events including mitochondrial outer membrane permeabilisation (MOMP), increased production of reactive oxygen species (ROS) and mitochondrial fission (Green and Kroemer, 2004; Jin and El-Deiry, 2005). Release of Ca^{2+} from the endoplasmic reticulum as a result cellular stress causes a rapid uptake of Ca^{2+} by the mitochondria. This, in turn results in the release of ROS and the opening of mitochondrial permeability transition (MPT) pores. The latter produces a massive mitochondrial inner transmembrane potential loss ($\Delta\Psi m$) and swelling which causes MOMP (Petrosillo *et al*., 2004; Klener Jr. *et al*., 2006). Thereafter, the outer membrane ruptures releasing cytochrome C and other pro-apoptotic molecules from the mitochondrial intermembrane space into the cytoplasm (Green and Kroemer, 2004; Jin and El-Deiry, 2005).

The changes in mitochondrial membrane permeability are both mediated and tightly controlled by Bcl-2 family members (Green and Kroemer, 2004). In mammals there are around 20 members of the Bcl-2 family, which are divided into three groups all of which contain between one and four Bcl-2 homology (BH) domains (Fesik, 2000; Klener Jr. *et al*., 2006). The first group consists of the anti-apoptotic molecules Bcl-2, Bcl-xL, Mcl-1 and A1, which have all four BH domains (BH1234) (Cheng *et al*., 2001; Jin and El-Deiry, 2005). Next are the pro-apoptotic effector Bcl-2 proteins, Bax, Bak and Bok, which contain three BH domains (BH123). The final group is made up of the pro-apoptotic damage sensing proteins, Bid, Bad, Bmf and Bim, which contain only the BH3 domain (Zong *et al*., 2001; Jin and El-Deiry, 2005).

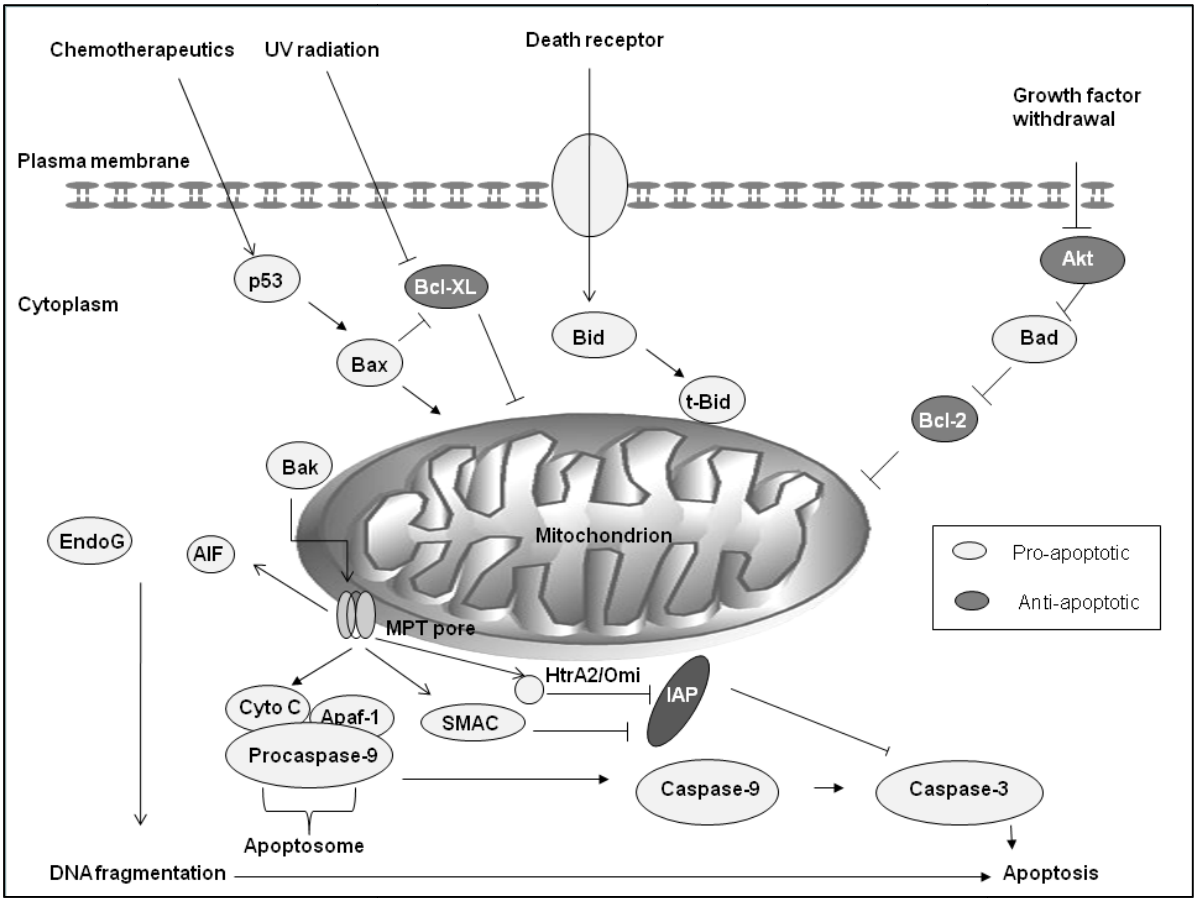

Figure 1.2: Overview of the intrinsic or mitochondrial apoptotic pathway. Stresses such as ultraviolet (UV) radiation or chemotherapeutic agents trigger the activation and accumulation of pro-apoptotic Bcl family proteins such as Bad, Bim, Bax and Bak. The latter two insert into the mitochondrial outer membrane causing mitochondrial permeability transition (MPT) pores from which death effectors such as cytochrome *c* (cyto C), procaspase-9, second mitochondrial activator of caspases (SMAC), HtrA2/Omi, endonuclease G (Endo G) and apoptosis initiating factor (AIF) are released to effect programmed cell death. Procaspase-9, cytochrome *c* and apoptotic protease-activating factor-1 (Apaf-1) form the apoptosome, which results in cleavage and activation of procaspase-9, which may then activate caspase-3 to initiate apoptosis. This process is inhibited by inhibitory apoptotic proteins (IAPs), which are themselves inhibited by SMAC and HtrA2/Omi. The effect of the survival pathway kinase, Akt, as well as intersection with the extrinsic pathway (death receptor) via the Bcl family protein, Bid, are also shown (adapted from Jin and El-Deiry, 2005).

Under stimulation by diverse death signals, certain BH3 family members are activated and induce oligomerization of BH123 proteins, allowing them to insert into the outer mitochondrial membrane and cause MOMP and release of pro-apoptotic factors. In addition, other BH3-only proteins act by releasing BH123 proteins from the anti-apoptotic Bcl-2 proteins which bind and inhibit them (Newmeyer and Ferguson-Miller, 2003). Cell death as a result of MOMP occurs in two ways; 1) by the release of soluble intermembrane proteins (SIMPs), such as cytochrome *c*

and 2) through disruption of mitochondrial function (Green and Kroemer, 2004). Once released into the cytoplasm, cytochrome *c*, in the presence of ATP, binds to apoptotic protease-activating factor-1 (Apaf-1) and undergoes a conformational change (Finucane *et al*., 1999; Klener Jr. *et al*., 2006). The caspase adaptor, Apaf-1, then recruits procaspase-9 to form a holoenzyme complex called the "apoptosome," which activates downstream executioner caspases including caspase-3, 6 and 7 (Figure 1.2) (Iannolo *et al*., 2008).

In addition to cytochrome *c*, the pro-apoptosis proteins, apoptosis inducing factor (AIF) and second mitochondrial activator of caspases/direct IAP binding protein with low pI (SMAC/DIABLO) and endonuclease G (endoG), are released from the mitochondrial intermembrane space upon MOMP (Klener Jr. *et al*., 2006). Upon release of AIF into the cytoplasm it migrates to the nucleus and induces chromatin condensation and large scale DNA fragmentation. Similarly, EndoG moves to the nucleus and facilitates oligonucleosomal DNA fragmentation (Susin *et al*., 2000). The role of SMAC/DIABLO and the related protein, HtrA2/Omi, is to competitively bind inhibitory apoptotic proteins (IAPs), thus preventing binding and inhibition of caspases (Figure 1.2) (Du *et al*., 2000; Jin and El-Deiry, 2005).

In the final stages of apoptosis, the cell is "dismantled" step by step, displaying DNA fragmentation; proteolysis of structural proteins such as lamin, actin and cytokeratin; and the formation of apoptotic vesicles from the shrinking cell (Savill and Fadok, 2000; Klener Jr. *et al*., 1997). During these final events, phosphotidylserine moves from the inner to the outer cell membrane. This serves as a signal to phagocytes, resulting in engulfment of the apoptotic cell. The efficient recognition and clearance of apoptotic cells is essential to prevent leakage of potentially cytotoxic cell contents and to maintain tissue homeostasis (Fadok *et al*., 1998; Jin and El-Deiry, 2005).

1.2.2 Targeting of the apoptotic pathway in cancer treatment

In recent years, the focus in the development of anti-cancer drugs which induce apoptosis has moved away from the non-specific DNA-damaging agents towards the targeting of individual molecules of the cell death machinery (Debatin and Krammer, 2004; Speir *et al*., 2011). In particular, studies have focused on the use of death ligands, SMAC agonists and both IAP and Bcl-2 anti-sense therapy (Ashkenazi *et al*., 1999; Debatin and Krammer, 2004; Jin and El-Deiry, 2005; Speir *et al*., 2011).

In terms of death ligands, research has been carried out on the suitability of TNF, CD95L and TRAIL as potential anti-cancer drugs (Nagata, 1997; Ashkenazi *et al*., 1999; Debatin and Krammer, 2004). This is an attractive option in cancer treatment since it triggers an apoptotic pathway that is independent of p53, a tumour suppressor which is the most commonly

mutated gene in human cancer (El-Deiry, 2001). Disappointingly, the prototypic death ligands, TNF and CD95L, showed high toxicity in clinical trials, with administration of TNF resulting in septic shock and CD95L treatment leading to hypertension, liver failure and, ultimately, death (Nagata, 1997; Jin and El-Deiry, 2005). On the other hand, TRAIL was able to induce apoptosis in a wide range of human cell lines, while displaying little toxicity, making it a more promising candidate for clinical application (Ashkenazi *et al*., 1999; Debatin and Krammer, 2004). Furthermore, what potential toxicity exists for this ligand, particularly in hepatocytes and brain tissue, can be overcome by removing the His-tag and optimizing its zinc content to prevent the formation of aggregates within the cells (Jo *et al*., 2000; LeBlanc and Ashkenazi, 2003). In this form, recombinant TRAIL is able to induce apoptosis *in vitro* in colon, prostate, breast, lung and pancreatic carcinoma; as well as *in vivo* in mouse xenograft models of colon and breast carcinoma, multiple myeloma and malignant glioma (Ashkenazi *et al*., 1999; Walczak *et al*., 1999; Chuntharapai *et al*., 2001; Ichikawa *et al*., 2001; LeBlanc and Ashkenazi, 2003). Although a large number of tumours are refractory to TRAIL, combination with chemotherapy and radiation is able to sensitize these cells to treatment with TRAIL (Chinnaiyan *et al*., 2000). TRAIL remains the central extrinsic apoptotic pathway-specific cancer treatment to date (Speir *et al*., 2011).

Anti-sense therapies targeted towards the anti-apoptotic molecules up-regulated in various tumours have received considerable interest as a means of pharmacological intervention in cancer treatment (Debatin and Krammer, 2004). In particular, Bcl-2 has been targeted due to its role in resistance to apoptosis induction by cytotoxic drugs (Reed, 1999). The anti-sense oligonucleotide G3139 (Genasense), which suppresses *bcl-2* expression, has been tested with some success both *in vitro* and *in vivo,* with clinical trials being carried out in Hodgkin's lymphoma, leukemia, multiple myeloma, prostate and lung cancers (Waters *et al*., 2000; Chi *et al*., 2001; Rudin *et al*., 2002). The IAP family of caspase inhibitors, in particular XIAP and survivin, have also been targeted by anti-sense therapy due to their overexpression in a number of tumours, including breast cancer, melanoma, leukemia and neuroblastoma (Debatin and Krammer, 2004). These agents have been shown to resensitize cells to both chemotherapeutic drugs and death receptor stimulation (Tamm *et al*., 2003).

1.2.3 Regulation of apoptosis

In line with its classification as a programmed form of cell death, apoptosis is stringently regulated (Jin and El-Deiry, 2005). This occurs not only via the balance of pro-apoptotic and anti-apoptotic Bcl-2 family mitochondrial-associated proteins, but also due to the interaction of numerous apoptotic signal transducers with elements of survival pathways such as the mitogen-activated protein kinase (MAPK) systems and the Akt/protein kinase B (PKB) pathway (Green and Kroemer, 2004; McCubrey *et al*., 2007; LoPiccolo *et al*., 2008).

1.3 Alterations in cellular survival pathways associated with cancer

In normal healthy tissues, survival pathways allow for cells to grow and proliferate in a way that ensures homeostasis, provided that these pathways are tightly controlled (Finnberg and El-Deiry, 2004; Grant, 2008). However, when survival cascades are aberrantly activated, cells replicate out of control and expand beyond their organ boundaries, which may result in diseases such as cancer (Finnberg and El-Deiry, 2004; Song *et al*., 2005). It has been reported that pathway activation is one of the most frequent molecular mutations in human cancers and represents an early event in carcinogenesis (Granville *et al*., 2006; LoPiccolo *et al*., 2008). Two of the most frequently dysregulated signal transduction cascades in cancer are the Raf/MEK (mitogen-activated ERK kinase)/ERK (extracellular signal-related kinase) and the Akt/PI3K (phosphatidy-linositide-3-kinase)/PTEN (phosphatase and tensin homologue deleted on chromosome 10) pathways (Grant, 2008).

1.3.1 The MAP kinase signalling pathways

The mitogen-activated protein kinase (MAPK) pathways are highly conserved kinase modules which are involved in mediating cellular responses to almost all environmental stimuli to facilitate membrane, cytoplasmic and nuclear signalling (Dhanasekeran and Johnson, 2007; Dhillon *et al*., 2007). Cellular processes regulated by MAPKs include growth, proliferation, differentiation, migration, apoptosis, chromatin remodeling and angiogenesis (Dunn *et al*., 2005; Yoon and Seger, 2006; Dhillon *et al*., 2007). All MAPK pathways are composed of a three-tier kinase module where a MAPK is phosphorylated and activated by a MAPK kinase (MAPKK) which in turn is activated by a MAPKK kinase (MAPKKK) (Figure 1.3) (Dhanasekeran and Johnson, 2007; Dhillon *et al*., 2007). At least eleven MAPKs, seven MAPKKs and 20 MAPKKKs have been reported (Dhanasekeran and Johnson, 2007). In mammals, the existing MAP kinases are classified into six groups, namely extracellular signal-regulated kinases (ERK) ERK1/2, ERK3/4, ERK5, ERK7/8, c-Jun N-terminal kinase (JNK)1/2/3 and the p38 isoforms α/β/γ(ERK6)/δ (Figure 1.3) (Schaeffer and Weber, 1999; Chen *et al*., 2001).

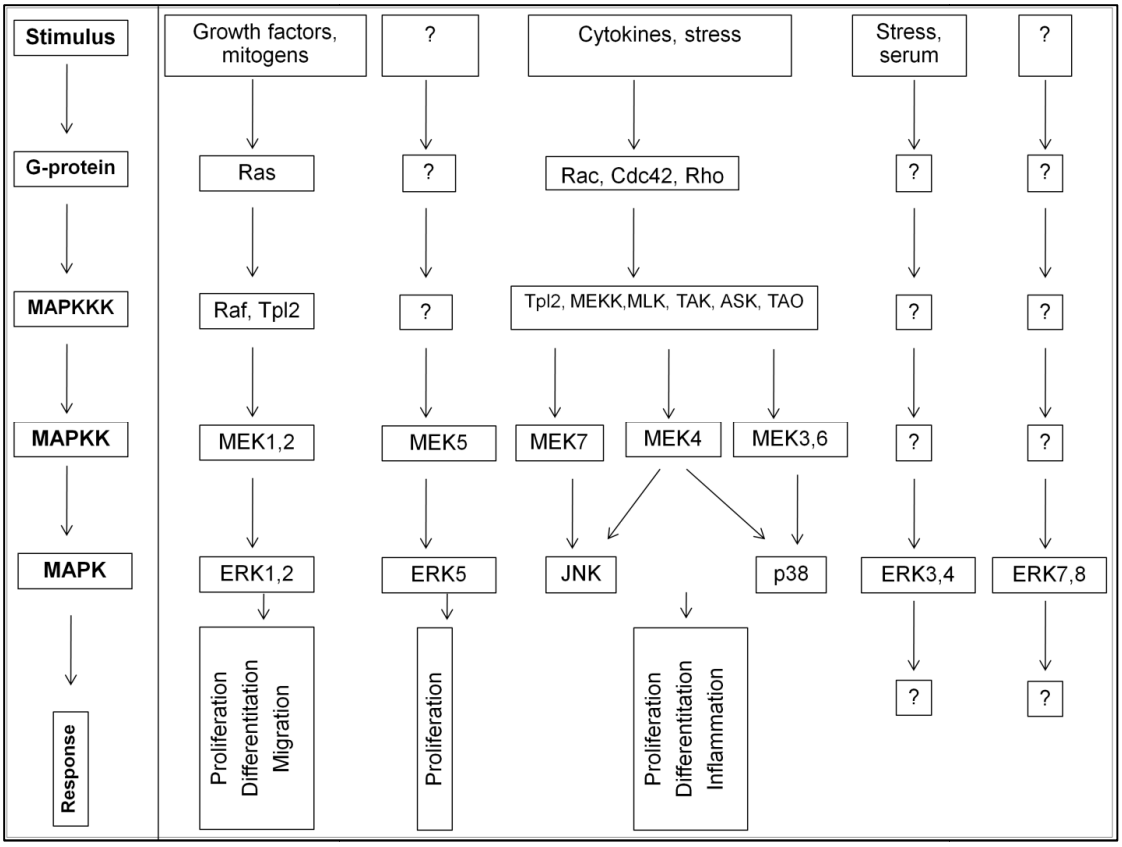

Figure 1.3: Overview and classification of the mitogen-activated protein kinase (MAPK) pathways in mammals. For each pathway, a MAPK such as extracellular signal-related kinase (ERK) is phosphorylated by a MAPK kinase (MAPKK) such as mitogen-activated ERK kinase (MEK) which in turn is activated by a MAPKK kinase (MAPKKK) such as Raf. The pathways are activated by a particular stimulus which activates a GTP-binding protein (G protein) associated with a particular cell surface receptor (Adapted from Dhillon *et al*., 2007).

The ERK pathway, depicted in Figure 1.4 is the best-studied of the MAPK pathways (Gough, 2011). In this pathway, ERK1/2 is phosphorylated by a mitogen-activated ERK kinase (MEK), MEK1/2, which is phosphorylated by Raf (Raf-1, A-Raf, B-Raf). The binding of growth factors and mitogens to receptor tyrosine kinases causes Ras to exchange GDP for GTP, allowing it to recruit Raf to the membrane for activation (reviewed in Dhillon *et al*., 2007). Ras GTPases function as molecular switches which regulate signalling via a number of pathways including the PI3K/Akt system, in addition to ERK (Downward, 2003; Dhillon *et al*., 2007). Four Ras proteins, Ha-Ras, N-Ras, Ki-Ras 4A and Ki-Ras 4B, have been reported (Yan *et al*., 1998; McCubrey *et al*., 2007). Ki-Ras shows a greater ability to activate the ERK pathway, whereas Ha-Ras shows a preference for the PI3K/Akt pathway (Yan *et al*., 1998; McCubrey *et al*., 2007).

Activation of Raf is complex and involves a series of five events, namely (1) recruitment to membrane by Ras (Yan *et al*., 1998), (2) dimerization (Luo *et al*., 1996), (3), phosphorylation or dephosphorylation of various domains (Fabian *et al*., 1993), (4) dissociation from the Raf kinase inhibitory protein (RKIP) (Yeung *et al*., 1999), (5) association with scaffolding proteins such as kinase suppressor of Ras (KSR) and SUR-8/SHOC-2, which modulate the activation of Raf-1 by Ras (Li *et al*., 2000; Lee Jr. and McCubrey, 2002). There are an impressive thirteen regulatory phosphorylation sites on Raf-1, which fine tune the activity of this serine/threonine kinase (McCubrey *et al*., 2007). Activity is further regulated by chaperones such as Bag1, 14-3-3 protein and heat shock protein 90 (Hsp90) (Fontl *et al*., 1994; Blagosklonny, 2004).

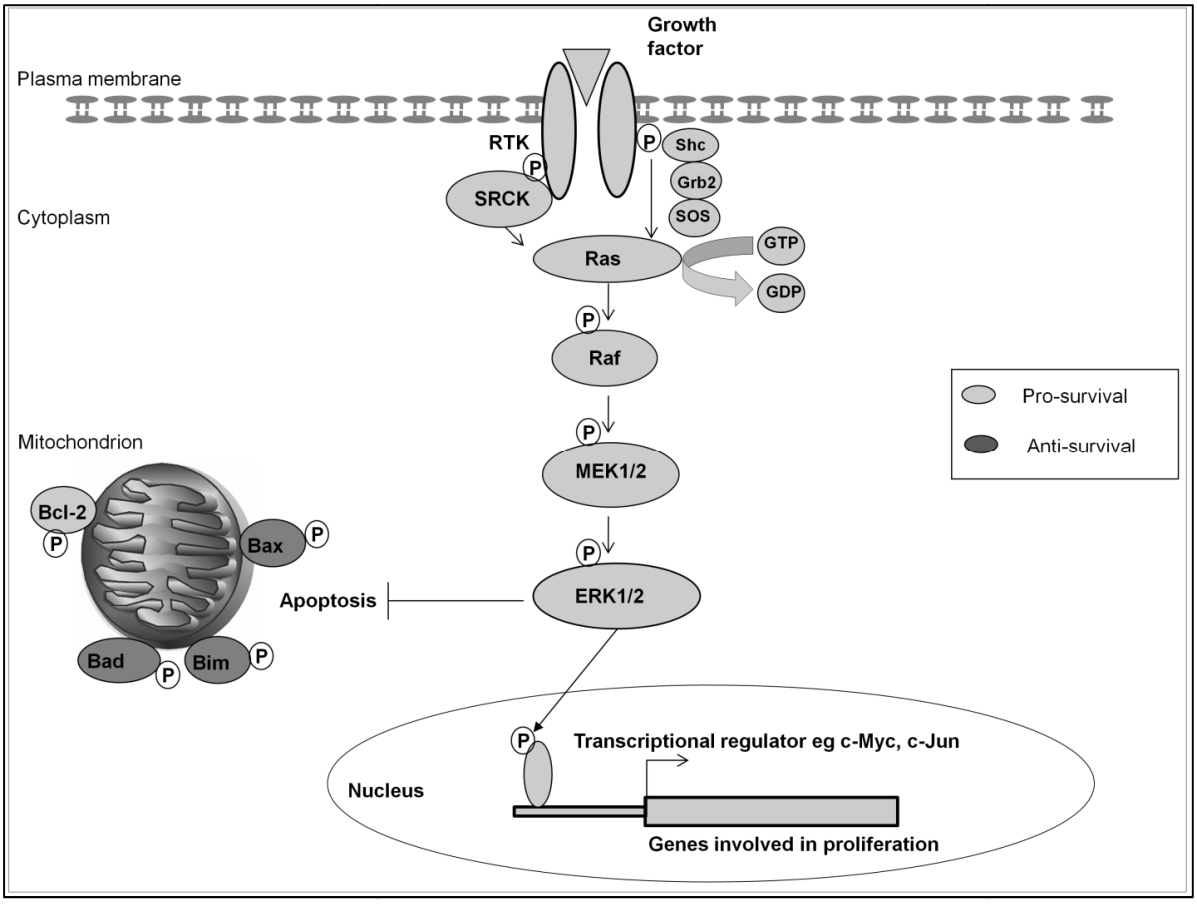

Figure 1.4: Schematic representation of the Raf/MEK/ERK pathway, the best studied of the MAPK signalling systems. Upon binding of a growth factor to its specific receptor, phosphorylation by the receptor (receptor tyrosine kinase, RTK) of either Ras, via a Src homology (SH)-2 containing protein -growth factor receptor bound 2-son of Sevenless (Shc-Grb2-SOS) coupling complex, or a SRC family kinase (SRCK) occurs. Either of these kinase enzymes may then phosphorylate and activate Raf. The kinase cascade continues via MEK1/2 and ERK1/2, before entering the nucleus and activating various transcription factors such as c-Myc and c-Jun to alter the pattern of gene expression. ERK1/2 also alters the activity of various mitochondrial apoptotic regulatory proteins in order to prevent apoptosis (modified from McCubrey *et al*., 2007).

Activated Raf phosphorylates MEK1 and MEK2 on conserved Ser218 and Ser222 residues within the catalytic domain in order to stimulate their dual specificity tyrosine and serine/threonine kinase activity (Alessi *et al*., 1994; McCubrey *et al*., 2007; Dhillon *et al*., 2007). While all three Raf isoforms are capable of activating MEK, they vary in the strength of their MEK kinase activity with B-Raf being the strongest activator, followed by Raf-1 and then A-Raf (Marais *et al*., 1997). Another MEK phosphorylation site, Ser212, has been reported to negatively regulate MEK activity (Gopalbhaj *et al*., 2003).

The downstream targets of MEK1 and MEK2 are ERK1 and ERK2, which phosphorylate more than 160 cytoplasmic and nuclear targets, including kinases, phosphatases, transcription factors and cytoskeletal elements (Yoon and Seger, 2006; McCubrey *et al*., 2007; Gough, 2011). ERK can enter the nucleus and directly phosphorylate transcription factors such as c-Myc, c-Jun and Ets-1; or it may activate the ribosomal kinase, $p90^{Rsk}$, which in turn activates the transcription factor, CREB (cAMP-response element binding protein) (Steelman *et al*., 2004; McCubrey *et al*., 2007).

In line with its role as a survival pathway, Raf/MEK/ERK interacts with a number of apoptotic regulatory molecules in order to prevent apoptosis (described in Section 1.2 above) (McCubrey *et al*., 2007). The first apoptotic molecule found to be influenced by this pathway was Bad, a mitochondrial-associated pro-apoptotic regulator (Zha *et al*., 1996). Phosphorylation of Bad on Ser112 by the Raf/MEK/ERK pathway results in its inactivation and sequestration by the chaperone protein, 14-3-3. As a result, the anti-apoptotic protein, Bcl-2, is able to dimerize and inhibit apoptosis. In addition, the pro-apoptotic regulator, Bim, is phosphorylated by ERK on Ser69 and targeted for degradation, allowing for the activation of the anti-apoptotic proteins, Bcl-2, Bcl-X_L and Mcl-1. This, in turn, prevents the dimerization and activation of the pro-apoptotic protein, Bax (Harada *et al*., 2004; McCubrey *et al*., 2007). Apart from mitochondrial apoptotic proteins, it has been shown that the Raf/MEK/ERK pathway can modulate the activity of the cytoplasmic executioner, caspase-9. Phosphorylation of caspase-9 on Tyr125 results in its inactivation, such that apoptosis is prevented (Allan *et al*., 2003). Caspase-9 may also be inactivated by Akt, highlighting the cross-talk between the Raf/MEK/ERK and PI3K/Akt survival pathways (McCubrey *et al*., 2007).

1.3.1.1 The role of Raf/MEK/ERK pathway mutations in cancer

At least 25 small molecule inhibitors targeting the MAPK pathways have reached clinical trials to date (Santarpia *et al*., 2012). The best studied of these pathways, the Raf/MEK/ERK system, is deregulated in one-third of all human cancers and has been the focus of anti-cancer drug discovery for at least 15 years (Dhillon *et al*., 2007). The rationale for targeting this pathway is that it is the convergence point for most, if not all, mitogenic signalling pathways (Kohno and

Pouyssegur, 2003). In addition, inhibition of the ERK pathway produces anti-metastatic effects, due to the effect of the pathway on motility in response to hepatocyte growth factor (HGF), and anti-angiogenic effects, as a result of stimulation of vascular endothelial growth factor (VEGF), a powerful angiogenic factor (Kohno and Pouyssegur, 2003). The main causes of constitutive activation of the pathway are mutations in components which lie upstream of ERK and thus the major targets for pathway inhibitors are Raf and MEK (Figure 1.5) (Dhillon *et al*., 2007).

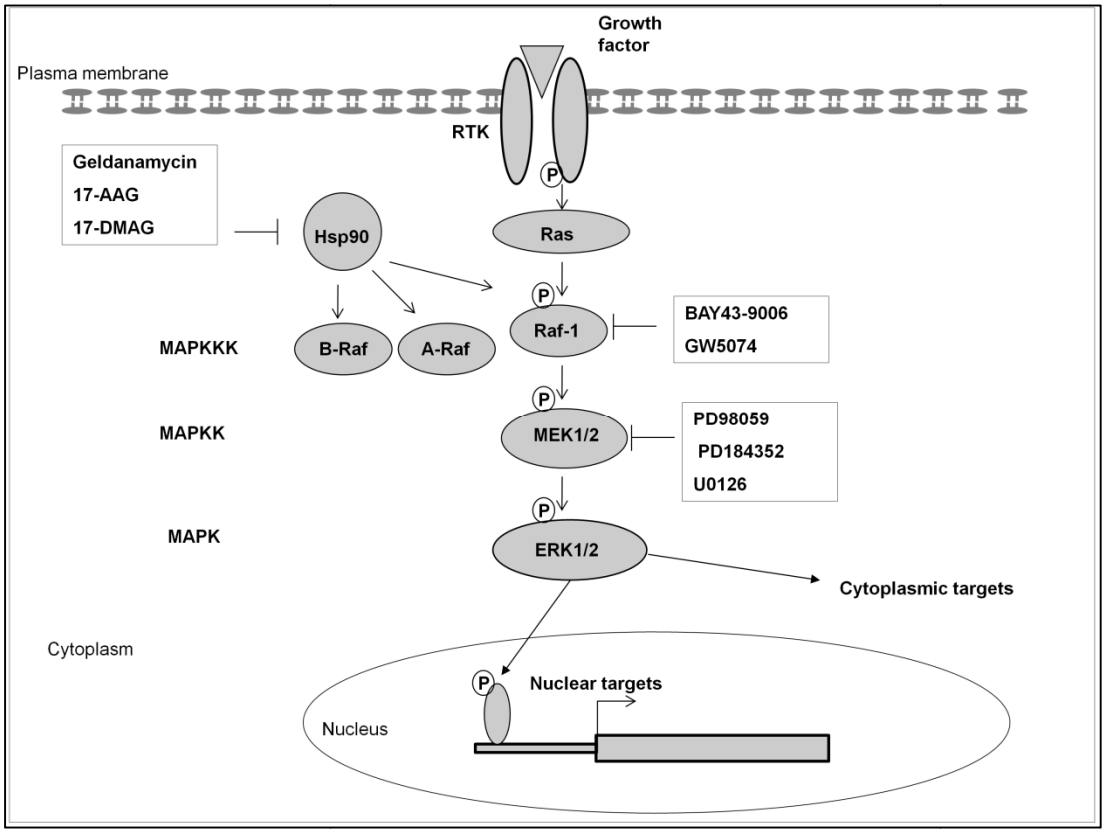

Figure 1.5: Targeting the Raf/MEK/ERK survival pathway in cancer treatment. Inhibitors have been developed to inhibit either Raf-1, all Raf isoforms via inhibition of the chaperone Hsp90 (geldanamycin, 17-AAG, 17-DMAG), or the MEK protein (modified from Kohno and Pouyysegur, 2003).

1.3.1.2 The development of specific Raf/MEK/ERK inhibitors

It has been reported that *B-Raf* is mutated in 7 % of all cancers, in particular such mutations are frequent in melanomas, as well as thyroid, colorectal and ovarian cancers (Garnett and Marais, 2004; Davies *et al*., 2002; McCubrey *et al*., 2007). Two powerful Raf-1 inhibitors, namely GW5074 and BAY43-9006 (Sorafenib) (Figure 1.6A and B, respectively), have been developed, both of which act as competitive inhibitors of the ATP-binding site of Raf (Lackey *et al*., 2000; Lyons *et al*., 2001; Kohno and Pouyssegur, 2003). These compounds display *in vitro* IC_{50} values

(concentration at which cell survival is 50 % of that of an untreated control) of 9 nm and 12 nm for GW5074 and BAY43-9006, respectively (Lackey *et al*., 2000; Lyons *et al*., 2001). In addition, *in vivo* inhibitory effects have been noted for BAY 43-9006 in colon, pancreatic and lung carcinomas and clinical trials in various locally advanced and metastatic cancers are underway, an example of which is the Phase II trial combining BAY 43-9006 with transarterial chemoembolization (TACE) and doxorubicin-eluting beads (DEB) in patients with advanced hepatocellular carcinoma (Lyons *et al*., 2001; Kohno and Pouyysegur, 2003; Pawlik *et al*., 2011). An alternate approach to targeting Raf is the use of specific anti-sense oligonucleotides which hybridize to the 3' UTR of the mRNA, thus inhibiting translation of a functional Raf enzyme (Monia *et al*., 1996; Kohno and Pouyysegur, 2003). The 20-base pair phosphorothioate oligodeoxynucleotide, ISIS 5132, is such a compound and displays IC_{50} values of between 50 and 100 nm *in vitro* in breast, lung and bladder cell lines, with tumour reduction being noted *in vivo* as well as early clinical trials (O'Dwyer *et al*., 1999; Stevenson *et al*., 1999; Fidias *et al*., 2009). An example of such a clinical trial is the Phase I study using ISIS 5132 in combination with carboplatin and paclitaxel in patients with advanced non-small cell lung cancer (Fidias *et al*., 2009).

Whereas the above agents target specific Raf isoforms, it is possible to inhibit all three isoforms using geldanamycin. This drug is a highly specific inhibitor of the molecular chaperone, heat-shock protein 90 (Hsp90), which is, in turn, an obligatory chaperone of the Raf enzymes (Chiosis, 2006; Dhillon *et al*., 2007). Raf is activated upon dimerization, a process which requires Hsp90; therefore inhibition of Hsp90 blocks activation of Raf by preventing these essential protein-protein interactions (Blagosklonny, 2002; McCubrey *et al.*, 2007). Geldanamycin and its derivatives (Figure 1.6C) have shown promising results as anti-cancer drugs in clinical trials for a variety of tumour types (Kim *et al*., 2009).

Since MEK1 and MEK2 interact with numerous upstream kinases but only ERK1 and ERK2 as downstream substrates, this convergence point is an attractive therapeutic target in the development of pathway-specific inhibitors (Kohno and Pouyssegur, 2003). The first MEK inhibitor to be identified was PD98059, followed by U0126 and the second generation inhibitor, PD184352 (Figure 1.6 D-F), the latter of which displays an IC_{50} value of 2 nm (Dudley *et al*., 1995; Favata *et al*., 1998; Sebolt-Leopold *et al*., 1999; Davies *et al*., 2000). These agents inhibit MEK indirectly by preventing phosphorylation by Raf (Kohno and Pouyysegur, 2003). In addition, a number of resorcylic acid lactones have been isolated from microbes, which display powerful anti-MEK activity. Examples include Ro 09-2210, which was isolated from fungal broth and L-783,277, extracted from a *Phoma* species (Figure 1.6 G and H, respectively). Here, inhibition is by competitive binding of ATP, with IC_{50} values of 60 nm and 4 nm for Ro 09-2210 and L-783,277, respectively (Williams *et al*., 1998). Finally, Compound 14, a 3-cyano-4-

(phenoxyanilino) quinolone (Figure 1.6I) was developed by Wyeth-Ayers and inhibits MEK *in vitro* with an IC_{50} value of 2.4 nm in human colon carcinoma cell lines (Zhang *et al*, 2000).

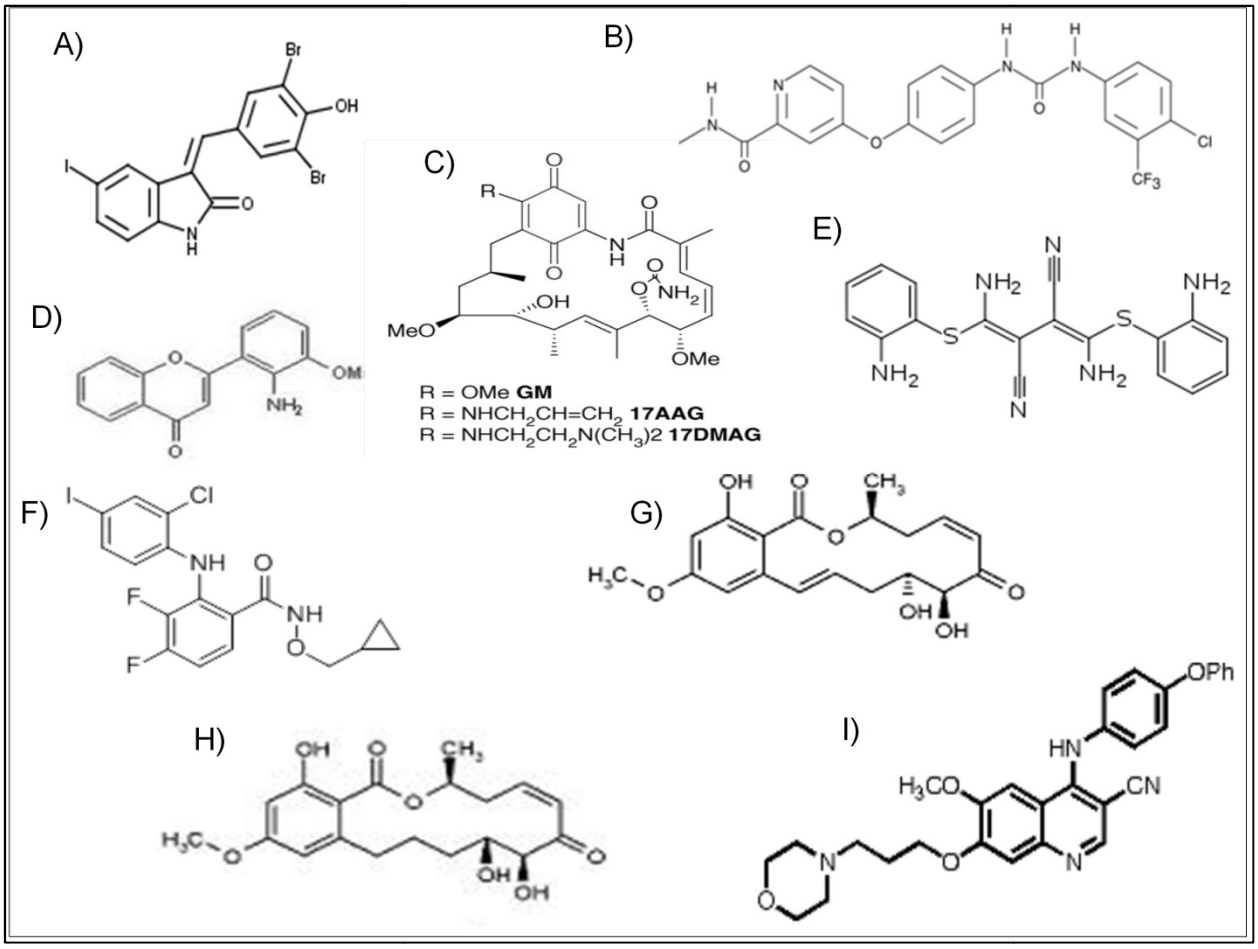

Figure 1.6: Chemical structures of several inhibitors of the Raf/MEK/ERK pathway. Raf-1-specific inhibitors include GW5074 (A) and BAY43-9006 (B). Inhibition of all three Raf isoforms has been achieved using the Hsp90 inhibitor geldanamycin and its analogues 17AAG and 17DMAG. Specific inhibitors of MEK1 and MEK2 include PD98059 (D), U0126 (E), the second generation inhibitor, PD184352 (F), the resorcylic acid lactones, Ro 09-2210 (G) and L-783,277 (H) and the 3-cyano-4-(phenoxyanilino)quinolone, Compound 14 (I), developed by Wyeth-Ayers (modified from Kohno and Pouyysegur, 2003).

It was only recently that specific inhibitors of ERK1/2, targeting the docking domain of the kinase, were reported, although their development has not yet reached clinical trials (Boston *et al*., 2011). Other promising strategies to target this pathway include the use of small interfering RNA (siRNA), which are roughly 19 nucleotides long and extremely powerful agents for the destruction of specific mRNA transcripts (Elbashir *et al*., 2001).

1.3.2. The Akt/PKB signal transduction pathway

The Akt pathway depicted in Figure 1.7 is a key survival pathway which regulates a number of processes including nutrient metabolism, cell growth, proliferation and survival (Granville *et al*., 2006; LoPiccolo *et al*., 2008). Activation of this pathway occurs at the cytoplasmic membrane (Andjelkovic *et al*., 1997; Song *et al*., 2005). Various growth factors, such as insulin-like growth factor (IGF) and epidermal growth factor (EGF), bind to their receptors, resulting in the activation of the lipid kinase PI3K (Burgering and Coffer, 1995; Robertson, 2005; LoPiccolo *et al*., 2008). Following activation, PI3K catalyzes the phosphorylation of phosphoinositides to produce the lipid second messengers, phosphoinositide-4,5-bisphosphate (PIP_2) and phosphoinositide-3,4,5-tri- phosphate (PIP_3) (Vanhausebroek and Alessi, 2000; Robertson, 2005). PIP_3 is able to recruit various proteins, such as Akt and PDK-1 (phosphoinositide-dependent kinase-1) to the cell membrane via their pleckstrin homology (PH) domains (Lemmon and Ferguson, 2000). This process is tightly regulated by the dual protein and lipid phosphatase, PTEN, which antagonizes PI3K by dephosphorylating 3'-phosphoinositides (Maehama *et al*., 1998; Blanco-Aparicio, 2007).

Activation of Akt occurs by two site-specific phosphorylation events at both its conserved threonine and serine residues (Alessi *et al*., 1996). Upon production of PIP_3 by PI3K, both Akt and PDK-1 are translocated to the cell membrane. Here, Akt is phosphorylated by PDK-1 at the conserved threonine residue within the central kinase domain of Akt (Alessi *et al*., 1996; Robertson, 2005). Full activation of Akt requires the additional phosphorylation of the conserved serine residue within the hydrophobic motif of its C-terminal domain (Alessi *et al*., 1996; LoPiccolo *et al*., 2008). This phosphorylation may be carried out by PDK-1, its homologue PDK-2, an integrin linked kinase (ILK), a DNA-dependent protein kinase or by Akt itself via its kinase domain (Stephens *et al*., 1998; Robertson, 2005; Song *et al*., 2005; LoPiccolo *et al*., 2008). Dephosphorylation of Akt by phosphatases such as protein phosphatase 2A (PP2A) results in the deactivation of Akt, thereby modulating downstream signalling by this key protein (Millward *et al*., 1999).

Activated Akt has numerous downstream targets, including those involved in the regulation of transcription, translation, cell cycle progression, glucose metabolism, apoptosis and many other cellular processes (Figure 1.7) (Robertson, 2005; Granville *et al*., 2006). Akt exerts its effect by phosphorylation of target proteins at a consensus sequence, RXRXX(S/T), that is surrounded by hydrophobic amino acids (Obenhauer *et al*., 2003). The best-characterised of the Akt targets is the mammalian target of rapamycin (mTOR) (Chiu *et al*., 1994). mTOR controls a variety of important cellular processes such as protein synthesis and degradation, transcription, protein kinase C signalling, cell cycle progression and angiogenesis (Sabers *et al*., 1995; Panwalkar *et al*., 2004; Granville *et al*., 2006).

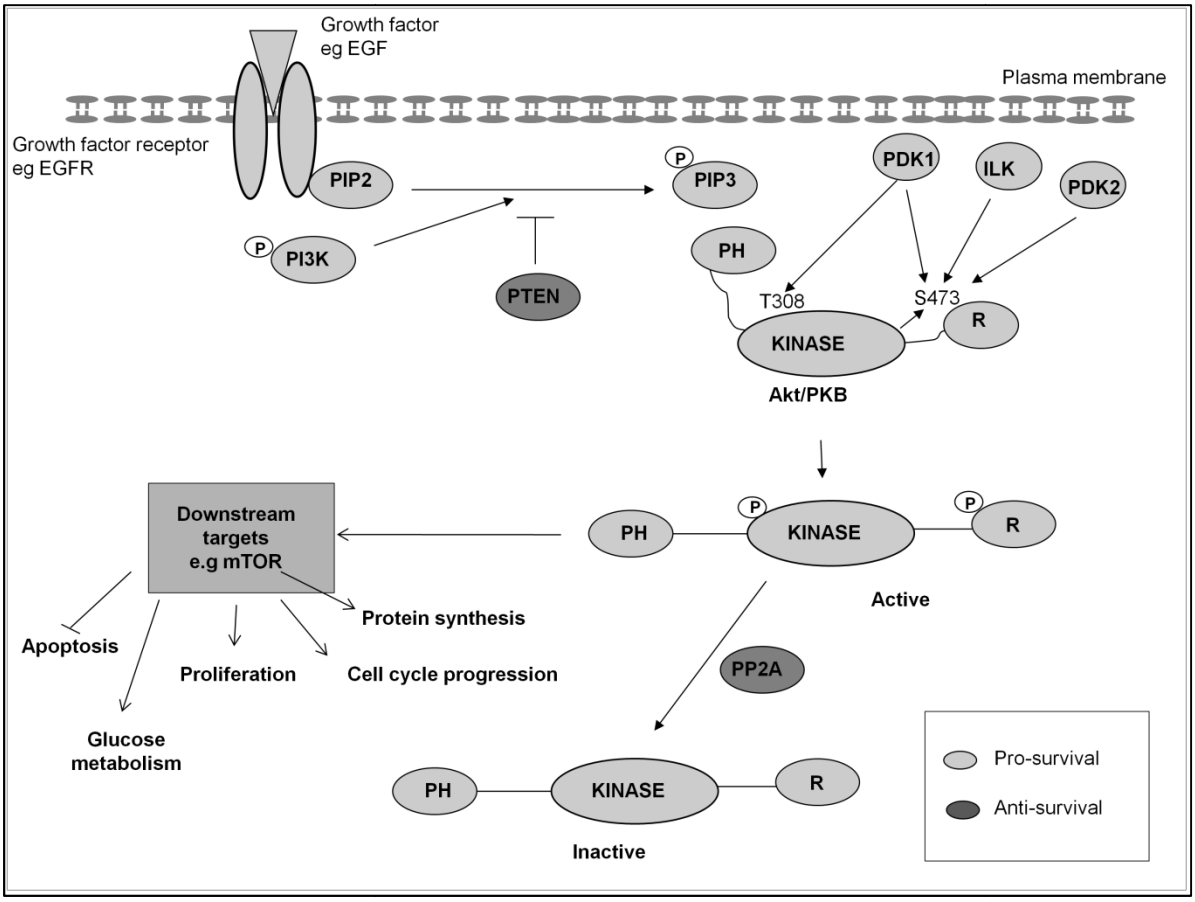

Figure 1.7: Cellular mechanism of activation of Akt. The binding of a growth factor to a particular receptor phosphorylates and activates PI3 kinase (PI3K), which phosphorylates PIP_2 (phosphoinositide-4,5-bisphosphate) to produce PIP_3 (phosphoinositide-1,4,5-triphosphate). This stimulates the phosphorylation and activation of Akt at the conserved threonine 308 (T308) residue in its kinase domain by phosphoinositide-dependent kinase (PDK1). This is followed by phosphorylation at the conserved serine 473 (S473) within the regulatory domain (R) of Akt by PDK1, PDK2, integrin linked kinase (ILK) or Akt itself. Akt is recruited to the plasma membrane via its pleckstrin homology (PH domain). Akt acts upon downstream targets such as the mammalian target of rapamycin (mTOR), so as to stimulate protein synthesis, proliferation, cell cycle progression and glucose metabolism, while inhibiting apoptosis (Modified from Song *et al*., 2005).

Other major functions of Akt include the regulation of nutrient metabolism and the evasion of apoptosis. The former effect is mediated via phosphorylation and inhibition of glycogen synthase kinase-3 (GSK-3), freeing glycogen synthase and resulting in storage of glucose as glycogen (Cross *et al*., 1995; Song *et al*., 2005). The inhibition of GSK-3 also has an effect on cell cycle progression, where inactivation of GSK-3 stabilizes cyclin D1 allowing the cell to advance through the cell cycle stages (Deihl *et al*., 1998; LoPiccolo *et al*., 2008). Cell cycle progression is stimulated via Akt through the phosphorylation and inhibition of the cyclin-dependent kinase (CDK) inhibitors, $p21^{WAF1/CIP1}$ and $p27^{KIP}$ (Zhou *et al*., 2001; Liang *et al*., 2002). In the latter effect,

activated Akt acts as a "survival signal" and allows cells to evade apoptosis. Examples of apoptotic molecules whose activity is modulated by Akt are the pro-apoptotic proteins, Bad and Caspase-9, whose ability to induce apoptosis is attenuated as a result of phosphorylation by activated Akt (Datta *et al*., 1997; del Peso *et al*., 1997; Donepudi *et al*., 2002; LoPiccolo *et al*., 2008).

1.3.2.1 Deregulation of the Akt pathway in cancer

The Akt/PKB (protein kinase B) survival cascade is subject to strict control via the C-terminal regulatory domain of Akt, which contains two regulatory phosphorylation sites (Alessi *et al*., 1996). The phosphorylation of these sites depends on the amount of PIP_3 generated by PI3 kinase, a process which is regulated by PTEN (Maehama *et al*., 1998). Loss of PTEN function is thus the most common cause of constitutive activation of the pathway and has been observed in human cancers of the brain, bladder, prostate, breast and endometrium (Li *et al*., 1997; Rasheed *et al*., 1997; Ali *et al*., 1999; Aveyard *et al*., 1999; Dahia, 2000; Dreyer *et al*., 2004; LoPiccolo *et al*., 2008). This may occur by mutation, deletion or epigenetic silencing of *PTEN* and is associated with poor prognosis (Smith *et al*., 2001; Bertram *et al*., 2006; LoPiccolo *et al*., 2008). Other events which trigger pathway dysregulation include overexpression and activation of growth factor receptors, PI3K and Akt (LoPiccolo *et al*., 2008). Alterations in PI3K activity occur by amplification, overexpression or mutations have been observed in 40 % of ovarian and 50% of cervical cancers (Shayesteh *et al*., 1999; Ma *et al*, 2000). Furthermore, it has been found that phosphorylation of the Ser473 residue of Akt is associated with poor prognosis in a wide range of human cancers (LoPiccolo *et al*., 2008).

1.3.2.2 Targeting the Akt survival pathway in cancer treatment

The Akt pathway is an attractive target in cancer treatment as it serves as a convergence point for numerous growth stimuli as well as controlling cellular processes which contribute to tumour development and maintenance via its large array of downstream substrates. As a result, a number of small molecule inhibitors of this pathway are in clinical trials. The main targets for pathway-specific therapies are PI3K, Akt and mTOR (Figure 1.8). These inhibitors are often combined with one another, with chemotherapy or radiation and with inhibitors of other pathways (LoPiccolo *et al*., 2008).

Research has shown that targeting of the pathway elements which lie furthest upstream is advantageous for inhibiting more distal components, as this has a broader effect on downstream signalling (LoPiccolo *et al*., 2008). With this in mind, the phosphoinositide kinase, PI3K, appears as an attractive target for therapy. The best-characterized PI3K inhibitors to date are wortmannin and 2-(4-morpholinyl)-8-phenyl-chromone (LY294002), (Figure 1.9 A and B, respectively) both of which affect the catalytic subunit of the enzyme (Granville *et al*., 2006;

LoPiccolo *et al*., 2008). Although both commercially available agents produce inhibitory effects *in vitro* and *in vivo*, poor solubility and high toxicity have limited their clinical application (Schultz *et al*., 1995; Semba *et al*., 2002; Granville *et al*., 2006; Isosaki et al., 2011).

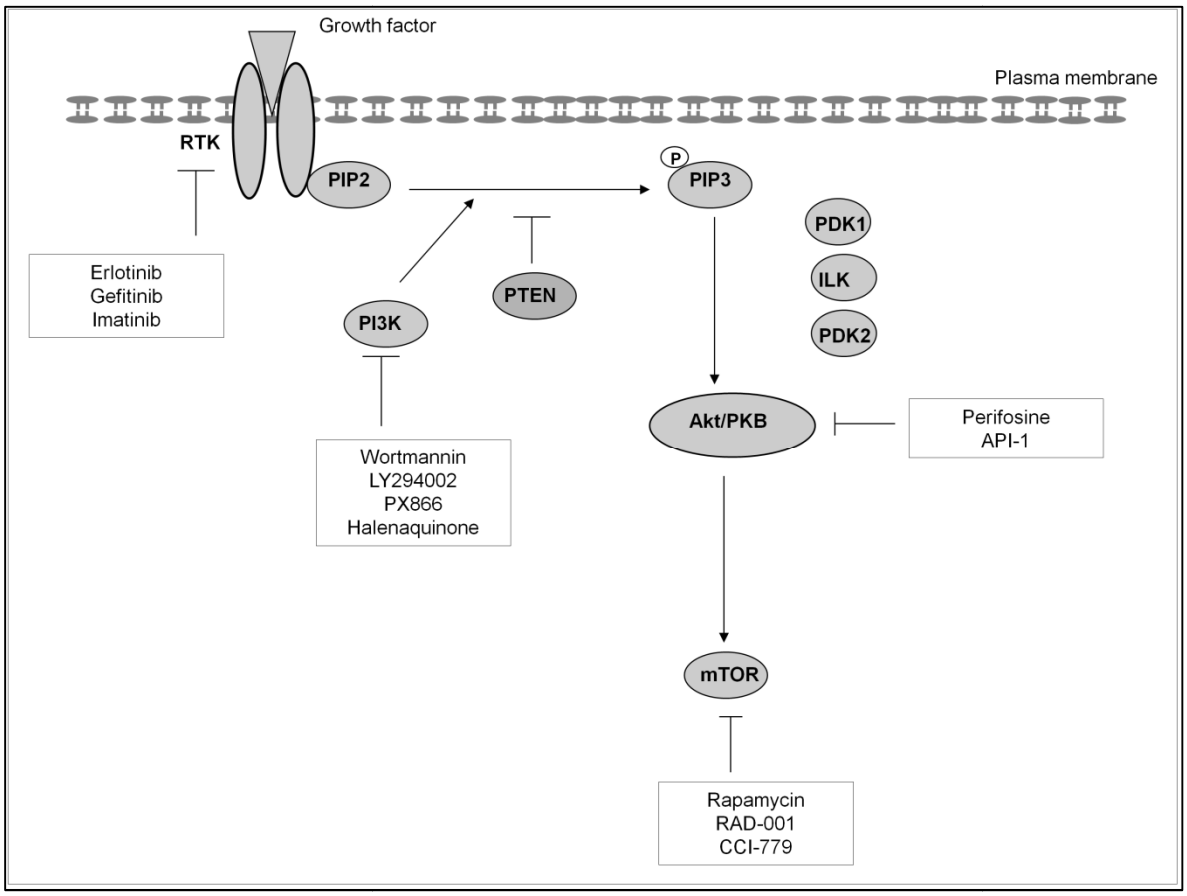

Figure 1.8: Summary of the targeted inhibition of the PI3K/Akt/mTOR pathway, including the best-characterized pathway-specific small molecule agonists. Inhibitors have been developed against the receptor tyrosine kinase (RTK), phosphoinoside-3 kinase (PI3K), Akt and mammalian target of rapamycin (mTOR) proteins in this pathway (Adapted from LoPiccolo *et al*., 2008).

Combining PI3K inhibitors with chemotherapy increases their efficacy, and the ability to use lower doses of the drugs reduces their toxicity (LoPiccolo *et al.*, 2008). In fact, most cancer drugs are used in combination therapies. Considering the multiple mutations inherent in cancer cells, it is prudent to target a number of potential aberrations in these cells in a single treatment (Dhillon *et al*., 2007). Wortmannin has successfully been combined with paclitaxel, cisplatin, etoposide, gemcitabine and 5-fluorouracil (Ng *et al*., 2000; Asselin *et al*., 2001; Hu *et al*., 2002; Wang *et al*., 2002; Reis *et al*., 2005). On the other hand, LY294002 has been shown to increase sensitivity to anti-microtubule agents such as taxanes and vinca alkaloids in a number

of human cancers (Hu *et al*., 2002). In addition, wortmannin and LY294002 have been reported to act as radiosensitizers, to augment radiation-induced cytotoxicity (LoPiccolo *et al*., 2008).

A number of new generation wortmannin and LY294002 derivatives have been developed which are more potent and less toxic than their predecessors (reviewed in LoPiccolo *et al*., 2008). An example of such a compound is the wortmannin derivative, PX-866 (Figure 1.9C), which has been tested *in vivo* in lung, colon and ovarian cancer (Ihle *et al*., 2005). Other PI3K inhibitors are continually being investigated. One such compound is halenaquinone, which was isolated from the marine sponge, *Xestospongia exigua* (Figure 1.9D) (Fujiwara *et al*., 2001; Granville *et al*., 2006).

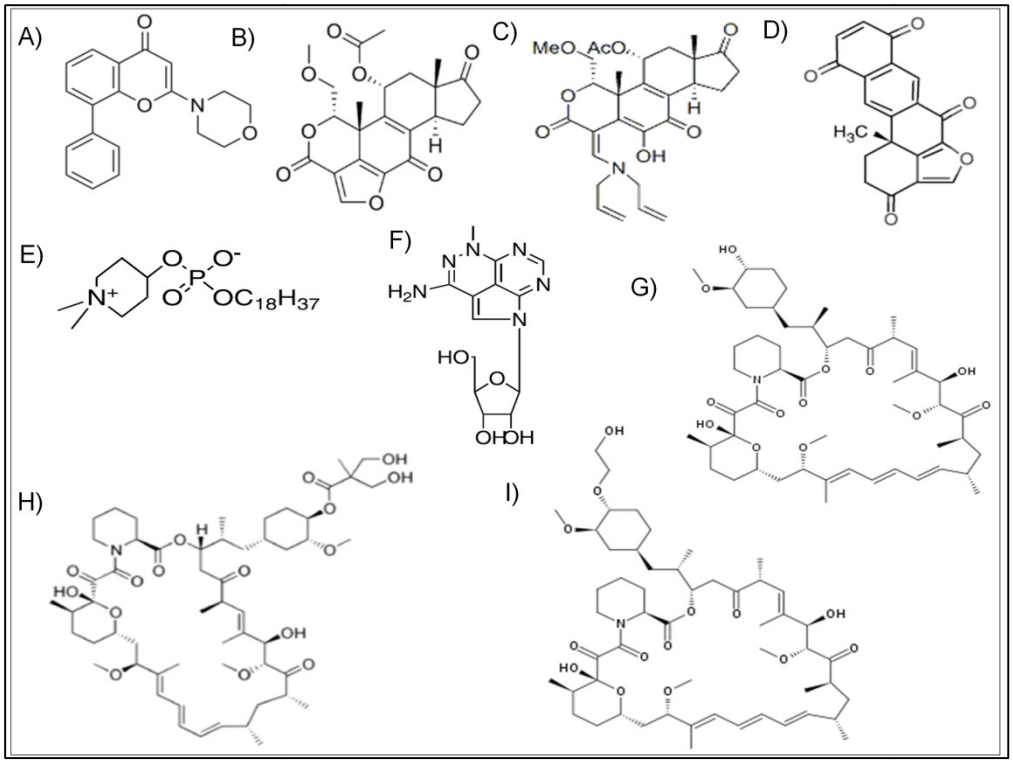

Figure 1.9: Chemical structures of various Akt pathway inhibitors. PI3K-specific inhibitors are depicted in A - D, Akt-specific inhibitors include E and F, and mTOR inhibitors are shown in G - I. A) LY294002, B) wortmannin and its derivative C) PX-866, D) halenaquinone, E) perifosine F) triciribine/AP-1, G) rapamycin, and its analogues, H) CCI-779 and I) RAD-001 (Adapted from Granville *et al*., 2006).

Clinical data has also been obtained for a number of specific Akt inhibitors (LoPiccolo *et al*., 2008; Chandarlapaty *et al*., 2011). One of the most developed Akt agonist to date is the lipid-based inhibitor, perifosine (Figure 1.9E), which inhibits the recruitment of Akt to the membrane, thus preventing its activation (Crul *et al*., 2002; Granville *et al*., 2006; Alexander,

2011). Phase I and II clinical trials of perifosine have been carried out for a number of different cancer types, including that of the pancreas, prostate and breast, as well as Phase III clinical trials in AML and colon cancer (Posadas *et al*., 2005; Leighl *et al*., 2007; Marsh Rde *et al*., 2007; LoPiccolo *et al*., 2008; Alexander, 2011). Unfortunately, low efficacy and toxicity concerns meant that it was not a good candidate for monotherapy. On the other hand, when combined with classic chemotherapeutic drugs or radiation, it showed promising inhibitory activity *in vitro*, with synergistic effects being noted with etoposide (in leukemia), doxorubicin (multiple myeloma) and temozolomide (glioma) (Momota *et al*., 2005; Hideshima *et al*., 2006; Nyakern *et al*., 2006;). The tricyclic nucleoside, triciribine or API-2 (Figure1.9F), acts by preventing phosphorylation at both the kinase and regulatory domains of Akt, thus abrogating its function (Mittelman *et al*., 1983). A number of phase I and II clinical trials using triciribine in various advanced cancer types were carried out in the 1980's and 1990's (LoPiccolo *et al*., 2008). In these studies, minimal efficacies and serious toxicities such as hepatotoxicity and hyperglycemia were observed (Feun *et al*., 1984; Feun *et al*., 1993; Hoffman *et al*., 1996). More recent phase I trials are in progress using lower doses of the drug, as well as combinations with tyrosine kinase inhibitors, including erlotinib and lapatinib or drugs targeting other molecules of the PI3K-AKT-pathway (Yang *et al*., 2004; Granville *et al*., 2006; LoPiccolo *et al*., 2008; Gloesenkamp *et al*., 2012).

By far the most developed class of Akt pathway inhibitors are the agonists of mTOR (Wander *et al*. 2011). Of these, rapamycin, the prototypic mTOR inhibitor was discovered in 1975. Rapamycin (Figure 1.9G) is produced by *Streptomyces hygroscopicus* and was found to be a potent fungicide (Sehgal *et al*., 1975). This compound is a Food and Drug Administration (FDA)-approved immunosuppressant (Granville *et al*., 2006). In addition, the compound displayed the ability to inhibit cancer cell proliferation, nearly 30 years ago (Douros and Suffness, 1981). The analogues RAD-001 (everolimus) and CCI-779 (temsirolimus) (Figure 1.9H and I, respectively) have been designed specifically as anti-cancer drugs and have shown great efficacy as both single agents and in combination with chemotherapy and radiation in phase I and II clinical trials (Raymond *et al*., 2000; O'Donnel *et al*., 2003; Granville *et al*., 2006). In addition, a phase III clinical trial has been carried out using CCI-779 to treat metastatic renal cell carcinoma and the success resulted in the FDA approval of CCI-779 as a front-line therapy for this disease (Hudes *et al*., 2007). Rapamycin and its analogues have displayed synergy with paclitaxel, vinblastine, doxorubicin, cisplatin and other standard chemotherapeutic drugs (Goerger *et al*., 2001; Marimpietri *et al*., 2005; Haritunians *et al*., 2007). Similar results have been obtained using rapamycin in combination with herceptin in breast cancer and Hsp90 inhibitors in multiple myeloma (MM) (Francis *et al*., 2006; Wang *et al*., 2007). As a result of the insight gained by clinical trials into the effects of treatment with mTOR inhibitors on various downstream signal

transduction pathways, second-generation drugs that target mTOR kinase activity, the mTOR kinase inhibitors (TOR-KIs) have recently been developed (Wander *et al*. 2011).

1.4 Breast cancer

Breast cancer is the most common malignancy among women, with 16 % of cancer-related deaths attributed to this form of the disease (WHO report, 2008). In Western countries, it remains the most fatal disease amongst women (Chodosh, 2011). In the United States, the prevalence of breast cancer has been reported as 111 cases per 100 000 women per year, with a mortality rate of 24 deaths per 100 000 women per year (Howe *et al*., 2001; Zhou *et al*., 2008). In South Africa, breast cancer makes up 16.6% of all malignancies diagnosed in women (Vorobiof *et al*., 2001). In fact, the 2000-2001 National Cancer Registry (NCR) report shows that South African women have a lifetime risk (LR) of 1 in 8 of getting cancer, with breast cancer predominating at a LR of 1 in 29 (NCR 200-2001 statistics available online, Internet 3). What is rarely published and thus largely unappreciated is that men can also develop breast cancer (Ravandi-Kashani and Hayes, 1998). In fact, male breast cancer accounts for approximately 1 % of all reported breast cancer cases worldwide. Interestingly, the male-to-female breast cancer ratio is higher in black populations than among white populations with male breast cancer being highly prevalent in some parts of Africa such as Zambia, where up to 15 % of all breast cancer cases occur in men, and Egypt, which reports up to 12 times more of such cases than the USA (Ravandi-Kashani and Hayes, 1998).

1.4.1 Breast cancer subtypes

Breast cancer is a histologically and genetically heterogeneous disease, a factor which has important clinical implications (Visvader, 2009). The vast majority of breast tumours arise within the lining of the ducts in breast tissue and are thus termed mammary ductal carcinomas (Keller *et al*., 2010). The mammary ducts are composed of a basal layer of contractile, myoepithelial cells and a luminal layer of specialized epithelial cells. Three important hormone receptors associated with mammary ductal tissue are estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER-2/neu) (Visvader, 2011). Breast cancers may be classified in a number of ways, according to the histopathology, receptor status, grade, genetic profile, prognosis or stage of the disease. The most common classification divides breast tumours into four subtypes based on their histopathological origin, receptor status and grade. These include the basal or triple-negative (ER^-, PR^- and $HER\text{-}2^-$), the ER^- $HER2^+$, the luminal A (ER^+, low grade) and the luminal B (ER^+, high grade) subtypes (Perou *et al*., 2000; Sorlie *et al*., 2001). These subtypes are linked to prognosis, with the greatest chance of relapse-free survival being associated with the luminal A subtype and the poorest prognosis being associated with the basal/triple-negative subtype (Perou *et al*., 2000; Dent *et al*., 2000).

Of particular importance in Africa, the more aggressive triple negative subtype of the disease has been shown to be more prevalent in black women (Morris *et al*., 2007; Lund *et al*., 2009). This form of the disease results in earlier age of onset, higher mortality and greater risk of metastasis (Dent *et al*., 2000). There is currently no effective therapy available for the triple negative subtype of breast cancer (Albeck and Brugge, 2011).

1.4.2 Treatment strategies for breast cancer

Current clinical practice in the treatment of the majority of cancers conventionally consists of surgical removal of the tumour(s), radiation, chemotherapy or combinations of the three (Recht and Solin, 2011). The chemotherapeutic drugs traditionally used in the treatment of breast cancer are paclitaxel (Taxol) and doxorubicin (Garcia *et al*., 2007). The shortcomings of such treatments are well known, namely the high toxicity of the drugs as well as their ineffectiveness at preventing recurrence of the disease (Widakowich *et al*., 2007). Hormone therapy is another avenue of treatment for breast cancer sufferers. The earliest developed hormone therapy for breast cancer is the drug Tamoxifen, which blocks the stimulation of breast cancer cells by estrogen via their estrogen receptors (ERs). This drug is used as a chemopreventative measure in patients which are considered to have a high risk of developing breast cancer and has been associated with decreased recurrence when administered after surgery (reviewed in Jordan, 2006).

Since the HER-2 receptor has been shown to be over-expressed in 20 % to 25 % of breast cancers and has been associated with poor clinical outcome due to increased aggressiveness and frequent metastases, specific HER-2 inhibitors such as trastuzumab (Herceptin) and lapatinib have been developed (Slamon and Pegram, 2001; Kakarala and Wicha, 2008). Although some success has been obtained with this form of therapy in clinical trials, especially when used in conjunction with traditional chemotherapeutic agents such as doxorubicin, 50 % of patients experienced relapse of the disease (Slamon and Pegram, 2001; Kakarala and Wicha, 2008). In addition, the above therapies such as Tamoxifen and Herceptin are completely reliant on the presence of the relevant receptors and are thus ineffective against the receptor-negative subtypes of the disease. There is a need for novel therapies which are able to treat these more aggressive receptor-negative subtypes of breast cancer (Lund *et al*., 2009).

Worldwide, there has been a 25 % reduction in breast cancer-related deaths between 1990 and 2004 (Heron *et al*., 2007). This may be attributed to earlier detection by mammography screening as well as improved adjuvant therapies administered in the early stages of the disease (Calvocoressi *et al*., 2008; Kakarala and Wicha, 2008). Despite these advances, there has been no significant change in the survival rates for women diagnosed with metastatic breast cancer during this time frame (Takebe *et al*., 2011). The reasons for this are intimately

linked to the cancer stem cell theory described below (Kakarala and Wicha, 2008). The identification of cancer stem cells within breast tumours has resulted in a major shift in focus in terms of the development of novel therapies to treat this disease. It is now thought that specific anti-CSC therapies are needed to remove the source of the tumour and prevent metastasis and relapse (Takebe *et al*., 2011).

Despite numerous advances in the understanding of the etiology of breast cancer, there is still no recognized "cure" for the disease (Chodosh, 2011). Many of the new generation pathway inhibitors have been hampered in their clinical application due to a number of undesirable traits such as poor solubility, unexpected side effects and toxicities as well as limited effectiveness (LoPiccolo *et al*., 2008). Furthermore, a major challenge associated with therapies targeting cancer stem cells is the fact that they share a number of pathways with normal, healthy stem cells, in particular the self-renewal pathways (Kakarala and Wicha, 2008). This, together with molecular complexity of signal transduction pathways and the reality of compensatory mutations in these pathways, suggest that the road to a cure for breast cancer will be a long and bumpy one (Grant *et al*., 2008)!

1.5 The cancer stem cell theory

Stem cells are undifferentiated cells that are defined by their ability to self-renew, in addition to being able to differentiate into the cell types which make up a particular organ in the body (Sell, 2004; Massard *et al*., 2006). There are three types of stem cells: embryonic stem cells, which arise from the earliest divisions of a fertilized egg and are able to give rise to all the cell types in the body; germinal stem cells, able to produce eggs and sperm in reproduction; and somatic or adult stem cells, which display a more limited differentiation ability and are associated with a particular organ (Blagosklonny, 2005).

The concept of a "cancer stem cell" (CSC) was first proposed about 150 years ago by Virchow and Conheim and was based on the similarities between the self renewal properties seen in fetal development and those in certain tumours. Advances in stem cell biology have resulted in scientists revisiting this theory in order to describe the origin and spread of cancers (Korkaya and Wicha, 2007). The revised CSC theory calls into question the traditional monoclonal model of cancer development and consists of two important and related hypotheses: 1) that tumours arise from and are maintained by a unique subset of cells which possess features of both cancer cells and stem cells; and 2) that these cells are capable of asymmetric division in that they can both self-renew to produce more CSCs as well as differentiate to generate the other cells types found within a tumour (Pardal *et al*., 2003; Wicha *et al*., 2006; Lawson *et al*., 2009).

Cancer stem cells were first identified in acute myelogenous leukemia in 1994 (Lapidot *et al*., 1994; Zhou *et al*., 2007). In 1997, it was discovered that the ability to transfer human leukemia into non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice was due to a small subpopulation of cells displaying the cell surface markers $CD34^{+}CD38^{-}$, which comprised less than 1 in 10 000 cells; while the bulk of the cells were non-tumourigenic (Dick *et al*., 1997). The first solid tumour stem cells were identified in breast cancer a decade later, where it was demonstrated that a $CD44^{+}CD24^{-}$ marker-bearing subpopulation could regenerate a tumour from as little as 100 cells, whereas tens of thousands of cells from the bulk population failed to do so (Al-Hajj *et al*., 2003). The identification of specific surface markers has resulted in the characterization of CSCs in brain tumours as well as cancers of the prostate, pancreas, neck, colon, lung, skin, liver and ovary (Singh *et al*., 2004; Patrawala *et al*., 2006; Li *et al*., 2007; O'Brien *et al*., 2007; Prince *et al*., 2007; Ricci-Vittiani *et al*., 2007; Eramo *et al*., 2008; Zhang *et al*., 2008; Marotta and Polyak, 2009).

1.5.1 Implications of the CSC theory on treatment of cancer

The CSC theory has numerous implications for cancer treatment (Massard *et al*., 2006). The original monoclonal model of carcinogenesis can be described as a stochastic and random system, where every cell in an organ has an equal ability to be transformed and become tumourigenic. Furthermore, the model suggests that each cell within a tumour is equally malignant (Martinez-Climent *et al*., 2006). Thus, traditional treatments, such as chemotherapy and radiation, were aimed at killing the rapidly-dividing bulk population of cells within the tumour (Kakarala and Wicha, 2008). However, while these therapies are able to shrink the tumour, the effects are usually transient and recurrence remains a reality for a substantial proportion of sufferers (Massard *et al*., 2006; Kakarala and Wicha, 2008). The reason for this is that the highly tumourigenic CSC subpopulation, like normal stem cells, is more resistant to traditional therapies and remains behind after chemotherapy or radiation treatment (Massard *et al*., 2006). In particular, these cells may possess multidrug resistance transporters which are able to efflux chemotherapeutic drugs and specialized enzymes such as aldehyde dehydrogenase, which are able to degrade the drugs (Karkarala and Wicha, 2008). Furthermore, CSCs often display increased expression of anti-apoptotic molecules such as the Bcl-2 family and survivin, which prevent the drugs from initiating programmed cell death (Litingtung *et al*., 1999; Smalley and Clarke, 2005; Marotta and Polyak, 2009). Since these cells are capable of both self-renewal and differentiation, they can then repopulate the original tumour (Pardal *et al*., 2003).

Cancer stem cells have also been implicated in metastasis or spreading of the disease to other sites in the body, a process which accounts for over 90 % of lethality in cancer patients (Weigelt *et al*., 2005). Cancer cell metastasis consists of six steps: 1) the cells must detach and

extravasate from the primary tumor; 2) invade the extracellular matrix (ECM) and the endothelium to reach the blood stream; 3) survive in the bloodstream; 4) attach at another site in the body by adhesion to a particular endothelium; 5) extravasate across the endothelium and ECM once more and 6) initiate and maintain growth at this new site (Siclari *et al*, 2006).

The concept of "seeds and soil" was proposed by Paget over a century ago and states that metastasis is dependent on the characteristics of both the tumour (seeds) and the environment at the distal site (soil) (Wicha, 2006). It has been suggested that the "seeds" in this model are CSCs. The ability of CSC to undergo asymmetrical cell division together with their capability of anchorage-independent growth and higher adaptability due to increased genetic instability lends weight to the argument that secondary tumours are most likely derived from the CSC subpopulation (Li *et al*., 2007). Furthermore, functional genomic studies have suggested that a minor population of cancer cells within a heterogeneous breast tumor are already programmed to preferentially metastasize and these cells are termed metastatic CSCs (mCSCs) (Ramaswamy *et al*., 2003, Li *et al*., 2007). To date, however, the exact mechanisms by which cancer stem cells metastasize and populate secondary sites are not completely understood (Takebe *et al*., 2011). Either the original CSC, or a new stem cell derived from the original, undergoes a variety of genetic and epigenetic changes which result in these cells becoming more invasive in a process termed an epithelial-mesenchymal transition (EMT). This has been observed at the invading edge of a number of tumours (Mani *et al*., 2008). Once metastasized, the CSCs may remain dormant until they receive the appropriate environmental signals, resulting in recurrence even after many years (Wicha *et al*., 2006).

There is therefore a need for new therapies which are able to specifically target the cancer stem cells in order to eliminate the cause of the tumour and prevent recurrence and metastasis. There have been some reports of the success associated with using apparently CSC-specific agents in cancer therapy (Marotta and Polyak, 2008). One such approach is to induce differentiation of the malignant stem cells (Korkaya and Wicha, 2007). In one study, the administration of all-*trans*-retinoic acid (tretinoin) in conjunction with chemotherapy increased the long-term survival rate of AML patients (Bruserud and Gjertsen, 2000). Similarly, the delivery of bone morphogenic protein 4 (BMP4) *in vivo* has been reported to significantly reduce the proportion of stem-like precursors in human glioblastoma (Piccirillo *et al*., 2006). In another report, the combination of parthenolide and rapamycin appeared to target cancer stem cells in AML, while the normal haematopoietic cells were unaffected by this treatment (Yilmaz and Morrison, 2008). Another important strategy in developing CSC-specific treatments is to target the self-renewal pathways operating in these cells. Furthermore, an understanding of these pathways may provide a clearer picture of how dysregulation of these pathways leads

to malignancies in addition to providing targets for cancer prevention and therapy (Kakarala and Wicha, 2008).

1.5.2 Self-renewal signalling pathways in normal and cancer stem cells

Self-renewal is an essential feature of stem cells allowing them to last the lifetime of the organism (Massard *et al*., 2006). Stem cells may either divide symmetrically to produce two daughter stem cells (self renewal), or they may undergo asymmetrical division to produce a stem cell and a progenitor cell which is destined to differentiate, an ability termed multipotency (Weissman, 2000; Dontu *et al*., 2004). In healthy tissues, homeostasis is maintained by the tight regulation of the balance between these processes. This also ensures that mature, differentiated cells may be derived from stem cells without depleting the stem cell subpopulation (Korkaya and Wicha, 2007). Thus, all tissues should consist of a hierarchy comprising a) stem cells able to generate all progeny, b) committed progenitors and terminally differentiated cells (Dontu *et al*., 2004).

Alterations in the self-renewal pathways found in stem cells may result in their transformation to a malignant state, a concept which is at the centre of the cancer stem cell hypothesis (Pardal *et al*., 2003). The bulk differentiated cells within a tumour are unable to undergo self-renewal, thus inhibition of these pathways is a means of specifically targeting the stem cell component. Clearance of the bulk tumour cells require treatment with agents which either induce apoptosis, such as paclitaxel, or target survival pathways, such as rapamycin, in conjunction with CSC-specific drugs (Kakarala and Wicha, 2008). The main self-renewal pathways whose dysregulation has been implicated in carcinogenesis are the Wnt, Notch and Hedgehog systems, the description of which is beyond the scope of this review (Wicha *et al*., 2006). Inhibitors specific to the self-renewal pathways Wnt, Notch and Hedgehog have displayed efficacy against breast cancer, in particular the use of gamma secretase inhibitors (GSI's) in combination with chemotherapy has been evaluated in clinical trials (Kakarala and Wicha, 2008).

1.6 Drug discovery in cancer

There are two major approaches when attempting to develop novel anti-cancer compounds. One is to focus on deregulated pathways within the cell and then develop specific inhibitors to target key signal transduction molecules within a particular pathway. This approach is called the "target to hit" strategy, examples of which are described in Section 1.2. Conversely, the second method involves screening a range of novel compounds for cytotoxicity towards a malignant cell and then attempting to characterize the anti-proliferative action by identifying the pathways that are deregulated by the "hit compound." This strategy is known as the "hit to target" approach (Korkaya and Wicha, 2007; LoPiccolo *et al*, 2008). The current study will make use of the latter approach as a means of identifying novel hit compounds of marine algal origin.

1.6.1 The marine environment as a source of biomedical compounds

In the last century, the bioactive secondary metabolites produced by living organisms have provided many compounds of pharmacological importance (Ioannou, 2004). The vast majority of novel natural products were originally derived from terrestrial plants due to the ease of sample collection (Carté, 1996). However, since the ocean is thought to host at least 80 % of the Earth's plant and animal species, this meant that, potentially, the largest source of therapeutically beneficial compounds remained untapped (Jha and Zi-rong, 2004). In particular, deep-sea environments exceeding depths of 400 m represent 91 % of the total bottom surface area of the ocean. It wasn't until the 1960s that the development of SCUBA allowed researchers to begin probing the rich biodiversity of the ocean floor for organisms which produce novel bioactive compounds (Carté, 1996). Since then, thousands of structurally unique compounds have been isolated from seaweeds, sponges, corals, sea slugs and marine microbes, many of which have displayed biological activity of pharmacological interest (Jha and Zi-rong, 2004). Marine natural products have been used in the development of antibiotics, anti-viral, anti-inflammatory, and anti-cancer drugs (Carté, 1996).

South Africa is ranked as the country with the fifth richest species endemism and biodiversity in the world (Le Roux *et al*., 2002). In terms of marine biodiversity, South African waters host more than 10 000 species, making up 15 % of the total number of species on Earth (Le Roux *et al*., 2002). This high species biodiversity has been shown to be associated with high secondary metabolite diversity (Davies-Coleman, 2004).

1.6.2 Halogenated monoterpenes from marine algae

Marine algae, in particular those belonging to the Chlorophyta (green algae), Rhodophyta (red algae) and Phaeophyta (brown algae), have provided many structurally unique compounds of pharmacological importance (Carté, 1996). Two brown algal compounds which have received attention in recent years are the carotenoid, fucoxanthin, and the tetraprenylated quinone, sargaquinoic acid. The former is the best-characterized among the algal compounds to date and has been isolated from a range of species including *Laminaria japonica*, *Undaria pinnatifida* and *Sargassum heterophyllum* (Peng *et al*., 2011). Fucoxanthin has displayed an unprecedented range of therapeutic properties including anti-malarial, antioxidant, anti-diabetic, anti-obesity, anti-angiogenic and anti-cancer activities, all of which have been demonstrated in *in vivo* systems (reviewed in Peng *et al*., 2011). The anti-cancer activity of fucoxanthin has been demonstrated in a wide range of human cell lines including those for leukemia, prostate, bladder and colon cancer, and has been associated with the induction of apoptosis in cancer cells *in vitro* (Hosokawa *et al*., 1996; Hosokawa *et al*., 2004; Das *et al*., 2005; Kotake-Nara *et al*., 2005a; Zhang *et al*., 2008). Sargaquinoic acid, on the other hand, has been isolated from various species of the Sargassum genus and has displayed neurite outgrowth promoting and anti-cholinesterase activity, suggesting a potential role for the compound in the treatment of Alzheimer's disease (Kamei and Tsang, 2003; Choi *et al*., 2007). In addition, the compound has been shown to induce apoptosis in human skin cancer cells (Hur *et al*., 2008).

Red algae or Rhodophyta are interesting from a biopharmacological point of view due to their tendency to incorporate halogen moieties into their secondary metabolites (Carté, 1996). Several studies have reported the isolation of novel halogenated monoterpenes from various species of marine red algae (Rhodophyta) such as those belonging to the *Plocamium* and *Portiera* genera (König *et al*., 2000; Blunt *et al*., 2003; Knott *et al*., 2005). These secondary metabolites are thought to be produced by the alga as a protective mechanism against, for example, reef herbivores (König *et al*., 1990). In addition, the compounds have been found to display anti-tubercular and anti-cancer activities (Fuller *et al*., 1992; König *et al*., 2000). Monoterpenes are naturally occurring hydrocarbons produced by the condensation of two isoprenes (Gould, 1997). It has been reported that these compounds are able to successfully treat cancers of the liver, lungs, brain, breast and pancreas in both the early and advanced stages (Haag *et al*., 1992; Crowell *et al*, 1994; Stark *et al*., 1995). It is hypothesized that they act in the initiation and progression stages of cancer by stimulating the overexpression of various cell surface receptors (Kornfeld, 1992). This alters the pattern of signal transduction and facilitates cell cycle arrest, apoptosis and redifferentiation (Crowell *et al*., 1994; Gould, 1997).

A number of novel polyhalogenated monoterpenes have been isolated from the red algae *Portieria hornemannii* (Fuller *et al*., 1992; Fuller *et al*., 1994; Gunatilaka *et al*., 1999). A subset of

these were tested for cytotoxicity towards various cancer cell lines obtained from the U.S. National Cancer Institute, with the greatest inhibitory effects occurring in the brain, renal and colon tumour cell lines (Fuller *et al*., 1992). One compound, the pentahalogenated monoterpene halomon, displayed the highest and most specific cytotoxicity in the NCI's panel of cancer cell lines and was selected for preclinical drug development (Fuller *et al*., 1992; Fuller *et al*., 1994). However, work on this compound has not proceeded past mouse models (Egorin *et al.*, 1996; Adrianoloso *et al*., 2006). Another red algae species, *Plocamium cartilagineum*, has yielded a large number of new halogenated monoterpene compounds (König *et al*., 1990; Darias *et al*., 2001; de Inés *et al*., 2004). In one study, the cytotoxic effects of the compounds were tested in a number of cell lines including that from a mouse colon adenocarcinoma (CT26) as well as from human colon (SW480) and cervical carcinomas (HeLa) and a malignant melanoma (SkMel28) (de Inés *et al*., 2004). Although a handful of marine natural products have reached clinical trials, research on halogenated monoterpenes remains at the pre-clinical phase (Sashidhara *et al*., 2009).

Research into the presence of halogenated monoterpenes in South African marine red algal samples has recently been carried out (Knott *et al*., 2005; Mann *et al*., 2007). One such study reports the isolation of a number of polyhalogenated monoterpenes from the *Plocamium corallorhiza* and *Plocamium cornutum* species, which were collected along the west coast of South Africa (Knott *et al*., 2005). The novel compounds were tested for cytotoxicity towards the human oesophageal cell carcinoma line WHC01 and displayed IC_{50} values between 9.3 and 33.8 µM, respectively, which was comparable to that of the chemotherapeutic drug cisplatin (13.0 µM) in the same study (Knott *et al*., 2005). The cytotoxicity of the compounds was determined by crystal violet staining of apoptotic cells (Knott *et al*., 2005). A later report by the same group describes the isolation of novel halogenated monoterpene aldehydes from *Plocamium corallorhiza,* this time harvested from the south-east coast of South Africa (Mann *et al*., 2007). Cytotoxicity tests against the oesophageal cancer line WHC01 were carried out for all the compounds using an MTT assay and produced IC_{50} values of between 7.5 and 64.8 µM (Mann *et al*., 2007). The group later reported the isolation of the quinone sargaquinoic acid from the brown alga *Sargassum heterophyllum* (Afolayan *et al*., 2008).

2. KNOWLEDGE GAP

A number of novel compounds of marine algal origin have been found to display cytotoxic effects towards cancer cell lines. A recent report describes the isolation of a series of polyhalogenated monoterpenes and halogenated monoterpene aldehydes from the marine red algae *Plocamium corollorhiza* and *Plocamium cornutum* (Knott *et al*., 2005; Mann *et al*., 2007). A number of these compounds displayed cytotoxicity in the low micromolar range against the oesophageal cancer cell line, WHC01 (Knott *et al*., 2005; Mann *et al*., 2007). However, the precise mechanism by which this cytotoxicity occurs was not determined. In addition, the effect of these and other compounds isolated by the same group on metastatic breast cancer cell lines is unknown. Of particular interest is whether the inhibitory compounds induce apoptosis of the cells and, if so, which signalling pathways and molecules mediate this response.

Although breast cancer stem cells were the first solid tumour stem cells to be discovered, this research is still in its infancy. A major stumbling block is the limited ability to identify and isolate these cells. Furthermore, there are very few reports of drugs which specifically target breast cancer stem cells and their clinical significance is, as yet, unestablished (Marotta and Polyak, 2009).

3. RESEARCH QUESTION

What is the potential of South African marine red and brown alga to yield novel lead compounds with anti-breast cancer activity using a “hit to target” approach, and can the specific mode of action of these compounds be elucidated using standard biochemical assays?

4. AIMS

To screen a library of novel marine algal compounds for anti-breast cancer and anti-breast cancer stem cell activity and to analyze the mechanisms involved in mediating the cytotoxic effects of such compounds on breast cancer cells and their cancer stem cell subpopulation *in vitro.*

4. OBJECTIVES

1. Screening of novel marine algal compounds against metastatic ER^- PR^- $HER2neu^-$ breast cancer cells *in vitro* (described in CHAPTER 2)

2. Assessment of the effect of selected novel algal compounds on the survival of non-cancerous breast epithelial cells *in vitro* in order to identify "hit" compounds with differential toxicity against breast cancer but not normal breast epithelial cells (CHAPTER 2)

3. Preliminary pharmacological profiling of potential hit compounds (CHAPTER 2)

4. Elucidation of the mode of action of selected marine algal compounds against metastatic breast cancer cells *in vitro* in terms of the type of cell death induced and the effect on cell cycle of these compounds (CHAPTER 3)

5. Characterization of the molecular mechanisms mediating toxicity of the novel algal compounds sargaquinoic acid and fucoxanthin against breast cancer cells *in vitro* in terms of the involvement of key signal transducer molecules (CHAPTER 4)

6. Screening of novel marine algal compounds for putative anti-cancer stem cell activity *in vitro* using a mammosphere assay system (CHAPTER 5)

CHAPTER 2

SCREENING OF NOVEL MARINE ALGAL COMPOUNDS AGAINST METASTATIC BREAST CANCER CELLS *IN VITRO*

2.1 INTRODUCTION

The marine environment has proven to be a valuable source of structurally unique bioactive compounds, with many lead compounds being derived from seaweeds, sponges, corals and marine microorganisms (Carté, 1996, Jha and Zi-rong, 2004). In particular, metabolites from the highly abundant marine algae represent a wealth of novel compounds with biomedical potential (Carté, 1996). The majority of these pharmacologically important compounds are derived from species belonging to the Chlorophyta (green algae), Rhodophyta (red algae) or Phaeophyta (brown algae) (Carté, 1996). An example of a brown algal metabolite which is currently commercially available is the carotenoid, fucoxanthin. This novel compound has been isolated from a range of algal species including *Laminaria japonica*, *Undaria pinnatifida* and *Sargassum heterophyllum*. Fucoxanthin has been demonstrated to possess a vast array of therapeutically beneficial properties, including anti-oxidant, anti-obesity, anti-diabetic, anti-angiogenic, anti-inflammatory, anti-malarial, and anti-cancer activities in studies which have proceeded past cell culture (reviewed in Peng *et al*., 2011). Of particular interest to this study, the compound was found to display cytotoxicity against human leukemia as well as colon and prostate cancer cells *in vitro* (Hosokawa *et al*., 1996; Das *et al*., 2005; Kotake-Nara *et al*., 2005b).

In the case of the Rhodophyta group, it was discovered that these red algae produced a number of metabolites containing one or more halogen moieties (Carté, 1996). In particular, several studies reported the isolation of novel halogenated monoterpenes from various species belonging to the *Plocamium* and *Portiera* genera (König *et al*., 2000; Blunt *et al*., 2003; Knott *et al*., 2005). Two decades ago, a pentahalogenated monoterpene was isolated from *Portiera hornemanni*, which was found to be highly toxic against a range of cancer cell lines, including those of the brain, kidneys and colon (Fuller *et al*., 1992). As a result, halomon was selected for pre-clinical drug development. The effect of the compound on non-cancerous cells was not determined. In addition, very little is known about the mechanism of action of the compound, although it has been suggested that the anti-cancer activity may be due to DNA methyltransferase inhibition (Andrianasolo *et al*., 2006).

The South African coastline is reported to have one of the richest displays of marine biodiversity in the world, representing 15 % of the total number of marine species on Earth (le Roux, 2002). This biodiversity has, in turn, been associated with high secondary metabolism diversity (Davies-Coleman, 2004). In a recent report, a number of polyhalogenated monoterpenes were isolated from South African marine algae of the *Plocamium corallorhiza* and *Plocamium cornutum* species (Knott *et al*., 2005; Mann *et al*., 2007). The same group later reported the isolation of a previously described quinone derivative, sargaquinoic acid, from the brown alga *Sargassum heterophyllum* (Afolayan *et al*., 2008). The *Plocamium* compounds

displayed cytotoxicity in the low micromolar range against the oesophageal cancer cell line, WHC01 (Knott *et al*., 2005; Mann *et al*., 2007). Nothing is known about the effect of any of the indigenous algal compounds described above on metastatic breast cancer cell lines or their non-cancerous counterparts.

The aims of this chapter were to identify potential "hit" compounds from a library of novel South African algal metabolites by screening for differential cytotoxicity against metastatic breast cancer cells but not non-cancerous breast epithelial cells *in vitro* and, furthermore, to assess the "druggability" of these hit compounds by preliminary pharmacological profiling.

2.2 MATERIALS AND METHODS

2.2.1 Reagents

Dulbecco's Modified Eagle Medium (DMEM) with Glutamax™, Ham's F10 medium with Glutamax™, fetal calf serum (FCS) and Penicillin-streptomycin-amphotericin (PSA) were obtained from Gibco (Invitrogen, UK). Epidermal growth factor (EGF) and hydrocortisone (HC) were obtained from Sigma-Aldrich. Recombinant human insulin was obtained from NovoRapid (Novo Nordisk Pharmaceuticals, Denmark). Cell Proliferation Kit I for MTT assays was from Roche (Switzerland). Marine algal compounds were obtained as part of a collaboration with Dr Denzil Beukes (Pharmacy Faculty, Rhodes University). The structures of the novel compounds were verified by nuclear magnetic resonance (NMR) and stocks dissolved in dimethyl sulphoxide DMSO (Sigma-Aldrich, Germany). Paclitaxel and fucoxanthin were from Sigma-Aldrich (Germany).

2.2.2 Cell lines and culture conditions

The breast cancer cell line, MDA-MB-231 (ATCC: HTB-26) was obtained as a gift from Dr Sharon Prince (Department of Human Biology, Faculty of Health Sciences, University of Cape Town), while the Hs578T line (ATCC: HTB-126) was purchased from The American Tissue Culture Collection (ATCC). The MCF12A breast epithelial cell line (ATCC: CRL-10782) was a kind gift from Dr Anna-Mart Engelbrecht (Department of Physiology, Stellenbosch University). All cell lines were shown to be negative for mycoplasma infection using PCR.

Table 2.1: Description of the human cell lines used in this study

Cell line	Origin	Receptor status	Molecular phenotype
MDA-MB-231	Pleural effusion, metastatic carcinoma	$ER^-PR^-Her2^-$	Basal
MCF-7	Pleural effusion, metastatic carcinoma	$ER^-PR^-Her2^-$	Luminal
Hs578T	Primary carcinoma of the breast	$ER^+PR^+Her2^-$	Basal
MCF-12A	Spontaneously immortalized breast epithelium	$ER^-PR^-Her2^-$	Basal

(taken from Subik *et al.*, 2010).

Breast cancer cells were maintained in culture in DMEM containing Glutamax™ and supplemented with either 5 % (v/v) (MDA-MB-231) or 10 % (v/v) (Hs578T) heat-inactivated FCS, 100 U/ml penicillin, 100 µg/ml streptomycin and 12.5 µg/ml amphotericin (PSA) at 37 ºC in a humidified 9 % CO_2 incubator. The immortalized non-cancerous MCF12A cells were maintained in a 1:1 ratio of Ham's F10 medium and DMEM, both containing Glutamax™, supplemented with 10 % (v/v) heat-inactivated FCS, 100 U/ml penicillin, 100 µg/ml streptomycin and 12.5 µg/ml amphotericin (PSA), 20 ng/ml EGF, 500 ng/ml hydrocortisone and 10 µg/ml insulin.

2.2.3 Cell proliferation assay

The effect of paclitaxel as well as the algal compounds of interest on cellular survival and proliferation in the MDA-MB-231, Hs578T and MCF12A cell lines was determined by means of the MTT assay according to manufacturer's instructions. Cells were seeded at 6000 cells/well in a 96-well plate and allowed to settle overnight before treating with the DMSO vehicle control (final concentration of 1.03 % in culture) or a range of concentrations (as indicated in figure legends, DMSO content between 0.017 and 1.03 %) of the compounds and incubating for 96 hours. After treatment, 10 µL of a 5 mg/mL MTT solution was added to each well and the plate incubated for a further 4 hours before solubilizing the resultant precipitate in sodium dodecyl sulphate (SDS) buffer overnight. The assay measures the conversion of the yellow tetrazolium compound to blue formazan crystals by metabolically active cells which can be quantified by the characteristic absorbance of the crystals at 550 nm and used as an indicator of cells which are metabolically active. The absorbance at 550 nm was determined using a Powerwave spectrophotometer (BioTek) and the percentage survival calculated relative to the vehicle-treated control. The half-maximal inhibitory concentrations (IC_{50}) were calculated over a linear

concentration range using the linear regression software in Microsoft Excel 2007. The experiment was carried out in triplicate on each of three plates.

2.2.4 Trypan blue staining

The viability of MDA-MB-231 and Hs578T breast cancer cells after treatment with paclitaxel and selected algal compounds was assessed by their ability to exclude the dye trypan blue. In each case, cells were seeded at 6000 cells/well in a 96-well plate, allowed to settle overnight and treated with either 0.3 % v/v DMSO or a single concentration of compound (equivalent to the IC_{50} value obtained by MTT assay) over 96 hours. Following treatment, both floating and adherent cells were collected for staining. Adherent cells were lifted with a 1 % (v/v) trypsin, 0.3 % (w/v) ethalene diamine tetra-acetic acid (EDTA) solution, pooled with the media fraction containing the floating cells, and a portion representing 1 % of the total cells stained with an equivalent volume of trypan blue (Sigma-Aldrich, Germany). The number of stained and unstained cells was counted using a haemocytometer slide and the percentage viability calculated as the ratio of unstained (trypan blue negative) cells relative to the total in each case. Each treatment was carried out in triplicate and each sample counted a minimum of three times.

2.2.5 Preliminary pharmacological profiling

The drug-likeness of the algal compounds was assessed using an amended version of the "Rule of 5" as described by Lipinski (Lipinski *et al*., 2001). Lipophilicity, in terms of cLogP values, was calculated using ALOGPS 2.1 software from VCC labs (available at http://www.vcclab.org.). Analyses were carried out on canonical SMILE formats of the compounds obtained from the Pubchem database.

2.3 RESULTS

2.3.1 Optimization of the MTT cell proliferation kit for cytotoxicity assays

In order to assess the effect of the algal compounds on cellular survival and proliferation, the MTT assay was used. The assay was validated by treatment with the commercially available compound paclitaxel (Ptx), which was selected due to the fact that the IC_{50} value against breast cancer cell lines using this assay has previously been reported (Dwight *et al*., 1997). Ptx is also currently used as a chemotherapeutic drug, which would serve as a means of comparison in terms of cytotoxicity between potential "hit" compounds and a known drug.

Ptx is a large and complex aromatic compound, the structure of which is depicted in Figure 2.1A. Cells were treated with an increasing concentration of Ptx and a drop in cell

survival was detected as a dose-dependent decrease in absorbance at 550 nm. The concentration at which cell viability was calculated to be half of that of DMSO-treated cells (IC_{50}) was calculated from the linear regression of the survival curve. Using this method it was found that Ptx displayed IC_{50} values of 104.36 ± 3.90 nM cells and 29.17 ± 1.58 nM against MDA-MB-231 and Hs578T breast cancer cells, respectively (Figure 2.1B). Although only one survival curve is shown for each of the two cell lines, the experiment was carried out in triplicate, with the IC_{50} values being calculated from independent experiments and the standard deviation reported.

The kinetics of the observed anti-cancer activity of Ptx were assessed by determining the effect of 25, 50, 100 and 200 nM concentrations of the drug on the survival of MDA-MB-231 and Hs578T cells after 24, 48, 72 and 96 hours of treatment (Figure 2.1C and Figure 2.1D, respectively). In the case of the MDA-MB-231 line, although a noticeable decrease in cell survival was seen after 24 hours of treatment with all of the doses of Ptx, it was only after 48 hours that the effect of the drug was clearly evident, with a further drop in survival at 72 and 96 hours (Figure 2.1C). The effect of Ptx on MDA-MB-231 cell survival after 72 hours of treatment was very similar to that observed after 96 hours, with the exception of that for treatment with the lowest dose (25 nM) of the drug, for which the decrease in cell survival was greater after 96 hours of treatment. On the other hand, the pattern of susceptibility of Hs578T cells to Ptx over time displayed a marked difference when compared to that of the MDA-MB-231 cell line (Figure 2.1D vs. Figure 2.1C). In the former, the effect of Ptx on cell survival was almost negligible after 24 hours of treatment, with a steady increase in susceptibility to the drug when treated for increased periods of time over the aforementioned dose range (Figure 2.1D). Although the differences in the survival curves of the Hs578T cell line between treatment for 72 and 96 hours was less than that observed between both 24 and 48 hours as well as between 48 and 72 hours, this difference was nonetheless greater than that seen in the MDA-MB-231 line between the last two treatment intervals. (Figure 2.1C vs. Figure 2.1D). Based on this kinetic study for paclitaxel, it was decided that screening of the MDA-MB-231 and Hs578T cells with the novel algal compounds would be carried out over 96 hours.

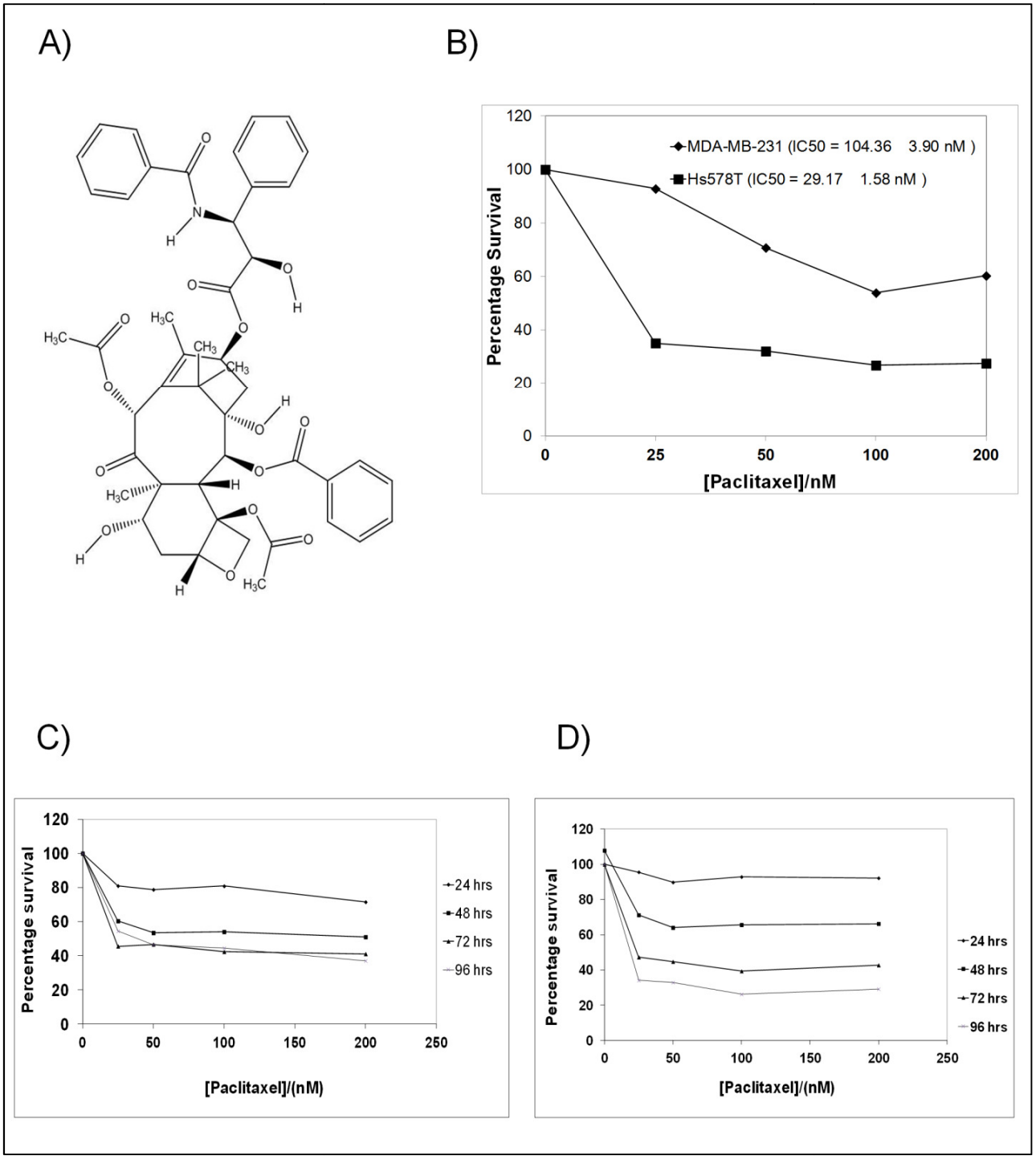

Figure 2.1: Optimization of the MTT cell proliferation assay using the chemotherapeutic paclitaxel. A) Chemical structure of paclitaxel drawn using ChemSketch. B) Survival curve for MDA-MB-231 and Hs578T breast cancer cells treated with either 0.013 % v/v DMSO vehicle control or paclitaxel for 96 hours. The half maximal inhibitory concentration (IC_{50}) of the compound in each cell line is displayed above the curves. For each curve, the standard deviations are given where n = 3. C) and D) depict the kinetic study for treatment of MDA-MB-231 and Hs578T cells, respectively with paclitaxel over 24, 48, 72 and 96 hours.

2.3.2 Screening of algal compounds in metastatic ER$^-$ PR$^-$ HER2neu$^-$ breast cancer cells *in vitro*

The library of compounds isolated from *Plocamium* and *Sargassum* algae consisted of sixteen novel linear polyhalogenated monoterpenes (RU002, RU004, RU007, RU008, RU015, RU016, RU017, RU018, RU019, RU048, RU054, RU075, RU076, RU077, RU078 and RU079) three novel cyclic polyhalogenated monoterpenes (RU050, RU065 and RU066), as well as the tetraprenylquinones, sargaquinoic acid (SQA) and sargahydroquinoic acid (SHQA) (Figure 2.2). The carotenoid, fucoxanthin (FXN), was obtained commercially, yet has reportedly been isolated from *Sargassum heterophyllum* and *Sargassum elegans* (Afolayan *et al*., 2008). The monoterpene compounds all contained between two and five halogen moieties, specifically chlorine and/or bromine, and could be grouped into structural series whose terpene backbone was identical but in which the halogen substitutions varied from compound to compound. These included the RU007, RU008, RU048 and RU054 monoterpene aldehyde group and the RU015, RU016, RU017, RU018 and RU019 series (Figure 2.2).

Throughout the course of this study, the selection of compounds for testing in the various assays was made largely on the basis of novelty. However, these decisions were influenced by a limited supply of verified compound and, where it was not possible to carry out particular assays on all compounds, an attempt was made to either compare between the previously described algal compounds (SQA and FXN) and the novel Rhodes University compounds (RU002-RU081), or to make comparisons between the two more readily available, known compounds (SQA and FXN).

All twenty-two of the algal compounds (Figure 2.2) were tested against the metastatic ER$^-$ PR$^-$ HER2neu$^-$ MDA-MB-231 breast cancer cell line, with a number displaying cytotoxicity in the micromolar range against these cells (Figure 2.3). The half-maximal inhibitory concentrations (IC_{50} values) were calculated in the same way as for Ptx (Section 2.3.1) Of the novel polyhalogenated monoterpenes, compounds RU002, RU004, RU007, RU008, RU015, RU065, RU066, RU077 and RU078, were toxic to MDA-MB-231 cells with IC_{50} values ranging from 6.2 ± 0.13 µM (RU015) to 82.7 ± 5.18 µM (RU008) (Figure 2.3A). Compounds for which the IC_{50} value was greater than 200 µM were considered non-toxic (NT). The compounds RU017 and RU018 are stereoisomers (Figure 2.2) and were tested in MDA-MB-231 cells both as a racemic mixture and separately and were non-toxic to the cells in all cases (Figure 2.3A). The previously described compounds, sargaquinoic acid and fucoxanthin, also decreased the survival of MDA-MB-231 cells. The tetraprenylquinone, sargaquinoic acid, displayed an IC_{50} of 67.4 ± 5.92 µM against MDA-MB-231 cells, while the reduced form, sargahydroquinoic acid, gave an IC_{50} of 139.5 ± 4.41 µM (Figure 2.3A) The commercially sourced carotenoid, fucoxanthin, displayed an IC_{50} of 11.07 ± 0.56 µM in this cell line (Figure 2.3A).

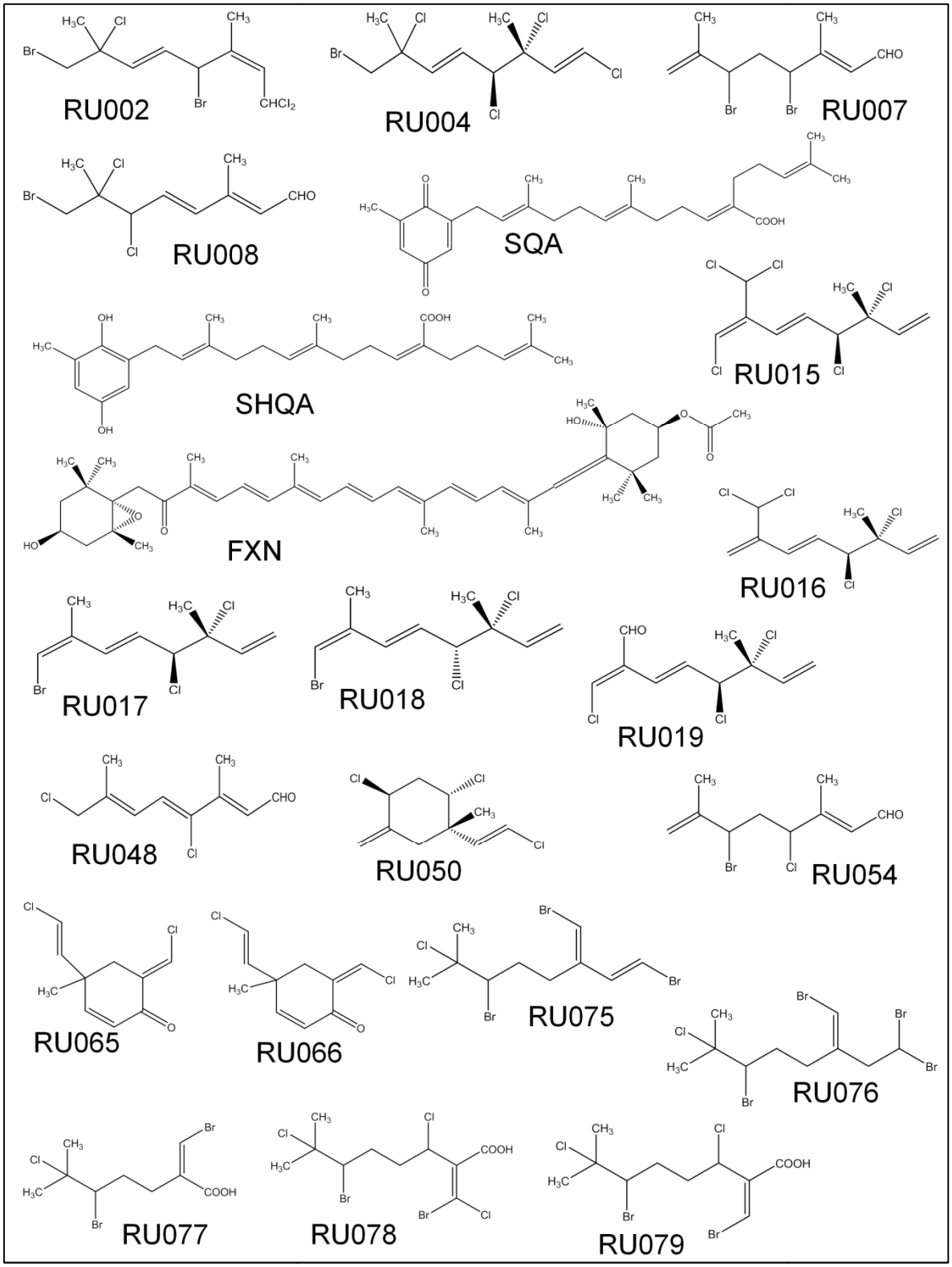

Figure 2.2: Chemical structures of compounds isolated from marine algae of the *Plocamium* and *Sargassum* genera. Compound structures were generated in ChemSketch. The previously described compounds, sargaquinoic acid, sargahydroquinoic acid and fucoxanthin are represented by SQA, SHQA and FXN, respectively.

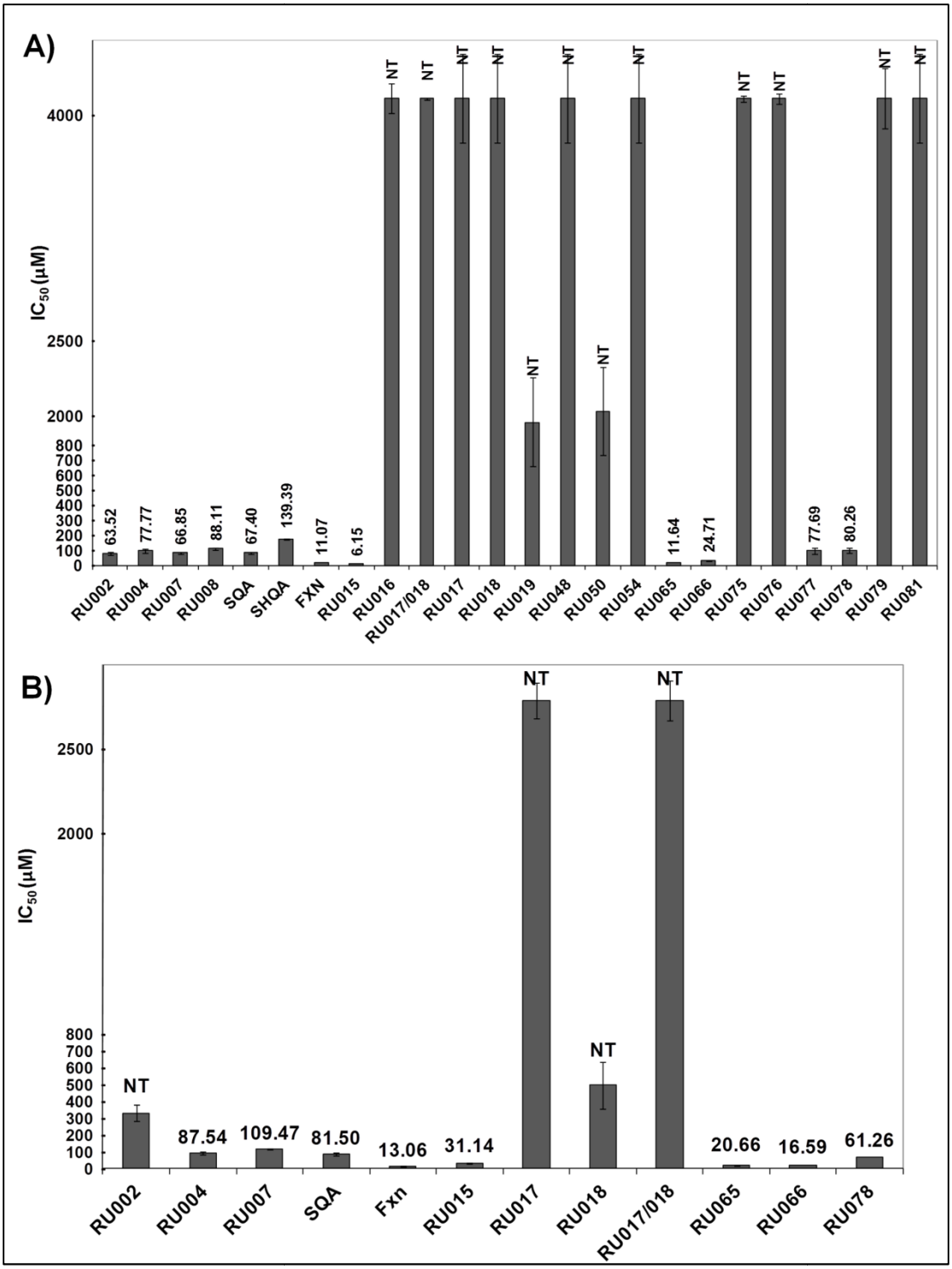

Figure 2.3: Screening of marine algal compounds for anti-proliferative effects against metastatic breast cancer cells *in vitro*. Anti-cancer activity was determined in both the MDA-MB-231 (A) and Hs578T (B) cell lines. Cells were treated with a range of concentrations of compound over 96 h and the effect on cell proliferation determined by MTT assay. The previously described compounds, sargaquinoic acid, sargahydroquinoic acid and fucoxanthin are represented by SQA, SHQA and FXN, respectively. Values quoted are the half maximal-inhibitory concentrations (IC_{50}) of each compound in µM calculated relative to a 1.03% v/v DMSO vehicle control. Concentrations greater than 200 µM are considered non-toxic (NT). Error bars indicate the standard error of the mean where n=3.

A number of the algal compounds were screened in a different metastatic ER^- PR^- $HER2neu^-$ breast cancer cell line, namely the Hs578T cell line. These included the compounds RU002, RU004, RU007, RU015, RU017, RU018, RU065, RU066, RU078, sargaquinoic acid and fucoxanthin (Figure 2.3B). With the exception of RU002, the compounds displayed the same pattern of cytotoxicity against Hs578T cells as for MDA-MB-231 cells, namely that compounds RU004, RU007, RU015, RU065, RU066, RU078, sargaquinoic acid and fucoxanthin were toxic to the Hs578T cells in the micromolar range, while RU017 and RU018 were not (Figure 2.3A and 3B). It was observed, however, that the absolute IC_{50} values of the compounds as well as the ranking of the cytotoxic compounds from least to most toxic differed between the two breast cancer cell lines (Figure 2.3A vs. Figure 2.3B).

2.3.3 Trypan blue assessment of cell viability for selected compounds

As an alternative method of assessing the cytotoxicity of selected algal compounds and in order to validate the results obtained using the MTT assay, trypan blue staining was carried out to assess cellular viability of MDA-MB-231 and Hs578T cells after treatment with a subset of the algal compounds. Unlike the MTT assay, this method allows for both the live and the dead cells to be counted. In the MDA-MB-231 cell line, the DMSO vehicle-treated control sample displayed a percentage viability of close to 100 % (98 %; Figure 2.4A), while the percentage viabilities of the SQA- and FXN-treated cells at their respective IC_{50} values were 55 % and 48 %, respectively (Figure 2.4A). The percentage viability of the Ptx-treated sample was 62 %, while cells treated with RU004 and RU007 displayed percentage viability values of 81 % and 78 %, respectively (Figure 2.4A). In the Hs578T cell line, the percentage viability calculated for the DMSO-treated control sample was 85 %, while cells treated with the chemotherapeutic drug Ptx displayed a cellular viability of 38 % (Figure 2.4B). Treatment of Hs578T cells with the algal compounds SQA and FXN resulted in percentage viability values of 59 % and 25 %, respectively (Figure 2.4B).

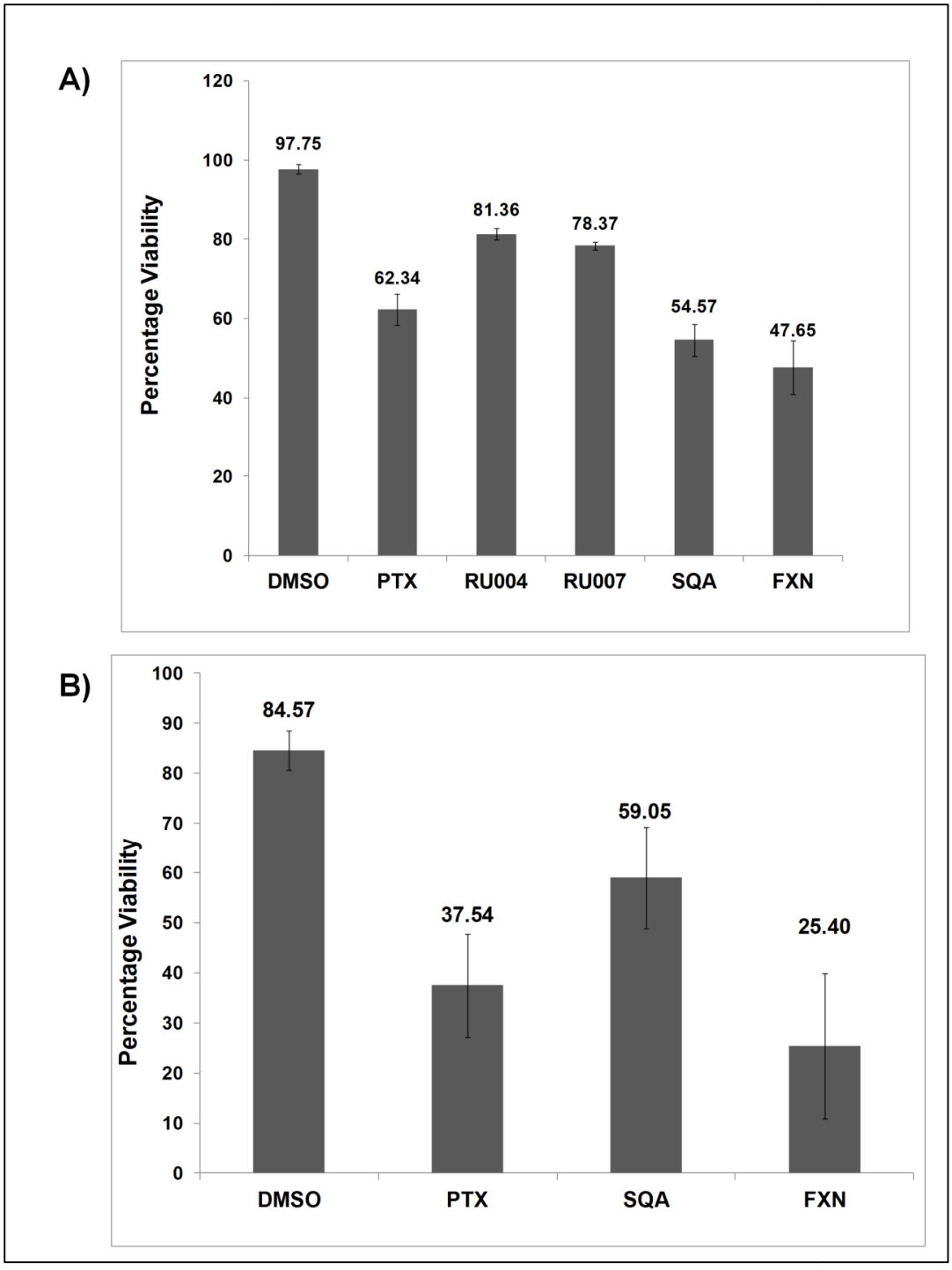

Figure 2.4: Assessment of cell viability by trypan blue staining of breast cancer cells treated with paclitaxel and selected marine algal compounds. For both MDA-MB-231 (A) and Hs578T (B) metastatic breast cancer cell lines, treatment was carried out over 96 h with with either 0.3 % v/v DMSO (vehicle control) or relevant algal compound at a concentration equivalent to the IC_{50} value as obtained via MTT assay. The effect on cellular viability was determined by staining a proportion of the cells equivalent to 1 % of the total with a 1:1 (v/v) ratio of trypan blue. Values quoted represent the percentage of the total number of cells which were not stained by trypan blue and therefore considered to be viable. Error bars indicate the standard error of the mean where n=3.

2.3.4 The effect of selected algal compounds on non-cancerous breast epithelial cells

Selected compounds were tested for their ability to specifically inhibit growth of breast cancer cells, while leaving the immortalized non-cancerous breast epithelial cell line, MCF12A, relatively unaffected. In each case, MCF12A breast cells were treated with a concentration equivalent to the IC_{50} value of the compound against MDA-MB-231 breast cancer cells. At a concentration of 100 nM, the chemotherapeutic drug, Ptx, reduced survival and proliferation of the MCF12A cells to 69 % (69.40 ± 3.82 %, Table 2.2). Of the novel algal compounds, RU004, RU007 and SQA were found to be non-toxic to MCF12A cells (percentage survival values of 111.55 ± 2.30 %, 106.53 ± 1.74 % and 154.14 ± 1.93 %, respectively; Table 2.2), while FXN was relatively cytotoxic to the MCF12A cell line (percentage survival of 71.32 ± 9.20 %, Table 2.2). The halogenated monoterpene stereoisomers RU017 and RU018 had very little effect on the survival of MCF12A cells, even at the very high dose of 300 µM (percentage survival values of 98.11 ± 4.56 % and 95.15 ± 3.38 %, respectively; Table 2.2).

Table 2.2: Differential cytotoxicity screening of paclitaxel and novel algal compounds in MCF12A breast epithelial cells

Compound	Concentration*	Percentage survival determined by MTT assay
Paclitaxel	100 nM	69.40 ± 2.8 %
RU004	75 µM	111.55 ± 2.3 %
RU007	75 µM	106.53 ± 1.7 %
Sargaquinoic acid	75 µM	154.14 ± 1.9 %
Fucoxanthin	10 µM	71.32 ± 9.2 %
RU017	300 µM	98.11 ± 4.6 %
RU018	300 µM	95.15 ± 3.4 %

*The IC_{50} value of each compound in MDA-MB-231 cells

2.3.5 Preliminary pharmacological profiling of "hit compounds"

Potential "hit" compounds, selected on the basis that they displayed cytotoxicity against metastatic breast cancer cells (Figure 2.3 and 2.4), while resulting in greater than 95 % survival in non-cancerous breast epithelial cells (Table 2.2), were scored for their "drug-likeness" in terms of their lipophillicity according to Lipinski's Rule of 5 (Ro5) and compared to the commonly used therapeutic, Ptx (Table 2.2). These included compounds RU004, RU007 and SQA. Of these, only RU007 displayed a favourable cLogP value of below 5 (4.07, Table 2.3), while RU004 and SQA had cLogP values of 5.41 and 5.74, respectively (Table 2.3). In the case of

Ptx, the compound displayed a cLogP value below 5 (4.2; Table 2.3); however the size of the molecule as well as the number of oxygen and hydrogen atoms did not meet the favourable druggability criteria defined in the Rule of 5.

Table 2.3: Comparison of drugability of novel monoterpene compounds with that of paclitaxel using the Rule of 5 (Ro5)

Compound	Assessment of favourable druggable characteristics			
	[1]cLogP < 5 ?	[2]MW < 500?	[3]# of H-bond donors	[4]O + N atoms < 10?
RU004	No (5.41)	Yes (278.05)	0	Yes (0)
RU007	Yes (4.07)	Yes (310.03)	0	Yes (1)
SQA	No (5.74)	Yes (424.57)	1	Yes (4)
PTX	Yes (4.2)	No (853.906)	22	No (15)

[1]cLogP values are indicative of lipophillicity, which, together with compound size ([2]MW), affect the bioavalaibility and absorption of the compound in terms of its ability to cross the intestinal wall and blood-brain barrier.[3]An excessive number of hydrogen bond donor groups impairs permeability across a membrane bi-lipid layer as does an excessive hydrogen bond accepting ability measured by [4]the number of oxygen and nitrogen atoms (Lipinski *et al*., 2001).

2.4 DISCUSSION

In order to screen a set of novel indigenous marine algal metabolites for anti-cancer activity, MTT assays were carried out on breast cancer cells treated with these compounds. The commonly used chemotherapeutic agent, Ptx, was used to validate the MTT assay. Ptx is a large and complex molecule which was first isolated from the Pacific yew tree *Taxus brevifolia* (Newman *et al*., 2003) and exerts its effect as an anti-mitotic agent by interacting with tubulin in order to inhibit microtubule dynamics (Bharadwaj *et al*., 2004; Brito *et al*., 2008). In clinical trials, breast cancer patients received 175 mg/m^2 of body surface area after adjuvant therapy and showed increased overall survival rates compared to untreated control patients (Sparano *et al*., 2008). *In vitro*, the compound has been tested against MCF-7 breast cancer cell (Dwight *et al*., 1997). The IC_{50} of paclitaxel obtained in the MDA-MB-231 line in this study (104 nM) was very similar to the 100 nM value previously reported for the drug in the MCF-7 breast cancer line (Dwight *et al*., 1997). With an IC_{50} value that is three times lower, Hs578T cells appeared to be more susceptible to paclitaxel than both MDA-MB-231 and MCF-7 breast cancer cells. Since, like MDA-MB-231 cells, the Hs578T cell line was derived from an aggressive ER^- PR^- $HER2neu^-$ metastatic breast carcinoma (Hackett *et al*., 1977), the lower IC_{50} for Ptx in Hs578T cells was unexpected.

Before analyzing the data provided by the assay, it is essential to reiterate what the MTT system is designed to quantify, namely that the absorbance values obtained are a representative of metabolically active cells which are able to convert the tetrazolium substrate to a coloured formazan product (Berridge and Tan, 1993). Therefore, although commonly used as a measure of the "cytotoxicity" of particular compounds (for example in de Inés *et al*., 2004; Das *et al*., 2005 and Kotake-Nara *et al*., 2005b), it is important to bear in mind that the kit does not discriminate between dead and senescent cells or those which are alive but not metabolically active and thus it is more accurate to state that the assay measures the "anti-proliferative" or "growth-inhibitory" activity of compounds of interest. This is a potential shortcoming of the MTT assay as well as other systems which detect metabolic activity as a measure of proliferation of cells yet are used to determine the toxicity of a particular compound. For the purposes of this study, as is the case in the vast majority of reports in the literature, the term "cytotoxic" will be used to describe the anti-proliferative activity of a compound irrespective of whether the cells in question are dead or senescent.

Screening of a library of twenty-two novel algal compounds against the metastatic ER^- PR^- $HER2neu^-$ MDA-MB-231 breast cancer cell line identified twelve compounds which were cytotoxic to the breast cancer cells with IC_{50} values in the micromolar range from 6 to 140 µM. Although IC_{50} values in the nanomolar range are preferred in terms of drug development, it is important to bear in mind that once a hit halogenated monoterpene compound has been identified, rational chemical modification could be carried out to enhance the cytotoxicity of the compound of interest (Li and Vederas, 2009). The cytotoxic compounds identified were the halogenated monoterpenes RU002, RU004, RU007, RU008, RU015, RU065, RU066, RU077 and RU078, as well as the previously described compounds sargaquinoic acid (SQA), sargahydroquinoic acid (SHQA) and fucoxanthin (FXN). The most toxic compound was the linear polyhalogenated monoterpene RU015, while the least toxic was the prenylated quinone, SHQA. Interestingly, it was found that the IC_{50} of this reduced form of SQA, SHQA, was almost double that of SQA, indicating that reduction had a substantial effect on the cytotoxicity of the compound. The bioactive form and potential metabolites of a compound are important factors to bear in mind when carrying out further characterization of promising anti-cancer agents (Peng *et al*., 2011).

An earlier study within the Biomedical Biotechnology Research Unit (BioBRU) assessed the cytotoxicity of a selection of the marine algal compounds in Figure 2.2 on the non-metastatic ER^+ PR^- $HER2neu^-$ MCF-7 breast cancer cell line by MTT assay (Lawson, 2009; MSc thesis). In this assay, compounds RU002, RU004, RU007, RU008, RU015, sargaquinoic acid and a racemic mixture of RU017 and RU018 displayed identical trends in cytotoxicity against MCF-7 cells as reported for MDA-MB-231 cells in the current study; namely that all but RU017/RU018 were

toxic to the breast cancer cells in the micromolar range, with RU015 being the most toxic (Figure A1, Appendix). This indicates that the algal compounds were equally effective against ER^+ (MCF-7) and ER^- (MDA-MB-231) breast cancer cells *in vitro*.

A subset of eleven of the algal compounds screened in MDA-MB-231 cells in this study were also assessed by MTT assay against a different metastatic ER^- PR^- $HER2neu^-$ breast cancer cell line, namely the Hs578T line. In general, the cytotoxicity results obtained in the Hs578T cell line agreed with those observed in the MDA-MB-231 line. This served to validate the findings obtained for MDA-MB-231 cells due to the shared characteristics of the MDA-MB-231 and Hs578T cell lines. However, the actual IC_{50} values as well as the ranking of compounds from least to most toxic varied between the MDA-MB-231 and Hs578T cell lines. In addition, the IC_{50} values of the compounds were, in general, higher in the Hs578T cell line when compared to those in the MDA-MB-231 cell line. Considering the greater susceptibility of the Hs578T cells to Ptx, this was unexpected. It is important to remember, however, that since testing in the two cell lines was not always carried out at the same time, it cannot be ruled out that the changes in cytotoxicity may be due to decreased stability of the compounds over time. This was most likely the case for compound RU002 (IC_{50} of 309 µM i.e. non-toxic in Hs578T *vs* IC_{50} of 64 µM in MDA-MB-231), which was tested in MDA-MB-231 cells a year before the testing was carried out in Hs578T cells. Very little is known about the long-term stability of these novel polyhalogenated monoterpene compounds. It was encouraging, however, that for all but one of the compounds, the anti-cancer activity of the monoterpenes after storage at -80 ^oC for periods between six and twelve months is retained to the same order of magnitude against the metastatic breast cancer cell lines. A further factor to bear in mind is that once a hit halogenated monoterpene compound has been identified, chemical modifications could be carried out to improve the stability of the compound.

As a further indication of the cytotoxicity of selected algal compounds against metastatic breast cancer cells, trypan blue staining was carried out on treated MDA-MB-231 and Hs578T cells. As opposed to the MTT assay, which is able to detect both senescent cells as well as those undergoing the initial stages of apoptosis, trypan blue will only stain cells which are either late apoptotic or necrotic and whose cell membranes have become compromised (Perry *et al.*, 1997). Thus a comparison of the results obtained by the MTT and trypan blue assays could provide information regarding the mechanism of toxicity of a particular compound. However, it is important to remember that the trypan blue assay relies on manual counting of cells and thus has a much larger margin for human error than the spectroscopic MTT assay.

In the MDA-MB-231 cell line, the percentage viabilities determined by trypan blue staining after treatment with SQA or FXN at their respective IC_{50} values were within the expected range of around 50 % (48 % and 55 %, respectively). Percentage viability of Ptx as assessed by trypan

blue staining was slightly higher than anticipated (62 % viability at the IC_{50} of Ptx) but, considering the larger margin for error with this technique, not out of range. The much higher viabilities observed in the RU004 and RU007-treated samples (81 % and 78 %, respectively) could indicate that the majority of the cells, while susceptible to the monoterpene compounds, are in the earlier stages of apoptosis and thus still able to exclude the dye, or may simply be attributed to the inaccuracy of the technique. Since treatment was carried out over 96 hours, the former is less likely. A further possibility is that the number of live cells detected by trypan blue staining was overestimated due to the complete disintegration of the dead cells after 96 hours of treatment, such that these cells were not visible when counting.

For the Hs578T cell line, a much lower viability was calculated for the DMSO vehicle-treated control (85 %) as compared to that in the MDA-MB-231 cell line (98 %), a factor which must be taken into account when interpreting the percentage viability data in the Hs578T samples treated with the compounds of interest. Considering this, the lower percentage viabilities calculated for Hs578Tcells treated with Ptx and FXN (38 % and 25 %, respectively), when compared to those in MDA-MB-231 cells (62 % and 48 %, respectively), were not unexpected. The standard deviations calculated for the IC_{50} values of the compounds in the Hs578T cell line were much greater than those obtained in the MDA-MB-231 line. A simple explanation for this is that Hs578T cells, unlike MD-MB-231 cells, tend to clump together, which makes accurate counting difficult and can affect the reproducibility of the cell counts since the cells are not evenly distributed in solution. On the whole, however, the trypan blue assay results validated those obtained by MTT assay, in that the compounds which were found to be cytotoxic to breast cancer cells in the MTT assay also reduced cellular viability as assessed by trypan blue staining

Measurement of cellular viability by trypan blue staining has previously been carried out in HL-60 leukemia cells treated with fucoxanthin (Hosokawa *et al*., 1996). In this report, the number of viable HL-60 cells was counted after treatment with a range of concentrations of fucoxanthin over 24 hours and used to derive an IC_{50} value of 22.6 µM. The cellular viability was also determined after treatment with a concentration equivalent to the IC_{50} of the compound for 24, 48, 72 and 96 hrs (Hosokawa *et al*., 1996). In the current study, previous attempts to determine the effect of algal compounds on cell viability by trypan blue staining after 24 hours were unsuccessful, since almost 100 % of the cells were found to be viable (unstained) for each of the treatments at this time point (data not shown). However, treatment was carried out with only a single concentration, namely the IC_{50} obtained by MTT. Should the fucoxanthin dose have been increased from 11 and 13 µM (IC_{50} in MDA-MB-231 and Hs578T, respectively, Figure 2.3) to 22.6 µM as in Hosokawa *et al*, it is possible that an effect on cellular viability would have been observed after 24 hours of treatment. Indeed, in the Hosokawa study, a much greater difference in HL-60 viability between the vehicle- and fucoxanthin-treated samples after 96

hours as compared to 24 hours was observed (Hosokawa *et al*., 1996). In our study, increasing the treatment period to 96 hours had the added benefit of allowed for comparison of the results obtained using the MTT assay with those derived from the trypan blue staining method.

The library of novel halogenated monoterpenes screened in this study by MTT assay (and, in some cases, trypan blue staining), contained two structural series, in which the monoterpene backbone was identical but for which the halogen substitutions varied between compounds, namely the RU007, RU008, RU048 and RU054 monoterpene aldehyde group and the RU015, RU016, RU017, RU018 and RU019 series (Figure 2.2). When screened against the MDA-MB-231 breast cancer cell line, these structural series gave preliminary information as to structure-function relationships and the potential role of the various halogen moieties on the molecule in mediating cytotoxicity. In the RU007/008/048/054 series, it appeared that the aldehyde group which was common to all four compounds was unlikely to be responsible for toxicity, since, while RU007 and RU008 were toxic to MDA-MB-231 cells, RU048 and RU054 were not. Interestingly, the substitution of just one bromine atom in RU007 for a chlorine atom in RU054 rendered the compound non-toxic to the cells (Figure 2.2 and Figure 2.3A).

In the RU015/016/017/018/019 group, despite their similarities, one of the compounds in this series displayed the highest toxicity towards MDA-MB-231 breast cancer cells of all the algal compounds screened in this study, while the other four molecules were non-toxic to these cells (Figure 2.3A). It would appear that a higher number of halogen atoms in this series resulted in a more toxic compound, as there were five halogen moieties in the highly toxic RU015 compound, while the non-toxic compounds RU016, RU017, RU018 and RU019 had either three or four halogen atoms (Figure 2.2). It did not appear that the position of the chlorine moieties in RU015 contributed to its toxicity. Interestingly, when the structure of RU015 was compared to those of the other toxic novel compounds which contained five halogens, it was found to be the only one which contained chlorine alone, with no bromine atoms (Figure 2.2). All further attempts at deriving a correlation between halogen number, as well as chlorine and bromine content of the compounds, with cytotoxicity, were unsuccessful in elucidating a clear trend (data not shown). However, based on the high toxicity of RU015, it could tentatively be predicted that substitution of a monoterpene with as many chlorine atoms as possible would increase the toxicity towards metastatic MDA-MB-231 breast cancer cells. Expansion of the compound library by systematic chemical substitutions could shed more light on how the various atoms in the novel monoterpene compounds contribute to their cytotoxicity.

Since the algal compounds SQA and FXN have previously been described in the literature, this provided the opportunity to compare the cytotoxicity of these compounds in various cancer cell lines (Choi *et al*., 2007; Hur *et al*., 2008; Hosokawa *et al*., 1996; Das *et al*., 2005; Kotake-Nara *et al*., 2005a). The quinone derivative, SQA, has reportedly been isolated from samples of

Sargassum sagamianum collected off the coast of Korea (Choi *et al*., 2007). The compound was tested against the human keratinocyte HaCaT cell line by MTT assay and it was found to reduce cellular proliferation to roughly 20 % at a concentration of 23 µM (Hur *et al*., 2008). In our studies, the compound supplied was isolated from a different species, namely *Sargassum heterophyllum*, (Afoloyan *et al*., 2008) and displayed IC_{50} values of 67.40 and 81.50 µM against the breast cancer cell lines MDA-MB-231 and Hs578T, respectively, suggesting that the compound had a greater effect on proliferation of human skin cancer HaCaT cells than metastatic breast cancer cells *in vitro*.

Previous studies have reported the isolation of FXN from the brown alga *Laminaria japonica*, which showed a 50 % reduction in cell proliferation at approximately 100 µM in human colon carcinoma cells (Hosokawa *et al*., 1996). In addition, FXN extracted from *Undaria pinnatifida* completely abolished growth of HL-60 leukemia cancer cells at a concentration of 22.6 µM (Das *et al*., 2005). Furthermore, the compound has been demonstrated to decrease growth of PC-3 human prostate cancer cells by approximately 50 % when treatment with 20 µM of the compound was carried out over 72 hours (Kotake-Nara *et al*., 2005a). In this study, the supplied compound was isolated from *Undaria pinnatifida* and displayed IC_{50} values of 11.06 and 13.06 µM against the breast cancer cell lines MDA-MB-231 and Hs578T, respectively. It would appear, therefore, that metastatic breast cancer cells were more susceptible to FXN than human colon cells and similarly sensitive to the compound when compared to both human leukemia and prostate cancer cells *in vitro* (Hosokawa *et al*., 1996; Das *et al*., 2005; Kotake-Nara *et al*., 2005a).

Selected algal compounds were further assessed for differential toxicity against breast cancer but not non-cancerous breast epithelial cells. The commonly used chemotherapeutic drug, Ptx, was found to be highly toxic to both breast cancer and non-cancerous breast cells. It is precisely this non-selective toxicity which is responsible for the adverse side-effects of anti-cancer drugs such as Ptx (Garcia *et al*., 2007). While compounds RU004, RU007, SQA and, to a lesser extent, FXN, displayed greater cytotoxicity against the MDA-MB-231 and Hs578T cancer cell lines than the MCF12A cell line, the stereoisomers RU017 and RU018 had no effect on the survival of any of the cell lines, even at the very high dose of 300 µM. To the best of our knowledge, this is the first report of the testing of halogenated monoterpene compounds or sargaquinoic acid in a non-cancerous cell line, apart from other studies carried out within our research group (Lawson, 2009) and the first study of fucoxanthin's effect on a non-cancerous cell line. As part of a MSc research project, Lawson screened compounds RU002, RU004, RU007, RU008, sargaquinoic acid, RU015 and a racemic mixture of RU017 and RU018 for cytotoxicity against the non-cancerous breast epithelial line, MCF12A (Figure A1, Appendix). An important difference between the cytotoxicity testing described in the Lawson study and the current report is that in the former, MCF12A cells were cultured in the presence of cholera toxin. A

further difference between the Lawson study and the current work is that, in the former, cytotoxicity was carried out over a range of concentrations for each compound in MCF12A cells, in order to obtain an IC_{50} value; whereas, in the current study, a single-point cytotoxicity test was carried out for each compound in the MCF12A line at a concentration equivalent to the IC_{50} of the particular compound in MDA-MB-231 cells. Despite these differences, the data generated in the current study validated those in the Lawson report, in that the compounds RU004, RU007 and sargaquinoic acid were selected as potential "hits" in both cases due to the finding that their IC_{50} values in MCF12A cells were more than three times higher than in MCF-7 cells (Lawson, 2009; Figure A1).

The differential toxicity of a hit compound is a very important consideration in the screening of novel compounds for anti-cancer activity, since failure to discriminate between cancer cells and healthy tissue could result in adverse side-effects of the compound *in vivo*. It is not uncommon in the literature for researchers to carry out detailed studies of the mechanisms of action of novel compound without first determining the toxicity of the compound in question towards non-cancerous cells. An example of this is the characterization of the anti-cancer activity of the pentahalogenated monoterpene halomon, isolated from *Portieria hornemannii* (Fuller *et al*., 1992). The compound displayed strong cytotoxicity against brain, renal and colon cancer cell lines in the National Cancer Institute's *in vitro* screening panel and was selected for preclinical drug development (Fuller *et al*., 1992). Although the mechanism of cytotoxicity of halomon is thought to be due to DNA methyltransferase inhibition, the effect on non-malignant cells was not determined (Andrianasolo *et al*., 2006). Such differential toxicity is an important consideration, as has been demonstrated by the Lawson study discussed above, where the most toxic compound in our library, RU015, (Figure 2.2, Figure 2.3A) was also found to be highly toxic to normal breast epithelial cells (Lawson, 2009; Figure A1).

The pharmacological potential of the hit compounds identified in this study, namely RU004, RU007 and SQA, was assessed using Lipinski's Rule of 5 (Ro5). Although only four characteristics of a compound are assessed in the Ro5, the name is derived from the cutoffs for each of the four parameters, which are all close to 5 or a multiple of 5 (Lipinski *et al*., 2001).The chemotherapeutic drug, Ptx, was included in the analysis as a means of comparison with the hit algal compounds in terms of their 'druggability." What is immediately apparent when comparing their chemical structures is that the three algal compounds, in particular the two halogenated monoterpenes RU004 and RU007, display much smaller and simpler structures than the 850 g/mol aromatic molecule, paclitaxel. In terms of the "drug-likeness" of the hit compounds, RU007 displayed favourable pharmacological properties for all the criteria assessed by Lipinski's Rule of 5. While RU004 and sargaquinoic acid had cLogP values which were considered to be out of the "druggable" range, they did meet the other requirements outlined in the Ro5. On the other hand, although Ptx displayed a favourable cLogP value, it did

not meet the other druggability criteria defined in the Rule of 5, despite being in clinical use. Indeed, this compound has been reported to display "poor biopharmaceutical properties" including low aqueous solubility and low bioavailability, such that the drug must currently be delivered intravenously in an ethanolic solution (Khandavilli and Pnachagnula, 2007). Therefore, the therapeutic potential of a compound does not appear to be dependent solely on a favourable cLogP value, highlighting the importance of the remaining elements in Lipinski's Ro5. Since compound RU007 met all the druggability requirements outlined in the Ro5, this predicted that the compound would display favourable *in vivo* solubility and bioavailability properties. Finally, one must bear in mind that the cLogP values of RU004 and sargaquinoic acid could again be improved by chemical modification, as for their stability and toxicity, should the compounds warrant further investigation.

2.5 CONCLUSIONS

In summary, this study reports the screening of twenty-two algal compounds against ER^- PR^- $HER2neu^-$ metastatic breast cancer cells *in vitro*. These included nineteen novel polyhalogenated monoterpenes as well as the previously described compounds, sargaquinoic acid, sargahydroquinoic acid and fucoxanthin. All twenty two compounds were screened in the MDA-MB-231 cell line, of which twelve were toxic in the micromolar range. A subset of the compounds were tested in a different ER^- PR^- $HER2neu^-$ metastatic breast cancer line, Hs578T, and for the most part mirrored the cytotoxicity profile obtained for the MDA-MB-231 cells. From these, priority compounds were selected based on their differential toxicity when tested against healthy, non-cancerous MCF12A cells, namely RU004, RU007 and sargaquinoic acid. In contrast to paclitaxel, the chemotherapeutic agent often used to treat breast cancer *in vivo* and which was found to be almost equally toxic to breast cancer cells as to normal breast cells in this study, the algal compounds RU004, RU007 and sargaquinoic acid had no adverse effect on normal breast epithelial cells at concentrations which were toxic to breast cancer cells *in vitro*.

The differential toxicity of the hit compounds, together with their novel and relatively simple chemical structures, justify further studies on these algal secondary metabolites. The three compounds, RU004, RU007 and sargaquinoic acid, together with the commercially sourced carotenoid, fucoxanthin, were selected for further characterization in terms of their mechanism of action in metastatic ER^- PR^- $HER2neu^-$ breast cancer cells *in vitro*.

CHAPTER 3

ELUCIDATION OF THE MODE OF ACTION OF SELECTED MARINE ALGAL COMPOUNDS AGAINST METASTATIC BREAST CANCER CELLS *IN VITRO*

3.1 INTRODUCTION

One of the defining features of cancer cells is their ability to evade apoptosis (Iannolo *et al.,* 2008). This process of "programmed cell death" is the body's way of removing irreversibly damaged and unwanted cells in a manner which does not result in an immune response (Jin and El-Deiry, 2005;). On the other hand, necrosis is described as an unregulated and irreversible process, which occurs in response to a "major insult" (Fink and Cookson, 2005; Klener Jr. *et al.,* 2006). During necrosis, the cell ruptures and releases its contents into the surrounding tissues, causing a massive inflammatory response (Leist and Jaattela, 2001). Apoptosis occurs via two major pathways, the extrinsic or receptor-mediated pathway and the intrinsic or mitochondrial pathway (Nagata, 1997; Kroemer and Reed, 2000). These pathways are interconnected at a number of points and the main role-players in both pathways are caspases (Iannolo *et al.,* 2008). The extrinsic and intrinsic pathways converge on the effector caspase, caspase-3, which initiates the cleavage of key molecules in order to execute the final stages of apoptosis (Jina nd El-Deiry, 2005; Debatin and Krammer, 2004). One such target of caspase-3 action is poly(ADP-ribose) polymerase (PARP), an enzyme which is involved in DNA repair. The cleavage of PARP allows for cellular disassembly and is traditionally used as a marker of apoptotic cells (Boulares *et al.,* 1999). However, PARP has recently been shown to be cleaved during necrosis of cells (Chaitanya and Babu, 2009). It has since been reported that it is the specific PARP cleavage products generated, and not PARP cleavage in general, which can be used to distinguish between the processes of apoptosis and necrosis. While an 85 kDa PARP fragment is produced as a result of caspase-3-mediated cleavage and is indicative of apoptosis, a 50 kDA product is generated upon cathepsin B action and is typical of necrosis (Chaitanya and Babu, 2009).

Although a high toxicity towards cancer cells is desirable in a potential chemotherapeutic, it is crucial that the mechanism of toxicity be elucidated. Whereas apoptosis is the preferred means of eliminating cancer cells, other forms of cell death that are not programmed, such as necrosis, cause massive inflammatory and immune responses and compounds which induce such a response may not be therapeutically useful (Klener Jr. *et al.,* 2006). Literature reports a limited number of studies attempting to elucidate the mechanism of anti-cancer activity of novel marine algal compounds in terms of cell death. These include the findings that apoptosis is the mode of cell death induced both by sargaquinoic acid in human skin cancer cells and by fucoxanthin in leukemia as well as prostate and colon cancer cells *in vitro* (Hur *et al.,* 2008; Kotake-Nara *et al.,* 2001a; Hosokawa *et al.,* 1999; Hosokawa *et al.,* 2004).

In addition to the induction of cell death, many cytotoxic compounds cause cell cycle arrest (Das *et al.,* 2005; Palozza *et al.,* 2002). During normal division, a cell passes through four defined phases of the cell cycle. The G_1 phase, during which cells increase in size in preparation for division; the DNA synthesis or S phase, in which DNA replication occurs; the G_2 phase in

which the cells continue to increase in size; and the M or mitotic phase, in which the cell divides into two daughter cells. Two checkpoints exist within the cycle: the G_1/S checkpoint and the G_2/M checkpoint, which regulate the progression of cells through the cell cycle and prevent entry to the next phase until the cell has met the requirements for that phase. The checkpoints also ensure that DNA damage may be repaired. When cells exit the cell cycle and no longer divide, they are said to be in the G_0 phase or resting state (reviewed in Smith and Martin, 1973).

The anti-cancer activities of novel chemotherapeutic agents are often mediated through cell cycle arrest by interacting with various cell cycle-associated proteins. These include cyclin-dependent kinases (cdks) as well as their activators and inhibitors, namely cyclins, $p21^{WAF1/Cip1}$ and $p27^{Kip1}$ (Das *et al.,* 2005; Palozza *et al.,* 2002). Carotenoids, in particular, have been shown to affect the activity of the aforementioned proteins. Other examples of natural products which interfere with cell cycle proteins include the modification of cyclin D3 and cdk4 in breast cancer MCF-7 cells by retinoic acid, as well as the reduction of cyclin D levels and activation of the inhibitor $p27^{Kip1}$ in breast and endometrial cancer cells by lycopene (Zhu *et al.,* 1997; Nahum *et al.,* 2001). The marine algal carotenoid, fucoxanthin, has been demonstrated to cause G_0-G_1 phase arrest in WiDr and HCT116 human colon cancer cell lines as well as neuroblastoma GOTO cells (Das *et al.,* 2005; Okuzumi *et al*., 1990). Furthermore, this effect of fucoxanthin on the colon cancer cell lines was shown to be mediated by upregulation of the cdk inhibitor $p21^{WAF1/Cip1}$ (Das *et al.,* 2005).

The aim of this body of work was to determine the mode of action of the priority algal compounds RU004, RU007, RU015, sargaquinoic acid and fucoxanthin in breast cancer cells *in vitro*. In particular, the type of cell death induced by the compounds as well as their ability to cause cell cycle arrest was investigated.

3.2 MATERIALS AND METHODS

3.2.1 Reagents

The mouse anti-human poly(ADP-ribose) polymerase (PARP) primary antibody was from Cell Signalling Technologies (Massachusetts, USA), while the rabbit anti-β-actin primary antibody as well as the goat anti-mouse and donkey anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibodies were obtained from Sigma-Aldrich (Germany), Kirkegaard and Perry Laboratories (Maryland, USA) and Amersham Biosciences (UK), respectively. Hoechst 33342 and propidium iodide for flow cytometric analyses were from Invitrogen (UK) and Sigma-Aldrich (Germany), respectively. RNAse-A and Triton X-100 for cell cycle analyses were from Sigma-

Aldrich (Germany). All other chemicals were of reagent grade. Tissue culture vessels were from Corning (Pennsylvania, USA).

3.2.2 Cell lines and culture conditions

The breast cancer cell lines, MDA-MB-231 (ATCC: HTB-26) and Hs578T (ATCC: HTB-126), were cultured as described in Chapter 2 (Section 2.2.2).

3.2.3 Western blot analysis

MDA-MB-231 cells (1×10^6) were seeded into 60 mm plastic tissue culture dishes and allowed to settle overnight. The cells were treated with either 0.5 % v/v DMSO vehicle control, 17-allylamino-17-demethoxygeldanamycin (17-AAG) (5 nM), sargaquinoic acid (0.6, 6 or 60 µM) or RUMB-015 (0.3, 3 or 30 µM) over the course of 8, 24, 48 or 96 hrs at 37 ^{o}C and 9 % CO_2. Thereafter, whole cell lysates were prepared by scraping the adherent cells into the media, spinning at 2000 rpm for 3 minutes and resuspending in 100 µM sodium dodecyl sulphate (SDS) sample buffer [125 mM Tris-HCl pH 6.8, 2 % (w/v) SDS, 20 % (v/v) glycerol, 0.2 % (w/v) bromophenol blue], before boiling for 5 minutes. Lysates from the equivalent of 1×10^5 cells were separated by electrophoresis using 12 % SDS polyacrylamide gels, transferred onto nitrocellulose membranes and probed with a 1 in 1000 dilution of mouse anti-human PARP primary antibody at 4 ^{o}C overnight. The HRP-conjugated goat anti-mouse and donkey anti-rabbit secondary antibodies (diluted 1 in 5000) were detected using the ECL Advanced Western Blotting Kit (GE Healthcare, UK) and visualized using the Molecular Imager ChemiDoc XRS System (BioRad, California, USA). Lysates were probed with a 1 in 2000 dilution of rabbit anti-β-Actin antibody as a loading control. All antibody dilutions were made up using a 5 % (w/v) non-fat milk powder solution in Tris-buffered saline (TBS) (50 mM Tris, 150 mM NaCl, pH 7.5).

3.2.4 Cytotoxicity detection by flow cytometry

Flow cytometric analysis of cells stained with Hoechst 33342 and propidium iodide was used to distinguish between the processes of apoptosis and necrosis within treated breast cancer cells, according to the protocol described by Dive and colleagues (Dive *et al.*, 1992). MDA-MB-231 or Hs578T cells (3×10^5) were seeded into 25 cm^2 flasks and allowed to settle and grow over 24 hrs before dosing with the relevant compounds overnight. Cells were lifted carefully using I x trypsin [1% (w/v) trypsin solution made up in 0.3 % (w/v) ethelene diamine tetra-acetic acid (EDTA)], washed and resuspended in a pre-warmed 2 % (w/v) solution of bovine serum albumin (BSA) in phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4 pH 7.4). Hoechst 33342 was added to a final concentration of 1 µg/ml, the suspension vortexed and incubated at 37 ^{o}C for 7 minutes. Propidium iodide (PI) was added to a final concentration of 1 µg/ml, the cells incubated on ice for 10 minutes and analyzed on a FACS Aria II flow cytometer. Hoechst 33342 was excited by the ultraviolet laser at 355 nm and emission

detected in the 450/50 channel, while PI was excited by the blue laser at 488 nm and detected in the 610/20 channel. Data was analysed using FlowJo software Version 7.6.1. (Tree Star Inc., 2010).

3.2.5 Cell cycle analysis by propidium iodide staining

The effect of selected algal compounds on the distribution of MDA-MB-231 cells in the various phases of the cell cycle was determined by flow cytometry with propidium iodide staining (according to Nunez *et al*., 2001). Cells were seeded at 2.5 x 10^5 and, after 24 hours, were treated with either 0.024 % v/v DMSO (vehicle control), paclitaxel (50 nM), sargaquinoic acid (25, 75, 120 µM) or fucoxanthin (10, 25, 50 µM) for a period of 16 hours. Cells were harvested by treatment with 1 x trypsin and resuspended in PBS before fixation with 70 % ethanol. Cells were collected by centrifugation and stained in a PI solution (50 µg/ml, containing 0.1 mg/ml RNAse A and 0.05 % (v/v) Triton X-100), followed by incubation at 37 ^{o}C for 40 minutes. Thereafter cells were collected by centrifugation, resuspended in PBS and analysed by flow cytometry as described above. Doublet discrimination was carried out to eliminate false positives due to cell aggregates.

3.3 RESULTS

3.3.1 Discrimination between the induction of apoptosis and necrosis of breast cancer cells by marine algal compounds via flow cytometry

The marine algal compounds sargaquinoic acid (SQA), RU004 and RU007 were selected as potential "hit" compounds based on their differential toxicity towards breast cancer cells, but not non-cancerous breast cells (Figure 2.3 and Table 2.2, Chapter 2). These compounds, together with the indiscriminately toxic monoterpene RU015, the commercially available brown algal metabolite fucoxanthin (FXN) and the chemotherapeutic agent paclitaxel (Ptx), were selected as priority compounds for further study. In order to ascertain whether the cytotoxicity of the algal compounds could be attributed to apoptosis or necrosis, flow cytometry using dual Hoechst 33342 (H33342) and propidium iodide (PI) staining was carried out on both MDA-MB-231 and Hs578T cells treated with a range of concentrations of the compounds. Both cell lines were selected for flow cytometric analyses in order to compare the mode of action of selected algal compounds against two different breast cancer cell lines. The H33342/PI assay involves the fluorescent detection of compacted chromatin in apoptotic cells (Sun *et al.,* 1992). The vital bisbenzimidazole dye, H33342, stains the compacted chromatin of apoptotic cells more intensely than the chromatin of live cells; while the DNA-intercalating agent, PI, is impermeable to cells with an intact membrane, namely live cells and those in the early stages of apoptosis. As a result, cells that are H33342-positive but PI-negative may be

alive or undergoing apoptosis, but those that are both H33342-positive and PI-positive are either in the late stages of apoptosis or are necrotic (Sun *et al.,* 1992).

3.3.1.1 Assessment of whether selected novel algal compounds induce apoptosis or necrosis in MDA-MB-231 cells

For the MDA-MB-231 breast cancer cell line, Figure 3.1A depicts the H33342/PI staining for selected samples, represented by dot-plots generated using FlowJo software (Tree Star Inc., 2010). These include the fluorescence data detected for the DMSO vehicle-treated samples as negative controls (i-iv), the Triton X-100 necrosis positive control sample (viii), as well as a dose-response study for SQA as a representative of the novel marine algal compounds (v-vii). In each case, the dot-plot is divided into four quadrants or "gates" which defined whether a particular "dot" or fluorescence "event", representing the emission from a single cell, was "positive" or "negative" for either H33342 or PI. The four quadrants are characterized as follows: The upper left hand (UL) quadrant defines cells which were PI-positive, but H33342-negative; the lower left hand (LL) quadrant represents cells which were H33342-negative and PI-negative; the lower right hand (LR) quadrant defines cells which were H33342-positive, but PI-negative; and the upper right hand (UR) quadrant represents cells which were both H33342-positive and PI-positive. The latter gates were set according to the dot-plots obtained for the vehicle-treated control samples which were stained for H33342 and/or PI, and the gates copied to all other samples.

Figure 3.1B was derived by plotting the percentage of cells for each sample in Figure 3.1A which were both H33342-positive and PI-positive and thus located in the UR quadrant of the dot-plots. The latter proportion of cells is depicted as being "late apoptotic or necrotic" in the y-axis of Figure 3.1B. In addition, Figure 3.1B represents a summary of all the flow cytometry experiments performed in MDA-MB-231 cells, including those not represented in Figure 3.1A, but for whom the "percentage late apoptotic/necrotic" value was derived from dot-plots of the fluorescence data obtained (histograms not shown). These included cells treated with a range of concentrations of either Ptx, RU004, RU007, RU015 or FXN.

For the DMSO-treated samples, it was observed that the entire population in the unstained control (i.e. 100 % of cells) was located in the H33342-negative, PI-negative LL quadrant (Figure 3.1Ai). In the case of the DMSO control where cells were stained with H33342 only, the vast majority of cells were detected in the H33342-positive, PI-negative LR quadrant (99.9 %, Figure 3.1Aii); while DMSO-treated cells stained with PI alone were located largely in the H33342-negative, PI-negative LL quadrant (97.3 %, Figure 3.1Aiii); and cells stained with both H33342 and PI were detected mainly in the H33342-positive PI-negative LR quadrant (98.6 %, Figure 3.1Aiv).

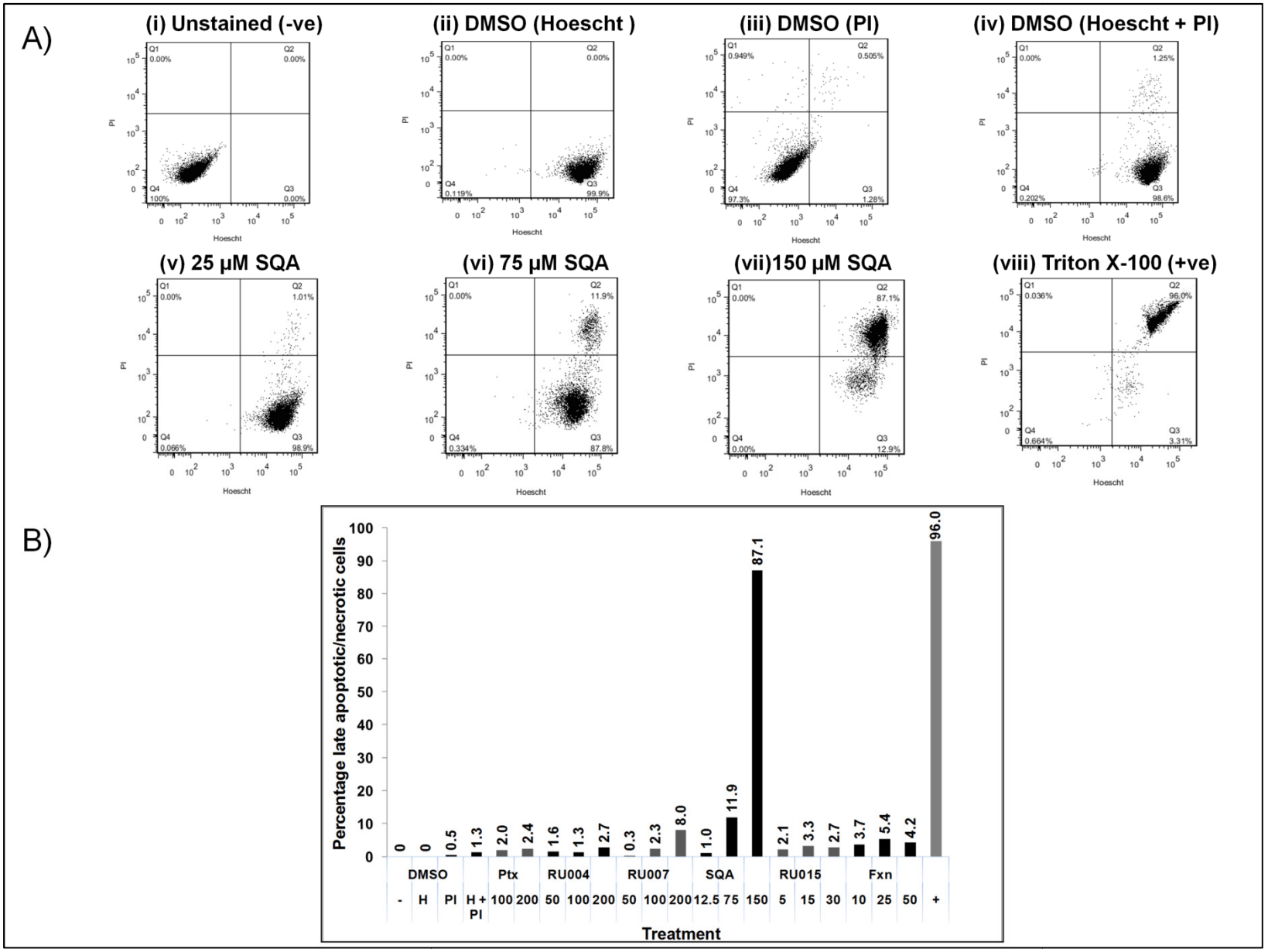
A)
(i) Unstained (-ve)
(ii) DMSO (Hoescht)
(iii) DMSO (PI)
(iv) DMSO (Hoescht + PI)
(v) 25 µM SQA
(vi) 75 µM SQA
(vii)150 µM SQA
(viii) Triton X-100 (+ve)
Q1 0.00%
Q2 0.00%
Q4 100%
Q3 0.00%
Q1 0.00%
Q2 0.00%
Q4 0.119%
Q3 99.9%
Q1 0.949%
Q2 0.505%
Q4 97.3%
Q3 1.28%
Q1 0.00%
Q2 1.25%
Q4 0.202%
Q3 98.6%
Q1 0.00%
Q2 1.01%
Q4 0.066%
Q3 98.9%
Q1 0.00%
Q2 11.9%
Q4 0.334%
Q3 87.8%
Q1 0.00%
Q2 87.1%
Q4 0.00%
Q3 12.9%
Q1 0.036%
Q2 96.0%
Q4 0.664%
Q3 3.31%
PI
Hoescht
B)
Percentage late apoptotic/necrotic cells
0 10 20 30 40 50 60 70 80 90 100
0 0 0.5 1.3 2.0 2.4 1.6 1.3 2.7 0.3 2.3 8.0 1.0 11.9 87.1 2.1 3.3 2.7 3.7 5.4 4.2 96.0
DMSO Ptx RU004 RU007 SQA RU015 Fxn
- H PI H + PI 100 200 50 100 200 50 100 200 12.5 75 150 5 15 30 10 25 50 +
Treatment

Figure 3.1: Characterisation of the mode of cell death induced by paclitaxel and novel marine algal compounds in MDA-MB-231 breast cancer cells. Cells were treated with 0.3 % v/v DMSO (vehicle control) or compounds of interest for 15 h, stained with Hoechst 33342 and/or propidium iodide (PI) and analysed by flow cytometry. Cells that are Hoechst-positive may be alive or undergoing apoptosis, but those that are both Hoechst-positive and PI-positive are either in the late stages of apoptosis or are necrotic. A) Fluorescence dot-plots depicting the effect of DMSO (i-iv) and sargaquinoic acid (SQA) (v-vii) on the viability of MDA-MB-231 cells as measured by Hoechst/PI staining. Unstained DMSO-treated cells (i) served as a negative control (-) and were used to set the gates for each dot-plot, while Triton X-100 (viii) was used to permeabilize cells and served as a positive control (+) for the induction of necrosis. B) Comparison of the proportion of late apoptosis and/or necrosis of the cells in response to treatment with paclitaxel (Ptx), RU004, RU007, RU015, sargaquinoic acid (SQA), fucoxanthin (FXN) and Triton X-100, derived from the percentage of cells in each sample located in the Hoechst-positive and PI-positive upper right-hand quadrant of dot-blots generated using FlowJo software (Tree Star Inc., 2010). Note: not all of the data in 3.1B is represented by dot-plots in 3.1A, however the values were derived in the same manner as for the DMSO- and SQA-treated samples in 3.1A . Concentrations given on the y-axis below each compound name are in nM for Ptx and µM for the algal compounds. Flow cytometric analyses were repeated at least twice and the same trends in terms of mechanism of cell death were observed for all treatments.

The gating strategy derived from the DMSO-treated control allowed for the quantification of the proportion of cells treated with the compounds of interest which were potentially apoptotic or necrotic. The staining profiles for the negative controls indicated that the experimental system was free of background staining or auto-fluorescence which would confound the results obtained for the test compounds.

Treatment with SQA caused a dose-dependent shift in staining towards the H33342-positive, PI-positive UR quadrant representing late apoptotic or necrotic cells (1.0 %, 11.9 % and 87.1 % for treatment with 25, 75 and 150 µM, respectively; Figure 3.1Av - vii). This shift to the UR quadrant via the LR quadrant upon treatment with a compound is typical of apoptosis (Dive *et al*., 1992). In contrast to SQA, treatment of MDA-MB-231 cells with Triton X-100 caused a shift in staining directly to the H33342-positive, PI-positive UR quadrant, appearing as a "comet-shaped" population on the fluorescence dot plot (96 % the UR H33342-positive, PI-positive quadrant; Figure 3.1Aviii). Triton X-100 is a detergent which permeabilizes cells by disruption of their phospholipid membrane, allowing PI to enter the cells, such that this treatment represented a positive control for H33342-positive PI-positive necrotic cells (Dive *et al*., 1992). Indeed, these cells displayed a characteristic H33342-positive, PI-positive necrotic staining pattern with the majority of cells being detected in the UR quadrant of the dot-plot (Figure 3.1A).

As mentioned previously, the fluorescence data plotted for the treatment of MDA-MB-231 cells with Ptx, RU004, RU007, RU015 and FXN (Figure 3.1B) were obtained from the percentage of cells located in the H33342-positive/PI-positive UR quadrant of their respective dot blots (data not shown) in the same manner as for SQA-treated cells. While treatment of cells with the novel polyhalogenated monoterpene, RU007, resulted in a similar flow cytometric profile to that seen for treatment with SQA, namely a dose-dependent increase in late-apoptotic or necrotic cells; treatment with the compounds Ptx, RU004, RU015 and FXN did not produce many cells which were detected as late apoptotic or necrotic at any of the concentrations tested (Figure 3.1B).

3.3.1.2 Determination of whether selected novel algal compounds induce apoptosis or necrosis in Hs78T breast cancer cells

In the case of the Hs578T cell line, flow cytometric detection of H33342 and PI staining was carried out for cells treated with Ptx, RU004, RU007 and FXN (Figure 3.2) in the same manner as for the MDA-MB-231 cell line (Figure 3.1). For the DMSO vehicle-treated negative controls, the unstained population was detected as being both H33342-negative and PI-negative (99.7 % of cells in the LL quadrant, Figure 3.2Ai), the H33342-stained cells exhibited H33342-positive and PI-negative staining (99.7 % of cells in the LR quadrant, Figure 3.2Aii), the PI-stained cells were negative for both H33342 and PI (89.3 % of cells in the LL quadrant, Figure 3.2Aiii) and the

double-stained H33342 and PI cells were detected as H33342-positive and PI-negative (95.6 % of cells in the LR quadrant, Fig3.2Aiv).

As for MDA-MB-231 cells, the controls represented the expected staining patterns, indicating the absence of autofluorescence or background levels of H33342 and PI staining (Figure 3.2A). The positive control sample treated with Triton X-100 also displayed the expected characteristic necrotic staining pattern in the UR quadrant of the H33342/PI dot plot (94.3 % H33342-positive PI-positive cells, Figure 3.2Aviii).

For the Hs578T cell line, Figure 3.2A depicts the fluorescence dot-plots generated for the vehicle-treated negative controls as well those for cells treated with a range of concentrations of the halogenated monoterpene, RU007, as a representative of the novel marine algal compounds; while Figure 3.2B is derived by plotting the percentage of cells in the UR quadrant of the dot blots for each sample (including those not shown in Figure 3.2A) as a "percentage of late apoptotic/necrotic cells". The monoterpene RU007 was selected as a model compound due to its differential toxicity and favourable pharmacological profile (Figure 2.3B, Table 2.2; Chapter 2). Treatment with RU007 resulted in a dose-dependent shift in staining of the Hs578T cells towards the H33342-positive PI-positive UR quadrant (4.4 %, 5.8 % and 23.9 % for treatment with 50, 100 and 200 µM, respectively; Figure 3.2Av-vii). A similar staining pattern was observed for cells treated with Ptx (4.3 %, 10.8 % and 14 % late apoptotic/necrotic cell for treatment with 5, 25 and 125 nM, respectively), as well as for cells treated with the novel monoterpene RU004 (9.4 %, 11.1 % and 24.9 % late apoptotic/necrotic cells for treatment with 50, 100 and 200 µM, respectively) (Figure 3.2B). For both of these compounds, the dose-dependent H33342/PI staining patterns were different between the MDA-MB-231 and Hs578T breast cancer cell lines.

In the case of the carotenoid, FXN, Hs578T cells treated with the compound displayed very low levels of late apoptotic/necrotic cells, even at 25 µM (0.8 %; Figure 3.2B), a concentration almost double the IC_{50} of the compound (13 µM, Figure 2.3B; Chapter 2), which agreed with the results obtained in the MDA-MB-231 cell line (Figure 3.1B)

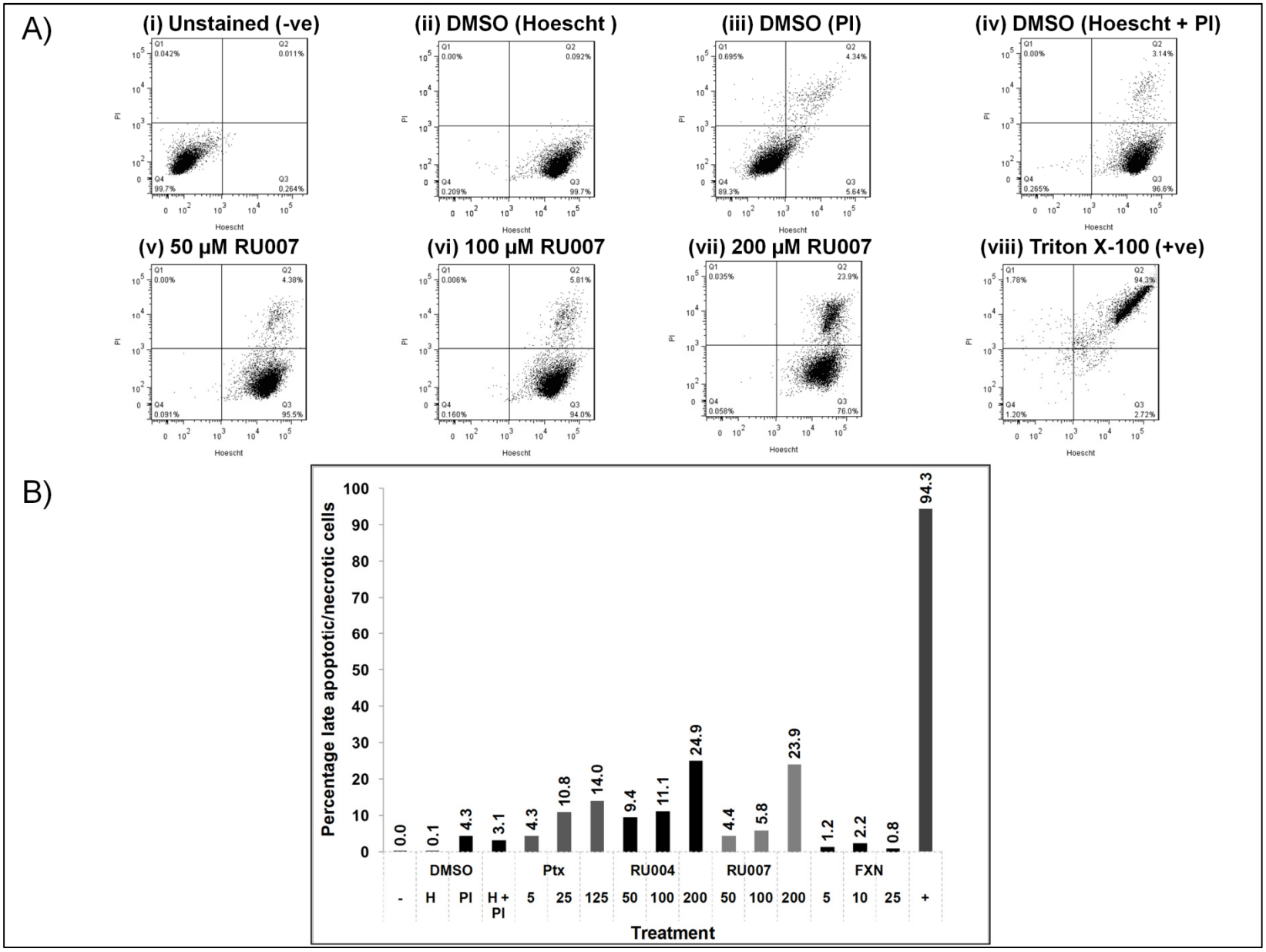
A)
(i) Unstained (-ve)
(ii) DMSO (Hoescht)
(iii) DMSO (PI)
(iv) DMSO (Hoescht + PI)
(v) 50 µM RU007
(vi) 100 µM RU007
(vii) 200 µM RU007
(viii) Triton X-100 (+ve)
PI
Hoescht
B)
Percentage late apoptotic/necrotic cells
0.0
0.1
4.3
3.1
4.3
10.8
14.0
9.4
11.1
24.9
4.4
5.8
23.9
1.2
2.2
0.8
94.3
DMSO
Ptx
RU004
RU007
FXN
-
H
PI
H + PI
5
25
125
50
100
200
50
100
200
5
10
25
+
Treatment

Figure 3.2: Determination of the mode of cell death induced by paclitaxel and novel marine algal compounds in the Hs578T breast cancer cell line. Treatment of cells was with 0.3 % v/v DMSO (vehicle control) or compound of interest for 16 h, followed by staining with Hoechst 33342 and/or propidium iodide (PI) and analysis by flow cytometry. A) The effect of DMSO (i-iv) and RU007 (v-vii) on the viability of Hs578T cells as assessed by Hoechst and PI staining. Unstained DMSO-treated cells (i) served as a negative control (-) and were used to set the gates in each dot-plot, while Triton X-100 treated cells (viii) served as a positive control (+) for the induction of necrosis. B) Comparison of the proportion of late apoptotic and/or necrotic cells in response to treatment with paclitaxel (Ptx), RU004, RU007, RU015, fucoxanthin (FXN) and Triton X-100 (+) derived from the percentage of cells which are detected as Hoechst-positive and PI-positive and thus located in the upper right-hand quadrant of dot-plots generated using FlowJo software (Tree Star Inc., 2010). Concentrations given on the y-axis below each compound name are in nM for Ptx and µM for the algal compounds. Note: not all of the corresponding dot-plots for each treatment are depicted in A. The experiment was repeated at least twice, with similar results being obtained in terms of the mode of cell death observed after treatment with the compounds of interest.

3.3.2 Elucidation of the mode of action of SQA and RU015 in MDA-MB-231 cells by the PARP cleavage assay

Whereas the Hoechst/propidium iodide staining and flow cytometric analyses represented a broader screening-type approach, the PARP cleavage assay was selected as a more focused apoptosis detection assay. Since insufficient amounts of compound were available to perform the latter assay for all compounds, one of the previously characterized algal compounds (ie. SQA and FXN) and one of the novel Rhodes University algal compounds (RU002-081) were selected. The PARP cleavage assay is a more specific protein-based method for the detection of apoptosis and can be used to distinguish between apoptosis and necrosis according to the size of the cleavage products produced; an 85 kDa PARP fragment is typical of apoptosis, while a 50 kDa fragment is indicative of necrosis (Chaitanya and Babu, 2009). The experiment was optimized using the Hsp90 inhibitor, 17-allylamino-17-demethoxygeldanamycin (17-AAG), which is known to induce apoptosis in the MCF-7 breast cancer cell line (Chiosis, 2006). It was determined that treatment with 17-AAG induced PARP cleavage to produce an 85 kDa product, which was evident after 8 hours of treatment (Figure 3.3A). Thereafter, all PARP assays were carried out after 8 hours of treatment with the compound of interest. Cleavage products of 85 kDa for PARP were observed after treatment of MDA-MB-231 cells with increasing concentrations of SQA (Figure 3.3B), while treatment with the novel halogenated monoterpene RU015 generated 50 kDa PARP cleavage products (Figure 3.3C).

The membranes in Figure 3.3B and 3.3C were probed for β-actin as a loading control and indicated that the levels of protein loaded per well in the SQA-treated samples were roughly equivalent (Figure 3.3B), while for the RU015-treated samples, the β-actin signals varied somewhat between samples (Figure 3.3C). The latter changes in the levels of β-actin were reflected in the levels of PARP and its cleavage products (Figure 3.3C). Despite the differences in quantities of protein loaded, PARP cleavage was observed for treatment with all three concentrations of RU015, albeit that this effect was not necessarily dose-dependent.

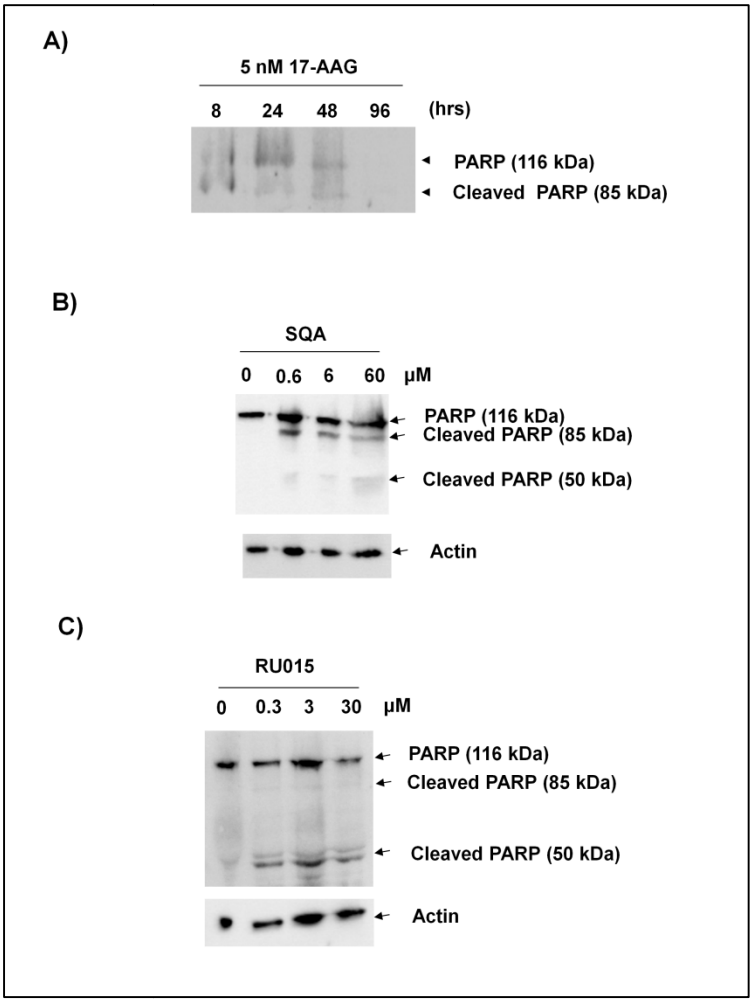

Figure 3.3: Elucidation of the mechanism of cell death induced by sargaquinoic acid and RU015 in MDA-MB-231 breast cancer cells by PARP assay. Western analyses were carried out on whole cell lysates, with cleavage of poly(ADP-ribose) polymerase (PARP) used as an indicator of either apoptosis or necrosis. A) Optimization of assay by treatment with the apoptosis-inducing Hsp90 inhibitor, 17-allylamino-17-demethoxygeldanamycin (17-AAG) over 8, 24, 48 and 96 h. Thereafter assays were carried out over 8 h. B) PARP cleavage products upon treatment with either 0.5 % v/v DMSO (vehicle control), sargaquinoic acid (SQA) or (C) RU015. In each case, detection of β-actin was used to ensure equal loading of protein between samples. The Western analyses were carried out on freshly treated lysates a minimum of two times in order to confirm the results obtained.

3.3.3 Determination of the effects of SQA and FXN on cell cycle distribution in MDA-MB-231 cells by flow cytometry

To determine if either SQA or FXN arrest MDA-MB-231 breast cancer cells in a certain phase of the cell cycle, cells were treated with a range of concentrations of the compounds and their DNA content assessed by flow cytometry with propidium iodide (PI) staining. In addition, this protocol measures the extent of apoptosis according to the DNA content of the cells, such that those cells whose DNA content is less than the G_0-G_1 proportion in the untreated population

are considered to be apoptotic. Figure 3.4A depicts the doublet gating strategy, while Figure 3.4B shows the fluorescence histograms depicting the cell cycle profiles for MDA-MB-231 cells treated with either DMSO vehicle control (i), Ptx (ii), or a range of concentrations of SQA (iii-v), and Figure 3.4C represents a summary of the data in Figure 3.4B obtained by plotting the percentage distribution of the cells in each phase of the cell cycle for each treatment.

In Figure 3.4A, gating of the population was carried out to allow for doublet discrimination. In the analysis of cell cycle distribution by DNA content, it is essential to carefully gate the population of cells on a PI:Area vs. PI:Width dot plot. The reason for this is that the PI Pulse Area (PI:A) signal is unable to distinguish between two cells with a G_1 phase DNA content which are clumped together (2 x n), and a single cell which has twice the DNA content ie G_2-M phase (1 x 2n). Since the PI Pulse Width (PI:W) signal is a representative of the size of the cells, the PI:A vs. PI:W plot allows for the discrimination between cell clumps and cells which are in the G_2-M phase (Nunez *et al.,* 2001).

In Figure 3.4Bi and 3.4Bii, the histograms depicting the cell cycle profiles for the DMSO vehicle-treated negative control and Ptx–treated positive control samples were used to define the gates for each cell cycle phase. Ptx treatment was used as a control since this is known to cause G_2-M phase arrest in breast cancer cells (Tzu-Hao *et al*., 2000). Gating was carried out by assigning the dominant peak as G_0-G_1 phase (PI:A values between 80K and 120K) and defining the G_2-M phase as a peak at a PI:A value along the X-axis that was roughly double that for G_0-G_1 and that corresponded to the dominant peak in the Ptx-treated control (PI:A values between 140K and 240K). The remainder of the histogram was divided up into the sub-G_0 apoptotic and S-phase gates and copied to all samples. For the positive control, treatment with Ptx resulted in a shift from 39.8 % of the population in the G_2/M phase in untreated MDA-MB-231 cells to 45.2 % in cells treated with 50 nM Ptx (Figure 3.4Bi and Figure 3.4Bii, respectively).

In the case of SQA, treatment with various concentrations of the quinone compound resulted in an altered cell cycle profile when compared to the vehicle treated control (Figure 3.4Biii-v and 3.4Bi, respectively, Figure 3.4C). In addition to a dose-dependent increase in the sub-G_0 apoptotic population (1.7 %, 7.4 % and 14.9 % for treatment with 25, 75 and 120 µM, respectively), an increase in the G_0-G_1 phase (63.8 %, 55.6 % and 56.7 %, respectively) as well as a dose-dependent decrease in the G_2-M phase (27.8 %, 25.9 % and 17.7 %, respectively) was observed upon treatment with SQA (Figure 3.4Biii-v and C).

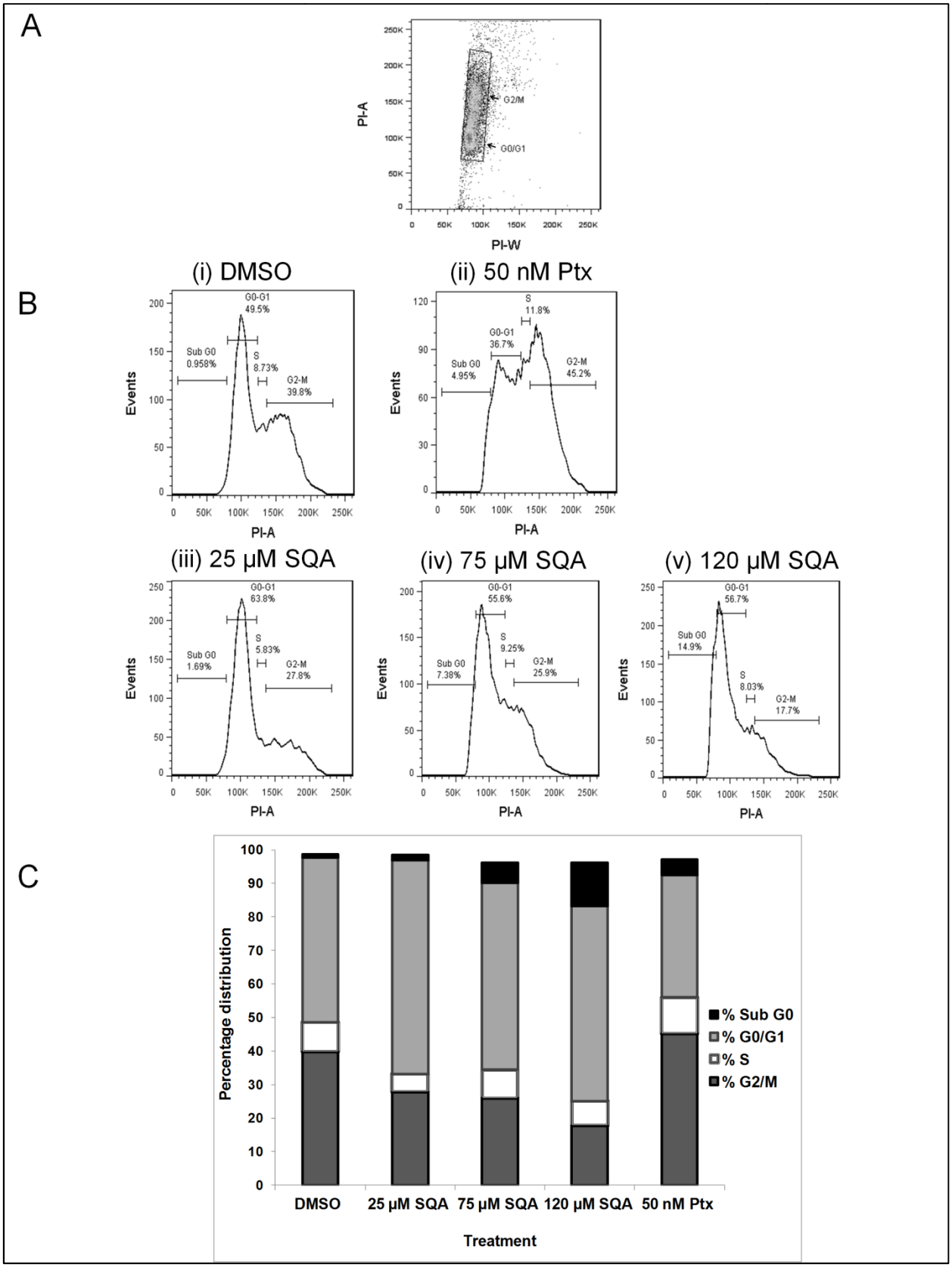
A
PI-A
PI-W
G2/M
G0/G1
B
(i) DMSO
G0-G1
49.5%
Sub G0
0.958%
S
8.73%
G2-M
39.8%
Events
PI-A
(ii) 50 nM Ptx
S
11.8%
G0-G1
36.7%
Sub G0
4.95%
G2-M
45.2%
(iii) 25 μM SQA
G0-G1
63.8%
Sub G0
1.69%
S
5.83%
G2-M
27.8%
(iv) 75 μM SQA
G0-G1
55.6%
Sub G0
7.38%
S
9.25%
G2-M
25.9%
(v) 120 μM SQA
G0-G1
56.7%
Sub G0
14.9%
S
8.03%
G2-M
17.7%
C
Percentage distribution
DMSO
25 μM SQA
75 μM SQA
120 μM SQA
50 nM Ptx
Treatment
% Sub G0
% G0/G1
% S
% G2/M

Figure 3.4: Analysis of the effect of sargaquinoic acid on cell cycle in MDA-MB-231 breast cancer cells. Cells were treated with vehicle control (0.24 % v/v DMSO), 25, 75 or 120 μM sargaquinoic acid (SQA) or 50 nM paclitaxel (Ptx) for 16 h, stained with propidium iodide (PI) and analysed by flow cytometry. (A) Gating of cell population to allow for doublet discrimination, in which false positives due to cell aggregates are eliminated. The singlet gate was copied to all samples such that subsequent analyses were carried out only for cells within the gate. (B) Histograms showing the cell cycle profile of MDA-MB-231 cells upon treatment with either the DMSO vehicle control (i), Ptx (ii) or a range of concentrations of SQA (iii-v). The phases of the cell cycle were gated according to the DMSO-treated control such that the dominant peak represented G_0-G_1 phase and the G_2-M gate was defined for a peak at a PI-A value along the X-axis that was roughly double of that for G_0-G_1. The remainder of the histogram was divided up into the sub-G_0 apoptotic and S-phase gates. (C) Summary of the alterations in cell cycle distribution and apoptosis induction by SQA and Ptx as depicted in B. The assay was repeated twice, with the same trends in terms of changes in cell cycle distribution upon treatment being observed.

For treatment of MDA-MB-231 cells with FXN, Figure 3.5 is laid out in a similar manner as for SQA treatment (Figure 3.4), where Figure 3.5A represents the doublet gating strategy, Figure 3.5B depicts the individual fluorescence histograms for each treatment and Figure 3.5C represents a summary of the data in Figure 3.5B in graph form. The gating of the various cell cycle phases according to the DMSO and paclitaxel controls in Figure 3.5Bi was carried out using the same criteria to that in Figure 3.4Bi, although the position of the gates is not identical due to variations in the controls between the two data sets. This demonstrated the importance of carrying out the gating separately for each flow cytometry experiment. In Figure 3.5B, the G_0-G_1 gate was set at PI:A values between 80K and 100K, while the G_2-M gate was set between 140K and 240K, allowing for the assignment of the sub-G_0 and S phases. These gates were copied to all the samples and variations in cell cycle distribution noted. Treatment with 50 nM Ptx revealed a shift in PI staining for the G_2-M phase from 13.2 % in untreated cells to 45.2 % in treated cells (Figure 3.5Bi and Figure 3.5Bii, respectively). For the carotenoid, FXN, a similar alteration in cell cycle distribution was observed in treated cells to that observed for SQA (Figure 3.5B vs. Figure 3.4B), namely a dose-dependent increase in both the Sub-G_0 apoptotic population (5 %, 8 % and 12 % for treatment with 10, 25 and 50 µM, respectively) and the G_0-G_1 phase (23 %, 33 % and 40 %, respectively), as well as a dose-dependent decrease in both the S (43 %, 36 % and 30 %, respectively) and G_2-M phases (30 %, 23 % and 18%, respectively) of the cell cycle (Figure 3.5Biii-v vs. Figure 3.4Biii-v).

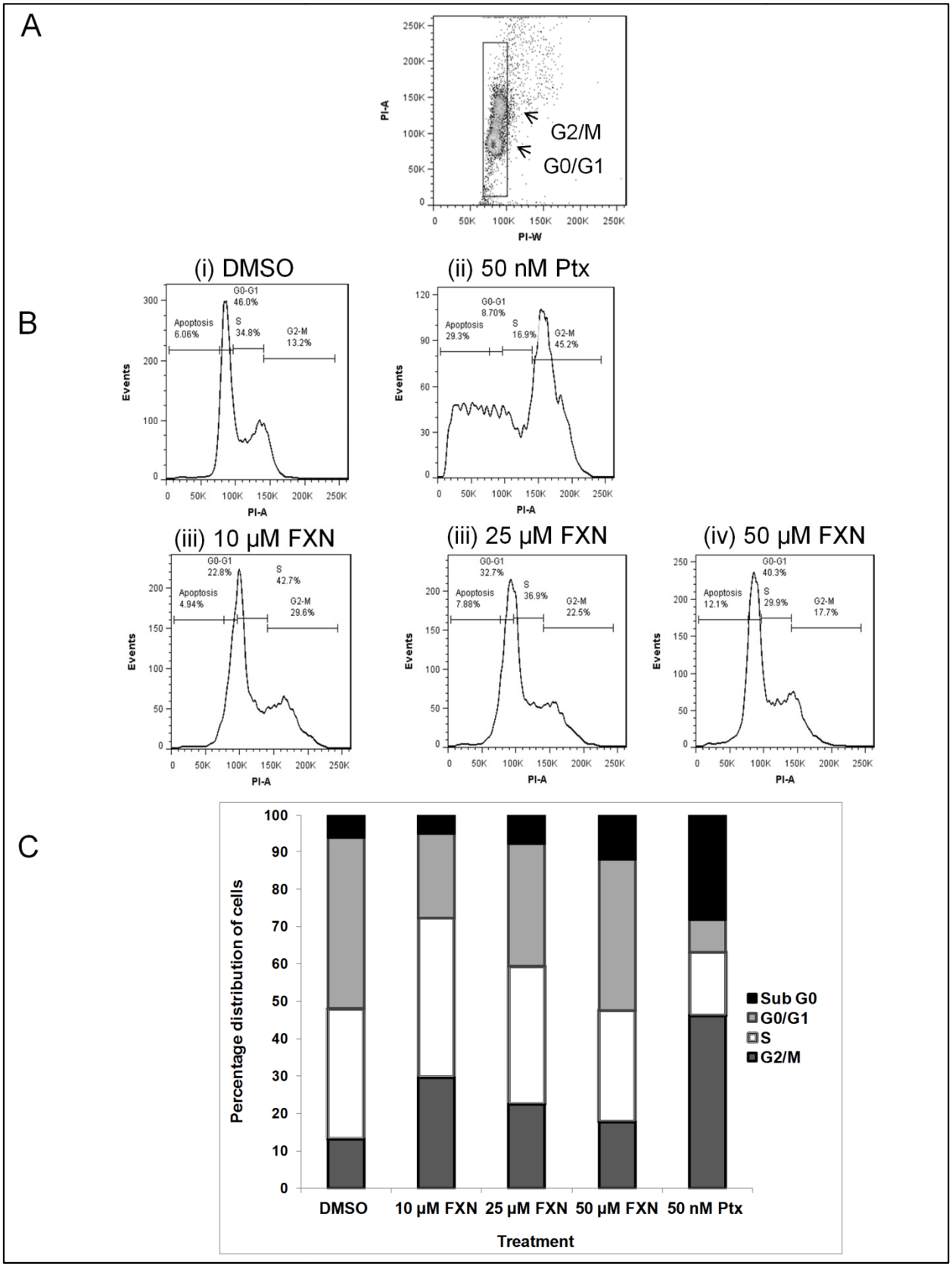
A
PI-A
PI-W
G2/M
G0/G1
B
(i) DMSO
G0-G1 46.0%
Apoptosis 6.06%
S 34.8%
G2-M 13.2%
(ii) 50 nM Ptx
G0-G1 8.70%
Apoptosis 29.3%
S 16.9%
G2-M 45.2%
(iii) 10 μM FXN
G0-G1 22.8%
Apoptosis 4.94%
S 42.7%
G2-M 29.6%
(iii) 25 μM FXN
G0-G1 32.7%
Apoptosis 7.88%
S 36.9%
G2-M 22.5%
(iv) 50 μM FXN
G0-G1 40.3%
Apoptosis 12.1%
S 29.9%
G2-M 17.7%
Events
PI-A
C
Percentage distribution of cells
DMSO
10 μM FXN
25 μM FXN
50 μM FXN
50 nM Ptx
Treatment
Sub G0
G0/G1
S
G2/M

Figure 3.5: Determination of the effect of fucoxanthin treatment on cell cycle in MDA-MB-231 cells. Cells were treated with vehicle control (0.24 % v/v DMSO), 10, 25 or 50 µM fucoxanthin (FXN) or 50 nM paclitaxel (Ptx) for 16 h, stained with propidium iodide (PI) and analysed by flow cytometry. (A) Gating of cell population to allow for doublet discrimination. The singlet gate eliminates false positives due to cell aggregates and was copied to all samples. Further analyses were carried out on populations contained within the gate.(B) Histograms representing the cell cycle profile of MDA-MB-231 cells upon treatment with either DMSO (i), Ptx (ii) or a range of concentrations of FXN (iii-v). The phases of the cell cycle were gated with respect to the DMSO-treated control such that the dominant peak was defined as the G_0-G_1 phase and the G_2-M gate was set for a peak whose PI-A value along the X-axis was roughly double of that for G_0-G_1. The remainder of the histogram was divided into the sub-G_0 apoptotic and S-phase gates. (C) Summary of changes in cell cycle distribution and apoptosis induction by FXN and Ptx derived from the histograms in B. The assay was repeated and similar alterations in cell cycle distribution observed.

3.4 DISCUSSION

The present study aimed to elucidate the mode of action of selected novel marine algal compounds shown to display cytotoxicity against breast cancer cells *in vitro*. In the first instance, the mode of cell death was established for selected compounds by means of flow cytometry with Hoechst and propidium iodide staining in order to distinguish between the induction of programmed cell death (apoptosis) and necrosis. The former is preferred in terms of drug development since necrosis would result in an undesirable inflammatory response *in vivo* (Klener Jr. *et al.,* 2006).

In the case of the tetraprenylated quinone SQA, shown to display differential toxicity against breast cancer but not non-cancerous breast cells (Chapter 2), the dose-dependent increase in treated cells which were stained positive for both H33342 and PI suggested that the compound induced apoptosis in MDA-MB-231 breast cancer cells (Dive *et al*., 1992). This finding was consistent with that in a previous study in which SQA was reported to induce apoptosis in the human keratinocyte cell line HaCaT using flow cytometry with PI staining alone (Hur *et al.,* 2008). The current study was encouraging since, at 75 µM, a concentration near the IC_{50} of SQA (67 µM, Chapter 2; Figure 2.3A), only 11 % of treated cells were detected as being late apoptotic or necrotic.

As was described for SQA, the staining profile of cells treated with RU007 suggested the induction of apoptosis by this compound. It was expected that the chemotherapeutic, paclitaxel (Ptx), known to induce apoptosis in breast cancer cells *in vitro*, (Tzu-Hao *et al*., 2000) would display a similar dose-dependent increase in the percentage of late apoptotic/necrotic cells (derived from the upper right hand (UR) quadrant of a PI vs. H33342 dot plot). This was, however, not observed in our study (2.0 % and 2.4 % late apoptotic/necrotic cells after treatment with 100 nM and 200 nM, respectively). It must be noted at this point that failure of a compound to produce an increase in the proportion of late-apoptotic/necrotic cells does not necessarily indicate that the compound was not inducing apoptosis of the cells, since both live and early apoptotic cells are stained H33342-positive PI-negative. Since treatment of cells for flow cytometry was carried out overnight, as opposed to the 96 hour treatment interval used for cytotoxicity screening of the marine algal compounds (Chapter 2), it is possible that the cells were in the early stages of apoptosis and thus indistinguishable from live cells. This represented a potential shortcoming of the flow cytometric assay in that, although a dose-dependent increase in H33342-positive/PI-positive staining (or the proportion of late apoptotic/necrotic cells) has been associated with apoptosis induction in the literature (Dive *et al*., 1992), the assay was originally designed largely for the detection of necrotic cells, which are stained PI-positive. More accurate methods exist for the detection of apoptosis, such as the PARP cleavage and TUNEL assays.

For all the compounds tested in MDA-MB-231 cells, none displayed the characteristic necrotic staining pattern exhibited by Triton X-100. In the case of the potential "hit" compound RU004, the fact that the compound did not appear to induce necrosis was encouraging and described a further favourable characteristic of the compound, in terms of its development as a lead compound. For RU015, the most toxic compound in the library of novel marine algal compounds towards breast cancer cells (Chapter 2, Figure 2.3) and which had previously been shown to be equally toxic to cancerous MCF-7 and non-cancerous MCF12A cells (Lawson, MSc thesis, 2009; Figure A1), one might expect that the compound would induce necrosis of the cells and thus display a H33342/PI staining pattern similar to that of Triton X-100-treated cells. This was, however, not observed in the flow cytometry profiles for RU015-treated MDA-MB-231 cells (2.1 %, 3.3 % and 2.7 % late apoptotic/necrotic cell for treatment with 5, 15 and 30 µM, respectively).

In the case of the Hs578T cell line, H33342/PI staining was carried out on cells treated with either RU004, RU007, FXN or Ptx in order to compare the modes of action of priority compounds between this and the MDA-MB-231 cell line. For Hs578T cells, treatment with RU007 resulted in a dose-dependent shift in staining the towards the H33342-positive PI-positive UR quadrant, suggesting that the compound induces apoptosis of the cells, a result which was similar to that obtained for the compound in MDA-MB-231 cells. A dose-dependent shift in staining the towards the H33342-positive PI-positive UR quadrant was also observed for cells treated with either Ptx or RU004, once more suggesting that the compounds induced apoptosis of the cells. For both of these compounds, the dose-dependent H33342/PI staining patterns were different between the MDA-MB-231 and Hs578T breast cancer cell lines. These variations could be point to different modes of action of the compounds in the different breast cancer cell lines, a phenomenon which has previously been observed for resveratrol in MCF-7 versus MDA-MB-231 cells (Pozo-Guisadoa *et al*., 2002). On the other hand, the differences in staining patterns could be attributed to the inability of the flow cytometric technique to distinguish between live and early apoptotic cells, such that an apoptotic effect of the compounds in MDA-MB-231 cells was not detected.

In the case of the carotenoid FXN, Hs578T cells treated with the compound displayed a very low proportion of late apoptotic/necrotic (H33342-positive/PI-positive) cells, even at 25 µM, a concentration almost double the IC_{50} of the compound (13 µM, Chapter 2, Figure 2.3B), which agreed with the results obtained in the MDA-MB-231 cell line.

As mentioned previously, the PARP cleavage assay is a more accurate and definitive measure of cells undergoing apoptosis. Two of the priority compounds used in the H33342/PI flow cytometry studies were further analyzed in terms of their ability to induce apoptosis or necrosis of MDA-MB-231 cells by means of the PARP cleavage assay. In this assay, 85 kDa PARP

cleavage products were observed for cells treated with a range of concentrations of SQA, indicating that the compound induced apoptosis in MDA-MB-231 cells. Apoptotic PARP fragments have been reported in human HaCaT skin cancer cells treated with 23.6 µM of SQA, a concentration at which cell viability was reduced to 20 % as assessed by MTT assay (Hur *et al.,* 2008). Interestingly, in the latter, no PARP fragments were detected at lower concentrations of the compound, while in the current study, apoptotic PARP fragments were detected at a concentration one hundred times lower than the IC_{50} of the compound in MDA-MB-231 cells (67 µM, Figure 2.3A).

It was thought that the novel halogenated monoterpene RU015 may be having an effect on the cells which was not detectable by flow cytometry and further mechanistic studies were carried out on the compound. Indeed, treatment with RU015 generated a 50 kDa PARP cleavage product in MDA-MB-231 cells. Such cleavage products are typical of degradation by cathepsin B (Chaitanya and Babu, 2009) and suggested that necrosis was responsible for the high cytotoxicity of the compound in these cells and potentially in both Hs578T breast cancer and MCF12A non-cancerous breast cells. PARP fragments of 50 kDa and 62 kDa have been observed during necrosis in Jurkat cells, while 89, 50, 40 and 35 kDa fragments have been reported in necrotic HL60 cells (Soldani and Scovassi, 2002).

Since it has been reported that many compounds exert their cytotoxic effects by causing cell cycle arrest, in addition to the induction of apoptosis (Palozza *et al*., 2002) the effects of both SQA and FXN treatment on cell cycle distribution was assessed in the MDA-MB-231 cell line. The assay was validated by treatment with Ptx, which is known to cause G_2-M phase arrest in breast cancer cells (Tzu-Hao *et al*., 2000), and resulted in an increase in the proportion of MDA-MB-231 cells in G_2-M phase compared to the DMSO vehicle treated control. In the case of SQA, the dose-dependent increase in the Sub-G_0 apoptotic population for treated cells confirmed the findings obtained by H33342/PI staining and PARP cleavage assays, namely that SQA appeared to induce apoptosis in MDA-MB-231 cells. At the sub-lethal SQA dose of 25 µM (IC_{50} of 67 µM; Figure 2.3A, Chapter 2), an increase in G_0-G_1 phase from 49 % to 64 % was observed, indicating cell cycle arrest at that phase. This effect decreased with increased SQA concentration, when apoptosis became the main mode of action of the compound (as opposed to cell cycle arrest). In addition, SQA caused a dose-dependent decrease in the G_2-M phase, a trend which was opposite to that observed for paclitaxel-treated cells and points to a different mechanism of action of the quinone. It has been found that the twenty carbon aromatic compound retinoic acid, which is structurally similar to SQA (Figure A2), causes G_1 arrest in MCF-7 breast cancer cells (Zhu *et al.,* 1997), which is consistent with our findings in metastatic MDA-MB-231 cells.

Treatment of MDA-MB-231 cells with the carotenoid FXN also resulted in an altered cell cycle profile compared to the DMSO-treated control. The dose-dependent increase in the Sub-G_0

apoptotic population provided the first evidence that FXN may be inducing apoptosis in the MDA-MB-231 breast cancer cells, since this was not distinguishable by the H33342/PI flow cytometry analyses. FXN has previously been demonstrated to induce apoptosis in leukemia as well as prostate and colon cancer cells *in vitro* (Kotake-Nara *et al.,* 2001a and b; Hosokawa *et al.,* 1999; Hosokawa *et al.,* 2004). In addition, the G_0-G_1 phase arrest observed for FXN in breast cancer cells is consistent with that reported for the compound in both WiDr and HCT116 human colon cancer cell lines, as well as GOTO neuroblastoma cells in previous studies (Das *et al.,* 2005; Okuzumi *et al*., 1990). In the former study, the effect of FXN on cell cycle was further characterized and found to be mediated through the up-regulation of $p21^{WAF1/Cip1}$ (Das *et al.,* 2005). This allows for the tentative prediction that the compound may display a similar mechanism of action in human colon and breast cancer cells *in vitro*.

The similarity in cell cycle alterations induced by SQA and FXN could point to similar mechanisms of action of the two compounds. This would not be unexpected considering that their structures are much alike, consisting of either one (SQA) or two (FXN) aromatic rings linked to a sixteen carbon long aliphatic chain (Figure 2.2, Chapter 2). The cell cycle data obtained for treatment of MDA-MB-231 cells with either SQA or FXN could be validated by treatment with a compound known to cause G_0-G_1 phase arrest in breast cancer cells. The similarities between the cell cycle distribution after FXN treatment in this study and those reported in the literature for other cancer cell lines (Das *et al.,* 2005; Okuzumi *et al*., 1990) are, however, encouraging.

What is immediately apparent is that the untreated controls vary greatly in terms of their cell cycle distribution between the two different cell cycle studies (Figure 3.4 vs. Figure 3.5). The cell cycle distribution is dependent on the growth rate of the cells in culture overnight, which may fluctuate from experiment to experiment and highlights the importance of comparing the cell cycle distribution of treated cells in each experiment to an untreated control from the same experiment, as opposed to assuming that the cell cycle distribution of the untreated population is constant.

3.5 CONCLUSIONS

In summary, the data herein describes the further characterization of selected novel algal compounds in terms of their mode of action. While there have been several reports describing the anti-cancer activity of marine algal metabolites, this is one of a limited number attempting to determine the manner in which novel algal compounds exert their cytotoxic effects (Jha and Zi-Rong, 2004; Fuller *et al*., 1994). Of the compounds selected for further analysis in this study, RU007 and SQA appeared to induce apoptosis in MDA-MB-231 cells, while the flow cytometric profile of both RU004 and RU007 were suggestive of apoptosis in the Hs578T cell line. The ability of RU004 to induce apoptosis in MDA-MB-231 cells could not be ruled out using the H33342/PI staining protocol. The more informative PARP assay was utilized to determine the mode of cell death induced by SQA and RU015 in MDA-MB-231 cells and it was determined that the differentially toxic "hit" compound, SQA , induced apoptosis in MDA-MB-231 cells; while the indiscriminately toxic halogenated monoterpene, RU015, induced necrosis in these cells, resulting in its exclusion from further characterization in this study. The former result for SQA treatment represented the first report as to the mode of cell death induced by the compound in breast cancer cells.

In addition to investigation into the type of cell death induced by novel marine algal compounds, the present study examined the effect on cell cycle distribution in MDA-MB-231 breast cancer cells of both SQA and FXN. Interestingly, the latter compounds exhibited similar effects on cell cycle in MDA-MB-231 cells, namely an arrest in G_0-G_1 phase with an increase in the Sub-G_0 apoptotic population, a trend which is both consistent with the previous findings for FXN in human colon cancer and neuroblasoma cells, and mimics that of the structurally similar compound, retinoic acid (Das *et al.,* 2005; Zhu *et al.,* 1997). To the best of our knowledge, this is the first report of the effect of SQA acid on cell cycle distribution in cancer cells and the first study of the effect of either SQA or FXN on cell cycle in breast cancer cells.

The aforementioned studies into their mode of action validated the selection of the "hit" compounds RU004, RU007 and SQA in Chapter 2. Furthermore, the data generated in this body of work justified further study of these novel marine algal compounds in terms of the specific signaling pathways and molecules involved in mediating their cytotoxicity in metastatic breast cancer cells.

CHAPTER 4

CHARACTERIZATION OF THE MOLECULAR MECHANISMS MEDIATING TOXICITY OF THE NOVEL ALGAL COMPOUNDS SQA AND FXN AGAINST BREAST CANCER CELLS *IN VITRO*

4.1 INTRODUCTION

Cancer cells are characterized by multiple alterations in key signal transduction pathways in order to achieve uncontrolled proliferation and evasion of apoptosis (Martin, 2003; Dhillon *et al*., 2007). The deregulation of apoptosis by either the decreased expression of pro-apoptotic molecules or increased expression of anti-apoptotic molecules has been reported for many cancers, including breast cancer (Jin and El-Deiry, 2005). As a result, the apoptotic machinery has become an important target for drug development (Debatin and Krammer, 2004). Cysteine aspartic acid-specific proteases or caspases play a key role in both in the initiation and execution of apoptosis (Green and Kroemer, 1998; Jin and El-Deiry, 2005). Caspases are expressed as inactive precursors which are activated by proteolytic cleavage giving rise to cascades where each caspase cleaves and activates the next in the chain. These proteases are traditionally classified into two main groups; those that mediate apoptosis (caspase-2, -3, -6, -7, -8, -9, 10) and those that participate in inflammation (caspase-1, -4, -5, -11, -12, -13 and -14) (Thornberry *et al*., 1997; Martinon *et al*., 2002; Jin and El-Deiry, 2005; Klener Jr. *et al*., 2006). The apoptotic caspases are further divided into initiator and effector caspases. The initiator caspases (2, 8, 9 and 10) activate the effector caspases (3, 6 and 7), which in turn cleave various cellular substrates in order to induce cell death (Jin and El-Deiry, 2005).

Apoptosis occurs via two main signaling cascades, namely the extrinsic and the intrinsic pathways. In the extrinsic or receptor-mediated pathway, a death ligand such as tumour necrosis factor alpha (TNFα) binds to its cognate death receptor, in this case TNF receptor (TNFR), resulting in the recruitment and subsequent activation of caspase-8, the initiator caspase of this pathway (Nagata, 1997). On the other hand, the intrinsic mitochondrial pathway is induced in response to cellular stresses including exposure to cytotoxic agents, DNA damage and growth factor deprivation, which induce mitochondrial outer membrane permeabilisation (MOMP) and subsequent release of cytochrome C. The end result of this process is the cleavage and activation of the initiator caspase, caspase-9 (Jin and El-Deiry, 2005; Klener Jr. *et al*., 2006; Iannolo *et al*., 2008). MOMP is tightly regulated by Bcl-2 family proteins, which are classified as either pro-apoptotic (Bax, Bak, Bim, Bad, Bid) or anti-apoptotic (Bcl-2, Bcl-XL, Mcl-1) (Jin and El-Deiry, 2005). The extrinsic and intrinsic pathways are interconnected via the pro-apoptotic Bid protein and converge on an executioner caspase, such as caspase-3 (Debatin and Krammer, 2004).

In order to progress through the phases of drug development, the precise cellular pathways and target molecules mediating the activity of a “hit” anti-cancer compound must be elucidated (Li and Vanderas, 2009). Of particular interest are the effects that such an agent may have on the apoptotic and survival pathways described above. In the case of algal anti-cancer lead compounds, few reports exist in which the cellular targets of the novel compounds have

been identified. One rare example includes the finding that sargaquinoic acid (SQA) induces apoptosis via caspase-3, -8 and -9 in human skin cancer cells *in vitro* (Hur *et al*., 2008). Perhaps the best characterized algal metabolite is the carotenoid, fucoxanthin (FXN), isolated from a range of brown algal species including *Laminaria japonica*, *Sargassum heterophyllum* and *Undaria pinnatifida* (Peng *et al*., 2011). Several preliminary investigations into the molecular mechanisms mediating the cytotoxicity of the compound have been carried out and have identified caspase-3, -7, -8 and -9, as well as the Bcl-2 proteins Bax, Bcl-2 and Bcl-X_L as potential targets of FXN. However, these data are fragmented since studies were carried out in a range of different cancer cell lines using different techniques, with few corroborating results (Kotake-Nara *et al*., 2005a and b; Hosokawa, 1999; Das *et al*., 2005; Ishikawa *et al*., 2008; Peng *et al*., 2011). Thus, to date, the precise molecular mechanism of action of both SQA and FXN are incompletely understood

The broad aim of the following section entailed the identification of specific pathways and signal transducer molecules involved in mediating the cytotoxicity of the previously described algal compounds sargaquinoic acid and fucoxanthin in breast cancer cells. In particular, the potential role of a number of caspases, together with the anti-apoptotic molecule, in mediating the action of FXN and/or SQA was assessed.

4.2 METHODS AND MATERIALS

4.2.1 Reagents

The rabbit anti-human caspase-9 primary antibody was obtained from Cell Signaling Technologies (Massachusetts, USA), while the rabbit anti-β-actin and mouse anti-human Bcl-2 primary antibodies were from Sigma-Aldrich (Germany) and Abcam (UK), respectively. The donkey anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibody was obtained from Amersham Biosciences (UK), while the donkey anti-mouse fluorescein isothiocyanate (FITC)-conjugated secondary antibody was from Invitrogen (UK). The caspase inhibitor sampler kit and MTT cell proliferation kit were obtained from R and D Systems (Minneapolis, USA) and Roche (Switzerland) respectively. Tumour necrosis factor alpha (TNFα), paclitaxel and fucoxanthin were from Sigma-Aldrich (Germany). Tissue culture vessels were from Corning (Pennsylvania, USA). All other chemicals were of reagent grade.

4.2.2 Cell lines and culture conditions

The breast cancer cell lines, MDA-MB-231 (ATCC: HTB-26) and Hs578T (ATCC: HTB-126), were cultured as described in Chapter 2 (Section 2.2.2). The MCF-7 breast cancer cell line (ATCC: HTB-22) was a gift from Dr Sharon Prince (Department of Human Biology, Faculty of Health

Sciences, University of Cape Town) and was cultured in the same manner as the MDA-MB-231 cell line.

4.2.3 Caspase inhibitor studies

MCF-7, MDA-MB-231 or Hs578T cells were seeded at a density of 6000 cells per well in a 96-well plate and allowed to settle overnight, followed by pre-treatment with 500 nM, 5 µM or 50 µM of the caspase inhibitor (dissolved in DMSO, max concentration 0.5 % v/v) of interest for 4 hours. Thereafter, cells were treated with either DMSO vehicle control (0.5 % v/v) or 200 ng/ml TNF or 75 µM sargaquinoic acid or 15 or 25 µM fucoxanthin for 96 hours. Treated cells were assessed for viability by MTT assay as described in Chapter 2 (Section 2.2.3).

4.2.4 Western analyses

MDA-MB-231 cells (1×10^6) were seeded into 60 mm plastic tissue culture dishes and allowed to settle overnight. The cells were treated with either DMSO vehicle control (0.5 % v/v) or sargaquinoic acid (0.6, 6 or 60 µM) for 8 hours at 37 ^oC and 9 % CO_2. Thereafter, whole cell lysates were prepared by scraping the adherent cells into the media, spinning at 2000 rpm in an Eppendorf 5415 R centrifuge using a F45-24-11 rotor (Eppendorf, Germany) for 3 minutes and resuspending in 100 µM sodium dodecyl sulphate (SDS) sample buffer [125 mM Tris-HCl pH 6.8, 2 % (w/v) SDS, 20 % (v/v) glycerol, 0.2 % (w/v) bromophenol blue) before boiling for 5 minutes. Lysates from the equivalent of 1×10^5 cells were separated by electrophoresis using a 12 % SDS polyacrylamide gel, transferred onto a nitrocellulose membrane and probed for caspase-9. Incubation with rabbit anti-human caspase-9 antibody (1 in 1000 dilution) was carried out at 4 ^oC overnight. Following incubation with the HRP-conjugated donkey anti-rabbit secondary antibody (1 in 5000) for I hour at room temperature, detection was carried out using the ECL Advanced Western Blotting Kit (GE Healthcare, UK) and visualized using the Molecular Imager ChemiDoc XRS System (BioRad, California). Lysates were probed with rabbit anti-β-Actin antibody as a loading control. All antibody dilutions were carried out using a 5 % (w/v) non-fat milk powder solution in Tris-buffered saline (TBS) (50 mM Tris, 150 mM NaCl, pH 7.5).

4.2.5 Quantification of Bcl-2 protein levels using flow cytometry

MDA-MB-231 or Hs578T cells were seeded at a density of 3 x 10^5 cells/ml into 25 cm^2 culture flasks and allowed to settle and grow over 24 hours before dosing with the DMSO vehicle control (0.02 % v/v) or relevant compounds (as indicated in figure legends) overnight. Cells were lifted with 1 % (v/v) trypsin solution made up in 0.3 % (w/v) ethelene diamine tetra-acetic acid (EDTA), washed twice in ice-cold phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 2 mM KH_2PO_4, 10 mM Na_2HPO_4, pH 7.4) and collected by centrifugation at 4000 rpm in a Eppendorf 5804 R centrifuge with a A-4-44 rotor (Eppendorf, Germany). The resulting pellet was resuspended to a density of 5 X 10^6 cells/ml in 0.01 % (v/v) formaldehyde and fixed on ice

for 10 minutes. Cells were collected by centrifugation and resuspended to a density of 5 X 10^6 cells/ml in permeabilisation buffer (PB) [0.1 % (v/v) Triton X-100 in PBS containing 0.5 % (w/v) BSA] prior to incubation in the dark for 15 minutes on ice. Thereafter cells were collected by centrifugation and resuspended in PB to the same density as before. Immunostaining of cells was carried out using 1 µg of mouse anti-human Bcl-2 primary antibody in 100 µL of cell solution and incubated at 4 ^{o}C for 1 hour. Cells were washed twice with permeabilisation wash buffer (PWB) [0.1 % (v/v) Triton X-100 in PBS, pH 7.4], followed by incubation with 2 µL of donkey anti-mouse FITC-conjugated secondary antibody for 45 minutes at 4 ^{o}C in the dark. Cells were washed in PWB, resuspended in 100 µL PBS and analysed by flow cytometry. Excitation of FITC was achieved at 488 nm and emission detected at 530 nm. Data was analysed using FlowJo software Version 7.6.1. (Tree Star Inc., 2010).

4.3 RESULTS

4.3.1 Identification of caspases involved in mediating the cytotoxicity of paclitaxel and selected algal compounds in breast cancer cells *in vitro*

In an attempt to characterize the molecular mechanism of action of the commonly used chemotherapeutic drug paclitaxel (Ptx), as well as the marine algal compounds sargaquinoic acid (SQA) and fucoxanthin (FXN) in breast cancer cells, the ability of various caspase inhibitors to reverse the cytotoxicity of these compounds against breast cancer cells was investigated. Although a single graph is represented in each case, the assays were repeated and consistent results obtained.

4.3.1.1 Optimization of caspase inhibitor-coupled MTT assay using tumour necrosis factor alpha (TNF-α)-treated MCF-7 breast cancer cells

Since a wide range of effective concentrations had been reported for the caspase inhibitor kit utilized in this study, optimization experiments were carried out for two of the inhibitors, namely the general caspase inhibitor (Z-VAD-FMK) and the caspase-8 inhibitor (Z-IETD-FMK). MCF-7 breast cancer cells were pre-treated with 500 nM, 5 µM or 50 µM of the caspase inhibitors, followed by treatment with 200 ng/ml of TNF-α and assessed in terms of percentage survival by MTT assay. All of the samples depicted in Figure 4.1A, with the exception of the DMSO control, were treated with TNFα, which was selected as a positive control due to the fact that it is known to bind to its cognate receptor TNFR1 at the cell surface and initiate the extrinsic apoptotic pathway via caspase-8 (Nagata, 1997).

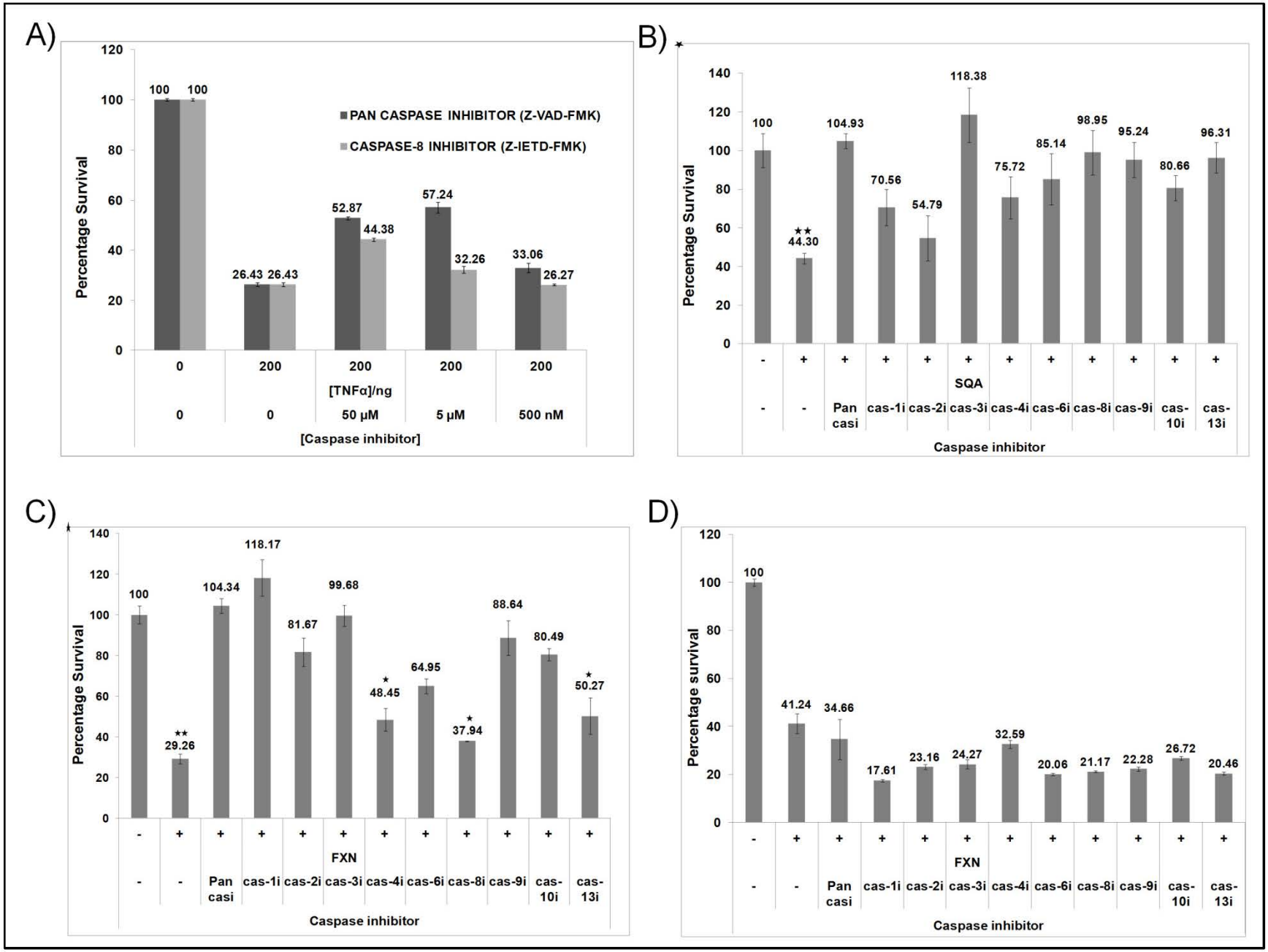
A)
Percentage Survival
PAN CASPASE INHIBITOR (Z-VAD-FMK)
CASPASE-8 INHIBITOR (Z-IETD-FMK)
100 100
26.43 26.43
52.87 44.38
57.24 32.26
33.06 26.27
0 200 200 200 200
[TNFα]/ng
0 0 50 μM 5 μM 500 nM
[Caspase inhibitor]
B)
Percentage Survival
100 ★★44.30 104.93 70.56 54.79 118.38 75.72 85.14 98.95 95.24 80.66 96.31
SQA
- - Pan casi cas-1i cas-2i cas-3i cas-4i cas-6i cas-8i cas-9i cas-10i cas-13i
Caspase inhibitor
C)
Percentage Survival
100 ★★29.26 104.34 118.17 81.67 99.68 ★48.45 64.95 ★37.94 88.64 80.49 ★50.27
FXN
- - Pan casi cas-1i cas-2i cas-3i cas-4i cas-6i cas-8i cas-9i cas-10i cas-13i
Caspase inhibitor
D)
Percentage survival
100 41.24 34.66 17.61 23.16 24.27 32.59 20.06 21.17 22.28 26.72 20.46
FXN
- - Pan casi cas-1i cas-2i cas-3i cas-4i cas-6i cas-8i cas-9i cas-10i cas-13i
Caspase inhibitor

Figure 4.1: Identification of specific caspases involved in mediating the cytotoxicity of sargaquinoic acid (SQA) and fucoxanthin (FXN) in breast cancer cells by MTT assay. A) Optimization of caspase inhibitor kit using MCF-7 cells treated with tumour necrosis factor alpha (TNFα). Cells were pre-incubated with either 500 nM, 5 or 50 µM of either the pan caspase inhibitor (Z-VAD-FMK) or the caspase-8 inhibitor (Z-IETD-FMK) for 4 h before treating with 200 ng/ml of TNFα for 96 h and assessing cellular survival by MTT assay. Percentage survival was calculated relative to the 0.5 % v/v DMSO treated vehicle control (no TNFα or caspase inhibitor added). Subsequent MTT assays were carried out by pre-treatment of cells with 50 µM of the relevant caspase inhibitors (casi) for 4 h, followed by treatment with algal compound at the relevant IC_{50}, either 75 µM of SQA (B) or 25 µM Fxn (C) in the case of MDA-MB-231 cells, or 15 µM Fxn in the case of Hs578T cells (D) for 96 h. The general or pan caspase inhibitor is indicated by "Pan casi" while the specific caspase inhibitors for caspase-1 to caspase-13 are represented by cas-xi. The presence (+) or absence (-) of a particular compound is indicated for each sample. Error bars indicate the standard error of the mean where n = 3. Statistical significance of the differences in percentage survival relative to the DMSO control were calculated using a two-tailed Students t-test (**$p < 0.01$, *$p < 0.05$). For all treatments in A) and D), $p < 0.05$.

Thus, treatment with both a general caspase inhibitor as well as a caspase-8-specific inhibitor should reduce the toxicity of TNF-α towards breast cancer cells. The percentage survival of MCF-7 cells treated with TNF-α alone was 26 % (26.43 ± 0.65 %, Figure 4.1A) relative to that of the DMSO vehicle treated-control (regarded as 100 % survival). For the general caspase inhibitor, pre-incubation of cells with 500 nM, 5 µM or 50 µM of the inhibitor prior to treatment with TNF-α increased cell survival to 33, 57 and 53 %, respectively (33.06 ± 1.87 %, 57.24 ± 2.12 % and 52.87 ± 0.67 %, respectively; Figure 4.1A), indicating that Z-VAD-FMK was most effective at reducing TNF-α-mediated cytotoxicity when cells were pre-treated with 5 µM of this inhibitor. On the other hand, pre-treatment with 500 nM of the caspase-8 inhibitor was unable to alter the viability of TNF-α-treated cells (26.27 ± 0.38 %, Figure 4.1A), while pre-treatment with 5 and 50 µM of the inhibitor increased cell survival from 26 % to 32 % and 44 %, respectively (32.26 ± 1.34 % and 44.38 ± 0.59 %, respectively; Figure 4.1A). The latter indicated that the caspase-8 inhibitor was most effective when pre-treatment was carried out using 50 µM of the inhibitor.

Taken together, these data led to a decision that the maximum concentration of caspase inhibitors (i.e. 50 µM) be used in subsequent assays for all ten of the caspase inhibitors in both MDA-MB-231 and Hs578T cells as a means of ensuring activity of the inhibitors.

4.3.1.2 Identification of specific caspases involved in mediating the cytotoxicity of SQA against MDA-MB-231 breast cancer cells

In order to indentify caspases which may have played a role in mediating the observed cytotoxicity of SQA in MDA-MB-231 cells (Chapter 2), pre-treatment of cells with a range of ten caspase inhibitors was carried out as optimized in 4.3.1.1 above, followed by treatment with a concentration equivalent to the IC_{50} of SQA in MDA-MB-231 cells (67 µM, Fig 2.3; Chapter 2). The MTT assay revealed that treatment with the marine algal quinone SQA alone reduced cell survival to 44 % (44.30 ± 2.67 %, Figure 4.1B), an effect which was completely reversed by pre-treatment with the general caspase inhibitor Z-VAD-FMK (percentage survival of 104.93 ± 3.97 %, Figure 4.1B). The inhibitors against caspase-3, -6, -8, -9, -10 and -13 all increased cell survival to above 80 % when cells were treated with these compounds prior to treatment with SQA (percentage cell survival values of 118.38 %, 85.14 %, 85.59 %, 95.24 %, 80.66 % and 96.31 % for pre-treatment with caspase-3, -6, -8, -9, -10 and -13 inhibitors, respectively; Figure 4.1B). The caspase inhibitors which had the least effect on cell survival after treatment with SQA were those inhibiting caspase-2 (percentage survival of 54.79 ± 11.67 %), caspase-1 (percentage survival of 70.56 ± 9.33 %) and caspase-4 (percentage survival of 75.72 ± 10.90 %), (Figure 4.1B).

4.3.1.3 Investigation of the role of various caspases in mediating the toxicity of FXN in MDA-MB-231 cells

In order to gain insight into the molecular mechanism of action of FXN, the ability of various caspase inhibitors to reverse the cytotoxicity of the brown algal carotenoid against MDA-MB-231 breast cancer cells was assessed. Treatment of MDA-MB-231 cells with 25 µM of FXN resulted in a decrease in cell survival to 29 % (29.26 ± 7.35 %, Figure 4.1C), relative to the DMSO treated control (taken as 100 % survival). On the other hand, pre-treatment with the general caspase inhibitor increased the survival of cells treated with FXN to 89 % (89.07 ± 7.35 %, Figure 4.1C). In the case of pre-treatment with inhibitors against caspase-1, -2, -3 -9 and -10, cell survival was increased to above 80 % in all cases, compared to treatment with FXN alone (percentage cell survival values of 85.95 %, 81.67 %, 85.63 %, 88.64 % and 80.49 % for pre-treatment with caspase-1 -2, -3, -9, -10 and -13 inhibitors, respectively; Figure 4.1C). The caspase inhibitors for which pre-treatment resulted in the smallest increases in cell survival upon FXN treatment were those for caspase-4 (48.45 ± 5.68 %), caspase-13 (50.27 ± 9.05 %), caspase-8 (54.77 ± 7.40 %) and caspase-6 (64.95 ± 3.61 %) (Figure 4.1C).

4.3.1.4 Identification of the caspases involved in mediating the cytotoxicity of FXN against Hs578T breast cancer cells

In order to compare the mechanism of action of FXN in two different breast cancer cell lines, the role of various caspases in mediating the cytotoxicity of the carotenoid compound was investigated in Hs578T cells. Treatment of Hs578T cells with FXN alone at a concentration equivalent to the IC_{50} of FXN in these cells (13 µM, Chapter 2; Fig 2.3) decreased cell survival to 41 % (41.24 ± 4.12 %, Figure 4.1D), relative to the DMSO treated control. None of the caspase inhibitors tested were able to increase the percentage survival of FXN-treated cells; in fact, cell survival was decreased upon pre-treatment with the general caspase inhibitor as well as for pre-treatment with the inhibitors of caspase-1, -2, -3, -4, -6, -8, -9, -10 and -13 (Figure 4.1D).

4.3.2 Determination of the effect of SQA on caspase-9 cleavage in MDA-MB-231 cells by Western analysis

As an alternative method of assessing the potential involvement of a caspase, in this case caspase-9, in mediating the cytotoxicity of SQA, MDA-MB-231 cells were treated with a range of concentrations of SQA and Western analysis carried out on treated whole cell lysates. As depicted in Figure 4.2A, treatment with 0.6, 6 and 60 µM of SQA appeared to cause a dose-dependent increase in the detection of the 37 and 17 kDa cleavage products of caspase-9 compared to that observed for the DMSO-treated control, a result which was validated by performing densitometry on the signals detected by Western analysis (Fig 4.2B).

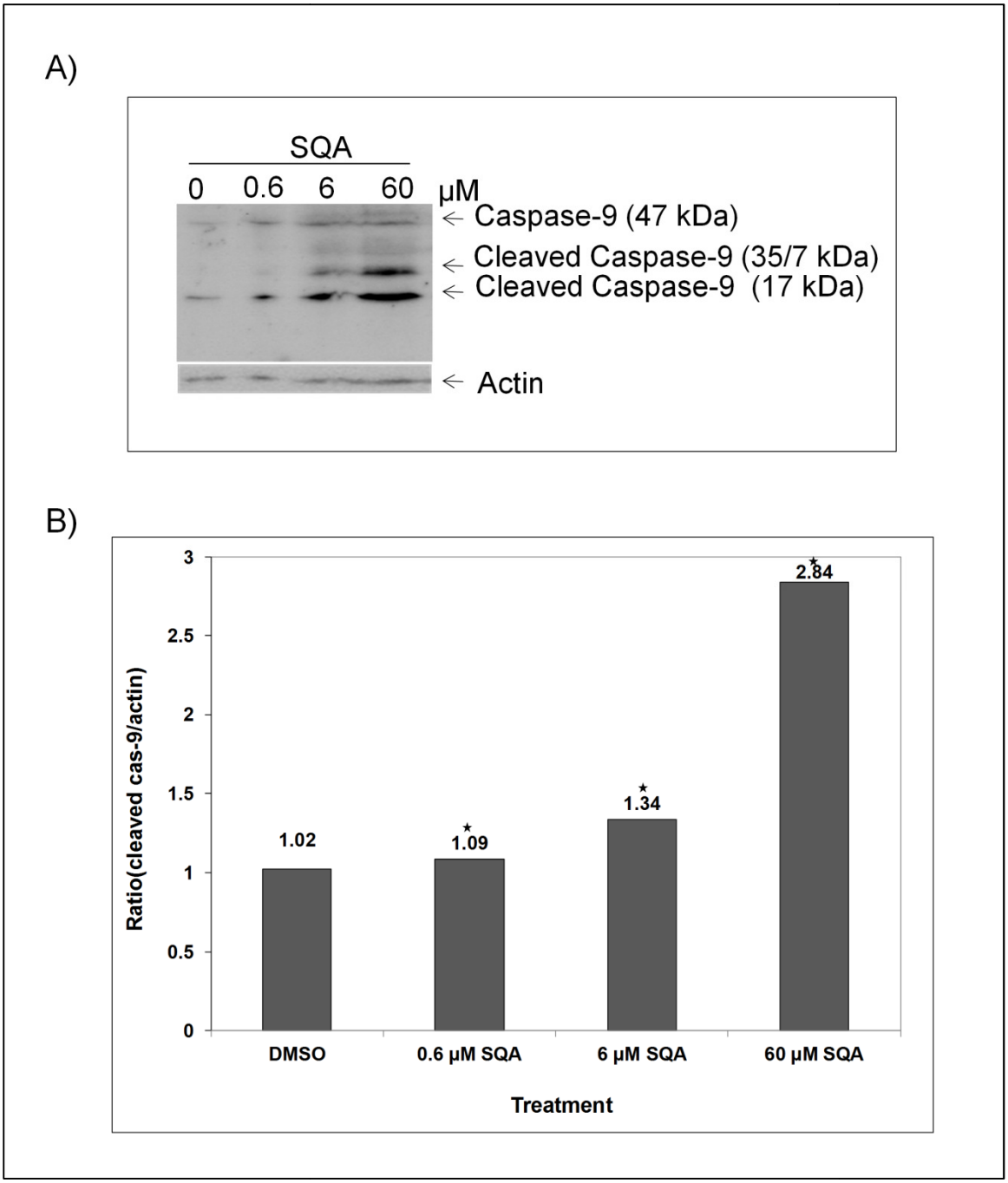

Figure 4.2: Western analysis of the cleavage of caspase-9 in MDA-MB-231 cells upon treatment with sargaquinoic acid. Cells were treated with either the 0.5 % (v/v) DMSO vehicle control (0) or 0.6, 6 or 60 μ M of sargaquinoic acid (SQA) for 8 hours after which whole cell lysates (WCLs) were probed for cleaved and uncleaved caspase-9. A) Western blot for the detection of caspase-9 in treated MDA-MB-231 WCLs. Lysates were probed for β-actin as a loading control. B) Densitometric analysis of signals detected in A) using ImageJ. For each sample, the ratio of the mean signals for cleaved caspase-9 to that of actin was calculated. The differences in the ratio of the mean signals for cleaved caspase-9 to that of actin between the DMSO control and cells treated with SQA was found to be statistically significant (*) using a two-tailed unpaired Student's t-test for each of the treatments ($p < 0.05$, confidence interval of 95 %). The experiment was repeated three times and the same trends in caspase-9 cleavage observed.

The ratio of the signals detected for both of the caspase-9 cleavage products to that for the β-actin loading control, calculated using ImageJ (National Institute of Health Freeware), was used as a measure of caspase-9 activation (Fig 4.2B).

4.3.3. Analysis of the effect of Ptx, SQA and FXN on Bcl-2 protein levels in breast cancer cells *in vitro* by flow cytometry

In order to investigate whether the induction of apoptosis by the algal compounds SQA and FXN in breast cancer cells was linked to the ability to alter the expression levels of anti-apoptotic proteins, the levels of the anti-apoptotic mitochondrial protein Bcl-2 in treated cells were compared to that in the DMSO vehicle-treated control sample for two breast cancer cell lines. In all cases, flow cytometry experiments were repeated at least twice and similar trends in Bcl-2 levels observed in each case.

4.3.3.1 Identification of alterations in Bcl-2 protein levels between untreated MDA-MB-231 cells and those treated with Ptx, SQA and FXN

MDA-MB-231 cells were treated with either DMSO vehicle control or 100 nM Ptx, 75 µM SQA or 25 µM FXN (concentrations equivalent to the IC_{50} value) and the Bcl-2 protein levels in treated cells quantified by flow cytometry. Figure 4.3A depicts histogram representations generated using FlowJo software (Tree Star Inc., 2010) of the fluorescence data detected for each sample, while Figure 4.3B is a summary of the data generated by plotting the percentage Bcl-2 positive events from each histogram in Figure 4.3A (events within the gate). The secondary antibody control sample was immunostained with the fluorescently labeled secondary antibody alone (i.e. not the anti-Bcl-2 primary antibody) and was used both to determine the level of background immunostaining and, in conjunction with the vehicle-treated control, in the gating of cell populations to define the Bcl-2 positive population. The proportion of Bcl-2 positive events was used as a comparative measure of the levels of Bcl-2 protein in cells treated with either DMSO or the selected cytotoxic compounds. The level of immunostaining detected in the secondary antibody control was set at a proportion of 5.60 % Bcl-2 positive events (Fig 4.3Ai and Fig 4.3B) and was considered to be an acceptable level of background staining in this and subsequent experiments. The vehicle-treated control sample displayed a proportion of 29.80 % Bcl-2 positive events, which was reduced to 22.30 % following treatment of the cells with 100 nM Ptx (Fig 4.3Aii and 4.3Aiii, respectively; Fig 4.3B). Treatment with 75 µM SQA and 25 µM FXN decreased the Bcl-2 protein levels to 17.40 % and 5.66 % Bcl-2 positive events, respectively, relative to the DMSO-treated control (Fig 4.3Aiv and 4.3Av, respectively; Fig 4.3B).

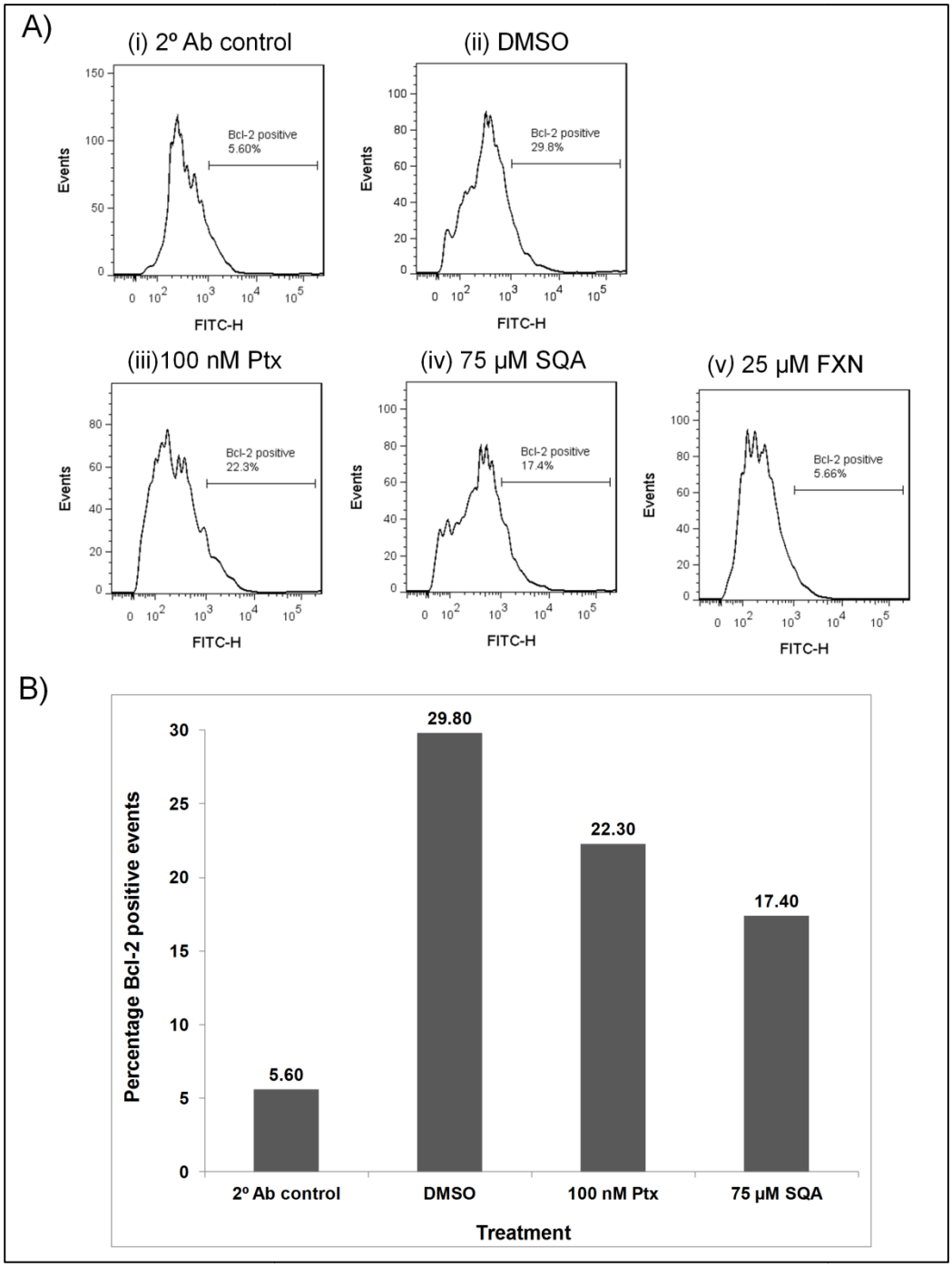

Figure 4.3: Analysis of the effect of paclitaxel, sargaquinoic acid and fucoxanthin on the levels of the anti-apoptotic protein Bcl-2 in MDA-MB-231 breast cancer cells. Cells were treated with vehicle control (DMSO, 0.02 % v/v) or compound at a concentration equivalent to the IC_{50} value ie. (ii), 100 nM paclitaxel (Ptx) (iii), 75 µM sargaquinoic acid (SQA) (iv) or 25 M fucoxanthin (FXN) (v) for 16 h, immunostained using an anti-Bcl-2 primary antibody and FITC-conjugated secondary antibody and analysed by flow cytometry. Excitation of FITC was achieved at 488 nm and emission detected at 530 nm. A) Histograms generated using FlowJo software Version 7.6.1 showing the shift in Bcl-2 positive events between the DMSO-treated control and cells treated with Ptx, SQA or FXN. Gating was carried out according to the secondary antibody control (2º Ab control) (i) to define the Bcl-2-positive and -negative populations and copied to all samples. B) Summary of the alterations in Bcl-2 levels by Ptx, SQA and FXN depicted in (A) in terms of the percentage Bcl-2 positive events detected for each treatment.

4.3.3.2 Comparison of Bcl-2 protein levels in untreated Hs578T cells with those treated with Ptx and FXN

As a means of comparison of the molecular mechanisms mediating the cytotoxicity of Ptx and FXN between two breast cancer cell lines, Hs578T cells were treated with 30 nM and 15 µM, respectively (according to the IC_{50} values of the compounds) and the effects on Bcl-2 protein levels analyzed by flow cytometry as for MDA-MB-231 cells. Figure 4.4A represents the fluorescence data obtained by flow cytometry depicted as histograms for each sample, while Figure 4.4B is an overview of this data obtained by plotting the percentage Bcl-2 positive events in Figure 4.4A. In this experiment the secondary antibody control was used to set the gate for Bcl-2-positive events, giving rise to a background immunostaining level of 5.24 %, which was comparable with that set for the secondary antibody control in the MDA-MB-231 cell line (Fig 4.4Ai vs. Fig 4.3Ai). Treatment of the Hs578T cells with Ptx and FXN reduced the proportion of Bcl-2 positive events from 41.5 % in the DMSO control to 23.9 % and 25.4 %, respectively (Fig 4.4Aiii and 4.4Aiv, respectively; Fig 4.4B).

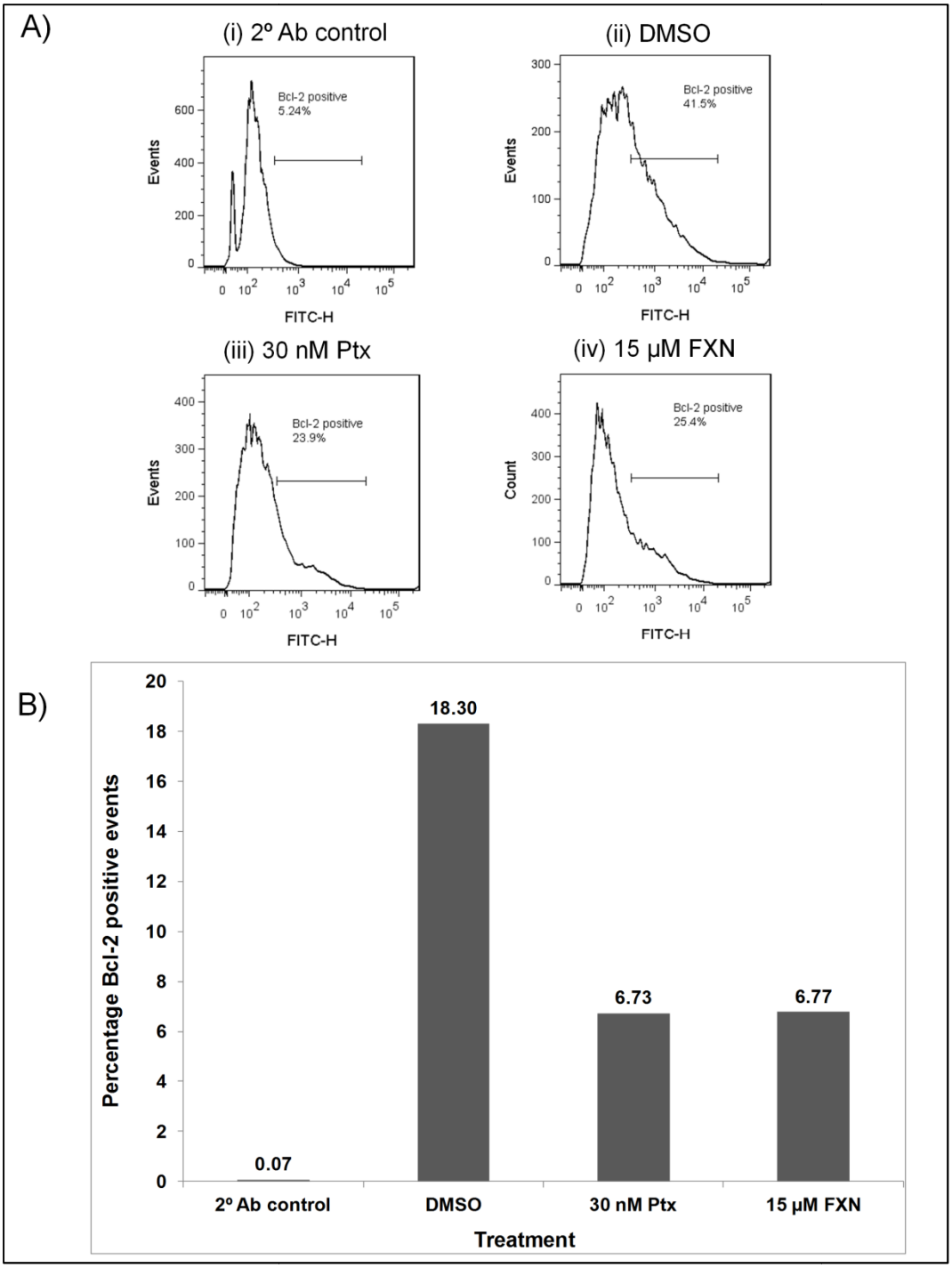

Figure 4.4: Determination of the effect of paclitaxel and fucoxanthin on Bcl-2 levels in Hs578T breast cancer cells. Cells were treated with vehicle control (DMSO, 0.02 % v/v) (ii), or compound at concentrations equivalent to the IC_{50}, namely 30 nM paclitaxel (Ptx) (iii) or 15 M fucoxanthin (FXN) (iv) for 16 h, immunostained with anti-Bcl-2 primary antibody followed by FITC-conjugated secondary antibody and the fluorescence detected by flow cytometry. Excitation of the fluorophore was achieved at 488 nm and emission detected at 530 nm. A) Flow cytometric data represented as histograms generated using FlowJo software Version 7.6.1. Gating in order to define the Bcl-2 positive and negative populations was carried out on the secondary antibody control (2º Ab control) (i) and copied to all subsequent histograms. B) Summary of the alterations in Bcl-2 levels induced by Ptx and FXN as depicted in (A).

DISCUSSION

As a means of further elucidation of the mechanism of action of marine algal compounds against breast cancer cells, the involvement of specific signal transducer molecules in mediating their activity was assessed. Unfortunately, there were unsufficient quantities of the novel algal "hit" compounds (RU004, RU007) on which to carry out these analyses. However, the previously described algal compounds SQA and FXN were more readily available and allowed for the comparison, both between the two known compounds in terms of their molecular mechanism of action, as well as comparisons between the data generated in this study and that published in previous reports.

The prenylated quinone SQA was found to be cytotoxic against a range of breast cancer cell lines, while having no adverse effects on the growth of non-cancerous breast epithelial cells when added at a dose equivalent to the IC_{50} in breast cancer cells, resulting in its designation as a "hit" compound in this study (Fig 2.3 and Table 2.2, Chapter 2; Fig A1, Appendix). Furthermore, the compound appeared to induce apoptosis in MDA-MB-231 breast cancer cells as evidenced by flow cytometric and PARP cleavage studies and caused a G_0-G_1 arrest in these cells (Fig 3.1, 3.3 and 3.4, Chapter 3). These data justified further studies as to the molecular mechanisms of action of SQA. On the other hand, although the commercially available carotenoid FXN was more toxic than SQA to breast cancer cells, the compound also displayed cytotoxicity against non-cancerous breast cells (Fig 2.3 and Table 2.2, Chapter 2). While there was no conclusive data as to the mode of cell death induced by the carotenoid, the compound was shown to cause G_0-G_1 arrest in MDA-MB-231 cells in the same manner as SQA. Thus, although not designated as a "hit" compound, FXN was included in subsequent mechanistic studies as a means of comparison with SQA due to their structural similarities (Fig 2.2, Chapter 2) and potential parallels in molecular mechanisms of action.

In the first instance, the role of specific caspases in mediating the cytotoxicity of SQA and FXN in breast cancer cells *in vitro* was analyzed using a caspase-inhibitor-coupled MTT assay. Optimization of the assay using TNFα as a positive control in terms of caspase-mediated cytotoxicity was carried out in MCF-7 cells due to previous reports that MDA-MB-231 and, to a lesser extent Hs578T cells, were resistant to TNFα (Pirianov and Colston, 2001). The latter finding was confirmed in this study (data not shown). The effect of caspase-inhibitor alone on MCF-7 cell survival was also assessed and revealed that, at the concentration range used in the assay, the inhibitors were non-toxic to the breast cancer cells (data not shown). Thus, the caspase-inhibitor negative control was eliminated from future assays in MDA-MB-231 and Hs578T cells.

Following optimization of the caspase-inhibitor assay, screening for the involvement of various caspases in mediating the anti-cancer activities of SQA and FXN was carried out. Treatment of MDA-MB-231 cells with 75 µM SQA alone produced a percentage survival of 44 %, a result which was in alignment with the previously determined IC_{50} value of 67 µM. This cytotoxicity was completely reversed by pre-treatment with the general caspase inhibitor Z-VAD-FMK, which was consistent with the previous findings both in this study (Fig 3.1 and 3.3, Chapter 3) and that reported in the literature, that the compound is able to induce apoptosis in human cancer cells *in vitro* (Hur *et al*., 2008). Furthermore, the data herein suggested a role for caspase-3, -6, -8, -9, -10 and -13 in mediating the anti-cancer activity of SQA in MDA-MB-231 cells due to the ability of the inhibitors of these caspases to substantially reduce SQA-induced toxicity. The findings that pre-treatment with the inhibitors of caspase-3, caspase-9 and, to a lesser extent, caspase-8 were able to reverse the cytotoxic effects of SQA were consistent with those obtained by Western analyses in HaCaT human skin cancer cells (Hur *et al*., 2008). On the other hand, a role for caspase-6, -10 and -13 in mediating the cytotoxicity of SQA in cancer cells has not previously been reported. The discovery that both caspase-8 and caspase-9 inhibition reverse the cytotoxicity of SQA points to the involvement of the extrinsic as well as the intrinsic apoptotic pathways in mediating the activity of SQA.

Pre-treatment of MDA-MB-231 cells with the inhibitors of caspase-1 and caspase-4 was unable to increase cell survival to above 76 % upon treatment with SQA, although these differences in percentage survival relative to the DMSO control were not found to be statistically significant. This may indicate either partial involvement of these caspases in mediating the toxicity of SQA or inability of the inhibitors to completely block the activity of the caspases under the specific experimental conditions. Since optimization of the two inhibitors was not carried out using a positive control compound known to require these inhibitors for its toxicity, the latter possibility cannot be ruled out. It is tempting, however, to conclude that caspase -1 and -4 do not play a role in mediating the action of SQA, since these caspases are involved in the initiation of an inflammatory response (Klener Jr *et al*., 2006). In the case of caspase-2, pre-treatment with the inhibitor of this protein appeared to have little effect on the toxicity of SQA against MDA-MB-231 cells. However, it was determined that the difference in percentage survival for cells pre-treated with caspase-2 relative to the DMSO control were not statistically significant, suggesting that it was possible that the caspase did play a role in mediating the anti-cancer activity of SQA.

In the case of the brown algal metabolite FXN, treatment of MDA-MB-231 cells with 25 µM of the compound decreased cell survival to 29 %. Since the IC_{50} value obtained for FXN in MDA-MB-231 cells was 11 µM (Figure 2.3A, Chapter 2) the low percentage survival when treated with 25 µM was not unexpected. As was the case for SQA, pre-treatment with the general

caspase inhibitor was able to reverse the cytotoxicity of FXN to a large extent. This result represented the first compelling evidence in the current study that the carotenoid induced apoptosis in breast cancer cells, as has been reported in the literature for leukemia as well as prostate and colon cancer cells *in vitro* (Kotake-Nara *et al.,* 2005a; Hosokawa *et al.,* 1999; Hosokawa *et al.,* 2004). Furthermore, pre-treatment of MDA-MB-231 cells with inhibitors against caspase-1, -2, -3, -9 and -10 increased cell survival to above 80 % in all cases, compared to treatment with FXN alone. The involvement of caspase-3 and caspase-9 in mediating the cytotoxicity of FXN is consistent with that obtained in previous studies in leukemia, prostate and bladder cancer cells *in vitro* (Kotake-Nara *et al.,* 2005a and b; Zhang *et al*., 2008). The finding that inhibition of caspase-9, but not caspase-8, is able to reverse the cytotoxicity of FXN suggests that the compound acts via the intrinsic and not the extrinsic apoptotic pathway in these cells, a result which differs from that observed in leukemia cells where both pathways were implicated in mediating the cytotoxicity of FXN (Ishikawa *et al*., 2008). Prior to the current study, the involvement of caspase-1, -2, or -10 in facilitating the anti-cancer activity of FXN had not been reported. In addition, this study revealed that pre-treatment with the inhibitors of caspase-4, -6 or -13 appeared unable to reverse the cytotoxicity of FXN in MDA-MB-231 cells, evidence of which has not been reported elsewhere. Since caspase-4 and -13 have been implicated in the initiation of the inflammatory response (Klener Jr *et al*., 2006), the latter result was encouraging, particularly since the difference in percentage survival relative to the DMSO control for cells pre-treated with inhibitors against caspase-4 or caspase-13 were found to be statistically significant.

While the proposed role of caspase-3, -9 and -10 in mediating the cytotoxicity of FXN is consistent with the findings for SQA, the involvement of caspase-2 in facilitating the toxicity of FXN differs from that observed for SQA. Furthermore, while caspase-8 and caspase-13 were found to play a role in mediation of the cytotoxicity of SQA, this was not the case for FXN. These data suggest that while similarities exist in the molecular mechanism of action of SQA and FXN, the two compounds do not activate identical signal transducer molecule targets.

Unexpectedly, in the Hs578T breast cancer cell line, pre-treatment with caspase inhibitors was unable to reverse the cytotoxicity of FXN for all ten of the caspases tested. Since the effect of the caspase inhibitors alone on cell survival was not carried out for the Hs578T cell line, but only the MCF-7 line, it is possible that the caspase-inhibitors themselves were toxic to Hs578T cells. On the other hand, the failure of the caspase inhibitors to reverse the cytotoxicity of FXN to Hs578T cells could indicate either that the carotenoid compound displays a different mode of action in the Hs578T cell line as compared to that in the MDA-MB-231 cell line, or that the caspase inhibitors were not functioning optimally under the selected experimented conditions, in terms of the concentrations of inhibitor utilized and the period of pre-treatment. Since

optimization was not carried out for each caspase-inhibitor in the Hs578T cell line using a positive control, the latter interpretation cannot be excluded. However, differences in the mechanism mediating the cytotoxicity of a compound in different breast cancer cell lines have previously been reported. In particular, it has been demonstrated that the mode of action of resveratrol varies between MDA-MB-231 and MCF-7 cell lines (Pozo-Guisadoa *et al*., 2002).

The role of the intrinsic pathway initiator, caspase-9, in mediating the cytotoxicity of SQA in MDA-MB-231 cells was confirmed by Western analysis as evidenced by a dose-dependent increase in the levels of cleaved and thus active form of the protein upon treatment with the quinone. As stated previously, a similar study had been carried out in human skin cancer cells and a comparable result obtained, although only a single caspase-9 cleavage product was shown in the latter report (Hur *et al*., 2008). The correlation between the findings obtained by Western analysis and caspase-inhibitor assay in terms of the potential involvement of caspase-9 in mediating the cytotoxicity of SQA in MDA-MB-231 cells validated the results obtained by caspase inhibitor assay.

Since Bcl-2 has been demonstrated to be up-regulated in a range of cancers, the levels of this anti-apoptotic mitochondrial protein have been correlated with tumour progression (Jin and El-Deiry, 2005). The ability of a compound to decrease expression of Bcl-2 would thus be therapeutically useful. The capability of the novel algal compounds SQA and FXN as well as a clinically approved anti-cancer drug, namely Ptx, to decrease Bcl-2 levels was assessed by flow cytometry. In DMSO-treated MDA-MB-231 cells, Bcl-2 levels were quantified to be 29.80 %, in terms of positive fluorescence events. The latter percentage is derived from the gating of the secondary antibody control, which was set at a value considered to represent an acceptable level of background. Thus, it is not the absolute value of Bcl-2 positive events which is important in these experiments, but rather the comparison of these levels between identically gated samples. Treatment of MDA-MB-231 cells with Ptx reduced the levels of Bcl-2 to 22.30 %, a result which agreed with previous reports that the mechanism of action of the compound involves inhibition of this anti-apoptotic protein (Srivastava *et al*., 1999). In addition, treatment with SQA and FXN decreased Bcl-2 levels to 17.40 % and 5.65 %, respectively, suggesting that the novel algal compounds were more effective than the chemotherapeutic agent Ptx in down-regulating Bcl-2 expression. In particular, FXN treatment was able to decrease Bcl-2 levels to the equivalent of the secondary antibody baseline control (5.60 %). The greater effect on Bcl-2 levels by FXN was, however, most likely due to the fact that the compound was used at a concentration more than twice the IC_{50} value (11 µM, Figure 2.3A, Chapter 2), while Ptx and SQA were added at concentrations equivalent to their IC_{50} values.

The identification of alterations in Bcl-2 levels induced by SQA and FXN in MDA-MB-231 cells suggest that the compounds act by inducing the intrinsic apoptotic pathway, which is consistent with the findings above in the caspase inhibitor studies and, in the case of SQA, the caspase-9 cleavage assay. While the decrease in Bcl-2 levels in MDA-MB-231 cells treated with FXN in this study correlated positively with previous reports for leukemia and prostate cancer cells *in vitro* (Kotake-Nara *et al*., 2005a and b), the reduced Bcl-2 levels in breast cancer cells described here after treatment with SQA differed from that reported for human skin cancer cells, where the compound had no effect on Bcl-2 expression (Hur *et al*., 2008). While one may make tentative conclusions as to the similarities and differences in molecular mechanisms of action of FXN and SQA in this study with reports in the literature for other cancer cell lines, it is important to bear in mind the differences in methods of quantification of Bcl-2 i.e. while the current study made use of flow cytometry to quantify Bcl-2 protein levels, the aforementioned reports in literature performed Western analyses in order to assess Bcl-2 levels.

The effect of treatment with either Ptx or FXN on Bcl-2 levels was determined in the Hs578T cell line as a means of comparison of the mechanism of action of the compounds in two different breast cancer cell lines. The decrease in Bcl-2 levels from 41.5 % Bcl-2-positive events to 23.9 % and 25.4 % after treatment with Ptx and FXN is consistent with the results obtained in the MDA-MB-231 cell line that the compounds were able to down-regulate Bcl-2 expression. Furthermore, in the Hs578T cell line, it was observed that treatment with FXN at a concentration close to the IC_{50} value of the compound resulted in a decrease in Bcl-2 levels which was comparable to that produced by Ptx. This was in contrast as to the much higher concentration of FXN used in the MDA-MB-231 cell line, which caused a much greater decrease in Bcl-2 levels as compared with Ptx.

An interesting study which was not carried out in this body of work would be to compare the Bcl-2 levels in untreated breast cancer cells, such as the Hs578T and MDA-MB-231 cell lines, to those in non-cancerous breast epithelial cells, such as the MCF12A cell line. This would confirm whether the protein is indeed up-regulated in breast cancer cells, as has been reported in the literature (Jin and El-Deiry, 2005).

CONCLUSIONS

In summary, the aims in this study were achieved by the identification of specific pathways and signal transducer molecules which played a role in mediating the cytotoxicity of both sargaquinoic acid (SQA) and fucoxanthin (FXN) in breast cancer cells. The “hit” compound SQA was shown to require the activity of caspase-3, -8, -9, -6, -10 and -13 for its cytotoxicity against MDA-MB-231 cells. Furthermore, the compound was found to be able to decrease the level of the anti-apoptotic protein Bcl-2 in treated cells. These findings allude to a mechanism of action of the quinone involving both the intrinsic and the extrinsic apoptotic pathways. On the other hand, the cytotoxicity of FXN was shown to be dependent on the activity of caspase-1, -2, -3, -9 and -10, suggesting a more specific mechanism of action of the carotenoid involving the intrinsic apoptotic pathway alone. The ability of FXN to decrease the expression of Bcl-2 was consistent with this proposed mechanism of action. This body of work represents the first in-depth study of the molecular mechanisms mediating the cytotoxicity of both SQA and FXN in breast cancer cells *in vitro*.

CHAPTER 5

SCREENING OF NOVEL MARINE ALGAL COMPOUNDS FOR POTENTIAL ANTI-CANCER STEM CELL ACTIVITY *IN VITRO*

5.1 INTRODUCTION

The cancer stem cell theory states that tumours are initiated and maintained by a subpopulation of cells with both cancer and stem cell characteristics, termed "cancer stem cells" (CSCs). Most importantly the theory purports that CSCs are able to undergo asymmetrical division, meaning that the cells can both self-renew to produce more stem cells and differentiate to give rise to the various cell types within a tumour (Pardal *et al*., 2003; Wicha *et al*., 2006). The first solid tumour stem cells were identified in breast cancer (Al-Hajj *et al*., 2003). Breast cancer stem cells (BCSCs), comprising 1 % to 10 % of the total population have been isolated from both primary patient samples and cell lines such as MCF-7 and SKBR3, thus breast cancer has been described as a "stem-cell disease" (Al-Hajj *et al*., 2003; Patrawala *et al*., 2005; Ponti *et al*., 2005; Dontu *et al*., 2005).

Cancer stem cells can be isolated in a number of ways. BCSCs have been identified and extracted from the bulk population using fluorescence-activated cell sorting (FACS) based on their specific cell surface markers, such as the $CD44^{+}CD24^{-}lin^{-}$ phenotype (Al-Hajj *et al*., 2003; Kakarala and Wicha, 2008; Zhou *et al*., 2008). The increased expression of aldehyde dehydrogenase enzyme, detected by the Aldefluor assay, can also be used to isolate BCSCs (Armstrong *et al*., 2004; Kakarala and Wicha, 2008). Recently, the term "side population" (SP) has been used to describe a subpopulation of cancer cells that is able to exclude the fluorescent dye, Hoechst 33342, in flow cytometry. This ability is due to the presence of a number of ATP-binding cassette (ABC) transmembrane proteins, such as breast cancer resistance protein 1 (BCRP1 or ABCG2), which actively pump the dye out of these cells (Kondo *et al*., 2004; Patrawala *et al*., 2005; Zhou *et al*., 2007). These SP cells are known to be enriched for CSC-like cells in a variety of tumour cell lines and those extracted from the MCF-7 cell line have displayed higher tumourigenicity than the bulk MCF-7 population both *in vitro* and *in vivo* (Zhou *et al*., 2007).

Cancer stem-like cells have also been isolated based on their functional characteristics, in particular their ability to grow anchorage-independently in serum-free non-adherent conditions. In these culture conditions non-stem cancer cells undergo anoikis, a programmed cell death associated with loss of adhesion, thus selecting for the CSC-enriched subpopulation (Reynolds and Rietze, 2005; Dontu *et al*, 2003). These CSC-like cells form tumourspheres in suspension *in vitro* and have been shown to be capable of *in vivo* tumour formation at limiting cell dilutions (Ponti *et al*, 2005).

The identification of CSCs within breast tumours has resulted in a major shift in focus in terms of the development of novel therapies to treat this disease (Kakarala and Wicha, 2008). It is now thought that complete eradication and prevention of relapse requires the removal of the CSC subpopulation within a tumour (Massard *et al*., 2006). Therefore, the race is on to find

agents which are able to specifically target CSCs. To date, most potential anti-cancer stem cell agents have been designed to target this population either by the inhibition of the self-renewal pathways upregulated in the cells or by inducing differentiation of the CSC (Korkaya and Wicha, 2007). Self-renewal target pathways include the Wnt, Notch and Hedgehog signaling cascades, which are activated in a range of malignancies as well as in CSCs (Korkaya and Wicha, 2007). Examples of specific pathway inhibitors include the γ-secretase inhibitor, GSI-18, used to block the Notch pathway; as well as the natural product alkaloid, cyclopamine, which is able to suppress the Hedgehog pathway (Fan *et al*., 2006; Chen *et al*., 2002). In the second approach to anti-CSC drugs, clinical studies have made use of the FDA-approved combination therapy of all *trans* retinoic acid (tretinoin) together with the histone deacetylase inhibitor, sodium phenylbutyrate, in the treatment of various forms of leukemia (Tallman *et al*., 1997; Bruserud and Gjertsen, 2000; Zhou *et al*., 2002). However, to date there have been no reports of a compound which is able to target CSCs while leaving both normal healthy cells and bulk tumour cells unaffected.

While attention in the past decades has turned towards marine natural products as a source of lead anti-cancer compounds, marine algae have received considerably less attention in terms of their potential for bioactive metabolites than other marine organisms such as sponges, Cnidarians and cyanobacteria (Jha and Zi-rong, 2004). In addition, very few studies of the biological activity of algal metabolites go beyond the standard *in vitro* cytotoxicity screening tests (Carte, 1996; Adrianosolo *et al*., 2006). Recently, a number of polyhalogenated monoterpene compounds were isolated from the red alga, *Plocamium corallorhiza*, collected from the South African coastline, which were cytotoxic to oesophageal cancer cells *in vitro* (Knott *et al*., 2005; Mann *et al*., 2007). The effect of these compounds on normal, non-cancerous cells as well as their activity against other cancer cell lines and their respective CSC subpopulation were unknown.

The aims in this chapter included the screening of novel marine algal compounds for potential anti-CSC activity using the mammosphere assay, comparison of this activity to that of a commonly used chemotherapeutic and, finally, preliminary studies into the mechanism of action of identified putative anti-CSC agents.

5.2 MATERIALS AND METHODS

5.2.1 Reagents

Dulbecco's Modified Eagle Medium (DMEM) containing Glutamax™, fetal calf serum (FCS) and penicillin-streptomycin amphotericin (PSA) were obtained from Gibco (Invitrogen, UK). Recombinant human epidermal growth factor (EGF), basic fibroblast growth factor (bFGF) and heparin were from Sigma-Aldrich (Germany), while the B-27 supplement was from Invitrogen (UK). Recombinant human insulin was obtained from NovoRapid (Novo Nordisk Pharmaceuticals, Denmark). The WST-1 Cell Proliferation kit was from Roche (Switzerland). The algal compounds sargaquinoic acid, RU017 and RU018 were obtained as part of a collaboration with Dr Denzil Beukes (Pharmacy Faculty, Rhodes University). Paclitaxel and fucoxanthin were from Sigma-Aldrich (Germany).

5.2.2 Cell lines and culture conditions

The breast cancer cell line MCF-7 (ATCC: HTB-22) was maintained in culture either in anchorage-dependent or anchorage-independent conditions. In the former, cells were cultured as for the MDA-MB-231 and Hs578T breast cancer cell lines (described in Chapter 2, Section 2.2.2) in either T25 tissue culture flasks (Corning, Pennsylvania) or regular 96-well plates (Nunc, Thermo Scientific, USA). Anchorage-independent growth conditions were modified from Dontu *et al*., 2003 and are described in the mammosphere assay protocol.

5.2.3 Mammosphere assay

The capability for anchorage-independent growth was assessed by the "mammosphere" assay, modified from that previously described (Dontu *et al*., 2003). Cells were lifted with trypsin-EDTA solution, washed with phosphate-buffered saline (PBS) and passed through a 40 µM cell strainer (BD Biosciences, Belgium) to achieve a single cell suspension. Cell were seeded at a density of either 1000 or 3000 cells per well in ultralow attachment 96-well plates (Corning, Pennsylvania) containing DMEM with Glutamax™ supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin and 12.5 µg/ml amphotericin (PSA), 2% (v/v) B-27 supplement, 20 ng/ml EGF and bFGF, 4 ng/ml heparin and 10 µg/ml insulin. Treatment with either 0.61 % v/v DMSO vehicle control or paclitaxel, fucoxanthin, RU017 or RU018 at concentrations indicated in figure legends was carried out either upon seeding (Day 0) or four days after seeding (Day 4) for quintuplicate samples. The cultures were supplemented with fresh medium, which was added to exisiting media every 48 hrs, and the resultant mammospheres were photographed using a Nikon camera (Coolpix 990) attached to a light microscope at 100x magnification. Images were analyzed and scale bars calculated using ImageJ (NIH freeware).

5.2.4 Cell proliferation assay

Cell viability upon treatment with the compounds was assessed by means of the WST-1 assay according to manufacturer's instructions. After growth of the mammosphere cultures for 8 days, 10 µL of a 5 mg/mL WST-1 reagent was added to each well and incubated for a further 4 hours at 37 ^{o}C before reading the absorbance at 450 nm using a Powerwave spectrophotometer (BioTek). Cell survival was determined by the ability of live cells to convert the WST-1 reagent to a soluble coloured formazan product and percentage survival calculated according to the absorbance of the treated samples at 450 nm compared to that of the DMSO vehicle-treated control. All treatments were carried out in quintuplicate.

5.3 RESULTS

5.3.1 Observation of the formation and development of MCF-7 mammospheres in anchorage-independent serum-free culture conditions

The mammosphere assay was carried out for the MCF-7 breast cancer cell line in order to enrich for cancer stem cell-like cells, which are able to grow in anchorage-independent serum-free conditions (Dontu *et al*., 2003). The development of non-adherent mammospheres or tumourspheres (Dontu *et al*., 2003) under these conditions after seeding of 1000 MCF-7 cells per well in a 96-well plate was observed under a light microscope over the course of one week. As depicted in Figure 5.1, the single-cell suspension seeded on Day 0 had formed small, irregular cell "clumps" by Day 1 (Figure 5.1A), which had developed into small suspended colonies representative of mammospheres (as described by Dontu *et al*., 2003) by Day 3 (Figure 5.1B). By Day 5, the mammospheres had increased in size to approximately 0.1 mm (100 µM) in diameter and displayed a more regular spherical shape (Figure 5C). After seven days growth in anchorage-independent serum-free conditions, the mammospheres, while remaining roughly the same size as Day 5, began to exhibit different morphologies (Figure 5.1D). The most striking feature of the Day 7 cultures was the formation of hollow mammospheres; empty bubble-like structures surrounded by one or more cells or small mammospheres attached to what appeared to be an outer membrane of the hollow spherical bodies (Figure 5.1D, inset bottom right).

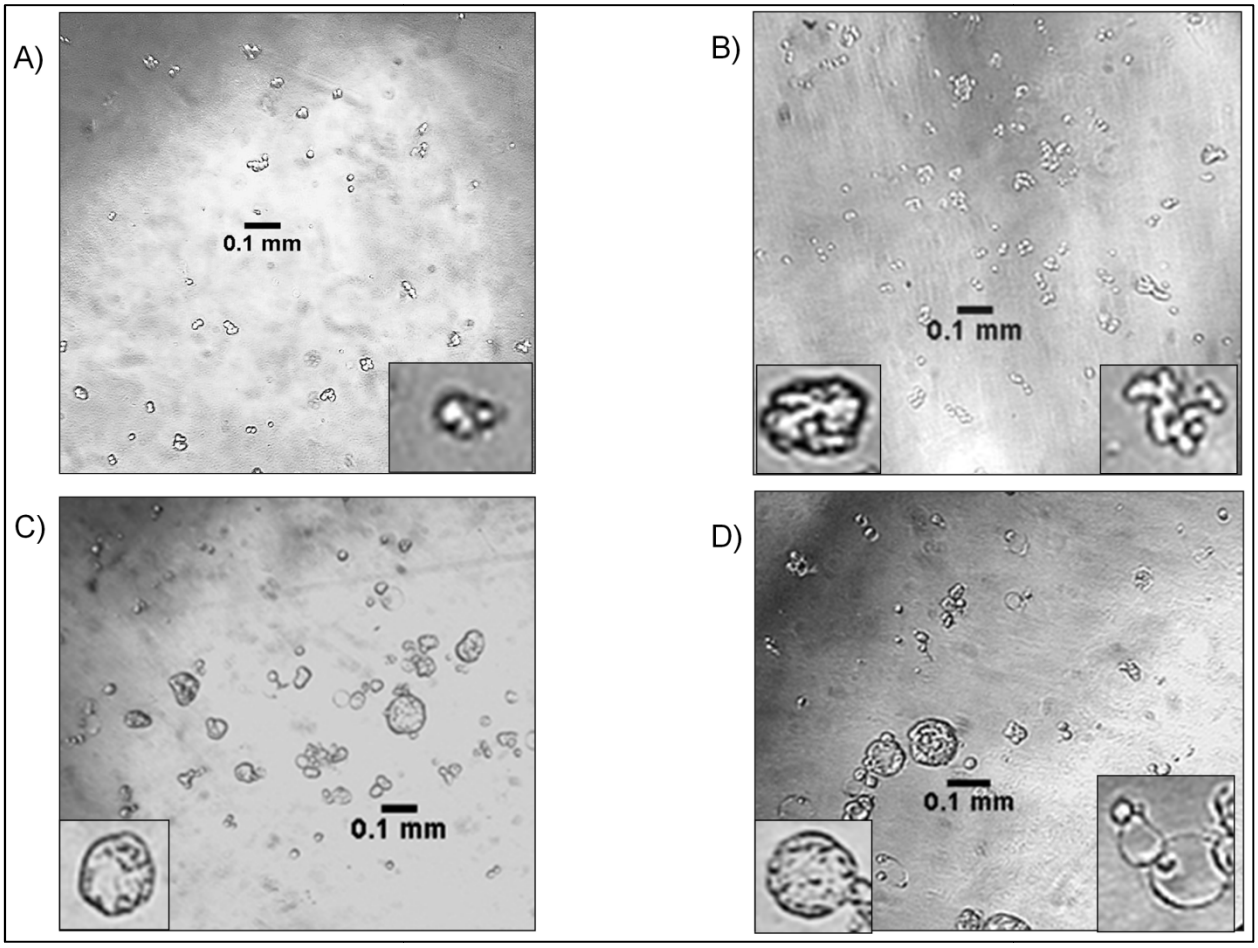

Figure 5.1: Time-course study of the formation of tumourspheres in the MCF-7 breast cancer cell line in anchorage-independent serum-free culture conditions. Alterations in number, size and shape of tumourspheres was observed over the course of seven days. Photographs were taken at Day 1 (A), Day 3 (B), Day5 (C) and Day 7 (D). Images are representative of at least three randomly selected fields. Insets show specific structural features of MCF-7 tumourspheres at various stages of their development. Images were captured under a light microscope at 100x magnification using a Nikon Coolpix 990 camera. Images were analyzed and scale bars inserted in ImageJ. Note: Insets not set to scale. Scale bars representative of 0.1 mm.

These observations in terms of the formation and development of MCF-7 mammospheres were consistent over the course of many experiments for 0.16 % v/v DMSO-treated cells (Figure 5.2 – 5.8) and were used as a means of comparison with treated MCF-7 cells cultured in anchorage-independent conditions over the same time period.

The mammosphere assay has been validated within the Biomedical Biotechnology Research Unit (BioBRU) by flow cytometry to detect specific CSC markers, revealing that MCF-7 mammospheres were enriched for $CD44^{high}/CD44^{low}$ CSC-like cells and express the stem cell marker Oct4 (unpublished data, Jason Sterrenberg, 2011).

Repeated attempts within the research group to culture both MDA-MB-231 and Hs578T breast cancer cells under the anchorage-independent, serum-free conditions described above were unsuccessful (Personal communication: Dr Mugdha Sukhthankar and Jason Sterrenberg).

5.3.2 Screening of marine algal compounds for potential anti-cancer stem cell activity using the MCF-7 mammosphere assay

The effect of the previously described marine algal compounds sargaquinoic acid (SQA) fucoxanthin (FXN), as well as the novel halogenated monoterpenes RU017 and RU018, on the formation and development of MCF-7 mammospheres was assessed by addition of these compounds to the culture medium either at seeding (Day 0) or after four days growth in anchorage-independent conditions (Day 4). The compounds were added at a concentration equivalent to the IC_{50} values in MDA-MB-231 cells as determined by MTT assay (Chapter 2, Figure 2.3). In the case of the novel non-toxic compounds RU017 and RU018, a concentration of 300 μM was selected due to the preliminary findings within the research group that a racemic mixture of the compounds appeared to reduce the $ABCG2^+$ "side population" as assessed by flow cytometry at that concentration (Figure A3, Appendix). This high concentration of the compounds was not, however, toxic to MCF-7 cancer or non-cancerous MCF12A breast epithelial cells under regular adherent conditions (Figure A1 and Table 2.2).

The effect of other novel algal "hit" compounds described in Chapters 2 through 4 (eg. RU004 and RU007) on mammosphere formation could not be assessed due to insufficient quantities of these compounds.

5.3.2.1 Analysis of the effect of selected algal compounds on MCF-7 mammosphere formation and development when added upon seeding

When added to the anchorage-independent serum-free culture conditions at Day 0, the prenylated quinone SQA, though able to slow down mammosphere formation compared to the DMSO control by Day 3, was unable to prevent the formation of MCF-7 mammospheres by Day 6 (Figure 5.2, second column vs. first column). In fact, the compound appeared to stimulate the formation of the more mature hollow mammosphere structures as compared to the DMSO-treated control sample after 6 days growth (inset in image in third row of Figure 5.2). In the case of the carotenoid, FXN, while the compound was able to reduce the number and size of the mammospheres both at Day 3 and Day 6 compared to the control, mammosphere formation was not completely inhibited by FXN (third column of Figure 5.2). On the other hand, for both of the novel halogenated monoterpene stereoisomers RU017 and RU018, the MCF-7 mammospheres were eliminated by Day 3, leaving only single cells or clusters of two or three cells in the treated samples (fourth and fifth columns in Figure 5.2), despite having no effect on MCF-7 cells under adherent conditions at the same concentration (Figure A1).

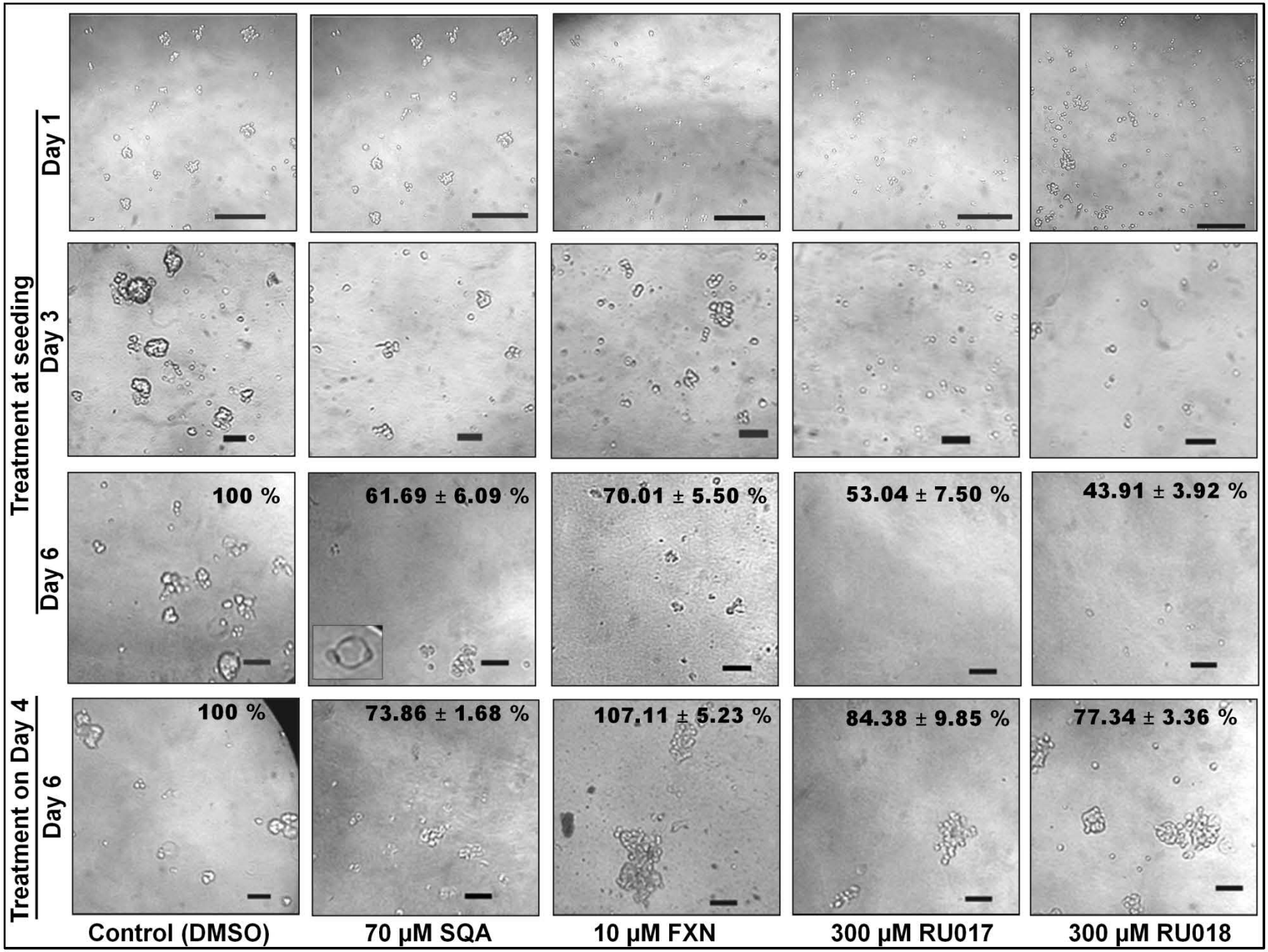
Treatment at seeding
Day 1
Day 3
Day 6
Treatment on Day 4
Day 6
100 %
61.69 ± 6.09 %
70.01 ± 5.50 %
53.04 ± 7.50 %
43.91 ± 3.92 %
100 %
73.86 ± 1.68 %
107.11 ± 5.23 %
84.38 ± 9.85 %
77.34 ± 3.36 %
Control (DMSO)
70 µM SQA
10 µM FXN
300 µM RU017
300 µM RU018

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Figure 5.2: Screening of marine algal compounds for the ability to inhibit the formation and development of MCF-7 mammospheres. MCF-7 breast cancer cells were seeded as a single cell suspension into anchorage-independent serum-free culture conditions. Treatment of cultures was with either the DMSO vehicle control (0.61 % v/v) or 70 µM sargaquinoic acid (SQA), 10 µM fucoxanthin (FXN), or 300 µM of either of the halogenated monoterpene stereoisomers RU017 or RU018 (treatments indicated beneath each column of images). The latter concentrations were derived from the IC_{50} values in MDA-MB-231 breast cancer cells grown in anchorage-dependent conditions in the case of SQA and FXN. For RU017 and RU018, the compounds were non-toxic to either MCF-7 or MDA-MB-231 cells at 300 µM in adherant conditions, but were shown to decrease the $ABCG2^+$ cancer stem cell-enriched "side population" in a previous study. The top three rows represent images captured on Day 1, Day 3 and Day 6 after treatment with the relevant compounds upon seeding, whereas the bottom row depicts images obtained on Day 6 of mammospheres treated with compounds four days after seeding (treatment Day 4). Images were captured under a light microscope at either 400x or 100x magnification using a Nikon Coolpix 990 camera and were representative of at least three randomly selected fields for each treatment. Analysis of images and calculation of scale was carried out using ImageJ (NIH freeware). Scale bars representative of 0.1 mm. The inset in the image of mammospheres treated with SQA upon seeding shows the hollow mammosphere phenotype and is not set to the same scale. Values given on images represent the percentage survival of the samples as determined by Wst-1 assay on Day 8, together with the standard error of the mean where n = 5.

5.3.2.2 Elucidation of the effect of algal compounds on MCF-7 mammosphere development when added after four days growth in anchorage-independent culture conditions

For all of the algal compounds tested, the effect of the compounds on mammosphere development when added at Day 4 differed from that obtained when the compounds were added upon seeding of the MCF-7 cells (Figure 5.2, third vs. fourth row). When added to existing MCF-7 mammospheres on Day 4, none of the compounds screened were able to remove the existing MCF-7 mammospheres or prevent their further development (fourth row of Figure 5.2). In particular, SQA appeared to have little, if any, effect on the development of the mammospheres when added at Day 4 as compared to the DMSO control (second image vs. first image in fourth row of Figure 5.2). On the other hand, the addition of FXN after four days resulted in the formation of much larger, irregularly shaped mammospheres than those observed in the DMSO-treated MCF-7 cells (third image in the third row of Figure 5.2). In contrast to the effects when added upon seeding, neither of the stereoisomers RU017 or RU018 were able to eliminate the MCF-7 mammospheres or affect their further development when added at Day 4 (fourth and fifth images, respectively, in the third row of Figure 5.2).

In order to gain information as to the mode of action of the novel marine algal compounds against MCF-7 mammospheres, and as an attempt to quantify the changes in mammosphere number and size, the WST-1 cell proliferation assay was carried out after eight days growth in anchorage-independent culture conditions. The percentage survival values relative to the DMSO-treated negative control for each sample are indicated on the relevant images in Figure 5.2. In this assay, SQA was found to decrease cell survival to 62 % when added at seeding and to 74 % when added at Day 4. While FXN reduced cellular survival to 70 % when added upon seeding, the compound was unable to decrease cell survival when added four days after seeding. The halogenated monoterpenes RU017 and RU018 decreased cellular survival to 53 % and 44 %, respectively, when added at Day 0; with a slight reduction in survival to 84 % and 77 %, respectively, when added at Day 4.

The screening of SQA, FXN, RU017 and RU018 described in Section 5.3.2 above was carried out in a mammosphere assay where cells were seeded at a density of 1000 cells per well. These experiments were repeated for mammospheres obtained by seeding with 3000 MCF-7 cells and treating with the compounds upon seeding. The results obtained in terms of the effects of the compounds on mammosphere formation and viability were largely consistent with those observed when seeding with 1000 cells (Figure A4, Appendix). All subsequent assays were carried out using 1000 MCF-7 cells for seeding into mammosphere conditions.

5.3.3 Dose-response study of the effect of paclitaxel and selected algal compounds on MCF-7 mammosphere formation

The effects of the algal compounds Fxn, RU017 and RU018 on the formation and development of MCF-7 mammospheres were more thoroughly investigated by determining whether the observed alterations to the mammospheres (Section 5.3.2) were dose-dependent. In addition, the effect of various concentrations of the chemotherapeutic agent, paclitaxel (Ptx), on mammosphere formation was assessed. In all cases, the compounds were added upon seeding of 1000 MCF-7 cells and the tumoursphere assay carried out as before.

5.3.3.1 The effect of various doses of RU017, RU018, FXN and Ptx on mammosphere formation and development

For both of the novel monoterpene stereoisomers, RU017 and RU018, treatment with 25 μM of the compound did not have a noticeable effect on the number or size of the MCF-7 mammospheres formed after six days; however, the mammospheres were observed to be more irregular in shape when compared to the DMSO-treated control (first and second columns of Figure 5.3; first and second row, respectively). In addition, the latter concentration did not reduce cellular survival of the treated mammospheres compared to the DMSO control as determined by WST-1 assay. At a concentration of 50 μM, treatment with either RU017 or RU018 resulted in a decrease in mammosphere size compared to the control, while having no effect on cell survival (third column in Figure 5.3, first and second row respectively). Treatment with 100 μM inhibited mammosphere formation almost completely but had little effect on percentage survival relative to the control (fourth columns in Figure 5.3, first and second row respectively). As was observed in Section 5.3.2, treatment of MCF-7 cells upon seeding in anchorage-independent conditions with 300 μM of either RU017 or RU018 prevented mammosphere formation entirely and substantially reduced cell survival (fifth column of Figure 5.3; first and second row, respectively).

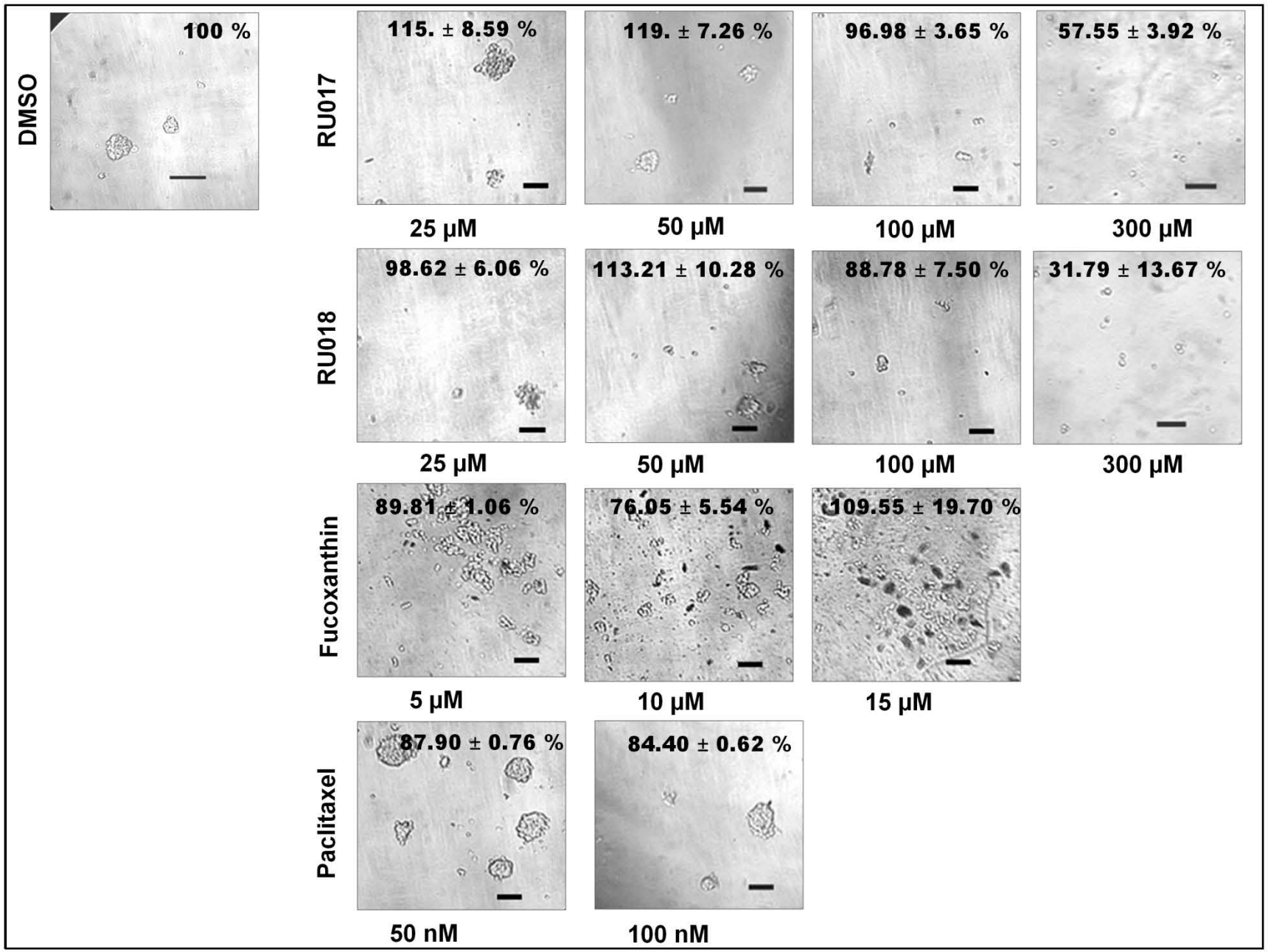

DMSO
100 %
RU017
115. ± 8.59 %
119. ± 7.26 %
96.98 ± 3.65 %
57.55 ± 3.92 %
25 µM
50 µM
100 µM
300 µM
RU018
98.62 ± 6.06 %
113.21 ± 10.28 %
88.78 ± 7.50 %
31.79 ± 13.67 %
25 µM
50 µM
100 µM
300 µM
Fucoxanthin
89.81 ± 1.06 %
76.05 ± 5.54 %
109.55 ± 19.70 %
5 µM
10 µM
15 µM
Paclitaxel
87.90 ± 0.76 %
84.40 ± 0.62 %
50 nM
100 nM

Figure 5.3: Dose-response study of the effect of paclitaxel and the marine algal compounds fucoxanthin, RU017 and RU018 on MCF-7 mammosphere formation *in vitro.* MCF-7 cells were seeded as a single cell suspension into anchorage-independent serum-free conditions containing either the DMSO vehicle control (0.61 % v/v) or a range of concentrations of either of the halogenated monoterpene stereoisomers RU018 or RU018 (25, 50, 100 or 300 µM), fucoxanthin (5, 10 or 15 µM) or paclitaxel (50 or 100 nM). Cells were photographed on Day 6 under a light microscope at 100x magnification. All images are representative of at least three randomly selected fields and were set to the same scale using ImageJ. Scale bars are representative of 0.1 mm. Values given on images represent the percentage survival of the samples as determined by Wst-1 assay on Day 8, together with the standard error of the mean where n = 5.

In the case of the carotenoid compound FXN, none of the concentrations tested were able to completely eliminate mammosphere formation when added to MCF-7 cells upon seeding into anchorage-independent conditions, although a dose-dependent decrease in mammosphere size was observed (third row of Figure 5.3). On the other hand, the cytotoxicity of FXN against MCF-7 mammospheres was not dose-dependent, with only the 10 μM treatment effectively reducing cell survival relative to the DMSO control (third row of Figure 5.3). The dark granular structures depicted in the FXN-treated samples were undissolved FXN crystals which appear orange under the microscope (data not shown).

The chemotherapeutic drug Ptx appeared to increase the number of MCF-7 mammospheres when 50 nM was added upon seeding, while treatment with 100 nM had little effect on mammosphere formation compared to the DMSO-treated control (fourth row of Figure 5.3). This was despite the latter concentration being reported as the IC_{50} value for MCF-7 cells under adherent conditions (Dwight *et al*., 1997). On the other hand, Ptx was only mildly toxic towards MCF-7 mammospheres when added at both 50 and 100 nM in anchorage-independent mammosphere conditions (Figure 5.3).

5.3.4 Analysis of the effect of pre-treatment with either Ptx or selected novel algal compounds on MCF-7 tumoursphere formation

As a final strategy to characterize the observed effects of RU017, RU018, FXN and Ptx on the formation, development and survival of MCF-7 mammosphere, the compounds were used to pre-treat MCF-7 cells in regular anchorage-dependent conditions overnight prior to seeding into anchorage-independent tumoursphere conditions. The consequences of such treatment on mammosphere development and viability were compared to those obtained for treatment at Day 0 and Day 4.

5.3.4.1 Analysis of the effects on MCF-7 mammosphere formation as a result of pre-treatment with RU017, RU018, FXN or Ptx

For all four of the compounds RU017, RU018, FXN and Ptx, pre-treatment of MCF-7 cells in anchorage-dependent culture conditions prior to seeding into anchorage-independent conditions in the tumoursphere assay was unable to either prevent mammosphere formation or effectively reduce cell survival relative to the DMSO control (first row in Figure 5.4). In particular, pre-treatment with the chemotherapeutic agent Ptx appeared to produce larger, more regular shaped mammospheres when compared to the DMSO vehicle-treated control, while having little effect on cell survival (first vs. last image in the first row of Figure 5.4).

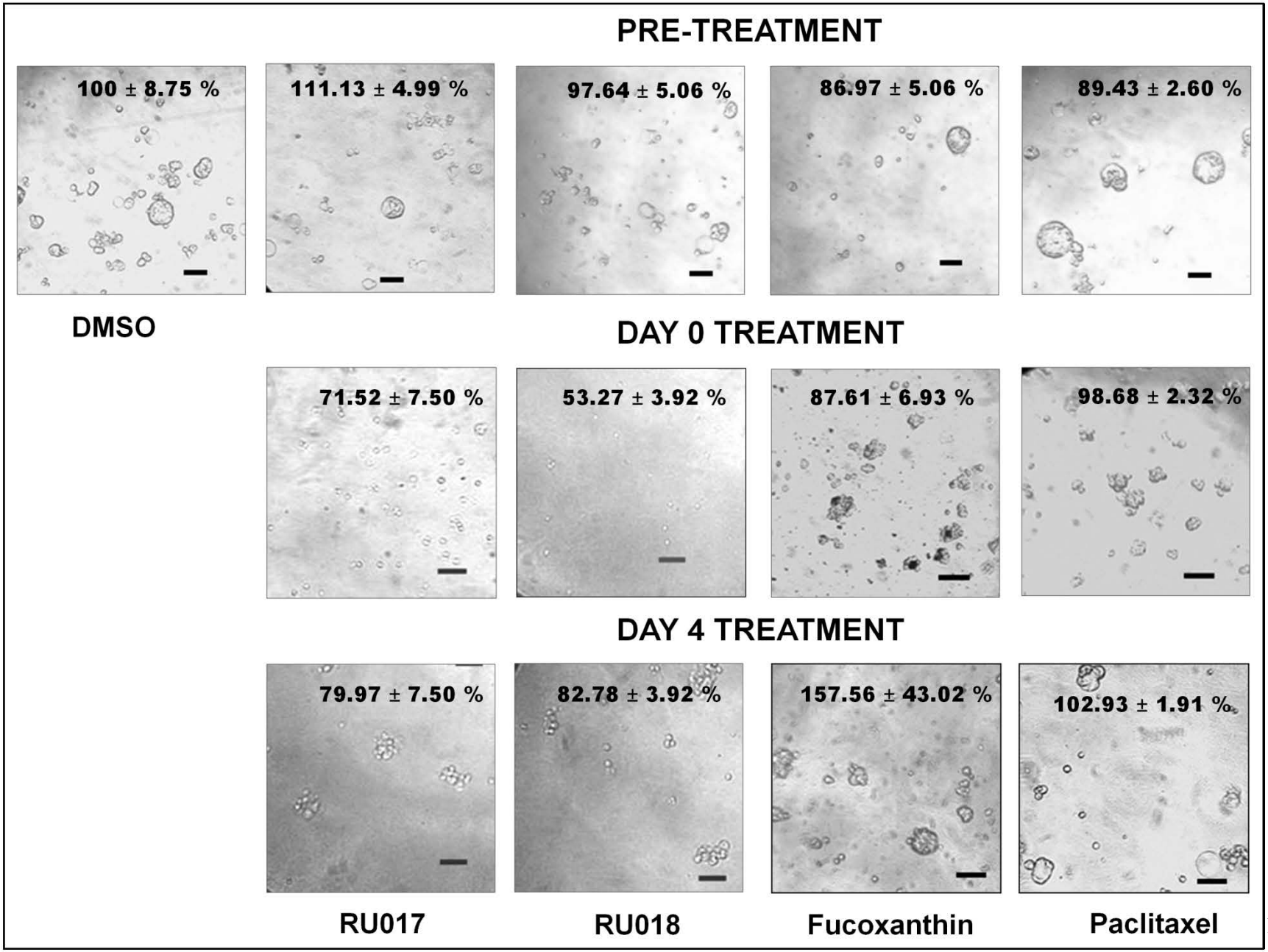

PRE-TREATMENT
100 ± 8.75 %
111.13 ± 4.99 %
97.64 ± 5.06 %
86.97 ± 5.06 %
89.43 ± 2.60 %
DMSO
DAY 0 TREATMENT
71.52 ± 7.50 %
53.27 ± 3.92 %
87.61 ± 6.93 %
98.68 ± 2.32 %
DAY 4 TREATMENT
79.97 ± 7.50 %
82.78 ± 3.92 %
157.56 ± 43.02 %
102.93 ± 1.91 %
RU017
RU018
Fucoxanthin
Paclitaxel

Figure 5.4: Kinetic study of the effect of paclitaxel and the marine algal compounds fucoxanthin, RU017 and RU018 on MCF-7 mammosphere formation *in vitro*. Treatment of MCF-7 cells was with the DMSO vehicle control (0.61 % v/v) or either of the halogenated monoterpene stereoisomers RU017 or RU018 (100 µM), fucoxanthin (10 µM) or paclitaxel (100 nM). For pre-treatment, MCF-7 cells were cultured in regular anchorage-dependent culture conditions containing the compound of interest overnight, before trypsinizing and transferring to anchorage-independent serum-free mammosphere conditions as a single cell suspension. The Day 0 and Day 4 samples refer to treatment of MCF-7 cells either upon seeding or after 4 days growth, respectively, in anchorage-independent serum-free culture conditions. Images were captured on Day 6 under a light microscope at 100x magnification. Each image is representative of at least 3 randomly selected fields. Images were set to the same scale using ImageJ. Scale bars are equivalent to 0.1 mm. Values given on each image represent the percentage survival for each treatment as determined by Wst-1 assay on Day 8 together with the standard error of the mean.

As before, treatment with Ptx on Day 0 was unable to prevent either mammosphere formation or decrease cellular viability (last image in the second row of Figure 5.4 and Figure 5.3). When added to existing mammospheres on Day 4, Ptx was unable to remove the mammosphere or reduce cell survival and resulted in the formation of a greater number of hollow mammospheres by Day 6 when compared to the DMSO-treated control (last image in third row of Figure 5.4).

The effect of the algal compounds RU017, RU018 and FXN on mammosphere formation and development when added either at Day 0 or Day 4 were consistent with those observed in previous assays. That is that RU017 and RU018 completely inhibited mammosphere formation when added at Day 0, but had little effect on the viability or further development of existing mammospheres when added on Day 4; while FXN was unable to prevent mammosphere formation or effectively reduce cell viability when added either at Day 0 or Day 4 (second and third row of Figure 5.4).

5.4 Analysis of the effect of RU017, RU018, FXN and Ptx on the ability of MCF-7 mammospheres to revert to anchorage-dependent growth

The effect of treatment with RU017, RU018, FXN and Ptx on the ability of MCF-7 mammospheres to attach and grow when transferred back into regular growth conditions without compound was assessed. For each compound, similar results were obtained for all the concentrations tested (data not shown) and thus only a single example is shown for each of the treatments (experiments carried out in in quadruplicate). For the algal compounds RU017, RU018 and FXN (at concentrations of 50 μM for RU017 and RU018 and 10 μM for FXN), none of the treatments appeared to inhibit the ability of the MCF-7 mammospheres to attach and grow in regular anchorage-dependent growth conditions (Figure 5.5). It was observed, however, that those samples containing larger and more numerous mammospheres took a greater amount of time to fully attach to the culture vessels, as was the case for FXN-treated mammospheres (Figure 5.5). The chemotherapeutic agent, Ptx, had a significant impact on the MCF-7 mammospheres once they were placed in anchorage-dependent culture conditions, being able to prevent adhesion and/or growth, even after four days incubation (Figure 5.5).

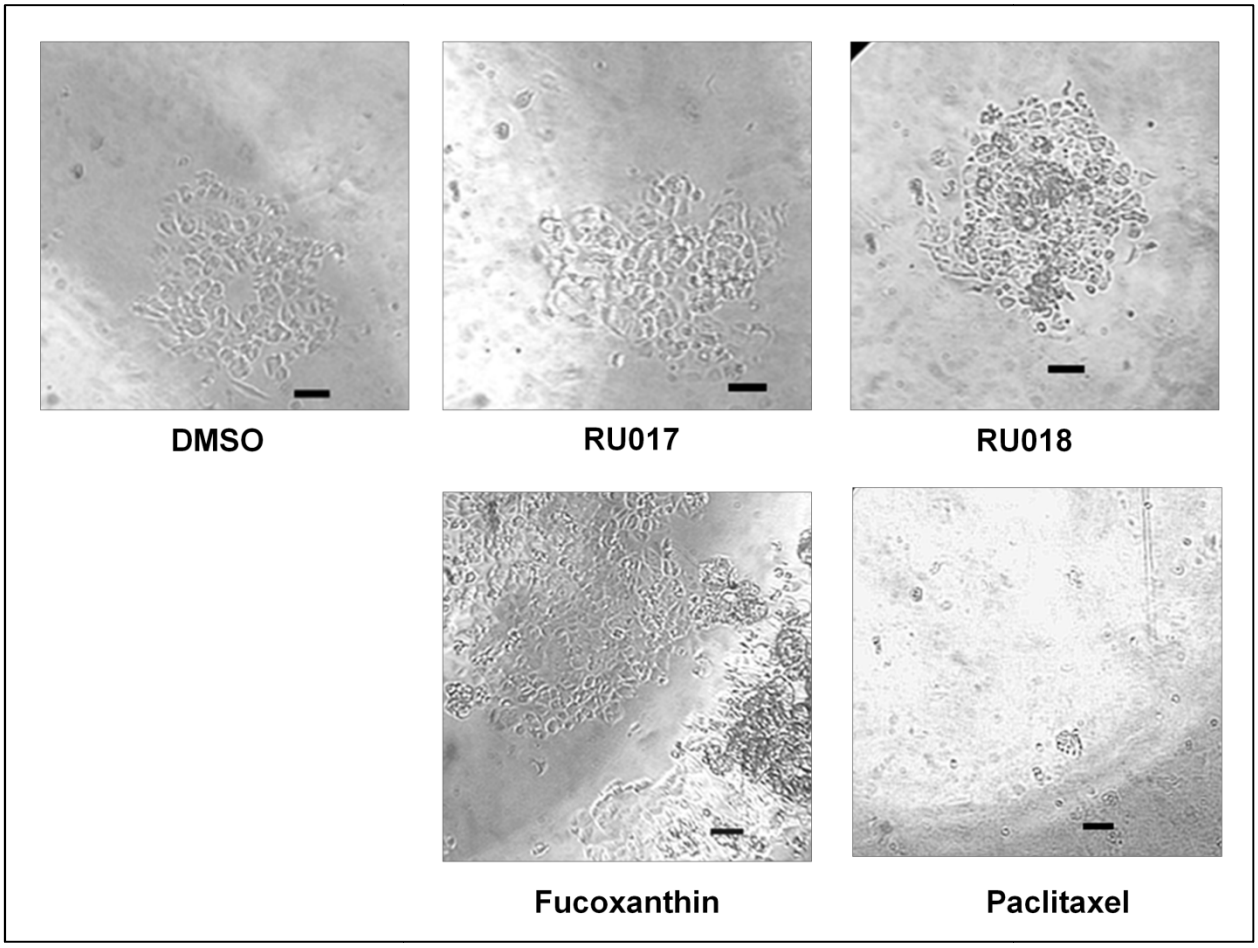


Figure 5.5: Assessment of the ability of treated MCF-7 tumourspheres to re-attach and grow when returned to anchorage-dependent culture conditions. Cells were originally treated upon seeding with either the DMSO vehicle control (0.61 % v/v) or either of the halogenated monoterpene stereoisomers RU017 or RU018, fucoxanthin or paclitaxel in anchorage-independent serum-free conditions. For each sample, mammospheres were transferred from anchorage-independent conditions after 8 days and placed into regular anchorage-dependent culture conditions without compound. Images were taken on Day 4 under a light microscope at 100x magnification and set to a common scale in ImageJ. Scale bars equivalent to 0.1 mm. Each image was representative of at least 3 randomly selected fields.

DISCUSSION

The mammosphere cultivation system developed by Dontu *et al*. allows for the *in vitro* propagation of mammary stem and progenitor cells based on their ability to grow in suspension in anchorage-independent serum-free conditions (Dontu *et al*., 2003). This technique was modified and optimized within the Biomedical Biotechnology Research Unit (BioBRU, Rhodes University) to allow for the *in vitro* cultivation of the cancer stem cell-like subpopulation of the MCF-7 breast cancer cell line (unpublished data; Mugdha Sukthankar and Jason Sterrenberg,

2011). In the current study, the mammosphere system was used to develop an assay which allowed for the screening of novel marine algal compounds for potential anti-cancer stem cell (CSC) activity.

The formation and development of MCF-7 mammospheres was observed after seeding of one thousand cells into anchorage-independent serum-free conditions over the course of one week. As reported by Dontu *et al*. in the case of dissociated breast epithelial cells, "floating spherical colonies" or mammospheres were observed to develop under the anchorage-independent assay conditions (Dontu *et al*., 2009). While the aforementioned study reported the formation of mammospheres after five to seven days, the MCF-7 breast cancer cell line generated mammospheres after only three days (Dontu *et al*., 2003). The more rapid development of the mammospheres by a cancer cell line compared to that by dissociated breast epithelial cells could be attributed to the more rapid growth of cancer cell lines in general, as suggested by Fillmore and Kupperwasser (2009).

The mammospheres observed in the suspension growth conditions were not uniform in appearance, varying in both size and morphology. The larger MCF-7 mammospheres generated in anchorage-independent conditions have previously been reported to have a diameter of between 100 and 150 μm (0.1 – 0.15 mm) after seven days growth in suspension (Sansone *et al*., 2007), a size which is consistent with those observed in this study. In addition, the mammospheres have been demonstrated to consist of a "ball" of cells as opposed to being hollow in the centre (Sansone *et al*., 2007; Fillmore and Kupperwasser, 2009). However, in the current study, hollow and apparently empty mammospheres were observed after seven days growth in suspension, and were thought to represent a more mature phenotype. There are no reports in the literature describing such a phenotype for either mammospheres or tumourspheres in general, and the closest resemblance of the hollow mammospheres is to the *zona pellucida*, the spherical glycoprotein shell surrounding a developing oocyte (Connor *et al*., 2005). Further characterization of the various morphological sub-types of the MCF-7 mammospheres would be required in order to understand their significance.

The mammosphere assay was used to determine whether the marine algal compounds SQA, FXN, RU017 and RU018 displayed potential anti-CSC activity in terms of their ability to prevent both formation and development/maturation of the MCF-7 mammospheres. While the addition of the test compounds at the stage of seeding of MCF-7 cells into anchorage-independent culture conditions assessed their effect on mammosphere formation, dosing with the compounds to existing mammospheres on Day 4 measured their ability to remove the mammospheres or alter their further development. Furthermore, assessment of the cellular viability of mammospheres after treatment with the algal compounds was carried out as a measure of cytotoxicity of the compounds to MCF-7 cells in anchorage-independent serum-free

culture conditions and in an attempt to quantify the inhibitory effects of the compounds on mammosphere formation and development.

The prenylated quinone, SQA, was found to be differentially toxic to metastatic MDA-MB-231 and Hs578T breast cancer cells, while having no adverse effect on healthy breast epithelial MCF12A cells and was thus identified as a potential "hit" compound (Chapter 2, Figure 2.3 and Table 2.2). The quinone was, however, unable to prevent formation of MCF-7 mammospheres either when added upon seeding or after four days growth in anchorage-independent conditions. In fact, when added upon seeding, the compound appeared to speed up the maturation of the mammospheres as evidenced by an increase in hollow mammospheres. Although unable to inhibit MCF-7 mammosphere formation, SQA was nonetheless found to reduce the viability of treated mammospheres when added either at seeding or on Day 4. This, together with the apparent ability of SQA to speed up mammosphere maturation, could indicate a mechanism whereby the lifespan of the mammospheres may be reduced by the quinone. The latter possibility could be tested by culturing the mammospheres for longer than the standard eight day incubation and comparing the SQA-treated mammospheres with those in the vehicle-treated control.

More in-depth studies in terms of the effect on mammosphere formation and development were carried out for the algal compounds RU017, RU018 and FXN as well as the chemotherapeutic drug Ptx. These studies assessed the dose-dependency and potential mode of action responsible for the effects on MCF-7 mammospheres. As a means of gaining insight into the specific cell sub-populations targeted by the compounds of interest, the effect of pre-treatment of MCF-7 cells with the compounds in anchorage-dependent culture conditions prior to seeding into mammosphere conditions was assessed. The rationale behind the latter approach was that, should a compound specifically target the CSC sub-population, this effect would be evident by a decrease in mammosphere formation when the treated cells were transferred into anchorage-independent serum-free culture conditions. In addition, further functional studies involved the assessment of the ability of the selected compounds to prevent attachment and growth of mammospheres when transferred into regular anchorage-dependent culture conditions. This *in vitro* assay mimics the *in vivo* process of metastasis and regeneration of a tumour at a distant site, since in the latter case, the cells would need to detach from the extracellular matrix (ECM), survive in suspension in the blood stream, attach at another site in the body and then initiate and maintain growth at the new site (Siclari *et al*, 2006). The ability to survive and grow in anchorage-independent conditions, as well as the capability of asymmetric cell division has led to the hypothesis that CSCs are responsible for metastasis of cancers (Li *et al*., 2007; Lawson *et al*., 2009).

The carotenoid compound, FXN, was shown to be highly toxic to MDA-MB-231 and Hs578T breast cancer cells and, to a lesser extent, non-cancerous MCF12A breast epithelial cells (Chapter 2, Figure 2.3 and Table 2.2). However, the compound was unable to prevent the formation of MCF-7 mammospheres when added upon seeding or at Day 4 or when used to pre-treat MCF-7 cells prior to seeding into anchorage-independent mammosphere conditions. The carotenoid did, however, reduce the size of the mammospheres when added upon seeding and, when added to existing mammospheres in suspension on Day 4, appeared to alter further development, resulting in larger and more irregular shaped mammospheres by Day 6. In terms of the viability of the treated mammospheres, FXN displayed moderate toxicity against MCF-7 mammosphere cells when added to the cultures upon seeding, but not when added to existing mammospheres on Day 4. Dose-response studies revealed that, even at a concentration which was more than double the IC_{50} value against breast cancer cells (Chapter 2), FXN remained unable to inhibit either mammosphere formation or viability when added upon seeding. In addition, the compound had no effect of the ability of the mammospheres to attach and grow when transferred into regular anchorage-dependent culture conditions. This was despite the toxicity of FXN against breast cancer cells under regular adherent culture conditions (Chapter 2, Figure 2.3), but agreed with the low cytotoxicity of the compound under anchorage-independent conditions. A possible interpretation of these data could be that the specific cell sub-type(s) able to survive in anchorage-independent mammosphere conditions, and enriched for by this assay, are resistant to FXN.

In the case of the novel halogenated monoterpene stereoisomers RU017 and RU018, both of which were shown to have no effect on the survival and proliferation of either breast cancer cells or healthy breast cells *in vitro* (Chapter 2, Figure 2.3 and Table 2.2), the compounds were able to completely inhibit mammosphere formation when added to MCF-7 cells upon seeding in the mammosphere assay. This agreed with the preliminary results obtained within the research group, in which a racemic mixture of the compounds was shown to decrease the $ABCG2^+$ MCF-7 "side population," thought to be enriched for CSCs (Lawson, 2009; MSc thesis). On the other hand, when added to existing mammospheres on Day 4, neither of the compounds was able to remove the mammospheres or affect their further development.

In terms of the effect of the compounds on cell viability, RU017 and RU018 appeared to substantially decrease cell survival when added upon seeding and, to a lesser extent, when added on Day 4. Interestingly, in both cases, mammosphere cell viability was reduced to a greater extent in RU018-treated cells compared to those treated with RU017, indicating that the stereoisomers differ in their cytotoxicity against the cell sub-type(s) enriched by the mammosphere assay. However, considering that the RU017- and RU018-treated samples did not contain any visible mammospheres by Day 8, the percentage survival values of roughly 50 % relative to the DMSO control were higher than expected. This raised questions as to the

suitability of the WST-1 assay for either the assessment of mammosphere cell viability or as a means of quantifying the mammospheres present in each sample. One of the problems associated with assessing cellular viability in the mammosphere assay is the large variation in both the number and size of the mammospheres between samples. For the larger mammospheres, it is not known whether the WST-1 reagent is able to penetrate the mammospheres or whether the absorbance from the buried cells within the mammospheres would be detectable. Thus, it was not possible to conclude whether the values obtained indicate toxicity against the total population of the mammospheres or simply those exposed on the surface. Dissociation of the mammospheres to a single cell suspension prior to cytotoxicity assays could potentially overcome this problem, providing that this was carried out in such a way that cell viability would not be affected. Since the tumoursphere assay is a relatively new technique (Fillmore and Kupperwasser, 2007), cytotoxicity testing for the screening of compounds in this system has not been optimized and thus the results obtained using the WST-1 assay in this study were interpreted with caution.

The inhibitory effect of the halogenated monoterpene stereoisomers RU017 and RU018 on mammosphere formation when added upon seeding into anchorage-independent conditions was further characterized in terms of the dose-dependency of the anti-mammosphere activity. The 300 μM concentration of the compounds used in the initial screening, though non-toxic to breast cancer and healthy breast cells in regular adherent conditions, was considered to be unfavourably high in terms of a hit compound, whose activity should ideally fall between 1 and 50 μM (Keseru and Makara, 2006). Therefore, the ability of lower doses of the compounds to inhibit mammosphere formation was assessed. For both compounds, a three times lower concentration than that used in screening (ie. 100 μM) was able to effectively prevent mammosphere formation. As a result, the latter concentration of the compounds was used in subsequent studies, albeit that this dose displayed only mild cytotoxicity in the WST-1 assay.

Pre-treatment of MCF-7 cells with either RU017 or RU018 prior to seeding into anchorage-independent conditions had no effect on the ability of MCF-7 cells to form mammospheres under these conditions. One might expect that pre-treatment with the algal monoterpene stereoisomers would decrease or prevent mammosphere formation due to the ability of the compounds to completely inhibit mammosphere formation when added upon seeding. A possible explanation for this discrepancy is that the effect of the compounds on the MCF-7 sub-population enriched by the mammosphere assay may be specific to growth in anchorage-dependent conditions. The latter sub-population thought to be targeted by RU017 and RU018 was predicted to represent CSCs. If this hypothesis was correct and since CSCs are thought to undergo both morphological and phenotypic changes enabling them to grow in suspension (Fillmore and Kupperwasser, 2007), it is not unlikely that RU017 and RU018 was targeting such adaptations of the CSCs. When transferred to anchorage-dependent conditions,

mammospheres treated with either RU017 or RU018 were able to attach and grow as adherent monolayers, indicating that this property of the mammosphere-enriched cell sub-type was not inhibited by the halogenated monoterpenes.

The commonly used chemotherapeutic agent, Ptx, was analysed for the ability to inhibit mammosphere formation and viability. Ptx was unable to prevent mammosphere formation when added at a concentration which was toxic to both breast cancer and normal breast cells under adherent conditions (Chapter 2). Furthermore, neither of the concentrations tested were found to be particularly toxic to MCF-7 mammospheres, being unable to reduce cellular viability to below 84 % as assessed by WST-1 assay. The effect of 10 ng/L (12 pM) of Ptx has previously been reported to have no effect on MDA-MB-231 mammosphere survival (Fillmore and Kupperwasser, 2009). Since CSCs have been reported to be resistant to Ptx and other chemotherapeutic agents due to the presence of high levels of ABC drug transporters which are able to efflux the drugs, thus protecting the CSCs (Dean *et al*., 2009), this lent strength to the hypothesis that the cells comprising the mammosphere may be CSCs. In addition, when added at a concentration equivalent to half the IC_{50} value determined in MD-MB-231 cells (Figure 2.1; Chapter 2), Ptx appeared to stimulate mammosphere formation. This agreed with published reports which have suggested that, since the compound is cytotoxic to the bulk population of tumours and not the CSC sub-population, chemotherapy with this and similar drugs may effectively enrich for CSCs (Lawson *et al*., 2009). This CSC enrichment theory could also explain the observation that pre-treatment with Ptx appeared to speed up mammosphere growth and development as evidenced by the larger more regularly-shaped mammospheres arising from pre-treated MCF-7 cells.

In adherence studies, mammospheres treated with Ptx did not produce an adherent culture when transferred to regular anchorage-dependent conditions, even after four days incubation in these conditions. Rather, the large mammospheres were replaced by small floating cell clumps in adherent conditions. This was thought to have occurred in one of two ways: either the mammosphere cells were unable to attach and thus underwent anoikis; or the cells were able to attach to the culture vessel, but were subsequently rapidly eliminated by traces of Ptx transferred along with the mammospheres into the new culture conditions (Ptx was diluted by a factor of ten when transferred into regular media). The latter possibility could be explored by washing the mammospheres prior to transferring into adherent conditions to completely remove the drug. Since no such adherence study has been reported in the literature for treatment of mammospheres with Ptx, there was no data with which to compare the results obtained in this body of work.

For all of the treatment described above, further experiments to determine the cellular sub-type composition of the treated mammospheres could shed light onto the specific cells

targeted by the compounds. For example, the ability of RU017 and RU018 to inhibit mammosphere formation when added at Day 0, but not when added at Day 4 could indicate that the compounds targeted the self-renewal and differentiation capabilities of CSCs, while leaving their progenitors unaffected.

CONCLUSIONS

Finally, the body of work described in this chapter reports the screening of the novel marine algal compounds SQA, FXN, RU017 and RU018 in the mammosphere assay and revealed that the latter monoterpene stereoisomers displayed putative anti-CSC activity. This was suggested by the ability of both RU017 and RU018 to completely inhibit mammosphere formation in anchorage-independent conditions even after six days incubation. In stark contrast, the commonly used chemotherapeutic drug Ptx appeared to enhance both the formation and maturation of MCF-7 mammospheres. The inability of SQA and FXN to inhibit mammosphere formation and development revealed that the putative anti-CSC activity was not a feature shared by all compounds of marine algal origin. This is the first report of the screening of novel marine algal compounds against BCSCs and indeed against CSCs in general. It is also the first report of compounds which are able to selectively inhibit CSCs while leaving both the bulk cancer cells and non-cancerous breast epithelial cells unaffected. More work is required to determine the specific molecular mechanism mediating the CSC-specific activity of the halogenated monoterpenes as well as their respective cellular targets.

CHAPTER 6
FINAL DISCUSSION

6.1 SUMMARY OF RESULTS

The rich biodiversity of the marine environment of South Africa, in particular that of marine algae, has proven to be an abundant source of structurally unique natural products (Le Roux *et al.,* 2002; Davies-Coleman, 2004). Indeed, the library of compounds from red and brown algae analyzed in this study contained a large number of novel polyhalogenated monoterpenes and monoterpene aldehydes, as well as the previously described tetraprenylated quinone sargaquinoic acid (SQA) and the carotenoid (FXN) (see Figure 2.2, Chapter 2 for structures) (Knott *et al.,* 2005; Mann *et al.,* 2007; Afoloyan *et al.,* 2008). Algal secondary metabolites such as these have been demonstrated to possess a range of pharmacologically beneficial properties including anti-bacterial, anti-fungal, anti-viral, anti-malarial and anti-cancer activities (Carté, 1996; Peng *et al.,* 2011). While a subset of the novel compounds in the current study had previously been subject to preliminary cytotoxicity screening in WHC01 oesophageal cancer cells *in vitro* (Mann *et al.,* 2007), the majority of the algal metabolites had not been assessed for biological activity. In particular, none of the algal compounds had been tested for cytotoxicity against metastatic ER^-PR^-HER2/neu^- breast cancer cells or the putative breast cancer stem cells (BCSCs) contained in MCF-7 mammospheres *in vitro*.

The aims of this study included the screening of novel compounds of South African marine algal origin against metastatic ER^-PR^-HER2/neu^- breast cancer cells, non-cancerous breast epithelial cells and BCSC, as well as the characterization of the molecular mechanisms of action of identified hit compounds. The aims were achieved using a range of biochemical and cell biological techniques including MTT cytotoxicity assays, Western analyses, flow cytometry studies using vital dyes and antibodies, PARP cleavage assays, caspase inhibitor studies and MCF-7 mammosphere assays. The results from this body of work are summarized in Table 6.1.

The novel algal compounds were divided into four groups based on their differential toxicity when screened against breast cancer, non-cancerous breast and putative BCSCs (Table 6.1). Group 1 contained the compound with the highest toxicity against MDA-MB-231 breast cancer cells and which was demonstrated in a previous study within the Biomedical Biotechnology Research Unit (BioBRU) to be equally toxic to non-cancerous MCF12A breast epithelial cells (Lawson, 2009; MSc thesis). The mode of cell death induced by RU015 was investigated and the 50 kDa PARP cleavage products generated within treated cells were suggestive of the induction of necrosis; however this was not confirmed by flow cytometry using the vital dyes Hoechst 33342 (H33342) and propidium iodide (PI).

Table 6.1: Overview of the results obtained

GROUP	COMPOUNDS	CYTOTOXICITY (MTT ASSAY)	CHARACTERIZATION OF MECHANISM OF ACTION
1	RU015	• Highly toxic to breast cancer (MDA-MB-231 and Hs578T) cells • Highly toxic to non-cancerous (MCF12A) cells (Lawson, 2009; MSc thesis)	• **Mode of cell death** a) Hoechst 33342 (H33342)/propidium iodide (PI) flow cytometry data inconclusive b) PARP cleavage products suggestive of necrosis
2	RU004 RU007 SQA	• Differential toxicity against breast cancer (MDA-MB-231 and Hs578T) BUT NOT non-cancerous (MCF12A) cells. • Toxicity in MDA-MB-231 and Hs578T cells confirmed by trypan blue staining. • Mildly cytotoxic to MCF-7 mammospheres (SQA) as assessed by Wst-1 assay	• **Mode of cell death** a) H33342/PI flow cytometry data suggestive of apoptosis b) PARP cleavage products indicative of apoptosis (SQA) • **Cell cycle analysis** Flow cytometry with PI staining suggestive of G_0-G_1 arrest with sub-G_0 apoptotic population (SQA) • **Involvement of caspases** a) Caspase-inhibitor studies identified caspase-3, -6, -8, -9, -10 and -13 as required for cytotoxicity (SQA) b) Western analysis showed dose-dependent increase in cleavage of caspase-9 (SQA) • Decrease in Bcl-2 levels in treated cells (SQA) • Dose-dependent decrease in levels of p-ERK1/2 in treated cells (SQA) • **Potential anti-breast cancer stem cell (BCSC) activity** (SQA) Unable to inhibit mammosphere formation or development
3.	FXN	• More toxic to breast cancer (MDA-MB-231 and Hs578T) than non-cancerous (MCF12A) cells • Toxicity in MDA-MB-231 and Hs578T cells confirmed by trypan blue staining. • Non-toxic to MCF-7 mammospheres as determined by Wst-1 assay	• **Mode of cell death** H33342/PI flow cytometry data inconclusive • **Cell cycle analysis** Flow cytometry with PI staining suggestive of G_0-G_1 arrest with sub-G_0 apoptotic population • **Involvement of caspases** Caspase-inhibitor studies identified caspase-3, -6, -8, -9, -10 and -13 as required for cytotoxicity • Decrease in Bcl-2 levels in treated cells • **Potential anti-BCSC activity** Unable to inhibit mammosphere formation or development
4.	RU016, RU017,RU018, RU019,RU048, RU050,RU054, RU075,RU076, RU079, RU081	• Non-toxic to MDA-MB-231 cells (all) • Non-toxic to Hs578T cells (RU017/18) • Non-toxic to MCF12A cells (RU017/18) • Wst-1 assay indicated potential cytotoxic effect against MCF-7 mammospheres (RU017/18)	**Potential anti-BCSC activity (RU017, RU018)** a) Inhibition of mammosphere formation when added upon seeding of 1000 or 3000 MCF-7 cells into anchorage-independent conditions b) No effect on mammospheres when added four days after seeding of MCF-7 cells into anchorage-independent conditions or when used to pre-treat MCF-7 cells prior to assay c) No effect on ability of mammospheres to revert to anchorage-dependent growth

* Hit compounds underlined.

Group 2 included the compounds RU004, RU007 and SQA, which were found to be toxic to breast cancer but not healthy breast epithelial cell lines. Flow cytometric analysis of treated breast cancer cells stained with the aforementioned vital dyes were suggestive of the induction of apoptosis for all three compounds, a result which was confirmed for SQA by the presence of 85 kDa PARP cleavage products in treated cells. The latter compound was also demonstrated to cause G_0-G_1 cell cycle arrest and produce a sub-G_0 apoptotic population as determined by flow cytometry with PI staining. Further studies into the specific molecular mechanisms mediating the cytotoxicity of SQA revealed that the compound potentially required the activity of caspase-3, -6, -8, -9 and -13 for its activity and was able to decrease the levels of both the anti-apoptotic protein Bcl-2 and the activated phosphorylated form of the survival kinase ERK1/2. Although SQA was mildly toxic against the putative BCSCs contained within MCF-7 mammospheres, the compound was unable to prevent mammosphere formation or development in anchorage-independent culture conditions.

Group 3 contained the previously described carotenoid FXN, which was demonstrated to be highly toxic to breast cancer cell lines and, to a lesser extent, the non-cancerous MCF12A breast epithelial cell line. In terms of the mode of action of FXN, while flow cytometry studies with H33342/PI staining were inconclusive, the sub-G_0 population detected in cell cycle analyses was suggestive of the induction of apoptosis. This was verified by the finding that the caspases -1, -3, and -9 were potentially required for the cytotoxicity of FXN, together with the observed ability of the compound to decrease the levels of the anti-apoptotic Bcl-2 protein. FXN, like SQA, also appeared to cause G_0-G_1 cell cycle arrest in treated cells in flow cytometry experiments. In terms of potential anti-BCSC activity, FXN was non-toxic to MCF-7 mammospheres and was unable to prevent mammosphere formation *in vitro*.

Finally, Group 4 contained the novel algal polyhalogenated monoterpenes which were found to be non-toxic to breast cancer cells. Of these, the stereoisomers RU017 and RU018 were also demonstrated to be non-toxic to healthy MCF12A breast cells. On the other hand, these compounds were found to be able to completely inhibit mammosphere formation *in vitro*, indicating potential anti-BCSC activity.

These findings together represent a rigorous interrogation of the biological activity of the library of novel compounds extracted from South African marine algae and assessed a number of characteristics of the compounds which are not often addressed in the field of natural product research. In particular, cytotoxicity against healthy non-cancerous cells for the vast majority of novel natural products is not routinely performed. As seen in this and previous studies within our research group, toxicity against non-cancerous cells is an important factor to consider, since the compound which displayed the most promising activity against breast cancer cells was also toxic to healthy breast cells (Lawson, 2009; MSc thesis). It is not

uncommon in the literature for researchers to carry out studies into the mechanism of action of novel compounds without first assessing whether the observed cytotoxicity is specific to cancer and not healthy cells (Andrianosolo *et al.,* 2006). On the other hand, it is also evident from the literature that few studies report the investigation of the mode of action of novel compounds after the initial screening for biological activity.

6.2 PROPOSED MODEL FOR MOLECULAR MECHANISM OF ACTION OF SQA AND FXN

Two of the best-characterized novel marine algal compounds to date are the quinone SQA, isolated from various species of the *Sargassum* genus, and the carotenoid FXN, extracted from species belonging the *Sargassum*, *Laminaria* and *Undaria* genera (Choi *et al.,* 2007; Hur *et al.,* 2008; Peng *et al.,* 2011). The data previously reported for these compounds provided the opportunity for comparison between the molecular mechanisms uncovered in the current study in breast cancer cells with those in other human cancer cell lines.

The precise target(s) to which SQA and FXN bind in breast cancer cells was not determined in this study and could represent either an extracellular receptor or an intracellular protein. However, a number of proteins were implicated in mediating the anti-cancer activities of the compounds. Figure 6.1 represents an overview of the interconnected apoptotic and survival pathways within human cells and indicates those signal transducer proteins identified in this study as potential role-players in mediating the toxicity of SQA and FXN against MDA-MB-231 breast cancer cells. The majority of proteins identified as potential mediators for the activity of SQA and FXN in this study are caspases. Since it has been predicted that, in most cases, the activity of anti-cancer therapies will involve caspases (Fulda and Debatin, 2006), this was not unexpected.

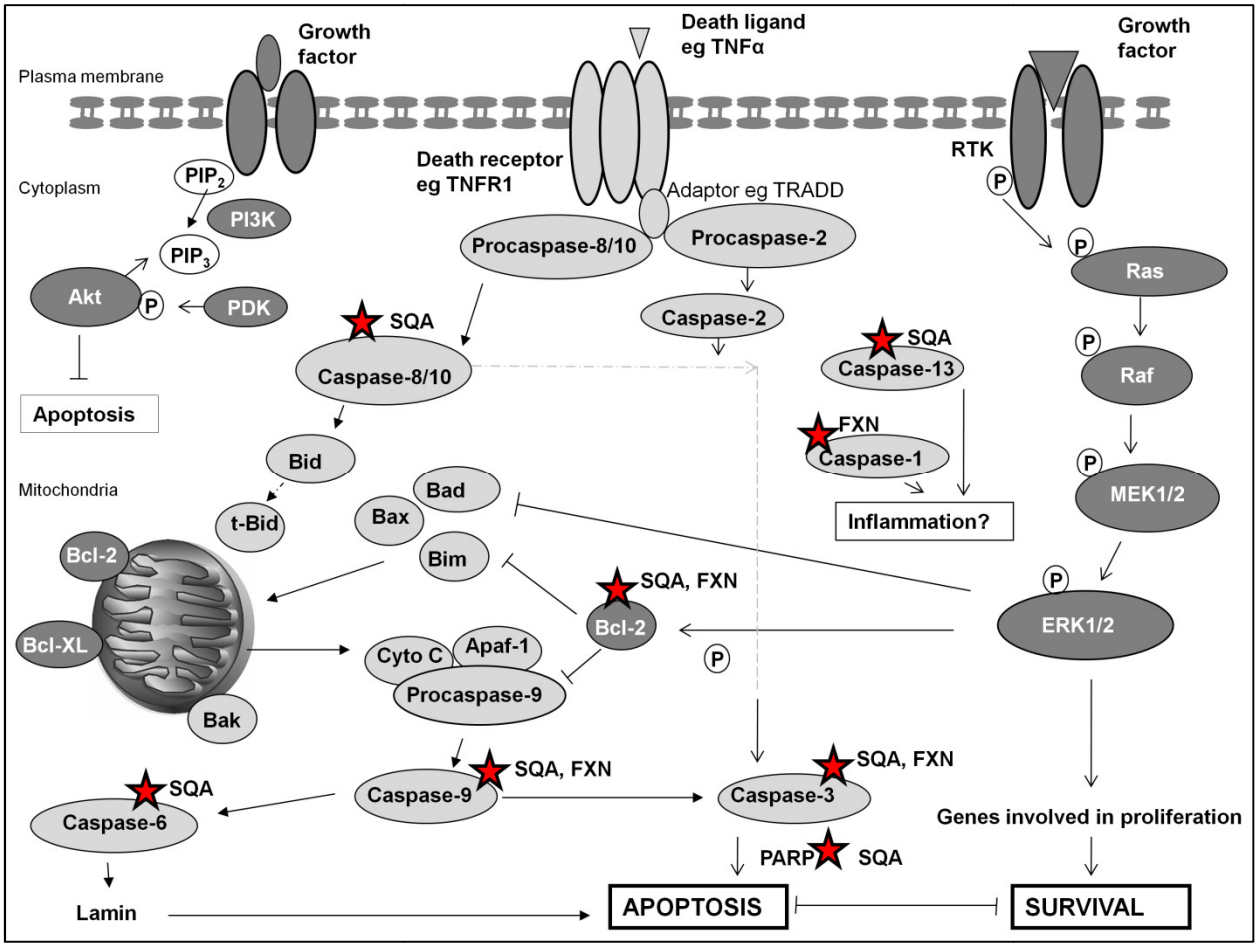


Figure 6.1: Signal transducer pathways and molecules thought to be involved in mediating the cytotoxicity of sargaquinoic acid (SQA) and fucoxanthin (FXN) against MDA-MB-231 breast cancer cells in this study. Proteins which are pro-survival and anti-apoptotic are indicated by dark grey ovals with white text, while pro-apoptotic molecules are.represented by light grey ovals with black text. Red stars indicate signal transducer molecules identified as potential mediators of the cytotoxicity of SQA and/or FXN. Phosphorylation events are indicated by the letter P. (Adapted from Klener Jr. *et al.,* 2006 and McCubrey *et al.,* 2007).

What is immediately apparent from Figure 6.1 is that, while SQA and FXN require a number of the same signal transducer molecules to mediate their cytotoxicity, the specific mechanisms of action are not identical for these compounds. Whereas the cytotoxicity of FXN appears to be mediated exclusively by the intrinsic apoptotic pathway, the mechanism of action of SQA appears to be less specific, involving caspases from both the extrinsic and intrinsic pathway. The latter phenomenon may be explained by the fact that the two apoptotic pathways are interconnected at various points (Klener Jr. *et al.,* 2006) and may point to a common upstream signal transducer. Furthermore, a similar result has previously been reported for SQA in human skin cancer cell lines and, unlike the findings in this study, for FXN in human leukemia cells (Hur *et al.,* 2008; Ishikawa *et al.,* 2008). A further possibility is that the cytotoxicity of SQA and/or

FXN may be mediated by a global regulator of transcription, translation or protein folding which impacts on multiple signal transduction pathways.

The finding that caspase-1 and -13 may be required for the activity of FXN and SQA, respectively, was unexpected, since these caspases are traditionally classified as being involved in the induction of inflammatory cell death programs and not apoptosis (Thornberry *et al.,* 1997). This could indicate a mixed mode of cell death induced by the compounds. Although this study assessed the ability of the hit compounds to induce apoptosis, recent reports have controversially suggested that necrosis and autophagy may be equally useful as a means of removing cancer cells (Fulda and Debatin, 2006). It is further possible that the compounds may be pro-inflammatory, resulting in the release of specific cytokines which, in turn, bind to cell surface receptors in an autocrine signaling cascade to facilitate the observed apoptosis of treated cells (Sporn and Roberts, 1992).

6.3 DRUG DEVELOPMENT: EVOLUTION OF A HIT COMPOUND TO A DRUG

Despite the almost casual use of the term “drug” in research laboratories, the reality is that numerous costly steps are involved between the initial identification of a hit compound in *in vitro* studies and the marketing of a clinically tested drug (Dimasi, 2002). What follows is an overview of the process of drug development and the procedures which would be involved in developing the compounds identified as promising hits in the current study into *bona fide* drugs. It is appreciated, however, that of the 5000 to 10000 compounds that start this process, only one will emerge as an approved drug (Dimasi, 2001). Nonetheless, should a molecule itself fail to reach drug status, there remains the potential for the compound to act as a scaffold for rational drug design (Keseru and Makara, 2006).

Research activities in the pharmaceutical industry involve both drug discovery and drug development phases. In the drug discovery phase, a hit compound displaying favourable pharmacological properties in initial screening experiments is identified. Following this, the lengthy process of drug development begins and includes pre-clinical, clinical and post-clinical phases. In the pre-clinical phase of development, the so-called “New Chemical Entity” will be subject to further rigorous testing (Bleicher *et al.,* 2003; Meadows, 2006). Before large amounts of capital are invested in the development of a hit compound into a drug, potential infringements on intellectual property (IP) rights must be ruled out and the patentability assessed. Both SQA and FXN, analyzed in the current study, have patents associated with their use as part of plant extracts. SQA, as part of an extract of African nutmeg is patented for use as an antioxidant (US patent 737413), while both the extraction procedure to obtain FXN from

Undaria pinnatifida as well as the dietery supplement obtained thereby are patented (US patent 7892580). Since neither of these patents describes the use of the compounds in pure form or as anti-cancer agents, this should not represent a violation of IP regulations. Finally, the feasibility of synthesis of a hit compound must also be carefully assessed (Dimasi, 2001; Meadows, 2006).

Once the above criteria have been satisfied, identification of the precise targets and biophysical testing of the strength and specificity of binding to the target will be carried out (Bleicher *et al.,* 2003; Keseru and Makara, 2006). The biologically active form of the compound and toxicity of potential metabolic derivatives must also be determined (Meadows, 2006; Li and Vederas, 2009). The finding in the current study that the reduced form of SQA was only half as toxic as the original compound against breast cancer cell lines highlights the importance of this step.

A promising lead compound is defined as one which, besides displaying significant biological activity, is highly selective, both membrane permeable and water soluble and is not rapidly metabolized (Dimasi, 2001; Lipinski *et al.,* 2001). Preliminary pharmacological profiling was carried out in this report using Lipinski's Rule of 5 (Ro5) (Lipinski *et al.,* 2001) and it was determined that the hit compound RU007 met all of the criteria included in this assessment. While the lipophillicity of RU004 and SQA, in terms of cLogP values, were considered to be out of the druggable range, the compounds met all the remaining requirements outlined in the Ro5.

Once verified, lead compounds are altered in subsequent "lead optimization" experiments where sequential chemical modifications are performed and the structure activity relationships (SAR) of the derivatives determined to generate analogues which display enhanced potency, improved biophysical properties and fewer potential disadvantages (Dimasi, 2001; Meadows, 2006; Li and Vederas, 2009). Preliminary SAR data was obtained in this study as a result of the presence of structural series in the library of compounds, whose monoterpene backbone was identical but which differed in the number and position of halogen atoms. Though a direct relationship between structure and activity was not identified, it did appear that substitution of a monoterpene with numerous chlorine moieties made for a highly toxic compound, as was the case for RU015.

Testing of an optimized lead product *in vivo* in animal models forms the final pre-clinical step in drug development (Bleicher *et al.,* 2003; Meadows, 2006). The information generated from these studies may be used to apply for "Investigational New Drug (IND)" status from the relevant regulatory bodies and, if granted, the compound will move into the clinical phase of testing. Three phases exist for human testing from Phase I, in which safety is determined usually in healthy subjects, through Phase II, where the safety and efficacy are assessed in a

small number of sick patients and finally to Phase III, the large scale clinical trial phase (Meadows, 2006). Should the IND perform well in these clinical trials, the final phase in drug development would involve the registration of the drug with the appropriate authorities in order to obtain a license to market the drug (Dimasi, 2001; Meadows, 2006). In the case of FXN, the fact that the compound is already marketed as a dietary supplement (Peng *et al.,* 2011) could indicate that at least some of the safety testing and licensing for FXN has already been carried out. This could represent significant savings in terms of time and expenditure in the marketing of FXN as an anti-cancer drug.

6.4 IMPORTANCE OF CANCER STEM CELL-SPECIFIC THERAPIES

The hypothesis that cancers arise from and are maintained by cancer stem cells (CSCs), which are capable of asymmetric cell division and are resistant to traditional chemotherapy drugs, has caused a major shift in focus in the development of new therapies to treat the disease (Lawson, 2009; Massard *et al.,* 2006). It is now thought that effective treatment and prevention of recurrence and metastasis requires the removal of the CSC component of a tumour (Massard *et al.,* 2006; Marotta and Polyak, 2008). It has been proposed that an effective cure for cancer will require two steps: first the rapidly proliferating bulk tumour cells must be removed and, second, elimination or differentiation therapies must remove the CSC subpopulation (Massard *et al.,* 2006). Both of these steps must occur without causing generalized toxicity to healthy cells (Widakowich *et al.,* 2007). There is to date no report of a compound able to specifically target BCSCs while leaving both breast cancer and non-cancerous breast cells unaffected.

In the current study, the novel algal monoterpene stereoisomers RU017 and RU018 were demonstrated to inhibit MCF-7 mammosphere formation *in vitro* despite being non-toxic to both breast cancer and healthy breast epithelial cell lines. Since the MCF-7 mammospheres were shown to be enriched for BCSC-like cells (Jason Sterrenberg, unpublished data), the results in this study indicate potential specific anti-BCSC activity for RU017/18. The potential of an agent capable of targeting BCSCs is that treatment with such a compound, in conjunction with a drug displaying specific activity against cancer cells and which is not toxic to healthy tissues, could represent the two-step cure proposed by Massard *et al*. (2006).

6.5 OPPORTUNITIES FOR FUTURE STUDY

The novelty and structural simplicity of the algal hit compounds analyzed in this body of work, together with their promising biological activity, justify further biochemical characterization of these compounds. Despite the fact that this body of work represents one of the few in-depth studies into the biological activity of novel natural products, there remain numerous opportunities for further analysis of the algal hit compounds. The induction of apoptosis by RU004, RU007, SQA and FXN in breast cancer cell lines could be confirmed using Annexin V staining, TUNEL tests and DNA fragmentation analyses. In addition, the role of specific caspases identified as being involved in mediating the cytotoxicity of SQA and FXN could be verified by Western analyses as performed for caspase-9 in this study. While the levels of the anti-apoptotic molecule Bcl-2 were found to be decreased by both SQA and FXN, it would be interesting to assess the levels of other anti-apoptotic regulators such as Bcl-xL, as well as the pro-apoptotic proteins Bad, Bid, Bim, Bax and Bak. Furthermore, the effect of SQA, FXN and the other hit algal compounds on the Raf/MEK/ERK and Akt/PI3K/PTEN survival pathways are unknown.

The putative anti-BCSC activity of RU017 and RU018 as assessed by mammosphere assay represent preliminary findings and certainly requires validation and further characterization. In particular, the effect of treatment with RU017/18 on the proportion of BCSC in breast cancer cell lines could be determined using flow cytometry to detect specific stem cell and CSC markers. The levels and activation state of key signal transducer molecules forming part of the self-renewal, survival and apoptotic pathways in treated mammosphere-derived cells could also be investigated by means of Western analyses.

Further studies into the mechanism of action of the novel compounds have been hampered by the availability of these algal metabolites. In particular, in-depth investigation into the molecular mechanisms of action of the novel polyhalogenated monoterpenes RU004 and RU007 was not possible in this study due to challenges in sourcing the compounds. Apart from the fact that collection of the algal samples is expensive and time-consuming, the secondary metabolites of interest are not always present, despite the alga being collected from the same area (Li and Vederas, 2009). Seasonal variations in algal secondary metabolites have previously been reported. For example, attempts to re-isolate a series of halomon-related compounds from the red algae *Portiera hornemannii*, collected from a specific location in the Phillipines, proved unsuccessful just one month after the initial collection and purification of the compounds (Fuller *et al.,* 1994). As with all natural products, while it may be appropriate for initial screening studies to extract the compounds from the seaweed, the conservation of the marine ecosystem must also be considered should large amounts of the seaweed be required (Li and Vederas, 2009). Of the marine algal metabolites analyzed in this study, FXN was the only

commercially available compound. FXN is marketed as a dietery supplement to aid weight loss and as a natural anti-oxidant (Peng *et al.,* 2011). Taken together, these factors highlight the need to develop synthetic strategies to produce priority compounds to enable further characterization or hit to lead refinement (Li and Vederas, 2009).

Synthetic strategies for the mass production of a compound may be either chemical or biological in nature. In the former, synthetic chemists make use of established reagents or structurally similar precursor molecules in multiple non-enzymatic steps to generate a particular compound, while the latter approach involves genetic engineering to allow for the heterologous expression of those enzymes involved in producing the compounds of interest in the alga (Li and Vederas, 2006). While both chemical and biological synthetic methods have been developed for FXN (Yamano and Ito, 1994; Eonseon et al., 2002), no such techniques exist for either SQA or the novel monoterpenes analysed in this study.

CONCLUDING REMARKS

The development process from hit to lead to drug outlined in section 6.3 above takes on average 10 to 15 years and has been estimated to cost anything from $50 million to over $2 billion (Dimasi, 2001; Dimasi *et al.,* 2003; Adams and Brantner, 2006). Since the majority of this cost is incurred in the later stages of development, increasing emphasis is being placed on pre-clinical studies and the comprehensive characterization of hit and lead compounds *in vitro*, to avoid the investment of large amounts of capital on a compound which ultimately fails in clinical trials (Dimasi, 2002; Dimasi *et al.,* 2003). The current study is an example of such increasingly important *in vitro* investigations and could serve as a useful starting point for further pre-clinical analyses.

APPENDIX

APPENDIX FIGURES

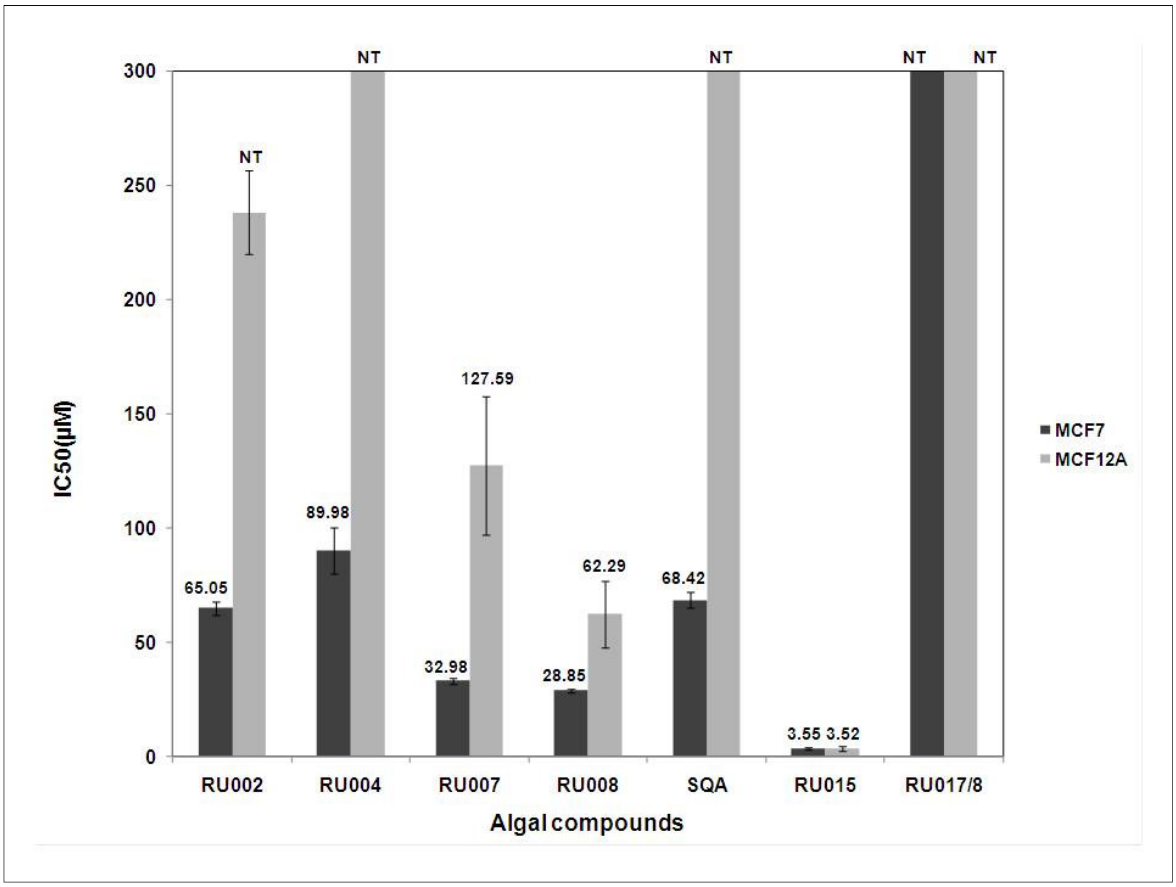


Figure A1: Differential toxicity screening of selected marine algal compounds in MCF7 cancer and MCF12A non-cancerous breast cells (Lawson, 2009; MSc thesis). Both cell lines were treated with a range of concentrations of algal compound for 96 h and their viability assessed by MTT assay. Sargaquinoic acid is represented by SQA. Values quoted are the half maximal-inhibitory concentrations (IC_{50}) of each compound in μM. Concentrations greater than 200 μM are an extrapolation and considered non-toxic (NT). Error bars indicate the standard error of the mean where n=3.

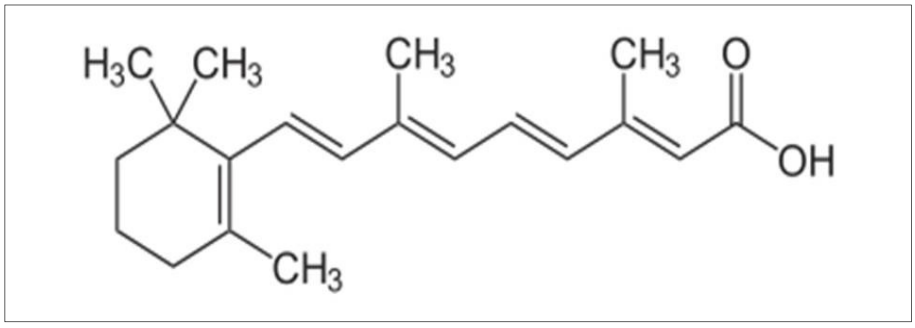


Figure A2 :Chemical structure of the vitamin A metabolite, retinoic acid

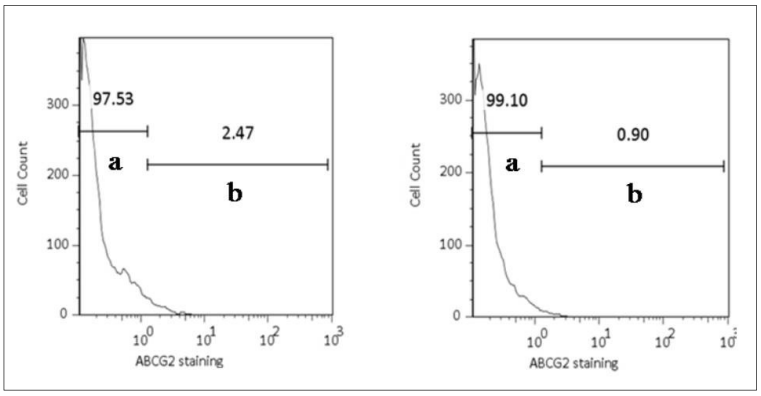
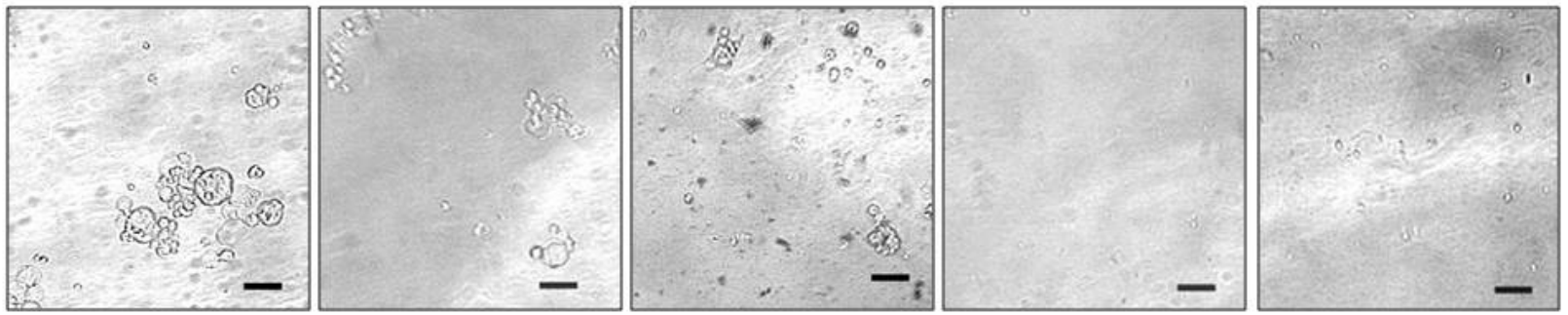


Figure A3: Flow cytometric analysis of the effect of a racemic mixture of the novel halogenated monoterpenes RU017 and RU018 on the proportion of the MCF-7 side population using ABCG2 staining (Lawson, 2009; MSc thesis). Cells were treated with either DMSO vehicle control (A) or 300 µM of RU017/RU018 (B) for 96 hours, stained for the drug transporter ABCG2 and analysed by flow cytometry. Gating of the bulk cells (a) and "side population" cells (b) was carried out on the DMSO control and copied to the treated sample.

A)


DMSO
70 µM SQA
10 µM FXN
300 µM RU017
300 µM RU018

B)

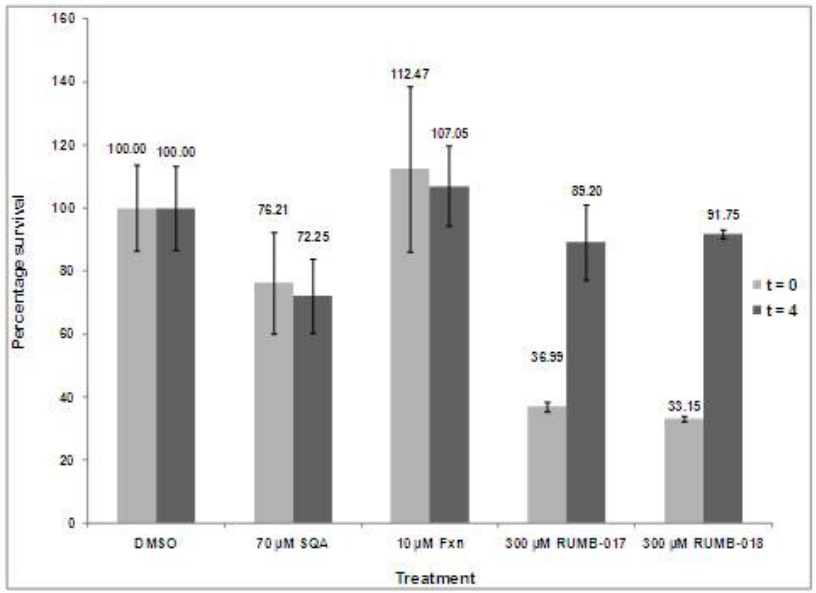

Percentage survival
160
140
120
100
80
60
40
20
0
100.00
100.00
76.21
72.25
112.47
107.05
36.99
89.20
33.15
91.75
t = 0
t = 4
DMSO
70 µM SQA
10 µM Fxn
300 µM RUMB-017
300 µM RUMB-018
Treatment

Figure A4: Screening of marine algal compounds for the ability to inhibit the formation and development of MCF-7 tumourspheres when seeded at 3000 cells per well. MCF-7 breast cancer cells were seeded as a single cell suspension into anchorage-independent serum-free culture conditions containing either the DMSO vehicle control (0.6 % v/v) or 70 µM sargaquinoic acid (SQA), 10 µM fucoxanthin (FXN), or 300 µM of either of the halogenated monoterpene stereoisomers RU018 or RU018. The latter concentrations were derived from the IC_{50} values in MDA-MB-231 breast cancer cells grown in anchorage-dependent conditions in the case of SQA and FXN. For RU017 and RU018, the compounds were non-toxic to either MCF-7 or MDA-MB-231 cells at 300 µM in adherant conditions, but were shown to decrease the $ABCG2^{+}$ cancer stem cell-enriched "side population" in a previous study. Cells were photographed on Day 6 (A). Images were captured under a light microscope at 100x magnification using a Nikon Coolpix 990 camera. Images were set to scale using ImageJ (NIH freeware). Scale bars represent 0.1 mm. B) The viability of treated mammospheres on Day 8 as assessed by Wst-1 assay. Error bars represent the standard error of the mean where n = 5.

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