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Anti-tumour Actions of ω-3 Epoxyfatty Acid Analogues in Breast Cancer Cells

By

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A thesis submitted in the fulfilment of the requirement for the degree of Doctor of Philosophy

Discipline of Pharmacology
School of Medical Sciences
and Bosch Institute
The University of Sydney
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Declaration

I, Herryawan Ryadi Eziwar Dyari declare that the work presented in this thesis is original except as acknowledged in the text. I hereby declare that I have not submitted this material, either in full or part, for a degree at this or any other institution.

Signature: ____________________________  Date: 30/08/2014
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### Abbreviations

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<th>Description</th>
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</thead>
<tbody>
<tr>
<td>AA</td>
<td>Arachidonic acid</td>
</tr>
<tr>
<td>AIF</td>
<td>Apoptosis-inducing factor</td>
</tr>
<tr>
<td>ASK1</td>
<td>Apoptosis signal-regulating kinase-1</td>
</tr>
<tr>
<td>AUDA</td>
<td>12-(3-adamantan-1-yl-ureido)dodecanoic acid</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>B-cell lymphoma-2</td>
</tr>
<tr>
<td>BH3</td>
<td>Bcl-2 homology 3</td>
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<tr>
<td>CARD</td>
<td>Caspase-recruitment domain</td>
</tr>
<tr>
<td>CDK</td>
<td>Cyclin-dependent kinase</td>
</tr>
<tr>
<td>COX</td>
<td>Cyclooxygenase</td>
</tr>
<tr>
<td>CSC</td>
<td>Cancer stem cells</td>
</tr>
<tr>
<td>CTRL</td>
<td>Control</td>
</tr>
<tr>
<td>CYP</td>
<td>Cytochromes P450</td>
</tr>
<tr>
<td>DHA</td>
<td>Docosahexaenoic acid</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s Medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DN</td>
<td>Dominant-negative</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>EPA</td>
<td>Eicosapentaenoic acid</td>
</tr>
<tr>
<td>FADD</td>
<td>Fas-associated death domain</td>
</tr>
<tr>
<td>HB-EGF</td>
<td>Heparin-binding EGF</td>
</tr>
<tr>
<td>HETE</td>
<td>Hydroxyeicosatetraenoic acid</td>
</tr>
<tr>
<td>HIF-1</td>
<td>Hypoxia-inducible factor 1</td>
</tr>
<tr>
<td>HPETE</td>
<td>Hydroperoxides</td>
</tr>
<tr>
<td>IAP</td>
<td>Inhibitor of apoptosis</td>
</tr>
<tr>
<td>IARC</td>
<td>International Agency for Research on Cancer</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>LO</td>
<td>Lipoxygenase</td>
</tr>
<tr>
<td>M</td>
<td>Mitosis</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MKK/MP2K</td>
<td>MAPK kinase</td>
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</table>

XX
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
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<tbody>
<tr>
<td>MP3K</td>
<td>MAPK kinase kinases</td>
</tr>
<tr>
<td>MTT</td>
<td>(3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>MUFA</td>
<td>Monounsaturated fatty acid</td>
</tr>
<tr>
<td>NRG1</td>
<td>Neuregulin 1</td>
</tr>
<tr>
<td>PARP</td>
<td>Poly (ADP-ribose) polymerase</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PG</td>
<td>Prostaglandin</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide-3-kinase</td>
</tr>
<tr>
<td>PLA₂</td>
<td>Phospholipase A₂</td>
</tr>
<tr>
<td>pRB</td>
<td>Retinoblastoma protein</td>
</tr>
<tr>
<td>PTK</td>
<td>Protein-tyrosine kinase</td>
</tr>
<tr>
<td>PUFA</td>
<td>Polyunsaturated fatty acid</td>
</tr>
<tr>
<td>RIP</td>
<td>Receptor-interacting protein</td>
</tr>
<tr>
<td>RITA</td>
<td>Reactivation of p53 and induction of tumour cell apoptosis</td>
</tr>
<tr>
<td>RNase A</td>
<td>Ribonuclease A</td>
</tr>
<tr>
<td>SCC</td>
<td>Squamous cell carcinoma</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
</tr>
<tr>
<td>Smac</td>
<td>Second mitochondria-derived activator of caspases</td>
</tr>
<tr>
<td>tBid</td>
<td>Truncated Bid</td>
</tr>
<tr>
<td>TBS-T</td>
<td>Tris buffered saline-Tween 20</td>
</tr>
<tr>
<td>TGF-α</td>
<td>Transforming growth factor-α</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>TNF-R1</td>
<td>Tumour necrosis factor receptor 1</td>
</tr>
<tr>
<td>TRADD</td>
<td>The TNF-R1-associated death domain</td>
</tr>
<tr>
<td>TRAF2</td>
<td>TNF-R1-associated factor 2</td>
</tr>
<tr>
<td>TRAIL</td>
<td>TNF-related apoptosis-inducing ligand</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
</tbody>
</table>
Abstract

Epidemiological studies suggest an inverse relationship between cancer incidence and the intake of polyunsaturated fatty acids. ω-3 polyunsaturated fatty acids intake decrease cancer risk while the intake of ω-6 counterparts may increase cancer risk. Observations from animal studies have produced similar findings to human epidemiological studies.

A significant recent finding was that the unique ω-3-17,18-epoxide of eicosapentaenoic acid decreased cell growth and activated apoptosis. Epoxide formed at the unique ω-3 double bond in eicosapentaenoic acid, but not the regioisomeric epoxides, decreases the rate of cell proliferation and increases apoptosis. An important cellular target for ω-3 17,18-epoxy-EPA is cyclin D1; its down-regulation is mediated by activation of the p38 MAPK. Because the epoxide isomers activated cell growth, a series of ω-3 epoxides of long chain saturated fatty acids (C20-C22) were synthesized for this project and evaluated for the ability to decrease the viability of breast cancer cells. As epoxides are prone to degradation and have low stability in vivo, stable bioisosteric urea analogues (C20-C22) were also synthesized and tested as inhibitors of breast cancer proliferation. Studies were conducted in four breast cancer cell lines with different characteristics.

A pronounced decrease in the viability of the highly aggressive epithelial adenocarcinoma-derived MDA-MB-231 cell line occurred after treatment with the epoxides. Somewhat less aggressive cell lines, including another epithelial adenocarcinoma-derived line (MDA-MB-468), the estrogen receptor-positive MCF-7 and the multiple hormone receptor-positive T47D cells, were less
sensitive towards these molecules. The C20 epoxide was most effective, followed by the C21 analogue, while the C22 epoxide was least active. Findings from flow cytometry were consistent with these observations. In MDA-MB-231 cells the proportion of cells in G0/G1 phase increased, especially after C20 epoxide treatment, while those in later phases (S and G2/M) decreased. The expression of cyclin D1 and cyclin E proteins was also decreased, which is consistent with the apparent pause in cell cycle progression and the failure of treated cells to complete the cell cycle. Decreased mitosis appears to be a significant contributor to the loss of MDA-MB-231 cell viability produced by the epoxides. In contrast, the C20-C22 ureas were ineffective in four different breast cancer cell lines.

The pan-caspase inhibitor, z-VAD-fmk, effectively reversed the decrease in cell viability of MDA-MB-231 cells elicited by treatment with the epoxides. In contrast, co-treatment of MDA-MB-231 cells with the necroptosis inhibitor, necrostatin-1, and the autophagy inhibitors 3-methyladenine and chloroquine did not alter the effects of the epoxides on cell viability. Together, these observations suggest that the C20-C22 epoxides induce apoptosis in MDA-MB-231 cells, but not necroptosis or autophagy. It was also found that epoxide treatment markedly increased the population of MDA-MB-231 cells showing apoptotic characteristics such as cleavage of the DNA repair enzyme poly (ADP-ribose) polymerase, increase in cells in sub-G1 phase and increased population of cells that were stained with annexin V.

The synthetic C20 ω-3 epoxide activates apoptosis in MDA-MB-231 human breast cancer cells by the dual mechanisms of death receptor activation and mitochondrial targeting. The treatment of MDA-MB 231 cells with the C20 ω-3 epoxide triggered mitochondrial disruption, cytochrome c release and
caspase activation. Mitochondrial disruption is due to the increased expression of the pro-apoptotic Bcl-2 proteins Bax and Bak and decreased expression of the pro-survival Bcl-2 and Bcl-XL. In gene silencing experiments MKK4 and MKK7, but not the alternate MAPK kinases MKK3 and MKK6, contributed to C20 ω-3 epoxide-dependent caspase 3 cleavage. Thus, the involvement of both MKK4 and MKK7 suggests that JNK is the predominant MAPK that regulates the C20 ω-3 epoxide apoptotic pathway. Expression of the phosphorylated form of the MAPK kinase kinase ASK1 increased in C20 ω-3 epoxide-treated cells and implicated death receptors in the apoptotic mechanism of the C20 epoxide. Caspase 3 activation was inhibited by knockdown of the prototypic death receptor TNF-R1 and the adaptor proteins TRADD and TRAF2. However, knockdown of Fas, DR4 and DR5, and an alternate adaptor protein FADD, did not alter the increase in caspase 3 cleavage produced by the C20 ω-3 epoxide in MDA-MB-231 cells.

Findings in the present project suggest that the C20 ω-3 epoxide selectively activates TNF-R1, recruits TRADD and TRAF-2 and activates downstream ASK1-MKK4/MKK7-JNK/p38 MAPK signalling to elicit the killing of breast cancer cells. Activation of Bid was an important event in C20 ω-3 epoxide-mediated apoptosis and JNK activation was important in Bid truncation.

The present ω-3 epoxide analogues, but not the urea isosteres, have promising properties as potential anti-tumour agents. Further research is warranted for the future application of these ω-3 epoxide analogues in cancer chemotherapy.
**Publications**


HRE Dyari, J Dwyer, SE Allison, T Rawling and M Murray. Cell killing elicited by the synthetic ω-3 epoxy-eicosanoic acid is mediated by the tumour necrosis factor-a receptor and downstream ASK1. In preparation.

Presentations

T Rawling, P Cui, HRE Dyari and M Murray. Identification of a novel, omega-3 fatty acid derived drug scaffold for the development of new breast cancer chemotherapeutics. RACI Biomolecular Division Conference, Torquay, VIC, December 4-8, 2011.


1. Literature review

“While there are several chronic diseases more destructive to life than cancer, none is more feared”

(Mayo & Hendricks, 1926)

1.1. Cancer: early evidence

According to the Oxford Dictionary, cancer is a disease caused by uncontrolled division of abnormal cells (Oxford Dictionaries, 2014). The Merriam-Webster Dictionary has extended the definition to ‘a serious disease caused by cells that are not normal and that can spread to one or many parts of the body’ (Merriam-Webster Dictionary, 2014).

Evidence of cancer has been found in fossilized bone tumours and Egyptian mummies, and has been described in ancient manuscripts (Shimkin, 1977). The oldest recorded description of cancer dates back to around 3000 BC in an ancient Egyptian textbook on trauma surgery.

1.2. Origin of cancer

The multistep development of cancer involves the acquisition of new cellular capabilities. Known as the six hallmarks of cancer, these capabilities include growth factor-independent growth, low response to growth-inhibition signals, inhibition of apoptosis, uncontrolled replication, sustained angiogenesis, and acquisition of invasion and metastatic characteristics (Hanahan & Weinberg, 2011). Mutations in certain genes that confer selective growth advantage on cells
lead to formation of cancers. The Cancer Genome Project database has identified more than 350 genes that can cause cancer if mutated (Greenman, Stephens, Smith, et al., 2007). Mutations that occur in cancer development are termed somatic mutations (Stratton, Campbell & Futreal, 2009). Such mutations occur during DNA replication, and include nucleotide base substitutions, insertions and deletions, rearrangements, modifications of copy number or epigenetic changes (Pleasance, Cheetham, Stephens, et al., 2010). Exogenous or endogenous mutagens also cause somatic mutation (Greenman, Stephens, Smith, et al., 2007). Examples include aflatoxins and ultraviolet light (Olivier, Hussain, Caron de Fromentel, et al., 2004), and viruses such as human papilloma virus, Epstein Barr virus and hepatitis B virus among others (International Agency for Research on Cancer, 1995, 1994). Somatic mutation in cancer cells can be divided into two types: driver mutations and passenger mutations. Only driver mutations are causally implicated in cancer development (Pleasance, Cheetham, Stephens, et al., 2010).

The cell-of-origin is the first cell to acquire somatic mutation and is important in cancer initiation. Once a tumour is initiated, cancer stem cells are responsible for self-propagation (Figure 1-1) (Visvader, 2011). Stem cells are cells that can undergo self-renewal and generate mature cells through differentiation (Reya, Morrison, Clarke, et al., 2001). Similarly, cancer stem cells have the capacity for self-renewal and division to sustain malignant growth (Bjerkvig, Tysnes, Aboody, et al., 2005). Cancer stem cells are multipotent cells that give rise to progenitors and differentiated cells causing tumour heterogeneity, and whose migration results in metastasis (Mathonnet, Perraud, Christou, et al., 2014). Although only a small proportion of cells within a tumour
(<2.5%) are endowed with tumour propagation, cancer stem cells have the ability to reproduce the parental tumour *in vivo* (Mathonnet, Perraud, Christou, *et al.*, 2014). The accumulation of genetic and/or epigenetic damage in progenitor cells leads to the acquisition of increased migratory behaviour as cells undergo the epithelial-mesenchymal transition; this is required for migration to distant metastatic sites (Mimeault, Hauke, Mehta, *et al.*, 2007).

![Diagram of cancer stem cell process](image)

**Figure 1-1:** Common progenitor cells can become the cell-of-origin by acquisition of mutations. Cancer stem cells (CSCs) are capable of self-renewal and contribute to tumour propagation (Visvader, 2011).

Phenotypic and functional heterogeneity occurs in tumours. Two major mechanisms are likely: different genetic or epigenetic mutations resulting in different tumour phenotypes (Figure 1-2a), and different tumour subtypes
that arise from distinct cells within the tissue and that serve as the originating cell (Figure 1-2b) (Visvader, 2011). The interaction between tumour cells and other cells in the local tumour microenvironment is important for malignant growth (Tlsty & Coussens, 2006). Certain oncogenes and tumour-suppressor genes, especially the prosurvival phosphoinositide-3-kinase (PI3-kinase)/Akt cascade, RAS and p53 genes, are frequently mutated in diverse cancer types (Visvader, 2011).
Figure 1-2: A| The mutation model suggests that alternate mutations produce different tumour phenotypes. B| The cell-of-origin model suggests that identical mutations could occur in different cell populations and produce different tumour phenotypes (Visvader, 2011).

Metastasis (Figure 1-3) is the spread of cells from the primary neoplasm to distant organs (Fidler, 2003) and can be viewed as a process in which tumour cells overcome physiological barriers to escape their local microenvironment and relocate to distant microenvironments (Nguyen & Massagué, 2007). Most mortality from cancer is related to metastasis (Fidler, 2003). The development of treatments for metastatic disease has been slow and is limited to those used for primary tumours or to palliative care (Sleeman, Gomes & Higginson, 2012). Metastatic cancer stem cells are thought to be involved in the propagation of cancer at secondary locations (Li, Tiede, Massagué, et al., 2007). Heterogeneity of
cancer cells has been the major obstacle for the development of anti-metastatic treatments (Fidler, 2003). Heterogeneity is due to the instability of cancer genomes as a result of accumulating DNA mutations, chromosomal rearrangements and epigenetic alterations; this selects for populations that have traits that facilitate invasion of distant microenvironments (Gupta & Massagué, 2006). It has been found that highly metastatic clones have a higher rate of genetic mutability than non-metastatic clones from the same tumour (Nowell, 1976).

**Figure 1-3: Overview of metastasis.** A| Cellular transformation and tumour growth. Tumours obtain nutrients through diffusion. B| Angiogenesis forms capillaries within the surrounding tissue to provide nutrients for proliferation. C| Tumour cells penetrate lymphatic vessels or capillaries which are a common route for entry into the circulation. D| Tumour cells are detached and adhere to
capillaries in distant organs. E| Extravasation, which resembles invasion. F| Proliferation within distant organs and angiogenesis support growth (Fidler, 2003).

The formation of new vasculature, or angiogenesis, is an essential process in tumour development, progression, and metastasis (Mathonnet, Perraud, Christou, et al., 2014). In normal adult tissues, angiogenesis is transiently activated. In tumours, however, angiogenesis promotes new blood vessel formation in response to the need for nutrients and oxygen by the tumour and for the elimination of metabolic waste (Mathonnet, Perraud, Christou, et al., 2014). During progression, angiogenesis is activated causing quiescent vasculature to sprout new vessels and sustain expanding neoplastic growth (Hanahan & Weinberg, 2011). The resultant blood vessels, however, in the tumour are abnormal (Nagy, Chang, Shih, et al., 2010).

1.3. Cancer as a public health problem

According to GLOBOCAN 2012 project of International Agency for Research on Cancer (IARC)/World Health Organization (WHO) (International Agency for Research on Cancer, 2012), cancer is among the leading causes of death worldwide. About 8.2 million deaths in 2012 were attributed to cancer, while an estimated 32.6 million others live with cancer. By 2030 cancer deaths are projected to rise to an estimated 13.1 million deaths per year. WHO has suggested that there will be a higher mortality rate in relation to incidence in less developed countries, most likely due to the lack of early detection, inadequate diagnosis and less treatment facilities (Fig. 1-4) (World Health Organization,
North America, Europe and Australia have the highest incidence of breast cancer compared to other parts of the world (Fig. 1-5) (World Health Organization, 2014).

**Figure 1-4:** Estimated age-standardized rates of cancer incidence and their mortality rates worldwide (per 100,000 individuals). Rates are higher in more developed regions than in less developed regions. Mortality rates are
similar in all regions (http://globocan.iarc.fr/Pages/fact_sheets_cancer.aspx) (International Agency for Research on Cancer, 2012).

Figure 1-5: Estimated age-standardized incidence of breast cancer and mortality rates worldwide (per 100,000 individuals). Although incidence is higher in developed countries, the mortality to incidence ratio is greater in less developed countries (http://globocan.iarc.fr/Pages/fact_sheets_cancer.aspx) (International Agency for Research on Cancer, 2012).
Breast cancer is the second most common cancer in the world behind lung cancer and is the most frequent among women. It has been estimated that 1.67 million new cases were reported in 2012 (International Agency for Research on Cancer, 2012). The National Cancer Institute has estimated that there will be over 200,000 new cases and 40,000 deaths from breast cancer in 2014 in the United States (Howlader, Noone, Krapcho, et al., 2014). This places breast cancer second behind prostate cancer and accounts for 14% of all new cancers in that country (Figure 1.6). When the breast cancer has metastasized to distant locations, patient survival decreases to ~25%, compared to 98.5% when the cancer is confined (Howlader, Noone, Krapcho, et al., 2014).

<table>
<thead>
<tr>
<th>Common Types of Cancer</th>
<th>Estimated New Cases 2014</th>
<th>Estimated Deaths 2014</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Prostate Cancer</td>
<td>233,000</td>
<td>29,480</td>
</tr>
<tr>
<td>2. Breast Cancer (Female)</td>
<td>232,670</td>
<td>40,000</td>
</tr>
<tr>
<td>3. Lung and Bronchus Cancer</td>
<td>224,210</td>
<td>159,260</td>
</tr>
<tr>
<td>4. Colon and Rectum Cancer</td>
<td>136,830</td>
<td>50,310</td>
</tr>
<tr>
<td>5. Melanoma of the Skin</td>
<td>76,100</td>
<td>9,710</td>
</tr>
<tr>
<td>6. Bladder Cancer</td>
<td>74,690</td>
<td>15,580</td>
</tr>
<tr>
<td>7. Non-Hodgkin Lymphoma</td>
<td>70,800</td>
<td>18,990</td>
</tr>
<tr>
<td>8. Kidney and Renal Pelvis Cancer</td>
<td>63,920</td>
<td>13,860</td>
</tr>
<tr>
<td>9. Thyroid Cancer</td>
<td>62,980</td>
<td>1,890</td>
</tr>
<tr>
<td>10. Endometrial Cancer</td>
<td>52,630</td>
<td>8,590</td>
</tr>
</tbody>
</table>

**Figure 1-6: Comparison of common types of cancer: incidence and mortality in United States (estimated age-standardized).** (Howlader, Noone, Krapcho, et al., 2014).
Similarly in Australia, breast cancer is also the commonest cancer in women (Figure 1-7) and is the second leading cause of cancer deaths after lung cancer (Figure 1-8). Increasing awareness and screening has led to early detection. Although early detection has increased the incidence of breast cancer, the mortality rate has decreased due to increased survival (Figure 1-9). Women diagnosed with smaller tumours (≤10mm) have a five-year relative survival of 98%, while women with larger tumours (≥30mm) have five-year relative survival of 73%.

Figure 1-7: In Australia, breast cancer is the most diagnosed cancer in women and significantly higher than the next most common (bowel cancer). (Cancer Australia, 2012)
Figure 1-8: Mortality from breast cancer is the second highest in Australian women after lung cancer. (Cancer Australia, 2012).

Figure 1-9: Between 1982-2007, the breast cancer incidence increased but mortality decreased. (Cancer Australia, 2012).
1.4. Breast cancer

Breast cancer is a major cause of cancer death in women in the developed and developing world. According to WHO, the incidence of breast cancer is increasing in developing countries due to increased life expectancy, increased urbanization and adoption of western lifestyles (World Health Organization, 2014).

In the breast, tumours can be benign or malignant. Fibrosis, cysts, fibroadenomas and intraductal papilloma are all types of benign breast tumours (American Cancer Society, 2013). Ductal carcinoma \textit{in situ} is a malignant tumour that is initiated as clonal proliferation of cells in the basement membrane-bound structure of the breast but is minimally invasive. In contrast, invasive ductal and invasive lobular carcinomas are the main forms of invasive breast cancer (Pinder, 2010). Invasive ductal carcinoma represents up to ~80% of all invasive breast cancer cases worldwide (Zhao, Langerød, Ji, \textit{et al.}, 2004). Invasive ductal and invasive lobular carcinoma show similar increases in expression profile of proliferative genes, such as the cell-cycle regulator cyclin D1, and pro-survival genes, such as the anti-apoptotic gene bcl-2 (Geradts & Ingram, 2000; Coradini, Pellizzaro, Veneroni, \textit{et al.}, 2002).

Metastatic primary breast cancers frequently produce tumours in bone, liver, lung and brain (Chambers, Groom & MacDonald, 2002). Microarray studies suggest that the gene-expression profile of metastatic and localized breast tumours differ (van ’t Veer, Dai, van de Vijver, \textit{et al.}, 2002). An integrated model of breast cancer metastasis has been proposed based on the expression of prognostic markers, including matrix degrading genes (Figure 1-10) (van ’t Veer, Dai, van de Vijver, \textit{et al.}, 2002). The potential of breast cancer cells to metastasize to the brain is increased by over-expression of cyclooxygenase 2 (COX-2),
epidermal growth factor receptor (EGFR) ligands and α2,6-sialyltransferase (Bos, Zhang, Nadal, et al., 2009).

Figure 1-10: The integrated model of breast cancer metastasis in which mutations precede the development of breast cancer stem cells. Stem cells give rise to ‘poor-prognosis’ tumours that degrade the extracellular matrix (Umeda, Eguchi, Okino, et al., 1997). Stem cell variants can also express genes for homing to tissues such as bone or lung. At the distant site, disseminated breast cancer stem cells may induce a similar stromal response as in the primary tumour. In well-differentiated progenitor cells, some mutations may have a better prognosis (van ‘t Veer, Dai, van de Vijver, et al., 2002).

The most common hereditary breast cancers are due to mutations in the tumour suppressor genes BRCA1 and BRCA2 (King, Marks & Mandell, 2003). Individuals carrying such mutations have a 40-80% greater risk of developing
breast cancer (Claus, Risch & Thompson, 1991; Easton, Ford & Bishop, 1995; Schubert, Lee, Mefford, et al., 1997; Fackenthal & Olopade, 2007). Mutation in these genes also increases the risk of developing ovarian cancer (Miki, Swensen, Shattuck-Eidens, et al., 1994; Fackenthal & Olopade, 2007). However, there are other gene mutations that are also associated with inherited breast cancers. The ATM gene is involved in DNA repair, and inheritance of a mutated copy has been linked to an increased breast cancer risk (Painter & Young, 1980). The TP53 gene encodes another tumour suppressor p53, which prevents the growth of cells containing damaged DNA. Inherited p53 mutations cause Li-Fraumeni syndrome and increase the risk of numerous cancers, including breast cancer, leukaemia, brain cancer and sarcomas. Other genes that have also been implicated include CHEK2 (Vahteristo, Tamminen & Karvinen, 2001) and PTEN (Li, 1997), CDH1 (Oka, Shiozaki, Kobayashi, et al., 1993) and STK11 (Jenne, Reumann, Nezu, et al., 1998).

1.5. Breast cancer treatment

1.5.1. Surgery and radiation therapy

Human breast tumours have a diverse natural history and responsiveness to treatments (Perou, Sørlie, Eisen, et al., 2000). In the past, localized breast cancer was treated with radical mastectomy and axillary lymph node dissection (Moulder & Hortobagyi, 2008). Present strategies consist of surgical resection to remove the primary tumour and pathologic evaluation of ipsilateral axillary nodal basin (Moulder & Hortobagyi, 2008). To further reduce the risk of metastasis, radiation therapy to the remaining breast tissue and draining of
lymph nodes after surgery is added to the treatment regime (Moulder & Hortobagyi, 2008). Systemic adjuvant therapy is also recommended for patients with higher risk of metastasis (Early Breast Cancer Trialists’ Collaborative Group (EBCTCG), 2005; Moulder & Hortobagyi, 2008). These include chemotherapy, hormonal therapy and biologic therapy given before (neoadjuvant) or after (adjuvant) surgery.

1.5.2. Drugs used in the treatment of breast cancer

The era of chemotherapy began in the 1940s with the introduction of nitrogen mustards and antifolate drugs (Chabner & Roberts Jr, 2005). Chemotherapy is used widely to palliate symptoms in patients with advanced breast cancer (Moulder & Hortobagyi, 2008).

Important chemotherapeutic agents used currently in the treatment of breast cancer include the taxanes, anthracyclines, estrogen-receptor antagonists and aromatic inhibitors, and others; drugs may either be used as single agents or in combination therapies.

Taxanes bind to β-tubulin, stabilize microtubule formation, and block cells undergoing mitosis (Zhong, 2002). This results in cell cycle arrest in G2/M phase of the cell cycle, which prevents mitosis. Important taxanes used in the treatment of breast cancer include paclitaxel (Taxol) and docetaxel (Taxotere) (Crown & O’Leary, 2000; Sparano, 2000). Treatment with taxanes produces side effects including hypersensitivities, stomatitis, mucositis, peripheral neuropathy, bone marrow suppression, fatigue, alopecia and diarrhea (Crown & O’Leary, 2000; Markman, 2003).
Paclitaxel was previously obtained from the bark of Pacific Yew tree (*T. brevifolia*) (Chabner & Roberts Jr, 2005), but is now prepared semi-synthetically from the natural precursor 10-deacetylbaccatin III (Zhong, 2002). Docetaxel is a semi-synthetic analogue derived from the needles of the European yew tree (Herbst & Khuri, 2003). Despite sharing the same microtubule binding site, docetaxel is believed to have distinct effects on microtubule dynamics (Andreu, Díaz, Gil, *et al.*, 1994; Díaz, Valpuesta, Chacón, *et al.*, 1998; Herbst & Khuri, 2003).

In addition to their actions on microtubules, both taxanes have been reported to cause apoptosis and inhibit angiogenesis (Wang, Liu, Kreis, *et al.*, 1999; Herbst & Khuri, 2003).

Anthracyclines are another group of cytotoxic drugs used in the treatment of breast cancer (Roché, Fumoleau, Spielmann, *et al.*, 2006). Anthracyclines, such as doxorubicin (adriamycin) and its 4’-epimer epirubicin (ellence), have been isolated from pigment-producing *Streptomyces* (Zagotto, Gatto, Moro, *et al.*, 2001). Their mode of action is controversial and, while there is evidence that doxorubicin intercalates DNA, these drugs also generate free radicals and inhibit topoisomerase II. The semiquinone radical and reactive oxygen species can induce DNA damage. Because topoisomerase II maintains the correct structure of DNA during replication its inhibition by anthracyclines promotes the accumulation of tangled DNA as well as DNA breaks (Walker & Nitiss, 2002; Cortés-Funes & Coronado, 2007; Moulder & Hortobagyi, 2008). Anthracyclines also produce severe side effects such as alopecia, nausea, cardiotoxicity, leukopenia and stomatitis (Kaklamani & Gradishar, 2003). Redox cycling of doxorubicin and formation of reactive oxygen species causes dose-dependent cardiotoxicity (Zhang, Liu, Bawa-Khalfe, *et al.*, 2012). Redox cycling leads to an
uneven consumption of $O_2$ and cellular reducing equivalents. Eventually, the formation of reactive oxygen species cause oxidative stress (Cohen & d’Arcy Doherty, 1987).

Tamoxifen is a nonsteroidal triphenylethylene derivative that has a high binding affinity for estrogen receptor, which blocks the proliferative actions of estrogen on the mammary epithelium (Jordan, Collins, Rowsby, et al., 1977). However, as a partial agonist of the estrogen receptor the drug has both estrogenic and anti-estrogenic actions (Osborne, Elledge & Fuqua, 1996). Aromatase inhibitors can be a valuable alternative and decrease the synthesis of estrogens from their androgenic precursors. Those in current use include the steroidal agent exemestane, and the non-steroidal inhibitors aminoglutethimide, fadrozole, anastrozole and letrozole (Goss & Strasser, 2005; Smith & Dowsett, 2003).

Chemotherapeutic agents are more effective than alternative therapies for metastatic cancers, especially when used in combination therapy (Chabner & Roberts Jr, 2005; Moulder & Hortobagyi, 2008); combination therapy is more effective than sequential administration (Sledge, Neuberg, Bernardo, et al., 2003). Thus, the combination of a taxane and doxorubicin produced a 94% response rate, as indicated by the number of patients whose tumours regressed (Gianni, Munzone, Capri, et al., 1995). The combination of paclitaxel and doxorubicin was quite active in term of response rate against metastatic breast cancers (Valero & Hortobagyi, 2003; Cortés-Funes & Coronado, 2007). Addition of paclitaxel to the doxorubicin/cyclophosphamide combination produced further improvements in disease-free survival - that is, the length of time the patient remained cancer free - but had no impact in overall survival (Mamounas, Bryant, Lembersky, et al.,
A significant time to progression with manageable toxicity has been achieved with the addition of capecitabine (Xeloda) to docetaxel in cases of metastatic breast cancer (O’Shaughnessy, Miles, Vukelja, et al., 2002). Capecitabine is approved for patients with metastatic breast cancer after anthracycline- and taxane-treatment, but has quite a low response rate (~15-26%) (Fumoleau, Largillier, Clippe, et al., 2004; O’Shaughnessy, 2005). Capecitabine is converted to 5-fluorouracil selectively in tumours through a cascade of three enzymes (Miwa, Ura, Nishida, et al., 1998). Other drugs used in combination therapy are gemcitabine (Gemzar) and vinorelbine (Navelbine) (O’Shaughnessy, 2005).

### 1.5.3. Targeted therapies

In the late 1980s, molecular and genetic studies identified signaling networks that regulate cell proliferation and survival (Chabner & Roberts Jr, 2005). In cancer cells, many of these pathways are dysregulated; this has led to the development of so-called targeted therapies directed against these molecular defects in tumour cells (Chabner & Roberts Jr, 2005). Thus, growth factors, signaling molecules, cell cycle proteins, and modulators of apoptosis and angiogenesis are increasingly targeted by such therapies (Hanahan & Weinberg, 2011). Because these drugs are directed against molecular targets in cancer cells, and have lower capacity to interrupt signalling in normal cells, their toxicity profiles tend to be superior to cytotoxic agents (Segota & Bukowski, 2004). Targeted therapies can include monoclonal antibodies and small molecule drugs (Segota & Bukowski, 2004).
Protein-tyrosine kinases (PTKs) regulate cell signaling, cell growth, differentiation, adhesion, motility, and cell death (Robinson, Wu & Lin, 2000). Over-active PTKs may increase tumour cell proliferation, decrease apoptosis, and promote angiogenesis and metastasis (Arora & Scholar, 2005). PTKs mediate the transfer of a phosphate from ATP to suitable protein substrates and may be either receptor or non-receptor tyrosine kinases (Hubbard & Till, 2000). Receptor tyrosine kinase ligands bind to their receptors, elicit autophosphorylation of their cytoplasmic domains and activate tyrosine kinase activity (Arora & Scholar, 2005). Non-receptor tyrosine kinases on the other hand relay signals along signaling cascades (Arora & Scholar, 2005). Receptor tyrosine kinases include receptors for EGF, fibroblast growth factor, platelet-derived growth factor, vascular endothelial growth factor and nerve growth factor.

In the context of tumour cell proliferation possibly the most important receptor tyrosine kinase is the EGFR (Figure 1-11A). Upon autophosphorylation, downstream signal transduction pathways, especially the Ras/Raf/mitogen-activated protein kinase (MAPK) and the phosphoinositol 3'-kinase/Akt (PI3K/Akt) are activated (Arora & Scholar, 2005). The Ras/Raf/MAPK and PI3K/Akt signaling pathways are critical for proliferation by activating cyclin D1 expression and cell cycle progression (Ciardiello & Tortora, 2001). Dysregulation of these tightly controlled pathways promote malignant transformation (Mendelsohn & Baselga, 2003).

ErbB-2 (HER2/neu, referred as HER2) is an EGFR-family member that is amplified 100-fold in tumours from ~30% of patients with metastatic breast cancer (Salomon, Brandt, Ciardiello, et al., 1995; Roskoski Jr., 2004).
Amplification decreases overall survival and results in differential responses to several chemotherapeutic and hormonal agents (Hudis, 2007). HER2 activates the PI3K/Akt prosurvival pathway in tumours, as well as the proliferative Raf/Raf/MAPK pathway (Figure 1-11A). These pathways also activate vascular endothelial growth factor that promotes angiogenesis and tumour progression (Figure 1-11A). HER2 over-expression can cause malignant transformation in the absence of growth factor, which suggests that it has a high level of ligand-independent activity (Yarden & Sliwkowski, 2001; Roskoski Jr., 2004). Down-regulation, or inhibition, of HER2 produces cytostatic effects in tumours that primarily stop cell growth, rather than by activating cell killing.

Monoclonal antibodies bind to tumour cells so that they may be detected by the immune system, or they may bind to plasma membrane receptors that drive tumour growth, or they may more efficiently deliver anti-tumour agents to the cancer cell (Segota & Bukowski, 2004). The development of humanized antibodies has significantly improved the problem of immune responses raised against immunoglobulins from heterologous species, such as the mouse (Harris, 2004). Bevacizumab (Avastin) is a recombinant humanized antibody that binds to vascular endothelial growth factor, which has a major role in angiogenesis (Hurwitz, Fehrenbacher, Novotny, et al., 2004). The EGFR, which is up-regulated in a high proportion of colorectal cancers, is targeted by Cetuximab (Erbitux) (Cunningham, Humblet, Siena, et al., 2004).

Trastuzumab (Herceptin) (Figure 1-11B) is a humanized mouse monoclonal antibody approved for the treatment of breast cancer characterized by HER2 over-expression (Carter, Presta, Gorman, et al., 1992; Roskoski Jr., 2004). The antibody binds to the extracellular domain of HER2 to impair the
proliferation and survival of HER2-dependent tumours (Hudis, 2007). Trastuzumab partially prevents dimerization of HER2 (Figure 1-11D) and the recruitment of immune effector cells (Figure 1-11E) and also promotes HER2 down-regulation by stimulating endocytosis (Figure 1-11F) (Franklin, Carey, Vajdos, et al., 2004). Clinical treatment with the antibody following adjuvant chemotherapy in HER2-positive breast cancers improves disease-free survival in patients (Piccart-Gebhart, Procter, Leyland-Jones, et al., 2005). Single-agent trastuzumab was initially proposed to be an effective first-line treatment in metastatic breast cancers that over-express HER2 (Vogel, Cobleigh, Tripathy, et al., 2002). Pertuzumab, another humanized monoclonal antibody, binds at a different epitope on HER2 to prevent dimerization with other ligand-activated HER receptors (Baselga, Bradbury, Eidtmann, et al., 2012). The combination of trastuzumab and pertuzumab appears to produce greater anti-tumour activity and more prolonged progression-free survival without cardiotoxicity (Baselga, Cortés, Kim, et al., 2012).

Other important monoclonal antibodies used in cancer chemotherapy include rituximab (Rituxan) that binds to CD20 on mature B-cells in B-cell non-Hodgkin lymphoma (Edwards, Szczepanski, Szechinski, et al., 2004), gemtuzumab (Myelotarg) that binds to CD33 found on leukemic myeloid cells (Hamann, Hinman, Hollander, et al., 2002), and alemtuzumab (Campath) that binds to CD52 on B and T lymphocytes (Enblad, Hagberg, Erlanson, et al., 2004).

Some antibodies may be conjugated with radioisotopes, toxins, enzymes or drugs (Harris, 2004). Ibritumomab tiuxetan (Zevalin) consists of a murine anti-CD20 antibody and a high-affinity chelation site for the radionuclide yttrium-90 and can be used in the treatment of poorly vascularized tumours.
(Witzig, Flinn, Gordon, et al., 2002). Others include tositumomab (Bexxar), which is a murine CD20 antibody that can be labeled with iodine-131 (Kaminski, Tuck, Estes, et al., 2005).
Figure 1-11: Signal transduction by EGFRs and the mechanism of action of trastuzumab. A| The EGFR family. HER2 dimerization activates PI3K/Akt and Ras/Raf/MAPK cascades that support cell survival and promote angiogenesis. B| trastuzumab has an antigen binding region and a humanized Fc (Fragment, crystallized) region. C| Trastuzumab inhibits the cleavage and activation of HER2,
D| the dimerization of HER2, and E| recruits immune effector cells to tumour cells that over-express HER2 which elicits cell death. F| Trastuzumab also down-regulates HER2 by endocytosis (Hudis, 2007).

HER2 may also be targeted by small-molecule drugs, such as the tyrosine kinase inhibitor drug lapatinib (N-{3-chloro-4-[[3-fluorobenzyl]oxy]-phenyl}-6-[5-([2-(methylsulfonyl)ethyl]amino)methyl]-2-furyl]-4-quinazolinamine).

Lapatinib is a substituted quinazoline, as are a number of other EGFR-linked tyrosine kinase inhibitors (Figure 1-12) (Rusnak, Affleck, Cockerill, et al., 2001; Medina & Goodin, 2008). The drug is used effectively in combination with capecitabine (Xeloda) in the treatment of patients with HER2-positive metastatic breast cancer and in patients who previously received anthracyclines and trastuzumab (Medina & Goodin, 2008). The antiproliferative effect of lapatinib is observed in many breast cancer cell lines (including MDA-MB-175, SK-BR-3, MDA-MB-361, UACC-893, UACC-812, T47D, MDA-MB-468, MCF-7, MDA-MB-435, MDA-MB-231, KPL1, EFM19, SUM225 and others) with varying effectiveness (IC50s 0.01 – 20 µM) (Konecny, Pegram, Venkatesan, et al., 2006). Lapatinib was found to inhibit HER2, Raf, Akt and Erk phosphorylation in those cells (Konecny, Pegram, Venkatesan, et al., 2006). The trastuzumab and lapatinib combination was shown recently to be more effective than single agent treatment (Nahta, Yuan, Du, et al., 2007).
**Figure 1-12: Mechanism of action of lapatinib.** Lapatinib inhibits EGFR and HER2. HER2 is activated by homodimerization or heterodimerization with other EGFRs. Ligand binding to EGFR (by EGF, transforming growth factor-α (TGF-α), amphiregulin, β-cellulin, heparin-binding EGF (HB-EGF) and epiregulin) or HER3 (heregulin and neuregulin 1 (NRG1)) causes HER2 to activate downstream signaling (PI3K/Akt and Ras/Raf/MAPK cascades), that is inhibited by lapatinib (Medina & Goodin, 2008).

**1.6. The molecular mechanism of tumourigenesis**

The altered balance between cell proliferation and apoptosis promotes uncontrolled progression in cancer (Chang, Ormerod, Powles, *et al.*, 2000). In cancer cells, pathways that control normal cell proliferation are defective (Evan & Vousden, 2001). Hence, uncontrolled cell proliferation is a major distinguishing feature of cancer cells compared with normal cells (Sandal, 2002).
This frequently occurs due to mutation of critical regulators such as Ras and the cell cycle controller retinoblastoma protein (pRB) (Harbour & Dean, 2000; Evan & Vousden, 2001). Ras regulates the cell cycle by activating the cyclin D1 promoter (Amanatullah, Zafonte, Albanese, et al., 2001). pRB is a tumour suppressor that inhibits cell cycle progression (Weinberg, 1995). Mutation of Ras may render the encoded protein constitutively active, which is a common defect in many human cancers (Jo, Zhang, Zhang, et al., 2000; Bos, 1989). The p53 gene regulates growth and apoptosis in cells (Woods & Vousden, 2001). It inhibits transcriptionally cell survival genes such as Bcl-2, survivin and Mcl-1 (Ho & Benchimol, 2003; Pietrzak & Puzianowska-Kuznicka, 2008). Cellular stresses such as genotoxic damage, oncogene activation and hypoxia are all able to activate p53 (Giaccia & Kastan, 1998). Activated p53 then induces apoptosis through both the mitochondrial and death receptor pathways (Levine, 1997; Green & Kroemer, 2009). Suppression of apoptosis is a major characteristic of cancer cells that is frequently due to mutations in p53 (in ~50% of human cancers) (Evan & Vousden, 2001; Wang & Sun, 2010). Indeed, p53 itself has been targeted in anticancer therapy. Thus, hemoradiation, gene therapy, synthetic peptides and small molecules are used to activate endogenous wild-type p53 (Wang & Sun, 2010). However, from *in vitro* and *in vivo* studies, tumours with mutated p53 are less sensitive to chemoradiation (Lowe, Ruley, Jacks, *et al.*, 1993; Lowe, Bodis, McClatchey, *et al.*, 1994). Approaches to reintroduce wild-type p53 in cancers, for example by gene therapy, may eventually be effective (Fujiwara, Grimm, Mukhopadhyay, *et al.*, 1993). Alternatively the small molecule RITA (reactivation of p53 and induction of tumour cell apoptosis) is used in p53 activation. RITA causes transcriptional repression of prosurvival proteins Mcl-1,
Bcl-2 and survivin, and blocks the Akt pathway (Grinkevich, Nikulenkov, Shi, et al., 2009). Other types of small molecules act by reactivation of p53 mutants back to wild-type. CP-31398, PRIMA-1, MIRA-1, WR-1065 and P53R3 are small molecules that rescue p53 (Foster, Coffey, Morin, et al., 1999; Bykov, Issaeva, Shilov, et al., 2002; Bykov, Issaeva, Zache, et al., 2005; Weinmann, Wischhusen, Demma, et al., 2008; Grinkevich, Nikulenkov, Shi, et al., 2009).

1.6.1. The cell cycle and the regulation of cell proliferation

The cell cycle begins with DNA replication, followed by division of the nucleus and cytoplasm to yield two daughter cells (Massagué, 2004). There are two stages in the cell cycle: mitosis (M) and interphase (Figure 1-13) (Vermeulen, Van Bockstaele & Berneman, 2003). Mitosis is the process by which the nucleus divides, while interphase is the gap between successive M phases. Interphase is subdivided further to G\textsubscript{1}, S and G\textsubscript{2} phases (Norbury & Nurse, 1992). During G\textsubscript{1} the cell prepares for DNA synthesis, DNA replication occurs in S-phase and G\textsubscript{2} phase prepares the cells for mitosis. Cells in G\textsubscript{1} can commit to DNA replication or enter the resting G\textsubscript{0} state, where they exit the cell cycle and do not proliferate (Vermeulen, Van Bockstaele & Berneman, 2003).

Transitions between phases of the cell cycle are tightly controlled by cyclin-dependent kinases (CDKs) (Figure 1-13). These are serine/threonine protein kinases that form regulatory subunits with cyclin proteins (Malumbres & Barbacid, 2009). CDK protein levels remain relatively constant during the cell cycle but cyclin expression fluctuates significantly (Pines, 1991; Vermeulen, Van Bockstaele & Berneman, 2003). Cyclins are synthesized and degraded at specific
times during the cell cycle to tightly regulate CDK activity (Malumbres & Barbacid, 2005, 2009). The most important CDKs are CDK4 and CDK6, that regulate progression though G₁ phase, CDK2, that controls both the G₁/S transition and progression through S phase, and CDK1 that regulates G₂/M transition and progression through M phase (Figure 1-13). Cyclin D1 regulates CDK4 and CDK6 activities, cyclins A and E regulate the activity of CDK2, while both cyclins A and B control CDK1 activity (Figure 1-13).

Figure 1-13: The cell cycle consists of the mitotic (M) phase and interphase (G₁, S and G₂) (Vermeulen, Van Bockstaele & Berneman, 2003).

Restriction points and checkpoints for cell cycle progression regulate quality control in mitosis. The restriction point is the point of no return in G₁, where the cell is committed to enter the cell cycle (Vermeulen, Van Bockstaele &
Cells that are growth factor-depleted before reaching the restriction point (e.g. during serum starvation) enter G0-like phase, while cells that are serum starved after the restriction point progress to mitosis (Pardee, 1974). Activation of checkpoint control, especially due to DNA damage, causes cell cycle arrest via altered regulation of CDK activity (Malumbres & Barbacid, 2005, 2009). This protects cells from attack by exogenous and endogenous genotoxins, including foreign chemicals, free radicals, ionizing radiation or cytotoxic drugs (Kastan & Bartek, 2004). DNA repair may occur during a checkpoint phase but if repair fails because DNA damage is excessive, or because of a defect in the checkpoint or the DNA repair machinery, cells will undergo apoptosis (Malumbres & Barbacid, 2009). If DNA damage is not controlled, accumulation of DNA damage can lead to genetic instability, which results in cell transformation and oncogenesis (Kastan & Bartek, 2004).

G1 is the phase in which cells make the determination as to whether or not to proceed through the cell cycle (Massagué, 2004). G1 is a DNA damage checkpoint to enable repair of DNA damage or replication errors. G1 phase is controlled principally by CDK4, CDK6 and cyclin D, that act in concert. It has been reported that cyclin D1 is over-expressed in about half of human breast cancers (Barnes & Gillett, 1998). Inactivation of the tumour suppressor gene pRb may produce dysfunction of cell cycle inhibitor proteins and cause unrestrained cell cycle progression; pRb is an important substrate for CDK during G1 phase (Vermeulen, Van Bockstaele & Berneman, 2003). Induction of cyclin D1 in T47D breast cancer cells shortens G1 phase and induces arrested cells to complete the cell cycle (Musgrove, Lee, Buckley, et al., 1994). Because a large number of breast
tumours exhibit increased cyclin D1 expression, this may be a target for development of novel drugs (Arnold & Papanikolaou, 2005). Flavopiridol (L86-8275) is a CDK4 inhibitor that arrests breast cancer cells in $G_1$ phase by impairing the function of cyclin D1 (Carlson, Dubay, Sausville, et al., 1996).

### 1.6.2. ATP production in cancer

Cell proliferation requires nutrients, energy and biosynthetic activity to replicate cell macromolecules during cell cycle progression (DeBerardinis, Mancuso, Daikhin, et al., 2007). Tumour cells typically exhibit altered energy metabolism with increased glycolysis under aerobic, rather than anaerobic, conditions (Moreno-Sánchez, Rodríguez-Enríquez, Marín-Hernández, et al., 2007). Known as the Warburg effect, high glycolytic rates are typical of aggressive cancer cells (Zu & Guppy, 2004). Because aerobic glycolysis is now an accepted hallmark of cancer cells (Hsu & Sabatini, 2008), positron emission tomography with the glucose analogue $[^{18}\text{F}]$fluorodeoxyglucose can be used to detect aggressive tumours (Mankoff, Eary, Link, et al., 2007).

The PI3K/Akt/mTOR signaling pathway is strongly pro-survival and is constitutively activated in most cancers (LoPiccolo, Blumenthal, Bernstein, et al., 2008). This pathway is important for cell cycle progression and is the main regulator of tumour glycolysis (Gao, Zhang, Jiang, et al., 2003; DeBerardinis, Mancuso, Daikhin, et al., 2007). PI3K up-regulates the glucose transporter Glut1 to enhance the uptake of glucose, which is required to meet the cellular requirements of ATP by aerobic glycolysis (Barata, Silva, Brandao, et al., 2004). Cell proliferation also requires a consistent supply of lipid and lipid precursors.
that are necessary for protein modification; the PI3K/Akt pathway participates in these processes (Bauer, Hatzivassiliou, Zhao, et al., 2005). Besides regulating ATP and lipid metabolism in cancer cells, the PI3K/Akt/mTOR pathway also activates hypoxia-inducible factor 1 (HIF-1) (Shafee, Kaluz, Ru, et al., 2009).

Hypoxia is a well-recognized characteristic of solid tumours (Gordan & Simon, 2007). Hypoxia induces HIF-1 that reprograms metabolism by upregulating genes that enhance glucose uptake, glucose conversion to lactate and shunts pyruvate away from the mitochondrion, which minimizes oxidative phosphorylation (Semenza, 2010). HIF-1 also promotes angiogenesis, invasion and metastasis by activating vascular endothelial growth factor expression (Semenza, 2003; Gordan & Simon, 2007). Inhibition of HIF-1 has significant effects on tumour growth (Semenza, 2003).

1.6.3. Prosurvival pathway

The PI3K/Akt signaling pathway is frequently dysregulated in cancer (Fresno Vara, Casado, de Castro, et al., 2004). PI3K is a lipid kinase that is activated by insulin, the oncogene products Ras and HER2, and by growth factors via their receptor tyrosine kinases (Liang & Slingerland, 2003). In breast cancer cells, survival signaling is activated via EGFR/HER2/PI3K/Akt (Kallergi, Agelaki, Kalykaki, et al., 2008). In parallel, apoptosis is inhibited at multiple levels (discussed further in section 1.6.4) (Zhou, Li, Meinkoth, et al., 2000). The PI3K/Akt/mTOR/p70S6K1 signaling cascade also mediates tumour cell progression through G1 phase by increasing cyclin D1 expression and by phosphorylating and inhibiting the CDK inhibitor p27 (Shin, Yakes, Rojo, et al.,
Together these mechanisms accelerate the growth of tumour cells by inactivating growth control pathways.

Raf/MKK/ERK is another prosurvival signaling cascade that regulates cell proliferation and differentiation and inhibits apoptosis by several mechanisms (Chen, Fujii, Zhang, et al., 2001; Roberts & Der, 2007). The Raf serine/threonine kinase is the MAPK kinase kinase at the top of the cascade that in turn activates a MAPK kinase (M KK ) downstream that can then activate the ERK itself (Roberts & Der, 2007). ERK protects cells from apoptotic signaling initiated by death receptors by phosphorylating and inactivating caspase 9 (Tran, Holmstrom, Ahonen, et al., 2001; Allan, Morrice, Brady, et al., 2003). This pathway also regulates mitosis by impairing the function of the CDK inhibitors p21 and p27 (Woods, Parry, Cherwinski, et al., 1997; Gysin, Lee, Dean, et al., 2005).

### 1.6.4. Apoptosis

Apoptosis is a major mechanism of programmed cell death and defects in apoptosis are common in cancer. Apoptotic cells exhibit characteristic features of chromatin condensation, nuclear fragmentation, plasma membrane blebbing and cell shrinkage (Kerr, Wyllie & Currie, 1972). Apoptosis is activated through two principal pathways: the intrinsic or mitochondrial pathway, and the extrinsic or death receptor-mediated pathway (Figure 1-14) (Brenner & Mak, 2009). The intrinsic pathway is activated by a range of stimuli, such as growth factor deprivation, damage to nuclear DNA, and cytotoxic insults such as reactive oxygen species and cytotoxic drugs (Brenner & Mak, 2009). The extrinsic pathway is activated by ligand binding to death receptors (Khan, Afaq & Mukhtar,
Death receptors belong to the tumour necrosis factor (TNF) receptor 1 (TNF-R1) family of plasma membrane receptors that also includes the FAS-receptor (CD95) and TNF-related apoptosis-inducing ligand (TRAIL) receptors known as TRAIL-RI and TRAIL-RII (Khan, Afaq & Mukhtar, 2007). Both pathways of apoptosis converge on caspases, especially caspase 3 (Wang & Youle, 2009).

**Figure 1-14: Major pathways of apoptosis.** The extrinsic pathway involves ligand binding to plasma membrane death receptors that activate caspase 3 via caspase 8. The intrinsic pathway is activated by intracellular stresses. Caspase 8 can also activate the mitochondrial pathway by cleavage of BID to form tBID (Brenner & Mak, 2009).

Caspases are cysteine proteases that cleave their protein substrates at aspartic acid residues (Alnemri, Livingston, Nicholson, *et al.*, 1996). All caspases
are inactive zymogen and require proteolytic activation (Shi, 2002). Caspases are initiators or effectors of apoptosis, which involves activation of downstream caspases or cell execution, respectively. Initiator caspases include caspases 2, 8, 9 and 10, while caspases 3, 6 and 7 are effectors (Shi, 2002). In the intrinsic pathway caspase 9 activates caspases 3 and 7 (Shi, 2002) while caspase 8 undergoes autocleavage and activation in the extrinsic pathway (Donepudi, Mac Sweeney, Briand, et al., 2003). Caspase 8 also directly activates caspase 3 independent of the intrinsic pathway, thus providing a link between the two pathways (Reed, 2001). Caspase 3 is the major executioner caspase, although some cell lines such as MCF-7 utilise caspase 7 (Liang, Yan & Schor, 2001; Walsh, Cullen, Sheridan, et al., 2008). Formation of the apoptosome complex between the initiator caspase 9 and the mitochondrial proteins cytochrome c and Apaf-1 (Figure 1-15) activates apoptosis (Riedl & Salvesen, 2007).
**Figure 1-15: Structure of the apoptosome.** A| Apaf-1 has an N-terminal caspase-recruitment domain (CARD), which binds caspase 9, a nucleotide-binding and oligomerization domain (NB-ARC), that binds ATP and a WD40 region for cytochrome c binding. B| In the absence of an apoptotic signal, Apaf-1 is in an inhibited state. Binding of cytochrome c and ATP activates the complex (Riedl & Salvesen, 2007).

There are additional modulatory factors that regulate apoptosis. Thus, members of the Inhibitor of apoptosis (IAP) family bind and inhibit caspases 3 and 7 (Roy, Deveraux, Takahashi, et al., 1997; Deveraux, Roy, Stennicke, et al., 1998). Second mitochondria-derived activator of caspases (Smac, also known as DIABLO) is also released from the mitochondrion in response to an apoptotic stimulus (Chai, Du, Wu, et al., 2000). Smac/DIABLO binds to and neutralizes IAPs, which derepresses the inhibition of caspases 3, 7 and 9 (Shiozaki & Shi, 2004).
Mitochondrial integrity is controlled by the B-cell lymphoma-2 (Bcl-2) family of proteins (Brenner & Mak, 2009). These proteins fall into three classes: the anti-apoptotic Bcl-2, Bcl-XL and Mcl-1, the pro-apoptotic Bax and Bak, and the Bcl-2 homology 3 (BH3)-only proteins that include Bad, Bik, Bid, Bim, NOXA and PUMA (Youle & Strasser, 2008). Bax and Bak are responsible for inducing permeabilisation of the outer mitochondrial membrane and the subsequent release of cytochrome c and Smac/DIABLO (Youle & Strasser, 2008). The anti-apoptotic family Bcl-2 and Bcl-XL interact directly with Bax and Bak to inhibit apoptosis but the interaction is also modulated by BH3-only proteins (Youle & Strasser, 2008).

The balance between pro-apoptotic and anti-apoptotic Bcl-2 family members within the mitochondrial membrane dictates the fate of the cell (Brenner & Mak, 2009). During apoptosis Bax and Bak form a pore on the mitochondrial membrane that enables the release of apoptotic proteins (Korsmeyer, Wei, Saito, et al., 2000). Bcl-2 and Bcl-XL inhibit apoptosis by preventing the interaction of the proapoptotic Bax and Bak. As mentioned above, Bcl-2 and Bcl-XL also recruit BH3-only proteins, which can inhibit Bax-Bak-mediated apoptosis (Cheng, Wei, Weiler, et al., 2001). Among the BH3-only proteins Bid plays an important role. Stimulation of the extrinsic pathway activates caspase 8 that mediates Bid cleavage (Yin, 2000); the proapoptotic JNK and p38 MAPK pathways regulate this step. The truncated active form of Bid (tBid) then translocates to mitochondria and promotes cytochrome c release by creating membrane pores (Kuwana, Mackey, Perkins, et al., 2002).

The MAPK are modular signaling cascades that transduce extracellular stimuli in cells. The proliferative and pro-survival ERK pathway is frequently
upregulated in cancers while the c-Jun N-terminal kinase (JNK) and p38 MAPK cascades are repressed (Wagner & Nebreda, 2009). These cascades generally antagonize cell proliferation but this is subverted in many tumours (Figure 1-16) (Wagner & Nebreda, 2009). MAPK signaling is activated by a series of upstream kinases in response to a range of cell stresses. Upstream from the JNK are the MAPK kinases M KK4 and M KK7, while these kinases are responsive to MAPK kinase kinases that are further upstream, such as the apoptosis signal-regulating kinase-1 (ASK1) (Asaoka & Nishina, 2010). On the other hand, the p38 MAPK is activated by the MAPK kinases M KK3, M KK4 and M KK6, and by several MAPK kinase kinases, including ASK1 (Brancho, Tanaka, Jaeschke, et al., 2003).

Mitochondria respond to pro-apoptotic signals transduced through the JNK and p38 MAPK pathways (Tournier, Hess, Yang, et al., 2000). Once activated, JNK has been reported to phosphorylate and inactivate Bcl-2 and Bcl-XL (Yamamoto, Ichijo & Korsmeyer, 1999; Kharbanda, Saxena, Yoshida, et al., 2000). ASK1 is activated in response to cytotoxic stress and can in turn activate JNK and p38 MAPKs through their respective MAPK kinases (Tobiume, Matsuzawa, Takahashi, et al., 2001). The p38 MAPK can also inhibit cell cycle progression at G_1/S and G_2/M phases by downregulating cyclin D1 and upregulating CDK inhibitors (Lavoie, L’Allemain, Brunet, et al., 1996; Wagner & Nebreda, 2009).
Figure 1-16: The JNK and p38 MAPK cascades. MAPK are activated by environmental stresses, growth factor and cytokines at the level of the MAPK kinase kinases (MAP3Ks) that then activate the downstream MAPK kinases (MAP2Ks) before activating the JNK and p38 MAPKs themselves. The MAPKs then regulate genes that participate in cell proliferation, survival and differentiation (Wagner & Nebreda, 2009).

The extrinsic apoptosis pathway is initiated by activation of death receptors, including the Fas receptor, TNF-R1 and TRAIL receptors. These receptors contain an intracellular protein interaction region known as the death domain (Thorburn, 2004). In the cases of the Fas and TRAIL receptors the Fas-associated death domain (FADD) protein is recruited to this domain, which then promotes caspase 8 activation (Salvesen & Dixit, 1999; Suliman, Lam, Datta, et al., 2001). The TNF-R1-associated death domain (TRADD) protein recruits
additional adapters, including receptor-interacting protein (RIP), TNF-R1-associated factor 2 (TRAF2) and FADD to the intracellular death receptor complex (Figure 1-17) (Chen & Goeddel, 2002).

Recruitment of these proteins to the complex enables ASK1 activation and subsequent downstream activation of JNK, which ultimately modulates the expression of proapoptotic genes and proteins (Nishitoh, Saitoh, Mochida, et al., 1998; Chen & Goeddel, 2002).

**Figure 1-17: Overview of the TNF-R1-mediated signal transduction pathway.** Binding of the cytokine TNF-α to TNF-R1 recruits TRADD, TRAF2, RIP and FADD to the intracellular death domain (Chen & Goeddel, 2002).
1.7. **Fatty acids: dietary factors that modulate tumourigenesis**

Fatty acids are essential components of the human diet that play important roles in cell membrane assembly and, more generally, in many aspects of cell homeostasis, including the regulation of proliferation and apoptosis. Sources of fatty acids include fruits, vegetable oils, seeds, nuts, animal fats, and fish oils. Naturally occurring long-chain fatty acids are 18-22 carbons in length (Figure 1-18). Fatty acids are stored in cell membranes esterified as triglycerides or phospholipid esters. Fatty acids belong to three major classes: saturated (have no carbon-carbon double bonds), monounsaturated (have a single carbon-carbon double bond) and polyunsaturated (that have multiple carbon-carbon double bonds) (Table 1-1) (Rennison & Van Wagoner, 2009; White, 2009).
Table 1-1: Typical saturated, monounsaturated and polyunsaturated fatty acids.

<table>
<thead>
<tr>
<th>Name</th>
<th>Carbon atoms</th>
<th>Type</th>
<th>Essential fatty acid</th>
<th>Common sources</th>
</tr>
</thead>
<tbody>
<tr>
<td>Palmitic acid</td>
<td>16</td>
<td>Saturated</td>
<td>No</td>
<td>Palm oil</td>
</tr>
<tr>
<td>Stearic acid</td>
<td>18</td>
<td>Saturated</td>
<td>No</td>
<td>Animal fat</td>
</tr>
<tr>
<td>Oleic acid</td>
<td>18</td>
<td>Monounsaturated</td>
<td>Yes</td>
<td>Olive oil</td>
</tr>
<tr>
<td>Linoleic acid</td>
<td>18</td>
<td>Polyunsaturated</td>
<td>Yes</td>
<td>Sunflower oil</td>
</tr>
<tr>
<td>Linolenic acid</td>
<td>18</td>
<td>Polyunsaturated</td>
<td>Yes</td>
<td>Soybean Oil</td>
</tr>
<tr>
<td>Arachidonic acid</td>
<td>20</td>
<td>Polyunsaturated</td>
<td>Yes</td>
<td>Meat, Dairy</td>
</tr>
<tr>
<td>Eicosapentaenoic acid</td>
<td>20</td>
<td>Polyunsaturated</td>
<td>Yes</td>
<td>Fish oil</td>
</tr>
<tr>
<td>Docosahexaenoic acid</td>
<td>22</td>
<td>Polyunsaturated</td>
<td>Yes</td>
<td>Fish oil</td>
</tr>
</tbody>
</table>
Figure 1-18: Chemical structures of saturated, monounsaturated and polyunsaturated fatty acids. Saturated fatty acids (eg palmitic acid) have no double bonds. Monounsaturated fatty acids (MUFAs) (eg oleic) have one double bond. Polyunsaturated fatty acids (PUFAs) (eg linolenic, arachidonic, eicosapentaenoic and docosahexaenoic acids) have multiple double bonds. Numbers indicate the carbon atom at which the double bond is located relative to the terminal methyl group (Rennison & Van Wagoner, 2009).

1.7.1. Essential fatty acids

Diets that were low in fatty acid content produced severe dermatological and organ damage in rats that was reversed by dietary fat replacement (Burr & Burr, 1929). However, it was found subsequently that only essential fatty acids (eg linoleic and linolenic acids) produced these health benefits (Burr & Burr, 1930).
Certain omega-3 (ω-3) and omega-6 (ω-6) PUFA are essential fatty acids that cannot be synthesized in mammalian tissues and that must be obtained from the diet (Connor, 2000). The names of these PUFA are derived from the location of the first double bond furthest from the carboxyl group. The terminal methyl group is in the ω position. The first double bond from the ω end of linoleic acid and linolenic acid is located at the sixth and third carbon, respectively. Hence, linoleic acid is an ω-6 PUFA and linolenic acid is an ω-3 PUFA. Biotransformation of essential fatty acids occurs in mammalian cells. For example, γ-linolenic and arachidonic acids (AAs) are synthesized from linoleic acid, (Fig. 1-19) while α-linolenic acid is the precursor of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Fig. 1-20). An additional nomenclature has also developed for PUFA that lists the number of carbons, double bonds and PUFA type (eg arachidonic acid is 20:4n-6 because it has 20 carbons, 4 double bonds and is an n-6 (or ω-6) PUFA) (Fig. 1-20)
**Figure 1-19: Metabolism of ω-6 linoleic acid to arachidonic acid.** Linoleic acid can be elongated and desaturated to γ linolenic acid and arachidonic acid. Subsequently, arachidonic acid can be converted to docosatetraenoic acid (Whelan & Fritsche, 2013).
Figure 1-20: Summary of ω-3 and ω-6 PUFA biotransformation pathways.

Desaturation and elongation occurs in the endoplasmic reticulum, after translocation to the peroxisome, β-oxidation shortens the chain by two carbon atoms, before translocation back into endoplasmic reticulum for esterification (Dyall & Michael-Titus, 2008).

A clear understanding of the fate of dietary PUFA could provide insight into their regulatory actions in cancer. As mentioned, long-chain fatty acids (18-22 carbons length) are esterified in lipid membranes as triglycerides or phospholipids. Triglycerides consist of a glycerol molecule in which each of the hydroxyl groups is esterified with a fatty acid. Most phospholipids consist of diglycerides, a phosphate group and a simple organic molecule such as choline (to produce phosphatidylcholine which is a major constituent of biological
membranes). PUFAs are stored esterified in membrane phospholipids and are released by the actions of phospholipases.

Once liberated in cells, free PUFA become substrates for three major enzymatic pathways. Thus, cyclooxygenase (COX) converts PUFA to prostaglandins (PGs), prostacyclin and thromboxane A₂, lipoxygenase (LO) generates hydroxyeicosatetraenoic acids (HETEs) and leukotrienes and cytochromes P450 (CYPs) produce epoxides and hydroxyfatty acids (Spector & Yorek, 1985; Oates, FitzGerald, Branch, et al., 1988; Oliw, 1994).

COX enzymes catalyse the synthesis of PGs from AA (Figure 1-21). The peroxidase activity of COX catalyses the insertion of molecular oxygen to form the unstable intermediate PGG₂ that is reduced by the same enzyme to PGH₂ (Subbaramaiah & Dannenberg, 2003). Specific isomerases convert PGH₂ to the range of biologically active PGs and thromboxane A₂ (Subbaramaiah & Dannenberg, 2003). There are two isoforms of COX. COX-1 is expressed constitutively in tissues and mediates the formation of PGs that modulate homeostasis in normal cells (Subbaramaiah & Dannenberg, 2003). In contrast, COX-2-dependent PGs have an important role in tumourigenesis by stimulating the growth and migration of tumour cells and neovascularization that increases the supply of nutrients to tumours (Masferrer, Leahy, Koki, et al., 2000). COX-2 is often over-expressed in many human cancers, including breast cancers that have high level HER2 expression (Ristimäki, Sivula, Lundin, et al., 2002).
**Figure 1-21: Arachidonic acid metabolism by COX.** Phospholipase A\(_2\) (PLA\(_2\)) releases arachidonic acid from membrane phospholipids. COX metabolizes arachidonic acid to prostaglandin H\(_2\) (PGH\(_2\)). PGH\(_2\) is converted to multiple eicosanoids by specific isomerases (Romano & Clària, 2003).

LO enzymes are non-heme iron dioxygenases that insert molecular oxygen into PUFAs to generate the hydroperoxides (HPETEs) and leukotriene A\(_4\) (Figure 1-22) (Pidgeon, Lysaght, Krishnamoorthy, *et al.*, 2007). One of the LO enzymes – 5-LO – is implicated in tumourigenesis: its expression is increased in tumour cells, where it promotes growth and angiogenesis (Jiang, Douglas-Jones & Mansel, 2003; Romano & Clària, 2003).
Figure 1-22: Arachidonic acid metabolism by 5-LO. 5-LO catalyses the oxygenation of arachidonic acid to 5(S)-hydroperoxyeicosatetraenoic acid (5(S)-HpETE), which is then reduced to 5(S)-HETE or converted to the unstable allylic epoxide LTA4. LTA4 then undergoes biotransformation to other leukotrienes (Romano & Clària, 2003).

CYPs were first reported in 1955 (Axelrod, 1955; Brodie, Axelrod, Cooper, et al., 1955). These enzymes catalyse reactions in which molecular oxygen is activated to a strongly pro-oxidant form using NADPH as the electron source (Mason, 1957; Estabrook, 2003). There are 57 human CYPs located mostly in the liver but also found in the intestine, kidney, lung and other tissues (Table 1-2) (Frye, 2004). The standard nomenclature consists of “CYP” followed by an arabic number to indicate the CYP family (members are ≥ 40% similar at the amino acid level), a letter indicates the subfamily (members are ≥ 55% similar at the amino
acid level) and another number indicates the individual CYP polypeptide. For example, CYP2J2 is the second CYP polypeptide from family 2 and subfamily 2J. While these enzymes are best known for their roles in drug metabolism and the elimination of foreign compounds (Farrell & Murray, 1990; Guengerich, 1990; Murray & Field, 1992), they also oxidise physiological substrates, including steroid hormones, fatty acids and vitamins (Nebert & Russell, 2002). CYPs are regulated by foreign compounds, dietary nutrients and in inflammation and cancer (Morgan, 1997; Murray, 2006).

**Table 1-2: Function of human CYP gene families.** Data from (Nebert & Russell, 2002).

<table>
<thead>
<tr>
<th>Family</th>
<th>subfamily</th>
<th>genes</th>
<th>Substrates (or function)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1</td>
<td>2</td>
<td>3</td>
<td>Foreign chemicals</td>
</tr>
<tr>
<td>CYP2</td>
<td>13</td>
<td>16</td>
<td>Foreign chemicals, fatty acids, steroids</td>
</tr>
<tr>
<td>CYP3</td>
<td>1</td>
<td>4</td>
<td>Foreign chemicals, steroids</td>
</tr>
<tr>
<td>CYP4</td>
<td>5</td>
<td>12</td>
<td>Fatty acids</td>
</tr>
<tr>
<td>CYP5</td>
<td>1</td>
<td>1</td>
<td>(Thromboxane A₂ synthase)</td>
</tr>
<tr>
<td>CYP7</td>
<td>2</td>
<td>2</td>
<td>Cholesterol (bile acid synthesis)</td>
</tr>
<tr>
<td>CYP8</td>
<td>2</td>
<td>2</td>
<td>(Prostacyclin synthase)</td>
</tr>
<tr>
<td>CYP11</td>
<td>2</td>
<td>3</td>
<td>Steroidogenesis</td>
</tr>
<tr>
<td>CYP17</td>
<td>1</td>
<td>1</td>
<td>Steroid 17α-hydroxylase, 17/20-lyase</td>
</tr>
<tr>
<td>CYP19</td>
<td>1</td>
<td>1</td>
<td>Aromatase</td>
</tr>
<tr>
<td>CYP20</td>
<td>1</td>
<td>1</td>
<td>Unknown</td>
</tr>
<tr>
<td>CYP21</td>
<td>1</td>
<td>1</td>
<td>Steroid 21-hydroxylase</td>
</tr>
</tbody>
</table>
The CYP2 gene family is the largest CYP family in man (Nebert & Russell, 2002). One of its members - CYP 2J2 - oxidises the ω-6 PUFA arachidonic acid to a series of epoxides, known as epoxyeicosatrienoic acids or EETs that have a number of important cellular functions, including regulation of ion channel activity, hormone release, cellular proliferation, apoptosis and angiogenesis (Morgan, 1997; Jiang, Chen, Card, et al., 2005; Chen, Li, Liao, et al., 2009). Human CYP2J2 was first reported in 1996 (Wu, Moomaw, Tomer, et al., 1996) and is probably the major epoxygenase in multiple tissues including heart, intestine, liver, lung, pancreas, kidney and blood vessels (Enayetallah, French, Thibodeau, et al., 2004). CYP2J2 also generates epoxides from ω-3 PUFA such as EPA. In this regard, it was found recently by (Cui, Petrovic & Murray, 2011) that the unique ω-3 epoxide of EPA decreased cell proliferation, which is in contrast to the proliferative actions of other isomeric epoxides of ω-3 EPA and the ω-6 EETs.
1.7.2. **ω-3 and ω-6 PUFA and their metabolites influence tumourigenesis**

Epidemiological studies suggest that there is an inverse relationship between cancer incidence and the intake of ω-3 PUFAs (Jing, Wu & Lim, 2013) and that intake of ω-6 PUFAs may increase cancer risk (Maillard, Bougnoux, Ferrari, *et al.*, 2002). Thus, observations in Japanese, Inuit and Norwegians populations, whose diets have a high content of oily fish, suggest a lower cancer incidence (Hirose, Takezaki, Hamajima, *et al.*, 2003). There was an increased incidence of colon and breast cancer in Japanese who migrated to North America and adopted Western diets relative to their counterparts who remained in Japan and ingested fish-containing diets (Tomatis & Aitio, 1990).

More recent studies have shown that the risk of breast cancer appears to be increased in premenopausal women with increasing ω-6 PUFA intake (Chajès, Torres-Mejía, Biessy, *et al.*, 2012). In another study, diets that were high in ω-3 PUFA (ingestion of up to 40g fish per day) were associated with a decreased breast cancer risk of up to 25% (Gago-Dominguez, Yuan, Sun, *et al.*, 2003). Rather than the absolute amount of individual PUFAs in diets, the ratio of ω-6: ω-3 PUFA may be more important (de Lorgeril & Salen, 2012). This ratio has increased from 1.5:1 around 200 years ago to 16:1 or higher due to widespread acceptance of western diets (Simopoulos, 2002a). This has been supported by measurements of PUFA content in adipose tissue of European women (Simonsen, van't Veer, Strain, *et al.*, 1998).

Cancers other than breast cancers may also be influenced by PUFA intake. High dietary ω-6 PUFA content appeared to increase prostate tumour proliferation (Aronson, Kobayashi, Barnard, *et al.*, 2011). A lower incidence of
metastatic prostate cancer has been reported with increased consumption of oily fish and flaxseed oil (Terry, Lichtenstein, Feychting, et al., 2001; Augustsson, Michaud, Rimm, et al., 2003; Demark-Wahnefried, Polascik, George, et al., 2008). In addition, a recent case-control study suggested that long-chain ω-3 PUFA from fish and shellfish may be protective in prostate cancer (Fradet, Cheng, Casey, et al., 2009). Similar findings have been made in colon cancer (Ziegler, Hoover, Pike, et al., 1993; Caygill & Hill, 1995).

Observations from animal studies have produced similar findings to human epidemiological studies. For example, ω-3 PUFA were found to have anti-cancer actions in rodent models of breast and colon cancer (Reddy & Maruyama, 1986; Cannizzo & Broitman, 1989; Rose, Connolly, Rayburn, et al., 1995). The growth and migration characteristics of human breast cancer cell xenografts in nude mice were increased when the animals ingested diets that were high in ω-6 PUFA and the opposite was noted when diets contained high levels of ω-3 PUFA (Rose, Connolly & Liu, 1995). Experiments in fibrosarcoma-implanted rats that received an ω-3 EPA-supplemented diet found that tumour volume was decreased, relative to those that received the control diet, and expression of the proangiogenic growth factor VEGF was also lower (Tevar, Jho, Babcock, et al., 2002).

There is evidence to support several potential mechanisms that could account for the decrease in tumourigenesis produced by ω-3 PUFA. Firstly, ω-3 PUFA have been found to decrease the formation of ω-6 PUFA-derived eicosanoids. Tumour formation was decreased in nude mice carrying prostate cancer cell xenografts and that ingested ω-3 PUFA or fish oil. Decreased growth was attributed to decreased formation of the protumourigenic PGE₂ (Rose &
Cohen, 1988; Kobayashi, Barnard, Henning, et al., 2006; Berquin, Min, Wu, et al., 2007). \(\omega\)-3 PUFA were also found to activate apoptosis in human breast tumour xenografts in nude mice by inhibiting the formation of the anti-apoptotic PGE\(_2\) and 12-HETE (Rose & Connolly, 1997). Thus, decreased formation of pro-tumourigenic \(\omega\)-6 PUFA metabolites could normalise cell growth and apoptosis.

Secondly, the long chain \(\omega\)-3 PUFA EPA were found to decrease the proliferation of hepatocellular carcinoma cells by down regulating COX-2 that, in turn, inhibited the formation of PGE\(_2\) formation (Lim, Han, Dai, et al., 2009). In CaCo-2 cells, DHA down-regulated COX-2 and cell cycle genes, and upregulated caspases (Narayanan, Narayanan, Simi, et al., 2003). \(\omega\)-3 PUFA also inhibited PGE\(_2\)-mediated ERK phosphorylation and HIF-1 protein expression in colon cancer (Calviello, Di Nicuolo, Gragnoli, et al., 2004) and mammary gland (Liu, Pu, Wang, et al., 2007). Thus, signalling pathways that promote the expression of tumourigenic genes, or that stimulate cell survival, may be inhibited by \(\omega\)-3 PUFA.

More recently, it has been found that \(\omega\)-3 PUFA metabolites may themselves have beneficial direct actions in preventing cancer development. Eicosanoids of \(\omega\)-3 PUFAs are anti-inflammatory and inhibit cancer cell proliferation, metastasis, and angiogenesis (Abou-el-Ela, Prasse, Farrell, et al., 1989; Rose & Connolly, 2000; Brown, Hart, Gazi, et al., 2006). While \(\omega\)-6 PGE\(_2\) formation was decreased in \(\omega\)-3 EPA-treated colorectal cells formation of the analogous eicosanoid metabolite - PGE\(_3\) - was increased (Hawcroft, Loadman, Belluzzi, et al., 2010). PGE\(_3\) has been found to suppress angiogenesis directly by impairing the VEGF-mediated induction of angiopoietin-2 (Szymczak, Murray & Petrovic, 2008). A significant recent finding was that the unique \(\omega\)-3-17,18-
epoxide of EPA decreased cell growth and activated apoptosis (Cui, Petrovic & Murray, 2011); this is in clear contrast to the proliferative and anti-apoptotic actions of other EPA-epoxide isomers and the ω-6 EETs (Jiang, Chen, Card, et al., 2005; Chen, Li, Liao, et al., 2009).

1.8. Lipids and lipid analogues as potential anticancer agents

There is evidence that a number of lipid-based molecules have the potential for development as anticancer agents. Short-chain fatty acids, such as the C4-butyric acid, cause cell cycle arrest and activate apoptosis in colon cancer cells in vitro (Heerdt, Houston & Augenlicht, 1997). Apoptosis involved activation of the pro-apoptotic Bcl-2 protein Bak (Hague, Diaz, Hicks, et al., 1997; Ruemmele, Dionne, Qureshi, et al., 1999). The C16-saturated palmitic acid activated the mitochondrial release of cytochrome c, cleavage of poly-ADP ribose polymerase (PARP) and fragmentation of DNA (de Pablo, Susin, Jacotot, et al., 1999). Similarly, the branched-chain 13-methyltetradecanoic acid induced apoptosis by disrupting mitochondrial integrity in cancer cells (Yang, Liu, Chen, et al., 2000; Wongtangtintharn, Oku, Iwasaki, et al., 2005; Lin, Yin, Cai, et al., 2012). This fatty acid inhibited the PI3K/Akt survival cascade, activated pro-apoptotic JNK and p38 MAPKs, down-regulated the pro-survival Bcl-2, up-regulated Bax, induced Cytochrome c release and activated caspases (Lin, Yin, Cai, et al., 2012). Because 13-methyltetradecanoic acid had minimal toxicity in nude mice it is a potential candidate as a chemotherapy drug (Yang, Liu, Chen, et al., 2000).

Several PUFA also have properties that may be useful in anti-cancer drug development. There is evidence that these fatty acids promote lipid peroxidation, which may activate apoptosis (Cao, Pearman, Zimmerman, et al., 2000).
Conjugated linoleic acid has been shown to inhibit proliferation and activated apoptosis in mammary cancer cell line by decreasing anti-apoptotic Bcl-2 (Ip, Ip, Loftus, et al., 2000). Punicic acid is an ω-5 PUFA from pomegranate seed oil that activates apoptosis in human MDA-MB 231 breast cancer cells by a pro-oxidant mechanism causing DNA fragmentation (Grossmann, Mizuno, Schuster, et al., 2010). Jacaric acid is an isomer of linolenic acid that activated intrinsic apoptosis in LNCaP prostate cancer cells by promoting cleavage of caspases-3, 8, and 9, regulating Bcl-2 family proteins and increasing the cleavage of PARP. Importantly, however, jacaric acid had no effect on the viability of normal prostate epithelial cells (Gasmi & Sanderson, 2013).

Recently, a series of synthetic ω-3 monounsaturated fatty acids (C16-C22) was found to decrease proliferation and activate apoptosis in MDA-MB 468 human breast cancer cells that over-expressed COX-2 (Cui, Rawling, Bourget, et al., 2012). Longer chain analogues (C19-C22) were most active. The thiofatty acid tetradecylthioacetic acid exerted several potentially useful anticancer actions in cells, including the inhibition of proliferation, increased production of mitochondrial reactive oxygen species, glutathione depletion, and activation of mitochondrial apoptosis (Tronstad, Berge, Dyroy, et al., 2001; Tronstad, Gjertsen, Krakstad, et al., 2003). Increased vascularisation of colon cancer xenografts and improved survival occurred in xenografted mice that received a diet containing tetradecylthioacetic acid (Jensen, Berge, Bathen, et al., 2007).

The ether phospholipids are a promising class of anti-tumour lipids that act in part at the mitochondrion in cancer cells. Ether phospholipids have one or more glycerol carbons bonded to an alkyl chain via an ether linkage, as opposed to the usual ester linkage. The novel alkylphospholipid analog perifosine is a
PI3K/Akt inhibitor that inhibits growth at low micromolar concentrations and activates the intrinsic pathway of apoptosis in cells (Kondapaka, Singh, Dasmahapatra, et al., 2003). Interestingly, these agents are effective in rapidly proliferating cancer cells, but not quiescent normal cells.

Clinical evaluation of perifosine has been conducted, or is continuing, in patients with cancers of the endometrium, breast, prostate, bladder and other tissues. In 2010, perifosine was evaluated in phase II trials for metastatic colon cancer and extended the time taken for tumour progression. When used in combination with the multikinase inhibitor sorafenib, perifosine induced intrinsic apoptosis in cells and anti-tumour effects in NOD/SCID mice with Hodgkin lymphoma cell line xenografts (Locatelli, Giacomini, Guidetti, et al., 2013). In cell lines, this combination inhibited MAP kinase, activated PI3K/Akt phosphorylation and suppressed growth. In in vivo xenograft studies there was a reduction in tumour burden, increased survival time, increased apoptosis and necrosis in perifosine/sorafenib-treated animals compared with single agents (Locatelli, Giacomini, Guidetti, et al., 2013). Subsequently, treatment of human leukemia T-cells with perifosine and etoposide also effected synergistic induction of apoptosis by dual activation of intrinsic and extrinsic pathways (Nyåkern, Cappellini, Mantovani, et al., 2006). The combination produced a two-fold increase in caspase-8 activation, and a marked increase in caspase-9, caspase-3, and poly(ADP-ribose) polymerase cleavage, as well as increased Bim, Bid and Bcl-XL expression.

Edelfosine targets the mitochondrial membrane and inhibits mitochondrial respiration (Burgeiro, Pereira, Carvalho, et al., 2013). Edelfosine also activates the JNK MAP kinase pathway that induces apoptosis (Ruiter, Zerp,
Bartelink, et al., 1999; Nieto-Miguel, Gajate & Mollinedo, 2006). The mechanism involves direct activation of apoptosis by phosphorylation of the anti-apoptotic Bcl-XL (Kharbanda, Saxena, Yoshida, et al., 2000; Aoki, Kang, Hampe, et al., 2002). In regard to the apoptotic mechanism, no evidence of a role for reactive oxygen species was found. Instead it was suggested that edelfosine promoted a redistribution of lipid rafts from the plasma membrane to the mitochondrion (Mollinedo, Fernández, Hornillos, et al., 2011). In summary, ether phospholipids have potential for development as a novel class of anticancer agents that act in part by altering mitochondrial function.

Apart from the parent PUFA themselves some PUFA metabolites also modulated tumourigenesis. Over-expression of COX and CYP enzymes increased the formation of certain prostaglandin and epoxide metabolites that regulate cell proliferation and inhibit apoptosis (Tsujii, Kawano & DuBois, 1997; Jiang, Chen, Card, et al., 2005). Thus, CYP-dependent EETs, COX-mediated PGE2 and LO-mediated hydroxyeicosatetraenoic acids are pro-survival and proliferative (Avis, Hong, Martinez, et al., 2001; Koehne & Dubois, 2004; Chen, Li, Liao, et al., 2009). PGE2 and EETs promote cancer cells survival by activating EGFR-dependent ERK MAPK and PI3K/Akt signalling pathways (Chen, Li, Liao, et al., 2009). As mentioned, there is evidence that some metabolites derived from ω-3 PUFAs are capable of inhibiting cancer cell proliferation. PGE3, which is derived from COX-2-dependent biotransformation of the ω-3 EPA, is able to inhibit angiogenesis and cell migration characteristics (Szymczak, Murray & Petrovic, 2008). Epoxides of ω-3 PUFAs have also been found to inhibit proliferation and induce apoptosis in endothelial cells (Cui, Petrovic & Murray, 2011). The mechanism of action involved activation of the p38 MAPK and down-regulation of cyclin D1 (Cui,
Petrovic & Murray, 2011). Epoxide metabolites of the C22 ω-3 PUFA DHA were also anti-angiogenic (Zhang, Panigrahy, Mahakian, et al., 2013).

1.9. Aims of this project

Previous studies suggested that ω-3 PUFA metabolites have properties that could be useful for the development of anti-cancer agents. The epoxide formed at the unique ω-3 double bond in EPA, but not the isomeric epoxides, decreases the rate of cell proliferation and increases apoptosis (Cui, Petrovic & Murray, 2011). An important cellular target for ω-3 17,18-epoxy-EPA is cyclin D1; its down-regulation is mediated by activation of the p38 MAPK (Cui, Petrovic & Murray, 2011). Because the epoxide isomers activated cell growth, a series of ω-3 epoxides of long chain saturated fatty acids were synthesized for this project and evaluated for the ability to decrease the viability of breast cancer cells. Because epoxides are degraded and have low stability in vivo, stable bioisosteric urea analogues were also synthesized and tested as inhibitors of breast cancer proliferation. Breast cancer cell lines were selected for study because breast cancer is a major tumour type that is responsive to polyunsaturated fatty acid-based molecules.
2. General material and methods

2.1. Materials

2.1.1. Cell culture reagents

MDA-MB 231, MDA-MB 468, MCF-7 and T47D cells were purchased from American Type Culture Collection (Manassas, VA). HyClone® low glucose Dulbecco’s Modified Eagle’s Medium (DMEM), fetal bovine serum, L-glutamine, trypsin/EDTA, penicillin and streptomycin were from Thermo Fisher Scientific (Waltham, MA).

2.1.2. Antibodies

Anti-GAPDH, anti-DYKDDDDK Tag (anti-FLAG), anti-cyclin D1, anti-cyclin E, anti-Poly-(ADP-ribose) polymerase (PARP), anti-caspase 3, anti-caspase 9, anti-cytochrome c, anti-Bcl-2, anti-Bcl-XL, anti-Bax, anti-Bak, anti-Bid, anti-caspase 8, anti-JNK, anti-phospho-JNK, anti-p38 MAPK, anti-phospho-p38 MAPK, anti-PI3K, anti-phospho-PI3K, anti-MKK 3, anti-MKK 4, anti-MKK 6, anti-MKK 7, anti-phospho-MKK 7, anti-ASK1, anti-TRADD, anti-FADD, anti-TRAF2 and anti-GRB2 antibodies were purchased from Cell Signalling Technology (Beverly, MA). Anti-phospho-ASK1, anti-Crk-L, anti-Fas, anti-TNF-R1, anti-DR4 and anti-DR5 were purchased from Santa Cruz Biotechnology (Dallas, TX). IRDye conjugated goat anti-mouse, goat anti-rabbit and donkey anti-goat IgG secondary antibody were purchased from Li-Cor Biosciences (Lincoln, NE).
2.1.3. Molecular reagents

Opti-MEM ® 1 reduced serum medium was purchased from Life Technologies Australia Pty Ltd (Mulgrave, VIC). Dominant-negative (DN) mutants of p38 MAPK and JNK were generously provided by Dr. R.J. Davis, Howard Hughes Medical Institute, University of Massachusetts Medical School, Worcester, MA. DN mutants of M KK 3, M KK 6 and M KK 7 were generously provided by Dr Davis through Addgene (Cambridge, MA). Small interfering RNA (siRNA) directed against M KK 4, Bid, TRADD, FADD, TRAF2, GRB2, Crk-L, Fas, TNF-R1, DR4, DR5 (Santa Cruz), PI3K and Signal Silence control siRNA were from Cell Signalling Technology. X-tremeGENE HP DNA and X-tremeGENE siRNA and bovine serum albumin fraction V were purchased from Roche (Grenzach-Wyhlen, Germany).

2.1.4. Pharmacological inhibitors

The pan-caspase inhibitor z-VAD-fmk, necrostatin-1, 3-methyladenine and calpain inhibitor 1 were purchased from Cayman Chemical Company (Ann arbor, MI). SB203580, SP600125 and LY294002 were purchased from Alexis Biochemicals (through Sapphire Biosciences, Waterloo, NSW, Australia). NQDI1 and chloroquine diphosphate were purchased from Tocris Biosciences (Bristol, UK).

2.1.5. Electrophoresis reagents

Tween 20, glycine, sodium chloride, Tris, ammonium persulfate, sodium dodecyl sulphate and TEMED were from Amresco (Solon, OH). Whatman™ Protran BA83
0.2μm nitrocellulose transfer membrane was purchased from GE Healthcare Life Sciences (Rydalmere, NSW, Australia). Precision Plus Protein™ dual colour standards and 40% acrylamide/bis solution 29:1 were purchased from Bio-Rad (Richmond, CA). Dithiothreitol was purchased from Astral Scientific (Taren Point, NSW, Australia). Instant skim milk powder was purchased from local outlets.

2.1.6. Assay kits

Both CellTiter-Glo® Luminescent Cell Viability Assay and Caspase-Glo® 3/7 Assay were purchased from Promega (Annandale, NSW, Australia). BD Pharmingen™ annexin V-FITC apoptosis detection kit 1 was purchased from BD Biosciences (San Jose, CA).

2.1.7. Chemicals

Hydrochloric acid 32% was purchased from Merck (Whitehouse Station, NJ). Absolute ethanol was purchased from LabServ (Scoresby, VIC, Australia). Methanol was purchased from Fisher Scientific (Waltham, MA). Dimethyl sulfoxide (DMSO), Accustain® crystal violet solution, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)), bovine serum albumin, propidium iodide (PI), CaCl₂, glycerol, bromophenol blue, HEPES, trypan blue stain 0.4%, ribonuclease A (RNase A) were purchased from Sigma Aldrich (St. Louis, MO).
2.2. Methods

2.2.1. Synthesis of epoxides and ureas

ω-3 Epoxides (C20, C21 and C22) and ureas (C20, C21 and C22) and the ω-6 and ω-9 C20 epoxides were synthesized by Dr. Tristan Rawling (University of Technology - Sydney, Australia). Synthetic pathways are outlined in full in Appendix I. Briefly, epoxides C20-C22 were synthesized in the naturally occurring cis-configuration by epoxidation of the cis-ω-3 monounsaturated fatty acids with m-chloroperoxybenzoic acid. The ω-6 and ω-9 epoxides were synthesized similarly from the corresponding monounsaturated fatty acids. ω-3 Ureas C20-C22 were prepared in three-steps starting from the esterified nitriles. Selective reduction of the nitrile groups to amines was achieved using nickel boride, generated in situ using sodium borohydride and a catalytic amount of nickel (II) chloride. Reaction of the resulting crude amines with ethyl isocyanate afforded the ethyl ester ureas, which were then saponified to yield the ω-3 ureas.

2.2.2. Cell culture

MDA-MB 231, MDA-MB 468, MCF-7 and T47D cells were maintained in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. Cells were grown in a humidified incubator at 37 °C under an atmosphere of 5% CO₂. Cells were routinely passaged at 80-90% confluence and harvested by trypsinisation. Cells were stained with trypan blue and counted using a Countess automated cell counter (Invitrogen, Mount Waverley, VIC, Australia). Epoxides and ureas were dissolved in DMSO and stored at -20 °C.
2.2.3. Microscopy and imaging

Cells were seeded at a density of 7.5 x 10^4 cells/well in 24-well plates and allowed to adhere overnight. After serum starvation for 24 hours, cells were treated with various concentrations of the epoxides or ureas in serum-free DMEM for 48 and 120 hours. Medium was removed and cells were fixed with 500 µl of 95% ethanol for 10 minutes at -20 °C. Cells were then stained with 500 µl 1% crystal violet in 2% ethanol for 30 minutes at room temperature to enhance background contrast. Cells were then washed in water three times to remove residual stain. Multiple images of each well were taken using CKX41 inverted microscope (40x magnification) equipped with an Altra 20 camera (Olympus, Notting Hill, VIC, Australia) and analyzed with analySIS GetIT (Olympus) software.

2.2.4. Cell viability assays

2.2.4.1. MTT assay

The effect of epoxides and ureas on the viability of cells was assessed using the colorimetric assay for MTT reduction. Cells were seeded at a density of 10^4 cells/well in 96-well plates and 24 hours later were subject to serum starvation for a further 24 hours. Epoxides and ureas in serum-free DMEM were added at various concentrations and treatments were conducted for 24 and 48 hours. DMSO alone was used as the treatment control in all experiments. At the end of the treatments, 25 µl of MTT solution (2.5 mg/ml) was added to each well. Two hours later the purple formazan product was dissolved in 100 µl DMSO. Absorbance was measured at 540 nm using a Victor 3V 1420 multilabel counter.
(Perkin Elmer, Akron, OH). Percentage of inhibition is calculated from the difference between treatment and control absorbance.

2.2.4.2. ATP assay
ATP formation in cells was determined using the CellTiter-Glo® Luminescent Cell Viability Assay. Cells were seeded at a density of $10^4$ cells/well in 96-well plates and 24 hours later were serum starved for a further 24 hours. Epoxides and ureas in serum-free DMEM were added at various concentrations and treatments were conducted for 48 and 120 hours. DMSO alone was used as the treatment control in all experiments. Following treatment, the plates were allowed to equilibrate at room temperature for 30 minutes and 100 µl of the CellTiter-Glo® reagent was added to each well. Cells were lysed on an orbital shaker for 2 minutes. The plates were incubated at room temperature for 10 minutes. Luminescence readings were recorded using a Victor 3V 1420 multilabel counter. Percentage of inhibition is calculated from the difference between treatment and control absorbance.

2.2.5. Caspase-3/7 activity assay
Caspase-3 activity was determined using the Caspase-Glo® 3/7 Assay. Cells were seeded at a density of $10^4$ cells/well in 96-well plates and 24 hours were serum starved for a further 24 hours. Epoxides and ureas were added to cells in serum-free DMEM at various concentrations and treatments were carried out for 24 and 48 hours. Following treatment, the plates were allowed to equilibrate at room temperature for 30 minutes and 100µl of the Caspase-Glo® 3/7 reagent was
added to each well. After lysis and mixing on an orbital shaker for 30 seconds, plates were incubated at room temperature for 30 minutes. Luminescence readings were recorded using a Victor 3V 1420 multilabel counter.

2.2.6. Transfection of siRNAs and DN-negative plasmids

Cells were seeded at a density of $15 \times 10^4$ cells/well in 6-well plates and allowed to adhere overnight. Using XtremeGENE HP DNA or XtremeGENE siRNA, 1 µg of DN mutant or 1 µg siRNA, respectively, was mixed in Opti-MEM to form a transfection complex. After 20 minutes, the complex was added to cells in DMEM with fetal bovine serum, but not penicillin or streptomycin. Four hours later, the transfection medium was replaced with serum-free DMEM. After 24 hours of serum starvation, the cells were treated with various concentrations of test compounds for 48 hours.

2.2.7. Pre-treatment with pharmacological inhibitors

All inhibitors (z-VAD-fmk 10 µM, 20 µM and 40 µM; necrostatin-1 10 µM, 20 µM and 40 µM; 3-methyladenine 1 mM, 5 mM and 10 mM; chloroquine 1 µM, 10 µM and 20 µM, NQDI-1 5 µM and 10 µM, SB203580 10 µM and 20 µM, SP600125 10 µM and 20 µM, and LY294002 10 µM and 20 µM) were diluted to the required concentration using serum-free DMEM. The inhibitors were added to the cells two hours prior to treatment with epoxides; an equivalent volume of solvent was added to control cells.
2.2.8. Sodium dodecyl sulphate (SDS)/polyacrylamide gel electrophoresis and Western blotting

Cells were seeded at a density of 15 x 10⁴ cells/well in 6-well plates and allowed to adhere overnight. Cells were serum starved for another 24 hours. The cells then were treated with various concentrations of test compounds for 24, 48 or 120 hours. Following treatment, cells were washed with phosphate buffered saline (PBS) and lysed in Laemmli buffer (62.5 mM Tris-HCl, pH 6.8; 2% (w/v) SDS; 25% glycerol; 0.01% bromophenol blue; 350mM Dithiothreitol). Cell lysates were sonicated for 10 seconds and then heated for 5 minutes at 100°C. Equal amounts of proteins were loaded and separated on sodium dodecylsulphate-polyacrylamide gels before transfer to nitrocellulose membrane. Membranes were blocked with 5% non-fat dry milk or bovine serum albumin in Tris buffered saline-Tween 20 (TBS-T) buffer (10mM Tris; 100mM NaCl; 0.1% Tween 20) for 1 hour at room temperature. After three washes with TBS-T (5 minutes) on a shaker, blots were washed in TBS-T and then incubated overnight at 4 °C with primary antibodies. Detection was performed using IRDye conjugated goat anti-mouse or goat anti-rabbit IgG secondary antibody at 1:10000 dilution for 1 hour at room temperature and analyzed using the Odyssey Infrared Imaging System (Li-Cor Biosciences).

2.2.9. Sub-G1 and cell cycle analysis

Apoptotic cells (sub-G1) and the distribution of cells in different phases of the cell cycle were evaluated by determining the DNA content after propidium iodide staining. Cells were seeded at a density of 15 x 10⁴ cells/well in 6-well plates and
allowed to adhere overnight. After serum starvation for 24 hours, the cells were treated with various concentrations of epoxide and ureas in serum-free DMEM for 24, 48 or 120 hours. Cells were harvested by trypsinisation and washed twice with PBS. After centrifugation at 300g and 20°C for 5 minutes, cells were fixed using 80% ethanol overnight. For sub-G1 analysis, cells were washed again with PBS before centrifugation at 1200g and 20°C for 5 minutes and were then resuspended in 200µl incubation buffer (100µg/ml DNase-free RNase A; 0.1% Nonidet NP40). Cells were then incubated in the dark for 1 hour at room temperature with propidium iodide (final concentration 50µg/ml). Samples were analyzed in a FC 500 flow cytometer and using MXP software (Beckman Coulter Australia, Lane Cove, NSW).

2.2.10. Annexin V binding assay

Cells were seeded at a density of 15 x 10⁴ cells/well in 6-well plates and allowed to adhere overnight. After serum starvation for 24 hours, the cells were treated with various concentrations of epoxides and ureas in serum-free DMEM for 24 or 48 hours. Cells were harvested by trypsinisation using EDTA-free trypsin and washed twice with PBS. After centrifugation at 300g and 20°C for 5 minutes, cells were resuspended in 200µl Annexin V binding buffer (10mM HEPES, pH 7.4; 0.14M NaCl; 2.5mM CaCl₂). Annexin V-FITC and propidium iodide were added to the cells according to the manufacturer's protocol and cells were incubated for 15 minutes at room temperature in the dark. Samples were analyzed within 1 hour in a FC 500 flow cytometer and using MXP software (Beckman Coulter).
2.2.11. Statistical analysis

Data were derived in experiments that were conducted at least in triplicate, unless otherwise stated, and are presented throughout as mean ± SD. Data from multiple treatments were analyzed by one-way ANOVA in combination with Fisher’s Protected Least Significant Difference test to locate differences between groups. The p-value below 0.05 is considered as significant.
3. Synthetic ω-3 epoxyfatty acids as novel anti-proliferative agents in human breast cancer cells

3.1. Background

Evidence from epidemiological observations and experimental studies suggests that ω-3 PUFA have potential as anti-cancer agents. The ω-3 PUFA EPA decreases the growth and development of breast tumors, while their ω-6 PUFA counterparts enhance tumorigenesis (Rose, Connolly, Rayburn, et al., 1995; Rose & Connolly, 2000; Simopoulos, 2002b; Chajès, Torres-Mejía, Biessy, et al., 2012). Although the mechanisms by which PUFAs modulate tumor progression have not been fully elaborated, several studies suggest that PUFA-derived eicosanoid metabolites may be important contributors (Tapiero, Ba, Couvreur, et al., 2002; Szymczak, Murray & Petrovic, 2008; Cui, Petrovic & Murray, 2011).

In cells COX, LO and CYP enzymes convert the ω-6 PUFA AA to eicosanoids, some of which, such as PGE₂ and the CYP-derived EETs, enhance tumor growth (Chen, Capdevila & Harris, 2001; Jiang, Chen, Card, et al., 2005). EETs promote the neoplastic phenotype of carcinoma cells by activating proliferation and cyclin D1 expression, and by inhibiting apoptosis (Potente, Michaelis, Fisslthaler, et al., 2002; Jiang, Chen, Card, et al., 2005). Although less intensively studied, the corresponding epoxides formed from ω-3 PUFAs have different biological potencies.
Studies have revealed the biotransformation of ω-3 PUFAs to certain metabolites may enhance anti-cancer activity. It was found recently that the CYP-mediated ω-3-17,18-epoxy-EPA impaired endothelial cell proliferation by down-regulating cyclin D1, preventing cell cycle progression through S-phase and activating apoptosis (Cui, Petrovic & Murray, 2011). However, these actions were specific to ω-3-17,18-epoxy-EPA because the regioisomeric epoxides formed at the alternate ω-3 olefinic bonds in EPA stimulated proliferation and did not activate apoptosis. The present chapter evaluated the activity of molecules that were designed to keep the anti-proliferative and pro-apoptotic actions of ω-3-17,18-epoxy-EPA whilst minimizing the potential protumourigenic effects of the regioisomeric epoxides. These were the novel ω-3 epoxides of long chain saturated fatty acids (C20-C22) (Refer to Appendix A; Scheme A-1).

Several studies have shown that epoxide bioisosteres retain the pharmacological activity of ω-6 EETs but have greater metabolic stability (Falck, Kodela, Manne, et al., 2009; Falck, Wallukat, Puli, et al., 2011). Therefore, urea bioisosteres that corresponded to the ω-3 epoxyfatty acids were also prepared and evaluated in this chapter (Refer to Appendix A; Scheme A-2).

In cancer cells, pathways that control the proliferation in normal cells are dysregulated (Evan & Vousden, 2001). Hence, uncontrolled cell proliferation is one of the major features that distinguishes cancer cells from normal cells (Sandal, 2002). Cell proliferation begins with DNA replication, followed by nuclear division to yield a pair of daughter cells (Massagué, 2004). The cell cycle consists of mitosis (or M phase) and interphase (Figure 1-13) (Vermeulen, Van Bockstaele & Berneman, 2003). Mitosis is the process of nuclear division, while interphase is the time between M phases and consists of G1, S and G2 phases.
(Norbury & Nurse, 1992). Cells in G₁ can commit to DNA replication or enter a resting state called G₀; non-proliferating cells are in G₀ phase (Vermeulen, Van Bockstaele & Berneman, 2003).

Progression between phases is controlled by regulatory cyclin proteins, that are synthesized and degraded at specific times during the cell cycle (Pines, 1991; Vermeulen, Van Bockstaele & Berneman, 2003; Malumbres & Barbacid, 2009). It has also been reported that cyclin D1 is over-expressed in many human cancers; together, cyclins D1 and E are rate limiting for cell cycle progression (Vermeulen, Van Bockstaele & Berneman, 2003). The CYP-mediated ω-3 epoxide of EPA has been found to down-regulate cyclin D1 (Cui, Petrovic & Murray, 2011).

The cell cycle is energy-dependent, and a decrease in ATP production prevents proliferation (Petty, Sutherland, Hunter, et al., 1995; DeBerardinis, Mancuso, Daikhin, et al., 2007). The studies in this chapter characterized the anti-proliferative actions of the synthetic ω-3 epoxides and their urea bioisosteres in breast cancer cells.

3.2. Results

3.2.1. Effects of ω-3 epoxides on the proliferation of breast cancer cells

3.2.1.1. Microscopy
Epoxide cytotoxicity was observed as a change in cell confluence of four breast cancer cell lines: MDA-MB-231, MDA-MB-468, MCF-7 and T47D.
Figure 3-1: Cytotoxicity of epoxides in MDA-MB-231 breast cancer cells. A) 48-hour treatment B) 120-hour treatment. (Microscopy imaging with 40x magnification)
Treatment of MDA-MB-231 cells with epoxides decreased cell confluence in a concentration- and time-dependent manner (Figure 3-1A and 3-1B). Decreased MDA-MB-231 cell confluence (48 hours) (Figure 3-1A) was enhanced by treatment with the epoxides for 120 hours (Figure 3-1B). The C22 epoxide also decreased confluence but was less effective than the other epoxides (Figure 3-1A and 3-1B).

The C20 epoxide also decreased the confluence of MDA-MB-468 cells, although not to the same extent as in MDA-MB-231 cells (Figure 3-2A and 3-2B). Treatment with the C20 epoxide (10 µM; 48 hours) decreased confluence compared to control, but was not enhanced by longer treatments (10 µM; 120 hours). Decreased confluence was more pronounced at higher concentrations of the C20 epoxide (40 µM; 48 hours) that was further accentuated at 120 hours. The C21 and C22 epoxides (10 µM; 48 hours and 120 hours) were less effective. A decrease was observed at 40 µM but longer treatment over 120 hours did not increase this effect.
Figure 3-2: Cytotoxicity of epoxides in MDA-MB-468 breast cancer cells. A) 48-hour treatment B) 120-hour treatment. (Microscopy imaging with 40x magnification)
Figure 3-3: Cytotoxicity of epoxides in MCF-7 breast cancer cells. A) 48-hour treatment  B) 120-hour treatment. (Microscopy imaging with 40x magnification)
In contrast with findings in MDA-MB-231 and MDA-MB-468 cells, the epoxides did not markedly decrease confluence in MCF-7 cells, even at higher concentrations or for longer treatments (Figure 3-3A; Figure 3-3B). Similarly, the confluence of T47D cells was unresponsive to the epoxides at any concentration and at either time point (Figure 3-4A and 3-4B).

Thus, the C20-C22 epoxides exhibited different capacities to decrease the confluence of MDA-MB-231, MDA-MB-468, MCF-7 and T47D breast cancer cells. The most aggressive MDA-MB-231 cells were more susceptible to the epoxides, MDA-MB-468 cells were also sensitive, but less so than their more aggressive counterparts. The hormone-responsive MCF-7 and T47D lines were essentially resistant to the epoxides.
Figure 3-4: Cytotoxicity of epoxides in T47D breast cancer cells. A) 48-hour treatment  B) 120-hour treatment. (Microscopy imaging with 40x magnification)
3.2.1.2. MTT reduction

The anti-proliferative effects of the epoxides were tested in the same four breast cancer cell lines. The MTT assay was used for this purpose. This assay determines the intracellular reduction of a yellow tetrazolium salt to an insoluble purple formazan in viable cells by dehydrogenase enzymes.

![Figure 3-5: Effect of epoxides on MDA-MB-231 cell viability based on MTT assay at 24 hours.](image)

Data are mean ± SD of 3 independent experiments. Different from control: * p<0.05, ** p<0.01 and *** p<0.001.
Figure 3-6: Effect of epoxides on MDA-MB-231 cell viability based on MTT assay at 48 hours. Data are mean ± SD of 3 independent experiments. Different from control: *** p<0.001.

Treatment of MDA-MB-231 cells with all epoxides at concentrations of 1 and 10 µM (24 hours) decreased cell viability (Figure 3-5). Similar effects were noted after 48 hours of treatment: at 50 µM concentrations decreases in cell viability were to 29 ± 2%, 47 ± 2% and 47 ± 2% of control, respectively (Figure 3-6).

Treatment of the less aggressive MDA-MB-468 cells with epoxides (1 µM and 10 µM; 24 hours) also altered cell viability (Figure 3-7). At 50 µM concentrations the epoxides decreased cell viability to 38 ± 5%, 37 ± 1% and 42 ± 1% of control, respectively (Figure 3-7). Corresponding treatments with 50 µM epoxide for 48 hours decreased cell viability to 27 ± 6%, 29 ± 7% and 24 ± 1% of control, respectively (Figure 3-8).
Figure 3-7: Effect of epoxides on MDA-MB-468 cell viability based on MTT assay at 24 hours. Data are mean ± SD of 3 independent experiments. Different from control: ** p<0.01 and *** p<0.001.

Figure 3-8: Effect of epoxides on MDA-MB-468 cell viability based on MTT assay at 48 hours. Data are mean ± SD of 3 independent experiments. Different from control: ** p<0.01 and *** p<0.001.
Figure 3-9: Effect of epoxides on MCF-7 cell viability based on MTT assay at 24 hours. Data are mean ± SD of 3 independent experiments. Different from control: * p<0.05 and *** p<0.001.

Figure 3-10: Effect of epoxides on MCF-7 cell viability based on MTT assay at 48 hours. Data are mean ± SD of 3 independent experiments. Different from control: *** p<0.001.
Treatment of estrogen-dependent MCF-7 cells with epoxides (1, 10 and 50 μM; 24 hours) slightly decreased MTT reduction, but this did not appear to be concentration dependent (Figure 3-9). Longer treatments (1, 10 and 50 μM; 48 hours) were slightly more effective (Figure 3-10). At the 50 μM concentration, 48 hours of epoxide treatment decreased MTT reduction to 52 ± 1%, 62 ± 6% and 56 ± 1% of control, respectively.

**Figure 3-11:** Effect of epoxides on T47D cell viability based on MTT assay at 24 hours. Data are mean ± SD of 3 independent experiments. Different from control: * p<0.05, ** p<0.01 and *** p<0.001.
Figure 3-12: Effect of epoxides on T47D cell viability based on MTT assay at 48 hours. Data are mean ± SD of 3 independent experiments. Different from control: ** p<0.01 and *** p<0.001.

T47D cells were less responsive to the epoxides. At 24 hours decreases in MTT reduction were unrelated to concentration (Figure 3-11); decreases were slightly more pronounced after 48 hours of treatment (Figure 3-12).

Overall the epoxides decreased the viability, as reflected by MTT reduction, especially in MDA-MB-231 and MDA-MB-468 cells. The hormone-dependent MCF-7 and T47D lines were less responsive. These findings were in accord with assessments from cell confluence experiments.

3.2.1.3. ATP formation

Cellular ATP formation was estimated because this may more closely indicate cell viability. The effects of the epoxides on viability were again assessed in the four cell lines, MDA-MB-231, MDA-MB-468, MCF-7 and T47D.
Figure 3-13: Effect of epoxides on MDA-MB-231 cell viability based on ATP production at 48 hours. Data are mean ± SD of 3 independent experiments. Different from control: * p<0.05, ** p<0.01 and *** p<0.001.

Figure 3-14: Effect of epoxides on MDA-MB-231 cell viability based on ATP production at 120 hours. Data are mean ± SD of 3 independent experiments. Different from control: * p<0.05 and *** p<0.001.
All epoxides (C20-C22) effectively decreased ATP production in MDA-MB-231 cells in a concentration- and time-dependent fashion consistent with the impairment of energy metabolism and viability (Figure 3-13 and Figure 3-14). At the highest concentration the C20 epoxide (40 µM) in particular decreased ATP formation to 45 ± 5% of control after 48 hours, that was considerably more pronounced after 120 hours of treatment (3.2 ± 1% of control; Figure 3-14). The C21 and C22 epoxides both decreased ATP production to 72 ± 6% and 78 ± 6% of control, respectively, at 48 hours and to 35 ± 15% and 66 ± 26% of control, respectively, at 120 hours.

Figure 3-15: Effect of epoxides on MDA-MB-468 cell viability based on ATP production at 48 hours. Data are mean ± SD of 3 independent experiments. Different from control: * p<0.05, ** p<0.01 and *** p<0.001.
The C20 epoxide decreased ATP production in MDA-MB-468 cells most effectively. At the 40 µM concentration ATP production was decreased to 65 ± 10% of control (48 hours; Figure 3-15) and to 50 ± 20% of control (120 hours; Figure 3-16). However, the C21 and C22 epoxides minimally decreased ATP production at 48 and 120 hours (Figure 3-15 and Figure 3-16). Longer treatment times (120 hours) with lower concentrations (1 and 10 µM) of epoxides did not appear to enhance these effects (Figure 3-16).

The C20-C22 epoxides also decreased ATP production in MCF-7 cells in a concentration-dependent manner (1, 10 and 50 µM; 48 hours) (Figure 3-17). At the 40 µM concentration the C20, C21 and C22 epoxides (48 hours) decreased ATP formation to 57 ± 4%, 59 ± 3% and 55 ± 6% of control, respectively. Longer treatment times (120 hours) did not appear to enhance these effects (Figure 3-17).
At 120 hours the C20, C21 and C22 epoxides (40 μM) decreased ATP formation to 58 ± 5%, 63 ± 3% and 61 ± 9% of control, respectively.

Figure 3-17: Effect of epoxides on MCF-7 cell viability based on ATP production at 48 hours. Data are mean ± SD of 3 independent experiments. Different from control: *** p<0.001.
Figure 3-18: Effect of epoxides on MCF-7 cell viability based on ATP production at 120 hours. Data are mean ± SD of 3 independent experiments. Different from control: ** p<0.01 and *** p<0.001.

Figure 3-19: Effect of epoxides on T47D cell viability based on ATP production at 48 hours. Data are mean ± SD of 3 independent experiments. Different from control: ** p<0.01 and *** p<0.001.
In T47D cells the C20-C22 epoxides (40 µM, 48 hours) decreased ATP formation somewhat to 65 ± 6%, 70 ± 7% and 70 ± 6% of control, respectively (Figure 3-19), but this had diminished by 120 hours (Figure 3-20).

In summary, in agreement with impaired confluence and MTT reduction, ATP production was responsive to the epoxides in the MDA-MB-231 cell line. Some treatments were also effective in MDA-MB-468 cells, but MCF-7 and T47D cells were partially responsive to the epoxides.

3.2.2. Effects of ω-3 urea bioisosteres on the proliferation of breast cancer cells

3.2.2.1. Microscopy

The effects of the urea isosteres on the confluence of MDA-MB-231, MDA-MB-468, MCF-7 and T47D cells was also assessed. Treatment with ureas (C20-C22)
minimally decreased the confluence of MDA-MB-231 cells at 48 hours (Figure 3-21A) and at 120 hours (Figure 3-21B).

Ureas (C20-C22) were also less effective than the epoxides against MDA-MB-468 cells at both 48 hours and 120 hours. No apparent decrease in cell confluence was observed (Figure 3-22A and Figure 3-22B).

Similarly, treatment with ureas for 48 or 120 hours minimally altered the confluence of MCF-7 cells (Figure 3-23A; Figure 3-23B) or T47D cells (Figure 3-24A and Figure 3-24B).
Figure 3-21: Cytotoxicity of ureas in MDA-MB-231 breast cancer cells. A) 48-hour treatment B) 120-hour treatment. (Microscopy imaging with 40x magnification)
Figure 3-22: Cytotoxicity of ureas in MDA-MB-468 breast cancer cells. A) 48-hour treatment B) 120-hour treatment. (Microscopy imaging with 40x magnification)
Figure 3-23: Cytotoxicity of ureas in MCF-7 breast cancer cells. A) 48-hour treatment B) 120-hour treatment. (Microscopy imaging with 40x magnification)
Figure 3-24: Cytotoxicity of ureas in T47D breast cancer cells. A) 48-hour treatment  B) 120-hour treatment. (Microscopy imaging with 40x magnification)
3.2.2.2. MTT reduction

To further evaluate possible effects of the urea isosteres on viability their effects on MTT reduction was assessed in MDA-MB-231, MDA-MB-468, MCF-7 and T47D cells. In MDA-MB-231 cells the ureas significantly decreased MTT reduction at 24 and 48 hours (Figure 3-25 and Figure 3-26). Decreases in MTT reduction to 29 ± 3%, 33 ± 4% and 30 ± 5% of control were produced, respectively, by the C20, C21 and C22-ureas 50 µM at 24 hours (Figure 3-25). Similar decreases were recorded at 48 hours (C20: 29 ± 3%; C21: 34 ± 4%; C22: 32 ± 2% of control, respectively) (Figure 3-26).

![Figure 3-25: Effect of ureas on MDA-MB-231 cell viability based on MTT assay at 24 hours.](image-url)

Data are mean ± SD of 3 independent experiments. Different from control: *** p<0.001.
Figure 3-26: Effect of ureas on MDA-MB-231 cell viability based on MTT assay at 48 hours. Data are mean ± SD of 3 independent experiments. Different from control: *** p<0.001.

Figure 3-27: Effect of ureas on MDA-MB-468 cell viability based on MTT assay at 24 hours. Data are mean ± SD of 3 independent experiments. Different from control: *** p<0.001.
Figure 3-28: Effect of ureas on MDA-MB-468 cell viability based on MTT assay at 48 hours. Data are mean ± SD of 3 independent experiments. Different from control: *** p<0.001.

MTT reduction was also decreased in MDA-MB-468 cells after treatment with the ureas. Maximal decreases to 22 ± 2%, 29 ± 1% and 27 ± 3% of control were produced, respectively, by the C20, C21 and C22-ureas 50 µM at 24 hours (Figure 3-27). Similar decreases were recorded at 48 hours (C20: 27 ± 2%; C21: 29 ± 3%; C22: 24 ± 1% of control, respectively) (Figure 3-28).

In MCF-7 cells, the ureas also decreased MTT reduction (Figure 3-29 and Figure 3-30). At the 50 µM concentration the C20-C22 ureas decreased MTT reduction at 24 hours to 52 ± 1%, 60 ± 3% and 51 ± 2% of control, respectively. The decreases were more pronounced after 48 hours of treatment with C20-C22 ureas; at 50 µM the inhibition of MTT reduction was to 38 ± 3%, 43 ± 3% and 38 ± 3% of control, respectively.
Figure 3-29: Effect of ureas on MCF-7 cell viability based on MTT assay at 24 hours. Data are mean ± SD of 3 independent experiments. Different from control: *** p<0.001.

Figure 3-30: Effect of ureas on MCF-7 cell viability based on MTT assay at 48 hours. Data are mean ± SD of 3 independent experiments. Different from control: *** p<0.001.
Figure 3-31: Effect of ureas on T47D cell viability based on MTT assay at 24 hours. Data are mean ± SD of 3 independent experiments. Different from control: * p<0.05, ** p<0.01 and *** p<0.001.

Figure 3-32: Effect of ureas on T47D cell viability based on MTT assay at 48 hours. Data are mean ± SD of 3 independent experiments. Different from control: *** p<0.001.
MTT reduction in T47D cells was decreased by the C20-C22 ureas but to a lesser extent than seen in the other cell lines (Figure 3-31 and Figure 3-32).

In summary, the C20-C22 ureas decreased MTT reduction in all cell lines. MDA-MB-231 and MDA-MB-468 cells were most sensitive while MCF-7 and T47D cells were less responsive.

3.2.2.3. ATP assay

The effects of the ureas on ATP production by the MDA-MB-231, MDA-MB-468, MCF-7 and T47D cell lines were assessed.

![Graph showing ATP production](image)

**Figure 3-33: Effect of ureas on MDA-MB-231 cell viability based on ATP production at 48 hours.** Data are mean ± SD of 3 independent experiments. Different from control: *p<0.05, and **p<0.01.
Figure 3-34: Effect of ureas on MDA-MB-231 cell viability based on ATP production at 120 hours. Data are mean ± SD of 3 independent experiments.

The C20-C22 ureas were less effective than the corresponding epoxides in decreasing ATP production in MDA-MB-231 cells. At the highest concentration (40 µM) the C20-C22 ureas (48 hours) decreased ATP production to 72 ± 10%, 72 ± 8% and 78 ± 12% of control, respectively (Figure 3-33), although these effects were diminished at 120 hours (Figure 3-34).

In MDA-MB-468 cells, 48 hours of treatment with the C22 urea produced minor decreases in ATP formation (Figure 3-35), but effects were again diminished by 120 hours (Figure 3-36).
Figure 3-35: Effect of urea on MDA-MB-468 cell viability based on ATP production at 48 hours. Data are mean ± SD of 3 independent experiments: * p<0.05.

Figure 3-36: Effect of urea on MDA-MB-468 cell viability based on ATP production at 120 hours. Data are mean ± SD of 3 independent experiments.
Figure 3-37: Effect of ureas on MCF-7 cell viability based on ATP production at **48 hours**. Data are mean ± SD of 3 independent experiments. Different from control: ** p<0.01 and *** p<0.001.

Figure 3-38: Effect of ureas on MCF-7 cell viability based on ATP production at **120 hours**. Data are mean ± SD of 3 independent experiments. Different from control: * p<0.05 and ** p<0.01.
The ureas decreased ATP production in MCF-7 cells but to a lesser extent than in MDA-MB-231 and MDA-MB-468 cells after 48 hours (Figure 3-37) and 120 hours of treatment (Figure 3-38). T47D cells were unresponsive to the ureas after 48 hours and 120 hours of treatment (Figure 3-39 and Figure 3-40).

In summary, like the C20-C22 epoxides, the C20-C22 ureas exhibited different capacities to decrease ATP formation in the different breast cancer cell lines.

![Bar chart showing luminescence (RLU) at different concentrations of ureas](image)

**Figure 3-39: Effect of ureas on T47D cell viability based on ATP production at 48 hours.** Data are mean ± SD of 3 independent experiments.
Figure 3-40: Effect of ureas on T47D cell viability based on ATP production at 120 hours. Data are mean ± SD of 3 independent experiments.

3.2.3. Effects of isomeric ω-3, ω-6 and ω-9 epoxyfatty acids on ATP production in breast cancer cells

To confirm the importance of the ω-3 epoxy moiety in decreasing cell viability, the corresponding ω-6 and ω-9 epoxides of the C20 saturated fatty acid (eicosanoic acid) were synthesized and tested (Refer to Appendix A; Scheme A-1). The possibility that epoxide activity might be decreased by soluble epoxide hydrolase-mediated biotransformation was also assessed in coinubcation experiments with the inhibitor 12-((3-adamantan-1-yl-ureido)dodecanoic acid (AUDA) (Dorrance, Rupp, Pollock, et al., 2005).
Figure 3-41: Effect of the ω-3 epoxide with and without AUDA (10 μM) on MDA-MB-231 cell viability based on ATP production at 48 hours. Data are mean ± SD of 3 independent experiments. Different from control: * p<0.05 and *** p<0.001.

Figure 3-42: Effect of the ω-6 epoxide with and without AUDA (10 μM) on MDA-MB-231 cell viability based on ATP production at 48 hours. Data are mean ± SD of 3 independent experiments.
Figure 3-43: Effect of the ω-9 epoxide with and without AUDA (10 µM) on MDA-MB-231 cell viability based on ATP production at 48 hours. Data are mean ± SD of 3 independent experiments.

Consistent with earlier findings, the ω-3 C20 epoxide decreased MDA-MB-231 cell viability (Figure 3-41). The epoxide hydrolase inhibitor AUDA did not markedly influence ATP formation in control cells, but slightly enhanced the apparent cytotoxicity of the ω-3 epoxide. In contrast, the ω-6 and ω-9 epoxides appeared to increase ATP formation, although non-significantly (Figure 3-42 and Figure 3-43); this was unaffected by AUDA.

3.2.4. Effects of C20-C22 epoxides and ureas on the cell cycle distribution in breast cancer cells

Cell proliferation is regulated by the cell cycle which can be followed by flow cytometry, which detects the binding of the DNA stain propidium iodide, so that cells may be divided into 3 major phases: G0/G1, S and G2/M phases. During G1
the cell prepares for DNA synthesis, DNA replication occurs in S phase and cells prepare for mitosis in G_2 phase. Cells in G_1 can commit to DNA replication or exit the cell cycle in G_0 phase. Non-proliferating cells are in G_0 phase.

In MDA-MB-231 cells, the C20 epoxide produced cell cycle arrest in G_0/G_1 phase in a concentration- and time-dependent manner (Figure 3-44). At 25 µM and 50 µM the increases in G_0/G_1 population were respectively 11 \pm 5\% and 13 \pm 4\% greater than in control (Figure 3-45). There were corresponding decreases in S and G_2/M phases that were more pronounced after 48 hours (Figure 3-46).
Figure 3-44: Cell cycle analysis of MDA-MB-231 cells treated with the C20 epoxide: concentration- and time-dependence.
Figure 3-45: Effect of the C20 epoxide on cell cycle distribution in MDA-MB-231 cells at 24 hours. Data are mean ± SD of 3 independent experiments. Different from control: *** p<0.001.

Figure 3-46: Effect of the C20 epoxide on cell cycle distribution in MDA-MB-231 cells at 48 hours. Data are mean ± SD of 3 independent experiments. Different from control: * p<0.05, p<0.01 and *** p<0.001.
In further studies, the treatment of MDA-MB-231 cells with C20-C22 epoxides altered the distribution of cells throughout phases of the cell cycle. Thus, the proportion of cells in G₀/G₁ phase was increased by 14 ± 5%, 11 ± 4% and 10 ± 7% over control by the C20-C22 epoxides (40 µM; Figure 3-47), while those in S- and G₂/M phases decreased; this is consistent with failure to complete the cell cycle.

Figure 3-47: Effect of epoxides (40 µM) on cell cycle distribution in MDA-MB-231 cells at 48 hours. Data are mean ± SD of 3 independent experiments. Different from control: *p<0.05, **p<0.01 and ***p<0.001.

There was a non-significant effect of epoxides on the cell cycle distribution in MDA-MB-468. The C20 epoxide minimally increased G₀/G₁ phase by 6 ± 5% over control (Figure 3-48). The small changes in G₀/G₁, S and G₂/M phase also did not reach statistical significance (Figure 3-48).
Figure 3-48: Effect of epoxides (40 µM) on cell cycle distribution in MDA-MB-468 cells at 48 hours. Data are mean ± SD of 3 independent experiments.

Figure 3-49: Effect of epoxides (40 µM) on cell cycle distribution in MCF-7 cells at 48 hours. Data are mean ± SD of 3 independent experiments.

MCF-7 and T47D cell lines were less responsive to treatments with the epoxides. No significant changes were observed in cell cycle distribution in treated cells (Figure 3-49 and Figure 3-50).
Figure 3-50: Effect of epoxides (40 µM) on cell cycle distribution in T47D cells at 48 hours. Data are mean ± SD of 3 independent experiments.

Table 3-1: Summary: effects of epoxides on cell cycle phases in breast cancer cells.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Effect on cell cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA-MB-231</td>
<td>C20-C22 epoxides increase G0/G1 and decrease S and G2/M</td>
</tr>
<tr>
<td>MDA-MB-468</td>
<td>Only the C20 epoxide increases G0/G1 with minimal changes in S and G2/M</td>
</tr>
<tr>
<td>MCF-7</td>
<td>No significant effects</td>
</tr>
<tr>
<td>T47D</td>
<td>No significant effects</td>
</tr>
</tbody>
</table>

The effects of the epoxides in breast cancer cell lines are summarized in Table 3-1. The most aggressive cell line MDA-MB-231 was markedly altered by
the epoxides with significant increases in $G_0/G_1$ phase, and decreases in $S$ and $G_2/M$ phases. MDA-MB-468 cell cycle distribution was only affected by the C20 epoxide and not the C21 or C22 epoxides. The cell cycle distribution in MCF-7 and T47D cells was unaffected by the epoxides.

![Graph showing cell cycle distribution](image)

Figure 3-51: Effect of ureas (40 µM) on cell cycle distribution in MDA-MB-231 cells at 48 hours. Data are mean ± SD of 3 independent experiments.

Treatment of MDA-MB-231 cells with the C20-C22 ureas did not alter the cell cycle distribution from control (Figure 3-51). Longer treatment over 120 hours with C21 and C22 ureas produced small but significant increases in $G_0/G_1$ phase to $11 ± 4\%$ and $11 ± 7\%$ of control, respectively (Figure 3-52). $G_2/M$ phase was decreased correspondingly by the C21 and C22 ureas.
Figure 3-52: Effect of ureas (40 μM) on cell cycle distribution in MDA-MB-231 cells at 120 hours. Data are mean ± SD of 3 independent experiments. Different from control: **p<0.01 and *** p<0.001.

Figure 3-53: Effect of ureas (40 μM) on cell cycle distribution in MDA-MB-468 cells at 48 hours. Data are mean ± SD of 3 independent experiments.
Figure 3-54: Effect of ureas (40 μM) on cell cycle distribution in MDA-MB-468 cells at 120 hours. Data are mean ± SD of 3 independent experiments.

C20-C22 ureas did not alter cell cycle distribution in MDA-MB-468 cells after treatment for 48 hours (Figure 3-53) or 120 hours (Figure 3-54).

Figure 3-55: Effect of ureas (40 μM) on cell cycle distribution in MCF-7 cells at 48 hours. Data are mean ± SD of 3 independent experiments.
Figure 3-56: Effect of ureas (40 µM) on cell cycle distribution in MCF-7 cells at 120 hours. Data are mean ± SD of 3 independent experiments. Different from control: *p<0.05.

Figure 3-57: Effect of ureas (40 µM) on cell cycle distribution in T47D cells at 48 hours. Data are mean ± SD of 3 independent experiments.
Figure 3-58: Effect of ureas (40 µM) on cell cycle distribution in T47D cells at 120 hours. Data are mean ± SD of 3 independent experiments.

In MCF-7 cells the cell cycle distribution was unchanged by urea treatment for 48 hours (Figure 3-55) but there were small increases to 8 ± 3 % and 9 ± 1% of control in G₀/G₁ phase with the C21 and C22 urea, respectively (Figure 3-56). In T47D cells no changes were evident at 48 or 120 hours (Figure 3-57 and 3-58).
### Table 3-2: Summary: effects of ureas on the cell cycle in breast cancer cells.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Effect on cell cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA-MB-231</td>
<td>C21 and C22 ureas increase G₀/G₁ and decrease G₂/M phase only (120 hours)</td>
</tr>
<tr>
<td>MDA-MB-468</td>
<td>No significant effects</td>
</tr>
<tr>
<td>MCF-7</td>
<td>C21 and C22 ureas increase G₀/G₁ only (120 hours)</td>
</tr>
<tr>
<td>T47D</td>
<td>No significant effects</td>
</tr>
</tbody>
</table>

The effects of ureas on breast cancer cell lines are summarized in Table 3-2. C20-C22 ureas did not alter cell cycle distribution at 48 hours but, after 120 hours, G₀/G₁ was increased by the C21 and C22 ureas. This only occurred in MDA-MB-231 and MCF-7 cells.

### 3.2.5. Modulation of cyclin expression

Cell cycle progression is controlled by the coordinated expression of cyclin proteins, with cyclins D1 and E having a major role in the progression from G₁ phase to S phase. Based on the findings from flow cytometry, the modulation of expression of cyclins D1 and E by C20-C22 epoxides and C20-C22 ureas was assessed in breast cancer cells.
Figure 3-59: A) Expression of cyclin D1 protein following treatment of MDA-MB-231 cells with the C20 epoxide. A representative immunoblot of 3 experiments is shown. B) Cyclin D1 protein expression in MDA-MB-231 cells after treatment with the C20 ω-3 epoxide. Expression of cyclin D1 was normalized to GAPDH expression, where control is set as 100%. Data are mean ± SD of 3 independent experiments. Different from control: *** p<0.001.

Treatment of MDA-MB-231 cells for 48 hours with the C20 epoxide decreased cyclin D1 expression in concentration-related fashion (Figure 3-59A and Figure 3-59B). Expression was decreased at 24 hours to 63 ± 11% (20 µM) and 73 ± 1% (40 µM), of control. At 48 hours, the decreases were more pronounced and were to 36 ± 15% (10 µM), 20 ± 13% (20 µM) and 18 ± 10% (40 µM) of control, respectively.
Figure 3-60: A) Expression of cyclin D1 protein following treatment of MDA-MB-231 cells with epoxides and ureas (40 μM). A representative immunoblot of 3 experiments is shown. B) Effects of epoxides and ureas (40 μM) on cyclin D1 expression.
D1 expression in MDA-MB-231 cells after 48 hours of treatment. C) Effect of ureas (40 µM) on cyclin D1 expression in MDA-MB-231 cells after 120 hours of treatment. Expression of cyclin D1 was normalized to GAPDH expression, where control is set as 100%. Data are mean ± SD of 3 independent experiments. Different from control: *** p<0.001.

Consistent with findings from flow cytometry, treatment of MDA-MB-231 cells with C20-C22 epoxides (40 µM) decreased cyclin D1 protein expression to 23 ± 5%, 24 ± 5% and 47 ± 17% of control, respectively; the C20-C22 ureas were inactive, even after 120 hours of treatment (Figure 3-60A, Figure 3-60B, Figure 3-60C).
Figure 3-61: A) Expression of cyclin D1 protein in MDA-MB-468 cells following treatment with epoxides and ureas. A representative immunoblot of 3 experiments is shown. B) Effect of epoxides and ureas (40 µM) on cyclin D1
expression in MDA-MB-468 cells after 48 hours of treatment. C) Expression of Cyclin D1 protein in MDA-MB-468 cells following treatment with ureas (40 µM) for 120 hours. Expression of cyclin D1 was normalized to GAPDH expression, where control is set as 100%. Data are mean ± SD of 3 independent experiments. Different from control: **p<0.01, ***p<0.001.

Treatment of MDA-MB-468 cells with the C20-C22 epoxides for 48 hours also decreased cyclin D1 expression to 44 ± 19%, 51 ± 22% and 65 ± 25% of control, respectively (Figure 3-61A). Again, the C20-C22 ureas were inactive, even after 120 hours of treatment (Figure 3-61A, Figure 3-61B, Figure 3-61C).

None of the epoxides or ureas significantly altered cyclin D1 expression in MCF-7 cells after 48 or 120 hours of treatment (Figure 3-62A, Figure 3-62B, Figure 3-62C).
Figure 3-62: A) Expression of cyclin D1 protein in MCF-7 cells following treatment with epoxides and ureas (40 µM). A representative immunoblot of 3 experiments is shown. B) Effect of epoxides and ureas (40 µM) on cyclin D1
expression in MCF-7 cells after 48 hours of treatment. C) Effect of ureas (40 µM) on cyclin D1 expression in MCF-7 cells at 120 hours. Expression of cyclin D1 was normalized to GAPDH expression, where control is set as 100%. Data are mean ± SD of 3 independent experiments.
Figure 3-63: A) Expression of cyclin D1 protein in T47D cells following treatment with epoxides and ureas. A representative immunoblot of 3 experiments is shown. B) Effect of epoxides and ureas (40 µM) on cyclin D1
expression in T47D cells at 48 hours. C) Effect of ureas (40 µM) on cyclin D1 expression in T47D cells after 120 hours of treatment. Expression of cyclin D1 was normalized to GAPDH expression, where control is set as 100%. Data are mean ± SD of 3 independent experiments.

The epoxides and ureas were without any effect on cyclin D1 expression in T47D cells at either 48 or 120 hours of treatment (Figure 3-63A, Figure 3-63B, Figure 3-63C).

Cyclin E expression was also assessed in the epoxide-responsive MDA-MB-231 cells. As shown in Figure 3-64A the epoxide decreased cyclin E expression to 78 ± 6% (20 µM) and 57 ± 1% (40 µM) of control at 24 hours, with further decreases to 50 ± 10% (10 µM), 40 ± 18% (20 µM) and 43 ± 11% (40 µM) of control produced by 48 hours of treatment (Figure 3-64B).
Figure 3-64: A) Expression of cyclin E protein in MDA-MB-231 cells after treatment with the C20 epoxide for 24 or 48 hours. A representative immunoblot of 3 experiments is shown. B) Cyclin E protein in MDA-MB-231 cells after treatment with the C20 epoxide. Expression of cyclin E was normalized to GAPDH expression, where control is set as 100%. Data are mean ± SD of 3 independent experiments. Different from control: *p<0.05 and *** p<0.001.
3.3. Discussion

Recent studies have implicated ω-3-17,18-epoxy-EPA, but not its regioisomeric EPA epoxides, in the inhibition of proliferation and activation of apoptosis (Cui, Petrovic & Murray, 2011). To retain these anti-tumor actions of ω-3-17,18-epoxy-EPA, without potential antagonism by regioisomeric EPA epoxides that may be formed in cells by CYP-dependent biotransformation, novel ω-3 epoxides of saturated long chain fatty acids were prepared and evaluated. Studies were conducted in four breast cancer cell lines with different characteristics (Table 3-3). MDA-MB-231 is a highly invasive breast cancer cell lines while MDA-MB-468, MCF-7 and T47D are weakly invasive cell lines (Wang, Navab, Iakovlev, et al., 2007).
Table 3-3: Characteristics of human breast cancer cell lines.

<table>
<thead>
<tr>
<th>Line</th>
<th>Tumor type$^1$</th>
<th>Estrogen Receptor</th>
<th>Vimentin$^2$</th>
<th>MTRGL$^3$</th>
<th>Nude mouse$^4$</th>
<th>INV$^5$</th>
<th>CTX$^6$</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA-MB-231</td>
<td>AC</td>
<td>-</td>
<td>+</td>
<td>Inv</td>
<td>LI</td>
<td>++++</td>
<td>++++</td>
</tr>
<tr>
<td>MDA-MB-468</td>
<td>IAC</td>
<td>-</td>
<td>-</td>
<td>Sph</td>
<td>P</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>MCF-7</td>
<td>IDAC</td>
<td>+</td>
<td>-</td>
<td>Sph</td>
<td>P</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>T47D</td>
<td>IDAC</td>
<td>+</td>
<td>-</td>
<td>Sph</td>
<td>N</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Source: Thompson & Dixon (1992)

$^1$Histopathologic diagnosis: AC, adenocarcinoma; IAC, Infiltrating adenocarcinoma; IDAC, infiltrating ductal adenocarcinoma.


$^3$Morphology in matrigel: Inv, invasive colony formation; Sph, spherical colony or non-invasive cluster formation.

$^4$Activity in the athymic nude mouse (NCR nu/nu): LI, local invasion through the peritoneum, colonization of visceral organs; P, primary tumor formation, no local invasiveness or metastasis; N, non-tumorigenic.

$^5$Activity in Boyden chamber chemoinvasion assay, graded as %MDA-MB-231: +, 0-20%; ++, 20-40%; ++++, 40-60%; ++++, 60-80%; +++++, >80%.

$^6$Activity in Boyden chamber chemotaxis towards fibroblast-conditioned medium, graded as %MDA-MB-231: +, 0-20%; ++, 20-40%; ++++, 40-60%; ++++, 60-80%; +++++, >80%.
In general, pronounced decreases in the viability of the highly aggressive epithelial adenocarcinoma-derived MDA-MB-231 cell line occurred after treatment with the epoxides. Somewhat less aggressive cell lines, including another epithelial adenocarcinoma-derived line (MDA-MB-468), the estrogen receptor-positive MCF-7 and the multiple hormone receptor-positive T47D cells, were less sensitive towards these molecules. The epoxides were consistently found to decrease viability, especially in the MDA-MB-231 cells, with confluence, MTT reduction and ATP production as endpoints. The C20 epoxide was most effective, followed by the C21 analogue, while the C22 epoxide was least active. Findings from flow cytometry were consistent with these observations. In MDA-MB-231 cells the proportion of cells in G0/G1 phase increased, especially after C20 epoxide treatment, while those in later phases (S and G2/M) decreased. The expression of cyclin D1 and cyclin E proteins was also decreased, which is consistent with the apparent pause in cell cycle progression and the failure of treated cells to complete the cell cycle. Decreased mitosis appears to be a significant contributor to the loss of MDA-MB-231 cell viability produced by the epoxides.

In contrast, the C20-C22 ureas were highly effective in four different breast cancer cell lines, when MTT reduction was the endpoint. Pronounced decreases in MTT reduction were most prominent in the highly aggressive MDA-MB-231 cells after urea treatment. However, the use of microscopy to assess cell confluence and ATP formation as an alternate indicator of cell viability produced findings that were not in agreement with MTT data. Less aggressive cell lines were again less sensitive towards the molecules.
In this chapter the reduction of the tetrazolium derivative MTT by cellular dehydrogenases was used to assess cell proliferation. The number of viable cells is considered to be directly proportional to the quantity of formazan formed in the assay. Several factors could influence findings with the MTT reduction assay. Certain compounds are known to interfere with MTT reduction, including ascorbate and sulphydryl reagents, as well as other redox active molecules (Berridge & Tan, 1993; Petty, Sutherland, Hunter, et al., 1995). Formazan production is also pH-dependent process, so that altered intracellular pH by the agents could account for the disparities (Plumb, Milroy & Kaye, 1989). However, in viable cells MTT reduction is known to be mediated by several dehydrogenases in different cellular compartments and is not linked exclusively to mitochondrial dehydrogenases that influence cell proliferation (Berridge & Tan, 1993).

ATP is important for cell cycle progression so that its production rates can be used as an indicator of cell proliferation. In this study, ATP formation data agreed more closely with findings from microscopy than those from MTT reduction assays. Effects of the epoxides on ATP formation were more pronounced than those of the ureas. Cell cycle suppression in epoxide-treated MDA-MB-231 cells occurred in G0/G1 phase, which is consistent with the inability of the cell to replicate DNA in S-phase, and the completion of mitosis in G2/M. Progression of the cell cycle is controlled by cyclin family proteins: the cellular content of cyclins varies with the phase of the cell cycle. Cyclins D1 and E are increased in cells that enter G1 phase, decreased in S-phase and re-established in G2 phase to promote mitogenesis (Stacey, 2003). Cyclin D1 expression is reported to be up-regulated in many human cancers including
breast cancer (Sweeney, Swarbrick, Sutherland, *et al.*, 1998; Vermeulen, Van Bockstaele & Berneman, 2003). From several studies cyclin D1 has emerged as a mediator of the actions of naturally occurring fatty acid epoxides. The proliferative and protumourigenic ω-6 EETs activated but ω-3-17,18-epoxy-EPA selectively decreased, cyclin D1 expression (Potente, Michaelis, Fisslthaler, *et al.*, 2002; Cui, Petrovic & Murray, 2011). Consistent with these findings, cyclin D1 and cyclin E immunoreactive proteins were strongly down-regulated in MDA-MB-231 cells by the C20-C22 epoxides that were available for this project, which may contribute to their anti-proliferative actions. Down-regulation of cyclins D1 and E is consistent with cell cycle arrest in G₀/G₁ phase.
4. Caspase-dependent apoptosis is an important cell killing mechanism in ω-3 epoxide-treated MDA-MB 231 cells

4.1. Introduction

Studies in Chapter 3 found that the C20-C22 epoxides strongly decreased the proliferation and viability of MDA-MB-231 cells, whereas the corresponding ureas were much less effective. Three other breast cancer cell lines were less responsive to the epoxides. The studies in Chapter 4 assessed the capacity of the molecules to elicit cell killing.

Programmed cell death is an important mechanism by which cell viability is decreased (Long & Ryan, 2012). Activation of apoptosis by anticancer agents has been studied intensively, but additional types of regulated cell death have been described more recently – especially necroptosis and autophagy. Apoptosis was first identified by Kerr, Wyllie & Currie, (1972) and is characterized by specific morphological and biochemical changes in cells. Apoptotic cells undergo shrinkage, nuclear condensation and fragmentation, membrane blebbing and finally the formation of apoptotic bodies (Kerr, Wyllie & Currie, 1972). Apoptotic cells also lose the capacity for adhesion to adjacent cells and the extracellular matrix (Nishida, Yamaguchi & Otsu, 2008). Characteristic biochemical changes in apoptotic cells include the externalization of the membrane phospholipid phophatidylserine (Fadok, Voelker, Campbell, et al., 1992) and the cleavage of a range of protein substrates (Martin & Green, 1995). Apoptosis occurs through
two major pathways termed the intrinsic and extrinsic pathways that are described in detail in Chapter 1. Activation along the intrinsic pathway is initiated intracellularly, while the extrinsic pathway involves activation of death receptors at the plasma membrane. Both pathways converge on cysteine proteases termed caspases that degrade protein substrates (Martin & Green, 1995; Degterev, Boyce & Yuan, 2003). Caspases are synthesized as inactive zymogens and undergo activation by cleavage of a regulatory domain (Hengartner, 2000). Caspases are also responsible for nuclear fragmentation by specific nucleases that cut the genomic DNA to generate small oligomers.

Necroptosis is a form of regulated necrosis that is activated by severe cell damage (Long & Ryan, 2012). Apoptosis and necroptosis can be activated simultaneously by death receptors (Christofferson & Yuan, 2010). Necroptotic and necrotic cells are characterized by plasma membrane permeabilisation that causes the cells to swell and rupture, which is distinct from apoptosis (Vandenabeele, Galluzzi, Vanden Berghe, et al., 2010). Release of the cellular contents can produce an inflammatory response and recruit immune cells to the damaged target cell (Long & Ryan, 2012). Necroptosis is mediated by receptor interacting protein kinases (RIP) that are inhibited by necrostatin-1 (Holler, Zaru, Micheau, et al., 2000; Degterev, Huang, Boyce, et al., 2005).

Autophagy is a process by which cells undergo self-digestion and can be important for housekeeping purposes, such as recycling of damaged organelles and cytoplasmic macromolecules (Yang & Klionsky, 2010). Although autophagy is an important regulatory mechanism for energy efficiency and cell survival, excessive activation promotes autophagic cell death (Wang, Yu, Zhang, et al., 2011). A distinct characteristic of autophagy is the formation of double-
membrane bound vesicles termed autophagosomes. After a protein target that is to be recycled has been engulfed the autophagosome fuses with lysosomes to form autolysosome which mediates protein degradation (Mizushima, Ohsumi & Yoshimori, 2002). As with apoptosis and necroptosis, autophagy is also regulated by specific genes, including beclin-1 and other autophagy-related genes (Rosenfeldt & Ryan, 2011).

Although apoptosis, necroptosis and autophagy are distinct death mechanisms, a complex interplay appears to exist (Long & Ryan, 2012; Ouyang, Shi, Zhao, et al., 2012). Necroptosis is often activated along with apoptosis (Wu, Liu & Li, 2012) and, although the underlying reasons remain unclear, necroptosis may accompany autophagy (Degterev, Huang, Boyce, et al., 2005). ATP availability is considered to be a major driving force in the determination of the killing mechanism. Apoptosis requires energy and depletion of ATP has been found to inhibit apoptosis and activate necroptosis (Eguchi, Shimizu & Tsujimoto, 1997). The necrototic regulatory kinase RIP1 also inhibits ATP synthase during necroptosis, which diminishes energy metabolism (Temkin, Huang, Liu, et al., 2006). Conversely, if the ATP level is restored in ATP-depleted cells, necroptosis is inhibited, and the death mechanism reverts to apoptosis (Leist, Single, Castoldi, et al., 1997). Inhibition of autophagy leads to a bioenergetic crisis that favours necroptosis (Degenhardt, Mathew, Beaudoin, et al., 2006). However, as mentioned, autophagy is often activated together with necroptosis to enhance cell death (Ouyang, Shi, Zhao, et al., 2012).

ω-3 Epoxyfatty acids were found to decrease the viability of MDA-MB-231 breast cancer cells due at least in part to a decrease in proliferation following the down-regulation of regulatory cyclins and impaired cell cycle progression. To
further develop the mechanisms underlying the loss of cell viability, the capacity of epoxides to activate cell death mechanisms was assessed in Chapter 4.

**Table 4-1: Epoxide-mediated cell death - death pathway inhibitors.**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Pathway involved</th>
</tr>
</thead>
</table>
4.2. Results

4.2.1. The pan-caspase inhibitor z-VAD-fmk, but not the necroptosis inhibitor necrostatin-1, inhibits epoxide-mediated killing of MDA-MB-231 cells

Findings in the previous chapter showed that ω-3 epoxyfatty acids decrease the viability of breast cancer cells. The MDA-MB-231 cell line was found to be most sensitive to epoxide treatment. Three principal types of cell death (apoptosis, necroptosis and autophagy) were assessed in the present chapter to define how epoxides decreased breast cancer cell viability. These studies were conducted with the apoptosis inhibitor z-VAD-fmk, the necroptosis inhibitor necrostatin-1 and the autophagy inhibitors 3-methyladenine and chloroquine (as described in Tables 4.1 and 4.2).
Figure 4-1: The effect of the pan-caspase inhibitor z-VAD-fmk on killing of MDA-MB-231 cells by C20-C22 epoxides (40 µM; 48 hours). Images are representative of findings from duplicate experiments. (Microscopy imaging with 40x magnification)

Pre-treatment with z-VAD-fmk (10, 20 and 40 µM) was initiated 2 hours before addition of epoxides (40 µM). z-VAD-fmk produced a concentration-dependent protective effect against the epoxides (Figure 4-1). The protective effect was most pronounced at the highest z-VAD-fmk concentration of 40 µM.
Figure 4-2: The pan-caspase inhibitor z-VAD-fmk (10, 20, 40 µM) prevents the decrease in ATP production in MDA-MB-231 cells effected by C20-C22 epoxides (40 µM; 48 hours). Data are mean ± SD of 3 independent experiments: * p<0.05, ** p<0.01 and *** p<0.001 relative to control. †† p<0.01 and ††† p<0.001 relative to treatment in the absence of z-VAD-fmk.

The pan-caspase inhibitor z-VAD-fmk partially prevented the loss in ATP production produced by epoxides (Figure 4-2). Thus, the decreases produced by epoxides (C20-C22 to 45 ± 1%, 57 ± 1% and 67 ± 1% of control, respectively) were largely restored by z-VAD-fmk. At the highest concentration of z-VAD-fmk ATP production after the epoxides was 78 ± 5%, 57 ± 6% and 67 ± 3% of control.
Figure 4-3: The effect of the necroptosis inhibitor necrostatin-1 on killing of MDA-MB-231 cells by C20-C22 epoxides (40 μM; 48 hours). Images are representative of findings from duplicate experiments. (Microscopy imaging with 40x magnification)

The RIP-1 inhibitor necrostatin-1 was added 2 hours before 48-hour treatments with epoxides (40 μM). As shown in Figure 4-3, concurrent
necrostatin-1 had minimal effect on cell confluence even at the highest concentration that was tested (40 μM).

Figure 4-4: Effect of necrostatin-1 on ATP production by MDA-MB-231 cells in the presence of ω-3 epoxyfatty acids (40 μM; 48 hours). Data are mean ± SD of 3 independent experiments. Different from control: * p<0.05, ** p<0.01 and *** p<0.001.

The necroptosis inhibitor necrostatin-1 did not significantly alter the decrease in ATP production in MDA-MB-231 cells that were treated with the C20-C22 epoxides (40 μM). Thus, ATP formation after the C20-C22 epoxides was 45 ± 1%, 57 ± 1% and 67 ± 1% of control, respectively, and was 55 ± 5%, 62 ± 6% and 67 ± 2% of control, respectively, after co-treatment with necrostatin-1 (40 μM); these changes were not statistically significant (Figure 4-4).
4.2.2. The C20-C22 epoxides do not activate autophagy in MDA-MB-231 cells

![Figure 4-5: The effect of the autophagy inhibitor 3-methyladenine on killing of MDA-MB-231 cells by C20-C22 epoxides (40 µM; 48 hours). Images are representative of findings from duplicate experiments. (Microscopy imaging with 40x magnification)]
3-Methyladenine and chloroquine are inhibitors of autophagy, which is basically an energy-conservation mechanism in cells, but can also mediate cell death. 3-Methyladenine blocks the formation of autophagosomes, which are organelles that capture cellular components and deliver them to lysosomes for intracellular degradation. As shown in Figure 4-5, the decrease in cell confluence produced by the epoxides was similar in the presence 3-methyladenine (1, 5 and 10 mM). In agreement with these findings, epoxide-dependent decreases in ATP formation in MDA-MB-231 cells were unchanged by 3-methyladenine (Figure 4-6).

![Figure 4-6: Effect of 3-methyladenine on ATP production by MDA-MB-231 cells in the presence of ω-3 epoxyfatty acids (40 µM; 48 hours). Data are mean ± SD of 3 independent experiments. Different from control: ** p<0.01 and *** p<0.001.](image)

Data are mean ± SD of 3 independent experiments. Different from control: ** p<0.01 and *** p<0.001.
Figure 4-7: The effect of the autophagy inhibitor chloroquine on killing of MDA-MB-231 cells by C20-C22 epoxides (40 µM; 48 hours). Images are representative of findings from duplicate experiments. (Microscopy imaging with 40x magnification)

Chloroquine inhibits autophagy by preventing endosomal acidification. Pretreatment of MDA-MB-231 cells with chloroquine (1, 10 and 20 µM) had no effect on the decrease in cell viability elicited by the epoxides (Figure 4-7).
Similarly, chloroquine did not prevent the decline in ATP production effected by the C20-C22 epoxides (Figure 4-8).

![Graph showing luminescence levels](image)

**Figure 4-8: Effect of chloroquine on ATP production by MDA-MB-231 cells in the presence of ω-3 epoxyfatty acids (40 μM; 48 hours).** Data are mean ± SD of 3 independent experiments. Different from control: * p<0.05, ** p<0.01 and *** p<0.001.

4.2.3. **ω-3 epoxyfatty acids increase the activity of caspases 3 and 7 in MDA-MB 231 cells**

Treatment with the pan-caspase inhibitor z-VAD-fmk impaired the decrease in viability produced by the C20-C22 epoxides (Figure 4-1 and Figure 4-2). Consistent with these findings the C20 epoxide increased caspase 3/7 activity in MDA-MB-231 cells in a concentration- and time-dependent manner (Figure 4-9). At a concentration of 50 μM, caspase activity was increased to 233 ± 82% and 270 ± 56% after 24 and 48 hours of treatment, respectively. The increase was
more pronounced following treatment with 100 μM epoxide after 24 and 48 hours (to 508 ± 133% and 609 ± 90% of control, respectively).

Figure 4-9: Caspase 3/7 activity in MDA-MB-231 cells after treatment with the C20 epoxide. Data are mean ± SD of 3 independent experiments. Different from control: * p<0.05 and *** p<0.001.
Figure 4-10: Caspase 3/7 activity in MDA-MB-231 cells after treatment with C21 epoxide. Data are mean ± SD of 3 independent experiments. Different from control: ** p<0.01 and *** p<0.001.

The C21 epoxide also increased caspase 3 activity in MDA-MB-231 cells but the effect was less pronounced than with the C20 epoxide (Figure 4-10). Increases were to 207 ± 63% and 234 ± 27% of control, respectively, after 24 and 48 hours of treatment with C21 epoxide (50 µM). At the higher concentration (100 µM) activity was increased to 309 ± 97% and 359 ± 51% of control, respectively, after 24 and 48 hours of treatment.
Figure 4-11: Caspase 3/7 activity in MDA-MB-231 cells after treatment with C22 epoxide. Data are mean ± SD of 3 independent experiments. Different from control: * p<0.05 and *** p<0.001.

The C22 epoxide was much less active than either the C20 and C21 epoxide (Figure 4-11). Significant increases of caspase 3 activity were only observed at 100 µM (176 ± 20% of control after 24 hours; 160 ± 22% of control after 48 hours). The findings in chapter 3 indicated that the viability of MDA-MB-231 cells was strongly decreased by the epoxide, but that the MDA-MB-468, MCF-7 and T47D cells were less responsive. In further experiments, the activation of caspase 3 was assessed in each of the cell lines using the most potent analogue - the C20 epoxide.
As shown in figure 4-12, the C20 epoxide increased caspase 3/7 activity in MDA-MB-468 cells, but to a lesser extent than in MDA-MB-231 cells. Thus, at the higher concentration (50 µM and 100 µM) caspase activity was increased to 175 ± 3% and 176 ± 3% of respective control. In contrast, caspase activity was not increased by the C20 epoxide in MCF-7 cells (Figure 4-13) and T47D cells (Figure 4-14).
Figure 4-13: Caspase 3/7 activity in MCF-7 cells after treatment with the C20 epoxide at 48 hours. Data are mean ± SD of 3 independent experiments. Different from control: *p<0.05 and **p<0.01.

Figure 4-14: Caspase 3/7 activity in T47D cells after treatment with C20 epoxide at 48 hours. Data are mean ± SD of 3 independent experiments.
4.2.4. \( \omega-3 \) epoxylfatty acids activate additional apoptotic endpoints in MDA-MB-231 cells

The increase in caspase 3 activity in MDA-MB-231 cells produced by the C20 epoxide is consistent with activation of apoptosis. Other characteristics of apoptotic cells include inactivation of the DNA repair enzyme PARP, fragmentation of nuclear DNA and exposure of phosphatidylserine at the plasma membrane. These endpoints were assayed in further experiments.

4.2.4.1. The C20 epoxide induces PARP cleavage and inactivation

PARP is proteolysed and inactivated by caspase 3, PARP cleavage was increased in MDA-MB-231 cells by treatment with the C20 epoxide (Figure 4-15A). At a concentration of 40 \( \mu \text{M} \), total PARP decreased to 53 \( \pm \) 7\% and 12 \( \pm \) 1\% of control, after 24 and 48 hours of treatment, respectively. Cleaved PARP was evident after 24 hours of treatment (322 \( \pm \) 199\% of control, 40 \( \mu \text{M} \)) and was more pronounced after 48 hours (361 \( \pm \) 82\% of control at 20 \( \mu \text{M} \); 511 \( \pm \) 193\% of control at 40 \( \mu \text{M} \))(Figure 4-15B).
Figure 4-15: A) Expressions of total PARP and cleaved PARP protein after treatment of MDA-MB-231 cells with the C20 epoxide. A representative immunoblot from 3 experiments is shown. B) Total PARP protein expression in MDA-MB-231 cells after treatment with the C20 ω-3 epoxide. C) Cleaved PARP protein expression in MDA-MB-231 cells after treatment with the C20 epoxide.
**ω-3 epoxide.** Expression of total PARP and cleaved PARP was normalized to GAPDH expression, where control is set as 100%. Data are mean ± SD of 3 independent experiments. Different from control: * p<0.05, ** p<0.01 and *** p<0.001.

### 4.2.4.2. The C20 ω-3 epoxyfatty acid increases the sub-G1 population in treated MDA-MB-231 cells

Another important feature of apoptosis is fragmentation of chromosomal DNA due to activation of endonucleases that generate smaller oligomeric fragments. DNA fragmentation can be detected using flow cytometry because damaged cells have less DNA than viable cells. Such cells appear to the left of the G0/G1 population and are termed the sub-G1 population.

The C20 epoxide increased the percentage of MDA-MB-231 cells in sub-G1 phase (Figure 4-16). After 48 hours, the C20 epoxide (25 µM) increased the sub-G1 population to 17 ± 4% relative to 3 ± 1% in control (Figure 4-17). This increased to 24 ± 5% of cells in the sub-G1 population after treatment with C20 epoxide (50 µM) for 48 hours (Figure 4-17).
Figure 4-16: Sub-G1 analysis of MDA-MB-231 cells treated with the C20 epoxide: concentration- and time-dependence.
Figure 4-17: The sub-G1 population in MDA-MB-231 cells following treatment with the C20 epoxide. Data are mean ± SD of 3 independent experiments. Different from control: * p<0.05 and ** p<0.01.

4.2.4.3. Treatment of MDA-MB-231 cells with the C20 ω-3 epoxypatty acid increases phosphatidylserine exposure at the cell membrane

In normal cells, phosphatidylserine is restricted to the inner leaflet of the plasma membrane. In apoptosis the orientation of phosphatidylserine is inverted and appears on the external surface of the plasma membrane so that phagocytes can detect dying cells and facilitate their removal. Exposure of phosphotidylserine is due to a decrease in aminophospholipid translocase activity and activation of a calcium-dependent scramblase. Phosphatidylserine that is exposed on the outside surface of the cells can be stained with annexin V, which is a cellular
protein that binds to phosphatidylserine. In the annexin V assay flow cytometry is used in a dual staining procedure; propidium iodide is added along with annexin V. Early apoptotic cells (that have the plasma membrane still intact) are only stained with annexin V, but staining with both annexin V and propidium iodide detects necrotic cells and those in late apoptosis.

The C20 epoxide increased the proportion of cells that are stained with annexin V in a concentration- and time-dependent fashion (Figure 4-18). These increases were quantified and are presented in Figures 4-19 and 4-20. The C20 epoxide (40 μM) increased annexin V-FITC positive cells to 13 ± 3% relative to 6 ± 1% in control after 24 hours treatment (Figure 4-19). At 48 hours, annexin V-stained cells were increased to 8 ± 3% (10 μM), 20 ± 2% (20 μM) and 36 ± 3% (40 μM), respectively, relative to 4 ± 3% in control (Figure 4-20).
Figure 4-18: Annexin V/propidium iodide (PI) staining of MDA-MB-231 cells after treatment with the indicated concentrations of C20 epoxide for 24 or 48 hours. A representative of 3 independent experiments is shown.
Figure 4-19: Annexin V/PI positive MDA-MB-231 cells following treatment with the C20 epoxide (24 hours). Data are mean ± SD of 3 independent experiments. Different from control: *** p<0.001.

Figure 4-20: Annexin V/PI positive MDA-MB-231 cells following treatment with the C20 epoxide (48 hours). Data are mean ± SD of 3 independent experiments. Different from control: * p<0.05, * p<0.01 and *** p<0.001.
4.3. Discussion

The observations in chapter 3 indicate that ω-3 epoxyfatty acids inhibit the proliferation of breast cancer cells and had a pronounced effect on the viability of breast cancer cells, especially MDA-MB-231. There are several potential mechanisms by which chemicals may inhibit cell viability. While the importance of apoptosis to the anti-tumour actions of anticancer drugs has long been recognized, recent studies have also found that necroptosis and autophagy may be alternate cell death pathways.

Previous studies have shown that the naturally occurring ω-3 PUFA EPA also activates cell death, but that this may occur by different mechanisms, according to cell context. Thus, EPA induces apoptosis in the human Burkitt’s lymphoma Ramos cell line, but necroptosis in the similar Raji cell line (Heimli, Finstad & Drevon, 2001). EPA has also been reported to induce autophagy in the human pancreatic cancer MIA-PaCa-2 and Capan-2 cell lines (Fukui, Kang, Okada, et al., 2013). The roles of these alternate mechanisms in cell killing induced by the synthetic C20-C22 fatty acid epoxides were studied in this chapter.

The pan-caspase inhibitor, z-VAD-fmk, effectively reversed the decrease in confluence of MDA-MB-231 cells elicited by treatment with epoxides (40 µM) for 48 hours. In agreement with these findings, ATP formation was also restored by z-VAD-fmk treatment in a concentration dependent manner. In contrast, co-treatment of MDA-MB-231 cells with the necroptosis inhibitor, necrostatin-1, had no apparent effect on cell confluence or the decrease in ATP formation produced by C20-C22 epoxides even at the highest concentration (40 µM). Similarly, the autophagy inhibitors 3-methyladenine and chloroquine did not alter the effects of the epoxides on cell viability endpoints at any concentration. Together, these
observations suggest that the C20-C22 epoxides induce apoptosis in MDA-MB-231 cells, but not necroptosis or autophagy.

The DNA repair enzyme PARP undergoes cleavage in cells that contain activated caspase 3. In accord with the increase in caspase 3 cleavage in MDA-MB-231 cells by C20 epoxide treatment there was also an increase in cleaved PARP. This deactivation of a major DNA repair mechanism could account for the increase in cells in sub-G1 phase in C20 epoxide-treated cells. Thus, following caspase 3 activation and PARP deactivation DNA fragmentation was increased.

Although the strongly proliferative and highly metastatic MDA-MB-231 cell line was highly responsive to the present ω-3 epoxyfatty acids, three other breast cancer cell lines were not. Previous studies have also found that parent ω-3 fatty acids activate caspases in MDA-MB-231 cells (Schley, Jijon, Robinson, et al., 2005). The pro-apoptotic actions of ω-3 PUFA are not restricted to breast cancer cells. EPA was shown to increase early and late apoptosis in premalignant keratinocytes (Nikolakopoulou, Shaikh, Dehlawi, et al., 2013). This was antagonized by serum albumin, possibly due to a decrease in the free concentration of lipid (Huang, Dass & Kim, 2005). ω-3 EPA and DHA, but not the ω-6 linoleic acid, increased the number of apoptotic cells in the human preadipocyte cell line AML-1, as reflected by annexin V staining (Hanada, Morikawa, Hirota, et al., 2011). EPA and DHA also effected an increase in the annexin V positive population in human colorectal cancer stem-like cells (SW620; Yang, Fang, Zhang, et al., 2013). In the human pancreatic cancer cell lines, MIA-PaCa-2 and Capan-2, cleavage of caspase 3 and PARP were increased by EPA (Fukui, Kang, Okada, et al., 2013).
Naturally occurring saturated fatty acids, such as C16-palmitic and C18-stearic acids, induced both caspase-dependent and caspase-independent apoptosis in the rat pheochromocytoma PC-12 cell line (Ulloth, Casiano & De Leon, 2003). In that study, the activation of caspases 3 and 8 led to increased cleavage of PARP. However, the pan-caspase inhibitor z-VAD-fmk did not prevent cell death thus implicating caspase-independent mechanisms in cell killing. The fatty acid derivative ceramide has also been reported to induce apoptosis without activating caspase (Thon, Möhlig, Mathieu, et al., 2005). Again, the pan-caspase inhibitor z-VAD-fmk did not suppress ceramide-induced apoptosis in MCF-7 cells. These findings suggest that different fatty acid derivatives are able to activate alternate death mechanisms in a cell-type specific manner. PUFA and other fatty acids are now recognized to activate apoptosis pathway in multiple cell types.

Staining with annexin V and propidium iodide was used to confirm apoptosis in epoxide-treated MDA-MB-231 cells. The redistribution and reorientation of phosphatidylserine to the outer plasma membrane occurs early in apoptosis and before plasma membrane integrity is compromised (Martin, Reutelingsperger, McGahon, et al., 1995; Brumatti, Sheridan & Martin, 2008). Following the externalization of phosphatidylserine, macrophages and other immune cells are more readily recruited to the cell membrane, which facilitates the deletion of damaged cells (Fadok, Voelker, Campbell, et al., 1992). It was found that epoxide treatment markedly increased the population of MDA-MB-231 cells that were stained with annexin V and, to a lesser extent, with propidium iodide. This is consistent with the activation of apoptosis by the C20 epoxide and a lesser activation of necrosis. However, the inability of necroptosis and
autophagy inhibitors to modulate the decrease in viability produced by epoxides suggests that these two alternate forms of programmed cell death are not involved. More extensive studies could now be done to supplement the findings in this project.
5. Mechanism of caspase-dependent apoptosis induced by the synthetic C20 ω-3 fatty acid epoxide in MDA-MB 231 cells

5.1. Introduction

The findings in Chapter 4 indicated that the C20 ω-3 epoxide activated apoptosis in MDA-MB-231 breast cancer cells. In Chapter 5 the pathways underlying the activation of apoptosis were investigated in this cell line. To provide a rationale for these experiments a brief summary of apoptotic mechanisms is outlined; a more extensive background is provided in Chapter 1.

A hallmark of apoptosis is activation of the cysteine proteases known as caspases (Strasser, O'Connor & Dixit, 2000). Caspase 3 is a major cell executioner that is activated by cleavage of the inactive zymogen procaspase 3. This activation step may be achieved either directly, or indirectly by the action of upstream initiator caspases, such as caspase 8 or caspase 9 (Hengartner, 2000). The activation of caspase 3 is a critical event in apoptosis and can be mediated by either of the principal pathways of apoptosis, which are termed the intrinsic and extrinsic pathways.

The intrinsic pathway of apoptosis is activated in response to intracellular stimuli, such as reactive oxygen species, viral infections, ionizing radiation and DNA damage (Elmore, 2007). This is also termed the mitochondrial pathway of apoptosis because, in addition to their well established role in the production of ATP, mitochondria also regulate programmed cell death (Reed, 2001). Bcl-2
family proteins are the central regulators of intrinsic apoptosis and consist of anti-apoptotic proteins (eg Bcl-2, Bcl-XL and Mcl-1), pro-apoptotic proteins (eg Bax, Bak and Bad) and BH3-only pro-apoptotic proteins (eg Bid, Bik and Bim) (Tsujimoto & Shimizu, 2000). Pro-apoptotic Bax- and Bak-containing oligomers form pores in the outer mitochondrial membrane that enable the release of mitochondrial proteins into the cytosolic fraction of the cell (Bratton & Salvesen, 2010).

Homo- or heterodimerisation of Bcl-2 proteins modulates the release of cytochrome c, second mitochondria-derived activator of caspases (Smac) and apoptosis-inducing factor (AIF) from the mitochondrion (Kroemer & Reed, 2000). Cytochrome c is a hemoprotein that normally participates in mitochondrial electron transport, but is released in response to an apoptotic stimulus. In the cytosolic fraction of the cell it forms the apoptosome complex that activates the initiator caspase 9 that then activates executioner caspases, such as caspase 3. AIF is a flavoprotein required for optimal apoptotic activity and Smac modulates the activity of the apoptosome (Susin, Lorenzo, Zamzami, et al., 1999; Reed, 2001).

In contrast to the intrinsic pathway, the extrinsic pathway of apoptosis is mediated by death receptors (Reed, 2001). As detailed in Chapter 1, the TNF-α-receptor (TNF-R1) and related receptors transduce extracellular cell death signals received at the plasma membrane that lead to the activation of the JNK and p38 MAPK signaling cascades that regulate cell death (Aggarwal, 2000). Other important death receptors that participate in apoptosis include the Fas receptor and the TNF-related apoptosis-inducing ligand (TRAIL) receptors; there are two TRAIL receptors - TRAIL-R1 and TRAIL-R2 - also termed DR4 and DR5.
The Fas and TRAIL-dependent pathways rapidly transduce pro-apoptotic signals by recruiting the adaptor Fas-associated protein with the death domain (also termed FADD) and other proteins to their intracellular binding domains (Scaffidi, Kirchhoff, Krammer, et al., 1999). FADD then recruits and activates procaspase 8 that leads ultimately to downstream activation of executioner caspase 3 (Micheau & Tschopp, 2003). The TNF-R1 signaling system is similar, except that TRADD (an abbreviation for TNF-R1-associated via death domain) and TRAF2 (TNF-R1-associated factor 2) are essential protein components of intracellular death receptor complexes after activation (Chen & Goeddel, 2002).

Recruitment and activation of ASK-1 to death receptor complexes is also important because this is the most upstream component of the MAPK cascades that transduce apoptotic signals in cells (Figures 1-16, 1-17). MAPKs are modular signal transduction pathways in which a signal is propagated in cells by a sequence of phosphorylation events. Thus, phosphorylation of upstream signaling intermediates enables their interaction with downstream partner proteins. The proliferative and prosurvival ERK cascade is the best described MAPK pathway, but there is also a deal of information on the upstream JNK and p38 MAPK pathways that mediate apoptosis (Figure 1-16).

The JNK and p38 MAPK pathways respond to a range of stimuli that activate either the extrinsic or intrinsic pathways of apoptosis, including UV and g-radiation, reactive oxygen species, inhibitors of protein synthesis such as anisomycin, hyperosmolarity, a range of toxins, ischemia/reperfusion injury, heat shock, anticancer drugs such as cisplatin, adriamycin, or etoposide, ceramide, and proinflammatory cytokines (Yada, Shimamoto, Hampton, et al., 2004). ASK1 is one of the MAPK kinase kinases at the top of MAPK signaling
cascades that are activated in apoptosis (Ichijo, Nishida, Irie, et al., 1997). Immediately downstream from ASK-1 are the MAPK kinases (MKKs) MKK3, MKK4, MKK6 and MKK7, that in turn phosphorylate and activate the downstream JNK and p38 MAPKs (Ichijo, Nishida, Irie, et al., 1997; Haeusgen, Herdegen & Waetzig, 2011). MKK3 and MKK6 phosphorylate the p38 MAPK, while MKK7 exclusively activates the JNK MAPK and MKK4 mediates the phosphorylation of both kinases (Ichijo, Nishida, Irie, et al., 1997); the participation of both MKK4 and MKK7 is required for full activation of JNK (Weston & Davis, 2002). The JNK and the p38 MAPKs promote apoptosis by regulating the release of cytochrome c and other mitochondrial factors from that organelle (Aoki, Kang, Hampe, et al., 2002; Chauhan, Li, Hideshima, et al., 2003). Thus, the extrinsic and intrinsic pathways of apoptosis converge on the activation of executioner caspases.

The naturally occurring ω-3 17,18-epoxide of EPA has been found previously to decrease the viability of endothelial cells by activating the p38 MAPK (Cui, Petrovic & Murray, 2011). In earlier chapters of this thesis the synthetic C20 ω-3 epoxide, which is structurally similar to ω-3 17,18-epoxy EPA, also decreased the viability of breast cancer cells, as shown by an increase in caspase 3 activity. In this chapter the apoptotic mechanism underlying cell killing by the C20 ω-3 epoxide was studied in greater detail in MDA-MB-231 cells.
Table 5-1: Signaling inhibitors to inhibit caspase activation by the C20 epoxide.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Target molecule</th>
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<tbody>
<tr>
<td>SP600125</td>
<td>JNK (Bennett, Sasaki, Murray, et al., 2001; Kim, Choi, Kim, et al., 2008).</td>
</tr>
<tr>
<td>SB203580</td>
<td>p38 MAPK (Bartucci, Morelli, Mauro, et al., 2001; Kim, Choi, Kim, et al., 2008).</td>
</tr>
<tr>
<td>LY294002</td>
<td>PI3K (Bartucci, Morelli, Mauro, et al., 2001; Sliva, Rizzo &amp; English, 2002).</td>
</tr>
</tbody>
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5.2. Results

5.2.1. Caspase 3 and caspase 9 proteins are activated in MDA-MB-231 cells by treatment with the C20 ω-3 epoxide

The findings in chapter 4 indicated that the C20 ω-3 epoxide activated caspase 3 in MDA-MB-231 cells and that apoptosis was a major feature underlying the loss of cell viability. In chapter 5 the apoptotic mechanism was evaluated in detail. Treatment of MDA-MB 231 cells with the C20 ω-3 epoxide increased caspase 3 cleavage in a time- and concentration-dependent fashion (Figure 5-1A). Thus, 24 hours of treatment at the 40 μM concentration increased the expression of cleaved caspase 3 to 220 ± 41% of control (P<0.01; Figure 5-1B). Longer treatments (48 hours) effected more pronounced increases in cleaved caspase 3
expression (10 μM: 204 ± 82% of control; 20 μM: 365 ± 30% of control and 40 μM: 465 ± 50% of control; all P<0.001; Figure 5-1B).

Figure 5-1: **A)** The C20 ω-3 epoxide increases the expression of cleaved caspase 3 protein in MDA-MB-231 cells. A representative immunoblot of 3 experiments is shown. **B)** Cleaved caspase 3 protein expression in MDA-MB-231 cells after treatment with the C20 epoxide. Expression of cleaved caspase 3 was normalized to GAPDH expression, where control is set as 100%. Data are mean ± SD of 3 independent experiments. Different from control: ** p<0.01 and *** p<0.001.
Treatment of MDA-MB 231 cells with the C20 ω-3 epoxide also increased the expression of cleaved caspase 9 protein (Figure 5-2A). Whereas treatment for 24 hours did not alter expression (Figure 5-2B), treatment for 48 hours produced marked increases (10 μM: 144 ± 26% of control; 20 μM: 285 ± 12% of control and 40 μM: 332 ± 9% of control; all P<0.001; Figure 5-2B).

Figure 5-2: A) The C20 ω-3 epoxide increases the expression of cleaved caspase 9 protein in MDA-MB-231 cells. A representative immunoblot of 3 experiments is shown. B) Cleaved caspase 9 protein expression in MDA-MB-231 cells after treatment with the C20 epoxide. Expression of cleaved caspase 9 was normalized to GAPDH expression, where control is set as 100%. Data are mean ± SD of 3 independent experiments. Different from control: *** p<0.001.
5.2.2. Involvement of the mitochondrion in the mechanism of apoptosis elicited by the C20 ω-3 epoxide in MDA-MB-231 cells

Because the C20 ω-3 epoxide produced caspase 3/9 activation the potential involvement of the mitochondrion in the apoptotic mechanism was evaluated. An important indicator of mitochondrial damage is the release of cytochrome c into the cytosolic fraction of cells. Thus, cytochrome c protein was estimated in the cytosolic fraction of treated cells by immunoblotting. No protein was detected in cytosolic fractions after 24 hours of treatment with the C20 ω-3 epoxide but was readily detected by 48 hours (Figure 5-3A). Thus, cytosolic cytochrome c expression was increased to 121 ± 3%, 152 ± 6% and 190 ± 7% of control by the C20 ω-3 epoxide at 10, 20 and 40 µM concentration, respectively (Figure 5-3B).
Figure 5-3: A) The C20 ω-3 epoxide increases the release of cytochrome c protein into the cytosol of MDA-MB-231 cells. A representative immunoblot of 3 experiments is shown. B) Cytochrome c protein expression in MDA-MB-231 cells after treatment with the C20 ω-3 epoxide. Expression of cytosolic cytochrome c was normalized to GAPDH expression, where control is set as 100%. Data are mean ± SD of 3 independent experiments. Different from control: ** p<0.01 and *** p<0.001.

Cytochrome c release from the mitochondrion occurs as a result of membrane disruption following exposure to proapoptotic stimuli. The Bcl-2 family of proteins are important constituents of mitochondrial channels and the
ratio of pro- and anti-apoptotic Bcl-2 proteins is used as an indicator of mitochondrial integrity.

Expression of the pro-apoptotic proteins Bax and Bak was increased by treatment of MDA-MB-231 cells with the C20 ω-3 epoxide (Figure 5-4A). After 24 hours Bax immunoreactive protein expression increased to $161 \pm 25\%$ of control after treatment with C20 ω-3 epoxide (40 μM; $P<0.01$; Figure 5-4B). The increase in expression was somewhat more pronounced after 48 hours treatment: to $161 \pm 14\%$ and $222 \pm 45\%$ of control produced by 20 and 40 μM C20 ω-3 epoxide, respectively ($P<0.001$; Figure 5-4B). Similarly, Bak protein was increased in MDA-MB-231 cells after treatment with the C20 ω-3 epoxide. At concentrations of 20 and 40 μM Bak protein expression was increased to $129 \pm 17\%$ and $132 \pm 8\%$ of control, respectively, after 24 hours (Figure 5-4C). This increase was more pronounced at 48 hours (10 μM: $156 \pm 11\%$ of control; 20 μM: $226 \pm 5\%$ of control and 40 μM: $294 \pm 14\%$ of control; all $P<0.001$; Figure 5-4C).
Figure 5-4: A) The C20 ω-3 epoxide increases the expression of Bax and Bak proteins in MDA-MB-231 cells. A representative immunoblot of 3 experiments.
is shown. **B) Bax protein expression in MDA-MB-231 cells after treatment with the C20 ω-3 epoxide. C) Bak protein expression in MDA-MB-231 cells after treatment with the C20 ω-3 epoxide.** Expression of Bax and Bak was normalized to GAPDH expression, where control is set as 100%. Data are mean ± SD of 3 independent experiments. Different from control: ** p<0.01 and *** p<0.001.

Expression of the pro-survival proteins Bcl-2 and Bcl-XL in MDA-MB-231 cells was also affected by treatment with the C20 ω-3 epoxide (Figure 5-5A). Thus, expression of Bcl-2 protein decreased to 55 ± 2% and 57 ± 1% of control when cells were treated with the C20 ω-3 epoxide at 20 and 40 μM concentrations over 24 hours (P<0.001; Figure 5-5B). Expression was decreased to 75 ± 11% (epoxide concentration 20 μM; P<0.01) and 48 ± 4% (epoxide concentration 40 μM; P<0.001) of control after 48 hours (Figure 5-5B). In contrast, Bcl-XL protein expression was less responsive to C20 ω-3 epoxide treatment. The only significant decrease in Bcl-XL expression occurred after 48 hours of treatment with the highest concentration of the C20 ω-3 epoxide (to 67 ± 13% of control at 40 μM; P<0.001; Figure 5-5C).
Figure 5-5: A) The C20 ω-3 epoxide decreases the expression of Bcl-2 and Bcl-XL proteins in MDA-MB-231 cells. A representative immunoblot of 3 experiments is shown. B) Bcl-2 protein expression in MDA-MB-231 cells after
treatment with the C20 ω-3 epoxide. C) Bcl-XL protein expression in MDA-MB-231 cells after treatment with the C20 ω-3 epoxide. Expression of Bcl-2 and Bcl-XL was normalized to GAPDH expression, where control is set as 100%. Data are mean ± SD of 3 independent experiments. Different from control: ** p<0.01 and *** p<0.001.

As discussed in section 1.6.4, the ratio of pro- to anti-apoptotic Bcl-2 family protein expression may be used as an index of cell viability in apoptotic cells. In this study the Bax:Bcl-2 ratio in MDA-MB-231 cells that were treated with the C20 ω-3 epoxide for 24 hours increased to 2.5 ± 0.4-fold and 2.8 ± 0.5-fold of control at concentrations of 20 μM and 40 μM, respectively (P<0.001; Figure 5-6). Similarly, the Bak:Bcl-2 ratio was increased to 2.5 ± 0.1-fold and 2.3 ± 0.1-fold of control at epoxide concentrations of 20 μM and 40 μM, respectively (P<0.001; Figure 5-6). However, at 24 hours the Bax:Bcl-XL ratio was only altered at the highest concentration of the C20 ω-3 epoxide (to 1.6 ± 0.4-fold of control) and the Bak:Bcl-XL ratio was unchanged at any concentration (Figure 5-6).
Figure 5-6: Ratios of normalized pro-apoptotic Bax and Bak expressions to normalized pro-survival Bcl-2 and Bcl-XL expressions in MDA-MB-231 cells following C20 ω-3 epoxide treatment for 24 hours. Data are mean ± SD of 3 independent experiments. Different from control: ** p<0.01 and *** p<0.001.

These ratios were also determined after 48 hours of treatment with the C20 ω-3 epoxide: more pronounced changes were noted (Fig 5-7). Thus, C20 ω-3 epoxide treatment for 48 hours markedly increased the ratio of Bax:Bcl-2 protein expression (at the 20 μM concentration to 2.2 ± 0.1-fold of control, P<0.001; and at the 40 μM concentration to 4.6 ± 0.5-fold of control, P<0.001). The Bax:Bcl-XL ratio was also increased to 1.9 ± 0.2-fold of control at the 20 μM concentration and to 3.4 ± 0.5-fold of control at the 40 μM concentration (P<0.001). Pronounced increases in the Bak:Bcl-2 ratio to 3.0 ± 0.4-fold of control and 6.2 ± 0.5-fold of control were noted at 20 and 40 μM, respectively, while the Bak:Bcl-XL ratio increased to 2.7 ± 0.1 and 4.5 ± 0.8-fold of control at 20 μM and 40 μM of control (Figure 5-7).
Figure 5-7: Ratios of normalized pro-apoptotic Bax and Bak expressions to normalized pro-survival Bcl-2 and Bcl-XL expressions in MDA-MB-231 cells following C20 ω-3 epoxide treatment for 48 hours. Data are mean ± SD of 3 independent experiments. Different from control: * p<0.05, ** p<0.01 and *** p<0.001.
5.3.3. The JNK and p38 MAPK cascades mediate the activation of caspase 3 in MDA-MB-231 cells by the C20 ω-3 epoxide

Figure 5-8: Effect of MAPK inhibitors on caspase 3 activity in MDA-MB-231 cells after treatment with the C20 ω-3 epoxide (20 μM, 48 hours). Data are mean ± SD of 3 independent experiments: *** p<0.001 relative to control in the absence of C20 ω-3 epoxide; †††p<0.001 relative to C20 ω-3 epoxide in the absence of inhibitor.

Co-treatment of MDA-MB-231 cells with the JNK inhibitor SP600125 and p38 MAPK inhibitor SB203580, but not the PI3K inhibitor LY294002, significantly decreased caspase 3 activity effected by the C20 ω-3 epoxide (20 μM; Figure 5-8). Caspase 3 activity was found to be decreased by SP600125 (10
μM: 101 ± 2%; 20 μM: 104 ± 3%) and SB203580 (10 μM: 139 ± 13%; 20 μM: 112 ± 2%) compared to 164 ± 4% of control (Figure 5-8).

These findings were confirmed by gene silencing. Transfection of MDA-MB-231 cells with the DN-JNK and DN-p38 MAPK significantly decreased caspase 3 activity effected by the C20 ω-3 epoxide (40 μM; Figure 5-9). However, knockdown of PI3K with a specific siRNA did not prevent the epoxide-mediated increase in caspase 3 activity (Figure 5-9). DN-JNK and DN-p38 MAPK decreased

Figure 5-9: Effect of MAPK gene silencing on caspase 3 activity in MDA-MB-231 cells after treatment with the C20 ω-3 epoxide (40 μM, 48 hours). Data are mean ± SD of 3 independent experiments: *** p<0.001 relative to pcDNA or scrambled siRNA control in the absence of C20 epoxide; ††† p<0.001 relative to pcDNA or scrambled siRNA control in the presence of C20 epoxide.
caspase 3 activity to 102 ± 10% and 107 ± 16% compared to 156 ± 10% of control (Figure 5-9).

Because treatment with pharmacological inhibitors of the proapoptotic JNK and p38 MAPK cascades were able to prevent C20 ω-3 epoxide-mediated killing in MDA-MB-231 cells further studies were undertaken to characterize the upstream signaling events. Transfection of MDA-MB-231 cells with DN-MKK7, but not DN-MKK3 or DN-MKK6, significantly decreased caspase 3 cleavage effected by the C20 ω-3 epoxide (40 μM; Figure 5-10A). Knockdown of MKK4 with a specific siRNA reagent (because a DN-mutant for this kinase was not available) also impaired epoxide-mediated caspase 3 cleavage (Figure 5-10B). After densitometry caspase 3 cleavage was found to be decreased by DN-MKK7 to 189 ± 12% compared to 433 ± 31% of control (empty plasmid alone; P<0.001; Figure 5-10C). MKK4 knockdown decreased caspase 3 cleavage to 138 ± 44% of control compared to 493 ± 84% in scrambled siRNA-transfected cells (Figure 5-10C).
Figure 5-10: A) Transfection of DN-MKK7 selectively impairs the increase in caspase 3 cleavage induced in MDA-MB-231 cells after 48 hours treatment with the C20 epoxide. A representative immunoblot of 3 experiments is shown. 
 B) Knockdown of MKK4 decreased the induction of caspase 3 cleavage by the C20 ω-3 epoxide in MDA-MB-231 cells at 48 hours. A representative immunoblot of 3 experiments is shown. C) Effect of MKK gene silencing on caspase 3 cleavage in MDA-MB-231 cells after treatment with the C20
epoxide (40 μM, 48 hours). Expression of cleaved caspase 3 was normalized to GAPDH expression, where control is set as 100%. Data are mean ± SD of 3 independent experiments: ** p<0.01 and *** p<0.001 relative to pcDNA or scrambled siRNA control in the absence of C20 epoxide; ††† p<0.001 relative to pcDNA or scrambled siRNA control in the presence of C20 epoxide.

Transfection of MDA-MB-231 cells with a DN-MKK7 mutant, but not DN-MKK3 or DN-MKK6, also decreased caspase 3 activity effected by the C20 ω-3 epoxide (40 μM; Figure 5-11). Knockdown of MKK4 similarly prevented the increase in caspase 3 activity (Figure 5-11). Thus, caspase 3 activity was decreased by the DN-MKK7 mutant to 160 ± 5% compared to 318 ± 18% of control (empty plasmid; Figure 5-11). Knockdown of MKK4 decreased caspase 3 activity to 187 ± 36% of control, compared to 308 ± 21% of control in scrambled siRNA-transfected cells (Figure 5-11).
Figure 5-11: Effect of MKK gene silencing on caspase 3 activity in MDA-MB-231 cells after treatment with the C20 ω-3 epoxide (40 μM, 48 hours). Data are mean ± SD of 3 independent experiments: *** p<0.001 relative to pcDNA or scrambled siRNA control in the absence of C20 ω-3 epoxide; ††† p<0.001 relative to pcDNA or scrambled siRNA control in the presence of C20 ω-3 epoxide.

Further studies tested whether the C20 epoxide activated MKK4 in MDA-MB-231 cells. At 20 μM and 40 μM concentrations the C20 ω-3 epoxide increased phospho-MKK4 expression to 174 ± 46% and 190 ± 65% of control respectively at 8 hours (Figure 5-12A; Figure 5-12B).
Figure 5-12: A) The C20 epoxide increases the expression of phospho-MKK4 protein in MDA-MB-231 cells. A representative of 2 experiments is shown. B) Phospho-MKK4 protein expression in MDA-MB-231 cells after treatment with the C20 ω-3 epoxide. Expression of phospho-MKK4 was normalized to GAPDH expression, where control is set as 100%. Data are mean ± SD of 2 independent experiments. Different from control: *** p<0.001.

Similarly, the C20 epoxide activated MKK7 in MDA-MB-231 cells (Figure 5-13A). After 8 hours phospho-MKK7 expression increased to 136 ± 24%, 209 ± 11% and 188 ± 22% of control, respectively, after treatment with 10, 20 and 40 μM epoxide (Figure 5-13B).
Figure 5-13: A) The C20 ω-3 epoxide increases the expression of phospho-MKK7 protein in MDA-MB-231 cells. A representative immunoblot of 2 experiments is shown. B) Phospho-MKK7 protein expression in MDA-MB-231 cells after treatment with the C20 ω-3 epoxide. Expression of phospho-MKK7 was normalized to GAPDH expression, where control is set as 100%. Data are mean ± SD of 2 independent experiments. Different from control: **p<0.01 and *** p<0.001.

Treatment with the C20 ω-3 epoxide also increased JNK activation in MDA-MB-231 cells (Figure 5-14A). After 24 hours phospho-JNK was increased to 171 ± 1%, 174 ± 2% and 178 ± 1% of control following treatment with the epoxide at 10 μM, 20 μM and 40 μM respectively (Figure 5-14B).
Figure 5-14: A) The C20 ω-3 epoxide increases the expression of phospho-JNK protein in MDA-MB-231 cells. A representative immunoblot of 2 experiments is shown. B) Phospho-JNK protein expression in MDA-MB-231 cells after treatment with the C20 epoxide. Expression of phospho-JNK was normalized to GAPDH expression, where control is set as 100%. Data are mean ± SD of 2 independent experiments. Different from control: *p<0.05 and *** p<0.001.

In addition, the C20 epoxide activated p38 MAPK in MDA-MB-231 cells (Figure 5-15A). After 24 hours phospho-p38 MAPK expression increased to 194 ± 22%, 200 ± 16% and 232 ± 7% of control, respectively, after treatment with 10, 20 and 40 μM epoxide (Figure 5-15B).
Figure 5-15: A) The C20 ω-3 epoxide increases the expression of phospho-p38 MAPK protein in MDA-MB-231 cells. A representative immunoblot of 2 experiments is shown. B) Phospho-p38 MAPK protein expression in MDA-MB-231 cells after treatment with the C20 epoxide. Expression of phospho-p38 MAPK was normalized to GAPDH expression, where control is set as 100%. Data are mean ± SD of 2 independent experiments. Different from control: *** p<0.001.

Upstream signaling intermediates at the MKKK level that have been linked with apoptotic signaling were evaluated. Co-treatment of MDA-MB-231 cells with the ASK-1 inhibitor NQDI-1 decreased caspase 3 activity effected by the C20 epoxide (40 μM) to 182 ± 8% (NQDI-1 5 μM) and 139 ± 17% of control (NQDI-1 10 μM) compared to 257 ± 54% of control (Figure 5-16).
Figure 5-16: Effect of NQDI-1 on caspase 3 activity in MDA-MB-231 cells after treatment with the C20 epoxide (40 μM, 48 hours). Data are mean ± SD of 3 experiments: * p<0.05 and *** p<0.001 relative to control in the absence of C20 epoxide; †p<0.05, ††p<0.01 relative to C20 epoxide in the absence of NQDI-1.

Treatment with the C20 epoxide also increased phospho-ASK-1 expression (Figure 5-17A) after 8 and 24 hours. The expression of phospho-ASK-1 increased to 170 ± 1%, 263 ± 11% and 439 ± 15% following treatment at 10, 20 and 40 μM, respectively (Figure 5-17B).
Figure 5-17: A) The C20 ω-3 epoxide increased the expression of phospho-ASK-1 protein in MDA-MB-231 cells. A representative immunoblot of 2 experiments is shown. B) Phospho-ASK-1 expression in C20 epoxide-treated MDA-MB-231 cells. Expression of phospho-ASK-1 was normalized to GAPDH expression, where control is set as 100%. Data are mean ± SD of 2 independent experiments. Different from control: *p<0.05 and *** p<0.001.

5.3.4. Caspase-dependent apoptosis in C20 epoxide-treated MDA-MB-231 cells is death receptor mediated

The involvement of ASK1 implicated death receptors in the apoptotic mechanism of the C20 epoxide. This possibility was tested further by knockdown of putative components of death receptor complexes.
Knockdown of the adaptor proteins TRADD and TRAF2, but not FADD, prevented the C20 ω-3 epoxide-mediated decreased in caspase 3 cleavage after 48 hours (Figure 5-18A). After densitometry these decreases were to 184 ± 29% and 244 ± 9% of respective control after transfection with TRADD and TRAF2 siRNA, relative to 433 ± 31% of control in scramble-siRNA-transfected cells (P<0.001; Figure 5-18B).

Figure 5-18: A) Knockdown of TRADD and TRAF2 prevent the C20 ω-3 epoxide-mediated cleavage of caspase 3 in MDA-MB-231 cells at 48 hours. A representative immunoblot of 3 experiments is shown. B) Effect of TRADD and
TRAF2 gene silencing on caspase 3 cleavage in MDA-MB-231 cells after treatment with the C20 epoxide (40 µM, 48 hours). Expression of cleaved caspase 3 was normalized to GAPDH expression, where control is set as 100%. Data are mean ± SD of 3 independent experiments: *** p<0.001 relative to scrambled siRNA control in the absence of C20 epoxide; ††† p<0.001 relative to scrambled siRNA control in the presence of C20 epoxide.

Knockdown of TNF-R1 selectively prevented C20 epoxide-dependent caspase 3 cleavage in MDA-MB-231 cells (40 µM, 48 hours; Figure 5-19A). The increase in cleaved caspase 3 was to 234 ± 55% of control, compared with 444 ± 30% in scrambled siRNA-transfected control (Figure 5-19B). In contrast, knockdown of FAS, DR4 or DR5 did not prevent the increase in caspase 3 cleavage (Figure 5-19A; Figure 5-19B).
Figure 5-19: A) Knockdown of TNF-R1, but not FAS, DR4 or DR5, impairs the induction of caspase 3 cleavage in MDA-MB-231 cells by the C20 epoxide (40 µM, 48 hours). The primary antibody dilution was 1:200. A representative immunoblot of 3 experiments is shown. B) TNF-R1 silencing selectively decreases caspase 3 cleavage in C20 epoxide-treated MDA-MB-231 cells (40 µM, 48 hours). Expression of cleaved caspase 3 was normalized to GAPDH expression, where control is set as 100%. Data are mean ± SD of 3 experiments: *** p<0.001 relative to scrambled siRNA control in the absence of C20 epoxide; ††† p<0.001 relative to scrambled siRNA control in the presence of C20 epoxide.
5.3.5. Activation of Bid by the C20 ω-3 epoxide links the extrinsic and intrinsic pathways of apoptosis in MDA-MB-231 cells

The dual involvement of TNF-R1 and the mitochondrion identified in this Chapter implicates both extrinsic and intrinsic pathways in C20 ω-3 epoxide-mediated apoptosis in MDA-MB-231 cells. Previous studies have shown that these pathways are linked by caspase-dependent activation of the BH3-interacting domain death agonist Bid to form truncated-Bid (tBid) (Huang, Zhang, Farahvash, et al., 2007). This possibility was tested as part of the present project.

Treatment of MDA-MB-231 cells with the C20 ω-3 epoxide was found to increase tBid protein expression (Figure 5-20A). Thus, at 20 µM and 40 µM concentrations the C20 ω-3 epoxide increased tBid expression to 152 ± 12% and 177 ± 7% of control, respectively, at 24 hours (Figure 5-20B). A more pronounced increase in tBid expression was noted at 48 hours (20 µM: 177 ± 28% and 40 µM: 375 ± 53% of control; Figure 5-20B).
Figure 5-20: A) Concentration and time-dependent increase in the expression of tBid immunoreactive protein in C20 ω-3 epoxide-treated MDA-MB-231 cells. A representative immunoblot of 3 experiments is shown. B) tBid protein expression in MDA-MB-231 cells after treatment with the C20 ω-3 epoxide. Expression of tBid was normalized to GAPDH expression, where control is set as 100%. Data are mean ± SD of 3 independent experiments: ** p<0.01 and *** p<0.001.

Consistent with these findings, knockdown of Bid was found to prevent caspase 3 cleavage after treatment of MDA-MB-231 cells with the C20 ω-3 epoxide (Figure 5-21A). Thus, the expression of cleaved caspase 3 after treatment with the epoxide (40 µM, 48 hours) decreased from 465 ± 50% of
control (scrambled siRNA only) to 200 ± 24% of control when Bid was knocked down (Figure 5-21B).

Figure 5-21: A) Knockdown of Bid decreases the cleavage of caspase 3 induced by treatment of MDA-MB-231 cells with C20 ω-3 epoxide (40 µM, 48 hours). A representative immunoblot of 3 experiments is shown. B) Bid gene silencing impairs caspase 3 cleavage in C20 ω-3 epoxide-treated MDA-MB-231 cells (40 µM, 48 hours). Expression of cleaved caspase 3 was normalized to GAPDH expression, where control is set as 100%. Data are mean ± SD of 3 independent experiments: ** p<0.01, *** p<0.001 relative to scrambled siRNA control in the absence of C20 ω-3 epoxide; †††p<0.001 relative to scrambled siRNA control in the presence of C20 ω-3 epoxide.
Further studies were undertaken to test the relationship between proapoptotic MAPKs and Bid truncation. As shown in Fig 5-22A, transfection of MDA-MB-231 cells with DN-JNK and DN-p-38 MAPK decreased the extent of caspase 3 cleavage produced by the C20 ω-3 epoxide (40 μM; 48 hours). Thus, the increase in caspase 3 cleavage in DN-JNK transfected and C20 ω-3 epoxide treated cells was to 200 ± 13% of untreated cells, and to 329 ± 28% of control in DN-p-38 MAPK transfected/epoxide-treated cells, compared to 465 ± 50% in cells that were transfected with empty plasmid (Figure 5-22B). This is consistent with a major role for JNK in caspase 3 cleavage.
Figure 5-22: A) Transfection of DN-JNK decreases caspase 3 cleavage induced by C20 ω-3 epoxide in MDA-MB-231 cells at 48 hours. A representative immunoblot of 3 experiments is shown. B) Caspase 3 cleavage in C20 epoxide-treated MDA-MB-231 cells (40 μM, 48 hours) after transfection with DN-JNK or DN-p-38 MAPK. Expression of cleaved caspase 3 was normalized to GAPDH expression, where control is set as 100%. Data are mean ± SD of 3 experiments. *** p<0.001 relative to empty plasmid control in the absence of C20 epoxide; ††† p<0.001 relative to empty plasmid control in the presence of C20 epoxide.
In view of the pronounced effect of the DN-JNK on caspase 3 cleavage by the epoxide further studies were conducted. The expression of tBid in MDA-MB-231 cells after treatment with the C20 ω-3 epoxide (40 μM, 48 hours) was diminished by DN-JNK transfection (Figure 5-23A). Thus, compared with tBid expression in epoxide-treated empty plasmid-transfected cells (411 ± 65% of control), expression was less responsive after epoxide treatment after transfection with DN-JNK (154 ± 30% of control; Figure 5-23B).

Figure 5-23: A) Transfection of DN-JNK decreases tBid immunoreactive protein in C20 ω-3 epoxide-treated MDA-MB-231 cells at 48 hours. A representative immunoblot of 3 experiments is shown. B) Effect of JNK gene silencing on tBid protein expression in MDA-MB-231 cells after treatment with the C20 ω-3 epoxide (40 μM, 48 hours). Expression of cleaved caspase 3
was normalized to GAPDH expression, where control is set as 100%. Data are mean ± SD of 3 independent experiments: *** p<0.001 relative to scrambled empty plasmid control in the absence of C20 ω-3 epoxide; ††† p<0.001 relative to empty plasmid control in the presence of C20 ω-3 epoxide.

5.4. Discussion

The findings in this chapter indicate that the synthetic C20 ω-3 epoxide activates apoptosis in MDA-MB-231 human breast cancer cells by the dual mechanisms of death receptor activation and mitochondrial targeting. The C20 ω-3 epoxide increased caspase 3 and caspase 9 cleavage. Caspases are synthesized as inactive procaspase zymogens and undergo cleavage by upstream initiator caspases to produce the active proteases (Earnshaw, Martins & Kaufmann, 1999). Thus, caspases-2, -8, -9 and -10 are important initiator caspases that are either recruited to intracellular death receptor complexes where they undergo activation (extrinsic pathway) or are activated by released mitochondrial factors, such as cytochrome c (intrinsic pathway). The activated initiator caspases in turn activate downstream executor caspases, such as caspase 3 and caspase 7, that are responsible for degradation of cellular proteins (Jiang & Wang, 2000; Kondoh, Araragi, Sato, et al., 2002). Caspase 3 is the major executioner, and has been shown to be essential for apoptosis-associated chromatin margination, DNA fragmentation, and nuclear collapse (Slee, Adrain & Martin, 2001).

Many anticancer agents and molecules of endogenous importance, including fatty acids, are able to activate caspases and apoptosis in tumour cells. Butyrate and other short-chain saturated fatty acids induce apoptosis by disrupting the mitochondrial membrane and activating caspase 3 in colon
carcinoma cells (Heerdt, Houston & Augenlicht, 1997; Hardy, El-Assaad, Przybytkowski, et al., 2003). The branched chain saturated fatty acid 13-methyltetradecanoic acid also disrupts the mitochondrion and activates apoptosis in several cancer cell lines (Yang, Liu, Chen, et al., 2000; Wongtangtintharn, Oku, Iwasaki, et al., 2005; Lin, Yin, Cai, et al., 2012). The PUFA linoleic acid decreases skin, gastric, breast and colon cancer cell viability by activating caspase 3 and apoptosis (Ip, Ip, Loftus, et al., 2000). The combination of ω-3 EPA and DHA strongly increased caspase activity in MDA-MB-231 cells (Schley, Jijon, Robinson, et al., 2005) and in human hepatocellular carcinoma Hep3B, Huh-7 and HepG2 cells (Lim, Han, Dai, et al., 2009). The N-acylated ceramide lipids accumulate in cells after treatment with anticancer agents or saturated fatty acids, such as palmitic acid (Merrill & Jones, 1990). Direct addition of ceramide to mitochondria disrupted the organelle and activated caspase 3 (García-Ruiz, Colell, Mari, et al., 1997).

Apart from the parent lipids, some fatty acid metabolites also inhibit aspects of tumourigenesis. For example, cyclooxygenase-2-derived ω-3 prostaglandin E₃ has been implicated in the anti-cancer effects of ω-3 PUFAs (Szymczak, Murray & Petrovic, 2008). The CYP-mediated 17,18-epoxide of EPA, but not its isomeric epoxides, inhibited cell proliferation and activated apoptosis in endothelial cells (Cui, Petrovic & Murray, 2011). Zhang, Panigrahy, Mahakian, et al., (2013) reported similar findings with epoxide metabolites of the C22 ω-3 fatty acid DHA.

Some information is available regarding the mechanisms by which ω-3 fatty acids and their metabolites produce cell death. There is evidence that fatty acids promote the release of cytochrome c from the mitochondrion, which
activates executioner caspases and promotes apoptosis. This occurred in the Hep3B hepatocellular carcinoma cell after treatment with the ω-3 fatty acid DHA (Lim, Han, Dai, et al., 2009) and in INS-1 pancreatic beta cells after palmitate and oleate treatment (Maestre, Jordán, Calvo, et al., 2003). In the present study the treatment of MDA-MB 231 cells with the C20 ω-3 epoxide triggered mitochondrial disruption, cytochrome c release and caspase activation. Loss of cytochrome c from mitochondria impairs electron transport and oxidative phosphorylation, which decreases ATP production and increases reactive oxygen species formation (Goldstein, Waterhouse, Juin, et al., 2000). The released cytochrome c is essential for formation of the apoptosome and cell killing (Zou, Li, Liu, et al., 1999; Jiang & Wang, 2000).

The relative expression of pro- and anti-apoptotic Bcl-2 proteins is an indicator of the cellular response to apoptotic stimuli (Finnegan, Curtin, Prevost, et al., 2001). Pro-apoptotic Bcl-2 proteins, such as Bax, Bak and Bid, dimerize and destabilize the outer mitochondrial membrane, whereas anti-apoptotic Bcl-2 proteins, such as Bcl-2 itself and Bcl-XL, stabilize the membrane. Addition of recombinant Bax or Bak to isolated mitochondria promoted cytochrome c release (Zong, Lindsten, Ross, et al., 2001). In this chapter, it was found that treatment of MDA-MB-231 cells with the C20 ω-3 epoxide increased the expression of the pro-apoptotic Bcl-2 proteins Bax and Bak and decreased the expression of the prosurvival Bcl-2 and Bcl-XL; the increase in the Bak/Bcl-2 ratio was most pronounced.

A number of studies have suggested that fatty acids may regulate the expression of Bcl-2 proteins. Thus, ω-3 EPA and DHA activated apoptosis in Jurkat T-cells by down-regulating the prosurvival Bcl-2 (Denys, Hichami & Khan,
An increase in the Bax/Bcl-2 ratio occurred after treatment of MCF-7 breast carcinoma cells with DHA (Chiu, Wong & Ooi, 2004). In addition, accumulation of the proapoptotic lipid ceramide in cells altered Bcl-2 protein that increased mitochondrial cytochrome c translocation and caspase 3 activation (García-Ruiz, Colell, Marí, et al., 1997). Dietary EPA and DHA also decreased Bcl-2 expression and increased Bax expression in an in vivo model of mammary carcinogenesis (Manna, Chakraborty, Ghosh, et al., 2008).

Bid is a pro-apoptotic BH3-only Bcl-2 protein that undergoes truncation to tBid and then interacts with Bax to induce opening of the mitochondrial voltage-dependent anion channel (Korsmeyer, Wei, Saito, et al., 2000). This enables cytochrome c release, formation of the apoptosome and activation of caspase 3. In this chapter the expression of tBid increased in a concentration- and time-dependent manner in MDA-MB-231 cells that were treated with C20 ω-3 epoxide. Knockdown of Bid decreased caspase 3 cleavage in C20 ω-3 epoxide-treated cells and knockdown of JNK inhibited tBid formation. Thus, activation of Bid was an important event in C20 ω-3 epoxide-mediated apoptosis and JNK activation was important in Bid truncation.

The p38 MAPK regulates apoptosis by ω-3 17,18-epoxy-EPA in bEND.3 microvascular endothelial cells (Cui, Petrovic & Murray, 2011). In this thesis, pharmacological inhibitors and gene silencing of both JNK and p38 MAPKs modulated caspase 3 cleavage in MDA-MB-231 cells that were treated with the synthetic C20 ω-3 epoxide. Co-involvement of both JNK and p38 MAPK in apoptotic pathways has been reported previously in several studies using different cells (Cuadrado, García-Fernandez, Gonzalez, et al., 2003; Ohtsuka, Buchsbaum, Oliver, et al., 2003; Kim, Ryu & Song, 2006). JNK also plays a role in
regulating pro- and anti-apoptotic Bcl-2 proteins that control mitochondrial membrane permeabilization and cytochrome c release (Blatt, Boitano, Lyssiotis, et al., 2008).

Upstream signalling intermediates in the apoptotic pathway were assessed in the present study to account for the apparent involvement of both MAPKs. Expression of phospho-JNK, phospho-MKK4 and phospho-MKK7 was increased in C20 ω-3 epoxide-treated MDA-MB-231 cells. In gene silencing experiments MKK4 and MKK7, but not the alternate MAPK kinases MKK3 and MKK6, contributed to C20 ω-3 epoxide-dependent caspase 3 cleavage. It is known that MKK4 can activate both JNK and p38 MAPK, while MKK7 exclusively activates JNK (Dhanasekaran & Reddy, 2008). In contrast, MKK3 and MKK6 both activate the p38 MAPK (Schaeffer & Weber, 1999). Thus, the involvement of both MKK4 and MKK7 suggests that JNK is the predominant MAPK that regulates the C20 ω-3 epoxide apoptotic pathway and that p38 MAPK activation may occur as a consequence of MKK4 activation.

Expression of the phosphorylated form of the MAPK kinase kinase ASK1 is increased in C20 ω-3 epoxide-treated cells. ASK1 is upstream of the MKKs 4 and 7 as well as JNK and p38 MAPK and is activated by diverse apoptotic stimuli such as TNF-α, reactive oxygen species, lipopolysaccharide and endoplasmic reticulum stress (Matsuzawa & Ichijo, 2001; Nakajima, Komazawa-Sakon, Takekawa, et al., 2006; Nagai, Noguchi, Takeda, et al., 2007). ASK1 is important for TNF-R1- and oxidative stress-induced sustained activations of JNK or p38 MAPK during apoptosis (Tobiume, Matsuzawa, Takahashi, et al., 2001).

Other fatty acids have been shown to activate MAPK pathways similar to the C20 ω-3 epoxide. The ω-3 fatty acids DHA and, to a lesser extent, EPA
activated JNK phosphorylation in oral squamous cell carcinoma (SCC)-25 cells (Nikolakopoulou, Shaikh, Dehlawi, et al., 2013) and 13-methyltetradecanoic acid activated the pro-apoptotic p38 and JNK MAPKs in human bladder cancer cells (Lin, Yin, Cai, et al., 2012). Inhibition of these MAPKs in Jurkat T-cells decreased ceramide-induced apoptosis by preventing the loss of mitochondrial transmembrane potential and inhibition of caspase activation (Chen, Chang, Huang, et al., 2008). Interestingly, the alkyl phosphocholine derivative perifosine, which is now used clinically, also activates these MAPKs in part to induce apoptosis (Ruiter, Zerp, Bartelink, et al., 1999; Nieto-Miguel, Gajate & Mollinedo, 2006).

The apparent involvement of ASK1 is consistent with a role for death receptors in C20 ω-3 epoxide-mediated apoptosis in MDA-MB-231 cells. This was tested in further experiments. Caspase 3 activation was inhibited by knockdown of the prototypic death receptor TNF-R1 and the adaptor proteins TRADD and TRAF2. However, knockdown of Fas, DR4 and DR5, and an alternate adaptor protein FADD, did not alter the increase in caspase 3 cleavage produced by the C20 ω-3 epoxide in MDA-MB-231 cells.

To date, targeting death receptors to activate extrinsic apoptosis in tumours has been largely restricted to protein drugs. However, those that activate TNF-R1 and Fas cause toxicity, while those that activate DR4 and DR5 are cleared rapidly or have low efficacy (Ashkenazi, 2002; Micheau, Shirley & Dufour, 2013). Very few small molecules have been shown to act via death receptors to elicit cell killing. Solamargine - a major steroidal alkaloid glycoside - induces apoptosis in human breast cancer cells by up-regulating TNF-R1 and TRADD, and activates a program of events resembling those of the C20 ω-3
epoxide (Shiu, Chang, Liang, et al., 2007). Very recently, Wang, Wang, Yu, et al., (2013) synthesized and evaluated a new agent - bioymifi – that targets the TRAIL-R2/DR5 receptor. Findings in the present project suggest that the C20 ω-3 epoxide selectively activates TNF-R1, recruits TRADD and TRAF-2 and activates downstream ASK1-MKK4/MKK7-JNK/p38 MAPK signalling to elicit the killing of breast cancer cells (Figure 5-24). Such epoxides could be developed further as alternatives to solamargine and bioymifi.
Figure 5-24: Apoptosis pathways assessed in this study. C20 ω-3 epoxide selectively activates TNF-R1, recruits TRADD and TRAF-2 and activates downstream ASK1-MKK4/MKK7-JNK/p38 MAPK signaling. Activation of Bid was
an important event in C20 ω-3 epoxide-mediated apoptosis and JNK activation was important in Bid truncation. Mitochondrial disruption is due to the increased expression of the pro-apoptotic Bcl-2 proteins Bax and Bak and decreased expression of the pro-survival Bcl-2 and Bcl-XL.
6. General discussion and future directions

Recent studies have implicated ω-3-17,18-epoxyEPA, but not the epoxides formed at the other EPA double bonds, in the inhibition of proliferation and activation of apoptosis in endothelial cells (Cui, Petrovic & Murray, 2011). The possibility that these potential anti-tumor actions of ω-3-17,18-epoxy-EPA could be retained in ω-3 epoxides of saturated long chain fatty acids was evaluated in this project. Studies were conducted in four breast cancer cell lines with different characteristics (Table 3-3). MDA-MB-231 cells proliferate rapidly and are highly invasive, while MDA-MB-468, MCF-7 and T47D cells are less aggressive (Wang, Navab, Iakovlev, et al., 2007).

The viability of the epithelial adenocarcinoma-derived MDA-MB-231 cell line was markedly decreased after treatment with the epoxides. The less aggressive cell lines, including another epithelial adenocarcinoma-derived line (MDA-MB-468), and the hormone-responsive MCF-7 and T47D cells were less sensitive to the epoxides. The C20 epoxide was most effective, followed by the C21 analogue while the C22 epoxide was least active. Findings from flow cytometry indicated an increase in the proportion of cells in G₀/G₁ phase, especially after C20 epoxide treatment, while those in later phases (S and G₂/M) were decreased. In agreement with this finding, the expression of cyclin D1 and E proteins in MDA-MB-231 cells was also decreased by the epoxides (Figure 6-1).
Figure 6-1: The ω-3 epoxides target cyclins D1 and E to produce a pause in cell cycle progression. Adapted from (Vermeulen, Van Bockstaele & Berneman, 2003).

In contrast, while the C20-C22 ureas decreased the rate of MTT reduction in breast cancer cell lines, they were much less effective than the epoxides in decreasing ATP production and cell cycle progression. This suggests that reliance on MTT reduction as a measure of cell viability could produce misleading findings. Redox-active compounds and intracellular pH are factors that are known to influence MTT reduction (Berridge & Tan, 1993; Plumb, Milroy & Kaye, 1989; Petty, Sutherland, Hunter, et al., 1995). However, MTT reduction in viable cells is also supported by several dehydrogenases so that it may not exclusively reflect the activity of mitochondrial dehydrogenases that influence cell proliferation (Berridge & Tan, 1993).
In this study, ATP formation in MDA-MB-231 cells was decreased by the epoxides but was relatively unaffected by the ureas; other cell types were also resistant to the ureas. In agreement, the effects of the ureas on cell cycle progression and cyclin expression in all cell types were minimal. Cyclin D1 expression is up-regulated in many human cancers, including breast cancer (Sweeney, Swarbrick, Sutherland, et al., 1998; Vermeulen, Van Bockstaele & Berneman, 2003). The epoxides, but not the urea isosteres, may be useful molecules to develop as anti-cyclin agents for impairment of cancer cell proliferation.

Previous studies have found that some ω-3 fatty acids activate apoptosis in MDA-MB-231 cells (Schley, Jijon, Robinson, et al., 2005), in pre-malignant keratinocytes (Nikolakopoulou, Shaikh, Dehlawi, et al., 2013), in human preadipocyte AML-I cells, (Hanada, Morikawa, Hirota, et al., 2011), in human colorectal SW620 cells (Yang, Fang, Zhang, et al., 2013), and in the MIA-PaCa-2 and Capan-2 pancreatic cancer cell lines (Fukui, Kang, Okada, et al., 2013). The ω-3 PUFA EPA activates cell death programs, although these occur by different mechanisms in different cell types. Apart from apoptosis, EPA also activated necroptosis in Raji cells and autophagy in pancreatic MIA-PaCa-2 and Capan-2 cells (Heimli, Finstad & Drevon, 2001; Fukui, Kang, Okada, et al., 2013).

In this project the synthetic C20-C22 ω-3 fatty acid epoxides activated apoptosis in the MDA-MB-231 cell line. The pan-caspase inhibitor, z-VAD-fmk, reversed the loss of confluence and ATP production produced by the epoxides in MDA-MB-231 cells. In contrast, co-treatment with the necroptosis inhibitor, necrostatin-1, or the autophagy inhibitors 3-methyladenine and chloroquine had no effect on the decrease in cell viability produced by the C20-C22 epoxides. No
evidence was found for other types of cell death effected by the epoxides in other breast cancer cell lines.

The activation of apoptosis in C20 epoxide-treated MDA-MB-231 cells was characterized by an increase in caspase 3 and caspase 9 cleavage, an increase in PARP inactivation and an increase in cells in sub-G1. Consistent with the role of apoptosis in cell death the present epoxides increased annexin V staining in MDA-MB-231 cells. This suggests that the epoxides cause the redistribution and reorientation of phosphatidylserine at the plasma membrane (Martin, Reutelingsperger, McGahon, et al., 1995; Brumatti, Sheridan & Martin, 2008). This enables the detection of damaged cells by macrophages, which facilitates their removal (Fadok, Voelker, Campbell, et al., 1992). The small increase in propidium iodide staining in epoxide-treated MDA-MB-231 cells is consistent with minimal activation of necrosis.

Apoptosis was activated in epoxide-treated MDA-MB-231 human breast cancer cells by the dual mechanisms of death receptor activation and mitochondrial targeting. The C20 epoxide triggered the released of cytochrome c from the mitochondrion, which activated executioner caspases and promoted apoptosis. Other fatty acids also promote the release of mitochondrial cytochrome c. Thus, the ω-3 fatty acid DHA enhanced its release in the Hep3B hepatocellular carcinoma cell (Lim, Han, Dai, et al., 2009). In INS-1 pancreatic beta cells the saturated palmitic and oleic fatty acids disrupted the mitochondrial membrane to release cytochrome c (Maestre, Jordán, Calvo, et al., 2003). To account for these findings the relative expression of pro- and anti-apoptotic Bcl-2 proteins was estimated as an indicator of the cellular response to apoptotic stimuli (Finnegan, Curtin, Prevost, et al., 2001). Like other fatty acids that
activate intrinsic apoptosis, the ω-3 epoxides increased the expression of Bak and Bax and decreased Bcl-2 and Bcl-XL (Figure 6-2). Similarly, ω-3 EPA and DHA activated apoptosis in T-cells by down-regulating the prosurvival Bcl-2 (Denys, Hichami & Khan, 2005). Dietary EPA and DHA decreased Bcl-2 and increased Bax expression in an in vivo breast cancer model (Manna, Chakraborty, Ghosh, et al., 2008). An increase in the Bax/Bcl-2 ratio occurred after treatment of MCF-7 breast carcinoma cells with DHA (Chiu, Wong & Ooi, 2004).

**Figure 6-2: Activation of the intrinsic pathway of apoptosis.** Adapted from (Dhanasekaran & Reddy, 2008; Brenner & Mak, 2009).

It was also found that the C20 epoxide acted via the TNF-R1 death receptor to activate extrinsic apoptosis in MDA-MB-231 cells (Figure 6-3). This appeared to be selective for TNF-R1, because the related Fas, DR4 and DR5 receptors were not involved. ASK1 is upstream of the MKKs 4 and 7 that are upstream of the JNK and p38 MAPKs (Matsuzawa & Ichijo, 2001; Nakajima,
ASK1 mediates TNF-R1 and oxidative stress-induced sustained activations of JNK or p38 MAPK during apoptosis (Tobiume, Matsuzawa, Takahashi, et al., 2001). The role for ASK1 and the adaptor proteins TRADD and TRAF2 in caspase activation in C20 epoxide-treated MDA-MB-231 cells is also consistent with involvement of death receptors.

The p38 MAPK was found to mediate apoptosis in microvascular endothelial cells that were treated with ω-3 17,18-epoxy-EPA (Cui, Petrovic & Murray, 2011). Co-involvement of JNK and p38 MAPK in apoptotic pathways has also been reported previously in several studies in multiple cell types (Cuadrado, Garcia-Fernandez, Gonzalez, et al., 2003; Ohtsuka, Buchsbaum, Oliver, et al., 2003; Kim, Ryu & Song, 2006). Indeed, the JNK pathway regulates pro- and anti-apoptotic Bcl-2 proteins that control mitochondrial membrane permeabilization and cytochrome c release (Blatt, Boitano, Lyssiotis, et al., 2008).

Other fatty acids have also been shown to activate MAPK pathways. ω-3 DHA and EPA activated JNK phosphorylation in oral squamous cell carcinoma cells (Nikolakopoulou, Shaikh, Dehlawi, et al., 2013), 13-methyltetradecanoic acid activated the JNK and p38 MAPKs in bladder cancer cells (Lin, Yin, Cai, et al., 2012) and inhibition of these MAPK pathways in T-cells decreased ceramide-induced loss of mitochondrial membrane potential and caspase activation (Chen, Chang, Huang, et al., 2008). The clinically useful alkyl phospholipid perifosine also activates these MAPKs and induces apoptosis (Ruiter, Zerp, Bartelink, et al., 1999; Nieto-Miguel, Gajate & Mollinedo, 2006).

The finding that death receptor and mitochondrial apoptotic pathways both contribute to epoxide-dependent breast cancer cell death can be explained
by the finding that the BH3-only Bcl-2 protein Bid is activated to tBid during treatment with recombinant caspase 8 and TNFα (Luo, Budihardjo, Zou, et al., 1998; Korsmeyer, Wei, Saito, et al., 2000; Deng, Ren, Yang, et al., 2003). Increased JNK activity catalyses Bid cleavage, which enables cytochrome c release from the mitochondrion and activation of caspase 3. Consistent with this finding, knockdown of Bid decreased caspase 3 cleavage and knockdown of JNK inhibited Bid truncation.

Figure 6-3: Involvement of TNF-R1 and the ASK/MKK/JNK/p38 MAPK cascades in extrinsic apoptosis mediated by the C20 epoxide. Adapted from (Dhanasekaran & Reddy, 2008; Brenner & Mak, 2009).

To date, few small molecule pharmacological agents have been shown to activate death receptors to elicit cell killing. Findings in the present project suggest that the C20 ω-3 epoxide selectively activates TNF-R1, recruits TRADD and TRAF-2 and activates downstream ASK1-MKK4/MKK7-JNK/p38 MAPK signalling to elicit the killing of breast cancer cells. However, there are
outstanding questions that could be evaluated in future studies. How the epoxide activates apoptosis requires further evaluation. Although the TNF-R1 death receptor is involved it is unclear whether the epoxides act as ligands, or whether they act indirectly. Some fatty acids and eicosanoids are ligands for nuclear receptors, such as the peroxisome proliferator-activated receptor α (Forman, Chen & Evans, 1997). Alternately, the composition of lipid rafts could be altered by the C20-C22 epoxides, to produce a sustained activation of death receptor/MAPK signalling (Stillwell, Shaikh, Zerouga, et al., 2005; Schley, Brindley & Field, 2007).

The relationship between apoptosis and altered cell cycle regulation in epoxide-treated cells should also be clarified. Deregulation of the cell cycle has been shown to induce mitotic catastrophe, and also may trigger apoptosis (King & Cidlowski, 1995). The finding that the epoxides produce cell cycle arrest in G0/G1 is consistent with activation of the p38 MAPK and resultant down-regulation of cyclin D1 expression, which has been noted previously (Molnar, Theodoras, Zon, et al., 1997). However, further studies to test this possibility should now be undertaken.

Although MDA-MB-231 cells were responsive to the ω-3 epoxyfatty acids, three other breast cancer cell lines were unresponsive. The reason for this observation is unclear but testing in further cell types could be done. Such information would be valuable to determine whether epoxides may have value in a broad or narrow range of tumour cell types. Cell type-dependent differences in the effectiveness of the epoxide in could be due to differential expression of target genes or target pathways. For example, in this project, anti-apoptotic members of the Bcl-2 protein family were found to be down-regulated in MDA-
MB-231 cells by the C20-C22 epoxides. Bcl-2 is implicated in reducing the effectiveness of 5-fluorouracil in colon cancer cells (Violette, Poulain, Dussaulx, et al., 2002). The novel Bcl-2 antagonist ABT-737 was more effective in small cell lung cancer cells that express higher relative levels of Bcl-2 and a lower level of Mcl-1, while the cells with lower levels of Bcl-2 and higher levels of Mcl-1 were resistant (Tahir, Yang, Anderson, et al., 2007). Similar findings were made with another small-molecule inhibitor of Bcl-2, known as compound 6 (Enyedy, Ling, Nacro, et al., 2001). The epoxides may similarly target regulatory proteins that are expressed at different levels in different cell types.

The present ω-3 epoxide analogues, but not the urea isosteres, have some promising properties as potential anti-tumour agents. Future studies could evaluate their \textit{in vivo} activities and toxicity in animal models. If the agents prove to be effective and well tolerated they may eventually find application in cancer chemotherapy, although a large amount of further research would be required to achieve this outcome.
References


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Appendix A. Synthesis of $\omega$-3 epoxyfatty acid and urea analogues

**General chemistry**

Monounsaturated fatty acids 1a-1c and nitriles 3a-3c were prepared by Dr Tristan Rawling, as described previously (Cui, Rawling, Bourget, *et al.*, 2012) and AUDA was synthesized by the method of (Morisseau, Goodrow, Newman, *et al.*, 2002). 14(Z)-Eicosenoic acid (1d) was purchased from Cayman Chemical (Ann Arbor, MI). 11 (Z)-Eicosenoic acid (1e) and all other reagents and anhydrous solvents were purchased from Sigma Aldrich (Castle Hill, NSW, Australia). The purity of all test compounds (2a-2e, 5a-5c) was confirmed by elemental analysis carried out in the Campbell Microanalytical Laboratory at the Department of Chemistry, University of Otago. All values were within ±0.4% of the calculated values. Dry Column Vacuum Chromatography was used to purify reaction products on silica gel with gradient elutions. TLC was performed on silica gel 60 F$_{254}$ plates. $^1$H and $^{13}$C NMR spectra were recorded on a Varian 400-MR instrument and were referenced internally to residual solvent (CDCl$_3$; $^1$H δ 7.26, $^{13}$C δ 77.10. d$_6$-DMSO; $^1$H δ 2.49, $^{13}$C δ 39.52). High resolution mass spectrometry (HRMS) was performed on an Agilent Technologies 6510 Q-TOF LCMS.

**Syntheses**

**General procedure for epoxidation**

A solution of 1a-1e (0.165 g, 0.53 mmol) in dichloromethane (5 mL) was added to $m$-chloroperbenzoic acid (77% max., 0.166 g, 0.74 mmol) in dichloromethane
(2 mL). The reaction was stirred at room temperature for 2 h, concentrated and then dissolved in ethyl acetate (30 mL). The solution was washed with sat. NaHCO₃ (3 x 30 mL) and 0.1M HCl (10 mL), then concentrated. The residue was purified on silica gel by stepwise gradient elution with dichloromethane/ethyl acetate (100:0 to 30:70), yielding 2a-2e (Scheme A-1).
Scheme A-1: Synthesis of ω-3, ω-6 and ω-9 epoxyfatty acids

16-[(Z)-3-ethyloxiran-2-yl]hexadecanoic acid (2a). White solid, mp = 61 – 62 °C. Yield = 68%. $^1$H NMR (400 MHz, CDCl$_3$): δ 2.93 (m, 1H), 2.89 (m, 1H), 2.35 (t, $J = 7.6$ Hz, 2H), 1.63 (p, $J = 7.2$ Hz, 2H), 1.59 – 1.40 (m, 6H), 1.38 – 1.20 (m, 22H),
1.04 (t, J = 7.2 Hz, 3H). $^{13}$C NMR (100.5 MHz, CDCl$_3$): δ 179.67, 58.48, 57.47, 34.01, 29.62 (2C), 29.61 (2C), 29.56, 29.53 (3C), 29.41, 29.22, 29.05, 27.65, 26.57, 24.67, 21.05, 10.58. HRMS calcd for C$_{20}$H$_{39}$O$_3$ ([M + H]$^+$) m/z: 327.2894; found 327.2898.

17-[[Z]-3-ethyloxiran-2-yl]heptadecanoic acid (2b). White solid, mp = 72 – 73 ºC. Yield = 62%. $^1$H NMR (400 MHz, CDCl$_3$): δ 2.92 (m, 1H), 2.88 (m, 1H), 2.35 (t, J = 7.6 Hz, 2H), 1.63 (p, J = 7.2 Hz, 2H), 1.59 – 1.40 (m, 6H), 1.38 – 1.20 (m, 24H), 1.04 (t, J = 7.2 Hz, 3H). $^{13}$C NMR (100.5 MHz, CDCl$_3$): δ 179.31, 58.47, 57.46, 33.95, 29.63 (3C), 29.61 (2C), 29.55 (2C), 29.54 (2C), 29.41, 29.22, 29.05, 27.66, 26.58, 24.68, 21.06, 10.58. HRMS calcd for C$_{21}$H$_{41}$O$_3$ ([M + H]$^+$) m/z: 341.3050; found 341.3056.

18-[[Z]-3-ethyloxiran-2-yl]octadecanoic acid (2c). White solid, mp = 68 – 69 ºC. Yield = 70%. $^1$H NMR (400 MHz, CDCl$_3$): δ 2.93 – 2.94 (m, 2H), 2.34 (t, J = 7.0 Hz, 2H), 2.34 (t, J = 7.0 Hz, 2H), 1.63 (p, J = 7.2 Hz, 2H), 1.59 – 1.40 (m, 6H), 1.38 – 1.20 (m, 26H), 1.04 (t, J = 7.6 Hz, 3H). $^{13}$C NMR (100.5 MHz, CDCl$_3$): δ 179.78, 58.49, 57.48, 34.04, 29.65 (4C), 29.62 (2C), 29.57, 29.54 (3C), 29.42, 29.23, 29.05, 27.64, 26.57, 24.68, 21.04, 10.57. HRMS calcd for C$_{22}$H$_{43}$O$_3$ ([M + H]$^+$) m/z: 355.3207; found 355.3200.

14-[[Z]-3-pentyloxiran-2-yl]tetradecanoic acid (2d). White solid, mp = 66 – 69 ºC. Yield = 65%. $^1$H NMR (400 MHz, CDCl$_3$): δ 2.97 – 2.94 (m, 2H), 2.34 (t, J = 7.0 Hz, 2H), 1.63 (p, J = 7.0 Hz, 2H), 1.55 – 1.45 (m, 6H), 1.40 – 1.20 (m, 26H), 0.90 (t, J = 7.0 Hz, 3H). $^{13}$C NMR (100.5 MHz, CDCl$_3$): δ 181.72, 59.98 (2C), 36.54, 34.38, 32.18 (2C), 32.17 (2C), 32.16, 32.04, 31.85, 31.69, 30.45, 30.42, 29.25, 28.92, 27.33, 25.24, 16.64. HRMS calcd for C$_{20}$H$_{39}$O$_3$ ([M + H]$^+$) m/z: 327.2894; found 327.2895.

11-[[Z]-3-octyloxiran-2-yl]undecanoic acid (2e). White solid, mp = 56 – 57 ºC. Yield = 71%. $^1$H NMR (400 MHz, CDCl$_3$): δ 2.95 – 2.85 (m, 2H), 2.34 (t, J = 7.0 Hz,
2H), 1.62 (p, J = 7.0 Hz, 2H), 1.55 – 1.45 (m, 6H), 1.40 – 1.20 (m, 26H), 0.88 (t, J = 7.0 Hz, 3H). $^{13}$C NMR (100.5 MHz, CDCl$_3$): $\delta$ 181.88, 59.97, 59.95, 36.55, 34.50, 32.20, 32.18, 32.14, 32.12, 31.95, 31.87, 31.83, 31.68, 30.46, 30.44, 29.24 (2C), 27.32, 25.32, 16.75. HRMS calcd for C$_{20}$H$_{39}$O$_3$ ([M + H]$^+$) m/z: 327.2894; found 327.2893.

**General procedure for urea synthesis.**

To a solution of NiCl$_2$.6H$_2$O (1.87 mmol) and 3a-3c (0.93 mmol) in methanol (20 mL) at 0°C was added NaBH$_4$ (9.53 mmol) over 30 min. The black reaction mixture was allowed to warm to room temperature, and was then stirred for 1.5 h. 1M HCl was added until the black precipitate disappeared. The mixture was made alkaline with concentrated NH$_4$OH solution and extracted with dichloromethane (3 x 30 mL). The combined extracts were dried over NaSO$_4$ and concentrated. The residue was then dissolved in anhydrous tetrahydrofuran (15 mL) under a nitrogen atmosphere. Ethyl isocyanate (0.93 mmol) was added and the mixture was stirred at room temperature for 2 h, and then concentrated. The residue was purified on silica gel by stepwise gradient elution with dichloromethane/ethyl acetate (100:0 to 75:25), yielding the products as a white solid (Scheme A-2).
Scheme A-2: Synthesis of ω-3 ureas 5a-c. Reagents and conditions: (i) NiCl₂·6H₂O, NaBH₄, 0 °C – rt, 2 h; (ii) ethyl isocyanate, anhydrous tetrahydrofuran, rt, 2 h; (iii) 1.5M NaOH, ethanol, rt, 3 h.

**Ethyl 16-[(ethylcarbamoyl)amino]hexadecanoate (4a).** White solid, mp = 91 – 93 °C. Yield = 93%. ¹H NMR (400 MHz, CDCl₃): δ 4.30 – 4.15 (m, 2H), 4.11 (q, J = 7.2 Hz, 2H), 3.20 (p, J = 7.2 Hz, 2H), 3.14 (q, J = 7.2 Hz, 2H), 2.28 (t, J = 7.6 Hz, 2H), 1.59 (p, J = 7.2 Hz, 2H), 1.48 (p, J = 7.2 Hz, 2H), 1.35 – 1.20 (m, 25H), 1.12 (t, J = 7.2 Hz, 3H). ¹³C NMR (100.5 MHz, CDCl₃): δ 173.98, 158.54, 60.14, 40.88, 35.63, 34.38, 29.90, 29.57 (3C), 29.52 (2C), 29.49, 29.40, 29.26, 29.22, 29.11, 26.81, 24.96, 15.20, 14.22. HRMS calcd for C₂₁H₄₃N₂O₃ ([M + H]⁺) m/z: 371.3268; found 371.3267.
Ethyl 17-[(ethylcarbamoyl)amino]heptadecanoate (4b). White solid, mp = 94 – 96 °C. Yield = 56%. $^1$H NMR (400 MHz, CDCl$_3$): $\delta$ 4.30 – 4.15 (m, 2H), 4.10 (q, $J$ = 7.2 Hz, 2H), 3.22 – 3.10 (m, 4H), 2.26 (t, $J$ = 7.6 Hz, 2H), 1.59 (p, $J$ = 7.2 Hz, 2H), 1.46 (p, $J$ = 7.2 Hz, 2H), 1.35 – 1.20 (m, 27H), 1.11 (t, $J$ = 7.2 Hz, 3H). $^{13}$C NMR (100.5 MHz, CDCl$_3$): $\delta$ 173.95, 158.11, 60.13, 40.65, 35.37, 34.38, 30.18, 29.60 (2C), 29.59 (2C), 29.54 (2C), 29.52, 29.41, 29.31, 29.23, 29.12, 26.87, 24.96, 15.46, 14.23. HRMS calcd for C$_{22}$H$_{45}$N$_2$O$_3$ ([M + H]$^+$) m/z: 385.3425; found 385.3429.

Ethyl 18-[(ethylcarbamoyl)amino]octadecanoate (4c). White solid, mp = 91 – 93 °C. Yield = 69%. $^1$H NMR (400 MHz, CDCl$_3$): $\delta$. 4.20 (m, 2H), 4.10 (q, $J$ = 7.2 Hz, 2H), 3.22 – 3.10 (m, 4H), 2.26 (t, $J$ = 7.6 Hz, 2H), 1.60 (p, $J$ = 7.2 Hz, 2H), 1.46 (p, $J$ = 7.2 Hz, 2H), 1.35-1.16 (m, 29H), 1.11 (t, $J$ = 7.2 Hz, 3H). $^{13}$C NMR (100.5 MHz, CDCl$_3$): $\delta$ 173.97, 158.11, 60.14, 40.67, 35.38, 34.38, 30.18, 29.62 (3C), 29.60, 29.55 (3C), 29.53, 29.44, 29.31, 29.24, 29.12, 26.87, 24.97, 15.46, 14.23. HRMS calcd for C$_{23}$H$_{47}$N$_2$O$_3$ ([M + H]$^+$) m/z: 399.3581; found 399.3577.

General procedure for ester hydrolysis.

To a solution of the ethyl ester (0.51 mmol) in ethanol (30 mL), was added 1M NaOH (10 mL). The solution was stirred at 40 °C for 3 h. The ethanol was removed under reduced pressure, and the aqueous residue was adjusted to pH 2 with 0.5M HCl. The resulting suspension was filtered and the solid product washed with water (10 mL) and ethanol (5 mL) (Scheme A-2).

16-[(ethylcarbamoyl)amino]hexadecanoic acid (5a). White solid, mp = 126 – 127 °C. $^1$H NMR (400 MHz, $d_6$-DMSO): $\delta$ 5.74 – 5.65 (m, 2H), 2.99 – 2.89 (m, 4H),
2.16 (t, J = 7.2 Hz, 2H), 1.46 (p, J = 7.2 Hz, 2H), 1.31 (p, J = 6.8 Hz, 2H), 1.25 – 1.10 (m, 22H), 0.94 (t, J = 7.2 Hz, 3H). $^{13}$C NMR (100.5 MHz, $d_6$-DMSO): δ 174.96, 158.42, 34.46, 34.11, 30.50, 29.50 (4C), 29.45 (2C), 29.35, 29.26, 29.19, 28.99, 26.85, 24.94, 16.18. HRMS calcd for C$_{19}$H$_{39}$N$_{2}$O$_{3}$ ([M + H]$^+$) m/z: 343.2955; found 343.2959.

**17-[(ethylcarbamoyl)amino]heptadecanoic acid (5b).** White solid, mp = 172 °C (decomp.). Yield = 65%. $^1$H NMR (400 MHz, $d_6$-DMSO): δ 5.70-5.63 (m, 2H), 2.95-2.88 (m, 4H), 2.14 (t, J = 7.2 Hz, 2H), 1.44 (p, J = 6.8 Hz, 2H), 1.29 (p, J = 6.4 Hz, 2H), 1.25-1.15 (m, 24H), 0.92 (t, J = 7.2 Hz, 3H). $^{13}$C NMR (100.5 MHz, $d_6$-DMSO): δ 175.03, 158.46, 34.51, 34.25, 30.55, 29.55 (4C), 29.53, 29.51, 29.49, 29.41, 29.32, 29.24, 29.04, 26.90, 24.99, 16.24. HRMS calcd for C$_{20}$H$_{41}$N$_{2}$O$_{3}$ ([M + H]$^+$) m/z: 357.3112; found 357.3119.

**18-[(ethylcarbamoyl)amino]octadecanoic acid (5c).** White solid, mp = 190 °C (decomp.). Yield = 79%. $^1$H NMR (400 MHz, $d_6$-DMSO): δ 5.83 – 5.70 (m, 2H), 2.95 – 2.85 (m, 4H), 2.08 (t, J = 7.2 Hz, 2H), 1.42 (p, J = 6.8 Hz, 2H), 1.29 (p, J = 6.8 Hz, 2H), 1.25 – 1.15 (m, 26H), 0.92 (t, J = 7.2 Hz, 3H). $^{13}$C NMR (100.5 MHz, $d_6$-DMSO): δ 174.96, 158.61, 34.53, 34.06, 30.62, 29.60 (4C), 29.53 (2C), 29.49, 29.45, 29.41, 29.36, 29.22, 29.01, 26.77, 25.04, 16.17. HRMS calcd for C$_{21}$H$_{43}$N$_{2}$O$_{3}$ ([M + H]$^+$) m/z: 371.3268; found 371.3273.
Appendix B. Gene silencing: effectiveness of dominant-negative plasmid and siRNA transfection

Silencing of most genes in the present project was done using knockdown by siRNA transfection. The effectiveness of knockdown was assessed by comparing the expression of proteins encoded by the corresponding target genes, with its expression after transfection with scrambled siRNA. Protein expression was determined densitometrically.

Silencing of several genes was performed using Flag-tagged DN-mutants, relative to control (empty pcDNA3 plasmid transfections). The expression of the activated (phosphorylated) form of the target protein was estimated relative to control. Transfection was confirmed by immunoblotting for the Flag tag using an anti-Flag antibody.
**Figure B-1:** Immunoblot analysis after knockdown with siRNA, relative to scrambled siRNA control.

**Table B-1:** Densitometric evaluation of gene knockdown by siRNA.

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Protein expression relative to control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fas</td>
<td>50%</td>
</tr>
<tr>
<td>TNF-R1</td>
<td>52%</td>
</tr>
<tr>
<td>DR4</td>
<td>51%</td>
</tr>
<tr>
<td>DR5</td>
<td>57%</td>
</tr>
<tr>
<td>FADD</td>
<td>70%</td>
</tr>
<tr>
<td>TRADD</td>
<td>69%</td>
</tr>
<tr>
<td>TRAF2</td>
<td>60%</td>
</tr>
<tr>
<td>MKK4</td>
<td>53%</td>
</tr>
<tr>
<td>PI3K</td>
<td>55%</td>
</tr>
<tr>
<td>Bid</td>
<td>54%</td>
</tr>
</tbody>
</table>
Figure B-2: Immunoblot analysis after transfection with DN-mutants.

Table B-2: Densitometric evaluation of gene silencing using DN-mutant plasmids.

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Percentage of decrease in expression of phosphorylated protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>MKK7</td>
<td>63%</td>
</tr>
<tr>
<td>p38 MAPK</td>
<td>66%</td>
</tr>
<tr>
<td>JNK</td>
<td>67%</td>
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</table>