Thesis for the degree Master of Pharmacy

Effect of 8,9-epoxy eicosapentaenoic acid on human breast cancer cell line MDA-MB-231

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Abbreviations

AA	Arachidonic acid
BCA-assay	Bicinchoninic-assay
CDK	Cyclin-dependent protein kinase
COX	Cyclooxygenase
CYP 450	Cytochrome P450
DCU	N,N'-dicyclohexylurea
DHA	Docosahexaenoic acid
DMEM	Dulbecco`s modified eagle medium
DMSO	Dimethyl sulfoxide
EET	Epxoyeicosatrienoic acid
EFA	Essential fatty acid
sEH	Soluble epoxide hydrolase
EHI	Epoxide hydrolase inhibitor
EPA	Eicosapentaenoic acid
8, 9-epoxy-EPA	8, 9-epoxy-eicosapentaenoic acid
FBS	Fetal bovine serum
HIF-1	Hypoxia-inducible factor-1
LOX	Lipooxygenase
MTT	Thiazolyl blue tetrazolium bromide
NFκB	Nuclear factor κB
PG	Prostaglandin
PUFA	Polyunsaturated fatty acid
VEGF	Vascular endothelial growth factor

Abstract

Studies have shown that fatty acids from fish and fish oils, like eicosapentaenoic acid (EPA) and docosapentaenoic acid (DHA) may have a beneficial role in cancer by inhibiting proliferation or inducing apoptosis of the cancer cells. Several mechanisms involving different metabolites (eicosanoids) of the fatty acids have been suggested for the anti-cancer actions, but the detailed effects are still not known. One of the eicosanoid groups of the fatty acids are epoxy-EPAs. Little have been investigated about epoxy-EPAs' effect on cancer cells.

This experiment is based on previous studies from this laboratory that have shown an anti-proliferative effect of epoxy-EPA on murine endothelial cells (unpublished data). In this study the effect of 8,9-epoxy-EPA was tested on human breast cancer cell line MDA-MB-231. The epoxide was tested alone and in combination with an epoxide hydrolase inhibitor, N'N-dicycyclohexylurea (DCU). Different cell viability assays were assessed to investigate the effect of 8,9-epoxy-EPA on cancer cell proliferation. Cell cycle and immunoblotting were carried out to test if cell cycle regulatory proteins were affected. 8,9-epoxy-EPA was found to increase cell viability of MDA-MB-231. No change in cell cycle regulatory proteins was found.

8,9-epoxy-EPA was also tested on the MDA-MB-231 cells together with a well known anti-cancer drug, paclitaxel. Cell viability assay and cell cycle analysis were assessed. An increase in cell viability of cell treated with both 8,9-epoxy-EPA and paclitaxel compared to cell treated with paclitaxel alone was found. No effect of 8,9-epoxy-EPA on cell cycle was observed.

Results from these experiments are not what we expected based on previous studies. Further studies on cell mechanism and apoptosis are required to investigate possible differences in cell response to epoxide n-3 fatty acids.

1. Introduction

1.1 Cancer

Cancer is a leading cause of death worldwide. In 2005 7.6 mill people died of cancer (13% of all deaths worldwide). The frequency of cancer increases with age, the range between 55 and 75 years being the age where most cancer mortalities occur. The cancer types with the highest mortality rates are lung, stomach, liver, colon and breast cancer.

About 1/3 of all cancer incidences could have been prevented by change of lifestyle (healthy diet, physical activity, less alcohol and tobacco) or by reduced exposure to cancer risks like chemicals, radiation and too much sunlight.

A cancer tumor can be defined as an abnormal mass of tissue that grows uncoordinated and more rapidly that of the normal tissue. The uncontrolled, rapid growth happens as a result of some sort of stimulus. The cancer cell proliferation does not respond normal to the regulatory influences that control normal cell growth.

A tumor is made up of two components, the parenchyma and the supporting, non-neoplastic cells. The latter part is made up of connective tissue and blood vessels. It supplies the parenchyma with nutrients via the blood stream and support the growth of the parenchyma cells. The parenchyma is made up of neoplastic cells. This part determines the tumors biological behavior.

Cancer tumors arise from one single cell, and originate in genetic damage. This genetic damage can be initiated by environmental agents, such as chemicals, radiation or viruses. It can also be inherited. The transformation from a normal cell to a tumor cell is a multistage process. Replication of the DNA damaged cell can results in somatic cells with mutations in the genome. This mutation can result in activation of growth-promoting oncogenes, alterations of genes that regulate apoptosis or inactivation of cancer suppressor genes. This leads to expression of altered genes, loss of regulatory gene products and the development of a malign tumor.

Some of the changes in cell physiology that occurs in cancer tumors compared to normal tissue are self-sufficiency in growth signals, the cells do not respond to growth-inhibitory signals, respond to programmed cell death, sustained angiogenesis and gain the ability to invade and metastasize.

Metastasis is when a cancer tumor spread and invades other parts of the body. Tumors can spread through seeding within body cavities, through the lymphatic system or the blood circulation [1, 2].

1.1.1 Cancer cell proliferation

Proliferation is the growth or cell division of cells. The proliferation of cancer cells is often more rapidly and less controlled than normal cells. Stimulation of a normal cell into proliferation depends upon the external signals of growth factors. They bind to receptors on the cell surface which initiates a series of biochemical changes within the cell. This eventually leads to cell division. Tumor cells are not dependent on external growth factors in the same way as normal cells. They are able to proliferate when the concentration of growth factors are much lower than those required by normal cells. Tumor cells also may secrete their own growth factors and stimulate their own proliferation. When normal dividing cells reach a certain density they stop proliferating. Cancer cells continue to replicate to much higher densities than normal cells [3].

1.1.2 Cell cycle

An important mechanism that regulates proliferation is the cell cycle. After a growth stimulus, cells may enter the cell cycle in G1 phase, pass through S phase, in which DNA synthesis is stimulated, and progress to G2 phase ready for mitosis (M phase). This progression involves the concerted interplay of cyclin proteins, the cyclin-dependent kinases (CDKs), which are the catalytic subunits, and the CDK-inhibitors. The cyclins binds to CDKs and make complexes that are involved in activating of the essential substrates required for DNA synthesis and mitosis. Thus, cyclin-CDK complexes regulate progression through the cell cycle. Cyclin D regulates the cells progression from G0 (quiescent phase) to G1 phase. At the end of G1 phase and into S phase, cyclin E is expressed. From S phase to G2, cyclin A is the regulatory

protein. And into M phase cyclin B regulatory proteins are expressed. CDK-inhibitors stops cell progression through the cell cycle. The relative levels of cyclins, CDKs and CDK-inhibitors change throughout the cell cycle [4].

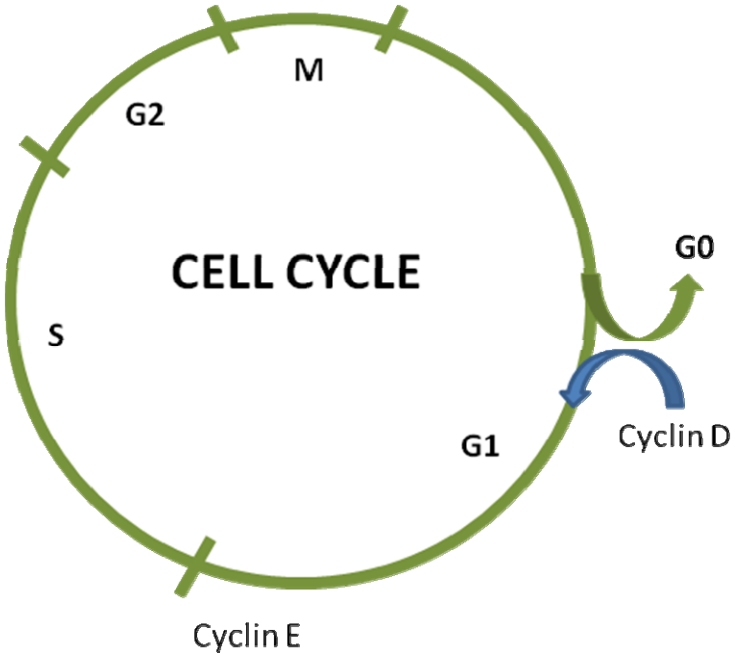

Figure 1.1: Cell cycle. The cells enter the cells cycle at G1 phase, DNA is replicated at S phase and cell division happens at M phase.

Flow cytometry is a method used to measure the proportion of cells in each phase of the cell cycle. The DNA of the cells is stained with a fluorescent dye before they are sent in a fluid stream through a laser beam. The scatter and emitted fluorescence from each cell is measured and directed to detectors by an optic filter. The electric system converts the light signals to electronic signals that can be processed by a computer.

It is cells size, granularity, internal complexion and fluorescence intensity that are measured. In cell cycle analysis the cells are sorted based on their DNA concentration. Cells in G1 phase have only one set of DNA, cells in G2 phase have two sets (double amount) and cells in S phase have something in between [5].

1.1.3 Angiogenesis

Cancer tumors cannot enlarge to a size beyond 1-2 mm unless they are vascularized. The vascularization of a tumor is called angiogenesis. 1-2 mm is the maximal distance which

oxygen and nutrients can diffuse from blood vessels. Thus, angiogenesis is required for continued tumor growth. Angiogenesis is also required for metastasis through blood.

Angiogenesis is induced by the tumor cells own production of angiogenic factors. Some of the most important angiogenic factors are vascular endothelial growth factor (VEGF) and fibroblast growth factor. When a tumor is subject to hypoxia, hypoxia-inducible factor-1 (HIF-1) is released. HIF-1 controls transcription of VEGF. Thus, hypoxia can induce apoptosis [2].

1.1.4 Breast cancer

Breast cancer (cancer mammae) is the most frequent type of cancer among women. About 502 000 people died of breast cancer worldwide in 2005. In Norway, 24488 new incidence of cancer were discovered in 2006. From these, 2673 incidences were breast cancer. There is no certain knowledge about the cause of breast cancer development, but heritage, gender and hormonal conditions are some of the risk factors.

There are different types of breast cancers. Some have rapidly growing tumors which metastasize early in the development. Others have slowly growing tumors that do not metastasize at all. A large proportion of breast cancers are estrogen dependent. These tumors express estrogen receptors which is an important target in the treatment of this type of breast cancer.

The prognosis for breast cancer is good when it is detected at an early stage and treated according to best evidence. The aim for treatment of breast cancer without metastases is curative. For breast tumors with metastasis there still is no curative treatment. In this case palliative care and keeping the development of the cancer to a minimum is the aim of the treatment [1, 6-8].

In this experiment the human breast cancer cell line MDA-MB-231 was used. MDA-MB-231 cells are estrogen independent epithelial cells. The cells express epithelial growth factor receptors. They are aggressive and rapidly growing. [9]

1.1.5 Anti-cancer drug paclitaxel

Paclitaxel is a complex diterpene derived from *Taxus brevifolia* or by synthesis. It belongs to the taxane group. It has anti-tumor activity against ovarian, breast, lung and prostate cancer. It acts by binding to β-tubulin and promote the formation of highly stable microtubules that resist depolymerization. This results in preventing normal cell division and cell cycle arrest in G2/M phase. Some of the most sever adverse effects are bone marrow depression and peripheral neuropathy. Paclitaxel is thought to be metabolized in the liver. Docetaxel is another taxane anti-cancer agent that is related to paclitaxel [10, 11].

1.2 n-3 and n-6 polyunsaturated fatty acids

Fatty acids are carboxylic acids containing long unbranched carbon chains. The chains can be either saturated (containing no double bonds) or unsaturated (containing one or more double bonds) and vary in length. Omega-3 (n-3) and omega-6 (n-6) fatty acids are families of polyunsaturated fatty acids (PUFA), which have more than one double bond. They are numbered according to the location of the double bond closest to the terminal methyl carbon.

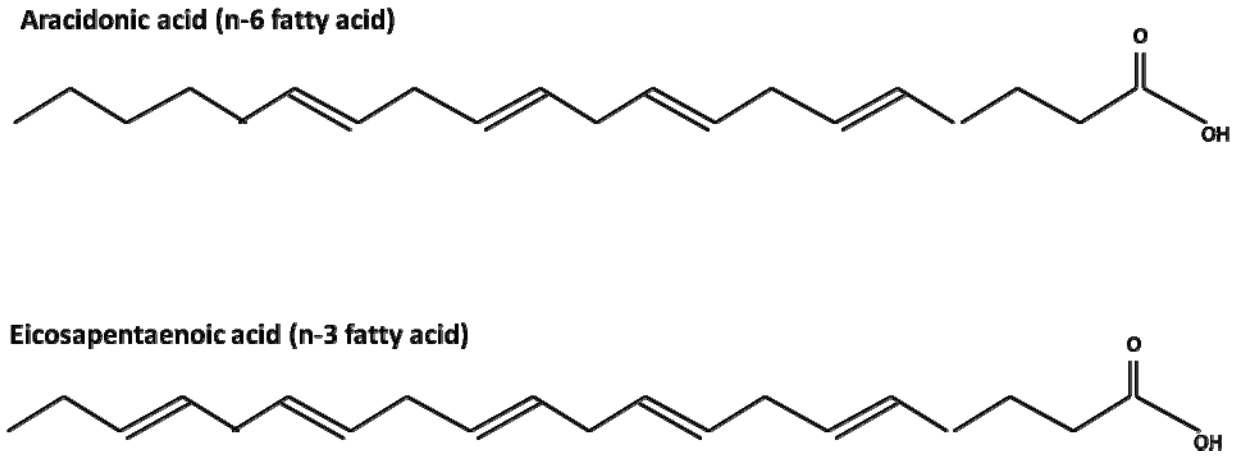

Figure 1.2: The n-3 fatty acid eicosapentaenoic acid and the n-6 fatty acid arachidonic acid. The double bond closest to the terminal methyl carbon starts at n-3 and n-6, respectively.

n-3 and n-6 PUFAs are essential fatty acids (EFA). The human body does not contain the enzymes necessary to synthesize the n-3 and n-6 double bonds; therefore they have to be obtained through the diet. n-3 and n-6 PUFAs are components of fish and fish oils, plant oils and animal tissue. n-3 PUFAs are the predominant fatty acids in fish and fish oil, and n-6 in plant oil and animal tissue.

n-3 PUFAs are synthesized from α-linolenic acid (18:3). Through desaturase and elongation enzymes n-3 PUFAs like eicosapentaenoic acid (20:5) and docosahexaenoic acid (22:6) are formed. The rate of conversion from α-linolenic acid to longer-chain metabolites in humans is very low, suggesting that a high proportion of the latter must come from the diet [12]. n-6 fatty acids are synthesized from linoleic acid (18:2). This n-6 fatty acid is a precursor for γ-linolenic acid (18:3) and arachidonic acid (20:4).

1.2.1 Eicosanoids of n-3 and n-6 polyunsaturated fatty acids

Eicosapentaenoic acid (EPA) and arachidonic acid (AA) are analogue fatty acids from the n-3 and n-6 families, respectively. In the body EPA and AA are incorporated into membrane phospholipids. They may subsequently be released from phospholipids and metabolized by cyclooxygenase (COX), lipoxygenase (LOX) and cytochrome P450 (CYP 450) enzymes to a range of different eicosanoids. COX metabolism leads to the formation of prostaglandins and thromboxanes, LOX leads to the formation of leukotrienes and CYP 450 hydroxylates or epoxidises the double bonds to make hydroxy- and epoxy-PUFAs.

1.2.2 Epoxy-PUFA is formed by CYP 450 metabolism

Both AA and EPA are metabolized through CYP 450. Some of the metabolites derived are monoepoxides. The epoxides is formed between all of the possible C=C double bonds of the fatty acid chain [13, 14]. The formation is mediated by several CYP 450 isoenzymes. AA is converted to 14,15-EET by CYP 2E1, 2J2 and 1A1, to 11,12-EET by CYP 2C8, 1A1 and 1A2 and to 8,9-EET by CYP 2C19, 2C18 and 3A4. EPA is converted to 17,18-epoxy-EPA by CYP 2D6, 1A2 and 1A1, 14,15-epoxy-EPA is formed by CYP 2C8 and 2C9, 11,12-epoxy-EPA is formed by CYP 2C9 and 2C18 and 8,9-epoxy-EPA is formed through CYP 2C9 and 2C19 [13]. The conversion from fatty acid to epoxide is also catalyzed by other CYP 450 isoenzymes [13].

1.3 Epoxide hydration to diols is mediated by the soluble epoxide hydrolase

Studies have shown that the epoxide formed when PUFAs are metabolized by CYP 450 may be rapidly hydrated to diols by epoxide hydrolase (EH) [13]. There are two types of epoxide

hydrolases, soluble epoxide hydrolase (sEH) present in the cytotsol and microsomal epoxide hydrolase. sEH has been shown to be the most efficient of the two [15]. Inhibition of sEH can prevent the epoxide from being hydrated to the diol. N'N-dicyclohexylurea (DCU) is an epoxide hydrolase inhibitor that has been shown to inhibit sEH effectively [16].

1.4 Effect of PUFA on cancer

The effects of polyunsaturated fatty acids on a range of cancer types, including breast cancer, have been tested in several studies, both epidemiological and experimental. The results have been somewhat inconsistent. Evidence from in vitro and animal studies of n-3 PUFA indicates that these fatty acids can slow down or inhibit cancer cell proliferation [17-21]. Epidemiological studies of n-3 fatty acids and cancer risk have not been able to show the same result. The conclusion among the latter is often that there is no significant reduction in cancer risk [22-24]. However, although the results are not significant there are trends that indicate beneficial effects of n-3 fatty acids on cancer development [22, 24]. Possible reasons for this inconsistency between epidemiological studies and animal and in vitro experiments could be epidemiological methodology issues such as sample size, adjustment for potentially confounding variables or detail and quality of the diet follow up. The laboratory models used in animal and *in vitro* experiment are not easily extrapolated to living humans, and there also might be a higher level of PUFA exposure to cancer cells in animal and *in vitro* models.

Some studies have investigated the effect of n-3 fatty acids versus n-6 fatty acids on cancer risk. They have found that an increased consumption or tissue ratio of n-3 fatty acids versus n-6 fatty acid may decrease the risk of developing cancer [25-29].

Although epidemiological studies and some experimental studies support a beneficial effect of n-3 diets on breast cancer the mechanism and detailed effects are unknown.

The effect of n-3 fatty acids on growth of the breast cancer cell line MDA-MB-231 have been investigated in several studies [17, 18, 20, 21]. Studies usually report a decrease in cell proliferation when MDA-MB-231 cells are exposed to n-3 fatty acids [17, 18, 20, 21]. Some differences have been found regarding the growth inhibiting effect of EPA and DHA. DHA seems to be more potent than EPA [18, 21].

1.5 Effect of epoxy-PUFA on cell proliferation

The monoepoxidized forms of arachidonic acid (EETs) have been found to promote the proliferation of endothelial [30, 31] and cancer cells [32]. The mechanism is not fully understood, but it has emerged that EETs protect cells from death pathways (apoptosis) [31, 32] and stimulate the growth of new blood vessels (angiogenesis) [30]. Angiogenesis could stimulate cancer growth by increasing the supply of nutrients via the blood stream. In contrast, little is known about the effect of monoepoxidized n-3 fatty acids on cell growth.

1.6 Mechanisms for n-3 fatty acid inhibition of cancer cell proliferation.

Several mechanisms for how n-3 fatty acids inhibit cancer proliferation have been suggested. The most prominent one might be n-3 fatty acid inhibition of the formation of AA derived eicosanoids [33, 34]. High amounts of ingested n-3 fatty acids could replace n-6 fatty acids in membrane phospholipids [35]. This may result in a decreased level of n-6 fatty acid derived products, such as prostaglandin E_2 (PGE_2) because of the increased supply of n-3 substrates [34].

PGE_2 is formed through COX metabolism of n-6 fatty acids [33]. COX enzymes are found to be over expressed in some tumors [36]. In addition, PGE_2 induces the production of COX-2 mRNA, which further increases the production of PGs [34]. Studies have shown that PGE_2 is important for cancer growth [33, 34, 37], possibly by protecting the cancer cells against apoptosis [38]. Metabolism of n-3 fatty acids by COX produces prostaglandin E_3 (PGE_3) [33]. PGE_3 have been found to inhibit cancer cell growth [33], but the mechanism is not clear. PGE_3 have also been found to be less potent in inducing the production of COX-2 mRNA compared to n-6 fatty acids [34]. This means that a higher level of n-3 fatty acids than n-6 fatty acids may reduce the induction of COX-2 and further, the amount of n-6 fatty acid eicosanoids.

These findings indicate that a higher level of n-3 fatty acids could modulate the n-6 fatty acid metabolism, membrane phospholipid incorporation of n-6 fatty acids and induction of COX by n-6 fatty acids. This may decrease the amount of n-6 fatty acid derived eicosanoids, resulting in a decreased cancer cell proliferation. Importantly, EPA has also been found to compete with AA for binding to CYP 450 enzymes [13].

n-6 EETs have been shown to stimulate cell proliferation by increasing cyclin D1 expression [39]. In recent study previously done in this laboratory, it has been found that epoxides of the n-3 EPA decrease cyclin D1 expression in murine endothelial cells (unpublished data). Whether the same process occurs in breast cancer cell lines has not yet been tested, and is a subject of this project.

As mention previously, an alternate mechanism for decreasing tumor growth is via inhibition of angiogenesis. A relationship might exist between COX-2 activity and angiogenesis in cancer cells [40]. Indeed, a recent study from this laboratory has investigated n-3 derived PG metabolites formed by COX on angiogenic processes in tumor cells [41]. Thus, COX-derived PGs from n-6 fatty acids stimulated angiogenesis whereas n-3 PGs did not.

Nuclear factor κB (NFκB) is a protein complex that regulates genes that control cell proliferation and cell survival. NFκB proteins are present as inactive forms in the cytoplasm in normal cells. They are bound to inhibitory proteins termed IκBs [42, 43]. Aberrant nuclear expression of NFκB has been found in breast cancer cells [42]. Inhibition of NFκB in breast cancer cells leads to apoptosis [42]. When IκB is phosphorylated, NFκB becomes free and can move towards the nucleus and initiate transcription of various genes. n-3 fatty acids decrease the amount of phosphorylated IκB [43]. The role of n-3 and n-6 derived eicosanoids formed by COX and CYP 450 enzymes in regulation of NKκB have not yet been assessed.

The mechanisms for n-3 fatty acid inhibition of cancer cell growth are not completely clear. However, it seems likely that the inhibition is a composite of several potential mechanisms.

1.7 Effect of anticancer drugs in combination with n-3 fatty acids

Studies have shown a decrease in cell viability when cancer cells are exposed to cytotoxic anticancer drugs in combination with n-3 PUFA, by comparison with the anticancer drug alone [44-46]. One study found that EPA decreased the dose of paclitaxel required to kill 50% of breast-tumor-derived MDA-MB-231 cells in culture (EPA reduced the IC_{50} of paclitaxel with 45%) [45]. Docosahexaenoic acid (DHA) also decreases MDA-MB-231 cell growth in combination with anticancer drugs paclitaxel and docetaxel [44]. A third study found a decrease in MDA-MB-231, but not in the alternate breast cancer cell line MCF-7, after exposure to DHA in combination with doxorubicin [46]. It has also been successful studies of

a combination drug of DHA and paclitaxel. When these are taken together, the required dose of paclitaxel and the toxicity that follows the treatment are both reduced [47].

Some of the suggested mechanisms for the enhanced cytotoxicity of anticancer drugs when combined with n-3 fatty acids are increased membrane fluidity due to the higher incorporation of n-3 PUFAs [48] or the involvement of lipid peroxidation [49].

1.8 The purpose of the study

The purpose of this study was to investigate the effect of epoxy-EPA on cancer cell growth. Little is known about the effect of the CYP 450 derived epoxy-EPAs and their role in cancer cell proliferation. MDA-MB-231 breast cancer cells where treated with 8,9-epoxy-EPA, alone and in combination with N'N-dicyclohexylurea (DCU), an epoxide hydrolase inhibitor. In further studies, MDA-MB-231 were also exposed to a combination of 8,9-epoxy-EPA and the well known anticancer drug paclitaxel, in order to investigate any synergistic or additive effects. Flow cytometry and Western blot analysis of cell cycle proteins was undertaken to investigate the impact of these treatments on cell growth and proliferation. Important endpoints included measurement of cell viability, cell cycle kinetics and cyclin D1 expression.

2. Materials and methods

2.1 Materials

2.1.1 Chemicals used in cell culture and experiments

Chemical	Purchased from
8,9-epoxy-EPA	Was kindly donated from Dr. Sarah Cui
Acrylamide/bis, 40%	Amresco (Solon, Ohio, USA)
Ammonuim persulfat (APS)	Univar, Ajax Chemicals, (Sydney, Australia)
Anti-β-actin IgG	Santa Cruz biotechnology (Santa Cruz, CA, USA)
Anti-cdk4 IgG	Santa Cruz biotechnology (Santa Cruz, CA, USA)
Ant-cdk6 IgG	Santa Cruz biotechnology (Santa Cruz, CA, USA)
Anti-cyclin D1 IgG	Santa Cruz biotechnology (Santa Cruz, CA, USA)
Anti-cyclin E IgG	Santa Cruz biotechnology (Santa Cruz, CA, USA)
Anti-p21 IgG	Santa Cruz biotechnology (Santa Cruz, CA, USA)
Anti-mouse IgG	Santa Cruz biotechnology (Santa Cruz, CA, USA)
Anti-rabbit IgG	Santa Cruz biotechnology (Santa Cruz, CA, USA)
Aprotinin	Sigma Chemical Company (St. Louis, MO, USA)
BCA Protein Assay Reagent kit	Pierce (Rockford, Illinois, USA)
Dicyclohexylurea (DCU)	Sigma Chemical Company (St. Louis, MO, USA)
Dimethyl sulfoxide (DMSO)	Sigma Chemical Company (St. Louis, MO, USA)

Diploma skim milk powder	Diploma, Australia
Dithiothreitol (DTT)	Sigma Chemical Company (St. Louis, MO, USA)
Dulbecco's modified eagle medium (DMEM)	Thermo Fisher Scientific (Waltham, MA, USA)
Fetal bovine serum (FBS)	Thermo Fisher Scientific (Waltham, MA, USA)
Glycine	Amresco (Solon, Ohio, USA)
N-(2-hydroxlethyl)piperazine-N'(2-ethanesulfonic acid) (HEPES)	USB Corporation (Cleveland, OH, USA)
Leupeptin	Sigma Chemical Company (St. Louis, MO, USA)
L-glutamine	Thermo Fisher Scientific (Waltham, MA, USA)
Nonidet P40 (Igepal)	Sigma Chemical Company (St. Louis, MO, USA)
Paclitaxel	Sigma Chemical Company (St. Louis, MO, USA)
Penicillin/Streptomycin	Thermo Fisher Scientific (Waltham, MA, USA)
Phenyl metyl sulfonyl fluoride (PMSF)	Sigma Chemical Company (St. Louis, MO, USA)
Phosphate buffer saline (PBS) tablets	Amresco (Solon, Ohio, USA)
Ponseau S red stain	ICN biomedical (Solon, Ohio, USA)
Prestained protein molecular weight marker	Fermentas (Canada)
Propidium iodide (PI)	Sigma Chemical Company (St. Louis, MO, USA)
Ribonuclease A (Rnase A)	Sigma Chemical Company (St. Louis, MO, USA)
Sodium deoxycholate	Sigma Chemical Company (St. Louis, MO, USA)
Sodium hydrogencarbonate ($NaHCO_3$)	APS Ajax Finechem (Auburn, NSW,

	Australia)
Sodium lauryl sulphate (SDS)	A.G. Scientific inc (San Diego, CA, USA)
TEMED	Amresco (Solon, Ohio, USA)
Thiazolyl blue tetrazolium bromide (MTT)	Sigma Chemical Company (St. Louis, MO, USA)
Tris	Amresco (Solon, Ohio, USA)
Trypan blue stain	Invitrogen (Mount Waverly, VIC, Australia)
Trypsin/EDTA	Thermo Fisher Scientific (Waltham, MA, USA)
Tween-20	Amresco (Solon, Ohio, USA)

2.1.2 Solutions for cell culture

Plain media, 900ml	
DMEM	9.87 g
Sodium hydrogencarbonate	1.2 g
HEPES	5.21 g
NaOH	Adjust pH to exactly 7.2
Baxter sterile water	900 ml

Mix on magnetic stirrer. Filter sterilize in tissue culture hood using VacuCap60 0.2µm. Store at 4°C.

Complete media (10% FBS), 255ml	
Plain media	225 ml
FBS	25 ml
L-glutamine	2.5 ml
Penicillin/streptomycin mix	2.5 ml

Make when needed. Store at 4°C.

0.5% FBS media, 255ml	
Plain media	225 ml
FBS	1.125 ml
L-glutamine	2.5 ml
Penicillin/streptomycin mix	2.5 ml

Phosphate buffered saline (PBS), pH 7.4, tissue culture grade	
PBS tablet	1
Baxter sterile water	100ml

Dissolve and sterilize at 121°C for 20 min in autoclave. Store at 4°C.

2.1.3 Buffers for flow cytometry

Incubation buffer, 10ml	
0.1M PBS	9.8 ml
10% Nonidet P40 (Igepal)	0.1 ml
10 mg/ml Rnase A	0.1 ml

Make on the day.

2.1.4 Buffers and solutions for Western blot analysis

Tris pH 8.8, 100ml	
Tris	18.15g
Deionized water	Make up to 100ml
HCl	Adjust pH to 8.8

Store at 4°C.

Tris pH6.8, 100ml	
Tris	6.0g
Deionized water	Make up to 100ml
HCl	Adjust pH to 6.8

Store at 4°C.

Sample buffer, 90ml	
Tris pH 6.8, 1M	25.0ml
Glycerol	23.0ml
SDS 10%	40.0ml
Bromophenol blue 0.1%	2.0ml
Deionized water	Make up to 90ml

Aliquot into 4.5ml. Add 0.5ml 0.5M dithiothereitol before use. Store at -20°C.

5 x running buffer, pH 8.3, 1L	
Tris	15.0 g
Glycine	72.0 g
SDS, 10%	50 ml
Deionized water	Make up to 1L

Store at 4°C.

1 x running buffer: 100ml 5 x running buffer + 400ml deionized water.

Transfer buffer, 2L	
Tris	6.06 g
Glycine	28.8 g
Methanol	400 ml
Deionized water	Make up to 2L

Store at 4°C.

10 x TBS stock, pH 7.4, 2L	
Tris	121.0 g
NaCl	233.8 g
Deionized water	Make up to 2L
HCL	Adjust pH to 7.4

Store at 4°C.

1 x TBS: 100ml 10 x TBS + 900ml deionized water

1 x TBS/Tween, 1L	
10 x TBS stock	100 ml
Deionized water	900 ml
Tween-20 (0.05%)	0.5 ml

Mix on magnetic stirrer. Store at room temperature.

Blocking milk solution, 5%, 100ml	
Diploma skim milk	5.0 g
1 x TBS/Tween	100 ml

Mix on magnetic stirrer.

2.1.5 Gels for Western blot analysis

12% separating gel, 30 ml	
Deionized water	11.4 ml
Acrylamide/bis, 40%	9 ml
TrisHCL pH 8.8, 2.5M	9 ml
SDS, 10%	300 µl
Ammonium persulphate, 10%	300 µl
TEMED	32 µl

5% stacking gel, 16 ml	
Deionized water	9.8 ml
Acrylamide/bis, 40%	2 ml
TrisHCL pH 6.8, 1.0M	4 ml
SDS, 10%	160 µl
Ammonium persulphate, 10%	80 µl
TEMED	16 µl

2.1.6 Instruments

Instruments	**Manufacturer**
Allegra 6R Centrifuge	Beckman
Altra 20 soft Imaging system CKx41	Olympus
Biorad GS-800 Calibrated Densitomenter	Biorad
Coulter flow cytometer	Beckman
Email Air Handling Biological Safety cabinet, class II	Email
Eppendorf Centrifuge 5417 R	Crown Scientific
Odyssey	LI-COR
Olympus CKx41	Olympus
Victor3 Wallac 1420 multilabel counter	Perkin Elmer

2.2 Methods

2.2.1 Cell culture

The MDA-MB-231 breast cancer cells line were kindly donated by Dr Nenad Petrovic of this laboratory. MDA-MB-231 is an epithelial breast cancer cell line isolated from the mammary gland of a 51-years old female caucasian. It is estrogen independent, aggressive and rapidly growing [9].

The MDA-MB-231 cells were cultured in Dulbecco's modified Eagle medium (DMEM), supplemented with 100units/ml penicillin, 100µg/ml streptomycin, 1% L-glutamine and 10%

fetal bovine serum (FBS). The cell were maintained in monolayer in T75 flasks, and incubated in 37°C in an atmosphere of 95% air and 5% CO_2. All cell work was carried out in a tissue culture hood (Email Air Handling Biological Safety cabinet, class II), using only sterile equipment in direct contact with the cells.

2.2.2 Passaging of MDA-MB-231 cells

The MDA-MB-231 cells were passaged every 3rd or 4th day (when they had reached 90-100% confluence). Media was removed by aspiration using sterile Pasteur pipette and a vacuum line. 10mL phosphate buffered saline (PBS) was added to the opposite side of where the cells were attached. The flask was gently rocked about 5 times to wash away media residues from the cells. PBS was removed by aspiration. 2mL trypsin/EDTA (1:250 w/o phenol red) was added directly to the cells and the flask was incubated for 5 min in 37°C, 5% CO_2. Cells were detached by gently tapping the flask. 10mL DMEM was added to the flask. Cells were suspended in the media, transferred to a 15mL tube and centrifuged at 1250 rpm in 5 min (Allegra 6R Centrifuge). Supernatant was removed by aspiration and the cell pellet was "flipped" to separate the cells. The cells were resuspended in 12mL DMEM and passaged into three T75 flasks containing 25mL DMEM each.

2.2.3 Thawing of MDA-MB-231 cells

Cells were kept in liquid nitrogen in cryotubes. Tubes were removed from the liquid nitrogen tank and into a 37°C water bath for about 30-60sec. The cells were transferred to a 15mL tube using 10mL heated DMEM and centrifuged at 1250 rpm for 5 min (Allegra 6R Centrifuge). Media was removed by aspiration and the cell pellet was "flipped" and resuspended in 10mL DMEM before transferred to a T75 flask containing 25mL DMEM.

2.2.4 Seeding of MDA-MB-231 cells

For experiments MDA-MB-231 were seeded in 24-well plates, 1mL in each well at a density of 1x10^{5}cells/ml. Media from one T75 flask was removed by aspiration. The cells were washed with PBS and incubated with trypsin/EDTA in 37°C, 5% CO_2 for 5 min. After detaching, the cells were suspended in 10mL media, transferred to a 15mL tube and

centrifuged at 1250 rpm for 5 min (Allegra 6R Centrifuge). The supernatant was removed, the cell pellet "flipped" and resuspended in 12mL media. Cell number was determined by cell counting using a hemacytometer (without trypan blue). The amount of cell suspension to make a $1x10^5$cells/ml dilution in media was calculated. Cells were seeded in 24-well plate, 1ml cell suspension in each well.

2.2.5 Harvesting of MDA-MB-231 cells

Media was removed by aspiration using sterile Pasteur pipette and vacuum line. 1ml PBS was added to each well and the plate was rocked gently to wash out media residues. PBS was removed by aspiration. 200µl trypsin/EDTA was added to each well, and the plate was incubated in 37°C, 5% CO_2 for 10 min. 0.5ml media containing 10% FBS was added to each well. Cells from each well were transferred to eppendorf tubes. Another 0.5 media was added to each well and transferred to the same tubes to make sure all the cells were harvested. The tubes were centrifuged at 1250 rpm 5-10 min (Eppendorf Centrifuge 5417 R). Supernatant was removed.

- For cell count:

Cell pellets were resuspended in 0.5ml media.

- For flow cytometry:

Cell pellets were resuspended in 0.5ml ice-cold PBS and centrifuged at max speed at 4 °C for 5 min. PBS was removed; cell pellets were "flipped" and another 0.5ml ice-cold PBS was added. Cells were then centrifuged at max speed at 4°C for 5 min. PBS was removed and cell pellets were resuspended in ice-cold 80% ethanol and transferred to -20°C for at least one hour.

- For western blotting:

Cell pellets were harvested into 10ml tubes (several wells containing cells that had received the same treatment into the same tube to obtain sufficient protein for the analysis). After removing media (supernatant), cell pellets were transferred directly to -80°C for storage.

2.2.6 Experiments using MDA-MB-231

For cell experiments, MDA-MB-231 cells were seeded in DMEM supplemented as described above. After 24 hr, the media was changed to DMEM containing the same amount of penicillin, streptomycin and L-glutamate, but with only 0.5% FBS. Old media was removed by aspiration, and immediately replaced with new media. After another 24 hr, media was replaced with treatment media. Treatment media contained DMEM, same amount of penicillin, streptomycin and L-glutamate, 0.5% FBS and the different treatments. Constant concentrations of 8,9-epoxy-EPA and DCU were used for all experiments (10μM). Because 8,9-epoxy-EPA is dissolved in DMSO, cells treated with only DMSO were used as controls. Paclitaxel was used at three different concentrations, 2nM, 5nM and 10nM.

2.2.7 Cell count and cell viability experiments on MDA-MB-231 cells

Cell count using hemacytometer

A hemacytometer is a graduated counting chamber that can be viewed under a microscope to determine the concentration of cells in a suspension. The central area of the instrument is defined by a set of grooves that form an "H" shape. Two counting areas with ruled grids are separated by the horizontal groove of the "H". In each counting area there are 9 squares (1.0 mm x 1.0 mm each). A glass coverslip is held at 0.1 mm above the surface of the counting area. Approximately 10μL of cell suspension is required to fill the counting chambers on each side [50].

In our experiments, the middle square in each counting chamber was counted.

Cell count with trypan blue

Trypan blue is a dye used to determine viable vs. non-viable cells. The dye is excluded from viable cells. Non-viable cells are colored dark blue.

Cell suspension was diluted 1:1 with trypan blue and pipetted up and down several times to ensure a uniform suspension. The suspension was loaded into both counting chambers of the hemacytometer, and the number of viable cells was counted by microscopy at 100x magnification. Cells in one square of each counting chamber were counted.

Number of cell/ml = [(total number of cells counted/number of squares counted) x 10^4cells/ml] / typan blue dilution

Cell count without trypan blue

This method was used for determining cell/ml in cell suspension when cells were seeded.

Cell suspension was loaded to each side of the hemacytometer, and cells in one square at each counting chamber were counted.

Number of cells/ml = (total number of cells counted/number of squares counted) x 10^4cells/ml

MTT-assay

MTT-assay is based on the cellular conversion of the tetrazolium salt, thiazolyl blue tetrazolium bromide (MTT), into a formazan product that is readily detected using a 96-well plate reader. The conversion is only carried out by viable cells. The absorbance is measured at 540nm and is directly proportional to the number of live cells [51].

2.5mg/ml MTT-solution was added to each well and incubated in 37°C, 5% CO_2 for 2 hr. Media was removed by aspiration. The cells were dissolved in 200µl DMSO and shaken on an orbital shaker for 30 min. (Plates were covered with aluminum foil to protect the crystals from light). 100ul or 50ul of solutions were transferred to 96-well plates and read in a Victor3 Wallac 1420 multilabel counter at 540nm.

2.2.8 Cell cycle analysis of MDA-MB-231 cells using flow cytometry

Flow cytometry measures the amount of DNA in cells. The cells flow in a fluid stream through a laser beam. Light scatter and fluorescence is measured and converted to electronic signals that are processed by a computer. By measuring the amount of DNA, the test is able to identify the proportions of cells in different parts of the cell cycle [5].

MDA-MB-231 cell pellets were fixed in 80% ethanol stored at -20°C for at least an hour and were then centrifuged at 2500 rpm at 4°C for 15 min (Eppendorf Centrifuge 5417 R). The ethanol was removed and the cell pellets were suspended in 0.5ml incubation buffer. 12.5µl 2mg/ml propidium iodide (PI) was added to each tube before they were incubated on ice for 1 hr. Cell cycle analysis were performed using a Beckman Coulter flow cytometer.

2.2.9 Western blot analysis of MDA-MB-231 cells

Western blot analysis is used to detect specific proteins in a sample. The proteins are separated by gel electrophoresis and then transferred electrophoretically to a nitrocellulose membrane. Antibodies are used to detect the specific proteins that are immobilized on the membranes.

MDA-MB-2312 cell pellets were lysed using lysis buffer and the amount of protein in the lysate was quantified using Bicinhoninic acid- assay (BCA-assay), (see Appendix). The amount of lysate containing the required amount of protein was diluted 1:1 with sample buffer and water. Samples were loaded onto 1.5mm 12% SDS polyacrylamide gels. Gels were run at 200V for about 2 hrs in 1 x running buffer. Proteins were transferred from the gel to nitrocellulose membranes (run at 100V for 1.5 hr in transfer buffer).

Development of blot:

The membranes were shaken in ponceau S dye on orbital shaker for 10 min and scanned to ensure efficiency of transfer. The membranes were shaken in blocking milk solution on an orbital shaker for 1 hr before removal to blocking milk solution containing primary antibodies and incubation at -4°C on an orbital shaker overnight. Primary antibodies were removed by washing in TBS/Tween before incubating membranes in blocking milk solution containing secondary antibodies on an orbital shaker for 1 hr. Secondary antibody was removed by washing in TBS/Tween and TBS only. Membranes were scanned using Odyssey (LI-COR) Table 2.1 shows the dilutions of the different commercial antibodies that were used.

Tabel 2.1: Antibody dilutions used in western blotting.

Antibodies	
Primary antibodies	**Dilutions**
β-actin	1:500
Cyclin D1	1.500
Cyclin E	1:500
Cdk 4	1:500
Cdk 6	1:1000
p21	1:1000
Secondary antibodies	
Anti-mouse	1:15000
Anti-rabbit	1:20000

2.3 Statistic analysis

All statistical analyses are evaluated through Stat View, ANOVA/t-test.

3. Results

3.1 Effect of FBS supplement and seeding density on growth of MDA-MB-231 cells.

In preliminary experiments the growth conditions of MDA-MB-231 cells were optimized. Cells were seeded in 24-well plates at densities of $1x10^5$cell/ml, $2x10^5$cells/ml and $3x10^5$cells/ml and were cultured in DMEM media supplemented with 10%, 2% or 0% FBS. After 48 hr, MDA-MB-231 cells seeded at an initial density of $1x10^5$cells/ml were about 100% confluent when grown in 10% or 2% FBS DMEM. For cells seeded at the same density, but grown in the absence of FBS, a somewhat lower confluence was observed. Cells that were seeded at densities of $2x10^5$cells/ml and $3x10^5$cells/ml were fully confluent at the same time point, and a large proportion of dead cells were observed.

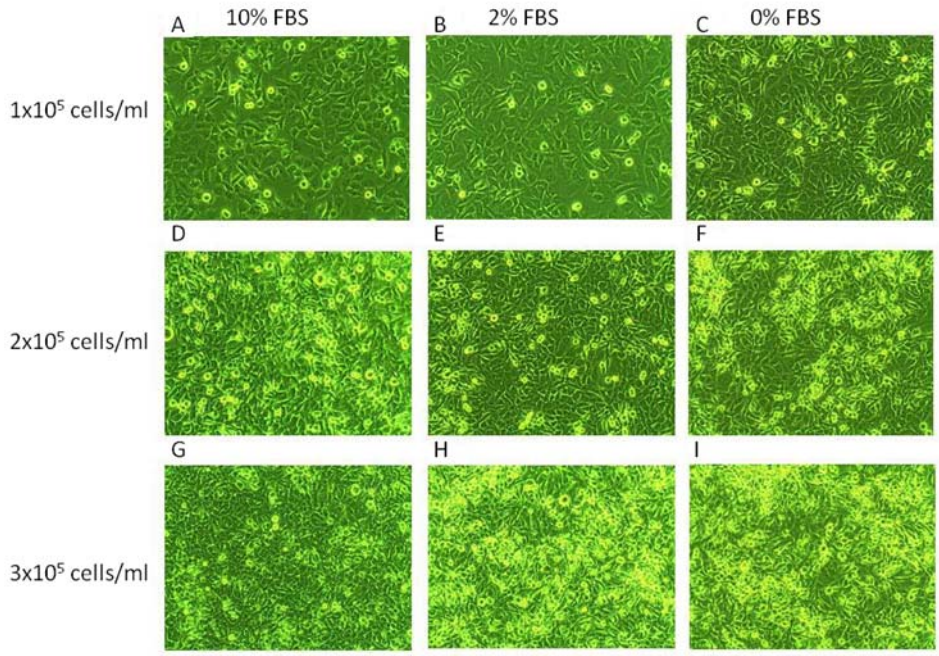

Figure3.2: Effect of FBS supplement and seeding density on growth of MDA-MB-231 cells after 24 hr.

In further experiments growth conditions in cultured MDA-MB-231 cells were assessed. Cells seeded in 24-well plates at densities of $0.7x10^5$cells/ml and $1x10^5$cells/ml were grown in 10%, 0.5% and 0% FBS supplemented DMEM. The cells were cultured for 72 hr and MTT assays were conducted at 24 hr intervals. Figure 3.2 A shows that the increase in viability (MTT assay) for cells seeded at densities of $0.7x10^5$ cells/ml after 24 hr was 66% (10% FBS), 39% (0.5% FBS) and 44% (0% FBS) compared to the 0 hr timepoint. At 48 hr the cell viability

further increased to 167% (10% FBS), 119% (0.5% FBS) and 105% (0% FBS) over the 0 hr timepoint. From 48-72 hr the viability of cells grown in 0.5% and 0% FBS supplemented DMEM was reduced to, 108% and 77% respectively, compared to control. In contrast cell proliferation in 10% FBS DMEM was increased further by 343% over control. The viability of cells seeded at $1x10^5$cell/ml increased 71% (10% FBS), 42% (0.5% FBS) and 52% (0% FBS) after the first 24 hr (figure 3.2 B). At 48 hr further increases to 162% (10% FBS), 121% (0.5% FBS) and 72% (0% FBS) of control were noted. Between 48-72 hr cell proliferation was decreased for cells grown in 0.5% and 0% FBS to 132% and 50% of control (0 hr) respectively. Cells that were cultured in 10% FBS supplemented DMEM exhibited increased viability of 278% compared to 0 hr.

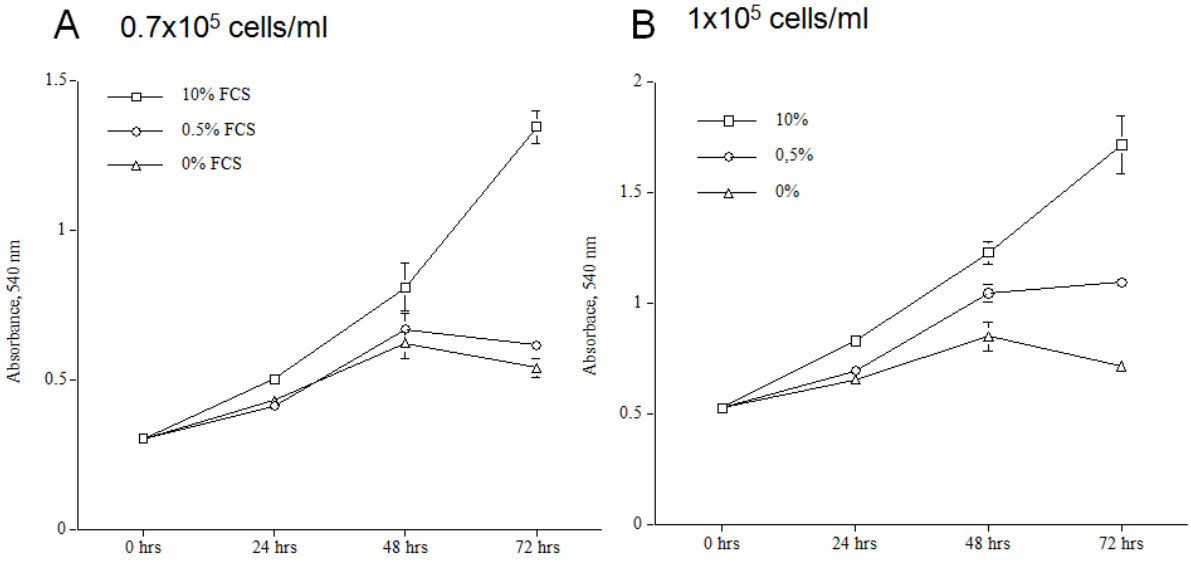

Figure 3.2: MDA-MB-231 cells were seeded in 24-well plates at densities of $0.7x10^5$cells/ml (A) and $1x10^5$cells/ml (B), were cultured in 10%, 0.5% and 0% FBS supplemented DMEM. Cell viability in 0.5% and 0% FBS supplemented media decreased after 48 hr. **A:** At 24 hr the percentage increase of viable cells compared to 0 hr was 66% (10% FBS), 39% (0.5% FBS) and 44% (0% FBS), at 48 hr 167% (10% FBS), 119% (0.5% FBS) and 105% (0% FBS) and at 72 hr 343% (10% FBS), 108% (0.5% FBS) and 77% (0% FBS). **B:** At 24 hr the percentage increases of viable cells compared to 0 hr were 71% (10% FBS), 42% (0.5% FBS) and 52% (0% FBS), at 48 hr 162% (10% FBS), 121% (0.5% FBS) and 72% (0% FBS) and at 72 hr 278% (10% FBS), 132% (0.5% FBS) and 50% (0% FBS). All data represent mean ±SE from n=2 independent experiments.

To avoid excessive stimulation of cell growth by FBS, the lowest possible serum concentration that maintained cell growth was selected for subsequent experiments. A density of $1x10^5$ cells/ml seeded in 24-well plates and cultured in 0.5% FBS supplemented DMEM was used in most of our experiments.

3.2 Effect of 8,9-epoxy-EPA on MDA-MB-231 cell proliferation

The effects of 8,9-epoxy-EPA (10μM) on MDA-MB-231 cells were assessed directly and in combination with the epoxide hydrolase inhibitor N,N'-dicyclohexylurea (10μM) for 24 hr. These conditions had been shown to be optimal in murine endothelial cell lines, and were adopted for the present experiment in human breast cancer cells. Cells treated with DMSO (vehicle control) and DCU alone were used as controls for 8,9-epoxy-EPA treated cells and 8,9-epoxy-EPA+DCU treated cells, respectively.

3.2.1 8,9-epoxy-EPA increases cell viability of MDA-MB-231 grown in 0.5% FBS supplemented DMEM.

The effect of 8,9-epoxy-EPA on MDA-MB-231 cell viability was evaluated in the presence of 0.5% FBS supplemented DMEM. As shown in figure 3.3, an increase in cell viability (MTT assay) was observed for both 8,9-epoxy-EPA alone (10.0±2.0%, $p=0.002$) and in combination with DCU (16.0±1.6, $p<0.0001$). Cells that were treated with either DMSO or DCU alone did not exhibit altered cell proliferation and viability.

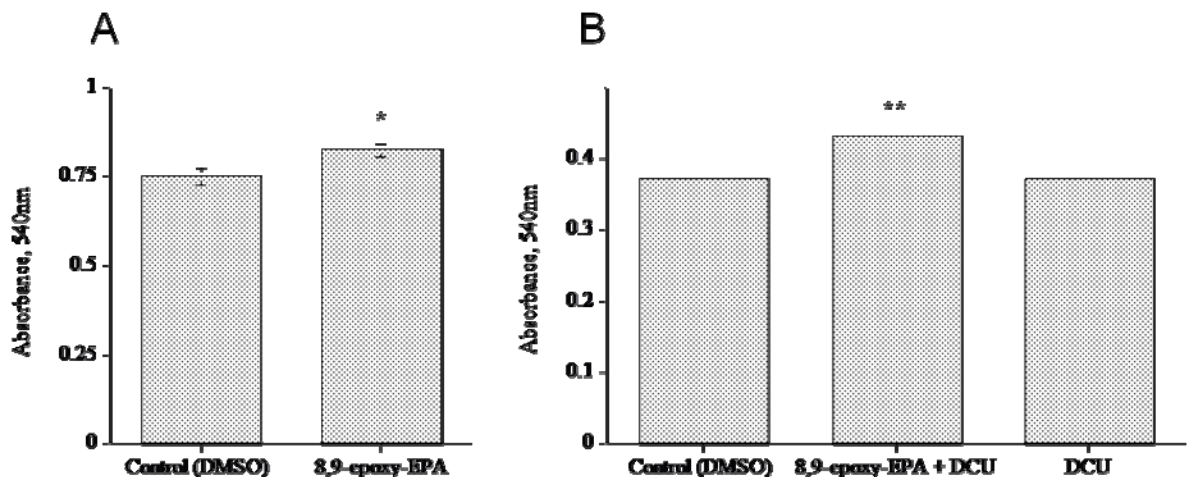

Figure 3.3: Effect of 24 hr 8,9-epoxy-EPA treatment (10µM) alone and in combination with N,N'-dicyclohexylurea (DCU) (10µM) on MDA-MB-231, cells cultured in 0.5% FBS supplemented DMEM. Cell viability was assessed using MTT assay. A: 8,9-epoxy-EPA increased cell viability significantly. * 10.0±2.0%, p=0.022. B: 8,9-epoxy-EPA in combination with DCU increased cell viability significantly. ** 16.0±1.6 versus DCU treated cells, p<0.0001. Data represent the mean ± SE, n=3

Consistent with the finding from MTT assay cell viability was also increased when evaluated by trypan blue exclusion assay. As shown in figure 3.4, 8,9-epoxy-EPA alone appeared to increase cell viability with 60.9±43.6% (versus DMSO treated cells), and in combination with DCU 15.9±13.1% (versus DCU treated cells). However, these data did not attain statistical significance because of the data variation.

Although the data did not reach statistical significance, the trend is consistent with findings from MTT-assay: an increased viability of MDA-MB-231 cells when exposed to 8,9-epoxy-EPA.

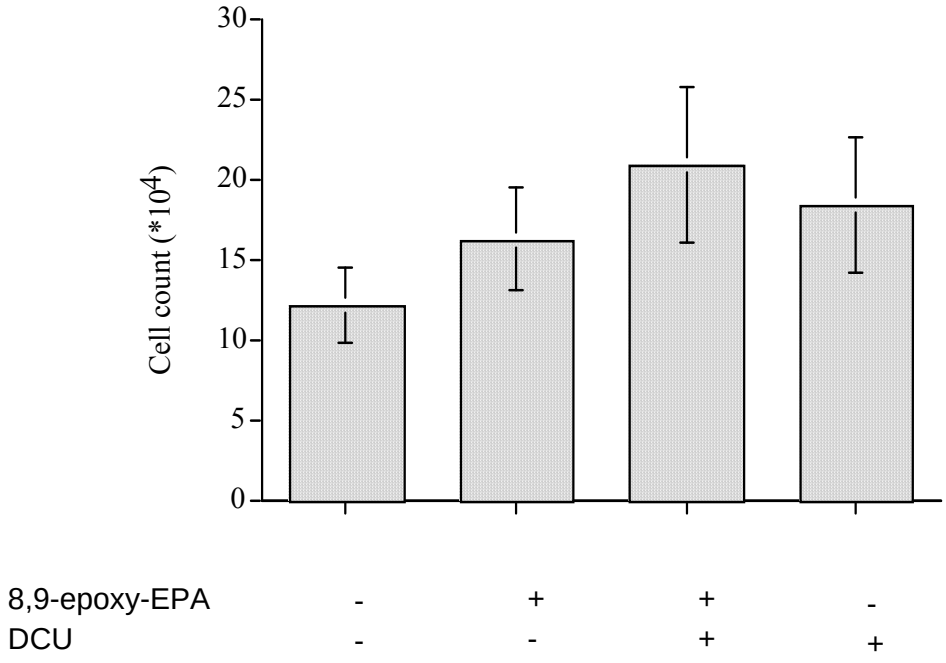

Figure 3.4: 8,9-epoxy-EPA alone and in combination with DCU increases viability of MDA-MB-231 cells. Viable cells were counted using trypan blue exclusion assay. 8,9-epoxy-EPA alone increases cell viability by 60.9±43.6%. 8,9-epoxy-EPA + DCU increases cell viability by 15.9±13.1% over DCU treated cells. Controls were incubated with DMSO or DCU alone. Data represent mean ±SE from n=3 independent experiments.

These results indicate that 8,9-epoxy-EPA might enhance cell proliferation and viability in MDA-MB-231 cells. The greater inter-assay variation in trypan blue exclusion data may have contributed to the lack of statistical significance.

3.2.2 Effect of 8,9-epoxy-EPA on viability of serum starved MDA-MB-231 cells.

We considered whether the effects of 8,9-epoxy-EPA on cell viability may be influenced by the presence of a growth stimulus (FBS). Thus, serum starved MDA-MB-231 cells were exposed to the same treatments as described above. No visible change in confluence or cell shape was found by studying the cells by microscope (figure 3.5). Live cells were again counted by trypan blue exclusion assay, and no change in cell proliferation and viability were found (figure 3.6).

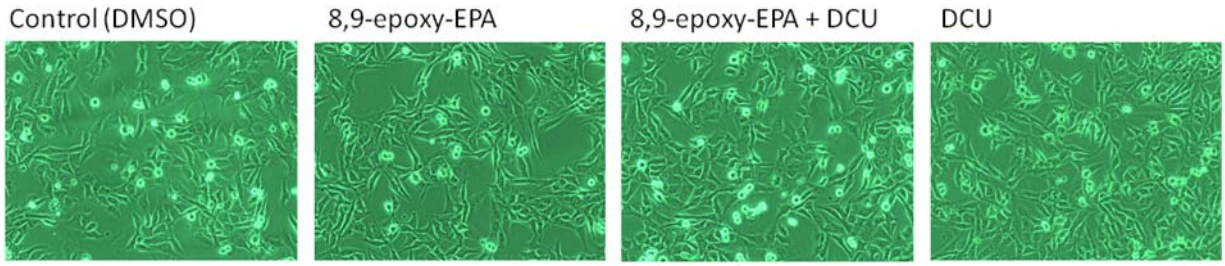

Figure 3.5: The effect of 8,9-epoxy-EPA on serum starved MDA-MB-231 cells did not show any visible difference from control when studied by microscopy.

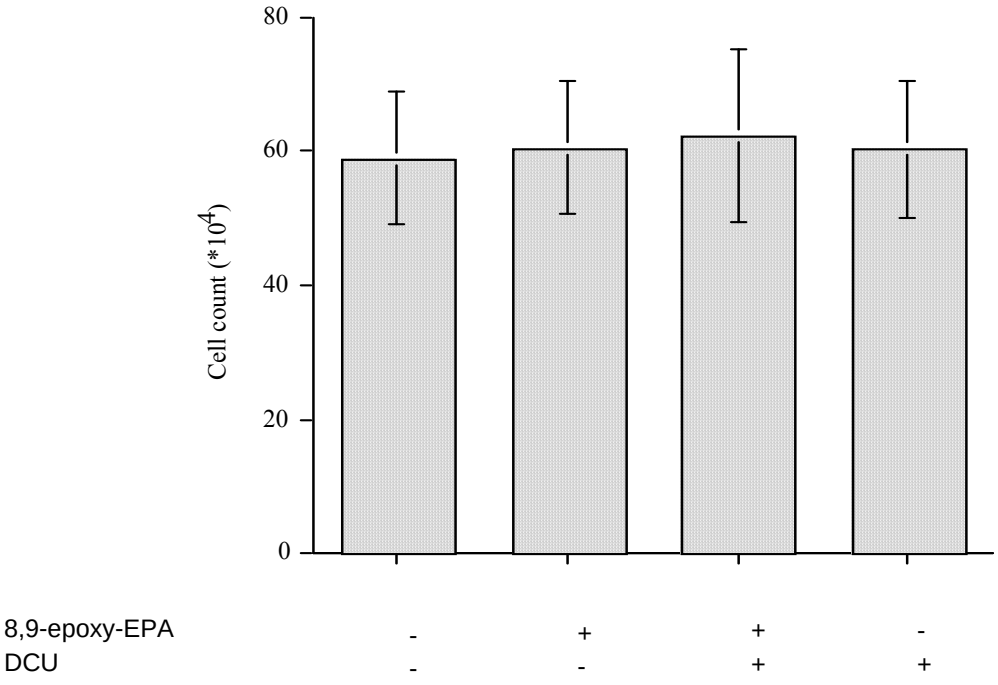

Figure 3.6: Treatment with 8,9-epoxy-EPA alone and in combination with DCU did not influence MDA-MB-231 cell viability when grown in serum free media. Data represent mean ±SE from n=3 independent experiments.

3.3 Effect of 8,9-epoxy-EPA on cell cycle in MDA-MB-231 cells.

The increased cell viability of MDA-MB-231 from MTT assay, when exposed to 8,9-epoxy-EPA suggested that there may be an acceleration in the rate of the cell cycle. To investigate

this further, the cells were cultured in 0.5% FBS supplemented media, and exposed to the treatments described above. Table 3.1 show that no significant change was found in any of the cell cycle phases.

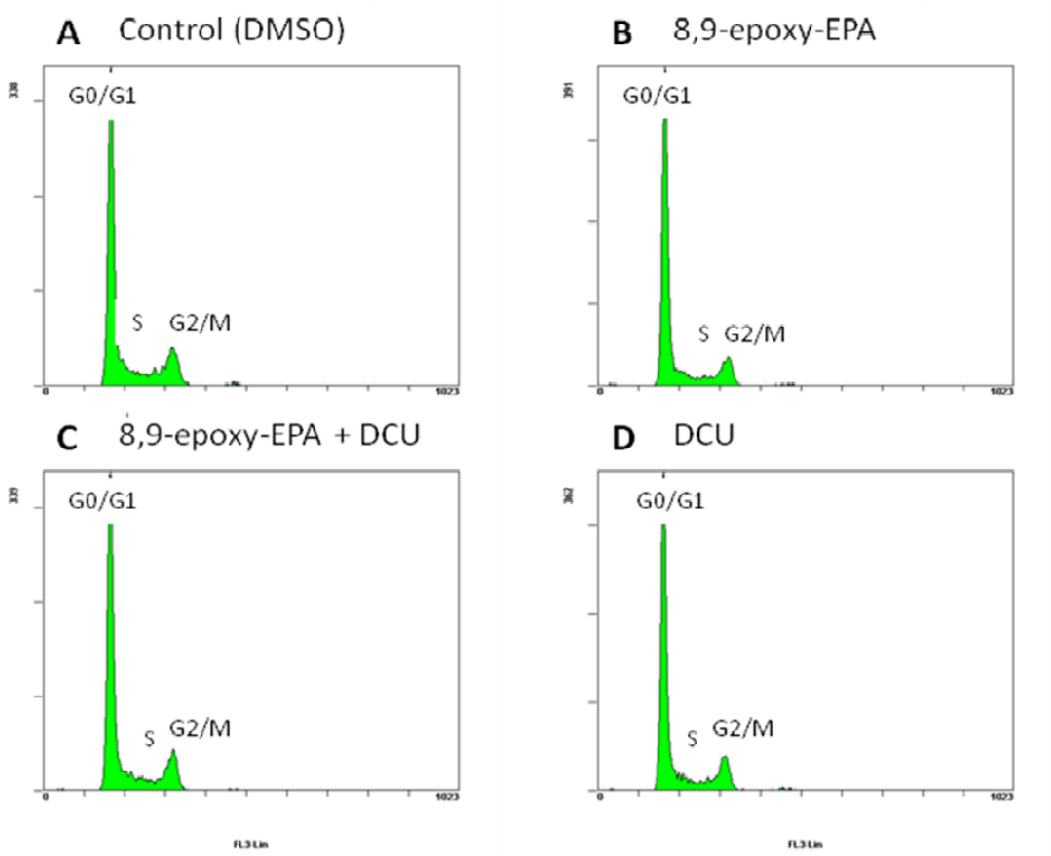

Figure 3.7: Flow measurement of MDA-MB-231 cells treated with DMSO only (A), 8,9-epoxy-EPA (B), 8,9-epoxy-EPA and DCU (C) and DCU alone (D). No difference in amount of cells in each phase of the cell cycle was found between each of the treatments

Table 3.1: Percentage of MDA-MB-231 cells in each cell cycle phase after treatment with 8,9-epoxy-EPA alone and in combination with DCU. No significant change was found. Data represent mean ±SE, n=3.

	G0/G1-phase (%)	S -phase(%)	G2/M-phase(%)
Control (DMSO)	53.14±1,78	16.76±1.20	18.99±0.59
8,9-epoxy-EPA	53.77±1.51	16.65±0.44	18.92±0.93
8,9-epoxy EPA + DCU	55.82±3.30	16.29±1.01	18.08±1.68
DCU	56.1.21±1.21	16.86±0.51	17.98±1.01

3.4 Effect of 8,9-epoxy-EPA on expression of cell cycle regulatory proteins in MDA-MB-231 cells

Next, we investigated the effect of 8,9-epoxy-EPA on cell cycle regulatory protein expression. Cells were cultured in 0.5% FBS supplemented DMEM, and exposed to 8,9-epoxy-EPA alone. The expression of the cell cycle related proteins cyclin D1 cyclin E, Cdk 4 Cdk 6, and p21 were analyzed by Western blot analysis. To confirm equivalence of protein loading, β-actin protein expression was also measured by immunoblotting of the same nitrocellulose filters that had been used for detection of cell cycle proteins. After 24 hr, a small, but non-significant increase in expression of these proteins was found. Thus, p21 is 119.2±10.0%, cyclin D1 111.8±13.8%, cyclin E 112.0±14.2%, cdk4 118.3±21.1% and cdk6 119.3±12.2% compared to DMSO treated cells (100%).

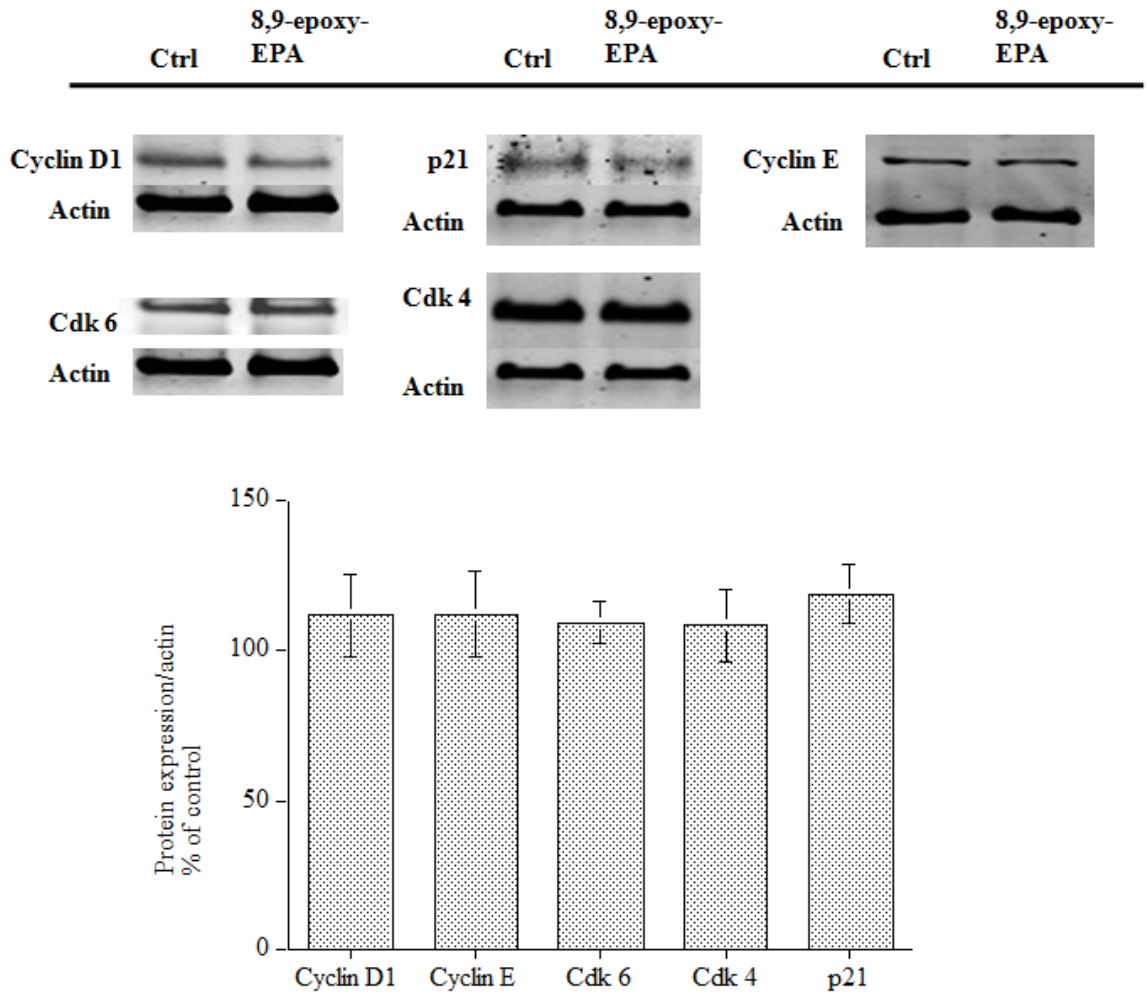

Figure 3.8: Effect of 8,9-epoxy-EPA on expression of cell cycle regulatory proteins. MDA-MB-231 were treated with 8,9-epoxy-EPA for 24 hr and were harvested as described under materials and methods. Equal amounts of cell lysates were analyzed by Western blot analysis. Data represent mean protein expression/mean actin expression as percentage of control ±SE, n=3 independent experiments.

3.5 Effect of 8,9-epoxy-EPA on MDA-MB-231 cells that had been exposed to the anticancer drug paclitaxel.

8,9-epoxy-EPA increases cell viability of MDA-MB-231 breast cancer cells as shown above. To test the importance of the potential cytoprotective effect of the n-3 fatty acid epoxide, cells were exposed to the established cytotoxic drug, paclitaxel, in combination with 8,9-epoxy-EPA.

3.5.1 Effect of paclitaxel on viability of MDA-MB-231 cells

The effects of different concentrations of paclitaxel on MDA-MB-231 cell viability are shown in figure 3.9. Cells were grown in 0.5% FBS supplemented DMEM, exposed to varying concentrations of paclitaxel for 24 hr and then analyzed by MTT assay. A reduction in the number of viable cells was found between 1-10nM paclitaxel (66.2±1.0% of control viability at 10nM paclitaxel).

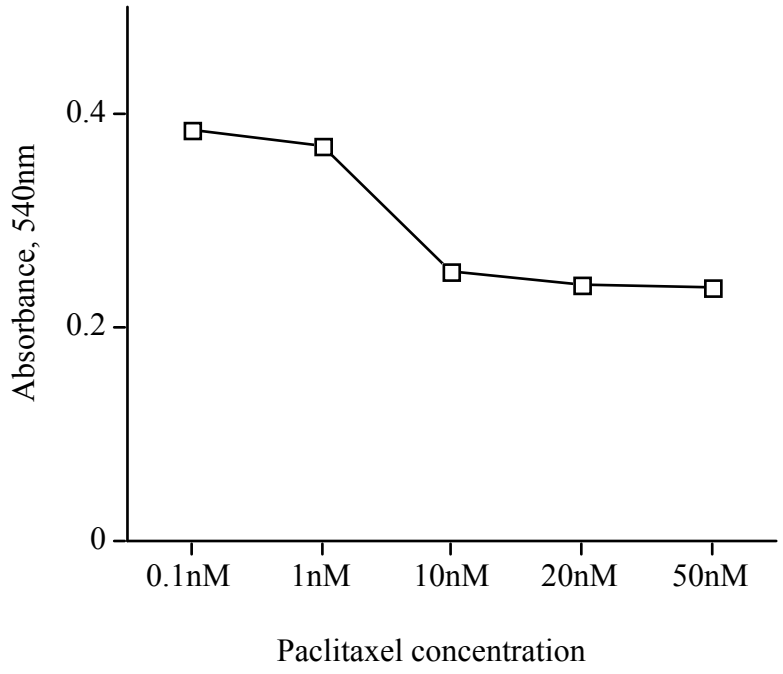

Figure 3.9: Concentration-dependent effect of paclitaxel on the viability of MDA-MB-231 cells. At 0,1nM paclitaxel viable cells are 100.5±3.2% compared to DMSO treated cells, and 97.12±3.4% at 1nM. At 10nM cell viability is reduced to 66.2±1.0% of control, and 62.5±0.5% and 62.17±2.0% at 20nM and 50nM, respectively. Data represent mean ±SE, n=3 independent experiment.

3.5.2 8,9-epoxy-EPA restore MDA-MB-231 viability in paclitaxel treated cells.

Cells grown in 0.5% FBS supplemented DMEM were exposed to 2nM, 5nM or 10nM paclitaxel in addition to 8,9-epoxy-EPA (alone and in combination with DCU) for 24 hr. Cell viability was assessed by MTT assay. As shown in figure 3.10, treatment with 8,9-epoxy-EPA either alone or in combination with DCU, increased viability of MDA-MB-231 in cells that also received paclitaxel. Paclitaxel (2nM) did not decrease cell viability significantly compared to DMSO treated cells. However, the increase in cell viability in cells exposed to 8,9-epoxy-EPA (10.6±1.4%, $p<0.0001$ versus paclitaxel treated cells) and 8,9-epoxy-EPA +

DCU (9.3±0.5%, p<0.0001, versus paclitaxel+DCU treated cells) was significant in both cases. In contrast paclitaxel (5nM and 10nM) decreased cell viability to 89.0±2.3% (p=0.043) and 74.0±4.3% (p<0.0001) of respective controls. In the case of 5nM paclitaxel, the increase in viability of cells treated with 8,9-epoxy-EPA and 8,9-epoxy-EPA + DCU, was 31.8±4.6% (p<0.0001) compared to paclitaxel treated cells, and 30.5±4.2% (p<0.0001) compared to paclitaxel+DCU treated cells. For 10nM paclitaxel treatment the increase was 39.8±10.0% (p<0.0001) for 8,9-epoxy-EPA alone and 44.17±6.7 (p<0.0001) for 8,9-epoxy-EPA in combination with DCU.

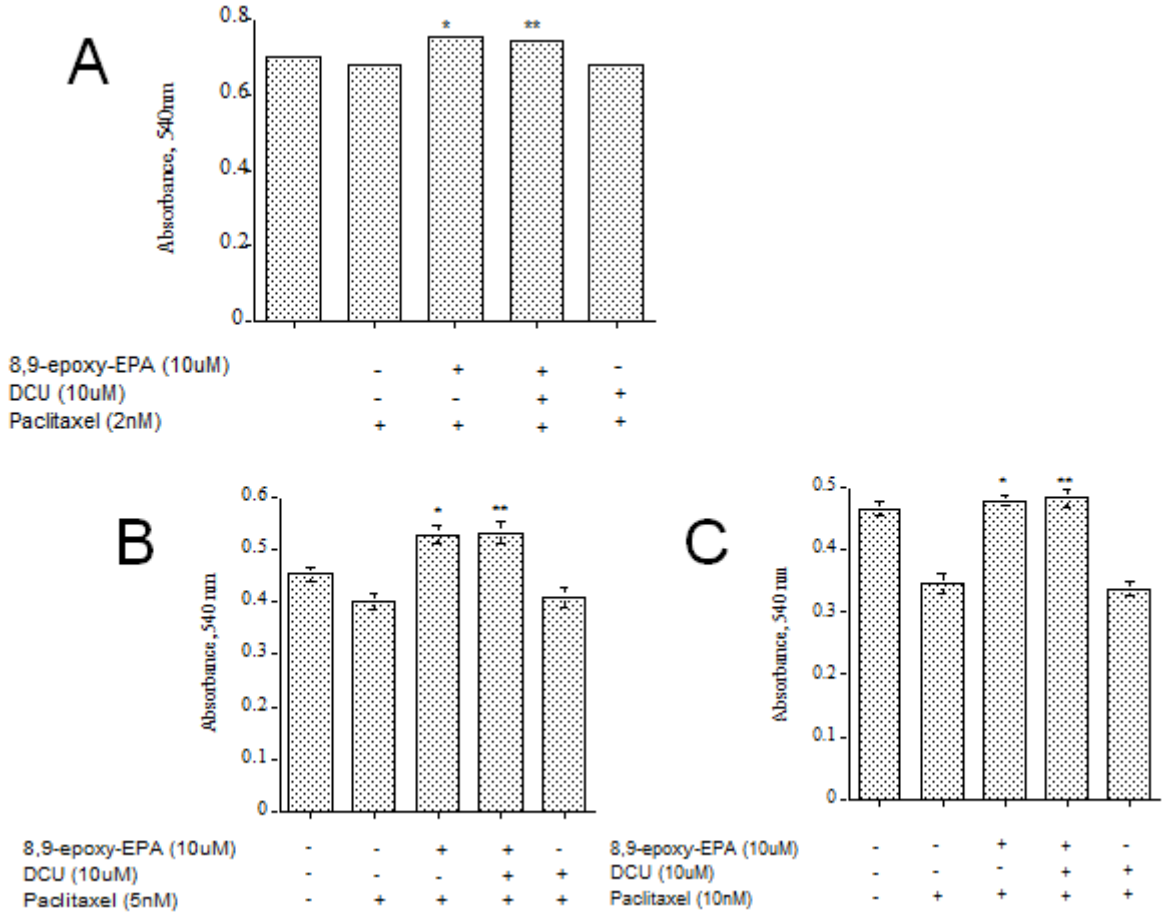

Figure 3.10: Effect of 2nM, 5nM and 10nM paclitaxel in combination with 8,9-epoxy-EPA and 8,9-epoxy-EPA + DCU on viability of MDA-MB-231 cells. **A:** 8,9-epoxy-EPA alone and in combination with DCU increases cell viability significantly: *10.6±1.4%, p<0.0001 versus paclitaxel alone. **9.3±0.5%, p<0.0001 versus paclitaxel+DCU treated cells. **B:** 5nM paclitaxel in combination with 8,9-epoxy-EPA and 8,9-epoxy-EPA + DCU increases cell viability significantly. . * 31.8±4.6% p<0.0001 versus paclitaxel alone. ** 30.5±4.2%,, p<0.0001 versus paclitaxel+DCU treated cells. **C:** 10nM paclitaxel in combination with 8,9-epoxy-EPA and 8,9-epoxy-EPA + DCU increases cell viability significantly: * 39.8±10.0%, p<0.0001versus paclitaxel alone. **

44.17±6.7, $p<0.0001$ versus paclitaxel+DCU treated cells. Data represent mean ±SE, n=3 independent experiments.

3.5.3 Effect of combined treatment with 8,9-epoxy-EPA and paclitaxel on cell cycle kinetics in MDA-MB-231 cells

To further investigate the increased viability observed when MDA-MB-231 cells were exposed to 8,9-epoxy-EPA in the presence of paclitaxel, cell cycle analysis by flow cytometry was carried out. The cells were exposed to 8,9-epoxy-EPA (10µM) alone and in combination with DCU (10µM) in the presence of 10nM paclitaxel for 6 and 24 hr. After 6 hr, a significant decrease of cells accumulated in G0/G1 phase was observed compared to cells treated with DMSO and cells treated with paclitaxel alone. No related increase in cell accumulation was found in any of the other phases. At 24 hr the same significant decrease of cells in phase G0/G1 phase were found. At this timepoint there also was a related accumulation of cells in G2/M phase.

Only one significant difference between 8,9-epoxy-EPA treated cells and control was found. In G0/G1 phase, cells treated with 8,9-epoxy-EPA in combination with paclitaxel decreased 24.05±0.796% ($p=0.0069$) compared to cells treated with only paclitaxel for 24 hr. Because cell viability experiments have shown that 8,9-epoxy-EPA restore the viability of MDA-MB-231 treated with paclitaxel, the same effect was expected to be found in cell cycle experiments. The reversal of paclitaxel effect in cell cycle would result in an increased amount of cells in G0/G1 phase. Thus, the significant decrease is not as expected.

No other significant differences was fount between MDA-MB-231 cells treated with 8,9-epoxy-EPA in combination with paclitaxel compared to control. Thus, 8,9-epoxy-EPA did not significantly alter the effect of paclitaxel on cell cycle.

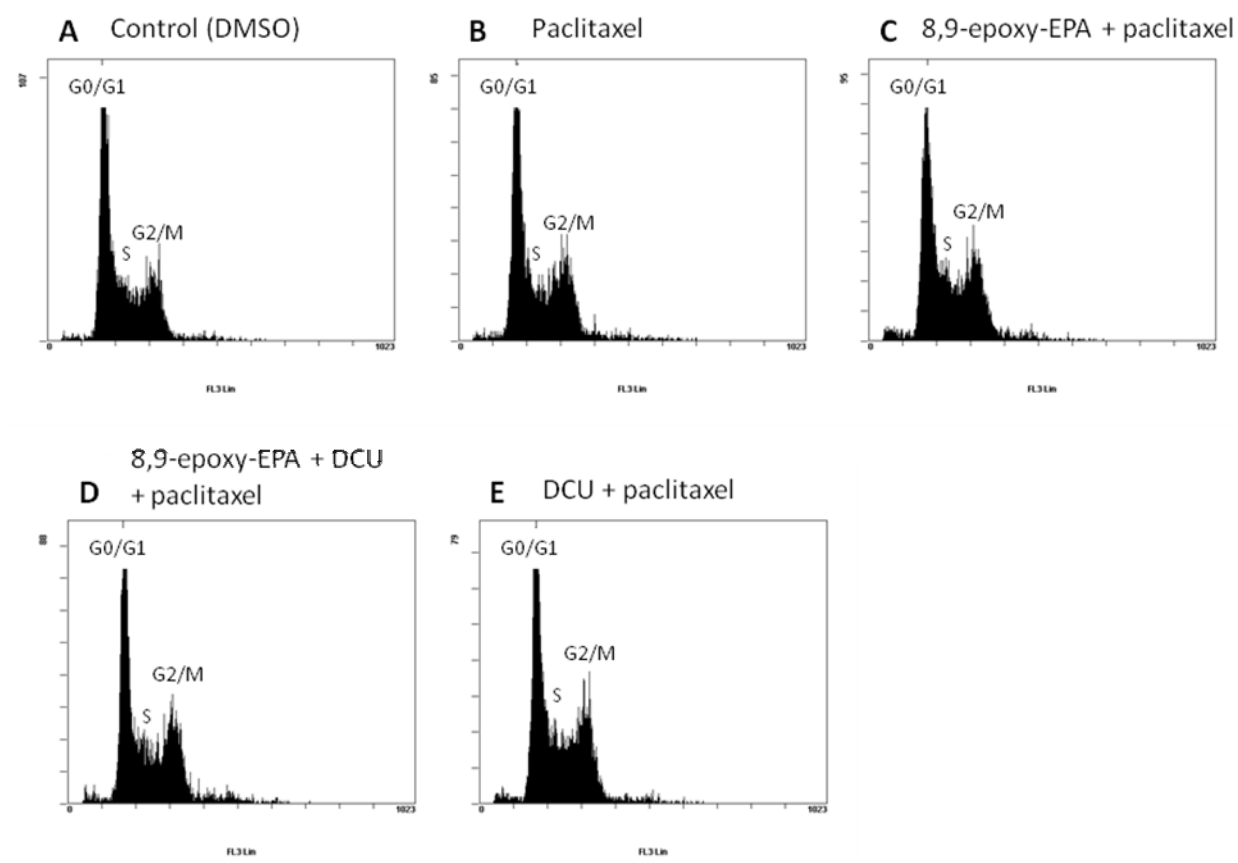

Figure 3.11: Flow cytomerty of MDA-MB-231 cells after 6 hr treatment of DMSO only (A), paclitaxel (B), paclitaxel and 8,9-epoxy-EPA (C), paclitaxel, 8,9-epoxy-EPA and DCU (D) and paclitaxel and DCU (E).

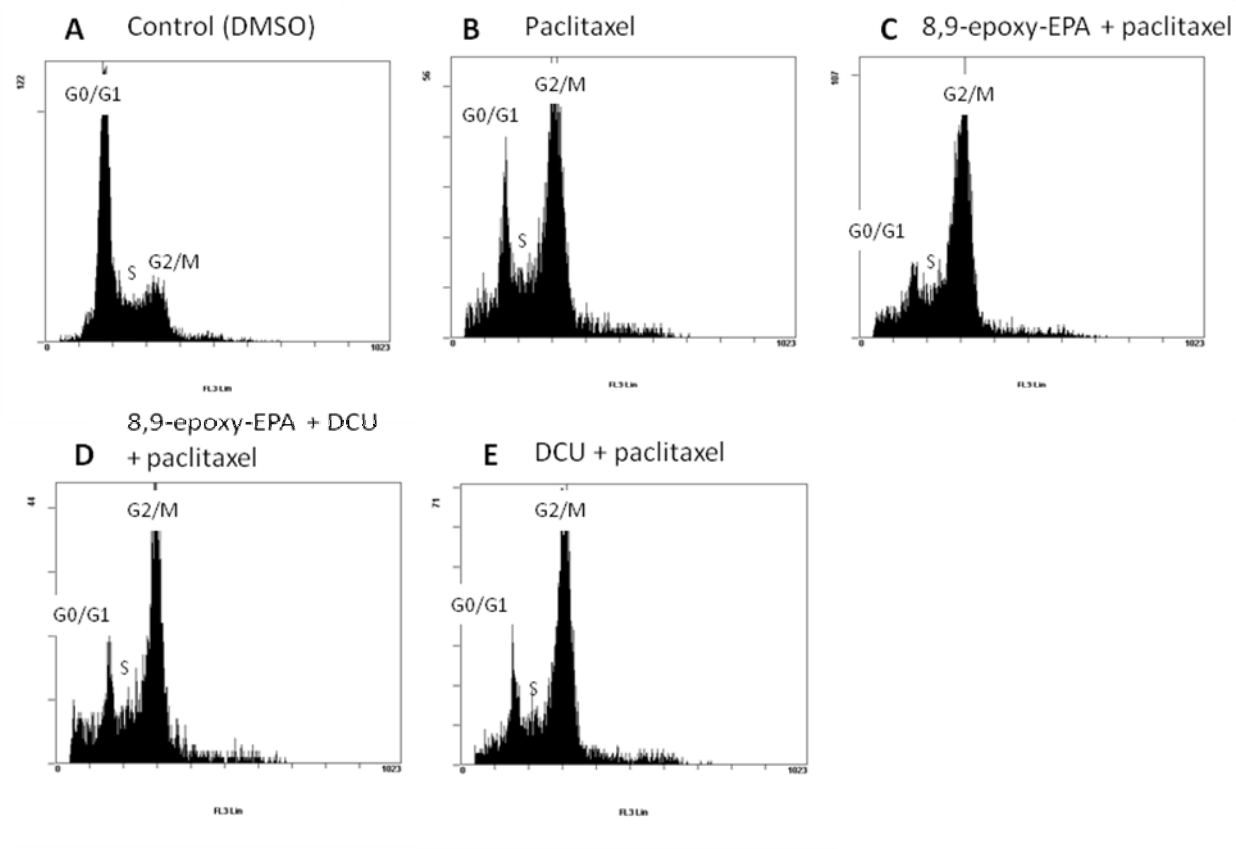

Figure 3.12: Flow cytomerty of MDA-MB-231 cells after 24 hr treatment of DMSO only (A), paclitaxel (B), paclitaxel and 8,9-epoxy-EPA (C), paclitaxel, 8,9-epoxy-EPA and DCU (D) and paclitaxel and DCU (E).

Table 3.2: Percentage of MDA-MB-231 cells in each cell cycle phase after treatment with 8,9-epoxy-EPA alone or in combination with DCU in the presence of paclitaxel after 6 hr. *Accumulation of cells in phase G0/G1 decreased 8.0±1.77 for paclitaxel (p=0.015) compared to DMSO treated cells. Data represent mean ±SE, n=3 independent experiments.

	G0/G1-phase (%)	**S -phase(%)**	**G2/M-phase(%)**
Control (DMSO)	52.88±0.07	17.18±1.31	24.56±1.66
Paclitaxel	48.67±1.70*	16.82±1.74	28.04±1.13
Paclitaxel + 8,9-epoxy-EPA	46.69±0.92**	17.89±2.16	28.74±2.55
Paclitaxel + 8,9-epoxy EPA + DCU	46.11±0.56***	19.37±1.58	28.04±1.23
Paclitaxel + DCU	47.25±1.05****	17.25±0.93	28.68±0.35

*p=0.015 compared to DMSO. ** p=0.0015 compared to DMSO. ***p=0.0008 compared to DMSO. ****p=0.0029 compared to DMSO.

Table 3.3: Percentage of MDA-MB-231 cells in each cell cycle phase after treatment with 8,9-epoxy-EPA alone or in combination with DCU in the presence of paclitaxel after 24 hr. Accumulation of cells decreased 68.4±1.55% ($p<0.0001$) in G0/G1 phase and increased 133.03±1.79% ($p=<0.0001$) in G2/M phase for paclitaxel compared to DMSO treated cells. 8,9-epoxy-EPA and paclitaxel treated cells decreased significantly compared to paclitaxel alone (24.05±0.796%, $p=0.0069$). Data represent mean ±SE, n=3 independent experiments.

	G0/G1-phase (%)	**S -phase(%)**	**G2/M-phase(%)**
Control (DMSO)	56.30±1.17	13.13±0.15	23.49±0.73
Paclitaxel	17.81±0.38[a]	10.52±0.69[f]	54.74±1.06[g]
Paclitaxel + 8,9-epoxy-EPA	13.52±0.42[b,e]	11.38±0.78	58.41±1.07[h]
Paclitaxel + 8,9-epoxy EPA + DCU	15.46±0.97[c]	11.34±0.62	55.43±2.14[i]
Paclitaxel + DCU	17.25±1.18[d]	10.77±1.10	55.06±1.20[j]

[a] $p<0.0001$ compared to DMSO. [b] $p<0.0001$ compared to DMSO. [c] $p<0.0001$ compared to DMSO. [d] $p<0.0001$ compared to DMSO. [e] $p=0.0069$ compared to paclitaxel.[f] $p=0.0306$ compared to DMSO.[g] $p<0.0001$ compared to DMSO. [h] $p<0.0001$ compared to DMSO. [i] $p<0.0001$ compared to DMSO. [j] $p<0.0001$ compared to DMSO.

4. Discussion

In this study we have investigated the effect of 8,9-epoxy-EPA in the human breast cancer cell line MDA-MB-231. The cells were treated with 8,9-epoxy-EPA alone or in combination with the epoxide hydrolase inhibitor N,N'-dicyclohexylurea (DCU). Studies have shown that fatty acid epoxids are hydrolysed to diols by epoxide hydrolase [16]. DCU was therefore added to the treatment to ensure the epoxide was kept stable long enough to detect possible effects on cell proliferation.

Studies have shown that EPA has anti-proliferative effects on breast cancer cells and other cancers [17, 18, 20, 21], but the mechanism is not fully understood. EPA is metabolized to several different eicosanoids in the body. 8,9-epoxy-EPA is one of these eicosanoids [13]. This compound is one of five monoepoxides formed when EPA is metabolized by cytochrome P450. No studies have previously been carried out in order to investigate the effect of epoxy-EPAs on cancer growth, which can be relevant for understanding how n-3 fatty acids are beneficial in cancer.

EPA is an analogue of arachidonic acid (AA). When AA is metabolized by CYP 450, epoxyeicosatrienoic acids (EETs), the monoepoxy forms of AA, are formed. Studies of EET on cancer cells have revealed a possible cancer growth stimulatory effect [30-32]. This effect may be related to protection of the cancer cells against apoptosis or control of angiogenesis [30-32]

This study consists of two parts. In the first part we tested the actions of 8,9-epoxy-EPA on cell viability and the cell cycle. In the second part we tested the effects of 8,9-epoxy-EPA on cancer cell viability and cell cycle in the presence of paclitaxel, an established cytotoxic agent. Previous studies have suggested that n-3 fatty acids my increase the cytotoxic effect of cytotoxic agents [44, 45]

4.1 Effect of 8,9-epoxy-EPA on MDA-MB-231 cell viability

MDA-MB-231 cells were exposed to 8,9-epoxy-EPA alone and in combination with N,N'-dicyclohexylurea (DCU) for 24 hr. The cell viability was assessed using MTT-assay and trypan blue counting. Based on studies showing an anti-proliferative effect of EPA on cancer

cells, we anticipated a possible decrease in MDA-MB-231 cell viability. In contrast to this, we found that the cell viability increased. The increase was consistent for both MTT-assay and trypan blue exclusion.

The increase in cell viability was slightly greater in the case of the combination with DCU, which indicates that DCU may increase the effect of 8,9-epoxy-EPA by preventing its hydrolysis by the soluble epoxy hydrolase (sEH) enzyme.

To test whether the potential confounding effect of growth factors on proliferation could be avoided, MDA-MB-231 cells were cultured in serum starved media and exposed to the same treatment as described above. No change in viability between cells treated with 8,9-epoxy-EPA compared to control were found in this experiment. This suggests that growth stimulation by FBS appears to be required for the effect of 8,9-epoxy-EPA on cell proliferation to be expressed.

Our results indicates a cytoprotective effect of 8,9-epoxy-EPA on MDA-MB-231 cells. Two different methods, MTT-assay and trypan blue counting, were used to show the same enhanced cell viability. Although the increased cell viability has not been anticipated based on the suggested anti-proliferative effect of EPA on cancer cells, it is consistent with previous reports of the effect of EET. EETs stimulate cyclin D1 expression, but 8,9-epoxy-EPA did not appear to exert a similar effect which would have been consistent with increased growth. However, EETs also increase viability by inhibiting apoptosis [31, 32]. Due to time constrains and the need to develop further new assays, this was not assessed in the present project. It is conceivable that 8,9-epoxy-EPA also inhibits cell death in MDA-MB-231.

4.2 Effect of 8,9-epoxy-EPA on cell cycle

The increased cell viability of MDA-MB-231 when exposed to 8,9-epoxy-EPA suggested that there may be an acceleration in the rate of the cell cycle. We exposed the cells to the same treatment as described earlier and measured cell cycle kinetics using flow cytometry. The results did not show any changes in cell cycle distribution between 8,9-epoxy-EPA treated cells and control.

We also tested the effect of 8,9-epoxy-EPA on cell cycle regulatory proteins using western blot analysis. For this experiment, the cells were only treated with 8,9-epoxy-EPA alone and

not in combination with DCU. The results from the immunoblotting did not show any significant changes in the expression of cell cycle regulatory proteins. These findings are consistent with the results from viability assay, but are not consistent with recent findings from this laboratory in murine endothelial cells. It is possible that a cell- and species- specific difference in cell response to epoxy-EPA occurs. Further studies are now required to assess this possibility.

4.3 8,9-epoxy-EPA restore MDA-MB-231 viability in paclitaxel treated cells

In the second part of this study we investigated the importance of the potential cytoprotective effect of 8,9-epoxy-EPA found by the cell viability experiments. MDA-MB-231 cells were exposed to 8,9-epoxy-EPA in the presence of paclitaxel, an established cytotoxic agent. Three different paclitaxel concentrations (2nM, 5nM and 10nM) were tested together with a constant concentration of 8,9-epoxy-EPA (10μM) alone and in combination with DCU (10μM). Cell viability was assessed using MTT-assay.

Paclitaxel at the two highest concentrations decreased cell viability. This was reversed when cells were treated with 8,9-epoxy-EPA in presence of paclitaxel.

The possible cytoprotective effect of 8,9-epoxy-EPA observed in these experiments are consistent with the results from the first experiments on cell viability. The increase in MDA-MB-231 viability occurs not only when cells are exposed to 8,9-epoxy-EPA alone, but also in the presence of paclitaxel. Again, this finding was in contrast with what had been expected from studies in endothelial cells [44, 45], but illustrates potential cell-specific effects of n-3 fatty acid epoxides.

4.4 Effect of combined treatment with 8,9-epoxy-EPA and paclitaxel on cell cycle kinetics in MDA-MB-231 cells.

Further, we investigated whether the possible cytoprotective effect of 8,9-epoxy-EPA on MDA-MB-231 cells in the presence of paclitaxel would show changes in cell cycle kinetics. The cells were exposed to 8,9-epoxy-EPA, alone and in combination with DCU, in the presence of paclitaxel. The treatments were conducted for 6hr and 24hr, and cell cycle kinetics was measured using flow cytometry.

After 6 hr a significant decrease of cells in G0/G1 occurred with paclitaxel. After 24 hr the decrease in the proportion of paclitaxel treated cells in G0/G1 phase occurred but was much more pronounced. At this timepoint there also was a related significant increase of accumulation of the same cells in G2/M phase. Thus, fewer cells are entering the cell cycle after 24 hr of paclitaxel treatment. The cell cycle distribution measures cell proportions, rather than absolute numbering. Those cells still in the cell cycle are distributed preferentially in G2/M, suggesting that they are arrested in G2/M phase and do not progress to mitosis. Based on the cell viability experiments, we expected to see a reversion of the paclitaxel effect on the cells when they were exposed to 8,9-epoxy-EPA in addition. This implied less accumulation of cells in G2/M phase. No such effect was observed or 8,9-epoxy-EPA alone or in combination with DCU. However, it is noteworthy that the proportions of cells in G0/G1 had decreased further, which suggests that even fewer were cycling than in the presence of paclitaxel. Considered together, the increase in viability produced by 8,9-epoxy-EPA in the presence of paclitaxel might have been due to an increase in quiescent cells, not in the cell cycle.

Because there is so little published literature regarding the effect of epoxide n-3 fatty acids on both normal cells and cancer cells, it is difficult to find any data that can be used to compare the results from these experiments with.

4.5 Summary

In summary, we found that 8,9-epoxy-EPA increased cell viability of MDA-MB-231. These results are contrary to the present view on EPAs anti-proliferative effect on cancer cells. Most likely CYP 450-derived epoxides are less important than EPA itself or other metabolites in altering cell proliferation.

The increased cell viability was again evident when cells were exposed to 8,9-epoxy-EPA in the presence of paclitaxel. This is consistent with our previous findings in the present cell line, but is not in accord with studies that have suggested that the combination of anticancer drugs and n-3 fatty acids offers advantages over one treatment alone.

The positive effect of 8,9-epoxy-EPA on MDA-MB-231 cell viability may be due to a similar mechanism as the apoptosis inhibiting effect of EET. Further studies may therefore include

apoptosis studies of 8,9-epoxy-EPA treated MDA-MB-231 cells. Further studies also need to address the signaling mechanisms by which n-3 epoxides control cyclin D1 expression in murine endothelial cells. With this information it will eventually be possible to understand the emerging difference between cell types in response to the epoxides. This information is important in understanding the limitations of the beneficial effects of n-3 fatty acids in cancer.

5. References

1. www.who.int. 2006, WHO.
2. Vinay Kumar, R.S.C., Stanley L. Robbins, *Robbins Basic Pathology*. 7th ed. 2003: Saunders.
3. Jonathan Phillips, P.M., Paul Kirk, *The Biology of Disease*. 2nd ed. 2001: Blackwell Science.
4. Christopher Potten, J.W., *Apoptosis. The life and death of cells.* 2004: Cambridge University Press.
5. *Introduction to Flow Cytometry: A learning guide, www.bova-university.org*. 2002, BD Bioscience.
6. *Brystkreft*. 2008, www.oncolex.no.
7. WHO, *http://www.who.int/mediacentre/factsheets/fs297/en/index.html*. 2006.
8. *www.kreftregisteret.no, mai 2008*, Kreftregisteret.
9. *www.lgcpromochem-atcc.com*. 2006, LGC Promochem.
10. *www.sigmaaldrich.com*. 2008, Sigma-Aldrich.
11. Martindale, W., *The extra pharmacopoeia.* 31th ed. 1996: Royal Pharmaceutical Society.
12. *Fatty acids: Methylene-interrupted double bonds, www.lipidlibrary.co.uk*. 2007.
13. Fer, M., et al., *Metabolism of eicosapentaenoic and docosahexaenoic acids by recombinant human cytochromes P450.* Arch Biochem Biophys, 2008. **471**(2): p. 116-25.
14. Chacos, N., et al., *The reaction of arachidonic acid epoxides (epoxyeicosatrienoic acids) with a cytosolic epoxide hydrolase.* Arch Biochem Biophys, 1983. **223**(2): p. 639-48.
15. Zeldin, D.C., et al., *Regio- and enantiofacial selectivity of epoxyeicosatrienoic acid hydration by cytosolic epoxide hydrolase.* J Biol Chem, 1993. **268**(9): p. 6402-7.
16. Fang, X., et al., *Pathways of epoxyeicosatrienoic acid metabolism in endothelial cells. Implications for the vascular effects of soluble epoxide hydrolase inhibition.* J Biol Chem, 2001. **276**(18): p. 14867-74.
17. Rose, D.P. and J.M. Connolly, *Effects of fatty acids and inhibitors of eicosanoid synthesis on the growth of a human breast cancer cell line in culture.* Cancer Res, 1990. **50**(22): p. 7139-44.
18. Schley, P.D., et al., *Mechanisms of omega-3 fatty acid-induced growth inhibition in MDA-MB-231 human breast cancer cells.* Breast Cancer Res Treat, 2005. **92**(2): p. 187-95.
19. Rose, D.P., J.M. Connolly, and M. Coleman, *Effect of omega-3 fatty acids on the progression of metastases after the surgical excision of human breast cancer cell solid tumors growing in nude mice.* Clin Cancer Res, 1996. **2**(10): p. 1751-6.
20. Wu M, H.K., Ruzmetov N, Welch ZR, Sech L, Jackson K, Stillwell W, Zaloga GP, Siddiqui RA., *Omega-3 polyunsaturated fatty acids attenuate breast cancer growth through activation of a neutral sphingomyelinase-mediated pathway.* Int J Cancer, 2005. **117**(3): p. 340-8.
21. Barascu, A., et al., *CDK1-cyclin B1 mediates the inhibition of proliferation induced by omega-3 fatty acids in MDA-MB-231 breast cancer cells.* Int J Biochem Cell Biol, 2006. **38**(2): p. 196-208.
22. Kuriki, K., et al., *Breast cancer risk and erythrocyte compositions of n-3 highly unsaturated fatty acids in Japanese.* Int J Cancer, 2007. **121**(2): p. 377-85.
23. Folsom, A.R. and Z. Demissie, *Fish intake, marine omega-3 fatty acids, and mortality in a cohort of postmenopausal women.* Am J Epidemiol, 2004. **160**(10): p. 1005-10.
24. Engeset, D., et al., *Fish consumption and breast cancer risk. The European Prospective Investigation into Cancer and Nutrition (EPIC).* Int J Cancer, 2006. **119**(1): p. 175-82.
25. Busstra MC, S.C., Grubben MJ, van Kranen HJ, Nagengast FM, van't Veer P, *Tissue levels of fish fatty acids and risk of colorectal adenomas: a case-control study (Netherlands).* Cancer Causes Control, 2003. **3**(14): p. 269-76.

26. Goodstine, S.L., et al., *Dietary (n-3)/(n-6) fatty acid ratio: possible relationship to premenopausal but not postmenopausal breast cancer risk in U.S. women.* J Nutr, 2003. **133**(5): p. 1409-14.
27. Gago-Dominguez, M., et al., *Opposing effects of dietary n-3 and n-6 fatty acids on mammary carcinogenesis: The Singapore Chinese Health Study.* Br J Cancer, 2003. **89**(9): p. 1686-92.
28. Wirfält E, M.I., Gullberg B, Johansson U, Olsson H, Berglund G, *Postmenopausal breast cancer is associated with high intakes of omega6 fatty acids (Sweden).* Cancer Causes Control, 2002. **10**(13): p. 883-93.
29. Newcomer LM, K.I., Wicklund KG, Stanford JL, *The association of fatty acids with prostate cancer risk.* Prostate., 2001. **4**(47): p. 262-8.
30. Wang, Y., et al., *Arachidonic acid epoxygenase metabolites stimulate endothelial cell growth and angiogenesis via mitogen-activated protein kinase and phosphatidylinositol 3-kinase/Akt signaling pathways.* J Pharmacol Exp Ther, 2005. **314**(2): p. 522-32.
31. Dhanasekaran, A., et al., *Protective effects of epoxyeicosatrienoic acids on human endothelial cells from the pulmonary and coronary vasculature.* Am J Physiol Heart Circ Physiol, 2006. **291**(2): p. H517-31.
32. Jiang, J.G., et al., *Cytochrome P450 2J2 promotes the neoplastic phenotype of carcinoma cells and is up-regulated in human tumors.* Cancer Res, 2005. **65**(11): p. 4707-15.
33. Yang, P., et al., *Formation and antiproliferative effect of prostaglandin E(3) from eicosapentaenoic acid in human lung cancer cells.* J Lipid Res, 2004. **45**(6): p. 1030-9.
34. Bagga, D., et al., *Differential effects of prostaglandin derived from omega-6 and omega-3 polyunsaturated fatty acids on COX-2 expression and IL-6 secretion.* Proc Natl Acad Sci U S A, 2003. **100**(4): p. 1751-6.
35. Lands, W.E., et al., *Maintenance of lower proportions of (n - 6) eicosanoid precursors in phospholipids of human plasma in response to added dietary (n - 3) fatty acids.* Biochim Biophys Acta, 1992. **1180**(2): p. 147-62.
36. Hwang, D., et al., *Expression of cyclooxygenase-1 and cyclooxygenase-2 in human breast cancer.* J Natl Cancer Inst, 1998. **90**(6): p. 455-60.
37. Chulada, P.C., et al., *Genetic disruption of Ptgs-1, as well as Ptgs-2, reduces intestinal tumorigenesis in Min mice.* Cancer Res, 2000. **60**(17): p. 4705-8.
38. Cheuk BL, C.S., Fiscus RR, Wong PY., *Cyclooxygenase-2 regulates apoptosis in rat epididymis through prostaglandin D2.* Biol Reprod, 2002. **66**(2): p. 374-80.
39. Potente, M., et al., *Cytochrome P450 2C9-induced endothelial cell proliferation involves induction of mitogen-activated protein (MAP) kinase phosphatase-1, inhibition of the c-Jun N-terminal kinase, and up-regulation of cyclin D1.* J Biol Chem, 2002. **277**(18): p. 15671-6.
40. Cianchi, F., et al., *Up-regulation of cyclooxygenase 2 gene expression correlates with tumor angiogenesis in human colorectal cancer.* Gastroenterology, 2001. **121**(6): p. 1339-47.
41. Szymczak, M., M. Murray, and N. Petrovic, *Modulation of angiogenesis by omega-3 polyunsaturated fatty acids is mediated by cyclooxygenases.* Blood, 2008. **111**(7): p. 3514-21.
42. Sovak MA, B.R., Kim DW, Zanieski GJ, Rogers AE, Traish AM, Sonenshein GE, *Aberrant Nuclear Factor kB/Rel Expression and the Pathogenesis of Breast Cancer.* J Clin Invest, 1997. **100**(12): p. 2952-60.
43. Novak TE, B.T., Jho DH, Helton WS, Espat NJ, *NF-kappa B inhibition by omega -3 fatty acids modulates LPS-stimulated macrophage TNF-alpha transcription.* Am J Physiol Lung Cell Mol Physiol, 2003. **284**(1): p. L84-9.
44. Menendez, J.A., R. Lupu, and R. Colomer, *Exogenous supplementation with omega-3 polyunsaturated fatty acid docosahexaenoic acid (DHA; 22:6n-3) synergistically enhances taxane cytotoxicity and downregulates Her-2/neu (c-erbB-2) oncogene expression in human breast cancer cells.* Eur J Cancer Prev, 2005. **14**(3): p. 263-70.

45. Menendez, J.A., et al., *Effects of gamma-linolenic acid and oleic acid on paclitaxel cytotoxicity in human breast cancer cells.* Eur J Cancer, 2001. **37**(3): p. 402-13.
46. Maheo, K., et al., *Differential sensitization of cancer cells to doxorubicin by DHA: a role for lipoperoxidation.* Free Radic Biol Med, 2005. **39**(6): p. 742-51.
47. Wolff, A.C., et al., *Phase I study of docosahexaenoic acid-paclitaxel: a taxane-fatty acid conjugate with a unique pharmacology and toxicity profile.* Clin Cancer Res, 2003. **9**(10 Pt 1): p. 3589-97.
48. Burns, C.P. and J.A. North, *Adriamycin transport and sensitivity in fatty acid-modified leukemia cells.* Biochim Biophys Acta, 1986. **888**(1): p. 10-7.
49. Germain, E., et al., *Enhancement of doxorubicin cytotoxicity by polyunsaturated fatty acids in the human breast tumor cell line MDA-MB-231: relationship to lipid peroxidation.* Int J Cancer, 1998. **75**(4): p. 578-83.
50. *Using a Hemacytometer*. 2002, Cascade Biologics.
51. *CellTiter96 Non-Radioactive cell proliferation Assay*. 2007, Promega.

6. Appendix

6.1 Western blotting protocol

Washing and lysis of cellpellets:

- Resuspend cellpellet in 0.5 ml ice-cold PBS and transfer to 1.5 ml eppendorf tubes.
- Add another 0.5 ml PBS to original tube to wash out cell rests, and transfer to the eppendorf tube.
- Spin down at highest rpm for 5 min at 4°C.
- Remove supernatant, flip the tube, and resuspend in 1 ml PBS.
- Spin down again, and remove supernatant.

Part A:

PBS	25.96 ml
1% Igepal	4 ml
0.5% Sodium deoxycholate	10 ml
0.1% SDS	0.2 ml
Total	40 ml

Lysis buffer = part A + phosphatase inhibitors

Part A	4992.5 µl
0.1 mM PMSF	2.5 µl
5 µg/ml aprotinin	2.5 µl
5 µg/ml leupeptin	2.5 µl
Total	5 ml

- Add lysis buffer to tubes (20 µl per one harvested well from 24-well plate).
- Pipette up and down when adding lysis buffer without make bubbles, vortex and incubate on ice for 30-40 min. Vortex a few times during incubation.
- Spin down at 10 000 g for 10 min at 4°C. The supernatant do now contain the proteins.

- Transfer 4 µl of the supernatant to a new tube, freeze the stock in -80°C (or remove all of supernatant to another new tube, freeze this and discard pellet).
- Put the tubes with 4 µl protein on ice.

BCA assay – quantification:

- To tube containing 4 µl protein; add 66 µl water. (= 70 µl, dilution factor = 17.5)
- Set up bovine serum albumin (BSA) standards as per table below using 2 mg/ml BSA and diluted lysis buffer (diluted 17.5 times). I.e. 400 µl lysis buffer + 6600 µl water = 17.5 times diluted lysis buffer.

BSA stock = 2 mg/ml X 1ml (from 1% BSA: 0.2 ml 1% BSA + 0.8 ml diluted buffer)

Tube	Diluted buffer, µl	BSA stock, µl	Final concentration, µl
A	0	300 of BSA stock	2000
B	125	375 of BSA stock	1500
C	325	325 of BSA stock	100
D	175	175 of B	750
E	325	325 of C	500
F	325	325 of E	250
G	325	325 of F	125
H	120	180 of G	75
I	300	200 of G	50
J	400	100 of G	25
K	150	150 of J	12,5
L	400	0	0 = Blank

- Prepare BCA working solution in ratio 50A:1B. I.e. 40 ml A + 0.8 ml B. (Reagent A and B from BCA protein assay kit). Vortex.
- Add 25 µl of BSA standards and test samples to clear 96-well plate in duplicates.
- Add 200 µl BCA working solution into each well using multi-channel pipette.
- Shake on orbital shaker for 30 seconds.

- Seal plate with parafilm (to prevent water from enter the wells) and incubate at 37ºC for 40 min.
- Cool at room temperature for 30 minutes.
- Read at 540 nm on plate reader.
- If there is moist on the lid after reading, cool a little longer and do another reading.
- If there is a big difference between the duplicates, also do a new reading. Make sure there are no bubbles in the wells.

Making the gel:

- Put together the gel cassette. For Mini-PROTEAN®3 Cell Assembly:
    - Place a short plate on top of a spacer plate.
    - Slide the two plates into the casting frame, keeping the shortplate facing the front. Insure both plates are flush at the bottom on a level surface.
    - Lock the pressure cams.
    - Tape the bottom of the glass plates to secure leaking.
    - Place the gel cassette assembly on the grey casting stand gasket with short plate facing you. (Use filter paper under and parafilm over gaskets for less leakage).
- Prepare 12% separating gel.
- Use a Pasteur pipette to apply the gel mix to the gel casting apparatus. Fill it up to the "green edge" (about 5 cm). If it starts leaking, keep adding more gel mix until it stops.
- Place about 5 drops of BuOH evenly across the gel to exclude air during polymerization.
- Allow the gel to set for 30-40 minutes.
- Wash off BuOH with water 5 times. Remove water from gel carefully by putting a filter paper between the glass plates.

- Prepare 5% stacking gel.
- Place the stacking gel on top of the separating gel with a Pasteur pipette and insert the comb.
- Allow to set for 30 minutes.

- Prepare test samples. Calculate how much sample is needed for the wanted concentration of protein beforehand. Add it to an equal amount of reducing buffer (in addition to water if necessary) in an eppendorf tube. Boil for 5 min (make hole in lid of tube with needle first!), and spin very short on a centrifuge.
    - Also prepare 1 x running buffer (electrophoresis buffer) from 5 x running buffer.
    - I.e. 100 ml 5 x running buffer + 400 ml deionized water.
- When the gel is ready, remove comb and wash with water. Remove water in the wells with a syringe.
- Remove gel cassette from the casting frame and place it into the electrode assembly with short plate facing inward.
- Apply 5 µl of marker on one side and apply samples in each well. (An evenly amount of sample must be loaded). There have to be samples in each well in order to make the running work properly.
- Slide the gel cassettes and electrode assembly into the clamping frame. Lower into the mini tank. Fill up with 1 x running buffer. Make sure it is very full in between the glass cassettes.
- Run the gels at 200V (Power PAC 200) for about 2 hr for 1.5 mm gels, until the bromophenyl blue reaches the end of the gel.

Transferring proteins from gel to nitrocellulose membrane:

- Soak nitrocellulose membrane in transfer buffer on shaker for at least 10 minutes.

- Prepare stack by assembling the components in the following order (in tray with transfer buffer). Start at the bottom:

  TOP

  Clear plastic holder

  1 pad

  3 x filter paper (soaked)

  Nitrocellulose

  Gel (remove excess gel on edges before stacking)

  3 x filter paper (soaked)

  1 pad

  Black plastic folder

  BOTTOM

Do not touch the middle of the gel! Smoothen over the filter paper on top of the membrane to reduce bubbles.

- Fasten folder and place into blotting module. Make sure the black side of the folder is facing the black side of the blotting module!
- Place into tank and fill with transfer buffer.
- Place tank in large esky surrounded by ice and fill ice container in tank with ice. Refill the ice in the ice container every 30 minutes.
- Run at 100V (Power Pac 200) for 1.5 hr at least for 1.5 mm gels.
- Put membrane between to filter papers and keep flat.

Development of blot:

- Remove blot from filter paper and put into a box containing the dye ponceus C.
- Shake on orbital shaker for 10 minutes.
- Wash quickly with water.
- Scan to see protein bands and ensure efficiency of transfer.
- Place membrane in box containing blocking milk solution.
- Shake for 1 hr.
- Move blot to new box with blocking milk solution and primary antibodies.
- Incubate on shaker in cold room overnight, 4 °C.
- Place membrane in new box for washing. Wash 5 x 5 min and 5 x 2.5 min with ~20 ml (enough to cover the membrane and a little more) TBS/Tween on shaker.
- Incubate in 10 ml blocking milk solution and secondary antibody for 1 hr on shaker. PROTECT FROM LIGHT IN THIS STEP AND FROM NOW ON!
- Remove secondary antibody.
- Wash blot with ~20 ml TBS/Tween for 5 x 5 min and 5 x 2.5 min, and then wash for 2 x 5 min with TBS (NO TWEEN).
- Dry blots between 2 filter papers. Wrap in foil to protect from light.
- Scan with Odyssey.