Thesis for the degree Master of Pharmacy

Effect of 8, 9-epoxy eicosapentaenoic acid on the growth of MCF-7 cells

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ABSTRACT

Epidemiologic studies have indicated that n-3 polyunsaturated fatty acids (PUFAs) supplementation may reduce the incidence of breast cancer, also *in vivo* and *in vitro* studies have shown that n-3 PUFAs have an inhibitory effect on breast cancer cells. One important n-3 PUFA are eiocosapentaenoic acid (EPA), which are metabolized to five regioisomeric epoxyeicosatetraenoic acids (epoxy-EPAs) by the cytochromes P450 system. Further, these epoxides are metabolized by epoxide hydrolase (EH), which can be inhibited by N, N`-dicyclohexylurea (DCU), an epoxide hydrolase inhibitor (EHI). The effect of these five epoxy-EPAs and the combination of epoxy-EPAs and EHI on breast cancer cells is not known.

In this study the effect of 8, 9-epoxy-EPA on the human estrogen responsive breast cancer cell line MCF-7 was tested. MCF-7 cells were treated with 8, 9-epoxy-EPA, DCU and a combination of 8, 9-epoxy-EPA + DCU. Both the treatment with 8, 9-epoxy-EPA and the combination of 8, 9-epoxy-EPA + DCU inhibited the growth of MCF-7 cells in the presence of FBS. Treatment with 8, 9-epoxy-EPA in the absence of FBS did not result in a change in cell viability, which indicates that the growth inhibition on MCF-7 cells by 8, 9-epoxy-EPA requires an additional proliferative stimulus. To examine the effects of 8, 9-epoxy-EPA on MCF-7 cell growth flow cytometry was used to monitor cell cycle progression. Cells treated with 8, 9-epoxy-EPA and the combination of 8, 9-epoxy-EPA + DCU for 16 and 24 hours were found to be arrested in G0/G1-phase, and did not progress through the cell cycle at the same rate as control cells. 8, 9-epoxy-EPA treatment for 24 hours also resulted in a corresponding decrease in G2/M-phase. The adding of DCU did not enhance the effect of the 8, 9-epoxy-EPA treatment on cell cycle progression significantly. Treatment in the absence of FBS showed no alteration in the progression of the cell cycle. The growth inhibition effect on MCF-7 cells of 8, 9-epoxy-EPA was further studied by examining changes in expression of different cell cycle regulatory proteins. The protein levels were found to be unaltered after 8, 9-epoxy-EPA treatment. The growth inhibition effect may be due to increased cell apoptosis as an alternative to decreased proliferation in MCF-7 cells. This possibility should be evaluated in further studies.

ABBREVIATIONS

AA	Arachidonic acid
BCA-assay	Bicinchoninic-assay
CDK	Cyclin-dependent protein kinase
COX	Cyclooxygenase
CYP-450	Cytochromes P450 system
DCU	N, N`-dicyclohexylurea
DHA	Docosahexaenoic acid
DHET	Dihydroxyeicosatrienoic acids
DMEM	Dulbecco`s modified eagle medium
DMSO	Dimethyl sulfoxide
EET	Epxoyeicosatrienoic acid
EH	Epoxide hydrolase
EHI	Epoxide hydrolase inhibitor
EPA	Eicosapentaenoic acid
8, 9-epoxy-EPA	8, 9-epoxy-eicosatetraenoic acid
FBS	Fetal bovine serum
HIF-1	Hypoxia-inducible factor-1
LA	Linoleic acid
LAF-bench	Laminar Air Flow bench
LOX	Lipooxygenase
MTT	Thiazolyl blue tetrazolium bromide
NFκB	Nuclear factor κB
PG	Prostaglandin
PUFA	Polyunsaturated fatty acid
VEGF	Vascular endothelial growth factor

1 INTRODUCTION

1.1 Cancer

In 2005 7.6 million people died of cancer, which accounts for 13% of the total deaths worldwide. The deaths caused by cancer are expected to continue to rise, and it is estimated that in 2015 nine million people will die of cancer. In Norway, there were 24 488 incident cases of cancer in 2006. Breast cancer is one of the major cancer forms among women, and each year 502,000 people die of breast cancer worldwide. In 2006, there were 2673 new cases of breast cancer in Norway. Factors that are associated with a higher risk of cancer are geographic and environmental factors, age, heredity and clinical conditions. In breast cancer there are also other risk factors like gender, hormonal relations (early menstruation and late menopause), atypical hyperplasia, long-lasting postmenopausal estrogen therapy, previous breast cancer, overweight and alcohol [1-3].

In cancer, cells do not respond to normal growth control because of changes in the genes that control the cell growth and repair. Changes in these genes can be caused by physical (UV, ionizing radiation), chemical (asbestos, tobacco smoke) or biological (infection by virus or bacteria) carcinogens. There are often mutations in oncogenes and tumor suppressor genes. Oncogenes are derived by mutations in protooncogenes, and promote cell growth in cancer cells in the absence of normal growth-promoting signals. Tumor supressor genes encode proteins that normally inhibit cell division [2, 3].

A neoplasm, also called a tumor, is an abnormal mass of tissue consisting of cells that are genetically altered, and do not respond to normal growth regulation. A tumor is completely depended on the host for nutrition and blood supply, and sometimes endocrine support. Tumors can be classified as benign or malign, based on its properties. A malign tumor have uncontrolled cell division, can invade adjacent tissues and spread to other parts of the body via lymph and blood (metastasis). Cancer cells can be well-differentiated, but are often less differentiated than normal cells. Differentiation is a process where cells undergo a change to perform a specific function. Benign tumors do not invade or infiltrate the surrounding normal tissue and cannot metastasis [3-5].

Tumors consist of two basic components called the parenchyma and the stroma. The parenchyma is made up of neoplastic cells, which determines the biologic behavior of the tumor. The stroma consists of connective tissue and blood vessels, which carries blood supply and provides support for growth of the tumor. The growth of the tumor is determined by cell kinetics (the rate of cell division and the rate of cells lost), blood supply, and some also depend on hormones. Breast cancer tumors can be regulated by steroid hormones (estrogens, progesterones, and androgens), growth factors, insulin and insulin-like growth factors (IGF-1) [1, 3].

1.1.1 Angiogenesis

Angiogenesis is the process by which new capillaries are formed in a tumor. A tumor that grows beyond 1-2 mm in diameters cannot enlarge unless formation of new capillaries, because 1-2 mm is the maximal distance oxygen and nutrients can diffuse from blood vessels. Tumor cells can produce and induce tumor-associated angiogenic factors, the two most important being vascular endothelial growth factor (VEGF) and basic fibroblast growth factor. When tumors are exposed to hypoxia, angiogenesis are induced by the release hypoxia-inducible factor-1 (HIF-1), which controls the transcription of VEGF. Since angiogenesis is important for the growth of the tumor it is a potential target for anticancer treatment [3].

1.2 Cell cycle

The eukaryotic cell cycle consists of four phases, called G1-, S-, G2- and M-phase as shown in figure 1.1. In the S-phase, synthesis-phase, the DNA-synthesis produces to copies of the DNA to both daughter cells. Between S-phase and cell division, G2–phase, RNA and proteins are synthesized. The cell divides into to genetically identical daughter cells in the M-phase, the mitotic phase. This phase consists of division of the cell nucleus, mitosis, and cytoplasm to produce two daughter cells, cytokinesis. After the cell division the daughter cells can enter G1-phase, where RNA and proteins are synthesized, and continue to division or a quiescent phase, G0 [6, 7].

1.2.1 Regulation of the cell cycle

The control of when a cell divide, differentiate or become permanently quiescent is important for the organism. The cell cycle is regulated by a family of protein kinases, which are

enzymes that phosphorylate specific proteins by catalyzing the transfer of a phosphate group from ATP. These kinases consist of a regulatory subunit, cyclin, and a catalytic subunit, cyclin-dependent protein kinase (CDK). When cyclin bindes to CDK and forms the cyclin-CDK complex, CDK becomes activated. There are four mechanisms that modulate the activity of specific CDKs. These mechanisms involve phosphorylation/dephosphorylation of CDKs, proteolytic breakdown of mitotic cyclins, regulation of the rate of synthesis of cyclin or CDK and the binding of specific protein inhibitors (for example p21) that inactivate CDK. During the cell cycle, different cyclins and CDKs form cyclin-CDK complexes. The cyclin D family is associated with the progression of cells from G0-phase to G1-phase, where cyclin D forms a complex with CDK4. When the cells approaches S-phase cyclin E bind to CDK2, and in the end of the S-phase when cyclin E is degraded cyclin A bind. During G2 phase cyclin B are formed [5-7].

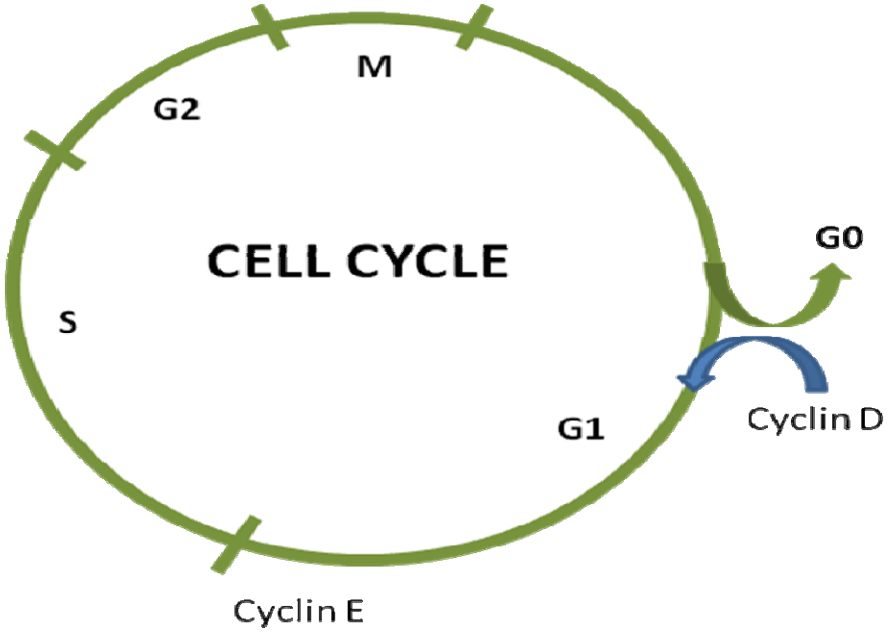

Figure 1.1: The cell cycle

1.3 Flow cytometry

To determine the proportion of cells in each of the cell cycle phases flow cytometry techniques can be used. Cells are fixed and labeled with a DNA-binding fluorescent probe (for example propidium iodide). The cell suspension passes through a narrow chamber as a single droplet with the size of one single cell. Lasers excite fluorescent dyes by sending out

monochromatic light of specific wavelengths, which are deflected or reflected depending on the size and density of the cell. The light emission from the dye is assayed by photon detectors, and computer software can analyze the data. The DNA-binding fluorescent probe will bind to the DNA, and the fluorescence is proportional with the amount of DNA in the cell. Cells in G0- and G1-phase should have the same amount of DNA, and when they are in G2-phase the amount of DNA is doubled. If cells have a DNA concentration between G1- and G2-phase they are assumed to be in S-phase. A typical frequency distribution of the DNA will show two peaks, the first one representing cells in G0/G1-phase and the other representing cells in G2/M-phase as shown in figure 1.2. Cells in S-phase are represented by the plateau between the peaks [5].

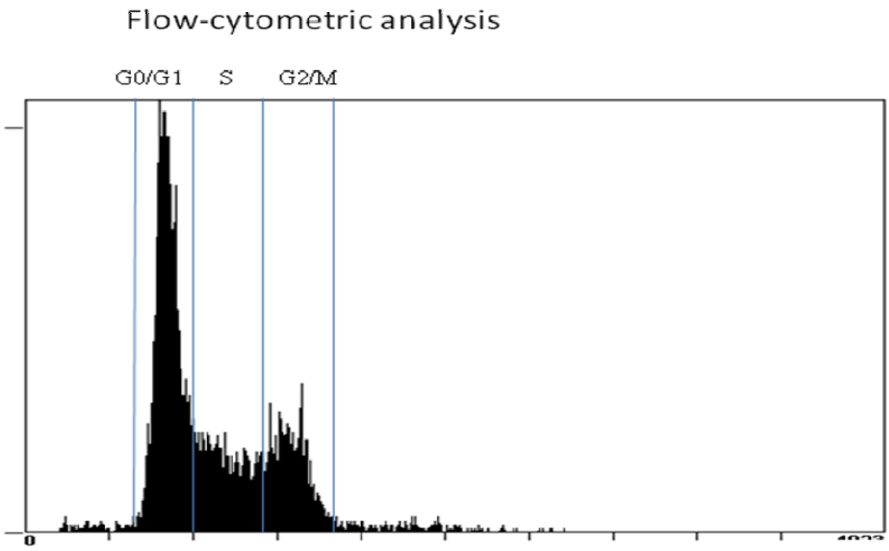

Figure 1.2: Flow-cytometric analysis

1.4 Apoptosis

Some of the same proteins that regulate the cell cycle also regulate the cells` time of death, the process of programmed cell death also called apoptosis. Apoptosis is important in many biological processes, and the result of an avoided apoptosis can be cancer. Apoptosis are triggered by cell death signals (for example tumor necrosis factor) or DNA damage (for example caused by ultraviolet light or radiation). These external signals and internal changes activate caspases, which are enzymes that mediate apoptosis. During the apoptosis the cells`

chromatin condenses and the cytoplasm shrinks. After the nucleus becomes fragmented and its DNA digested, the cell extends to numerous blebs. The small pieces of the cells, the apoptotic bodies, are ingested by phagocytic cells [5, 7].

1.5 Omega-3 and omega-6 fatty acids

Fatty acids are hydrocarbon chains that are substituted with a carboxyl group at one end. The carbon atoms are connected by either single or double bonds. Saturated fatty acids have only single bonds connecting the carbon atoms, and are fully saturated with hydrogen. Monounsaturated and polyunsaturated fatty acids (PUFAs) have one or more double bonds connecting the carbon atoms [8].

The omega-3 (n-3) and omega-6 (n-6) PUFAs are essential fatty acids, which mean that mammals cannot synthesize the n-3 and the n-6 double bond. Essential fatty acids must be consumed through diet, although short chain PUFAs can be converted to longer chain PUFAs. The n-6 fatty acid linoleic acid (LA, 18:2), which is found mostly in vegetable oils, can be converted to arachidonic acid (AA, 20:4). AA is found in meat, eggs and dairy products. In the body AA is found in phospholipids in cell membranes, and it is the precursor of eicosanoids. The n-3 fatty acid α-linoleic acid (ALA, 18:3) can be converted to eicosapentaenoic acid (EPA, 20:5) and docosahexaenoic acid (DHA, 22:6). EPA and DHA can be obtained by eating oily fish and fish oil [8].

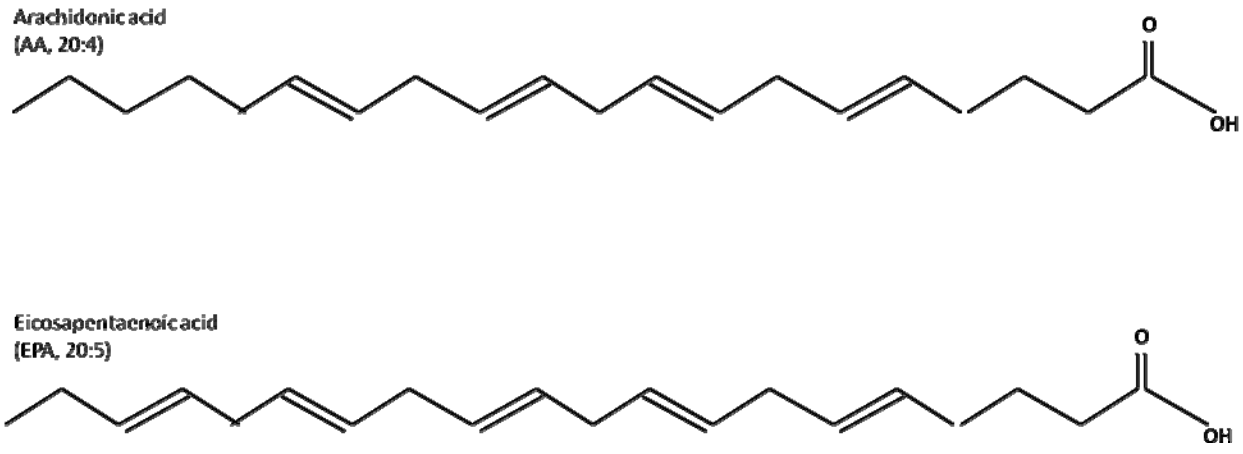

Figure 1.3

1.6 Metabolism of n-3 and n-6 fatty acids

AA, an n-6 fatty acid, can be metabolized by three major systems; cyclooxygenases (COX), lipoxygenases (LOX) and the cytochromes P450 system (CYP-450). COX oxygenates AA to prostaglandins (PGs), thromboxanes and prostacyclin, and the LOX system oxygenates AA to leukotrienes, hydroxyeicosatetraenoic acids (HETEs) and lipoxins. The CYP-450 system oxidizes AA to hydroxyl and epoxy fatty acids. EPA and DHA, n-3 fatty acids, can be metabolized by human CYP-450 families 1, 2 and 3 to epoxides, and also competitively inhibit the metabolism of AA by the CYP-450 system [9]. EPA can be oxidized at any of the five double bonds in the fatty acid structure to five regioisomeric epoxyeicosatetraenoic acids (5,6-, 8,9-, 11,12-, 14,15-, and 17,18-epoxy-EPAs) and hydroxylated to 19- and 20-OH-EPA by the CYP-450 system [9].

Further, AA-derived epoxides (EETs) are rapidly metabolized by hydration of the epoxide group to diols catalyzed by cytosolic epoxide hydrolase, or to shorter fatty acid epoxide chains by β-oxidation [10]. The conversion of EETs to the corresponding dihydroxyeicosatrienoic acids (DHETs) by epoxide hydrolase (EH), which is the main metabolic pathway, is inhibited by an epoxide hydrolase inhibitor (EHI) [10, 11], which stabilize the epoxide. The addition of N, N`-dicyclohexylurea (DCU), which is an EHI, results in an accumulation of EET in cells [10]. EPA-derived epoxides are also further metabolized by hydration of the epoxides to diols. This step is also mediated by epoxide hydrolase. The influence of inhibition of epoxide hydrolase on the amount and effect of EPA-derived epoxides is not known.

1.7 The effect of omega-3 fatty acids on cancer cells

The influence of dietary fat in breast cancer is not well understood, although epidemiologic data have indicated that n-3 fatty acid supplementation may reduce the incidence of breast cancer [12, 13]. However, it must be said that there are also studies that show no association between fish consumption and breast cancer [14, 15]. Thus, the precise details of dietary associations probably will come from findings in laboratory studies. Animal experiments have shown that n-3 PUFA have an inhibitory effect on breast cancer cells [16, 17] and other cancer cells [18]. In vitro experiments on breast cancer cells have indicated that n-3 fatty

acids inhibit proliferation [19-23]. This suggests that n-3 fatty acids may have value in nutritional therapy as a part of anti-cancer treatment regimen.

1.8 Mechanisms of the effect on cancer cell growth of n-3 and n-6 fatty acids

The n-3 fatty acids have been shown to inhibit the growth of cancer cells [19-22], while n-6 fatty acids have been shown to stimulate the growth of cancer cells [24]. Various mechanisms have been proposed to explain the effect of n-3 fatty acids and n-6 fatty acids on cancer cell growth. One proposed mechanism is that n-3 fatty acids may lower the risk of cancer through reducing the production of AA-derived eicosanoids. AA-derived and n-3 fatty acids-derived eicosanoids have different effects on cancer cells. AA-derived eicosanoids like PGE_2 have been found in higher concentrations in cancer cells compared to normal cells [25], and have been shown to have proliferative effects on cancer cells [26]. While PGE_3 produced from n-3 fatty acids have been found to inhibit proliferation of cancer cells [27]. Proliferation is the cell reproduction process. As mentioned PUFAs are metabolized to prostaglandins and thromboxanes by COX-enzymes. n-3 Fatty acids may also decrease PGE_3 production by decreasing the expression of COX and transcription nuclear factor κB (NFκB) [28]. Thus, n-3 fatty acids can reduce the proliferative effect of AA-derived eicosanoids on cancer cells by mechanisms involving decreased PGE_3 production by COX downregulation.

The potential use of epoxides formed by the action of CYPs on n-3 PUFA has not been explored. In previous studies from this laboratory it has been found that an epoxide of the n-3 PUFA EPA inhibited cell growth by decreasing proliferation. Thus, evidence has been produced that n-3 epoxides act in opposite fashion to n-6 PUFA epoxides (the EETs). EETs have been shown to inhibit the pronounced death of tumor cells (or apoptosis) and to stimulate proliferative cell growth.

Nuclear factor κB is a transcription factor that regulates genes that control cell proliferation and cell survival. Apoptosis can be blocked by NF-κB, which means that the cells do not die at the appropriate time. However, there are additional mechanisms. EETs inhibit apoptotic pathway in cells, but the mechanism is not fully understood [29]. Breast cancer cells have been shown to express high levels of functional nuclear NF-κB/Rel activity [30], and the n-3 fatty acids can decrease NF-κB activation [31]. Any involvement of CYP 450-derived

epoxides in effects on apoptosis remains to be explored. However, our laboratory have previously found that increased apoptosis by epoxy-EPA is minimal in endothelial cells. Therefore, the present study focused on the effects on cell proliferation.

In a recent report our laboratory have shown that n-3 derived eicosanoids inhibit angiogenesis [32]. Eicosanoids may also regulate the growth of new blood vessels, or angiogenesis. Angiogenesis is necessary for the cancer tumor to grow, and the inhibition of angiogenesis has been proposed to inhibit the cancer tumor growth. It has been suggested that PGE_2 can promote tumor angiogenesis [33]. Thus, treatment of human umbilical vein endothelial cells with n-3 EPA and PGE_3 inhibited the stimulatory effect of growth factor on angiogenesis. In unpublished experiment a role for CYP-450 derived epoxides was also noted. Thus, evidence suggests that n-3 PUFA and their biotransformation products may exert multiple effects on tumor cells and tumor genesis. Likely mechanisms include decreased proliferation, decreased angiogenesis and decreased metastasis (or tumor spread). The potential effects of CYP 450-derived n-3 epoxides on the proliferation of breast cancer cells, that have not previously been evaluated, are the major focus of the present project.

1.9 The MCF-7 breast cancer cell line

To study the effect of 8, 9-epoxy-eicosapentaeonic acid (8, 9-epoxy-EPA) on human breast cancer cells the MCF-7 cell line was chosen as cellular model. The MCF-7 cell line is estrogen responsive and contains the estrogen receptor [34, 35]. The cell line has been widely studied and exhibits relatively slow growth characteristics. Both EPA and DHA have been shown to have inhibitory effects on MCF-7 cell proliferation [16, 21]. In experiments AA have had a stimulatory effect on MCF-7 cells, and 8, 9-EET have been shown to have the same effect on a variety of human carcinoma cell lines [36]. The effects of EPA-derived epoxides on MCF-7 cells have not been studied to this date and will be the focus of this work.

1.10 The aim of the thesis

The aim of this project was to test the effect of 8, 9-epoxy-eicosapentaenoic acid on MCF-7 cells. Since the MCF-7 cell line is estrogen responsive, the influence of FBS on the effect of 8, 9-epoxy-EPA treatment was tested. Also the effect of the combination of 8, 9-epoxy-EPA and DCU, an epoxide hydrolase inhibitor, was assessed. The effects of 8, 9-epoxy-EPA were

assessed by examining cell viability, cell cycle progression and expression of cell cycle regulatory proteins in MCF-7 cells. After an optimization of growth effects, seeding density and FBS concentration, on MCF-7 cells, the effect of 8, 9-epoxy-EPA on cell viability was evaluated using macroscopic assessments and by counting cells manually by Trypan blue assay. To evaluate the effects of 8, 9-epoxy-EPA on cell cycle progression flow cytometric evaluation was used. Changes in the expression of cell cycle regulatory proteins were assessed by using western blotting.

2 MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals

Table 2.1: Chemicals

CHEMICALS	PRODUCER
8, 9-epoxy-eicosapentaenoic acid	Gift from Dr Sarah Cui
Acrylamide/Bis, 40% (29:1)	Amresco (Solon, Ohio, USA)
Ammonium persulphate	Univar, Aiax Chemicals (Sydney, Australia)
Aprotinin	Sigma Chemical Company (St. Louis, MO, USA)
BCA Protein Assay Reagent	Pierce (Rockford, Illinois, USA)
Blocking buffer	Rockland (Gilbertsville, PA, USA)
Bromophenol blue	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)
HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)	USB corporation (Cleveland, OH, USA)
Leupeptin	Sigma Chemical Company (St. Louis, MO, USA)
L-glutamine	Thermo Fisher Scientific (Waltham, MA, USA)

N,N`-dicyclohexylurea (DCU)	Sigma Chemical Company (St. Louis, MO, USA)
Nonidet P450 (Igepal)	Sigma Chemical Company (St. Louis, MO, USA)
Penicillin/Streptomycin	Thermo Fisher Scientific (Waltham, MA, USA)
Phosphate-buffered saline (PBS) tablets	Amresco (Solon, Ohio, USA)
PMSF (phenyl methyl sulfonyl fluoride)	Sigma Chemical Company (St. Louis, MO, USA)
Ponceau Stain	ICN biomedical (Solon, Ohio, U.S.A.)
Prestained protein molecular weight marker	Fermentas (Canada)
Propidium iodide	Chemical Company (St. Louis, MO, USA)
Ribonuclease A (Rnase A)	Chemical Company (St. Louis, MO, USA)
SDS (sodium dodecyl sulphate)	A.G. scientific inc (San Diego, CA, USA)
Sodium deoxycholate	Sigma Chemical Company (St. Louis, MO, USA)
Sodium hydrogen carbonate (NaHCO3)	Univar, APS Aiax, Finechem (Auburn, NSW, Australia)
TEMED	Amresco (Solon, Ohio, USA)
Thiazolyl blue tetrazolium bromide (MTT)	Chemical Company (St. Louis, MO, USA)
Tris	Amresco (Solon, Ohio, USA)
Trypan blue stain	Invitrogen (Mount Waverley, VIC, Australia)
Trypsin/EDTA	Thermo Fisher Scientific (Waltham, MA, USA)
Tween-20	Amresco (Solon, Ohio, USA)

2.1.2 Solutions for cell culture

Table 2.2

Plain media, 900 ml	
Dulbecco's modified eagle medium (DMEM)	9.87 g
Sodium hydrogen carbonate	1.2 g
HEPES	5.21 g
Baxter sterile water	Ad 900 ml

Table 2.3

Media with 10% FBS, 255 ml	
Plain media	220 ml
10% FBS	25 ml
1% L-glutamate	2.5 ml
1% Pencillin/Streptomycin Antibiotic Mix	2.5 ml

Table 2.4

Media with 2% FBS, 260 ml	
Plain media	250 ml
2% FBS	5 ml
1% L-glutamate	2.5 ml
1% Pencillin/Streptomycin Antibiotic Mix	2.5 ml

Table 2.5

Media without FBS, 225 ml	
Plain media	220 ml
1% L-glutamate	2.5 ml
1% Pencillin/Streptomycin Antibiotic Mix	2.5 ml

Table 2.6

Freezing media	
Media with 10% FBS	3.78 ml
20% FBS	1.08 ml
10% DMSO	0.54 ml

Table 2.7

Phosphate-buffered saline (PBS), pH7.4	
Phosphate-buffered saline (PBS) tablets	1 tablet
Baxter sterile water	100 ml

2.1.3 Solutions for flow cytometry

Table 2.8

Incubation buffer, 10 ml	
PBS	9.8 ml
Nonidet P450 (Igepal)	0.1 ml
Rnase A	0.1 ml

Table 2.9

Propidium iodide, 2 mg/ml	
Propidium idodie	10 mg
PBS	5 ml

2.1.4 Buffers for western blotting

Table 2.10

5x Electrophoresis buffer, pH 8.3	
Tris	15.0 g
Glycine	72.0 g
SDS	5.0 g
Deionized water	ad 1 L

Table 2.11

1x Electrophoresis buffer, pH 8.3	
Electrophoresis buffer 5x Tris-glycine pH 8.3 stock	100 ml
Deionized water	400 ml

Table 2.12

Sample (reducing) buffer	
1 M Tris pH 6.8 stock	25.0 ml
Glycerol	23.0 ml
10% SDS	40.0 ml
0.1% Bromophenol blue	2.0 ml
Deionized water	90 ml
0.5 M DTT	0.5 ml

Table 2.13

Transfer buffer, 2 L	
Tris	6.06 g
Glycine	28.8 g
Methanol	400.0 ml
Deionized water	Ad 2L

Table 2.14

10x TBS , 2 L	
Tris	121.0 g
NaCl	233.8 g
Adjust pH to 7.4 using concentrated HCl	
Deionized water	ad 2L

Table 2.15

1x TBS/Tween	
10x TBS	100 ml
Deionized water	900 ml
Tween-20	0.5 ml

Table 2.16

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

2.1.5 Gels for western blotting

Table 2.17

12 % separating gel, 2x1.5 mm gel	
Deionized water	11.4 ml
40% acrylamide/Bis	9 ml
2.5 M TrisHCl pH 8.8	9 ml
10% (w/v) SDS	150 ul
Ammonium persulphate (0.1g/ml)	300 ul
TEMED	32 ul

Table 2.18

5 % stacking gel, 2x1.5 mm gel	
Deionized water	9.8 ml
40% Acrylamide/Bis	2 ml
2.5 M TrisHCl pH 6.8	4 ml
10% (w/v) SDS	160 ul
Ammonium persulphate (0.1g/ml)	80 ul
TEMED	16 ul

Table 2.19

0.5 M Tris, pH 6.8	
Tris	6.0 g
Adjust pH to 6.8 with concentrated HCl	
Deionized water	ad 100 ml

Table 2.20

1.5 M Tris, pH 8.8 stock	
Tris	18.15 g
Adjust pH to 8.8 with concentrated HCl	
Deionized water	ad 100 ml

2.1.6 Equipment

Table 2.21

Equipment	**Manufacturer**
Allegra 6R Centrifuge	Beckman
Altra 20 soft Imaging system CKX41	Olympus
Biorad GS-800 Calibrated Densitometer	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. Cells and cell culture

2.2.1 Cell culture

MCF-7 cells isolated from a 69 years old Caucasian female were used [34]. Cells were maintained in monolayer culture in T75 flasks at 37^{o}C in DMEM containing 100 units/ml

penicillin, 100 µg/ml streptomycin, 1% L-glutamine and 10% fetal bovine serum (FBS) in an atmosphere of 95% air and 5% CO_2. All cell culture work was carried out on an aseptic LAF-bench, and all equipment used in direct contact with cells was sterilized or autoclaved.

2.2.2 Passaging MCF-7 cells

Cells were passaged when they were 90% confluent, about every fourth day. Media was removed by aspiration, and 10 ml of PBS were added to wash the media from the cells. To detach cells 2 ml of trypsin was added and cells were incubated at 37^{o}C for 5 minutes. The T75 flask was tapped gently against the palm to detach cells, and cells were checked to be detached under the microscope. Cells were transferred to a centrifuge tube by adding 10 ml DMEM, and centrifuged at 1250 rpm (Allegra 6R Centrifuge, Beckman) for 5 minutes. The supernatant was removed, and the tube was "flipped" to separate cells. Cells were resuspended in 10 ml of DMEM. After the resuspension half of the cell stock was transferred into a T75 flask containing 25 ml DMEM. Media, PBS and trypsin was heated to 37^{o}C before addition.

2.2.3 Thawing MCF-cells

10 ml of DMEM containing 10% FBS was added in a centrifuge tube. Cells kept in cryotubes in liquid nitrogen were quickly warmed in a water bath at 37^{o}C. DMEM containing 10% FBS was added, and cells were transferred to a centrifuge tube. Cells were centrifuged at 1250 rpm (Allegra 6R Centrifuge, Beckman) for 5 minutes, and the supernatant was removed by aspiration. The centrifuge tube was flipped to separate cells, and cells were resuspended in10 ml of DMEM. The cell stock was transferred into a T75 flask containing 25 ml DMEM.

2.3 Treatment with 8, 9-epoxy-EPA

In the experiments MCF-7 cells were treated with 10 µM 8, 9-epoxy-EPA dissolved in DMSO, and cells added DMSO was used as control cells. Cells were also treated with a mixture of 10 µM 8, 9-epoxy-EPA + 10 µM DCU, and cells treated with 10 µM DCU was used as control cells.

2.4 MCF-7 cell count

To prepare a cell suspension with the appropriate cell concentration for seeding, cells were counted using a Hemacytometer. A hemacytometer is a graduated counting chamber for determining the concentration of cells in a suspension. Approximately 10 µl of the cell suspension were loaded in both chambers. Cells were viewed under a microscope at 100x magnification, and cells contained within 1 mm^2 areas on both side were counted [37]. The total cell number was calculated as follows:

Cells/ml = Total cells counted in 2 mm^2 x 10^4 / Number of mm^2

Total number of cell = Cells/ml x Total volume of cell suspension (10 ml)

To determine the effect of 8, 9-epoxy-EPA on MCF-7 cell viability, cells were counted using a Hematocytometer. Cells were harvested, centrifuged at 1250 rpm (Eppendorf Centrifuge 5417 R, Crown Scientific) at 4°C for 5-10 minutes and resuspended in 1 ml DMEM. To determine the number of viable cells the cell suspension was diluted 1:1 with Trypan blue stain. The non-viable cells appeared as blue, while the viable cells were clear. The number of viable cells in 2 mm^2 was counted and the number of viable cells was calculated as follows:

Cells/ mm^2 = Viable cells counted in 2 mm^2 / Number of mm^2

Cells/ml = Cells/mm^2 x 10^4 / Dilution (1/2)

Total cells = Cells/ml x Total volume of cell suspension (1 ml)

2.5 Seeding of MCF-7 cells

Cells were seeded in 24-well plates at cell density $1x10^5$ or $2x10^5$ cells/well/ml. After 24 hours in media containing 10% FBS the media was changed to media containing the appropriate FBS concentration, which was 2% FBS or 0% FBS. Cells grown in media containing 2% or 0% FBS was given media containing the test compounds after 24 hours. Cells were treated for 24 and 48 hours, and media was replaced with fresh media every 24 hours.

2.6 Harvesting MCF-7cells.

Media was removed, and cells were washed with 1 ml/well PBS. 0.2 ml/well trypsin was added, and cells were incubated at 37°C for 10 minutes. The 24-well plates were tapped carefully to detach cells from the plate. Cells were harvested using 2x 0.5 ml DMEM, and transferred to 1.5 ml eppendorf tubes for counting, fixing or freezing.

2.7 MTT-assay

MTT-assay was used to determine the effect of seeding density and serum on MCF-7 cell growth. MTT-assay is a rapid and convenient method for determining viable cell number. Cells seeded in 24-well plates were added 125 µl/well of 2.5 mg/ml MTT-stock (thiazolyl blue tetrazolium bromide), and incubated at 37 °C for 2 hours. Active mitochondrial reductase enzymes in viable cells reduce the tetrazolium salt of the dye solution into a formazan product. Media with MTT-solution were removed and 0.2 ml/well of DMSO were added to dissolve the blue crystals that were formed. Plates were protected from light, and shaken for 30 minutes on a rotating platform. 100 µl of sample were transferred into 96-well plate, and the plate was measured at 540 nm to detect the formazan product using a Victor3 Wallac 1420 multilabel counter (Perkin, Elmer). The absorbance reading at 540 nm is directly proportional to the number of viable cells [38].

2.8 Flow cytometry

Flow cytometry is a method that can be used to quantify cells present in the different phases of the cell cycle. After harvesting cells were centrifuged at 1250 rpm (Eppendorf Centrifuge 5417 R, Crown Scientific) at 4°C for 10 minutes, and washed with 0.5 ml ice-cold PBS and resuspended. Cells were then centrifuged at 1250 rpm (Eppendorf Centrifuge 5417 R, Crown Scientific) at 4°C for 10 minutes. The supernatant was removed, the eppendorf tube was "flipped", and the cell pellet was washed with 0.5 ml PBS. The eppendorf tube was centrifuged at 1250 rpm (Eppendorf Centrifuge 5417 R, Crown Scientific) at 4°C for 10 minutes, and the supernatant was removed. 0.5 ml of pre-cooled 80% ethanol was added, and the cells were resuspended. Cells were stored in the -20 °C freezer for at least one hour, and then centrifuged at 1250 rpm (Eppendorf Centrifuge 5417 R, Crown Scientific) at 4°C for 15 minutes, and the ethanol was removed. 0.5 ml of incubation buffer was added, and cells were

resuspended. 12.5 µl of propidium iodide was added, and eppendorf tubes were vortexed. After 1 hour on ice cell samples were analyzed using a Coulter flow cytometer (Beckman).

2.9 Quantification of total lysate protein

Cell samples for western blotting was added the appropriate volume of lysis buffer, and the lysate protein content was determined by the Bicinchoninic-assay (BCA-assay). The absorbance at 540 nm was measured using a Victor3 Wallac multilabel counter (Perkin Elmer). A BCA standard curve was used to determine the total lysate protein content in cell samples (see Appendix, 6.1 Procedure for BCA-assay).

2.10 Western blotting

The expression of different cell cycle regulatory proteins in differently treated cells was measured by western blotting.

Protein lysate samples stored in the -80°C freezer were thawed. The appropriate amount, determined by quantification of total protein lysate (see Appendix, 6.1 Procedure for BCA-assay), was transferred to an eppendorf tube. Deionized water was added to adjust volumes. The samples were diluted 1:1 with sample buffer, and boiled at 100°C for 5 minutes, and then centrifuged for 1-2 minutes. The samples and the marker (Prestained protein molecular weight marker) were applied to the gel (see Appendix, 6.2 Procedure for western blotting). Gel electrophoresis, blotting and development of the blot were performed as described in Appendix, 6.2 Procedure for western blotting. The primary and secondary antibodies (Santa Cruz biotechnology, Santa Cruz, CA, USA) used in development are listed in table 2.22. Blots were scanned using an Odyssey LI-COR instrument.

Table 2.22

Primary antibody	**Dilution of primary antibody**	**Secondary antibody**	**Dilution of Secondary antibody**
Actin	1:500 1.200	Anti-mouse	1:15 000
CDK 4	1:500	Anti-mouse	1:15 000
CDK 6	1:1000	Anti-rabbit	1:15 000
Cyclin A	1:1000	Anti-rabbit	1:15 000
Cyclin B1	1:1000	Anti-mouse	1:15 000
Cyclin D1	1:500 1:200 1:150	Anti-mouse	1:15 000
Cyclin E	1:500	Anti-mouse	1:15 000

2.11 Statistical analysis

Data from experiments were analyzed by using the statistical program Statview, ANOVA/t-test.

3 RESULTS

3.1 Effect of seeding density and serum on MCF-7 cell growth

The effect of seeding density and serum (FBS) on MCF-7 cell growth was determined in preliminary experiments. Cells were seeded in 24-well plates, and were treated with 8, 9-epoxy-eicosapentaenoic acid (8, 9-epoxy-EPA; 10 μM). From previous experience in this laboratory the seeding density was tested in the range 2-4.8x10^5cells/ml. The lowest possible FBS concentration was considered desirable, because of its potent stimulatory effect on cell growth. The FBS range from 0% to 10% was tested. The effects of seeding density and serum were assessed by MTT-assay.

In figure 3.1 MCF-7 cells were seeded at 2x10^5 cells/well, 4x10^5 cells/well and 4.8x10^5 cells/well in media containing 0%, 5% and 10% FCS. Confluences at the seeding density of 2x10^5 cells/well in media containing 0%, 5% and 10% FBS were 20-30%, 40-50% and 50-60% at 24 hr, respectively. Cells that were seeded at 4x10^5 /well and 4.8x10^5 /well were fully confluent at 24 hr, therefore concentrations lower than 2x10^5 cells/ml would be most suitable. Cell growth was stimulated at concentrations higher than 0% of FBS, as expected (figure 3.1). As a result of these experiments 1x10^5 cells/ml were used in subsequent experiments. Thus, cells were seeded at 1x10^5 and 2x10^5 cells/well in media containing 0%, 2% and 5% FBS, and MTT-assay was performed at 0, 24, 48 and 72 hr. In serum-free media cell growth stopped after 24 hours (figure 3.2). At a seeding density of 1x10^5 cells/ml an increase of 67.2% (2% FBS) and 61.5% (5% FBS) was noted at 24 hr compared to 0 hr. At 48 hr the increases were 135.9% (2% FBS) and 142.8 % (5% FBS) compared control, and at 72 hr were 220.9% (2% FBS) and 214.0 % (5% FBS), respectively. Cells seeded at 2x10^5 cells/well were increased by 34.0% (2% FBS) and 37.5 (5% FBS) at 24 hr compared control at 0 hr. At 48 hr increases of 54.3% (2% FBS) and 57.8% (5% FBS) compared control, and at 72 hr to102.9% (2% FCS) and 74.9 % (5% FCS) compared control. Minimal differences in cell growth were observed between cells grown in the presence 2% and 5% FBS at both seeding densities. Two percent FBS concentration in media was selected for the 8, 9-epoxy-EPA experiments in MCF-cells (density 1x10^5 cells/ml).

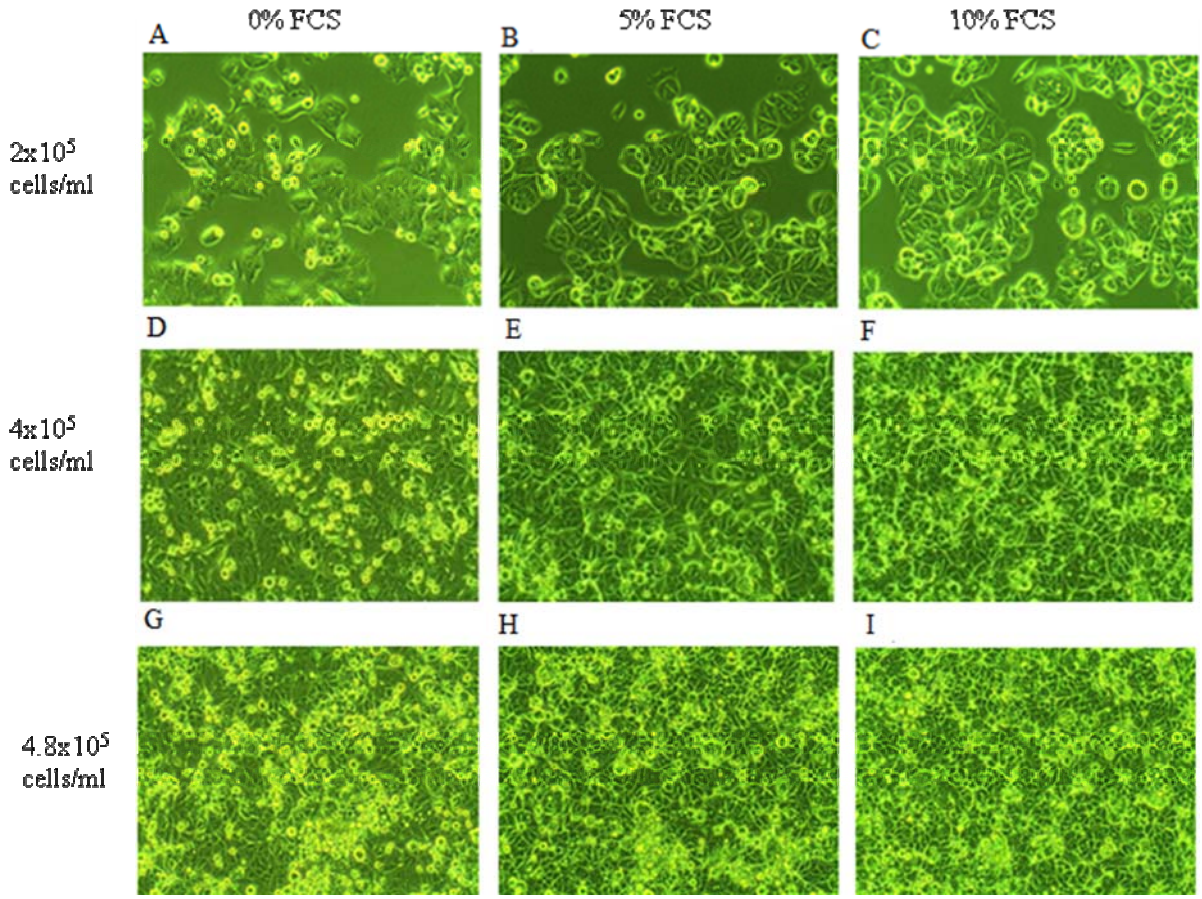

Figure 3.1: Effect of seeding density and serum on MCF-7 cell growth at 24 hr in 24-wellplates. Cells were seeded at $2x10^5$ cells/well in media containing 0% (A), 5% (B) and 10% FBS (C) or at $4x10^5$ cells/well in media containing 0% (D), 5% (E) and 10% FBS (F). Further cells were seeded at $4.8x10^5$ cells/well in media containing 0% (G), 5% (H) and 10% FBS (I). The confluence at seeding density 2x10^5 cells/well in media containing 0% FBS was 20-30%, 40-50% at 5% FBS and 50-60% at 10% FBS.

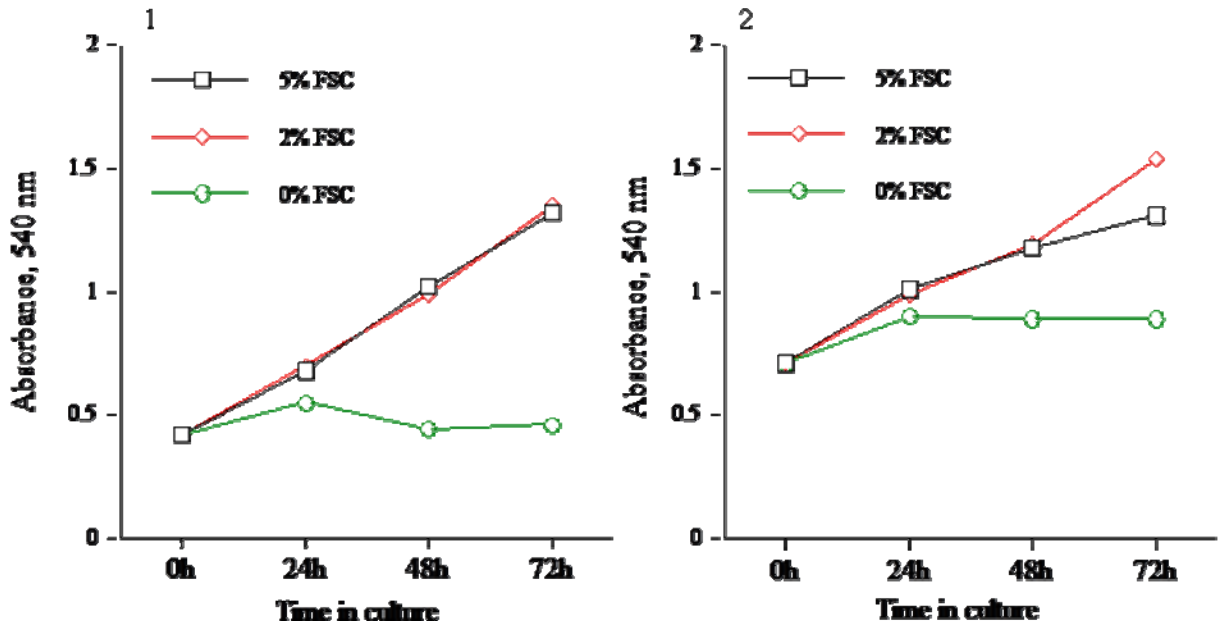

Figure 3.2: Effect of seeding density and serum on MCF-7 cell growth measured by MTT-assay. MCF-7 cells were seeded in 24-well plates at densities of $1x10^5$ (1) and $2x10^5$ (2) cells/well and in media containing 0%, 2% and 5% FBS. MTT assays were conducted at 0, 24, 48 and 72 hr. Data are expressed as means ± SE from two independent experiments. 1: At 24 hr the mean percentage compared to measurement at 0 hr are increased 31.8% (0% FBS), 67.2% (2% FBS) and 61.5% (5% FBS). At 48 hr the mean percentage compared to 0 hr is increased 5.0% (0% FBS), 135.9% (2% FBS) and 142.8 % (5% FBS). At 72 hr the mean percentage compared to 0 hr are increased 9.0% (0% FBS), 220.9% (2% FBS) and 214.0% (5% FBS).
2: At 24 hr the mean percentage compared 0 hr is increased 23.5% (0% FBS), 34.0% (2% FBS) and 37.5% (5% FBS). At 48 hr the mean percentage compared to 0 hr are increased 22.3% (0% FBS), 54.3% (2% FBS) and 57.8 % (5% FBS). At 72 hr the mean percentage compared to 0 hr is increased 22.5% (0% FBS), 102.9% (2% FBS) and 74.9% (5% FBS).

3.2 Effects of 8, 9-epoxy-EPA on cell viability of MCF-7 cells

The effect of 8, 9-epoxy-EPA on MCF-7 cell viability was assessed after 24 hours of treatment. The number of viable cells was manually counted using the Trypan blue assay.

3.2.1 Effects of 8, 9-epoxy-EPA on viability of MCF-7 cells grown in presence of FBS

MCF-7 cells were seeded at $1x10^5$cells/well in 24-well plates and exposed to 8, 9-epoxy-EPA (10µM), DCU (10µM) or the combination of 8, 9-epoxy-EPA (10 µM) + DCU (10 µM).

From figure 3.3 the confluences for cells treated with 8,9-epoxy-EPA and 8, 9-epoxy-EPA+DCU are significantly lower than for control cells (DMSO) and DCU-treated cells after 24 hr. 8, 9-Epoxy-EPA (10 µM) decreased viable cells by 21.5% ± 6.3% compared to control (figure 3.4 ; p=0.03). The combination of 8, 9-epoxy-EPA (10µM) and DCU (10µM) decreased viable cells by 26.3% ± 8.9% compared to control (p=0.03).

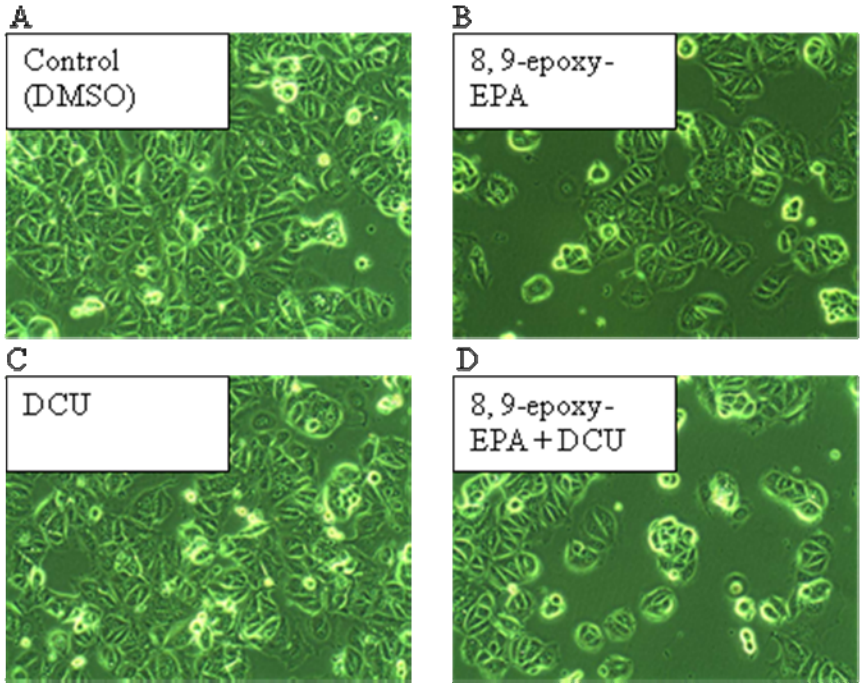

Figure 3.3: Growth inhibition of MCF-7 cells by 8, 9-epoxy-EPA. $1x10^5$cells/well were seeded in 24-well plates and cultured in media containing 2% FBS for 24 hr. Treatment consist of DMSO (A, control), 8, 9-epoxy-EPA (B), DCU (C) and 8, 9-epoxy-EPA + DCU (D). The confluences at 24 hr are approximately 80% (A, control), 20-30% (B, 8, 9-epoxy-EPA), 70-80% (C, DCU) and 20-30% (D, 8, 9–epoxy-EPA + DCU).

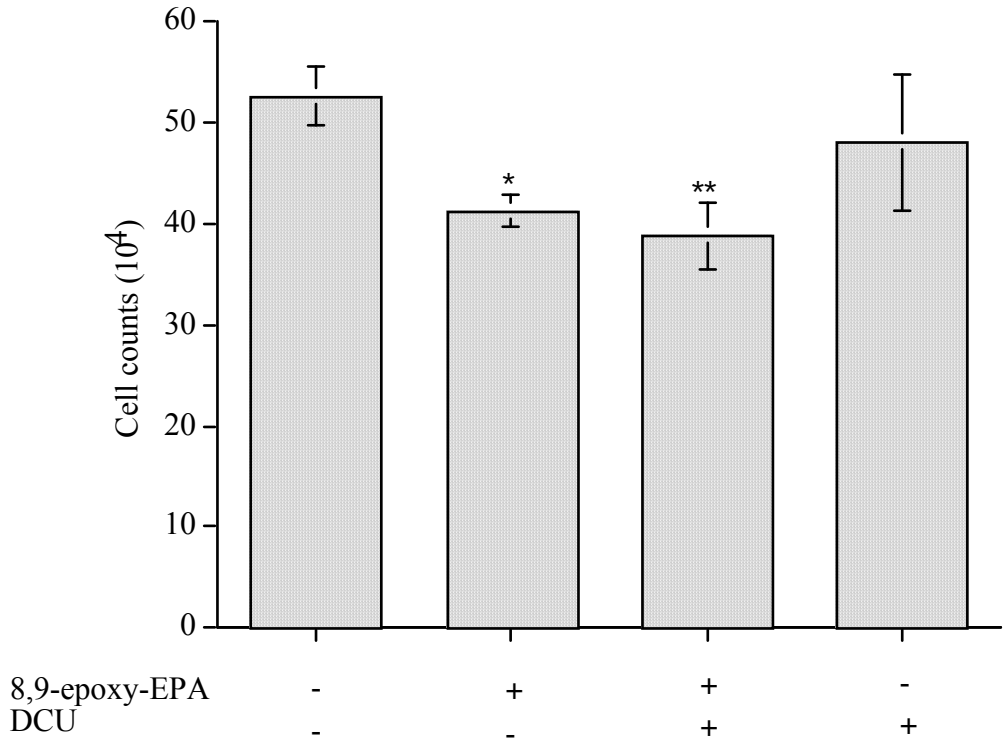

Figure 3.4: Effect of 8, 9-epoxy-EPA on the growth of MCF-7 cells. 1x10^5 cells/well were seeded in 24-well plates, to which DMSO (control), 8, 9-epoxy-EPA or DCU were added in media containing 2% FCS. Viable cell numbers were counted by Trypan blue assay at 24 hr. Data are expressed as means ± SE from three independent experiments. *Viable cell number decreased 21.2% ± 6.3 % for 8, 9-epoxy-EPA-treated cells compared to control cells (DMSO) (p = 0.03).**Viable cell number decreased 26.2% ± 8.9% for cells treated with 8, 9-epoxy-EPA+DCU compared to control cells (DMSO) (p = 0.03).

3.2.2 Effects of 8, 9-epoxy-EPA on viability of MCF-7 cells cultured in the absence of FBS

To determine whether the effect of 8, 9-epoxy-EPA on MCF-7 cells was dependent on the presence of FBS, an experiment with serum-free media was carried out. MCF-7 cells were seeded at $2x10^5$ cells/well in 24-well plates to which 8, 9-epoxy-EPA (10 μM), DCU (10 μM) or both 8, 9-epoxy-EPA (10 μM) + DCU (10μM) were added. Viable cells were counted by Trypan blue assay. 8, 9-epoxy-EPA-treatment did not alter viable cell number after 24 hours compared to control cells or DCU-treated cells (figure 3.5). Thus, it emerges that the effect of 8, 9-epoxy-EPA is dependent on the presence of serum. However, the combined effect of 8, 9-epoxy-EPA+DCU showed a trend toward a decrease. It is feasible that serum may have protected the epoxide against enzymic hydrolysis in the absence of DCU.

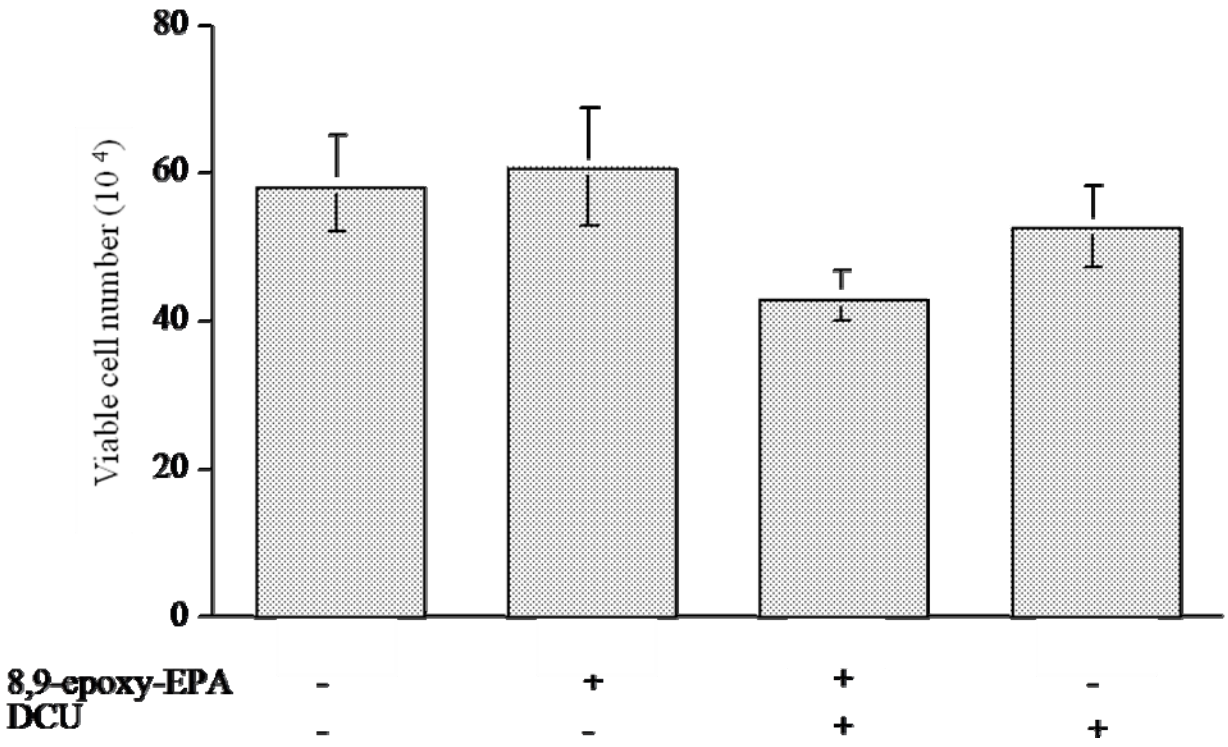

Figure 3.5: Effect of 8, 9-epoxy-EPA on the viability of MCF-7 cells. $2x10^5$ cells/well were seeded in 24-well plates, to which DMSO (control), 8, 9-epoxy-EPA, DCU or both were added in serum-free media. Viable cell numbers were counted by Trypan blue assay at 24 hr. Data are expressed as means ± SE from three independent experiments. The number of viable cells did not decrease significantly for 8, 9-epoxy-EPA-treated cells compared to control cells (DMSO) and DCU-treated cells.

3.3. Effects of 8, 9-epoxy-EPA on cell cycle progression in MCF-7 cells

To investigate the effects of 8, 9-epoxy-EPA on MCF-7 cell growth, we used flow cytometry and monitored cell cycle progression. MCF-cells were treated with 8, 9-epoxy-EPA for 6, 16 and 24 hours, and DNA was stained with propidium iodide. The cells were analyzed by flow cytometry for quantification of cells present in G0/G1, S and G2/M phase.

MCF-7 cells treated for 6 hours with 8, 9-epoxy-EPA, DCU and a combination of 8, 9–epoxy-EPA + DCU in presence and in absence of FBS did not show a significant change in the cell cycle progression compared with control (table 3.1a). The exception was the significant increase in the % of cells in G2/M-phase for cells grown in the absence of FBS and treated with a combination of 8, 9-epoxy-EPA +DCU, compared to control.

After 16 hours of treatment with 8, 9-epoxy-EPA in presence of FBS a significant 5.7%±1.2% (p=0.01) increase in % of cells in G0/G1 phase compared to control was seen (table 3.1b). The combination of 8, 9-epoxy-EPA + DCU increased the % of cells in G0/G1-phase by 8.3% ± 1.5% (p=0.002) compared to control, and cells treated with DCU increased by 8.7% ± 1.6% (p=0.002) compared to control. Cells grown in the absence of FBS and treated with 8, 9-epoxy-EPA + DCU showed a significant 27.4% ± 0.6% (p=0.01) decrease in % of cells in S-phase compared to cells treated with DCU (figure 3.1b).

24 hours of 8, 9-Epoxy-EPA-treatment in the presence of FBS increased the proportion of MCF-7 cells in G0/G1-phase by 3.8% ± 0.8% over control (p=0.002). Whereas 8, 9-epoxy-EPA+DCU increased the cells in G0/G1-phase by 6.0% ± 0.5 over control (p<0.0001) and 3.5% ± 0.7% over cells treated with DCU (p=0.003). A corresponding decrease in the proportion of cells in G2/M-phase for 8, 9-epoxy-EPA and 8, 9-epoxy-EPA+DCU was observed, as shown in table 3.1c. Figure 3.1c also shows that, in cells treated in the absence of FBS, the cell cycle progression did not change.

Table 3.1a: Effects of 8, 9-epoxy-EPA on cell cycle progression in MCF-7 cells. Flow cytometry analysis of MCF-7 cells treated with DMSO (control), 8, 9-epoxy-EPA, 8, 9-epoxy-EPA + DCU and DCU for 6 hours for G0/G1, S and G2/M phase quantification**.** Data are expressed as mean percentage of total cells ± SE from three independent experiments.

6 hours treatment						
	% of cells in G0/G1-phase		**% of cells in S-phase**		**% of cells in G2/M-phase**	
	2 % FCS	**0 % FCS**	**2 % FCS**	**0 % FCS**	**2 % FCS**	**0 % FCS**
Control	66.7 ± 0.5	89.0 ± 0.4	18.8 ± 0.7	6.1 ±0.4	13.5 ± 0.7	4.8 ± 0.9
8, 9-epoxy-EPA	66.0 ± 0.4	86.7 ± 0.9	19.6 ± 1.1	6.1 ± 0.1	12.7 ± 1.1	6.1 ± 0.4
8, 9-epoxy-EPA + DCU	65.1 ± 1.1	85.8 ± 0.8	19.4 ± 0.4	6.4 ± 0.8	13.5 ± 1.0	6.8 ± 0.2 *
DCU	66.3 ± 0.6	85.3 ± 1.4	19.0 ± 0.3	7.1 ± 1.3	13.2 ±0.7	6.4 ± 0.1

*Significant 41.9% ± 1.1% (p=0.02) increase of cells treated with 8, 9-epoxy-EPA +DCU in % of cells in G2/M-phase compared to control.

Table 3.1b: Effects of 8, 9-epoxy-EPA on cell cycle progression in MCF-7 cells treated for 16 hours. Flow cytometry analysis of MCF-7 cells treated with DMSO (control), 8, 9-epoxy-EPA, 8, 9-epoxy-EPA + DCU and DCU for 16 hr for G0/G1, S and G2/M phase quantification**.** Data are expressed as mean percentage of total cells ± SE from three independent experiments.

16 hours treatment						
	% of cells in G0/G1-phase		% of cells in S-phase		% of cells in G2/M-phase	
	2 % FCS	0 % FCS	2 % FCS	0 % FCS	2 % FCS	0 % FCS
Control	61.5 ± 0.6	85.0 ± 1.7	23.2 ± 1.7	7.0 ±0.6	13.2 ± 1.2	6.8 ± 1.1
8, 9-epoxy-EPA	65.0 ± 0.6 *	84.5 ± 1.1	19.7 ± 1.4	6.8 ± 0.7	12.4 ± 1.0	7.5 ± 0.4
8, 9-epoxy-EPA + DCU	66.7 ± 0.9 *	85.4 ± 1.0	20.1 ± 1.4	6.0 ± 0.3 #	12.0 ± 1.4	7.2 ± 0.1
DCU	66.8 ± 1.0 *	82.4 ± 0.1	19.2 ± 1.1	8.3 ± 0.3	12.0 ± 1.0	8.0 ±0.2

*Significant increase in % of cells in G1 phase compared to control; 5.7% ± 1.2% (8, 9-epoxy-EPA, p=0.01), 8.3% ± 1.5% (8, 9-epoxy-EPA + DCU, p=0.002), 8.7% ± 1.6% (DCU, p=0.002).
#Significant 27.4% ± 0.6% (p=0.01) decrease of cells treated with 8, 9-epoxy-EPA +DCU in % of cells in S-phase compared to cells treated with DCU.

Table 3.1c: Effects of 8, 9-epoxy-EPA on cell cycle progression in MCF-7 cells treated for 24 hours. Flow cytometry analysis of MCF-7 cells treated with DMSO (control), 8, 9-epoxy-EPA, 8, 9-epoxy-EPA + DCU and DCU for 24 hr for G0/G1, S and G2/M phase quantification**.** Data are expressed as mean percentage of total cells ± SE from three independent experiments.

24 hours treatment						
	% of cells in G0/G1-phase		% of cells in S-phase		% of cells in G2/M-phase	
	2 % FCS	0 % FCS	2 % FCS	0 % FCS	2 % FCS	0 % FCS
Control	61.7±0.2	83.2 ± 1.7	17.3±1.5	7.4 ± 0.7	17.0±1.1	7.5 ± 0.7
8, 9-epoxy-EPA	64.1±0.6 *	84.5 ± 1.6	18.9±0.8	7.3 ± 0.6	13.2±0.6 #	6.1 ± 0.6
8, 9-epoxy-EPA + DCU	65.4±0.3 *	84.6 ± 0.8	18.1±0.8	7.2 ± 0.5	12.7±0.5 #	7.0 ± 1.0
DCU	63.2±0.4*	83.2 ± 1.4	17.9±0.5	7.3 ± 0.9	14.7±0.5 #	8.0 ± 0.5

*Significant increase in % of cells in G0/G1-phase compared to control; 3.9% ± 0.8% (8, 9-epoxy-EPA, p=0.002), 6.0% ± 0.5% (8, 9-epoxy-EPA+DCU, p<0.0001), 2.4% ± 0.6% (DCU, p=0.04). Cells treated with 8, 9-epoxy-EPA was 3.5% ± 0.7% (p=0.003) increased in % of cells in G0/G1-phase compared to DCU.
#Significant decrease in % of cells in G2/M-phase compared to control: 22.6% ± 1.7% (8, 9-epoxy-EPA, p=0.0001), 25.6% ± 1.6% (8, 9-epoxy-EPA+DCU, p=0.0004), 13.8% ± 1.6% (DCU, p=0.03). Cells treated with 8, 9-epoxy-EPA were 13.7% ± 0.9% (p=0.047) lower in G2/M-phase compared to DCU.

3.4 Cell cycle protein expression in 8, 9-epoxy-EPA-treated MCF-7 cells

Since clear effects of 8, 9-epoxy-EPA on MCF-7 cell cycle progression were observed, we examined changes in expression of different cell-cycle regulatory proteins after treatment with 8,9-epoxy-EPA and 8, 9-epoxy-EPA + DCU.

3.4.1 Expression of different proteins in 8, 9-epoxy-EPA-treated MCF-7 cells

MCF-7 cells were treated with 8, 9-epoxy-EPA for 24 hours and then harvested for western blotting. The protein levels of cyclin D1, cyclin E, CDK4, CDK6 and p21 (figure 3.6) were found to be unaltered after 8, 9-epoxy-EPA treatment, as shown in figure 3.7. Actin was used as loading control.

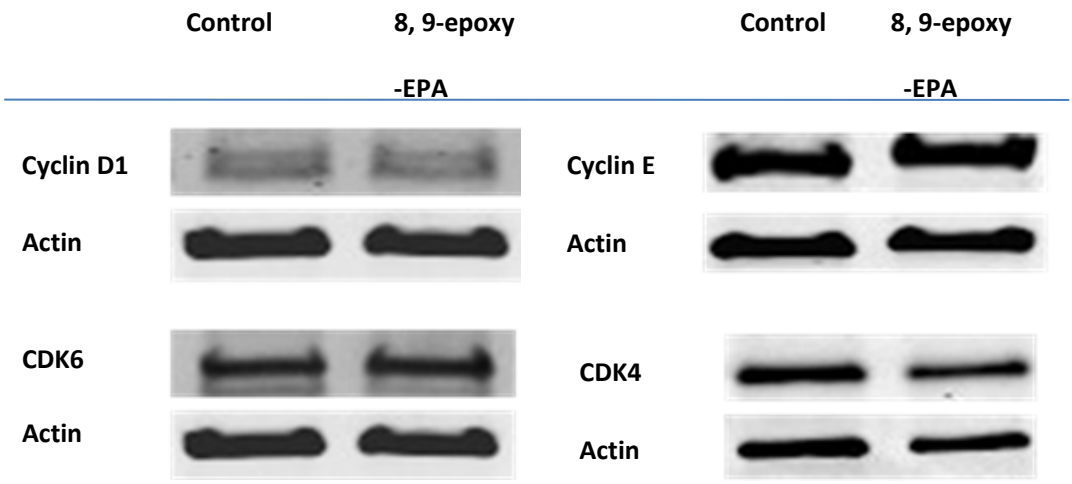

Figure 3.6: Effect of 8, 9-epoxy-EPA on cyclin D1, CDK6, cyclin E, CDK4 and p21 protein expression. MCF-7 cells were treated with 8, 9-epoxy-EPA and harvested at 24 hr. The relative protein levels of cyclin D1, CDK6, cyclin E, CDK4 and p21 were determined by western blot analysis.

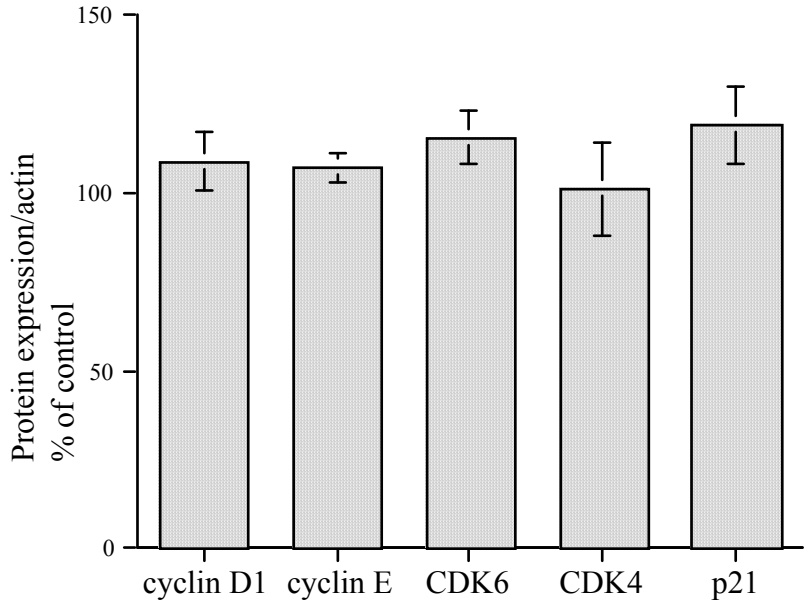

Figure 3.7: Relative expression of cell cycle regulatory proteins in 8, 9-epoxy-EPA-treated MCF-7 cells at 24 hr. Data are expressed as means ± SE from three independent experiments.

3.4.2 Cyclin D1 expression in 8, 9-epoxy-EPA-treated MCF-7 cells at 6, 16 and 24 hr

At our laboratory a decreased expression of cyclin D1 was observed in murine endothelial cells after n-3 EPA treatment, and the effect on cyclin D1 was also observed at earlier timepoints (at 2, 6 and 16 hr). Since the expression of different cell-cycle regulatory proteins at 24 hr did not account for the effects of 8, 9-epoxy-EPA observed on the cell cycle, we examined the expression of cyclin D1 at 6, 16 and 24 hr (the 24 hr timepoint is shown in figure 3.8). In this experiment the combined effect of epoxide + DCU was assessed. However, expression of cyclin D1 was unaltered after either 8, 9-epoxy-EPA or combined 8, 9-epoxy-EPA+DCU treatment for 6, 16 and 24 hr (figure 3.9 A-C). Thus, despite the growth inhibition effects of the epoxide and the relative accumulation of cells in G0/G1 phase, no clear evidence of cyclin D1 dysregulation was observed. It is possible that cyclin D1 dysregulation occurred at a very early timepoint after epoxide addition or that another regulatory cyclin may have been affected by epoxy-EPA.

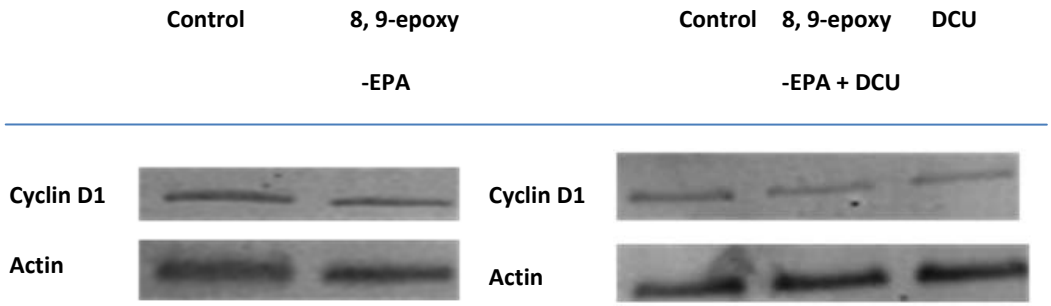

Figure 3.8: Effect of 8, 9-epoxy-EPA on cyclin D1expression. MCF-7 cells were treated with 8, 9-epoxy-EPA, DCU and the combination of 8, 9-epoxy-EPA+DCU and harvested at 24 hr. The relative protein level of cyclin D1 was determined by western blot analysis.

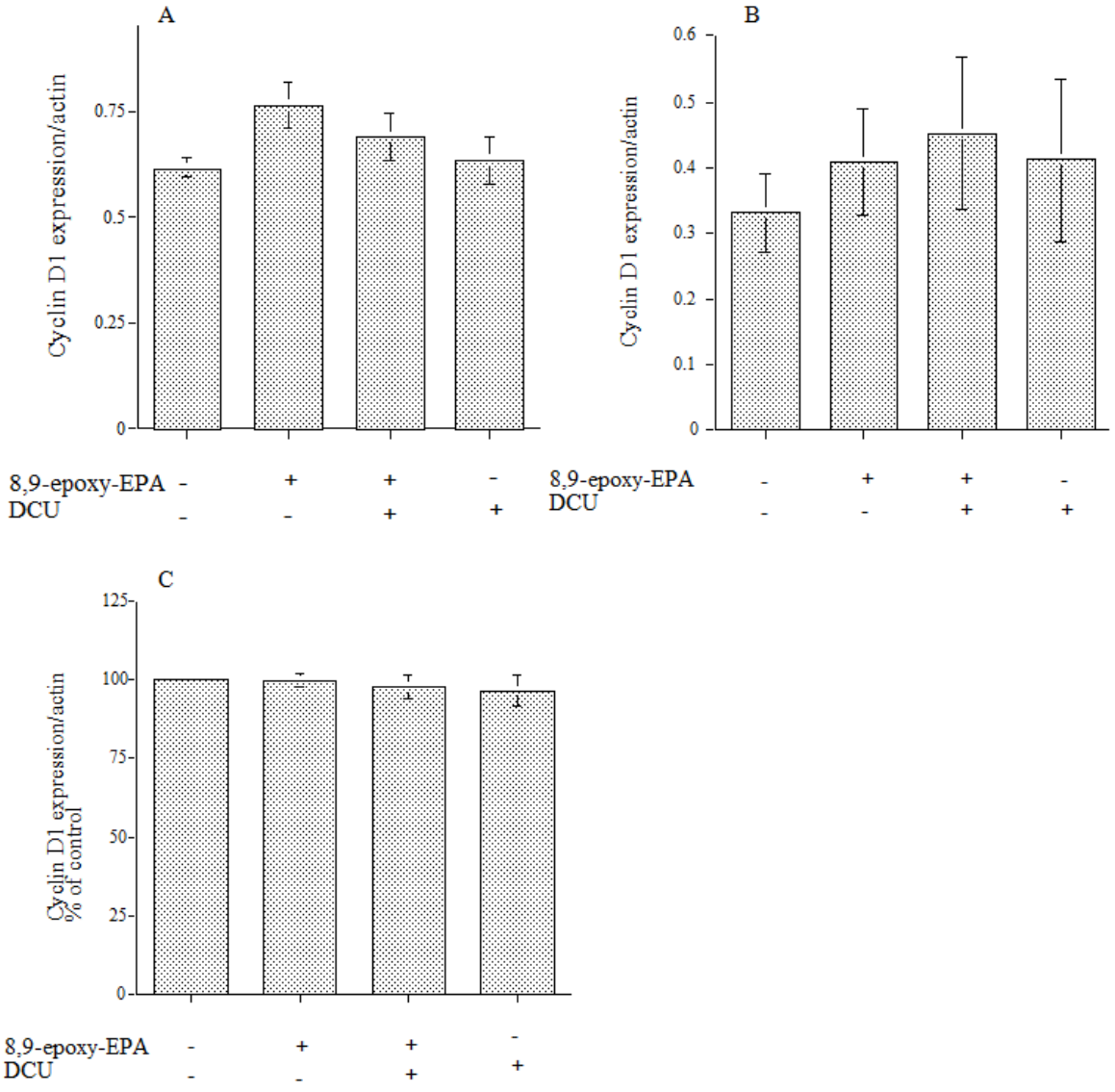

Figure 3.9: Expression of cyclinD1/actin (A and B) and cyclin D1 expression/actin % of control (C) in MCF-7 cells treated with 8, 9-epoxy-EPA and 8, 9-epoxy-EPA+DCU for 6 hr (A), 16 hr (B) and 24 hr (C). Data are expressed as means ± SE from three different experiments.

4 DISCUSSION

Several studies have been undertaken *in vivo* and *in vitro* that have suggested that n-3 PUFAs inhibit the growth and spread of cancer cells, including breast cancer cells [16-23]. However, the mechanism is not fully understood. Experiments have shown that the n-3 PUFA EPA inhibits growth of MCF-7 breast cancer cells [16, 21, 23]. Metabolism of EPA by the CYP-450 system results in five regioisomeric epoxyeicosatetraenoic acids (5,6-, 8,9-, 11,12-, 14,15-, and 17,18-epoxy-EPAs) [9]. Previous studies have shown that murine endothelial cells are stopped from proliferating by these epoxides (unpublished data). The present project tested whether 8, 9-epoxy-EPA might contribute to growth inhibition of EPA in human breast cancer cells. Positive findings would provide mechanistic insight into the anticancer properties of n-3 PUFA.

There were two major approaches in this study. The first was to test 8, 9-epoxy-EPA alone and in combination with DCU on MCF-7 cell proliferation. The second was to test the influence of serum on the effect of 8, 9-epoxy-EPA on MCF-7 cells. The reason for these approaches was to directly evaluate the potential of the epoxides to inhibit cancer growth and to explore some of their performance characteristics. Use of the epoxide hydrolase inhibitor DCU would provide information on the role of epoxide hydrolase in the duration of action, and serum provides a direct growth stimulus.

4.1 Effects of 8, 9-epoxy-EPA on cell viability

We first tested the effect of 8, 9-epoxy-EPA on cell viability. Our experiments showed a decrease in cell viability of MCF-7 cells treated with 8, 9-epoxy-EPA and with the combination of 8, 9-epoxy-EPA + DCU, compared to control. Chamras et al [21] tested among other things the influence of EPA and DHA on the proliferation of MCF-7 cells. They found that both EPA and DHA inhibited MCF-7 cell growth, which are in agreement with our findings by using a combination of cell count by Trypan blue assay and microscopy. Because the role of biotransformation was not evaluated in that study it is possible that eicosanoid metabolites may be important. The present findings indicate that 8, 9-epoxy-EPA may contribute to the beneficial effects of the n-3 polyunsaturated against breast cancer cells. This is in contrast with the growth stimulatory effects of the n-6 PUFA epoxide, termed EETs [36].

We also tested the effect of 8, 9-epoxy-EPA in combination with DCU. DCU is an epoxide hydrolase inhibitor, which decrease the conversion of EETs to the corresponding dihydroxyeicosatrienoic acids (DHETs) by epoxide hydrolase (EH). By adding an epoxide hydrolase inhibitor the enzymatic degradation of EET was inhibited, and the amount of EETs in cells enhanced [11].

Thus, some cells were treated with a combination of 8, 9-epoxy-EPA and DCU and control cells received DCU. Cells treated with the combination of 8, 9-epoxy-EPA+DCU showed slightly decreased viability 26.3% ± 8.9%, while cells treated with only 8, 9-epoxy-EPA showed a 21.5% ± 6.3% decrease compared to control, but these differences were not significant. However, it is noteworthy that MCF-7 cells, like murine endothelial cells, are subject to decreased viability in the presence of the parent n-3 PUFA EPA and its 8, 9-epoxide.

MCF-7 cells are estrogen responsive and contain the estrogen receptor [35]. Estrogen plays an important role in regulating proliferation and protein synthesis in MCF-7 cells. Serum is known to contain both estrogenic hormones and growth factors that will influence the growth of MCF-7 cells. The concentration of serum in the growing media is extremely important for the proliferation of MCF-7 cells. Treatment with 8, 9-epoxy-EPA and the combination of 8, 9-epoxy-EPA + DCU on cells grown in serum-free media did not alter cell viability. This was confirmed by microscopy, which showed that confluence was not altered. This indicates that the effect of 8, 9-epoxy-EPA on MCF-7 cells is dependent on the presence of serum and is important because it implies that growth inhibition by epoxides requires an additional proliferative stimulus. Thus, epoxides were ineffective in growth retarded cells but effective during breast cancer cell proliferation.

In this study media containing phenol red was used. Phenol red is a pH-indicator used in media, which have a structural resemblance to some nonsteriodal estrogens and therefore have estrogenic activity which can stimulate the growth of MCF-7 cells [39]. Since the mechanism of the growth inhibition effect of 8, 9-epoxy-EPA on MCF-7 is not known, and this study focused on cell cycle related mechanism media containing phenol red was used. If a mechanism involving estrogen activity or receptors was studied phenol-free media and charcoal stripped serum must have been used.

4.2 Effects of 8, 9 –epoxy-EPA on cell cycle progression in MCF-7 cells.

The effect of 8, 9-epoxy-EPA on MCF-7 cell viability was further investigated by monitoring cell cycle progression using flow cytometry. Cells were analyzed by flow cytometry for quantification of cells present in G0/G1, S and G2/M phase at 6, 16 and 24 hr.

Cells treated for 6 hours with 8, 9-epoxy-EPA, DCU and a combination of 8, 9–epoxy-EPA+DCU in the presence of FBS did not show a significant change in the cell cycle progression compared with control. Cells treated in the absence of FBS showed a slight decrease in the proportion of cells in G0/G1-phase, with a corresponding slight increase in G2/M-phase for cells treated with 8, 9-epoxy-EPA, DCU and the combination of 8, 9-epoxy-EPA+ DCU compared to control. Only the increase in % of cells in G2/M-phase for cells treated with the combination of 8, 9-epoxy-EPA+DCU was found to be significant compared to control.

The cell cycle analysis also showed that there was an increase of cells in G0/G1 phase for cells grown in the absence of FBS compared to cells grown in presence of FBS. This probably means that the cells are prevented from entering G1 phase, and the cell cycle progression is different compared to cells grown in presence of FBS. The alteration of the cell cycle progression for cells grown in the absence of FBS shows again the influence of FBS on MCF-7 cell growth. To further evaluate these effects longer term treatments were performed.

After 16 hours of treatment with 8, 9-epoxy-EPA, DCU and the combination of 8, 9-epoxy-EPA + DCU in the presence of FBS the proportion of cells in G0/G1-phase were significantly increased compared to control. However these effects were relatively small. This indicates that both treatment with 8, 9-epoxy-EPA and DCU alone has an effect on cell cycle progression at 16 hours. There was also a corresponding slight decrease in the proportion of cells in S-phase, but the decrease was not significant.

However, 24 hours of 8, 9-epoxy-EPA-treatment in the presence of FBS showed an accumulation of MCF-7 cells in G0/G1-phase compared to control. A corresponding decrease in the proportion of cells in G2/M-phase for 8, 9-epoxy-EPA and 8, 9-epoxy-EPA+DCU was also observed. A study which examined the effect of DHA on cell growth, cell cycle progression and expression of cell cycle regulatory proteins in KPL-1 cells, which also is a

human breast cancer cell line that is estrogen receptor positive, also found the same inhibitory effect on breast cancer cell growth, and an arrest of cells in G1-phase at 24 hours [22]. At 6 hours treatment they did not see any change in cell cycle progression, which is also consistent with our results.

The cell cycle analysis indicated that MCF-7 cells treated with 8, 9-epoxy-EPA for 16 and 24 hours were arrested in G0/G1 phase, and did not progress through the cell cycle at the same rate as control cells. It also showed that DCU alone increased the number of cells in G0/G1-phase compared to control, and in combination with 8, 9-epoxy-EPA the increase of the proportion of cells in G0/G1-phase was not significant higher than the treatment with 8, 9-epoxy-EPA alone. The adding of DCU does not seem to enhance the effect of the 8, 9-epoxy-treatment on cell cycle progression significantly, but may also contribute to growth inhibition by stabilizing endogenous epoxide in cells. The 24 hours treatment of MCF-7 cells in the absence of FBS did not alter the cell cycle progression. This is consistent with outcomes of experiments that tested the effect of 8, 9-epoxy-EPA on MCF-7 cell viability: the effect of 8, 9-epoxy-EPA is dependent on the presence of FBS.

4.3 Cell cycle protein expression in 8, 9-epoxy-EPA-treated MCF-7 cells.

The effects on cell viability and cell cycle progression were further investigated by examining changes in the expression of cell cycle regulatory proteins. Cell cycle progression is regulated by the cyclin system. Cyclin D1 is the master regulator of this process and is switched on in cells after a proliferative stimulus, e.g. in MCF-7 cells by exposure to estrogen and growth factors present in serum. Cyclin D1 acts together with cyclin-dependent kinases (CDKs), which phosphorylate downstream proteins to activate gene expression in the nucleus. CDK-inhibitors (CDKIs), such as p21 and p27, interact with the cyclin-CDK complex to regulate target protein phosphorylation. In this study we tested whether the small changes in cell cycle distribution might occur along with altered expression of cyclins, CDKs and CDKIs.

We found the expression of cyclin D1, cyclin E, CDK4, CDK6 and p21 to be unchanged in 8, 9-epoxy-EPA-treated MCF-7 cells. Inclusion of DCU did not alter this finding. n-3 Epoxy EPA has been found to strikingly decrease the expression of cyclin D1 protein and mRNA expression in murine endothelial cells. A decrease in CDK4, which forms a functional

complex with cyclin D1, was also noted but other cyclins, and CDKIs were unaffected. The present findings that cyclin D1 expression was unchanged by epoxide treatment in the presence or absence of the epoxide hydrolase inhibitor DCU suggests that the cyclin is regulated differently in MCF-7 cells. At this point the signaling mechanisms in cells involving n-3 epoxy EPA are unclear. After these have been clarified it may be possible to account for these cell-type-specific effects. Another potential mechanism is that epoxy EPA may exert an apoptotic effect in MCF-7 cells. That is, they may stimulate programmed cell death rather than decreasing cell proliferation. This could account for the present finding that cell viability and cell cycle distribution are impaired by the epoxide in the absence of cyclin D1 effect.

In summary we found have that 8, 9-epoxy-EPA inhibited the growth of MCF-7 cells, which is in agreement with other studies that have shown similar inhibitory effects of EPA on MCF-7 cell growth [16, 21, 23]. The effect of 8, 9-epoxy-EPA on MCF-7 cell viability has been shown to be dependent on the presence of FBS, which is also required for the effect of linoleic acid on MCF-7 cell growth [40]. We also studied the mechanism in relation to cell cycle by using flow cytometry and western analysis. The results from the cell cycle analysis showed a significant increase in the proportion of cells in the G0/G1-phase for cells treated for 16 hours with 8, 9-epoxy-EPA, DCU and 8, 9-epoxy-EPA+DCU compared to control. After 24 hours there was an accumulation of treated cells in the G0/G1-phase with a corresponding decrease in G2/M-phase compared to control. Treatment in the absence of FBS showed no alteration in the progression of the cell cycle. The expression of cell cycle regulatory proteins did not show any change between treated cells and control. Thus, the growth inhibition effect may be due to increased cell apoptosis as an alternative to decreased proliferation in MCF-7 cells. This possibility should be evaluated in further studies.

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6 APPENDIX

6.1 Procedure for BCA-assay

Total protein lysate preparation:

- Prepare part A (table 6.1) and lysis buffer (table 6.2).
- Add 2x 0.5 ml of ice-cold PBS to cell samples, stored in -80 °C freezer, to transfer cells to an eppendorf tube.
- Centrifuge cell samples at 10 000 g at 4 °C for 5 minutes.
- Remove supernatant.
- Add 20 µl of lysis buffer per cell sample harvested from one well in a 24-well plate, and vortex samples.
- Leave samples on ice for 40 min, and centrifuge at 10 000 g at 4 °C for 10 minutes.
- Transfer 4 µl of the supernatant to a BCA eppendorf tube, and store at -80°C.

Table 6.1

Part A in lysis buffer, 10 ml	
1x PBS	6.49 ml
1% Igepal	1 ml
0.5% Sodium deoxycholate	2.5 ml
0.1% SDS	0.05 ml

Table 6.2

Lysis buffer (part A + phosphatase inhibitors), 5 ml	
Part A	4992.5 ml
0.1 mM PMSF	2.5 ml
5 ug/ml Aprotinin	2.5 ml
5 ug/ml Leupeptin	2.5 ml

Quantification of protein:

- Dilute 4 µl of protein samples from total protein lysate with 66 µl of deionized water (dilution factor 17.5).

- Dilute 400 µl lysis buffer with 6600 µl deionized water (dilution factor 17.5).
- Prepare BSA (bovine serum albumin) standards as per table 6.3 using the BSA stock 2mg/ml and diluted lysis buffer.

Table 6.3: BSA-standards

Tube	**Diluted lysis buffer, (µl)**	**BSA stock, 2 mg/ml (µl)**	**Final concentration (µg/ml)**
A	0	300 of 1x stock	2000
B	125	375 of 1x stock	1500
C	325	325 of 1x stock	1000
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 working solution by using BCA Protein Assay Reagent kit (Pierce), and mix 40 ml of reagent A and 0.8 ml of reagent B (50A : 1B).
- Add 25 µl of each BSA standard and test sample in duplicate into a 96 well plate.
- Add 200 µl of working solution into each well using a multi channel-pipette.
- Put the 96-well plate on an orbital shaker for 30 seconds.
- Seal the plate with parafilm and incubate at 37 °C for 40 min, and leave the plate to cool at room temperature for 30 minutes.
- Change the lid to avoid water, and read at 540 nm on Victor3 Wallac 1420 multilabel counter.
- Calculate the amount of protein in each well (sample) by plotting a standard curve.

6.2 Procedure for western blotting

Gel casting:

- Put gel casting apparatus together.
- Prepare 15 ml of 12% separating gel (see Materials and methods, table 2.17)
- Apply 5 cm of 12% separating gel into the gel casting apparatus.
- Apply 5 drops of water-saturated BeOH evenly across the gel to exclude air during polymerization.
- Leave the gel to set for 30-40 minutes, and wash the gel 5x deionized water to remove the BeOH.
- Prepare 8 ml of 5% stacking gel (see Materials and methods, table 2.18).
- Apply the stacking gel on top of the separating gel, and insert 1.5 mm 10 well combs.
- Leave the stacking gel to set for 30 minutes.
- Carefully remove combs, and wash the gel with deinonized water.
- Carefully remove water with a syringe.

Gel electrophoresis:

- Apply 5 µl marker and prepared samples (see Material and methods, 2.10 Western blotting) on gel.
- Prepare 1x electrophoresis buffer (see Material and methods, table 2.10 and 2.11).
- Add the electrophoresis buffer in the tank, and fill up the wells.
- Run at 150V using the Power PAC 200.
- Leave the tank in an icekit.
- Run the gel until the bromophenol blue completely have reached the end of the gel.

Transfer:

- Prepare the transfer buffer (see Material and methods, table 2.13)
- Activate the nitrocellulose membrane by soaking it for 10 minutes in transfer buffer on an orbital shaker.

- Prepare the stacks by assembling the components in the following order:

  TOP
  - Clear plastic holder
  - 1 pad
  - 3x Whatman filterpaper 3 mm (8x10 cm)
  - Nitrocellulose membrane
  - Gel
  - 3x Whatman filterpaper 3 mm (8x10 cm)
  - 1 pad
  - Black plastic folder

  BOTTOM
- Put the stack into the blotting module.
- Add transfer buffer in to the tank, and put in the blotting module.
- Set blotting on 100V for 1.5 hour, and fill ice container with ice and replace every 30 minutes.
- After the transfer mark the outline of the gel on the membrane with a pencil to orientate.
- Color the membrane with Ponceau Stain for 10 minutes on an orbital shaker.
- Quickly wash of Ponceau Stain with deinonized water, and scan the membrane on a Biorad GS-800 Calibrated Densitometer.

Development of the blot:

- Incubate the blot in a tray with 10 ml of 5% blocking milk solution (see Materials and methods, table 2.16) for 1 hour on the shaker.
- Pour out the blocking solution, and incubate the blot in a tray with 10 ml 5% blocking milk solution and the appropriate primary antibody overnight at 4 °C on an orbital shaker.
- Wash the blot 5x 5 minutes and 5x 2.5 minutes with 20 ml 1x TBS/Tween (see Materials and Methods, table 2.13 and 2.14) on an orbital shaker.
- Place the blot in a tray with 10 ml of 5% blocking milk solution and the appropriate secondary antibody for 1 hour on the shaker.

- Protect the blot from light by using a lid covered in foil.

- Remove the secondary antibody and wash the blot 5x 5 minutes and 5x 2.5 minutes with 1x TBS/Tween, and then 2x 5 minutes with TBS (no Tween).
- Dry the blot between to filter papers.
- Scan the blot by using Odyssey LI-COR.