Potentiation of Drug-Induced Cytotoxicity by Conjugated Linoleic Acids (CLA) in *In Vitro* models of Drug-Resistant Cancer

Ph.D. thesis

By

Isobel O'Reilly B.Sc. (Hons.)

Project Supervisor: Dr. Rosaleen Devery

September 2009

Declaration

"I hereby certify that this material, which I now submit for the assessment on the programme of study leading to the award of Doctor of Philosophy is entirely my own work, I have exercised reasonable care to ensure that the work is original and does not to the best of my knowledge breach any law of copyright, and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work."

Signed: _____

ID No: 52171825

Date: 18th September 2009

Dedicated to the memory of my Mother

Mary O'Reilly

Acknowledgements

First and foremost, I would like to thank my supervisor Dr. Rosaleen Devery, whose patience, attention to detail and wisdom were boundless throughout my time here.

I would like to thank all the academic staff, technicians and researchers in the School of Biotechnology for their support, encouragement and friendship over the years. Especially: David Cunningham for his help with the HPLC and GC. Shafiqur Rahman and Anna Kuchta in the lab for their friendship and support –it was always great have someone to bounce new ideas off in the lab or over coffee. The many other coffee culprits old and new; Sharon, Conor, Aisling, Pam, Zelda, Clair, Catherine, Sinead, Eva, Carolyn, Mark and Ann-Marie.

This research project was funded by the National Institute for Cellular Biotechnology (NICB). Thanks to Prof. Martin Clynes for making that possible, and for his continued support and encouragement throughout. To Dr. Robert O'Connor (NICB) for all his help throughout the project, particularly with the drug-work and the LC-MS analysis. Dr. Sharon Glynn (NICB) for her help in the early stages. Aoife Devery and Dr. Rachel Wall (NICB) for their help with the LC-MS work.

To the UCD crowd (Eleanor, Esther, Nova, Louise, Rosanne, Padraig, Fearghal, Deirdre, Neal, Maria, Jon, Orla, Grania and others I may have left out) and Anna, Kate and Bibi – thanks for being a shoulder or great laugh when needed –and for the many dinner/house parties when we were too broke to go out!

A very special word of thanks to Laura for sticking with me through it all –let the good times roll!

Finally my family, without whom nothing is possible. My brothers Philip and Henry, sisters Gemma and Linda, but most especially my Dad, James O'Reilly for his love and support all these years.

Table of Contents

Abstract

Preclinical studies indicating that conjugated isomers of linoleic acid suppress mitosis, restore apoptosis, induce differentiation and suppress angiogenesis in animal models of cancer formed the rationale for an *in vitro* investigation of their potential benefits in targeting drug-resistant cancer cells. Additionally, several lines of evidence implicate a role for sphingolipids in multiple drug resistance. The objective of this study was to evaluate the effects of conjugated linoleic acids (CLA) and various anticancer drugs on growth of MCF-7 and MCF-7/cis cell lines and to evaluate the effects of co-treatments of CLA and doxorubicin (Dox) on cell growth, intracellular drug accumulation, sphingolipid content and cellular lipid composition in a drug-resistant melanoma model. Initially an investigation of the potential of co-treatment with a commercial mixture of CLA isomers (CLA-mix) and two single isomers *c*9,*t*11-CLA and *t*10,*c*12-CLA to enhance the cytotoxic effects of Doxorubicin (Dox) on a drug naive MDA-MB435-S-F (MDA) cancer cell line was carried out. It was found that each preparation of CLA enhanced cytotoxicity of Dox in a dose dependant manner with an increase of over 3-fold with 6 μg/ml CLA-mix. Similarly, both *c*9,*t*11- and *t*10,*c*12-CLA purified isomers yielded an increase in doxorubicin cytotoxicity of up to 3 fold. Toxicity assays carried out on a Dox-pulsed MDA cell line, MDA-MB435-S-F/Adr10p10p (A10p10p), demonstrated a similar pattern of enhanced toxicity with up to 5, 7 and 12-fold increase when co-treated with CLA-mix, *c*9,*t*11-CLA and *t*10,*c*12-CLA respectively. Dox retention as determined by liquid chromatography tandem mass spectrometry (LCMS) was significantly enhanced by the CLA-mix in A10p10p cells and by *c*9,*t*11-CLA in MDA and A10p10p cells. Analysis of sphingomyelin metabolites by High Performance Liquid Chromatography (HPLC) revealed that CLA treatments in combination with Dox increased ceramide levels in both cell lines compared with Dox alone. Treatment of both MDA and A10p10p cells with CLA-mix resulted in down-regulation of Her2/*neu* coded p185 $^{\text{c-erbB2/c-}neu}$ oncoprotein expression. Eicosapentanoic acid (EPA) and docosahexanoic acid (DHA) resulted in similar down-regulation of this protein in the A10p10p cell line. Fatty acid composition of MDA and A10p10p cells was analysed by gas chromatography (GC). There was a marked increase in the unsaturation index of both cell lines following all fatty acid treatments, suggesting inhibition of fatty acid synthase (FAS) activity, a key enzyme in the *de novo* synthesis of fatty acids. With the exception of the CLA-mix in the resistant line, all fatty acids in both cell lines caused a decrease in the ratio of C18:1/18:0 suggesting the Δ 9 desaturase enzyme stearoyl-CoA desaturase (SCD) as a potential target for inhibition by fatty acid treatments. The data suggest that CLA-induced changes in ceramide status, intracellular drug accumulation and lipid profiles may underlie the enhanced chemosensitivity of the resistant cell line and represent a new avenue to develop chemotherapy regimes for patients with drug-resistant tumours leading to improved response and survival rates in these patients.

Communications

Publication **(in preparation)**

O'Reilly, I., Glynn, S., O'Connor, R., Devery, R. Enhancement of Doxorubicin Cytotoxicity by Conjugated Linoleic Acid (CLA) in a cellular model of Drug-Resistant Cancer. Manuscript in preparation.

Abstract

O'Reilly, I., Devery, R., 2004. A comparative study of effects of conjugated linoleic acid isomers on proliferation in MCF-7 and drug-resistant MCF-7 breast cancer cells. *Anticancer Research 24: 536*

Oral

Potentiation of Drug-Induced Cytotoxicity by Conjugated Linoleic Acids (CLA) in In Vitro models of Breast Cancer. II International Congress on Conjugated Linoleic Acid (CLA): From Experimental Models to Human Application, Villasimius, Cagliari, Italy, September 2007.

An investigation of the role of conjugated linoleic acids (CLA) in chemotherapy. Annual meeting of the Canadian section of the American Oil Chemist Society (CAOCS), Halifax, Nova Scotia, Canada, October 2004.

The adjuvant role of lipids in chemotherapy. School of Biotechnology seminar series, DCU, Dublin, March 2004.

Poster

O'Reilly, I. and Devery, R., 2009. Modulation of Lipid Profile by Conjugated Linoleic Acid (CLA) isomers in an in vitro model of Drug-Resistant Cancer. School of Biotechnology Research Day, DCU, January 2009.

O'Reilly, I., Glynn, S., O'Connor, R., Devery, R., 2005. Potentiation of Adriamycin-Induced Cytotoxicity by Conjugated Linoleic Acid (CLA) isomers in an in vitro model of breast cancer. 2nd International Conference on Tumor Progression and Therapeutic Resistance, Boston, MA, USA.

O'Reilly, I., Glynn, S., O'Connor, R., Devery, R., 2005. An investigation of the role of conjugated linoleic acids (CLA) in drug-induced cytotoxicity. Annual meeting of the Irish Association for Cancer Research (IACR), Kilkenny.

O'Reilly, I., Glynn, S., O'Connor, R., Devery, R., 2004. Conjugated linoleic acids (CLA) in cancer therapy. The first annual Open Day for National Institute for Cellular Biotechnology (NICB), Dublin.

O'Reilly, I. and Devery, R., 2003. A comparative study of effects conjugated linoleic acid on proliferation in MCF-7 and drug resistant MCF-7 breast cancer cells. National Institute for Cellular Biotechnology: Biotechnology, Cancer & Drug Resistance New Targets, New Diagnostics & New Treatments for Tumours Resistant to current therapies, Dublin.

Abbreviations

5-FdUDP	5-fluorodeoxyuridine diphosphate
5-FdUMP	5-fluorodeoxyuridine monophosphate
5-FdUrd	5-fluorodeoxyuridine
5-FdUTP	5-fluorodeoxyuridine triphosphate
5-FU	5-fluorouracil
5-FUDP	5-fluorouridine diphosphate
5-FUMP	5-fluorouridine monophosphate
5-FUrd	5-fluorouridine
5-FUTP	5-fluorouridine triphosphate
AA	arachidonic acid
Abs	absorbance
ALA	α-linolenic acid
BF_3	boron trifluoride
BRCA1	Breast cancer gene 1
BRCA2	Breast cancer gene 2
BSA	Bovine serum albumin
BSS	Balanced salt solution
c	*cis*
CDK	cyclin dependent kinases
CLA	conjugated linoleic acid
CO_2	carbon dioxide
COX	cyclooxygenase
DCIS	ductal carcinoma *in situ*
DGLA	dihomo-γ-linolenic acid
dH_2O	distilled water
DHA	docosahexaenoic acid
DMBA	7,12-dimethyl-benz[a]anthracene
DMEM	Dulbecco's Minimum Essential Medium

DMH	dimethylhydrazine
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
dUMP	deoxyuridine monophosphate
ECM	extracellular matrix
EDTA	ethylene diamine tetraacetic acid
EFA	essential fatty acid
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ELISA	enzyme linked immunosorbant assay
EPA	eicosapentaenoic acid
ER	oestrogen receptor
ErbB2	erythroblastic leukemia viral oncogene homolog 2
ERE	oestrogen response element
ERK	extracellular signal regulated kinases
FA	fatty acid
FAME	fatty acid methyl ester
FAS	fatty acid synthase
FCS	foetal calf serum
FFA	free fatty acid
FID	flame ionisation detector
G1	growth phase 1
G2	growth phase 2
GC	gas chromatography
GLA	γ-linolenic acid
HCl	hydrochloric acid
HEPES	N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
HER1	human epithelial growth factor receptor 1
HER2	human epithelial growth factor receptor 2
HIC-1	hypermethylated in cancer-1

HPLC	high performance liquid chromatography
HRG	heregulin
IGF	insulin-like growth factors
IMS	industrial methylated spirit
IP_3	inositol triphosphate
LA	linoleic acid
LCIS	lobular carcinoma *in situ*
LC-MS	liquid chromatography-mass spectrometry
LCSFA	long chain saturated fatty acid
MAP	mitogen-activated protein
MAPK	mitogen-activated protein kinase
MEK	MAP kinase kinase
MeOH	methanol
mRNA	messenger ribonucleic acid
MTS	[3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfonyl)-2H-tetrazolium]
MUFA	monounsaturated fatty acid
NaCl	sodium chloride
NADH	nicotinamide adenine dinucleotide
$NaHCO_3$	sodium bicarbonate
NCRI	national cancer registry Ireland
NICB	National Institute for Cellular Biotechnology
NMR	nuclear magnetic resonance
OPA	O-phthalaldehyde
PBS	phosphate buffered saline
PBST	PBS containing tween 20
PGE_2	prostaglandin E_2
P-gp	P-glycoprotein
PhIP	2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine
PI3K	phosphoinositide-3-kinase
PL	phospholipid

PMS	phenazine methosulfate
PMSF	phenazine methosulfate fluoride
pNP	p-nitrophenol
pNPP	p-nitrophenol phosphate
PPAR	peroxisome proliferator-activated receptor
PPRE	peroxisome proliferator response element
pRb	retinoblastoma protein
PS	phosphatidylserine
PUFA	polyunsaturated fatty acid
Rb	retinoblastoma
RNA	ribonucleic acid
SCD	Stearoyl CoA desaturase
SCID	severe combined immuno deficient
SD	standard deviation
SEM	standard error of the mean
SFA	saturated fatty acid
SM	sphingomyelin
SP1	sphingosine 1 (Standards following extraction by alkaline hydrolysis)
SP2	sphingosine 2 (Standards following deacylation step)
SP_N	sphingosine $_{Neat}$
Strep	streptomycin
t	*trans*
TG	triglyceride
t-VA	trans-vaccenic acid

Units

Å	Angstrom
d	day
g	gram
h	hour
kDa	kilodalton
L	litre
M	molar
mg	milligram
min	minute
mL	millilitre
mM	millimolar
mol	mole
µg	microgram
µL	microlitre
µM	micromolar
nm	nanometre
ng	nanograms
^{o}C	degrees centigrade
U	unit of enzyme activity
v/v	volume per volume
w/v	weight per volume
w/w	weight per weight

Chapter 1

General Introduction

1.1 Cancer

Cancer is a group of diseases characterised by uncontrolled growth and spread of genetically abnormal cells. If spread is not controlled it can lead to death. The word "cancer" is synonymous with malignant tumours. It is a Latin term, literally translated from the Greek "Karkinos" meaning crab, which refers to the metaphorical claws reaching out to invade surrounding tissues. In the ancient Hippocratic school of medicine "Karkinos" was also used to describe a non-healing ulcer, whereas "Karkinoma" referred to a malignant tumour.

In Ireland there were over 19000 new cases of cancer reported each year up to 2001, with 7627 cancer deaths (Campo *et al,* 2004). Figures for the years 2000-2004 show that incidence rates rose to over 29000 (21500 cases excluding non-melanoma skin cancer) with the average number of cancer deaths at 11261 (National Cancer Registry, 2007; Donnelly *et al.,* 2009). This shows that while cancer incidence is rising sharply in Ireland, death rates are slower indicating an improvement in cancer survival, either through early detection, improved treatment or both.

Age-specific rates of incidence for men and women aged between 80 to 84 y are two to three times higher than rates for those aged 60 to 64 y, and rates for men and women aged 60-64 y are roughly four to ten times higher than for the 40-44 y age group. Given the fact that more and more people are living well past 65 y, the number of cancer cases is sure to continue to rise. However, risk factors other than age are modifiable. It is known that, approximately one third of all cancers are caused by tobacco; one third by diet (high fat/ low fruit and vegetables); and most of the remaining third by other lifestyle choices such as excessive drinking, lack of regular exercise, sexual and reproductive patterns and frequent sunburns. Occupational exposures account for the remaining cancer risk, while the final –and very small-outstanding proportion of risk relates to toxins in the environment (Campo *et al,* 2004).

A few worrying trends have been identified in a relatively recent report by the Irish Cancer Registry. Mortality rates are higher for both men and women in Ireland than in the US even though the incidence is lower. Men have higher incidence (20 %) and mortality (40 %) rates than women in Ireland. However, while the rates for men are equivalent to those in the EU, the incidence and mortality rates for women in Ireland are significantly higher than in the EU (Campo *et al.,* 2004; Donnelly *et al.,* 2009). This highlights the scope for interdisciplinary and inter-institutional collaboration and communication promoting a free-flow of information to aid translation of new treatments from the laboratory bench to the patient's bedside.

1.1.1 Cancer Development

Cancer represents a large group of diseases characterised by uncontrolled growth of an abnormal cell to produce a population of cells that have acquired the ability to multiply and invade surrounding and distant tissues. In terms of nomenclature most adult cancers are carcinomas that are derived from epithelial cells (breast, colon, skin, lung, etc.). Sarcomas are derived from mesenchymal tissues while leukaemias and lymphomas are derived from bone marrow cells and lymphoid tissue. Cancer is essentially a genetic disease because it can be traced to activation of specific genes (oncogenes) that promote cell proliferation and to inactivation of specific genes (tumour suppressor genes) that normally restrain cell proliferation. The latter act as brakes in preventing cells from becoming malignant whereas the former function like an accelerator speeding up cell proliferation and producing malignant tumours that invade surrounding healthy tissue. Human cancers develop by stepwise accumulation of mutations involving oncogenes and tumour suppressor genes. Epidemiologists have interpreted the age-related exponential increase in cancer incidence to denote a small number (three to seven) of critical mutations or 'hits' within a single somatic cell during an individual's lifetime

(Lodish *et al.,* 2004).

Oncogenes arise as a result of critical mutations in proto-oncogenes that are involved in the positive regulation of cell growth in normal cells. Oncogenes encode proteins that belong to one of seven basic functional groups involved in growth factor signalling: growth factors, growth factor receptors, plasma membrane G proteins, protein kinases, transcription factors, cdk-cyclins and apoptotic proteins. Tumour suppressor genes encode proteins involved in DNA repair or chromosome sorting. When mutated they encode proteins that are mainly inactive. Most cancers are monoclonal in origin, being derived from a single cell. Initially a somatic mutation occurs in a critical gene and this provides a growth advantage to the cell and results in expansion of the mutant clone. Each additional critical mutation provides a further selective growth advantage resulting in clonal expansion of cells with mutations in critical genes. Under normal conditions DNA repair may revert the cell back to a normal cell. In other cases programmed cell death or apoptosis is initiated. Failure in each case results in carcinogenesis (Lodish *et al.,* 2004). These stages are illustrated in Figure 1.1.1 below.

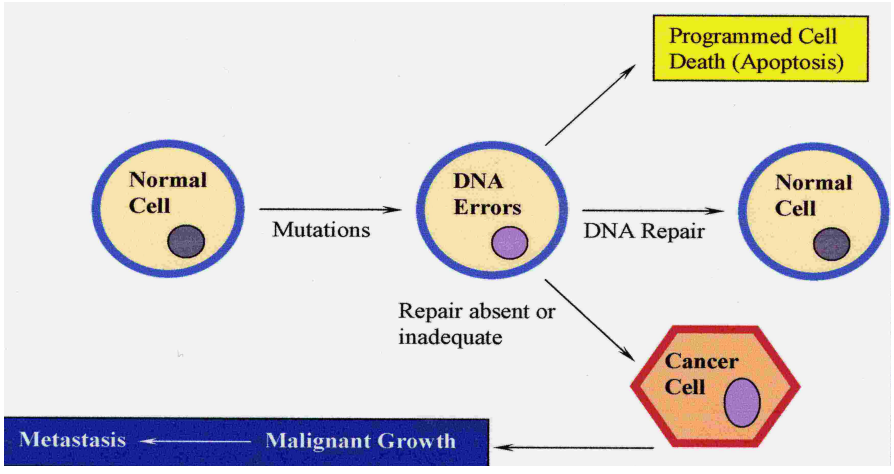


Figure 1.1.1 Schematic showing development of cancer cells (adapted from McGrath, 2003)

Apoptosis is characterised by cell shrinkage, chromatin condensation, membrane blebbing and DNA fragmentation (Arends and Wylie, 1991). A cascade of cysteine proteases (caspases) are activated in the final stages of apoptosis, which act on key proteins including poly (ADP-ribose) polymerase (PARP) (Alnemri, 1997). This is followed by endonuclease cleavage of DNA (DNA laddering) into small fragments typically 180-200 bp and subsequent absorption of apoptotic bodies into macrophages (Raff *et al*., 1993). Activation of caspases occurs as a result of exposure to chemotherapeutic agents, radiation or growth factor withdrawal. Mechanisms of action of chemotherapeutic agents will be discussed in greater detail in later sections.

Figure 1.1.2 takes the example of breast cancer and shows a schematic of the probable stages in breast cancer development. Mutations in *BRCA*1 and *BRCA*2 tumour suppressor genes have been implicated in the majority of hereditary forms of breast cancer, which in turn constitute approximately 10 % of breast cancers (Polyak, 2001).

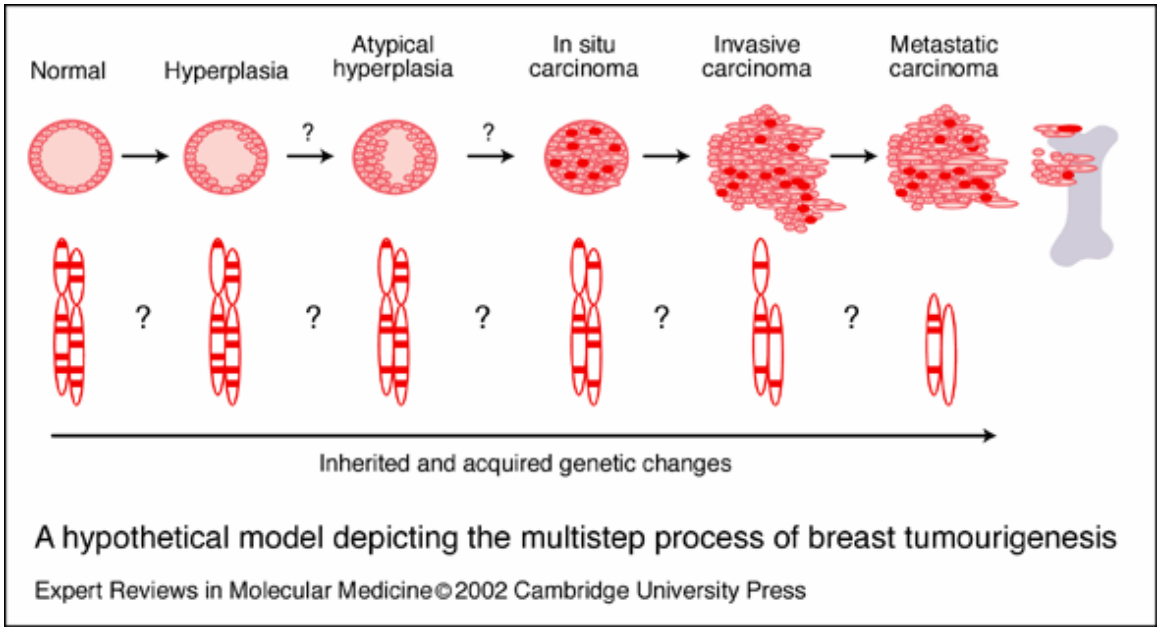


Figure 1.1.2 Hypothetical model depicting the multi-step process of breast tumourigenesis (Polyak, 2002)

They (*BRCA*1 and *BRCA*2) normally repair breaks in double stranded DNA. If DNA damage is not repaired, a checkpoint is activated that leads to an increase in p53 transcription factor. Mutations in *TP53*, the tumour suppressing gene encoding p53, are found in 15-34 % of sporadic breast cancers. P53 normally acts as a "molecular policeman" in monitoring genome integrity (Voet *et al.,* 1999). It accumulates in the cell following DNA damage, directing the cell to halt in G1 phase of the cell cycle until the damage is repaired or to apoptosis if the damage is extensive (Ingvarsson, 1999). Excessive activity of certain proteins and genes in breast cancer cells can switch on molecular signalling pathways that stimulate cell proliferation and survival. Her2, for example is a member of the epidermal growth factor receptor family which when activated dimerises with a neighbouring EGFR (HER1, 3 or 4) causing activation of tyrosine kinase activity that initiates a signal cascade to the cell nucleus (Franklin et al., 2004). Activation of growth factor receptors essentially switches on ras and/or PI3K activity which lead to a cascade of protein interactions that lead to cell growth and suppression of cell death by apoptosis. Mutations in any gene along these signalling pathways can promote cell growth and survival making these genes and their encoded proteins important targets for anti tumour therapies (Lodish *et al.,* 2004).

1.1.2 Breast Cancer

Despite the major advances that have been made in the past 25 years in understanding the biological and clinical nature of breast cancer it still is the most prevalent type of cancer among women in the developed world and its incidence has shown a continuous rise in recent decades (Hanklin, 1993). The National Cancer Institute (NCI) in America estimates that more than one in eight women born today will develop breast cancer at some point in their lifetime, based on statistics for the years 2000 to 2002. This means

that estimated lifetime risk of breast cancer has gone up gradually over the past several decades, from one in ten women in the 1970's. Breast cancer also occurs in men but the probability is about one hundred times less than for women (Ries, 2005).

In Ireland, the incidence rate for breast cancer in women is higher than the average rate for women in the EU. Also, the mortality rate for women with breast cancer is significantly higher than in both the EU and US. Mortality rates in Northern Ireland, where nationally sponsored screening programs are well-established, have fallen by more than 20 % between 1994 and 2000. However, in the Republic of Ireland, the breast cancer mortality rate was the same in 2000 as in 1994. This suggests the need for improved mammography screening services in the Republic (Campo *et al.,* 2004).

Among the numerous histological types of breast carcinomas, the majority (nearly 90 %) arise in the ducts and are referred to as ductal carcinomas. They are further classified according to cell types e.g. medullary, tubular, mucinous and "NOS" (not otherwise specified), the most common of the ductal carcinomas. Approximately 5 % of breast cancers arise in the lobules and are referred to as lobular carcinomas. The remainder are classified as Paget's disease, which involve the nipple and the inflammatory carcinomas (Cooper, 1992).

Stages of breast cancer range from Stage 0 to IV. Stage 0 is used to describe non-invasive breast cancers, such as ductal carcinoma *in situ* (DCIS) and lobular carcinoma *in situ* (LCIS). In stage 0, there is no evidence of cancer cells or non-cancerous abnormal cells breaking out of the part of the breast in which they started, or of getting through to or invading neighbouring normal tissue. Stages I to IV describe degrees of advanced invasive breast cancer (cancer cells are breaking through to or invading neighbouring normal tissue). Stage IV describes invasive breast cancer in which the cancer has spread to other organs of the body, usually the lungs, liver, bone or brain. "Metastatic at presentation" means that the breast cancer has spread beyond the breast and nearby lymph nodes, even though this is the first diagnosis of breast cancer. The

reason for this is that the primary breast cancer was not found when it was only inside the breast. Metastatic cancer is considered stage IV (www.breastcancer.org).

The statistics listed above and in section 1.1 highlight the need for continued advancement in both the early diagnosis and treatment of breast cancer. It is clear from the comparison of incidence and mortality rates between Ireland and the UK (including Northern Ireland), that many women would benefit from an early diagnosis as facilitated by well-organised screening programs (Campo *et al.,* 2004; Allgar and Neal, 2005; Donnelly *et al.,* 2009). To complement this, continued fervour in the research and development of new and improved cancer treatment schedules is also of vital importance.

1.2 Cancer Treatment

There are four treatment options available when a patient presents with a tumour, surgery, radiation, chemotherapy and molecular-based therapy. If the tumour is confined to its initial location surgery almost always results in a cure. If cancer cells have broken away from the parent mass and have entered the lymphatic or vascular circulation so as to have spread to other parts of the body and established lethal secondary tumours (metastases) surgical removal of primary tumour is insufficient. Radiation treatment destroys actively proliferating cells that have metasatasised to distant locations. Chemotherapy uses drugs that, like radiation treatment, selectively destroys dividing cells (Smith *et al.*, 2007). There are a range of classes of chemotherapy drugs in current use including cytotoxic antibiotics (e.g. Doxorubicin), mitotic inhibitors (e.g. Taxol), antimetabolites (e.g. 5-Fluorouracil) and alkylating agents (e.g. Cisplatin) (Smith *et al.*, 2000; Serin *et al.*, 2005; Sparreboom *et al.*, 2005; Wright *et al.*, 2005; Smith *et al.*, 2007). These drugs enter the bloodstream and travel throughout the body, killing cancer cells wherever they reside. The problem with both radiation and

chemotherapy is that they kill normal dividing cells as well as cancer cells, resulting in toxic side effects (susceptibility to infections as a result of destruction of bone marrow cells). Because of this shortcoming scientists are currently developing other less toxic ways to treat metastatic cancer (Smith *et al.*, 2007). The field of cancer chemotherapy, as with other areas of cancer detection and treatment, is ever evolving. Breast cancer was the first solid tumour cancer to be treated with the monoclonal antibody trastuzumab (herceptin) when it was approved for human use in 1998. It is a humanised antibody that targets a specific cell surface receptor protein on tumour cells called Her2, blocking its growth factor binding site, preventing it from being activated and stimulating its internalisation. HER2 is a member of the erbB family of receptor tyrosine kinases, all of which bind extracellular growth factor ligands initiating intracellular signalling pathways regulating diverse biological responses including proliferation, differentiation, cell motility and survival (Marmor *et al.,* 2004). In 30 % of patients with breast cancer the ERBB2 receptor gene has been found to be significantly overexpressed. Amplifications of the gene have also been reported in other cancers including ovarian (reviewed in Sahin and Wiemann, 2009). Overexpression of the HER2 oncogene has been shown to confer resistance to chemotherapeutic drugs and to be indicative of poor prognosis in patients with advanced breast cancer (Colomer *et al.,* 2000). However, the use of targeted drugs such as the monoclonal antibody, trastuzumab (herceptin), has led to improved treatment results in this subset of patients (Smith *et al.,* 2007).

A massive research effort over the last decade to understand the cellular and molecular basis of cancer by clinicians and researchers alike looks set to yield promising results in the war against cancer.

1.2.1 Targeted Cancer Therapy

Conventional approaches to combating cancer (surgery, chemotherapy, radiation) often

cannot rid a patient of all cancer cells. Advances in the disciplines of genetics and biochemistry in the last decade have revolutionised our understanding of cancer cell biology, so much so as to provide new insights into how cancer may be treated. DNA microarray analysis is a powerful chip technology that has impacted on the way cancer is diagnosed and treated. The expression of tens of thousands of genes may be determined simultaneously, permitting complex tumour phenotypes to be defined at the molecular genetics level. Analysis of expression patterns has revealed subtle differences between tumour cells and has potential to distinguish between primary tumours and metastatic tumours, thereby providing cancer researchers with a list of genes to look at more closely as potential targets for therapeutic drugs. Indeed, variations in the genes that are altered may lead to differences in how breast cancers of individual patients behave. The development of proteomics tools to determine the protein profile of a patient's tumour has greatly enhanced the potential for identifying differences between cancer cells and normal cells and has provided potential new leads for drug development.

Basic biology research has led to the rational design of treatments for selectively destroying cancer cells. At the present time a large number of clinical trials are being conducted to test a variety of new anticancer strategies that may be divided into 4 groups: those that depend on antibodies or immune cells to attack tumour cells, those that introduce a gene that either kills tumour cells or causes them to regain normal properties, those that inhibit the activity of cancer-promoting proteins and those that prevent the growth of blood vessels that nourish tumours. The success of trastuzumab has led to development of other antibody-based therapies that target different sites on Her2, preventing dimerisation (eg pertuzumab) or that have the potential to deliver a toxin to cancer cells (Agus *et al.,* 2002). Other examples of monoclonal antibodies include cetuximab (Erbitux), which is used in the treatment of metastatic colorectal cancer and advanced head and neck squamous cell carcinoma (Vincenzi *et al.,* 2008).

Growth factor receptors are also being targeted by small molecule tyrosine kinase

inhibitors that prevent the transmission of growth signals into a cell. Lapatinib is a dual EGFR/Her2 tyrosine kinase inhibitor that has shown promise in inducing growth arrest and cell death in breast cancer cell lines that overproduce Her2. Other kinase inhibitors target insulin like growth factor receptor 1 protein and vascular endothelial growth factor (VEGF) receptor protein involved in forming blood vessels for tumours. Small molecular weight chemical inhibitors such as erlotinib (Tarceva) and gefitinib (Iressa) have been developed which compete for the ATP binding sites of the receptors' tyrosine kinase domain. These are in current use for the treatment of non-small cell lung carcinoma (NSCLC) (Sahin and Wiemann, 2009). Since angiogenesis, or the ability to develop new blood vessels to supply nutrients and oxygen and to remove waste products, is a key step in the progression of tumour development, this represents another ideal drug target to treat such tumours. Bevacizumab (Avastin), also a monoclonal antibody, was developed by Genentech as a VEGF inhibitor and is in use in the treatment of metastatic colorectal cancer and more recently breast cancer (Calfa *et al.,* 2006; Cameron and Bell, 2008). Other VEGF inhibitors such as sunitinib (Sutent) are undergoing clinical trials (Eichelberg *et al.,* 2008).

Hormonal therapy includes the agonist Tamoxifen which binds ERβ and blocks oestrogen-dependent transcription and cell proliferation (Lazo and Larner, 1998). When a cancer arises in a tissue whose growth requires a certain hormone, it may be treated using drugs that block the action of that hormone. Degarelix (Firmagon) is an example of a luteinising-hormone releasing-hormone (LHRH or gonadotropin-releasing hormone/GnRH) antagonist which suppresses testosterone in prostate cancer cells –in effect causing chemical castration (Gittleman *et al.,* 2008). Anti-androgens such as Flutamide (Eulexin) on the other hand, block androgen receptors in prostate cancer cells inhibiting growth (Labrie *et al.,* 1988).

The remainder of this review will focus on three conventional chemotherapeutic drugs in particular; Doxorubicin, Taxol and 5-Fluorouracil and their related families. All three drugs have been used extensively, either as single agents or in combinations with other

drugs, for decades in the treatment of various types of cancer including breast cancer and continue to form part of standard therapies to this day (Smith *et al.*, 2000; Serin *et al.*, 2005; Sparreboom *et al.*, 2005; Wright *et al.*, 2005).

1.2.2 Doxorubicin

Doxorubicin (or Adriamycin) is a member of the anthracycline drug family, which is in turn a member of the rhodomycin group of antibiotics isolated from different strains of *Streptomyces* (Woodruff and Waksman, 1960) (Figure 1.2.2.1).

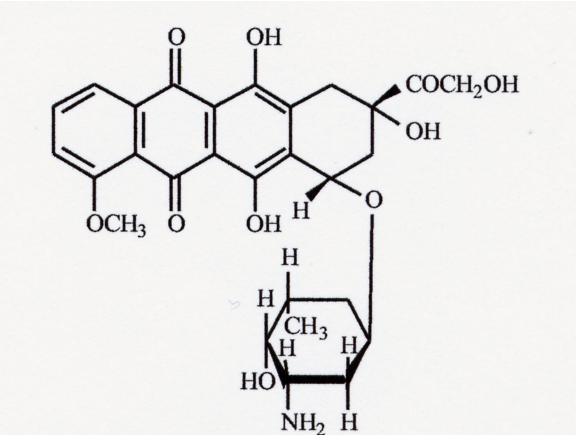


Figure 1.2.2.1 Structure of Doxorubicin (Smith *et al.,* 2000)

Epirubicin is another example of an anthracycline (Figure 1.2.2.2). Both drugs consist of a flat anthraquinone linked by a glycosidic bond to an amino sugar (Robert, 1994).

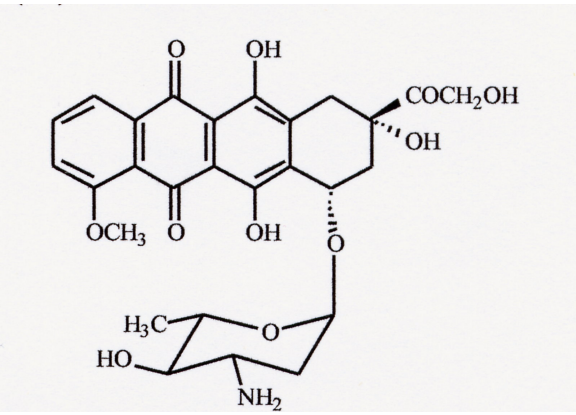


Figure 1.2.2.2 Structure of Epirubicin (Smith *et al.,* 2000)

The bulk of doxorubicin (Dox) administered to sensitive tumour cells is known to rapidly localise in the nucleus and its high affinity for DNA provides the driving force for further nuclear uptake (Gigli *et al,* 1988). Dox first binds to DNA by intercalation – inserting between the stacked paired bases of the super-coiled double helix. This results in uncoiling of the helix followed by impairment of topoisomerase II activity (Liu, 1989) an essential enzyme in controlling the supercoiling of DNA, allowing it to function normally in replication and transcription. This enzyme creates and repairs nicks in two adjacent parent DNA strands (Epstein, 1988). These nicks permit the release of the daughter DNA strand following replication and therefore prevent the parent and daughter DNA strands from becoming inextricably entangled. Inhibition of topoisomerase results in permanent DNA cleavage (D'Arpa and Liu, 1989).

Anthracyclines are among the most efficient drugs used in cancer chemotherapy, but a significant risk of cardiotoxicity limits their use (Olson and Mushlin, 1990; Singal *et al.,* 1997). Other common side effects associated with the drug are anorexia, nausea, vomiting, thinning and ulceration of mucous membranes, bone marrow suppression, leukopenia and alopecia (Abraham *et al.*, 1996). Despite these side effects, there is a general consensus that the most efficacious chemotherapy regimens for the treatment of breast cancer are those that contain one member of the anthracycline drug family (Smith

et al., 2000).

1.2.3 Taxanes

Following the isolation of Taxol (or Paclitaxel) from the stem bark of the Pacific yew tree *Taxus brevifolia* and its discovery as an antileukemic agent by Wani *et al.* in 1971, the taxanes have garnered considerable attention. Taxanes have been used with success in the clinical treatment of a range of human cancers including ovarian, metastatic breast and non-small cell lung carcinomas (Rowinski *et al.,* 1992). Unfortunately, *Taxus* bark is not a renewable source and a 100-year-old tree can only provide approximately one gram of taxol. And so, attention turned to the development of semisynthetic derivatives. The production of these derivatives followed the discovery that the taxol molecule (Figure 1.2.3.1, Smith *et al.,* 2000) consisted of two parts, a complex central core (a diterpene with a taxane ring system linked to a four-membered oxetane ring at positions C-4 and C-5) connected to a structurally simpler ester side chain at the C-13 position.

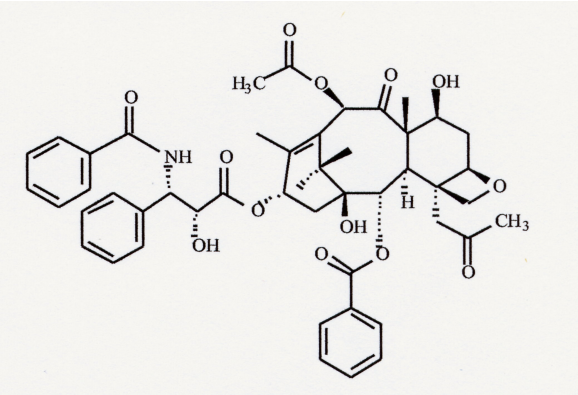


Figure 1.2.3.1 Structure of Taxol™ (paclitaxel) (Smith *et al.,* 2000)

It was found that the central core of the taxol molecule could be obtained from the

needles of the European yew tree, *Taxus braccata* (Figure 1.2.3.2) and that the side chain could be synthesised *de novo* and attached to the core (Nicolaou *et al.,* 1996). Paclitaxel (Taxol™; Bristol-Myers Squibb Oncology, Princeton, NJ) was the first semisynthetic taxane produced. The second clinically used taxane, docetaxel (Taxotere™; Rhone-Poulenc Rorer, Antony, France) (Figure 1.2.3.3) differs from paclitaxel by the composition of the ester side chain, which accounts for the different activities, potencies and toxicity profiles of the two agents (Smith *et al.,* 2000).

Figure 1.2.3.2 *Taxus braccata*

Following the discovery by Horwitz and her team that the cellular target was tubulin (Schiff *et al.,* 1979), it has been established that taxanes share a unique mechanism of action, altering the function of cellular microtubules. The dynamic polymerisation and de-polymerisation of microtubules is essential for normal cellular function. This is because these structures compose the mitotic spindle apparatus and are involved in a number of essential interphase functions, for example, maintenance of shape, motility, anchorage, mediation of signals between cell surface receptors and the nucleus and intracellular transport (Rowinski *et al.,* 1992; reviewed in Smith *et al.,* 2000).

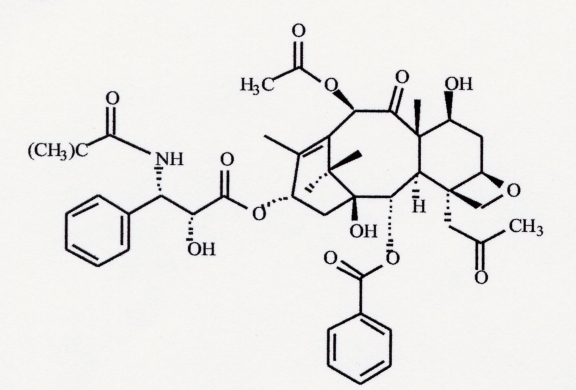


Figure 1.2.3.3 Structure of Taxotere™ (docetaxel) (Smith *et al.,* 2000)

Upon binding to the β-subunit of tubulin, Taxol promotes the formation of structurally abnormal microtubules that consist of only 12 subunits that are relatively resistant to de-polymerisation i.e. they are abnormally stable. Taxotere exerts a similar influence, the resulting microtubules consisting of 13 subunits that are structurally normal, but also abnormally stable. This explains the comparative effects of the two agents; taxotere has a greater affinity for the microtubule binding site (Diaz and Andreu, 1993) and double the activity as a microtubule stabiliser (Gueritte-Voegelein *et al.,* 1991).

Adverse events associated with taxane therapy include myelosuppression, mucositis, alopecia, asthenia, arthralgia and paraesthesia. However, there are also adverse events that are specific to taxane therapy. Hypersensitivity reactions, ranging from mild erythematous skin rashes to anaphylaxis have been observed with both taxol and taxotere. These reactions are more frequently associated with the administration of taxol, thought to be due to the use of Cremophor EL to solubilise the drug (Smith *et al.,* 2000).

1.2.4 5-Fluorouracil

The anti-metabolite 5-Fluorouracil (5-FU) (Figure 1.2.4.1) was first synthesised in 1957 and has been used widely in the treatment of various cancers, including breast cancer, for over 40 years (Ansfield *et al.,* 1969; Pinedo and Peters, 1988; Smith *et al.,* 2000). 5-FU an analogue of uracil, the naturally occurring pyrmidine, inhibits the enzyme thymidylate synthase. After delivery to cells it is metabolised to nucleoside forms by the addition of ribose or deoxyribose by enzymes that normally act on uracil and thymine. Phosphorylation then leads to the active fluorinated nucleotides 5-FUTP and 5-FdUMP. One of the pathways responsible for the cytotoxic events associated with 5-FU occurs upon the catabolism of the false deoxyribotide –FdUMP. This competes with the naturally occurring molecule dUMP, resulting in inhibition of thymidylate synthase therefore depleting dTMP required for DNA synthesis through mis-incorporation of uracil leading to DNA strand breaks (Ladner et al., 2000).

Another major mechanism of action is through the incorporation of the nucleotide FUTP into RNA, particularly nuclear RNA. This apparently disturbs the normal maturing of nuclear, ribosomal and messenger RNA and may cause other errors of base pairing during transcription (Siu and Moore, 2005).

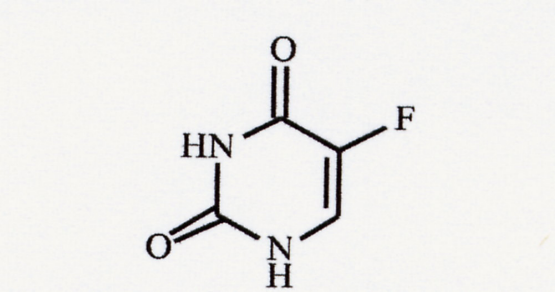


Figure 1.2.4.1 Structure of 5-Fluorouracil (Smith *et al.,* 2000)

5-FU is usually administered as a single dose by intravenous bolus injection because of its erratic bioavailability following oral treatment. Adverse events associated with 5-FU are significant and varied and a major problem in treating patients due to its lack of tumour specificity. They include myelosuppression, megaloblastic anaemia, leucopoenia, thrombocytopenia, alopecia and gastro-intestinal toxicities. To try to circumvent these problems, analogues of 5-FU have been developed, e.g. Capecitabine (Figure. 1.2.4.2). This can be administered orally and has better tumour specificity leading to a lower toxicity profile (Smith *et al.,* 2000).

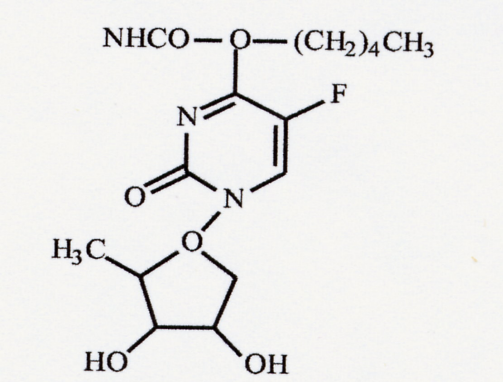


Figure 1.2.4.2. Structure of Capecitabine

1.3 Multidrug Resistance

The ability of tumour cells to develop cross resistance to many chemotherapeutic drugs (termed multidrug resistance or MDR) poses a major obstacle to successful chemotherapy. Its development relates to either inadequate drug exposure and or changes in cancer cells themselves. Poor drug bioavailability and distribution, insufficient dosage and poor tumour vascularisation account for why cells may not be exposed to effective doses of drugs during chemotherapy. An increase in drug efflux or

a decrease in drug uptake by cancer cells, increased drug metabolism and excretion, changes in drug metabolising enzymes and altered apoptotic genes in tumour cells may all contribute to reduced intracellular concentrations of anticancer drugs in tumour cells (Gaudiano *et al.,* 2000). Although chemotherapy drugs (anthracyclins, taxanes, vinca alkaloids) may have entirely different structures and mechanisms of cytotoxicity they share a common transport mechanism; they are actively pumped out of the cell as a consequence of expression of ABC transporter proteins. The ABC superfamily comprises several hundred different transport proteins specific for either single substrates or groups of related substrates (Moscow and Cowan, 1988; Gottesman, 1993). The genetic basis for the phenotypic expression of drug resistance has been elucidated in tumour cell lines for many drugs. Amplification of the *mdr1* gene in multidrug resistant tumour cells leads to a large overproduction of MDR1 protein, more commonly known as P-gp. It was the first eukaryotic ABC protein identified in 1976 and is a significant component of anthracycline resistance in tumour cells that showed resistance to tumour drugs. It uses the energy of ATP hydrolysis to export drugs from the cytosol to the extracellular medium. It is also involved in physiological functions, for example lipid transport and cholesterol trafficking (Johnstone *et al.,* 2000; Moscow and Cowan, 1988; Gottesman, 1993). Its expression on gut epithelia, liver cells, kidney tubules and at blood tissue barriers is consistent with drug removal role, but its selective expression in the adrenal gland, haematopoietic stem cells, natural killer cells, dendritic cells, T and B lymphocytes suggest P-gp also has important functions in immunology and apoptosis. Evidence suggests that P-gp can cause drug resistance by inhibiting caspases, downstream effectors of apoptosis (Moscow and Cowan, 1988; Gottesman, 1993).

Other ABC transporter proteins include MRP-1, a multidrug resistance associated protein (Schrenk *et al.,* 2001), Lung resistance-related protein (LRP) (Scheper *et al.,* 1993) and BCRP, breast cancer resistance protein Mao and Unadkat, 2005. MRP-1 shares homology with P-gp, although it has a more limited substrate specificity (Seelig *et al.,* 2000). Lung resistance-related protein (LRP) is a major vault protein widely distributed in normal tissues, and over-expressed in multidrug-resistant cancer cells.

LRP was originally found to be over-expressed in a non small cell lung cancer cell line selected for doxorubicin resistance that did not express P-gp (Scheper *et al.,* 1993). The *LRP* gene encodes a 110 kDa multi-subunit with a barrel-like structure thought to be involved in nucleo-cytoplasmic transport. Substrates for LRP include doxorubicin, cisplatin, etoposide and mitoxantrone, although transport of such substrates is also dependent on expression of associated minor vault proteins such as vault poly-(ADP-ribose) polymerase (VPARP) and telomerase-associated protein (TEP1) (Mossink *et al.,* 2003). Breast cancer resistance protein (BCRP or ABCG-2) is an ABC transporter protein that is expressed and functionally active in the mitochondria of MDR-positive cancer cells (Solazzo *et al.,* 2009). As the name suggests, this 72 kDa protein was first discovered in breast cancer cells, specifically the MCF-7 cell line, and has since been reported in colon, gastric and ovarian carcinoma and fibrosarcoma cells. Its substrates include anthracyclins, mitoxantrone, camptothecins and methotrexate (Mao and Unadkat, 2005).

Glutathione upregulation represents another common mechanism of resistance in tumour cells. This endogenous antioxidant is believed to cause resistance by providing protection against free radical damage (Lutzky *et al.,* 1989). A related mechanism relies on the ability of resistant tumour cells to efficiently promote glutathione S-transferase (GST)-catalyzed GSH conjugation of antitumour drugs (O' Brien and Tew, 1996; Tew, 1994). Although Gaudiano *et al.* (2000) failed to demonstrate GSH conjugation in doxorubicin-resistant human breast cancer MCF-7/DOX cells, there was however a 14-fold increase of the GST P1–1 activity in resistant cells relative to the sensitive MCF-7 cells suggesting that the overexpression of GST P1–1 in MCF-7/DOX cells may be an endogenous defense against oxidative stress induced by anthracyclines. Liu and colleagues (2001) suggested that GST P1–1 functions together with drug efflux pumps such as MRP-1, MRP-2 and/or P-gp to reduce cellular cytotoxic drug accumulation. Similar observations were reported by Harbottle and coworkers (2001) in HEp2 human cancer cells.

Other mechanisms for chemoresistance relate to changes in topoisomerase II activity, to expression of important apoptosis-associated proteins such as bcl-2 family of proteins (Reed, 1995; Clynes *et al.,* 1998), the tumour suppressor protein p53 (Mueller and Eppenberger, 1996), the synthesis of vaults (Kickhoefer *et al.,* 1998), overexpression of caveolae (Lavie *et al.,* 1998) and dysfunctional metabolism of ceramide (Senchenkov *et al.,* 2001).

Many attempts have been made to use current knowledge of drug resistance mechanisms to reverse MDR in cancer and to extend patient survival. Agents which inhibit cancer resistance mechanisms are termed resistance modulators or chemosensitising agents. These include pump inhibitors (e.g. cyclosporin A, verapamil, resveratrol, quercetin), pump bypass agents (inclusive drugs) i.e. drugs that are not substrates of drug transporters (e.g. flavopridol, epothilone, inhibitors of MAPK and PI3K signalling pathways) and exclusive protectors (a protective agent that is a substrate of drug transporters and therefore retained at a higher level in normal cells; e.g. caspase inhibitor Z-DEVD-fmk, low dose anthracyclines) (Blagosklonny, 2003). Specifications for an agent that would be therapeutically most useful to modulate MDR were listed by Clynes *et al.* (1998) and include i) it should inhibit at a low and physiologically relevant concentration, ii) be selectively toxic for tumour tissue, iii) irreversibly inhibit the resistance mechanism, iv) permeate through all cells in a tumour at an effective concentration, v) have no significant effect on the pharmacokinetics of chemotherapy drugs, vi) be non toxic on its own, vii) be available as a pure chemical with well described consistent pharmacokinetics and viii) it should not increase the toxicity of the chemotherapy in other parts of the body.

1.4 Improving Chemotherapy

While cancer continues to be a leading cause of morbidity and mortality, there will

always be room for improvement in cancer chemotherapy; the need is obvious and has been stated at length in previous sections. Numerous studies have focused on trying to improve the efficacy of the anti-cancer drugs in present use. One such study tested the potential of a number of non steroidal anti-inflammatory drugs (NSAIDs) (indomethacin, sulindac, tolmetin, acemetacin, zomepirac, mefenamic acid, meclofenamic acid, diclofenac, naproxen, fenoprofen, phenylbutazone, flufenamic acid, flurbiprofen, ibuprofen and ketoprofen) to enhance the cytotoxic effect of a catalogue of chemotherapeutic agents. Specific NSAIDs (including indomethacin and sulindac) enhanced the efficacy of the anthracycline group and structurally related anti-cancer drugs. These drugs are substrates for MRP-1 (multidrug resistance related protein). The synergistic combination was effective in cell lines in which the multi-drug resistance (MDR) was due to MRP-1 over-expression and in drug sensitive cell lines that were shown to express MRP (Duffy *et al.,* 1998). Other groups have more recently directed their attention toward various polyunsaturated fatty acids (PUFA).

1.5 Polyunsaturated Fatty Acids (PUFA)

1.5.1 Enhancement of chemotherapy cytotoxicity by PUFA

Experimental studies in animal models of chemical carcinogenesis, animals bearing transplanted tumours and in animals representing genetic models of tumourigenesis have indicated that dietary treatments with omega-3 (n-3) polyunsaturated fatty acids (PUFA) exert anti-tumoural effects. Similarly, research has shown that n-3 PUFA inhibited the growth of various cancer cells in culture (Guthrie and Carroll, 1999).

Numerous studies have shown that n-3 PUFA protect against carcinogenesis, help prevent formation and growth of aberrant crypt foci in the colon, and inhibit tumour metastasis including that of mammary tumours (Bougnoux, 1999). While the growth of

tumors is inhibited in animals fed diets rich in omega-3 fish oils, it is often reported that tumour growth is stimulated by diets rich in n-6 vegetable oils, particularly Linoleic acid. However, exceptions exist in the n-6 family of PUFA's that show great promise as antitumour agents, two of which are γ-linolenic acid (GLA) and an isomer of linoleic acid (LA) known as conjugated linoleic acid (CLA) (Burns and Spector, 1993).

The fact that certain essential fatty acids (EFA's) and their derivatives, of both the n-3 and n-6 series show selective cytotoxicity without harming normal cells, makes them ideal candidates for further research as possible chemo-sensitisers (Burns and Spector, 1994). For example, Das *et al.* (1998) found that not only did various EFA's (γ-linolenic acid (GLA), eicosapentanoic acid (EPA), arachidonic acid (AA) and docosahexanoic acid (DHA)) enhance chemotherapy drug efficacy in cancer cell lines, they also increased the sensitivity of drug-resistant cells (KB-Ch^R-8-5 (HeLa variant) human cervical carcinoma cells) to the cytotoxic action of vincristine *in vitro.* Further, it was found that these fatty acids and DGLA (dihomo gamma-linolenic acid) also increased the uptake and decreased the efflux of radiolabelled vincristine in KB-3-1 (HeLa variant), KB-Ch^R-8-5 and HeLa cell lines.

Tsai *et al.* (1997) found EPA increased the sensitivity of the tumourigenic cell line derived from immune mouse fibroblastic NIH3T3 cells to Mitomycin C; also that EPA demonstrated a protective function against Mitomycin C in the non-malignant variant of the NIH3T3 cell line. DHA was shown to enhance doxorubicin cytotoxicity in MDA-MB-231 breast cancer cells by Germain *et al.* (1998). This effect was nullified by Vitamin E, indicating the role of lipid oxidation pathways. It has also been shown by Bougnoux *et al.* (1999) that an increased level of DHA in breast adipose tissue directly correlated with increased responses (both partial and complete) to chemotherapy.

Animal studies have also corroborated these findings. In one such study, human A549 lung cancer cells were implanted subcutaneously on the backs of nude mice. Following an incubation period to allow the tumours to develop and grow, the mice were divided

into groups fed on diets containing 20 % corn oil, or 19 % fish oil/1 % corn oil. They were then sub-divided into those treated with/without doxorubicin. It was found that there was significant tumour regression in the mice that consumed fish oil diets before and during Dox treatment, but not in the mice that consumed corn oil diets before and during Dox treatment (Hardman *et al.*, 2000). Subsequent studies by this group showed that fish oil concentrate increased efficacy of Dox against MDA-MB-231 human breast cancer xenografts in athymic mice (Hardman *et al.*, 2001) and that the cytotoxic action of these fatty acids is through mechanisms including lipid peroxidation, although suppression of glutathione peroxidase (GPX) was found to be a more important factor in increasing sensitivity to Dox (Hardman *et al.*, 2005).

Furthermore, Hardman *et al.* (2002) demonstrated that a concentrated omega-3 product containing 55 % EPA at 2 % in the diet ameliorated the harmful side-effects of CPT-11 chemotherapy in mice. Beneficial effects were obtained with a volume of concentrate that was lower than a 6 % fish oil diet that contained the same amount of EPA and DHA. The ability of fish oils to enhance the antitumour activity of CPT-11 in breast carcinoma xenografts was confirmed in a separate study in rats bearing the Ward colon tumour (Xu *et al.,* 2007). Colas *et al.* (2006) also showed that dietary DHA supplementation turned a malignant tumour from being anthracycline-resistant to anthracycline-sensitive.

In experiments conducted by Davies *et al.* (1999) it was found that cellular uptake of various anthracyclines including doxorubicin increased following a 24 h incubation of multidrug resistant MGH-U1/R bladder and MCF-7/R breast cancer cells with GLA. GLA was administered as the clinical formulation Lithium-GLA (LiGLA) at 20 μg/ml, which was a sub-lethal dose. These results demonstrate that one of the mechanisms of action in enhancement of drug cytotoxicity is by increasing drug uptake into the cells thereby increasing intracellular concentrations of the drug. Menendez *et al.* (2002), found that co-incubation with GLA in various combinations enhanced the cytotoxic action of vinorelbine in breast cancer cell lines. Potential synergy was evaluated following exposure of cells simultaneously to vinorelbine and GLA, or sequentially to

GLA followed by vinorelbine. The cell lines used were MDA-MB231, T47D and SK-Br3 breast cancer cells. In all cell lines it was found that the level of synergism was independent of treatment schedule and exposure time.

More recently, a soluble fatty acid formulation (meglumine EPA,) was demonstrated to enhance the efficacy of both epirubicin and mitomycin in bladder cancer cells *in vitro* (Mackie *et al.,* 2006). It is apparent that conjugation of fatty acid with glutamine to form soluble quaternary amine complexes has potential to improve treatment regimes based on unstable highly oxidisable PUFA like EPA.

1.5.2 Mechanisms of Action of PUFA

Several studies have implicated lipid peroxidation as one mechanism by which n-3 PUFA potentiate the cytotoxic action of chemotherapy drugs. In the study by Germain and colleagues (1998), described above it was concluded that the increased cytotoxicity of doxorubicin by DHA in human breast cancer cells *in vitro* was due to oxidative stress which was reduced by the addition of α-tocopherol. Similarly the increased sensitisation by DHA of chemically induced mammary carcinoma to epirubicin in Sprague-Dawley rats was cancelled out by the addition of α-tocopherol to the diet (Colas *et al.,* 2006).

n-3 PUFA have been shown to cause a reduction in the production of eicosanoids from arachidonic acid (AA) (Rose *et al.,* 1995). Production of such eicosanoids (e.g. prostaglandin E_2) has been shown to be up-regulated in cancer cells (Karmali, 1987). EPA has the ability to compete with AA for the enzymes cyclooxygenase (COX) and lipooxygenase (LOX) which are involved in the production of these eicosanoids, resulting in the production of compounds which are less biologically active and in some cases antiproliferative in cancer cells (Yang *et al.,* 2004).

PUFA are the basic constituents of membrane phospholipids and their alterations in membranes may modify cellular membrane fluidity and consequently modulate enzyme activities, carriers and membrane receptors (Calviello *et al.,* 2006). DHA incorporation into the plasma membrane of T27A murine leukaemia cells has been shown to enhance membrane permeability (Stillwell *et al.,* 1993) and alter membrane structure, increasing susceptibility of the cells to destruction by the immune system (Pascale *et al.,* 1993). Supplementing the diet of mice with fish oil markedly altered the lipid composition of colonic caveolae/lipid rafts and EPA and DHA were incorporated in the fatty acyl groups of caveola phospholipids and decreased the caveolar content of cholesterol and caveolin-1, an important structural component. Perturbation of these lipid micro-domains was found to displace resident proteins H-Ras but not K-Ras in colonic caveolae both *in vitro* and *in vivo*, altering their functionality and subsequent downstream signalling (Ma *et al.,* 2004).

Several transcription factors such as NFκB have been shown to be modulated by n-3 fatty acids. Constitutive activation of NFκB has been implicated in the progression of cancer in humans having roles in the regulation of cell proliferation, migration and apoptosis (Calviello *et al.,* 2006). EPA and DHA have been shown to down-regulate NFκB activity in various cell lines including human T-cells, colon and prostate cancer cells (Denys *et al.,* 2005; Narayanan *et al.,* 2003; Ross *et al.,* 2003) and in kidney cells of transgenic rats (Theuer *et al.,* 2005). Several studies now indicate that inhibition of NFκB can sensitise tumour cells to chemotherapeutic drugs (Reviewed in Biondo *et al.,* 2008). n-3 PUFA have also been reported to modulate the activity of other transcription factors such as peroxisome proliferator activated receptors (PPAR), p53, c-myc AP-1, HIF-1 and retinoid X receptors (RXR) (Calviello *et al.,* 2006).

Recent evidence suggests that the phosphoinositol 3-kinase (PI3K)/Akt signalling pathway is constitutively active in many types of human cancer and overexpression has

been linked to development of multi-drug resistance. EPA and DHA have been demonstrated to inhibit Akt phosphorylation and activation in various types of cancer cells, thereby explaining in part the increased chemotherapeutic response by these fatty acids in tumour cells (Biondo *et al.,* 2008). Another protein kinase overexpressed in various mammary cancers is mitogen activated protein kinase (MAPK). n-3 PUFA are reported to decrease phosphorylation and activity of the MAPK extracellularly regulated kinases (ERK) in tumour cells (Calviello *et al.,* 2006).

It is widely accepted that human cancer cells have the ability to synthesise their own supply of fatty acids, seemingly independent of the regulatory signals that down-regulate fatty acid synthesis in normal cells (Kuhajda, 2000). Fatty acid synthase (FAS), previously known as oncogenic antigen 519 (OA-519), is a key enzyme in the *de novo* synthesis of fatty acids and has been identified as a tumour marker in breast cancer indicating a poor prognosis (Kuhajada *et al.,* 1989; 1994). FAS is down-regulated in normal human cells by the intake of small amounts of fat in the diet, but is highly expressed in human cancers and is probably one of the most common molecular changes in cancer cells (Kuhajda, 2000; Menendez *et al.,* 2005). In fact it has recently been discovered that elevated FAS expression contributes to increased drug resistance in breast cancer cells (Liu *et al.,* 2008). DHA and alpha-linolenic acid (ALA) have been found to inhibit FAS activity in human breast cancer cells (Menendez *et al.,* 2004).

Other modulatory effects of DHA and EPA which explain an increased sensitivity to chemotherapy drugs in tumour cells include an increase in the induction of apoptosis through reduction in expression of the anti-apoptotic protein bcl-2 (Chiu *et al.,* 1999; 2004). In addition, DHA treatment increased expression of the pro-apoptotic protein Bax in HL-60 human leukaemia cells (Chiu *et al.,* 2004) while EPA induced apoptosis and increased caspase 3 activation in human pancreatic cell lines (Shirota et al., 2005). Work by Calviello and colleagues (2005), supports the hypothesis that n-3 PUFA enhance the pro-apoptotic effect of anticancer drugs. DHA was shown to increase the cytotoxicity of 5-FU in a colon cancer cell line by decreased expression of anti-apoptotic

proteins bcl-2 and bcl-X_L. An understanding of the mechanisms of action of n-3 PUFA in combination therapy will drive translational research towards developing their use as dietary adjuvants to chemotherapy for patients.

1.6 Conjugated Linoleic Acids (CLA)

1.6.1 CLA Structure and Origin

Conjugated linoleic acid (CLA) refers to a group of polyunsaturated fatty acids that exist as positional and geometric isomers of the n-6 essential fatty acid linoleic acid (LA) (*c*9,*c*12; octadecadienoic acid), which contain a conjugated double bond system and occur naturally in edible fats derived from ruminant animals. Several isomers of CLA exist depending on which double bonds are relocated to either *cis* or *trans* configurations. The *c*9,*t*11 and *t*10,*c*12-CLA isomers account for up to 90 % of synthetic CLA mixtures combined (Lin *et al*., 1995).

*c*9,*t*11-CLA is the predominant isomer naturally occurring in dairy products constituting up to 90 % of the total CLA present, with *t*10,*c*12-CLA making up the majority of the remainder. CLA content in food varies from 0.2 mg/g in corn and peanut oils, 17 mg/g in beef and 30 mg/g in milk fat (O'Shea *et al*., 1998). The CLA content of milk can be improved greatly when dairy cows are fed supplemental fish and vegetable (safflower) oils (Murphy *et al.,* 2008).

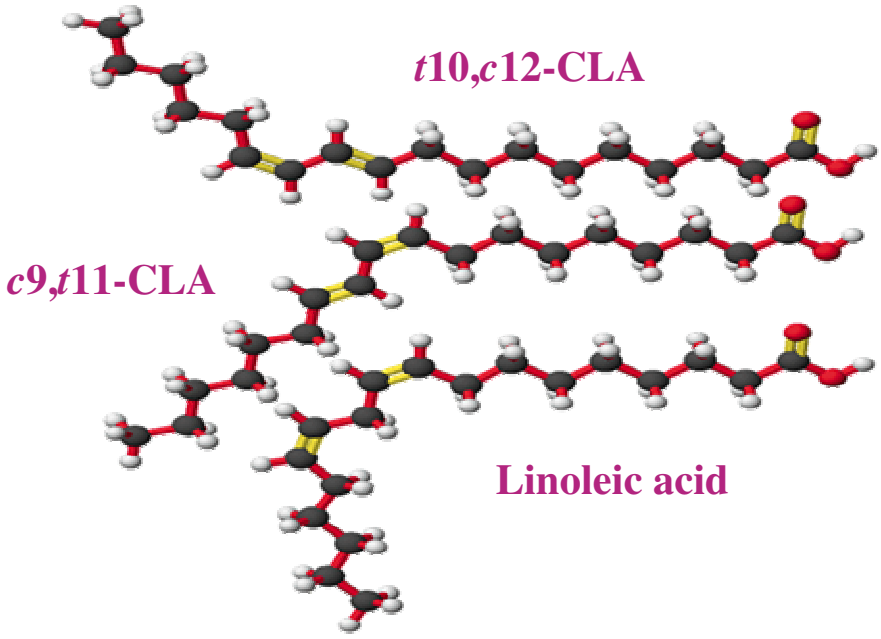


Figure 1.6.1.1 Structure of *c*9,*t*11 and *t*10,*c*12-CLA and Linoleic acid (LA) (Pariza, 2001).

*c*9,*t*11-CLA is formed in the rumen as an intermediate product in the biohydrogenation of LA by linoleic acid isomerase enzyme of the anaerobic bacteria *Butyrivibrio fibrisolvens* (Kepler et al., 1970). More recently it has also been found to be produced in tissues by the desaturation of *trans*-vaccenic acid (*t*-VA) by Δ9 desaturase (Yurawecz *et al.*, 1998). This was confirmed by Miller and co-workers in breast and colon cancer cells (2003) and more recently by Reynolds *et al.* (2008), in Caco2 colon cancer cells.

Other isomers are formed at much lower amounts, such as the *t*10,*c*12-CLA isomer which is produced when the pH in the rumen drops with a low fibre diet (Griinari *et al.*, 1998). A second pathway involving the bacterium *Megasphaera elsdenii* YJ-4 is responsible for producing the *t*10,*c*12-CLA isomer from LA (Kim *et al.*, 2002). Figure 1.6.1.2 illustrates the production of *c*9,*t*11-CLA from LA in the rumen and vaccenic acid in the tissues and the role of Δ9-desaturase (Bauman and Griinari, 2000).

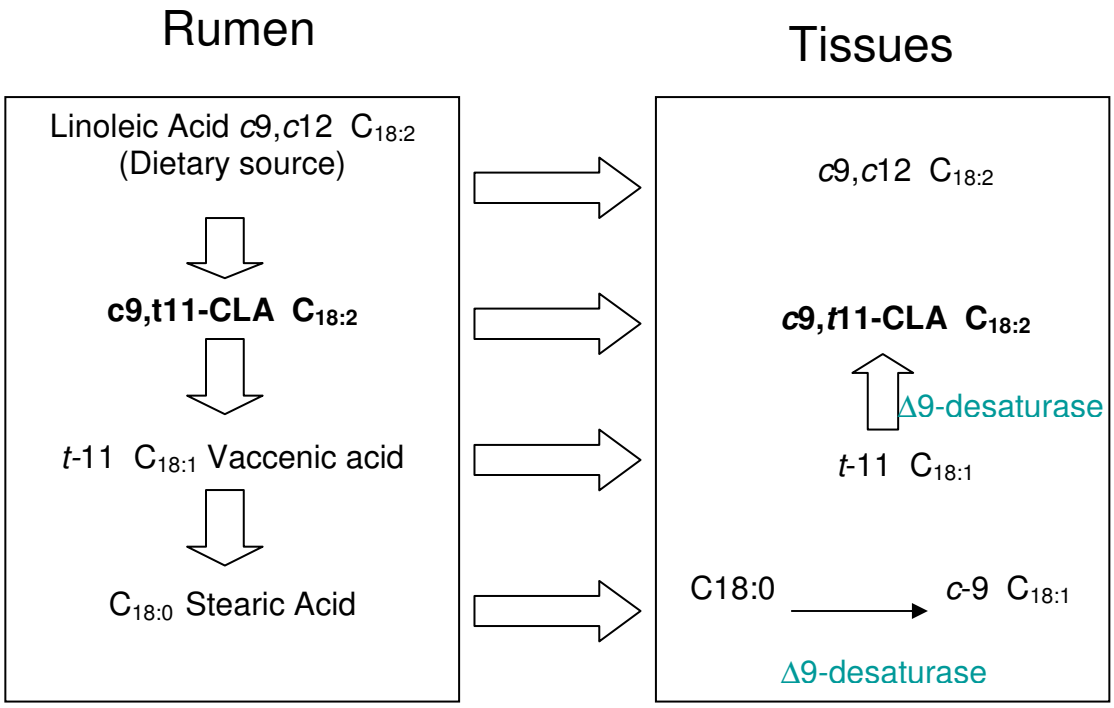


Figure 1.6.1.2 Production of *c*9,*t*11-CLA from LA in the rumen and vaccenic acid in the tissues. Also showing the role of Δ9-desaturase. (Adapted from Bauman and Griinari, 2000)

A dairy diet represents the main source of CLA in humans (McGuire *et al.,* 1999), although CLA, principally *c*9,*t*11-CLA, has recently been reported to be produced by intestinal *Bifidobacteria* from free linoleic acid (Coakley *et al.,* 2006). This was postulated to account for the beneficial effects of bifidobacteria present in the human gastrointestinal tract. Dietary intake of CLA has been estimated to range between 52-350 mg CLA/day in women and 137-430 mg CLA/day in men (Ritzenthaler *et al.,* 1998; Fritsche and Steinhart, 1998; Ens *et al.,* 2001). However, one study in Australia estimated a daily intake of up to 1500 mg CLA by its participants (Parodi *et al.,* 2003).

1.6.3 Biological Effects of CLA and Mechanisms of Action

CLA was first found to have anticarcinogenic properties when extracted from cooked minced beef (Ha *et al.*, 1987). Since then CLA has been shown to exhibit a number of physiological effects in animal models, having activity as an anti-adipogenic agent (Park *et al.,* 1997), an anti-atherogenic agent (Lee *et al.,* 1994; Navarro *et al.,* 2007) and having immuno modulatory effects (Cook *et al.,* 1993; Miller *et al.,* 1994; Loscher *et al.,* 2005; Reynolds *et al.,* 2008). Clement and Margot Ip and co-workers have reported extensively on the anticancer effects of CLA in various rat and murine mammary cancer models *in vitro* and *in vivo* (Ip *et al.,* 1991, 1994, 1995, 1996, 1997ab, 1999ab, 2000 and 2001). Dietary supplementation with 1 % CLA in the diet has been shown to inhibit chemically induced cancer development in mammary glands of rats. When CLA was administered to pre-pubescent animals, i.e. during active morphogenesis of the mammary gland from weaning at 21 days to 50 d there was persistent protection against subsequent tumour development following withdrawal of CLA from the diet (Ip *et al.,* 1995). However, when they administered CLA to a more mature rat model at 55 d, it was found that a continuous intake of CLA was necessary to inhibit tumour development (Thompson *et al.,* 1997). More recently they reported that the *t*10,*c*12-CLA isomer induced apoptosis and G_1 arrest of p53 mutant TM4t murine mammary tumour cells (Ou *et al.,* 2007). Chemically-induced cancer in the mammary glands of rodents has proved to be a good model to replicate breast cancer tumourigenesis in humans as both occur primarily in the undifferentiated, rapidly proliferating epithelium at the distal end of the terminal end buds and terminal ducts of breast tissue (Medina, 1996).

CLA induced apoptosis of murine mammary tumour cells via release of cytochrome c from the mitochondria to the cytosol, decreased expression of anti-apoptotic protein Bcl-2 and activation of the caspase-9/caspase-3 proteolytic cascade (Ou *et al.,* 2007). Other studies have also demonstrated a similar reduction in Bcl-2 levels in colon (Miller *et al.,* 2002), prostate (Ochoa *et al.,* 2004) and breast cancer cell lines (Majumder *et al.,* 2002).

In MDA-MB-231 breast cancer cells the anti-proliferative effect of CLA was associated with up-regulation of the pro-apoptotic protein Bak, reduced levels of anti-apoptotic Bcl-x_L, release of cytochrome c from the mitochondria, cleavage of pro-caspase-9 and pro-caspase-3 and finally a reduction in ERK1/2 leading to interruption of ERK/MAPK signalling (Miglietta *et al.,* 2006).

Dietary CLA has been shown to inhibit angiogenesis *in vivo* and *in vitro*, and to decrease vascular endothelial growth factor (VEGF) and its receptor, Flk-1 concentrations in the mouse mammary gland. The *t*10,*c*12-CLA isomer was demonstrated to have the additional ability to decrease serum leptin, a proangiogenic growth factor in CD2F1Cr mice. It is widely accepted that the ability of CLA isomers to inhibit angiogenesis *in vivo* may contribute to their ability to inhibit carcinogenesis (Masso-Welch *et al.,* 2004).

ErbB2 (HER2) and ErbB3 have been implicated in the development of various cancers. In a study in the HT-29 colon cancer cell line, CLA decreased ErbB2 and ErbB3 mRNA and protein levels in a dose-dependent manner. Cho *et al.* (2003) concluded that CLA inhibits cell proliferation and stimulates apoptosis in HT-29 cells by its ability to downregulate ErbB3 signalling and the PI3-kinase/Akt pathway.

Tanmahasamut and colleagues (2004) demonstrated that CLA inhibited growth of the oestrogen receptor (ER)-positive breast cancer cell line MCF-7, but not ER- negative MDA-MB-231 breast cancer cells. This was attributed to the ability of CLA to directly affect oestrogenic activity in the ER positive cells, down regulating ERα expression at both mRNA and protein levels and interfering with binding of a nuclear protein to the oestrogen response element.

In addition to the anticarcinogenic effects, CLA has demonstrated other positive health benefits. CLA was first found to have immune modulatory effects in chickens and rats (Cook *et al.,* 1993). Since then, Loscher and colleagues demonstrated that *c*9,*t*11-CLA suppressed NF-κB activation and enhanced transcription and production of the anti-

inflammatory cytokine IL-10 in dendritic cells, while inhibiting the Th1-promoting cytokine IL-12 (Loscher *et al.,* 2005). As described above constitutive activation of NFκB has been implicated in the progression of cancer in humans having roles in the regulation of cell proliferation, migration and apoptosis, therefore suppression of NFκB activity may explain in part the anticarcinogenic effects of CLA. This was supported by Hwang and co-workers (2007), in a study which found that CLA inhibited NF-κB driven-COX-2 expression by blocking the IκK and PI3K-Akt signalling in hairless mouse skin *in vivo*.

It has also been shown that inhibition of lipid accumulation induced by *t*10,*c*12-CLA treatment during adipocyte differentiation is associated with a tight regulatory cross-talk between early (PPARγ and CCAAT/enhancer binding protein a (C/EBPa)) and late (liver X receptor a; a fatty acid binding protein, aP2; CD36, a fatty acid translocase; adipogenic marker genes (Granlund *et al.,* 2005). Activation of PPAR gamma and delta by conjugated linoleic acid was also found to have a protective effect in experimental inflammatory bowel disease in mice (Bassaganya-Riera *et al.,* 2004).

In a study of the effect of CLA on atherosclerosis in cholesterol-fed rabbits, the LDL cholesterol to HDL cholesterol ratio and total cholesterol to HDL cholesterol ratio were significantly reduced in CLA-fed rabbits. Also, examination of the aortas of CLA-fed rabbits showed less atherosclerosis than controls (Lee *et al.,* 1994). *t*10,*c*12-CLA feeding in hypercholesterolaemic hamsters was demonstrated to reduce cholesterol esterification in liver and decrease serum VLDL-cholesterol, but did not result in a hypocholesterolaemic effect (Navarro *et al.,* 2007).

It has been suggested that CLA competes with LA for desaturation and elongation enzymes involved in the conversion of long chain metabolites. These same enzymes are involved in the metabolism of n-3 fatty α-linolenic acid, ALA. Elongation and desaturase metabolites of CLA, notable for their conservation of the conjugated diene

structure, have been detected and identified by Banni and coworkers in various tissues including mammary tissue, rat liver and lamb tissue (1996, 1999 and 2001). In rat liver and adipose tissue *c*9,*t*11-CLA was found to be mainly converted into a C20:3 conjugated fatty acid, while the *t*10,*c*12-CLA isomer was metabolised into a conjugated C18:3 or C16:3 fatty acid (Sebedio *et al.,* 2001). The presence of these metabolites in different lipid fractions of tissues (Banni *et al.,* 2004) may account for some of the differing and sometimes conflicting physiological effects of the different CLA isomers.

These diverse physiological actions of CLA make it an ideal candidate for further study of the anticancer potential of CLA in multi-drug resistant tumours. The work described in this thesis is the first study to test the efficacy of CLA alone or in combination with chemotherapeutic drugs in drug-resistant cancer cell lines. Following an evaluation of the cytotoxic effects of PUFA and doxorubicin (Dox) as single agents, the overall objective of this project was to characterise the effects of co-administering PUFA including CLA with the anticancer drug Dox in a cellular model of multi-drug resistant cancer and to determine mechanisms by which fatty acids may modulate drug resistance or sensitivity.

1.7 Thesis Objectives

- To characterise the cytotoxic effects of single treatments of various PUFA including CLA and three chemotherapeutic agents (Doxorubicin, Taxotere and 5-Fluorouracil) in drug sensitive and resistant cancer cell lines using a cost effective high through-put cytotoxicity assay.

- To evaluate the potential of co-treatment with a commercial mixture of CLA isomers (CLA-mix) and two single isomers *c*9,*t*11-CLA and *t*10,*c*12-CLA to enhance the cytotoxic effects of Doxorubicin (Dox) on a drug naive MDA-MB435-S-F (MDA) melanoma cell line and a multidrug resistant variant MDA-MB435-S-F/Adr10p10p (A10p10p).

- To determine the effect of CLA-mix, *c*9,*t*11 and *t*10,*c*12-CLA on net uptake/accumulation of Dox in cells and retention after efflux by liquid chromatography tandem mass spectrometry (LC-MS) methods.

- To determine differences if any between ceramide and basal sphingosine levels in untreated MDA and A10p10p cells and investigate the effects of CLA, EPA and DHA, alone and in combination with Dox, on basal sphingosine and ceramide levels in both cell lines.

- To measure and compare the expression of her2/neu expression in untreated MDA and A10p10p cells and elucidate the effects of CLA, EPA and DHA treatments on her2/neu expression in both cell lines.

- To determine the differences if any between the lipid profiles of untreated MDA and A10p10p cells and investigate the effects of CLA, EPA and DHA, on the total cellular lipid fatty acid profiles of both MDA and A10p10p cell lines.

Chapter 2

A comparative study of effects of conjugated linoleic acid and three chemotherapeutic drugs on proliferation of human cancer cells.

2.0 Abstract

Conjugated linoleic acid (CLA) has been shown to reduce tumour incidence and progression *in vivo* and to be cytotoxic toward a broad range of tumour cells *in vitro* including breast, colon, prostate and skin cancer cell lines. The effect of CLA on drug resistant tumour cells however, has yet to be characterised. The aim of this work was to characterise the effects of three preparations of CLA (*c*9,*t*11 and *t*10,*c*12-CLA isomers and a synthetic mixture of CLA isomers) complexed with BSA or as free fatty acids and three common chemotherapy drugs (taxotere, doxorubicin and 5-fluorouracil) in cellular models of clinically significant drug-resistant cancer. Cytotoxicity was determined by MTS and acid phosphatase assays and expressed as percentage inhibition relative to control. Significant inhibition ($p<0.05$) of cell growth was observed after 5 and 7 days of treatments with each CLA preparation. Cytotoxicity of CLA was dependent on isomer composition, concentration and duration of exposure. MCF-7 cisplatin resistant cells were more sensitive to inhibition by *t*10,*c*12-CLA and the CLA mixture than parental MCF-7 cells. Drug resistance of MDA-MB435S-F/Tax10p (Tax10p) and MDA-MB435S-F/Tax10p4p (Tax10p4p) was demonstrated only when cells were treated with taxotere, doxorubicin and 5-fluorouracil in a short term exposure assay; negligible differences were observed following long term drug exposure. There was no significant difference in sensitivities of the drug naïve MDA-MB435S-F (MDA) cancer cell line and two taxol pulsed, drug resistant variants Tax10p and Tax10p4p to the CLA mixture of isomers. MDA cells however, were more sensitive to inhibition by *c*9,*t*11 and *t*10,*c*12-CLA at concentrations exceeding 12 μg/mL than the Tax10p or Tax10p4p cell lines. Toxicity data obtained in this chapter will be used in the design of an experiment using sub-optimal concentrations of both fatty acids and drug to investigate synergistic interactions in melanoma cells.

2.1 Introduction

Several isomers of CLA both in pure form and as a mixture have been shown to be cytotoxic at micromolar concentrations for a variety of breast, colon, prostate and skin tumour cell lines (Belury, 2002). Cytotoxicity of CLA was found to be concentration-dependent and isomer specific in the MCF-7 human breast cancer cell line (O'Shea *et al.,* 2000). Cell numbers were significantly reduced following treatment for 8 days with synthetic preparations of a mixture of CLA isomers and the single isomer *c*9,*t*11 -CLA; and CLA enriched milk fat. Interestingly, while the *c*9,*t*11-CLA isomer, mixture of CLA isomers and CLA enriched milk fat were found to significantly reduce cell numbers, treatment with the *t*10,*c*12-CLA isomer resulted in no significant difference relative to untreated controls. Linoleic acid (LA) was also tested in this study and found to increase cell numbers. The same authors found in another study that CLA-induced cytotoxicity against MCF-7 and the colon cancer cell line SW480 was related to the extent of lipid peroxidation of CLA treated cells; CLA-induced antioxidant enzymes failed to protect these cells from cytotoxic lipid peroxidation products (O'Shea *et al.,* 1999).

The overall objective of the work described in this chapter was to assess in a pilot study the antiproliferative effects of various preparations of CLA in two *in vitro* models of drug-resistant cancer, MCF-7 and a cisplatin-resistant variant MCF-7/cis and MDA-MB-435S-F (MDA) and two taxol-resistant variants MDA-MB-435S-F/Taxol-10p (Tax10p) and MDA-MB-435S-F/Taxol-10p4p (Tax10p4p). MCF-7 and MDA cell lines and variants have been shown previously to be ER positive cell lines (Tanmahasamut *et al.,* 2004; Glynn *et al.,* 2004). There is a consensus in current scientific literature that the MDA cell line has been mis-identified as a metastatic breast cancer line for over 25 years. It is now known to express several genes commonly transcribed in melanocytes but not in commonly used breast cancer cell lines (Rae *et al.,* 2004; 2007). For this reason, the MDA-MB-435 cell line is designated a melanoma cell line in this thesis.

This is the first study to examine the effects of CLA in drug-resistant cancer cell lines. The growth inhibitory effects of three common chemotherapeutic drugs taxotere, 5-fluorouracil and doxorubicin were also evaluated in the three MDA derived cell lines. Toxicity data obtained in this chapter was used in the design of an experiment using sub-optimal concentrations of both fatty acids and drug to investigate synergistic interactions in human cancer cells.

2.1.1 Specific Objectives

- To compare the effects of three preparations of CLA (*c*9,*t*11 and *t*10,*c*12-CLA isomers and a synthetic mixture of CLA isomers) and LA, delivered as sodium salts complexed with bovine serum albumin (BSA) in a 2:1 w/w ratio, on an MCF-7 breast cancer cell line and the cisplatin resistant variant, MCF-7/cis using the MTS microplate assay.

- To establish and optimise a more cost effective high through-put cytotoxicity assay and to investigate an alternative cancer cell line whose drug resistance is better characterised.

- To determine the cytotoxic effects of single treatments of five fatty acids (three preparations of CLA, LA and GLA) complexed with bovine serum albumin (BSA) in a 2:1 molar ratio and three chemotherapeutic agents (Doxorubicin, Taxotere and 5-Fluorouracil) on three MDA melanoma cell lines (MDA, Tax10p and Tax10p4p) using the Acid Phosphatase assay.

- To determine the cytotoxic effects of the above fatty acids without BSA i.e. as free fatty acid solutions to eliminate a source of variation in the system.

- To determine the cytotoxic effects of the above anticancer drugs in a more clinically relevant assay i.e. to run the assays with a short term exposure (4 h exposure to the drug, with a 7 day recovery) in place of long term (7 day) exposure.

- To design an experimental model based on IC_{50} values, to elucidate the possible synergistic interactions between CLA and Dox in combination treatments.

2.2 Materials and Methods

2.2.1 Materials

Cell culture media, supplements and related solutions were purchased from Sigma-Aldrich, Dublin, Ireland, unless otherwise stated. The MCF-7 human breast cancer cell line was obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA). MCF-7/cis a cisplatin-resistant variant of MCF-7 was developed and gifted by Dr. Yizheng Liang (formerly of National Institute of Cellular Biotechnology (NICB), Dublin, Ireland). The MDA-MB-435-S-F (MDA) human cancer cell line and its taxol-resistant variants MDA-MB-435-S-F/ Taxol 10p (Tax10p) and MDA-MB-435-S-F/ Taxol 10p4p (Tax10p4p) were developed and kindly donated by Dr. Sharon Glynn (formerly of NICB, Dublin). Chemotherapy agents Doxorubicin (Dox), 5-Fluorouracil (5-FU) [Farmitalia Carlo Erba, Milton Keynes, UK] and Taxotere (Tax) [Aventis, France] were kindly donated by Dr. O'Connor (NICB, Dublin).

Conjugated Linoleic Acid (CLA) mixture of isomers (99% pure, approximately comprising: 44 % *t*10,*c*12-CLA; 41 % *c*9,*t*11/*t*9,*c*11-CLA; 10 % *c*10,*c*12-CLA and minor amounts of *t*9,*t*11; *t*10,*t*12 & *c*9,*c*11-CLA) (Cat: UC-59A) and single preparations (90 % pure) of isomers *c*9,*t*11- and *t*10,*c*12-CLA (Cat: UC-60A, UC-61A) were from NuChek-Prep, Elysian, MN, USA. Linoleic Acid (LA) and γ-Linolenic Acid (GLA) were purchased from Sigma-Aldrich, Dublin (Cat: L1012; L2378). All fatty acid preparations were dissolved in sterile filtered ethanol: 1 g fatty acid in 10 mL ethanol to yield solutions of 99 mg/mL CLA mixture, LA and GLA; and 90 mg/mL *c*9,*t*11- and *t*10,*c*12- CLA. These were then divided into 1 mL aliquots and stored at -20 ^{o}C.

Fatty acids used in the cytotoxicity tests on the MCF-7 and MCF-7/cis cell lines, were delivered as sodium salts in bovine serum albumin (BSA) (Cat: A8918) (in a 2:1 w/w ratio, equivalent to 473:1 molar ratio) containing medium and were prepared as follows: Equimolar amounts of CLA and NaOH (1 mL CLA stock at 0.1 g/mL or 0.35 M and 1

mL 0.35 M NaOH) were mixed. This was diluted to 20 mg/mL CLA with 3 mL DMEM containing BSA to yield a BSA concentration of 10 mg/mL. The solution was incubated for 15 minutes at 37 ^{o}C, then at 50 ^{o}C for a further 15 min. These stocks were stored at -20 ^{o}C (Ip, *et al*, 1999). Working stock solutions of these fatty acid sodium salts were prepared in medium at concentrations of 142 μM fatty acid: 0.32 μM BSA and were stored for up to two weeks at -20 ^{o}C if necessary.

For the first phase of the single treatment cytotoxicity testing on the MDA cell lines (MDA, Tax10p and Tax10p4p), stock solutions of fatty acids were prepared in RPMI medium containing bovine albumin (BSA) (Cat: A1595) in a 2:1 molar ratio. A fatty acid free formulation of BSA was sourced and used for this stage to reduce variation in the assays. In the second phase of single treatments, fatty acids were made up as free fatty acid solutions in RPMI medium fresh for each experiment.

Chemotherapy drug treatments were also prepared in basal medium, fresh for each experiment. Drugs were first diluted 1:1000 (e.g. 10 μL in 10 mL) in medium, then diluted further to 2X the final desired concentration. The three drugs were supplied in liquid form (at concentrations of 2 mg/mL Dox, 6 mg/mL Tax and 25 mg/ml 5-FU) aliquoted into 1.5 mL screwcap vials (by Dr. Glynn, NICB); this eliminated the dangers involved in handling the powdered forms and the use of syringes. All work using the drugs was carried out in a class II laminar air-flow cabinet (Gelaire 85, BSB4 laminar air-flow cabinet). Safety measures included wearing lab coats that fastened covering the neck, which also had elasticated cuffs. Nitrile disposable gloves were worn stretched to overlap the elasticated cuffs of the lab coat and safety goggles were worn at all times when working with the drugs. Tax and 5-FU were stored at room temperature and Dox at 4 ^{o}C, all in locked compartments.

All sterile disposable plastic-ware was from Sarstedt Ltd., Wexford, Ireland. Phosphate buffered saline (PBS) (Lennox, Cat: BR14) was prepared by dissolving five tablets in 500 mL ultra-distilled water (dH_2O). This was then autoclaved at 115 ^{o}C for 20 min.

All water used in cleaning or for maintaining humidity in the incubator was also dH_2O autoclaved. PBS and sterile water were both stored at room temperature. Trypsin/EDTA solution (T/E) was made up as follows: 50 mL of 10X Trypsin (Sigma, Cat: T4549) and 10 mL of 1% w/v EDTA (Cat: E6511) were added to 440 mL PBS. This was aliquoted into sterile universal containers and stored at -20 ^{o}C. A stock solution of 1% EDTA can be made up in advance and stored at 4 ^{o}C.

2.2.2 Cell Culture

2.2.2.1 Media Preparation

Cell culture media was prepared as follows: For MCF-7 and MCF-7/cis, 25 mL (5 % v/v) Foetal calf serum (FCS) (Sigma, Cat: F7524); 5 mL (1 unit/ml) Penicillin/Streptomycin (Pen/Strep) (Cat: P0781); 5 mL (10 mM) Sodium Pyruvate (Cat: S8636) and 0.5 mL (1 mM) HEPES (Cat: H0887) were added to Dulbecco's Minimum Essential Medium (DMEM) (Cat: D5796). Basal medium for the MDA, Tax10p and Tax10p4p cell lines was RPMI-1640 (Cat: R7388) to which supplements 50 mL (10 % v/v) FCS, 5 mL Pen/Strep and 5 mL sodium pyruvate were added. Complete media was stored at 4 ^{o}C for up to two weeks.

2.2.2.2 Feeding

All cell lines were grown in a ShelLab, IR2424 model CO_2 humidified Incubator at 37^{o}C. Cell culture work was carried out in a class II laminar airflow cabinet (Gelaire 85, BSB4 laminar air-flow cabinet). Protocol for maintenance of cell lines was adapted from O'Shea *et al*, 1999. The relevant complete media was incubated at 37 ^{o}C for 20 min in a water-bath prior to use. Industrial methylated spirits (IMS) (Lennox, Cat:

1170) diluted to 70 % with dH_2O was used to spray all internal surfaces of the laminar prior to use. All bottles, plastics etc brought into the laminar, as well as gloves were also sprayed with IMS. Waste media was drawn off from the flask with a pipette and transferred to a waste bottle. The flask was then rinsed with PBS (Lennox, Cat: BR14), 3 mL for T25 flask, 7 mL for T75 flask and again transferred to the waste bottle. The appropriate fresh complete media was then added, 3-5 mL for T25 or 10-15 mL for T75 flasks. The flask was then sprayed with IMS and replaced in the incubator. When finished working in the laminar all surfaces were washed down with Virkon solution (Lennox, Cat: 222/0154/01). This was then rinsed using tissue paper damped with sterile water. Once dry all surfaces were then sprayed down with IMS and allowed to dry again. This cleaning operation was also performed between any work involving different cell lines.

2.2.2.3 Subculturing

Media and trypsin/EDTA (T/E) solution were incubated at 37 ^{o}C for 20 min in a water-bath. Waste media was drawn off and the flask rinsed with PBS as per feeding method. T/E was then added, 2 mL for T25's or 4 mL for T75's and incubated until all the cells were detached from the base of the flask (2-5 min). This solution was then transferred to a universal tube. The flask was then rinsed with PBS, 8 mL for T25's or 6 mL for T75's and added to the universal. This cell suspension along with a counter balance of another universal containing 10 mL was centrifuged at 1000 rpm for 5 min using a Labofuge 400 centrifuge, Heraeus Instruments (supplied by Foss Electric, Dublin). The supernatant was removed and the pellet resuspended in 15 mL complete media or appropriate amount. Following a cell count the appropriate amount of this cell suspension or stock was then used to re-seed a new flask at the required cell density.

2.2.3 Cell Counting and Viability Assays

2.2.3.1 Cell Counting with Trypan Blue

A cell suspension was made as per Subculturing method i.e. a flask of cells was trypsinised, spun and resuspended. 1 mL of this suspension was then transferred to a microtube, into which 200 µL trypan blue (Sigma, Cat: T8154) was added. This was then mixed and 10 µL of this mixture was pipetted to the side of the chamber of the haemocytometer enclosed by a cover slip and was drawn in by capillary motion.

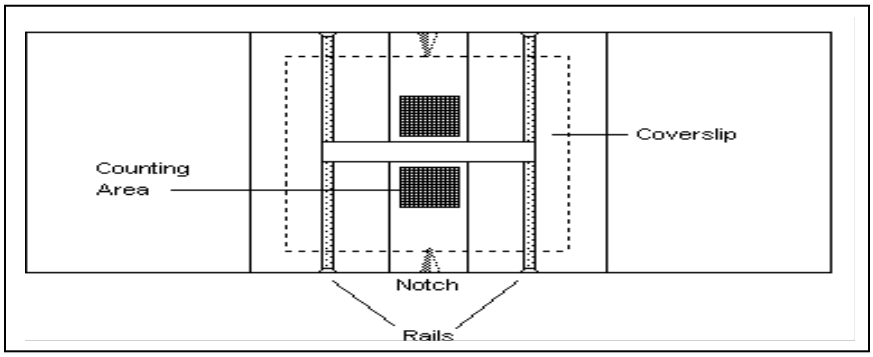


Figure 2.3.1.1. Diagram of Haemocytometer with coverslip.

Cells were counted from the four large corner quadrants and the centre square as observed under the 10X objective. This total number was divided by 5 to give the average cell number per square. This was multiplied by the dilution factor of 1.2 and then by $10^{4,}$ which results in the total cell number per mL. Viable cells appear clear and do not stain, whereas non-viable cells stain blue from the influx of trypan blue across breached membranes. % viability can thus be determined.

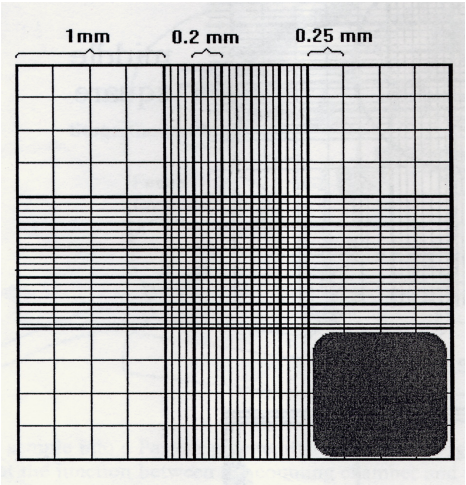


Figure 2.3.1.2. Illustration of squares on a haemocytometer, showing one of the corner quadrants shaded. The volume underneath the coverslip of this area (or one square) is 0.1 mm^3 or 10^{-4} ml.

2.2.3.2 MTS Microplate Assay

The MTS microplate assay (CellTiter 96^R Aq_{ueous} One Solution Cell Proliferation Assay, Promega, distributed by Medical Supply Company (MSc), Dublin, Ireland, Cat: G3581) is a modification of the original tetrazolium assay, the MTT assay, involving fewer steps because the formazan product is soluble in culture medium unlike the product of MTT reduction, which is a crystalline precipitate that requires the addition of a solubilisation solution and incubation overnight before the spectrophotometric absorbance can be measured on a microplate reader. This method is therefore also safer, eliminating the need for handling volatile organic solvents. The MTS assay is a colourimetric non-radioactive assay system, which is used widely to quantify cell proliferation and viability. The MTS assay solution contains a tetrazolium compound, 3-(4,5-diamethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl) -2H-tetrazolium, inner salt (MTS) and the electron coupling reagent phenazine ethosulfate (PES)

(Promega Technical Bulletins; No's 169 and 245).

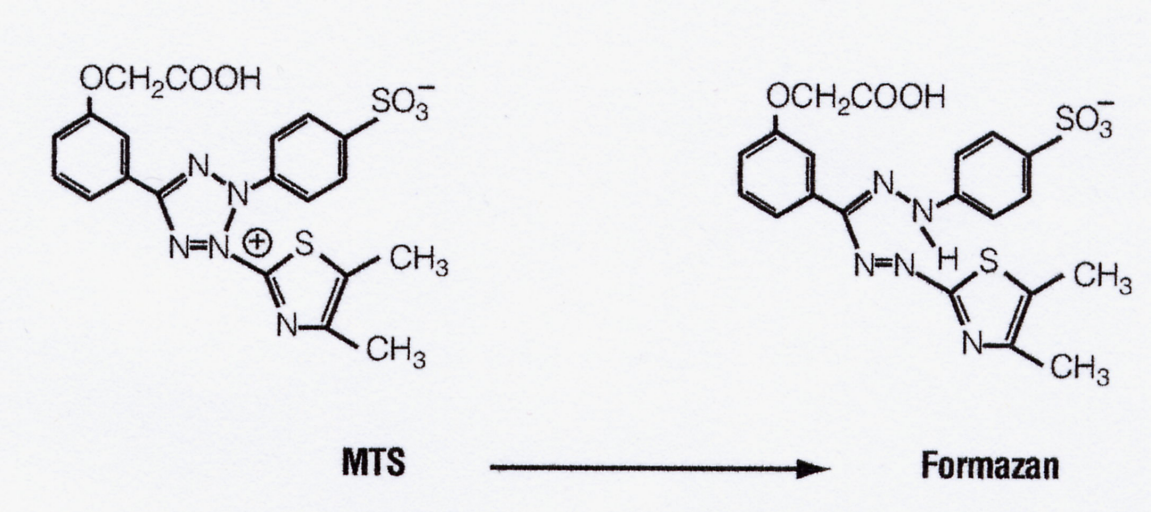


Figure 2.3.2.1. Conversion and structures of MTS tetrazolium to the formazan product (Promega, Technical Bulletin No.245)

The assay is based on the cleavage or bio-reduction of the yellow tetrazolium salt (MTS) into a soluble form of the purple formazan product by mitochondrial dehydrogenases in metabolically active cells. This bio-reduction involves the pyridine nucleotide co-factors NADH and NADPH produced by those enzymes. The absorbance of the formazan product read at 490 nm is directly proportional to the number of living cells in the well/culture (Promega, Technical Bulletin No.245).

A confluent flask was trypsinised and cell suspension made as per Subculturing method. A cell count was performed using trypan blue according to the Cell Counting method and stocks were calculated and made up at the required cell density i.e. $5x10^4$ cells/mL for 1 and 3 day assay time points and $1x10^4$ cells/mL for 5 and 7 day assays. Sterile 96-well microplates were seeded as follows; using a multi-channel micropipette, 100 µL of the appropriate cell stock was pipetted into each well. Wells to be used as blanks were

left empty. Plates were then cultured for 24 h to allow cells to attach to the substratum before addition of 100 µL fatty acid treatment/media. Control and blank wells were supplemented with medium containing the vehicle -BSA at the same concentration as the maximum in the experimental wells. All treatments were set up in triplicate. Plates were incubated for the required time period i.e. 1, 3, 5 and 7 days after which cytotoxicity was assessed.

20 µL of One Solution Cell Proliferation Assay (Promega, distributed by the Medical Supply Company –MSC, Dublin; Cat: G3581) was added to each well and incubated for 4 h. Following this the plates were read on a Rosys Anthos 2010 microplate reader (Anthos Labtec Instruments, Austria) at 492nm. Cytotoxicity was expressed as percentage inhibition compared to control. Data was obtained from at least five experiments in which treatments and time points were set up in triplicate. Statistical analysis was carried out using the Student's *t*-test.

2.2.3.3 Acid Phosphatase Assay

A confluent flask was trypsinised and cell suspension made as per Subculturing method. A cell count was performed using trypan blue according to the Cell Counting method and a stock was made up at a cell density of $1x10^4$ cells/ mL. Plates were seeded with 100 µL of cell stock in each well. These were then cultured for 24 h following which 100 µL of treatments/media was added to corresponding wells. Cytotoxicity was assessed after 7 days.

All assay reagents were pre-prepared in advance. PBS was made up as previously listed, as was a 1 M NaOH solution and both were stored at room temperature. Sodium acetate buffer was prepared at a concentration of 0.1 M, containing 0.1 % Triton X-100 and adjusted to pH 5.5 using glacial Acetic acid. This was stored at 4 ^oC in the dark for up

to one month. The *p*-nitrophenyl phosphate (pNPP) (Sigma-Aldrich, Cat: P5869) was added immediately prior to performing the assay to yield a 10 mM solution.

After the required incubation time (7 days) all media was removed from the plates by flicking the plates upside-down over a waste container. They were then rinsed with 100 µL PBS per well, which was also removed by flicking plates upside-down and 100 µL sodium acetate buffer containing the substrate (pNPP) was added. This was gently mixed, then incubated for 2 h. After this period 50 µL 1M NaOH was added to each well. This causes an electrophilic shift in the pNP chromophore thus producing the yellow colour. NaOH also kills the cells upon addition, therefore stopping the reaction and preserving the results. Plates were usually left at least one day at 4 ^{o}C before reading absorbance; this allows any bubbles that may have formed in the wells through pipetting solutions to die down, allowing a more accurate result. Absorbance was read at 405 nm on a Tecan A-5082 Sunrise microplate reader (Tecan, Austria) (O'Connor, 1998; Martin and Clynes, 1991). Statistical analysis was carried out using the Student's *t*-test.

2.2.4 Cytotoxicity Testing

2.2.4.1 Fatty Acids (as sodium salts with BSA 2:1 w/w ratio) on MCF-7 and MCF-7/cis

MCF-7 and MCF-7/cis cells were seeded as listed in Section 2.2.3.2 MTS Microplate Assay. Fatty acid stock solutions were prepared as sodium salts complexed with BSA in a 2:1 w/w ratio (equivalent to 473:1 molar ratio) as described above. After 24 h, 100 µL of medium containing fatty acids was added in increments to yield final concentrations in the range 2 µg/mL to 20 µg/mL (or 7.1 µM to 71 µM) and a maximum BSA concentration of 0.16 µM. Fatty acids tested at this stage were Linoleic Acid (LA), CLA mixture of isomers (CLA-mix) and pure isomers *c*9,*t*11- and *t*10,*c*12-CLA. Control and

blank wells were supplemented with medium containing BSA at a final concentration of 0.16 µM as in experimental wells. All treatments were set up in triplicate. Plates were incubated for the required time period i.e. 1, 3, 5 and 7 days after which cytotoxicity was assessed using the MTS assay as listed in Section 2.3.2 MTS Microplate Assay.

2.2.4.2 Fatty Acids (with BSA 2:1 molar ratio) on MDA cell lines

MDA-MB-435-S-F (MDA), MDA-MB-435-S-F/ Taxol 10p (Tax10p) and MDA-MB-435-S-F/ Taxol 10p4p (Tax10p4p) cells were seeded as listed in Section 2.3.3 Acid Phosphatase Assay. Stock solutions of fatty acids were prepared in medium containing bovine albumin in a 2:1 molar ratio. After 24 h 100 µL of medium containing treatments was added to corresponding wells in 10 % increments to a maximum concentration of 40 µg/mL for CLA mix, *c*9,*t*11 and *t*10,*c*12-CLA; 50 µg/mL for LA and GLA. 10 concentrations of each treatment were examined per plate with 6 replicates for each concentration and each plate was set up in duplicate. Cells treated with medium containing an equivalent concentration of bovine albumin served as controls in this experiment. Cytotoxicity was assessed after 7 days using the Acid Phosphatase Assay listed in section 2.3.3. Data was obtained from at least three experiments.

2.2.4.3 Free Fatty Acids on MDA cell lines

MDA, Tax10p and Tax10p4p cells were seeded at a density of $1x10^4$ cells/mL as previously listed. Stock solutions of free fatty acids were prepared in medium without BSA and made fresh for each experiment. As above treatments were added after 24 h in 10 % increments with maximum final concentrations for CLA mix, *c*9,*t*11 and *t*10,*c*12-

CLA at 40 µg/mL and LA and GLA at 50 µg/mL. 10 concentrations of each treatment were examined per plate with 8 replicates for each concentration and each plate was set up in duplicate. Cells treated with medium containing an equivalent concentration of ethanol served as controls in this experiment. Cytotoxicity was assessed after 7 days using the Acid Phosphatase Assay described above. Data was obtained from at least three experiments.

2.2.4.4 Chemotherapy drugs Dox, Tax and 5-FU on MDA cell lines in long term exposure assays

MDA, Tax10p and Tax10p4p cells were seeded at a density of $1x10^4$ cells/mL. Drug treatments were made up in basal medium and were prepared fresh for each experiment. After 24 h 100 µL of medium containing treatments was added to corresponding wells in 10 % increments to a maximum concentration of 50 ng/mL, 10 ng/ml and 400 ng/mL for Doxorubicin (Dox), Taxotere (Tax) and 5-Fluorouracil (5-FU) respectively. 10 concentrations of each treatment were examined per plate with 6 replicates for each concentration and each plate was set up in duplicate. Cells treated with basal medium served as controls for this experiment. Cytotoxicity was assessed after 7 days using the Acid Phosphatase Assay listed in section 2.3.3. Data was obtained from at least three experiments.

2.2.4.5 Chemotherapy Drugs on MDA cell lines in short term exposure assay

MDA, Tax10p and Tax10p4p cells were seeded at a density of $1x10^4$ cells/mL as previously listed. Drug Treatments were made up in basal medium and prepared fresh

for each experiment. Treatments were added after 24 h in 10 % increments with maximum final concentrations up to 500 ng/mL, 50 ng/mL and 10 µg/mL for Dox, Tax and 5-FU respectively. In this assay, drug treatments were left on for a 4 h exposure following which media/treatments were removed. Plates were then rinsed with PBS and 200 µL fresh media added to each well for a 7 day recovery period. 10 concentrations of each treatment were examined per plate with 8 replicates for each concentration and each plate was set up in duplicate. Cells treated with basal medium were controls for this experiment. Cytotoxicity was assessed after 7 days using the Acid Phosphatase Assay listed in section 2.2.3.3 Acid Phosphatase Assay. Data was obtained from at least three experiments.

2.3 Results

2.3.1 Fatty acid single treatments on MCF-7 and MCF-7/cis cell lines

Fatty acids used in the cytotoxicity tests on the MCF-7 and MCF-7/cis cell lines, were delivered as sodium salts in BSA in a 2:1 w/w ratio (equivalent to 473:1 molar ratio) and incubated for 1, 3, 5 and 7 days after which cytotoxicity was assessed using the MTS assay as listed above. Fatty acids tested at this stage were Linoleic Acid (LA), CLA mixture of isomers (CLA-mix) and pure isomers *c*9,*t*11- and *t*10,*c*12-CLA. MCF-7 cisplatin resistant cells (MCF-7/cis) were more sensitive to inhibition by the CLA-mix than the MCF-7 parental cell line after the 7 day incubation, illustrated in Figure 2.3.1.1. These differences were found to be significant with $p<0.05$, as determined by the Student's *t*-test. IC_{70} values for MCF-7/cis and MCF-7 were 9.6 $\pm$ 0.5 and 13.4 $\pm$ 0.5 µg/mL, while IC_{50} values were 6.8 $\pm$ 0.9 and 10.1 $\pm$ 1.0 µg/mL. Linoleic acid (LA) treatment caused no significant inhibition relative to control in MCF-7 cells. However, significant inhibition of 41.8 $\pm$ 10.0 % relative to control was seen at the maximum dose

of 20 μg/mL in MCF-7/cis cells (p=0.025) after 7 days (Figure 2.3.1.2). *c*9,*t*11-CLA was similarly effective in inhibiting cell growth in both cell lines after a 7 day treatment. IC_{50} values for MCF-7/cis and MCF-7 were 11.1 ± 1.3 and 13.5 ± 1.7 μg/mL *c*9,*t*11-CLA respectively as seen in Figure 2.3.1.3. MCF-7/cis cells were more sensitive to inhibition by *t*10,*c*12-CLA than the MCF-7 parental cell line. IC_{50} values for MCF-7/cis and MCF-7 were 5.1 ± 1.2 and 13.2 ± 2.0 μg/mL *t*10,*c*12-CLA (Figure 2.3.1.4).

All fatty acids in both cell lines followed a similar pattern of toxicity with a 5 day incubation to that seen after 7 days. In MCF-7 cells, significant inhibition was seen with the CLA-mix at as low as 6 μg/mL (p=0.032) and at 16 and 14 μg/mL (p=0.01 and 0.048) for *c*9,*t*11 and *t*10,*c*12-CLA respectively. LA caused no significant inhibition in MCF-7 cells for the concentrations used (Figure 2.3.1.5). In MCF-7/cis cells, growth inhibition by *t*10,*c*12-CLA was significant for all treatments in the range 0-20 μg/mL ($p<0.05$). Inhibition by CLA-mix and *c*9,*t*11-CLA were found to be significant for treatments of and above 4 and 8 μg/mL respectively ($p<0.05$). LA showed significant inhibition at 18 and 20 μg/mL (p=0.02 and 0.0004), but at a maximum of 40.5 ± 3.6 % inhibition (Figure 2.3.1.6).

In the MCF-7 parental line, 3 days incubation resulted in significant inhibition for *c*9,*t*11-CLA at 20 μg/mL and *t*10,*c*12-CLA at 16 to 20 μg/mL. LA and CLA-mix caused no significant inhibition after 3 days in range tested (Figure 2.3.1.7). In the MCF-7/cis cell line, incubation of the CLA mixture and both isomers *c*9,*t*11 and *t*10,*c*12-CLA for 3 days resulted in significant inhibition at the highest concentration, 20 μg/mL only and did not reach 50 % inhibition in any case. LA did not result in significant inhibition at any concentration in MCF-7/cis cells (Figure 2.3.1.8).

Following the 1 day incubation significant inhibition was observed in the MCF-7 parental line with the CLA mixture and *c*9,*t*11-CLA for the 1 day incubation at 18 and 20 μg/mL, whereas *t*10,*c*12-CLA and LA caused no significant inhibition (Figure

2.3.1.9). In the MCF-7/cis cell line no significant inhibition was seen with any fatty acid for the 1 day incubation (Figure 2.3.1.10).

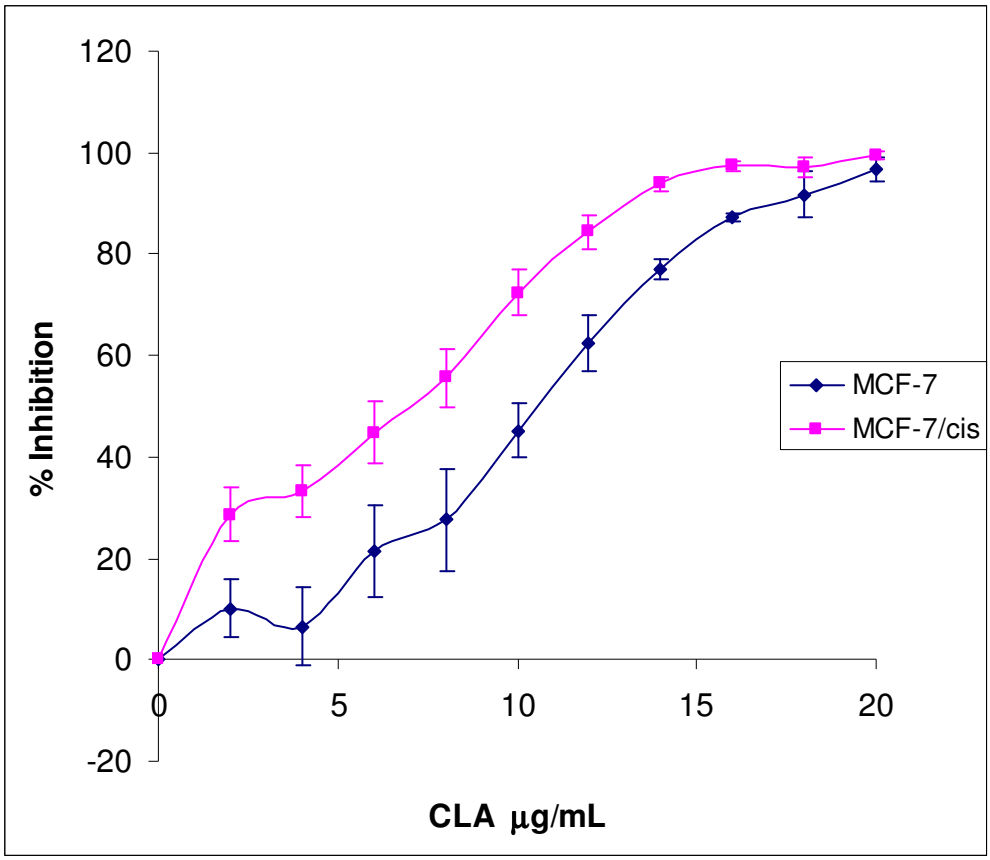


Figure 2.3.1.1 Growth inhibition in MCF-7 and MCF-7/cis drug resistant cells incubated with CLA mixture (0-20 µg/mL) for 7 days. Cytotoxicity was assessed following the 7 day incubation using the MTS microplate assay as described in Section 2.3.2. Growth inhibition was expressed as a percentage relative to control, which was taken to be 0 %. Each concentration was set up with 6 replicates per plate and each plate was set up in duplicate. Data was obtained from at least three experiments in which treatments and time points were set up in triplicate. Error bars represent standard error of the mean. Cells without treatment served as controls for this experiment.

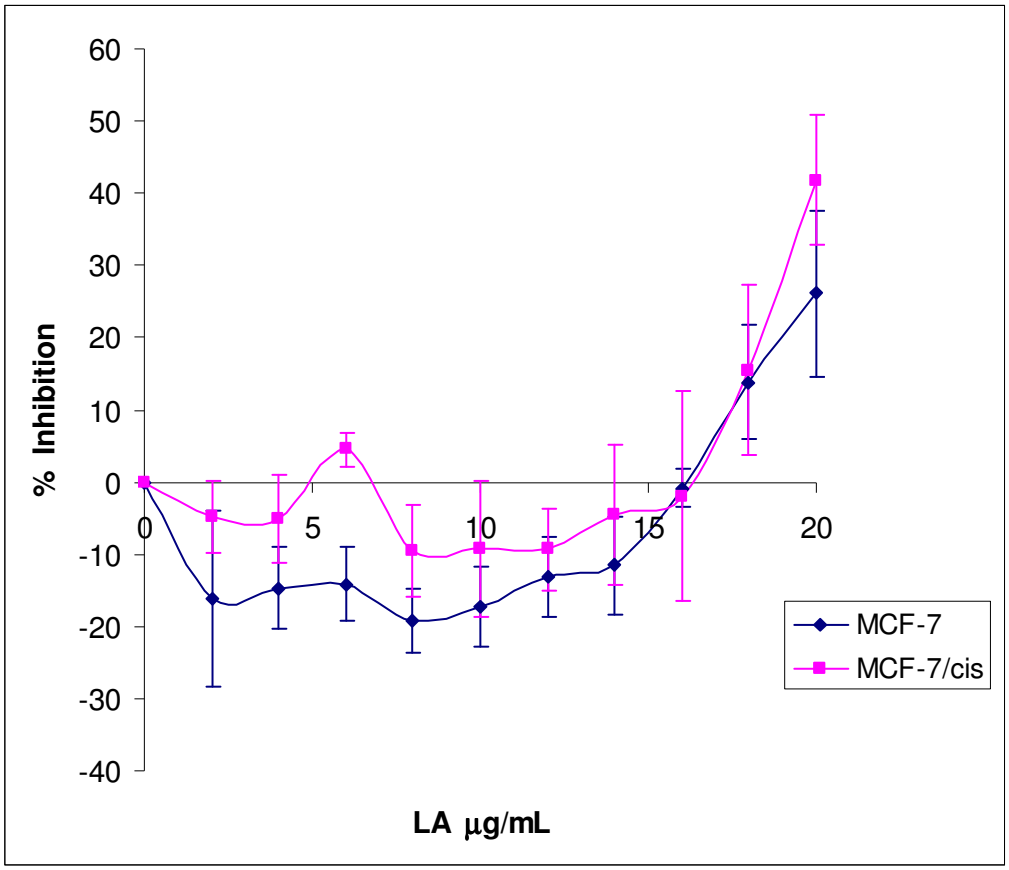


Figure 2.3.1.2 Effects of LA (0-20 µg/mL) on growth of MCF-7 and MCF-7/cis drug resistant cells for 7 days. Growth was assessed following the 7 day incubation using the MTS microplate assay as described in Section 2.3.2 and expressed as a percentage relative to control, which was taken to be 0 %. Data was obtained from five experiments in which treatments and time points were set up in triplicate. Error bars represent standard error of the mean. Cells without treatment served as controls for this experiment.

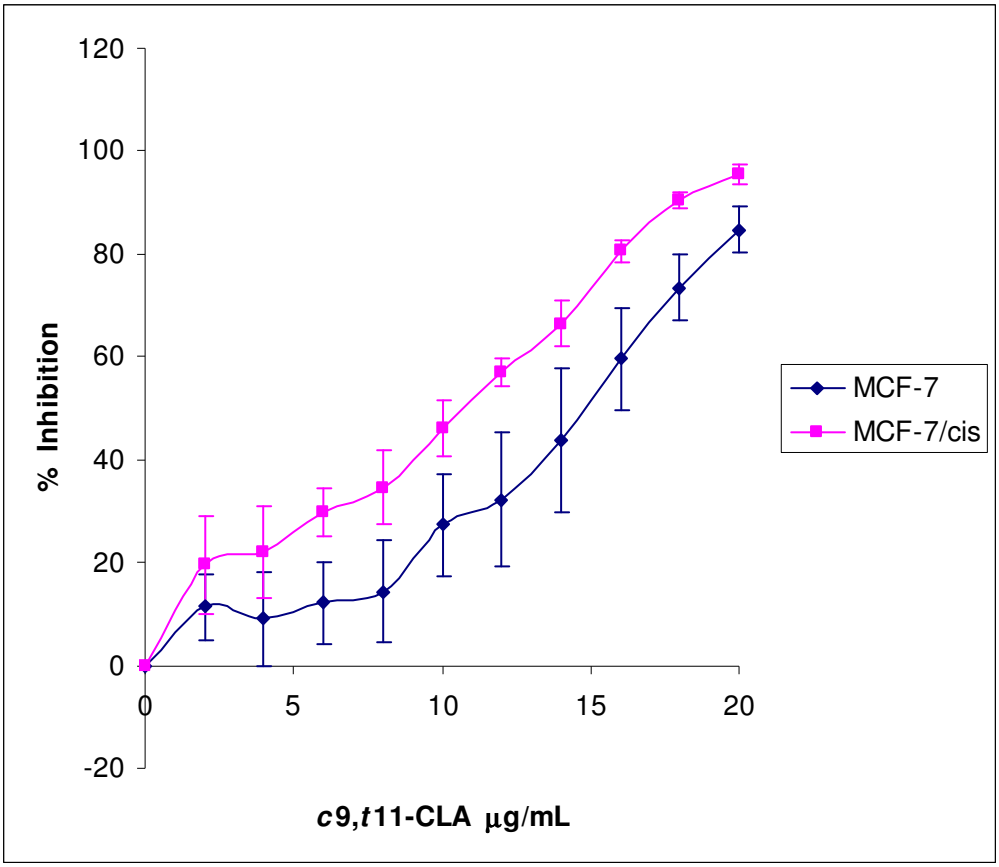


Figure 2.3.1.3 Growth inhibition in MCF-7 and MCF-7/cis drug resistant cells incubated with *c*9,*t*11-CLA (0-20 µg/mL) for 7 days. Cytotoxicity was assessed following the 7 day incubation using the MTS microplate assay as described in Section 2.3.2. Growth inhibition was expressed as a percentage relative to control, which was taken to be 0 %. Data was obtained from at least three experiments in which treatments and time points were set up in triplicate. Error bars represent standard error of the mean. Cells without treatment served as controls for this experiment.

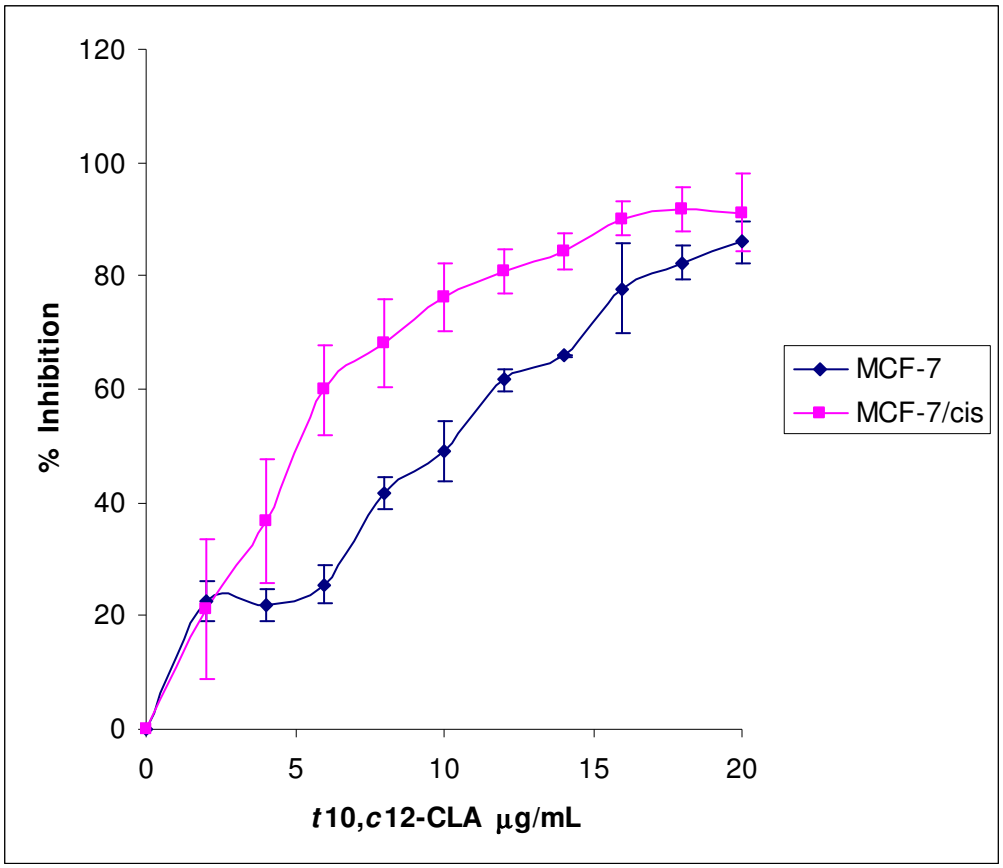


Figure 2.3.1.4 Growth inhibition in MCF-7 and MCF-7/cis drug resistant cells incubated with *t*10,*c*12-CLA (0-20 µg/mL) for 7 days. Cytotoxicity was assessed following the 7 day incubation using the MTS microplate assay as described in Section 2.3.2. Growth inhibition was expressed as a percentage relative to control, which was taken to be 0 %. Cells without treatment served as controls for this experiment. Data was obtained from at least three experiments in which treatments and time points were set up in triplicate. Error bars represent standard error of the mean.

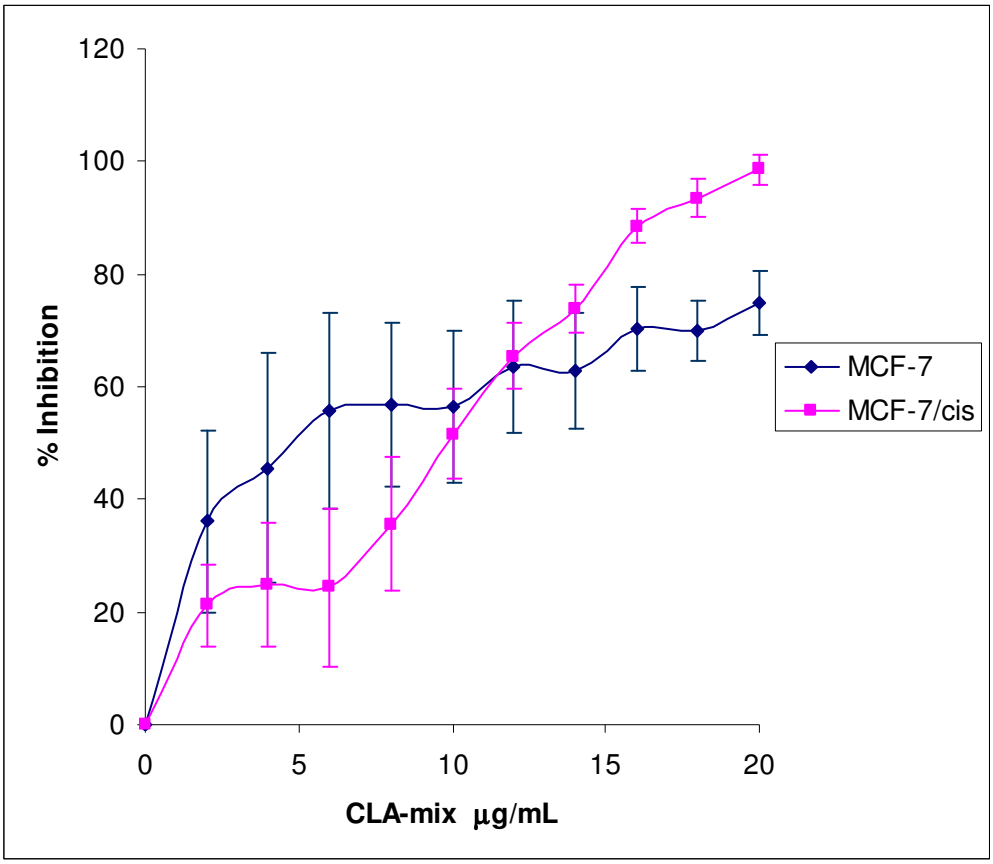


Figure 2.3.1.5 Growth inhibition in MCF-7 and MCF-7/cis drug resistant cells incubated with CLA mixture (0-20 µg/mL) for 5 days. Cytotoxicity was assessed following the 5 day incubation using the MTS microplate assay as described in Section 2.3.2. Growth inhibition was expressed as a percentage relative to control, which was taken to be 0 %. Each concentration was set up with 6 replicates per plate and each plate was set up in duplicate. Data was obtained from at least three experiments in which treatments and time points were set up in triplicate. Cells without treatment served as controls for this experiment. Error bars represent standard error of the mean.

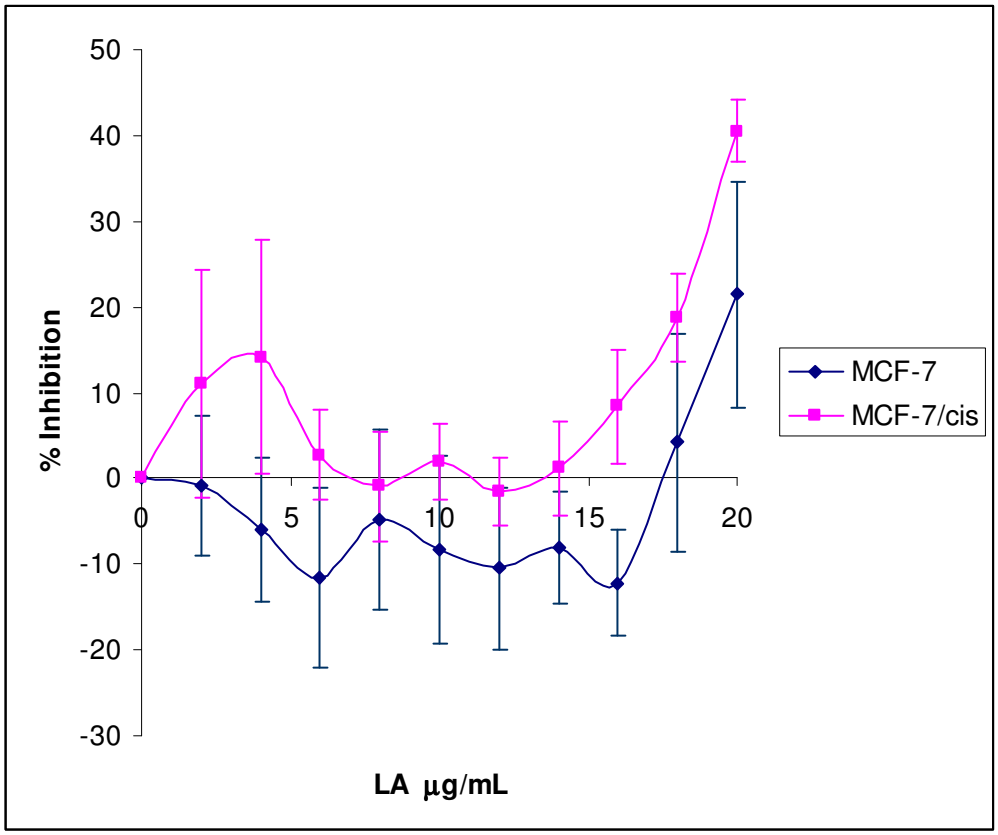


Figure 2.3.1.6 Effects of LA (0-20 µg/mL) on growth of MCF-7 and MCF-7/cis drug resistant cells for 5 days. Growth was assessed following the 5 day incubation using the MTS microplate assay as described in Section 2.3.2 and expressed as a percentage relative to control, which was taken to be 0 %. Each concentration was set up with 6 replicates per plate and each plate was set up in duplicate. Data was obtained from at least three experiments in which treatments and time points were set up in triplicate. Cells without treatment served as controls for this experiment. Error bars represent standard error of the mean.

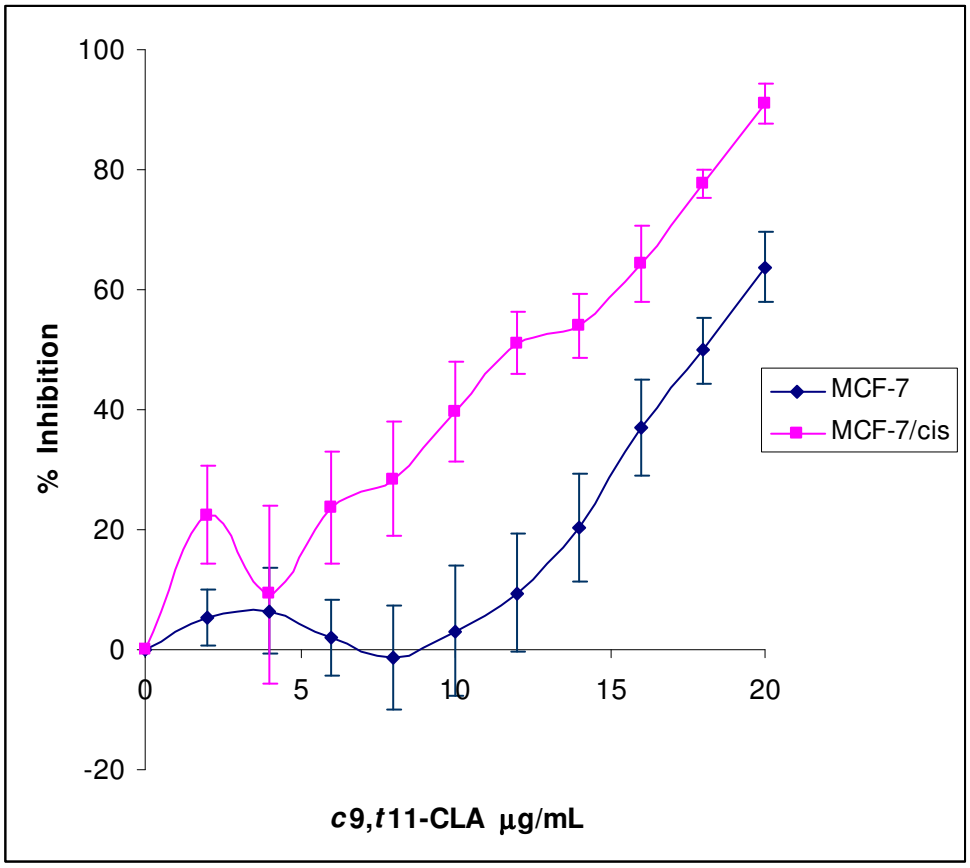


Figure 2.3.1.7 Growth inhibition in MCF-7 and MCF-7/cis drug resistant cells incubated with *c*9,*t*11-CLA (0-20 µg/mL) for 5 days. Cytotoxicity was assessed following the 5 day incubation using the MTS microplate assay as described in Section 2.3.2. Growth inhibition was expressed as a percentage relative to control, which was taken to be 0 %. Each concentration was set up with 6 replicates per plate and each plate was set up in duplicate. Data was obtained from at least three experiments in which treatments and time points were set up in triplicate. Cells without treatment served as controls for this experiment. Error bars represent standard error of the mean.

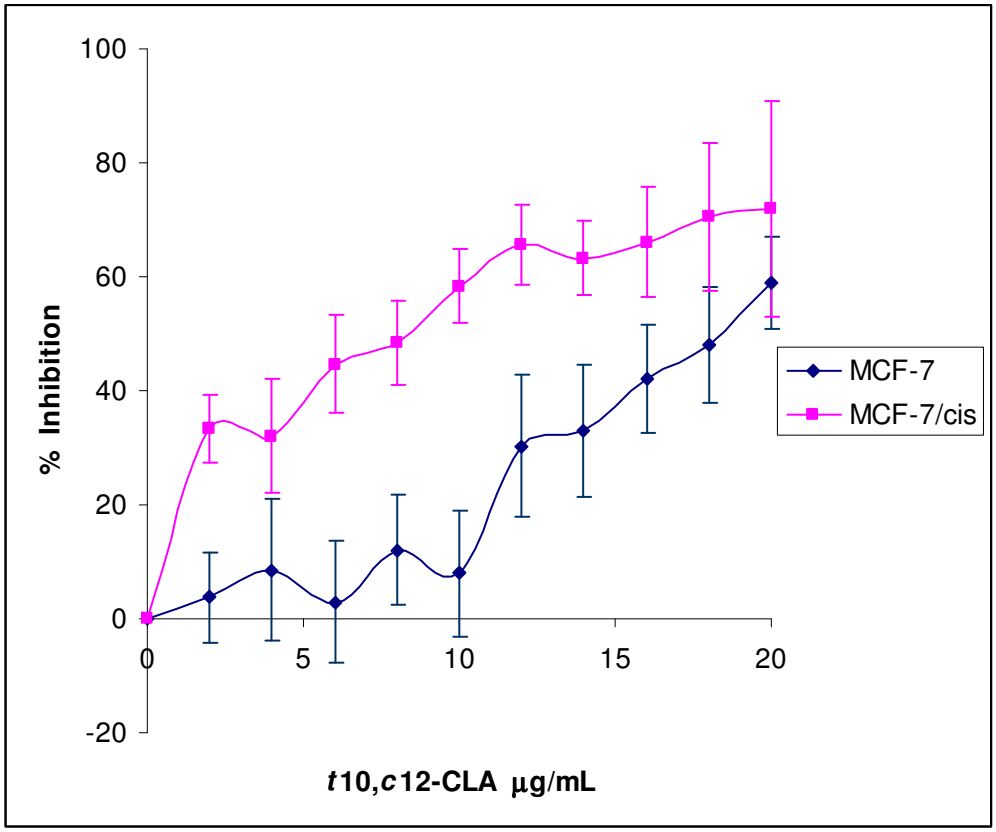


Figure 2.3.1.8 Growth inhibition in MCF-7 and MCF-7/cis drug resistant cells incubated with *t*10,*c*12-CLA (0-20 µg/mL) for 5 days. Cytotoxicity was assessed following the 5 day incubation using the MTS microplate assay as described in Section 2.3.2. Growth inhibition was expressed as a percentage relative to control, which was taken to be 0 %. Each concentration was set up with 6 replicates per plate and each plate was set up in duplicate. Data was obtained from at least three experiments in which treatments and time points were set up in triplicate. Cells without treatment served as controls for this experiment. Error bars represent standard error of the mean.

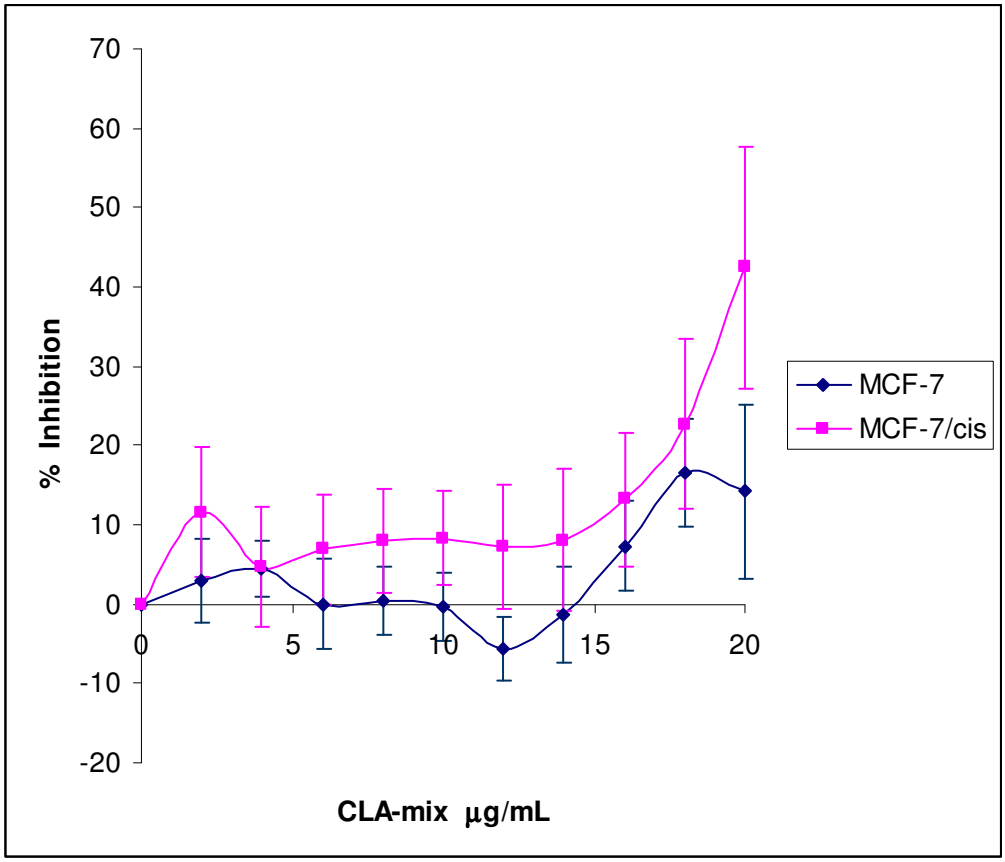


Figure 2.3.1.9 Growth inhibition in MCF-7 and MCF-7/cis drug resistant cells incubated with CLA-mix (0-20 µg/mL) for 3 days. Cytotoxicity was assessed following the 3 day incubation using the MTS microplate assay as described in Section 2.3.2. Growth inhibition was expressed as a percentage relative to control, which was taken to be 0 %. Each concentration was set up with 6 replicates per plate and each plate was set up in duplicate. Data was obtained from at least three experiments in which treatments and time points were set up in triplicate. Cells without treatment served as controls for this experiment. Error bars represent standard error of the mean.

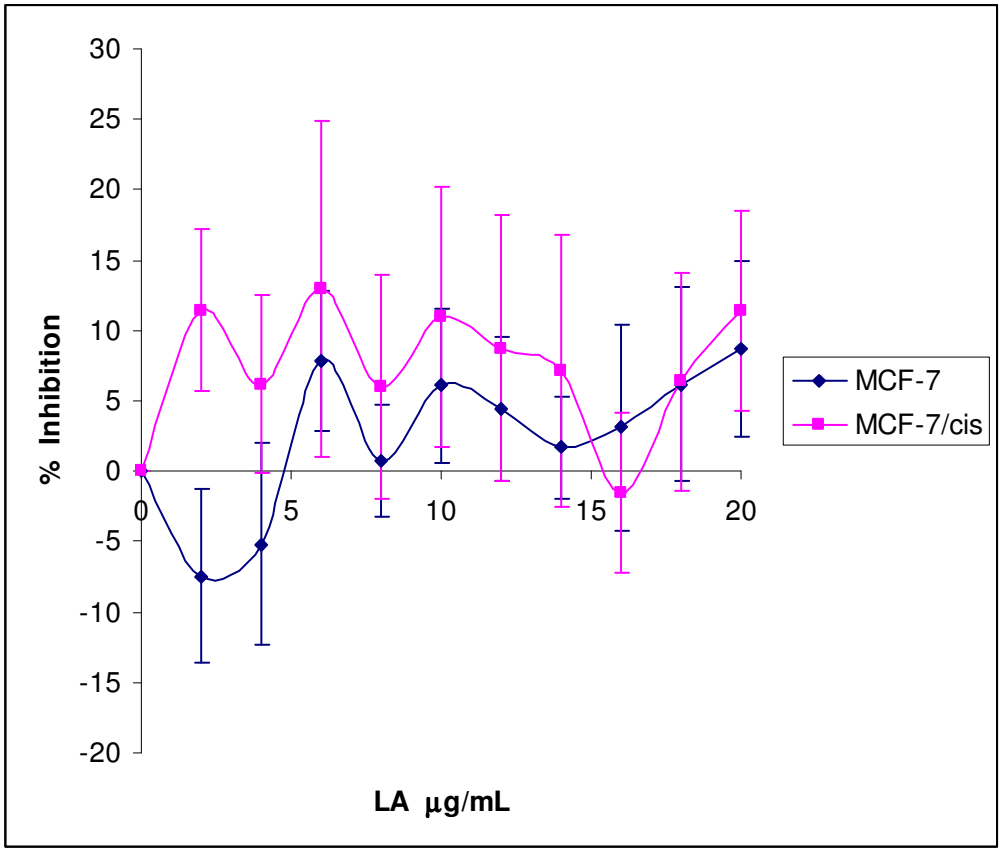


Figure 2.3.1.10 Effects of LA (0-20 µg/mL) on growth of MCF-7 and MCF-7/cis drug resistant cells for 3 days. Growth was assessed following the 3 day incubation using the MTS microplate assay as described in Section 2.3.2 and expressed as a percentage relative to control, which was taken to be 0 %. Each concentration was set up with 6 replicates per plate and each plate was set up in duplicate. Data was obtained from at least three experiments in which treatments and time points were set up in triplicate. Cells without treatment served as controls for this experiment. Error bars represent standard error of the mean.

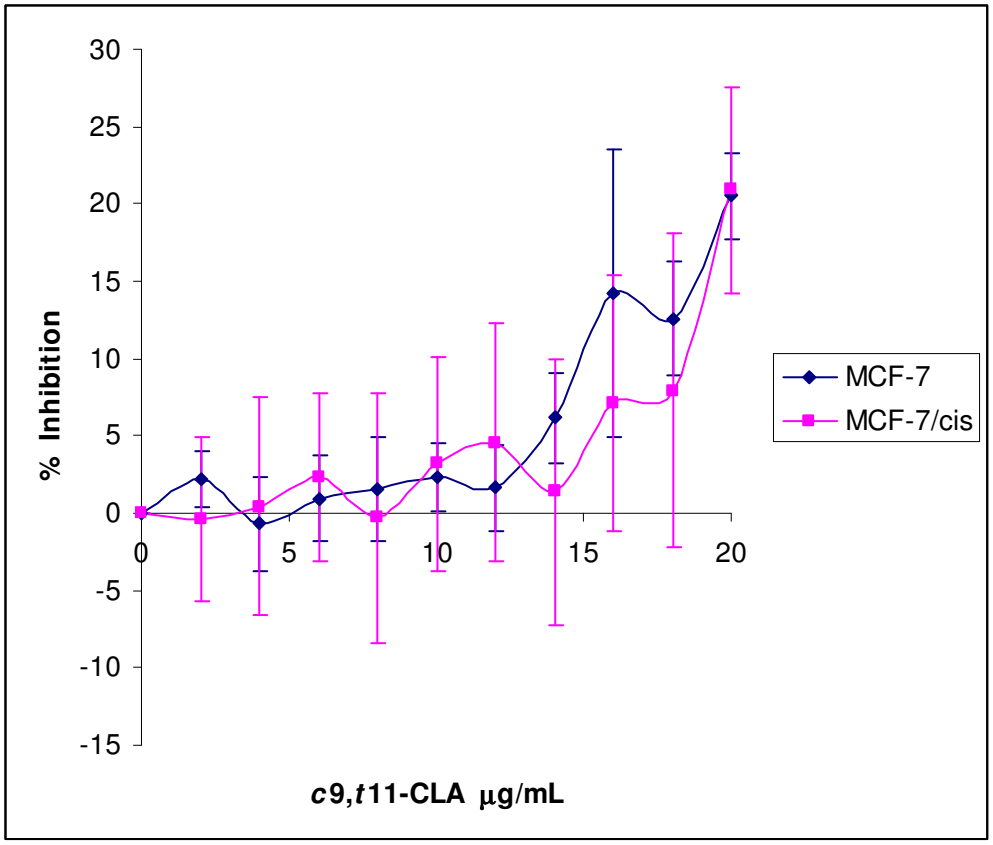


Figure 2.3.1.11 Growth inhibition in MCF-7 and MCF-7/cis drug resistant cells incubated with *c*9,*t*11-CLA (0-20 µg/mL) for 3 days. Cytotoxicity was assessed following the 3 day incubation using the MTS microplate assay as described in Section 2.3.2. Growth inhibition was expressed as a percentage relative to control, which was taken to be 0 %. Each concentration was set up with 6 replicates per plate and each plate was set up in duplicate. Data was obtained from at least three experiments in which treatments and time points were set up in triplicate. Cells without treatment served as controls for this experiment. Error bars represent standard error of the mean.

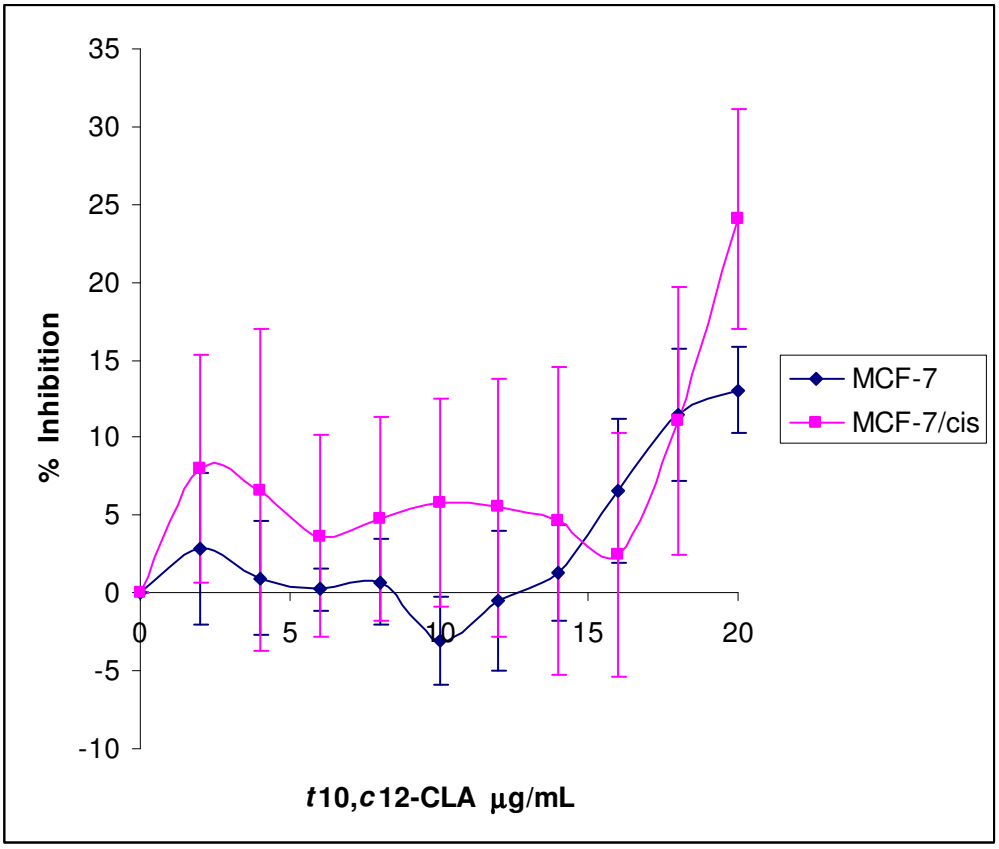


Figure 2.3.1.12 Growth inhibition in MCF-7 and MCF-7/cis drug resistant cells incubated with *t*10,*c*12-CLA (0-20 µg/mL) for 3 days. Cytotoxicity was assessed following the 3 day incubation using the MTS microplate assay as described in Section 2.3.2. Growth inhibition was expressed as a percentage relative to control, which was taken to be 0 %. Each concentration was set up with 6 replicates per plate and each plate was set up in duplicate. Data was obtained from at least three experiments in which treatments and time points were set up in triplicate. Cells without treatment served as controls for this experiment. Error bars represent standard error of the mean.

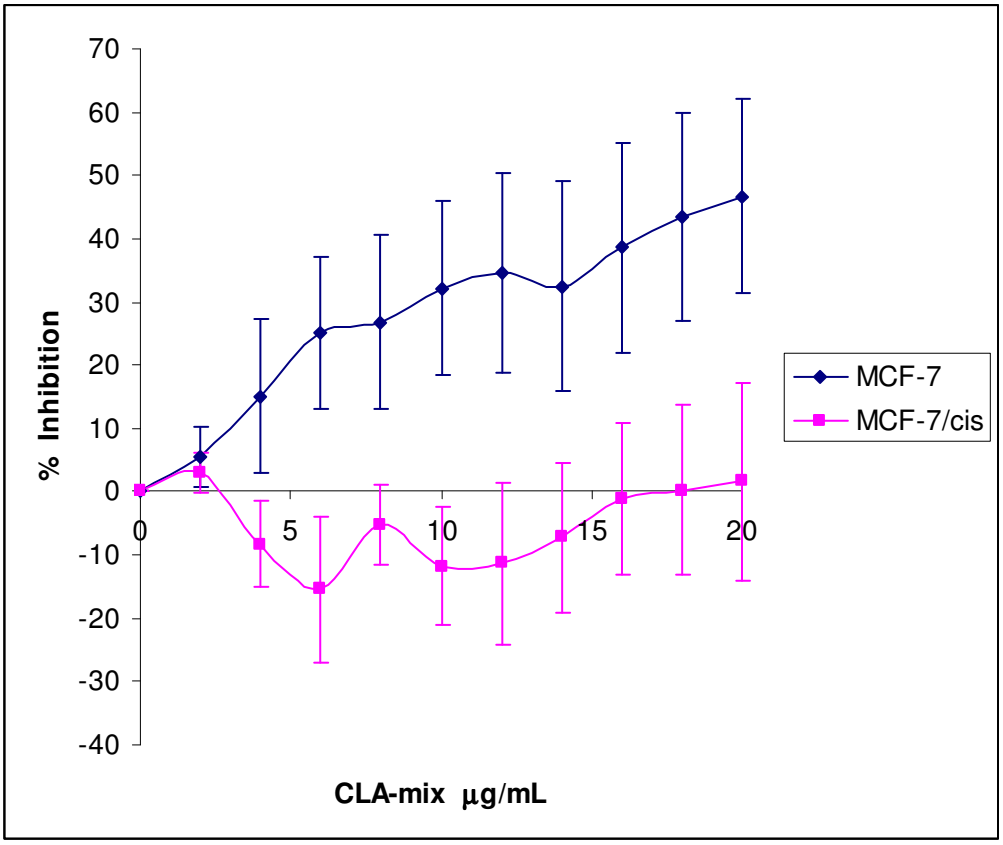


Figure 2.3.1.13 Growth inhibition in MCF-7 and MCF-7/cis drug resistant cells incubated with CLA-mix (0-20 µg/mL) for 1 day. Cytotoxicity was assessed following the 1 day incubation using the MTS microplate assay as described in Section 2.3.2. Growth inhibition was expressed as a percentage relative to control, which was taken to be 0 %. Each concentration was set up with 6 replicates per plate and each plate was set up in duplicate. Data was obtained from at least three experiments in which treatments and time points were set up in triplicate. Cells without treatment served as controls for this experiment. Error bars represent standard error of the mean.

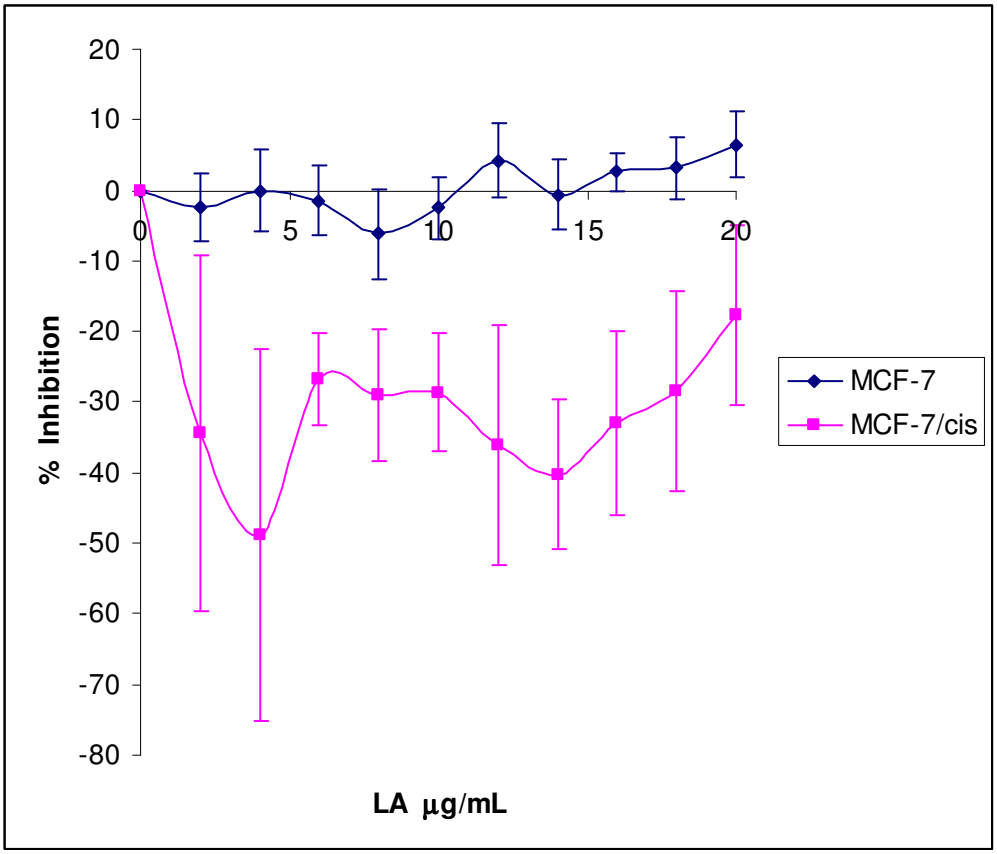


Figure 2.3.1.14 Effects of LA (0-20 µg/mL) on growth of MCF-7 and MCF-7/cis drug resistant cells for for 1 day. Growth was assessed following the 1 day incubation using the MTS microplate assay as described in Section 2.3.2 and expressed as a percentage relative to control, which was taken to be 0 %. Each concentration was set up with 6 replicates per plate and each plate was set up in duplicate. Data was obtained from at least three experiments in which treatments and time points were set up in triplicate. Cells without treatment served as controls for this experiment. Error bars represent standard error of the mean.

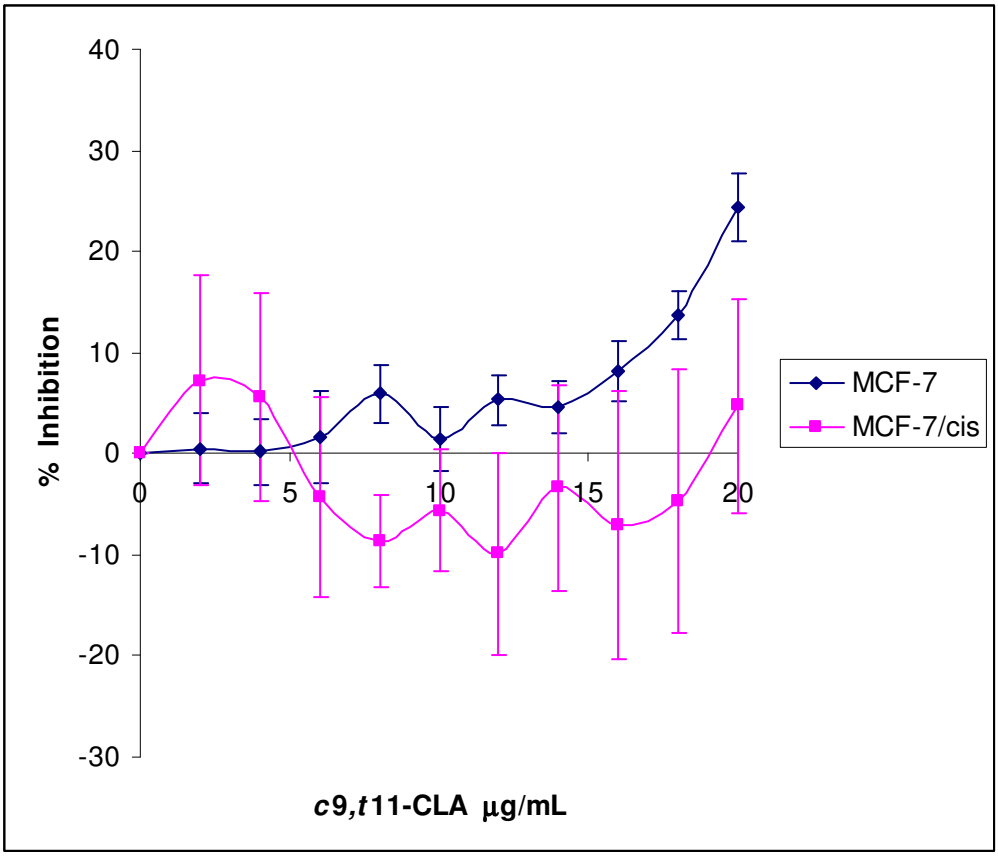


Figure 2.3.1.15 Effects of *c*9,*t*11-CLA (0-20 µg/mL) on growth of MCF-7 and MCF-7/cis drug resistant cells for 1 day. Growth was assessed following the 1 day incubation using the MTS microplate assay as described in Section 2.3.2 and expressed as a percentage relative to control, which was taken to be 0 %. Each concentration was set up with 6 replicates per plate and each plate was set up in duplicate. Data was obtained from at least three experiments in which treatments and time points were set up in triplicate. Cells without treatment served as controls for this experiment. Error bars represent standard error of the mean.

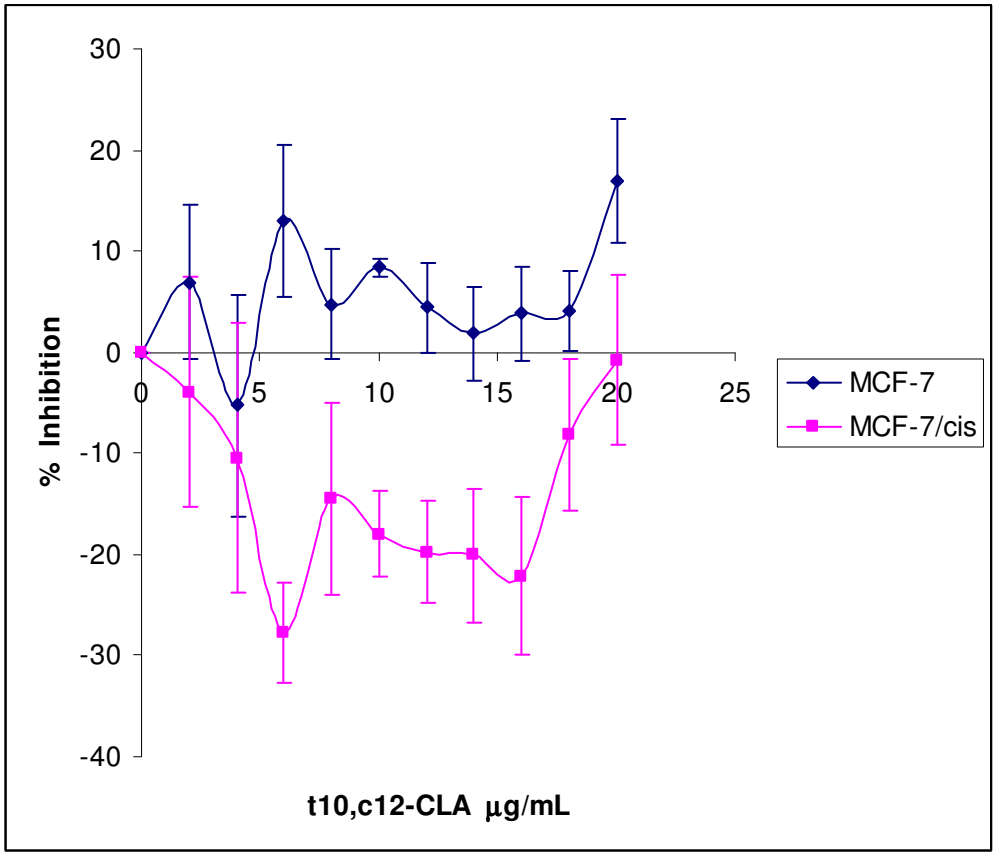


Figure 2.3.1.16 Effects of *t*10,*c*12-CLA (0-20 µg/mL) on growth of MCF-7 and MCF-7/cis drug resistant cells for 1 day. Growth was assessed following the 1 day incubation using the MTS microplate assay as described in Section 2.3.2 and expressed as a percentage relative to control, which was taken to be 0 %. Each concentration was set up with 6 replicates per plate and each plate was set up in duplicate. Data was obtained from at least three experiments in which treatments and time points were set up in triplicate. Cells without treatment served as controls for this experiment. Error bars represent standard error of the mean.

2.3.2 Single Treatments of Fatty Acids (with BSA 2:1 molar ratio) on MDA, Tax10p and Tax10p4p cell lines

With a view to addressing the synergistic potential of fatty acid treatments with chemotherapy drugs, it was decided that it would be more beneficial to use a cellular model where the resistance and other qualities of the cell lines in question were better characterised to gain a better understanding of the interactions. For this reason it was decided to repeat the single treatments of fatty acids on a new range of cell lines and to use the 7 day exposure only. This proved the most effective time point for delivering accurate significant inhibition results. MDA-MB435S-F (MDA) melanoma cell line and its taxol resistant variants MDA-MB435S-F/Taxol 10p (Tax10p) and MDA-MB435S-F/Taxol 10p4p (Tax10p4p) were developed and kindly donated by Dr. Sharon Glynn (NICB, Dublin) (Glynn *et al.,* 2004).

In this experimental design, stock solutions of fatty acids were prepared in RPMI medium containing bovine serum albumin (BSA) in a 2:1 molar ratio. After seeding for 24 h, MDA, Tax10p and Tax10p4p cells were treated up to a maximum concentration of 40 µg/mL for CLA-mix, *c*9,*t*11 and *t*10,*c*12-CLA; 50 µg/mL for LA and GLA; and 50 ng/mL, for a 7 day exposure. Cytotoxicity was assessed after 7 days using the Acid Phosphatase Assay. Data was obtained from at least three experiments.

While the Tax10p cell line appears to be more sensitive to inhibition by the CLA mixture than the MDA and Tax10p4p lines, this difference was only statistically significant with the 36 & 40 µg/mL treatments ($p < 0.05$), illustrated in Figure 2.3.2.1. IC_{50} values for MDA, Tax10p and Tax10p4p were 26.7, 21.2 & 26.2 µg/mL respectively. LA showed no significant inhibition with respect to control in any cell line at the concentrations used (0-50 µg/mL), illustrated in Figure 2.3.2.2. Furthermore, significant growth stimulation by LA was found at 5 and 45 µg/mL in the Tax10p4p cell

line and at 5 μg/mL in the Tax10p cell line.

Tax10p cells were more sensitive to inhibition by *c*9,*t*11-CLA than the MDA and Tax10p4p lines, this difference was statistically significant with treatments above 20 μg/mL ($p < 0.005$) (Figure 2.3.2.3). IC_{50} values for MDA, Tax10p and Tax10p4p were 31, 19.5 & 27 μg/mL respectively. *t*10,c12-CLA was equally effective at inhibiting cell growth in MDA, Tax10p and Tax10p4p lines at the concentrations used (Figure 2.3.2.4). IC_{50} values for MDA, Tax10p and Tax10p4p were 28.3, 24.5 & 27.5 μg/mL respectively.

GLA treatment on the three cell lines (Figure 2.3.2.5) yielded generally lower inhibition values than the three CLA preparations even though a higher concentration range was used (0-50 μg/mL). The treatment was most effective on the MDA parental line reaching a maximum inhibition of 50.6 % at 50 μg/mL. Maximum inhibition values on Tax10p and Tax10p4p were 25.1 & 40.1 μg/mL respectively.

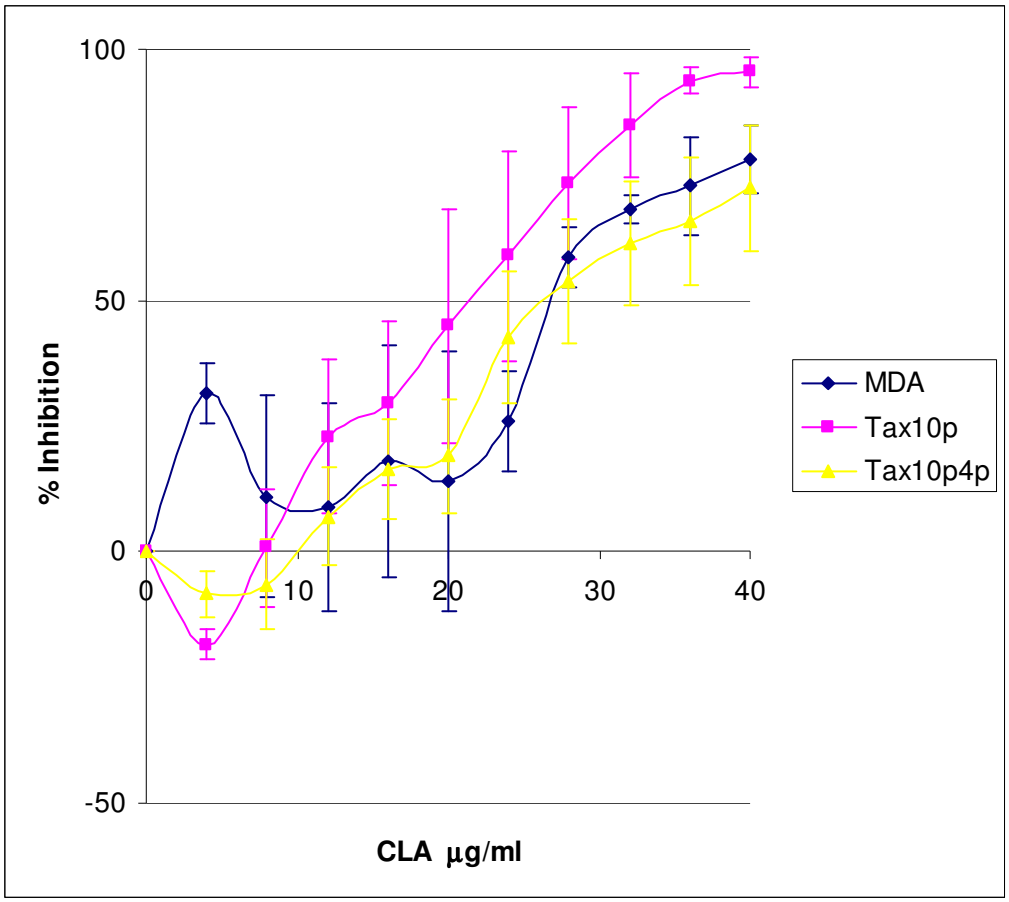


Figure 2.3.2.1 Growth inhibition in MDA, Tax10p and Tax10p4p cells incubated with CLA-mix in the range 0-40 µg/mL, for 7 days. CLA was prepared in medium containing bovine albumin in a 2:1 molar ratio. Cytotoxicity was assessed following the 7 day incubation using the Acid Phosphatase assay as described in Section 2.3.3. Growth inhibition was expressed as a percentage relative to control, which was taken to be 0 %. Each concentration was set up with 6 replicates per plate and each plate was set up in duplicate. Data was obtained from three experiments. Cells without treatment served as controls for this experiment. Error bars depict standard error of the mean.

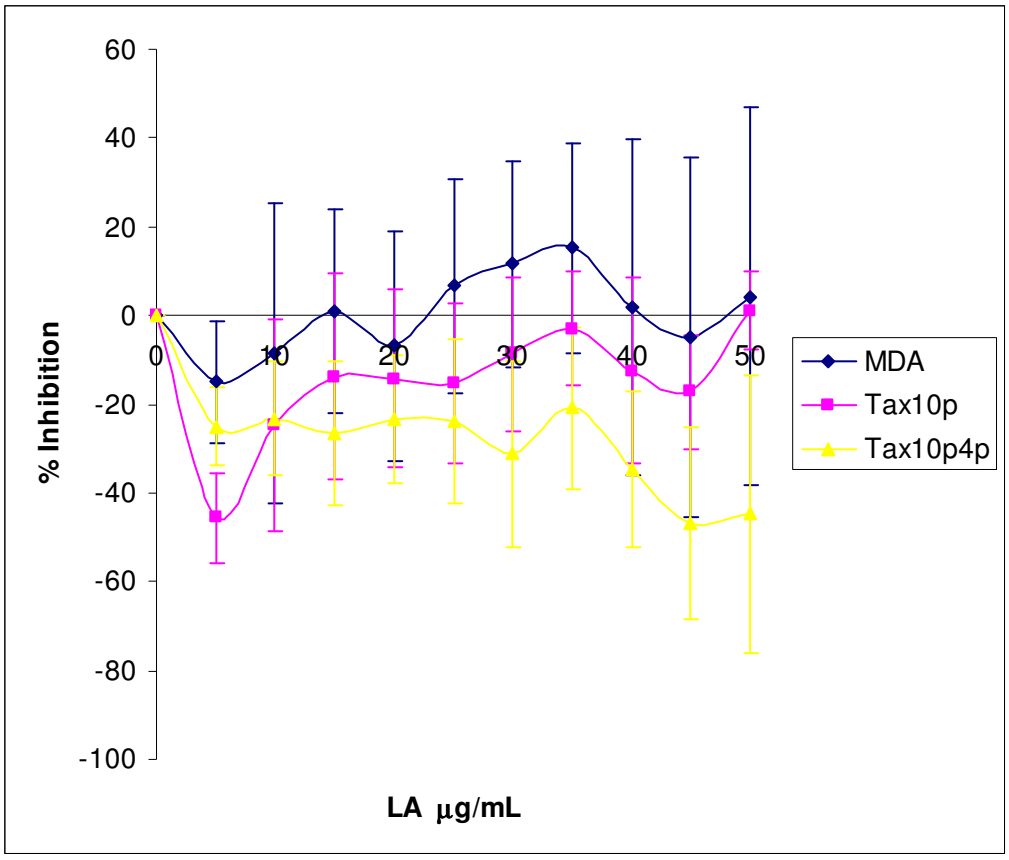


Figure 2.3.2.2 Effects of LA (0-50 µg/mL) on growth of MCF-7 and MCF-7/cis drug resistant cells for 7 days. LA was prepared in medium containing bovine albumin in a 2:1 molar ratio. Growth was assessed following the 7 day incubation using the Acid Phosphatase assay as described in Section 2.3.3. Growth inhibition was expressed as a percentage relative to control, which was taken to be 0 %. Each concentration was set up with 6 replicates per plate and each plate was set up in duplicate. Data was obtained from three experiments. Cells without treatment served as controls for this experiment. Error bars depict standard error of the mean.

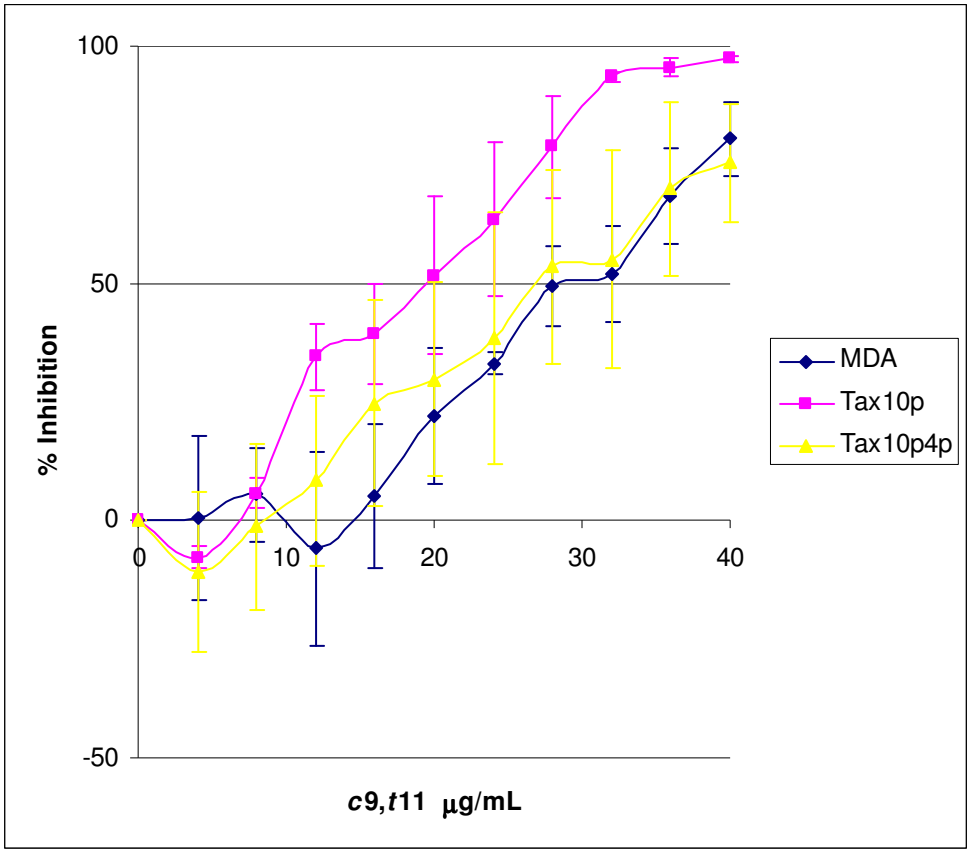


Figure 2.3.2.3 Growth inhibition in MDA, Tax10p and Tax10p4p cells incubated with *c*9,*t*11-CLA in the range 0-40 µg/mL, for 7 days. CLA was prepared in medium containing bovine albumin in a 2:1 molar ratio. Cytotoxicity was assessed following the 7 day incubation using the Acid Phosphatase assay as described in Section 2.3.3. Growth inhibition was expressed as a percentage relative to control, which was taken to be 0 %. Each concentration was set up with 6 replicates per plate and each plate was set up in duplicate. Data was obtained from three experiments. Cells without treatment served as controls for this experiment. Error bars depict standard error of the mean.

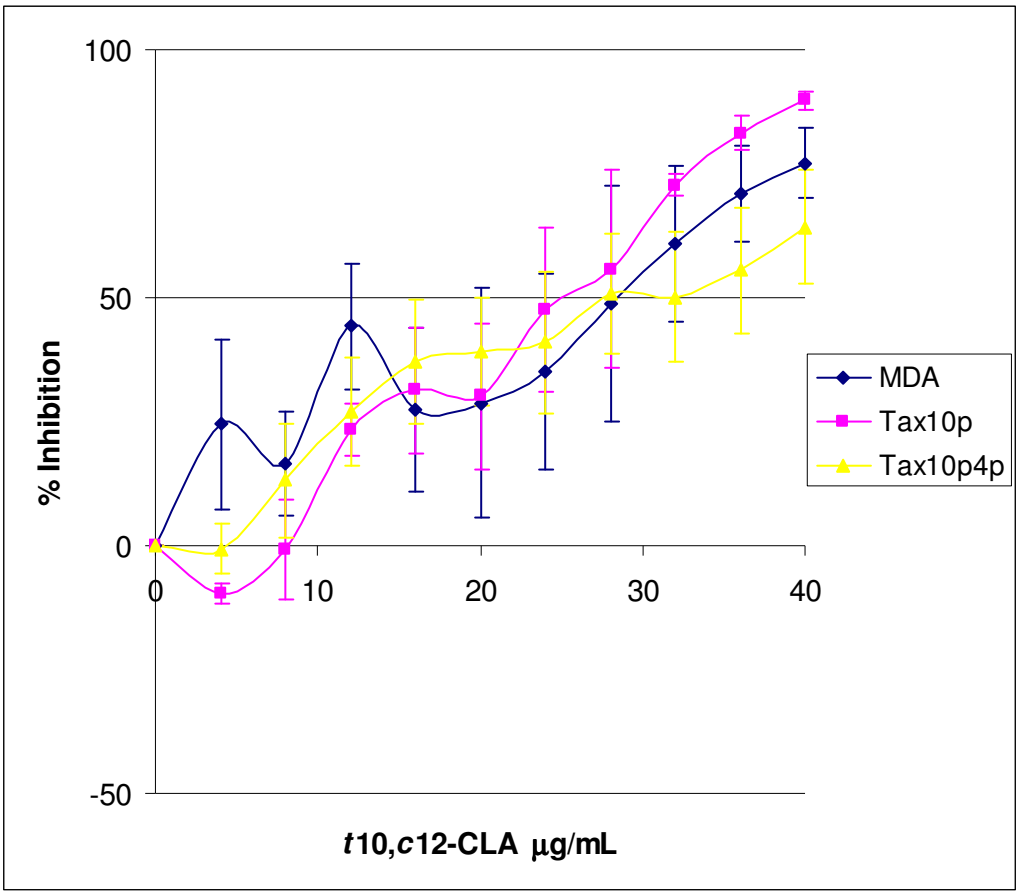


Figure 2.3.2.4 Growth inhibition in MDA, Tax10p and Tax10p4p cells incubated with *t*10,*c*12-CLA in the range 0-40 µg/mL, for 7 days. CLA was prepared in medium containing bovine albumin in a 2:1 molar ratio. Cytotoxicity was assessed following the 7 day incubation using the Acid Phosphatase assay as described in Section 2.3.3. Growth inhibition was expressed as a percentage relative to control, which was taken to be 0 %. Each concentration was set up with 6 replicates per plate and each plate was set up in duplicate. Data was obtained from three experiments. Cells without treatment served as controls for this experiment. Error bars depict standard error of the mean.

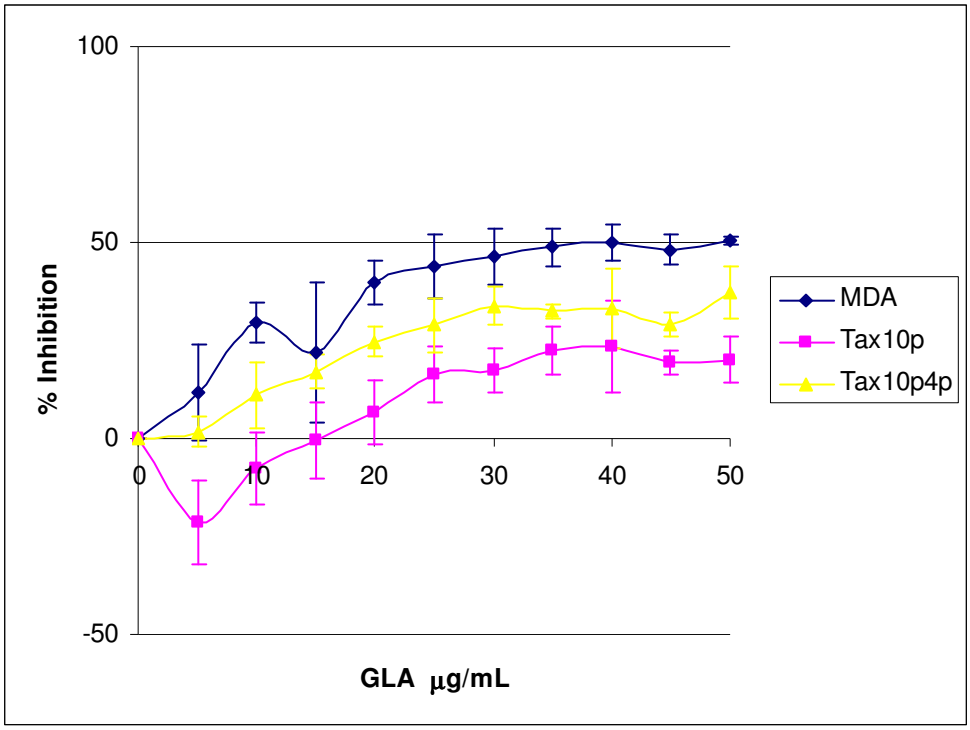


Figure 2.3.2.5 Growth inhibition in MDA, Tax10p and Tax10p4p cells incubated with GLA in the range 0-50 µg/mL, for 7 days. GLA was prepared in medium containing bovine albumin in a 2:1 molar ratio. Cytotoxicity was assessed following the 7 day incubation using the Acid Phosphatase assay as described in Section 2.3.3. Growth inhibition was expressed as a percentage relative to control, which was taken to be 0 %. Each concentration was set up with 6 replicates per plate and each plate was set up in duplicate. Data was obtained from three experiments. Cells without treatment served as controls for this experiment. Error bars depict standard error of the mean.

2.3.3 Single Treatments of Free Fatty Acids on MDA, Tax10p and Tax10p4p cell lines

One of the problems encountered when using bovine serum albumin (BSA) as the vehicle for delivery of fatty acids was that it was observed that BSA introduced considerable variation to the system which was made manifest in high standard deviations and % co-efficient of variations (% CV). For this reason it was decided to repeat the fatty acid assays using free fatty acids instead of that complexed with BSA.

After seeding for 24 h, MDA, Tax10p and Tax10p4p cells were treated in 10 % increments up to a maximum concentration for CLA-mix, *c*9,*t*11 and *t*10,*c*12-CLA at 40 µg/mL and LA and GLA at 50 µg/mL, for a 7 day exposure. Stock solutions of free fatty acids were prepared in basal medium without BSA. Cytotoxicity was assessed after 7 days using the Acid Phosphatase Assay. Data was obtained from at least three experiments.

The free fatty acid CLA treatment resulted in a similar pattern of toxicity in each of the three cell lines (Figure 2.3.3.1). Growth was significantly inhibited in each cell line, but IC_{50} values were not statistically different relative to each other as determined by the Student's *t*-test. IC_{50} values for MDA, Tax10p and Tax10p4p were 15.2, 21.7 & 19.7 µg/mL respectively. Treatment with LA yielded IC_{50} values of 44.0 µg/mL for the MDA cell line and 44.0 and 46.5 µg/mL for Tax10p and Tax10p4p (Figure 2.3.3.2).

Both *c*9,*t*11 and *t*10,*c*12-CLA resulted in a similar pattern of toxicity on the three cell lines, with both the parental MDA cell line and Tax10p4p being more susceptible to inhibition than Tax10p. IC_{50} values for *c*9,*t*11 and *t*10,*c*12-CLA on MDA cells were 12.8 and 10.0 µg/mL; 23.5 and 17.0 µg/mL on Tax10p; and 10.4 and 4.3 µg/mL on Tax10p4p respectively (Figures 2.3.3.3 and 2.3.3.4). Treatment with GLA on the other

hand, resulted in IC_{50} values of 35, 34.3 and 32 µg/mL for MDA, Tax10p and respectively (Figure 2.3.3.5).

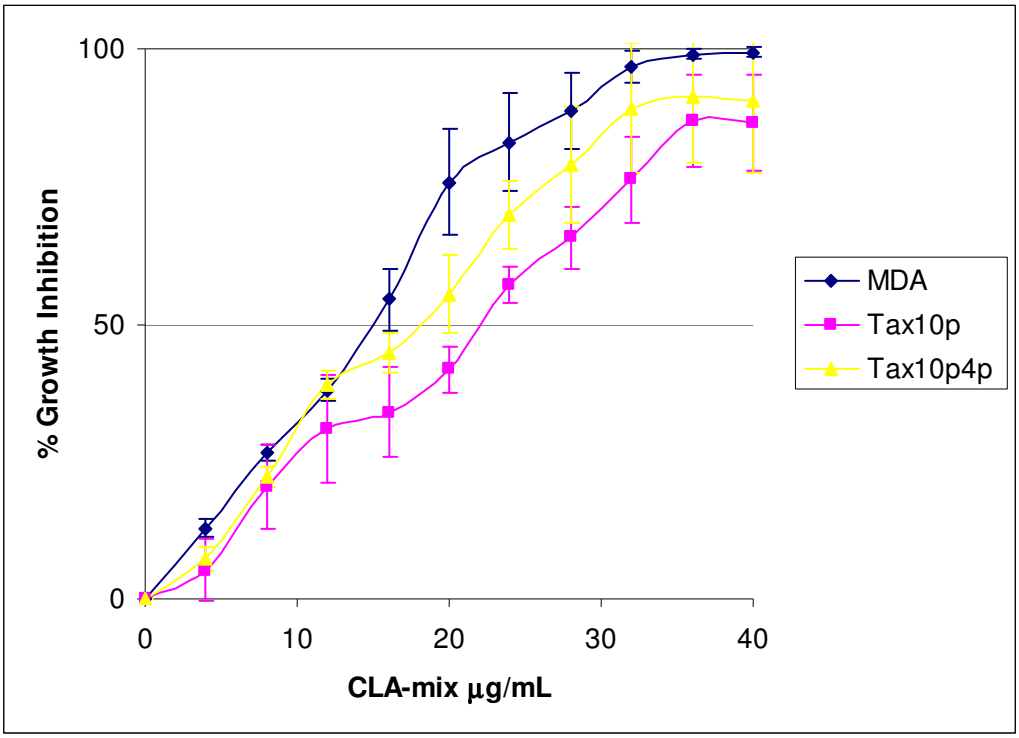


Figure 2.3.3.1 Growth inhibition in MDA, Tax10p and Tax10p4p cells incubated with CLA in the range 0-40 µg/mL, for 7 days. CLA was prepared fresh for each experiment in basal medium without BSA. Cytotoxicity was assessed following the 7 day incubation using the Acid Phosphatase assay as described in Section 2.3.3. Growth inhibition was expressed as a percentage relative to control, which was taken to be 0 %. Each concentration was set up with 8 replicates per plate and each plate was set up in duplicate. Data was obtained from at least three experiments. Cells without treatment served as controls for this experiment. All datapoints for the MDA cell line were significant ($p<0.005$) with respect to control and all above 4 and 8 µg/mL were significant for Tax10p ($p<0.005$) and Tax10p4p ($p<0.01$) respectively, as determined by the Student's *t*-test. Error bars depict standard error of the mean.

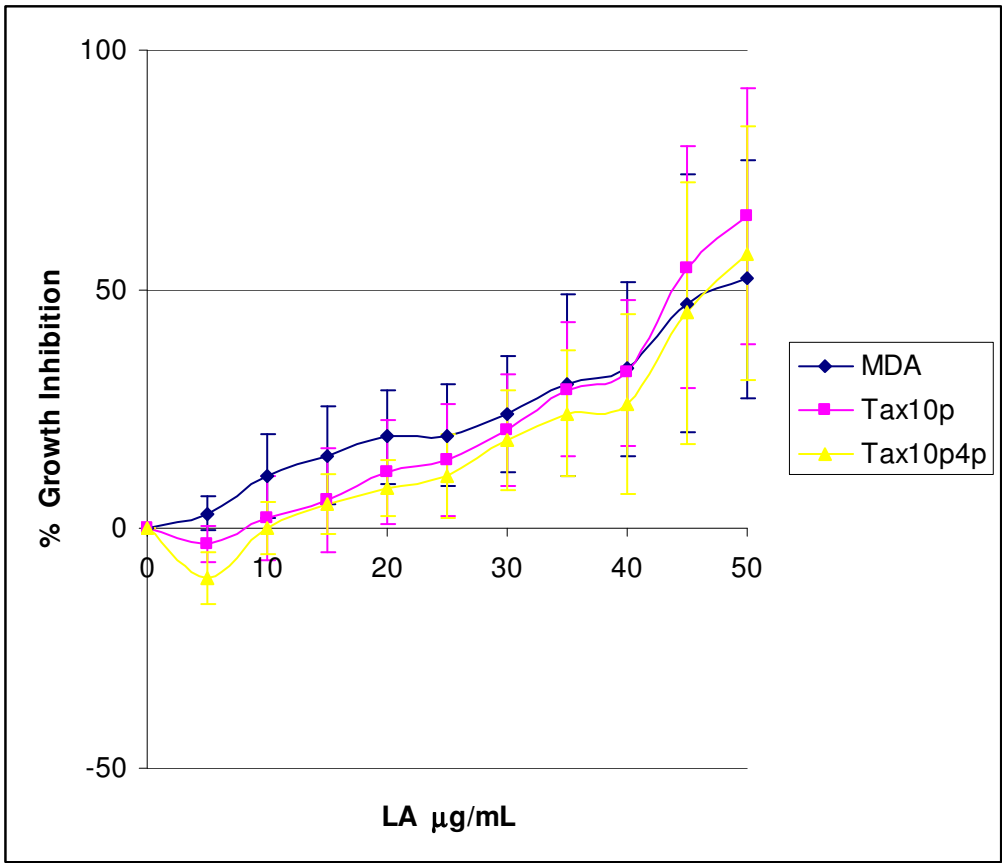


Figure 2.3.3.2. Growth inhibition in MDA, Tax10p and Tax10p4p cells incubated with LA in the range 0-50 µg/mL, for 7 days. LA was prepared fresh for each experiment in basal medium without BSA. Cytotoxicity was assessed following the 7 day incubation using the Acid Phosphatase assay as described in Section 2.3.3. Growth inhibition was expressed as a percentage relative to control, which was taken to be 0 %. Each concentration was set up with 8 replicates per plate and each plate was set up in duplicate. Data was obtained from three experiments. Cells without treatment served as controls for this experiment. All datapoints for the MDA cell line above 15 µg/mL were significant with respect to control and all above 25 µg/mL were significant for Tax10p and Tax10p4p, as determined by the Student's *t*-test ($p<0.05$). Error bars depict standard error of the mean.

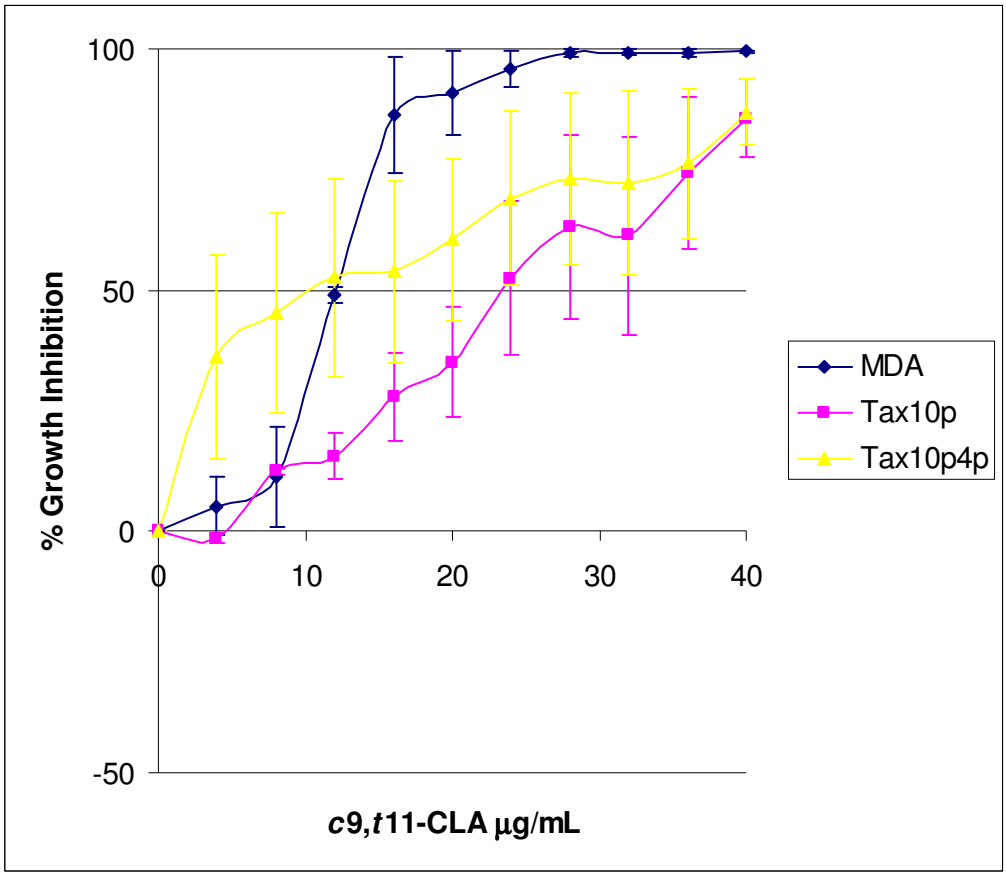


Figure 2.3.3.3 Growth inhibition in MDA, Tax10p and Tax10p4p cells incubated with *c*9, *t*11-CLA in the range 0-40 µg/mL, for 7 days. *c*9,*t*11-CLA was prepared fresh for each experiment in basal medium without BSA. Cytotoxicity was assessed following the 7 day incubation using the Acid Phosphatase assay as described in Section 2.3.3. Growth inhibition was expressed as a percentage relative to control, which was taken to be 0 %. Each concentration was set up with 8 replicates per plate and each plate was set up in duplicate. Data was obtained from four experiments for the MDA cell line, three in the case of Tax10p and Tax10p4p. Cells without treatment served as controls for this experiment. All datapoints above 4 µg/mL were significant with respect to control for the MDA cell line and Tax10p and all were significant for Tax10p4p cells ($p<0.05$), as determined by the Student's *t*-test. Error bars depict standard error of the mean.

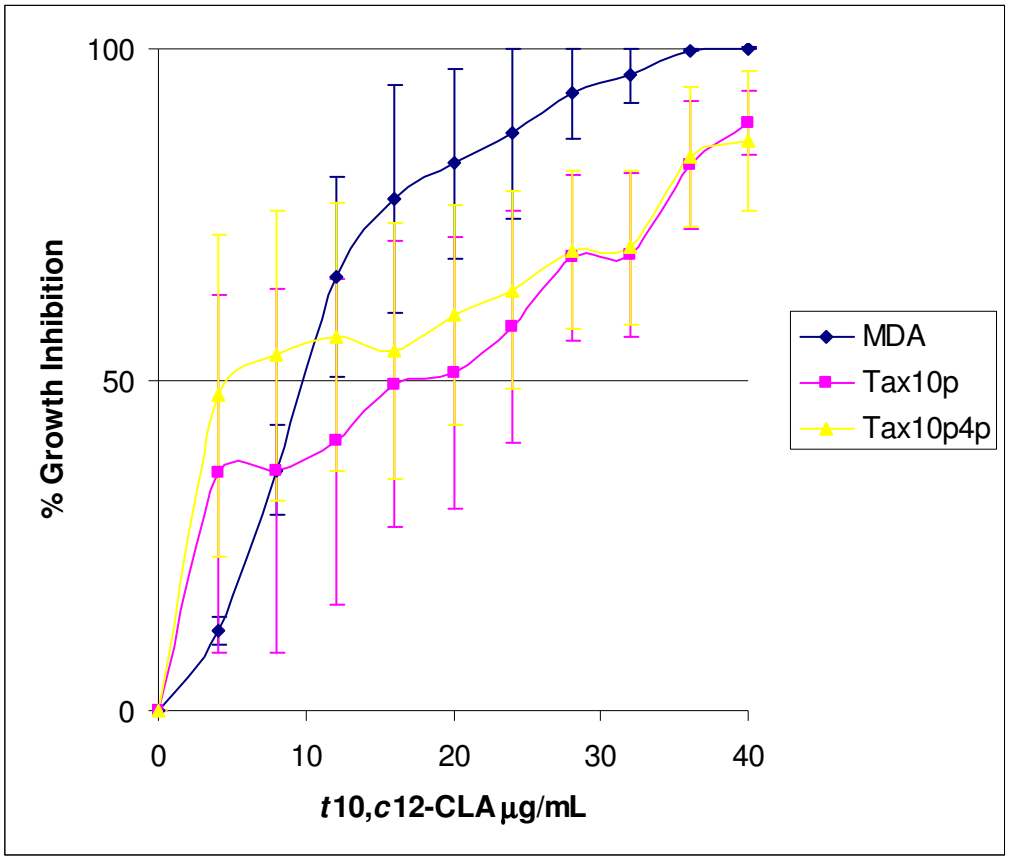


Figure 2.3.3.4 Growth inhibition in MDA, Tax10p and Tax10p4p cells incubated with *t*10, *c*12-CLA in the range 0-40 µg/mL, for 7 days. *t*10,*c*12-CLA was prepared fresh for each experiment in basal medium without BSA. Cytotoxicity was assessed following the 7 day incubation using the Acid Phosphatase assay as described in Section 2.3.3. Growth inhibition was expressed as a percentage relative to control, which was taken to be 0 %. Each concentration was set up with 8 replicates per plate and each plate was set up in duplicate. Data was obtained from three experiments for the MDA cell line and two in the case of Tax10p and Tax10p4p. Cells without treatment served as controls for this experiment. All datapoints were significant with respect to control for the MDA cell line ($p \leq 0.001$); all except 8 µg/mL for Tax10p ($p<0.05$) and all above 8 µg/mL for Tax10p4p ($p<0.005$), as determined by the Student's *t*-test. Error bars depict standard error of the mean.

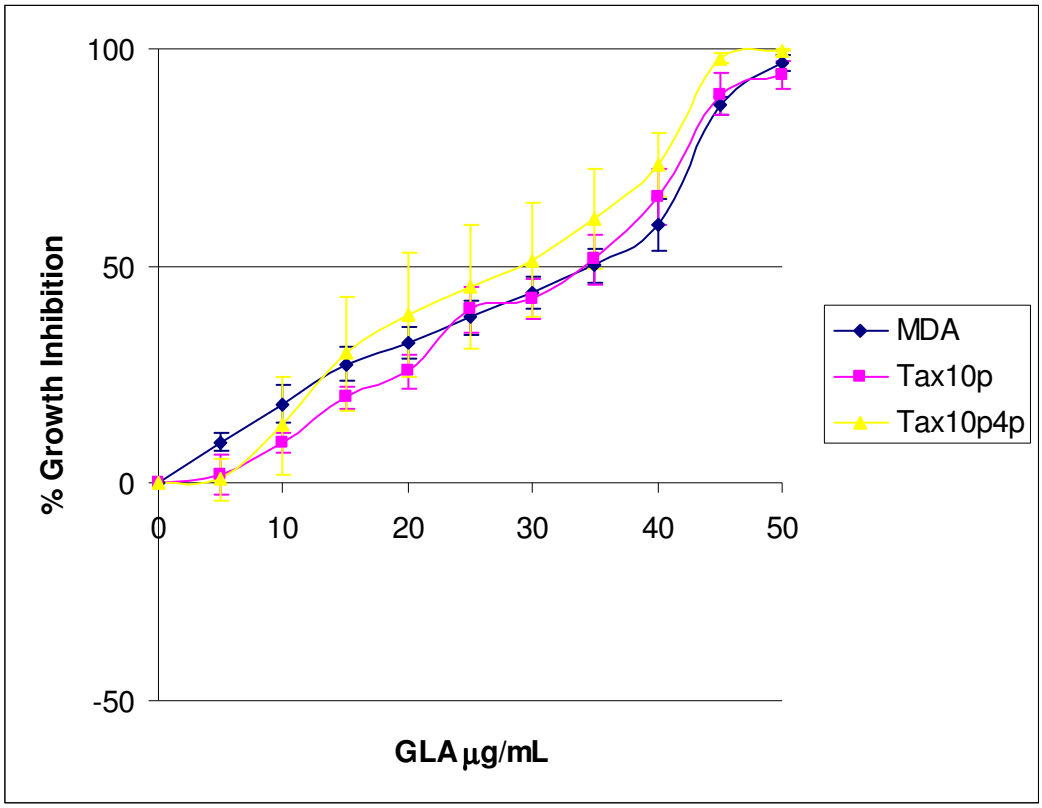


Figure 2.3.3.5 Growth inhibition in MDA, Tax10p and Tax10p4p cells incubated with γ-Linolenic Acid (GLA) in the range 0-40 µg/mL, for 7 days. GLA was prepared fresh for each experiment in basal medium without BSA. Cytotoxicity was assessed following the 7 day incubation using the Acid Phosphatase assay as described in Section 2.3.3. Growth inhibition was expressed as a percentage relative to control, which was taken to be 0 %. Each concentration was set up with 8 replicates per plate and each plate was set up in duplicate. Data was obtained from three experiments for the MDA cell line and two in the case of Tax10p and Tax10p4p. Cells without treatment served as controls for this experiment. All datapoints were significant with respect to control for the MDA cell line ($p < 0.001$) and all above 5 µg/mL and 10 µg/mL for Tax10p ($p<0.01$) and Tax10p4p ($p<0.05$) respectively, as determined by the Student's t-test. Error bars depict standard error of the mean.

2.3.4 Single Treatments of Chemotherapy Drugs on MDA, Tax10p and Tax10p4p cell lines in long term exposure assays

After seeding for 24 h, MDA, Tax10p and Tax10p4p cells were treated in 10 % increments up to a maximum concentration of 50 ng/mL, 10 ng/ml and 400 ng/mL for doxorubicin (Dox), taxotere (Tax) and 5-fluorouracil (5-FU) respectively for a 7 day exposure. Stock solutions of drug treatments were prepared in basal medium. Cytotoxicity was assessed after 7 days using the Acid Phosphatase Assay. Data was obtained from at least three experiments.

Doxorubicin was equally effective at inhibiting growth in the parental MDA and Tax10p and Tax10p4p lines (Figure 2.3.4.1). IC_{50} values for MDA, Tax10p and Tax10p4p were 12.3, 12.4 & 12.7 ng/mL respectively. All points above 5 ng/mL were significant with $p<0.005$. Taxotere was also found to be similarly effective at inhibiting growth in the MDA, Tax10p and Tax10p4p lines. IC_{50} values for MDA, Tax10p and Tax10p4p were 0.65, 0.64 & 0.94 ng/mL respectively (Figure 2.3.4.2). All points were significant with $p<0.005$. Response to 5-Fluorouracil treatment followed a similar pattern in all three cell lines. IC_{50} values for MDA, Tax10p and Tax10p4p were 109.9, 119.3 and 117 ng/mL respectively (Figure 2.3.4.3). All points were significant with $p<0.05$.

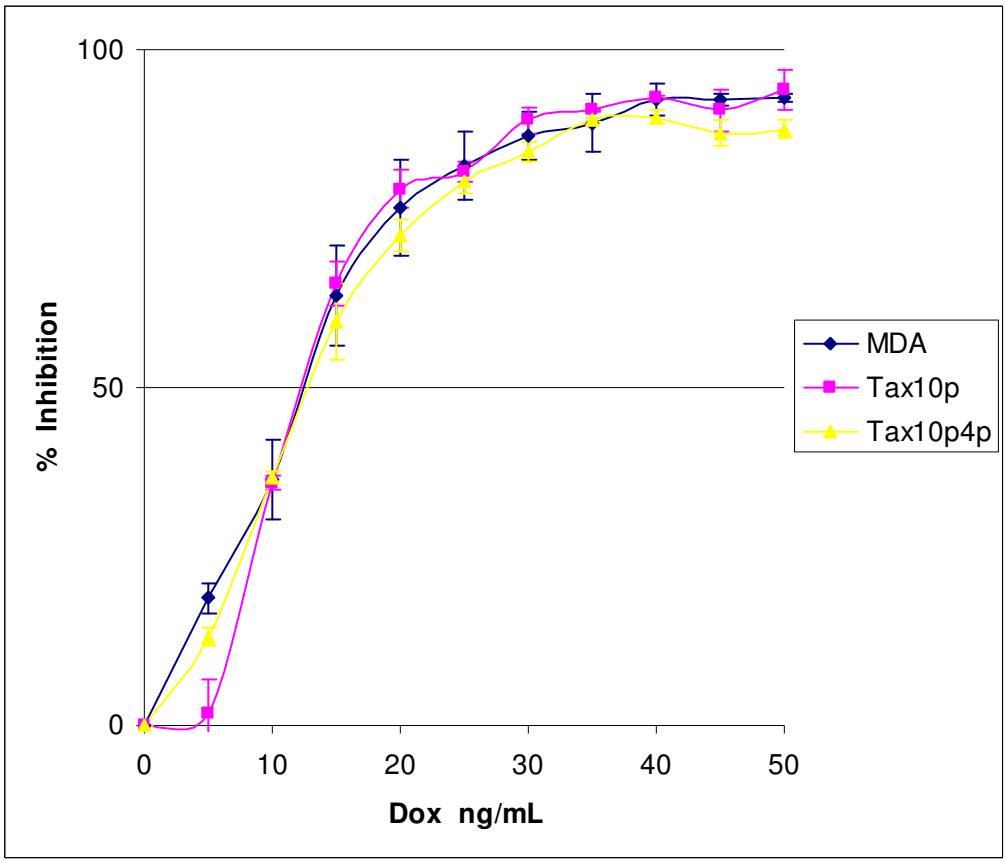


Figure 2.3.4.1 Growth inhibition in MDA, Tax10p and Tax10p4p cells incubated with Doxorubicin (Dox) in the range 0-50 ng/mL, for 7 days. Dox was prepared fresh for each experiment in basal medium. Cytotoxicity was assessed following the 7 day incubation using the Acid Phosphatase assay as described in Section 2.3.3. Growth inhibition was expressed as a percentage relative to control, which was taken to be 0 %. Each concentration was set up with 6 replicates per plate and each plate was set up in duplicate. Data was obtained from three experiments. Cells without treatment served as controls for this experiment. Error bars depict standard error of the mean.

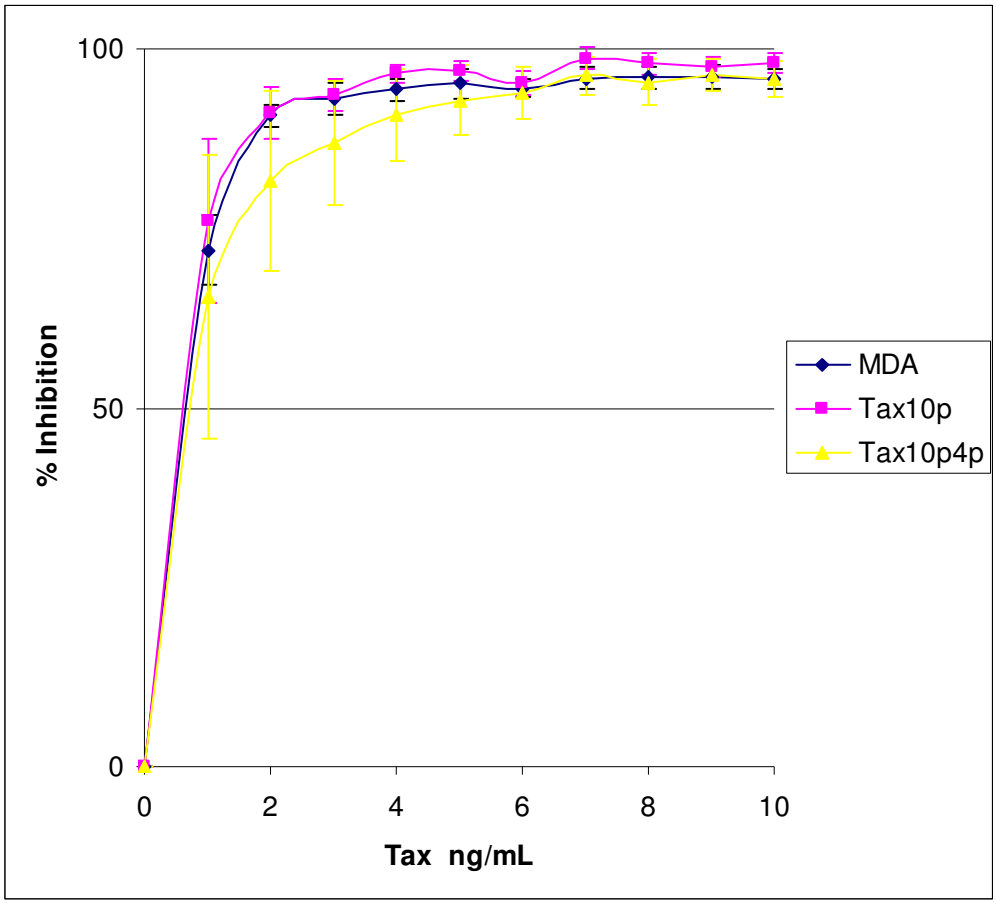


Figure 2.3.4.2 Growth inhibition in MDA, Tax10p and Tax10p4p cells incubated with Tax in the range 0-10 ng/mL, for 7 days. Tax was prepared fresh for each experiment in basal medium. Cytotoxicity was assessed following the 7 day incubation using the Acid Phosphatase assay as described in Section 2.3.3. Growth inhibition was expressed as a percentage relative to control, which was taken to be 0 %. Each concentration was set up with 6 replicates per plate and each plate was set up in duplicate. Data was obtained from three experiments. Cells without treatment served as controls for this experiment. Error bars depict standard error of the mean.

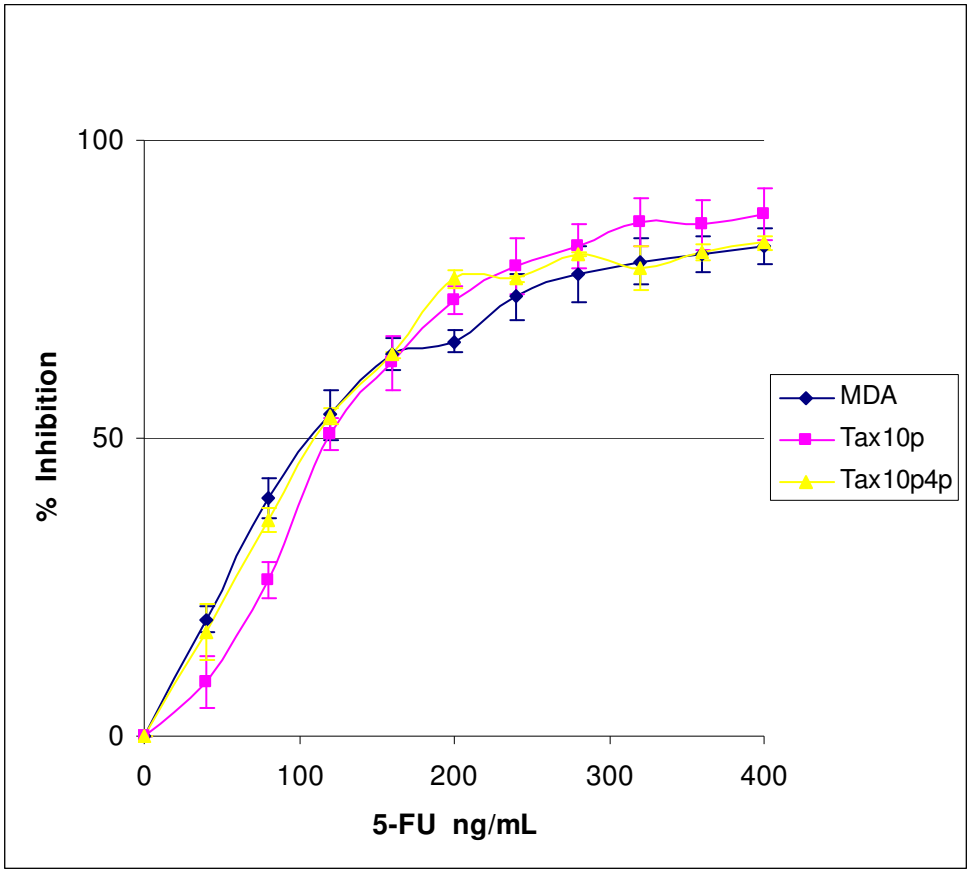


Figure 2.3.4.3 Growth inhibition in MDA, Tax10p and Tax10p4p cells incubated with 5-FU in the range 0-400 ng/mL, for 7 days. 5-FU was prepared fresh for each experiment in basal medium. Cytotoxicity was assessed following the 7 day incubation using the Acid Phosphatase assay as described in Section 2.3.3. Growth inhibition was expressed as a percentage relative to control, which was taken to be 0 %. Each concentration was set up with 6 replicates per plate and each plate was set up in duplicate. Data was obtained from three experiments. Cells without treatment served as controls for this experiment. Error bars depict standard error of the mean.

2.3.5 Single Treatments of Chemotherapy Drugs on MDA, Tax10p and Tax10p4p cell lines in short term exposure assays

In the previous section, no significant difference was observed in the sensitivity of the three cell lines to taxotere (Tax), doxorubicin (Dox) and 5-fluorouracil (5-FU) – suggesting that resistance of the Tax10p and Tax10p4p lines to the drugs would be better observed in short term assays. To this end it was decided to run the drug assays as 4 h exposure, 7 day recovery in place of long term (7 day) exposure –with a view to mimicking the clinical situation more closely (Glynn *et al*, 2004).

After seeding for 24 h, MDA, Tax10p and Tax10p4p cells were treated in 10 % increments up to a maximum concentration for 500 ng/mL, 50 ng/mL and 10 µg/mL for Dox, Tax and 5-FU respectively for 4 h exposure followed by a 7 day recovery period. Stock solutions of drug treatments were prepared in basal medium. Cytotoxicity was assessed after 7 days using the Acid Phosphatase Assay. Data was obtained from at least three experiments.

Doxorubicin treatment for the 4 h exposure, 7 day recovery resulted in IC_{50} values of 228, 274 and 334 ng/mL for the MDA, Tax10p and Tax10p4p cell lines respectively. All Dox treatments above 50 ng/mL were significant relative to control with $p \leq 0.0001$ for the MDA cell line and $p < 0.05$ for Tax10p4p and Tax10p as determined by the Student's *t*-test (Figure 2.3.5.1). Treatment with Taxotere showed that the parental MDA was more susceptible to drug cytotoxicity than the Tax10p and Tax10p4p cell lines with IC_{50} values of 13.0, 40.0 and 50.5 ng/mL respectively. All concentrations of Tax resulted in significant inhibition for the MDA cell line, whereas all above 5 and 25 ng/mL were significant for Tax10p and Tax10p4p cells respectively ($p \leq 0.05$) (Figure 2.3.5.2). Finally, with 5-Fluorouracil exposure there was a similar order of susceptibility with IC_{50} values on MDA, Tax10p and Tax10p4p being 6, 9.75 and 9.9 µg/mL respectively. All concentrations above 1 µg/mL 5-FU yielded significant inhibition

relative to control for the MDA cell line, whereas 4 and 7-10 µg/mL 5-FU resulted in significant inhibition for Tax10p and all above 3 µg/mL were significant for Tax10p4p cells, with $p<0.05$ (Figure 2.3.5.3).

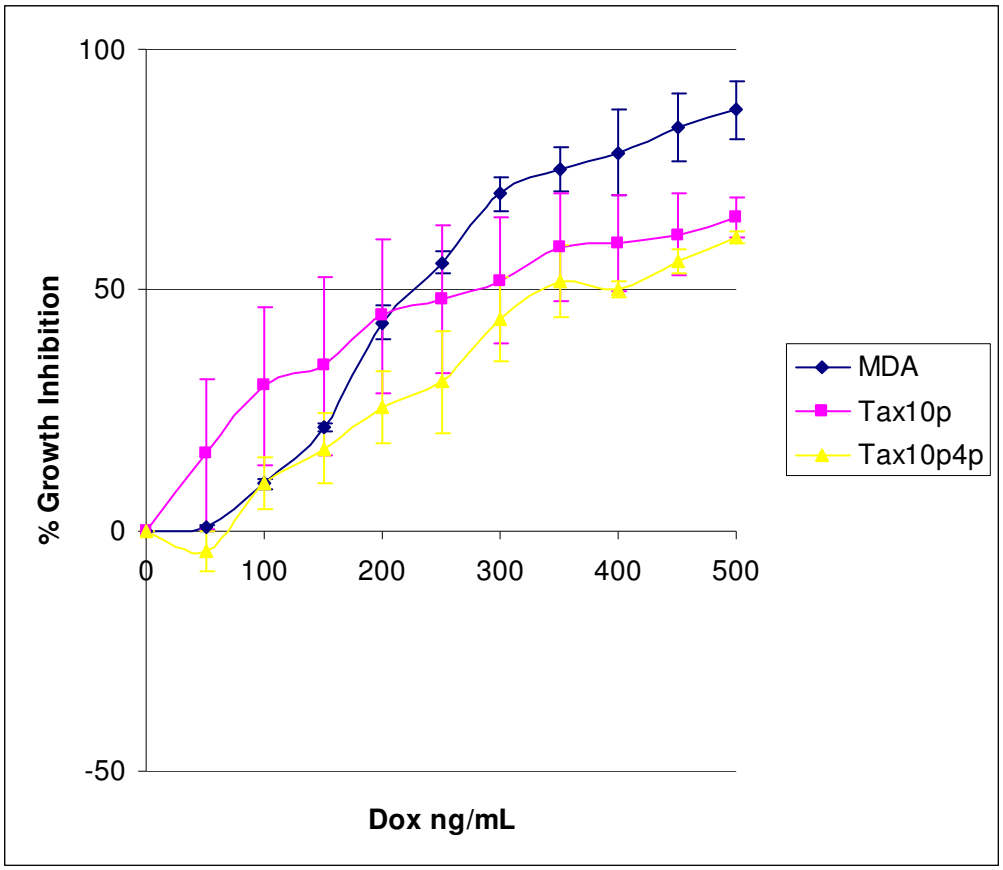


Figure 2.3.5.1 Growth inhibition in MDA, Tax10p and Tax10p4p cells incubated with Doxorubicin (Dox) in the range 0-500 ng/mL for 4 h followed by a 7 day recovery. Dox was prepared fresh for each experiment in basal medium. Cytotoxicity was assessed following the 7 day incubation using the Acid Phosphatase assay as described in Section 2.3.3. Growth inhibition was expressed as a percentage relative to control, which was taken to be 0 %. Each concentration was set up with 8 replicates per plate and each plate was set up in duplicate. Data was obtained from three experiments. Cells without treatment served as controls for this experiment. Error bars depict standard error of the mean.

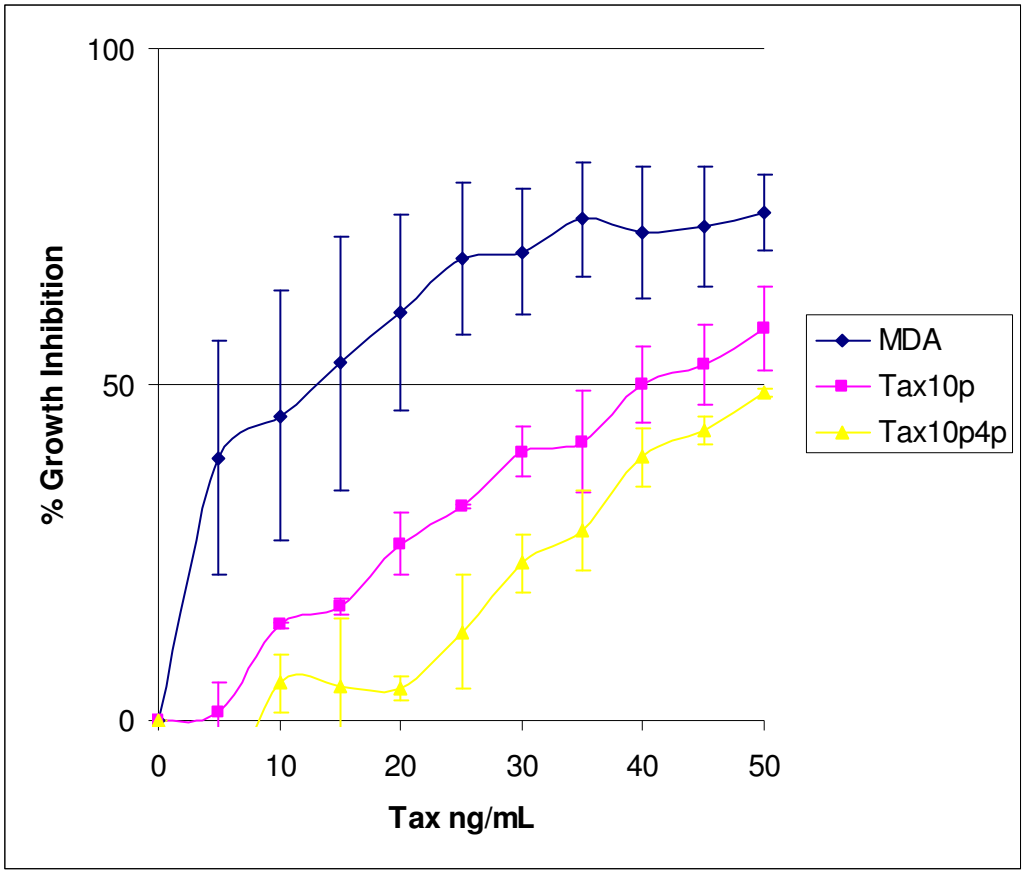


Figure 2.3.5.2 Growth inhibition for MDA, Tax10p and Tax10p4p cells incubated with Taxotere (Tax) in the range 0-50 ng/mL for 4 h followed by a 7 day recovery. Tax was prepared fresh for each experiment in basal medium. Cytotoxicity was assessed following the 7 day incubation using the Acid Phosphatase assay as described in Section 2.3.3. Growth inhibition was expressed as a percentage relative to control, which was taken to be 0 %. Each concentration was set up with 8 replicates per plate and each plate was set up in duplicate. Data was obtained from three experiments. Cells without treatment served as controls for this experiment. Error bars depict standard error of the mean.

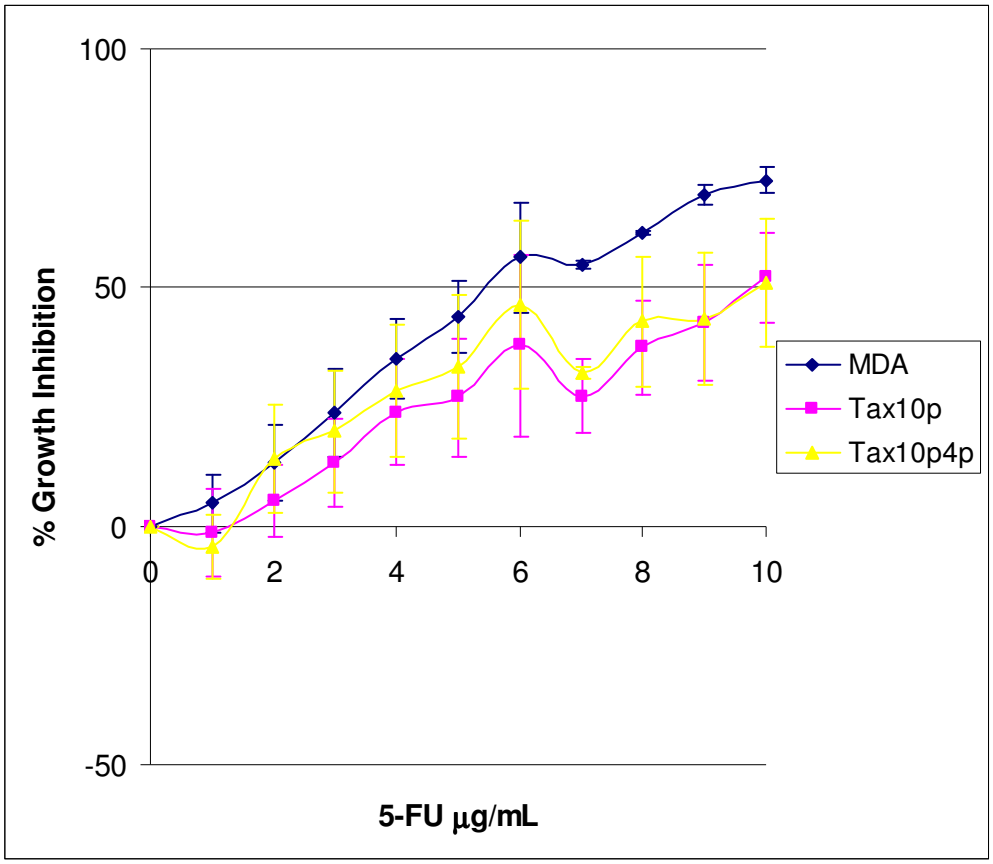


Figure 2.3.5.3 Growth inhibition in MDA, Tax10p and Tax10p4p cells incubated with 5-Fluorouracil (5-FU) in the range 0-10 µg/mL for 4 h, followed by a 7 day recovery. 5-FU was prepared fresh for each experiment in basal medium. Cytotoxicity was assessed following the 7 day incubation using the Acid Phosphatase assay as described in Section 2.3.3. Growth inhibition was expressed as a percentage relative to control, which was taken to be 0 %. Each concentration was set up with 8 replicates per plate and each plate was set up in duplicate. Data was obtained from three experiments. Cells without treatment served as controls for this experiment. Error bars depict standard error of the mean.

2.4 Discussion

In the first set of single treatments, MCF-7 and MCF-7/cis cells were treated with fatty acids (conjugated linoleic acid mixture (CLA-mix), LA, *c*9,*t*11 and *t*10,*c*12-CLA) delivered as sodium salts in medium containing BSA in a 2:1 w/w ratio (473:1 molar ratio). Cytotoxic effects of CLA-mix, *c*9,*t*11 and *t*10,*c*12-CLA were time and dose-dependent in both MCF-7 and MCF-7/cis cell lines. Cytotoxicity of CLA was found to be dependent on isomer composition, concentration and duration of exposure. This is in agreement with findings by O'Shea *et al.* (2000) that the cytotoxic effects of CLA were concentration dependant in the MCF-7 cell line. However little is known about the action of CLA on a drug-resistant cell line. Here we found MCF-7 cisplatin resistant cells were more sensitive to inhibition by *t*10,*c*12-CLA and the CLA mixture than parental MCF-7 cells. Linoleic acid (LA) displayed a much lower cytotoxicity profile than the three CLA preparations as expected, with negligible effects on growth following exposure of both cell lines to linoleic acid at concentrations less than 18 μg/mL.

With a view to moving on to investigate the synergistic potential of fatty acid treatments with chemotherapy drugs, it was decided that it would be more beneficial to use a model where the resistance and other qualities of the cell lines in question were better characterised to gain a better understanding of the interactions. MDA-MB435S-F (MDA) human melanoma cell line and taxol resistant variants MDA-MB435S-F/Taxol 10p (Tax10p) and MDA-MB435S-F/Taxol 10p4p (Tax10p4p) were developed and kindly donated by Dr. Sharon Glynn (NICB, Dublin) (Glynn *et al*, 2004). The single treatments of fatty acids were tested on the new range of cell lines in the 7 day exposure only. This proved the most effective time point for delivering accurate significant inhibition results.

In this experimental design, stock solutions of fatty acids were prepared in RPMI

medium containing BSA in a 2:1 molar ratio. Of the fatty acid treatments, CLA-mix and *t*10,*c*12-CLA were similarly effective at inhibiting growth in all three cell lines. *c*9,*t*11-CLA however, at the higher concentrations (36 & 40 μg/mL) appeared to be more effective at inhibiting cell growth in Tax10p cells than in the MDA or Tax10p4p cell lines. LA showed no significant inhibition in any cell line at the concentrations used (0-50 μg/mL). GLA on the other hand, while the resulting inhibition was significant relative to control, the inhibition observed was much lower than that of all three CLA preparations.

It was also decided to repeat the fatty acid assays using free fatty acids instead of that complexed with bovine serum albumin (BSA). One of the problems encountered when using BSA as the vehicle for delivery was that it introduced considerable variation to the system which was made manifest in high standard deviations and % co-efficient of variations (% CV). Fatty acids were made up as free fatty acid solutions in basal RPMI medium, fresh for each experiment. Delivery of the fatty acids as free fatty acid solutions eliminated one source of variation in the experiments, resulting in statistically significant inhibition at lower concentrations –including with LA, which was showing negligible effects complexed with BSA. The IC_{50} values of all fatty acid treatments were reduced compared to the treatments containing BSA in a 2:1 molar ratio, suggesting that BSA conferred a protective effect on the cells. The MDA cell line appeared to be more susceptible to inhibition by the three CLA preparations (CLA-mix, *c*9,*t*11 and *t*10,*c*12-CLA) than Tax10p4p, which was in turn more susceptible than, Tax10p although these differences were not statistically significant.

The chemotherapeutic agents doxorubicin (Dox), taxotere (Tax) and 5-fluorouracil (5-FU) were also tested on MDA, Tax10p and Tax10p4p cells in a 7 day exposure. Drug treatments were made up in medium and were prepared fresh for each experiment. Cytotoxicity was assessed after a 7 day incubation. No significant difference was observed in the sensitivity of the three cell lines to Tax, Dox or 5-FU –suggesting that resistance of the Tax10p and Tax10p4p lines to Tax and other drugs would be better

observed in short term assays. To this end it was decided to run the drug assays as 4 h exposure, 7 day recovery in place of long term (7 day) exposure –also with a view to mimicking the clinical situation more closely (Glynn *et al*, 2004). Drug treatments were again prepared fresh for each experiment in basal medium.

With Dox, Tax and 5-FU, the change in design from treatments for a 7 day exposure to the 4 h exposure, 7 day recovery resulted in a dramatic increase in IC_{50} values as expected. Differences emerged between the cell lines in terms of drug sensitivities. When comparing IC_{50} values, Tax10p and Tax10p4p cells were 1.6 and 1.7 fold more resistant to 5-FU treatment than MDA cells respectively. Similarly, Tax10p and Tax10p4p were 1.2 and 1.5 fold more resistant to Dox treatment and 3.1 and 3.9 fold more resistant to Tax treatment than the MDA cell line. Not surprisingly, of the two cell lines that had previously been taxol pulsed; Tax10p4p, which had four extra rounds of pulses of Tax, displayed higher resistance to each of the three drugs than Tax10p. This finding supports data from the original characterisation of these cell lines (Glynn *et al*, 2004). IC_{50} values obtained in this section will be used in the design of an experiment using sub-optimal concentrations of both fatty acids and drugs to investigate synergistic interactions in melanoma cells.

Chapter 3

Enhancement of Doxorubicin Cytotoxicity by Conjugated Linoleic Acid (CLA) in a cellular model of Drug-Resistant Cancer

3.0 Abstract

The effects of two isomers of conjugated linoleic acid (CLA) and a mixture of isomers on doxorubicin (Dox) cytotoxicity and intracellular drug accumulation were evaluated in a human melanoma cell line MDA-MB435S-F (MDA) and a Dox resistant variant MDA-MB435S-F/A10p10p (A10p10p). CLA enhanced cytotoxicity of Dox in a dose dependant manner in MDA cells and enhanced the toxicity with greater effect in the resistant A10p10p cells. The *c*9,*t*11-CLA isomer significantly increased uptake of Dox ($p<0.05$) in both MDA and A10p10p cell lines. CLA mixture of isomers also resulted in significantly greater retention of Dox in A10p10p cells ($p<0.05$) after 3 h of efflux, while *c*9,*t*11-CLA again significantly increased Dox retention in both cell lines after 3 h ($p<0.05$). These data demonstrate the potential of CLA for further use in modulating tumour drug-sensitivity in pre-clinical trials.

3.1 Introduction

Multidrug resistant (MDR) cancer cells accumulate chemotherapeutic drugs to a lesser degree than sensitive cells. It is believed that ability to keep intracellular drug levels below a cell killing threshold is principally the result of pumping out of cytotoxic drugs by transport proteins resident in the lipid bilayer of cells. Targeting multidrug resistance (by co-administering pump inhibitors, pump bypass agents or membrane active agents) has been a goal of cancer biologists during the past 35 years. However recent studies (reviewed in Hendrich and Michalak 2003) have shown that acquisition of the MDR phenotype in general is accompanied by up-regulation of specific membrane lipids notably glucosylceramide, cholesterol and phospholipids containing saturated fatty acids. The development of doxorubicin resistance in breast and colon cancer cells has been associated with decreased fluidity of lipid bilayers and increased degree of fatty acid saturation of cholesteryl esters (Santini *et al*, 2001). These lipids constitute liquid-ordered membrane micro-domains which house members of the ABC (ATP binding cassette) transporter superfamily (Pgp, MRP1) responsible for drug efflux.

Considering the importance of lipid phase in modulating membrane structure and function in tumour cells, it is not surprising that many studies have been carried out to elucidate the role of polyunsaturated fatty acids in modulating tumour drug sensitivity. Several *in vitro* studies and limited *in vivo* investigations have shown that select polyunsaturated fatty acids enhance the cytotoxicity of several anticancer agents. Gamma linolenic acid was shown to synergistically enhance the cytotoxicity of lipophilic taxane drugs against human breast cancer cells (Menendez *et al.,* 2001, 2002, 2004). Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) were found to enhance taxol and taxotere efficacy in various breast cancer cell lines including MDA-MB-231 (Germain *et al.*, 1998; Menendez *et al.*, 2002 & 2005), T47D and SK-Br3 (Menendez *et al.*, 2002 & 2005). DHA has also been reported to reverse taxane (taxol and taxotere) resistance in BT-474 taxane resistant breast cancer cell lines (Menendez *et*

al., 2005). Supplementation of diet with fish oil containing EPA and DHA potentiated efficacy of doxorubicin against lung and breast cancer xenografts (Hardman *et al.*, 1999, 2000, 2001). A pro-drug formed by covalent linkage of DHA with paclitaxel (Taxoprexin; Protarga, Inc), improved safety and efficacy of paclitaxel in a Phase 1 study (Wolff *et al.*, 2003).

All of the fatty acids that enhance sensitivity of drug-resistant human tumour cell lines cited above contain double bonds in the cis configuration. Current evidence suggests that susceptibility of PUFA's to oxidation and generation of cytotoxic hydroperoxides in drug resistant cancer cells may reverse the antioxidant tone that characterises drug resistant cells in which glutathione and antioxidant enzyme activities are elevated to protect the cell against drug-induced free radical aggression (Mahéo *et al.*, 2005).

Interestingly a wealth of literature now exists to support a role for conjugated isomers of these fatty acids also being metabolised and active in cancer cell models (Cesano *et al.*, 1996; Ha *et al.*, 1987 & 1990; Hubbard *et al.*, 2000, 2003, 2006; Ip *et al.*, 1999; Liew *et al.*, 1995; Miglietta *et al.*, 2006; Tsujita-Kyutoku *et al.*, 2004; Tsuzuki *et al.*, 2004; Visonneau *et al.*, 1998) Conjugated linoleic acid (CLA) refers to a group of positional and geometric isomers of octadecadienoic acids (C18:2) with two conjugated double bonds. The double bonds of CLA can be located at the 9,11 or 10,12 positions of C18:2 and in *cis* and/or *trans* spatial configurations (Lin *et al*., 1995). CLA has been shown to be an anticancer agent in a number of rodent and human tumour model systems, including carcinogen-induced and transplantable mammary tumour models. Several isomers of CLA (*c*9*t*11-, *t*10*c*12- and *t*9*t*11-CLA) both in pure form and as a mixture have been shown to be cytotoxic at micromolar concentrations for a variety of breast, colon, prostate and skin tumour cell lines (Belury, 2002a). *c*9*t*11- and *t*10*c*12-CLA have been shown to alter a wide array of targets involved in regulation of cell proliferation, apoptosis, angiogenesis, tumour invasion and differentiation (proteins of BCL-2 family, cyclins and cyclin dependent kinase inhibitors, protein-kinases and phosphatases (Miglietta *et al.*, 2006), COX-2 (Nugent *et al.*, 2005), 5-LOX (Ochoa *et al.*, 2004),

VEGF and matrix-metalloproteinases (Wang *et al.,* 2005; Hubbard *et al.,* 2007) and alkaline phosphatase (Lampen *et al.*, 2005)). In addition they have been shown to act as transcription regulators, being able to modulate the activity of different transcription factors, including NFkB (Goua *et al.*, 2008); peroxisome proliferator-activated receptors (Belury *et al.,* 2007), retinoid X receptors (Lee *et al.*, 2008/9) and SREBP (Ecker *et al.,* 2007; Purushotham *et al.*, 2008). Enzymes regulating lipid metabolism, such as fatty acid synthase and stearoyl-CoA desaturase (SCD), are further molecular targets critical in the growth and survival of cancer cells which have been modulated by CLA (Choi *et al.,* 2000, 2002; Ntambi *et al.,* 2004).

Previous work in this lab showed a concentration dependent cytotoxic effect of CLA in human breast cancer (MCF-7) and colon cell lines (SW480, HT29) which was associated with peroxidative damage (O'Shea *et al.*, 2000), alterations in antioxidant enzymes (O'Shea *et al.*, 1999), rapid cellular uptake and modulation of membrane phospholipid composition (Miller *et al.*, 2001; 2003) and induction of apoptosis (Miller *et al.*, 2002). The discovery by Fite *et al.* (2007) that CLA isomers (40 μM) can significantly potentiate the cytotoxicity of docetaxel in breast cancer cells is of considerable importance as it was the first study to show the potential role of CLA to improve chemotherapy effects when drug treatments are accompanied by CLA isomers. The effective cytotoxic concentration of docetaxel was reduced by up to 70 % when the *t*10,*c*12-CLA isomer was present and by 59 % when a 50:50 isomer mix of *t*10,*c*12-CLA and *c*9,*t*11-CLA was present. CLA's similarity to docetaxel in terms of modulating similar key oncogenes involved in the apoptotic pathway suggests CLA may be a new adjuvant agent for chemotherapy. The effect of CLA on the process of intracellular drug accumulation (uptake, retention, distribution and efflux) in breast cancer cells remains to be determined.

The cytotoxic activity of a CLA mixture of isomers (CLA-mix) and two single isomers, *c*9*t*11 and *t*10*c*12-CLA (all present at 1 and 6 μg/ml equivalent to 3.6 and 21.4 μM) in combination with doxorubicin (37.5-115 ng/mL or 65-200 nM), in the MDA-MB-435S-

F human melanoma cell line and a doxorubicin-pulsed variant was assessed. Net uptake/accumulation of Dox in cells following 2 h treatment and retention after 3 h of efflux were determined by liquid chromatography tandem mass spectrometry (LC-MS) methods. The study provided evidence that the co-administration of CLA at as low a concentration as 21.4 μM and doxorubicin (65 nM) can result in better chemosensitivity and enhanced drug retention in resistant cells than higher concentrations of drug alone and may represent novel approach for improving therapeutic management of cancer.

3.1.1 Specific Objectives

- To evaluate the potential of co-treatment with a commercial mixture of CLA isomers (CLA-mix) and two single isomers *c*9,*t*11-CLA and *t*10,*c*12-CLA to enhance the cytotoxic effects of Doxorubicin (Dox) on a drug naive MDA-MB435-S-F (MDA) human melanoma cell line and a multidrug resistant variant MDA-MB435-S-F/Adr10p10p (A10p10p)

- To determine the effect of CLA-mix, *c*9,*t*11 and *t*10,*c*12-CLA on net uptake/accumulation of Dox in cells and retention after efflux by liquid chromatography tandem mass spectrometry (LC-MS) methods

3.2 Materials and Methods

3.2.1 Materials

Cell culture media, supplements and related products were purchased from Sigma-Aldrich, Dublin, Ireland, unless otherwise stated. MDA-MB-435S-F (MDA) human melanoma cell line and its doxorubicin resistant variant MDA-MB-435S-F/Adr10p10p (A10p10p) were developed and donated by Dr. Sharon Glynn (NICB, Dublin). Chemotherapy agents, doxorubicin (Dox) and daunorubicin were kindly gifted by Dr. Robert O'Connor (NICB, Dublin) (sourced originally from Farmitalia Carlo Erba (Milton Keynes, UK)). All solvents used were of LC-MS grade and purchased from Sigma-Aldrich, Dublin, Ireland.

Conjugated Linoleic Acid (CLA-mix) mixture of isomers (99 % pure, approximately comprising: 44 % *trans* 10, *cis* 12 (*t*10*c*12)-CLA; 41 % *cis* 9, *trans* 11 (*c*9*t*11)/ *trans* 9, *cis* 11 (*t*9*c*11)-CLA; 10 % *cis* 10, *cis* 12 and minor amounts of *trans* 9, *trans* 11; *trans* 10, *trans* 12; *cis* 9, *cis* 11-CLA) (Cat: UC-59A) and single preparations of isomers *c*9*t*11-CLA and *t*10*c*12-CLA (90 % pure, Cat: UC-60A, UC-61A) were from NuChek-Prep, Elysian, MN, USA. Eicosapentaenoic acid (EPA) and Docosahexaenoic acid (DHA) were purchased from Sigma-Aldrich, Dublin (purity: 99 and 98 %, Cat: E2011 and D2534 respectively). All fatty acid preparations were dissolved in sterile filtered ethanol and stored at -20 ^{o}C.

3.2.2 Cytotoxicity assays

Cells were grown and maintained in RPMI-1640 culture medium, supplemented with 10 % (v/v) foetal calf serum, penicillin/streptomycin (1 unit/mL) and 10 mM sodium

pyruvate. 96-well plates were seeded at a cell density of $1x10^4$ cells/mL in each well. These were then cultured for 24 h following which 100 µL of treatments/serum-free media was added to corresponding wells. Fatty acids (FA) examined were CLA mixture of isomers (CLA-mix), purified isomers *c*9*t*11-CLA and *t*10*c*12-CLA and omega-3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Three concentrations of Dox (37.5, 57.5 and 115 ng/mL, equivalent to 65, 100 and 200 nM) and two concentrations of CLA (1 and 6 µg/mL or 3.6 and 21.4 µM) were examined per plate with 8 replicates for each treatment combination and each plate was set up in duplicate. The concentrations of EPA and DHA used in this study were 1 & 6 µg/mL (corresponding to 3.3 and 19.8 µM EPA and 3 and 18.2 µM DHA). Each experiment was divided into three treatment regimes: Dox and fatty acid (FA) for 4 h exposure, followed by a 7 day recovery [4 h], Dox and FA for 4 h, followed by 7 day FA exposure [7 day] and finally cells treated as in the 7 day group but pre-treated with FA for 24 h [pre-treat]. Cytotoxicity was assessed after 7 days using the acid phosphatase assay [25]. Data was obtained from at least three experiments and expressed as mean $\pm$ standard error from the mean.

3.2.3 Cellular Doxorubicin Content: Sample Preparation

The flasks were seeded at $6x10^5$ cells/flask with or without FA pre-treatment, and then cultured for 24 h to allow cells to attach to the substratum. Following this, flasks were treated in triplicate with combinations of Dox (2 µM) and fatty acid (6 µg/mL) for 2 h and divided into two groups for influx and retention measurements. To measure the influx of Dox into cells, treatment/media was removed after the 2 h incubation and centrifuged at 1000 g for 5 min using a Labofuge 400 centrifuge, Heraeus Instruments (supplied by Foss Electric, Dublin) to collect the floating dead cells. The flasks were then rinsed with basal media and the attached live cells were harvested, combined with floating cells, counted and spun to form a pellet for later extraction. To measure

retention after efflux the second group of flasks were rinsed with basal media following the 2 h treatment and incubated with fatty acid/control media for a further 3 h after which flasks were harvested, counted and pelleted as above. Cells treated with Dox and ethanol (at the same concentration as in the fatty acid stock) and Dox alone were used as controls for this experiment.

3.2.4 Cellular Doxorubicin Content: Extraction and Analysis

Pellets were extracted and analysed by LC-MS according to Wall *et al.,* 2007. Briefly, pellets were re-suspended in 200 µL ultrapure water, transferred to labelled plastic extraction tubes (Sarstedt, Cat: 60.551) making sure that all cells were removed and 20 µL of 33 % (w/v) silver nitrate added. A range of doxorubicin standards were prepared in microtubes at 10X concentrations of 50, 100, 500, 1000, 5000 ng/mL. 100 µL of each standard was added in duplicate to extraction tubes containing the untreated cells. Control (cells, no treatment) and blank (cells, no treatment, no IS) samples were also prepared in extraction tubes containing untreated cell pellets. Following this 100 µL internal standard daunorubicin (excluding blank), 700 µL ice cold isopropanol and 100 µL 1 M ammonium formate buffer were added and mixed. After careful addition of 1400 µL chloroform, tubes were mixed by inverting for 5 min and centrifuged at 4000 rpm for 5 min in a Heraeus Labofuge 400 (Foss Electric, Dublin). The bottom layer containing the drug was removed (1.1 mL) using a glass Pasteur pipette (Lennox, Dublin, Cat: APP490.33) and placed in an autosampler vial (AGB, Dublin, Cat: 11090417). The samples were then dried in a vacuum rotary evaporator at -80 ^{0}C.

Samples were reconstituted in 50 µL of LC mobile phase and 20 µL was injected automatically using the instrument autosampler. The LC-MS system used was a GE Healthcare (Amersham Biosciences) Ettan^{TM} Multi-dimensional liquid chromatographic

system (MDLC) interfaced to Thermo Finnigan™ PDA detector and LTQ™ mass spectrometer. The chromatographic separation was performed on a Phenomenex® Prodigy ODS(3) 100 Å, 5 µm, 150 mm x 2.1 mm column with a mobile phase of water, acetonitrile and formic acid in the ratio 72:28:0.1 (v/v/v), delivered isocratically at a flow-rate of 200 µL/min. The monitoring wavelength was 254 nm and mass spectral data were collected in the scan range 200-700 *m/z*. Selected reaction monitoring (SRM) mode was used for quantitation using the transition ion: *m/z* 544-397 for the $[M + H^+]$ doxorubicin adduct and 528-363 for the $[M + H^+]$ daunorubicin adduct.

3.2.5 Statistical analysis

Differences between means were analysed for significance using the Student's two-tailed, paired *t*-test, unless otherwise stated.

3.3. Results

3.3.1 Cytotoxicity Assays: Single Treatments

Tables 3.3.1- 3.3.3 summarise data obtained from the [4 h], [7 day] and [pre-treat] treatment regimes respectively. Each table presents percentage growth inhibition of five fatty acids (CLA-mix, *c*9*t*11 and *t*10*c*12-CLA, EPA and DHA) alone and in combination with Dox in the MDA parental cell line and the drug resistant variant, A10p10p. In the [4 h] treatment regime (Table 3.3.1), CLA-mix, *t*10*c*12-CLA and DHA at 1 µg/mL significantly inhibited growth ($p < 0.05$) of the MDA cell line by 16.8 $\pm$ 3.2, 12.6 $\pm$ 1.5 and 20.5 $\pm$ 3.3 % respectively. In A10p10p cells, only *c*9*t*11-CLA at 1 µg/mL caused significant inhibition (17.0 $\pm$ 2.7 %). At 6 µg/mL however, the CLA mixture, *c*9*t*11- & *t*10*c*12-CLA and EPA significantly inhibited growth ($p < 0.05$) of the MDA cell line by

23.1 ± 3.2, 12.3 ± 3.0, 25.1 ± 7.7 and 25.6 ± 2.3 % respectively. In A10p10p cells, only *c*9*t*11-CLA at 6 µg/mL caused significant inhibition (26.5 ± 5.6 %).

In the [7 day] treatment regime (Table 3.3.2), *t*10*c*12-CLA and DHA at 1 µg/mL significantly inhibited growth ($p < 0.05$) of the MDA cell line. Growth inhibition was 13.4 ± 2.9 and 34.0 ± 5.9 % relative to ethanol control. Only the CLA-mix at 1 µg/mL resulted in significant inhibition of 26.8 ± 6.4 % in the A10p10p cell line. At 6 µg/mL however, *c*9*t*11- and *t*10*c*12-CLA and DHA yielded significant inhibition alone in MDA cells. Percentage growth inhibition relative to control for *c*9*t*11- and *t*10*c*12-CLA and DHA was 18.1 ± 5.6 %, 25.8 ± 4.4 % and 38.9 ± 6.9 % respectively. Again, the CLA mixture, at 6 µg/mL was the only fatty acid to cause significant inhibition of 34.6 ± 5.1 % ($p < 0.05$) in A10p10p cells. Treatment for 7 days with either of the two single isomers of CLA, EPA or DHA had no significant effect on growth in the resistant cell line.

As in the 7 day treatment regime *t*10*c*12-CLA (1 µg/mL) significantly inhibited growth ($p < 0.05$) of the MDA cell line in the [pre-treat] group with 9.9 ± 0.4 % inhibition (Table 3.3.3). None of the fatty acid treatments at 1 µg/mL inhibited growth in A10p10p cells. At 6 µg/mL, only the CLA mixture (21.7 ± 3.6 %) yielded significant inhibition ($p < 0.05$) in MDA cells. In A10p10p cells, only inhibition by EPA and DHA at 6 µg/ml reached statistical significance ($p < 0.05$). Percentage growth inhibition relative to control for EPA and DHA was 72.7 ± 10.4 % and 79.4 ± 7.7 % respectively.

The effects of Dox on growth of MDA and A10p10p cells are also listed in Tables 3.3.1-3.3.3. There was no difference in exposure time of Dox across the three treatment regimes. Growth inhibition by Dox alone is shown to be dose dependent across the three concentrations (65, 100 and 200 nM) used. As expected, there was higher growth inhibition at each drug concentration across all treatments regimes in the parental MDA cell line than in the A10p10p cells. % inhibition was 7.6 ± 1.1, 18.6 ± 1.1 and 40.9 ± 0.9

% for 65, 100 and 200 nM Dox respectively in the MDA cell line. Corresponding values for 65, 100 and 200 nM Dox in A10p10p were 4.1 ± 1.8, 12.5 ± 1.7 and 28.9 ± 1.2 % respectively, thus confirming doxorubicin resistance in the A10p10p cell line (Glynn *et al.,* 2004).

In summary, the effects of the CLA mixture, *c*9*t*11-CLA, *t*10*c*12-CLA, EPA and DHA on cell growth were dose dependent in both cell lines. The effects of the CLA mixture, *t*10*c*12-CLA, EPA and DHA were time dependent only in the A10p10p cell line. Growth inhibition by Dox was also dose dependant in both cell lines.

3.3.2 Cytotoxicity Assays: Combination Treatments during [4 h] regime

CLA at 1 μg/mL enhanced by a factor of 2.3 the toxicity of Dox (65 nM) in the [4 h] regime in MDA cells, from 9.2 ± 3.4 to 21.3 ± 4.8 % inhibition (Table 3.3.1). At higher drug concentrations CLA at 1 μg/mL had no significant effect on Dox toxicity. The CLA mixture (1 μg/mL) had negligible effect on Dox toxicity in the A10p10p cell line. At the higher concentration (6 μg/mL), CLA mixture enhanced by factors of 2.8 and 1.4 the toxicity of Dox at 65 and 100 nM in the MDA cell line but had no effect at the higher drug concentration of 200 nM. Interestingly, while the CLA mixture apparently enhanced the toxicity of Dox from -9.6 ± 1.5 to 23.9 ± 14.6 %, 5.8 ± 7.0 to 32.3 ± 17.1 % and 23.0 ± 9.4 to 41.1 ± 15.5 % at 65, 100 and 200 nM respectively in A10p10p cell line, the differences did not reach statistical significance according to the Student's *t*-test.

Treatment with the *c*9*t*11-CLA isomer (1 μg/mL) for 4 h enhanced Dox toxicity in MDA cells by factors of 1.3 and 1.2 at 100 and 200 nM respectively. Similarly, 1 μg/mL *c*9*t*11-CLA increased the toxicity of Dox by a factor of 1.6 at 100 nM in A10p10p cells.

At the higher concentration (6 μg/mL) the *c*9*t*11-CLA isomer had no effect on Dox toxicity in MDA cells but did enhance ($p < 0.05$) toxicity of Dox at 100 nM in A10p10p cells. Inhibition of growth in the resistant cell line increased from 21.1 $\pm$ 7.2 % in the presence of drug alone to 40.0 $\pm$ 11.6 % (sensitivity factor of 1.9) in the presence of drug/fatty acid combination (Table 3.3.1).

Treatment with the *t*10*c*12-CLA isomer at 1 μg/mL did not significantly affect Dox toxicity in MDA cells. Treatment with *t*10*c*12-CLA at 6 μg/mL significantly enhanced the toxicity of 65, 100 and 200 nM Dox by factors of 2.4, 1.8 and 1.4 respectively. In A10p10p cells, *t*10*c*12-CLA at 1 μg/mL enhanced toxicity of Dox at 65 nM by a factor of 2.6. 6 μg/mL *t*10*c*12-CLA did not significantly affect Dox toxicity at any concentration in this cell line.

Treatment with EPA at 1 μg/mL for 4 h significantly enhanced toxicity of Dox at 200 nM by a factor of 1.1 in MDA cells. 6 μg/mL EPA did not significantly affect Dox toxicity at any concentration in this cell line. In A10p10p cells, neither 1 nor 6 μg/mL EPA caused significant enhancement of Dox toxicity at any concentration. DHA at 1 or 6 μg/mL did not significantly enhance Dox toxicity at any concentration in either MDA or A10p10p cells.

3.3.3 Cytotoxicity Assays: Combination Treatments [7 day] regime

Increasing the duration of treatment with CLA mixture of isomers (6 μg/mL) from 4 h to 7 days significantly enhanced the toxicity of Dox at 100 nM in MDA cells by a factor of 2.2 (Table 3.3.2). However CLA-mix at 1 μg/mL did not yield a significant increase in Dox toxicity in the MDA cell line. In A10p10p cells, 1 μg/mL CLA-mix did yield significant increased Dox toxicity at 65, 100 and 200 nM by factors of 2.5, 1.9 and 1.5

respectively. At 6 μg/mL CLA-mix significantly enhanced the toxicity of Dox at 100 and 200 nM by factors of 3.1 and 2.2 respectively.

Interestingly, a concentration as low as 1 μg/mL *c*9*t*11-CLA caused significant enhancement of toxicity of Dox at 65, 100 and 200 nM in the MDA cell line in the [7 day] regime, by factors of 2.1, 1.7 and 1.3 respectively. At 6 μg/mL, *c*9*t*11-CLA resulted in significant enhancement of toxicity of Dox at all three concentrations, by factors of 2.5, 1.8 and 1.4 respectively. In A10p10p cells, only 6 μg/mL *c*9*t*11-CLA significantly enhanced the toxicity of Dox at 200 nM by a factor of 2.2.

Combination of *t*10*c*12-CLA at 6 μg/mL significantly enhanced ($p<0.05$) toxicity of Dox at 100 nM by a factor of 1.5 in the MDA cell line. No other combination of *t*10*c*12-CLA with drug significantly enhanced Dox toxicity in MDA cells. In A10p10p cells, 1 μg/mL *t*10*c*12-CLA resulted in significant enhancement of toxicity of Dox at 65 and 200 nM, from -1.5 $\pm$ 12.3 to 20.6 $\pm$ 6.5 % and 23.6 $\pm$ 16.6 to 45.4 $\pm$ 19.1 % inhibition respectively. 6 μg/mL *t*10*c*12-CLA resulted in significant enhancement of toxicity of Dox at all three concentrations, by factors of 2.5, 1.8 and 1.4 respectively.

EPA at 1 μg/mL significantly enhanced ($p<0.05$) toxicity of 65 and 200 nM Dox in MDA cells (by factors of 3.3 and 1.1) and that of 100 and 200 nM Dox at 6 μg/mL EPA (by factors of 2.2 and 1.5). In A10p10p cells, treatments of 1 μg/mL EPA did not yield a significant increase in Dox toxicity at any concentration of Dox. 6 μg/mL EPA significantly enhanced ($p<0.05$) the toxicity of Dox at concentrations of 65, 100 and 200 nM (by factors of 10.7, 7.4 and 2.2 respectively).

Combination of 1 μg/mL DHA significantly enhanced ($p<0.05$) Dox toxicity at 100 and 200 nM Dox in the MDA cell line (by factors of 1.6 and 1.2 respectively), while 6 μg/mL DHA significantly enhanced Dox toxicity at all three concentrations of Dox (by factors of 5.4, 2.0 and 1.3 respectively). However, in A10p10p cells, neither 1 nor 6

μg/mL DHA significantly enhanced toxicity of Dox at any concentration of Dox.

3.3.4 Cytotoxicity Assays: Combination Treatments [pre-treat] regime

Pre-treatment of cells with the mixture of CLA isomers (CLA-mix) at 1 μg/mL for 24 h prior to 4 h drug treatment and recovery in the presence of CLA-mix for 7 days did not significantly affect the toxicity of Dox in MDA cells (Table 3.3.3). At 6 μg/mL however, the CLA-mix significantly enhanced Dox toxicity at 65 and 100 nM Dox (by factors of 4.6 and 1.9 respectively). In the A10p10p cell line, 1 μg/mL CLA-mix yielded significant increased Dox toxicity at all three Dox concentrations (by factors of 2.8, 2.0 and 1.7), while 6 μg/mL CLA-mix yielded significant increased Dox toxicity at 100 and 200 nM Dox (by factors of 2.8 and 2.1 respectively).

*c*9*t*11-CLA at 1 μg/mL did not significantly affect the toxicity of Dox in MDA or A10p10p cells. 6 μg/mL *c*9*t*11-CLA significantly enhanced ($p<0.05$) Dox toxicity of 200 nM in MDA cells by a factor of 1.3, while the same combination in A10p10p significantly enhanced ($p<0.05$) Dox toxicity by a factor of 2.6. Similarly, *t*10*c*12-CLA at 1 μg/mL did not significantly affect the toxicity of Dox in MDA or A10p10p cells. 6 μg/mL *t*10*c*12-CLA significantly enhanced ($p<0.05$) toxicity of Dox at 65 and 200 nM in MDA cells only, by factors of 3.3 and 1.5 respectively.

EPA at 1 μg/mL did not significantly affect the toxicity of Dox in MDA or A10p10p cells. 6 μg/mL EPA significantly enhanced ($p<0.05$) toxicity of 100 and 200 nM Dox in MDA cells (by factors of 6.8 and 1.7) and that of all Dox concentrations in A10p10p cells (by factors of 10.2, 5.3 and 2.9). DHA treatments did not significantly affect the toxicity of Dox in MDA cells. In A10p10p cells, 1 μg/mL DHA significantly enhanced ($p<0.05$) toxicity of Dox at 65 nM in A10p10p cells (by a factor of 5.6), while 6 μg/mL

DHA significantly enhanced ($p<0.05$) toxicity of Dox at all concentrations (by factors of 9.0, 5.6 and 2.6).

In summary, it was apparent that across all three treatment regimes that the CLA mixture of isomers and the two single pure isomers were as potent as EPA and DHA in enhancing sensitivity of both cell lines to Dox.

Table 3.3.1 Relative growth inhibition values following exposure to Dox with various fatty acids in the [4 h] treatment regime on the MDA and A10p10p melanoma cell lines.

FA µg/ml	Dox nM	CLA-mix		*c*9,*t*11-CLA		*t*10,*c*12-CLA		EPA		DHA	
		MDA	A10p10p	MDA	A10p10p	MDA	A10p10p	MDA	A10p10p	MDA	A10p10p
0	0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
1	0	16.8 (3.2)*	3.3 (5.8)	6.8 (4.7)	17.0 (2.7)*	12.6 (1.5) *	23.1 (9.3)	10.7 (7.9)	10.1 (6.0)	20.5 (3.3)*	8.9 (9.5)
6	0	23.1 (3.2)*	19.0 (12.5)	12.3 (3.0)*	26.5 (5.6)*	25.1 (7.7) *	34.5 (15.3)	25.6 (2.3)*	17.3 (7.5)	25.7 (12.7)	20.3 (19.6)
0	65	9.2 (3.4)	-9.6 (1.5)	7.6 (5.3)	7.2 (4.1)	7.1 (5.0)	7.9 (13.1)	6.2 (6.7)	-3.9 (5.4)	3.0 (6.9)	4.7 (7.3)
1	65	21.3 (4.8)+	9.8 (10.8)	16.1 (0.5)	21.1 (5.6)	16.9 (6.0)	20.2 (14.6)+	11.2 (2.9)	10.3 (11.3)	10.0 (6.8)	17.6 (19.4)
6	65	25.9 (3.7)+	23.9 (14.6)	19.1 (3.6)	33.2 (14.1)	16.9 (4.1)+	33.4 (21.5)	21.5 (5.0)	21.8 (21.6)	13.0 (4.7)	39.7 (19.7)
0	100	22.6 (5.4)	5.8 (7.0)	21.3 (3.4)	21.1 (7.2)	15.9 (1.9)	20.7 (16.8)	17.9 (2.5)	4.7 (9.3)	13.9 (10.3)	10.7 (10.3)
1	100	26.2 (4.1)	18.0 (9.1)	27.5 (2.6)+	33.0 (7.5)+	25.4 (4.4)	31.0 (20.2)	15.3 (1.9)	23.9 (14.2)	18.2 (10.8)	30.3 (15.9)
6	100	30.6 (4.6)+	32.3 (17.1)	25.8 (1.9)	40.0 (11.6)+	28.1 (3.2)+	41.7 (22.8)	29.3 (6.5)	32.1 (19.7)	21.8 (6.4)	43.5 (19.9)
0	200	41.7 (8.6)	23.0 (9.4)	43.4 (8.8)	36.4 (9.0)	34.7 (5.9)	31.7 (16.4)	36.2 (7.5)	22.0 (8.7)	39.4 (20.8)	28.7 (10.1)
1	200	47.9 (7.2)	32.1 (7.9)	50.6 (7.7)+	45.8 (8.5)	46.3 (8.7)	42.8 (20.6)	41.9 (8.0)+	34.5 (13.9)	43.9 (21.7)	43.6 (16.9)
6	200	48.7 (4.7)	41.1 (15.5)	48.2 (8.5)	51.0 (13.7)	49.3 (6.6)+	45.7 (25.2)	50.4 (4.6)+	38.8 (18.4)	43.3 (15.7)	47.3 (22.2)

Growth inhibition expressed as a percentage relative to control, which was taken to be 0 %. Data was obtained from at least three experiments (Standard Error of Mean).

* denotes significant inhibition by fatty acids relative to control with $p \leq 0.05$

+ denotes significant inhibition by fatty acid-drug combination relative to corresponding treatment of Dox alone with $p \leq 0.05$

Table 3.3.2 Relative growth inhibition values following exposure to Dox with various fatty acids in the [7 d] treatment regime on the MDA and A10p10p melanoma cell lines.

FA	Dox	CLA-mix		*c*9,*t*11-CLA		*t*10,*c*12-CLA		EPA		DHA	
µg/ml	ng/ml	MDA	A10p10p	MDA	A10p10p	MDA	A10p10p	MDA	A10p10p	MDA	A10p10p
0	0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
1	0	13.4 (6.9)	26.8 (6.4)*	10.0 (4.6)	11.0 (5.6)	13.4 (2.9)*	25.8 (9.4)	17.5 (10.1)	25.3 (17.7)	34.0 (5.9)*	23.9 (23.3)
6	0	13.6 (4.8)	34.6 (5.1)*	18.1 (5.6)*	37.2 (13.2)	25.8 (4.4)*	41.2 (11.4)	46.1 (16.8)	44.3 (18.1)	38.9 (6.9)*	56.3 (20.7)*
0	65	13.1 (5.4)	9.3 (1.7)	14.1 (7.5)	-6.6 (4.2)	7.9 (2.1)	-1.5 (12.3)	6.3 (2.8)	5.4 (9.0)	5.1 (2.0)	3.4 (4.1)
1	65	26.4 (2.7)	23.1 (3.6)$^{+}$	30.0 (6.6)$^{+}$	15.7 (13.1)	20.6 (6.5)	20.6 (16.1)$^{+}$	20.5 (4.5)$^{+}$	31.0 (16.8)	19.6 (4.5)	31.4 (16.3)
6	65	28.4 (4.8)	47.1 (14.1)	34.8 (2.9)$^{+}$	41.7 (21.8)	30.0 (6.3)	43.6 (19.2)$^{+}$	42.0 (12.2)	57.8 (16.3)$^{+}$	27.4 (3.9)$^{+}$	55.0 (19.7)
0	100	19.0 (3.7)	17.0 (6.5)	19.4 (8.1)	4.6 (5.3)	18.9 (3.5)	8.9 (12.5)	24.1 (6.2)	8.3 (9.3)	19.5 (4.5)	10.1 (10.7)
1	100	28.0 (4.6)	32.9 (6.5)$^{+}$	32.1 (8.9)$^{+}$	19.4 (12.8)	28.9 (2.9)$^{+}$	30.0 (19.2)	29.6 (7.3)	32.4 (14.4)	31.9 (2.7)$^{+}$	39.1 (16.8)
6	100	41.8 (4.0)$^{+}$	53.4 (14.1)$^{+}$	35.8 (7.6)$^{+}$	42.5 (23.2)	38.2 (5.8)	48.4 (20.5)$^{+}$	52.5 (13.5)$^{+}$	61.7 (14.6)$^{+}$	39.7 (1.2)$^{+}$	58.0 (21.1)
0	200	43.2 (8.8)	30.5 (7.7)	37.1 (10.2)	27.3 (11.6)	34.6 (9.2)	23.6 (16.6)	44.2 (10.9)	30.4 (7.8)	44.8 (8.6)	28.1 (6.6)
1	200	51.9 (8.8)	46.3 (8.4)$^{+}$	50.0 (10.1)$^{+}$	38.5 (14.2)	44.8 (5.9)	45.4 (19.1)$^{+}$	50.1 (10.4)$^{+}$	48.0 (14.0)	54.7 (7.1)$^{+}$	47.9 (17.9)
6	200	57.6 (7.1)	66.8 (13.0)$^{+}$	51.8 (9.6)$^{+}$	59.5 (17.0)$^{+}$	55.4 (4.9)	57.2 (20.1)$^{+}$	64.4 (11.8)$^{+}$	67.7 (14.2)$^{+}$	59.7 (5.0)$^{+}$	61.1 (21.4)

Growth inhibition expressed as a percentage relative to control, which was taken to be 0 %. Data was obtained from at least three experiments (Standard Error of Mean).

* denotes significant inhibition by fatty acids relative to control with $p \leq 0.05$

$^{+}$ denotes significant inhibition by fatty acid-drug combination relative to corresponding treatment of Dox alone with $p \leq 0.05$

Table 3.3.3 Relative growth inhibition values following exposure to Dox with various fatty acids in the [pre-treat] treatment regime on the MDA and A10p10p melanoma cell lines.

FA µg/ml	Dox ng/ml	CLA-mix		*c*9,*t*11-CLA		*t*10,*c*12-CLA		EPA		DHA	
		MDA	A10p10p	MDA	A10p10p	MDA	A10p10p	MDA	A10p10p	MDA	A10p10p
0	0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
1	0	18.8 (6.7)	28.5 (7.9)	8.1 (7.8)	-23.6 (35.6)	9.9 (0.4)*	25.0 (14.4)	7.9 (8.5)	44.6 (16.4)	7.1 (5.1)	38.7 (14.9)
6	0	21.7 (3.6)*	49.4 (17.0)	16.8 (7.2)	16.3 (24.6)	23.3 (2.7)	47.0 (12.3)	44.5 (20.1)	72.7 (10.4)*	24.9 (9.4)	79.4 (7.7)*
0	65	8.3 (5.2)	16.6 (6.1)	12.0 (5.2)	8.4 (2.9)	12.7 (1.0)	4.5 (5.9)	0.2 (3.8)	6.7 (2.4)	-0.5 (6.4)	8.8 (7.3)
1	65	19.5 (7.4)	45.7 (10.4)$^{+}$	15.7 (7.5)	33.7 (11.1)	9.9 (11.5)	43.2 (13.5)	11.6 (5.4)	57.8 (20.3)	-5.8 (13.7)	49.1 (12.8)$^{+}$
6	65	38.2 (6.2)$^{+}$	64.7 (12.2)	27.2 (7.2)$^{+}$	46.4 (27.2)	42.3 (3.8)$^{+}$	56.6 (13.7)	49.8 (17.0)	68.5 (13.6)$^{+}$	0.3 (20.8)	79.6 (8.8)$^{+}$
0	100	23.3 (4.4)	25.7 (8.4)	18.0 (5.4)	7.5 (5.3)	15.9 (9.8)	14.9 (5.0)	8.6 (3.2)	13.7 (3.3)	9.8 (5.0)	14.1 (4.0)
1	100	32.3 (5.6)	51.8 (10.4)$^{+}$	20.4 (6.7)	38.7 (9.8)	14.0 (6.0)	52.0 (14.9)	14.3 (5.5)	63.9 (17.5)	1.4 (13.6)	55.1 (13.3)
6	100	43.6 (3.5)$^{+}$	71.4 (10.9)$^{+}$	31.2 (6.8)$^{+}$	53.5 (24.3)	36.6 (7.1)	64.3 (13.8)	58.6 (17.0)$^{+}$	72.1 (12.5)$^{+}$	21.9 (14.0)	81.1 (8.7)$^{+}$
0	200	42.5 (8.1)	38.7 (6.5)	44.0 (5.8)	28.0 (11.6)	38.2 (9.9)	28.3 (5.3)	37.0 (7.5)	26.3 (7.1)	35.9 (11.4)	31.0 (2.6)
1	200	46.3 (6.1)	65.8 (3.7)	46.9 (7.9)	54.8 (9.0)	40.8 (8.5)	62.6 (8.3)	41.1 (10.5)	73.5 (13.5)	42.5 (7.3)	62.2 (9.8)
6	200	63.2 (7.5)	81.1 (6.9)	55.3 (9.0)$^{+}$	73.9 (13.7)$^{+}$	55.5 (10.2)$^{+}$	77.0 (7.3)	61.9 (14.0)$^{+}$	77.3 (10.2)$^{+}$	47.2 (6.1)	81.9 (7.9)$^{+}$

Growth inhibition expressed as a percentage relative to control, which was taken to be 0 %. Data was obtained from at least three experiments (Standard Error of Mean).

* denotes significant inhibition by fatty acids relative to control with $p \leq 0.05$

$^{+}$ denotes significant inhibition by fatty acid-drug combination relative to corresponding treatment of Dox alone with $p \leq 0.05$

3.3.5 Calculation of Intracellular Dox levels

LC-MS detection results in chromatogram peaks, the area of which are given in peak area counts and are proportional to the amount of Dox/daunorubicin in the sample. Standard solutions of doxorubicin (Dox) in the range 0-500 ng were run with each set of samples extracted. The peak area ratio (PAR) was calculated by dividing the peak area of a given concentration of Dox by the corresponding IS peak area in the same sample. The PAR calculation is used to correct for any variation that may occur as a result of the extraction process. Graphs were plotted of Dox concentration over PAR in the MDA and A10p10p cell lines, illustrated in Figure 3.3.5.1. This reverse plot for each extraction was used to generate the equation of the line. The equation is used in Excel to calculate from the PAR data the mass in nanograms (ng) of Dox in each experimental sample. From these values percentage Dox per million cells relative to control, which was taken to be 100 %, was calculated.

3.3.6 Analysis of Intracellular Drug levels

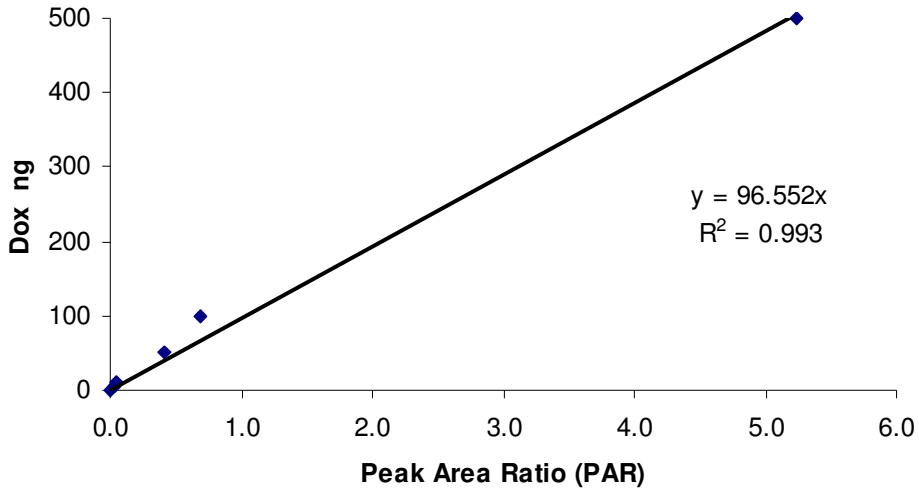
Figure 3.3.6.1 represents the proportion of intracellular Dox concentrations remaining in MDA and A10p10p cells after pulsing with drug (2 μM Dox) in the absence and presence of CLA preparations (6 μg/mL) for 2 h and after a 3 h recovery from drug treatment. It is apparent that irrespective of fatty acid treatments, the A10p10p variant exhibited an increase in Dox efflux compared with the parental cell line. % cellular Dox in control MDA cells was reduced from 100 $\pm$ 1.1 % Dox at 0 h to 77.5 $\pm$ 34.7 % at 3 h and from 100 $\pm$ 0.5 % at 0 h to 44.8 $\pm$ 19.4 % in A10p10p cells at 3 h.

When drug was combined with *c*9*t*11-CLA, there was a significant increase ($p < 0.001$) in Dox accumulation in MDA cells relative to Dox alone at time 0 h and

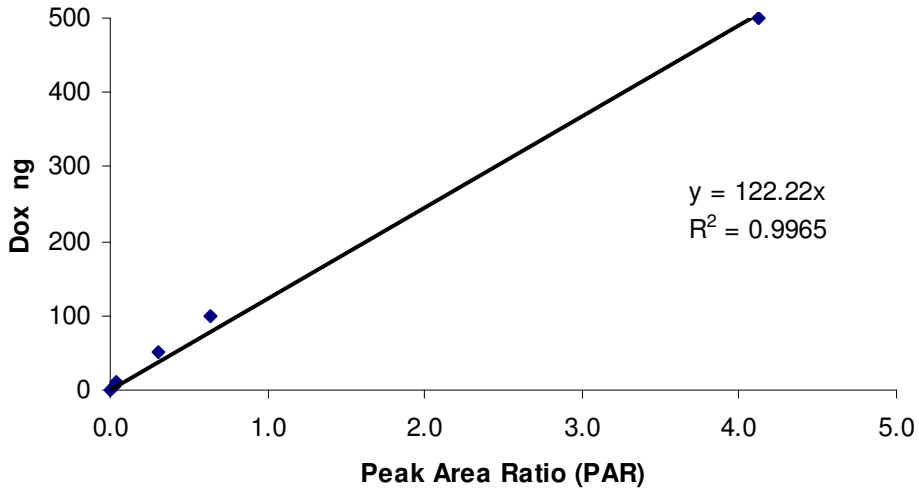
retention after 3 h in presence of the fatty acid (Figure 3.3.6.1 a). Both the CLA mixture of isomers and *t*10*c*12-CLA also increased Dox accumulation in MDA cells but this did not reach statistical significance ($p = 0.260$ and 0.088 respectively).

*c*9*t*11-CLA also significantly increased ($p < 0.05$) % cellular drug level (178.1 $\pm$ 16.7 % relative to control) in resistant A10p10p cells at time 0 h (Figure 3.3.6.1 b). Following 3 h recovery in the presence of *c*9*t*11-CLA, % Dox was retained at a higher level (69.7 $\pm$ 20.5 %) relative to control (44.8 $\pm$ 19.4 %) ($p = 0.008$). The CLA mixture significantly increased Dox retention to 58.0 $\pm$ 21.9 % ($p = 0.04$) after 3 h relative to control. Both the CLA mixture of isomers and *t*10*c*12-CLA also increased Dox accumulation at time 0 h in A10p10p cells from 100.31 $\pm$ 0.5 to 144.5 $\pm$ 32.7 and 177.4 $\pm$ 28.4 % respectively, but this did not reach statistical significance ($p = 0.305$ and 0.110 respectively). The *t*10*c*12-CLA isomer also increased drug retention in A10p10 cells from 44.8 $\pm$ 19.4 to 91.3 $\pm$ 35.9 %, but again did not reach statistical significance ($p = 0.148$).

In summary, untreated MDA cells retain more Dox after 3 h efflux following drug treatment than A10p10p cells. When drug was combined with *c*9*t*11-CLA there was a significant increase in cellular Dox accumulation at 0 h and retention after 3 h in both MDA and A10p10p cell lines. Also, the CLA mixture significantly increased the intracellular Dox retention after 3 h in A10p10p cells.

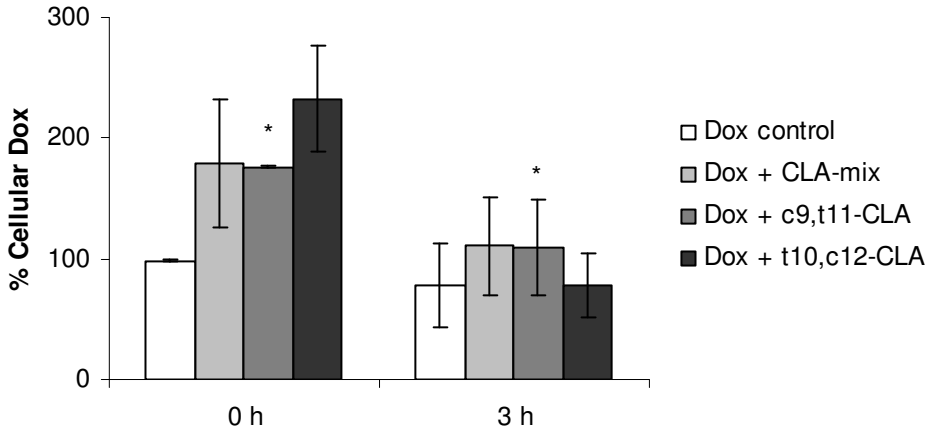


a)

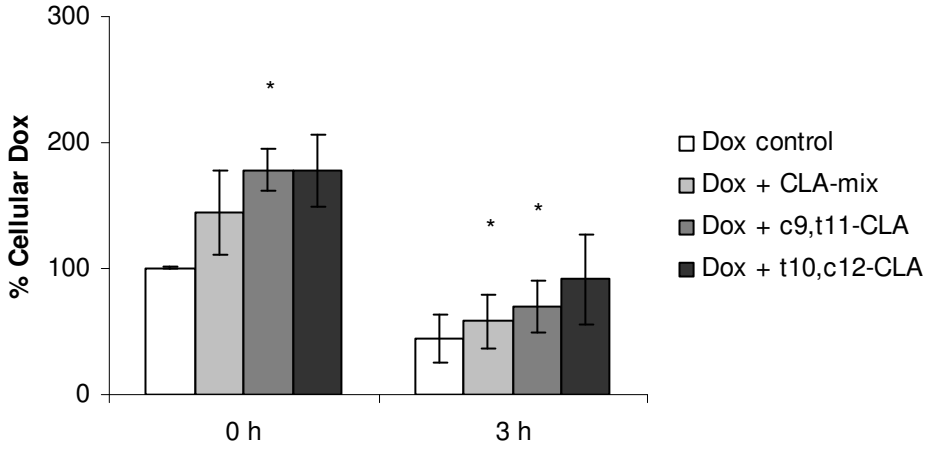


b)

Figure 3.3.5.1. Standard curves of Dox over peak area ratio (PAR) in the MDA (a) and A10p10p (b) cell lines. PAR was calculated by dividing the peak area of a given concentration of Dox by the corresponding internal standard (IS) peak area in the same sample.



a)



b)

Figure 3.3.6.1. Effect of fatty acid preparations on doxorubicin influx and retention after efflux in a) MDA and b) A10p10p melanoma cells. Following 24 h fatty acid pre-treatment, cells were treated in triplicate with combinations of Dox (2 µM) and fatty acid (6 µg/mL) for 2 h. Half were harvested (0 h) and half were washed and re-incubated with fatty acid/control media for a further 3 h. Data represent the mean $\pm$ SEM of three experiments. * denotes significance relative to control with $p \leq 0.05$.

3.4 Discussion

Drug resistance and toxicity are significant factors that limit the effectiveness of current chemotherapeutic drugs. Despite a significant risk of cardiotoxicity (Olson and Mushlin, 1990; Singal *et al.,* 1997) and multiple side effects including cachexia, nausea, vomiting, thinning and ulceration of mucous membranes, bone suppression and alopecia (Abraham *et al.,* 1996), doxorubicin (or Adriamycin), a member of the anthracycline drug family of antibiotics, is a chemotherapeutic agent that has been used extensively, either as a single agent or in combinations with other drugs for decades in the treatment of various types of cancer including breast cancer (Smith *et al.,* 2000; Serin, *et al.,* 2005; Sparreboom *et al.,* 2005; Wright *et al.,* 2005). Several mechanisms have been proposed to explain the antitumor property of doxorubicin which include intercalation into DNA and inhibition of topoisomerase II (Liu, 1989; D'Arpa and Liu, 1989), lipid peroxidation and bioreductive activation leading to the formation of drug and oxygen free radicals and alkylating species (reviewed in Quiles *et al.*, 2002). Pre-clinical nutritional intervention studies with DHA (Ohira *et al.,* 1996) have provided valuable new insights into how to address the problem of attenuating side effects while enhancing tumour drug responses and reversing inherent or acquired multiple drug resistance.

Emerging mechanisms of action of bioactive CLA isomers in cancer cells (affecting cell proliferation, apoptosis, angiogenesis, tumour invasion and differentiation (Miglietta *et al.*, 2006; Nugent *et al.*, 2005; Ochoa *et al.*, 2004; Wang *et al.,* 2005; Hubbard *et al.,* 2007; Lampen *et al.*, 2005)) provided the impetus in this study to investigate the potential of CLA to predispose resistant cancer cells to increased drug sensitivity. Apart from work published by Fite *et al.* (2007) this is the only other study showing CLA to augment the response of human cancer cell lines to chemotherapy drugs and the only to report CLA reversing drug resistance in a drug-resistant cancer cell line.

In this report we provide evidence that the three preparations of CLA used (CLA mixture of isomers and purified isomers *c*9*t*11- and *t*10*c*12-CLA) were similarly

effective to two omega-3 polyunsaturated fatty acids EPA and DHA at enhancing the cytotoxicity of doxorubicin in MDA-MB435S-F (MDA) melanoma cells. Data show that CLA enhanced cytotoxicity of Dox on the MDA cell line in a dose dependant manner in each of the three treatment groups with significant enhancement ($p<0.05$) seen with as low as 1 µg/mL of each of the fatty acids in combination with Dox. The most effective regime over all was found to be pre-exposure to fatty acid followed by concurrent treatment with Dox and a 7 day fatty acid exposure in the [pre-treat] group. All fatty acids enhanced the efficacy of Dox in the Dox resistant cell line MDA-MB435S-F/Adr10p10p (A10p10p) to a greater extent than the corresponding treatments in the MDA cell line. Further, all five fatty acids in combination with Dox rendered the A10p10p cells more sensitive to the drug than the parental drug sensitive MDA cells with higher levels of percentage inhibition for corresponding combination treatments. The fatty acids are therefore in effect reversing the Dox-resistance in the A10p10p cells. The *c*9*t*11-CLA isomer was found to enhance the uptake of Dox into the cells in both cell lines possibly by increasing the fluidity of the membrane allowing more passive diffusion and/or flip flop of the drug across the membrane (Hendrich and Michalak 2003). Also, the mixture of CLA isomers (CLA-mix) enhanced drug retention after treatment in the A10p10p line, while the *c*9*t*11-CLA isomer enhanced retention in both cell lines.

The mechanism of action of CLA may relate to an altered rate of cellular lipid synthesis. Previous work in the lab has shown that CLA isomers and its precursor *t*-VA are rapidly taken up into cancer cells (O'Shea *et al.,* 2000; Miller *et al.,* 2003b,). Agatha *et al.* (2004) reported that CLA modulated membrane FA composition and PUFA metabolism (FA desaturation and elongation), in four cultured human leukemia cell lines representing a profile of possible variations of leukemic tumor differentiation. The increased membrane unsaturation index as a result of CLA incorporation would likely provide more abundant targets for reactive oxygen species (ROS) generated by Dox metabolism. Products of lipid peroxidation such as hydroperoxides, hydroxynonenal, malondialdehyde and other aldehydes modulate signalling cascades involved in cell replication and cell death and thus may account for increased drug efficacy.

The ability to enhance chemosensitivity of drug resistant tumour cells by administering CLA prior to or simultaneously with chemotherapeutics is of potential benefit for treatment of cancer. Although multiple sources of CLA exist (O'Shea *et al.,* 1998); a mixture of the *c*9*t*11 and *t*10*c*12-CLA isomers is widely available as dietary supplement in health food shops. The *c*9*t*11 isomer of CLA is the major form of naturally occurring CLA and is found in milk fat, cheese and ruminant meats. *c*9*t*11 isomer of CLA is also synthesised endogenously in humans after supplementation with its precursor vaccenic acid through the stearoyl CoA desaturase (SCD)/Delta 9 desaturase pathway (Miller *et al.,* 2003b). Blood levels of CLA isomers are reported to be in range 20-70 μM, of which *c*9*t*11-CLA and *t*10*c*12-CLA make up approximately 80 % and 10 % respectively (Salminen *et al.,* 1998). Studies to determine the extent to which physiological levels of CLA alter composition of lipid rafts, functionality of ABC transporters and prooxidant activity of drugs may provide an explanation for the enhanced chemosensitivity in drug resistant cells. Optimal drug treatment reflects a compromise between effects on the cancer and toxicity to normal tissues. Hence, it is of critical importance to demonstrate that such treatments do not enhance the chemosensitivity of normal non malignant cells. Long term supplementation with a commercial 50:50 mixture of the two main CLA isomers showed it was well tolerated in healthy, overweight and obese human subjects and did not adversely affect blood safety parameters including inflammatory and diabetogenic markers (Gaullier *et al.,* 2007). This bodes favourably for undertaking translational research as suggested by Fite *et al.* (2007) to determine if CLA may be capable of reversing multiple drug resistance *in vivo*. To address the problem of MDR cancer, the role of CLA in modulating the efficacy of other anticancer drugs in drug resistant models of cancer deserves further study.

Chapter 4

Effects of conjugated linoleic acid (CLA) on cellular ceramide levels and expression of Her2/*neu* in drug-resistant and sensitive melanoma cells.

4.0 Abstract

Growth factor receptors and proteins that activate apoptosis are amongst several types of proteins encoded by protooncogenes, which if selectively blocked could stop the uncontrolled growth of cancer cells. Polyunsaturated fatty acids are known to exert important regulatory effects on genes controlling apoptotic proteins and growth factor receptors. Such effects may be mediated either by binding directly to transcription factors or indirectly through effects on specific enzyme-mediated pathways. Amongst the latter is the sphingomyelinase signal transduction pathway that generates ceramide, a pro-apoptotic lipid whose rapid conversion to glucosylceramide is a hallmark of drug-resistant cancer. Direct effects on gene transcription have recently been reported to explain the down-regulation by DHA and GLA of the HER2/*neu* oncoprotein, a growth factor receptor that is over-expressed in approximately 30 % of breast cancer cases. The aim of this study was to determine if anticancer activity of CLA and n-3 PUFA in drug-resistant cells is mediated by modulating levels of ceramide and/or HER2/*neu*. MDA and A10p10p cells were treated in the presence or absence of Dox at 115 ng/mL with CLA-mix; *c*9,*t*11-CLA; *t*10,*c*12-CLA; EPA or DHA at 6 µg/mL. Ceramide levels, as determined by HPLC were lower in untreated A10p10p cells than in the MDA cell line, suggesting that dysfunctional metabolism of ceramide may protect drug-resistant cells from cytotoxic effects of doxorubicin (Dox). Of the fatty acids studied, only *c*9,*t*11- and *t*10,*c*12-CLA in the presence of Dox significantly increased ceramide in A10p10p. HER2/*neu* expression as determined by ELISA was elevated in A10p10p cells compared with MDA cells and was down-regulated by CLA-mix in both MDA and A10p10p cells and by EPA and DHA in the A10p10p cell line. These data suggest that the cytotoxic action of Dox and *c*9,*t*11-CLA combination and the Dox and *t*10,*c*12-CLA combination in A10p10p cells is related in part to an increase in cellular ceramide-mediated apoptosis levels and that the cytotoxic action of the Dox and CLA-mix is related in part to a decrease in HER2/*neu*-mediated proliferation.

4.1 Introduction

Despite the major advances that have been made in the past 25 years in understanding the biological and clinical nature of breast cancer it still is the most prevalent type of cancer among women in the developed world and its incidence has shown a continuous rise in recent decades (Hanklin, 1993). Nutrition is a key factor in breast cancer development and a relationship between incidence of breast cancer and diet has been revealed by numerous epidemiological and experimental studies (Miglietta *et al.,* 2006). In chapter 2 and 3 the cytotoxic effect of CLA both as a single treatment and in combination with doxorubicin (Dox) on the multidrug resistant and sensitive melanoma cell lines (originally derived from a patient with metastatic ductal adenoma of the breast) was reported. However, the precise cellular mechanisms by which CLA exert these anticancer effects remains to be fully elucidated.

Ip and colleagues reported that CLA induced apoptosis in various rodent mammary cancer cell lines via reduction in expression of the antiapoptotic, proto-oncogene bcl-2 (Ip *et al.,* 1999; 2007; Banni *et al.,* 1999). CLA also induced apoptosis and increased the expression of the tumour suppressive proteins in the p53-dependent apoptotic pathway (p53, p21WAF1/CIP1) whilst inhibiting the expression of the anti-apoptotic bcl-2 in MCF-7 (oestrogen-positive cells) in this pathway (Wahle and Heys, 2004). Miglietta and co-workers (2006) demonstrated that CLA induced apoptosis in oestrogen unresponsive breast cancer cells (MDA-MB-231) via a mitochondrial dependent pathway involving ERK/MAPK signalling and the release of cytochrome *c* from the mitochondrial intramembrane space to the cytoplasm. It has also been shown previously that various anti-cancer drugs including doxorubicin induce apoptosis via this same mitochondrial dependent pathway (Wang *et al.,* 2001).

Other endogenous biological factors associated with fatty acid-induced apoptosis and differentiation are sphingolipid metabolites such as ceramide and sphingosine. Sphingomyelinase is an enzyme that catalyzes the hydrolysis of sphingomyelin (SM)

to ceramide. A variety of studies have shown that ceramide is ubiquitously produced during cellular stress and is associated with apoptosis. Furthermore, treating cells with synthetic short-chain ceramide has been shown to induce cell-cycle arrest and apoptosis. Ceramide levels also changed during progression through the cell cycle and have been shown to enhance expression of p21, a cellular inhibitor of cdk2 kinase that is involved in cell-cycle arrest via hypophosphorylation of retinoblastoma protein (pRb) (Wu *et al.,* 2005). Direct evidence for an involvement of sphingolipid signalling in growth arrest by polyunsaturated fatty acids was provided recently when omega-3 polyunsaturated fatty acids attenuated breast cancer growth through activation of a neutral sphingomyelinase-mediated pathway (Wu *et al.,* 2005). However data in the literature is not conclusive; while some reports have indicated that n-3 PUFA such as DHA and EPA result in increased ceramide production in cancer cells (Siddiqui *et al.,* 2003; Wu *et al.,* 2005), others have reported decreases in ceramide levels in response to n-3 PUFA treatment (Jolly *et al.,* 1997; McMurray *et al.,* 2000). Whether sphingolipid signalling plays an important role in CLA-mediated induction of apoptosis of MDA and A10p10p breast cancer cells has yet to be assessed.

Other significant effects induced by CLA include downregulation of target genes of the APC-β-catenin-TCF-4 and PPAR δ signalling pathways in CaCo2 cells. Expression of c-myc, c-jun, β-catenin, PPARδ, cyclin D1 and promoter activities of c-myc and AP1 were decreased in a concentration-dependent manner (Lampen *et al.,* 2005). Interestingly, the *t*10,*c*12-CLA isomer was found to stimulate tumourigenesis in transgenic mice over-expressing erbB2 oncogene in the mammary epithelium (Ip *et al.,* 2007; Meng *et al.,* 2008), while DHA was found by Menendez and colleagues (2005c) to downregulate *c*-erbB2 (her2/*neu*) oncogene expression in human breast cancer cells. HER2 is a member of the erbB family of receptor tyrosine kinases all of which bind extracellular growth factor ligands initiating intracellular signalling pathways regulating diverse biological responses including, proliferation, differentiation, cell motility and survival (Reviewed in Marmor *et al.,* 2004). The effects of the various fatty acids on expression of her2/*neu* in MDA and A10p10p cells remains to be elucidated.

One objective of this chapter was to determine the effects of various preparations of CLA alone and in combination with doxorubicin on ceramide levels in MDA and A10p10p cells, thus identifying a potential mechanism of apoptosis induction. Another objective was to elucidate the effects of the various fatty acids on expression of her2*/neu* in MDA and A10p10p cells.

4.2 Specific Objectives

- To validate a high performance liquid chromatography (HPLC) procedure for the analysis of basal sphingosine and ceramide levels in MDA and A10p10p cells.

- To determine differences if any between basal sphingosine and ceramide levels in untreated MDA and A10p10p cells.

- To investigate the effects of CLA mixture of isomers (CLA-mix), two single isomers (*c*9,*t*11 and *t*10,*c*12-CLA) and two omega-3 fatty acids (EPA and DHA), alone and in combination with doxorubicin (Dox), on basal sphingosine and ceramide levels in the MDA cell line.

- To investigate the effects of CLA-mix, *c*9,*t*11 and *t*10,*c*12-CLA, EPA and DHA, alone and in combination with Dox, on basal sphingosine and ceramide levels in the A10p10p cell line.

- To measure and compare the expression of her2/neu oncoprotein in untreated MDA and A10p10p cells.

- To elucidate the effects of the various fatty acid treatments on her2/neu oncoprotein expression in both cell lines.

4.3 Materials & Methods

4.3.1 Materials

MDA-MB-435-S-F (MDA) human melanoma cell line and its Doxorubicin resistant variant MDA-MB-435-S-F/Adr10p10p (A10p10p) were developed and kindly donated by Dr. Sharon Glynn (NICB, Dublin). Chemotherapy agent Doxorubicin (Dox) was kindly donated by Dr. Robert O'Connor (NICB, Dublin) (sourced originally from Farmitalia Carlo Erba (Milton Keynes, UK)). Conjugated Linoleic Acid (CLA) mixture of isomers (99 % pure, approximately comprising: 44 % *t*10,*c*12-CLA; 41 % *c*9,*t*11/*t*9,*c*11-CLA; 10 % *c*10,*c*12-CLA and minor amounts of *t*9,*t*11; *t*10,*t*12 and *c*9,*c*11-CLA) (Cat: UC-59A) and single preparations (90 % pure) of isomers *c*9,*t*11- and *t*10,*c*12- CLA (Cat: UC-60A, UC-61A) as listed previously were from NuChek-Prep, Elysian, MN, USA. All sterile disposable plastic-ware was from Sarstedt Ltd., Wexford, Ireland. Cell culture media, supplements and reagents were purchased from Sigma-Aldrich. Sphingosine, N-acetyl-D-sphingosine (ceramide) and O-phthaldehyde (OPA) were also purchased from Sigma, as were all other chemicals and solvents listed in this section unless otherwise specified. The Calbiochem c-erbB2/c-neu (HER2/neu) Rapid Format ELISA kit (Cat # QIA10) was purchased from Merck Chemistry Ltd, Nottingham, UK. Pepstatin (# 516481) was also purchased from Merck. Leupeptin(# L-2884), ethylenediamine-tetraacetic acid (EDTA) (#E-6511) and phenylmethylsulfonyl fluoride (PMSF) (#P-7626) were purchased from Sigma.

4.3.2 Ceramide Analysis

4.3.2.1 Reagent preparation

Solvents were HPLC grade or higher. All solutions were prepared in advance unless otherwise stated and stored in the solvent press or refrigerator as appropriate.

- Chloroform: methanol: 1 *N* HCl solution; 100: 100: 1 v/v/v
- Balanced salt solution (BSS) (135 mM NaCl, 4.5 mM KCl, 1.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5.6 mM glucose, 10 mM HEPES, pH 7.2)
- 100 mM solution of EDTA sodium salt at pH 7.5 was prepared and mixed with the BSS in a ratio of 9: 1 BSS: EDTA
- 0.1 and 1 *N* KOH and 1 *N* HCl in 100 % methanol
- 1 M NaCl in water
- Mobile phase consisted of 90 % methanol and 10 % 5 mM K_2HPO_4 (to pH 7.0 using KOH)
- OPA reagent was prepared fresh each day by mixing 24.86 mL 3 % boric acid in water (pH 10.5 with KOH), 125 µL ethanol containing 100 mg/mL OPA (o-phthalaldehyde, Cat: H5030, Sigma) and 12.5 µL 2-mercaptoethanol (Cat: M6250, Sigma).

4.3.2.2 Cell Culture

4.3.2.2.1 Single treatment Fatty Acids on MDA and A10p10p

Cell lines were grown and maintained as listed previously in a ShelLab, IR2424 model CO_2 humidified Incubator at 37°C. Cell culture work was carried out in a class II laminar airflow cabinet (Gelaire 85, BSB4 laminar air-flow cabinet). T-75 flasks were seeded with MDA or A10p10p cells at a density of $3x10^6$ cells/flask and pre-treated in triplicate with CLA mixture of isomers (CLA-mix); *c*9,*t*11-CLA; *t*10,*c*12-CLA; EPA or DHA at a final concentration of 6 µg/mL. The flasks were then cultured for 24 h to allow the cells to attach. Following 24 h, flasks were rinsed with PBS and 12 mL fresh media containing fatty acid/control was added for a 3 day exposure period. For controls, flasks were treated with media containing ethanol at the same concentration as in experimental flasks (< 0.01 %). After 3 days, floating cells were collected by centrifuging the media in the flasks at 1000 g for 5 min using a Labofuge 400 centrifuge, Heraeus Instruments (supplied by Foss Electric, Dublin) and adherent cells were trypsinised as listed previously. Floating and adherent cells

were pooled and a cell count performed using the Trypan Blue exclusion method. Cells were centrifuged as above and the pellets frozen until extraction at a later date.

4.3.2.2.2 Combinations of Fatty Acids and Dox on MDA and A10p10p

T-75 flasks were seeded with MDA or A10p10p cells at a density of $3x10^6$ cells/flask and pre-treated in triplicate with CLA mixture of isomers (CLA-mix); *c*9,*t*11-CLA; *t*10,*c*12-CLA; EPA or DHA at a final concentration of 6 µg/mL. The flasks were then cultured for 24 h to allow the cells to attach, following which media was removed and replaced with fresh media containing fatty acid/control plus Dox at 115 ng/mL for 4h. Flasks were then rinsed with PBS and 12 mL fresh media containing fatty acid/control added for a 3 day recovery period. For controls, flasks were treated with Dox plus media containing ethanol at the same concentration as in experimental flasks (<0.01%). After 3 days, floating cells were collected by spinning down the media in the flasks and adherent cells were trypsinised as listed previously. These were pooled and a cell count performed using the Trypan Blue exclusion method. Cells were centrifuged at 1000 g for 5 min using a Labofuge 400 centrifuge, Heraeus Instruments (supplied by Foss Electric, Dublin) and the pellets frozen until extraction at a later date.

4.3.2.3 Cellular Lipid Extraction

Frozen cell pellets were removed from the freezer and 2 mL Chloroform: methanol: 1 *N* HCl solution was added followed by 0.6 mL of BSS/EDTA. The solution was then vortexed and centrifuged at 800 g for 5 min using a Labofuge 400 centrifuge, Heraeus Instruments (supplied by Foss Electric, Dublin). The lower organic phase was transferred to two different sets of tubes; marked 'basal', on which an alkaline hydrolysis step was carried out to detect the basal level of sphingosine and 'total', on which the cellular ceramide was deacylated converting it to sphingosine. 'Basal' represents the normal level of sphingosine in the cells. 'Total' represents ceramide

levels plus 'basal' sphingosine levels. Both sets of samples were dried down under a stream of nitrogen gas. At this point it was possible to freeze the samples at -20°C until a later date as required (Santana et al., 1996).

4.3.2.4 Sphingosine extraction by alkaline hydrolysis

Sphingosine (SP_1) standards (0-2000 pmol) were made up in chloroform: methanol (1:1, v/v) and dried down under nitrogen. 500 µL of 0.1 M KOH was added to the lipid film of each standard and basal sample tube. These tubes were then vortexed and incubated for 1 h in a 37 ºC water-bath (Grant Instruments, Cambridge, UK). Following this, 500 µL of chloroform and 300 µL BSS/EDTA solution were added to all tubes to extract the sphingolipids. The tubes were then vortexed and centrifuged at 800 g for 5 min using a Labofuge 400 centrifuge, Heraeus Instruments (supplied by Foss Electric, Dublin). The lower organic phase was transferred to a new set of tubes and dried down under nitrogen. At this point it was possible to freeze the samples until a later date as required (Santana et al., 1996).

4.3.3.5 Deacylation of ceramide

Sphingosine (SP_2) and ceramide standards (0-2000 pmol) were dissolved in chloroform: methanol (1:1, v/v) and dried down under nitrogen. These were used later to calculate the overall recovery of sphingolipids and the efficiency of the deacylation procedure. 500 µL of 1 M KOH in methanol was added to the lipid film of each standard and 'total' sample tubes to deacylate the ceramide. The tubes were then capped, vortexed and incubated at 100ºC for 1.5 h. At this point the tubes were allowed to cool and then neutralized with 500 µL 1 M HCl in methanol. The sphingoid base was extracted by addition of 1 mL chloroform and 900 µL 1 M NaCl, vortexed and centrifuged at 800 g for 5 min using a Labofuge 400 centrifuge, Heraeus Instruments (supplied by Foss Electric, Dublin). The upper phase was

removed and the remaining lower phase dried down under nitrogen. At this point it was possible to freeze the samples until a later date as required (Santana et al., 1996).

4.3.3.6 Derivatisation with O-Phthalaldehyde (OPA)

Neat sphingosine (SP_N, in the range 0-2000 pmol) was dissolved in chloroform: methanol (1:1, v/v) and dried down under nitrogen. All standards and samples were derivatised at this stage with OPA reagent. The sphingoid base in each tube was redissolved in 50 μL of methanol, to which 50 μL of OPA reagent (prepared as above) was added and incubated at room temperature for 10 min. 500 μL of methanol: 5 mM potassium phosphate (9:1, v/v), pH 7.0 was added to the tubes, which were then centrifuged at 1500 g for 30 s using a Labofuge 400 centrifuge, Heraeus Instruments (supplied by Foss Electric, Dublin) to clarify the samples. Of this 500 μL was transferred to HPLC vials. This derivatisation step converts the sphingosine to a fluorescent compound which can be detected and quantitated by HPLC (Santana et al., 1996).

4.3.3.7 HPLC separation and Quantitation

The derivatised sphingosine was separated by HPLC (Varian 9012) fitted with a Dynamix® AI-200 automatic sample injector with a 20 μL injection loop and quantitated using a Varian 9075 fluorescence detector. A Nova Pack® C18 column (Waters, Milford, MA, USA) was used for the separation. A mobile phase of methanol: 5 mM potassium phosphate, pH 7.0 (9:1, v/v) and flow rate of 0.6 mL/min were used to elute the samples. An excitation wavelength of 340 nm and an emission wavelength of 455 nm were used (Santana et al., 1996).

4.3.3.8 Calculation of cellular ceramide content

Calculations for ceramide cellular content were adapted from Santana *et al.,* (1996) and were carried out as follows:

pmol ceramide/ 10^6 cells = [(pmol total x recovery 1 x recovery 2) – pmol basal] / million cells in each pellet

pmol total = pmol in the total sample (deacylated) read against the ceramide standard curve.
Recovery 1 = averaged peak areas of each point in ceramide standard curve / peak areas of SP_N. This is a measure of the efficiency of deacylation.
Recovery 2 = averaged peak areas of each point in SP_2 standard curve/peak areas of SP_1. This is a measure of the efficiency of sphingolipid recovery.
pmol basal = pmol in the basal sample (alkaline hydrolysis) read against the SP_1 standard curve.

4.3.4 HER2/neu Assay

4.3.4.1 Cell Culture

Cell lines were grown and maintained as listed previously in a ShelLab, IR2424 model CO_2 humidified Incubator at 37°C. Cell culture work was carried out in a class II laminar airflow cabinet (Gelaire 85, BSB4 laminar air-flow cabinet). T-25 flasks were seeded with MDA or A10p10p cells at a density of $1x10^6$ cells/flask. The flasks were then cultured for 24 h to allow the cells to attach. Following 24 h flasks were treated with fresh media containing fatty acid/control in triplicate for a 3 day exposure period. Fatty acids used in this experiment were CLA mixture of isomers (CLA-mix); *c*9,*t*11-CLA; *t*10,*c*12-CLA; EPA and DHA at a final concentration of 6 µg/mL. For controls, flasks were treated in triplicate with media containing ethanol at the same concentration as in experimental flasks (<0.01%).

After 3 days, floating cells were collected by centifuging the media in the flasks at 800 g for 5 min using a Labofuge 400 centrifuge, Heraeus Instruments (supplied by Foss Electric, Dublin) and adherent cells were trypsinised as listed previously. Floating and adherent cells were pooled and a cell count performed using the Trypan Blue exclusion method. Cells were centrifuged as above and the pellets frozen until extraction at a later date.

4.3.4.2 Reagent preparation

Immediately prior to extracting the cell lysates, the following reagents were prepared.

- Resuspension buffer provided in the assay kit was adjusted to contain 5 mM EDTA, 0.2 mM PMSF, 1 µg/mL pepstatin and 0.5 µg/mL leupeptin.
- Wash buffer was prepared by addition of 25 mL of 20X concentrated solution (provided in the kit) to 475 mL dH_20. This was mixed and transferred to a wash bottle.
- A lyophilised c-erbB2/c-neu standard (provided in the kit) was re-constituted in dH_20 and allowed to sit at room temperature for 15 min.
- Serial dilutions of this standard were made in labelled tubes to contain 3, 1.5, 0.75, 0.375, 0.188, 0.094 and 0 ng/mL c-erbB2/c-neu using sample diluent (provided in the kit).
- The 500X Conjugate was diluted 1:500 with conjugate diluent (both provided); i.e. 24 µL in 12 mL. This was gently mixed and filtered using a 0.2 µm syringe filter.

4.3.4.3 Cell Lysate Extraction

Frozen cell pellets were removed from the freezer and allowed to thaw. Resuspension buffer prepared above was added to each pellet at approximately 1 mL/1x10^6 cells. For every 100 µL of cell suspension, 20 µL of antigen extraction agent (AEA) (provided in the kit) was added. These were mixed and placed on ice for 30 min with occasion vortexing throughout. The extracts were transferred to microcentrifuge tubes and centrifuged for 5 min at 1000 g using a Labofuge 400 centrifuge, Heraeus Instruments (Foss Electric, Dublin). The resulting clear lysates were transferred to clean tubes. An aliquot of each was set aside and frozen for a protein assay to be done at a later date (Calbiochem user protocol QIA10 Rev. 16).

4.3.4.4 c-erbB2/c-neu ELISA protocol

The appropriate number of wells were removed from the foil pouch (provided in the kit). Unused wells were returned to the pouch, re-sealed and stored at 4°C. 100 µL of each standard in duplicate and each sample (already in triplicate) was pipetted into the appropriate wells. The plate was covered with a plate-sealer and incubated at room temperature for 2 h, after which the wells were washed three times with the wash buffer prepared above. 100 µL of detector antibody (provided in the kit) was pipetted into each well, covered with a plate-sealer and incubated at room temperature for 1 h. Again the wells were washed three times with wash buffer, following which 100 µL of conjugate (prepared above) was added. The plate was covered with a plate-sealer and incubated at room temperature for 30 min. Wells were washed three times with wash buffer, after which the entire plate was flooded with dH_20. The water was removed by inverting over the sink and tapping the plate on paper towels. 100 µL of substrate solution (provided) was added to each well and incubated in the dark at room temperature for 30 min. 100 µL of stop solution (provided) was added to the wells in the same order as previous additions. The absorbance of each well was measured using a Tecan A-5082 Sunrise microplate

reader (Tecan, Austria) at dual wavelengths of 450/595 nm within 30 min of adding the stop solution. The c-erbB2/c-neu content of each sample was calculated using the equation of the line from the standard curve which was linear in the range used. This yielded values in ng/mL; these were multiplied by a conversion factor of 400 to yield values of c-erbB2/c-neu in human neu units (HNU) (Calbiochem user protocol QIA10 Rev. 16).

4.3.4.5 Bio-rad Protein Assay

Bio-Rad protein assay dye reagent was prepared by addition of 4 mL dye reagent concentrate to 16 mL dH_20. This was mixed by inverting. A protein standard solution was prepared using BSA and diluted using dH_20 to yield a range of (0-2100 µg/mL) standards of BSA. 10 µL of each sample (aliquots of cell lysate for each sample set aside earlier) and standard was pipetted in triplicate into appropriate wells of a 96-well plate. 200 µL of dye reagent was added to each well and mixed gently. The plate was incubated at room temperature for 5 min (max 1 h). Absorbance was read on a Tecan A-5082 Sunrise microplate reader (Tecan, Austria) at a wavelength of 620 nm. The protein content of each sample was calculated by extrapolation from the standard curve. These values were then used to adjust the p185 c-erbB2/c-neu HNU values to p 185 c-erbB2/c-neu HNU/mg protein.

4.3.5 Statistical Analysis

Differences between means were analysed for significance using the Student's two-tailed, paired *t*-test, unless otherwise stated.

4.4 Results

4.4.1 Ceramide Analysis

4.4.1.1 Standard curves

Four standard curves were set up as per "Materials and Methods" section. All standard curves were prepared in the range 0- 2000 pmol. Each standard was prepared and analysed for sphingosine and ceramide (converted to sphingosine) content using HPLC to obtain reliable linear correlations between sphingosine concentration and peak area. A sphingosine standard curve (SP_1) was set up and subjected to an alkaline hydrolysis step in addition to derivatisation and was quantified using HPLC, illustrated in Figure 4.4.1.1.1 (b). A second sphingosine standard curve (SP_2) was set up and subjected to the steps involved in deacylation and derivatisation, and then quantified using HPLC (Figure 4.4.1.1.2 (a)). The purpose of these two steps was to calculate the overall recovery of sphingosine (Recovery 2) from the extraction procedure, which would then be used when quantifying basal sphingosine levels in the MDA and A10p10p cell extracts. The Recovery 2 value was calculated to be 81.9 % (n= 4).

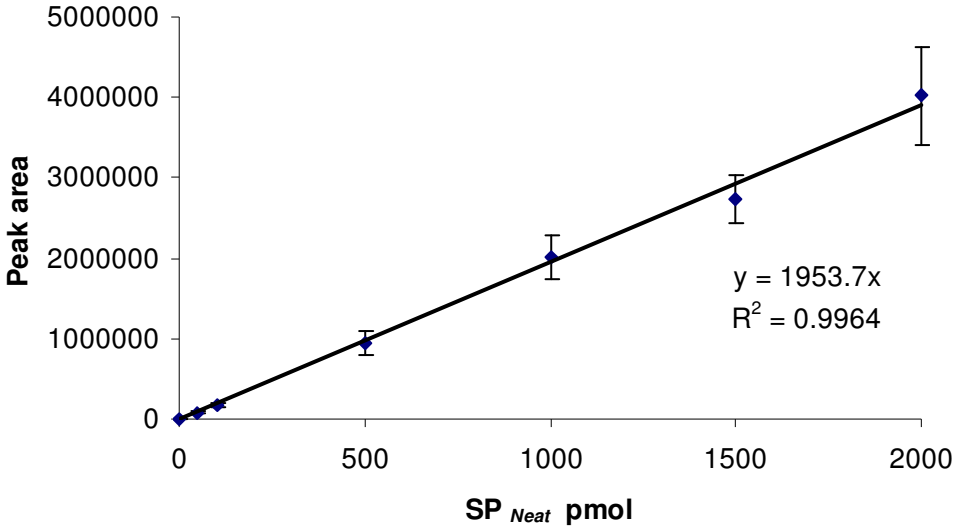
Figure 4.4.1.1.2 (b) illustrates a standard curve in which ceramide standards were deacylated to form sphingosine, derivatised and quantified using HPLC. Finally, a sphingosine standard curve (SP_N) was set up and subjected to derivatisation with OPA and was quantified using HPLC (Figure 4.4.1.1.1 (a)). By comparing the SP_N standard curve with the ceramide standard curve, it is possible to calculate the efficiency of the deacylation procedure (Recovery 1). The Recovery 1 value was calculated to be 89.9 % (n= 4). The ceramide is converted to sphingosine via the deacylation procedure and thus a ceramide standard curve allows for quantification of total sphingosine (basal sphingosine levels plus deacylated ceramide levels) in the cell samples. A simple subtraction of basal sphingosine levels from total sphingosine levels will subsequently yield cellular ceramide levels. Figure 4.4.1.1.3 depicts a typical HPLC chromatogram of (a) SP_{NEAT} at a concentration of 1000 pmol and (b) ceramide standard at a concentration of 500 pmol having undergone deacylation, where ceramide is converted to sphingosine, and derivatised with OPA. It can be

seen here that the retention time of the resultant sphingosine was 8.094 min in (a) and 7.919 min in (b) and the OPA reagent eluted between 1.5- 2.5 min in both.

4.4.1.2 Basal sphingosine levels in untreated MDA and A10p10p cells

T-75 flasks were seeded with MDA or A10p10p cells at a density of $3x10^6$ cells/flask. Following 24 h flasks were rinsed with PBS and 12 mL fresh media was added for a 3 day incubation period. After 3 days, floating and adherent cells were collected and pooled as described in Materials and Methods. Lipids were extracted and subjected to alkaline hydrolysis and derivatisation steps and quantitated by HPLC as described in above sections.

(a)



(b)

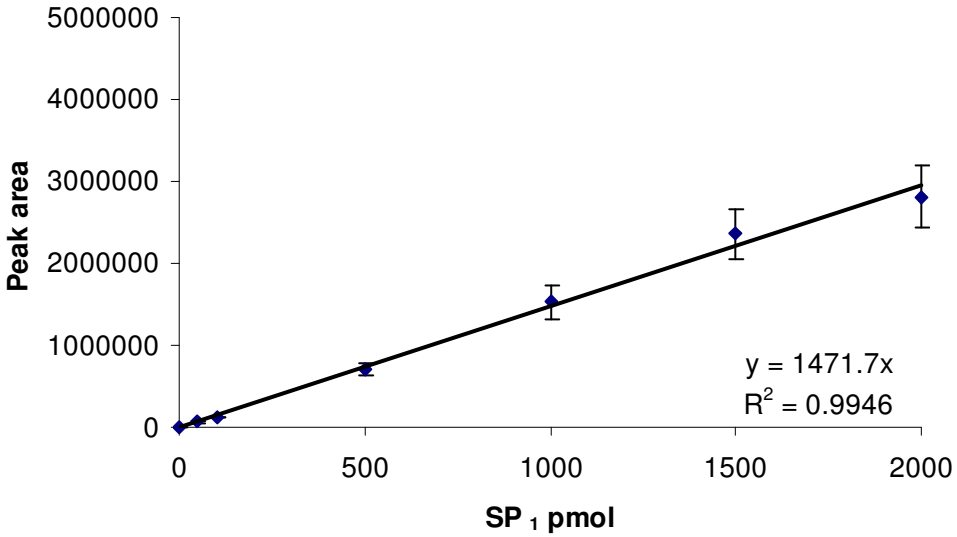
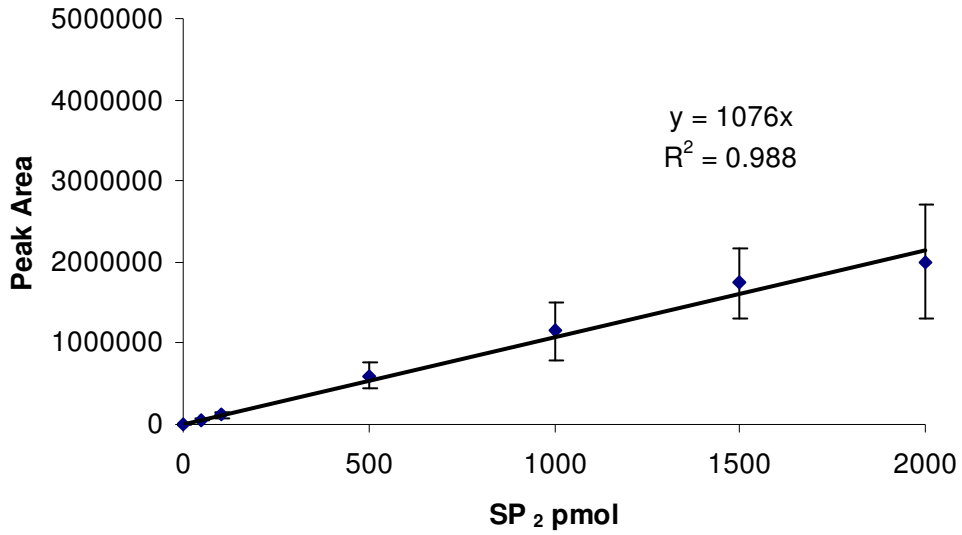


Figure 4.4.1.1.1 Standard curves of (a) SP_{NEAT} (neat sphingosine in the range 0-2000 pmol) which was derivatised with OPA and (b) SP_1 sphingosine standard (0-2000 pmol) which was hydrolysed and derivatised with OPA. All were separated by HPLC on a Nova Pack® C18 column and quantitated using a Varian 9075 fluorescence detector at an excitation wavelength of 340 nm and an emission wavelength of 455 nm (n = 4).

(a)



(b)

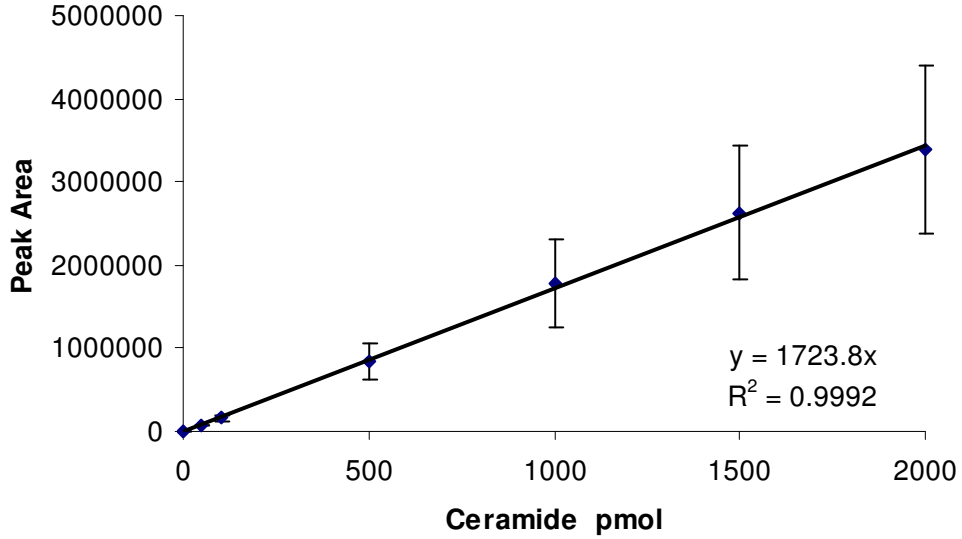


Figure 4.4.1.1.2. Standard curves of (a) SP_2 sphingosine standard (0-2000 pmol) and (b) Ceramide (0-2000 pmol) both of which were deacylated (to convert ceramide to sphingosine), derivatised and derivatised with OPA. All were separated by HPLC on a Nova Pack® C18 column and quantitated using a Varian 9075 fluorescence detector at an excitation wavelength of 340 nm and an emission wavelength of 455 nm (n = 4).

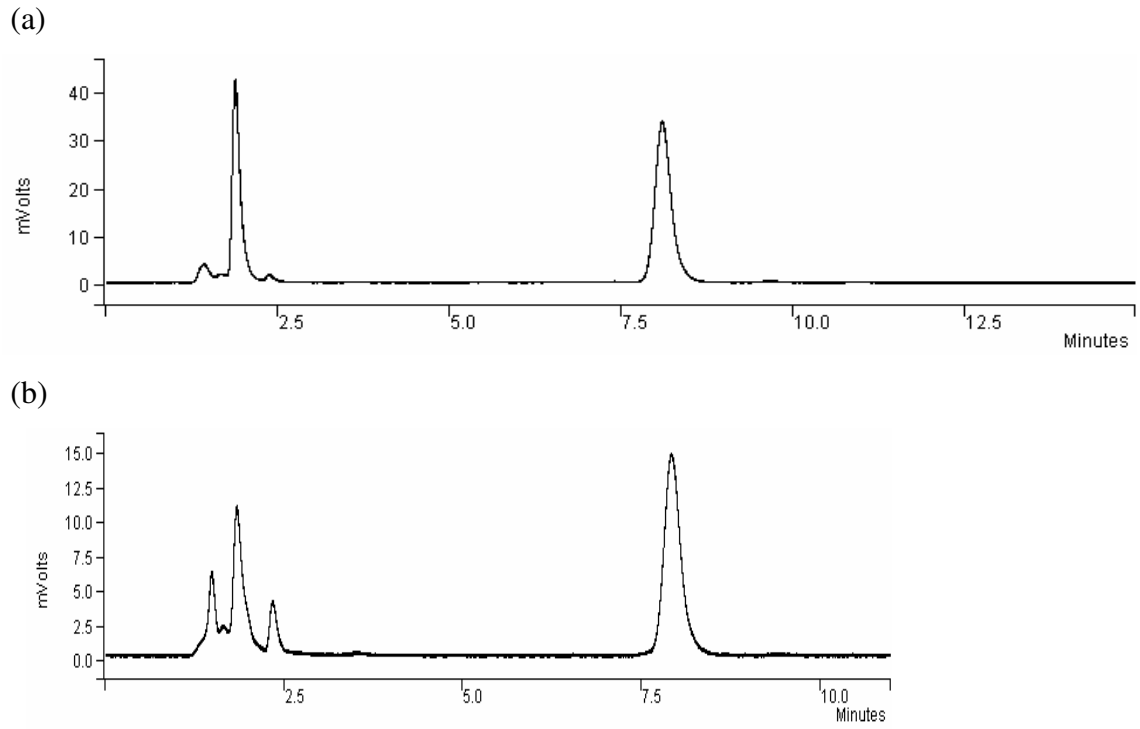


Figure 4.4.1.1.3. Typical HPLC chromatogram of (a) SP_{NEAT} at a concentration of 1000 pmol and (b) ceramide standard at a concentration of 500 pmol having undergone deacylation, where ceramide is converted to sphingosine, and derivatised with OPA. The retention time of the sphingosine was 8.094 in (a) and 7.919 min in (b) and the OPA reagent eluted between 1.5- 2.5 min in both.

Levels of basal sphingosine in MDA and A10p10p cells were compared and illustrated in Figure 4.4.1.2. There was no trace of basal sphingosine detected in the untreated MDA cells and while it was detected in some A10p10p samples 0.56 ± 0.4 pmol, the variation was such that the levels were not deemed statistically significant with respect to the MDA cells. The fact that sphingosine was only detected in the A10p10p cells twice out of three experiments suggests that the levels of sphingosine found in A10p10p cells are close to the limit of detection for this method.

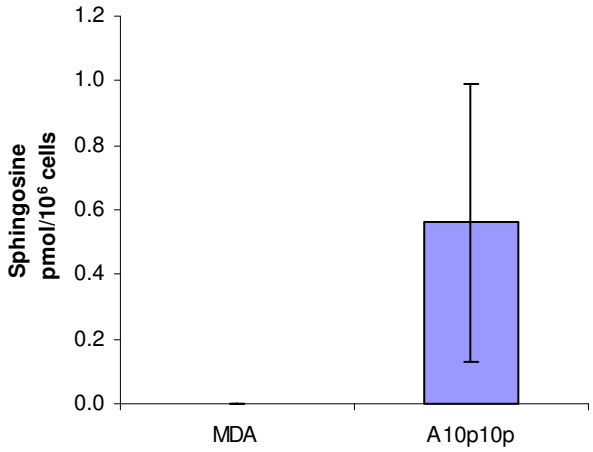


Figure 4.4.1.2. Basal Sphingosine in the MDA and A10p10p cell lines. T-75 flasks were seeded with MDA or A10p10p cells at a density of $3x10^6$ cells/flask. Following 24 h the flasks were washed with PBS, treated with control media for 72 h, extracted and analysed as above. Data represents results from three experiments. Error bar depicts SEM.

4.4.1.3 Ceramide levels in untreated MDA and A10p10p cells

T-75 flasks were seeded with MDA or A10p10p cells at a density of $3x10^6$ cells/flask. Following 24 h flasks were rinsed with PBS and 12 mL fresh media was added for a 3 day incubation period. After 3 days, floating and adherent cells were collected and pooled as described in Materials and Methods. Lipids were extracted and subjected to deacylation and derivatisation steps and quantitated by HPLC as described in above sections. Levels of ceramide in MDA and A10p10p cells were compared and illustrated in Figure 4.4.1.3. While there was a clear apparent difference in the levels of ceramide in MDA and A10p10p cells at 253.1 $\pm$ 40.4 and 91.0 $\pm$ 32.8 pmol respectively, the difference was not found to be statistically significant.

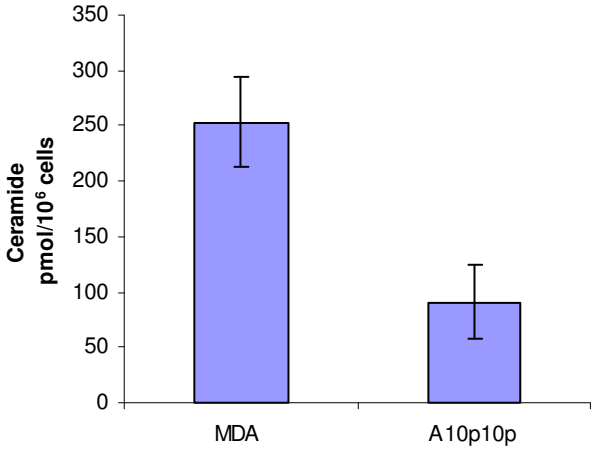


Figure 4.4.1.3 Ceramide levels in the MDA and A10p10p cell lines. T-75 flasks were seeded with MDA or A10p10p cells at a density of $3x10^6$ cells/flask. Following 24 h the flasks were washed with PBS and treated with control media for 72 h. Data represents results from three experiments. Error bars depict SEM.

4.4.1.4 Effects of fatty acid treatments alone or in combination with Dox on basal sphingosine levels in MDA cells

T-75 flasks were seeded with MDA cells at a density of $3x10^6$ cells/flask and pre-treated in triplicate with CLA-mix; *c*9,*t*11-CLA; *t*10,*c*12-CLA; EPA or DHA at a final concentration of 6 µg/mL as listed in above sections. Following 24 h, media containing fatty acid alone/control or fatty acid/control plus Dox at 115 ng/mL was added. After 3 days, floating and adherent cells were pooled and a cell count performed as listed previously. Lipids were extracted and subjected to alkaline hydrolysis and derivatisation steps and quantitated by HPLC as described in above sections. The effects of fatty acids/control alone or in combination with Dox on cellular basal sphingosine levels in the MDA cell line are illustrated in Figure 4.4.1.4 (a) and (b) respectively.

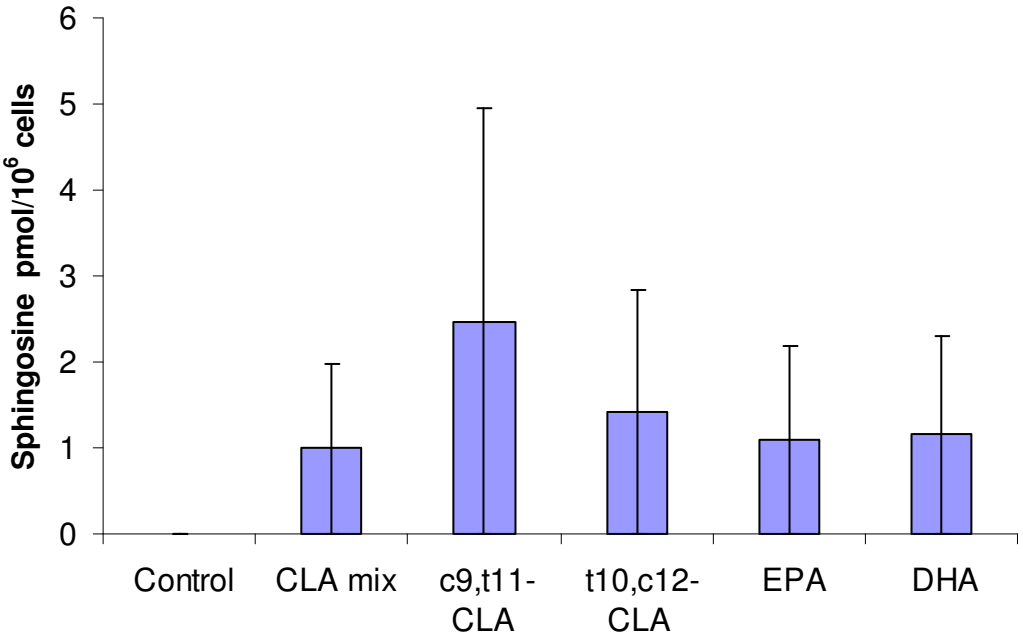
Each of the five fatty acid treatments alone caused an increase in basal sphingosine levels at 1.0 $\pm$ 1.0, 2.5 $\pm$ 2.5, 1.4 $\pm$ 1.4, 1.1 $\pm$ 1.1, 1.2 $\pm$ 1.2 pmol with CLA mix,

*c*9,*t*11 and *t*10,*c*12-CLA, EPA and DHA respectively. However, none were deemed significantly different from control cells in which sphingosine was undetectable. Sphingosine was only detected following fatty acid alone treatments in one out of three experiments, hence Mean = SEM in each case. When Dox was combined with CLA mix, *c*9,*t*11 and *t*10,*c*12-CLA there was a significant increase in basal sphingosine levels (22.4 $\pm$ 5.0, 20.6 $\pm$ 7.5 and 15.3 $\pm$ 5.5 pmol sphingosine respectively) relative to respective fatty acid alone treatments listed above. These combinations were also found to be significantly different relative to untreated control. However, relative to Dox alone which yielded 32.4 $\pm$ 13.7 pmol sphingosine, the combination of Dox plus any of the five fatty acids was not found to be significantly different.

4.4.1.5 Effects of fatty acid treatments alone or in combination with Dox on ceramide levels in MDA cells

T-75 flasks were seeded with MDA cells at a density of $3x10^6$ cells/flask and pre-treated in triplicate with CLA-mix; *c*9,*t*11-CLA; *t*10,*c*12-CLA; EPA or DHA at a final concentration of 6 µg/mL as listed in above sections. Following 24 h, media containing fatty acid alone/control or fatty acid/control plus Dox at 115 ng/mL was added. After 3 days, floating and adherent cells were pooled and a cell count performed as listed previously. Lipids were extracted and subjected to deacylation and derivatisation steps and quantitated by HPLC as described in above sections. The effects of fatty acids/control alone or in combination with Dox on ceramide levels in the MDA cell line are illustrated in Figure 4.4.1.5 (a) and (b) respectively.

a)



b)

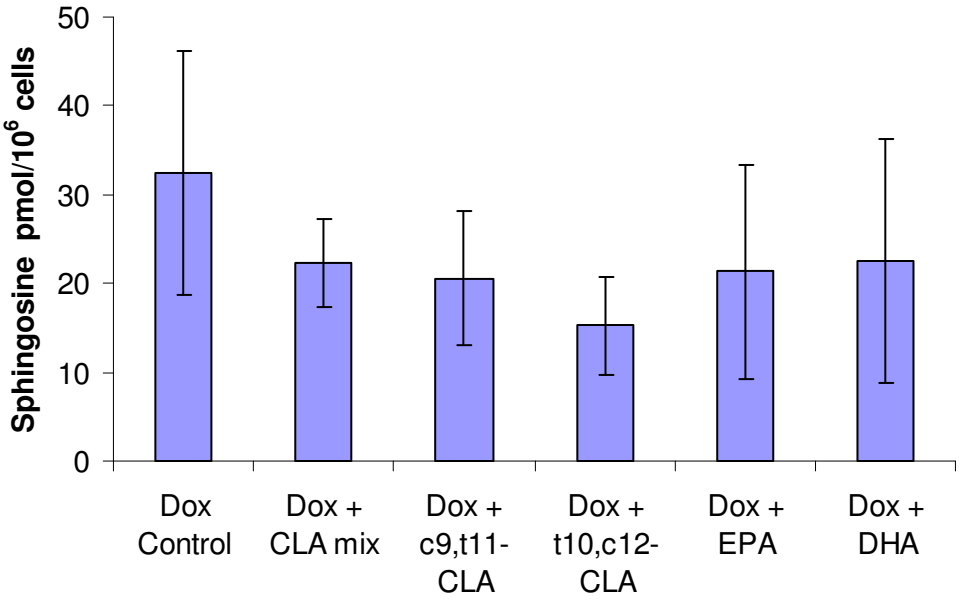
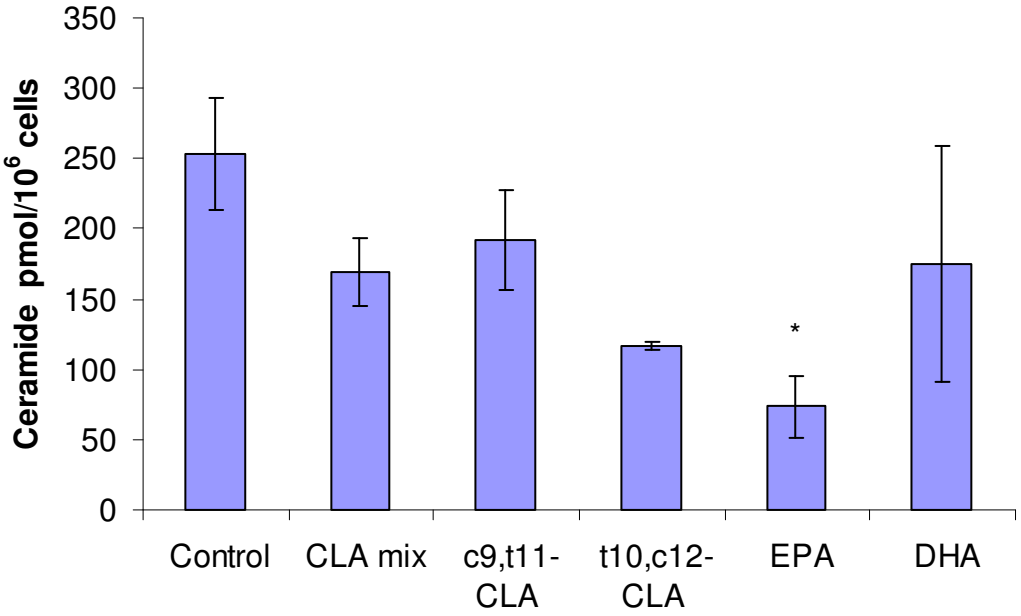


Figure 4.4.1.4 Effects of fatty acids/control alone or in combination with Dox on cellular basal sphingosine levels in the MDA cell line. T-75 flasks were seeded with MDA cells and pre-treated in triplicate with CLA, *c*9,*t*11, *t*10,*c*12, EPA or DHA at a final concentration of 6 µg/ mL. Following 24 h the flasks (FA alone) were washed with PBS and treated with fatty acid for 72 h. Flasks (Dox + FA) were treated with fatty acid/control plus Dox at 115 ng/mL for 4h, followed by 72 h with fatty acid/control. Data represents results from at least three experiments. Error bars depict SEM.

(a)



(b)

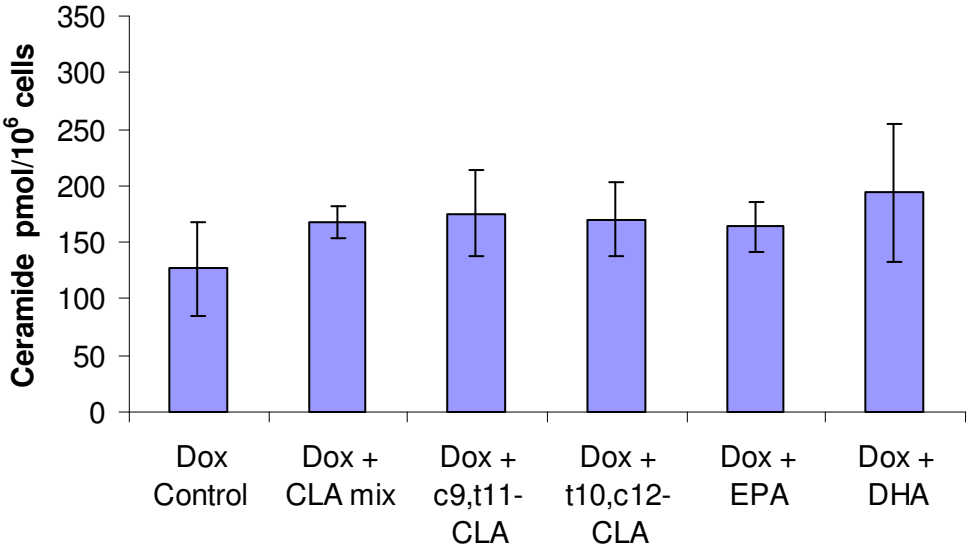


Figure 4.4.1.5 Effects of fatty acids/control (a) alone or (b) in combination with Dox on total cellular ceramide levels in the MDA cell line. T-75 flasks were seeded with MDA cells at a density of $3x10^6$ cells/flask and pre-treated in triplicate with CLA, *c*9,*t*11, *t*10,*c*12, EPA or DHA at a final concentration of 6 µg/ mL. Following 24 h the flasks (a) were washed with PBS and treated with fatty acid for 72 h. Flasks (b) were treated with fatty acid/control plus Dox at 115 ng/mL for 4h followed by 72 h with fatty acid/control. Data represents results from at least three experiments. Error bars depict SEM. *Denotes results which are significantly different to control/Dox control cells ($p<0.05$).

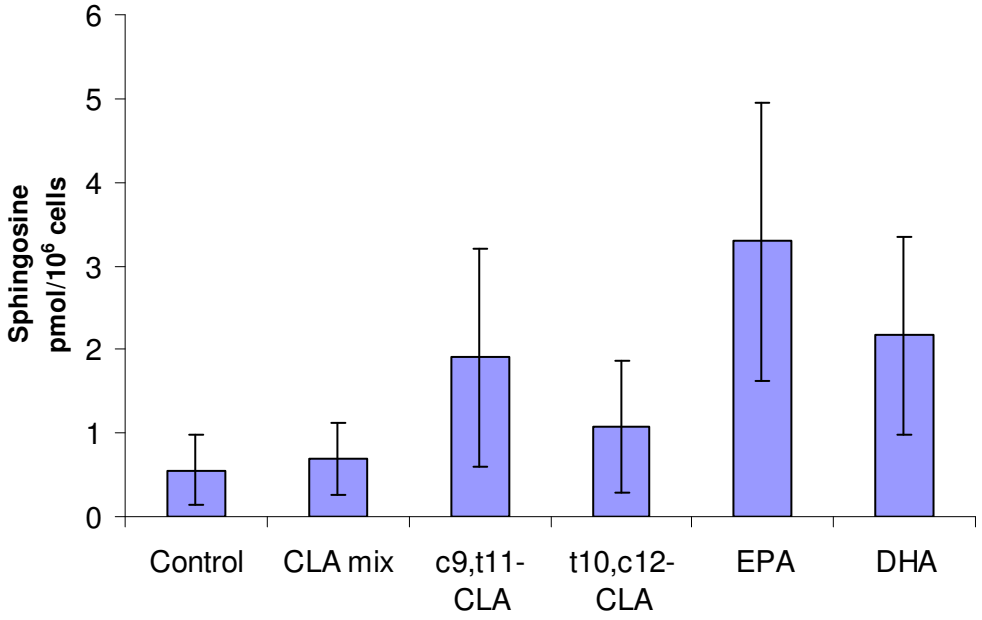
The levels of cellular ceramide were found to be 169.7 ± 33.9, 192.0 ± 50.2, 117.1 ± 4.3, 73.3 ± 31.1 and 174.6 ± 118.6 pmol in MDA cells treated with CLA mix, *c*9,*t*11 and *t*10,*c*12-CLA, EPA and DHA respectively. With the exception of EPA, these values were not deemed to be significant relative to untreated controls at 253.1 ± 40.4 pmol. In cells treated with combinations of Dox plus fatty acid, corresponding values were 168.6 ± 14.2, 175.2 ± 37.9, 170.5 ± 32.4, 163.6 ± 22.1 and 193.6 ± 60.4 pmol respectively. Again, these values were not deemed to be significant relative to Dox alone at 126.6 ± 40.9 pmol or to untreated controls and respective fatty acid alone treatments (listed above).

4.4.1.6 Effects of fatty acid treatments alone or in combination with Dox on basal sphingosine levels of A10p10p cells

T-75 flasks were seeded with A10p10p cells at a density of $3x10^6$ cells/flask and pre-treated in triplicate with CLA-mix; *c*9,*t*11-CLA; *t*10,*c*12-CLA; EPA or DHA at a final concentration of 6 µg/mL as listed in above sections. Following 24 h, media containing fatty acid alone/control or fatty acid/control plus Dox at 115 ng/mL was added. After 3 days, floating and adherent cells were pooled and a cell count performed as listed previously. Lipids were extracted and subjected to alkaline hydrolysis and derivatisation steps and quantitated by HPLC as described in above sections. The effects of fatty acids/control alone or in combination with Dox on basal sphingosine levels in the A10p10p cell line are illustrated in Figure 4.4.1.6 (a) and (b) respectively.

While all five fatty acids appeared to cause an increase in basal sphingosine from 0.6 ± 0.4 pmol in the untreated control to 0.7 ± 0.4 pmol with CLA mix, 1.9 ± 1.3 pmol with *c*9,*t*11-CLA, 1.0 ± 0.8 pmol with *t*10,*c*12-CLA, 3.3 ± 1.7 pmol with EPA and 2.2 ± 1.2 pmol sphingosine with DHA, none were found to be statistically significant.

(a)



(b)

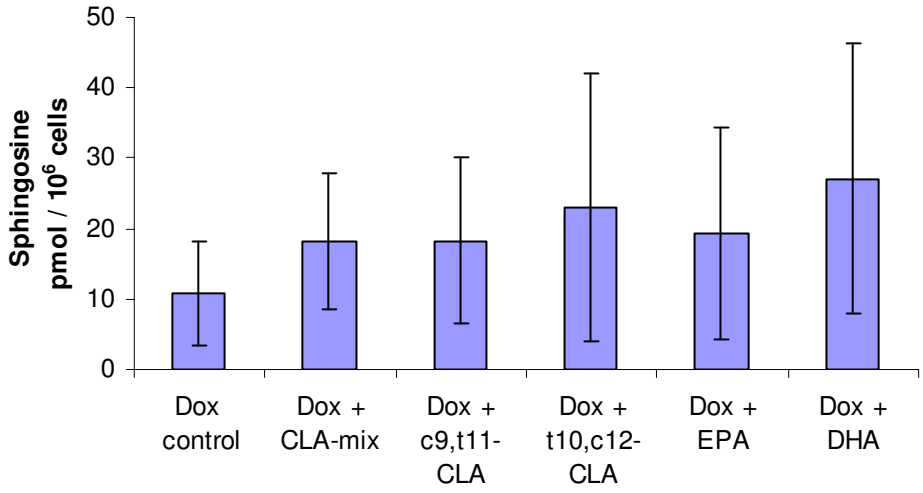


Figure 4.4.1.6 Effects of fatty acids/control (a) alone or (b) in combination with Dox on cellular basal sphingosine levels in the A10p10p cell line. T-75 flasks were seeded with A10p10p cells at a density of $3x10^6$ cells/flask and pre-treated in triplicate with CLA, *c*9,*t*11, *t*10,*c*12, EPA or DHA at a final concentration of 6 µg/ mL. Following 24 h the flasks (a) were washed with PBS and treated with fatty acid for 72 h. Flasks (b) were treated with fatty acid/control plus Dox at 115 ng/mL for 4 h followed by 72 h with fatty acid/control. Data represents results from at least three experiments. Error bars depict SEM.

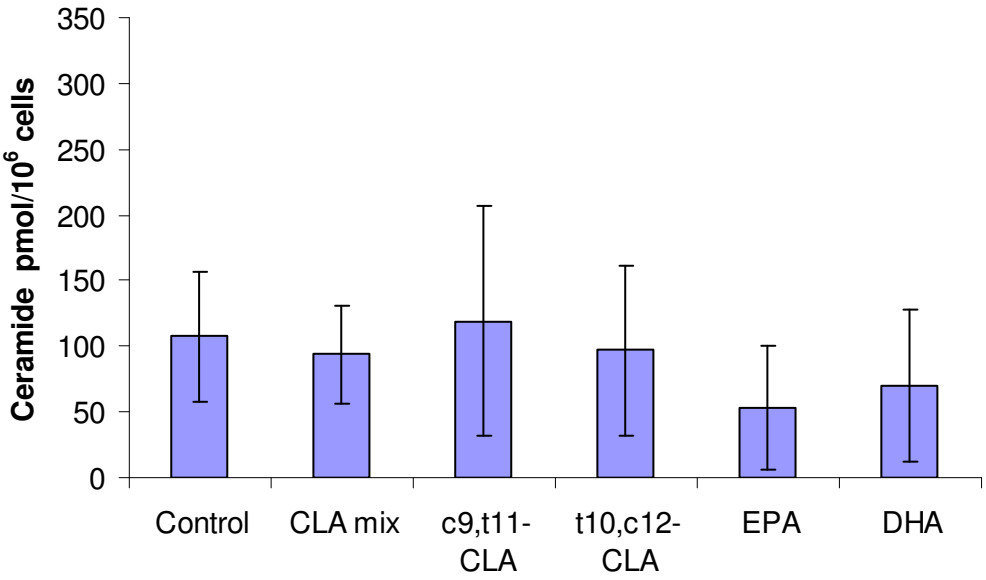
There was a similar trend in the combinations with Dox relative to Dox alone with increases in basal sphingosine from 10.9 ± 7.4 pmol in the Dox control to 18.1 ± 9.7 pmol in combination with CLA mix, 18.3 ± 11.8 pmol with *c*9,*t*11-CLA, 23.0 ± 19.0 pmol with *t*10,*c*12-CLA, 19.3 ± 15.0 pmol with EPA and 27.1 ± 19.1 pmol with DHA, although again not statistically significant (Figure 4.4.1.6 (a)).

4.4.1.7 Effects of fatty acid treatments alone or in combination with Dox on ceramide levels of A10p10p cells

T-75 flasks were seeded with A10p10p cells at a density of $3x10^6$ cells/flask and pre-treated in triplicate with CLA-mix; *c*9,*t*11-CLA; *t*10,*c*12-CLA; EPA or DHA at a final concentration of 6 µg/mL as listed in above sections. Following 24 h, media containing fatty acid alone/control or fatty acid/control plus Dox at 115 ng/mL was added. After 3 days, floating and adherent cells were pooled and a cell count performed as listed previously. Lipids were extracted and subjected to deacylation and derivatisation steps and quantitated by HPLC as described in above sections. The effects of fatty acids/control alone or in combination with Dox on basal sphingosine levels in the A10p10p cell line are illustrated in Figure 4.4.1.7 (a) and (b) respectively.

Treatments of fatty acids alone caused no significant effect on the cellular ceramide in A10p10p cells. The combination of Dox plus *t*10,*c*12-CLA yielded a significant increase in ceramide levels from 100.30 ± 0.08 pmol in the Dox control to 276.49 ± 17.16 pmol following combination treatment. This was found to be significant relative to untreated control listed above. Similarly, the combination of Dox plus *c*9,*t*11-CLA yielded a significant increase in ceramide levels to 226.8 ± 55.0 pmol following treatment. Combinations with the remaining three fatty acids, CLA-mix, EPA and DHA, caused an apparent but not significant increase in ceramide levels to 221.2 ± 106.4, 138.4 ± 55.2 and 116.6 ± 22.9 pmol ceramide respectively following treatments.

(a)



(b)

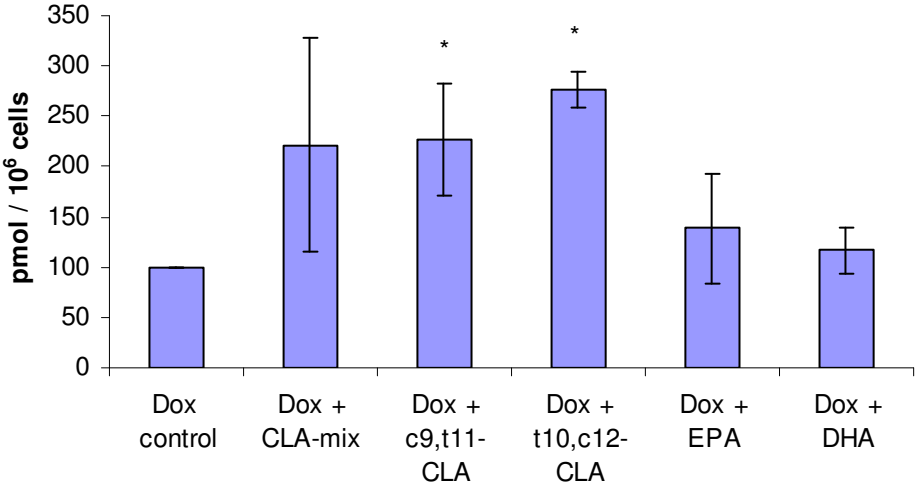


Figure 4.4.1.7 Effects of fatty acids/control (a) alone or (b) in combination with Dox on total cellular ceramide levels in the A10p10p cell line. T-75 flasks were seeded with A10p10p cells at a density of $3x10^6$ cells/flask and pre-treated in triplicate with CLA, *c*9,*t*11, *t*10,*c*12, EPA or DHA at a final concentration of 6 µg/ mL. Following 24 h the flasks (a) were washed with PBS and treated with fatty acid for 72 h. Flasks (b) were treated with fatty acid/control plus Dox at 115 ng/mL for 4h followed by 72 h with fatty acid/control. Data represents results from at least three experiments. *Denotes results which are significantly different to control/Dox control cells ($p<0.01$). Error bars depict SEM.

4.4.2 HER2/neu Assay

4.4.2.1 Bio-Rad Protein Assay

A bovine serum albumin (BSA) standard curve in the range 0-2100 µg/mL was set up as per "Materials and Methods" section. By extrapolation from this standard curve, the protein content of fatty acid/control treated MDA and A10p10p cells was calculated (Table 4.4.2.1).

Table 4.4.2.1 Protein levels in MDA and A10p10p cells following various fatty acid treatments

Treatment	MDA cells µg/mL	A10p10p cells µg/mL
Control	1450	1550
CLA-mix	1500	1500
*c*9,*t*11-CLA	1200	800
*t*10,*c*12-CLA	1350	1450
DHA	1725	1775
EPA	700	1550

4.4.2.2 c-erbB2/c-neu ELISA

T-25 flasks were seeded with MDA or A10p10p cells at a density of $1x10^6$ cells/flask. Following 24 h flasks were treated with fresh media containing fatty acid/control in triplicate for a 3 day exposure period. Fatty acids used in this experiment were CLA mixture of isomers (CLA-mix); *c*9,*t*11-CLA; *t*10,*c*12-CLA; EPA and DHA at a final concentration of 6 µg/mL. Floating and adherent cells were pooled, the cell lysate extracted and an aliquot of each sample was assayed for protein content as described above. The p185 c-erbB2/c-neu Calbiochem ELISA

was performed as listed in Methods. A standard curve of c-erbB2/c-neu in range 0-3 ng/mL was performed and illustrated in Figure 4.4.2.2.1.

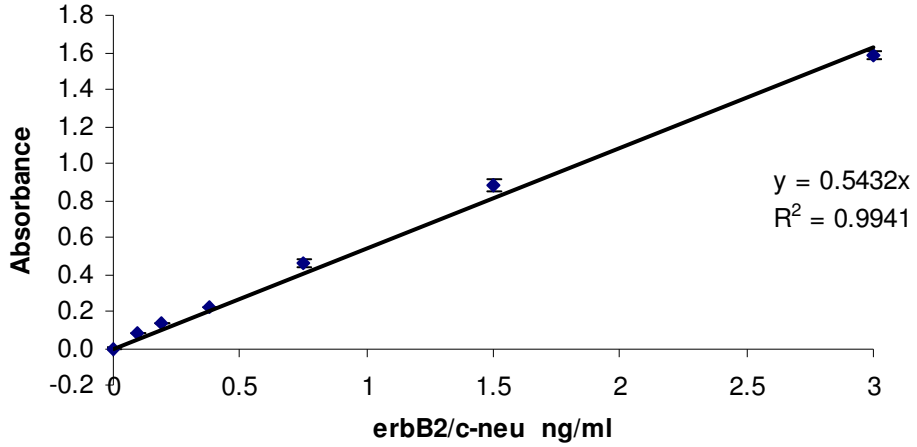


Figure 4.4.2.2.1 Standard Curve of p185 c-erbB2/c-neu in the range 0-3 ng/mL. The p185 c-erbB2/c-neu Calbiochem ELISA was performed as listed in Methods. Error bars depict SEM.

The effects of fatty acids/control on the expression of c-erbB2/c-neu in MDA and A10p10p cells were determined using the equation of the line obtained from the standard curve and is illustrated in Figure 4.4.2.2.2. In MDA cells CLA-mix treatment resulted in a significant reduction in c-erbB2/c-neu expression relative to untreated MDA control from 30.9 $\pm$ 6.0 HNU/mg protein in control MDA cells to 8.4 $\pm$ 1.7 HNU/mg protein following CLA-mix treatment. Conversely, c-erbB2/c-neu expression was significantly upregulated to 64.1 $\pm$ 8.6 and 56.8 $\pm$ 7.3 HNU/mg protein following treatment with *c*9,*t*11 and *t*10,*c*12-CLA respectively. DHA and EPA treatment did not result in a significant change in c-erbB2/c-neu expression in MDA cells at levels of 36.5 $\pm$ 3.0 and 13.1 $\pm$ 1.2 HNU/mg protein respectively.

CLA-mix also resulted in significant down-regulation of c-erbB2/c-neu expression in A10p10p cells relative to untreated A10p10p control from 82.9 $\pm$ 4.3 to 36.6 $\pm$ 1.0 HNU/mg protein following treatment. As in MDA cells, *c*9,*t*11-CLA caused a significant upregulation of c-erbB2/c-neu expression in A10p10p cells relative to control with a level of 145.6 $\pm$ 5.3 HNU/mg protein following treatment. Both DHA and EPA treatment resulted in significant down-regulation of c-erbB2/c-neu expression in A10p10p cells relative to untreated A10p10p control at levels of 71.6 $\pm$ 4.2 and 67.2 $\pm$ 2.8 HNU/mg protein respectively. *t*10,*c*12-CLA treatment did not result in significant change at 79.0 $\pm$ 3.9 HNU/mg protein following treatment.

In untreated samples of MDA and A10p10p, there was a significant up-regulation c-erbB2/c-neu expression in the multidrug resistant A10p10p cells relative to MDA cells. Also, this difference between the cell lines remained following treatments of CLA-mix, *c*9,*t*11-CLA, EPA and DHA with a significant increase in c-erbB2/c-neu levels relative to corresponding treatments in the MDA cell line.

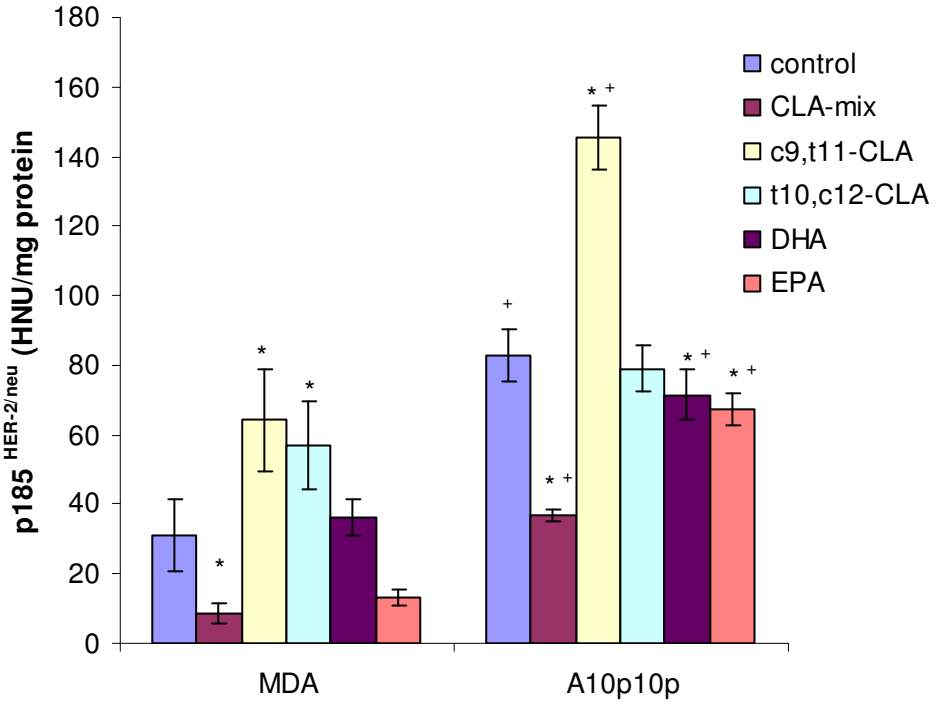


Figure 4.4.2.2 (a) Standard Curve of p185 c-erbB2/c-neu in the range 0-3 ng/mL and (b) level of p185 c-erbB2/c-neu (HNU/mg protein) in MDA and A10p10p cells treated with fatty acid/control. Fatty acids used were CLA-mix; *c*9,*t*11-CLA; *t*10,*c*12-CLA; EPA and DHA at 6 µg/mL for 3 days. The p185 c-erbB2/c-neu Calbiochem ELISA was performed as listed in Methods. *Denotes samples statistically different relative to control MDA or A10p10p cells as determined by the Student's *t*-test with $p<0.05$. $^{+}$Denotes A10p10p samples statistically different relative to corresponding MDA samples with $p<0.05$. Error bars depict SEM.

4.5 Discussion

One hypothesis being tested in this work is that fatty acids including CLA induce toxicity in MDA and A10p10p melanoma cells by mechanisms involving an increase in ceramide production in the cells. Ceramide belongs to highly bioactive class of molecules known as sphingolipids that are used by cells to regulate growth, differentiation, apoptosis and other cellular functions. They are located in lipid-rich structures such as the extracellular leaflet of the cell membrane and are critical for the maintenance of membrane structure, especially that of "microdomains" (such as caveolae) (Harder and Simons, 1997); they modulate the behaviour of growth factor receptors and extracellular matrix proteins (Hakomori, 1991). Sphingolipids function as "second messengers" for growth factors, cytokines, differentiation factors and growing list of agonists and toxins (Kolesnick, 1998; Merrill *et al.,* 1997; Riboni *et al.,* 1997; Spiegel and Merrill, 1996). Sphingolipid turnover into different bioactive metabolites depends on activation of sphingomyelin hydrolysis to ceramide by sphingomyelinase, which is further metabolized by ceramidase and sphingosine kinase to sphingosine and sphingosine-1-phosphate. Agents that activate only sphingomyelinase (which results in ceramide accumulation) have profound effects on the behaviour of cells because sphingosine-1-phosphate is a potent mitogen and an inhibitor of apoptosis (Cuvillier *et al.,* 1998; Olivera and Spiegel, 1993), whereas sphingosine and ceramide inhibit growth and/or induce apoptosis (Hannun, 1994; Sweeney *et al.,* 1998).

In this study an *in vitro* model of drug sensitive and resistant melanoma cells characterised by Glynn *et al.,* (2004) was used. MDA and A10p10p cells were treated in the presence or absence of Dox, with CLA-mix; *c*9,*t*11-CLA; *t*10,*c*12-CLA; EPA or DHA. Ceramide and basal sphingosine were analysed and quantitated by HPLC as described in above sections. Ceramide levels were found to be lower in A10p10p cells than in the MDA cell line, which points to one mechanism by which the drug resistant A10p10p cells protect themselves from anticancer drugs. It has been shown that decreased levels of endogenous ceramide by over-expression of glucosylceramide synthase, which clears ceramide levels by incorporating it into

glucosylceramide, results in the development of a multidrug resistant phenotype in human cancer cells (Ogretmen and Hannun, 2001).

EPA single treatments caused a significant reduction in ceramide levels in both cell lines, suggesting that the cytotoxic action of EPA is not through a pathway initiated by ceramide production. In contrast, all fatty acids plus Dox resulted in an increase in ceramide levels relative to Dox alone in both cell lines although this was only significant in the case of *c*9,*t*11 and *t*10,*c*12-CLA plus Dox in A10p10p cells. This points to a trend indicating that perhaps ceramide production is one of many pathways by which these fatty acids enhance the cytotoxic action of anticancer drugs.

Interestingly, Dox treatments alone and in combination with fatty acids caused an increase in sphingosine levels relative to respective single treatments of fatty acid/ control in the MDA cell line. This is the first study to report this finding, although it was only significant with CLA-mix, *c*9,*t*11 and *t*10,*c*12-CLA. In the A10p10p cell line, all fatty acids alone caused an increase in sphingosine levels relative to control. Treatments of Dox alone and in combination with each fatty acid resulted in a further although not significant increase. Sweeney and co-workers (1998) established that sphingosine also induces apoptosis –independently from ceramide, acting in an earlier part of the apoptotic pathway than ceramide.

The p185 c-erbB2/c-neu Calbiochem ELISA was performed on MDA and A10p10p cells treated with CLA-mix; *c*9,*t*11-CLA; *t*10,*c*12-CLA; EPA or DHA. In untreated cells, the A10p10p cell line had significantly higher levels of Her2/*neu* coded p185 $^{c\text{-}erbB2/c\text{-}neu}$ oncoprotein present than in MDA cells. This indicates another potential mechanism by which the A10p10p cell line maintains resistance to conventional anticancer drugs. Overexpression of the Her2/*neu* oncogene has been shown previously to confer resistance to chemotherapeutic drugs in breast cancer cells (Colomer *et al.,* 2000). Treatment of both MDA and A10p10p cells with CLA-mix resulted in down-regulation of Her2/*neu* expression. Also, both EPA and DHA resulted in down-regulation in the A10p10p cell line. Menendez *et al*. (2005) also demonstrated that DHA down-regulated Her2/*neu* expression in breast cancer cells overexpressing the gene. However, there were opposing results with treatments of

the single isomers *c*9,*t*11 and *t*10,*c*12-CLA. Both isomers caused significant up-regulation of Her2/*neu* expression in A10p10p and *c*9,*t*11-CLA also caused significant up-regulation in MDA cells. Since these two studies are the only to date to investigate the effects of these fatty acids on Her2/*neu* expression in melanoma cells, further work is necessary to elucidate the mechanisms behind and confirm these opposing effects.

Chapter 5

Modulation of lipid profile by conjugated linoleic acid (CLA) in drug-resistant and sensitive melanoma cells.

5.0 Abstract

It is widely accepted that human cancer cells have the ability to synthesis their own supply of fatty acids, seemingly independent of the regulatory signals that down-regulate fatty acid synthesis in normal cells. Also, the development of doxorubicin resistance in breast and colon cancer cells has been associated with decreased fluidity of lipid bilayers and increased degree of fatty acid saturation of cholesterol esters. The previous chapters showed that exogenous fatty acids had a significant inhibitory effect on growth in breast cancer cell lines. One proposed mechanism for this anticarcinogenic activity is the alteration of the fatty acid composition of the cell membrane phospholipids by CLA isomers resulting in reduced synthesis of arachidonic acid (AA) and AA-derived eicosanoids associated with stimulation of cancer cell proliferation. Fatty acid profiles of untreated MDA-MB-435S-F (MDA) melanoma cells and a doxorubicin resistant variant MDA-MB435S-F/Adr10p10p (A10p10p) were analysed by gas chromatography. There was a statistically significant increase in the proportion of total saturated fatty acids (SFA) and decrease in the ratio of unsaturated fatty acids (UFA) to SFA in the resistant A10p10p cell line relative to the MDA line, suggesting an up-regulation in fatty acid synthase (FAS) activity. Uptake of CLA by MDA cells was dose dependent; treatment with *c*9,*t*11-CLA and *t*10,*c*12-CLA (75 μM) for 72 h resulted in significant uptake into MDA cells (29.44 $\pm$ 3.63 and 26.37 $\pm$ 5.53 % total fatty acid methyl ester (FAME) respectively). Less uptake by the CLA mixture of isomers into MDA cells was observed. A similar pattern of CLA uptake was observed in drug resistant cells. There was a definite trend, although not always significant, of a decrease in the long chain saturated fatty acids (LCSFA) and an increase in the unsaturation index of both cell lines following all fatty acid treatments, possibly due to FAS inhibition. With the exception of the CLA-mix in the resistant line, all fatty acids in both cell lines caused a decrease in the ratio of C18:1/18:0 suggesting the Δ 9 desaturase enzyme stearoyl-CoA desaturase (SCD) as a potential target for inhibition by fatty acid treatments. These data highlight the potential of fatty acids to modulate lipid composition of tumour cells and the need for further study to fully elucidate the specific interactions with the enzymes involved.

5.1 Introduction

Analysis of the fatty acid composition of cellular lipids has indicated an altered balance of saturated to monounsaturated fatty acids in tumours relative to normal non malignant cells (Fermor *et al.,* 1992). A study by Merchant *et al.* (1991) using ^{31}P NMR spectroscopy found that levels of the major lipid classes (phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI) and sphingomyelin (SM)) were different in normal and transformed breast tissue cells. In particular, there was an elevated level of PI in the malignant breast cells. In a following study, it was found that PC levels in colon cancer cells were significantly different when comparing moderately and poorly differentiated tumours (Merchant *et al.,* 1995).

Studies have also reported differences in lipid composition between sensitive and multidrug resistant cancer cells. In P388 murine leukaemia cells it was found that the sub-line resistant to doxorubicin displayed altered PC/SM ratio caused by the decrease in membrane PC and an increase in SM, with the membranes being more ordered. Also in Leukemic T-lymphoblasts, cell membranes from the vinblastine resistant line had significantly higher cholesterol and phospholipid levels and raised protein/lipid ratio compared to the drug sensitive cells leading to a more ordered and therefore less fluid membrane (reviewed in Hendrich and Michalak, 2003).

Cancer cells and indeed all cells have only two options available to source the biologically active fatty acids they require: host diet/circulation or *de novo* synthesis (Spector and Burns, 1987). It is widely accepted that human cancer cells have the ability to synthesis their own supply of fatty acids, seemingly independent of the regulatory signals that down-regulate fatty acid synthesis in normal cells (Kuhajda, 2000). Fatty acid synthase (FAS), previously known as oncogenic antigen 519 (OA-519), is a key enzyme in the *de novo* synthesis of fatty acids and has been identified as a tumour marker in breast cancer indicating a poor prognosis (Kuhajada *et al.,* 1989; 1994). FAS is a multi-enzyme complex comprising an intertwined dimer with two lateral semicircular reaction chambers, each containing a full set of catalytic

domains required for fatty acid elongation. *De novo* synthesis by FAS involves a cyclic set of chemical reactions leading to the formation of palmitate (C16:0) from acetyl- and malonyl-CoA. Palmitate is released from the final domain, thioesterase (TE) and is then available to be processed by other desaturases or elongase to form other fatty acid molecules (Maier *et al.,* 2006). FAS is down-regulated in normal human cells by the intake of small amounts of fat in the diet, but is highly expressed in human cancers and is probably one of the most common molecular changes in cancer cells (Kuhajda, 2000; Menendez *et al.,* 2005). Of interest is the recent discovery that elevated FAS expression contributes to increased drug resistance in breast cancer cells (Liu *et al.,* 2008).

Another important enzyme in *de novo* fatty acid synthesis is stearoyl-CoA desaturase (SCD). SCD is responsible for catalyzing the formation of monounsaturated fatty acids (MUFA), particularly oleic (C18:1) and palmitoleic (C16:1) acid. The MUFA synthesised can then be used in the synthesis of major lipid classes such as phospholipids, triglycerides and cholesterol esters (Ntambi *et al.,* 2004). Hardy *et al* (2000) found that oleic acid stimulated proliferation in breast cancer cell lines, while palmitate, a product of FAS, inhibited growth. It is probable that although production of palmitate is upregulated in cancer cells and further up regulated in drug resistant cells as described above, it is quickly metabolised by SCD to form oleic acid vital for survival and proliferation of cancer cells.

There is a large body of evidence both *in vivo* and *in vitro* indicating that tumour cell growth can be modulated by individual fatty acids (Burns and Spector, 1993; Bougnoux, 1999). In particular, CLA has been shown to exert a wide range of health benefits including anticarcinogenic activity (Belury, 2002; Wahle *et al.,* 2004; Nagao *et al.,* 2005). One proposed mechanism for this anticarcinogenic activity is the alteration of the FA composition of the cell membrane phospholipids by CLA isomers resulting in reduced synthesis of arachidonic acid (AA) and AA-derived eicosanoids associated with stimulation of cancer cell proliferation (Banni *et al.,* 1999; 2001).

It has been suggested that CLA competes with LA for desaturation and elongation enzymes involved in the conversion of long chain metabolites (Banni *et al.*, 2004). These same enzymes are involved in the metabolism of the n-3 fatty acid α-linolenic acid, ALA (Figure 5.1.1).

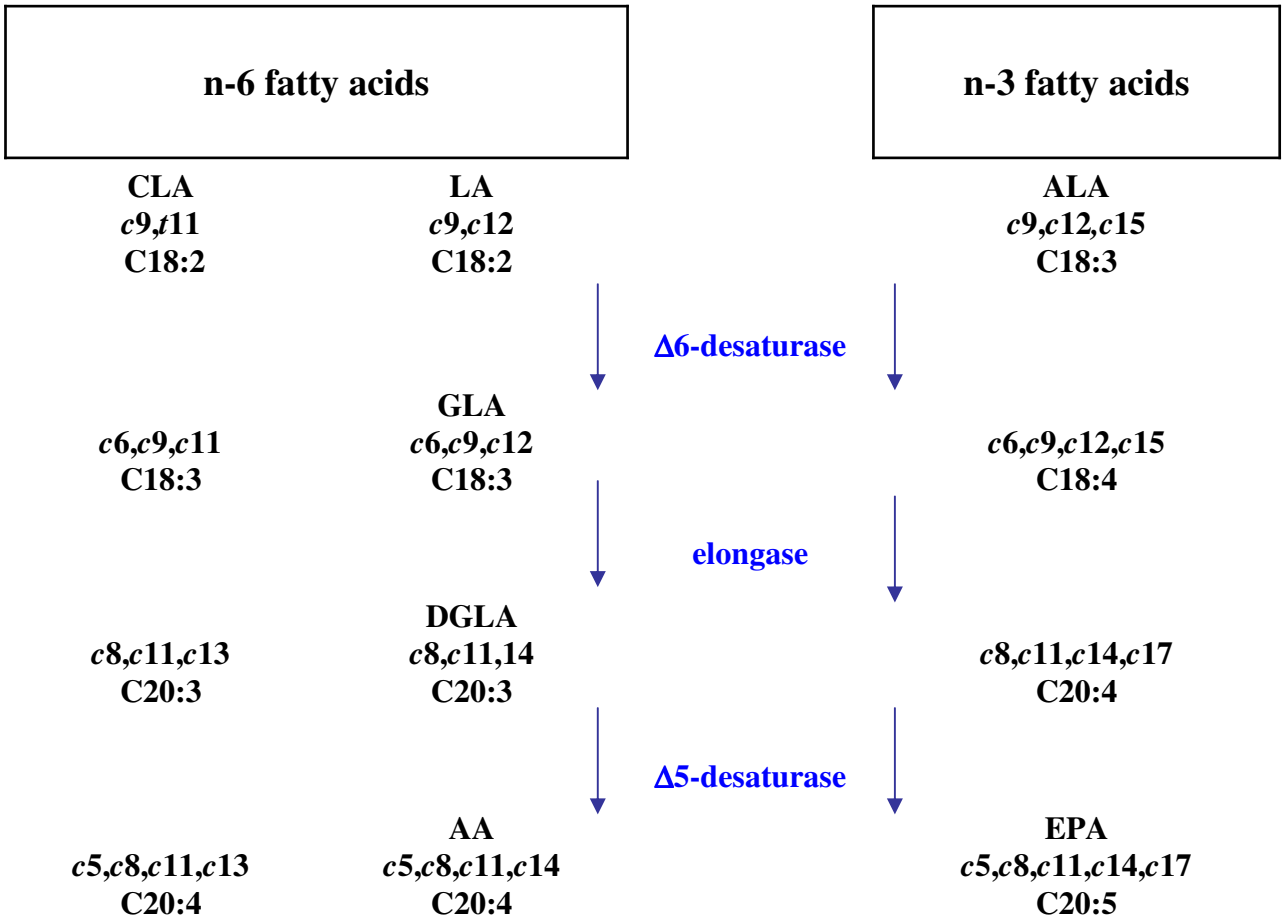


Figure 5.1.1. Schematic of pathways of the metabolism of CLA and LA and the n-3 ALA

Elongation and desaturase metabolites of CLA, notable for their conservation of the conjugated diene structure, have been detected and identified by Banni and coworkers in various tissues including mammary tissue, rat liver and lamb tissue (1996, 1999, 2001). In rat liver and adipose tissue *c*9,*t*11-CLA was found to be mainly converted into a C20:3 conjugated fatty acid, while the *t*10,*c*12-CLA isomer

was metabolised into a conjugated C18:3 or C16:3 fatty acid (Sebedio *et al.,* 2001). The presence of these metabolites in different lipid fractions of tissues (Banni *et al.,* 2001) may account for some of the differing and sometimes conflicting physiological effects of the different CLA isomers.

Examination of the lipid profiles of drug-resistant and sensitive melanoma cells following treatment with exogenous fatty acids including CLA may identify a potential therapeutic target to modulate the lipid environment and functionality of membrane related signalling proteins and drug efflux protein pumps. The overall objective of the work described in this chapter was to determine any differences between fatty acid profiles of untreated MDA-MB-435S-F (MDA) melanoma cells and a doxorubicin resistant variant MDA-MB435S-F/Adr10p10p (A10p10p) by gas chromatography (GC) methods. In addition, the extent to which CLA mixture of isomers (CLA-mix), purified isomers *c*9,*t*11 and *t*10,*c*12-CLA and omega-3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), can modulate the lipid composition of MDA and A10p10p were determined.

5.2 Specific Objectives

- To validate a gas chromatographic (GC) procedure for the analysis of the lipid profiles in total lipid extracts of drug sensitive MDA-MB435S-F (MDA) and drug resistant MDA-MB435S-F-Adr10p10p (A10p10p) melanoma cells.

- To determine the differences if any between the lipid profiles of untreated MDA and A10p10p cells.

- To investigate the effects of a mixture of CLA isomers (CLA-mix), two purified single isomers (*c*9,*t*11 and *t*10,*c*12-CLA) and two omega-3 fatty acids (EPA and DHA) on the total cellular lipid fatty acid profiles of both MDA and A10p10p cell lines.

5.3 Materials & Methods

5.3.1 Materials

MDA-MB-435-S-F (MDA) human melanoma cell line and its Doxorubicin resistant variant MDA-MB-435-S-F/ Adriamycin 10p10p (A10p10p) were developed and kindly donated by Dr. Sharon Glynn (NICB, Dublin). Chemotherapy agent Doxorubicin (Dox) was kindly donated by Dr. Glynn (NICB, Dublin). Conjugated Linoleic Acid (CLA) mixture of isomers (99 % pure) (Cat: UC-59A) and single preparations (90 % pure) of isomers *c*9,*t*11 and *t*10,*c*12- CLA (Cat: UC-60A, UC-61A) as listed previously were from NuChek-Prep, Elysian, MN, USA. A 7 FAME mixture comprising C14:0, C16:0, C16:1, C18:0, C18:1, C18:2 and C18:3 was also from NuChek-Prep. Fatty acid methyl esters (FAME) of single isomers *c*9,*t*11; *t*9,*t*11 and *t*10,*c*12- CLA and a mix of 37 FAME were purchased from Matreya Inc., Netherlands. Two FAME mixtures comprising; C8:0, C10:0, C12:0, C14:0 and C16:0, and C16:0, C18:0, C18:1 (Oleate), C18:2 (linoleate) and C18:3 (linolenate) along with single FAME standards of C20:0 (arachidate), C20:4 (arachidonate), C22:6 (docosahexanoate) and internal standard heptadecanoic acid were purchased from Sigma-Aldrich (Dublin, Ireland).

All sterile disposable plastic-ware was from Sarstedt Ltd., Wexford, Ireland. Cell culture media, supplements and reagents were purchased from Sigma-Aldrich. Boron trifluoride (BF_3/meth) (B1252) was also purchased from Sigma, as were all other chemicals and solvents listed in this section unless otherwise specified. All solutions were prepared in advance and stored in the solvent press or refrigerator as appropriate. Solvents were HPLC grade or higher.

5.3.2 Methods

5.3.2.1 Cell Culture

Cell lines were grown and maintained as listed previously in a ShelLab, IR2424 model CO_2 humidified Incubator at 37°C. T-75 flasks were seeded with MDA or A10p10p cells at a density of $2x10^6$ cells/flask and cultured for 24 h to allow the cells to attach. Flasks were then treated in triplicate with fatty acids (FA) at final concentrations of 21 and 75 μM. FA examined were CLA mixture of isomers (CLA), purified isomers *c*9,*t*11 and *t*10,*c*12-CLA and omega-3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Control flasks were treated with media containing ethanol at the same concentration as in experimental flasks (<0.01 %). After 3 days all flasks were harvested. Floating cells were collected by spinning down the media in the flasks and adherent cells were trypsinised as listed previously. These were pooled and a cell count performed using the Trypan Blue exclusion method. Cells were spun down at 1000 rpm for 5 min using a Labofuge 400 centrifuge, Heraeus Instruments (Foss Electric, Dublin) and the pellets frozen until extraction at a later date.

5.3.2.2 Cellular Lipid Extraction

Frozen cell pellets were removed from the freezer, allowed to thaw and total cellular lipids were extracted by a method modified from Folch *et al*, (1957) and Bligh and Dyer (1959). Briefly, 3.75 mL chloroform/methanol solution (2:1 v/v), 10 μL internal standard (IS) heptadecanoic acid (50mg/mL), 1.25 mL chloroform and 1.25 mL ultrapure water were added. The solution was vortexed well between each addition and centrifuged at 2000 rpm for 8 min (Heraeus Labofuge 400 centrifuge, Foss Electric, Dublin). The lower (organic) phase was collected, transferred to an extraction tube, dried under nitrogen and stored at -20 °C before methylation at a later date.

5.3.2.3 Methylation of Lipid Extract

The lipid extract containing free fatty acids was methylated in 14 % BF_3/meth according to Alonso *et al* (2004). To the extract 100 µL methanolic NaOH (1 M) was added. The solution was vortexed and incubated in a water bath at 70 ^oC. After 15 min 200 µL BF_3/meth was added and incubated at room temperature for a further 30 min, following which 200 µL hexane and 100 µL dH_20 were added. This was again vortexed and spun at 1000 rpm for 5 min in a centrifuge (Heraeus Labofuge 400 centrifuge supplied by Foss Electric, Dublin). The upper (organic) phase was transferred to a GC vial, dried under nitrogen and stored at -20 ^oC for later use. Prior to injection vials are thawed, 100 µL hexane added and vortexed. Using a Hamilton micro-syringe, 1 µL of sample/standard was injected into the gas chromatograph (GC).

5.3.2.4 Gas Chromatography Analysis

The fatty acid methyl esters (FAME) were analysed using a VARIAN CP-3800 gas chromatograph (GC) equipped with a flame ionization detector and a WCOT Fused Silica CP-Select CB column- 100 m x 0.25 mm ID, 0.2 µm film thickness (Chrompack, Middleburg, The Netherlands). The injector and the detector temperature were maintained at 250 ^oC. The column temperature was operated isothermally at 190 ^oC for 60 minutes after injection of samples and then raised from 190 ^oC to 225 ^oC at 4 ^oC /min with a final hold of 10 min at 225 ^oC. Nitrogen was used as carrier gas with column flow rate 0.7 mL/min. Samples were run in split (1:20) mode.

5.3.2.5 Identification of FAMEs

FAMEs were identified by retention time (RT) with reference to various standard FAMEs. Two FAME mixtures comprising; C8:0, C10:0, C12:0, C14:0 and C16:0, and C16:0, C18:0, C18:1 (*cis* 9), C18:2 (*cis* 9, *cis* 12) and C18:3 (*cis* 9, *cis* 12, *cis* 15) were initially analysed by GC and identified in the order listed (Figure 5.3.2.5.1 a & b). A 7 FAME mixture comprising C14:0, C16:0, C16:1, C18:0, C18:1, C18:2 and C18:3 was also analysed and cross referenced with the above to confirm identities (Figure 5.3.2.5.2).

Individual FAME standards of *c*9,*t*11, *t*9,*t*11 and *t*10,*c*12- CLA, C18:1 (*trans* 11) (vaccenic acid, *t*-VA), C20:0 (arachidate), C20:4 (arachidonate), C22:6 (docosahexanoate) and internal standard heptadecanoic acid (C17:0) were run and RT's recorded. A mixture of 37 FAME was then run and identified according to the supplier's specifications and cross referenced with the above (Figure 5.3.2.5.3 (a)). FAME standards of *c*9,*t*11, *t*9,*t*11 and *t*10,*c*12- CLA and *t*-VA were added to the 37 FAME mixture and again analysed (Figure 5.3.2.5.3 (b)). This mixture now contains all FAME to be investigated in samples and was injected with every experiment and used in the quantification of the various fatty acids present in the cellular lipid samples. As can be seen in Figure 5.3.2.5.3 (b) 18:1 *t*9, methyl elaidate and 18:1 *t*11, methyl vaccinate eluted together as one peak on the chromatogram. This combined peak also connects at its base with the peak for 18:1 *c*9, methyl oleate. Therefore, to remove ambiguity all three 18:1's were measured as one value. Table 5.3.2.5.1 contains the chemical name, abbreviation, retention time (RT), quantity present and peak area of FAME as detected from a typical injection of the 41 standard fatty acid mixture.

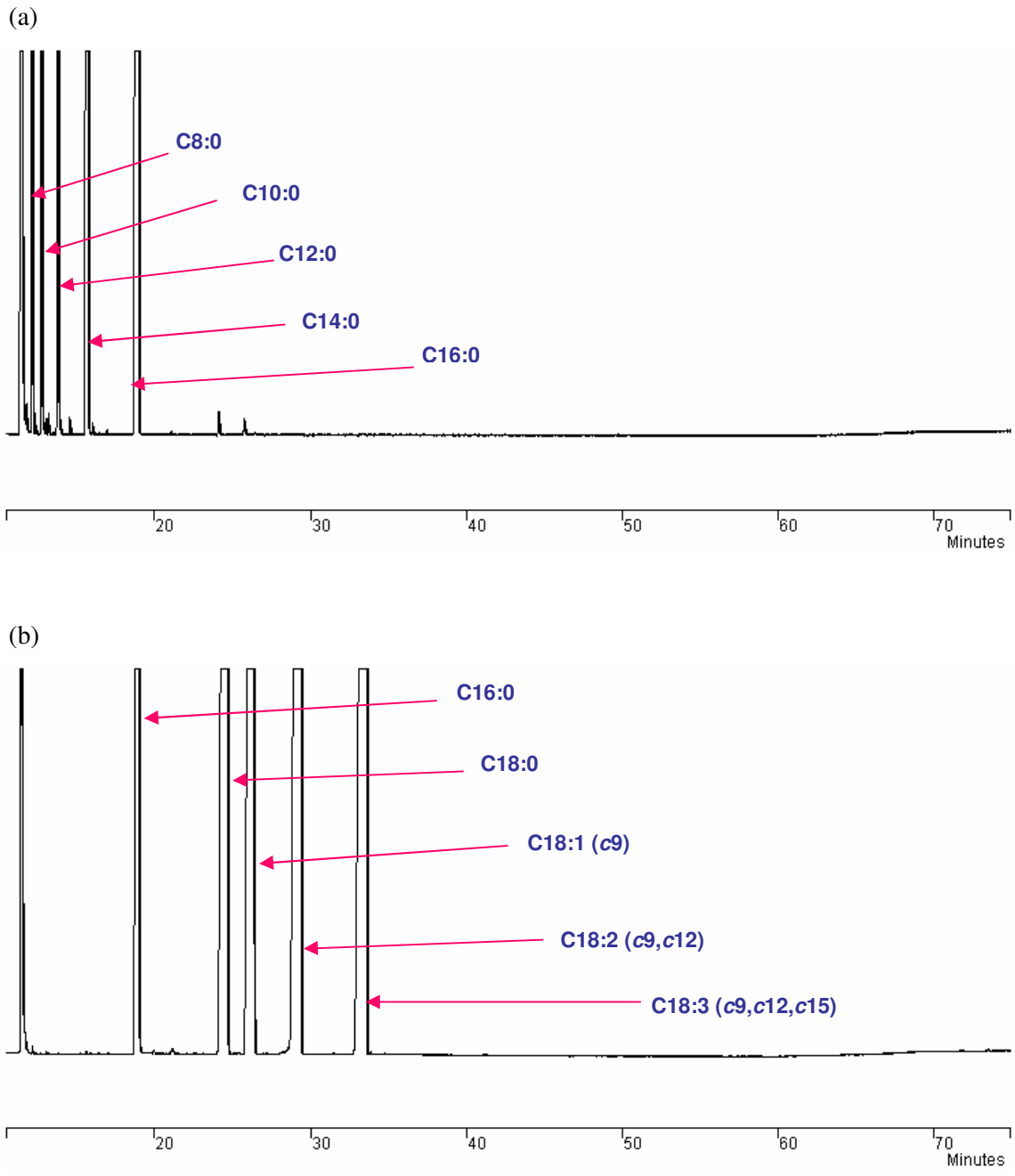


Figure 5.3.2.5.1. Sample chromatograms of (a) 5 FAME mix C8:0, C10:0, C12:0, C14:0 and C16:0, and (b) 5 FAME mix C16:0, C18:0, C18:1 (cis 9), C18:2 (cis 9, cis 12) and C18:3 (cis 9, cis 12, cis 15) analysed using a 100 m CP-Select CB capillary column. The solvent hexane is the first peak identified in each case eluting at approximately 11.37 min.

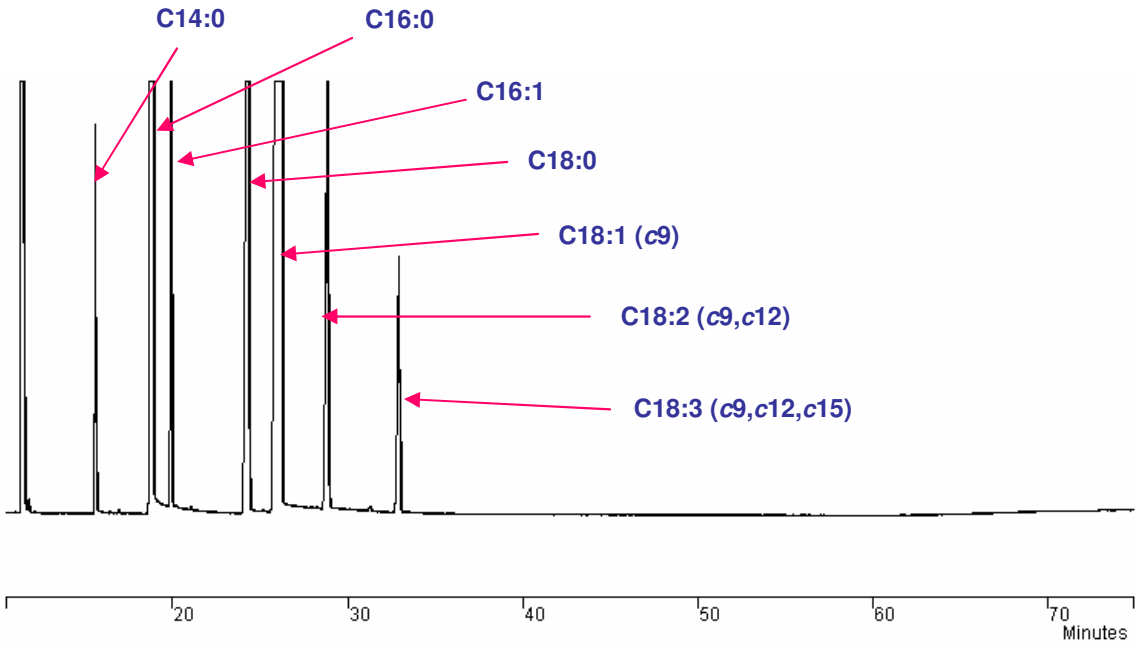
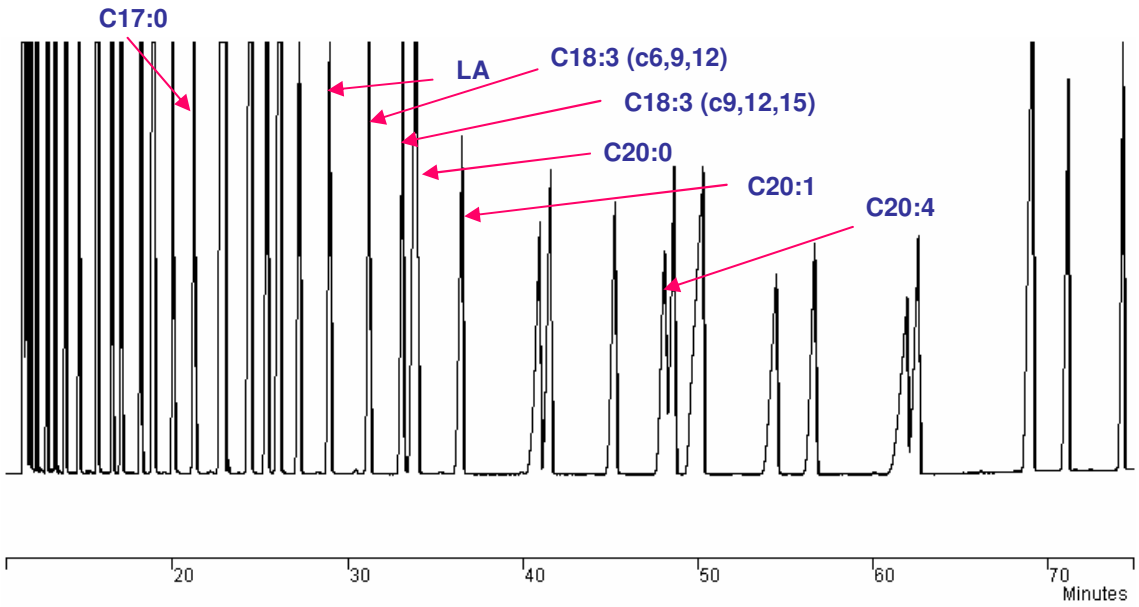


Figure 5.3.2.5.2. Sample chromatogram of a 7 FAME mix comprising C14:0, C16:0, C16:1, C18:0, C18:1 (cis 9), C18:2 (cis 9, cis 12) and C18:3 (cis 9, cis 12, cis 15) analysed using a 100 m CP-Select CB capillary column.

(a)



(b)

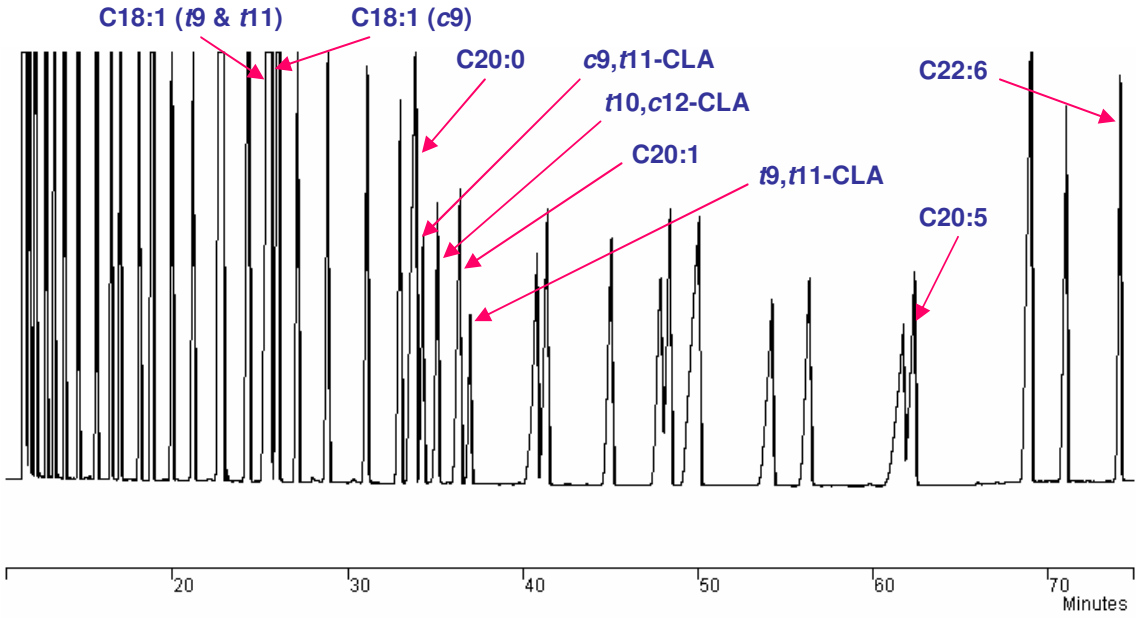


Figure 5.3.2.5.3. GC chromatogram of the methyl esters of (a) 37 fatty acid standard mixture and (b) 41 standard fatty acid mixture analysed using a 100 m CP-Select CB capillary column on a Varian CP 3800 GC.

Table 5.3.2.5.1. Notation, Chemical name, quantity present, retention time (RT), and peak area of a typical injection of the 41 standard fatty acid mixture analysed using a 100 m CP-Select CB capillary column on a Varian CP 3800 GC.

Notation	Chemical name	Amount mg/mL	RT Min	Peak Area
Solvent (Hexane)	Hexane		11.36	57484444
C4:0	Methyl butyrate	1.376	Unidentified	
C6:0	Methyl hexanoate	1.376	11.76	907432
C8:0	Methyl octanoate	1.376	12.11	1067428
C10:0	Methyl decanoate	1.376	12.71	1123277
C11:0	Methyl undecanoate	0.688	13.16	563010
C12:0	Methyl laurate	1.376	13.76	1174259
C13:0	Methyl tetradecanoate	0.688	14.54	586533
C14:0	Methyl myristate	1.376	15.58	1182762
C14:1	Methyl myristoleate	0.688	16.42	586266
C15:0	Methyl pentadecanoate	0.688	16.94	591187
C15:1	Methyl pentadecenoate	0.688	18.04	583906
C16:0	Methyl palmitate	2.064	18.80	1812848
C16:1	Methyl palmitoleate	0.688	19.89	587527
C17:0	**Methyl heptadecanoate**	**9.988**	21.11	**3767253**
C17:1	Methyl heptadecenoate	0.688	22.79	559917
C18:0	Methyl stearate	1.376	24.30	1213745
C18:1 (*t*9, *t*11 & *c*9)	Methyl elaidate, vaccinate (t-VA) & oleate	2.406	25.51	3503790
C18:2 (*t*9,*t*12)	Methyl linoelaidate	0.688	27.06	598750
C18:2 (*c*9,12)	Methyl linoleate (LA)	0.688	28.78	613373
C18:3 (*c*6,9,12)	Methyl gamma-linoleate (GLA)	0.688	31.01	588919
C18:3 (*c*9,12,15)	Methyl linolenate (ALA)	0.688	32.89	590350
C20:0	Methyl arachidate	1.376	33.79	1235250
C18:2 (*c* 9,*t*11)	Methyl *c*9,*t*11-CLA	1.03	34.19	365660
C18:2 (*t*10,*c*12)	**Methyl *t*10,*c*12-CLA**	**1.03**	35.02	**423112**
C20:1 (*c*11)	Methyl eicosanoate	0.688	36.30	603029
C18:2 (*t*9,*t*11)	Methyl *t*9,*t*11-CLA	0.52	36.88	255409
C21:0	Methyl heneicosanoate	0.688	40.70	607104
C20:2 (*c*11,14)	Methyl eicosadienoate	0.688	41.29	604843
C20:3 (*c*8,11,14)	Methyl dihomo-γ-linoleate (DGLA)	0.688	44.94	586727
C20:4 (*c*5,8,11,14)	Methyl arachidonate (AA)	0.688	47.74	598817
C20:3 (*c*11,14,17)	Methyl eicosatrienoate	0.688	48.28	624319
C22:0	Methyl behenate	1.376	49.95	1194218
C22:1 (*c*13)	Methyl erucate	0.688	54.08	609763
C23:0	Methyl tricosanoate	0.688	56.20	574299
C22:2 (*c*13,16)	Methyl docosadienoate	0.688	61.58	630700
C20:5 (*c*5,8,11,14,17)	Methyl eicosapentanoic acid (EPA)	0.688	62.22	569050
C24:0	Methyl lignocerate	1.376	68.98	1270807
C24:1 (*c*15)	Methyl nervonate	0.688	70.96	636075
C22:6 (*c*4,7,10,13,16,19)	Methyl docosahexanoic acid (DHA)	0.688	74.04	577297

5.3.2.6 Quantification of Cellular Lipid FAME

The first step in calculating quantities of fatty acids in a sample is to correct for variations in sample and injection volume and other errors which may occur during extraction and methylation. An internal standard at known concentrations in both sample and standard, in this case heptadecanoic acid or C17:0 is used to correct for these variations. A standard mixture of 41 fatty acids was run with each experiment and the values obtained were used to calculate levels of individual fatty acids in all samples within that experiment. Table 1 is an example of a typical standard injection. Using the following equation the corrected peak areas were determined for each fatty acid identified in a sample.

$$Ac_x = A_x \times \frac{A_{C17:0}\ \text{Standard}}{A_{C17:0}\ \text{Sample}} \times \frac{[\text{C17:0 Sample}]}{[\text{C17:0 Standard}]}$$

A_x denotes the area of the peak for the fatty acid identified in the chromatogram for the sample. Ac_x denotes the corrected peak area for the fatty acid in question. $A_{C17:0}$ standard and $A_{C17:0}$ sample denote the internal standard peak areas in the standard mixture and the sample, respectively. [C17:0 standard] and [C17:0 sample] denote the internal standard concentration in the standard solution and the sample respectively.

From these corrected peak areas (Ac_x) the concentration in mg/mL of specific fatty acid or [x] present in the cellular lipids of each sample can be calculated using the following equation.

$$[x] = Ac_x \times \frac{[x\ \text{Standard}]}{A_x\ \text{Standard}}$$

A_x Standard and [x Standard] denote the peak area and concentration of the standard of the particular fatty acid in question, x (Table 5.3.2.5.1).

5.3.2.7 An example of how [*t*10,*c*12-CLA] in a lipid extract may be quantified

Concentrations and peak area data from a typical injection of the standard mixture are presented in Table 5.3.2.5.1, including the internal standard C17:0 and an example of the *t*10,*c*12-CLA standard in bold. From this table the concentration and peak area of the internal standard C17:0 in the standard mixture were 9.988 mg/mL and 3767253 peak area units respectively. Corresponding values for *t*10,*c*12-CLA in the standard were 1.03 mg/mL and 423112 peak area units. Following analysis of a sample of MDA cells treated with the CLA mixture of isomers at 21 µg/mL for three days, the peak area for *t*10,*c*12-CLA was found to be 1848 peak area units. Corresponding values for C17:0 in the sample were 5 mg/mL and 976855 peak area units.

Using the first equation:

$$Ac_{t10,c12\text{-}CLA} = 1848 \times \frac{3767253}{976855} \times \frac{5 \text{ mg/mL}}{9.988 \text{ mg/mL}}$$

$$Ac_{t10,c12\text{-}CLA} = 3571.0$$

Using the second equation:

$$[t10,c12\text{-}CLA] = 3571.0 \times \frac{1.03 \text{ mg/mL}}{423112}$$

$$[t10,c12\text{-}CLA] = 0.0087 \text{ mg/mL}$$

$$= 8.7 \text{ µg/mL}$$

5.3.2.8 Validation of GC methods

To evaluate the percentage recovery of fatty acids, 21 µg of *t*10,*c*12-CLA was spiked into $2x10^6$ MDA cells with at least three replicates per experiment. Lipids were then extracted from these samples, methylated and analysed by GC as listed above (Figure 5.3.2.8 illustrates a typical chromatogram). Individual lipids were then quantified using the above equations. This calculation results in a concentration in mg/mL, therefore the value obtained is multiplied by the sample volume of 0.1 mL to give the total amount in mg present in the sample. Recovery was then calculated and was expressed as a percentage of the spiked amount, in this case 21 µg. Repeatability was assessed by calculating the above recoveries of spikes of 21 µg of *t*10,*c*12-CLA in $2x10^6$ MDA cells on four separate occasions.

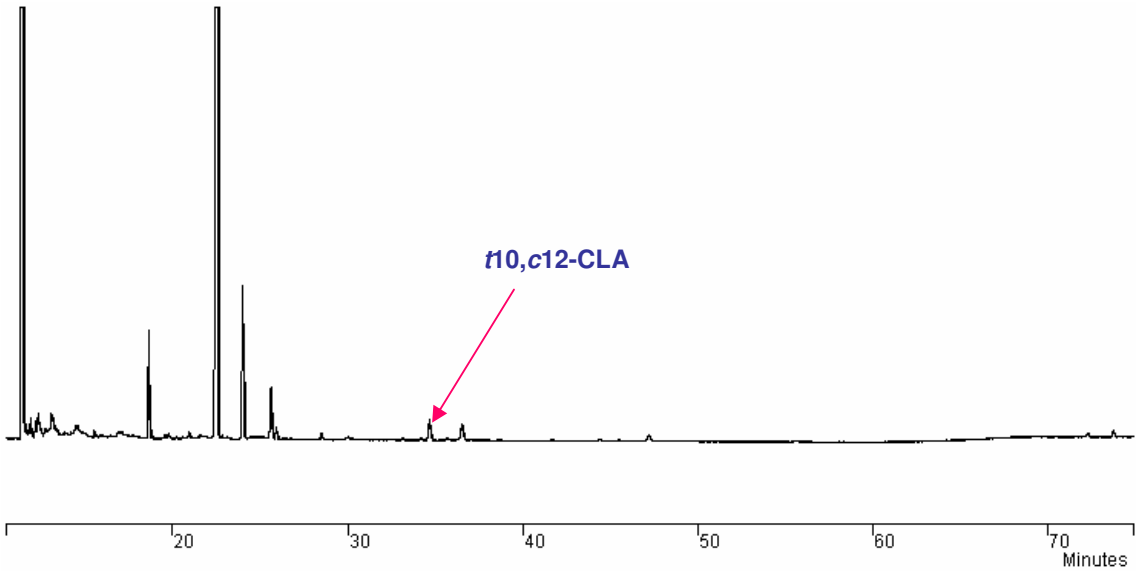


Figure 5.3.2.8. GC chromatogram of 21 µg of *t*10,*c*12-CLA spiked into $2x10^6$ MDA cells analysed using a 100 m CP-Select CB capillary column on a Varian CP 3800 GC.

5.3.3 Statistical Analysis

Differences between means were analysed for significance using the Student's two-tailed, paired *t*-test, unless otherwise stated.

5.4 Results

5.4.1 Validation of GC methods

To evaluate the percentage recovery of fatty acids, 21 µg of *t*10,*c*12-CLA was spiked into $2x10^6$ MDA cells with at least three replicates per experiment. Recovery was then calculated and expressed as a percentage of the spiked amount, in this case 21 µg. Table 5.4.1.1 lists values obtained over 4 days with at least three replicates per experiment. Intra-assay variation ranged from 4.9 to 41.2 % CV, while the amount recovered ranged from 19.7 to 23.1 µg corresponding to percentage recovery of 93.6 to 110.0 % over the 4 days.

Table 5.4.1.1. Intra-assay variation of recovered *t*10,*c*12-CLA spiked into MDA cells

	***t*10,*c*12-CLA Recovered (µg)**					**Mean ± SD**	**% CV**	**% Recovery**
Day 1	27.5	29.3	12.4	15.0	14.0	19.7 ± 8.1	41.2	93.6
Day 2	20.7	19.3	21.3			20.4 ± 1.0	4.9	97.3
Day 3	22.7	22.6	16.5			20.6 ± 3.5	17.1	98.1
Day 4	23.9	16.0	29.4			23.1 ± 6.7	29.0	110.0

Repeatability was assessed by calculating the above recoveries of spikes of 21 µg of *t*10,*c*12-CLA in $2x10^6$ MDA cells on four separate occasions, listed in table 5.4.1.2. Average recovery was 20.9 ± 1.5 µg of *t*10,*c*12-CLA, equating to an average percentage recovery of 99.7 %. Inter-assay variation was calculated to be 7.1 % CV.

Table 5.4.1.2. Inter-assay variation of recovered *t*10,*c*12-CLA over 4 days

*t*10,*c*12-CLA	1	2	3	4	Mean ± SD	% CV	% Recovery
µg	19.7	20.4	20.6	23.1	20.9	7.1	99.7
± SD	± 8.1	± 1.0	± 3.5	± 6.7	± 1.5		

5.4.2 Fatty acid profiles of untreated MDA and A10p10p cells

The fatty acid profile of untreated drug sensitive MDA cells was analysed as described earlier and compared to that of the drug resistant A10p10p cell line. Figure 5.4.2.1 illustrates typical chromatograms of the profiles of both cell lines. Table 5.4.2.1 lists the fatty acid composition of untreated MDA and A10p10p cells, while table 5.4.2.2 lists the summary of fatty acid composition and various fatty acid ratios of both cell lines. The most abundant fatty acid in both cell lines was the long chain saturated fatty acid (LCSFA), C18:0 stearic acid at 34.03 ± 0.93 % of total cellular FAME in MDA cells and a similar level in A10p10p cells at 34.98 ± 4.12 % of total. LCSFA were taken to be any saturated fatty acid (SFA) above a chain length of fourteen carbons.

Another predominant LCSFA, C16:0 methyl palmitate, was more abundant in MDA cells at 23.13 ± 0.26 % total lipid compared to 19.65 ± 1.92 % in A10p10p cells although this difference was not statistically significant. This apparent difference in methyl palmitate accounts for the overall raised level of LCSFA in the MDA cell line, with 61.80 ± 1.14 % in MDA cells and 58.25 ± 5.36 % of total FAME in A10p10p cells. However, total SFA were significantly higher in A10p10p cells than MDA with 73.27 ± 1.27 % FAME in MDA and 76.91 ± 1.91 % in A10p10p suggesting an up-regulation of the FAS enzyme followed by β-oxidation to produce short chain SFA (Table 5.4.2.2).

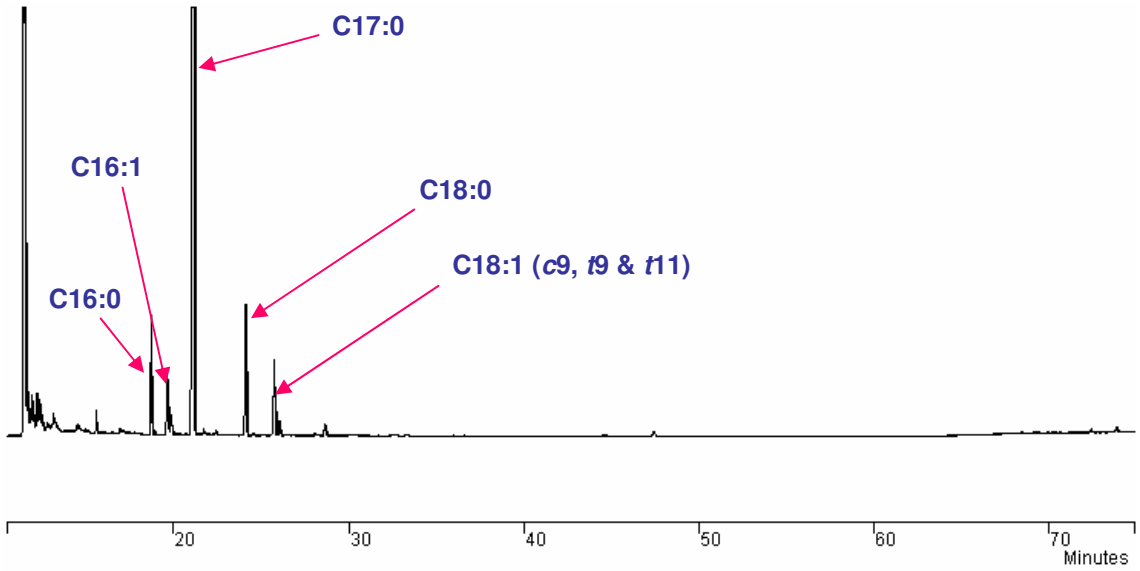
Other LCSFA were present in minor amounts in both cell lines including C14:0 methyl myristate, which was present at a significantly higher level in MDA (3.62 ± 0.13 % FAME) than A10p10p (2.19 ± 0.44 % FAME) (Table 5.4.2.1).

The predominant mono-unsaturated fatty acid (MUFA) in MDA cells was the group of three C18:1 fatty acids; elaidic (*t*-9), vaccenic (*t*-11), and oleic acid (*c*-9), which were indistinguishable from each other and eluted together at approximately 25.51 min in every run. Their combined proportion of total cellular lipids was 12.74 ± 0.56 % in MDA cells, whereas this decreased significantly to 7.60 ± 1.37 % in the resistant A10p10p cells. The predominant mono-unsaturated fatty acid (MUFA) in A10p10p cells was C16:1 methyl palmitoleate (palmitoleic acid) at 8.98 ± 2.01 % FAME. The corresponding proportion in MDA cells was 6.46 ± 1.31 % total lipid (Table 5.4.2.1).

Other UFA were present in both cell lines in minor amounts. C18:2 (*c*9,*c*12) linoleic acid (LA) comprised 3.08 ± 0.19 % FAME in MDA cells and 1.97 ± 0.62 % in A10p10p. C22:6 docosahexanoic acid (DHA) was significantly more abundant in MDA cells (1.29 ± 0.12 % FAME) than A10p10p cells (0.92 ± 0.19 % FAME). By contrast C18:3 α-linolenic acid (ALA), a precursor of DHA, was present in lower amounts in MDA control cells at 0.28 ± 0.12 % FAME compared to A10p10p cells at 0.63 ± 0.05 % FAME, possibly due to down-regulation in Δ5/Δ6 desaturase and/or elongase activities in A10p10p cells (Table 5.4.2.1).

The reduced level in the group of C18:1 MUFA contributed largely to an apparent decrease in overall MUFA from 19.40 ± 0.95 % in MDA to 17.30 ± 1.66 % of total lipid in A10p10p cells and in percentage total unsaturated fatty acids (UFA) from 26.73 ± 1.27 % in MDA cells to 23.09 ± 1.91 % in A10p10p ($p<0.05$) (Table 5.4.2.2).

(a)



(b)

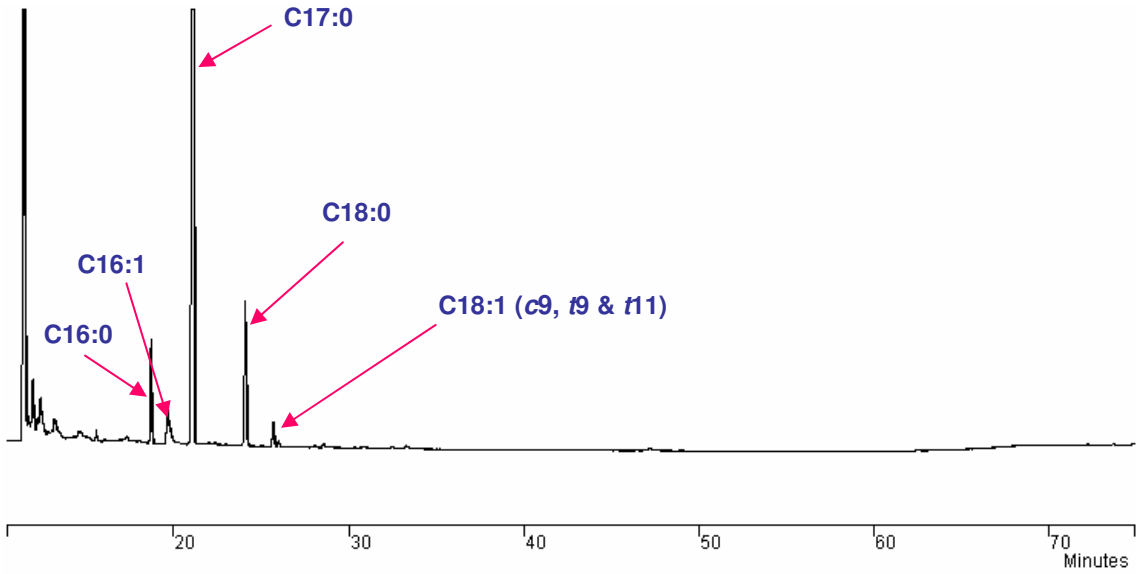


Figure 5.4.2.1. Typical chromatograms of fatty acid profiles of untreated (a) MDA and (b) A10p10p cells.

These apparent differences in the levels of LCSFA, MUFA and UFA in the MDA cell line compared to the A10p10p cell line cancelled each other out when calculated as ratios of UFA/LCSFA with similar values obtained in both cell lines, 0.43 $\pm$ 0.02 in MDA and 0.40 $\pm$ 0.02 in A10p10p cells. Corresponding values for the MUFA/LCSFA ratio were 0.31 $\pm$ 0.01 and 0.30 $\pm$ 0.02 for the MDA and A10p10p cell line respectively. However, there was a significant decrease in the unsaturation index of A10p10p compared to MDA cells when total UFA/SFA were calculated, yielding 0.37 $\pm$ 0.03 in MDA cells and 0.30 $\pm$ 0.03 in A10p10p (Table 5.4.2.2).

The ratio of C16:1/C16:0 apparently increased from 0.28 $\pm$ 0.06 in MDA cells to 0.48 $\pm$ 0.13 in A10p10p cells and while this difference did not reach statistical significance it points to trends in the data. However in the ratio of C18:1/C18:0 the decrease from 0.38 $\pm$ 0.03 in MDA to 0.22 $\pm$ 0.04 in A10p10p cells was deemed statistically significant. Since these ratios are thought to be governed by the same Δ9-desaturase enzyme, which introduces one double bond to the saturated fatty acid, they highlight opposing trends in activity of the enzyme (Table 5.4.2.2).

Table 5.4.2.1. Fatty acid composition of untreated drug sensitive MDA and drug resistant A10p10p cells.

Fatty Acid	MDA	A10p10p
C6:0	0.38 ± 0.20	0.38 ± 0.03
C8:0	3.83 ± 1.47	8.60 ± 4.71
C10:0	2.76 ± 0.27	1.61 ± 1.18
C11:0	3.25 ± 0.36	5.43 ± 1.60
C12:0	0.09 ± 0.06	0.09 ± 0.08
C13:0	1.16 ± 0.10	2.55 ± 0.95
C14:0	3.62 ± 0.13	2.19 ± 0.44*
C14:1	0.18 ± 0.09	0.38 ± 0.29
C15:0	0.50 ± 0.19	0.26 ± 0.08
C15:1	0.02 ± 0.02	0.06 ± 0.03
C16:0	23.13 ± 0.26	19.65 ± 1.92
C16:1	6.46 ± 1.31	8.98 ± 2.01
C18:0	34.03 ± 0.93	34.98 ± 4.12
C18:1 (*t*-9, *t*-11 & *c*-9)	12.74 ± 0.56	7.60 ± 1.37*
C18:2 LA	3.08 ± 0.19	1.97 ± 0.62
C18:3 GLA	0.00 ± 0.00	0.10 ± 0.10
C18:3 ALA	0.28 ± 0.12	0.63 ± 0.05
C20:0	0.25 ± 0.14	0.91 ± 0.19
C18:2 *c*9,*t*11-CLA	0.14 ± 0.14	0.03 ± 0.03
C18:2 *t*10,*c*12-CLA	0.05 ± 0.05	0.02 ± 0.01
C20:1 (*c*11)	0.10 ± 0.10	0.02 ± 0.02
C18:2 (*t*9,*t*11-CLA)	0.14 ± 0.14	0.10 ± 0.05
C20:2 (*c*11,14)	0.00 ± 0.00	0.13 ± 0.09
C20:3 DGLA	0.32 ± 0.16	0.16 ± 0.02
C20:4 AA	1.93 ± 0.23	1.54 ± 0.24
C22:1 (*c*13)	0.00 ± 0.00	0.10 ± 0.10
C23:0	0.27 ± 0.14	0.08 ± 0.04
C20:5 EPA	0.00 ± 0.00	0.18 ± 0.18
C24:0	0.00 ± 0.00	0.19 ± 0.19
C24:1 (*c*15)	0.00 ± 0.00	0.18 ± 0.16
C22:6 DHA	1.29 ± 0.12	0.92 ± 0.19*

Data represent Mean ± SEM of at least three experiments in triplicate expressed as a percentage of total cellular lipid composition. *Denotes results which are significantly different to MDA cells as determined by the Student's *t*-test ($p<0.05$)

Table 5.4.2.2. Fatty acid composition and ratios of untreated drug sensitive MDA and drug resistant A10p10p cells.

Fatty Acids	MDA	A10p10p
SFA	73.27 ± 1.27	76.91 ± 1.91*
LCSFA	61.80 ± 1.14	58.25 ± 5.36
UFA	26.73 ± 1.27	23.09 ± 1.91*
MUFA	19.40 ± 0.95	17.30 ± 1.66
UFA/SFA	0.37 ± 0.02	0.30 ± 0.03*
UFA/LCSFA	0.43 ± 0.02	0.40 ± 0.02
MUFA/LCSFA	0.31 ± 0.01	0.30 ± 0.02
C16:1/C16:0	0.28 ± 0.06	0.48 ± 0.13
C18:1(*t*9, *t*11 & *c*9)/C18:0	0.38 ± 0.03	0.22 ± 0.04*

Data represent Mean ± SEM of at least three experiments in triplicate expressed as a percentage of total cellular lipid composition. *Denotes results which are significantly different to MDA cells as determined by the Student's *t*-test ($p<0.05$).

5.4.3 Fatty acid profiles of MDA cells following treatments

5.4.3.1 Effects of CLA mixture on Fatty acid profiles of MDA cells

Flasks of $2x10^6$ MDA cells were seeded for 24 h before treatment with 21 and 75 µM CLA mixture, incubated for 3 days and extracted and analysed as described earlier. Figure 5.4.3.1 illustrates a chromatogram from a typical injection of an MDA sample treated with (a) 21 and (b) 75 µM CLA mixture of isomers (CLA-mix). Table 6 lists the fatty acid composition of untreated MDA control and MDA cells treated with 21 and 75 µM CLA-mix, while table 5.4.3.1.2 lists the summary of fatty acid composition and various fatty acid ratios of the above. The most abundant fatty acid in each treatment was the long chain saturated fatty acid (LCSFA) C18:0 stearic acid, at similar levels with each treatment, comprising 31.39 ± 1.93 % total cellular FAME in cells treated with 21 µM CLA-mix and 28.19 ± 1.33 % FAME following 75 µM

CLA-mix, compared to 34.03 ± 0.93 % in control MDA cells. Another predominant LCSFA C16:0 was apparently reduced from 23.13 ± 0.26 % FAME in control MDA cells to 21.40 ± 0.81 and 14.02 ± 4.10 % FAME following treatment with 21 and 75 µM CLA-mix respectively, although these differences were not deemed statistically significant. The decrease in both C16:0 and C18:0 suggests inhibition of FAS by the CLA-mix. Other LCSFA were present in minor amounts including C14:0 methyl myristate, which decreased significantly with 21 and 75 µM CLA treatments relative to control from 3.62 ± 0.13 % FAME to 3.39 ± 0.11 and 2.46 ± 0.15 % of total FAME respectively (Table 5.4.3.1.1).

The predominant MUFA was the group of three C18:1 fatty acids elaidic (*t*-9), vaccenic (*t*-11), and oleic acid (*c*-9). Their combined proportion of total cellular lipids of 12.74 ± 0.56 % in control MDA cells decreased to 11.31 ± 0.31 and 6.48 ± 0.70 % FAME following 21 and 75 µM CLA-mix treatment respectively although the difference was only significant following 75 µM CLA-mix treatment (Table 5.4.3.1.1). This decrease is possibly due to inhibition of Δ9-desaturase, which introduces a double bond into C18:0 stearic acid, producing C18:1 oleic acid.

There was a trend towards an increase in the mono-unsaturated fatty acid (MUFA) C16:1 methyl palmitoleate (palmitoleic acid) from 6.46 ± 1.31 % total lipid in control MDA cells to 6.92 ± 2.47 and 9.78 ± 5.66 % FAME following 21 and 75 µM CLA-mix treatment respectively. Other UFA were present in minor amounts. C18:2 (*c*9,*c*12) linoleic acid (LA) comprised 3.08 ± 0.19 % FAME in control MDA cells and 1.92 ± 0.96 and 1.25 ± 0.63 % FAME following 21 and 75 µM CLA-mix treatment respectively. C22:6 docosahexanoic acid (DHA) comprised 1.29 ± 0.12 % FAME in control MDA cells and 0.84 ± 0.42 and 0.57 ± 0.32 % FAME following 21 and 75 µM CLA-mix treatment. There was an apparent drop in C20:4 arachidonic acid (AA) levels from 1.93 ± 0.23 % FAME in MDA cells to 1.60 ± 0.60 and 0.83 ± 0.39 % FAME with 21 and 75 µM CLA-mix, suggesting inhibition of Δ5/Δ6 desaturase and/or elongase activities (Table 5.4.3.1.1).

The level of the *c*9,*t*11-CLA isomer was apparently increased from 0.14 ± 0.14 % FAME in control MDA cells to 0.72 ± 0.26 and 3.41 ± 2.04 % FAME following 21

and 75 μM CLA-mix treatment. Corresponding levels for the *t*10,*c*12-CLA isomer were 0.05 ± 0.05 % FAME in control MDA cells and 0.95 ± 0.56 and 6.08 ± 2.59 % FAME following 21 and 75 μM CLA-mix treatment. However, none of these differences were found to be statistically significant (Table 5.4.3.1.1).

The apparent reduction in C18:0 stearic acid and C16:0 methyl palmitate levels and significant reduction C14:0 methyl myristate levels following 21 and 75 μM CLA-mix treatment resulted in an apparent overall decreased level of LCSFA from 61.80 ± 1.14 % FAME in control MDA cells to 57.24 ± 2.43 % FAME following 21 μM CLA-mix treatment and a significant decrease to 45.93 ± 3.62 % FAME following 75 μM CLA-mix treatment. This suggests inhibition of FAS activity (Table 5.4.3.1.2).

The overall UFA level, at 26.73 ± 1.27 % FAME in control MDA cells was 28.30 ± 2.47 and 32.86 ± 7.52 % FAME following 21 and 75 μM CLA-mix treatment. Corresponding MUFA levels were 19.40 ± 0.95 % FAME in control MDA cells and 18.48 ± 2.50 and 16.52 ± 5.50 % FAME following 21 and 75 μM CLA-mix treatment. The apparent differences in the levels of LCSFA, MUFA and UFA in the lipid composition of control MDA cells compared to cells following treatment also resulted in a significant increase in the ratio of UFA/LCSFA from 0.43 ± 0.02 in control MDA cells to 0.49 ± 0.03 in cells following treatment with 21 μM CLA-mix and an apparent but not significant increase to 0.72 ± 0.16 with 75 μM CLA-mix treatment. Corresponding values for the MUFA/LCSFA ratio were 0.31 ± 0.01 in control MDA cells and 0.32 ± 0.03 and 0.36 ± 0.12 in cells following treatment with 21 and 75 μM CLA-mix respectively (Table 5.4.3.1.2).

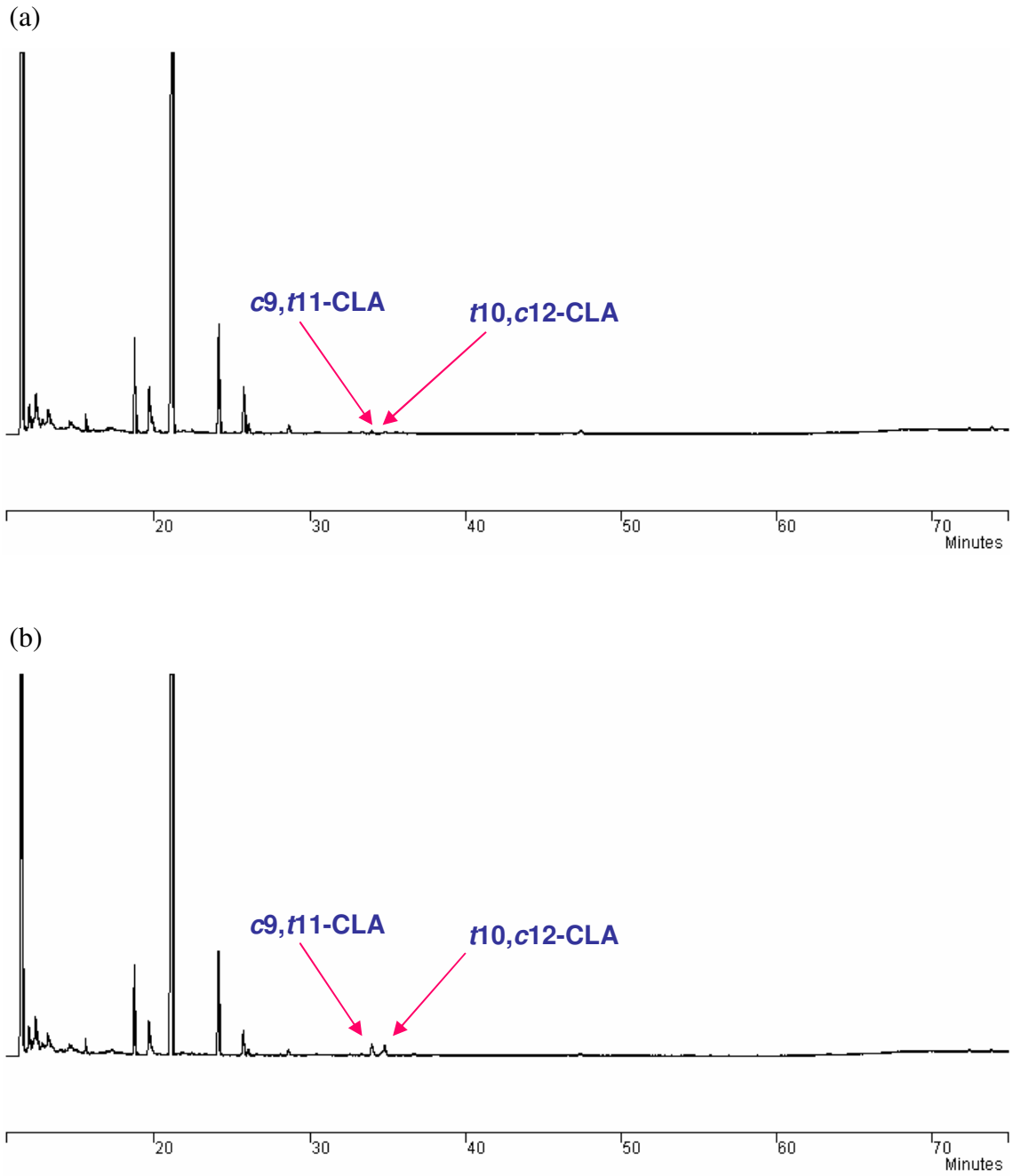


Figure 5.4.3.1. Typical chromatograms of fatty acid profiles of MDA cells treated with (a) 21 and (b) 75 µM CLA mixture.

Table 5.4.3.1.1. Fatty acid composition of MDA cells treated with CLA mix for 72 h.

Fatty Acid	Control	CLA 21 μM	CLA 75 μM
C6:0	0.38 ± 0.20	0.39 ± 0.20	0.55 ± 0.55
C8:0	3.83 ± 1.47	4.70 ± 2.28	1.55 ± 0.84
C10:0	2.76 ± 0.27	2.38 ± 0.51	3.04 ± 0.80
C11:0	3.25 ± 0.36	4.83 ± 1.63	8.46 ± 5.26
C12:0	0.09 ± 0.06	0.03 ± 0.03	0.29 ± 0.19
C13:0	1.16 ± 0.10	1.39 ± 0.19	4.88 ± 3.14
C14:0	3.62 ± 0.13	3.39 ± 0.11*	2.46 ± 0.15*
C14:1	0.18 ± 0.09	0.25 ± 0.25	0.07 ± 0.07
C15:0	0.50 ± 0.19	0.47 ± 0.14	0.91 ± 0.74
C15:1	0.02 ± 0.02	0.00 ± 0.00	0.14 ± 0.14
C16:0	23.13 ± 0.26	21.40 ± 0.81	14.02 ± 4.10
C16:1	6.46 ± 1.31	6.92 ± 2.47	9.78 ± 5.66
C18:0	34.03 ± 0.93	31.39 ± 1.93	28.19 ± 1.33
C18:1 (*t*-9, *t*-11 & *c*-9)	12.74 ± 0.56	11.31 ± 0.31	6.48 ± 0.70*
C18:2 (*c*9,12), LA	3.08 ± 0.19	1.92 ± 0.96	1.25 ± 0.63
C18:3 (*c*6,9,12), GLA	0.00 ± 0.00	0.88 ± 0.82	0.56 ± 0.50
C18:3 (*c*9,12,15)	0.28 ± 0.12	0.29 ± 0.23	0.67 ± 0.62
C20:0	0.25 ± 0.14	0.32 ± 0.04	0.25 ± 0.03
C18:2 *c*9,*t*11-CLA	0.14 ± 0.14	0.72 ± 0.26	3.41 ± 2.04
C18:2 *t*10,*c*12-CLA	0.05 ± 0.05	0.95 ± 0.56	6.08 ± 2.59
C20:1 (*c*11)	0.10 ± 0.10	0.41 ± 0.30	1.31 ± 0.79
C18:2 (*t*9,*t*11-CLA)	0.14 ± 0.14	0.31 ± 0.05	0.52 ± 0.30
C20:2 (*c*11,14)	0.00 ± 0.00	0.06 ± 0.06	0.04 ± 0.04
C20:3 DGLA	0.32 ± 0.16	0.32 ± 0.16	0.18 ± 0.09
C20:4 AA	1.93 ± 0.23	1.60 ± 0.60	0.83 ± 0.39
C22:1(*c*13)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
C23:0	0.27 ± 0.14	0.27 ± 0.14	0.10 ± 0.06
C22:2 (*c*13,16)	0.00 ± 0.00	0.06 ± 0.06	0.04 ± 0.04
C20:5 EPA	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
C24:0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
C24:1(*c*15)	0.00 ± 0.00	0.00 ± 0.00	0.05 ± 0.05
C22:6 DHA	1.29 ± 0.12	0.84 ± 0.42	0.57 ± 0.32

Data represent Mean ± SEM of at least three experiments in triplicate expressed as a percentage of total cellular lipid composition. *Denotes results which are significantly different to untreated/control cells as determined by the Student's *t*-test ($p<0.05$).

The ratio of C18:1/C18:0 decreased from 0.38 ± 0.03 in control MDA cells to 0.36 ± 0.03 in cells following treatment with 21 µM CLA-mix and decreased significantly to 0.23 ± 0.03 with 75 µM CLA-mix treatment, suggesting inhibition of oleic acid production by Δ9-desaturase in CLA-mix treated cells. However, the ratio of C16:1/C16:0 apparently increased from 0.28 ± 0.06 in control MDA cells to 0.32 ± 0.11 and 0.65 ± 0.30 in cells treated with 21 and 75 µM CLA-mix respectively, indicating that more complex interactions with the enzyme may be at play (Table 5.4.3.1.2).

Table 5.4.3.1.2. Fatty acid composition and ratios of MDA cells treated with CLA mix for 72 h.

Fatty Acids	Control	CLA mix 21 µM	CLA mix 75 µM
LCSFA	61.80 ± 1.14	57.24 ± 2.43	45.93 ± 3.62*
UFA	26.73 ± 1.27	28.30 ± 2.47	32.86 ± 7.52
MUFA	19.40 ± 0.95	18.48 ± 2.50	16.52 ± 5.50
UFA/LCSFA	0.43 ± 0.02	0.49 ± 0.03*	0.72 ± 0.16
MUFA/LCSFA	0.31 ± 0.01	0.32 ± 0.03	0.36 ± 0.12
C16:1/C16:0	0.28 ± 0.06	0.32 ± 0.11	0.65 ± 0.30
C18:1 (*t*9, *t*11 & *c*9)/C18:0	0.38 ± 0.03	0.36 ± 0.03	0.23 ± 0.03*

Data represent Mean ± SEM of at least three experiments in triplicate expressed as a percentage of total cellular lipid composition. *Denotes results which are significantly different to untreated/control cells as determined by the Student's *t*-test ($p<0.05$).

5.4.3.2 Effects of *c*9,*t*11-CLA treatments on Fatty acid profiles of MDA cells

Flasks of $2x10^6$ MDA cells were seeded for 24 h before treatment with the *c*9,*t*11-CLA isomer at 21 and 75 µM, incubated for 3 days and extracted and analysed as described earlier. Figure 5.4.3.2 illustrates a chromatogram from a typical injection of an MDA sample treated with (a) 21 and (b) 75 µM *c*9,*t*11-CLA.

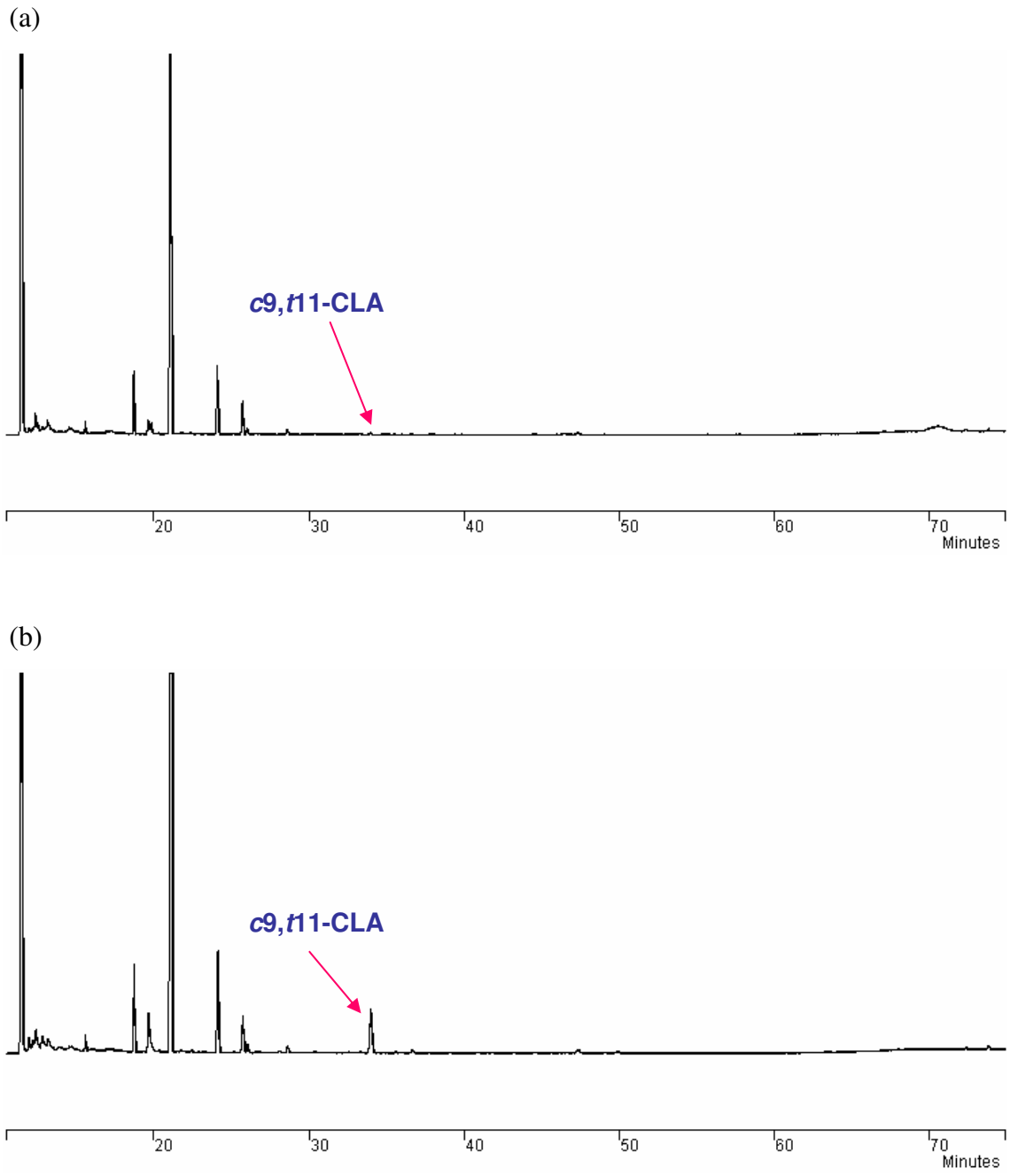


Figure 5.4.3.2. Typical chromatograms of fatty acid profiles of MDA cells treated with (a) 21 and (b) 75 µM *c*9,*t*11-CLA.

Table 5.4.3.2.1 lists the fatty acid composition of untreated MDA control and MDA cells treated with 21 and 75 μM *c*9,*t*11-CLA, while table 5.4.3.2.2 lists the summary of fatty acid composition and various fatty acid ratios of the above. The most abundant fatty acid in each treatment was the long chain saturated fatty acid (LCSFA) C18:0 stearic acid. This was apparently reduced from 34.03 ± 0.93 % total cellular FAME in control MDA cells to 28.14 ± 3.82 % FAME in cells treated with 21 μM *c*9,*t*11-CLA and significantly reduced to 21.09 ± 3.34 % FAME following 75 μM *c*9,*t*11-CLA. A similar trend was observed in another predominant LCSFA, C16:0 methyl palmitate. This was apparently reduced from 23.13 ± 0.26 % FAME in control MDA cells to 16.66 ± 5.53 % FAME in cells treated with 21 μM *c*9,*t*11-CLA and significantly reduced to 15.12 ± 1.85 % FAME following treatment with 75 μM *c*9,*t*11-CLA. These decreases suggest inhibition of FAS by the *c*9,*t*11-CLA isomer. Other LCSFA present in minor amounts include C14:0 methyl myristate, which decreased relative to control MDA cells from 3.62 ± 0.13 % FAME to 3.07 ± 0.40 % FAME with treatment of 21 μM *c*9,*t*11-CLA and decreased significantly with 75 μM *c*9,*t*11-CLA to 2.28 ± 0.18 % of total FAME (Table 5.4.3.2.1).

The predominant MUFA was the group of three C18:1 fatty acids elaidic (*t*-9), vaccenic (*t*-11), and oleic acid (*c*-9). Their combined proportion of total cellular lipids of 12.74 ± 0.56 % in control MDA cells decreased to 10.87 ± 1.47 % FAME with treatment of 21 μM *c*9,*t*11-CLA and decreased significantly to 6.79 ± 0.67 % FAME following 75 μM *c*9,*t*11-CLA treatment, suggesting inhibition of Δ9-desaturase. There was a trend towards an increase in the mono-unsaturated fatty acid (MUFA) C16:1 methyl palmitoleate (palmitoleic acid) from 6.46 ± 1.31 % total lipid in control MDA cells to 11.24 ± 4.75 and 12.15 ± 5.15 % FAME following 21 and 75 μM *c*9,*t*11-CLA treatment respectively (Table 5.4.3.2.1).

Other UFA were present in minor amounts. C18:2 (*c*9,*c*12) linoleic acid (LA) comprised 3.08 ± 0.19 % FAME in control MDA cells and 1.37 ± 0.84 % FAME following 21 *c*9,*t*11-CLA treatment and was significantly reduced to 1.69 ± 0.08 % FAME following treatment with 75 μM *c*9,*t*11-CLA. C22:6 docosahexanoic acid (DHA) comprised 1.29 ± 0.12 % FAME in control MDA cells and 2.30 ± 1.08 and 0.87 ± 0.08 % FAME following 21 and 75 μM *c*9,*t*11-CLA treatment. There was an

apparent drop in C20:4 arachidonic acid (AA) levels from 1.93 ± 0.23 % FAME in MDA cells to 1.24 ± 0.63 and 1.08 ± 0.07 % FAME with 21 and 75 µM *c*9,*t*11-CLA, suggesting inhibition of Δ5/Δ6 desaturase and/or elongase activities (Table 5.4.3.2.1).

The level of the *c*9,*t*11-CLA isomer was apparently increased from 0.14 ± 0.14 % FAME in control MDA cells to 1.55 ± 0.87 % FAME following 21 µM *c*9,*t*11-CLA treatment and increased significantly to 29.44 ± 3.63 % FAME with 75 µM *c*9,*t*11-CLA. Corresponding levels for the *t*10,*c*12-CLA isomer remained at trace levels of 0.05 ± 0.05 % FAME in control MDA cells and 0.67 ± 0.54 and 0.10 ± 0.03 % FAME following 21 and 75 µM *c*9,*t*11-CLA treatment (Table 5.4.3.2.1).

The reduction in C18:0 methyl stearic acid, C16:0 methyl palmitate and C14:0 methyl myristate levels following 21 and 75 µM *c*9,*t*11-CLA treatment resulted in an overall decreased level of LCSFA from 61.80 ± 1.14 % FAME in control MDA cells to 48.93 ± 9.33 % FAME following 21 µM *c*9,*t*11-CLA treatment and a significant decrease to 39.53 ± 5.13 % FAME with 75 µM *c*9,*t*11-CLA (Table 9). The overall UFA level, at 26.73 ± 1.27 % FAME in control MDA cells, increased to 32.85 ± 2.12 % FAME following 21 µM *c*9,*t*11-CLA treatment and increased significantly to 54.20 ± 6.37 % FAME with 75 µM *c*9,*t*11-CLA. Corresponding MUFA levels were 19.40 ± 0.95 % FAME in control MDA cells and 22.27 ± 3.64 and 19.05 ± 4.85 % FAME following 21 and 75 µM *c*9,*t*11-CLA treatment (Table 5.4.3.2.2).

The differences in the levels of LCSFA, MUFA and UFA in the lipid composition of control MDA cells compared to cells following treatment resulted in an increase in the ratio of UFA/LCSFA from 0.43 ± 0.02 in control MDA cells to 0.76 ± 0.22 and 1.47 ± 0.39 following 21 and 75 µM *c*9,*t*11-CLA treatment respectively, although the differences were not deemed statistically significant. Corresponding MUFA/LCSFA levels were 0.31 ± 0.01 in control MDA cells and 0.53 ± 0.21 and 0.52 ± 0.18 following 21 and 75 µM *c*9,*t*11-CLA treatment (Table 5.4.3.2.2).

Table 5.4.3.2.1. Fatty acid composition of MDA cells treated with *c*9,*t*11-CLA for 72 h.

Fatty Acid	Control	*c*9,*t*11-CLA 21 μM	*c*9,*t*11-CLA 75 μM
C6:0	0.38 ± 0.20	1.19 ± 0.70	0.42 ± 0.42
C8:0	3.83 ± 1.47	2.27 ± 1.20	1.29 ± 0.67
C10:0	2.76 ± 0.27	2.10 ± 0.49	2.23 ± 0.74
C11:0	3.25 ± 0.36	8.22 ± 5.30	1.97 ± 0.12
C12:0	0.09 ± 0.06	0.08 ± 0.08	0.04 ± 0.04
C13:0	1.16 ± 0.10	3.93 ± 2.75	0.31 ± 0.15*
C14:0	3.62 ± 0.13	3.07 ± 0.40	2.28 ± 0.18*
C14:1	0.18 ± 0.09	0.01 ± 0.01	0.00 ± 0.00
C15:0	0.50 ± 0.19	0.55 ± 0.45	0.40 ± 0.22
C15:1	0.02 ± 0.02	0.12 ± 0.12	0.00 ± 0.00
C16:0	23.13 ± 0.26	16.66 ± 5.53	15.12 ± 1.85*
C16:1	6.46 ± 1.31	11.24 ± 4.75	12.15 ± 5.15
C18:0	34.03 ± 0.93	28.14 ± 3.82	21.09 ± 3.34*
C18:1 (*t*9, *t*11 & *c*9)	12.74 ± 0.56	10.87 ± 1.47	6.79 ± 0.67*
C18:2 LA	3.08 ± 0.19	1.37 ± 0.84	1.69 ± 0.08*
C18:3 GLA	0.00 ± 0.00	0.97 ± 0.97	0.00 ± 0.00
C18:3(*c*9,12,15)	0.28 ± 0.12	0.38 ± 0.29	0.09 ± 0.04
C20:0	0.25 ± 0.14	0.36 ± 0.07	0.22 ± 0.10
C18:2 *c*9,*t*11-CLA	0.14 ± 0.14	1.55 ± 0.87	29.44 ± 3.63*
C18:2 *t*10,*c*12-CLA	0.05 ± 0.05	0.67 ± 0.54	0.10 ± 0.03
C20:1 (*c*11)	0.10 ± 0.10	0.21 ± 0.14	0.78 ± 0.37
C18:2 *t*9,11-CLA	0.14 ± 0.14	0.19 ± 0.10	0.88 ± 0.88
C20:2 (*c*11,14)	0.00 ± 0.00	0.00 ± 0.00	0.03 ± 0.03
C20:3 DGLA	0.32 ± 0.16	0.28 ± 0.16	0.18 ± 0.10
C20:4 AA	1.93 ± 0.23	1.24 ± 0.63	1.08 ± 0.07
C22:1(*c*13)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
C23:0	0.27 ± 0.14	0.15 ± 0.12	0.12 ± 0.06
C22:2 (*c*13,16)	0.00 ± 0.00	0.09 ± 0.09	0.00 ± 0.00
C20:5 EPA	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
C24:0	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
C24:1(*c*15)	0.00 ± 0.00	0.03 ± 0.03	0.12 ± 0.10
C22:6 DHA	1.29 ± 0.12	2.30 ± 1.08	0.87 ± 0.08

Data represent Mean ± SEM of at least three experiments in triplicate expressed as a percentage of total cellular lipid composition. *Denotes results which are significantly different to untreated/control cells as determined by the Student's *t*-test ($p<0.05$).

The ratio of C16:1/C16:0 apparently increased from 0.28 ± 0.06 in control MDA cells to 1.40 ± 1.10 and 0.90 ± 0.43 in cells treated with 21 and 75 µM *c*9,*t*11-CLA treatment. However in the ratio of C18:1/C18:0 remained relatively similar at 0.38 ± 0.03 in control MDA cells to 0.39 ± 0.00 and 0.33 ± 0.02 in cells following treatment with 21 and 75 µM *c*9,*t*11-CLA treatment respectively (Table 5.4.3.2.2).

Table 5.4.3.2.2. Fatty acid composition and ratios of MDA cells treated with *c*9,*t*11-CLA for 72 h.

Fatty Acids	Control	*c*9,*t*11-CLA 21 µM	*c*9,*t*11-CLA 75 µM
LCSFA	61.80 ± 1.14	48.93 ± 9.33	39.53 ± 5.13*
UFA	26.73 ± 1.27	32.85 ± 2.12	54.20 ± 6.37*
MUFA	19.40 ± 0.95	22.27 ± 3.64	19.05 ± 4.85
UFA/LCSFA	0.43 ± 0.02	0.76 ± 0.22	1.47 ± 0.39
MUFA/LCSFA	0.31 ± 0.01	0.53 ± 0.21	0.52 ± 0.18
C16:1/C16:0	0.28 ± 0.06	1.40 ± 1.10	0.90 ± 0.43
C18:1 (*t*9, *t*11 & *c*9)/C18:0	0.38 ± 0.03	0.39 ± 0.00	0.33 ± 0.02

Data represent Mean ± SEM of at least three experiments in triplicate expressed as a percentage of total cellular lipid composition. *Denotes results which are significantly different to untreated/control cells as determined by the Student's *t*-test ($p<0.01$).

5.4.3.3 Effects of *t*10,*c*12-CLA treatments on Fatty acid profiles of MDA cells

Flasks of $2x10^6$ MDA cells were seeded for 24 h before treatment with the *t*10,*c*12-CLA isomer at 21 and 75 µM, incubated for 3 days and extracted and analysed as described earlier. Figure 8 illustrates a chromatogram from a typical injection of an MDA sample treated with (a) 21 and (b) 75 µM *t*10,*c*12-CLA. Table 5.4.3.3.1 lists the fatty acid composition of untreated MDA control and MDA cells treated with 21 and 75 µM *t*10,*c*12-CLA.

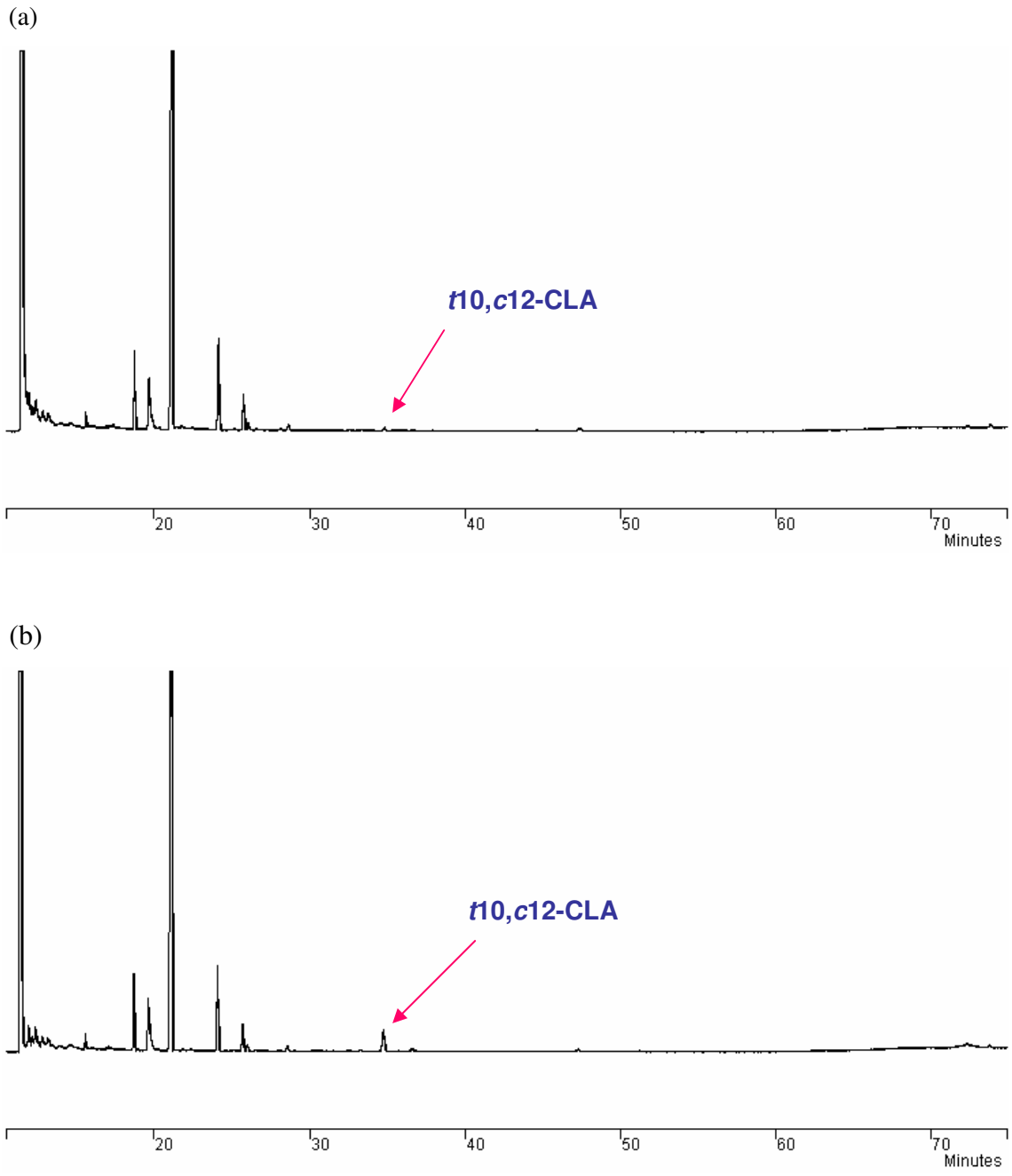


Figure 5.4.3.3. Typical chromatograms of fatty acid profiles of MDA cells treated with (a) 21 and (b) 75 µM *t*10,*c*12-CLA.

Table 5.4.3.3.2 lists the summary of fatty acid composition and various fatty acid ratios of untreated MDA control and MDA cells treated with 21 and 75 μM *t*10,*c*12-CLA. The most abundant fatty acid in each treatment was the long chain saturated fatty acid (LCSFA) C18:0 stearic acid. This was apparently reduced from 34.03 ± 0.93 % total cellular FAME in control MDA cells to 30.79 ± 1.73 % FAME in cells treated with 21 μM *t*10,*c*12-CLA and 23.32 ± 5.06 % FAME following 75 μM *t*10,*c*12-CLA. A similar trend was observed in another predominant LCSFA, C16:0 methyl palmitate. This was apparently reduced from 23.13 ± 0.26 % FAME in control MDA cells to 19.99 ± 1.12 and 15.65 ± 3.33 % FAME in cells treated with 21 μM and 75 μM *t*10,*c*12-CLA respectively. These decreases suggest inhibition of FAS activity by the *t*10,*c*12-CLA isomer. Other LCSFA present in minor amounts include C14:0 methyl myristate, which comprised 3.62 ± 0.13 % FAME in control MDA cells and 3.08 ± 0.38 and 2.67 ± 0.11 % FAME 21 μM and 75 μM *t*10,*c*12-CLA (Table 5.4.3.3.1).

The predominant MUFA was the group of three C18:1 fatty acids elaidic (*t*-9), vaccenic (*t*-11), and oleic acid (*c*-9). Their combined proportion of total cellular lipids of 12.74 ± 0.56 % in control MDA cells decreased to 9.58 ± 2.77 % FAME with treatment of 21 μM *t*10,*c*12-CLA and decreased significantly to 6.78 ± 0.54 % FAME following 75 μM *t*10,*c*12-CLA treatment, possibly due to inhibition of Δ9-desaturase. The mono-unsaturated fatty acid (MUFA) C16:1 methyl palmitoleate (palmitoleic acid) increased from 6.46 ± 1.31 % total lipid in control MDA cells to 16.01 ± 6.10 and 8.80 ± 3.12 % FAME following 21 and 75 μM *t*10,*c*12-CLA treatment respectively, although neither increase was significant (Table 5.4.3.3.1).

Other UFA were present in minor amounts. C18:2 (*c*9,*c*12) linoleic acid (LA) comprised 3.08 ± 0.19 % FAME in control MDA cells and 2.26 ± 0.17 % and 2.07 ± 0.14 % FAME following treatment with 21 and 75 μM *t*10,*c*12-CLA. C22:6 docosahexanoic acid (DHA) comprised 1.29 ± 0.12 % FAME in control MDA cells and 1.07 ± 0.04 % FAME with 21 μM *t*10,*c*12-CLA and was significantly reduced to 1.04 ± 0.12 % FAME 75 μM *t*10,*c*12-CLA treatment (Table 5.4.3.3.1).

Table 5.4.3.3.1. Fatty acid composition of MDA cells treated with t10,c12-CLA for 72 h.

Fatty Acid	Control	*t*10,*c*12-CLA 21 μM	*t*10,*c*12-CLA 75 μM
C6:0	0.38 ± 0.20	0.17 ± 0.09	0.15 ± 0.14
C8:0	3.83 ± 1.47	2.17 ± 0.83	1.99 ± 0.57
C10:0	2.76 ± 0.27	3.67 ± 1.03	2.52 ± 0.75
C11:0	3.25 ± 0.36	2.49 ± 0.43	1.72 ± 0.24
C12:0	0.09 ± 0.06	0.00 ± 0.00	0.02 ± 0.02
C13:0	1.16 ± 0.10	0.93 ± 0.14	0.76 ± 0.17
C14:0	3.62 ± 0.13	3.08 ± 0.38	2.67 ± 0.11
C14:1	0.18 ± 0.09	0.00 ± 0.00	0.00 ± 0.00
C15:0	0.50 ± 0.19	0.49 ± 0.25	0.31 ± 0.13
C15:1	0.02 ± 0.02	0.00 ± 0.00	0.00 ± 0.00
C16:0	23.13 ± 0.26	19.99 ± 1.12	15.65 ± 3.33
C16:1	6.46 ± 1.31	16.01 ± 6.10	8.80 ± 3.12
C18:0	34.03 ± 0.93	30.79 ± 1.73	23.32 ± 5.06
C18:1 (*t*9, *t*11 & *c*9)	12.74 ± 0.56	9.58 ± 2.77	6.78 ± 0.54*
C18:2 LA	3.08 ± 0.19	2.26 ± 0.17	2.07 ± 0.14
C18:3 GLA	0.00 ± 0.00	0.07 ± 0.04	0.00 ± 0.00
C18:3(*c*9,12,15)	0.28 ± 0.12	0.33 ± 0.22	0.29 ± 0.16
C20:0	0.25 ± 0.14	0.53 ± 0.15	0.66 ± 0.07
C18:2 *c*9,*t*11-CLA	0.14 ± 0.14	0.28 ± 0.10	0.17 ± 0.09
C18:2 *t*10,*c*12-CLA	0.05 ± 0.05	3.59 ± 1.12	26.37 ± 5.53*
C20:1 (*c*11)	0.10 ± 0.10	0.30 ± 0.16	0.95 ± 0.24
C18:2 *t*9,11-CLA	0.14 ± 0.14	0.20 ± 0.20	0.95 ± 0.95
C20:2 (*c*11,14)	0.00 ± 0.00	0.08 ± 0.04	0.09 ± 0.05
C20:3 DGLA	0.32 ± 0.16	0.24 ± 0.12	0.30 ± 0.05
C20:4 AA	1.93 ± 0.23	1.46 ± 0.08	1.57 ± 0.18
C22:1(*c*13)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
C23:0	0.27 ± 0.14	0.19 ± 0.08	0.12± 0.05
C22:2 (*c*13,16)	0.00 ± 0.00	0.00 ± 0.00	0.10 ± 0.10
C20:5 EPA	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
C24:0	0.00 ± 0.00	0.00 ± 0.00	0.32 ± 0.32
C24:1(*c*15)	0.00 ± 0.00	0.02 ± 0.02	0.13± 0.13
C22:6 DHA	1.29 ± 0.12	1.07 ± 0.04	1.04 ± 0.12*

Data represent Mean ± SEM of at least three experiments in triplicate expressed as a percentage of total cellular lipid composition. *Denotes results which are significantly different to untreated/control cells as determined by the Student's *t*-test ($p<0.05$).

The level of the *t*10,*c*12-CLA isomer was apparently increased from 0.05 ± 0.05 % FAME in control MDA cells to 3.59 ± 1.12 % FAME following 21 µM *t*10,*c*12-CLA treatment and increased significantly to 26.37 ± 5.53 % FAME with 75 µM *t*10,*c*12-CLA. Corresponding levels for the *c*9,*t*11-CLA isomer remained at trace levels of 0.14 ± 0.14 % FAME in control MDA cells and 0.28 ± 0.10 and 0.17 ± 0.09 % FAME following 21 and 75 µM *t*10,*c*12-CLA treatment (Table 5.4.3.3.1).

The apparent decreases in C18:0 stearic acid, C16:0 methyl palmitate and C14:0 methyl myristate levels following 21 and 75 µM *t*10,*c*12-CLA treatment resulted in an overall decreased level of LCSFA from 61.80 ± 1.14 % FAME in control MDA cells to 55.08 ± 2.79 and 43.24 ± 8.05 % FAME following 21 and 75 µM *t*10,*c*12-CLA treatment, although not deemed significant. The overall UFA level, at 26.73 ± 1.27 % FAME in control MDA cells, apparently increased to 35.48 ± 4.45 and 49.60 ± 9.01 % FAME with 21 and 75 µM *t*10,*c*12-CLA. Corresponding MUFA levels were 19.40 ± 0.95 % FAME in control MDA cells and 25.61 ± 3.68 and 15.71 ± 2.72 % FAME following 21 and 75 µM *t*10,*c*12-CLA treatment (Table 5.4.3.3.2).

Resultant ratio of UFA/LCSFA apparently increased from 0.43 ± 0.02 in control MDA cells to 0.65 ± 0.11 and 1.35 ± 0.55 following 21 and 75 µM *t*10,*c*12-CLA treatment respectively, although the differences were not deemed statistically significant. Corresponding MUFA/LCSFA levels were 0.31 ± 0.01 in control MDA cells and 0.47 ± 0.09 and 0.42 ± 0.17 following 21 and 75 µM *t*10,*c*12-CLA treatment (Table 5.4.3.3.2).

The ratio of C16:1/C16:0 apparently increased from 0.28 ± 0.06 in control MDA cells to 0.84 ± 0.33 and 0.74 ± 0.42 in cells treated with 21 and 75 µM *t*10,*c*12-CLA treatment. However the ratio of C18:1/C18:0 remained relatively similar at 0.38 ± 0.03 in control MDA cells to 0.30 ± 0.07 and 0.32 ± 0.07 in cells following treatment with 21 and 75 µM *t*10,*c*12-CLA treatment respectively (Table 5.4.3.3.2).

Table 5.4.3.3.2. Fatty acid composition and ratios of MDA cells treated with *t*10,*c*12-CLA for 72 h.

Fatty Acids	Control	*t*10,*c*12-CLA 21 μM	*t*10,*c*12-CLA 75 μM
LCSFA	61.80 ± 1.14	55.08 ± 2.79	43.24 ± 8.05
UFA	26.73 ± 1.27	35.48 ± 4.45	49.60 ± 9.01
MUFA	19.40 ± 0.95	25.61 ± 3.68	15.71 ± 2.72
UFA/LCSFA	0.43 ± 0.02	0.65 ± 0.11	1.35 ± 0.55
MUFA/LCSFA	0.31 ± 0.01	0.47 ± 0.09	0.42 ± 0.17
C16:1/C16:0	0.28 ± 0.06	0.84 ± 0.33	0.74 ± 0.42
C18:1 (*t*9, *t*11 & *c*9)/C18:0	0.38 ± 0.03	0.30 ± 0.07	0.32 ± 0.07

Data represent Mean ± SEM of at least three experiments in triplicate expressed as a percentage of total cellular lipid composition.

5.4.3.4 Effects of EPA treatments on Fatty acid profiles of MDA cells

Flasks of $2x10^6$ MDA cells were seeded for 24 h before treatment with eicosapentanoic acid (EPA) at 21 and 75 μM, incubated for 3 days and extracted and analysed as described earlier. Figure 5.4.3.4 illustrates a chromatogram from a typical injection of an MDA sample treated with (a) 21 and (b) 75 μM EPA. Table 5.4.3.4.1 lists the fatty acid composition of untreated MDA control and MDA cells treated with 21 and 75 μM EPA, while table 5.4.3.4.2 lists the summary of fatty acid composition and various fatty acid ratios of the above. The most abundant fatty acid in each treatment was the long chain saturated fatty acid (LCSFA) C18:0 stearic acid.

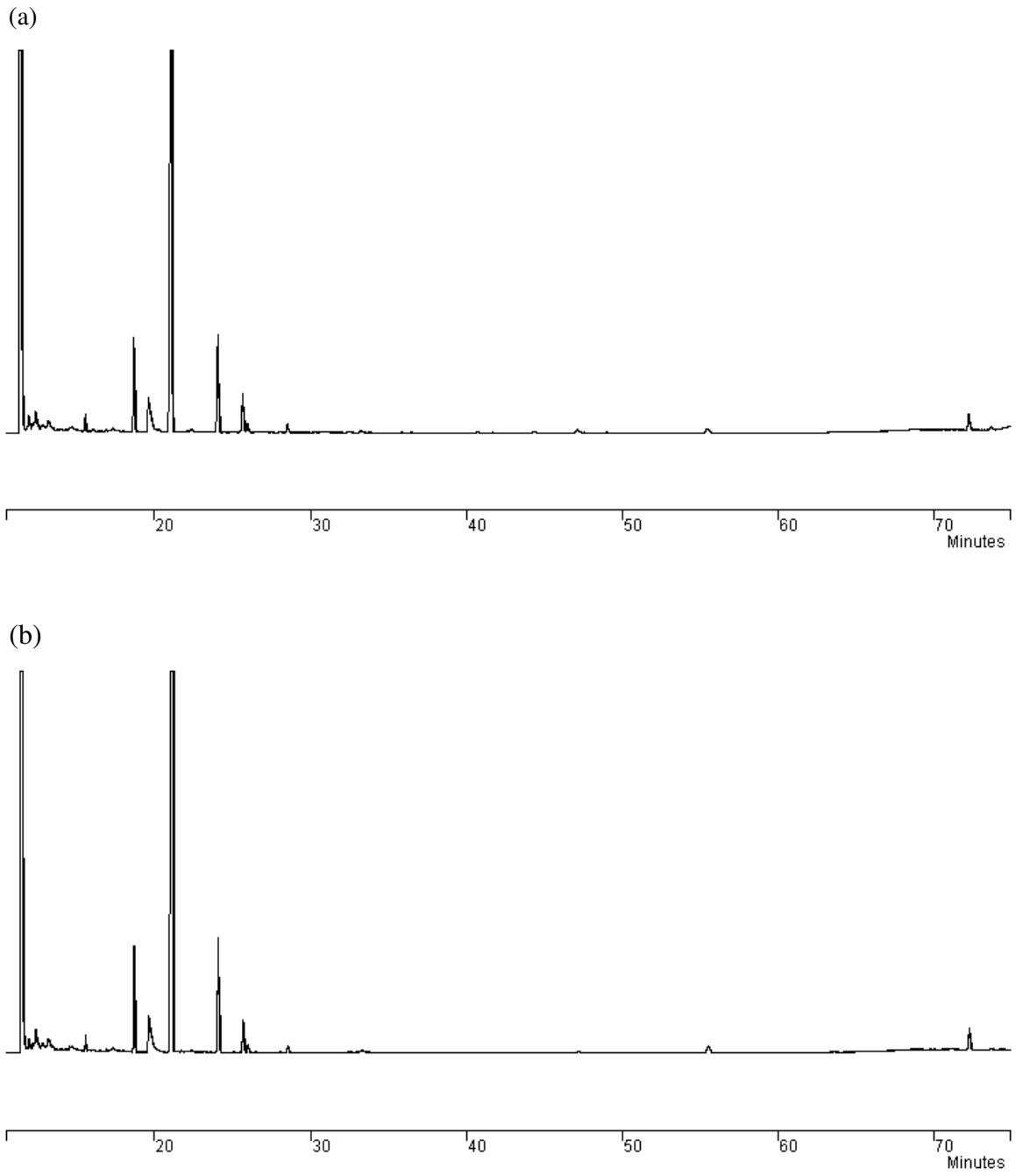


Figure 5.4.3.4. Typical chromatograms of fatty acid profiles of MDA cells treated with (a) 21 and (b) 75 μM EPA.

C18:0 stearic acid was relatively unchanged from 34.03 ± 0.93 % total cellular FAME in control MDA cells to 30.28 ± 2.37 % FAME in cells treated with 21 µM EPA and 34.15 ± 0.46 % FAME following 75 µM EPA. Another predominant LCSFA, C16:0 methyl palmitate was significantly reduced from 23.13 ± 0.26 % FAME in control MDA cells to 19.93 ± 0.51 % FAME in cells treated with 21 µM EPA and apparently reduced to 20.03 ± 0.95 % FAME with 75 µM EPA, perhaps due to inhibition of FAS activity by EPA (Table 5.4.3.4.1).

Other LCSFA present in minor amounts include C14:0 methyl myristate, which comprised 3.62 ± 0.13 % FAME in control MDA cells and decreased to 2.74 ± 0.45 with 21 µM EPA and decreased significantly to 2.07 ± 0.21 % FAME with 75 µM EPA. LCSFA C10:0 and C11:0 levels decreased significantly with treatments of 21 µM EPA relative to control MDA cells from 2.76 ± 0.27 to 1.08 ± 0.58 % FAME and from 3.25 ± 0.36 to 1.67 ± 0.19 % FAME. C10:0, C13:0 and C14:0 levels decreased significantly with treatments of 75 µM EPA from 2.76 ± 0.27 to 0.34 ± 0.29 %, 1.16 ± 0.10 to 0.06 ± 0.06 % and from 3.62 ± 0.13 to 2.07 ± 0.21 % FAME respectively. These decreases may be due to a reduced level of β-oxidation of longer chain fatty acids. C20:0 increased significantly from 0.25 ± 0.14 % FAME in the MDA control cells to 0.84 ± 0.06 with treatments of 75 µM EPA. Also, C24:0 increased significantly from undetectable levels in the MDA control cells to 0.12 ± 0.01 and 0.30 ± 0.07 with 21 and 75 µM EPA respectively (Table 5.4.3.4.1).

The predominant MUFA was the group of three C18:1 fatty acids elaidic (*t*-9), vaccenic (*t*-11), and oleic acid (*c*-9). Their combined proportion of total cellular lipids of 12.74 ± 0.56 % in control MDA cells decreased to 6.34 ± 2.29 % FAME with treatment of 21 µM EPA and decreased significantly to 3.15 ± 1.44 % FAME following 75 µM EPA treatment, suggesting inhibition of the Δ9-desaturase enzyme. The mono-unsaturated fatty acid (MUFA) C16:1 methyl palmitoleate (palmitoleic acid) increased from 6.46 ± 1.31 % total lipid in control MDA cells to 25.20 ± 3.95 and 27.15 ± 3.81 % FAME following 21 and 75 µM EPA treatment respectively. C20:1 methyl eicosanoate increased significantly from 0.10 ± 0.10 to 0.25 ± 0.06

with 75 μM EPA treatment, possibly formed as an intermediate in the oxidation/metabolism of the C20:5 EPA (Table 5.4.3.4.1).

Other UFA were present in minor amounts. C18:2 (*c*9,*c*12) linoleic acid (LA) comprised 3.08 ± 0.19 % FAME in control MDA cells and 2.05 ± 0.69 % following treatment with 21 μM EPA and was decreased significantly to 1.09 ± 0.34 % FAME with 75 μM EPA. C18:3 α-linolenic acid (ALA), a precursor of EPA, increased from 0.28 ± 0.12 to 0.51 ± 0.07 % FAME with 75 μM EPA, possibly due to negative feedback by EPA inhibiting Δ5/Δ6 desaturase and/or elongase activities. There was an apparent drop in C20:4 arachidonic acid (AA) levels from 1.93 ± 0.23 % FAME in MDA cells to 1.62 ± 0.51 and 0.88 ± 0.32 % FAME with 21 and 75 μM CLA-mix, also suggesting inhibition of Δ5/Δ6 desaturase and/or elongase activities (Table 5.4.3.4.1).

C20:2 methyl eicosadienoate, an oxidation intermediate of EPA, increased significantly from undetectable to 0.13 ± 0.02 % FAME with 75 μM EPA. C22:6 docosahexanoic acid (DHA) comprised 1.29 ± 0.12 % FAME in control MDA cells and 1.02 ± 0.28 % FAME with 21 μM EPA and was significantly reduced to 0.40 ± 0.06 % FAME following 75 μM EPA treatment. This indicates inhibition of Δ4-desaturase and/or elongase enzymes (Table 5.4.3.4.1).

The level of EPA apparently increased from undetectable in control MDA cells to trace levels of 0.14 ± 0.13 and 0.46 ± 0.43 % FAME following 21 and 75 μM EPA treatment (Table 5.4.3.4.1). This indicates that most of the EPA taken into the cells is quickly metabolised to other forms resulting in an apparently low uptake.

There was a significant decrease in the overall level of LCSFA from 61.80 ± 1.14 % FAME in control MDA cells to 56.76 ± 1.58 with 21 μM EPA, whereas the level remained relatively unchanged at 60.73 ± 1.61 % FAME following 75 μM EPA treatment. The overall UFA level, at 26.73 ± 1.27 % FAME in control MDA cells, increased significantly to 38.39 ± 0.61 and 35.66 ± 2.08 % FAME with 21 and 75 μM EPA. Corresponding MUFA levels increased significantly from 19.40 ± 0.95 %

FAME in control MDA cells to 31.70 ± 1.74 and 30.73 ± 2.43 % FAME following 21 and 75 µM EPA treatment (Table 5.4.3.4.2).

The ratio of UFA/LCSFA significantly increased from 0.43 ± 0.02 in control MDA cells to 0.68 ± 0.03 with 21 µM EPA, with an apparent but not significant increase to 0.59 ± 0.05 with 75 µM EPA. The MUFA/LCSFA ratio increased significantly from 0.31 ± 0.01 in control MDA cells to 0.56 ± 0.03 following 21 µM EPA treatment, with a corresponding level of 0.51 ± 0.05 following 75 µM EPA treatment (Table 5.4.3.4.2).

The ratio of C18:1/C18:0 apparently decreased from 0.38 ± 0.03 in control MDA cells to 0.22 ± 0.09 in cells following treatment with 21 µM EPA and significantly decreased to 0.09 ± 0.04 in cells with 75 µM EPA, possibly due to inhibition of Δ9-desaturase. The ratio of C16:1/C16:0 significantly increased from 0.28 ± 0.06 in control MDA cells to 1.27 ± 0.22 and 1.38 ± 0.24 in cells treated with 21 and 75 µM EPA (Table 5.4.3.4.2).

Table 5.4.3.4.1. Fatty acid composition of MDA cells treated with EPA for 72 h.

Fatty Acid	Control	EPA 21 μM	EPA 75 μM
C6:0	0.38 ± 0.20	0.09 ± 0.09	0.02 ± 0.02
C8:0	3.83 ± 1.47	1.52 ± 0.27	1.19 ± 0.23
C10:0	2.76 ± 0.27	1.08 ± 0.58*	0.34 ± 0.29*
C11:0	3.25 ± 0.36	1.67 ± 0.19*	2.00 ± 0.13
C12:0	0.09 ± 0.06	0.03 ± 0.01	0.00 ± 0.00
C13:0	1.16 ± 0.10	0.47 ± 0.27	0.06 ± 0.06*
C14:0	3.62 ± 0.13	2.74 ± 0.45	2.07 ± 0.21*
C14:1	0.18 ± 0.09	0.00 ± 0.00	0.00 ± 0.00
C15:0	0.50 ± 0.19	1.07 ± 0.29	0.66 ± 0.13
C15:1	0.02 ± 0.02	0.00 ± 0.00	0.00 ± 0.00
C16:0	23.13 ± 0.26	19.93 ± 0.51*	20.03 ± 0.95
C16:1	6.46 ± 1.31	25.20 ± 3.95*	27.15 ± 3.81*
C18:0	34.03 ± 0.93	30.28 ± 2.37	34.15 ± 0.46
C18:1 (*t*9, *t*11 & *c*9)	12.74 ± 0.56	6.34 ± 2.29	3.15 ± 1.44*
C18:2 LA	3.08 ± 0.19	2.05 ± 0.69	1.09 ± 0.34*
C18:3 GLA	0.00 ± 0.00	0.06 ± 0.02	0.12 ± 0.04
C18:3(*c*9,12,15)	0.28 ± 0.12	0.36 ± 0.10	0.51 ± 0.07
C20:0	0.25 ± 0.14	0.52 ± 0.05	0.84 ± 0.06*
C18:2 *c*9,*t*11-CLA	0.14 ± 0.14	0.16 ± 0.01	0.20 ± 0.04
C18:2 *t*10,*c*12-CLA	0.05 ± 0.05	0.15 ± 0.15	0.00 ± 0.00
C20:1 (*c*11)	0.10 ± 0.10	0.16 ± 0.04	0.25 ± 0.06*
C18:2 *t*9,11-CLA	0.14 ± 0.14	0.24 ± 0.12	0.19 ± 0.05
C20:2 (*c*11,14)	0.00 ± 0.00	0.14 ± 0.07	0.13 ± 0.02*
C20:3 DGLA	0.32 ± 0.16	0.32 ± 0.11	0.14 ± 0.09
C20:4 AA	1.93 ± 0.23	1.62 ± 0.51	0.88 ± 0.32
C22:1(*c*13)	0.00 ± 0.00	0.03 ± 0.03	0.23 ± 0.15
C23:0	0.27 ± 0.14	2.05 ± 0.75	2.64 ± 0.90
C22:2 (*c*13,16)	0.00 ± 0.00	0.27 ± 0.21	0.55 ± 0.19
C20:5 EPA	0.00 ± 0.00	0.14 ± 0.13	0.46 ± 0.43
C24:0	0.00 ± 0.00	0.12 ± 0.01*	0.30 ± 0.07*
C24:1(*c*15)	0.00 ± 0.00	0.13 ± 0.02*	0.20 ± 0.09
C22:6 DHA	1.29 ± 0.12	1.02 ± 0.28	0.40 ± 0.06*

Data represent Mean ± SEM of at least three experiments in triplicate expressed as a percentage of total cellular lipid composition. *Denotes results which are significantly different to untreated/control cells as determined by the Student's *t*-test ($p<0.05$).

Table 5.4.3.4.2. Fatty acid composition and ratios of MDA cells treated with EPA for 72 h.

Fatty Acids	Control	EPA 21 μM	EPA 75 μM
LCSFA	61.80 ± 1.14	56.76 ± 1.58*	60.73 ± 1.61
UFA	26.73 ± 1.27	38.39 ± 0.61*	35.66 ± 2.08*
MUFA	19.40 ± 0.95	31.70 ± 1.74*	30.73 ± 2.43*
UFA/LCSFA	0.43 ± 0.02	0.68 ± 0.03*	0.59 ± 0.05
MUFA/LCSFA	0.31 ± 0.01	0.56 ± 0.03*	0.51 ± 0.05
C16:1/C16:0	0.28 ± 0.06	1.27 ± 0.22*	1.38 ± 0.24*
C18:1 (*t*9, *t*11 & *c*9)/C18:0	0.38 ± 0.03	0.22 ± 0.09	0.09 ± 0.04*

Data represent Mean ± SEM of at least three experiments in triplicate expressed as a percentage of total cellular lipid composition. *Denotes results which are significantly different to untreated/control cells as determined by the Student's *t*-test ($p<0.05$).

5.4.3.5 Effects of DHA treatments on Fatty acid profiles of MDA cells

Flasks of $2x10^6$ MDA cells were seeded for 24 h before treatment with docosahexanoic acid (DHA) at 21 and 75 μM, incubated for 3 days and extracted and analysed as described earlier. Figure 5.4.3.5 illustrates a chromatogram from a typical injection of an MDA sample treated with (a) 21 and (b) 75 μM DHA. Table 5.4.3.5.1 lists the fatty acid composition of untreated MDA control and MDA cells treated with 21 and 75 μM DHA. Table 5.4.3.5.2 lists the summary of fatty acid composition and various fatty acid ratios of untreated MDA control and MDA cells treated with 21 and 75 μM DHA. The most abundant fatty acid in each treatment was the long chain saturated fatty acid (LCSFA) C18:0 stearic acid.

C18:0 stearic acid was relatively unchanged from 34.03 ± 0.93 % total cellular FAME in control MDA cells to 29.97 ± 2.19 % FAME in cells treated with 21 μM DHA and 30.22 ± 1.26 % FAME following 75 μM DHA. Another predominant

LCSFA, C16:0 methyl palmitate was apparently reduced from 23.13 ± 0.26 % FAME in control MDA cells to 19.39 ± 1.77 and 19.35 ± 2.06 % FAME in cells treated with 21 and 75 µM DHA respectively, although not statistically significant (Table 5.4.3.5.1). This indicates DHA had little or no effect on FAS activity in MDA cells.

The predominant MUFA was the group of three C18:1 fatty acids elaidic (*t*-9), vaccenic (*t*-11), and oleic acid (*c*-9). Their combined proportion of total cellular lipids of 12.74 ± 0.56 % in control MDA cells decreased significantly to 5.93 ± 1.80 % FAME with treatment of 21 µM DHA and resulted in an apparent decrease to 6.41 ± 1.48 % FAME following 75 µM DHA treatment, suggesting inhibition of Δ9-desaturase by DHA. The mono-unsaturated fatty acid (MUFA) C16:1 methyl palmitoleate (palmitoleic acid) apparently increased from 6.46 ± 1.31 % total lipid in control MDA cells to 24.40 ± 9.03 and 18.28 ± 3.98 % FAME following 21 and 75 µM DHA treatment respectively (Table 5.4.3.5.1).

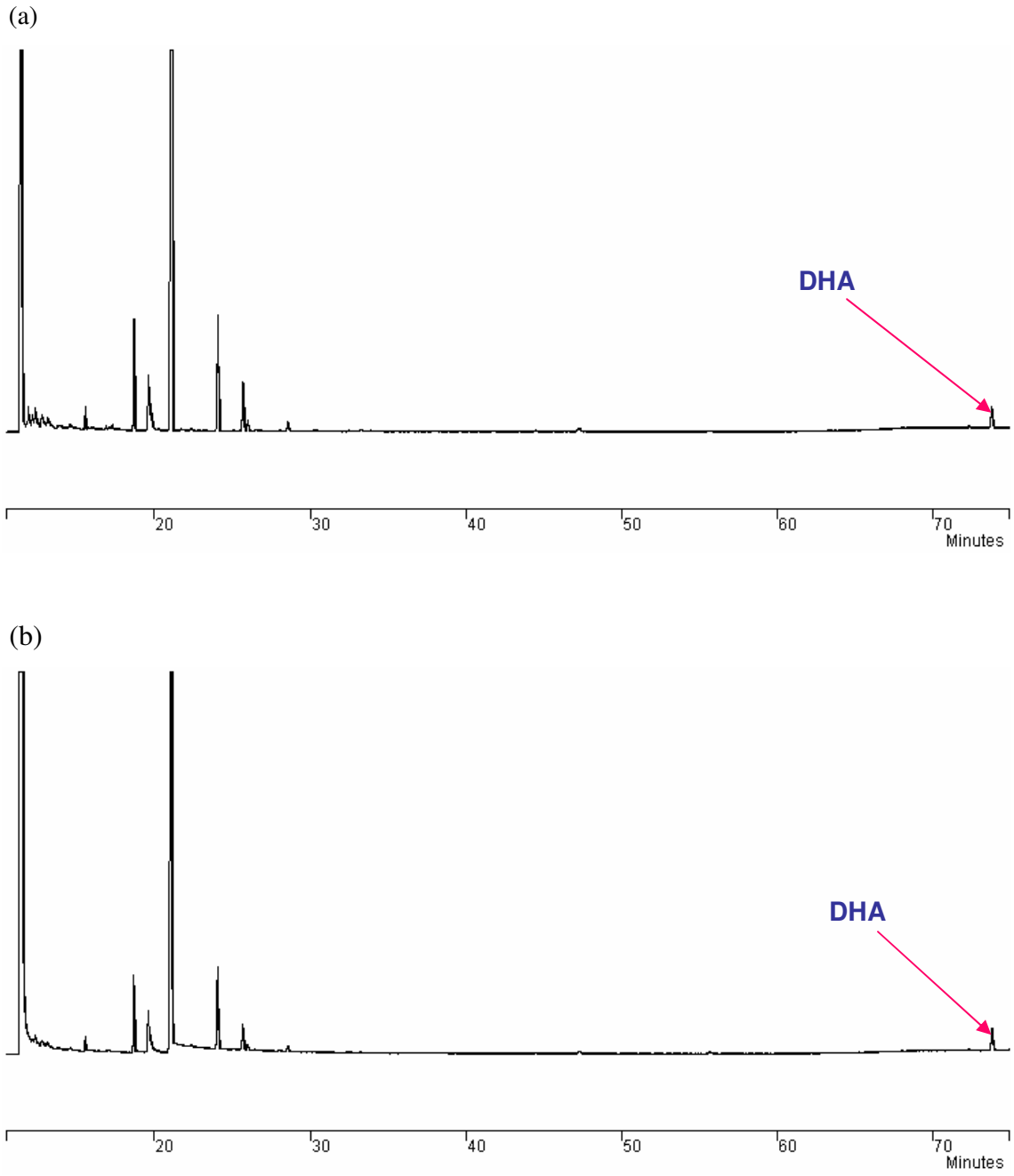


Figure 5.4.3.5. Typical chromatograms of fatty acid profiles of MDA cells treated with (a) 21 and (b) 75 µM DHA.

Other UFA were present in minor amounts. C18:2 (*c*9,*c*12) linoleic acid (LA) comprised 3.08 ± 0.19 % FAME in control MDA cells and 1.31 ± 0.78 and 1.92 ± 0.45 % FAME following treatment with 21 and 75 µM DHA. C18:3 α-linolenic acid (ALA) levels comprised 0.28 ± 0.12 % FAME in control MDA cells and 0.26 ± 0.17 and 0.20 ± 0.10 % FAME with 21 and 75 µM DHA (Table 5.4.3.5.1).

DHA treatment resulted in significant uptake of DHA into the cells with levels rising from 1.29 ± 0.12 % FAME in control MDA cells to 4.41 ± 1.37 and 8.06 ± 0.78 % FAME following treatment with 21 and 75 µM DHA respectively (Table 5.4.3.5.1).

There was an apparent decrease in the overall level of LCSFA from 61.80 ± 1.14 % FAME in control MDA cells to 52.78 ± 4.21 and 54.24 ± 2.53 % FAME following 21 and 75 µM DHA treatment. The overall UFA level, at 26.73 ± 1.27 % FAME in control MDA cells, apparently increased to 38.17 ± 7.10 and 36.75 ± 1.96 % FAME with 21 and 75 µM DHA. Corresponding MUFA levels apparently increased from 19.40 ± 0.95 % FAME in control MDA cells to 30.67 ± 8.46 and 24.99 ± 2.27 % FAME following 21 and 75 µM EPA treatment (Table 5.4.3.5.2). The increase in overall UFA and MUFA is mainly due to a combination of direct uptake of DHA and indirect actions leading to increases in C16:1 palmitoleic acid.

There was an apparent but not significant increase ratio of UFA/LCSFA from 0.43 ± 0.02 in control MDA cells to 0.77 ± 0.22 and 0.69 ± 0.06 with 21 and 75 µM DHA. The corresponding values for the MUFA/LCSFA ratio were 0.31 ± 0.01 in control MDA cells and 0.64 ± 0.23 and 0.47 ± 0.06 following 21 and 75 µM DHA treatment (Table 5.4.3.5.2).

The ratio of C18:1/C18:0 apparently decreased from 0.38 ± 0.03 in control MDA cells to 0.21 ± 0.04 in cells following treatment with 21 µM DHA and significantly decreased to 0.20 ± 0.06 in cells with 75 µM DHA, indicating inhibition of Δ9-desaturase. The ratio of C16:1/C16:0 apparently increased from 0.28 ± 0.06 in control MDA cells to 1.41 ± 0.60 and 1.05 ± 0.28 in cells treated with 21 and 75 µM DHA (Table 5.4.3.5.2).

Table 5.4.3.5.1. Fatty acid composition of MDA cells treated with DHA for 72 h.

Fatty Acid	Control	DHA 21 μM	DHA 75 μM
C6:0	0.38 ± 0.20	0.54 ± 0.31	0.19 ± 0.14
C8:0	3.83 ± 1.47	2.78 ± 1.23	1.94 ± 0.49
C10:0	2.76 ± 0.27	3.37 ± 1.49	2.95 ± 0.79
C11:0	3.25 ± 0.36	1.75 ± 0.65	2.96 ± 0.65
C12:0	0.09 ± 0.06	0.04 ± 0.04	0.02 ± 0.02
C13:0	1.16 ± 0.10	0.56 ± 0.29	0.96 ± 0.41
C14:0	3.62 ± 0.13	2.59 ± 0.51	2.76 ± 0.44
C14:1	0.18 ± 0.09	0.04 ± 0.04	0.09 ± 0.06
C15:0	0.50 ± 0.19	0.24 ± 0.12	0.68 ± 0.42
C15:1	0.02 ± 0.02	0.00 ± 0.00	0.01 ± 0.01
C16:0	23.13 ± 0.26	19.39 ± 1.77	19.35 ± 2.06
C16:1	6.46 ± 1.31	24.40 ± 9.03	18.28 ± 3.98
C18:0	34.03 ± 0.93	29.97 ± 2.19	30.22 ± 1.26
C18:1 (*t*9, *t*11 & *c*9)	12.74 ± 0.56	5.93 ± 1.80*	6.41 ± 1.48
C18:2 LA	3.08 ± 0.19	1.31 ± 0.78	1.92 ± 0.45
C18:3 GLA	0.00 ± 0.00	0.00 ± 0.00	0.04 ± 0.03
C18:3(*c*9,12,15)	0.28 ± 0.12	0.26 ± 0.17	0.20 ± 0.10
C20:0	0.25 ± 0.14	0.45 ± 0.16	0.39 ± 0.08
C18:2 *c*9,*t*11-CLA	0.14 ± 0.14	0.08 ± 0.05	0.04 ± 0.02
C18:2 *t*10,*c*12-CLA	0.05 ± 0.05	0.12 ± 0.05	0.11 ± 0.08
C20:1 (*c*11)	0.10 ± 0.10	0.09 ± 0.04	0.04 ± 0.02
C18:2 *t*9,11-CLA	0.14 ± 0.14	0.00 ± 0.00	0.00 ± 0.00
C20:2 (*c*11,14)	0.00 ± 0.00	0.01 ± 0.01	0.02 ± 0.02
C20:3 DGLA	0.32 ± 0.16	0.27 ± 0.15	0.30 ± 0.08
C20:4 AA	1.93 ± 0.23	0.93 ± 0.54	1.03 ± 0.25
C22:1(*c*13)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
C23:0	0.27 ± 0.14	0.14 ± 0.14	0.76 ± 0.22
C22:2 (*c*13,16)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
C20:5 EPA	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
C24:0	0.00 ± 0.00	0.00 ± 0.00	0.05 ± 0.04
C24:1(*c*15)	0.00 ± 0.00	0.31 ± 0.25	0.20 ± 0.11
C22:6 DHA	1.29 ± 0.12	4.41 ± 1.37*	8.06 ± 0.78*

Data represent Mean ± SEM of at least three experiments in triplicate expressed as a percentage of total cellular lipid composition. *Denotes results which are significantly different to untreated/control cells as determined by the Student's *t*-test ($p \leq 0.05$).

Table 5.4.3.5.2 Fatty acid composition and ratios of MDA cells treated with DHA for 72 h.

Fatty Acids	Control	DHA 21 μM	DHA 75 μM
LCSFA	61.80 ± 1.14	52.78 ± 4.21	54.24 ± 2.53
UFA	26.73 ± 1.27	38.17 ± 7.10	36.75 ± 1.96
MUFA	19.40 ± 0.95	30.67 ± 8.46	24.99 ± 2.27
UFA/LCSFA	0.43 ± 0.02	0.77 ± 0.22	0.69 ± 0.06
MUFA/LCSFA	0.31 ± 0.01	0.64 ± 0.23	0.47 ± 0.06
C16:1/C16:0	0.28 ± 0.06	1.41 ± 0.60	1.05 ± 0.28
C18:1 (*t*9, *t*11 & *c*9)/C18:0	0.38 ± 0.03	0.20 ± 0.06*	0.21 ± 0.04

Data represent Mean ± SEM of at least three experiments in triplicate expressed as a percentage of total cellular lipid composition. *Denotes results which are significantly different to untreated/control cells as determined by the Student's *t*-test ($p<0.05$).

5.4.4 Fatty acid profiles of A10p10p cells following treatments

5.4.4.1 Effects of CLA mixture treatments on Fatty acid profiles of A10p10p cells

Flasks of $2x10^6$ of drug resistant A10p10p cells were seeded for 24 h before treatment with 21 and 75 μM CLA mixture, incubated for 3 days and extracted and analysed as described earlier. Figure 5.4.4.1 illustrates a chromatogram from a typical injection of an A10p10p sample treated with (a) 21 and (b) 75 μM of the CLA mixture of isomers (CLA-mix). Table 5.4.4.1.1 lists the fatty acid composition of untreated A10p10p control cells and A10p10p cells treated with 21 and 75 μM CLA-mix, while table 5.4.4.1.2 lists the summary of fatty acid composition and various fatty acid ratios of the above. The most abundant fatty acid in each treatment was the long chain saturated fatty acid (LCSFA) C18:0 stearic acid.

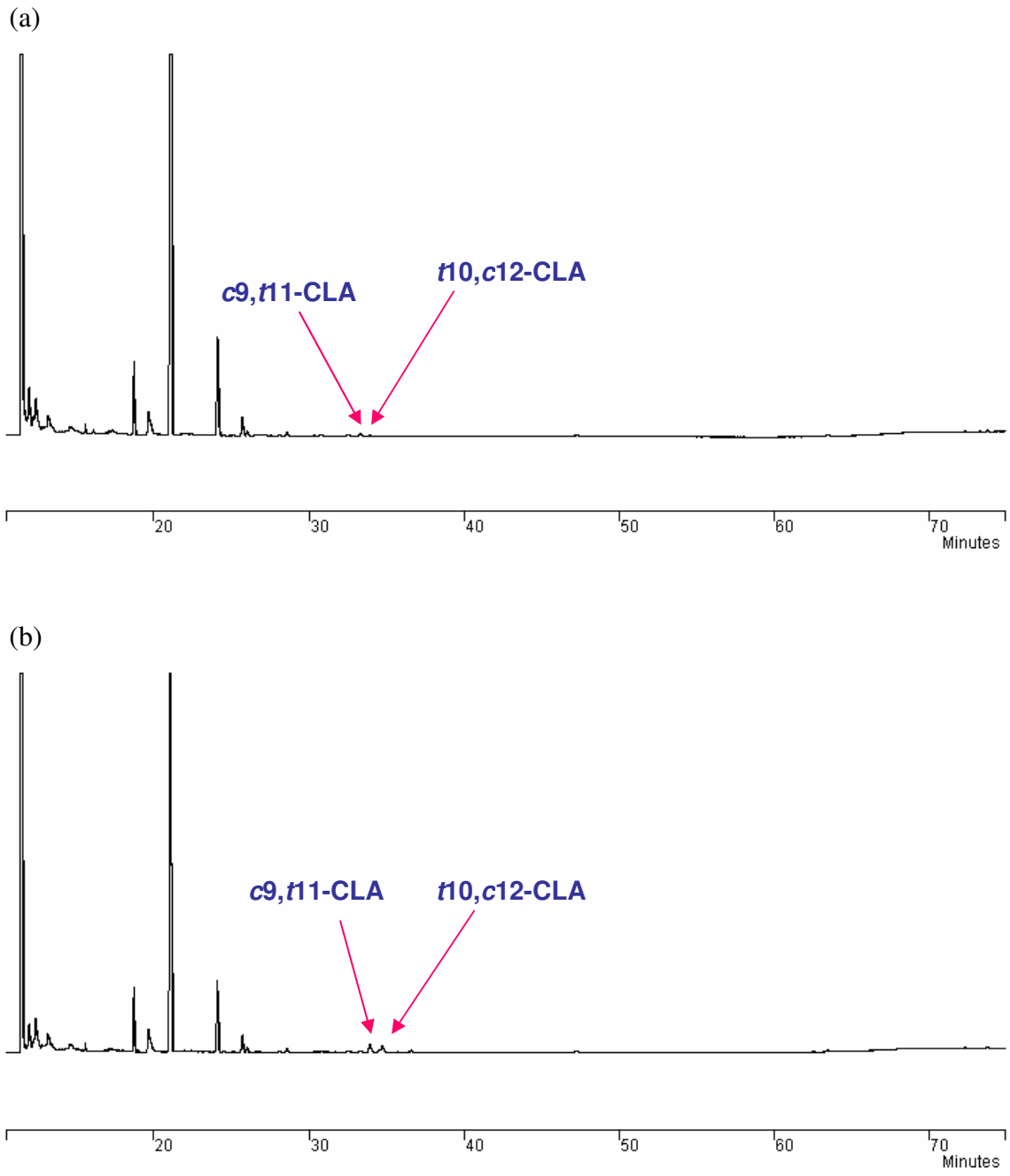


Figure 5.4.4.1. Typical chromatograms of fatty acid profiles of A10p10p cells treated with (a) 21 and (b) 75 µM CLA-mix.

C18:0 stearic acid was relatively unchanged from 34.98 ± 4.12 % total cellular FAME in control A10p10p cells to 37.53 ± 0.51 % FAME in cells treated with 21 µM CLA-mix and an apparent but not significant decrease to 24.69 ± 6.70 % FAME following 75 µM CLA-mix. Another predominant LCSFA, C16:0 methyl palmitate was apparently reduced from 19.65 ± 1.92 % FAME in control A10p10p cells to 18.94 ± 1.15 and 14.58 ± 2.75 % FAME in cells treated with 21 and 75 µM CLA-mix respectively, although not statistically significant (Table 5.4.4.1.1). These decreases suggest inhibition of FAS activity.

The predominant mono-unsaturated fatty acid (MUFA), C16:1 methyl palmitoleate (palmitoleic acid), apparently increased from 8.98 ± 2.01 % total lipid in control A10p10p cells to 10.25 ± 1.16 and 21.11 ± 12.32 % FAME following 21 and 75 µM CLA-mix treatment respectively, indicating a possible increase in Δ9-desaturase activity by CLA-mix. Another abundant MUFA was the group of three C18:1 fatty acids elaidic (*t*-9), vaccenic (*t*-11), and oleic acid (*c*-9). Their combined proportion of total cellular lipids of 7.60 ± 1.37 % in control A10p10p cells remained relatively unchanged with treatments of 21 and 75 µM CLA-mix, with levels of 7.83 ± 1.78 and 6.38 ± 1.89 % FAME respectively (Table 5.4.4.1.1).

Other UFA were present in minor amounts. C18:2 (*c*9,*c*12) linoleic acid (LA) comprised 1.97 ± 0.62 % FAME in control A10p10p cells and 2.15 ± 0.68 and 1.92 ± 0.95 % FAME following treatment with 21 and 75 µM CLA-mix. C18:3 α-linolenic acid (ALA) levels comprised 0.63 ± 0.05 % FAME in control A10p10p cells and 0.81 ± 0.26 and 0.58 ± 0.20 % FAME with 21 and 75 µM CLA-mix. Also, there was relatively no change in C20:4 arachidonic acid (AA) levels from 1.54 ± 0.24 % FAME in control A10p10p cells to 1.31 ± 0.34 and 1.32 ± 0.07 % FAME with 21 and 75 µM CLA-mix (Table 5.4.4.1.1).

While there was an apparent increase in *c*9,*t*11-CLA levels with both concentrations of CLA-mix from 0.03 ± 0.03 % FAME in control A10p10p cells to 0.66 ± 0.48 and 4.87 ± 3.62 % FAME, and in *t*10,*c*12-CLA levels from 0.02 ± 0.01 % FAME in control A10p10p cells to 0.41 ± 0.66 and 3.66 ± 6.06 % FAME following treatment with 21 and 75 µM CLA-mix, the differences were not found to be statistically

significant relative to control cells (Table 5.4.4.1.1). These low levels indicate that a higher proportion of both isomers are metabolised to other forms when delivered together in the CLA-mix than as single isomers.

There was relatively no change in the overall level of LCSFA from 58.25 ± 5.36 % FAME in control A10p10p cells to 60.32 ± 1.03 % FAME following 21 µM CLA-mix treatment and an apparent but not significant decrease to 45.37 ± 7.66 % FAME with 75 µM CLA-mix. The overall UFA level, at 23.09 ± 1.91 % FAME in control A10p10p cells, apparently increased to 26.66 ± 1.30 and 44.38 ± 11.06 % FAME with 21 and 75 µM CLA-mix. Corresponding MUFA levels apparently increased from 17.30 ± 1.66 % FAME in control A10p10p cells to 19.16 ± 0.21 and 28.18 ± 11.29 % FAME following 21 and 75 µM CLA-mix treatment (Table 5.4.4.1.2). The increases in both UFA and MUFA levels are mainly due to direct uptake of the CLA isomers, *c*9,*t*11 and *t*10,*c*12-CLA, and the increase in C16:1, palmitoleic acid.

There was a significant increase in the ratio of UFA/LCSFA from 0.40 ± 0.02 in control A10p10p cells to 0.44 ± 0.01 following treatment with 21 µM CLA-mix and an apparent but not significant increase in the ratio to 1.10 ± 0.37 with 75 µM CLA-mix. The corresponding values for the MUFA/LCSFA ratio were 0.30 ± 0.02 in control A10p10p cells and 0.32 ± 0.01 and 0.71 ± 0.35 following 21 and 75 µM CLA-mix treatment (Table 5.4.4.1.2).

The ratio of C16:1/C16:0 apparently increased from 0.48 ± 0.13 in control A10p10p cells to 0.54 ± 0.04 and 2.00 ± 1.48 in cells treated with 21 and 75 µM CLA-mix, suggesting up-regulation of the Δ9-desaturase enzyme. The ratio of C18:1/C18:0 remained relatively unchanged following CLA-mix treatment, with a value of 0.22 ± 0.04 in control A10p10p cells and 0.21 ± 0.05 and 0.26 ± 0.01 in cells treated with 21 and 75 µM CLA-mix (Table 5.4.4.1.2).

Table 5.4.4.1.1. Fatty acid composition of A10p10p cells treated with CLA-mix for 72 h.

Fatty Acid	Control	CLA 21 μM	CLA 75 μM
C6:0	0.38 ± 0.03	1.15 ± 0.76	0.39 ± 0.27
C8:0	8.60 ± 4.71	1.73 ± 1.00	2.08 ± 1.18
C10:0	1.61 ± 1.18	0.92 ± 0.72	1.26 ± 1.16
C11:0	5.43 ± 1.60	6.14 ± 1.39	4.42 ± 0.43
C12:0	0.09 ± 0.08	0.08 ± 0.05	0.10 ± 0.07
C13:0	2.55 ± 0.95	3.00 ± 1.02	2.00 ± 0.63
C14:0	2.19 ± 0.44	2.08 ± 0.68	2.34 ± 0.56
C14:1	0.38 ± 0.29	0.48 ± 0.44	0.04 ± 0.02
C15:0	0.26 ± 0.08	0.28 ± 0.10	0.27 ± 0.10
C15:1	0.06 ± 0.03	0.14 ± 0.08	0.03 ± 0.03
C16:0	19.65 ± 1.92	18.94 ± 1.15	14.58 ± 2.75
C16:1	8.98 ± 2.01	10.25 ± 1.16	21.11 ± 12.32
C18:0	34.98 ± 4.12	37.53 ± 0.51	24.69 ± 6.70
C18:1 (*t*9, *t*11 & *c*9)	7.60 ± 1.37	7.83 ± 1.78	6.38 ± 1.89
C18:2 LA	1.97 ± 0.62	2.15 ± 0.68	1.92 ± 0.95
C18:3 GLA	0.10 ± 0.10	0.36 ± 0.23	0.13 ± 0.07
C18:3(*c*9,12,15)	0.63 ± 0.05	0.81 ± 0.26	0.58 ± 0.20
C20:0	0.91 ± 0.19	0.84 ± 0.28	0.72 ± 0.22
C18:2 *c*9,*t*11-CLA	0.03 ± 0.03	0.66 ± 0.48	4.87 ± 3.62
C18:2 *t*10,*c*12-CLA	0.02 ± 0.01	0.42 ± 0.39	4.96 ± 2.97
C20:1 (*c*11)	0.02 ± 0.02	0.08 ± 0.04	0.08 ± 0.05
C18:2 *t*9,11-CLA	0.10 ± 0.05	0.32 ± 0.20	0.99 ± 0.80
C20:2 (*c*11,14)	0.13 ± 0.09	0.23 ± 0.12	0.17 ± 0.13
C20:3 DGLA	0.16 ± 0.02	0.17 ± 0.12	0.25 ± 0.14
C20:4 AA	1.54 ± 0.24	1.31 ± 0.34	1.32 ± 0.07
C22:1(*c*13)	0.10 ± 0.10	0.37 ± 0.19	0.52 ± 0.34
C23:0	0.08 ± 0.04	0.13 ± 0.08	0.51 ± 0.44
C22:2 (*c*13,16)	0.00 ± 0.00	0.03 ± 0.02	0.00 ± 0.00
C20:5 EPA	0.18 ± 0.18	0.15 ± 0.15	0.28 ± 0.27
C24:0	0.19 ± 0.19	0.51 ± 0.38	1.83 ± 1.71
C24:1(*c*15)	0.18 ± 0.16	0.08 ± 0.07	0.11 ± 0.00
C22:6 DHA	0.92 ± 0.19	0.80 ± 0.16	0.65 ± 0.26

Data represent Mean ± SEM of at least three experiments in triplicate expressed as a percentage of total cellular lipid composition. *Denotes results which are significantly different to untreated/control cells as determined by the Student's *t*-test ($p<0.005$).

Table 5.4.4.1.2. Fatty acid composition and ratios of A10p10p cells treated with CLA mix for 72 h.

Fatty Acids	Control	CLA mix 21 μM	CLA mix 75 μM
LCSFA	58.25 ± 5.36	60.32 ± 1.03	45.37 ± 7.66
UFA	23.09 ± 1.91	26.66 ± 1.30	44.38 ± 11.06
MUFA	17.30 ± 1.66	19.16 ± 0.21	28.18 ± 11.29
UFA/LCSFA	0.40 ± 0.02	0.44 ± 0.01*	1.10 ± 0.37
MUFA/LCSFA	0.30 ± 0.02	0.32 ± 0.01	0.71 ± 0.35
C16:1/C16:0	0.48 ± 0.13	0.54 ± 0.04	2.00 ± 1.48
C18:1 (*t*9, *t*11 & *c*9)/C18:0	0.22 ± 0.04	0.21 ± 0.05	0.26 ± 0.01

Data represent Mean ± SEM of at least three experiments in triplicate expressed as a percentage of total cellular lipid composition. *Denotes results which are significantly different to untreated/control cells as determined by the Student's *t*-test ($p<0.05$).

5.4.4.2 Effects of *c*9,*t*11-CLA on Fatty acid profiles of A10p10p cells

Flasks of $2x10^6$ of drug resistant A10p10p cells were seeded for 24 h before treatment with 21 and 75 μM *c*9,*t*11-CLA, incubated for 3 days and extracted and analysed as described earlier. Figure 5.4.4.2 illustrates a chromatogram from a typical injection of an A10p10p sample treated with (a) 21 and (b) 75 μM of the *c*9,*t*11-CLA isomer.

Table 5.4.4.2.1 lists the fatty acid composition of untreated A10p10p control cells and A10p10p cells treated with 21 and 75 μM *c*9,*t*11-CLA, while table 19 lists the summary of fatty acid composition and various fatty acid ratios of the above. The most abundant fatty acid in each treatment was the long chain saturated fatty acid (LCSFA) C18:0 stearic acid.

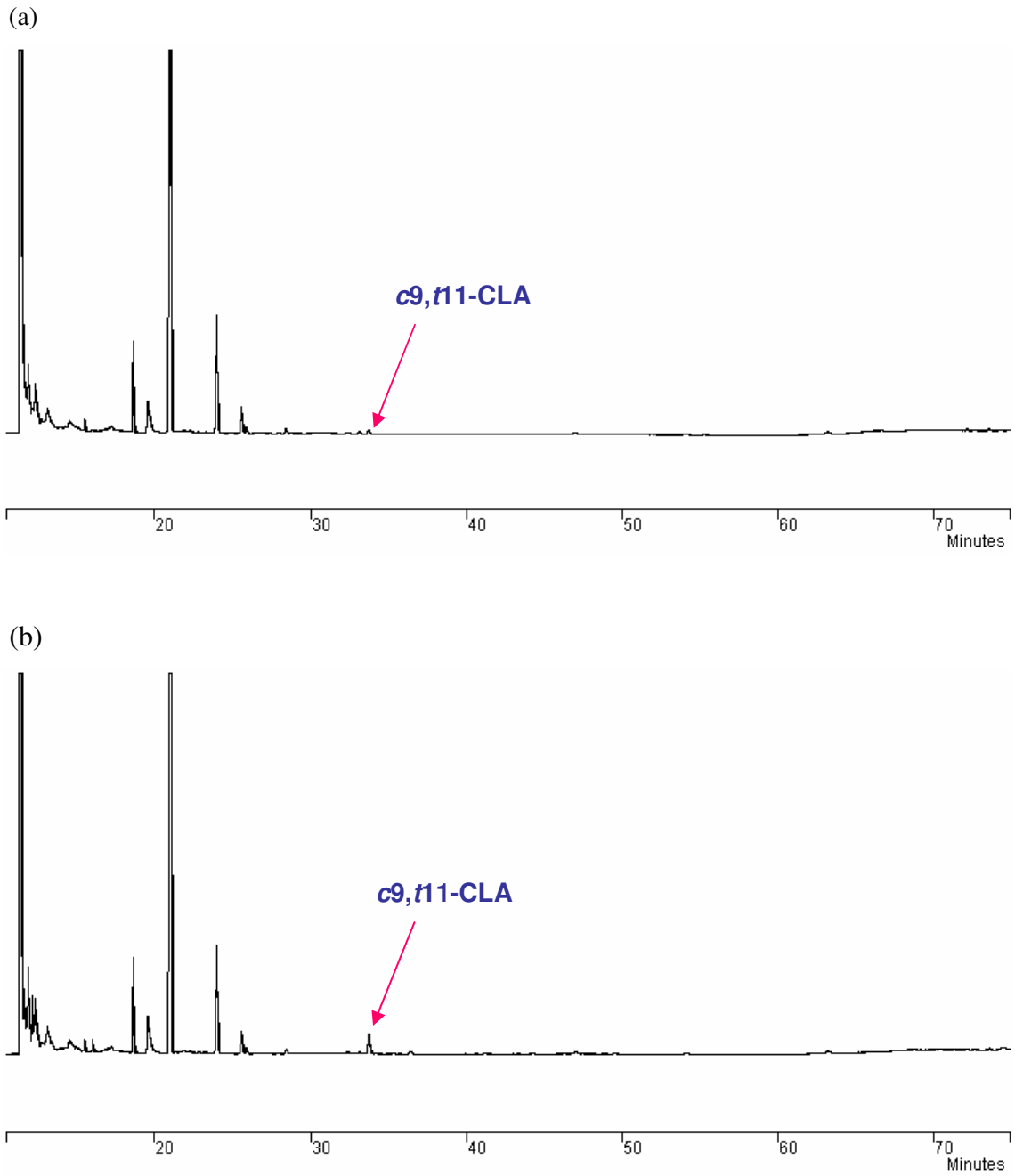


Figure 5.4.4.2. Typical chromatograms of fatty acid profiles of A10p10p cells treated with (a) 21 and (b) 75 µM *c*9,*t*11-CLA.

C18:0 stearic acid was relatively unchanged from 34.98 ± 4.12 % total cellular FAME in control A10p10p cells to 37.51 ± 4.25 % and 30.88 ± 3.20 % FAME in cells treated with 21 and 75 µM *c*9,*t*11-CLA. Another predominant LCSFA, C16:0 methyl palmitate was also relatively unchanged comprising 19.65 ± 1.92 % FAME in control A10p10p cells and 22.05 ± 0.84 and 18.14 ± 0.36 % FAME in cells treated with 21 and 75 µM *c*9,*t*11-CLA respectively. This indicates that the *c*9,*t*11-CLA isomer had little or no effect on FAS activity in A10p10p cells. Other LCSFA were present in minor amounts, including C14:0 methyl myristate at 2.19 ± 0.44 % FAME in control A10p10p cells and 2.20 ± 0.52 and 1.71 ± 0.31 % FAME following treatment with 21 and 75 µM *c*9,*t*11-CLA (Table 5.4.4.2.1).

The predominant mono-unsaturated fatty acid (MUFA), C16:1 methyl palmitoleate (palmitoleic acid), apparently increased from 8.98 ± 2.01 % total lipid in control A10p10p cells to 10.25 ± 1.16 % FAME following 21 µM *c*9,*t*11-CLA treatment, although reverting to 8.58 ± 3.50 % FAME with 75 µM *c*9,*t*11-CLA. Another abundant MUFA was the group of three C18:1 fatty acids elaidic (*t*-9), vaccenic (*t*-11), and oleic acid (*c*-9). Their combined proportion of total cellular lipids of 7.60 ± 1.37 % in control A10p10p cells remained relatively unchanged with treatments of 21 and 75 µM *c*9,*t*11-CLA, with levels of 7.52 ± 1.55 and 5.15 ± 0.64 % FAME respectively (Table 5.4.4.2.1).

Other UFA were present in minor amounts. C18:2 (*c*9,*c*12) linoleic acid (LA) comprised 1.97 ± 0.62 % FAME in control A10p10p cells and 2.02 ± 0.62 and 1.34 ± 0.17 % FAME following treatment with 21 and 75 µM *c*9,*t*11-CLA. At 75 µM *c*9,*t*11-CLA, there was a significant reduction in C20:3 dihomo-γ-linolenic acid (DGLA) from 0.16 ± 0.02 % in control A10p10p cells to 0.07 ± 0.04 % FAME. There was an apparent drop in C20:4 arachidonic acid (AA) levels from 1.54 ± 0.24 % FAME in A10p10p control cells to 1.11 ± 0.43 and 0.85 ± 0.47 % FAME with 21 and 75 µM *c*9,*t*11-CLA (Table 5.4.4.2.1). The decreases in both DGLA and AA levels suggest inhibition of Δ5/Δ6 desaturase and/or elongase activities. C18:3 α-linolenic acid (ALA) levels comprised 0.63 ± 0.05 % FAME in control A10p10p cells and 0.52 ± 0.26 and 0.40 ± 0.20 % FAME with 21 and 75 µM *c*9,*t*11-CLA (Table 5.4.4.2.1).

Following treatment with 21 and 75 µM *c*9,*t*11-CLA, there was an apparent but again not significant increase in *c*9,*t*11-CLA levels from 0.03 ± 0.03 % FAME in control A10p10p cells to 3.12 ± 0.94 and 20.19 ± 6.86 % of total cellular FAME respectively. There was also an apparent increase in *t*9,*t*11-CLA levels following treatment with 21 and 75 µM *c*9,*t*11-CLA, from 0.10 ± 0.05 % FAME in control A10p10p cells to 0.37 ± 0.20 and 1.00 ± 0.44 % FAME respectively (Table 5.4.4.2.1). This isomer is present in minor amounts in the *c*9,*t*11-CLA treatments.

There was relatively no change in the overall level of LCSFA from 58.25 ± 5.36 % FAME in control A10p10p cells to 63.09 ± 4.00 and 51.89 ± 3.51 % FAME following 21 and 75 µM *c*9,*t*11-CLA treatment. The overall UFA level, at 23.09 ± 1.91 % FAME in control A10p10p cells, apparently increased to 27.19 ± 3.24 and 39.51 ± 2.45 % FAME with 21 and 75 µM *c*9,*t*11-CLA. Corresponding MUFA levels were 17.30 ± 1.66 % FAME in control A10p10p cells and 18.30 ± 3.00 and 14.30 ± 3.76 % FAME following 21 and 75 µM *c*9,*t*11-CLA treatment (Table 5.4.4.2.2). The increases in both UFA and MUFA levels were due to direct uptake of the *c*9,*t*11-CLA isomer.

There was an apparent but not significant increase in the ratio of UFA/LCSFA from 0.40 ± 0.02 in control A10p10p cells to 0.44 ± 0.07 and 0.77 ± 0.09 with 21 and 75 µM *c*9,*t*11-CLA. The corresponding values for the MUFA/LCSFA ratio were 0.30 ± 0.02 in control A10p10p cells and 0.30 ± 0.06 and 0.27 ± 0.06 following 21 and 75 µM *c*9,*t*11-CLA treatment (Table 5.4.4.2.2).

The ratio of C16:1/C16:0 remained relatively unchanged following *c*9,*t*11-CLA treatment, with a value of 0.48 ± 0.13 in control A10p10p cells and 0.48 ± 0.20 and 0.48 ± 0.20 in cells treated with 21 and 75 µM *c*9,*t*11-CLA. The ratio of C18:1/C18:0 was also unchanged following *c*9,*t*11-CLA treatment, with a value of 0.22 ± 0.04 in control A10p10p cells and 0.21 ± 0.06 and 0.17 ± 0.02 in cells treated with 21 and 75 µM *c*9,*t*11-CLA (Table 5.4.4.2.2). Both ratios remaining unchanged indicates that the *c*9,*t*11-CLA isomer had no effect on the activity of the Δ9-desaturase enzyme in A10p10p cells.

Table 5.4.4.2.1. Fatty acid composition of A10p10p cells treated with *c*9,*t*11-CLA for 72 h.

Fatty Acid	Control	*c*9,*t*11-CLA 21 μM	*c*9,*t*11-CLA 75 μM
C6:0	0.38 ± 0.03	0.20 ± 0.15	0.22 ± 0.08
C8:0	8.60 ± 4.71	2.87 ± 1.52	2.97 ± 1.03
C10:0	1.61 ± 1.18	1.38 ± 1.07	1.24 ± 0.97
C11:0	5.43 ± 1.60	3.25 ± 1.64	2.49 ± 0.90
C12:0	0.09 ± 0.08	0.03 ± 0.02	0.13 ± 0.12
C13:0	2.55 ± 0.95	2.00 ± 0.55	1.55 ± 0.43
C14:0	2.19 ± 0.44	2.20 ± 0.52	1.71 ± 0.31
C14:1	0.38 ± 0.29	0.05 ± 0.05	0.13 ± 0.11
C15:0	0.26 ± 0.08	0.23 ± 0.05	0.13 ± 0.04
C15:1	0.06 ± 0.03	0.02 ± 0.02	0.02 ± 0.02
C16:0	19.65 ± 1.92	22.05 ± 0.84	18.14 ± 0.36
C16:1	8.98 ± 2.01	10.36 ± 3.89	8.58 ± 3.50
C18:0	34.98 ± 4.12	37.51 ± 4.25	30.88 ± 3.20
C18:1 (*t*9, *t*11 & *c*9)	7.60 ± 1.37	7.52 ± 1.55	5.15 ± 0.64
C18:2 LA	1.97 ± 0.62	2.02 ± 0.62	1.34 ± 0.17
C18:3 GLA	0.10 ± 0.10	0.16 ± 0.08	0.22 ± 0.13
C18:3 ALA	0.63 ± 0.05	0.52 ± 0.26	0.40 ± 0.20
C20:0	0.91 ± 0.19	0.71 ± 0.35	0.58 ± 0.20
C18:2 *c*9,*t*11-CLA	0.03 ± 0.03	3.12 ± 0.94	20.19 ± 6.86
C18:2 *t*10,*c*12-CLA	0.02 ± 0.01	0.00 ± 0.00	0.02 ± 0.02
C20:1 (*c*11)	0.02 ± 0.02	0.07 ± 0.04	0.08 ± 0.04
C18:2 *t*9,11-CLA	0.10 ± 0.05	0.37 ± 0.20	1.00 ± 0.44
C20:2 (*c*11,14)	0.13 ± 0.09	0.26 ± 0.15	0.26 ± 0.13
C20:3 DGLA	0.16 ± 0.02	0.14 ± 0.08	0.07 ± 0.04*
C20:4 AA	1.54 ± 0.24	1.11 ± 0.43	0.85 ± 0.47
C22:1(*c*13)	0.10 ± 0.10	0.31 ± 0.17	0.33 ± 0.19
C23:0	0.08 ± 0.04	0.06 ± 0.03	0.03 ± 0.01
C22:2 (*c*13,16)	0.00 ± 0.00	0.09 ± 0.09	0.11± 0.11
C20:5 EPA	0.18 ± 0.18	0.17 ± 0.17	0.18 ± 0.18
C24:0	0.19 ± 0.19	0.30 ± 0.23	0.34 ± 0.23
C24:1(*c*15)	0.18 ± 0.16	0.03 ± 0.02	0.10 ± 0.08
C22:6 DHA	0.92 ± 0.19	0.86 ± 0.23	0.51 ± 0.18

Data represent Mean ± SEM of at least three experiments in triplicate expressed as a percentage of total cellular lipid composition. *Denotes results which are significantly different to untreated/control cells as determined by the Student's *t*-test ($p \leq 0.05$).

Table 5.4.4.2.2. Fatty acid composition and ratios of A10p10p cells treated with *c*9,*t*11-CLA mix for 72 h.

Fatty Acids	Control	*c*9,*t*11-CLA 21 μM	*c*9,*t*11-CLA 75 μM
LCSFA	58.25 ± 5.36	63.09 ± 4.00	51.89 ± 3.51
UFA	23.09 ± 1.91	27.19 ± 3.24	39.51 ± 2.45
MUFA	17.30 ± 1.66	18.30 ± 3.00	14.30 ± 3.76
UFA/LCSFA	0.40 ± 0.02	0.44 ± 0.07	0.77 ± 0.09
MUFA/LCSFA	0.30 ± 0.02	0.30 ± 0.06	0.27 ± 0.06
C16:1/C16:0	0.48 ± 0.13	0.48 ± 0.20	0.48 ± 0.20
C18:1 (*t*9, *t*11 & *c*9)/C18:0	0.22 ± 0.04	0.21 ± 0.06	0.17 ± 0.02

Data represent Mean ± SEM of at least three experiments in triplicate expressed as a percentage of total cellular lipid composition. *Denotes results which are significantly different to untreated/control cells as determined by the Student's *t*-test ($p \leq 0.05$).

5.4.4.3 Effects of *t*10,*c*12-CLA on Fatty acid profiles of A10p10p cells

Flasks of $2x10^6$ of drug resistant A10p10p cells were seeded for 24 h before treatment with 21 and 75 μM *t*10,*c*12-CLA, incubated for 3 days and extracted and analysed as described earlier. Figure 5.4.4.3 illustrates a chromatogram from a typical injection of an A10p10p sample treated with (a) 21 and (b) 75 μM of the *t*10,*c*12-CLA isomer. Table 5.4.4.3.1 lists the fatty acid composition of untreated A10p10p control cells and A10p10p cells treated with 21 and 75 μM *t*10,*c*12-CLA, while table 21 lists the summary of fatty acid composition and various fatty acid ratios of the above. The most abundant fatty acid in each treatment was the long chain saturated fatty acid (LCSFA) C18:0 stearic acid.

C18:0 stearic acid was relatively unchanged from 34.98 ± 4.12 % total cellular FAME in control A10p10p cells to 40.28 ± 0.23 % and 30.55 ± 2.83 % FAME in cells treated with 21 and 75 μM *t*10,*c*12-CLA respectively (Table 5.4.4.3.1).

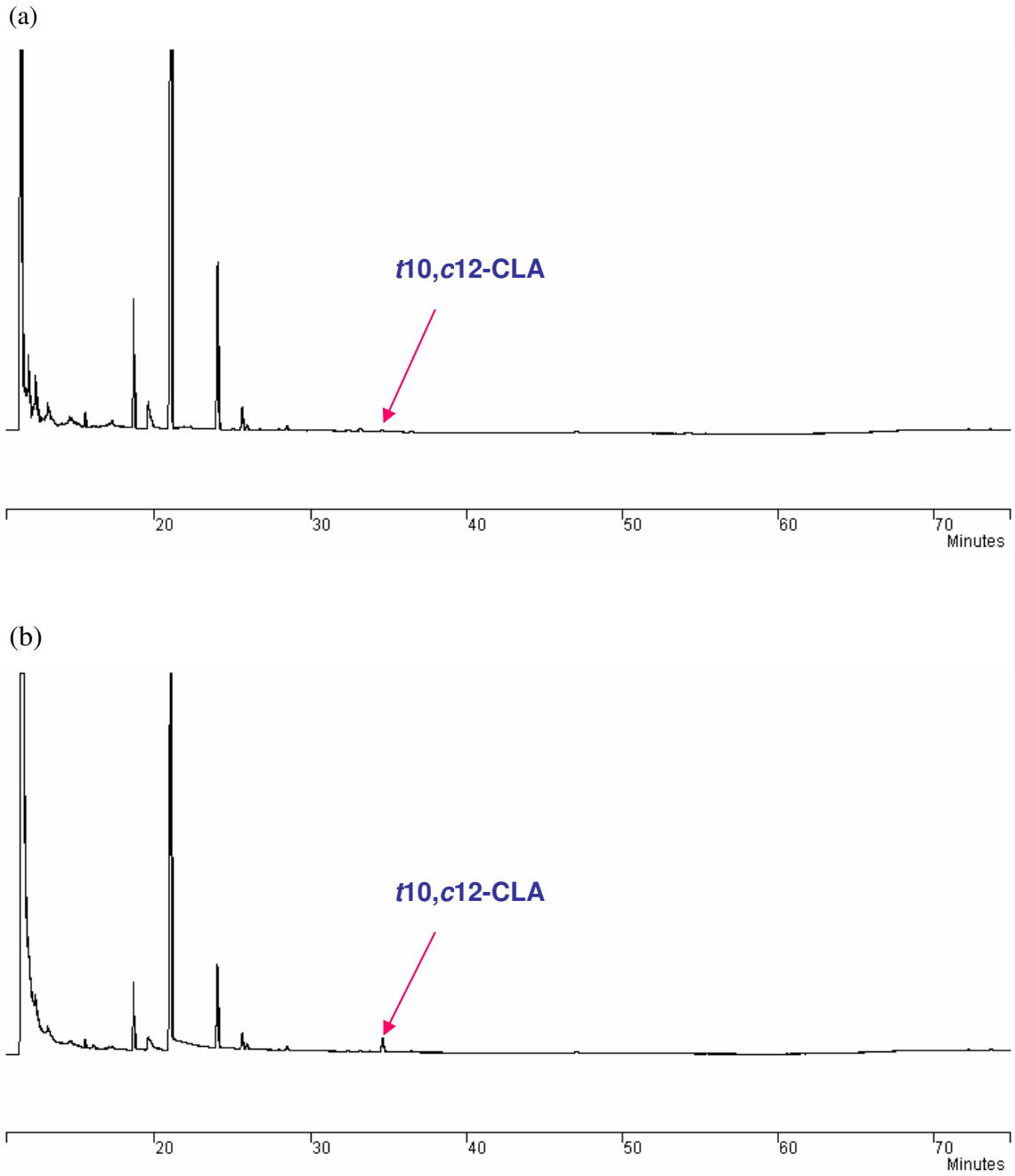


Figure 5.4.4.3. Typical chromatograms of fatty acid profiles of A10p10p cells treated with (a) 21 and (b) 75 μM *t*10,*c*12-CLA.

Another predominant LCSFA, C16:0 methyl palmitate was also relatively unchanged comprising 19.65 ± 1.92 % FAME in control A10p10p cells and 22.64 ± 0.83 and 18.05 ± 0.68 % FAME in cells treated with 21 and 75 µM *t*10,*c*12-CLA. This indicates *t*10,*c*12-CLA had little or no effect on FAS activity in A10p10p cells. Other LCSFA were present in minor amounts, including C6:0 methyl hexanoate at 0.38 ± 0.03 % FAME in control A10p10p cells, was significantly reduced to 0.10 ± 0.05 % FAME following treatment with 75 µM *t*10,*c*12-CLA (Table 5.4.4.3.1).

The predominant mono-unsaturated fatty acid (MUFA), C16:1 methyl palmitoleate (palmitoleic acid), remained relatively unchanged from 8.98 ± 2.01 % total lipid in control A10p10p cells to 10.28 ± 4.06 and 9.19 ± 4.04 % FAME following 21 and 75 µM *t*10,*c*12-CLA treatment respectively. Another abundant MUFA was the group of three C18:1 fatty acids elaidic (*t*-9), vaccenic (*t*-11), and oleic acid (*c*-9). Their combined proportion of total cellular lipids of 7.60 ± 1.37 % in control A10p10p cells was apparently reduced with 21 and 75 µM *t*10,*c*12-CLA to 5.37 ± 0.52 and 4.87 ± 0.49 % FAME respectively, suggesting inhibition of Δ9-desaturase by *t*10,*c*12-CLA (Table 5.4.4.3.1).

Other UFA were present in minor amounts. C18:2 (*c*9,*c*12) linoleic acid (LA) comprised 1.97 ± 0.62 % FAME in control A10p10p cells and 1.57 ± 0.13 and 1.46 ± 0.08 % FAME following treatment with 21 and 75 µM *t*10,*c*12-CLA. C18:3 α-linolenic acid (ALA) levels comprised 0.63 ± 0.05 % FAME in control A10p10p cells and 0.49 ± 0.25 and 0.42 ± 0.21 % FAME with 21 and 75 µM *t*10,*c*12-CLA (Table 5.4.4.3.1).

Treatments with 21 µM *t*10,*c*12-CLA resulted in direct uptake and significant increase in *t*10,*c*12-CLA levels from 0.02 ± 0.01 % in control cells to 1.96 ± 0.26 % of total cellular FAME and while not significant there was an apparent increase following 75 µM *t*10,*c*12-CLA treatment to 20.12 ± 6.48 % FAME. This indicates a lower rate of metabolism of the *t*10,*c*12-CLA isomer than when delivered as part of a CLA mixture of isomers and the lack of statistical significance is possibly due to this changeable rate of metabolism. There was a significant increase in levels of *t*9,*t*11-CLA following 75 µM *t*10,*c*12-CLA treatment from 0.10 ± 0.05 % to 2.04 ± 0.28 %

FAME (Table 5.4.4.3.1). The *t*9,*t*11-CLA isomer was present in minor amounts in the *t*10,*c*12-CLA treatment and clearly is not metabolised readily.

There was an apparent increase in the overall level of LCSFA from 58.25 ± 5.36 % FAME in control A10p10p cells to 66.89 ± 1.22 % FAME following treatment with 21 µM *t*10,*c*12-CLA and an apparent decrease with 75 µM *t*10,*c*12-CLA to 51.53 ± 3.69 % FAME. The overall UFA level, at 23.09 ± 1.91 % FAME in control A10p10p cells, remained unchanged at 23.18 ± 5.95 % FAME with 21 µM *t*10,*c*12-CLA and was increased significantly to 41.62 ± 1.34 % FAME with 75 µM *t*10,*c*12-CLA, due to direct uptake of *t*10,*c*12-CLA. Corresponding MUFA levels were 17.30 ± 1.66 % FAME in control A10p10p cells and 16.16 ± 4.78 and 14.50 ± 4.55 % FAME following 21 and 75 µM *t*10,*c*12-CLA treatment (Table 5.4.4.3.2).

While the ratio of UFA/LCSFA appeared to decrease from 0.40 ± 0.02 in control A10p10p cells to 0.35 ± 0.09 with 21 µM *t*10,*c*12-CLA treatment, the value increased significantly with 75 µM *t*10,*c*12-CLA to 0.82 ± 0.08, again due to direct *t*10,*c*12-CLA uptake. The corresponding values for the MUFA/LCSFA ratio were 0.30 ± 0.02 in control A10p10p cells and 0.24 ± 0.08 and 0.27 ± 0.07 following 21 and 75 µM *t*10,*c*12-CLA treatment (Table 5.4.4.3.2).

The ratio of C16:1/C16:0 remained relatively unchanged following *t*10,*c*12-CLA treatment, with a value of 0.48 ± 0.13 in control A10p10p cells and 0.47 ± 0.19 and 0.49 ± 0.21 in cells treated with 21 and 75 µM *t*10,*c*12-CLA. The ratio of C18:1/C18:0 apparently decreased following *t*10,*c*12-CLA treatment, with a value of 0.22 ± 0.04 in control A10p10p cells and 0.13 ± 0.01 and 0.16 ± 0.01 in cells treated with 21 and 75 µM *t*10,*c*12-CLA, suggesting possible inhibition of oleic acid production by Δ9-desaturase in cells treated with the *t*10,*c*12-CLA isomer (Table 5.4.4.3.2).

Table 5.4.4.3.1 Fatty acid composition of A10p10p cells treated with *t*10,*c*12-CLA for 72 h.

Fatty Acid	Control	*t*10,*c*12-CLA 21 μM	*t*10,*c*12-CLA 75 μM
C6:0	0.38 ± 0.03	0.66 ± 0.28	0.10 ± 0.05*
C8:0	8.60 ± 4.71	3.37 ± 1.33	1.20 ± 1.20
C10:0	1.61 ± 1.18	2.51 ± 2.10	1.71 ± 1.50
C11:0	5.43 ± 1.60	2.08 ± 1.45	2.38 ± 0.61
C12:0	0.09 ± 0.08	0.06 ± 0.05	0.04 ± 0.03
C13:0	2.55 ± 0.95	1.25 ± 0.32	1.44 ± 0.43
C14:0	2.19 ± 0.44	1.79 ± 0.11	1.84 ± 0.19
C14:1	0.38 ± 0.29	0.00 ± 0.00	0.01 ± 0.01
C15:0	0.26 ± 0.08	0.24 ± 0.11	0.20 ± 0.03
C15:1	0.06 ± 0.03	0.03 ± 0.03	0.08 ± 0.06
C16:0	19.65 ± 1.92	22.64 ± 0.83	18.05 ± 0.68
C16:1	8.98 ± 2.01	10.28 ± 4.06	9.19 ± 4.04
C18:0	34.98 ± 4.12	40.28 ± 0.23	30.55 ± 2.83
C18:1 (*t*9, *t*11 & *c*9)	7.60 ± 1.37	5.37 ± 0.52	4.87 ± 0.49
C18:2 LA	1.97 ± 0.62	1.57 ± 0.13	1.46 ± 0.08
C18:3 GLA	0.10 ± 0.10	0.22 ± 0.11	0.21 ± 0.13
C18:3 ALA	0.63 ± 0.05	0.49 ± 0.25	0.42 ± 0.21
C20:0	0.91 ± 0.19	0.85 ± 0.29	0.63 ± 0.26
C18:2 *c*9,*t*11-CLA	0.03 ± 0.03	0.16 ± 0.08	0.64 ± 0.32
C18:2 *t*10,*c*12-CLA	0.02 ± 0.01	1.96 ± 0.26*	20.12 ± 6.48^{+}
C20:1 (*c*11)	0.02 ± 0.02	0.04 ± 0.02	0.03 ± 0.03
C18:2 *t*9,11-CLA	0.10 ± 0.05	0.29 ± 0.16	2.04 ± 0.28*
C20:2 (*c*11,14)	0.13 ± 0.09	0.22 ± 0.12	0.07 ± 0.05
C20:3 DGLA	0.16 ± 0.02	0.14 ± 0.08	0.13 ± 0.07
C20:4 AA	1.54 ± 0.24	1.08 ± 0.55	1.06 ± 0.43
C22:1(*c*13)	0.10 ± 0.10	0.32 ± 0.17	0.29 ± 0.15
C23:0	0.08 ± 0.04	0.12 ± 0.06	0.09 ± 0.04
C22:2 (*c*13,16)	0.00 ± 0.00	0.03 ± 0.02	0.06 ± 0.06
C20:5 EPA	0.18 ± 0.18	0.16 ± 0.16	0.18 ± 0.18
C24:0	0.19 ± 0.19	0.97 ± 0.53	0.18 ± 0.11
C24:1(*c*15)	0.18 ± 0.16	0.15 ± 0.14	0.05 ± 0.04
C22:6 DHA	0.92 ± 0.19	0.65 ± 0.11	0.68 ± 0.03

Data represent Mean ± SEM of at least three experiments in triplicate expressed as a percentage of total cellular lipid composition. *Denotes results which are significantly different to untreated/control cells as determined by the Student's *t*-test ($p<0.05$). $^{+}$Denotes $p=0.088$

Table 5.4.4.3.2. Fatty acid composition and ratios of A10p10p cells treated with *t*10,*c*12-CLA for 72 h.

Fatty Acids	Control	*t*10,*c*12-CLA 21 μM	*t*10,*c*12-CLA 75 μM
LCSFA	58.25 ± 5.36	66.89 ± 1.22	51.53 ± 3.69
UFA	23.09 ± 1.91	23.18 ± 5.95	41.62 ± 1.34*
MUFA	17.30 ± 1.66	16.16 ± 4.78	14.50 ± 4.55
UFA/LCSFA	0.40 ± 0.02	0.35 ± 0.09	0.82 ± 0.08*
MUFA/LCSFA	0.30 ± 0.02	0.24 ± 0.08	0.27 ± 0.07
C16:1/C16:0	0.48 ± 0.13	0.47 ± 0.19	0.49 ± 0.21
C18:1 (*t*9, *t*11 & *c*9)/C18:0	0.22 ± 0.04	0.13 ± 0.01	0.16 ± 0.01

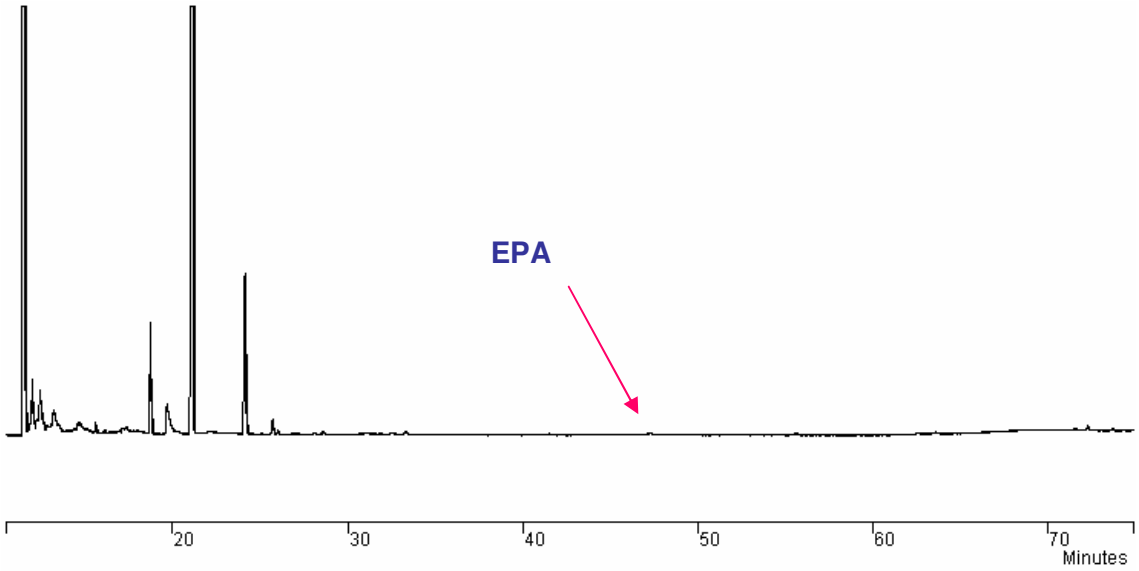
Data represent Mean ± SEM of at least three experiments in triplicate expressed as a percentage of total cellular lipid composition. *Denotes results which are significantly different to untreated/control cells as determined by the Student's *t*-test ($p \leq 0.05$).

5.4.4.4 Effects of EPA on Fatty acid profiles of A10p10p cells

Flasks of $2x10^6$ of drug resistant A10p10p cells were seeded for 24 h before treatment with 21 and 75 μM eicosapentanoic acid (EPA), incubated for 3 days and extracted and analysed as described earlier. Figure 5.4.4.4 illustrates a chromatogram from a typical injection of an A10p10p sample treated with (a) 21 and (b) 75 μM of EPA.

Table 5.4.4.4.1 lists the fatty acid composition of untreated A10p10p control cells and A10p10p cells treated with 21 and 75 μM EPA, while table 23 lists the summary of fatty acid composition and various fatty acid ratios of the above. The most abundant fatty acid in each treatment was the long chain saturated fatty acid (LCSFA) C18:0 stearic acid.

(a)



(b)

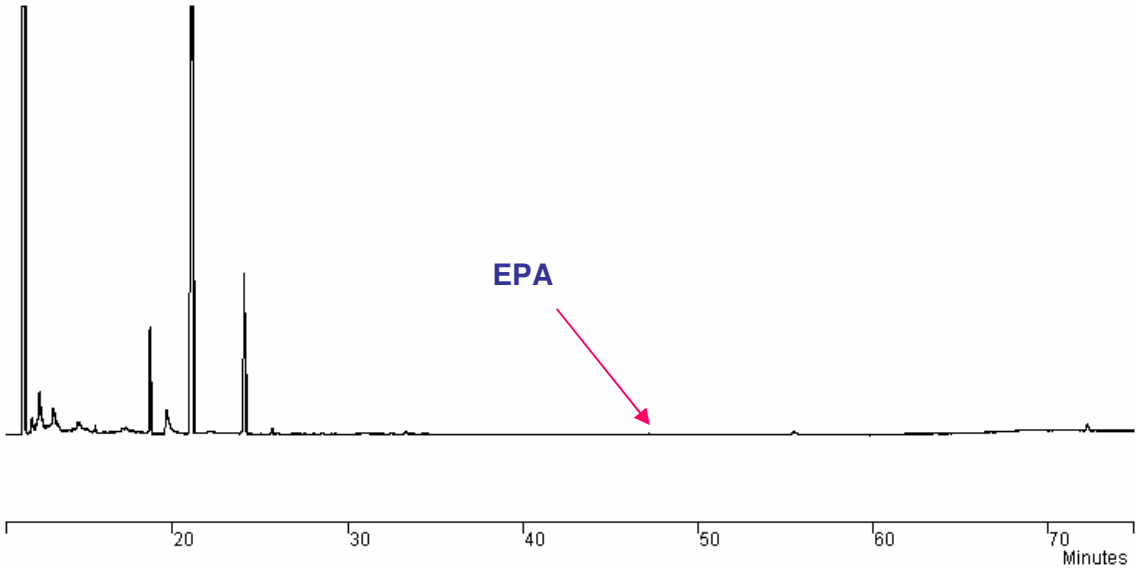


Figure 5.4.4.4. Typical chromatograms of fatty acid profiles of A10p10p cells treated with (a) 21 and (b) 75 µM EPA.

C18:0 stearic acid was relatively unchanged from 34.98 ± 4.12 % total cellular FAME in control A10p10p cells to 39.96 ± 0.92 % and 38.90 ± 3.39 % FAME in cells treated with 21 and 75 µM EPA respectively. Another predominant LCSFA, C16:0 methyl palmitate was significantly increased following 21 µM EPA comprising 19.65 ± 1.92 % FAME in control A10p10p cells and 23.19 ± 1.43 % FAME in cells treated with 21 µM EPA. However, the level was relatively unchanged following treatment with 75 µM EPA at 19.80 ± 1.58 % FAME (Table 5.4.4.4.1). This indicates EPA had little or no effect on FAS activity in A10p10p cells.

Other LCSFA were present in minor amounts. C6:0 methyl hexanoate at 0.38 ± 0.03 % FAME in control A10p10p cells, was significantly reduced to 0.07 ± 0.07 % FAME following treatment with 75 µM EPA. C14:0 methyl myristate levels were significantly reduced from 2.19 ± 0.44 % FAME in control A10p10p cells to 1.39 ± 0.43 % FAME with 75 µM EPA (Table 5.4.4.4.1).

The predominant mono-unsaturated fatty acid (MUFA), C16:1 methyl palmitoleate (palmitoleic acid), apparently increased from 8.98 ± 2.01 % total lipid in control A10p10p cells to 14.37 ± 4.10 and 18.64 ± 2.55 % FAME following 21 and 75 µM EPA treatment respectively. Another abundant MUFA was the group of three C18:1 fatty acids elaidic (*t*-9), vaccenic (*t*-11), and oleic acid (*c*-9). Their combined proportion of total cellular lipids of 7.60 ± 1.37 % in control A10p10p cells was apparently reduced to 5.53 ± 1.02 with 21 µM EPA and significantly reduced to 2.19 ± 0.49 % FAME following treatment with 75 µM EPA (Table 5.4.4.4.1). The increase in C16:1 and decrease in C18:1 suggests different substrate specificity of the Δ9-desaturase enzyme in A10p10p cells and diversion of monounsaturated fatty acid synthesis away from oleic acid towards palmitoleic acid.

Other UFA were present in minor amounts. C18:2 (*c*9,*c*12) linoleic acid (LA) comprised 1.97 ± 0.62 % FAME in control A10p10p cells and was apparently reduced to 1.66 ± 0.55 and 0.64 ± 0.29 % FAME following treatment with 21 and 75 µM EPA. C20:4 arachidonic acid (AA) levels were significantly reduced from 1.54 ± 0.24 % FAME in control A10p10p cells to 0.54 ± 0.15 % FAME with 75 µM EPA.

Table 5.4.4.4.1. Fatty acid composition of A10p10p cells treated with EPA for 72 h.

Fatty Acid	Control	EPA 21 μM	EPA 75 μM
C6:0	0.38 ± 0.03	0.06 ± 0.06	0.07 ± 0.07*
C8:0	8.60 ± 4.71	0.85 ± 0.69	0.51 ± 0.26
C10:0	1.61 ± 1.18	0.79 ± 0.79	0.00 ± 0.00
C11:0	5.43 ± 1.60	4.37 ± 0.22	4.33 ± 0.43
C12:0	0.09 ± 0.08	0.00 ± 0.00	0.00 ± 0.00
C13:0	2.55 ± 0.95	0.50 ± 0.24	0.56 ± 0.31
C14:0	2.19 ± 0.44	2.34 ± 0.50	1.39 ± 0.43*
C14:1	0.38 ± 0.29	0.13 ± 0.13	0.55 ± 0.55
C15:0	0.26 ± 0.08	0.15 ± 0.04	0.11 ± 0.06
C15:1	0.06 ± 0.03	0.00 ± 0.00	0.00 ± 0.00
C16:0	19.65 ± 1.92	23.19 ± 1.43*	19.80 ± 1.58
C16:1	8.98 ± 2.01	14.37 ± 4.10	18.64 ± 2.55
C18:0	34.98 ± 4.12	39.96 ± 0.92	38.90 ± 3.39
C18:1 (*t*9, *t*11 & *c*9)	7.60 ± 1.37	5.53 ± 1.02	2.19 ± 0.49*
C18:2 LA	1.97 ± 0.62	1.66 ± 0.55	0.64 ± 0.29
C18:3 GLA	0.10 ± 0.10	0.27 ± 0.16	0.38 ± 0.17
C18:3(*c*9,12,15)	0.63 ± 0.05	0.52 ± 0.19	0.71 ± 0.17
C20:0	0.91 ± 0.19	0.73 ± 0.25	0.96 ± 0.15
C18:2 *c*9,*t*11-CLA	0.03 ± 0.03	0.16 ± 0.10	0.11 ± 0.08
C18:2 *t*10,*c*12-CLA	0.02 ± 0.01	0.10 ± 0.06	0.09 ± 0.06
C20:1 (*c*11)	0.02 ± 0.02	0.03 ± 0.02	0.04 ± 0.01
C18:2 *t*9,11-CLA	0.10 ± 0.05	0.06 ± 0.03	0.13± 0.06
C20:2 (*c*11,14)	0.13 ± 0.09	0.24 ± 0.14	0.23 ± 0.15
C20:3 DGLA	0.16 ± 0.02	0.18 ± 0.08	0.08 ± 0.06
C20:4 AA	1.54 ± 0.24	0.84 ± 0.29	0.54 ± 0.15*
C22:1(*c*13)	0.10 ± 0.10	0.16 ± 0.08	0.17 ± 0.11
C23:0	0.08 ± 0.04	1.18 ± 0.41	2.66 ± 0.75
C22:2 (*c*13,16)	0.00 ± 0.00	0.22 ± 0.16	0.27 ± 0.17
C20:5 EPA	0.18 ± 0.18	0.33 ± 0.19	0.29 ± 0.26
C24:0	0.19 ± 0.19	0.34 ± 0.18	0.52 ± 0.21
C24:1(*c*15)	0.18 ± 0.16	0.04 ± 0.03	0.04 ± 0.02
C22:6 DHA	0.92 ± 0.19	0.69 ± 0.14	5.07 ± 4.89

Data represent Mean ± SEM of at least three experiments in triplicate expressed as a percentage of total cellular lipid composition. *Denotes results which are significantly different to untreated/control cells as determined by the Student's *t*-test ($p<0.05$).

C18:3 γ-linolenic acid (GLA) was apparently increased from 0.10 ± 0.10 % FAME in control A10p10p cells to 0.27 ± 0.16 and 0.38 ± 0.17 % FAME with 21 and 75 µM EPA (Table 5.4.4.4.1). These changes indicate inhibition of Δ5 desaturase and/or elongase enzyme activities by EPA or its metabolites.

C18:3 α-linolenic acid (ALA) levels comprised 0.63 ± 0.05 % FAME in control A10p10p cells and 0.52 ± 0.19 and 0.71 ± 0.17 % FAME with 21 and 75 µM EPA. Treatments with EPA did not result in direct uptake and EPA levels remained relatively unchanged from 0.18 ± 0.18 % in control A10p10p cells to 0.33 ± 0.19 and 0.29 ± 0.26 % of total cellular FAME with 21 and 75 µM EPA, indicating rapid metabolism following uptake (Table 5.4.4.4.1).

There was an apparent increase in the overall level of LCSFA from 58.25 ± 5.36 % FAME in control A10p10p cells to 67.89 ± 1.50 % FAME following treatment with 21 µM EPA and a significant increase with 75 µM EPA to 64.35 ± 5.05 % FAME. This increase was due to a combination of minor changes over a range of fatty acids. The overall UFA level, at 23.09 ± 1.91 % FAME in control A10p10p cells was apparently increased to 25.52 ± 3.25 and 30.19 ± 5.35 % FAME 21 and 75 µM EPA respectively. Total MUFA levels were apparently increased from 17.30 ± 1.66 % FAME in control A10p10p cells to 20.23 ± 3.13 and 21.58 ± 2.82 % FAME following 21 and 75 µM EPA treatment (Table 5.4.4.4.2). The increases in overall UFA and MUFA level were due to a combination of changes including the increase in C16:1 palmitoleic acid and not due to direct uptake of EPA.

While the ratio of UFA/LCSFA was relatively unchanged from 0.40 ± 0.02 in control A10p10p cells to 0.38 ± 0.05 with 21 µM EPA treatment, the value apparently increased with 75 µM EPA to 0.49 ± 0.12. The corresponding values for the MUFA/LCSFA ratio were 0.30 ± 0.02 in control A10p10p cells and 0.30 ± 0.05 and 0.34 ± 0.05 following 21 and 75 µM EPA treatment (Table 5.4.4.4.2).

The ratio of C16:1/C16:0 apparently increased following EPA treatment, with a value of 0.48 ± 0.13 in control A10p10p cells and 0.65 ± 0.19 and 0.95 ± 0.11 in cells treated with 21 and 75 µM EPA. The ratio of C18:1/C18:0 was significantly

reduced with EPA treatment, with a value of 0.22 ± 0.04 in control A10p10p cells and 0.14 ± 0.02 and 0.06 ± 0.01 in cells treated with 21 and 75 µM EPA (Table 5.4.4.4.2). The increase in the C16:1/C16:0 ratio and decrease in C18:1/C18:0 suggests different substrate specificity of Δ9-desaturase and inhibition of oleic acid modulation in favour of palmitoleic acid in EPA treated cells.

Table 5.4.4.4.2. Fatty acid composition and ratios of A10p10p cells treated with EPA for 72 h.

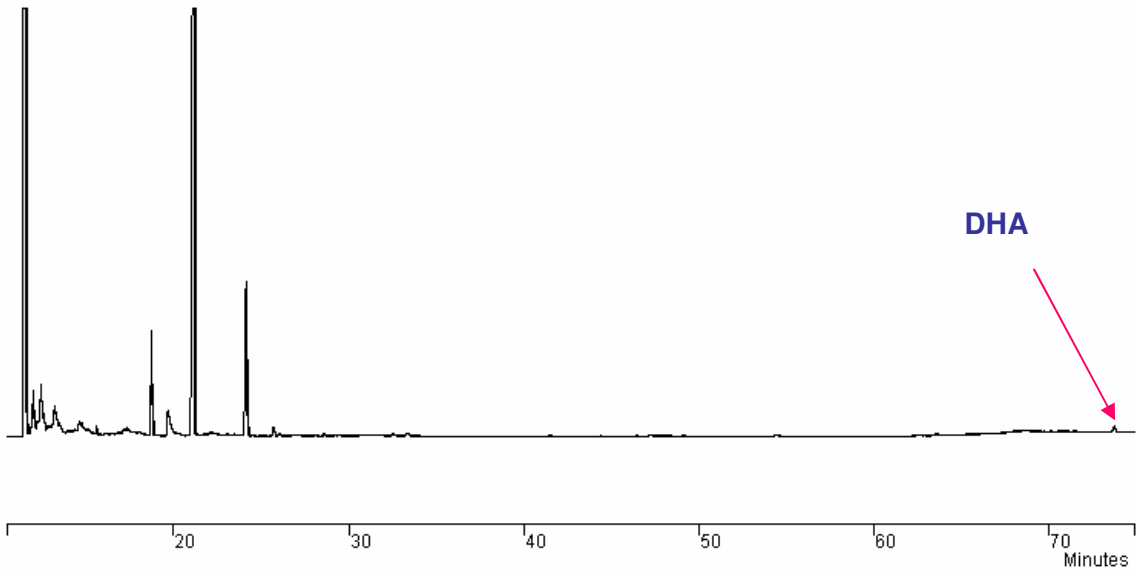
Fatty Acids	Control	EPA 21 µM	EPA 75 µM
LCSFA	58.25 ± 5.36	67.89 ± 1.50	64.35 ± 5.05*
UFA	23.09 ± 1.91	25.52 ± 3.25	30.19 ± 5.35
MUFA	17.30 ± 1.66	20.23 ± 3.13	21.58 ± 2.82
UFA/LCSFA	0.40 ± 0.02	0.38 ± 0.05	0.49 ± 0.12
MUFA/LCSFA	0.30 ± 0.02	0.30 ± 0.05	0.34 ± 0.05
C16:1/C16:0	0.48 ± 0.13	0.65 ± 0.19	0.95 ± 0.11
C18:1 (*t*9, *t*11 & *c*9)/C18:0	0.22 ± 0.04	0.14 ± 0.02*	0.06 ± 0.01*

Data represent Mean ± SEM of at least three experiments in triplicate expressed as a percentage of total cellular lipid composition. *Denotes results which are significantly different to untreated/control cells as determined by the Student's *t*-test ($p<0.05$).

5.4.4.5 Effects of DHA on Fatty acid profiles of A10p10p cells

Flasks of $2x10^6$ of drug resistant A10p10p cells were seeded for 24 h before treatment with 21 and 75 µM docosahexanoic acid (DHA), incubated for 3 days and extracted and analysed as described earlier. Figure 5.4.4.5 illustrates a chromatogram from a typical injection of an A10p10p sample treated with (a) 21 and (b) 75 µM of DHA. Table 5.4.4.5.1 lists the fatty acid composition of untreated A10p10p control cells and A10p10p cells treated with 21 and 75 µM DHA, while table 5.4.4.5.2 lists the summary of fatty acid composition and various fatty acid ratios of the above. The most abundant fatty acid in each treatment was the long chain saturated fatty acid (LCSFA) C18:0 stearic acid.

(a)



(b)

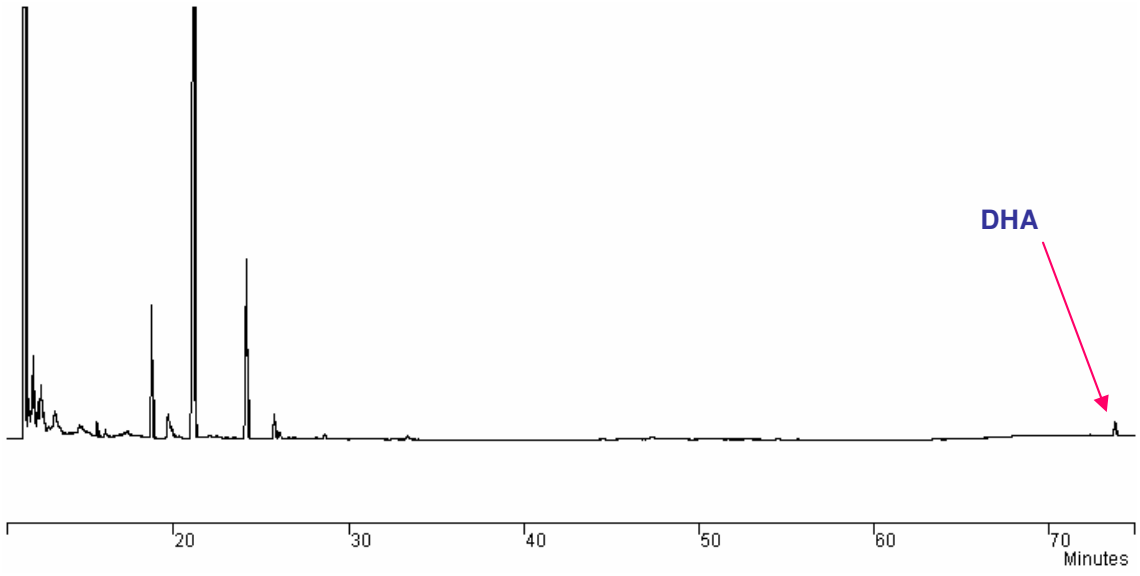


Figure 5.4.4.5. Typical chromatograms of fatty acid profiles of A10p10p cells treated with (a) 21 and (b) 75 µM DHA.

Table 5.4.4.5.1. Fatty acid composition of A10p10p cells treated with DHA for 72 h.

Fatty Acid	Control	DHA 21 μM	DHA 75 μM
C6:0	0.38 ± 0.03	0.17 ± 0.10	0.47 ± 0.47
C8:0	8.60 ± 4.71	1.78 ± 0.89	0.56 ± 0.56
C10:0	1.61 ± 1.18	0.00 ± 0.00	0.17 ± 0.09
C11:0	5.43 ± 1.60	4.60 ± 1.18	5.38 ± 1.05
C12:0	0.09 ± 0.08	0.00 ± 0.00	0.00 ± 0.00
C13:0	2.55 ± 0.95	1.11 ± 0.51	1.74 ± 0.85
C14:0	2.19 ± 0.44	1.45 ± 0.06	0.86 ± 0.36
C14:1	0.38 ± 0.29	0.32 ± 0.32	0.28 ± 0.21
C15:0	0.26 ± 0.08	0.16 ± 0.03	0.10 ± 0.02
C15:1	0.06 ± 0.03	0.08 ± 0.05	0.08 ± 0.08
C16:0	19.65 ± 1.92	20.35 ± 1.56	19.73 ± 2.40
C16:1	8.98 ± 2.01	18.86 ± 5.37	19.55 ± 6.97
C18:0	34.98 ± 4.12	36.73 ± 3.62	40.79 ± 4.26
C18:1 (*t*9, *t*11 & *c*9)	7.60 ± 1.37	4.12 ± 0.87	1.87 ± 0.67
C18:2 LA	1.97 ± 0.62	1.07 ± 0.20	0.40 ± 0.19
C18:3 GLA	0.10 ± 0.10	0.30 ± 0.16	0.42 ± 0.17
C18:3 AA	0.63 ± 0.05	0.86 ± 0.04	1.02 ± 0.05*
C20:0	0.91 ± 0.19	1.02 ± 0.09	1.06 ± 0.19
C18:2 *c*9,*t*11-CLA	0.03 ± 0.03	0.05 ± 0.03	0.11 ± 0.11
C18:2 *t*10,*c*12-CLA	0.02 ± 0.01	0.10 ± 0.10	0.03 ± 0.01
C20:1 (*c*11)	0.02 ± 0.02	0.00 ± 0.00	0.03 ± 0.02
C18:2 *t*9,11-CLA	0.10 ± 0.05	0.11 ± 0.09	0.03 ± 0.03
C20:2 (*c*11,14)	0.13 ± 0.09	0.20 ± 0.07	0.19 ± 0.10
C20:3 DGLA	0.16 ± 0.02	0.19 ± 0.06	0.13 ± 0.05
C20:4 AA	1.54 ± 0.24	1.26 ± 0.18	0.06 ± 0.07
C22:1(*c*13)	0.10 ± 0.10	0.52 ± 0.15	0.57 ± 0.18
C23:0	0.08 ± 0.04	0.31 ± 0.05	0.08 ± 0.07
C22:2 (*c*13,16)	0.00 ± 0.00	0.02 ± 0.02	0.05 ± 0.05
C20:5 EPA	0.18 ± 0.18	0.29 ± 0.17	0.23 ± 0.22
C24:0	0.19 ± 0.19	0.44 ± 0.09	0.64 ± 0.13
C24:1(*c*15)	0.18 ± 0.16	0.07 ± 0.04	0.04 ± 0.02
C22:6 DHA	0.92 ± 0.19	3.42 ± 0.69	3.02 ± 1.22

Data represent Mean ± SEM of at least three experiments in triplicate expressed as a percentage of total cellular lipid composition. *Denotes results which are significantly different to untreated/control cells as determined by the Student's *t*-test ($p<0.05$).

C18:0 stearic acid was relatively unchanged from 34.98 ± 4.12 % total cellular FAME in control A10p10p cells to 36.73 ± 3.62 % and 40.79 ± 4.26 % FAME in cells treated with 21 and 75 µM DHA respectively. Another predominant LCSFA, C16:0 methyl palmitate was also relatively unchanged following treatment, comprising 19.65 ± 1.92 % FAME in control A10p10p cells and 20.35 ± 1.56 and 19.73 ± 2.40 % FAME in cells treated with 21 and 75 µM DHA (Table 5.4.4.5.1). This indicates DHA had little or no effect on FAS activity in A10p10p cells.

Other saturated fatty acids were present in minor amounts. C13:0 methyl tetradecanoate at 2.55 ± 0.95 % FAME in control A10p10p cells, was apparently reduced to 1.11 ± 0.51 and 1.74 ± 0.85 % FAME following treatment with 21 and 75 µM DHA. C14:0 methyl myristate levels were apparently reduced from 2.19 ± 0.44 % FAME in control A10p10p cells to 1.45 ± 0.06 and 0.86 ± 0.36 % FAME with 21 and 75 µM DHA (Table 5.4.4.5.1).

The predominant mono-unsaturated fatty acid (MUFA), C16:1 methyl palmitoleate (palmitoleic acid), apparently increased from 8.98 ± 2.01 % total lipid in control A10p10p cells to 18.86 ± 5.37 and 19.55 ± 6.97 % FAME following 21 and 75 µM DHA treatment respectively (Table 5.4.4.5.1).

Another abundant MUFA was the group of three C18:1 fatty acids elaidic (*t*-9), vaccenic (*t*-11), and oleic acid (*c*-9). Their combined proportion of total cellular lipids of 7.60 ± 1.37 % in control A10p10p cells was apparently reduced to 4.12 ± 0.87 and 1.87 ± 0.67 % FAME following 21 and 75 µM DHA treatment (Table 5.4.4.5.1). The increase in C16:1 and decrease in C18:1 represent a decreased level of oleic acid production in favour of palmitoleic acid in DHA treated A10p10p cells.

Other UFA were present in minor amounts. C18:2 (*c*9,*c*12) linoleic acid (LA) comprised 1.97 ± 0.62 % FAME in control A10p10p cells and was apparently reduced to 1.07 ± 0.20 and 0.40 ± 0.19 % FAME following treatment with 21 and 75 µM DHA. C20:4 arachidonic acid (AA) levels were apparently reduced from 1.54 ± 0.24 % FAME in control A10p10p cells to 1.26 ± 0.18 and 0.06 ± 0.07 % FAME with 21 and 75 µM DHA, suggesting inhibition of Δ5/Δ6 desaturase and/or elongase

activities by DHA (Table 24). C18:3 α-linolenic acid (ALA) levels comprised 0.63 ± 0.05 % FAME in control A10p10p cells and was apparently increased to 0.86 ± 0.04 % FAME with 21 µM DHA and significantly increased to 1.02 ± 0.05 % FAME with 75 µM DHA treatment (Table 5.4.4.5.1). This also suggests inhibition of Δ5/Δ6 desaturase and/or elongase activities by DHA, possibly due to negative feedback, preventing conversion of ALA to more unsaturated longer chain metabolites including EPA and DHA.

Treatments with DHA did not result in significant direct uptake, although there was an apparent increase in DHA levels from 0.92 ± 0.19 % in control A10p10p cells to 3.42 ± 0.69 and 3.02 ± 1.22 % of total cellular FAME with 21 and 75 µM DHA treatments (Table 5.4.4.5.1). These levels indicate rapid metabolism of DHA in A10p10p cells.

There was an apparent increase in the overall level of LCSFA from 58.25 ± 5.36 % FAME in control A10p10p cells to 60.49 ± 5.32 following treatment with 21 µM DHA and an increase to 63.32 ± 6.80 % with 75 µM DHA. The overall UFA level, at 23.09 ± 1.91 % FAME in control A10p10p cells, was apparently increased to 31.85 ± 5.65 and 28.37 ± 8.02 % FAME following 21 and 75 µM DHA treatment respectively. Total MUFA levels were apparently increased from 17.30 ± 1.66 % FAME in control A10p10p cells to 23.97 ± 5.01 and 22.39 ± 6.83 % FAME following 21 and 75 µM DHA treatment (Table 5.4.4.5.2). These increases in UFA and MUFA levels were due to a combination of direct DHA uptake and range of minor changes in individual fatty acid levels.

The ratio of UFA/LCSFA was apparently increased from 0.40 ± 0.02 in control A10p10p cells to 0.55 ± 0.16 and 0.49 ± 0.20 with 21 and 75 µM DHA treatments. The corresponding values for the MUFA/LCSFA ratio were 0.30 ± 0.02 in control A10p10p cells and 0.42 ± 0.13 and 0.39 ± 0.17 following 21 and 75 µM DHA treatment (Table 5.4.4.5.2).

The ratio of C16:1/C16:0 apparently increased following DHA treatment, with a value of 0.48 ± 0.13 in control A10p10p cells and 0.98 ± 0.36 and 1.13 ± 0.56 in

cells treated with 21 and 75 μM DHA. The ratio of C18:1/C18:0 was appartently reduced with DHA treatment, with a value of 0.22 ± 0.04 in control A10p10p cells and 0.11 ± 0.02 and 0.05 ± 0.02 in cells treated with 21 and 75 μM DHA (Table 5.4.4.5.2).

The increase in the C16:1/C16:0 ratio and decrease in C18:1/C18:0 suggests different substrate specificity of Δ9-desaturase and inhibition of oleic acid modulation in favour of palmitoleic acid in DHA-treated A10p10p cells (Table 5.4.4.5.2).

Table 5.4.4.5.2. Fatty acid composition and ratios of A10p10p cells treated with DHA for 72 h.

Fatty Acids	Control	DHA 21 μM	DHA 75 μM
LCSFA	58.25 ± 5.36	60.49 ± 5.32	63.32 ± 6.80
UFA	23.09 ± 1.91	31.85 ± 5.65	28.37 ± 8.02
MUFA	17.30 ± 1.66	23.97 ± 5.01	22.39 ± 6.83
UFA/LCSFA	0.40 ± 0.02	0.55 ± 0.16	0.49 ± 0.20
MUFA/LCSFA	0.30 ± 0.02	0.42 ± 0.13	0.39 ± 0.17
C16:1/C16:0	0.48 ± 0.13	0.98 ± 0.36	1.13 ± 0.56
C18:1 (*t*9, *t*11 & *c*9)/C18:0	0.22 ± 0.04	0.11 ± 0.02	0.05 ± 0.02

Data represent Mean ± SEM of at least three experiments in triplicate expressed as a percentage of total cellular lipid composition.

Discussion

The hypothesis being tested in this work is that uptake and incorporation of fatty acids including CLA into cells can alter their lipid composition such that cellular processes controlling cancer cell growth are modulated. One proposed mechanism for the anticarcinogenic activity of these fatty acids is that alteration of the fatty acid composition of the cell membrane phospholipids by CLA isomers results in reduced synthesis of arachidonic acid (AA) and AA-derived eicosanoids associated with

stimulation of cancer cell proliferation (Banni *et al.,* 1999; 2001). The overall objective of the work described in this chapter was to determine the extent to which CLA mixture of isomers (CLA-mix), purified isomers *c*9,*t*11 and *t*10,*c*12-CLA and omega-3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), can modulate the lipid composition of MDA-MB-435S-F (MDA) melanoma cells and a doxorubicin resistant variant MDA-MB-435S-F/Adr10p10p (A10p10p).

The Gas chromatography (GC) technique employed used a long (100 m) capillary column to separate fatty acids as fatty acid methyl esters (FAME). The method separated FAME including CLA *cis/trans, trans/cis* and *trans/trans* isomers with good resolution on the basis of chain length, geometric configuration and numbers of double bonds. However, 18:1 (*t*9) methyl elaidate and 18:1 (*t*11) methyl vaccinate eluted together as one peak on the chromatogram. This combined peak also connected at its base with the peak for 18:1 (c9) methyl oleate. Therefore, to remove ambiguity all three 18:1's were measured as one value.

All FAMEs were quantitated by reference to an internal standard heptadecanoic acid (C17:0) which was added to cell pellets before lipid extraction. Methylation was a two stage procedure involving alkali-catalysed hydrolysis in methanol for derivatising bound fatty acids in lipid extracts and BF_3 /methanol for methylation of free fatty acids. This procedure was previously shown to suppress artificial isomerisation of *cis/trans* CLA and *trans/cis* CLA to *trans/trans* CLA (Igarashi *et al.,* 2004; Koritala and Rohwedder 1972; Kramer *et al.,* 1997).

As presented above, the recovery of CLA was in the range 93.6-110.0 % with intra-assay variation ranging between 4.9-41.2 % CV. A possible reason for the high variation in some of the runs was degradation of the GC column due to age and the extent to which it had been used prior to this project. This was further confirmed by the finding that peaks for the three C18:1's were blending together at the base and had to be measured as one. In previous work in the lab these had eluted separately.

Overall reproducibility was 99.7 % and inter-assay variation was 7.1 % CV. The data reported here is consistent with those reported in the literature when NaOH-BF_3

reagent was used for methylation (Kim *et al.,* 2000; Jiang *et al.,* 1996; Alonso *et al.,* 2004). The former reported 83 % recovery of CLA and 6.6 % CV for repeatability using capillary GC analysis while the latter reported 89.4 % recovery with 3.6 % CV. The method was therefore considered advantageous for analysis of CLA isomers in MDA cells.

The first hypothesis being tested was whether there were any differences between the lipid profiles of the drug-sensitive MDA cells and the drug-resistant A10p10p cell line. The fatty acid profile of untreated MDA and A10p10p cells was analysed as described in earlier sections. Both cell lines were characterised by higher proportions of long chain saturated fatty acids (LCSFA) such as C18:0 stearic acid and C16:0 methyl palmitate compared with unsaturated fatty acids (LCSFA>UFA), suggesting that these cells were capable of synthesising their own supply of fatty acids via fatty acid synthase (FAS). While these long chain saturated fatty acids (LCSFA) were slightly reduced in the drug resistant A10p10p cell line compared to drug sensitive MDA cells; the total saturated fatty acids (SFA) were significantly higher in A10p10p cells than in MDA cells. This is suggestive of an up-regulation of the fatty acid synthase (FAS) enzyme followed by β-oxidation resulting in production of short chain saturated FA. As described above, FAS is a key enzyme in the de novo synthesis of fatty acids which has been identified as a tumour marker in breast cancer indicating a poor prognosis (Kuhajda, 2000) and recently discovered to contribute to increased drug resistance in breast cancer cells (Liu *et al.,* 2008).

C16:1 palmitoleic acid and the group of three C18:1 fatty acids; elaidic (*t*-9), vaccenic (*t*-11), and oleic acid (*c*-9) represent the major monounsaturated fatty acids (MUFA) of both cell lines. C16:1 palmitoleic acid was the predominant MUFA in A10p10p cells, while the C18:1 group was predominant in MDA cells. This increase in C16:1 and decrease in C18:1 in A10p10p cells relative to MDA cells represent conflicting evidence on the activity of the Δ9-desaturase enzyme, stearoyl-CoA desaturase (SCD). This enzyme introduces one double bond to the corresponding saturated fatty acid. Originally, C18:0 stearic acid was thought be the sole substrate, but more recently C16:0 was also found to be a substrate for the enzyme (Ntambi, 1999). Attie *et al*. (2002) recently described the desaturation index or ratio of

C18:1/C18:0 as a marker of SCD activity in plasma, seemingly ignoring the C16:1/C16:0 ratio. Further, Miyasaki et *al* (2002) identified C18:0 as the preferred substrate for the SCD1 isoform of the enzyme in mouse models. Following this model the reduced C18:1/C18:0 ratio indicates downregulation of SCD activity in the A10p10p cell line. Alternatively, it could be that the enzyme shows an altered specificity in A10p10p cells, favouring production of C16:1.

The polyunsaturated fatty acid (PUFA) C22:6 docosahexanoic acid (DHA) was significantly more abundant in MDA cells than A10p10p cells, while C18:3 α-linolenic acid (ALA), a precursor of DHA, was less abundant in MDA control cells compared to A10p10p cells. This suggest a possible down-regulation in Δ5/Δ6 desaturase and/or elongase activities in A10p10p cells.

The second hypothesis being tested was whether there was an alteration of the fatty acid composition of the cell membrane phospholipids by the exogenous fatty acids evidenced by reduced synthesis of arachidonic acid (AA) and AA-derived eicosanoids (prostaglandin E_2 (PGE_2)) associated with stimulation of cancer cell proliferation. The data presented above shows that although only significant with treatments of EPA at 75 μM in the A10p10p cell line, all treatments reduced the levels of AA to a similar extent relative to control in both cell lines. This points to a clear trend and suggests these fatty acids inhibit Δ5, Δ6 desaturase and/or elongase activities through competition for the same pathways in both MDA and A10p10p cells. Also, in MDA cells only, the CLA-mix, *c*9,*t*11 and *t*10,*c*12-CLA and EPA treatments caused a reduction in the cellular levels of DHA, which is consistent with the theory that these fatty acids inhibit Δ5, Δ6 desaturase and/or elongase activities. The fact that EPA caused a reduction in DHA also points to inhibition in MDA cells of Δ6 desaturase, the enzyme responsible for one of the steps in converting EPA to DHA. Other possible markers of inhibition of these enzymes would be increased levels of alpha linolenic acid (ALA), dihomo-gamma-linolenic acid (DGLA), gamma-linolenic acid (GLA) and linoleic acid (LA) relative to control (Figure 5.1.1). DGLA and GLA were present in very low amounts, just above trace levels and therefore not reliable predictors of enzyme activity. LA however, was reduced with all fatty acid treatments in both cell lines suggesting a contradiction to this

hypothesis. It is possible that in addition to competing for the same metabolic enzymes, these fatty acid treatments also compete with LA for uptake from the medium and incorporation into the cells resulting in reduced cellular levels of LA and subsequent production of AA (and AA derived eicosanoids).

The CLA-mix, *c*9,*t*11 and *t*10,*c*12-CLA treatments all reduced the levels of LCSFA, particularly C16:0 palmitate and C18:0 stearic acid in the MDA cell line suggesting inhibition of FAS, although only the CLA-mix was effective in this regard in A10p10p cells. Consistent with these observations Belury and co-workers found that CLA reduced FAS expression in wistar rats (Purushotham *et al.,* 2007). Also, Menendez *et al*. (2005b) concluded that the selective anticancer action of gamma-linolenic acid (GLA), a plant derived n-6 PUFA, in human breast cancer cells was due in part to the inhibition of FAS activity. EPA and DHA treatments by comparison caused minimal reduction of LCSFA in MDA and both actually resulted in an increase in LCSFA levels in A10p10p cells suggesting that while these fatty acids may result in some degree of inhibition, they are not as effective lowering saturated fatty acid levels as the CLA preparations. As with CLA, there are some animal studies which show the EPA and DHA in the diet causes a reduction in FAS expression/activity (Kim *et al.,* 2004), however this is the first study to include both CLA preparations and omega-3 fatty acids allowing a direct comparison.

Using the C18:1/C18:0 ratio as an indicator of SCD Δ9 desaturase activity, all fatty acid treatments in the MDA cell line and *t*10,*c*12-CLA, EPA and DHA caused inhibition of SCD in A10p10p cells. However, all fatty acid treatments in both cell lines caused an increase in C16:1 levels and in the C16:1/C16:0 ratio, highlighting the need for further investigation into the interaction of these fatty acids and the SCD enzyme. CLA was found previously to reduce both C18:1/C18:0 and C16:1/C16:0 ratios in the study mentioned above in Wistar rats (Purushotham *et al.,* 2007).

In terms of direct uptake measurement, EPA treatments resulted in the lowest level of uptake of the five fatty acids in both cell lines. However, there were significant changes in a range of individual fatty acid levels, including increases in derivatives of EPA, suggesting that EPA may be very susceptible to oxidation and that the

enhancement of the cytotoxic effect of doxorubicin observed in chapter 3 was perhaps due to a combination of the action of these derivates, the increase in membrane fluidity and competition with LA leading to reduced production of AA.

DHA treatment also resulted in low cellular uptake relative to the CLA preparations, although there was some significant DHA uptake detected in the MDA cell line. DHA in general was the least effective fatty acid at modulating the lipid profiles in both cell lines, suggesting that the enhancement of the cytotoxic effect of doxorubicin observed above was due to a more direct cytotoxic mechanism. It was noted in chapter 3 that DHA was more cytotoxic as a single treatment than the other fatty acids tested. Recently, the sensitisation of breast cancer cells to doxorubicin by DHA was found to be due to a loss of glutathione peroxidase response, a crucial enzyme for protection against hydrogen and lipid peroxides (Vibet *et al.,* 2008).

Both *c*9,*t*11 and *t*10,*c*12-CLA treatments resulted in significantly higher cellular uptake levels than the other fatty acids including the CLA-mix, even taking into account the lower concentrations of each contained in the mixture of isomers compared to pure isomer treatments. This lower direct uptake in the CLA mixture may be due to a lower level of incorporation into the cells, or a higher rate of metabolism of the isomers once incorporated leading to a lower detection. Considering the range of effects on individual and overall fatty acid levels in both cell lines and the fact that the CLA-mix was the only treatment found to have an effect on a measure of FAS activity in the A10p10p cell line, the latter is more likely. Banni *et al.,* (2004) described different patterns of incorporation into cellular lipids and rates of metabolism of the *c*9,*t*11 and *t*10,*c*12-CLA isomers, perhaps when delivered as a mixture there is some interaction allowing one to facilitate the other yielding more positive results than the single isomers alone.

Finally, one question remains, why are the combinations of fatty acid and drug more effective, both in terms of enhanced drug efficacy and total cell kill, in the drug resistant A10p10p cells than in the parental drug sensitive MDA cell line? First, looking at the observations of enzyme activity through alterations in lipid profiles, both cell lines contained reduced cellular levels of LA and AA (and AA derived

eicosanoids) by the fatty acid treatments. The A10p10p cell line was more resistant to alterations of markers of FAS activity by the fatty acid treatments than the MDA cell line. While all fatty acid treatments in the MDA cell line caused inhibition of SCD evidenced by a reduction in the C18:1/C18:0 ratio, only *t*10,*c*12-CLA, EPA and DHA caused inhibition of SCD in A10p10p cells. It would appear that from this point of view that the drug-resistant cell line A10p10p is more resistant to modification of its lipid metabolic pathways than the MDA cell line. Also, taking a more fundamental approach, with the exception of the CLA-mix, each fatty acid treatment caused an increase in the fluidity of cell membranes, by reduced LCSFA and/or increased UFA levels, to a similar or lesser degree in A10p10p cells than the corresponding treatment in MDA cells.

To conclude, while these data may explain some of the mechanisms by which these fatty acids enhance chemotherapy in melanoma cells (reduction in markers of FAS activity, reduced AA and AA derived eicosanoid formation (PGE_2) and increased fluidity of cell membranes by increasing the UFA/LCSFA ratio), the mechanism(s) by which they result in a greater cytotoxic effect in drug-resistant cells remains elusive. Santini *et al.* (2001) found drug resistant breast cancer cells contained more neutral lipids than their drug sensitive counterparts and a range of differences in individual fatty acid levels of the various lipid fractions, perhaps further study is required focusing on the effects and incorporation of fatty acid treatments on and into these lipid fractions within the cells. Also, it has been known for quite some time that the function of the ABC-transporter, P-glycoprotein (P-gp), a trans-membrane drug transport pump overexpressed in multidrug resistant cancer cells, is dependent on the surrounding lipid in the membrane. Delipidated P-gp loses ATP-ase activity, which can be restored after reconstitution of P-gp into liposomes (Doige *et al.,* 1993). It seems likely that alterations in the lipid domains of membranes by exogenous fatty acids could also alter the function of these and other transporter proteins such as MRP1, thus explaining how these treatments can render multidrug-resistant cells more sensitive to chemotherapy drugs than their drug-sensitive counterparts and represents another potential target area for further research.

Chapter 6

Final Discussion and Conclusions

Final Discussion and Conclusions

Cancer is a group of diseases characterised by uncontrolled growth, impaired cell death and spread of abnormal cells. If the spread is not controlled it can result in patient death. It has been estimated by Lopez *et al.* (2006) that there were approximately 12 million new cases of cancer worldwide in 2007. Of this number 1.3 million were breast cancer, with just under 0.5 million deaths associated with breast cancer, making it the leading cause of cancer death among women worldwide. The five-year survival of women with breast cancer is 89 % in the United States, but 76 % in Europe, perhaps due to a lack of an established mammography screening program in some countries. The stage at diagnosis is the most important prognostic variable, and while the overall prognosis for breast cancer is relatively favourable in the US, five-year survival drops to 26 % for patients presenting with advanced disease (American Cancer Society, 2007). This highlights tremendous scope for improving current and developing new treatment regimes outlined in Chapter 1.

Several isomers of CLA both in pure form and as a mixture have been shown to be cytotoxic at micromolar concentrations for a variety of breast, colon, prostate and skin tumour cell lines (Belury, 2002a; Miller *et al.,* 2001; 2003; O'Shea *et al.,* 2000). Following method development described in chapter 2 (including an evaluation of the cytotoxic effects of PUFA including CLA and doxorubicin (Dox) as single agents), the overall objective of this project was to characterise the effects of co-administering PUFA including CLA with the anticancer drug Dox in a human melanoma cellular model of multi-drug resistance and to determine mechanisms by which fatty acids may modulate drug resistance or sensitivity.

In the first set of single treatments, MCF-7 and MCF-7/cis cells were treated with the fatty acids; CLA mixture of isomers (CLA-mix), LA, *c*9,*t*11 and *t*10,*c*12-CLA delivered as sodium salts in medium containing BSA in a 2:1 w/w ratio FA:BSA (473:1 molar ratio). BSA, or bovine serum albumin, is a carrier protein in the blood stream designed to bind to and transport molecules such as fatty acids, facilitating the transport of large quantities of these relatively insoluble molecules throughout the body (Spector, 1975). Therefore, delivery of the above fatty acids as sodium salts in

medium containing BSA in a 2:1 w/w ratio was designed to mimic the biologic situation (Ip, *et al.*, 1999). Cytotoxic effects of CLA-mix, *c*9,*t*11 and *t*10,*c*12-CLA were time and dose-dependent in both MCF-7 and MCF-7/cis cell lines. Cytotoxicity of CLA was found to be dependent on isomer composition, concentration and duration of exposure. This was consistent with findings by O'Shea *et al.* (2000) that the cytotoxic effects of CLA were concentration dependant in the MCF-7 cell line. However little is known about the action of CLA on a drug-resistant cell line. Here the MCF-7 cisplatin resistant cells were found to be more sensitive to inhibition by *t*10,*c*12-CLA and the CLA mixture than parental MCF-7 cells.

Following this, it was decided to test the cytotoxic action of PUFA including CLA on multi-drug resistant cell lines in which the resistance was better characterised. MDA-MB435S-F (MDA) human melanoma cell line and taxol resistant variants MDA-MB435S-F/Taxol 10p (Tax10p) and MDA-MB435S-F/Taxol 10p4p (Tax10p4p) were developed and kindly donated by Dr. Sharon Glynn (NICB, Dublin) (Glynn *et al.*, 2004). In light of reports indicating a 2:1 molar ratio of fatty acids to BSA represented a better delivery solution to mimic the biological situation more closely (Evans *et al.,* 2001; 2002; Iwakiri *et al.,* 2002) stock solutions of CLA-mix, *c*9,*t*11 and *t*10,*c*12-CLA, LA and GLA were prepared in RPMI medium containing BSA in a 2:1 molar ratio. This ratio is based on evidence indicating BSA contains two strong binding sites for long chain fatty acids (Reed, 1986).

Of the fatty acid treatments, CLA-mix and *t*10,*c*12-CLA were similarly effective at inhibiting growth in all three cell lines. *c*9,*t*11-CLA however, at the higher concentrations (36 & 40 μg/mL) appeared to be more effective at inhibiting cell growth in Tax10p cells than in the MDA or Tax10p4p cell lines. LA showed no significant inhibition in any cell line at the concentrations used (0-50 μg/mL). GLA (0-50 μg/mL) on the other hand, while the resulting inhibition was significant relative to control, the inhibition observed was much lower than that of all three CLA preparations.

One of the problems encountered when using BSA as the vehicle for delivery was that it introduced considerable variation to the system which was made manifest in high standard deviations (SD). Also, there was an apparent lack of consensus among those using BSA as the delivery vehicle for fatty acid treatments, with molar ratios ranging from approximately 667:1 to 2:1 and many in between (Moya-Camarena *et al.,* 1999; Brown *et al.,* 2003; Kim *et al.,* 2003; Evans *et al.,* 2001; 2002; Iwakiri *et al.,* 2002). Increasingly, other authors opted to omit BSA altogether from the experimental design and deliver the fatty acids as free fatty acids (FFA) in a media solution (Chamras *et al.,* 2002; Tanmahasamut *et al.,* 2004; Maheo *et al.,* 2005; Wu *et al.,* 2005). Also Richieri (1993) demonstrated that not all serum fatty acids are bound to albumin in solution, some remain as FFA. These fluctuations in the level of fatty acid binding to albumin in solution could account for the high SD observed in the cytotoxicity assays described above. For these reasons, it was decided to repeat the fatty acid assays using FFA instead of that complexed with BSA.

Delivery of the fatty acids as FFA solutions eliminated one source of variation in the experiments, resulting in statistically significant inhibition at lower concentrations – including with LA, which was showing negligible effects complexed with BSA. The IC_{50} values of all fatty acid treatments were reduced compared to the treatments containing BSA in a 2:1 molar ratio, suggesting that BSA conferred a protective effect on the cells. The MDA cell line appeared to be more susceptible to inhibition by the three CLA preparations; CLA-mix, *c*9,*t*11 and *t*10,*c*12-CLA followed by Tax10p4p and least susceptible, Tax10p although these differences were not statistically significant.

The outcomes of these experiments are: that multidrug resistant cell lines are more (MCF-7/cis) or similarly (Tax10p and Tax10p4p) susceptible to the cytotoxic action of CLA as their drug sensitive counterparts. This is the first report to demonstrate this phenomenon and represents an attractive new approach to developing more effective treatment regimes to counteract multi-drug resistance in tumours. Other outcomes relate to experimental design; that long term exposures (7 day) and free fatty acid treatments yield more consistent results with lower SD and better statistical significance than shorter term assays or fatty acids complexed with BSA.

The chemotherapeutic agents doxorubicin (Dox), taxotere (Tax) and 5-fluorouracil (5-FU) were also tested on MDA, Tax10p and Tax10p4p cells in a 7 day exposure. No significant difference was observed in the sensitivity of the three cell lines to Tax, Dox or 5-FU –suggesting that resistance of the Tax10p and Tax10p4p lines to Tax and other drugs might be better observed in short term assays. To this end it was decided to run the drug assays as 4 h exposure followed by 7 day recovery, in place of long term (7 day) exposure. This is also with a view to mimicking the clinical situation more closely (Glynn *et al.*, 2004).

With Dox, Tax and 5-FU, the change in design from treatments from a 7 day exposure to the 4 h exposure, 7 day recovery resulted in a dramatic increase in IC_{50} values as expected. Differences emerged between the cell lines in terms of drug sensitivities. When comparing IC_{50} values, Tax10p and Tax10p4p cells were 1.6 and 1.7 fold more resistant to 5-FU treatment than MDA cells respectively. Similarly, Tax10p and Tax10p4p were 1.2 and 1.5 fold more resistant to Dox treatment and 1.7 and 2.5 fold more resistant to Tax treatment than the MDA cell line. Not surprisingly, of the two cell lines that had previously been taxol pulsed; Tax10p4p, which had four extra rounds of pulses of Tax, displayed higher resistance to each of the three drugs than Tax10p. This finding supports data from the original characterisation of these cell lines (Glynn *et al.*, 2004).

IC_{50} values of both fatty acid and drugs obtained above were used in the design of an experiment using sub-optimal concentrations (below IC_{50}) of both fatty acids and drugs to investigate synergistic interactions in melanoma cells. The MDA drug sensitive cell line and a Dox pulsed variant MDA-MB-435S-F/Adr-10p10p (A10p10p) were used in this experiment. A10p10p was shown previously to have increased levels of Pgp and MRP1 drug efflux proteins and while the parental MDA line was shown to be highly invasive, the pulsed A10p10p variant displayed a more aggressively invasive phenotype, termed “superinvasive” (Glynn *et al.,* 2004).

At this point the potential of five free fatty acids (CLA-mix, *c*9,*t*11 and *t*10,*c*12-CLA, EPA and DHA) to enhance the cytotoxicity of doxorubicin in the above melanoma

model was investigated. The three preparations of CLA used (CLA-mix, *c*9,*t*11 and *t*10,*c*12-CLA) were similarly effective to the omega-3 fatty acids, EPA and DHA at enhancing the cytotoxicity of doxorubicin in MDA-MB435S-F (MDA) melanoma cells. Data show that CLA enhanced cytotoxicity of Dox on the MDA cell line in a dose dependant manner in each of the three treatment groups with significant enhancement ($p<0.05$) seen with as low as 1 μg/mL (21 μM) of each of the fatty acids in combination with Dox. The most effective regime over all was found to be pre-exposure to fatty acid followed by concurrent treatment with Dox and a 7 day fatty acid exposure in the [pre-treat] group. The report by Fite *et al.* (2007) that CLA isomers (40 μM) can significantly potentiate the cytotoxicity of docetaxel in breast cancer cells is the only other report to demonstrate the effect of CLA on the efficacy of chemotherapeutic drugs in cancer cell lines.

All fatty acids enhanced the efficacy of Dox in the multi-drug resistant cell line A10p10p to a greater extent than the corresponding treatments in the MDA cell line. Further, all five fatty acids in combination with Dox rendered the A10p10p cells more sensitive to the drug than the parental drug sensitive MDA cells with higher levels of percentage inhibition for corresponding combination treatments. The fatty acids are therefore in effect reversing the Dox-resistance in the A10p10p cells. This is the first study to report the effects of CLA on the efficacy of chemotherapeutic drugs in multi-drug resistant cancer cell lines.

The *c*9,*t*11-CLA isomer was found to enhance the uptake of Dox into the cells in both cell lines possibly by increasing the fluidity of the membrane allowing more passive diffusion and/or flip flop of the drug across the membrane (Hendrich and Michalak, 2003). Also, the CLA mixture of isomers enhanced drug retention after treatment in the A10p10p line, while *c*9,*t*11-CLA enhanced retention in both cell lines.

The ability to enhance chemosensitivity of drug resistant tumour cells by administering CLA prior to or simultaneously with chemotherapeutics is of potential benefit for treatment of cancer. The mechanism of action of CLA may be through a combination of many different pathways. Studies in breast cancer (Wu *et al.,* 2005)

and leukaemic (Siddiqui *et al.,* 2003) cells have shown that omega-3 polyunsaturated fatty acids attenuated cancer growth through activation of a neutral sphingomyelinase-mediated pathway leading to increased ceramide production. However, data in the literature is not conclusive; others have reported decreases in ceramide levels in response to n-3 PUFA treatment (Jolly *et al.,* 1997; McMurray *et al.,* 2000).

Ceramide and basal sphingosine levels were analysed and compared in untreated MDA and A10p10p cells. Ceramide levels were found to be higher in A10p10p cells than in the MDA cell line, which points to one mechanism by which the drug resistant A10p10p cells protect themselves from anticancer drugs. It has been shown that decreased levels of endogenous ceramide by over-expression of glucosylceramide synthase results in the development of a multidrug resistant phenotype in cancer cells. Glucosylceramide synthase clears ceramide levels by incorporating it into less toxic glucosylceramide (Ogretmen and Hannun, 2001).

MDA and A10p10p cells were treated in the presence or absence of Dox, with CLA-mix; *c*9,*t*11-CLA; *t*10,*c*12-CLA; EPA or DHA. EPA single treatments caused a significant reduction in ceramide levels in both cell lines, suggesting that the cytotoxic action of EPA is not through a pathway initiated by ceramide production. In contrast, all fatty acids plus Dox resulted in an increase in ceramide levels relative to Dox alone in both cell lines although this was only significant in the case of *c*9,*t*11- and *t*10,*c*12-CLA in A10p10p cells. This points to a trend indicating that perhaps ceramide production is one of many pathways by which these fatty acids enhance the cytotoxic action of anticancer drugs.

Interestingly, Dox treatments alone and in combination with fatty acids caused an increase in sphingosine levels relative to respective single treatments of fatty acid/control in the MDA cell line, although this was only significant with CLA-mix, *c*9,*t*11 and *t*10,*c*12-CLA. In the A10p10p cell line, all fatty acids alone caused an increase in sphingosine levels relative to control. Treatments of Dox alone and in combination with each fatty acid resulted in a further although not significant increase. While evidence in the literature is lacking to support this finding, Sweeney

and co-workers (1998) established that sphingosine also induces apoptosis – independently from ceramide, acting in an earlier part of the apoptotic pathway than ceramide.

CLA has been shown to alter a wide array of targets (outlined in chapter 1). Relatively recently, DHA was found by Menendez and colleagues (2005c) to downregulate *c*-erbB2 (Her2/*neu*) oncogene expression in Her2/*neu* over-expressing human breast cancer cells. Her2/*neu* normally encodes a transmembrane tyrosine kinase glycoprotein which if overexpressed, is part of a signalling cascade that involves the activation of PI3K/Akt and MAPK pathways and the promotion of cell survival. It was postulated that perhaps the cytotoxic action of CLA may relate to an effect on the expression of Her2/*neu* in MDA &/or A10p10p cells. In untreated cells, the A10p10p cell line had significantly higher levels of Her2/*neu* coded p185 $^{c\text{-}erbB2/c\text{-}neu}$ oncoprotein present than in MDA cells. This indicates another mechanism by which the A10p10p cell line maintains resistance to conventional anticancer drugs. Overexpression of the Her2/*neu* oncogene has been shown previously to confer resistance to chemotherapeutic drugs in breast cancer cells (Colomer *et al.,* 2000).

Treatment of both MDA and A10p10p cells with CLA-mix resulted in down-regulation of Her2/*neu* expression. Also, both EPA and DHA resulted in down-regulation in the A10p10p cell line. This supports the finding by Menendez *et al*. (2005) that DHA down-regulated Her2/*neu* expression in breast cancer cells overexpressing the gene. However, there were opposing results with treatments of the purified single isomers *c*9,*t*11 and *t*10,*c*12-CLA. Both isomers caused significant up-regulation of Her2/*neu* expression in A10p10p and *c*9,*t*11-CLA also caused significant up-regulation in MDA cells. Since this study is the only to date to demonstrate Her2/*neu* in melanoma cells and the first to investigate the effects of these fatty acids on Her2/*neu* expression in melanoma cells, further work is necessary to elucidate the mechanisms behind and confirm these unexpected and opposing effects.

Previous studies in the lab have shown that CLA isomers and its precursor *t*-VA are rapidly taken up into cancer cells (O'Shea *et al.,* 2000; Miller *et al.,* 2003b). Further, Agatha *et al.* (2004) reported that CLA modulated membrane FA composition and PUFA metabolism (FA desaturation and elongation), in four cultured human leukemia cell lines representing a profile of possible variations of leukemic tumor differentiation.

Fatty acid profiles of untreated MDA and A10p10p were analysed by gas chromatography in chapter 5. Both MDA and A10p10p cell lines were characterised by higher proportions of long chain saturated fatty acids (LCSFA) such as C18:0 methyl stearate and C16:0 methyl palmitate compared with unsaturated fatty acids, suggesting that these cells were capable of synthesising their own supply of fatty acids via fatty acid synthase (FAS). Furthermore, the level of total saturated fatty acids (SFA) was significantly higher in A10p10p cells than in MDA cells, suggestive of an up-regulation of the fatty acid synthase (FAS) enzyme followed by β-oxidation resulting in production of shorter chain saturated FA.

As described above, FAS is a key enzyme in the *de novo* synthesis of fatty acids but is downregulated in most normal human tissues because of the fat in human diet. In contrast, FAS is often highly expressed in human cancers; has been identified as a tumour marker in breast cancer indicating a poor prognosis (Kuhajda, 2000) and recently discovered to contribute to increased drug resistance in breast cancer cells (Liu *et al.,* 2008).

The CLA-mix, *c*9,*t*11 and *t*10,*c*12-CLA treatments all resulted in reduction in saturated fatty acids (LCSFA), a marker of FAS activity, although only the CLA-mix was effective in this regard in A10p10p cells. Consistent with these observations Belury and co-workers found that CLA reduced FAS expression in Wistar rats (Purushotham *et al.,* 2008). Also, Menendez *et al*. (2005b) concluded that the selective anticancer action of gamma-linolenic acid (GLA), a plant derived n-6 PUFA, in human breast cancer cells was due in part to the inhibition of FAS activity. EPA and DHA treatments by comparison caused minimal reduction in LCSFA in MDA and both actually resulted in an increase in LCSFA in A10p10p cells

suggesting that while these fatty acids may result in growth inhibition, they are not as effective at inhibiting FAS as the CLA preparations. However, there are some animal studies which show that EPA and DHA in the diet of rats caused a reduction in FAS expression/activity (Kim *et al.,* 2004).

This is the first study to demonstrate the effect of CLA on a marker of FAS activity in drug-resistant cancer cells and to compare its effects with omega-3 fatty acids. Inhibition of fatty-acid synthesis by CLA could be a means to enhance cytotoxic effects of chemotherapeutic agents in proliferating cells with high levels of FAS. This strategy would likely target cancer cells and leave the normal proliferating cells in the human body intact. It would be interesting to confirm data obtained here by examining the direct effect of these fatty acids on the activity of FAS, by measurement of incorporation of [14C] malonyl-CoA into cellular fatty acids as described by Knowles *et al.* (2007), and effects on FAS protein expression by Western-blot analysis (Vazquez-Martin *et al.,* 2008).

C16:1 palmitoleic acid and the group of three C18:1 fatty acids; elaidic (*t*-9), vaccenic (*t*-11), and oleic acid (*c*-9) represent the major monounsaturated fatty acids (MUFA) of both cell lines. C16:1 palmitoleic acid was the predominant MUFA in A10p10p cells, while the C18:1 group was predominant in MDA cells. The reduced C18:1/C18:0 ratio indicates downregulation of stearoyl-CoA desaturase (SCD) activity towards stearate in the A10p10p cell line. Using the C18:1/C18:0 ratio as an indicator of SCD Δ9 desaturase activity, all fatty acid treatments in the MDA cell line and *t*10,*c*12-CLA, EPA and DHA caused inhibition of SCD in A10p10p cells. CLA was found previously to reduce both C18:1/C18:0 and C16:1/C16:0 ratios in the study mentioned above in Wistar rats (Purushotham *et al.,* 2008). However, the results presented here are anomalous when combined with data demonstrating that all fatty acid treatments in both cell lines caused an increase in C16:1 levels and in the C16:1/C16:0 ratio, highlighting the need for further investigation into the interaction of these fatty acids and the SCD enzyme. A direct measurement of SCD activity would be benefical, in which cells are incubated with 3 μM (0.25 microcurie Ci/dish) [^{14}C] stearic acid as described by Scaglia *et al.* (2005). The Δ9 desaturating activity of SCD can then be estimated by conversion of [^{14}C] stearic acid to [^{14}C] oleic acid.

This combined with a measurement of SCD protein expression by immunoblot analysis (Scaglia *et al.,* 2005) would shed more light on the altered function of SCD in drug-resistant cell lines and the effect of these fatty acids on drug-sensitive and resistant cell lines.

Another mechanism proposed for the anticarcinogenic activity of these fatty acids was the alteration of the fatty acid composition of the cell membrane phospholipids by CLA isomers resulting in reduced synthesis of arachidonic acid (AA) in turn leading to reduced synthesis of AA-derived eicosanoids associated with stimulation of cancer cell proliferation (Banni *et al.,* 1999; 2001). Although only significant with treatments of EPA at 75 μM in the A10p10p cell line, all treatments reduced the levels of AA to a similar extent relative to control in both cell lines. This points to a clear trend and suggests these fatty acids inhibit Δ5, Δ6 desaturase and/or elongase activities through competition for the same pathways in both MDA and A10p10p cells. Also, in MDA cells only, the CLA-mix, *c*9,*t*11 and *t*10,*c*12-CLA and EPA treatments caused a reduction in the cellular levels of DHA, which is consistent with the theory that these fatty acids inhibit Δ5, Δ6 desaturase and/or elongase activities. The fact that EPA caused a reduction in DHA also points to inhibition in MDA cells of Δ6 desaturase, the enzyme responsible for one of the steps in converting EPA to DHA. It is possible that in addition to competing for the same metabolic enzymes, these fatty acid treatments also compete with LA for uptake from the medium and incorporation into the cells resulting in reduced cellular levels of LA and subsequent production of AA (and AA derived eicosanoids such as PGE_2).

In terms of direct uptake measurement, EPA treatments resulted in the lowest level of uptake of the five fatty acids in both cell lines. However, there were significant changes in a range of individual fatty acid levels, including increases in derivatives of EPA, suggesting that EPA is very susceptible to oxidation and that the enhancement of the cytotoxic effect of doxorubicin observed in chapter 3 was due to a combination of the action of these derivatives, the increase in membrane fluidity through greater unsaturation of membrane fatty acids and the competition with LA leading to reduced production of AA.

DHA treatment also resulted in low cellular uptake relative to the CLA preparations, although there was some significant DHA uptake detected in the MDA cell line. DHA in general was the least effective fatty acid at modulating the lipid profiles in both cell lines, suggesting that the enhancement of the cytotoxic effect of doxorubicin observed above was due to a more direct cytotoxic mechanism. It was noted in chapter 3 that DHA was more cytotoxic as a single treatment than the other fatty acids tested. Recently, the sensitisation of breast cancer cells to doxorubicin by DHA was found to be due to a loss of glutathione peroxidase response, a crucial enzyme for protection against hydrogen and lipid peroxides (Vibet *et al.,* 2008).

Both *c*9,*t*11 and *t*10,*c*12-CLA treatments resulted in significantly higher cellular uptake levels than the other fatty acids including the CLA-mix, even taking into account the lower concentrations of each contained in the mixture of isomers compared to pure isomer treatments. This may be due to a lower level of incorporation into the cells, or a higher rate of metabolism of the isomers once incorporated leading to a lower detection rate. Considering the range of effects on individual and overall fatty acid levels in both cell lines and the fact that the CLA-mix was the only treatment found to have an effect on markers of FAS activity in the A10p10p cell line, the latter is more likely. Banni *et al.,* (2004) described different patterns of incorporation into cellular lipids and rates of metabolism of the *c*9,*t*11 and *t*10,*c*12-CLA isomers, perhaps when delivered as a mixture there is some interaction allowing one to facilitate the other yielding more positive results than the single isomers alone. The increased membrane unsaturation index as a result of CLA incorporation would likely provide more abundant targets for reactive oxygen species (ROS) generated by Dox metabolism. Products of lipid peroxidation such as hydroperoxides, hydroxynonenal, malondialdehyde and other aldehydes interfere with intracellular signalling cascades involved in cell replication and cell death and thus may account for increased drug efficacy. The degree of lipid peroxidation induced by the combination of CLA and doxorubicin could be determined by measurement of the production of hydroperoxides, hydroxynonenal and malondialdehyde according to methods described by Requena *et al.* (1997).

Another issue to address at this point is regarding the MDA cell line. Although originally derived from a 31 year old female with metastatic, ductal adenocarcinoma of the breast it is now accepted that the cell line is in fact a melanoma derived cell line (Ellison *et al.,* 2002; Rae *et al.,* 2007). This in no way invalidates the research described in these chapters, but rather what is lost from the breast cancer research arena is gained by the melanoma research arena. Further, it is probable that since the action of the fatty acids described here is focused on cellular processes common to many diverse cancer cell lines, the combination of CLA and chemotherapeutic drugs may be similarly efficacious in many different cancer cell lines and perhaps many types of tumours *in vivo*.

In summary, this study has identified many mechanisms by which a multi-drug resistant cancer cell line maintains its resistance to chemotherapeutic drugs. The first characteristic of a multidrug resistant phenotype in cancer cells observed was that ceramide levels were lower in A10p10p cells than in the parental MDA cell line. The A10p10p cell line had significantly higher levels of Her2/*neu* coded p185 $^{c\text{-}erbB2/c\text{-}neu}$ oncoprotein present than in MDA cells. Also, metabolic markers of β-oxidation and FAS were shown to be elevated in A10p10p relative to MDA cells and there were alterations in markers for SCD which remain to be fully elucidated.

These differences between the cell lines represent attractive therapeutic targets by which to selectively kill multi-drug resistant tumour cells while causing minimal damage to surrounding normal proliferating cells. While single treatments of the fatty acids failed to cause an alteration in ceramide levels, all fatty acids in combination with Dox resulted in an increase in ceramide levels in both cell lines, indicating that ceramide production is one of many pathways by which these fatty acids enhance the cytotoxic action of anticancer drugs. Interestingly, all fatty acids in combination with Dox resulted in an increase in sphingosine levels in both cell lines, with a more marked effect in the A10p10p cells line. Effects of CLA on Her2/*neu* expression were not as conclusive. CLA-mix treatment in both MDA and A10p10p cells and EPA and DHA in A10p10p cells resulted in down-regulation of Her2/*neu* expression. There were however, opposing results with treatments of the single isomers *c*9,*t*11 and *t*10,*c*12-CLA. Both isomers caused significant up-

regulation of Her2/*neu* expression in A10p10p and *c*9,*t*11-CLA also caused significant up-regulation in MDA cells. The CLA-mix, *c*9,*t*11 and *t*10,*c*12-CLA treatments all resulted in reduction of markers of FAS activity, although only the CLA-mix was effective in A10p10p cells. Finally, all treatments reduced the level of arachidonic acid (AA) to a similar extent in both cell lines suggesting these fatty acids inhibit Δ5, Δ6 desaturase and/or elongase activities through competition for the same pathways in both MDA and A10p10p cells.

6.1 Future Work

The obvious area to explore following this work is to determine whether CLA can also potentiate the cytotoxic effect of Dox and other chemotherapeutic drugs in other types of cancer cell lines.

Also, it would be interesting to elucidate whether these effects are due in part to an alteration in the activity of the function of the ABC-transporter, P-glycoprotein (P-gp), a trans-membrane drug transport pump overexpressed in multidrug resistant cancer cells. These and other transporter proteins such as breast cancer resistance protein (BCRP) in breast cancer cells are localised to specific cholesterol-rich domains termed membrane rafts and their function is dependent on the surrounding lipid in the membrane rafts (Dos Santos *et al.,* 2007; Storch *et al.,* 2007). By isolating membrane rafts from cancer cells using Brij-96 as described by Radeva *et al*., (2005) and extracting the lipids as in chapter 5, the local effects of CLA treatments can be determined in these rafts. In combination with this approach, the direct effects of CLA on protein expression of P-gp, MRP1 and caveolin-1 (a signalling protein associated with specialised rafts called caveolae (Liu *et al.,* 2002)) can be determined by western blot techniques (Gouaze *et al.,* 2005; Storch *et al.,* 2007).

Immunoblot analysis can be used to determine fatty acid synthase (FAS) protein expression and activity. Cells for this experiment would be lysed in SDS sample buffer, separated by SDS-PAGE and transferred to nitrocellulose as described by Vazquez-Martin *et al.* (2008). FAS activity may be determined by measurement of incorporation of [14C] malonyl-CoA into cellular fatty acids as described by Knowles *et al.* (2007).

A direct measurement of SCD activity would be benefical in light of conflicting evidence for the effect of CLA treatment on the markers of SCD activity (C18:1/C18:0 and C16:1/C16:0 ratios). The Δ9 desaturating activity of SCD may be estimated by conversion of [^{14}C] stearic acid to [^{14}C] oleic acid. Effects on SCD

protein expression can be assessed by immunoblot analysis according to Scaglia *et al.* (2005).

The degree of lipid peroxidation induced by the combination of CLA and doxorubicin could be determined by measurement of the production of hydroperoxides, hydroxynonenal, malondialdehyde according to methods described by Requena *et al.* (1997). Alternatively, a direct measurement of lipid hydroperoxide levels is possible by utilising redox reactions with ferrous ions as in the Calbiochem Lipid Hydroperoxide (LPO) Assay kit. The resulting ferric ions can then be detected by spectrophotometry using thiocyanate ion as the chromogen (Morrow and Roberts, 1997).

7.0 Bibliography

Abraham, R, Basser, R.L., Green, M.D., 1996. A risk benefit assessment of anthracycline antibiotics in antineoplastic therapy. *Drug Safety 15: 406-429*

Agatha, G., Voigt, A., Kauf, E., Zintl, F., 2004. Conjugated linoleic acid modulation of cell membrane in leukemia cells. *Cancer Letters* 209: 87-103

Agus, D.B., Akita, R.W., Fox, W.D., Lewis, G.D., Higgins, B., Pisacane, P.I., Lofgren, J.A., Tindell, C., Evans, D.P., Maiese, K., Scher, H.I., Sliwkowski, M.X., 2002. Targeting ligand-activated ErbB2 signaling inhibits breast and prostate tumor growth. *Cancer Cell 2(2): 127-137*

Allgar, V.L. and Neal, R.D., 2005. Delays in the diagnosis of six cancers: analysis of data from the 'National Survey of NHS Patients: Cancer'. *British Journal of Cancer 92: 1959-70*

Alnemri, E.S., 1997. Mammalian cell death proteases: a family of highly conserved aspartate specific cysteine proteases. *Journal of Cellular Biochemistry 64: 33-42*

Alonso, L., Cuesta, E.P., Gilliland, S.E., 2004. Gas chromatographic method for analysis of conjugated linoleic acids isomers (*c*9, *t11*, *t*10, *c12* and *t*9, *t11*) in broth media as application in probiotic studies. *Journal of Chromatographic Science* 42: 167-170

American Cancer Society, 2007. Global cancer facts and figure 2007. Accessed Nov'08
http://www.cancer.org/downloads/STT/Global_Facts_and_Figures_2007_rev2.pdf

Ames, B.N., Gold, L.S., Willet, W.C., 1995. The causes and prevention of cancer. *Proceedings of the National Academy of Sciences, USA 92: 5258-5265*

Ansfield, F., Ramirez, G., Mackman, S., Bryan, G.T., Curreri, A.R., 1969. A 10-year study of 5-Fluorouracil in disseminated breast cancer with clinical results and survival time. *Cancer Research 29: 1062-66*

Arends, M., Wylie, A., 1991. Apoptosis: mechanisms and role of pathology. *International Review of Experimental Pathology 32: 223-254*

Aro, A., Mannisto, S., Salminen, I., Ovaskainen, M-L., Kataja, V., Uusitupa, M., 2000. Inverse association between dietary and serum conjugated linoleic acid and risk of breast cancer in postmenopausal women. *Nutrition and Cancer 38(2): 151-157*

Attie, A.D., Krauss, R.D., Gray-Keller, M.P., Brownlie, A., Miyazaki, M., Kastelein, J.J., Lusis, A.J., Stalenhoef, A.F.H., Stoehr, J.P., Hayden, M.R., Ntambi, J.M., 2002. Relationship between stearoyl-CoA desaturase activity and plasma triglycerides in human and mouse hypertriglyceridemia. *Journal of Lipid Research 43: 1899-1907*

Banni, S., Carta, G., Contini, M.S., 1996. Characterisation of conjugated diene fatty acids in milk, dairy products and lamb tissues. *Journal of Nutritional Biochemistry 7: 150-155*

Banni, S., Angioni, E., Casu, V., Melis, M.P., Carta, G., Corongiu, F.P., Thompson, H., Ip, C., 1999. Decrease in linoleic acid metabolites as a potential mechanism in cancer risk reduction by conjugated linoleic acid. *Carcinogenesis 20: 1019-1024*

Banni, S., Carta, G., Angioni, E., Murru, E., Scanu, P., Melis, M.P., Bauman, D.E., Fischer, S.M., Ip, C., 2001. Distribution of conjugated linoleic acid and metabolites in different lipid fractions in the rat liver. *Journal of Lipid Research 42: 1056-1061*

Banni, S., Petroni, A., Blasevich, A., Carta, G., Cordeddu, L., Murru, E., Melis, M.P., Mahon, A., Belury, M.A., 2004. Conjugated linoleic acids (CLA) as precursors of a distinct family of PUFA. *Lipids 39: 1143-1146*

Bassaganya-Riera, J., Reynolds, K., Martino-Catt, S., Cui, Y., Hennighausen, L., Gonzalez, F., Rohrer, J., Benninghoff, A.U., Hontecillas, R., 2004. Activation of PPAR gamma and delta by conjugated linoleic acid mediates protection from experimental inflammatory bowel disease. *Gastroenterology 127(3): 994-6*

Bauman, D.E., Griinari, J.M., 2000. Regulation and nutritional manipulation of milk fat: low-fat milk syndrome. *Livestock Production Science 70: 15-29*

Belury M.A., 2002a. Dietary conjugated linoleic acid in health: Physiological effects and mechanisms of action. *Annual Review Nutrition 22: 505-531*

Belury, M.A., 2002b. Inhibition of carcinogenesis by conjugated linoleic acid: Potential mechanisms of action. *American Society for Nutritional Sciences: Recent Advances in Nutritional Sciences.*

Belury, M.A., Kavanaugh, C.J., Liu, K-L., 2007. Conjugated linoleic acid modulates phorbol ester–induced PPAR-δ and K-FABP mRNA expression in mouse skin. *Nutrition Research 27(1): 48-55*

Biondo, P.D., Brindley, D.N., Sawyer, M.B., Field, C.J., 2008. The potential for treatment with dietary long-chain polyunsaturated n-3 fatty acids during chemotherapy. *Journal of Nutritional Biochemistry 19(12): 787-796*

Blagosklonny, M.V., 2003. Targeting cancer cells by exploiting their resistance. *Trends in Molecular Medicine 9(7): 307-312*

Blankson, H., Stakkestad, J.A., Fagertun, H., Thom, E., Wadstein, J., Gudmundsen, O., 2000. Conjugated Linoleic Acid Reduces Body Fat Mass in Overweight and Obese Humans. *Journal of Nutrition 130: 2943-2948*

Bligh, E. and Dyer, W.J., 1959. A rapid method of total lipid extraction and purification. *Canadian Journal of Biochemical Physiology 37: 911-917*

Bougnoux, P., 1999. n-3 polyunsaturated fatty acids and cancer. *Current Opinion in Clinical Nutrition and Metabolic Care 2: 121-126*

BreastCancer.org. Symptoms and diagnosis. (Accessed 2008) *http://www.breastcancer.org/symptoms/diagnosis/staging.jsp*

Brown, J.M., Boysen, M.S., Jensen, S.S., Morrison, R.F., Storkson, J., Lea-Currie, R., Pariza, M., Mandrup, S., McIntosh, M.K., 2003. Isomer-specific regulation of metabolism and PPAR gamma signaling by CLA in human preadipocytes. *Journal of Lipid Research 44(7): 1287–1300*

Burns, C.P., Spector, A.A., 1993. Biochemical effects of lipids on cancer therapy. *Journal of Nutritional Biochemistry 5: 114-123*

Buzder, A.M., Ibrahim, N.K., Francie, D., Booser, D.J., Thomas, E.S., Theriault, R.L., Pusztai, L., Green, M.C., Arun, B.K., Giordano, S.H., Cristofanilli, M., Frye, D.K., Smith, T.L., Hunt, K.K., Singletary, S.E., Sahin, A.A., Ewer, M.S., Buchholz, D., Hortobagyi, G.N., 2005. Significantly higher pathologic complete remission rate after neoadjuvant therapy with Trastuzumab, paclitaxel, and epirubicin chemotherapy: Results of a randomized trial in human epidermal growth factor receptor 2- Positive operable breast cancer. *Journal of Clinical Oncology 23: 3676-85*

Calfa, C.I., Rosenblatta, J.D., Choa, H-M., Websterb, K., Shin, S-U., 2006. Antibodies and antibody-fusion proteins as anti-angiogenic, antinext term-tumor agents. *Update on Cancer Therapeutics 1(2): 159-173*

Cameron, D., Bell, R., 2008. Future use of bevacizumab and other anti-angiogenic agents in breast cancer. *European Journal of Cancer Supplements 6(6): 40-50*

Campo, J., Comber, H., Gavin, A.T., 2004. All Ireland Cancer Statistics 1998-2000. *Northern Ireland Cancer Registry/ National Cancer Registry 2004.*

Cantwell, H., Devery, R., O'Shea, M., Stanton, C., 1999. The effect of conjugated linoleic acid on the antioxidant enzyme defense system in rat hepatocytes. *Lipids 34(8): 3-9*

Calviello, G., Di Nicuolo, F., Serini, S., Picconi, E., Boninsegna, A., Maggiano, N., Raneletti, F.O., Palozza, P., 2005. Docosahexaenoic acid enhances the susceptibility of human colorectal cancer cells to 5-fluorouracil. *Cancer Chemotherapy and Pharmacology 55: 12-20*

Calviello, G., Serini, S., Palozza, P., 2006. n-3 polyunsaturated fatty acids in signal transduction modulators and therapeutical agents in cancer. *Current Signal Transduction Therapies 1: 255-71*

Cesano, A., Visonneau, S., Scimeca, J.A., Kritchevsky, D., Santoli, D., 1998. Opposite effects of linoleic acid and conjugated linoleic acid on human prostatic cancer in SCID mice. *Anticancer Research 18: 833-838*

Chamras, H., Ardashian, A., Heber, D., Glaspy, J.A., 2002. Fatty acid modulation of MCF-7 human breast cancer cell proliferation, apoptosis and differentiation. *Journal of Nutritional Biochemistry 13: 711-716*

Chiu, L.C., Wan, J.M., 1999. Induction of apotosis in HL-60 cells by eicosapentanoic acid (EPA) is associated with down-regulation of bcl-2 expression. *Cancer Letters 145: 17-27*

Chiu, L.C., Wong, E.Y., Ooi, V.E., 2006. Docosahexaenoic acid from a cultured microalga inhibits cell growth and induces apoptosis by up-regulating Bax/Bcl-2 ratio in human breast carcinoma MCF-7 cells. *Annals of the New York Academy of Sciences 1030: 361-8*

Chiu, L.C., Wong, E.Y., Ooi, V.E., 2004. Docosahexaenoic acid modulates different genes in cell cycle and apoptosis to control growth of human leukaemia HL-60 cells. *International Journal of Oncology 25: 737-744*

Cho, H.J., Kim, W.K., Kim, E.J., Jung, K.C., Park, S., Lee, H.S., Tyner, A.L., Park, J.H., 2003. Conjugated linoleic acid inhibits cell proliferation and ErbB3 signaling in HT-29 human colon cell line. *American Journal of Physiology –Gastrointestinal and Liver Physiology 284: G996-G1005*

Choi, Y., Kim, Y.C., Han, Y.B., Park, Y., Pariza, M.W., Ntambi, J.M., 2000. The trans-10,cis-12 isomer of conjugated linoleic acid downregulates stearoyl-CoA desaturase 1 gene expression in 3T3-L1 adipocytes. *Journal of Nutrition 130: 1920-1924*

Choi, Y., Park, Y., Storkson J.M., Pariza M.W., Ntambi J.M., 2002. Inhibition of stearoyl-CoA desaturase activity by the *cis*-9, *trans*-11 isomer and the *trans*-10, *cis*-12 isomer of conjugated linoleic acid in MDA-MB-231 and MCF-7 human breast cancer cells. *Biochemical and Biophysical Research Communications* 294: 785-790

Clynes, M., Daly, C., NicAmhlaoibh, R., Cronin, D., Elliott, C., O'Connor, R., O'Doherty, T., Connolly, L., Howlett, A., Scanlon, K., 1998. Recent developments in drug resistance and apoptosis research. *Critical Reviews in Oncology/Hematology 28 181-205*

Coakley, M., Johnson, M.C., McGrath, E., Rahman, S., Ross, R.P., Fitzgerald, G.F., Devery, R., Stanton, C., 2006. Intestinal Bifidobacteria that produce trans-9, trans-11 conjugated linoleic acid: a fatty acid with antiproliferative activity against human colon SW480 and HT-29 cancer cells. *Nutrition and Cancer 56(1): 95-102*

Colas, S., Paon, L., Denis, F., Prat, M., Louisot, P., Hoinard, C., Le Floch, O., Ogilive, G., Bougnoux, P., 2004. Enhanced radiosensitivity of rat autochothonous mammary tumors by dietary docosahexaenoic acid. *International Journal of Cancer 109: 449-454*

Colas, S., Maheo, K., Denis, F., Goupille, C., Hoinard, C., Champeroux, P., Tranquart, F., Bougnoux, P., 2006. Sensitization by dietary docosahexaenoic acid of

rat mammary carcinoma to anthracycline: a role for tumor vascularization. *Clinical Cancer Research 12: 5879-86*

Colomer, R., Montero, S., Lluch, A., Ojeda, B., Barnadas, A., Casado, A., Massutí, B., Cortés-Funes, H., Lloveras, B., 2000. Circulating HER2 Extracellular Domain and Resistance to Chemotherapy in Advanced Breast Cancer. *Clinical Cancer Research 6: 2356-62*

Cook, M.E., Jerome, D.L., Park, Y., Pariza, M.W., 1993. Immune modulation by altered nutrient metabolism: Nutritional control of immune-induced growth depression. *Poultry Science 72: 1301-1305*

Cooper, G.M., 2000. *The Cell: A Molecular Approach, 2nd edn.* Sunderland, Massachusetts, USA: Sinauer Associates, Inc.

Cuvillier, O., Rosenthal, D.S., Smulson, M.E., Spiegel, S., 1998. Sphingosine 1-phosphate inhibits activation of caspases that cleave poly(ADP-ribose) polymerase and lamins during Fas- and ceramide-mediating apoptosis in Jurkat T lymphocytes. *Journal of Biological Chemistry 273: 2910-2916*

D'Arpa, P., Liu, L.F., 1989. Topoisomerase-targeting antitumour drugs. *Biochemica et Biophysica Acta 989: 163-177*

Das, U.N., Madhavi N., Sravan Kumar G., Padma M., Sangeetha P., 1998. Can tumour cell drug resistance be reversed by essential fatty acids and their metabolites? *Prostaglandins, Leukotrienes and Essential Fatty Acids* 58 (1): 39-54

Davies, C.L., Loizidou, M., Cooper, A.J., Taylor, I., 1999. Effect of γ-linoleic acid on cellular uptake of structurally related anthracyclines in human drug sensitive and multidrug resistant bladder and breast cancer cell lines. *European Journal of Cancer 35 (10): 1534-40*

Denys, A., Hichami, A., Khan, N.A., 2005. n-3 PUFAs modulate T-cell activation via protein kinase C-alpha and –epsilon and the NF-kappaB signaling pathway. *Journal of Lipid Research 46: 752-8*

Diaz, J.F., Andreu J.M., 1993. Assembly of purified GDP-tubulin into microtubules induced by taxol and taxotere, reversibility, ligand stoichiometry and competition. *Biochemistry 32: 2747-55*

Doige, C.A., Yu, X., Sharom, F.J., 1993. The effects of lipids and detergents on ATPase-active P-glycoprotein. *Biochimica et Biophysica Acta (BBA) 1146(1): 65-72*

Donnelly, D.W., Gavin, A.T., Comber, H., 2009. Cancer in Ireland: a summary report. *Northern Ireland Cancer Registry/National Cancer Registry, Ireland; 2009* *http://ncri.ie/pubs/pubfiles/ALL_IRELAND_1994-2004_SUMMARY.pdf*

Dos Santos, S.M., Weber, C.C., Franke, C., Muller, W.E., Eckert, G.P., 2007. Cholesterol: Coupling between membrane microenvironment and ABC transporter activity. *Biochemical and Biophysical Research Communications 354(1): 216-21*

Doyle, L.A., Ross, D.D., 2003. Multidrug resistance mediated by the breast cancer resistance protein BCRP (ABCG2). *Oncogene 22(47):7340-58*

Druker, B.J., Talpaz, M., Resta, D.J., Peng, B., Buchdunger, E., Ford, J.M., Lydon, N.B., Kantarjian, H., Capdeville, R., Ohno-Jones, S., Sawyers, C.L., 2001. Efficacy and safety of a specific inhibitor of the BCR/ABL tyrosine kinase in chronic myeloid leukemia. *The New England Journal of Medicine 344 (14): 1031-37*

Duffy, C.P., Elliott, C.J., O'Connor, R.A., Heenan, M.M., Coyle, S., Cleary, I.M., Kavanagh, K., Verhaegen, S., O'Loughlin, C.M., NicAmhlaoibh, R., Clynes, M., 1998. Enhancement of chemotherapeutic drug toxicity to human tumour cell *in vitro* by a subset of non-steroidal anti-inflammatory drugs (NSAIDs). *European Journal of Cancer 34(8): 1250-1259*

Ecker, J., Langmann, T., Moehle, C., Schmitz, G., 2007. Isomer specific effects of Conjugated Linoleic Acid on macrophage ABCG1 transcription by a SREBP-1c dependent mechanism. *Biochemical and Biophysical Research Communications 352(3): 805-811*

Eichelberg, C., Heuera, R., Chuna, F.K., Hinrichsa, K., Zachariasa, M., Hulanda, H., Heinze, H., 2008. Sequential Use of the Tyrosine Kinase Inhibitors Sorafenib and Sunitinib in Metastatic Renal Cell Carcinoma: A Retrospective Outcome Analysis. *European Urology 54(6): 1373-1378*

Ellison, G., Klinowska, T., Westwood, R.F.R., Docter, E., French, T., Fox, J.C., 2002. Further evidence to support the melanocytic origin of MDA-MB-435. *Molecular Pathology 55: 294-299*

Ens, J.G., Ma, D.W.L., Cole, K.S., Field, C.J., Clandinin, M.T., 2001. An assessment of c9,t11 linoleic acid intake in a small group of young Canadians. *Nutrition Research 21: 955-960*

Epstein, R.J., 1988. Topoisomerase in human disease. *Lancet 1: 521-523*

Evans, M., Park, Y., Pariza, M., Curtis, L., Kuebler, B., McIntosh, M., 2001. Trans-10,cis-12 conjugated linoleic acid reduces triglyceride content while differentially affecting peroxisome proliferator activated receptor gamma2 and aP2 expression in 3T3-L1 preadipocytes. *Lipids 36(11): 1223-32*

Evans, M., Lin, X., Odle, J., McIntosh, M., 2002. Trans-10, cis-12 conjugated linoleic acid increases fatty acid oxidation in 3T3-L1 preadipocytes. *Journal of Nutrition 132(3): 450-455*

Fermor, B.F., Masters, J.R., Wood, C.B., Miller, J., Apostolov, K., Habib, N.A., 1992. Fatty acid composition of normal and malignant cells and cytotoxicity of stearic, oleic and sterculic acids *in vitro*. *European Journal of Cancer 28: 1143-47*

Fite, A., Goua, M., Wahle, K.W.J., Schofield, A.C., Hutcheon, A.W., Heys, S., 2007. Potentiation of the anti-tumour effect of docetaxel by conjugated linoleic acids (CLAs) in breast cancer cells in vitro. *Prostaglandins, Leukotrienes and Essential Fatty Acids 77: 87-96*

Folch J., Lees M., Sloane-Stanley G.H., 1956. A simple method for the isolation and purification of total lipids from animal tissues. *Journal of Biological Chemistry* 497-509

Fritsche and Steinhart, 1998. Amounts of conjugated linoleic acid (CLA) in German foods and evaluation of daily intake. *Zeitschrift fur Lebensmittel-Untersuchung und -Forschung. A, European food research and technology 206: 77-82*

Ganapathi, R. and Grabowski, D., 1983. Enhancement of the sensitivity of Adriamycin in resistant P3888 leukemia by the calmodulin inhibitor trifloperazine. *Cancer Research 43: 3696-3699*

Gaudino, G., Koch, T.H., Lo Bello, M., Nuccetelli, M., Ravagnan, G., Serafino, A., Sinibaldi-Vallebona, P., 2000. Lack of glutathione conjugation to adriamycin in human breast cancer MCF-7/DOX cells. *Biochemical Pharmacology 60: 1915-23*

Gaullier, J.M., Halse, J., Hoivik, H.O., Hoye, K., Svvertsen, C., Nureminiemi, M., Hassfeld, C., Einerhand, A., O'Shea, M., Gudmundsen, O., 2007. Six months supplementation with conjugated linoleic acid induces regional-specific fat mass decreases in overweight and obese. *British Journal of Nutrition 97(3): 550-560*

Germain, E., Chajes, V., Cognault, S., Lhuillery, C., Bougnoux, P., 1998. Enhancement of doxorubicin cytotoxicity by polyunsaturated fatty acids in the human breast tumour cell line MDA-MB-231: relationship to lipid peroxidation. *International Journal of Cancer 75: 578-583*

Gigli, M., D, Doglia S. M., Millot J.M., Valentini L., Manfait, M., 1988. *Biochimica et Biophysica Acta 950: 13-20*

Gittleman, M., Pommerville, P.J., Persson, B-E, Jenson, J-K, Olesen, T.K., 2008. A 1-Year, Open Label, Randomized Phase II Dose Finding Study of Degarelix for the Treatment of Prostate Cancer in North America. *The Journal of Urology 180(5): 1986-1992*

Glynn, S.A., Gammell, P., Heenan, M., O'Connor, R., Liang, Y., Keenan, J., Clynes, M., 2004. A new superinvasive in vitro phenotype induced by selection of human breast carcinoma cells with the chemotherapeutic drugs paclitaxel and doxorubicin. *British Journal of Cancer 91: 1800-1807*

Goua, M., Mulgrew, S., Frank, J., Rees, D., Sneddon, A.A., Wahle, K.W.J., 2008. Regulation of adhesion molecule expression in human endothelial and smooth muscle cells by omega-3 fatty acids and conjugated linoleic acids: Involvement of the transcription factor NF-κB? *Prostaglandins, Leukotrienes and Essential Fatty Acids 78(1): 33-43*

Gouaze, V., Liu, Y.Y., Prickett, C.S., Yu, J.Y., Giuliano, A.E., Cabot, M.C., 2005. Glucosylceramide synthase blockade down-regulates P-glycoprotein and resensitizes multidrug-resistant breast cancer cells to anticancer drugs. *Cancer Research 65: 3861-67*

Gottesman, M.M., 1993. How cancer cells evade chemotherapy: Sixteenth Richard and Hinda Rosenthal Foundation Award Lecture. *Cancer Research 53: 747-754*

Granlund, L., Pedersen, J.I., Nebb, H.I., 2005. Impaired lipid accumulation by trans 10, cis 12 CLA during adipocyte differentiation is dependent on timing and length of treatment. *Biochimica et Biophysica Acta. Molecular and cell biology of lipids 1687(1-3): 11-22*

Griinari, J.M., Dwyer, D.A., McGuire, M.A., Bauman, D.E., Palmquist, D.L., Nurmela, K.V.V., 1998. Trans-Octadecenoic Acids and Milk Fat Depression in Lactating Dairy Cows. *Journal of Dairy Science 81 (5): 1251-61*

Gueritte-Voegelein, F., Guenard, D., Lavelle F., Le Goff, M.T., Mangatal, L., Potier, P., 1991. Relationships between the structure of taxol analogues and their antimitotic activity. *Journal of Medicinal Chemistry 34: 992-928*

Guthrie, N., Carroll K.K., 1999. Specific versus non-specific effects of dietary fat on carcinogenesis. *Progress in Lipid Research 38: 261-271*

Ha, Y.L., Grimm, N.K., Pariza, M.W., 1987. Anticarcinogens from ground beef: heat-altered derivatives of linoleic acid. *Carcinogenesis 8: 1881-7*

Ha, Y.L., Grimm, N.K., Pariza, M.W., 1990. Inhibition of benzo(a)pyrene-induced mouse forestomach neoplasia by conjugated dienoic derivatives of linoleic acid. *Cancer Research 50: 1097-1101*

Hakomori, S., 1992. The functional role of glycosphingolipids in tumor progression. *The Tohoku Journal of Experimental Medicine 168(2): 211-222*

Hanklin, J.H., 1993. Role of nutrition in women's health: diet and breast cancer. *Journal of the American Dietetic Association 93: 994-999*

Hannun, Y.A., 1994. The sphingomyelin cycle and the second messenger function of ceramide. *Journal of Biological Chemistry 269: 3125-8*

Harbottle, A., Daly, A.K., Atherton, K., Campbell, F.C., 2001. Role of glutathione S-transferase P1, P-glycoprotein and multidrug resistance-associated protein-1 in acquired doxorubicin resistance. *International Journal of Cancer 92: 777-83*

Harder, T., Simons, K., 1997. Caveolae, DIGs, and the dynamics of sphingoslipid-cholesterol microdomains. *Current Opinions in Cell Biology 9: 534-542*

Hardman, W.E., Moyer, M.P. and Cameron, I.L., 1999. Fish oil supplementation enhanced CPT-11 (irinotecan) efficacy against MCF7 breast carcinoma xenografts and ameliorated intestinal side-effects. *British Journal of Cancer 81 (3): 440-448*

Hardman, W.E., Moyer, M.P. and Cameron, I.L., 2000. Dietary fish oil sensitizes A549 lung xenographs to doxorubicin chemotherapy. *Cancer Letters 151: 145-151*

Hardman, W.E., Avula, C.P.R., Fernandes, G., Cameron, I.L., 2001. Three percent dietary fish oil concentrate increased efficacy of doxorubicin against MDA-MB 231 human breast cancer xenografts. *Clinical Cancer Research 7: 2041-2049*

Hardman, W.E., 2002. Role of lipid peroxidation and antioxidant enzymes in omega-3 fatty acids induced suppression of breast cancer xenografts growth in mice. *Cancer Cell International 2:* http://www.cancerci.com/content/2/1/10

Hardman, W.E., Moyer, M.P. and Cameron, I.L., 2002. Consumption of an omega-3 fatty acids product, INCELL AAFA, reduced side-effects of CPT-II (irinotecan) in mice. *British Journal of Cancer 86(6): 983-988*

Hardman, W.E., Sun, L., Short, N., Cameron, I.L., 2005. Dietary omega-3 fatty acids and ionizing irradiation on human breast xenografts growth and angiogenesis. *Cancer Cell International 5:12:* http://www.cancerci.com/content/5/1/12

Hardy, S., Langelier, Y., Prentki, M., 2000. Oleate activates phosphatidylino-sitol 3-kinase and promotes proliferation and reduces apoptosis of MDA-MB-231 breast cancer cells, whereas palmitate has opposite effects. *Cancer Research 60: 6353–6358*

Hendrich, A.B. and Michalak, K., 2003. Lipids as a target for drugs modulating multidrug resistance of cancer cells. *Current Drug Targets 4: 23-30*

Hubbard, N.E., Lim, D., Summers, L., Erickson, K.L., 2000. Reduction of murine mammary tumor metastasis by conjugated linoleic acid. *Cancer Letters 150: 93-100*

Hubbard, N.E., Lim, D., Erickson, K.L., 2003. Effect of separate conjugated linoleic acid isomers on murine mammary tumorigenesis. *Cancer Letters 190: 13-19*

Hubbard N.E., Lim, D., Erickson, K.L., 2006. Beef tallow increases the potency of conjugated linoleic acid in the reduction of mouse mammary tumor metastasis. *Journal of Nutrition 136: 88-93*

Hubbard N.E., Lim, D., Erickson, K.L., 2007. Conjugated linoleic acid alters matrix metalloproteinases of metastatic mouse mammary tumor cells. *Journal of Nutrition 137(6): 1423-9*

Hwang, D.M., Joydeb, K.K., Shin, J-W., Lee, J-C., Hyong, J.L., Surh, Y-J., 2007. cis -9,trans -11 -conjugated linoleic acid down-regulates phorbol ester-induced NF-κB activation and subsequent COX-2 expression in hairless mouse skin by targeting IκB kinase and PI3K-Akt. *Carcinogenesis 28(2): 363-371*

Igarashi, M., Tsuzuki, T., Kambe, T., Miyazawa, T., 2004. Recommended methods of fatty acid methyl ester preparation for conjugated dienes and trienes in food and biological samples. *Journal of Nutritional Science and Vitaminology (Tokyo) 50(2): 121-128*

Ingvarsson, S., 1999. Molecular genetics of breast cancer progression. *Seminars in Cancer Biology 9: 277-288*

Ip, C., Chin, S.F., Scimeca, J.A., Pariza M.W., 1991. Mammary cancer prevention by conjugated dienoic derivative of linoleic acid. *Cancer Research 51: 6118-24*

Ip, C., Singh M., Thompson, H.J., Scimeca, J.A., 1994. Conjugated linoleic acid suppresses mammary carcinogenesis and proliferative activity of the mammary gland in the rat. *Cancer Research 54: 1212-15*

Ip, C., Scimeca, J.A., Thompson, H., 1995. Effect of timing and duration of dietary conjugated linoleic acid on mammary cancer prevention. *Nutrition and Cancer 24: 241-247*

Ip, C., Briggs, S.P., Haegele, A.D., Thompson, H.J., Storkson, J., Scimeca, J.A., 1996. The efficacy of conjugated linoleic acid in mammary cancer prevention is independent of the level or type of fat in the diet. *Carcinogenesis 17: 1045-50*

Ip, C., Jiang, C., Thompson, H.J., Scimeca, J.A., 1997. Retention of conjugated linoleic acid in the mammary gland is associated with tumour inhibition during the post-initiation phase of carcinogenesis. *Carcinogenesis 18: 755-759*

Ip, C., Scimeca, J.A., 1997. Conjugated linoleic acid and linoleic acid are distinctive modulators of mammary carcinogenesis. *Nutrition and Cancer 27: 131-135*

Ip, M.M., Masso-Welch, P.A., Shoemaker, S.F., Shea-Eaton, W.K., Ip, C., 1999. Conjugated linoleic acid inhibits proliferation and induces apoptosis of normal rat mammary epithelial cells in primary culture. *Experimental Cell Research 250: 22-34*

Ip, C., Banni, S., Angioni E., Carta, G., McGinley, J., Thompson, H.J., Barbano, D., Bauman, D., 1999. Conjugated linoleic acid-enriched butter fat alters mammary gland morphogenesis and reduces cancer risk in rats. *Journal of Nutrition 129: 2135-42*

Ip, C., Loftus, T., Shoemaker, S.F., Shea-Eaton, W.K., 2000. Induction of apoptosis by conjugated linoleic acid in cultured mammary tumor cells and premalignant lesions of the rat mammary gland. *Cancer Epidemiology Biomarkers & Prevention 9: 689-696*

Ip, M.M., McGee, S.O., Masso-Welch, P.A., Ip, C., Meng, X., Ou, L., Shoemaker, S.F., 2007. The *t*10,*c*12 isomer of conjugated linoleic acid stimulates mammary

tumorigenesis in transgenic mice over-expressing erbB2 in the mammary epithelium. *Carcinogenesis. 28: 1269-1276.*

Ip, C., Dong, Y., Thompson, H.J., Bauman, D., Ip, M.M., 2001. Control of rat mammary epithelium proliferation by conjugated linoleic acid. *Nutrition and Cancer 39: 233-238*

Iwakiri, Y., Sampson, D.A., Allen, K.G.D., 2002. Suppression of cyclooxygenase-2 and inducible nitric oxide synthase expression by conjugated linoleic acid in murine macrophages. *Prostaglandins Leukotrienes and Essential Fatty Acids 67(6):435–443*

Jiang, J., Bjoerck, L., Fonden, R., Emanuelson, M., 1996. Occurrence of conjugated cis-9, trans-11-octadecadienoic acid in bovine milk: effects of feed and dietary regimen. *Journal of Dairy Science 79: 438-445*

Jolly, C.A., Jiang, Y.H., Chapkin, R.S., McMurray, D.N., 1997. [n-3] polyunsaturated fatty acids suppress murine lymphoproliferation, interleukin-2 secretion, and the formation of diacylglycerol and ceramide. *Journal of Nutrition 127: 37-43*

Johnstone, R.W., Ruefli, A.A., Smyth, M.J., 2000. Multiple physiological functions for multidrug transporter P-glycoprotein? *Trends in Biochemical Sciences 25(1):1-6*

Karmali, R.A., 1987. Eicosanoids in neoplasia. *Preventative Medicine 16: 493-502*

Kemp, M.Q., Jeffy, B.D., Romagnolo, D.F., 2003. Conjugated linoleic acid inhibits cell proliferation through a p53-dependent mechanism: Effects on the expression of G1-restriction points in breast and colon cancer cells. *Journal of Nutrition 133(11): 3670-3677*

Kepler, C.R., Tucker, P.W., Tove, S.B., 1970. Biohydrogenation of unsaturated fatty acids. IV. Substrate specificity and inhibition of linoleate delta-12-cis, delta-11-trans-isomerase from Butyrivibrio fibrisolvens. *Journal of Biological Chemistry 245 (14): 3612-20*

Kickhoefer, V.A., Rajavel, K.S., Scheffer, G.L., Dalton, W.S., Scheper, R.J., Rome, L.H., 1998. Vaults are up-regulated in multidrug-resistant cancer cell lines. *The Journal of biological chemistry 273(15): 8971-4*

Kim, E., Kang, I.J., Cho, H.J., Kim, W.K., Ha, Y.L., Park, J.H.Y., 2003. Conjugated linoleic acid downregulates insulin-like growth factor-I receptor levels in HT-29 human colon cancer cells. *Journal of Nutrition 133: 2675-81*

Kim, H.K., Choi, S., Choi, H., 2004. Suppression of hepatic fatty acid synthase by feeding α-linolenic acid rich perilla oil lowers plasma triacylglycerol level in rats. *Journal of Nutritional Biochemistry 15: 485-492*

Kim, Y., Liu, R.H., 2000. Selective increase in conjugated linoleic acid in milk fat by crystallization. *Journal of Food Science 5: 792-795*

Kim, Y.L., Liu, R.H., Rychlik, J.L., Russell, J.B., 2002. The enrichment of a ruminal bacterium (*Megasphaera elsdenii* YJ-4) that produces the trans-10, cis-12 isomer of conjugated linoleic acid. *Journal of Applied Microbiology 92: 976-982*

Knowles, L.M., Smith, J.W., 2007. Genome-wide changes accompanying knockdown of fatty acid synthase in breast cancer. *BMC Genomics 8: 168-180*

Kolesnick, R.N., Kronke, M., 1998. Regulation of ceramide production and apoptosis. *Annual Reviews in Physiology 60: 643-665*

Koritala, S. and Rohwedder, W.K., 1972. Formulation of an artifact during methylation of conjugated fatty acids. *Lipids 7: 274-278*

Kramer, J.K.G., Kellner, V., Dugan, M.E.R., Sauer, F.D., Mossoba, M.M., Yurwecz, M.P., 1997. Evaluating acid and base catalysts in the methylation of milk and rumen fatty acids with special emphasis on conjugated dienes and total trans fatty acids. *Lipids 32: 1219-1228*

Kuhajada, F.P., Piantadosi, S., Pasternack, G.R., 1989. Haptoglobin-related protein (Hpr) epitopes in breast cancer as a predictor of recurrence of the disease. *New England Journal of Medicine 321: 636-641*

Kuhajada, F.P., Jenner, K., Wood, F.D., Henigar, R.A., Jacobs, L.B., Dick, J.D., Pasternack, G.R., 1994. Fatty acid synthesis: a potential selective target for antineoplastic therapy. *Proceedings of the National Academy of Sciences of the United States of America 91: 6379-83*

Kuhajda, F.P., 2000. Fatty acid synthase and human cancer: new perspectives on its role in tumor biology. *Nutrition 16: 202-208*

Labrie, F., Duponta, A., Cusana, L., Giguerea, M., Beroerona, N., Borsanyia, J-P., Lacourcierea, Y., Belangera, A., Emonda, J., Monfettea, G., Bouchera, H., Lachancea, R., 1988. Combination therapy with flutamide and castration (LHRH agonist or orchiectomy) in previously untreated patients with clinical stage D2 prostate cancer: Today's therapy of choice. *Journal of Steroid Biochemistry 30: 107-117*

Ladner, R.D., Lynch, F.J., Groshen, S., Xiong Y.P., Sherrod, A., Caradonna, S.J., Stoehlmacher, J., Lenz, H-J., 2000. dUTP nucleotidohydrolase isoform expression in normal and neoplastic tissues: association with survival and response to 5-Fluorouracil in colorectal cancer. *Cancer Research 60: 3493-3503*

Lampen, A., Leifheit, M., Voss, J., Nau, H., 2005. Molecular and cellular effects of *cis*-9, *trans*-11-conjugated linoleic acid in enterocytes: Effects on proliferation, differentiation, and gene expression. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids 1735(1): 30-40*

Lavie, Y., Cao, H-T., Bursten, S.L., Giuliano, A.E., Cabot, M.C., 1998. Accumulation of Glucosylceramides in Multidrug-resistant Cancer Cells. *Journal of Biological Chemistry 271(32): 19530-36*

Lazo, J.S., Larner, L.M., 1998. Chapter 45: Individual antineoplastic drugs. *Human Pharmacology: Molecular to Clinical.* Mosby 3rd Edition. Editors: Brody, T.M., Larner, J., Minneman, K.P.

Lee, K.N., Kritchevsky, D., Pariza, M.W., 1994. Conjugated linoleic acid and atherosclerosis in rabbits. *Atherosclerosis 108: 19-25*

Lee, Y., Thompsom, J.T., de Lera, A.R., Vanden Heuvel, J.P., 2008. Isomer-specific effects of conjugated linoleic acid on gene expression in RAW 264.7. *Journal of Nutritional Biochemistry* Article in Press, available online Nov '08. http://dx.doi.org/10.1016/j.jnutbio.2008.07.013

Liew, C., Schut, H.A.J., Chin, S.F., Pariza, M.W., Dashwood, R.H., 1995. Protection of conjugated linoleic acids against 2-amino-3-methylimidazol[4,5-f]quinoline-induced colon carcinogenesis in the F344 rat: a study of inhibitory mechanisms. *Carcinogenesis 16: 3037-43*

Lim, D.Y., Tyner, A.L., Park, J-B., Lee, J-Y., Choi, Y.H., Park, J.H.Y., 2005. Inhibition of colon cancer cell proliferation by the dietary compound conjugated linoleic acid is mediated by the CDK inhibitor $^{p21CIP1/WAF1}$. *Journal of Cellular Physiology 205: 107-113*

Lin, H., Boylston, T.D., Chang, M.J., Luedecke, L.O., Shultz, T.D., 1995. Survey of the conjugated linoleic acid contents of dairy products. *Journal of Dairy Science 78: 2358-65*

Liu, H., Liu, Y., Zhang, J-T., 2008. A new mechanism of drug resistance in breast cancer cells: fatty acid synthase overexpression-mediated palmitate overproduction. *Molecular Cancer Therapeutics 7(2): 263-270*

Liu, J., Chen, H., Miller, D.S., Saavedra, J.E., Keefer, L.K., Johnson, D.R., Klaasson, C.D., Waalkes, M.P., 2001. Over expression of glutathione S-transferase II and multidrug resistance transport proteins is associated with acquired tolerance to inorganic arsenic. *Molecular Pharmacology 60: 302-309*

Liu, L.F., 1989. DNA Topoisomerase Poisons as Antitumor Drugs. *Annual Review of Biochemistry 58: 351-375*

Liu, P., Rudick, M., Anderson, R.G., 2002. Multiple functions of caveolin-1. *Journal of Biological Chemistry 277(44): 41295-8*

Lodish, H., Berk, A., Matsudaira, P., Kaiser, C.A., Krieger, M., Scott, M.P., Zipursky, S.L., Darnell, J., 2004. *Molecular Cell Biology, 4th edn.* New York, USA: W.H. Freeman and Company

Lopez, A.D., Mathers, C.D., Ezzati, M., Jamison, D.T., Murray, C.J., 2006. Global and regional burden of disease and risk factors, 2001: systematic analysis of population health data. *The Lancet 367(9524): 1747-57*

Loscher, C.E., Draper, E., Leavy, O., Kelleher, D., Mills, K.G.H., Roche, H.M., 2005. Conjugated linoleic acid suppresses NF-kappa B activation and IL12 production in dendritic cells through ERK mediated IL10 induction. *Journal of Immunology 175 (8): 4990-8*

Ma, D.W., Seo, J., Davidson, L.A., Callaway, E.S., Fan, Y-Y., Lupton, J.R., Chapkin, R.S., 2004. *n*-3 PUFA alter caveolae lipid composition and resident protein localization in mouse colon. *FASEB Journal 18: 1040-2*

Mackie, S.J., Sharma, D.M., Cooper, A.J., Harris, N.M., Lwaleed, B.A., 2006. Meglumine Eicosapentaenoic acid (MeEPA) a new soluble omega-3 fatty acid formulation: In vitro bladder cancer cytotoxicity tests in combination with epirubicin and Mitomycin. *Prostaglandins, Leukotrienes and Essential Fatty Acids 75: 367-73*

Mahéo, K., Vibet, S., Steghens, J.P., Dartigeas, C., Lehman, M., Boughnoux, P., Goré, J., 2005. Differential sensitization of cancer cells to doxorubicin by DHA: A role for lipoperoxidation. *Free Radical Biology & Medicine 39: 742-751*

Maier, T., Jenni, S., Ban, N., 2006. Architecture of mammalian fatty acid synthase at 4.5 Å resolution. *Science 311: 1258-62*

Majumder, B., Wahle, K.W., Moir, S., Schofield, A., Choe, S.N., Farquharson, I., Grant, I., Heys, S.D., 2002. Conjugated linoleic acids (CLAs) regulate the expression of key apoptotic genes in human breast cancer cells. *FASEB Journal 16: 1447-9*

Mao, Q., Unadkat, J.D., 2005. Role of the breast cancer resistance protein (ABCG2) in drug transport. *AAPS J. 7(1): 118-33*

Marmor, M.D., Skaria, K.B., Yarden, Y., 2004. Signal transduction and oncogenesis by ErbB/HER receptors. *International Journal of Radiation Oncology *Biology *Physics 58(3): 903-913*

Martin, A. and Clynes, M., 1991. Acid phosphatase: Endpoint for *in vitro* toxicity tests. *In Vitro Cell. Dev. Biol. 27A: 183-184*

Masso-Welch, P.A., Zangani, D., Ip, C., Vaughan, M.M., Shoemaker, S.F., Oflazoglu McGee, S., Ip, M.M., 2004. Isomers of Conjugated Linoleic Acid Differ in Their Effects on Angiogenesis and Survival of Mouse Mammary Adipose Vasculature. *Journal of Nutrition 134: 399-407*

McGrath, E., 2003. *Biological Production of CLA and Investigation of Anticarcinogenic Activity. M.Sc. Thesis.* Dublin City University.

McGuire, M.A, McGuire, M.K., Parodi, P.W., Jensen, R.G., 1999. Conjugated linoleic acids in human milk. In Advances in Conjugated Linoleic Acid Research, Vol. 1. Ed. M.P. Yurawecz, M.M. Mossoba, J.K.G. Kramer, M.W. Pariza, G.J. Nelson pp. 296-306 AOCS Press Champaign, IL.

McMurray, D.N., Jolly, C.A., Chapkin, R.S., 2000. Effects of dietary n-3 fatty acids on T-cell activation and T cell receptor-mediated signalling in a murine model. *Journal of Infectious Diseases 182: S103-7*

Medina, D., 1996. The mammary gland: a unique organ for the study of development and tumourigenesis. *Journal of the Mammary Gland and Neoplasia 1: 5-19*

Menendez, J.A., del Mar Barbacid, M., Montero, S., Seville, E., Escrich, E., Solanas, M., Cortes-Funes, H., Colomer, R., 2001. Effects of gamma-linolenic acid and oleic acid on paclitaxel cytotoxicity in human breast cancer cells. *European Journal of Cancer 37: 402-413*

Menendez, J.A., Ropero, S., del Mar Barbacid, M., Montero, S., Solanas, M., Escrich, E., Cortes-Funes, H., Colomer, R., 2002. Synergistic interaction between vinorelbine and gamma-linolenic acid in breast cancer cells. *Breast Cancer Research and Treatment 72: 203-219*

Menendez, J.A., Lupu, R., Colomer, R., 2004. Inhibition of tumor-associated fatty acid synthase hyperactivity induces synergistic chemosensitization of HER-2/neu-overexpressing human breast cancer cells to docetaxel (taxotere). *Breast cancer research and treatment 84(2): 183-195*

Menendez, J.A., Ropero, S., Mehimi, I., Atlas, E., Colomer, R., Lupu, R., 2004. Overexpression and hyperactivity of breast cancer-associated fatty aicd synthase

(oncogenic antigen-519) is insensitive to normal arachidonic fatty acid-induced suppression in lipogenic tissues but it is selectively inhibited by tumoricidal alpha-linolenic and gamma-linolenic fatty acids: a novel mechanism by which dietary fat can alter mammary tumorigenesis. *International Journal of Oncology 24: 1369-83*

Menendez, J.A., Colomer, R., Lupu, R., 2005. Why does tumor-associated fatty acid synthase (oncogenic antigen-519) ignore dietary fatty acids? *Medical Hypotheses 64: 342-349*

Menendez, J.A., Colomer, R., Lupu, R., 2005b. Inhibition of fatty acid synthase-dependent neoplastic lipogenesis as the mechanism of γ-linolenic acid-induced toxicity to tumor cells: an extension to Nwankwo's hypothesis. *Medical Hypotheses 64: 337-341*

Menendez, J.A., Lupu, R., Colomer, R., 2005c. Exogenous supplementation with ώ-3 polyunsaturated fatty acid docosahexanaenoic acid (DHA; 22:6*n*-3) synergistically enhances taxane cytotoxicity and downregulates Her2/*neu* (c-*erb*B-2) oncogene expression in human breast cancer cells. *European Journal of Cancer Prevention 14: 263-270*

Menendez, J.A., Vellon, L., Colomer, R., Lupu, R., 2005. Effect of γ-linolenic acid on the transcriptional activity of the her-2/*neu* (erbB-2) oncogene. *JNCI Journal of the National Cancer Institute 97(21):1611-1615*

Meng, X., Shoemaker, S., McGee, S.O., Ip, M.M., 2008. *t*10,*c*12-Conjugated linoleic acid stimulates mammary tumor progression in her2/erbB2 mice through activation of both proliferative and survival pathways. *Carcinogenesis*, in press.

Merchant, T.E., Meneses, P., Gierke, L.W., Den Otter, W., Glonek, T., 1991. 31P magnetic resonance phospholipid profiles of neoplastic human breast tissues. *British Journal of Cancer 63(5): 693-698*

Merchant, T.E., Diamantis, P.M., Lauwers, G., Haida, T., Kasimos, J.N., Guillem, J., Glonek, T., Minsky, B.D., 1995. Characterization of malignant colon tumors with 31^{p} nuclear magnetic resonance phospholipid and phosphatic metabolite profiles. *Cancer 76(10): 1715-1723*

Merrill, A.H. Jr., Schelz, E.M., Dillehay, D.L., Spiegel, S., Shayman, J.A., Schroeder, J.J., Riley, R.T., Voss, K.A., Wang, E., 1997. Sphingolipids: the enigmatic lipid class: biochemistry, physiology, and pathophysiology. *Toxicology and Applied Pharmacology 142: 208-225*

Miglietta, A., Bozzo, F., Bocca, C., Gabriel, L., Trombetta, A., Belotti, S., Canuto, R.A., 2006. Conjugated linoleic acid induces apoptosis in MDA-MB-231 breast cancer cells through ERK/MAPK signalling and mitochondrial pathway. *Cancer Letters 234: 149-157*

Miller, A., Stanton, C., Devery, R., 2001. Modulation of arachidonic acid distribution by conjugated linoleic acid isomers and linoleic acid in MCF-7 and SW480 cancer cells. *Lipids 36(1): 1161-68*

Miller, A., Stanton, C., Devery, R., 2002. Cis 9, trans 11- and trans 10, cis 12-conjugated linoleic acid isomers induce apoptosis in cultured SW480 cells. *Anticancer Research 22: 3879-88*

Miller, A., Stanton, C., Murphy, J., Devery, R., 2003a. Conjugated linoleic acid (CLA)-enriched milk fat inhibits growth and modulates CLA-responsive biomarkers in MCF-7 and SW480 human cancer cell lines. *British Journal of Nutrition 90: 877-885*

Miller, A., McGrath, E., Stanton, C., Devery, R., 2003b. Vaccenic acid (*t*11-18:1) is converted to *c*9,*t*11-CLA in MCF-7 and SW480 cancer cells. *Lipids 38(6): 623-632*

Miller, C.C., Park. Y., Pariza, M.W., Cook, M.E., 1994. Feeding conjugated linoleic acid to animals partially overcomes catabolic responses due to endotoxin injection. *Biochem. Biophys. Res. Commun. 198: 1107-12*

Miyasaki, M., Gomez, F.E., Ntambi, J.M., 2002. Lack of stearoyl-CoA desaturase-1 function induces a palmitoyl-CoA Δ6 desaturase and represses the stearoyl-CoA desaturase-3 gene in the preputial glands of the mouse. *Journal of Lipid Research 43: 2146-2154*

Morrow, J.D., Roberts, L.J., 1997. The isoprostanes: Unique bioactive products of lipid peroxidation. *Progress in Lipid Research 36: 1-21*

Moscow, J.A., Cowan, K.H., 1988. Multidrug resistance. *Journal of the National Cancer Institute 80: 14-20*

Mossink, M.H., van Zon, A., Scheper, R.J., Sonneveld, P., Wiemer, E.A.C., 2003. Vaults: a ribonucleoprotein particle involved in drug resistance? *Oncogene 22: 7458–67*

Moya-Camarena, S.Y., Vanden Heuvel, J.P., Belury, M.A., 1999. Conjugated linoleic acid activates peroxisome proliferator-activated receptor α and β subtypes but does not induce hepatic peroxisome proliferation in Sprague–Dawley rats. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids 1436(3): 331-342*

Mueller, H., Eppenberger, U., 1996. The dual role of mutant p53 protein in chemosensitivity of human cancers. *Anticancer research. 16(6B):3845-8.*

Murphy, M.M., Coakley, M., Stanton, C., 2008. Supplementation of dairy cows with a fish oil containing supplement and safflower oil to increase the CLA content in milk produced at pasture. *Livestock Science 116: 332-337*

Nagao, K., Yanagita, T., 2005. Conjugated fatty acids in food and their health benefits. *Journal of Bioscience and Bioengineering 100(2): 152-157*

Narayanan, B.A., Narayanan, N.K., Simi, K., Reddy, B.S., 2003. Modulation of inducible nitric oxide synthase and related proinflammatory genes by the omega-3 fatty acid docosahexanoic acid in human colon cancer cells. *Cancer Research 63: 972-9*

National Cancer Registry, Ireland, 2007. Cancer in Ireland 1994-2005 a summary. *http://www.ncri.ie/pubs/pubfiles/summary2007.pdf*

Navarro, V., Macarulla, M.T., Fernandez-Quintela, A., Rodriquez, V.M., Simon, E., Portillo, M.P., 2007. Effects of trans-10,cis-12 conjugated linoleic acid on cholesterol metabolism in hypercholesterolaemic hamsters. *European Journal of Nutrition 46(4): 213-219*

Nicolaou, K.C., Guy, R.K., Potier, P., 1996. New weapons against cancer. *Scientific American 84:88*

Ntambi, J.M., Miyazaki, M., 2004. Regulation of stearoyl-CoA desaturases and role in metabolism. *Progress in Lipid Research 43: 91-104*

Nugent, A.P., Roche H.M., Noone E.J, Long, A., Kelleher, D.K., Gibney, A.M., 2005. The effects of conjugated linoleic acid supplementation on immune function in healthy volunteers. *European Journal of Clinical Nutrition* 59*: 742-750*

O'Brien, M.L., Tew, K.D., 1996. Glutathione and related enzymes in multidrug resistance. *European Journal of Cancer 32A: 967–978*

Ochoa, J.J., Farquharson, A.J., Grant, I., Moffat, L.E., Heys, S.D, Wahle, K.W.J., 2004. Conjugated linoleic acids (CLAs) decrease prostate cancer cell proliferation: different molecular mechanisms for *cis*-9, *trans*-11 and *trans*-10, *cis*-12 isomers. *Carcinogenesis 25(7): 1185-91*

O'Connor, R., Heenan, M., Duffy, C., Clynes, M., 1998. Miniaturized in vitro methods in toxicity testing. In *Animal cell culture techniques*, Chap. **22,** 423-433. Berlin; New York: Springer.

Ogilvie, G.K., Fettman, M.J., Mallinckrodt, C.H., Walton, J.A., Hansen, R.A., Davenport, D.J., Gross, K.L., Richardson, K.L., Rogers, Q., Hand, M.S., 2000. Effect of fish oil, arginine, and doxorubicin chemotherapy on remission and survival time for dogs with lymphoma. A double-blind, randomized placebo-controlled study. *Cancer 88(8): 1916-28*

Ogretman, B., Hannun Y.A., 2001. Updates on functions of ceramide in chemotherapy-induced cell death and in multidrug resistance. *Drug Resistance Updates 4(6): 368-377*

Ohira, T., Nishio, K., Ohe, Y., Arioka, H., Nishio, M., Funayama, Y., Ogasawara, H., Kukada, M., Yazawa, K., Kato, H., Saijo, N., 1996. Improvement by eicosanoids in cancer cachexia induced by LLC-IL6 transplantation. *Journal of Cancer Research and Clinical Oncology 122(12): 711-715*

Olivera, A., Spiegel, S., 1993. Sphingosine-1-phosphate as second messenger in cell proliferation induced by PDGF and FCS mitogens. *Nature 365: 557-560*

Olson, R.D. and Mushlin, P.S., 1990. Doxorubicin cardiotoxicity: analysis of prevailing hypotheses. *FASEB Journal 4: 3076-3086*

O'Shea M., Lawless F., Stanton C., Devery R., 1998. Conjugated linoleic acid in bovine milk fat: a food-based approach to cancer chemoprevention. *Trends in Food Science & Technology 9: 192-196*

O'Shea, M., Stanton, C., Devery, R., 1999. Antioxidant enzyme defence responses of human MCF-7 and SW480 cancer cells to conjugated linoleic acid. *Anticancer Research 19: 1953-1960*

O'Shea, M., Devery, R., Lawless, F., Murphy, J., Stanton, C., 2000. Milk fat conjugated linoleic acid (CLA) inhibits growth of human mammary MCF-7 cancer cells. *Anticancer Research 20: 3591-3602*

Ou, L., Ip, C., Lisafeld, B., Ip, M.M., 2007. Conjugated linoleic acid induces apoptosis of murine mammary tumor cells via Bcl-2 loss. *Biochemical and Biophysical Research Communications 356: 1044-9*

Pariza, M.W., Park, Y., Cook, M.E., 2001. The biologically active isomers of conjugated linoleic acid. *Progress in Lipid Research 40: 283–298*

Park, Y., Albright, K.J., Storkson, J.W., Liu, W., Cook, M.E., Pariza, M.W., 1997. Effect of conjugated linoleic acid on body composition in mice. *Lipids 32: 853-8*

Park, J.H., Cho, H.J., Kim, W.K., Kim, E.J., Tyner, A.L., 2003. Conjugated linoleic acid (CLA) induces apoptosis and inhibits coupling of heregulin (HRG) signaling to phosphatidylinositol 3-kinase (PI3K) and Akt via ErbB3 next term phosphorylation in human colon cancer Ht-29 cells. *Gastroenterology 124(4): A281*

Parodi, P.W., 2003. *In: Advances in conjugated linoleic acid research Vol. 2.* Editors: Sebedio, J.L., Christie, W.W., Adlof, R. AOCS Press, Champaign (IL); 2: 189-217

Pascale, A.W., Ehringer, W.D., Stillwell, W., Sturdevant, L.K., Jenski, L.J., 1993. Omega-3 fatty acid modification of membrane structure and function. II. Alteration by docosahexanoic acid of tumor cell sensitivity to immune cytolysis. *Nutrition and Cancer 19: 147-57*

Pinedo, H.M., Peters, G.F.J., 1988. Fluorouracil: biochemistry and pharmacology. *Journal of Clinical Oncology 6: 1653-1664*

Polyak, K., 2001. On the birth of breast cancer. *Biochimica et Biophysica Acta 1552: 1-13*

Polyak, K., 2002. Breast cancer gene discovery. *Expert Reviews in Molecular Medicine 15 August, http://www.expertreviews.org/0200491Xh.htm*

Powis, G., Prough, R.A., 1997. Metabolism and Action of Anticancer Drugs. *London: Taylor and Frances. Pp. 211-260*

Purushotham, A., Shrode, G.E., Wendel, A.A., Li-Fen Liu, L-F., Belury, M.A., 2007. Conjugated linoleic acid does not reduce body fat but decreases hepatic steatosis in adult Wistar rats. *Journal of Nutritional Biochemistry 18(10): 676-684*

Quiles, J.L., Huertas, J.R., Battino, M., Mataix, J., Ramírez-Tortosa, M.C., 2002. Antioxidant nutrients and adriamycin toxicity. *Toxicology 180(1): 79-95*

Radeva, G., Perabo, J., Sharom, F.J., 2005. P-glycoprotein is localized in intermediate density membrane microdomains distinct from classical lipid rafts and caveolar domains. *FEBS Journal 272: 4924-37*

Rae, J.M, Ramus, S.J., Waltham, M., Armes, J.E., Campbell, I.G., Clarke, R., Barndt, R.J., Johnson, M.D., Thompson, E.W., 2004. Common origins of MDA-MB-435 cells from various sources with those shown to have melanoma properties. *Clinical & Experimental Metastases 21: 543-552*

Rae, J., Creighton, C., Meck, J., Haddad, B., Johnson, M., 2007. MDA-MB-435 cells are derived from M14 Melanoma cells--a loss for breast cancer, but a boon for melanoma research. *Breast Cancer Research and Treatment 104(1): 13-19*

Raff, M., Barres, B., Burne, J., Coles, H., Ishizaki, Y., Jacobson, M., 1993. Programmed cell death and the control of cell survival: lessons from the nervous system. *Science 262: 695-700*

Ramu, A., Glaubiger, D., Weintraub, H., 1984. Differences in lipid composition of doxorubicin-sensitive and -resistant P388 cells. *Cancer Treatment Reports 68(4): 637-641*

Reed, J.C., 1995. Bcl-2: prevention of apoptosis as a mechanism of drug resistance. *Hematology/Oncology Clinics of North America 9(2):451-73*

Reed, R.G., 1986. Location of long chain fatty acid-binding sites of bovine serum albumin by affinity labelling. *Journal of Biological Chemistry 261(33): 15619-24*

Requena, J.R., Fu, M.X., Ahmed, M.U., Jenkins, A.J., Lyons, T.J., Baynes, J.W., Thorpe, S.W., 1997. Quantification of malondialdehyde and 4-hydroxynonenal adducts to lysine residues in native and oxidized human low-density lipoprotein. *Biochemistry Journal 322(1): 317-25*

Reynolds, C.M., Loscher, C.E., Moloney, A.P., Roche, H.M., 2008. Cis-9, trans-11-conjugated linoleic acid but not its precursor trans-vaccenic acid attenuate inflammatory markers in the human colonic epithelial cell line Caco-2. *British Journal of Nutrition 1: 1-5*

Riboni, L., Viani, P., Bassi, R., Prinetta, A., Tettamanti, A., 1997. The role of sphingolipids in the process of signal transduction. *Progress in Lipid Research 36(2-3): 153-195*

Richieri, G.V., Anel, A., Kleinfeld, A.M., 1993. Interactions of long-chain fatty acids and albumin: determination of free fatty acid levels using the fluorescent probe ADIFAB. *Biochemistry 32(29): 7574-80*

Ries, L.A.G., Eisner, M.P., Kosary, C.L., *et al.* (Eds) 2005. *SEER Cancer Statistics Review, 1975–2002*, National Cancer Institute. Bethesda, MD,

Riserus, U., Arner, P., Brismar, K., Vessby, B., 2002. Treatment with dietary *trans10cis12* conjugated linoleic acid causes isomer-specific insulin resistance in obese men with the metabolic syndrome. *Diabetes Care 25: 1516-1521*

Riserus, U., Vessby, B., Arnlov, J., Basu, S., 2004. Effects of cis-9, trans-11 conjugated linoleic acid supplementation on insulin sensitivity, lipid peroxidation, and proinflammatory markers in obese men. *American Journal of Clinical Nutrition 80: 279-283*

Ritzenthaler, K., McGuire, M.K., Falen, R., Schultz, T.D., McGuire, M.A., 1998. Estimation of conjugated linoleic acid (CLA) intake. *FASEB Journal 12: A527*

Robert, J., 1994. Clinical pharmacokinetics of epirubicin. *Clinical Pharmacokinetics 26:428-438*

Rose, D.P., Connolly, J.M., Rayburn, J., Coleman, M., 1995. Influence of diets containing eicosapentanoic or docosahexanoic acid on growth and metastasis of breast cancer cells in nude mice. *Journal of the National Cancer Institute 87: 587-92*

Ross, J.A., Maingay, J.P., Fearon, K.C., Sangster, K., Powell, J.J., 2003. Eicosapentanoic acid perturbs signalling via the NfkappaB transcriptional pathway in pancreatic tumour cells. *International Journal of Oncology 23: 1733-8*

Rowinski, E.K., Onetto, N., Canetta, R.M., Arbuck, S.G., 1992. The first of the taxanes, an important new class of antitumour agents. *Seminars in Oncology 19: 646-662*

Sahin, O., Wiemann, S., 2009. Functional genomics and proteomics approaches to study the ERBB network in cancer. *FEBS Letters 583: 1766-1771*

Salminen I., Mutanen M., Jauhiainen M., Aro A., 1998. Dietary *trans* fatty acids increase conjugated linoleic acid levels in human serum. *Nutritional Biochemistry 9: 93-98*

Santini M.T., Romano R., Rainaldi G., Filippini P., Bravo E., Porcu L., Motta A., Calcabrini A., Meschini S., Indovina P.L., Aranica G., 2001. The relationship between H-NMR mobile lipid intensity and cholesterol in two tumor multidrug resistant cell lines (MCF-7 and LoVo). *Biochimica et Biophysica Acta 1531: 111-131*

Scaglia, N., Caviglia, J.M., Igal, R.A., 2005. High stearoyl-CoA desaturase protein and activity levels in simian virus 40 transformed-human lung fibroblasts. *Biochimica et Biophysica Acta 1687(1-3): 141-51*

Scheper, R.J., Broxterman, H.J., Scheffer, G.L., Kaaijk, P., Dalton, W.S., van Heijningen, T.H., van Kalken, C.K., Slovak, M.L., de Vries, E.G., van der Valk, P., Meijer, C.J., Pinedo, H.M., 1993. Overexpression of a Mr 110,000 vesicular protein in non-P-glycoprotein-mediated multidrug resistance. Cancer Research 53(7): 1475–1479.

Schiff, P.B., Fant, J., Horwitz, S.B., 1979. Promotion of microtubule assembly in vitro by taxol. *Nature 277(5698): 665-667*

Schrenk, D., Baus, P.R., Ermel, N., Klein, C., Vorderstemann, B., Kauffmann, H-M., 2001. Up-regulation of transporters of the MRP family by drugs and toxins. *Toxicology Letters 120: 51-57*

Sebedio, J.L., Angioni, E., Chardigny, J.M., Gregoire, S., Juaneda, P., Berdeaux, O., 2001. The effect of conjugated linoleic acid isomers on fatty acid profiles of liver and adipose tissues and their conversion to isomers of 16:2 and 18:3 conjugated fatty acids in rats. *Lipids 36: 575-582*

Seelig, A., Blatter, X.L., Wohnsland, F., 2000. Substrate recognition by P-glycoprotein and the multidrug resistance-associated protein MRP1: a comparison. *International Journal of Clinical Pharmacology and Therapeutics 38(3): 111-21*

Serin, D., Verrill, M., Jones, A., Delozier, T., Coleman, R., Kreuser, E.D., Mross, K., Longerey, B. and Brandely, M., 2005. Vinorelbine alternating oral and intravenous plus Epirubicin in first-line therapy of metastatic breast cancer: results of a multicentre phase II study. *British Journal of Cancer 92: 1989-96*

Shirota, T., Haji, S., Yamasaki, M., Iwasaki, T., Hidaka, T., Takeyama, Y., Shiozaki, H., Ohyanagi, H., 2005. Apoptosis in human pancreatic cancer cells induced by eicosapentaenoic acid. *Nutrition 21(10): 1010-1017*

Siddiqui, R.A., Jenski, L.J. Harvey, K.A., Wiesehan, J.D., Stillwell, W., Zaloga, G.P., 2003. Cell-cycle arrest in Jurkat Leukemic cells: a possible role for docosahexanoic acid. *Biochemistry Journal 371: 621-9*

Singal, P.K., Iliskovic, N., Li, T., Kumar, D., 1997. Adriamycin cardiomyopathy: pathophysiology and prevention. *FASEB Journal 11: 931-936*

Sinha, B.K., Mimnaugh, E.G., Rajagopalan, S., Myers, C.E., 1989. Adriamycin activation and oxygen free radical formation in human breast tumor cells: protective role of glutathione peroxidase in adriamycin resistance. *Cancer Research 49 (14): 3844-3848*

Siu, L.L., Moore, M.J., 2005. Chapter 16, Pharmacology of Anticancer Drugs. In: *Tannock, I.F, Hill., R.P., Bristow, R.G., Harrington, L., The Basic Science of Oncology, 4th edn.* New York, USA: McGraw-Hill

Smith, I.C., Hutcheon, A.W., Heys, S.D., 2000. Current and Potential Chemotherapeutic Agents Used for Induction Chemotherapy in the Treatment of Breast Cancer. *Current Pharmaceutical Design 6: 327-343*

Smith, I., Procter, M., Gelber, R.D., Guillaume, S., Feyereislova, A., Dowsett, M., Goldhirsch, A., Untch, M., Mariani, G., Baselga, J., 2007. 2-year follow-up of trastuzumab after adjuvant chemotherapy in HER2-positive breast cancer: a randomised controlled trial. *Lancet 369(9555): 29-36*

Solazzo, M., Fantappiè, O., D'Amico, M., Sassoli, C., Tani, A., Cipriani, G., Bogani, C., Formigli, L., Mazzanti, R., 2009. Mitochondrial expression and functional activity of breast cancer resistance protein in different multiple drug-resistant cell lines. *Cancer Research (in press, available online 10.1158/0008-5472.CAN-08-4315)*

Song, H.J., Grant, I., Rotondo, D., Mohede, I., Sattar, N., Heys, S.D., Wahle, K.W.J., 2005. Effect of CLA supplementation on immune function in young healthy volunteers. *European Journal of Clinical Nutrition* 59*: 508-517*

Sparreboom, A., Scripture, C.D., Trieu, V., Williams, P.J., De, T., Yang, A., Beals, B., Figg, W.D., Hawkins, M. and Desai, N., 2005. Comparative preclinical and clinical pharmacokinetics of a Cremophor-free, nanoparticle albumin-bound Paclitaxel (ABI-007) and Paclitaxel formulated in Cremophor (Taxol). *Clinical Cancer Research 11(11): 4136-43*

Spector, A.A., Burns, C.P., 1987. Biological and therapeutic potential of membrane lipid modification in tumors. *Cancer Research 47: 4529-4537*

Spiegel, S., Merrill, A.H. Jr., 1996. Sphingolipid metabolism and cell growth regulation. *FASEB Journal 10: 1388-1397*

Stillwell, W., Ehringer, W., Jenski, L.J., 1993. Docosahexanoic acid increases permeability of lipid vesicles and tumor cells. *Lipids 28: 103-8*

Storch, C.H., Ehehalt, R., Haefeli, W.E., Weiss, J., 2007. Localization of the human breast cancer resistance protein (BCRP/ABCG2) in lipid rafts/caveolae and modulation of its activity by cholesterol in vitro. *The Journal of Pharmacology and Experimental Therapeutics 323(1): 257-264*

Sweeney, E., Inokuchi, J., Igarashi, Y., 1998. Inhibition of sphingolipid-induced apoptosis by caspases inhibitors indicates that sphingosine acts in an earlier part of the apoptotic pathway than ceramide. *FEBS Letters 425: 61-65*

Tanmahasamut, P., Liu, J., Hendry, L.B., Sidell, N., 2004. Conjugated linoleic acid blocks estrogen signalling in human breast cancer cells. *Journal of Nutrition 134: 674-680*

Tew, K.D., 1994. Glutathione-associated enzymes in anticancer drug resistance. *Cancer Research 54: 4313–4320*

Theuer, J., Shagdarsuren, E., Muller, D.N., Kaergel, E., Honeck, H., Park, J.K., Fiebeler, A., Dechend, R., Haller, H., Luft, F.C., Schunck, W.H., 2005. Inducible NOS inhibition, eicosapentaenoic acid supplementation, and angiotensin II–induced renal damage. *Kidney International 67: 248-258*

Thom, E., Wadstein, J., Gudmundsen, O., 2001. Conjugated linoleic acid reduces body fat in healthy exercising humans. *Journal of International Research 29: 392-396*

Thompson, H., Zhu, Z.J., Banni, S., Darcy, K., Loftus, T., Ip, C., 1997. Morphological and biochemical status of the mammary gland as influenced by conjugated linoleic acid –implication for a reduction in mammary cancer risk. *Cancer Research 57: 5067-5072*

Tsai, W.S., Nagawa, H. and Muto, T., 1997. Differential effects of polyunsaturated fatty acids on chemosensitivity of NIH3T3 cells and its transformants. *Int. J. Cancer 70: 357-61*

Tsujita-Kyutoku, M., Yuri, T., Danbara, N., Senzaki, H., Kiyosuka, Y., Uehara, N., Tadaka, H., Hada, T., Miyazawa, T., Ogawa, Y., Tsubura, A., 2004. Conjugated docosahexaenoic acid suppresses KPL-1 human breast cancer cell growth *in vitro*

and *in vivo*: potential mechanisms of action. *Breast Cancer Research 6(4): R291-R299*

Tsuzuki, T., Igarashi, M., Miyazawa, T., 2004. Conjugated eicosapentanoic acid (EPA) inhibits transplanted tumor growth via membrane lipid peroxidation in nude mice. *Nutrition and Cancer 134: 1162-66*

Van Oosterom, A.T., Judson, I., Verweij, J., Stroobants, S., di Paola, E.D., Dimitrijevic, S., Martens, M., Webb, A., Sciot, R., Van Glabbeke, M., Silberman, S., Nielson, O.S., 2001. Safety and efficacy of imatinib (STI571) in metastatic gastrointestinal stromal tumours: a phase I study. *The Lancet 358 (9291): 1421-1423*

Vazquez-Martin, A., Colomer, R., Menendez, J.A., 2008. Her-2/*neu*-induced "cytokine signature" in breast cancer. *Advances in Experimental Medicine and Biology 617: 311-9*

Vibet, S., Goupille, C., Bougnoux, P., Steghens, J-P., Goré, J., Mahéo, K., 2008. Sensitization by docosahexaenoic acid (DHA) of breast cancer cells to anthracyclines through loss of glutathione peroxidase (GPx1) response. *Free Radical Biology and Medicine 44(7): 1483-1491*

Vincenzi, B., Schiavona, G., Sillettaa, M., Santinia, D., Toninia, G., 2008. The biological properties of cetuximab. *Critical Reviews in Oncology/Hematology 68(2): 93-106*

Visonneau, S., Cesano, A., Tepper, S.A., Scimeca, J.A., Santoli, D., Kritchevsky, D., 1996. Effect of different concentrations of conjugated linoleic acid (CLA) on tumour growth in vitro. *FASEB Journal 10: A182*

Voet, D., Voet, J.G., Pratt, C.W., 1999. *Fundamentals of Biochemistry, 1st edn.* New York, USA: John Wiley & Sons Inc.

Wahle, K.W.J., Heys, S.D., 2002. Cell signal mechanisms, conjugated linoleic acids (CLAs) and anti-tumorigenesis. *Prostaglandins, Leukotrienes and Essential Fatty Acids 67(2-3): 183-186*

Wahle, K.W.J., Heys, S.D., Rotondo, D., 2004. *Review:* Conjugated linoleic acids: are they beneficial or detrimental to health? *Progress in Lipid Research 43: 553-587*

Wall, R., McMahon, G., Crown, J., Clynes, M., O'Connor, R., 2007. Rapid and sensitive liquid chromatography-tandem mass spectrometry for the quantitation of epirubicin and identification of metabolites in biological samples. *Talanta 72: 145-154*

Wang, G.Q., Gastman, B.R., Wieckowski, E., Goldstein, L.A., Gambotto, A., Kim, T.H., Fang, B., Rabinovitz, A., Yin, X-M., Rabinowich, H., 2001. A role for mitochondrial Bak in apoptotic response to anticancer drugs. *Journal of Biological Chemistry 276: 34307-17*

Wang, L-S., Huang, Y-W., Sugimoto, Y., Suling, L., Chang, H.L., Weiping, Y., Shu, S., Lin, Y.C., 2005. Effects of human breast stromal cells on conjugated linoleic acid (CLA) modulated vascular endothelial growth factor-A (VEGF-A) expression in MCF-7 cells. *Anticancer Research 25: 4061-8*

Wani, M.C., Taylor, H.L., Wall, M.E., Coggon P. and McPhail, A.T., 1971. Plant antitumour agents. VI. The isolation and structure of taxol, a novel antileukemic and antitumour agent from *Taxus brevifolia. Journal of the American Chemical Society 93: 2325-2327*

Wolff, A.C., Baker, S.D., Bowling, M.K., Carducci, M.A., Bradley, M.O., Anthony, F.H., Swindell, C.S., Witman, P.A., Webb, N.L., Donehower, R.C., 2003. Phase 1 study of docosahexaenoic acid-paclitaxel: a taxane-fatty acid conjugate with a unique pharmacology and toxicity profile. *Clinical Cancer Research 9: 3589-3597*

Woodruff, H.B., Waksman, S.A., 1960. The actinomycin –historical background. *Annals of the New York Academy of Sciences 89: 287*

Wright, M.A., Morrison, G., Lin, P., Leonard, G.D., Nguyen, D., Guo, X., Szabo, E., Hopkins, J.L., Leguizamo, J.P., Harold, N., Fioravanti, S., Schuler, B., Monahan, B.P., Saif, M.W., Quinn, M.G., Pang, J., Grem, J.L., 2005. A phase 1 pharmacologic and pharmacogenetic trial of sequential 24-hour infusion of irinotecan followed by leucovorin and a 48-hour infusion of fluorouracil in adult patients with solid tumors. *Clinical Cancer Research 11(11): 4144-50*

Wu, M., Harvey, K.A., Ruzmetov, N., Welch, Z.R., Sech, L., Jackson, K., Stillwell, W., Zaloga, G.P., Siddiqui, R.A., 2005. Omega-3 polyunsaturated fatty acids attenuate breast cancer growth through activation of a neutral sphingomyelinase-mediated pathway. *International Journal of Cancer 117(3): 340-348*

Xu, H., Sawyer, M.B., Field, C.J., Dieleman, L.A., Baracos, V.A., 2007. Nutritional modulation of antitumor efficacy and diarrhea toxicity related to irinotecan chemotherapy in rats bearing the ward colon tumor. *Clinical Cancer Research 13: 7146-54*

Yang, P., Chan, D., Felix, E., Cartwright, C., Menter, D.G., Madden, T., Klein, R.D., Fischer, S.M., Newman, R.A., 2004. Formation and antiproliferative effect of prostaglandin E3 from eicosapentaenoic acid in human lung cancer cells. *Journal of Lipid Research 45(6): 1030-39*

Yurawecz, M.P., Roach, J.A.G., Sehat, N., Mossoba, M.M., Kramer, J.K.G., Fritsche, J., Steinhart, H., Ku, Y., 1998. A new conjugated linoleic acid isomer, 7 *trans*, 9 *cis*-octadecadienoic acid, in cow milk, cheese, beef and human milk and adipose tissue. *Lipids 33: 803-809*