An investigation of the biochemical mechanisms underlying the growth-inhibitory effects of conjugated linoleic acid (CLA) isomers in cancer cell lines.

Ph.D. thesis

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Declaration

This thesis is submitted in fulfilment of the requirements for Doctor of Philosophy, by research and thesis. Except where otherwise acknowledged, this work was carried out by the author alone, on a full time basis between October 1998 and November 2002 at the School of Biotechnology, Dublin City University.

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Abstract

Conjugated linoleic acids (CLA) are a group of positional and geometric isomers of linoleic acid produced by ruminant animals. Despite animal studies showing that CLA is an effective agent in preventing mammary and colon tumour development there is a need to determine isomeric-specific effects of CLA and identify molecular targets in tumour tissues. Mammary (MCF-7) and colon (SW480) tumour cell lines were used in this study as *in vitro* models to investigate effects of CLA on growth and membrane-initiated signalling pathways. Both cell lines were sensitive to the growth-inhibitory effects of a CLA mixture of isomers and to the individual *t*10, *c*12- and *c*9, *t*11-CLA isomers at physiological levels. CLA isomers modulated arachidonic acid distribution among cellular lipids and altered the prostaglandin profile of both cell lines suggesting interference in an eicosanoid signaling pathway. Similar effects were observed in cells treated with CLA-enriched milk fat obtained from cows fed on rapeseed supplemented pasture. This study also provided evidence for cellular bioconversion of vaccenic acid to *c*9, *t*11 CLA isomer. Western blot analysis of a panel of apoptosis regulatory proteins (bcl-2, bax, Apaf-1 and caspase 3) in the SW480 cell line indicated that induction of apoptosis by CLA isomers contributed to growth inhibition in this cell line.

Publications and Presentations

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Abbreviations

AA	arachidonic acid
ACF	aberrant crypt foci
ALA	α-linolenic acid
ALA	α-linolenic acid
Apaf-1	apoptosis protease activation factor-1
Apc	adenomatous polyposis coli
BHT	butylated hydroxytoluene
BP	benzo(a)pyrene
BRAC1	breast cancer gene 1
BRAC2	breast cancer gene 2
BSA	bovine serum albumin
c	*cis*
CD	conjugated diene
CDKI	cyclin-dependent kinase inhibitors
Cdks	cyclin dependent kinases
Ckis	cyclin kinase inhibitors
CLA	conjugated linoleic acid
COX	cyclooxygenase
CYP450	cytochrome P450
DAG	diacylglycerol
DGLA	dihomo-γ-linolenic acid
DHA	docosahexaenoic acid
DMBA	7,12-dimethyl-benz[a]anthracene
DMEM	dulbecco's minimum essential medium
DMH	dimethylhydrazine
DMOX	dimethyoxazoline
DNA	deoxyribonucleic acid
DPM	disintegrations per minute
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EHS	engelbreth-holm-swarm
EPA	eicosapentaenoic acid
FAME	fatty acid methyl ester
FFA	free fatty acids
FFR	full fat rapseed
FFS	full fat soybeans
FID	flame ionisation detector
FTIR	fourier transformed infrared
GAPs	GTPase activating proteins
GDP	guanidine-diphosphate
GLA	γ-linolenic acid

GLC	gas liquid chromatography
GLC-MS	gas liquid chromatography mass spectroscopy
GSH	reduced glutathione
GST	glutathione s transferase
GTP	guanidine-triphosphate
HDL	high density lipoprotein
HEPES	N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
HETE	hydroxyeicosatetraenoic
5-HPETE	5-hydroperoxyeicosatetraenoate
HFCO	high fat dietary corn oil
HFFO	high fat fish oil
HMEC	human microvascular endothelial cell line
HPLC	high performance liquid chromatography
HRP	horseradish peroxidase
HSVEC	human saphenous vein endthelial cells
IDP	intraductal proliferation
IGF	insulin-like growth factors
IP_3	inositol triphosphate
IQ	2-amino-3-methyl-imidazo[4,5-f]-quinoline
LA	linoleic acid
LDL	low density lipoprotein
LOX	lipoxygenase
LPL	lipoprotein lipase
LPS	lipopolysaccharides
LTB_4	leukotriene B_4
MAP	mitogen-activated protein
MEK	MAP kinase kinase
MEO	mammary epithelial cell organoids
MG	monoglyceride,
MLH1	mutL homolog 1
MMP	matrix metalloproteases
MMTV	mouse mammary tumour virus
MNU	methylnitrosurea
MOS	v-mos moloney murine sarcoma viral oncogene homolog
mRNA	messenger ribonucleic acid
MROD	methoxyresorufin-o-deethylase
MSC	mammary stromal cells
MSH2	mutS homolog 2
MTAD	2-methyl-1,2,4-triazoline-3,5-dione
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(sulfophenyl)-2H-tetrazolium salt
NADPH	β-nicotinamide adenine dinucleotide phosphate, reduced form
NAT	N-acetyltransferase
NF-1	neurofibromatosis
NSAID	non-sterodialanti-inflammatory agent
OPT	o-phthalaldehyde

PBS	phosphate buffered saline
PBST	PBS containing tween 20
PC	phosphatidycholine
PCD	programmed cell death
PCNA	proliferating cell nuclear antigen
PDGF	platelet-derived growth factor
PE	phosphatidylethanolamine
PG	prostaglandin,
PGD_2	prostaglandin D_2
PGE_2	prostaglandin E_2
PGE_2	prostaglandin E_2
$PGF_{2\alpha}$	prostaglandin $F_{2\alpha}$
PhIP	2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine
PI	phosphatidylinositol
PI-PLC	phosphatidylinositol-specific phospholipase C
PKC	protein kinase C
PL	phospholipid
PLA_2	phospholipase A_2
PLC	phospholipase C
PLPC	1-palmitoyl-2-linoleoyl phosphatidylcholine
PMS	phenazine methosulfate
PMSF	phenylmethysulfonylfluoride
PP	peroxisome proliferators
PPAR	peroxisome proliferator-activated receptor
PPRE	peroxisome proliferator response elements
PS	phosphatidylserine
PUFA	polyunsaturated fatty acid
RB	retinolastoma
RBM	reconstituted basement membrane
RXR	retinoic acid-X receptor
SCID	severe combined immunodeficient mice
SD	standard deviation
SRD5A2	steroid 5 alpha-reductase type 2
t	*trans*
TBARS	thiobarbituric acid-reactive substances
TEB	terminal end buds
TG	triglyceride
TGFβ	transforming growth factor-beta
TLC	thin layer chromatography
TMG	tetramethylguanidine
TPA	12-O-tetradecanoylphorbal-13-acetate
TUNEL	terminal deoxynucleotidyl transferase biotin-dUTP end labeling
TVA	*trans*-vaccenic acid
VEGF	vascular endothelial growth factor
ZDF	zucker diabetic rat

Units

bp	Base pair
cpm	Counts per minute
°C	Degrees Celsius
U	Enzymes units
g	g force
g	Grams
h	Hours
L	Litres
m	Meters
mCi	Microcuries
µg	Micrograms
µL	Microliters
µM	Micromolar
µmol	Micromoles
min	Minutes
mmol	Millimoles
mg	Milligrams
ml	Milliliters
mol	Moles
ng	Nanograms
pg	Picograms
pmol	Picomole
s	Second
V	Volts

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CHAPTER 1

Literature Review

1.0 Overview

The role of diet in the development and prevention of cancer has been the focus of much scientific research during the past decade. The field of cancer chemoprevention has experienced a rapid growth in the identification and characterisation of a vast number of anticarcinogenic substances that are present naturally in many food sources. Among the more potent naturally occurring anticarcinogens to be identified is conjugated linoleic acid (CLA). Dairy products and other foods derived from ruminant animals are the main dietary sources of CLA. *In vitro* and experimental animal studies document a growing number of potential health benefits for CLA. Not only is CLA a powerful anticarcinogen but it also has been reported to have anti-atherogenic, immunomodulating, growth promoting, anti-diabetic and anti-obesity properties. The challenge now is to determine the effects of CLA in human subjects and to identify the specific physiological mechanism(s) by which different CLA isomers exert their unique biological effects. Such information will open the door for CLA-enriched dairy foods.

The aim of this chapter is to review the available literature on the role of dietary fats, in particular CLA, on cancer development and to provide a comprehensive background to the research work contained in this thesis.

1.1 Cancer

At the beginning of the third millennium, cancer remains the second leading cause of death in the developed world (Zhang, 2002). There are over one hundred different types of cancer, of which lung, colon, breast and prostate together account for over 50 % of

total cancer incidence. Cancer is a collection of different diseases with common features: excessive division of cells leading to uncontrolled cell growth, invasion of surrounding tissues, extravasation into circulation, migration, formation of tumor masses at distant sites and interference with the function of normal tissues and organs. Thus cancer is a disease involving dynamic changes in the genome. The molecular biology era has provided enormous insight into the accumulation of changes in critical genes that are the fundamental basis for the altered biological behaviour of cancer cells (Hursting *et al.*, 1999).

1.1.1 Development of Cancer

Normal cellular growth is tightly controlled by genes involved in the regulation of cell proliferation and cell death. Many of these genes encode proteins such as growth factors, receptors for growth factors, protein kinases and the proteins that activate them, proteins that regulate the cell cycle, proteins that either activate or inhibit apoptosis and DNA binding proteins. Deregulation of any of these proteins results in alterations to the finite balance controlling cell numbers and consequently results in altered cell growth (Holmgren *et al.*, 1995). Genetic mutations can cause stimulatory pathways to issue too many "go" signals or inhibitory pathways to issue too many "stop" signals (Figure 1.1). The birth of a tumor cell requires at least two successive events. Firstly, an initiator must strike the DNA of a cell and introduce a mutation into a gene that is involved in the regulation of normal cell growth. Unless the genetic change is corrected by specific DNA repair enzymes it will be passed to daughter cells during cell division leading to a localised collection of cells expressing the mutant gene (Eng and Ponder, 1993). The

second stage, termed promotion, is characterised by successive rounds of clonal expansion whereby tumor cells develop more aggressive biological behaviour (Fischer and DiGiovanni, 1995). During tumor promotion, further disruption of gene expression and multiple additional mutations develop due to progressive genomic instability (Pitot, 1989). Therefore, cancer is caused not by one mutation but by multiple mutations, which together allow the cell to escape normal control mechanisms. Molecular genetics has shown that cancer is a continuous evolving process involving the accumulation of a series of genetic alterations in genes controlling cellular proliferation (Ames *et al.*, 1995). Many genes have been identified, that when either turned on (oncogenes) or turned off (tumor suppressor genes), affect cell signalling pathways. Collectively, these two classes account for much of the uncontrolled cell proliferation seen in human cancer.

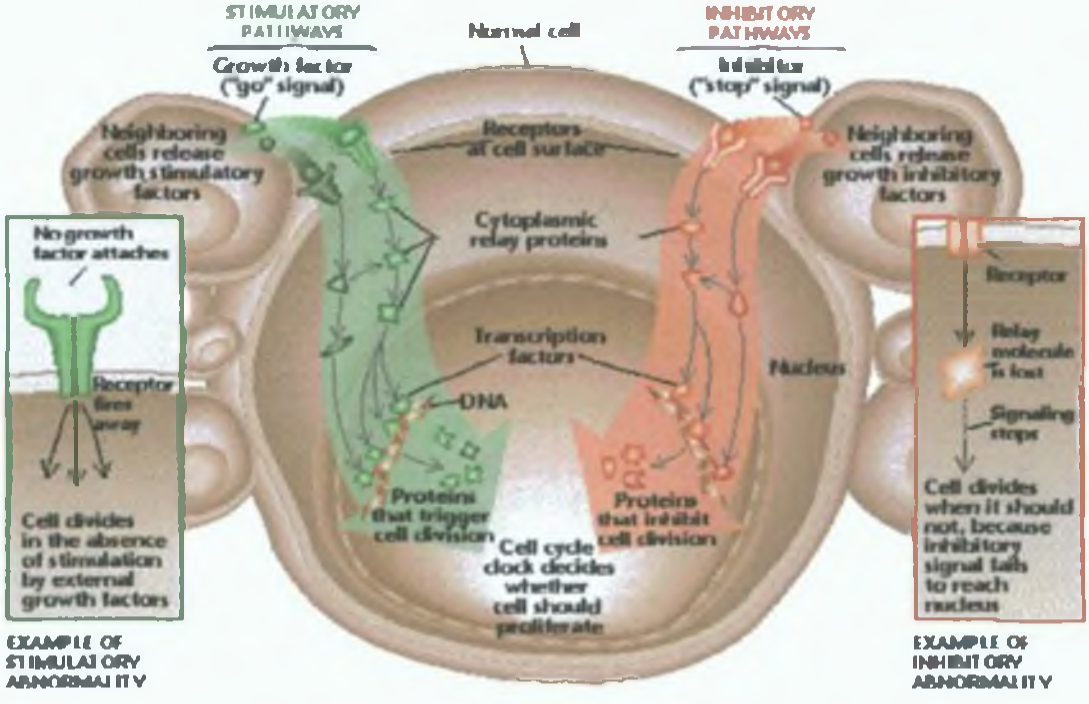


Figure 1.1 Signalling pathways in normal cells (Weinberg, 1996).

Oncogenes are mutated forms of normal cellular genes called proto-oncogenes. They promote excessive cell growth and thus tumor formation. The unmutated proto-oncogenes play important roles in controlling molecular processes inside the cell including growth stimulation by external ligands, transduction within the cell or progression through cell cycle (see Figure 1.2). Therefore, oncogenes have the potential to subvert the cell's elaborate biochemical circuitry and push the cell towards a malignant state. Oncogenes typically exert their action in an autosomal dominant mechanism resulting in the expression of a normally repressed function (Weinberg, 1994). Oncogenes may be activated by mutation in such a way that the gene products can no longer carry out normal activity. Alternatively, a mutation in a nearby regulatory sequence or a chromosomal rearrangement that brings a DNA sequence from a distant site in the genome into close proximity may alter expression of the proto-oncogene so that an excessive quantity of gene product or an alteration in its structure is produced.

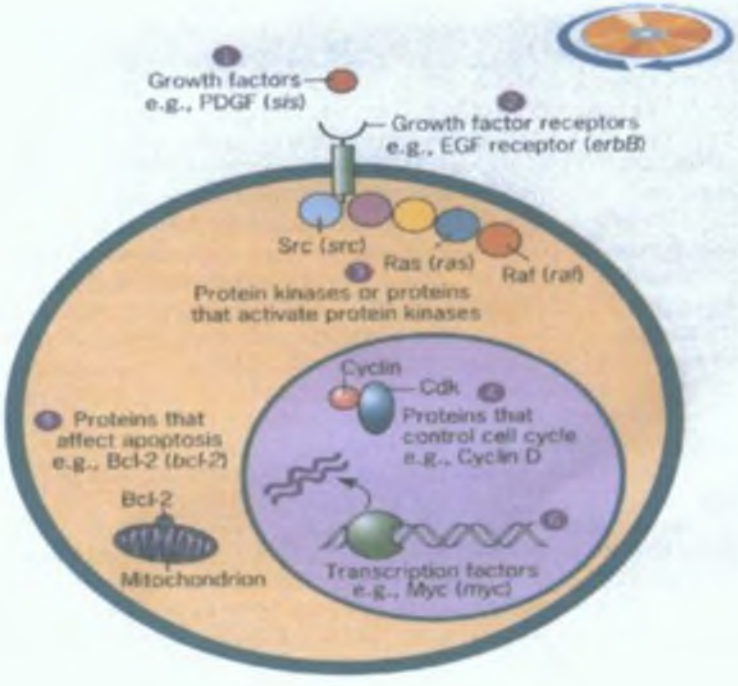


Figure 1.2 The types of proteins encoded by proto-oncogenes (Karp, 2001)

Although the number of oncogenes is large, they can be divided into several groups based on their molecular function in cells (Weinberg, 1995). Many oncogenes are involved in growth factor expression. Tumor cells may inappropriately produce their own growth factor (e.g. platelet-derived growth factor (PDGF), which is encoded by the gene *sis*) or growth factor receptors may be overexpressed (e.g. epidermal growth factor (EGF) which is encoded by the gene *c-erbB-2*). Oncogenes are involved in the phosphorylation of proteins with serine, threonine and tyrosine residues (Hunter, 1987). The proto-oncogene, *Raf*, for example encodes a serine-threonine protein kinase that activates the mitogen-activated protein (MAP) kinase cascade, the primary signalling pathway controlling growth in the cell. It phosphorylates MAP kinase kinase (MEK) which in turn phosphorylates cytosolic MAP kinases on threonine and tyrosine residues. Other protooncogenes within this family include v-mos Moloney murine sarcoma viral oncogene homolog (MOS), Maturation-promoting factor (MPF) and Protein kinase C (PKC). Oncogenes are involved in the transmission of signals by GTPases (Bourne *et al.*, 1990). The best understood example comes from the *ras* family which encodes a GTP-binding protein that functions as an on-off switch for a cascade of kinase-driven phosphorylation events that culminate in the activation of nuclear transcription factors controlling cell proliferation. Point mutations in codons 12,13 or 61 of oncogenic *ras* mutants typically encode a protein whose GTPase activity cannot be stimulated which leaves the molecule in an active GTP-bound form sending continuous proliferation signals along the pathway. Finally, oncogenes are involved in the control of transcription from DNA (Wasylyk *et al.*, 1990). These proteins, known as transcription factors, either bind to specific DNA sequences exerting an immediate effect (eg *ets*, *myp*) or form

complexes which in turn bind to DNA (eg *fos*, *jun*, *myc*). The activity of these transcription factors is regulated by phosphorylation. The *myc* onogene is one of the best studied oncogenes whose product acts as a transcription factor. The myc protein is one of the first proteins to appear when a cell has been stimulated by growth factors to leave the quiescent stage of the cell cycle and divide. Excess myc protein promotes progression of the cell through the cell cycle.

In contrast to the oncogenes discussed above, tumor suppressor genes act as the cell's brakes by encoding proteins that repress biochemical function and cell proliferation. They generally function in an autosomal recessive manner. When tumor suppressor genes are inactivated by mutations the cell is deprived of crucial brakes that prevent inappropriate growth. Examples include death factors, differentiation factors, receptors, signal transduction proteins, transcription factors and negative cell cycle regulators. Among the genes implicated in tumor suppression in humans are *p53*, retinoblastoma gene (*RB*) and neurofibromatosis gene (*NF-1*). The former has been described as the guardian of the genome. It encodes a polypeptide p53 having a molecular mass of 53000 daltons. It protects DNA by surveying for DNA damage and then co-ordinately blocking the cell cycle machinery, stimulating DNA repair and inducing damaged cells to die by a form of cell suicide known as apoptosis (Sturzbecher *et al.*, 1990). A mutation in p53 that abrogates its guardian function would allow cells to enter the S phase and attempt to replicate damaged DNA rather than repairing it first or undergoing apoptosis. *NF-1* encodes a protein that exerts negative control over Ras proteins by activating GTPase of Ras (Buchberg et al., 1990). The protein encoded by the *RB* gene, pRB serves as a brake

on the advancement of cells from the G_0/G_1 stages of the cell cycle into S stage where DNA synthesis occurs (Buchkovich *et al.*, 1989).

Human cancers grow inappropriately not only because signalling pathways are perturbed but also because the so called cell cycle clock becomes deranged. The cell cycle in mammalian cells is divided into 4 distinct phases: i) gap 1 (G_1) where most of control of cell proliferation occurs, ii) DNA synthesis phase (S), iii) gap2 (G_2) where molecular preparations for mitosis occur and iv) mitosis (M) (see Figure 1.3). A network of control mechanisms called "checkpoints" is responsible for ensuring that critical events such as DNA replication and chromosome segregation are completed correctly. Cells that have stopped dividing, whether temporarily or permanently, are present in a stage preceding the initiation of DNA synthesis. Cells that are arrested in this state are usually said to be in the G_0 state to distinguish them from the typical G_1 phase cells that must soon enter S phase.

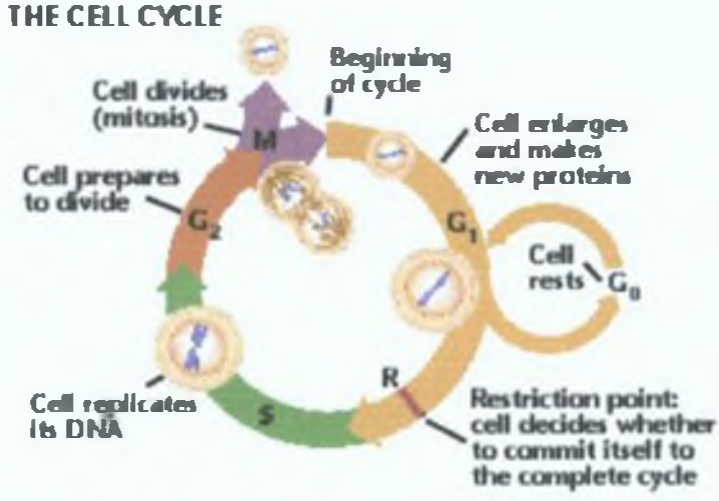


Figure 1.3 The Cell Cycle (Weinberg, 1996).

Each phase of the cell cycle is controlled by sequential activation of various cyclin-dependent kinases (Cdks). These kinases are known to phosphorylate various substrates whose activity is critical for cell cycle progression. In G_1 for example, cyclin D and later cyclin E combine and activate Cdk 4 or 6. The resulting complex phosporylates a powerful growth-inhibitory molecule, known as retinoblastoma pRb. This action releases the braking effect of pRB and enables the cell to progress into late G_1 and S phase. Factors that control the cell cycle can play a pivotal role in the development of cancer. Oncogenic processes exert their greatest effect by targeting particular regulators of G_1 progression.

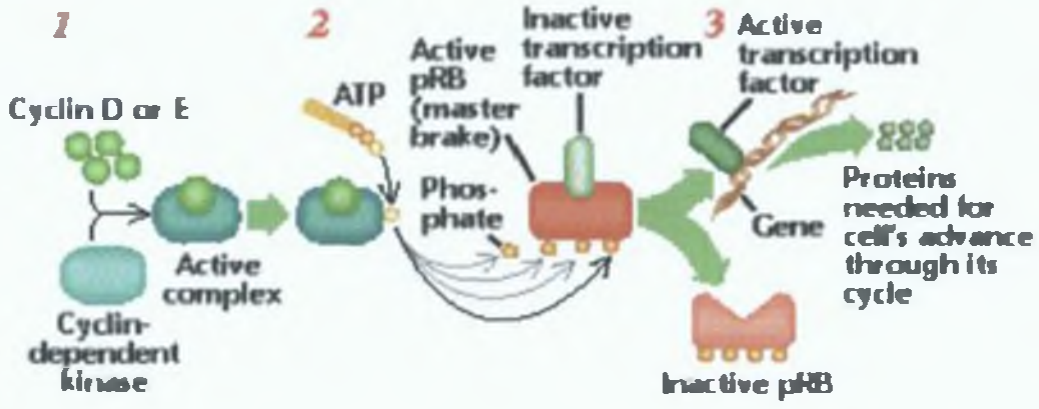


Figure 1.4 Regulation of G_1 phase by cyclin D and E (Weinberg, 1996).

A growth advantage is obtained by overcoming the inhibitory effect of pRB on the cell cycle. In 40 % of human cancers, this is accomplished by the direct mutation of the pRb gene (Weinberg, 1995). pRB is also inactivated by the hyperactivation of Cdks produced by overexpression of cyclins and by mutations/overexpression of the catalytic subunit (Delsal et al., 1996). Cdk inhibitors (Ckis) mediate cell cycle arrest in response to various antiproliferative signals. The family of inhibitors includes p15, p16, p18, p19, p20, p21, p27 and p53. It has been suggested that some of these Ckis may also be potential

oncogenes because their function is often altered in transformed cells. For example, p15 and p16 genes have been found mutated, deleted or inactivated in a large number of human malignancies (Sheaff and Roberts, 1995).

The growth of any tissue, whether normal or malignant, is determined by the quantitative relationship between the rate of cell proliferation and the rate of cell death. Certain promoting agents of carcinogenesis function not to enhance proliferation but rather to decrease the death of neoplastic-initiated cells (Isaacs, 1993). Cell death or apoptosis can involve processes that are equal in complexity and regulation to those involved in cell proliferation. Mutations and deletions of apoptotic genes play important roles in carcinogenesis and tumor growth (Saikumar *et al.*, 1999). Programmed cell death (PCD) or apoptosis, constitutes a system for the removal of unnecessary, aged or damaged cells that is regulated by the interplay of proapoptotic and antiapoptotic proteins of the Bcl-2 family.

The proapoptotic proteins Bax, Bad, Bid, Bik and Bim contain an alpha-helical BH3 death domain that fits the hydrophobic BH3 binding pocket on the antiapoptotic proteins Bcl-2 and Bcl-XL, forming heterodimers that block the survival-promoting activity of Bcl-2 and Bcl-XL. Thus, the relative abundance of proapoptotic and antiapoptotic proteins determines the susceptibility of the cell to programmed death. The proapoptotic proteins act at the surface of the mitochondrial membrane to decrease the mitochondrial trans-membrane potential and promote leakage of cytochrome c. In the presence of ATP, cytochrome c complexes with and activates apoptosis protease activation factor-1 (Apaf-

1). Upon activation, Apaf-1 binds to downstream caspases, such as procaspase-9 and processes them into proteolytically active forms. This begins a caspase cascade resulting in apoptosis (see figure 1.5).

Several genes that are part of the process of apoptosis have been found to be defective in tumor cells. The best characterised examples are the Bcl-2 and caspase-gene families which are overexpressed in about one third of all cancers tested. The tumor suppressor gene p53, which is found mutated in many cancers, does not directly participate in the apoptotic pathway but regulates a host of genes that lead to cell arrest and apoptosis. For example, the p53 protein can activate the expression of the bax gene, whose encoded product bax initiates apoptosis.

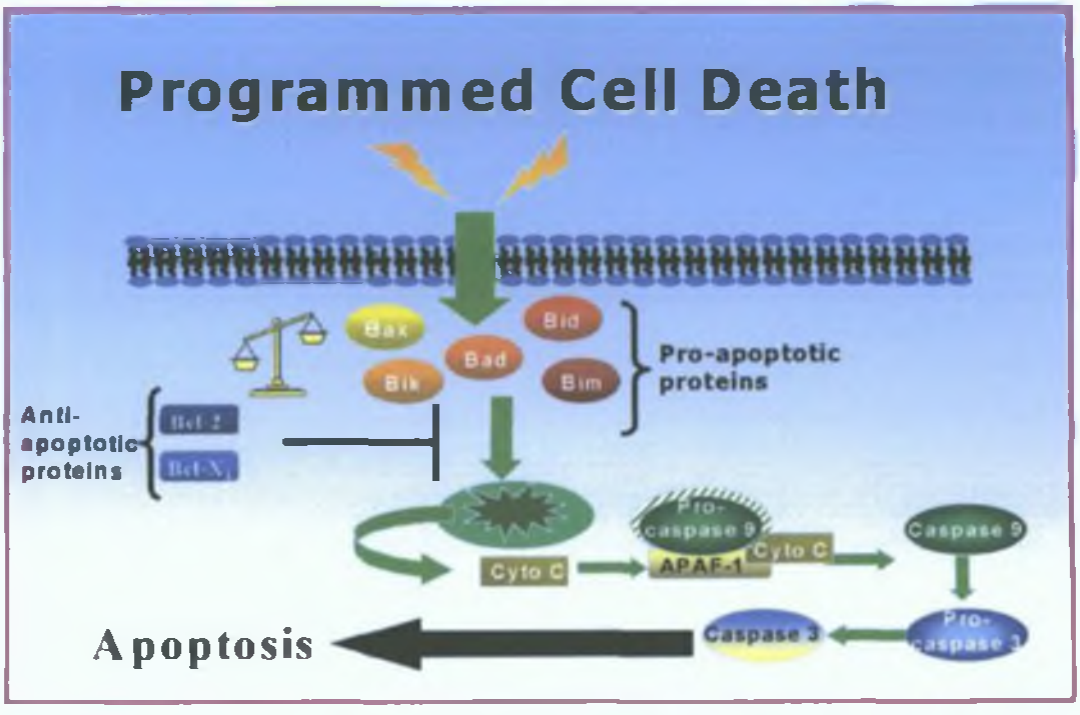


Figure 1.5 Schematic representation of Apoptosis

Other significant genetic lesions in cancer include germline mutations, many of which are associated with lost tumor suppressor function. Examples are familial adenomatous polyposis coli (Apc) leading to colorectal cancer and breast cancer gene 1 (BRCA1) and breast cancer gene 2 (BRCA2) which increase the risk of breast and ovarian cancers. Other cancer-predisposing genes such as mutL homolog 1 (MSH1) and mutS homolog 2 (MSH2) (both linked to hereditary nonpolyposis colon cancer) cause defective DNA repair. In addition, recent cancer epidemiology and pharmacogenetic studies have attributed importance to genetic polymorphisms of enzymes affecting the biotransformation of carcinogens e.g., glutathione S-transferase (GSTM1, GSTM2, GSTP1), N-acetyltransferase (NAT1, NAT2), cytochrome P450 (CYP450IAI) and steroid 5 alpha-reductase type II (SRD5A2) (Lai and Shields, 1999).

It is estimated that 5 percent of large bowel, breast and prostate cancers are due to genetic predisposition, 15 percent occur spontaneously and 80 percent are attributable to nutrition and lifestyle factors (Go *et al.*, 2001).

1.2 Diet and cancer chemoprevention.

Increasing knowledge about the 20 - 40 year process involved in the development of human carcinogenesis is providing many new opportunities for early intervention and prevention and specifically, for chemoprevention. Cancer chemoprevention may be defined as the use of specific chemical substances, many of which occur naturally in foods, to prevent cancer initiation and to inhibit or reverse the development of invasive cancer (Singletary, 2000). The goal of chemopreventive research with food constituents is to identify safe and effective agents for clinical use. Because food-derived chemopreventive substances or nutraceuticals are expected to be safe and because they are not perceived as "medicine", they may find widespread long-term use in the general population. A food-based chemopreventive strategy could prove particularily useful for individuals at high risk of certain cancers as determined by inherited susceptibilities. Chemopreventive agents could potentially prevent further damage that might enhance carcinogenesis or suppress the appearance of the cancer phenotype (Sporn, 1996).

Leads for the identification of putative chemopreventive agents have arisen from dietary epidemiological data and from laboratory studies. Several distinctive strategies are pursued in developing chemopreventive agents: (a) identifying and validating molecular biomarkers and early cell and tissue lesions that can be used instead of cancer incidence as an endpoint for measuring chemopreventive activity; (b) identifying and testing candidate agents based on considerations of mechanisms of action; (c) evaluating combinations of agents with potential for maximising efficacy and minimising toxicity; and (d) applying a systematic methodology for identifying and ranking candidate agents

at each stage of development to ensure discovery of the best agents and most effective use of available resources (Kelloff *et al.*, 1994).

Efficacy and toxicity are initially assessed using *in vitro* cell screening systems. Promising chemopreventive agents are then examined in site-specific *in vivo* assays. Agents that have high efficacy and low toxicity are prioritised for clinical evaluation (Kelloff *et al.*, 1994). Phase I clinical trials determine dose-related safety and toxicity in a limited number of human subjects. Phase II trials evaluate the agent in a larger group of subjects at high risk for specific cancers. Biochemical, genetic, cellular or tissue biomarkers which stimulate neoplastic progression are identified and it is determined if the chemopreventive agent can modulate these biomarkers. Examples of biomarkers include intraepithelial neoplasia, hyperproliferation, genomic instability, oncogene overexpression, tumor suppressor loss, growth factor and growth factor receptor overexpression, differentiation biomarkers (e.g. G-actin, cytokeratins) and biochemical changes (Greenwald *et al.*, 1999). Phase III trials involve thousands of subjects, may take years to complete and determine the long-term efficacy of the intervention.

To date, more than 40 diet-derived agents with significant chemopreventive potential for major cancers including breast, colon, prostate and lung have been identified. Examples include micronutrients (e.g. vitamins D and E, molybdenum, selenium, calcium) and phytochemicals (e.g. β-carotene and lycopene in fruit and vegetables, green and black tea polyphenols, soy isoflavones, sulforaphane, phenethyl isothiocyanate and indole-3-carbinol in cruciferous vegetables and curcumin, a carotenoid pigment in turmeric)

(Kelloff *et al.*, 2000). Table 1.1 lists examples of their mechanisms of action and possible molecular targets. Growth factors, telomerases, cyclooxygenases and caspases are among the many molecular targets for diet-derived chemopreventive agents. New DNA chip technology and functional proteomics will permit complex nutrient-gene interactions to be investigated. Such research will provide not only a greater understanding of mechanisms involved in prevention but will also improve the ability to conduct cancer surveillance.

Table 1. 1 Mechanisms for chemoprevention by diet-derived agents with possible molecular targets

Mechanism	Possible molecular targets	Representative agents
Antimutagenesis		
Inhibit carcinogen uptake	Bile acids (bind)	Calcium
Inhibit formation/activation of carcinogen	Cytochromes P450 (inhibit)	PEITC, tea, indole-3-carbinol, soy isoflavones
	PG synthase hydroperoxidase, 5-lipoxygenase (inhibit)	Curcumin
	Bile acids (inhibit)	Ursodiol
Deactivate/detoxify carcinogen	GSH/GST (enhance)	NAC, garlic/onion disulfides
Prevent carcinogen-DNA binding	Cytochromes P450 (inhibit)	Tea
Increase level or fidelity of DNA repair	Poly(ADP-ribosyl)transferase (enhance)	NAC, protease inhibitors (Bowman-Birk)
Antiproliferation/antiprogression		
Modulate hormone/growth factor activity	Estrogen receptor (antagonize)	Soy isoflavones
	Steroid 5-reductase (inhibit)	Tea
	IGF-I (inhibit)	Soy isoflavones
Inhibit oncogene activity	Farnesyl protein transferase (inhibit)	Perillyl alcohol, limonene, DHEA
Inhibit polyamine metabolism	ODC induction (inhibit)	Retinoids, curcumin, tea
Induce terminal differentiation	TGFß (induce)	Retinoids, vitamin D, soy isoflavones
Restore immune response	Cyclooxygenases (inhibit)	Tea, curcumin
	T, NK lymphocytes (enhance)	Selenium, tea
	Langherans cells (enhance)	Vitamin E
Increase intercellular communication	Connexin 43 (enhance)	Carotenoids (lycopene), retinoids
Induce apoptosis	TGFß (induce)	Retinoids, soy isoflavones, vitamin D
	RAS farnesylation (inhibit)	
	Telomerase (inhibit)	Perillyl alcohol, limonene, DHEA
	Arachidonic acid (enhance)	Retinoic acid
	Caspase (activate)	Curcumin, tea Retinoids
Inhibit angiogenesis	FGF receptor (inhibit tyrosine kinase)	Soy isoflavones
	Thrombomodulin (inhibit)	Retinoids
Correct DNA methylation imbalances	CpG island methylation (enhance)	Folic acid
Inhibit basement membrane degradation	Type IV collagenase (inhibit)	Protease inhibitors
Inhibit DNA synthesis	Glucose 6-phosphate dehydrogenase (inhibit)	DHEA

Abbreviations: PEITC, phenethyl isothiocyanate; PG, prostaglandin; GSH, glutathione; GST, glutathione *S*-transferase; NAC, *N*-acetyl-L-cysteine; IGF, insulin-like growth factor; DHEA, dehydroepiandrosterone; *ODC*, *ornithine* decarboxylase; TGFß, transforming growth factor ß; NK, natural killer; RAS, *ras* oncogene protein product; FGF, fibroblast growth factor.

(From Kelloff *et al.*, 2000)

1.3 Role of polyunsaturated fatty acids (PUFA) in cancer development

One of the perplexing questions to answer in nutritional oncology today is the inconsistency between animal studies and epidemiological studies on the association between dietary fat and cancer. Metaanalysis of animal studies involving data extracted from 97 reports of experiments and over 12000 rodents showed that n-6 PUFAs (such as linoleic and arachidonic acid) had a strong tumor enhancing effect whereas n-3 PUFAs (such as eicosapentaenoic and docosahexaenoic acid) had a small protective effect (Fay *et al.*, 1997). In contrast, pooled analysis of seven prospective cohort studies including more than 330,000 women and almost 5000 cases found no evidence of a positive association between total dietary fat and breast cancer risk (Hunter *et al.*, 1996). Epidemiological evidence in populations that have undergone lifestyle changes towards a more western culture during the past two decades clearly link consumption of fish oil with a protective effect against development of breast cancer. Fish oil contains mostly n-3 PUFA whereas vegetable oil (the major fatty acid in western diets) contains n-6 PUFA. A steady increase in breast cancer mortality rates in Japanese women accompanied a change in dietary fat preference away from fish consumption and towards increased use of linoleic acid-rich vegetable oils (Wynder *et al.*, 1991). Similar type changes in dietary habits of the Alaskan Inuit population may also have contributed to their increase in breast cancer rates (per 100,000 individuals) from 0.9 to 86.5 during a 20 year period (Lanier *et al.*, 1976). It has been suggested that different fatty acid types (n-6 or n-3) and particularly individual fatty acids may have different specific effects on breast cancer risk; therefore considering their effects together may result in an overall non significant effect.

1.3.1 Linoleic acid

In animal studies, the role of linoleic acid (LA) in tumour growth and metastasis has been extensively studied. A large amount of experimental data has shown that it promotes carcinogenesis, tumour growth and/or metastasis at numerous sites including mammary gland (Rose, 1997), prostate (Karmali *et al.*, 1987; Rose and Cohen, 1988; Zhou and Blackburn, 1997) and colon (Iigo *et al.*, 1997; Klurfeld and Bull, 1997, Reddy *et al.*, 1991). Colon tumor incidence and multiplicity were significantly reduced in rats fed a high fish oil diet while high fat corn oil had the opposite effect. (Reddy *et al.*, 1991). It has also been shown that LA has stimulatory effects on mammary cancer cell line growth *in vitro* (Rose and Connolly, 1989, 1990). However, LA has shown little correlation with cancer mortality in humans (Carroll *et al.*, 1986). Apart from the two populations mentioned above where cultures were westernised, few epidemiological studies have separated the effects of n-6 PUFAs and n-3 PUFAs from each other. Because linoleic acid contributes a large portion of total fatty acids in commonly consumed vegetable oils, more studies to clarify its role in human tumor growth and/or metastasis are needed.

1.3.2 Arachidonic acid

Arachidonic acid (AA) is the most biologically active PUFA in mammals, having a role in stress recognition, signal transduction, hormone regulation and gene regulation (Zhou and Blackburn, 1999). It is the most important PUFA associated with membrane phospholipids. It has been suggested that the pro-tumorigenic effects of dietary n-6 PUFA's are mediated via arachidonic acid metabolism. There are, however, limited studies investigating the association between dietary AA and tumorigenesis. *In vitro*

studies showed that AA was an effective stimulator of human prostate cancer cell growth and its growth stimulatory effect was mediated through its metabolism to 5-hydroxyeicosatetraenoic acid (5-HETE) series of eicosatetraenoids (Ghosh and Myers, 1997).

1.3.3 α-Linolenic acid

α-Linolenic acid (ALA) is a major n-3 PUFA found in vegetable oils. Animal studies have shown that ALA enriched diets inhibited growth and/or metastasis of mammary tumors (Fritsche and Johnson, 1990; Hirose *et al.*, 1990; Kamano *et al.*, 1989; Tinsley *et al.*, 1981). Klein *et al.* (2000) reported that low ALA levels in mammary adipose tissue were inversely correlated with increased mammary cancer risk in women. Previous work indicated that decreased ALA levels in patients with poor prognosis was likely to be a reflection of decreased dietary intake (Bougnoux *et al.*, 1994; Lhuillery *et al.*, 1995). These studies provide further support for the suggestion that the ratio of n-3:n-6 PUFA *in vivo* may play a protective role against development of mammary tumours.

1.3.4 Eicosapentaenoic and docosahexaenoic acid.

Fish oil supplementation has invaryingly been found to reduce tumour growth in virtually every animal model examined and at numerous sites including mammary (Kinoshita *et al.*, 1996; Kort *et al.*, 1987; Rose and Connolly, 1993; Rose *et al.*, 1995a), prostate (Karmali *et al.*, 1987; Rose and Cohen, 1988) and colon (Iigo *et al.*, 1997; Singh *et al.*, 1997a, 1998) although this effect in some studies did not reach significance (Fay *et al.*,

1997; Noguchi *et al.*, 1997). Fish oils have also been shown to be antimetastatic in a number of animal studies. Eicosapentaenoic acid (EPA) and/or docosahexaenoic acid (DHA) treatment significantly decreased lung metastases from primary tumors of colon (Iigo *et al.*, 1997; Singh *et al.*, 1998) or mammary gland (Kinoshita *et al.*, 1996; Rose and Connolly, 1993; Rose *et al.*, 1995a). Fatty acid analysis of tumor lipids revealed increased levels of EPA and DHA and decreased levels of arachidonic acid and eicosanoid metabolites, prostaglandin E_2 (PGE_2) and 12- and 15-HETE (Rose *et al.*, 1995a). Post-menopausal women with breast cancer had significantly lower dietary intake of EPA and DHA compared with those having benign breast disease (Zhu *et al.*, 1995).

1.4 Mechanisms underlying the effect of PUFAs on tumorigenesis.

1.4.1 Modulation of cell signalling pathways

Recent studies have attributed a mechanism to PUFAs that involves regulation of the activity of the EGFR/MAP kinase pathway, which is involved in regulating several oncogenes (*c-myc, c-fos, neu/c-erb-b2*) involved in the progression of cancer (Cowling and Shaker, 2001). The current view of the EGFR pathway is depicted in Figure 1.6. EGF binds to *trans*-membrane EGFR-tyrosine kinase, a membrane bound protein involved in signal transduction and growth stimulation of cells. Ligand-bound EGFR dimerise and activate a signal transduction cascade, thus inducing the activity of a variety of kinases, including a GTP-bound Ras, Raf-1, MEK and MAPK. The latter constitutes a family of serine/threonine kinases and may be the link that connects signal transduction

of EGFR to transcriptional activation in the nucleus. Dietary changes in PUFA composition has been shown to alter the EGFR/MAPK signalling cascade (Wang et al., 1992). Rats fed a high n-6 diet as corn oil showed increased MAPK activation and mammary tumor incidence compared with rats fed a low n-6 diet.

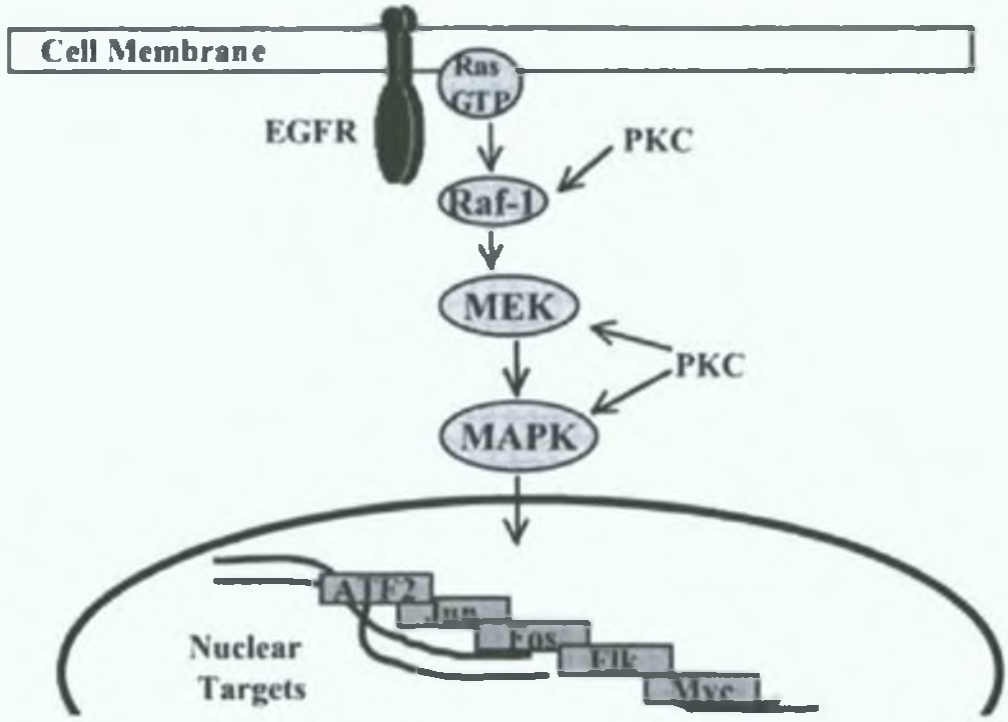


Figure 1.6 EGFR/MAPK signalling cascade (Cowing and Shaker, 2001).

Lipid modification of Ras is necessary for its localisation in membrane and for its interaction with the necessary molecules to initiate the MAPK signalling cascade. Researchers have demonstrated that feeding rodents n-6 PUFAs increased Ras farnesylation and expression in membranes during the promotion and progression stages of colon cancer development in rodents (Singh *et al.*, 1997a, 1998). In addition, arachidonic acid has been shown to inhibit GTPase-activating proteins (Tsai *et al.*, 1989), which are involved in the hydrolysis of GTP-bound (active) ras protein in the

EGFR/MAPK cascade. By inhibiting the GTPase activating protein, AA can prolong the signal transduction of EGFR to the nucleus, leading to increased growth stimulus. N-6 PUFAs and lipoxygenase metabolites have been implicated in the activation of several isoforms of protein kinase C (Lester, 1990, Fan *et al.*, 1990), which are effectors of MAPK signaling. N-6 PUFAs and lipoxygenase metabolites have been implicated in the activation of several isoforms of protein kinase C (Lester, 1990, Fan *et al.*, 1990), which are effectors of MAPK signaling. *In vivo*, PKC α and δ have been shown to activate Raf-1 and PKC β has been shown to activate MEK and subsequently MAPK (Toker, 1998). This provides evidence that n-6 PUFAs may influence MAPK mitogenesis of cells through a variety of mechanisms.

1.4.2 Modulation of eicosanoid production.

It is known that PUFAs exert their biological effects mainly via their regulation of eicosanoid metabolite production (Zhou and Blackburn, 1999). Dietary fatty acids undergo desaturation and elongation to yield a 20-carbon PUFA which is then oxygenated enzymatically by cyclooxygenase (COX) or Lipoxygenase (LOX) enzymes to form eicosanoids. Eicosanoids are potent bioregulatory compounds involved in a wide variety of cell signalling processes. These highly active substances are local modulators of a variety of intercellular and intracellular signals. They regulate many cell functions and play crucial roles in a variety of physiological and pathophysiological processes, one of which is the modulation of host immune fuctions.

Because AA is the most common fatty acid present in tissue lipids, the eicosanoids derived from it predominate in human tissue. Before AA is converted to eicosanoids, it

must be hydrolysed from phospholipids by a family of enzymes collectively known as phospholipase A_2 (Fonteh *et al.*, 1998). Prostaglandin (PG) synthase then catalyses two sequential reactions: first, the COX activity of the enzyme converts AA to PGG_2 and then the peroxidase activity reduces PGG_2 to PGH_2 (Kiefer *et al.*, 2001). However, in spite of these two distinct reactions, it has become commonplace for the complete entity to be referred to as COX. The PGH_2 from AA is then converted into the various 2-series prostanoids (PGs, thromboxanes and prostacyclins). AA is also a substrate for LOX which catalyse the biosynthesis of the hydroxyeicosatetraenoic acids (HETEs) and leukotrienes (LTs). Arachidonic acid can also undergo free radical peroxidation to form prostaglandin-like compounds known as isoprostanes. EPA is the precursor of the 3-series PGs and 5-series LTs. It can be metabolised by way of the COX and LOX pathways, which parallels the metabolism of AA. 1-series PGs and TXs are biosynthesised from dihomo-γ-linolenic acid (DGLA) (see figure 1.7).

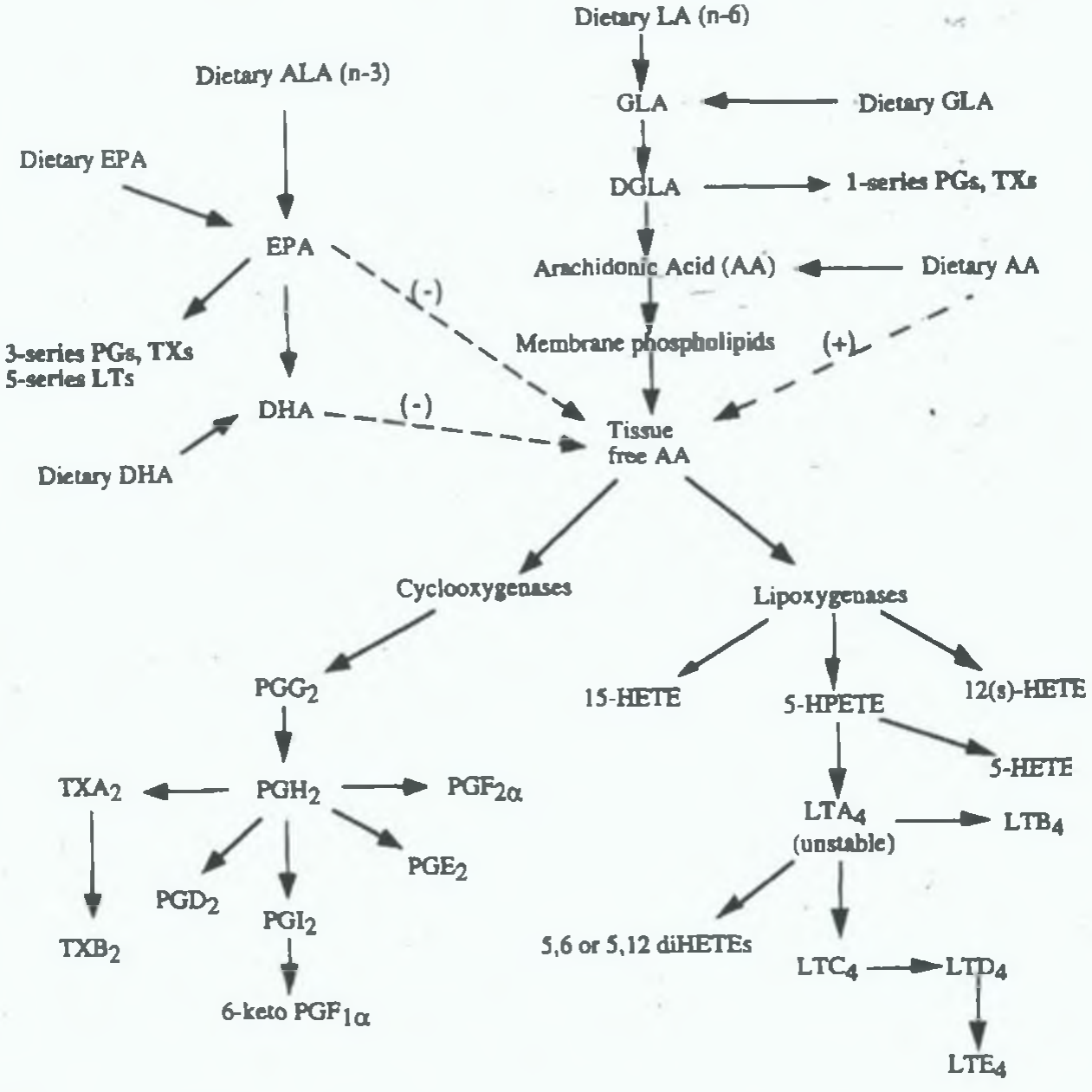


Figure 1.7 Metabolic pathways for biosynthesis of eicosanoids and their regulation by dietary fatty acids (Zhou and Blackburn, 1999).

Growing evidence indicates a causal relationship between cancer development and unscheduled eicosanoid signalling in many human and animal tumors and, therefore, these fatty-acid derived lipid mediators rank among the most attractive targets for cancer chemoprevention (Marks *et al.*, 2000). Tumors form more PGs and less AA than the normal tissue from which they arise (Lupulescu, 1996). Studies have shown that eicosanoids derived from AA increase cell proliferation, depress immune response and promote tumor cell invasion and metastases (Karmali *et al.*, 1987, Karmali, 1989). Intervention with pharmacological agents that inhibit eicosanoid synthesis, such as non-sterodial anti-inflammatory agents (NSAIDs), results in inhibition of tumorigenesis. Long-term ingestion of NSAIDs is associated with a reduced risk of colon cancer, a reduction in the number and size of colonic polyps and adenomas in patients with familial adenomatous polyposis and protection against chemically induced colon cancer in animal models (Reddy and Rao, 2002). There are two forms of COX, of which COX-1 is constitutively expressed in most tissues and is considered to generate PG for normal physiological function while COX-2 undergoes rapid induction in response to a variety of stimuli, including mitogens, cytokines and hormones (Herschman, 1994). COX-2 expression has been shown to be elevated in a number of cancers including pancreatic (Tucker *et al.*, 1999), colorectal (Kutchera *et al.*, 1996), gastric (Ristimaki *et al.*, 1997), lung (Hida *et al.*, 1998) and breast (Soslow *et al.*, 2000). Both COX-1 and COX-2 are targets of NSAIDs and the treatment with NSAIDs is associated with a decrease in COX-2 in colon tumors (Kargman *et al.*, 1995).

PUFAs of the n-6 class increase levels of PGs derived from AA (Karmali *et al.*, 1989) while n-3 PUFAs inhibit AA metabolism by competing with AA for cyclooxygenases and thereby inhibit tumor growth (Karmali *et al.*, 1997, 1998 and Reddy *et al.*, 1991). The n-3 PUFAs, EPA and DHA inhibit COX activity and AA metabolism (Lee *et al.*, 1985, Needleman *et al.*, 1979). Rats fed n-3 PUFAs showed selective incorporation of n-3 PUFAs with a concomitant reduction in n-6 PUFAs into membrane phospholipid pools of cells from various tissues (Hiller *et al.*, 1991). Studies suggest that n-6 PUFAs promote colon and mammary tumorigenesis by up-regulating the expression of COX-2 and p21 *ras*, whereas n-3 PUFAs may exert their antitumor effect by inhibiting COX-2 expression (Singh *et al.*, 1997a, Singh *et al.*, 1997b, and Badawi *et al.*, 1998). DHA was also shown to suppress polyp development in Apc knockout mice (a model for human familial adenomatous polyposis), possibly by inhibiting COX-2 (Oshima *et al.*, 1996).

A number of studies have demonstrated that AA derived eicosanoids play an important role in metastasis of tumors. In a rat metastasis model developed by injecting tumor cells into the portal vein, the administration of PGE_2 increased the number and size of metastatic tumor nodules in the liver and suppressed liver-associated immunity (Okuno *et al.*, 1995). The addition of the COX inhibitor indomethacin to the drinking water significantly reduced growth of metastasis of a human breast cancer cell line in nude mice (Connolly *et al.*, 1996). The relative reduction in metastases to the lungs in mice fed a high-fat low-LA diet compared with a high-fat high-LA diet was associated with reduced levels of tumor COX and LOX products (Connolly *et al.*, 1996).

1.4.3 Modulation of cell adhesion and proteolytic enzyme activities

Adhesion of tumors to endothelial cells is necessary for movement of tumor cells out of the interstitium to form metastatic deposits (Crissman *et al.*, 1988). Evidence suggests that PUFAs may affect tumor metastasis by regulating tumor-endothelial cell adhesion. Lipoxygenase products of AA metabolism may influence interactions between tumor and endothelial cells. 12(s)-HETE has been shown to stimulate tumor cell adhesion to endothelium and thus may stimulate tumor metastasis (Honn *et al.*, 1992). E-Cadherin is a calcium-dependent transmembrane cell to cell adhesion molecule which requires catenins (α, β and γ) to function normally. A reduction or complete loss of α-catenin has been observed in tumor tissues as well as in some tumor cell lines (Bongiorno *et al.*, 1995, Morton *et al.*, 1993, Shiozaki *et al.*, 1994). A specific n-6 PUFA, gamma linoleic acid (GLA) has been shown to increase α-catenin in most cell lines, while LA and AA had no effect (Jiang *et al.*, 1995a). GLA also induced E-cadherin expression in a range of human cancer cells (Jiang *et al.*, 1995b, c).

Another possible mechanism by which PUFAs modulate tumor metastasis is by regulating the expression and/or activity of matrix metalloproteolytic (MMP) enzymes via eicosanoids. Penetration of malignant tumor cells through basement membranes is facilitated by the action of these degrading MMP enzymes. *In vitro* studies indicate that LA increased tumor cell invasion and metastasis, increased production of eicosanoids and induced expression and activities of MMP-9 (Liu *et al.*, 1996, Liu and Rose, 1994). Dietary supplementation of GLA, which resulted in a higher incidence of macroscopic lung metastases and a larger total metastatic volume from a mammary tumor, was

associated with higher levels of AA and AA-derived eicosanoids and a higher activity of MMP-9 (Rose *et al.* 1995b).

1.5 Dairy products and cancer

Results from epidemiological studies on the consumption of dairy products and the risk of cancer have been controversial. Some of the studies found a significant inverse association, some a significant positive association and some no association (reviewed in Jain, 1998, Ursin *et al.*, 1990). The relationship between intake of dairy products and the risk of breast cancer was studied in 4679 initially cancer-free women in Finland. During a 25-year follow-up period food consumption data was collected. Results from this study found that women who went on to develop breast cancer had consumed less milk than cancer-free women (Knekt *et al.*, 1996). The relationship between the consumption of milk products and the occurrence of colorectal cancers was studied in 9959 men and women with no history of cancer. During a 24-year follow-up period 72 new cancers of the large bowel were detected and results indicated that individuals showing high consumption of milk had a potentially reduced risk of colon cancer (Jarvinen *et al.*, 2001). Research has shown that milk contains a number of potential anticarcinogenic lipid components including butyric acid, ether lipids, sphingomyelin and CLA (Parodi, 1999, Gill and Cross, 2000). CLA has been demonstrated to be a very efficient suppressor of chemically induced carcinogenesis in animal models (reviewed in Scimeca, 1999) and its presence in human serum has been associated with a decreased risk of breast cancer in postmenopausal women (Aro *et al.*, 2000).

1.6 Chemical structure of CLA

The acronym CLA refers to a mixture of positional and geometric isomers of the n-6 essential fatty acid linoleic acid (*c*9, *c*12, octadecadienoic acid) which contain a conjugated double bond system and occur naturally in edible fats derived from ruminant animals (Lin *et al.*, 1995). Unlike LA, which is a single unique molecule, several dozen different CLA isomers are possible depending on which double bonds are relocated and the resultant isomeric reconfigurations. The majority of research to date has been conducted using synthetic mixtures of CLA isomers. The *c*9, *t*11-CLA and the *t*10, c12-CLA isomers predominate in these mixtures (approximately 85-90%). In the last few years, research with individual CLA isomers has focused exclusively on these two isomers. The reason for this is that highly purified preparations of these two CLA isomers have been successfully produced, while the other isomers have yet to be acquired in sufficient quantities. Little has been done to determine the activity and mechanisms of isomers other than these two.

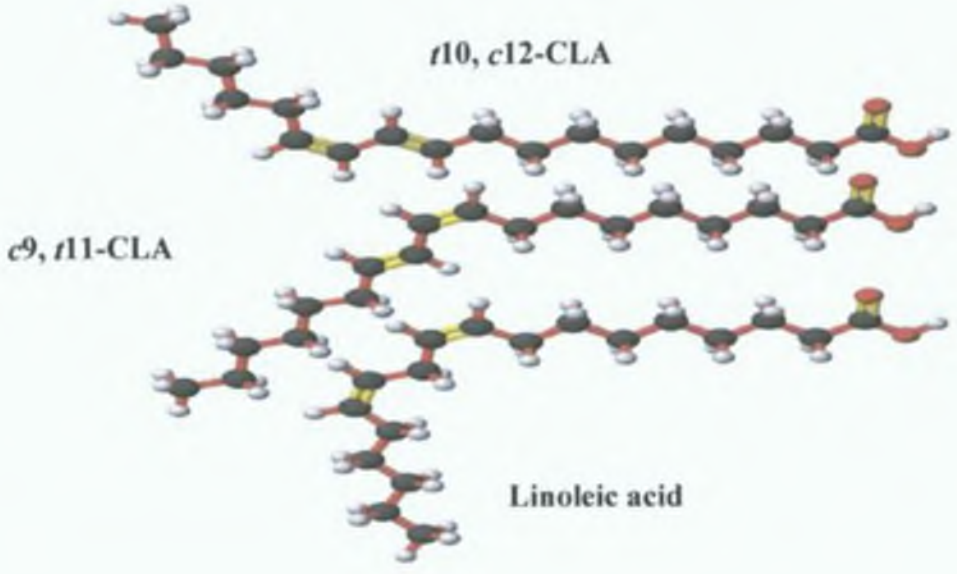


Figure 1.8 Chemical Structure of common CLA isomers and linoleic acid

1.7 The Analysis of CLA isomers.

The recognition of CLA as a natural fat component with a number of health-promoting properties has prompted increased interest in methods for their analysis. There are 56 possible positional and geometric isomers of CLA. It is known that many positional and geometric isomers of CLA are present in natural samples and in commercially manufactured material and it is emerging that these isomers may have different effects in biological systems. Desaturation and chain-elongation products of these isomers are also formed in tissues. In analysing CLA, it is therefore imperative to be able to separate and accurately quantify these positional and geometric isomers and identify CLA metabolites. Figure 1.9 shows different methods for CLA analysis.

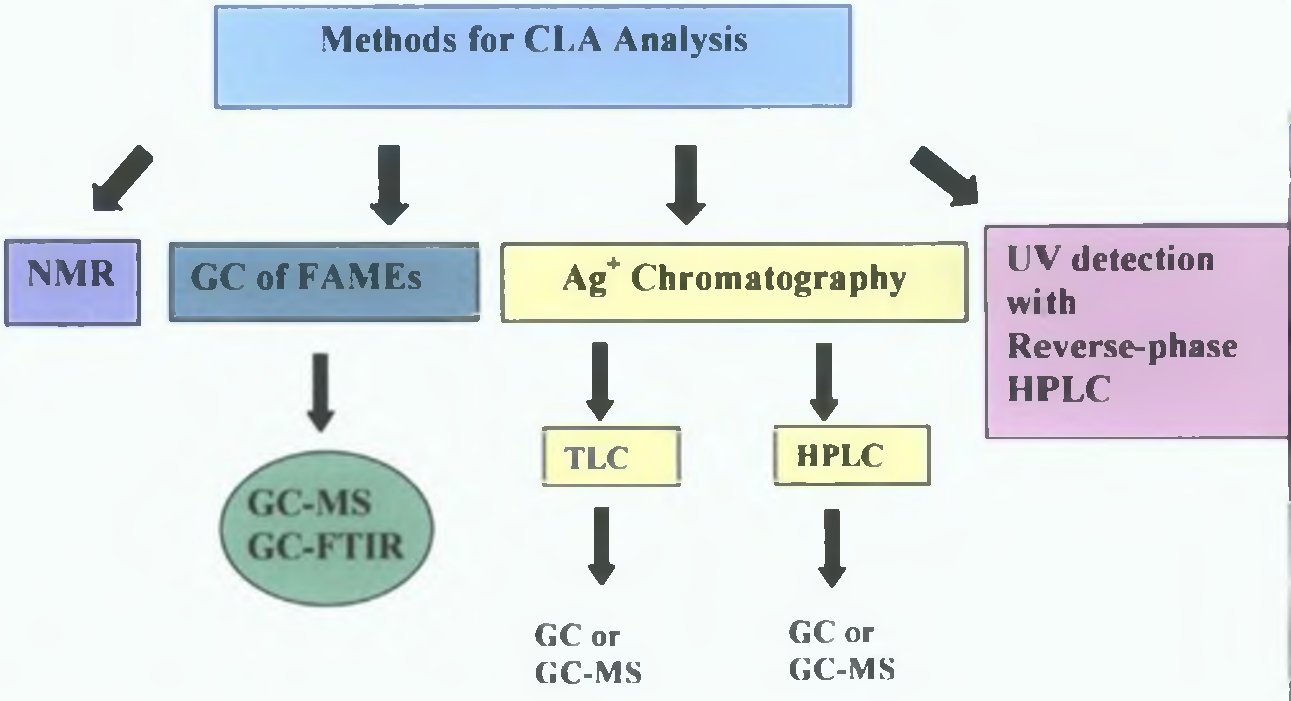


Figure 1.9 Different methods for CLA analysis.

The most common means of analysis has been gas chromatography (GC). Before fatty acids are analysed by GC, they must first be converted to fatty acid methyl esters (FAME). It has emerged that the selection of an appropriate methylation method is crucial to successfully quantifying acids such as CLA. Acid catalysed methylation is now seen as an unsuitable method for the preparation of methyl esters in biological samples as it causes stereomutation of the *cis/trans* diene system resulting in an increase in the relative proportions of *trans/trans* isomers (Kramer *et al.*, 1997). There is also the possibility of addition of methanol to a double bond leading to the formation of methoxy derivatives. By contrast, there appears to be no significant drawbacks associated with base-catalysed methylation of lipids. The use of tetramethylguanidine (TMG) as a base catalyst in the methylation appears to be the choice for biological samples (Yurawecz *et al.*, 1999).

GC is used for the separation of different FAMEs, which are detected by flame ionisation detection (FID). GC with highly polar stationary phases and longer than normal columns (100-120 m) are required for the resolution of CLA isomers with good separations reported for CP-Sil 88 and BPX-70 columns in 40-53 minutes. Using these columns the four major *cis/trans* isomers are separated and eluted first (in the order *c*9, *t*11-, *t*8, *c*10-, *c*11, *t*13- and *t*10, *c*12-CLA) followed by the *cis/cis* isomers and the 8, 10 through 10, 12 *trans/trans* isomers which remain unresolved. GC can also be coupled with fourier transformed infrared spectroscopic determination (FTIR) for analysis of CLA. GC-FTIR allows analysis of double bond configuration, is highly sensitive and therefore only a few ng of sample is required for quantitative analysis (Fritsche *et al.*,

1997). The use of a mass spectrometer in connection with GC has proved useful for CLA analysis in food and biological samples where minor isomers, CLA metabolites and other fatty acids may be present. GC-MS can be used to locate double bonds and also to identify minor isomers along with any nonconjugated fatty acids that co-chromatograph with those of interest. Different nitrogen containing derivatives have been used but of these, dimethyoxazoline (DMOX) derivatives have proved the most useful for conjugated dienes. A new derivative has been developed that is also highly specific for conjugated double bonds. A Diels-Alder reaction is used to form 2-methyl-1,2 ,4-triazoline-3,5-dione (MTAD) adducts which have excellent mass spectrometric properties and thus enable the location of conjugated double bonds. Prior to GC or GC-MS analysis, natural CLA samples like tissues, which contain CLA at very low levels, may require a preconcentration step. This can be achieved using silver ion chromatography or reverse phase high performance liquid chromatography (HPLC) (Christie *et al.*, 2001).

HPLC procedures using a single C_{18} or silica column is inadequate for separation of CLA isomeric mixtures (Chin *et al.*, 1992, Banni *et al.*, 1996, Sebedio *et al.*, 1997). In recent years a method has been developed which involves the use of Ag^{+}-HPLC. A mobile phase of hexane containing 0.1 % acetonitrile is used to separate methyl ester derivatives, using UV detection at 233 nm for detection and quantification of conjugated double bonds. This system allows for well-resolved separation of three groups of geometric isomers of CLA. *Trans/trans*-isomers elute first, followed by *cis/trans* and then *cis/cis* and within each group many positional isomers are clearly resolved. Separation of the positional isomers is facilitated by connecting Ag^{+}-HPLC columns in series (Sehet *et al.*, 1999, Ricket *et al.*, 1999). Figure 1.10 shows a separation using two Ag^{+}-HPLC columns.

The identity of CLA isomers can be determined using GC or GC-MS. The isolation of minor isomers in food has been achieved using silver ion HPLC with two to six columns in series. Ag^+-HPLC may also be adapted to analyse CLA as free fatty acids thus avoiding the need to methylate (Cross *et al.*, 2000).

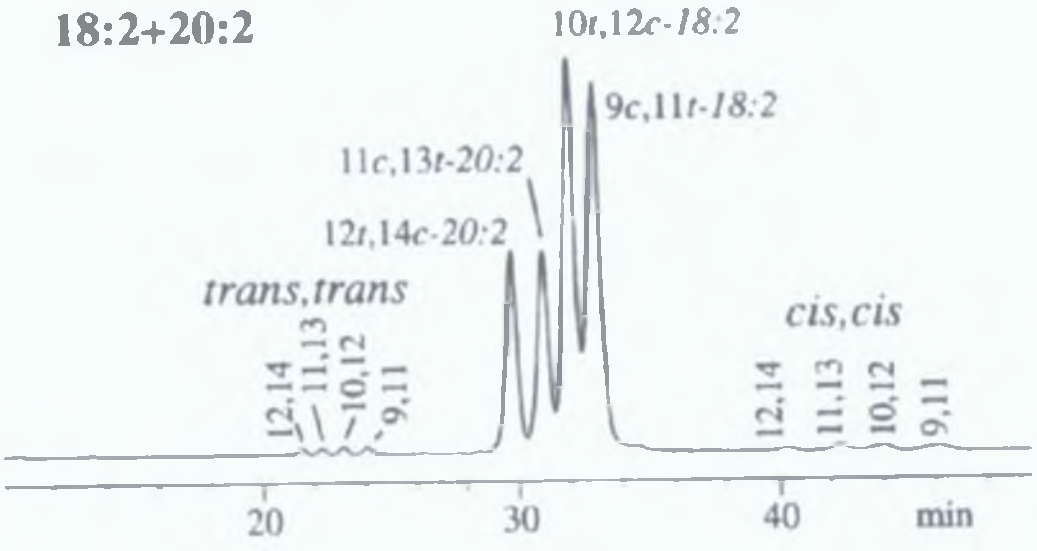


Figure 1.10 Ag^+-HPLC separation using two columns. (Sehat *et al.*, 1999).

Conjugated fatty acids have a distinct UV spectra absorbing at around 230-235 nm while methylene interrupted double bonds absorb at 200-210 nm. When conjugated bonds are the major component of the lipid sample their absorbance can easily be measured. However CLA is present at very low levels in tissue lipids and therefore absorbance of CLA may only be displayed as a shoulder on a broad peak at 200 nm due to residual absorption from the fatty acids in the 200-210 nm region. Corongiu and Banni (1994) developed a mathematical technique to overcome this difficulty. By taking the differential of the first derivative spectrum, a second derivative was obtained which

extracted a distinct peak from the shoulder. This second derivative spectroscopy afforded a more sensitive and accurate means to quantify conjugated dienes since the Beer-Lambert law is unaffected by differentiation. This technique can be used in tandem with reverse-phase HPLC to separate and quantify metabolites of CLA as well as CLA (Banni *et al.*, 1999).

Recent developments have indicated that useful analytical data can be obtained through nuclear magnetic resonance based on signals for the olefinic carbon atoms (Davis *et al.*, 1999). Each CLA isomer has signals for its four olefinic carbon atoms and chemical shifts have been identified for 20 different CLA isomers. This procedure avoids derivatisation and permits the identification and quantification of all the positional and geometric isomers present in commercial CLA preparations. Unfortunately, the methodology requires substantial amounts of sample and therefore, is not likely to be applicable to biological samples containing only low levels of CLA.

1.8 Origins of CLA in milk and meat

CLA is formed as an intermediate in biohydrogenation of LA by rumen bacteria and until recently it was generally accepted that CLA in ruminants originated from the incomplete biohydrogenation of LA (Fritsche and Steinhart, 1998). Complete biohydrogenation of LA in the rumen is a three step process, leading to the production of $C_{18:0}$ (Kepler *et al.*, 1966). CLA is formed as the first intermediate of this pathway by the action of LA isomerase, an enzyme of the anaerobic rumen bacteria *Butyrivibrio fibrisolvens* (Kepler *et al.*, 1970). The enzyme has been shown to exhibit maximum activity with the substrates LA and linolenic acid (Kepler and Tove, 1967). A portion of CLA formed escapes completes biohydrogenation and is incorporated into milk fat and body fat.

However this simplistic explanation for the presence of CLA in ruminant lipids does not adequately account for CLA levels in milk and body fat. It is proposed that *trans*-vaccenic acid (TVA) accumulates in the rumen and that a portion escapes further biohydrogenation (Griinari and Baumann, 1999). Following absorption from the digestive tract, TVA is utilised by different tissues where a portion is desaturated to CLA and incorporated into tissue and milk lipids. This 'desaturase hypothesis' has been proposed to explain the relatively constant ratio of TVA and CLA in bovine milk fat across a range of diets. The presence of *t*7, *c*9 CLA and *c*9, *t*13 $C_{18:2}$ supported the role of an active Δ^9 desaturase, an enzyme that introduces a *cis* double bond between carbons 9 and 10 (Ulberth and Henninger, 1994, Yurawecz *et al.*, 1998). Figure 1.11 illustrates the two pathways of CLA biosynthesis which together may account for the high CLA concentrations observed in milk fat even when cows are fed diets that are low in LA e.g.

pasture feeding or fish oil supplements. Griinari *et al.* (2000) demonstrated in a series of experiments that TVA is desaturated to CLA in lactating cows and estimated 64 % of *c*9, *t*11-CLA in milk fat was of endogenous origin. Abomasal infusion of TVA resulted in a 31 % increase in concentration of *c*9, *t*11-CLA in milk fat. Infusion of sterculic oil (source of cycolpropene fatty acids which specifically inhibit Δ^9 desaturase) decreased the concentration of CLA by 45 %.

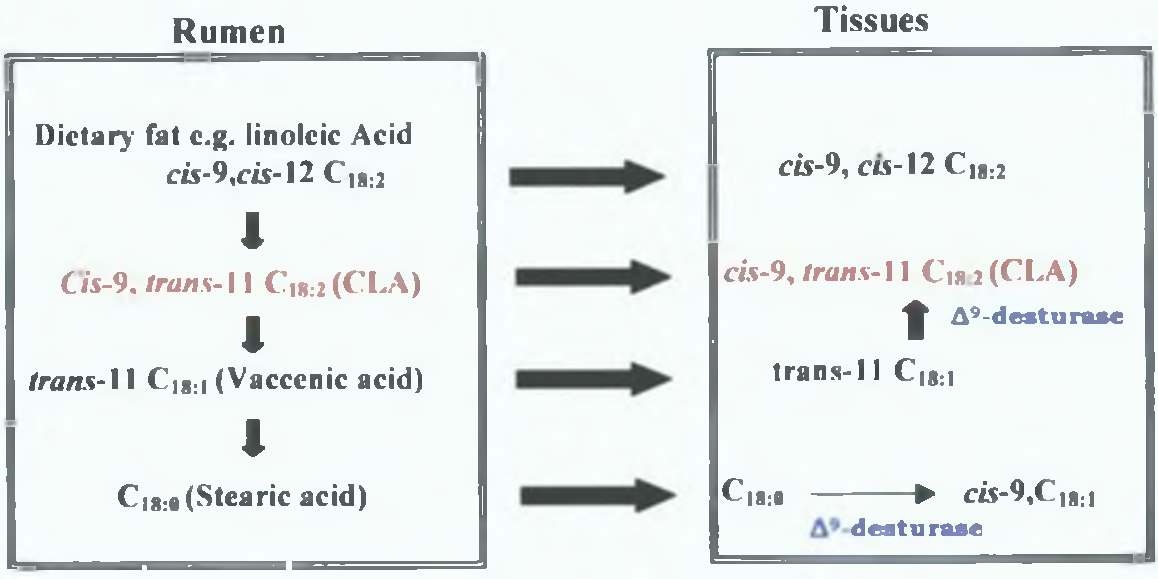


Figure 1.11 Role of rumen biohydrgenation and tissue Δ^9-desaturase in the production of *c*9, *t*11-CLA in ruminant fat. (Adapted from Bauman *et al.*. 1999)

The second most prevalent CLA isomer found in milk fat is the *t*7, *c*9-CLA isomer and is produced exclusively from endogenous synthesis by Δ^9 desaturation of *trans* 7-$C_{18:1}$. Other isomers, which are present at very low quantities in rumen fats, originate from ruman biohydrgenation (Unpublished data Bauman, 2002). When animals are fed a low-fibre diet the pH of the rumen environment drops and this shift in pH favours the

formation of *t*10-octadecenoic acid and increases the proportion of *t*10, *c*12-CLA in milk fat (Griinari *et al.*, 1998). Therefore it has been proposed that *t*10, *c*12-CLA is formed as a conjugated intermediate in the biohydrogenation of linoleic acid to *t*10-octadecenoic acid. Production of *t*10-octadecenoic acid would presumably involve a specific *c*9, *t*10 isomerase in rumen bacteria with the formation of *t*10, *c*12 conjugated bond structure as the first reaction (Grinari and Bauman, 1999). Changes in ruminal biohydrogenation, characterised by increased *c*9, *t*10 isomerization, were associated with a dramatic reduction in the rate of milk fat synthesis and a role for t10-octadecenoic acid and/or *t*10, *c*12-CLA as specific inhibitors of milk fat synthesis was proposed. Baumgard *et al.* (2001) have demonstrated that *t*10, *c*12-CLA isomer caused milkfat depression whereas the *c*9, *t*11-CLA isomer did not.

1.9 Dietary sources and intakes of CLA

Parodi (1977) first reported the presence of CLA in milk fat. Since then, numerous investigators have studied and documented the total CLA and *c*9, *t*11-CLA isomer concentrations of many foods (Table 1.2). CLA is mainly found in milk and meat from ruminant animals but is also found in plant oils and partially hydrogenated oils at low concentrations. The *c*9, *t*11-CLA isomer accounts for 80-95 % of total CLA isomers in dairy products in contrast to less than 50 % in vegetable oils (Chin *et al.*, 1992). The *t*7, *c*9-CLA isomer is generally prominent among the other minor CLA isomers found in dairy products (Yurawecz *et al.*, 1998). CLA is also detectable in non-ruminants but at very low levels. CLA levels between 3 and 10 mg CLA/g of fat were measured in meat from ruminants, while pork and poultry were shown to have a CLA content less than 1mg/g of fat (Chin *et al.*, 1992). The CLA content of cheeses varies considerably, ranging from 3 to 9 mg/g fat (Chin *et al.*, 1992). Seafood also contains low amounts of CLA with the highest value found in shrimp (0.6mg/g fat) (Chin *et al.*, 1992).

The major source of CLA in human tissues is the diet (McGuire *et al.*, 1999). Various methodologies have been used to estimate typical CLA intakes for humans. These include the use of food disappearance data, dietary recalls, food frequency questionnaires, weighed food records and biochemical analysis of food duplicates. All these methods have their own limitations but the biochemical analysis of food duplicates is considered the most accurate. The accuracy of the other indirect methods relies on the availability of a database containing the CLA contents of commonly consumed foods, which remains quite limited.

Table 1.2 Total CLA content and % *c*9, *t*11 CLA in food products

Food	Total CLA (mg/g fat)	*c*9,*t*11-CLA (% of total CLA)	Reference
Milk fat	2-30	90	Parodi, 1994
Butter	9.4-11.9	91	Shantha *et al.*, 1995
T-bone (cooked)	4.7-9.9	65	Shantha *et al.*, 1994a
Yogurt	5.1-9.0	82	Fritsche and Steinhart, 1998
Processed cheeses	3.2-8.9	17-90	Ha *et al.*, 1989, Chin et al., 1992, Garcia-Lopez *et al.*, 1994
Sour cream	7.5	78	Fritsche *et al.*, 1998
Condensed milk	7	90	Chin *et al.*, 1992
T-bone (raw)	4.4-6.6	59	Shantha *et al.*, 1994a
Cheddar cheese	5.1-5.4	82-88	Werner *et al.*, 1992
Ice cream	3.8-4.9	73-76	Fritsche and Steinhart, 1998
Round beef	2.9	79	Ip *et al.*, 1991
Chicken	0.9	84	Chin et al., 1992
Pork	0.6	82	Chin et al., 1992
Non fat frozen dairy dessert	0.6	90	Chin *et al.*, 1992
Seafood	0.5	ND	Chin et al., 1992
Vegetable oils	0.2	45	Chin et al., 1992

ND= not detected

A summary of published data on CLA intake in humans is presented in Table 1.3. Using dietary records and a published database, Herbel *et al.*, (1998) reported that young men and women living in the United States consumed approximately 127 mg CLA/day. Somewhat similar values were obtained in another US study using similar methodology. This study estimated that the dietary intake of CLA in young men and woman was 137 and 52 mg/day, respectively (Ritzenthaler *et al.*, 1998). It is interesting to note that college-aged women have extremely low CLA intakes. The CLA intake in young Canadians (Ens *et al.*, 2001) has been estimated to be 94 mg/day. Eighteen out of the twenty-six subjects used in this study were female and this may account for the relatively low intake. Dietary intake of CLA in Germany was also estimated to be lower in women (350 mg CLA/day) than in men (430 mg CLA/day) (Fritsche and Steinhart, 1998) on the basis of the West German National Consumption Survey. In a more recent German study, daily intake was reported to be 246 and 323 mg CLA/day as measured by a newly developed food-frequency questionnaire and a 7-day estimated record, respectively. The differences in intake observed in the US and German studies can be accounted for by the fact that fat intake is higher in Germany than in the US (Aldolf, 1994) and by differences in the underlying CLA databases.

The methods used in the above studies may be somewhat inaccurate because of issues like under reporting and the use of inadequate CLA databases. Ritzenthaler *et al.*, (2001) compared indirect methods of CLA intake estimations (3-day recorded records and semiquantative food frequency questionnaire) with a more direct method involving the biochemical analysis of food duplicates. Total CLA intake using food duplicates was

estimated to be 212 and 151 mg/day for men and women respectively. Most of the CLA consumed was *c*9, *t*11-CLA (91 - 93 %) with the *t*10, *c*12-CLA isomer being the only other CLA isomer detectable in the analyses. Results indicated that 3-day dietary records and semiquantative food frequency questionnaire were not reliable estimators of CLA intake and may actually underestimate CLA intake. The authors also calculated on a dry weight basis that men and women in this study consumed diets containing approximately 0.03 g *c*9, *t*11-CLA /100g. To achieve an intake of 0.1 g/100g diet, the level of CLA that has been shown to significantly reduce tumors in animals (Ip *et al.*, 1994), the *c*9, *t*11-CLA intake would need to be 620 and 441 mg/day for men and women, respectively.

Dietary modifications can increase CLA concentration in human tissues. Specific intervention studies have shown that increasing the CLA content of the diet increased the CLA content in human milk (Park *et al.*, 1999a), plasma (Huang *et al.*, 1994) and adipose tissue (Jiang *et al.*, 1999). Feeding a high dairy fat diet containing 291 ± 75 mg CLA /day led to a 1.6 fold increase (13.5 ± 0.1 µmol/g fat) in CLA content of human milk (Park *et al.*, 1999). Plasma CLA increased 19-27 % to 9.6 ± 1.1 µmol/L when men were fed cheddar cheese containing 178.5 mg CLA each day for 4 weeks (Huang *et al.*, 1994). The amount of *c*9, *t*11-CLA in human adipose tissue was significantly related to milk fat intake (Jiang *et al.*, 1999).

Table 1.3 Published estimates of CLA Intake in Humans.

Country	Subjects	CLA intake (mg/d)	Method used	Reference
U.S.	Men and women n=12	127*	3-day dietary records + published values for CLA content in foods.	Herbel *et al.*, 1998
Germany	Males	430	National food intake survey + values for CLA in German foods.	Fritsche and Steinhart 1998
	Females	350		
U.S.	College aged subjects :		3-day dietary records + published values for CLA content in foods.	Ritzenthaler *et al.*, 1998
	Males (n=19)	137		
	Females (n=18)	52		
U.S.	Men (n=46)	212* 193	3-day dietary records + Biochemical analysis of food duplicates + Semiquantitative Food frequency questionnaire	Ritzenthaler *et al.*, 2001
	Women (n=47)	151* 140		
Canada	Men and women (n=22)	94	7-day dietary records + published values for CLA content in foods.	Ens *et al.*, 2001
Germany	Women (n=52)	246	Food frequency questionnaire	Fremann *et al.*, 2002
		323	7-day dietary records	

* Values represent intakes of all CLA isomers while others represent *c*9, *t*11- CLA

1.10 CLA intake in infants and children.

Studies have shown that human milk contains a variety of CLA isomers, with the *c*9, *t*11-CLA isomer being the predominant isomer (Fogerty *et al.*, 1988, McGuire *et al.*, 1997, Jensen *et al.*, 1998 and Park *et al.*, 1999). The amount of *c*9, *t*11-CLA present in human milk is surprisingly similar to that found in bovine milk. Maternal diet can influence the amount of CLA in human milk. A study has demonstrated that women consuming diets high in dairy fat produced milk with higher levels of *c*9, *t*11-CLA when compared to women with low dairy intakes (Park *et al.*, 1999). Infant formulas, on the other hand, contain negligible amounts of CLA (McGuire *et al.*, 1997). Therefore breast-fed babies potentially intake quite high levels of CLA throughout infancy while formula-fed infants consume no CLA before introduction of CLA-containing food into their diet. So what are the consequences for mother and child?

There is evidence to suggest that breast-feeding can afford protection to some women against breast cancer (Enger *et al.*, 1997). It may be a possibility that increased exposure to CLA during lactation may explain in part the protective effect of breast-feeding on breast cancer. Feeding CLA enriched butterfat to rats has been shown to diminish epithelial branching and decrease the population of terminal end buds, the primary sites for chemical induction of mammary carcinomas (Ip *et al.*, 1999a). The question remains whether CLA exposure could cause alterations in human breast morphology making them less susceptible to cancer. There is also an association between exposure to human milk in infancy and the risk of breast cancer in later life, results suggesting that breast fed infants are offered some protection against breast cancer risk (Titus-Ernstoff *et al.*, 1998).

Ip *et al.* (1995) have shown that feeding CLA to rats from weaning until carcinogen was administered offered protection against mammary cancer for life. In contrast, feeding CLA after carcinogen administration required continuous CLA feeding to achieve the same level of protection. McGuire *et al.* (1999) has suggested that it may be useful to feed rats varying levels of CLA during pregnancy and lactation. The different treatments could then be evaluated for protection against chemically induced mammary tumor incidence in the offspring.

Lusas (1991) proposed the concept of 'biological programming' which can be described as a process by which a stimulus (like nutrition) during fetal growth, infancy and childhood can potentially have important long term effects on physiological functions and might decrease risk for chronic diseases in later life. McGuire *et al.* (1999) has proposed that CLA intake in infants and children might impart beneficial effects on the immune system, nutrient partitioning, glycemic control and growth modulation in later life. There is, therefore, a need to document CLA intake in infants, children and adolescents. McGuire and co-worker have recently documented CLA intake in school children (5-15 yr). CLA intake was highest among the youngest children and girls consumed more CLA than did boys (184 versus 158 mg/d) (unpublished data from McGuire *et al.* 2002). CLA has been shown to reduce body fat and increase lean body mass in young growing animals (Chin *et al.*, 1994, Park *et al.*, 1997). Studies examining the effect of CLA on body composition in adults have so far proved some what disappointing. It may be speculated that CLA may have a more potent effect on body

composition in children and may provide a potential treatment and protection against childhood obesity. Studies in growing and /or obese children should be considered.

1.11 The Biosynthesis of CLA in man and rodent.

CLA has been identified in human blood, milk (Fogerty *et al.* 1988), adipose tissue (Ackman *et al.* 1981), bile and duodenal juices (Cawood *et al.* 1983) with *c*9, *t*11-CLA as the most predominant isomer present. The origin of CLA in human tissues is thought to be dietary as the consumption of CLA-containing foods such as cheese has been shown to increase plasma CLA levels (Britton *et al.*, 1992, Huang *et al.*, 1994). In 1994 Parodi proposed that dietary TVA, the predominant *trans* monounsaturated fatty acid in milk fat (Parodi, 1976) could be desaturated to *c*9, *t*11-CLA in humans. This hypothesis was based on the findings of Mahfouz *et al.*, (1980) and Pollard *et al.* (1980), who showed that a Δ^9 desaturase enzyme from rat liver microsomes produced CLA from TVA. Recently, Corl *et al.* (2001) demonstrated that endogenous synthesis is the major source of *c*9, *t*11-CLA in the milk fat of lactating cows. *Butyrivibrio fibriosolvens*, the anaerobic rumen bacteria responsible for the biohydrogenation of linoleic acid in ruminants, has also been found in the digestive tract of human subjects (Brown and Moore, 1960) and so it is also possible that CLA could be produced from dietary linoleic acid in humans. Thus, CLA in human tissues may reflect both dietary intake and endogenous synthesis.

Ip *et al.* (1999a) demonstrated that rats consuming CLA-enriched butterfat accumulated more total CLA in their tissues compared to those consuming either Matreya CLA or Nu-

Chek Prep CLA. The authors hypothesised that the availability of TVA in the high CLA butterfat may serve as the precursor for the endogenous synthesis of CLA via the Δ^9 desaturase reaction. Santora *et al.* (2000) reported and quantified the desaturation of TVA to CLA in mice. When equal quantities of TVA and CLA were fed to mice they reported that 12 % of the TVA consumed during a 2-wk feeding period was recovered in the carcass as CLA. Of the proportion of TVA in the tissues that was available for bioconversion, 48.8 % was desaturated. CLA was found in the carcass only when vaccenic acid or CLA was fed. CLA was found in both triglyceride and phospholipids when CLA was fed, but only in triglyceride when TVA was fed, suggesting that bioconversion occurred in the adipose tissue (Santora *et al.*, 2000).

The Δ^9 desaturase genes have been identified in tissues from human subjects (Zhang *et al.*, 1999). Salminen *et al.*, (1998) provided evidence that CLA in human serum has been derived in part from the diet and in part by conversion of dietary *trans* fatty acids. Serum CLA levels were significantly higher in subjects fed a high-dairy fat diet, rich in CLA and *trans*-fatty acids than when fed a CLA-poor stearic acid diet. Evidently, CLA was formed during consumption of the diet rich in *trans* fatty acids and incorporated into serum lipids. O'Shea *et al.*, (2000) examined the fatty acid composition of total cell lipids of MCF-7 human breast cancer cells, incubated in the presence of pure *c*9, *t*11-CLA (20 μg/ml) and with a CLA-enriched milk fat containing 20 μg/ml CLA. CLA uptake was approximately 6 fold more proficient from the milk fat than from the synthetic pure *c*9, *t*11-CLA source, supporting the study by Ip *et al.* (1999). This study suggested that CLA could be formed from TVA present in the milk fat by a Δ^9 desaturase enzyme present in

human breast cancer cells. Adlof *et al.*, (2000) showed that TVA was converted into CLA in humans, at a CLA enrichment of approximately 30%.

It has also been proposed that CLA may be synthesised from LA by intestinal flora or by free radical induced isomerisation. In normal rats, dietary linoleic acid gave rise to CLA in various tissues in proportion to the amount of linoleic acid fed, but this conversion was not evident in germ-free animals (Chin *et al.*, 1994). Salminen *et al.* (1998) suggested that their results did not favour the concept of production of CLA from linoleic acid in humans because significantly different levels of CLA were found in serum lipids from subjects fed three different dietary regimes that contained the same levels of LA. The consumption of LA in triglyceride form in sunflower oil did not increase plasma levels of esterfied CLA in the total lipids of human subjects (Herbel *et al.*, 1998). Adlof *et al.* (2000) found no evidence for the conversion of LA via bacterial isomerase enzymes to CLA in human subjects. The authors suggested that if CLA were to be formed in the colon it would be poorly absorbed in the lower intestine. Furthermore, human studies have shown ingested LA to be >96 % absorbed, which means that a very small percentage of LA would be available to bacteria in the intestine.

1.12 Metabolism of CLA

CLA isomers have been shown to undergo elongation, desaturation and β-oxidation processes similar to those that occur with linoleic acid while still maintaining the conjugated diene structure (Figure 1.12). Elongation and desaturase metabolites of CLA isomers (eg. conjugated 18:3, conjugated 20.3 and conjugated 20:4) have been detected in mammary tissue (Banni *et al.*,1999) and in the liver of rats (Banni *et al.*, 1995, Banni *et al.*, 2001), lamb tissue (Banni *et al.*, 1996) and in human plasma, adipose tissue and red blood cells (Lucchi *et al.*, 2000). Both *c*9, *t*11-CLA and *t*10, *c*12-CLA are converted to long-chain metabolites (Sebedio *et al.*, 1997, Sebedio et al., 2001). In rat liver and adipose tissue *t*10, *c*12-CLA is mainly metabolised into conjugated 18:3 while *c*9, *t*11-CLA is preferentially metabolised into a conjugated 20:3 isomer. Levels of *t*10, *c*12-CLA metabolites were higher suggesting that its turnover is higher than that of *c*9, *t*11-CLA (Sebedio *et al.*, 2001). The conjugated 20:4 metabolite (the expected main metabolite) was not detected in rat tissue (Sebedio *et al.*, 2001) or in human plasma and tissues (Lucchi *et al.*, 2000). The authors suggested that the linoleic acid in the diet shared desaturation and elongation enzymes with CLA and thus could compete for conjugated 20:4 formation (Lucchi *et al.*, 2000) or that conjugated 20:4 is formed and rapidly converted (Sebedio *et al.*, 2001). Another metabolite with 16 carbon atoms, conjugated 16:3 has been identified and is probably derived from peroxisomal β-oxidation of CLA. Interestingly, this metabolite was only found in liver lipids from rats fed *t*10, *c*12-CLA (Sebedio *et al.*, 2001).

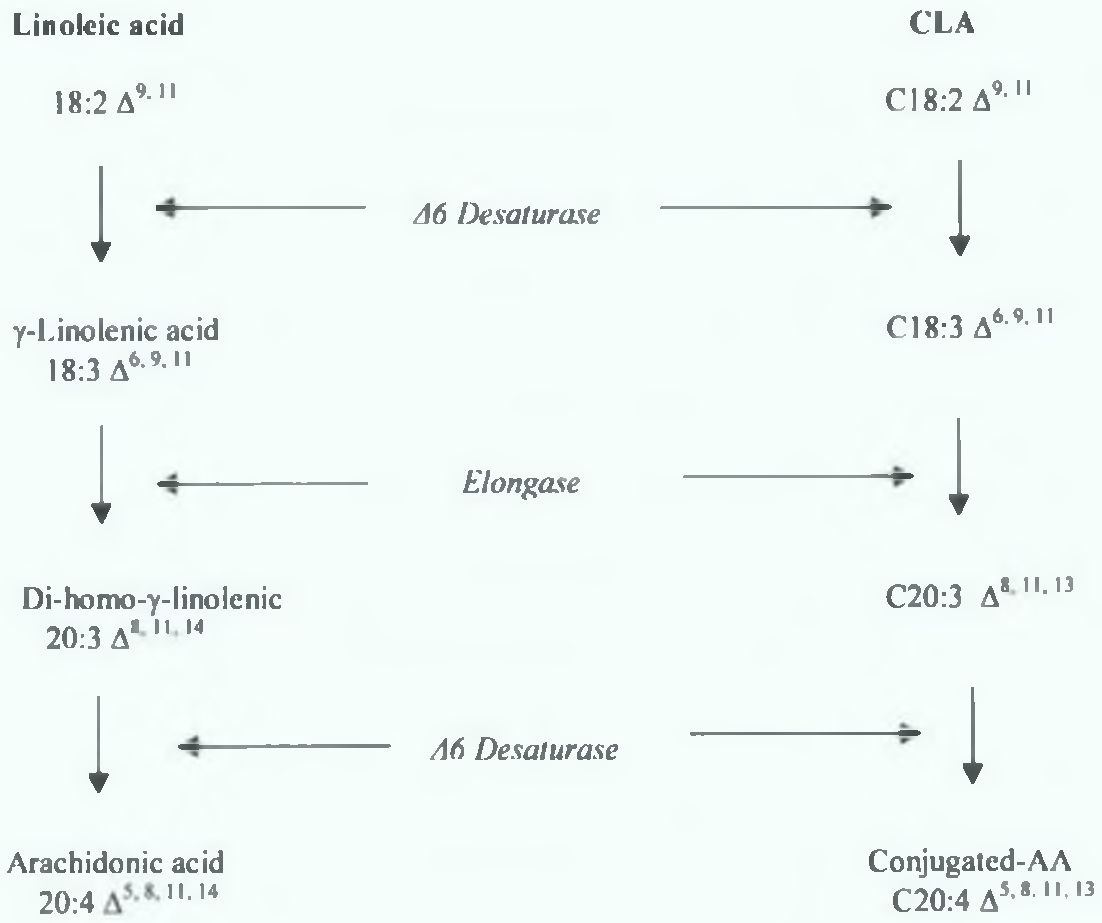


Figure 1.12 Pathway for desaturation and elongation of LA and CLA.

CLA metabolites display a distinct distribution pattern in rat tissues. Conjugated 18:3 and conjugated 20:3 is incorporated primarily in neutral lipids while conjugated 20:4 is preferentially enriched into specific phospholipids, mainly phosphatidylinositol and phosphatidylserine (Banni *et al.*, 2001a). The presence of this conjugated 20:4 metabolite in tissue phospholipids may influence linoleic acid metabolism and may compete with the parent compound in the biosynthesis of eicosanoids, and exert anti-inflammatory actions participating in the anticarcinogenic and possibility other physiological effects of CLA.

Whether or not CLA metabolites exert biological activity remains to be determined. It also needs to be elucidated whether the metabolites of CLA, rather than the fatty acid itself, may be responsible for the beneficial effects. Unfortunately, purified metabolites are currently unavailable in large enough quantities for use *in vitro* and *in vivo*.

1.13 Chemopreventive action of CLA

The most studied bioactivity of CLA is its anticancer effect. The surge of anticancer research involving CLA began when Ha *et al.*, (1987) found that CLA inhibited *in vivo* initiation of mouse epidermal tumors. Since then CLA has been shown to inhibit numerous cancer models in experimental animals and to inhibit the growth of a large variety of human cancer cells. The following sections describe the published literature that show an effect of CLA on cancer inhibition, primarily in animal models but also in studies utilising human cancer cell lines. Particular studies that have been conducted to elucidate mechanism(s) of chemoprotection by CLA will also be included.

1.13.1 Skin Cancer

The anticarcinogenic property of CLA was first identified in a mouse skin carcinogenesis model (Ha *et al.*, 1987) over fifteen years ago. Pariza and colleagues identified a fraction from grilled beef that could inhibit mutagenesis in bacteria and the initiation of epidermal carcinogenesis in mice by 7, 12-dimethylbenz[a]anthracene (DMBA) (Pariza and Hargraves, 1985). The fraction was purified and was shown to contain four isomeric derivatives of linoleic acid containing a conjugated double-bond system and so was named CLA (Ha *et al.*, 1987). They synthetically prepared a mixture of CLA isomers and tested it for anti-initiation activity in a two-stage mouse epidermal anticarcinogenesis system. The CLA mixture of isomers was topically applied to the dorsal area of mouse skin prior to initiation with DMBA and promotion with 12-O-tetradecanoylphorbal-13-acetate (TPA). Sixteen weeks after promotion, CLA treated mice had 50 % fewer papillomas and a ~15 % lower tumor incidence than control or LA-treated mice.

Belury *et al.* (1996) expanded the findings of Pariza's group by examining the effect of increasing levels of dietary CLA on skin tumor promotion in the same model. Female SENCAR mice were fed control diets during initiation and switched to diets supplemented with 0, 0.5, 1.0 or 1.5 % CLA during skin tumor promotion. Twenty-four weeks after tumor promotion began, diets containing 1.0 and 1.5 % CLA reduced tumor yield by 28 and 29 %, respectively. There was a modest reduction in skin tumor incidence of approximately 15 % for mice fed the 1.5 % diet. The data did not correlate with the reduction in tumor incidence observed in a mammary cancer model in which CLA at a dietary level of only 0.25 % reduced tumor incidence by ~37 % (Ip *et al.*, 1991). These results suggest that CLA may have less biopotency against mouse skin carcinogenesis and perhaps a less steep dose-response curve than for mammary cancer inhibition.

1.13.2 Mammary Cancer.

1.13.2.1 In vivo tumor inhibition

The effects of CLA on the rat mammary gland are the most extensively studied of all its effects on cells and tissues to date. In over eleven separate studies Ip and coworkers (2001, 1999a, 1999b, 1997a, 1997b, 1997c, 1996, 1995, 1994, 1991, 1985) have shown that dietary administration of CLA was an effective way of inhibiting chemically induced rat mammary tumors under a wide range of experimental conditions. CLA was effective at various doses, at various stages of carcinogenesis and for varying durations corresponding to particular stages of gland maturation, regardless of the level or type of fat or linoleic acid in the diet. In these studies, CLA inhibited cancer development at

levels of 1% CLA in the diet and below with no further beneficial effect at levels above 1 %. The timing of CLA administration has been found to be critical to mammary cancer prevention (Ip *et al.*, 1995). When CLA was administered to animals during active morphogenesis of the mammary gland (from weaning at day 21 until ~50 d of age), lasting protection against subsequent tumor development occurred. This group showed that exposure to CLA during maturation diminished epithelial branching and so reduced the formation of terminal end buds (TEB's) which are the primary sites for the chemical induction of mammary carcinogenesis. In contrast, when CLA was administered at a later age (55 d of age) and following the carcinogen, a continuous intake of CLA was necessary to inhibit tumors (Thompson *et al.*, 1997).

Ip and co-workers (1999a) demonstrated that milk-fat CLA feeding during the time of pubescent mammary gland development down-regulated morphological maturation of the mammary epithelium and reduced the risk of mammary cancer. This was the first study to show that CLA delivered in a food matrix had biological activities similar to those of the mixture of CLA isomers delivered as free fatty acids. Feeding butterfat (4 % CLA) to rats during the time of pubescent mammary gland development reduced mammary epithelial mass by 22 %, decreased the size of the TEB population by 30 %, suppressed the proliferation of terminal end bud cells by 30 % and inhibited mammary tumour yield by 53 % (Ip *et al.*, 1999a). Similar results were observed in rats fed the *c*9, *t*11-CLA isomer from Matreya or the CLA mixture of isomers from Nu-Chek, suggesting that the *c*9, *t*11-CLA isomer is biologically active as an anticarcinogen in the mammary gland. Furthermore, rats consuming CLA enriched butter fat consistently accumulated more *c*9, *t*11-CLA in the mammary gland and other tissues (liver, peritoneal fat and plasma)

compared with those consuming free fatty acid CLA. Despite the difference in CLA tissue levels achieved, both preparations were equally effective in modulating mammary gland morphogenesis and reduced cancer risk. The authors suggested that either other isomers of CLA had anticarcinogenic activity or that the *c*9, *t*11-CLA isomer had already achieved maximal effect at the tissue level of *c*9, *t*11-CLA achieved by feeding the Nu-Chek preparation.

In their most recent paper, these investigators examined the effect of CLA intake on the proliferation activity of the epithelium during mammary gland development. Rats were fed either a CLA mixture of isomers from Nu-Chek, the CLA enriched butter fat used in the previous study (Ip *et al.*, 2001) or *c*9, *t*11-CLA from Matreya as the mice matured from weaning to adult. Both the CLA mixture of isomers from Nu-Chek and the supply of *c*9, *t*11-CLA in food were equally effective at suppressing cell proliferation in the developing mammary gland while it underwent extensive morphogenesis during pubescence. They also showed that the mammary epithelium appeared to lose its sensitivity to CLA control of proliferation as it completely filled the fat pad and became quiescent. This suggests that the responsiveness of mammary gland epithelial cells to CLA intervention may be dependent on their proliferative status.

Ip's rodent model is a good model for human breast cancer as mammary cancer in rodents shows similarities to that in humans. The most striking similarities are in the overall pathogenesis and the site of origin of the majority of the breast cancers (Medina, 1996). Breast cancer in humans and in chemical carcinogen-treated mice and rats both

occur primarily in the undifferentiated, rapidly proliferating epithelium at the distal end of TEB and terminal ducts (Medina, 1996).

Administration of safflower oil (CFA-S), rich in a mixture of conjugated linoleic acid isomers (*c*9, *t*11/*t*9, *c*11 - 32.7 %, *t*10, *c*12 - 33.5 %), significantly decreased the incidence and multiplicity of mammary carcinomas in female Sprague-Dawley rats (Kimoto *et al.*, 2001). Rats were sequentially treated with 1,2-dimethylhydrazine and N-butyl-N-(4-hydroxybutyl)nitrosamine during the first three weeks for initiation and then fed diets containing 1 or 0.1 % CFA-S for 33 weeks. Both the 1 and 0.1 % CFA-S treatments significantly decreased the incidence and multiplicity of mammary carcinomas but a clear dose response was not observed.

Three studies have investigated the effect of CLA on mammary carcinogenesis without employing the chemically induced model. Human breast adenocarcinonoma cells (MDA-MB468 cells) were injected subcutaneously into severe combined immunodeficient mice (SCID) that were fed a diet containing CLA at a level of 1% of the diet for two weeks (Visonneau *et al.*, 1997). They continued to consume the CLA-containing diet until the end of the study at either week 8 or 14. Control animals were fed similar diets minus the CLA supplementation. CLA supplementation at 1 % of the diet significantly reduced tumor weight and area. CLA intake also inhibited the spread of breast cancer cells to the lung, peripheral blood and bone marrow indicating that CLA inhibited tumor metastasis via mechanisms independent of the host immune system.

In a second study, female Balb/c mice were fed a diet containing 0.1, 0.3 or 0.9 % CLA and injected with WAZ-2T metastatic mammary tumor cells (Wong *et al.*, 1997). Mice were fed experimental diets for 2 weeks prior to tumor cell injection. CLA failed to reduce mammary tumor growth, tumor latency or tumor incidence at any dose. However, the lack of an effect of CLA in this study may be explained by the use of an extremely metastatic cell line which was not hormone responsive. CLA had been previously shown to have no effect on the growth of estrogen negative MDA-MB-231 human breast cancer cell line suggesting some estrogen related interaction (Durgan and Fernandes, 1995).

In the final study female BALB/cAnN mice were fed 0, 0.1, 0.5 or 1.0 % CLA for three weeks and then injected subcutaneously with a mouse mammary tumor cell line 4526 and continued on the experimental diets for an additional 4 weeks (Hubbard *et al.*, 2000). Another group of mice were treated with indomethacin, a positive control and known suppresser of tumor growth and metastasis in this malignant model. CLA feeding increased latency and decreased the number of spontaneously metastatic pulmonary nodules as well as their total volume in the lung. As the level of CLA in the diet increased, the metastatic pulmonary tumor burden decreased proportionally, reaching levels lower than were achieved with indomethacin.

1.13.2.2 In vitro studies

CLA (4.5 - 36 µg/ml) has also been shown to inhibit cell growth of normal rat mammary epithelial cells organoids (MEO) in a time- and dose-dependent manner. CLA treatment also inhibited the survival of normal rat (MEO) colonies and the functional differentiation

of this cell line (Ip *et al.*, 1999b). CLA was shown to inhibit the growth of a rat mammary tumor cell line (NMU) after 3 and 4 days when added at a concentration of 9 μg/ml (Ip *et al.*, 2000)

Most of the other studies examining the *in vitro* effect of CLA on mammary epithelial cell growth have used the MCF-7 human breast cancer cell line. Shultz and co-workers were the first group to demonstrate the inhibitory response of MCF-7 cells to CLA in culture (Shultz *et al.*, 1992a). They exposed the MCF-7 cells to increasing levels of CLA (5, 10, 20 μg/ml) for 12 days of incubation. The CLA treatments exhibited an inhibitory effect as early as 2-6 days of incubation. CLA was shown to be inhibitory to cancer cell growth in a dose- and time-dependent manner. By day 12, CLA had reduced cancer cell growth by 54 % at 5 μg/ml and 100 % at the two higher concentrations. In this group's second study they compared the effect of LA and CLA on the same cell line (Shultz *et al.*, 1992b). Cells were incubated with either CLA or LA (5, 10, 20 μg/ml) and viability was assessed after days 4, 8 and 12. CLA inhibited cell growth at all concentrations and time tested while LA initially stimulated cell growth at 10 and 20 μg/ml but went on to exert an inhibitory effect at these two concentrations after 8 and 12 days of incubation. In this group's third study, they compared the antiproliferative effect of CLA (0.5 – 10 μg/ml) on MCF-7 cells with a normal human mammary cell line (HMEC). CLA inhibited (11-43 % of control) MCF-7 cell growth after 3 days of incubation as expected. However, CLA also inhibited the growth of normal HMEC (18-37 % of control) in a non-dose-dependent manner.

DesBordes and Lea (1995) examined the effect of CLA (at 28 and 140 µg/ml) on cell proliferation of the MCF-7 and the T47D mammary cancer cell lines after 24 hours incubation. At 28 µg/ml CLA failed to exert an inhibitory effect while a reduction (100 %) was observed at 140 µg/ml. The absence of an effect of CLA at 28 µg/ml may be due in part to the short 24 h incubation time. Durgam and Fernandes (1997) compared the effects of CLA on the MCF-7 estrogen-responsive and the MDA-BA-213 non-estrogen responsive cell line on cell viability as measured by viable cell count and thymidine incorporation over a 6 day incubation period. CLA (5-20 µg/ml) inhibited MCF-7 cell growth but did not inhibit the growth of the estrogen negative cell line suggesting that CLA may influence cell growth by interfering with molecules involved with the hormone regulated mitogenic pathway. They also examined whether the growth inhibitory effect observed in the MCF-7 cell line could be reversed upon replacement of CLA supplemented media with normal media. Results showed that after 4 days incubation with CLA cells began to proliferate upon return to normal media indicating that growth inhibition is only temporary and can be reversed. Park *et al.*, (2000) showed that CLA at lower concentrations (1-5 µg/ml) inhibited MCF-7 cell growth (11-20% of control).

Our own group has also extensively studied the effect of CLA on the MCF-7 cell line. O' Shea *et al.*, (1999) confirmed the dose- and time-dependent growth inhibitory response of MCF-7 cell line to CLA. Similar effects were observed in MCF-7 cells treated with the individual *c*9, *t*11-CLA isomer and with bovine milk fat enriched in CLA from animals fed pasture, rapeseed or soya (O'Shea *et al.*, 2000). The growth suppressive effects were

independent of the variable composition of the milk fat samples suggesting that CLA was the active cytotoxic agent in the milk fat.

1.13.3 Intestinal Cancer.

1.13.3.1 In vivo tumor inhibition

Evidence that CLA may have anti cancer activity against intestinal cancer first arose when CLA was shown to inhibit the formation of 2-amino-3-methyl-imidazo[4,5-f]-quinoline (IQ)-DNA adducts in a number of organs including the large intestine of CFD_1 mice (Zu and Schut, 1992). The heterocyclic amine IQ reacts with DNA to form carcinogen-DNA adducts, leading to mutation and subsequently, to the initiation of the carcinogenic process. Liew *et al.*, (1995) showed that CLA treatment reduced a number of early preneoplastic markers of carcinogenesis in rat colon. F344 rats were administered CLA (at 0.5 % of the diet) by gavage for a 4 week period. During week 3 and 4 rats were exposed to IQ in order to induce colon carcinogenesis. Rats were killed 6 hours after the final carcinogen dose in order to quantify IQ-DNA adducts or after 16 weeks to score aberrant crypt foci (ACF). ACF are preneoplastic lesions of colorectal carcinomas and are the earliest recognisable changes produced in the colon by carcinogens. CLA treatment caused a 74 % decrease in the number of colonic ACF compared with control animals given IQ without any dietary treatment. In addition, CLA treatment was associated with a significant reduction in the number of IQ-DNA adducts formed in the colon as determined by ^{32}P-postlabeling analysis.

In a study by Ealey and co-worker (2001) CLA did not inhibit the development of ACF in male Sprague-Dawley rats. In this study rats were given a single dose of azoxymethane (AOM), a carcinogen that induces colon tumors in rodents. A week later animals were randomised into two groups and fed a control diet or the control diet supplemented with CLA (1 % w/w). After 12 weeks the animals were sacrificed and ACF in their colons were scored. The total number of ACF per animal did not differ between the control and CLA group. Rats fed the 1 % CLA diet had significantly higher serum insulin levels at the time of sacrifice than those fed the control diet. The authors suggested that the promoting effects of elevated serum insulin on colon carcinogenesis might have counteracted an inhibitory effect of CLA.

Park *et al.*, (2001) demonstrated that dietary CLA at 1% of diet inhibited 1,2-dimethylhydrazine (DMH)-induced colon carcinogenesis in rats. Colon cancer was induced by injecting 6-week old, male, Sprague-Dawley rats with DMH twice for 6 weeks. During this time and for the following 24 weeks they were fed either 1 % CLA or a control diet *ad libitum*. After 30 weeks the animals were sacrificed and autopsies were performed on removed colons. The incidence of tumors was significantly lower in the rats fed the 1 % CLA diet compared to control rats.

CLA at 0.5% and 1% of the diet has been shown to significantly reduce the induction of mutations in distal colon of the Big Blue^{R} rat (a transgenic animal model developed for evaluation of mutagenicity of chemical compounds) (Yang *et al.*, 2002). In a study mimicking human dietary supplementation, the effect of timing of CLA feeding on mutagenesis was studied. CLA was added to the diet 1 week prior to exposure to 2-

amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) for 47 days or from weaning to age of 50 days, at which time they were then exposed to PhIP for 47 days. (Fig 1.13) Simultaneous administration of CLA with PhIP suppressed PhIP-induced mutations in the distal colon by 23%. Unlike the rat mammary gland model however, feeding CLA before carcinogen treatment did not protect the colon against subsequent mutagenesis. Consistent with inhibition of PhIP-induced mutation frequency, dietary CLA also inhibited aberrant crypt foci formation in male F344 rats given PhIP in basal diet (Yang *et al.*, 2002). In view of the presence of both PhIP and CLA in the typical western human diet, understanding the effects of CLA on mutagenesis and DNA repair will be necessary for development of strategies which can optimally impact on cancer control.

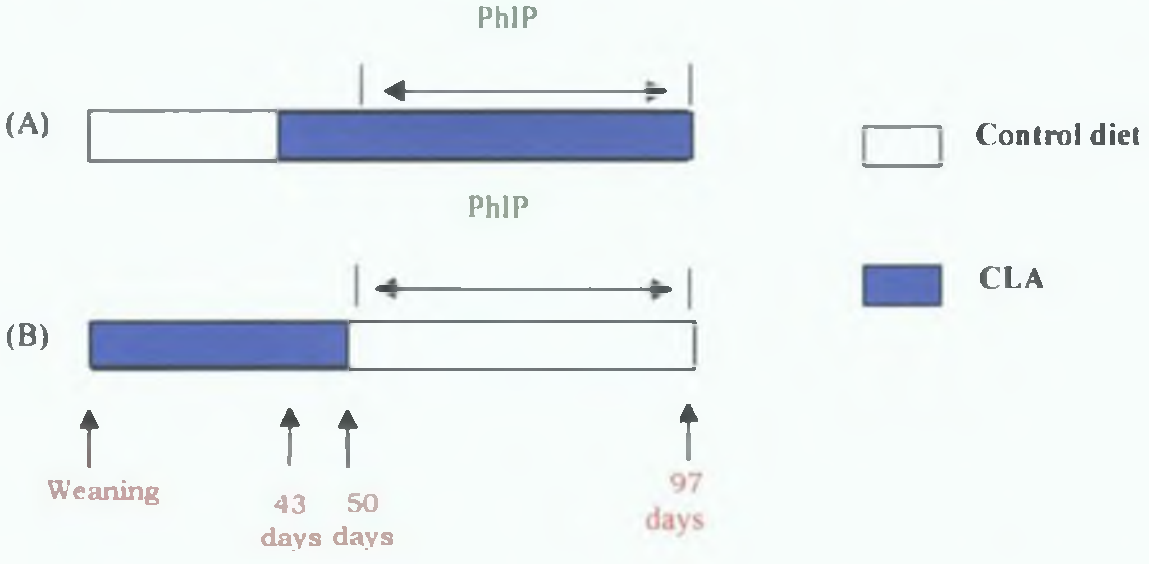


Figure 1.13 Experimental Design. (A) Male and female rats were given CLA starting at the age of 43 days. PhIP was incorporated into the diet and continued for 47 days. (B) Female rats were given CLA from weaning to the age of 50 days and then subjected to PhIP treatment for 47 days (From Yang *et al.*, 2002).

1.13.3.2 In vitro studies

CLA has proved to be effective in inhibiting the growth of a variety of colon tumor cell lines. Shultz *et al.* (1992b) showed a 47 % reduction by 5 μg/ml CLA on the growth of HT-29 human colorectal tumor cells. Increasing the concentration of CLA did not further enhance the growth inhibitory effect of CLA in this cell line. CLA also decreased cell viability of the SW480 colon cell in a dose and time dependent manner over a CLA concentration range of 5 –30 μg/ml (O'Shea *et al.*, 1999).

Unlike the *in vivo* experiments, all of which used a mixture of CLA isomers, some of the *in vitro* studies have provided some insight into the activities of specific CLA isomers on colon cancer cell growth. Kim *et al.* (2002) compared the individual potencies of the *c*9, *t*11- and the *t*10, *c*12-CLA isomers on the growth of the Caco-2 colon cell line. The *t*10, *c*12-CLA isomer decreased viable cell numbers in a dose dependent manner after 96 h while the *c*9, *t*11-CLA isomer had no effect. In a recent study the *t*10, *c*12-CLA isomer (at 14 and 28 μg/ml) exhibited the greatest potency against colorectal cancer proliferation of the HT-29 and MIP-101 cell lines (Palombo *et al.*, 2002). These studies all suggest that the *t*10, *c*12-CLA isomer may be the more biologically active isomer for inhibition of colon tumor cell proliferation *in vitro*.

1.13.4 Forestomach and prostate Cancer

Only one study has demonstrated the protective effect of CLA against forestomach tumors (Ha *et al.*, 1990). CLA or LA plus olive oil or olive oil alone was administered to mice by gavage twice weekly on Mondays and Wednesdays for 4 weeks. On the Friday of each week animals were given benzo(a)pyrene (BP) in olive oil. Animals were sacrificed 22 weeks after the first dose of BP. Mice treated with CLA developed only about half as many forestomach tumors/mice as compared with the number developed by mice in the control groups in three independent experiments. CLA also reduced tumor incidence in two out of three experiments compared with linoleic acid and olive oil controls.

Cesano et al. (1998) investigated the effects of dietary CLA and LA on the growth and progression of human prostatic carcinoma. Severe combined immunodeficient (SCID) mice were subcutaneous implantation with DU-145 human prostate cells. The rats were fed a standard diet or diets supplemented with LA or CLA (both at 1 % of the diet) for 2 weeks prior to subcutaneous implantation and for 12 weeks after. CLA fed mice showed significantly smaller local tumors and also an acute reduction in lung metastases as compared to the control and LA-fed groups. Visonneau *et al.* (1996) reported an inhibitory effect of CLA (at concentrations from (0.28 - 28 μg/ml) on a variety of cell lines including a prostate cell line. More recently, Palombo *et al.* (2002) showed that *c*9, *t*11-, *c*9, *c*11- and *t*10, *c*12-CLA isomers significantly decreased the proliferation of human prostate (PC-3) carcinoma cells at 28 μg/ml but not at 15 μg/ml.

1.14 Proposed mechanisms underlying the anticarcinogenic effect of CLA.

A number of studies have investigated the mechanistic role of CLA in modulating the three broad stages of carcinogenesis; initiation, promotion and progression. Given the modulating properties of CLA on this multi-stage process along with the fact that CLA is not a single molecule it is thought that multiple parallel mechanistic pathways are involved (Scimeca, 1999). Early studies focused on events associated with initiation and investigated the role of CLA as a possible antioxidant and its modulating effect on carcinogen activation and detoxification. More recent studies have focused on elucidating the mechanisms involved in the inhibitory effect of CLA on carcinogenesis during promotion and have investigated the effect of CLA isomers on cell proliferation, eicosanoid biosynthesis, apoptosis and gene expression. It is imperative to elucidate the molecular mechanisms responsible for the cancer preventive effect of CLA isomers at the level of nutrient-gene interactions and to identify specific CLA-responsive biomarkers which can be applied to biopsied human tissue samples in CLA intervention trials. In the following sections the various biological events will be treated separately as it is as yet impossible to present a unified mechanistic theory.

1.14.1 Modulation of free-radical induced oxidation.

Early studies postulated that CLA might prevent carcinogenesis by its ability to act as an antioxidant and scavenge free radicals generated from carcinogen exposure. CLA was shown to be more effective than α-tocopherol and as effective as butylated hydroxytoluene in reducing iron thiocyanate-induced peroxide and thiobarbituric acid

reactive substance (TBARS) formation *in vitro* (Ha *et al.*, 1990). TBARS is a biomarker used to assess oxidation in biological systems. Ip *et al.* (1991) reported that feeding CLA resulted in lower levels of malondialdehyde, an end product of lipid peroxidation, in the mammary gland but not in the liver of rats. However, CLA-feeding failed to change the levels of 8-hydroxyguanosine, a marker of oxidatively damaged DNA in mammary tissue. No differences in plasma TBARS levels were observed in rabbits fed a CLA-supplemented diet or control diet (Lee *et al.*, 1994).

Two test tube studies provided convincing evidence that CLA did not possess antioxidant activity. With the use of synthetic 1-palmitoyl-2-linoleoyl phosphatidylcholine (PLPC) membrane vesicles and precise analytical methods, CLA (1-50 μM / 0.28-14.08 μg/ml) did not act as a free radical scavenger and was not converted to a metal chelator under a variety of experimental oxidative stress conditions (Van den Berg *et al.*, 1995). Chen *et al.* (1997) showed that two forms of CLA, the free fatty acid form and its methyl ester form induced oxidation of heated canola oil in a dose dependent manner. Triglyceride bound-CLA had no protective effect against oxidation in the same model. This finding is important, as this is the form that CLA would naturally be found in dairy products (Chen *et al.*,1997). Analysis of conjugated diene hydroperoxy fatty acids using HPLC with a diode-array detector showed that conjugated diene fatty acids were more susceptible to oxidation than their parent non-conjugated fatty acids (Banni *et al.*, 1998). Another study has demonstrated that CLA may be oxidised by singlet oxygen yielding furan fatty acids upon decomposition (Yurawecz *et al.*, 1995).

It has been suggested that CLA induces cytotoxicity in cancer cell lines via pro-oxidant activity (Belury *et al.* 1995). Lipid peroxidation and its products regulate growth by inducing cytotoxicity and apoptosis in tumor cells (Grune *et al.*, 1994, Ben-Yoseph and Ross, 1994). A significant increase in lipid peroxidation as measured by MDA was observed after treatment of three lung adenocarcinoma cell lines (A-427, SK-LU-1, A-549) with CLA (Schonberg and Krokan, 1995). LA did not exert the prooxidant effect shown by CLA (Schonberg and Krokan, 1995). Hence, oxidation of CLA may play an important role in influencing cancer cell death. O'Shea *et al.*, (1999) indicated that reduction of cell growth by CLA against MCF-7 and SW480 cell lines was related to an increase in lipid peroxidation and activation of antioxidant defense enzymes such as superoxidase dismutase and glutathione peroxidase. Igarashi and Miyazawa (2001) investigated the growth inhibitory effect of CLA on human hepatoma HepG2 cells. CLA (1-11 μg/ml) inhibited the growth of these cells in a dose- and time-dependent manner. In order to evaluate the possible contribution of lipid perpoxidation exerted by CLA, α-tocopherol and butylated hydroxytoluene (BHT) were added to the medium with CLA. The addition of α-tocopherol and BHT to media with CLA did not restore cell growth. Furthermore, the CLA-supplementation did not alter TBARS and membrane phospholipid hydroperoxides, the parameters measured to assess lipid peroxidation.

Leung and Liu (2000) have shown that different isomers of CLA may display antioxidant or prooxidant activities, depending on concentration. The *t*10, *c*12-CLA isomer acted as an antioxidant at all concentrations tested (2-200 μM / 0.56-56 μg/ml) and was more effective than *c*9, *t*11-CLA and α-tocopherol at low concentrations (2-20 μM) as

measured by a total oxyradical scavenging capacity assay. In contrast, the *c*9, *t*11-CLA isomer possessed weak antioxidant activity at 2 and 20 μM and acted as a strong pro-oxidant at 200 μM. These data suggest that discrepancies in the results of previous studies on the antioxidant properties of CLA may be due to the balance of the antioxidant properties of *t*10, *c*12-CLA and the pro-oxidant properties of *c*9, *t*11-CLA under different oxidation conditions.

Basu and co-workers (2000a) have shown that 3-month CLA-supplementation in healthy human subjects caused an increase in urinary 8-iso-$PGF_{2\alpha}$, a marker of lipid peroxidation. They have also shown that CLA induced lipid peroxidation in men with abdominal obesity (Basu *et al.*, 2000b). The consequences of and the mechanisms involved in the increase in lipid peroxidation after CLA-supplementation in humans have yet to be determined.

1.14.2 Modulation of carcinogen-DNA adduct formation and carcinogen metabolism

Several studies have shown that CLA modulated DNA adduct formation at several organ sites in both mice and rats (Zu and Schut 1992, Liew *et al.*, 1995, Schut *et al.*, 1997, Josyula *et al.*, 1998, Yang *et al.*, 2002). DNA adducts are covalent DNA addition products formed when chemical carcinogens or their reactive intermediates bind to and react with DNA molecules resulting in mutations and tumor development. CLA treatment inhibited IQ-DNA formation in liver, lung, large intestine and kidney in mice (Zu and Schut 1992). Differential effects were noted for female and male mice. Using ^{32}P-post-

labeling CLA was shown to reduce IQ-DNA adduct labelling in the colon but not in the liver of male rats (Liew *et al.*, 1995). CLA was effective at inhibiting PhIP-DNA adduct formation in the liver and mammary gland of female mice (Schut *et al.*, 1997). Recently, Yang *et al.*, (2002) have shown that CLA-supplementation lowered PhIP induced mutation frequency in the distal colon of male rats. PhIP, is one of the most prevalent mutagenic heterocyclic amines in the Western diet, produced during the cooking process by the reaction of an amino acid with creatine (El-Bayoumy, 1992). To bind to DNA, PhIP must be metabolically N-hydroxylated by cytochrome P-450 1A1,1A2, and 1B activities in hepatic microsomes (Turteltaub *et al.*, 1990). N-Hydroxy-PhIP is further converted to its ultimate carcinogen via O-sulfation or O-acetylation which can bind covalently to DNA, reacting almost exclusively with guanines at the C8 position (Turesky *et al.*, 1991). One may surmise that CLA may act as a blocking agent upon several detoxifying enzymes, thereby inhibiting the metabolic activation of carcinogens in target organs.

Detoxifying enzymes catalyze metabolic detoxification of xenobiotics, drugs and carcinogens and thus, protect the cells against redox cycling and oxidative stress. Ip *et al.*, (1991) examined the effect of CLA on phase II detoxifying enzymes in female rats. Data indicated that CLA had no effect on glutathione-S-transferase and UDP-glucuronyl transferase activity in liver or mammary gland. Authors suggested that CLA may modulate carcinogen metabolism via phase I detoxifying enzymes i.e. cytochrome P450 isoenzymes. Inhibition of IQ activation was proposed as the mechanism of chemoprevention by CLA in colon tissue (Liew *et al.*, 1995). IQ must be activated to an aryl nitrenium ion by various enzymes which include cytochrome P4501A2,

prostaglandin H synthase, acetyltransferase and sulfotransferase before it reacts with guanine residues of DNA forming an adduct at the C-8 position leading to tumour production. Liew *et al.* (1995) showed that hepatic microsomes from CLA-treated rats exhibited lower methoxyresorufin O-deethylase (MROD) activity, an enzyme indicative of cytochrome P4501A2. Furthermore, their demonstration that CLA was antimutagenic in the presence of ram seminal vesicle microsomes, a rich source of prostaglandin H synthase (enzyme responsible for the production of prostaglandin H) is additional evidence that CLA might also inhibit prostaglandin-mediated activation of heterocyclic amines in extrahepatic tissues.

1.14.3 Modulation of cell proliferation by CLA

CLA has been shown to inhibit the proliferative activity of the mammary gland in the rat (see section 1.12.2.1) and the proliferation of a variety of tumor cells *in vivo* (see section 1.12.2.2). Cell cycle studies have revealed that a higher percentage of the CLA-treated MCF-7 cells remained in the G0/G1 phase (i.e. the resting and prereplication stage) compared to control cells or those treated with LA (Durgam and Fernandes, 1997). Therefore, it is possible that the decreased proliferation of MCF-7 cells in the presence of CLA may be due to delay of these cells in progressing through GO/G1 phases of the cells cycle. CLA also inhibited the expression of c-*myc* in MCF-7 cells (Durgam and Fernandes, 1997). *C-myc* is a transcriptional factor known to play a key role in biochemical pathways controlling cellular proliferation and is regulated by hormones. These authors concluded that CLA may inhibit MCF-7 cell growth by interfering with hormone regulated mitogenic pathway due to decreased expression of c-*myc*.

Dietary CLA (1 %) reduced proliferation of terminal end bud and lobuloalveolar bud structures of mammary epithelium as measured by histochemical analyses of bromodeoxyuridine staining in rats initiated with methylnitrosourea. This reduction in proliferation was accompanied by a decrease in the density of the TEB which are the primary target site for carcinogens that induce mammary tumors (*Ip et al.*, 1997). In a more recent study Ip and co-workers have shown that the reduction in proliferation of terminal bud structures by CLA was associated with a decrease in the levels of two cyclins known to regulate the cell cycle, cyclin A and cyclin D1 (Ip *et al.*, 2001).

Preincubation of human gastric adenocarcinoma SGC-7901 cells in media supplemented with different *c*9, *t*11-CLA concentrations (25-200 (μmol/L) at various times (24 and 48 h) significantly decreased the expression of proliferating cell nuclear antigen (PCNA) (Liu *et al.*, 2002). PCNA plays an essential role in both the replication and repair of DNA and is an essential component of the DNA replication machinery. This decrease in PCNA expression was accompanied by a decrease in the expression of Cyclins A, B_1 and D_1 whereas the expression of $p16^{ink4a}$ and $p21^{waf1}$, cyclin-dependent kinase inhibitors (CDKI), was increased (see tables 1.4 and 1.5). The authors concluded that the *c*9, *t*11-CLA isomer inhibited proliferation of SGC-7901 cells via blocking the cell cycle, with reduced expression of cyclin A, B_1 and D_1 and enhanced expression of CDKI's $p16^{ink4a}$ and $p21^{waf1}$.

Table 1. 4 Expression of cyclin A, B_1, and D_1 on SGC-7901 cells treated with c9, t11-CLA (%).

c9, t11-CLA(μmol/L)	24h			48h		
	Cyclin A	Cyclin B_1	Cyclin D_1	Cyclin A	Cyclin B_1	Cyclin D_1
0	10.7	4.2	9.5	5.9	5.1	6.0
25	11.0	4.8	3.6[b]	8.5	5.5	3.7[b]
50	7.9	2.5	3.5[b]	5.0	3.1[b]	3.7[b]
100	4.4[b]	2.6[b]	2.1[b]	1.3[b]	0.7[b]	0.6[b]
200	2.3[b]	1.8[b]	0.4[b]	0.5[b]	0.6[b]	0

[b] $P<0.01$ (From Liu *et al.*, 2002).

Table 1. 5 Expression of $p16^{ink4a}$ and $p21^{waf1}$ on SGC-7901 cells treated with c9, t11-CLA (%).

c9, t11-CLA (μmol/L)	24h		48h	
	$p16^{ink4a}$	$p21^{waf1}$	$p16^{ink4a}$	$p21^{waf1}$
0	1.0	0.2	0.8	0.6
25	0.7	1.4[b]	0.2	0.8
50	1.4	1.0[b]	3.0[b]	2.5[b]
100	2.8[b]	4.1[b]	4.6[b]	3.8[b]
200	3.6[b]	5.2[b]	5.0[b]	6.3[b]

[b] $P<0.01$ (From Liu *et al.*, 2002).

In contrast to the effects observed in mammary and gastric carcinogenesis, there was no relationship between dietary CLA and markers of cell proliferation in mouse epidermis (hyperplasia, ornitine decarboxylase activity or c-myc mRNA expression) (Kavanaugh *et al.*, 1999). These data suggest that inhibition of skin tumor promotion by CLA may not occur through inhibition of cell proliferation in mouse epidermis and that CLA's ability to reduce cell proliferation may be tissue specific.

1.14.4 CLA induces apoptosis.

Tumor growth is the net result of cell proliferation minus cell death. Therefore the induction of apoptosis or programmed cell death counterbalances the proliferating ability of cancer cells. Given the importance of apoptosis in cancer development, apoptosis-inducing agents could conceivably have an important role in adjunct anticancer therapy. Several genes that regulate the process of apoptosis have been found to be defective in tumour cells. The best characterised examples are the bcl-2 family genes which are overexpressed 30-50% in colon cancer (Zhang, 2002). Dietary CLA has been shown to induce apoptosis in a variety of tissues including mammary (Ip *et al.*, 2000), adipose (Tsuboyama-Kasaoka *et al.*, 2000), colon (Park *et al.*, 2001), liver (Lu *et al.*, 2002 in press) and also in cultured mammary epithelial cells (Ip *et al.*, 1999) and 3T3-L1 preadipocytes (Evans *et al.*, 2000).

CLA was first shown to induce apoptosis in normal differentiated rat mammary epithelial colonies as demonstrated by morphology criteria (the presence of apoptotic bodies and pyknotic nuclei) and the terminal deoxynucleotidyl transferase biotin-dUTP nick end

labeling (TUNEL) assay which detects double strand breaks in DNA (Ip *et al.*, 1999). In another study CLA induced apoptosis in cultured mammary tumor cells and in premaligant lesions known as intraductal proliferation (IDP) lesions in the rat mammary gland (see table 1.7) (Ip *et al.*, 2000). In this study, the induction of apoptosis by CLA was accompanied by a down regulation of anti-apoptotic bcl-2 protein. In contrast, CLA did not influence bak and bax, which suggested that these two inducers of apoptosis are not molecular targets in the action of CLA.

Table 1.6 Effect of CLA on bcl-2 expression in different mammary gland structures

Treatment (1 %)	Immunohistochemical score for bcl-2		
	Alveoli	TEB	IDP
Control	14.7 ± 1.1	15.1 ± 1.0	18.6 ± 1.1
CLA mixture	13.6 ± 0.7	14.4 ± 0.9	9.5 ± 0.7*
c9, t11-CLA	13.2 ± 0.6	13.7 ± 1.2	9.7 ± 0.8*

* Represents values significant different from control ($p<0.05$). From Ip *et al.*, 2000.

In a more recent study dietary CLA was shown to stimulate apoptosis in the colon mucosa of 1,2-dimethylhydrazine-treated rats as measured by the TUNEL technique (see table 1.7) (Park *et al.*, 2001).

Table 1.7 Effect of CLA on apoptotic index in the colonic mucosa of rats treated with 1,2-dimethylhydrazine.

Dietary Group (% CLA)	No. of rats	Apoptotic index	
		Mean	SD
0.0	5	0.1985	0.0664
0.5	6	0.4682*	0.1961
1.0	5	0.4577*	0.1486
1.5	5	0.5159*	0.0492

* Represents values significant different from control ($p<0.05$). From Park *et al.*, 2001.

1.14.5 CLA modulates eicosanoid formation.

Given the structural similarities between the CLA isomers and LA, an obvious avenue to investigate a possible mechanism for the anticarcinogenic effect of CLA is the determination of its effect on cicosanoid production. CLA had been shown to be incorporated into membrane phospholipids and neutral lipids in a number of tissues (Ha *et al.*, 1990, Ip *et al.*, 1996, Belury and Stempa-Steczko, 1997, Lui and Belury, 1998, Banni *et al.*, 1999, O'Shea *et al.*, 2000). The *c*9, *t*11-CLA isomer accumulated to a higher extent than the *t*10, *c*12-CLA isomer in tissue phospholipids of liver (Banni *et al.*, 2001, Belury *et al.*, 1997), skin (Kavanaugh *et al.*, 1999), and bone (Li and Watkins, 1998) of rodents. It is plausible that CLA through its incorporation into membrane phospholipids may modulate the fatty acid composition of membranes. More specifically, it is possible that CLA my exert its effect on carcinogenesis and on many of its other physiological functions (e.g., immunity, bone production and platelet aggregation) in part by

modulating the accumulation of arachidonic acid in phospholipids, resulting in a reduced arachidonic acid pool and reduced production of eicosanoids.

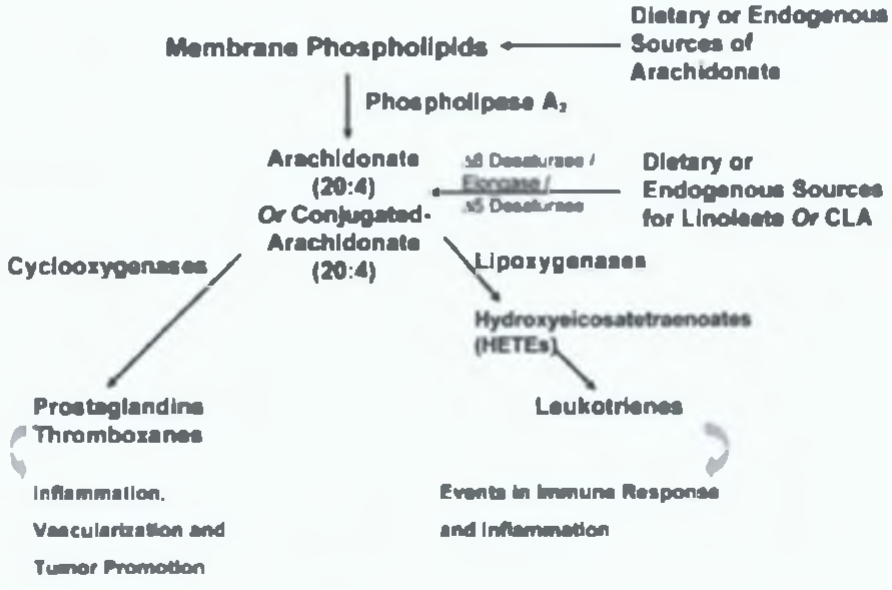


Figure 1.14 General schematic pathway for eicosanoid synthesis from arachidonic acid (Belury, 2002).

Belury and colleagues examined the hypothesis that CLA inhibited skin carcinogenesis via an eicosanoid-mediated mechanism. Using cultured murine keratinocytes this group showed that pre-treated of cells with CLA reduced the AA content by 50 % compared with cells pre-treatment with LA (Liu and Belury, 1997). Additionally, CLA decreased both uptake of ^{14}C-AA into cellular phosphatidycholine and the release of ^{14}C-AA compared with LA. 12-O-tetradecanoylphorbal-13-aceytate (TPA) -induced ^{14}C-PGE_2 production was also significantly reduced in cultures pre-treated with CLA compared with equimolar concentrations of LA (Liu and Belury, 1997). CLA was incorporated into phospholipids and neutral lipids in a dose dependent fashion in an *in vivo* study in which

mice were fed increasing amounts of CLA (0-1.5 %) in the diet (Kavanaugh *et al.*, 1999). PGE_2 synthesis was 50 % lower in mice fed 1.5 % CLA compared with mice fed a control diet (Kavanaugh *et al.*, 1999). These data suggest that the chemoprotective effect of CLA in skin carcinogenesis is mediated by a change in the composition of the lipid pool of the plasma membrane which alters the availability of AA for eicosanoid synthesis during tumor promotion.

Research from other laboratories also indicate that CLA effects AA levels and the synthesis of eicosanoids in a number of models. A number of studies have shown that dietary CLA displaced the arachidonic acid precursor, linoleic acid, in mouse liver (Belury and Kempo-Steczko, 1997a) and in mouse forestomach (Ha *et al.*, 1990) but not in mammary tissue (Banni *et al.*, 1999) or colon (Liew *et al.*, 1995). CLA has been reported to decrease PGE_2 in serum (Sugano *et al.*, 1997, Sugano *et al.*, 1998), bone (Li and Watkins, 1998) and in human saphenous vein endothelial cells (HSVEC) (Urquhart *et al.*, 2002) but not in small intestine tissue from Min mice (Petrik *et al.*, 2000) or spleen from rats (Sugano *et al.*, 1998). Furthermore, dietary CLA reduced accumulation of the lipoxygenase products LTB_4 and LTC_4 in spleen and lung (Sugano *et al.*, 1998) but not 12-hydroxyoctadecadienoic acid (Truitt *et al.*, 1999). These data suggest that the effects of CLA on eicosanoid production may be tissue specific.

Another mechanism for the reduction of AA-derived eicoanoids by CLA is through inhibition of the constitutive enzymes COX-1 and/or the inducible form COX-2. Bulgarella *et al.* (2001) demonstrated that specific CLA isomers decreased the rate of

oxygenation of AA by COX-1 in ram seminal vesicle microsomes with the *c*9, *t*11-CLA isomer having the most potent effect. The CLA mixture of isomers and individual isomers (*c*9, *t*11-CLA and *t*10, *c*12-CLA) were not found to alter the expression of COX-1 in HSVEC (Urquhart *et al.*, 2002). Whether CLA influences the expression of COX-2 has yet to be determined.

CLA was shown to be a substrate for liver microsomal Δ^6 desaturase, an enzyme that catalyses conversion of LA to AA in an *in vitro* study carried out by Belury and Kempa-Steczko (1997). Sebedio *et al.* (1997) have provided evidence indicating that both *c*9, *t*11-CLA and *t*10, *c*12-CLA are elongated and desaturated in a manner analogous to that of LA to form conjugated arachidonic acid and hence, may compete with LA for these enzymes and reduce the available AA for eicosanoid synthesis. Because COX requires a methylene group interrupted at carbon 13, it is unlikely that conjugated arachidonic acids are suitable substrates. However, it may be possible that these conjugated metabolites may interfere with COX activity.

1.14.6 CLA activates peroxisome proliferator-activated receptors (PPARs).

PPARs are ligand-activated transcription factors that increase transcription of target genes by binding to a specific nucleotide sequence in the gene's promoter and are members of the nuclear receptor superfamily (Issemann and Green, 1990). Three different PPAR isotopes can be distinguished: α, β and γ as a result of differential mRNA splicing. Each PPAR subtype has evolved to fulfil a different biological niche ranging

from lipid homeostasis to differentiation. As a result it has been proposed that PPARs may play a role in atherosclerosis, autoimmune diseases, diabetes, obesity and cancer and are key targets for therapeutic drugs.

PPAR ligands, known as peroxisome proliferators (PP), activate PPARs and the PPAR-ligand complex then recruits another hormone receptor, the retinoic acid-X receptor (RXR). The resulting complex binds to the PPAR-responsive elements (PPRE) on the target gene and drives transcription leading to alterations in gene expression that ultimately are responsible for changes in lipid metabolism and growth regulation (Vanden Heuvel, 1999). Depending on the cell type being examined, PPAR activation and regulation of growth regulatory and immediate early genes result in proliferation, apoptosis or differentiation (Vanden Heuvel, 1999) (Figure 1.15). Ligands for PPARs include the hypolipidemic drugs and insulin-sensitising triazolidinedione drugs (Lehmann *et al.*, 1995). Fatty acids and eicosanoids show structural and physiological characteristics similar to PPs and have been shown to be ligands for PPARs (Kliewer *et al.*, 1997). Therefore, it is plausible that PPARs may serve as sensors of dietary fatty acids, translating nutritional stimuli into changes in gene expression (Saez *et al.*, 1998). The potential role of CLA as an activator of PPARs may explain how CLA mediates its diverse biological effects.

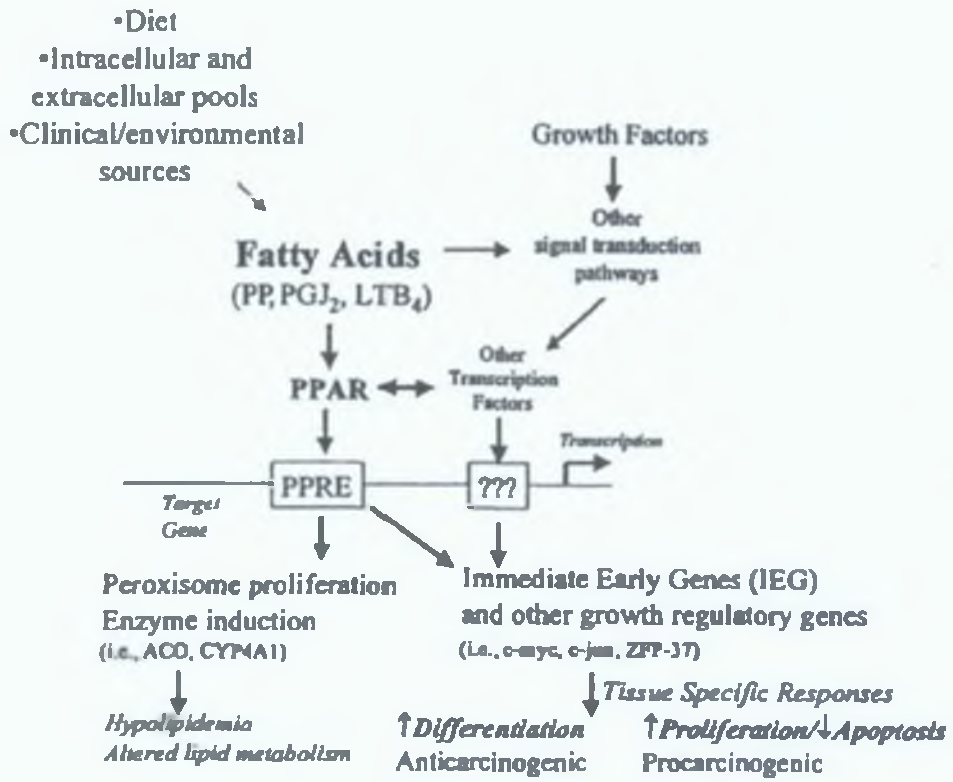


Figure 1.15 Basic mechanism of action of PPARs (Vanden Heuvel, 1999)

Several isomers of CLA are high affinity ligands and activators of PPARα (Moya-Camarena *et al.*, 1999). With the use of a scintillation proximity assay, CLA isomers were shown to be ligands for human PPARα (in the order of *c*9, *t*11 > *t*10, *c*12 > *t*9, *t*11). CLA was also shown to induce PPAR-responsive genes in the livers of SENCAR mice (Belury *et al.*, 1997) and in a cultured rat hepatoma cell line (Moya-Camarena *et al.*, 1999).

In a study to evaluate the possible involvement of PPARs in the skin tumorigenesis model, CLA and Wy-14643 (proven PPARα ligands) were applied topically to mice during an initiation-promotion tumor skin model (Thuillier *et al.*, 2000). Animals treated with these PPARα activators exhibited a 30% decrease in tumor yield compared to those treated with PPAR β and γ activators and control animals. The levels of all three PPAR subtypes were increased in tumors in contrast with normal epidermis. The PPARα protein was shown to be functional in the cultured keratinocyte cell line 308 and levels were found to be elevated during keratinocyte differentiation which was induced by high calcium levels. This suggests that induction of differentiation by PPARα may be a possible mechanism for inhibition of tumor growth in keratinocytes by CLA and other PPARα ligands.

Recent studies have focused on the interaction of CLA with PPARγ. PPARγ is expressed in diverse cell types including adipoctyes, hepatocytes, fibroblasts and epithelial cells. PPARγ activation appears to play a role in stimulation of adipocyte differentiation, stimulation of insulin, regulation of lipid metabolism, inhibition of tumor cell proliferation and diverse effects on inflammation (Houseknecht *et al.*, 2002) (figure 1.16). CLA has been shown to activate a dose-dependent transactivation of PPARγ in CV-1 cells co-transfected with PPARγ and PPRE X 3-luciferase reporter construct (Houseknecht *et al.*, 1998). Furthermore, dietary CLA (at 1.5 % of the diet) exerted an anti-diabetic action in Zucker diabetic fatty (fa/fa) rats in a similar manner to that of the PPARγ agonist troglitazone. Increased expression of aP2 mRNA in adipose tissue of

CLA-fed Zucker rats is consistent with the possibility that feasible dietary intakes of CLA can activate PPARγ *in vivo*.

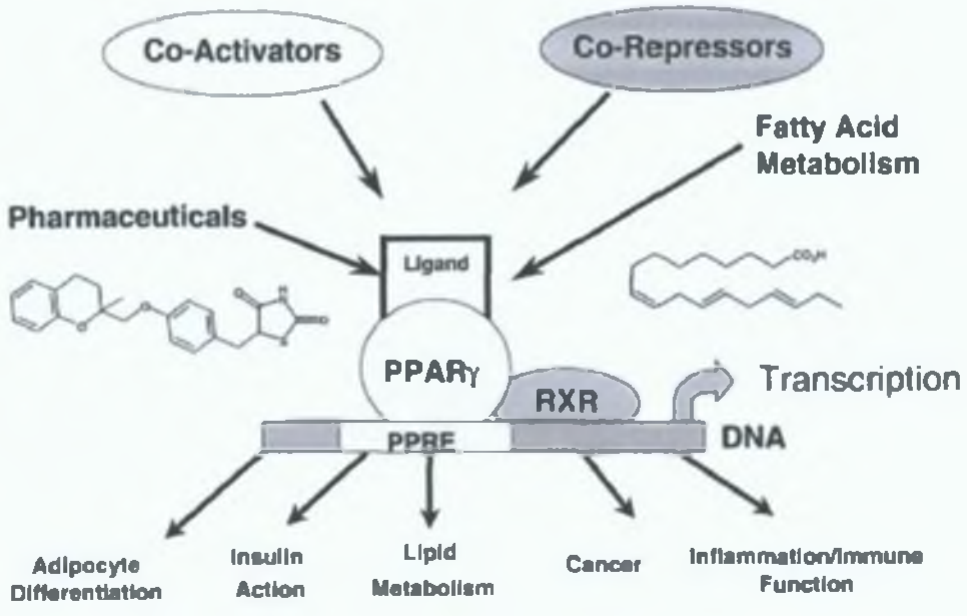


Figure 1.16 Activation of PPARγ regulates expression of genes involved in a myriad of physiological and pathophysiological states. (From Houseknecht *et al.*, 2002)

PPARγ regulates the expression of many genes relevant to carcinogenesis and now is an important target for development of new drugs for the prevention and treatment of cancer (Sporn and Mangelsdorf, 2001). Studies indicate that cell lines derived from human breast, prostate and colon cancers express PPARγ. Furthermore, the treatment of these cell lines *in vitro* with PPARγ agonists such as troglitazone slow clonal expansion and induces differentiation and/or apoptosis and slows the growth of these cell lines implanted in immmunodeficient mice (Mueller *et al.*, 1998, Elstner *et al.*, 1998. Kubota *et al.*, 1998, Sarraf *et al.*, 1998). Therefore it may be possible the anti-promotional and

cancer-retarding activity of CLA may be in part due to the activation of PPARγ. It has also been proposed that downstream metabolites of Δ^6 desaturase metabolism of *c*9, *t*11- or *t*10, *c*12-CLA may activate PPARγ but activation by these products has yet to be measured. A direct connection between the anticarcinogenicity of CLA and PPAR activation warrants further study. This possible connection is intriguing and may help explain the isomer-, tissue-, and sex-specific inhibition of tumors that has been observed.

1.14.7 Inhibition of angiogenesis by CLA

Angiogenesis has been recognised as an indispensable feature of neoplastic growth and the inhibition of this phenomenon has a profound effect on cancer growth (Folkman, 1997). Angiogenesis provides the tumor cell with access to the vascular circulatory system, thus establishing the potential for metastatic disease progression. Vascular endothelial cell proliferation, migration and capillary formation are stimulated by angiogenic growth factors, which include the proteins vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), transforming growth factor-beta (TGFβ) and eicosanoids (Rose and Connolly, 2000). Dietary CLA, because of its suppressive effects on eicosanoid biosynthesis, may to be antiangiogenic.

Masso-Welch *et al.*, (2002) have shown that CLA can inhibit angiogenesis. This group has shown that CLA modify mammary stroma by inducing differentiation of mammary stromal cells (MSC) to an adipocyte cell type. Significantly, concurrent with MSC differentiation, they found that CLA decreased the ability of MSC to form a microcapillary network *in vitro* on an EHS (Engelbreth-Holm-Swarm) tumor-derived

reconstituted basement membrane (RBM), and *t*10, *c*12-CLA was more effective than *c*9, *t*11-CLA (Masso-Welch *et al.*, 2002). The effect of CLA on angiogenesis *in vivo* was examined. CD2F1 mice were fed a diet with or without a mixture of CLA isomers (1% or 2 %) for 6 weeks and injected subcutaneously with an angiogenic gel substrate composed of RBM supplemented with βFGF and heparan sulfate. At one-week post injection, mice were sacrificed, serum collected, RBM pellets were harvested and histologically examined. Results indicated that functional angiogenesis i.e. formation of red blood cell containing vessels was decreased by ~80 %. Both serum and mammary gland concentrations of VEGF and its receptor flk-1 were also significantly decreased in CLA fed animals. The effect of individual isomers on angiogenesis and the effect of CLA on angiogensis during tumor development in the mouse or rat models warrant further research.

1.15 Other health benefits

Obesity is a major health problem and so CLA's ability to favourably influence body composition has received considerable attention. CLA has been shown to reduce body fat and increase lean mass in mice (Park *et al.*, 1997, West *et al.*, 1998), rats (Houseknecht *et al., 1998*, Sisk *et al.*, 1998), pigs (Dugan *et al.*, 1997, Ostrowska *et al.*, 1999) and hamsters (Gavino *et al.*, 2000). CLA's effect on body composition appears to be dose dependent and independent of dietary fat content. It is now emerging that this effect on body composition may be isomer specific with evidence suggesting that the *t*10, *c*12-CLA isomer could be responsible for the CLA-associated body compositional changes (Gavino *et al.*, 2000, Park *et al.*, 1999). A number of possible mechanisms have been proposed to explain how CLA affects body composition. These include an increase in metabolic rate and reduced resting energy expenditure (West *et al.*, 1998), an increase in fat oxidation (Park *et al.*, 1997), induced apoptosis in the adipose tissue (Tsuboyama-kasaoka *et al.*, 2000), inhibition of faty acid synthesis (Choi *et al.*, 2000) and inhibition of adipocyte differentiation (Brodie *et al.*, 1999). CLA supplementation (ranging from 3 to 7 g/day) had no significant affect on body weight in any of the human studies (review in Calder, 2002). In five out of ten studies, CLA supplementation decreased body fat while lean body mass was increased in two studies. There are several reasons to explain why CLA did not consistently affect body weight as was found in the animal studies. The dose of CLA provided was much lower per unit body weight than used in the animals. The CLA isomeric mixtures fed to the human subjects may not have provided sufficient amounts of the *t*10, *c*12-CLA isomer which has been shown to possess the anti-adipogenic activity. The human subjects were all adults rather than growing animals.

Also, none of the animal studies involved obese animals or restricted caloric intake. There also is a huge difference in the metabolic rate between mice and humans (Terpstra, 2001). It also has been suggested that CLA is a more powerful tool to 'treat the regain' rather than obesity.

Animal studies have provided evidence to suggest that CLA might benefit cardiovascular health (reviewed in Whigham *et al.*, 2000). Using rabbit (Lee *et al.*, 1994), hamster (Nicolosi *et al.*, 1997) and mouse (Munday *et al.*, 1999) models it was found that addition of 0.1 to 1 % (w/w) CLA to an atherogenic diet reduced blood levels of total cholesterol and low-density lipoproteins (LDL)-cholesterol and and triglycerides. In rabbits and hamsters, but not mice, feeding CLA up to 1 % (w/w) reduced the severity of aortic atherosclerotic lesions. Feeding CLA induced regression of pre-established lesions in rabbits (Kritchevsky *et al.*, 2000). There is evidence to suggest that t10, c12-CLA is more effective than c9, t11-CLA in reducing serum lipid levels (de Deckere *et al.*, 1999, Gavino *et al.*, 2000). Studies examining CLA's affect on blood lipid profile in humans have yielded varying results. Only in one study were significant reductions in LDL, high-density lipoproteins (HDL) and total cholesterol observed (Blankson *et al.*, 2000) but differences were not deemed clinically significant. In two other studies no significant changes in serum lipids were observed (Benito *et al.*, 2001, Riserus *et al.*, 2001).

Thiazolidinediones are a new class of drugs that act by improving insulin action, thereby lowering blood sugar levels in patients with diabetes (Lehmann *et al.*, 195). They are thought to trigger adipocyte differentiation and maturation, leading to improved glucose

uptake and a concomitant reduction in serum glucose levels via activation of the steroid hormone receptor PPARγ. It is now believed that CLA acts in a similar manner to thiazolidinediones and so may represent a new insulin-sensitising agent to aid in the management of type II diabetes. The Zucker diabetic fatty (ZDF) rat spontaneously develops diabetes at age 7 to 12 weeks and is used as an animal model for type II diabetes. CLA treatment normalised glucose tolerance, improved hyperinslinaemia and lowered circulating free fatty acids which prevented or delayed the onset of hyperglycemia in this ZDF rat model (Houseknecht *et al.*, 1998). A study by Ryder and co-workers (2001) indicate that the *t*10, *c*12-CLA isomer may be responsible for the beneficial effect on diabetes. Belury and colleagues are currently conducting a study in human subjects with type 2 diabetes taking CLA supplements.

CLA may enhance immune function via modulation of eicosanoid formation. Eicosanoids are produced by numerous types of immune cells and are thought to regulate cytokine synthesis and inflammation. Initial studies demonstrated that immune-induced weight loss (cachexia) in chickens and rats could be prevented by CLA (Cook *et al.*, 1993). CLA reduced antigen-induced histamine and PGE_2 in guinea pig tracheae suggesting that CLA may play a role in the regulating of type I hypersensitivity (Whigham *et al.*, 2001). In a mouse model of the autoimmune disorder, lupus erythematosis, dietary CLA exacerbated early stage but delayed late stage symptoms of lupus (Yang *et al.*, 2000). CLA has been shown to reduce the levels of certain macrophage and monocyte cytokines such as tumor necrosis factor-α (Turek *et al.*, 1998) and interleukin-1 (Wong *et al.*, 1997). A short-term study where healthy young women

were fed 3.9 g/day CLA found no beneficial or adverse effects on immune status (Kelley *et al.*, 2000).

CLA may have a positive effect on bone formation (reviewed in Watkins and Seifert, 2000). Watkins *et al.*, (1997) reported that butterfat (a natural source of CLA) led to a higher rate of bone formation in chickens compared with those given diets containing higher amounts of n-6 fatty acids. The higher bone formation was associated with a reduction in *ex vivo* PGE_2 (a bone absorption factor) production and an increase in insulin-like growth factor-1 (a bone growth factor). Bone organ cultures of tibia and femur from rats fed CLA (1%) showed a significant reduction in PGE_2 production compared to animals not fed CLA. Rat pups exposed to CLA (0.5 %), either *in utero* or during the first seven days of life, had significantly longer tail lengths (a measure of skeletal growth) compared with pups fed a diet without CLA (Poulos *et al.*, 2001).

To date toxicology studies in rats fed CLA (1.5 % of diet) have not revealed hematological abnormalities nor any evidence of histopathological damage to organs after 36 weeks of feeding (Scimeca, 1998). However, hepatomegala has been reported in some mice fed CLA (1 % of diet) (Delany *et al.*, 1999, 2000, Tsuboyama-Kasaoka *et al.*, 2000).

1.16 Aims

The aims of this research were to investigate specific mechanisms of action that may be responsible for the anticarcinogenic effect of synthetic CLA isomers in human cancer cell lines and to compare these effects with those induced by milk fat triglyceride bound CLA.

1.17 Specific objectives

- To evaluate the relative growth effects of a synthetic mixture of CLA isomers, pure *c*9, *t*11- and *t*10, *c*12- CLA on human breast (MCF-7) and colon (SW480) cancer cell lines by examination of viability.

- To investigate if the growth suppressive effects of CLA are modulated via alterations in arachidonic acid (AA) metabolism, i.e. AA uptake, distribution, release and conversion to eicosanoid classes in breast (MCF-7) and colon (SW480) human cancer cell lines.

- To elucidate whether apoptosis is the mode of death induced by CLA isomers in the colon (SW480) tumor cell line by examining the effect of CLA isomers on cell morphology, DNA laddering and a panel of apoptosis regulatory proteins.

- To examine the bioconversion capabilities of human breast (MCF-7) and colon (SW480) cancer cells to convert *trans*-vaccenic acid to *c*9, *t*11-CLA.

- To compare the relative growth effects of the synthetic *c*9, *t*11-CLA isomer with CLA-enriched milk fat, consisting primarily of the *c*9, *t*11 isomer present in triglyceride bound form, on human breast (MCF-7) and colon (SW480) cancer cells.

CHAPTER 2

Modulation of arachidonic acid distribution by conjugated linoleic acid isomers and linoleic acid in MCF-7 and SW480 cancer cells.[1]

[1] Published in Lipids 36: 1161-1168, 2001.

2.1 Introduction

Compelling evidence indicates that CLA, a derivative of linoleic acid, found in milk and ruminant fats, is among the more potent naturally occurring anticarcinogens. *In vivo* model studies of experimental carcinogenesis have revealed that the synthetic mixture of CLA isomers containing 21 % *c*11, *t*13, 29 % *t*10, *c*12, 29.5 % *c*9, *t*11 and 12.3 % *c*8, *t*10 (Sehat *et al.*, 1998) possess powerful inhibitory effects on mammary, colon, forestomach and skin carcinogenesis in rodents (Ip *et al.*, 1991, Ha *et al.*, 1990, Belury *et al.*, 1996, Ip *et al.* 1996, *Ip et al.*, 1997a, Ip, *et al.*, 1997b, Liew *et al.*, 1995). Other physiological benefits include a reduction in severity of atherosclerotic plaques, improvement of glucose tolerance in diabetic animals, body fat reduction, enhanced immune responses and positive effects on bone formation all of which have been well documented in numerous reviews (Pariza *et al.*, 2000, MacDonald, 2000, Pariza 1990, Cook and Pariza, 1998). The specific CLA isomers which possess biological activity have not yet been clearly identified. Most of the mechanistic work to explain the potent anticancer effects of CLA has involved a commercial free fatty acid preparation containing up to sixteen different CLA isomers (Sehat *et al.*, 1998). The availability of the pure isomers of *c*9, *t*11- and *t*10, *c*12-CLA has paved the way for determining the magnitude of biological responses of these isomers, which are predominantly present in the synthetic CLA mixture and which are produced by ruminants (Parodi, 1977) and consequently found in the human diet. A study by Ip *et al.* revealed that CLA enriched butterfat, containing predominantly the *c*9, *t*11-CLA isomer, had a powerful protective effect against the risk of mammary cancer development in rodents (Ip *et al.*, 1999).

The mechanisms by which CLA exerts its anticarcinogenic effects have not yet been fully elucidated. Induction of apoptosis by CLA via down-regulation of a membrane protein bcl-2 has been reported to be the mode of cell death in cultured mammary tumor cells and in differentiated colonies of mammary epithelial organoids (Ip *et al.*, 2000). The incorporation of CLA isomers into membrane phospholipids of breast, skin and liver tissue has been reported but with variable levels of displacement from membranes of LA and AA (Ip *et al.*, 1997b, Belury and Kempasteczko, 1997, Liu and Belury, 1998, Banni *et al.*, 1999, O'Shea *et al.*, 2000). This suggests that CLA may influence the fatty acid composition of cell membranes resulting in alterations in eicosanoid production and other signal transduction pathways downstream of the cell membrane. Eicosanoids, comprising prostaglandins and leukotrienes, are a family of membrane-derived lipid mediators that have been an attractive target for cancer chemoprevention (Marks *et al.*, 2000). Research has shown that CLA can affect the synthesis of eicosanoids, in particular PGE_2 (Belury and Kempasteczko, 1997), a prostanoid that has been shown to promote growth and metastasis in many experimental tumors (Fulton 1998).

The mammary (MCF-7) and colon (SW480) tumor cell lines have been used as *in vitro* models to investigate the mechanisms by which CLA may affect breast and colon cancer. The MCF-7 epithelial cell line retains several characteristics of differentiated mammary epithelium including the ability to process estradiol via cytoplasmic estrogen receptors (Brandes *et al.*, 1983). The SW480 cell line, which was established from a primary adenocarcinoma of the colon, is a dedifferentiated cell line which expresses elevated levels of the p53 protein, mutated ras and small amounts of carcinoembryonic antigen

(Leibovitz *et al.*, 1976). The mixture of CLA isomers induced a dose- and time-dependent cytotoxicty against both cell lines and this effect was accompanied by an increased lipid peroxidation (O'Shea *et al.*, 1999). That the anticancer effect of CLA may be due in part to a redistribution of AA among cellular lipids which might influence oxidative susceptibility of particular membrane phospholipids and/or alter eicosanoid synthesis during tumor growth.

2.2 Objectives

This study was undertaken to examine the modulatory effects of CLA isomers on cell viability in addition to AA uptake, distribution, release and conversion to eicosanoid classes in breast (MCF-7) and colon (SW480) human cancer cell lines. The effect of CLA or LA presented as 1) Fatty acids dissolved in ethanol, 2) Sodium salts of fatty acids or 3) Sodium salts of fatty acids complexed to BSA (10mg/ml) on cell growth was also evaluated

2.3 Materials and methods

2.3.1 Materials

^{14}C-AA (specific acitivity, 55mCi/mmol), Biotrak enzyme immunoassay kit for LTB_4 and radioreceptor kit for IP_3 were purchased from Nycomed Amersham (Little Chalfort, Buckinghamshire, UK). The CLA mixture (21 % *c*11, *t*13, 29 % *t*10, *c*12, 29.5 % *c*9, *t*11 and 12.3 % *c*8, *t*10) (Sehat *et al.*, 1998) was obtained from Nu Chek Prep (Elysian, MN, USA). Individual CLA isomers, *c*9, *t*11 and *t*10, *c*12 (both 95 % pure), were purchased from Matreya (Pleasant Gap, PA, USA). LA, authentic PGE_2, $PGF_{2\alpha}$, PGD_2, 5-HPETE,

phospholipid standards, Supelclean LC-18 SPE columns, trypan blue and bovine serum albumin (BSA) solution were all purchased from Sigma Chemical Co. (Poole, Dorset, UK). Silica Sep-Pak columns were obtained from Waters Corporation (Milford, MA, USA). The BIOXYTECH immunoassay kit for 8-epi-$PGF_{2\alpha}$ was obtained form Bio-Stat (Stockport, UK). DC-Alufliien Kiesegel 60 thin layer chromatography (TLC) plates were obtained from Lennox (Dublin, Ireland). The CellTitre®AQ_{ueous} Non-Radioactive Cell Proliferation Assay kit was purchased from Promega (Southampton, UK). All other chemicals and solvents used were HPLC grade.

2.3.2 Cell culture

Human breast (MCF-7) and colon (SW480) cancer cell lines were obtained from the American Type Culture Collection, (Manassas, VA). Culture media and supplements were purchased from GIBCOBRL (Paisley, Scotland). Both cell lines were maintained in Dulbecco's Minimum Essential Medium (DMEM) supplemented with fetal bovine serum (5 % v/v), 0.2 mM L-glutamine, 1 mM HEPES, and 1 unit/ml penicillin and streptomycin. The MCF-7 cells required an additional supplement of 10 mM sodium pyruvate. Cells were grown in Falcon T-25 cm^2 flasks and maintained as previously described (O'Shea *et al.*, 1999).

2.3.3 Comparison of fatty acid delivery methods

MCF-7 and SW480 cells were seeded in 6 well plates at densities of 1 x 10^5/well and 5 x 10^4/well respectively. The seeding densities used for both cell lines were selected from cell seeding optimisation studies for 4 days incubation. Cells were cultured for 24 h to allow the cells attach to the substratum. The medium was then replaced with medium supplemented with the CLA mixture of isomers (Nu-Chek Prep) or linoleic acid (LA) at a range of concentrations (5, 10, 16 and 20 μg/ml) presented in three different forms: 1) Fatty acids dissolved in ethanol, 2) Sodium salts of fatty acids or 3) Sodium salts of fatty acids complexed to BSA (10mg/ml). The Sodium salts and BSA complexes were prepared as outlined by Ip *et al.*, 1999. In brief, 1ml of CLA or LA stock (0.1g/ml dissolved in ethanol, this being a 0.35M solution) was mixed with 1ml of equimolar sodium hydroxide. The fatty acid concentration in the resulting solution was 50,000μg/ml. This solution was then diluted to a fatty acid concentration of 20,000 μg/ml in DMEM containing 10mg/ml BSA. This mixture was warmed to 37^{0}C, warmed to 50^{0}C and then further diluted in DMEM to achieve the CLA or LA concentrations required. Control wells for fatty acid treatments dissolved in ethanol were supplemented with an equivalent volume of ethanol (0.1 % v/v). Following 4 days of incubation, cells were harvested using phosphate buffered saline (PBS) containing 0.25 % (v/v) trypsin. Cell viability was determined using the trypan blue exclusion (0.4 % w/v) method. Trypan blue will stain dead or dying cells. Viable cells are able to exclude the dye and do not stain.

2.3.4 Quantification of cell numbers.

The comparative effects of four different fatty acid preparations on cell viability was evaluated: 1. the CLA mixture of isomers, 2. the pure *c*9, *t*11-CLA isomer, 3. the pure *t*10, *c*12-CLA isomer and 4. LA. MCF-7 and SW480 cells were seeded in 96 well plates at densities of 1 x 10^3/well and 5 x 10^2/well, respectively. The seeding densities used for both cell lines were selected from cell seeding optimisation studies in 96 well plates. Cells were cultured for 24 h to allow the cells attach to the substratum. The medium was then replaced with medium supplemented either the CLA mixture of isomers, the pure *c*9, *t*11-CLA, the pure *t*10, *c*12-CLA, or LA at two different lipid concentrations: 5 and 16 μg/ml corresponding to 17.8 μM and 57 μM, respectively. The CLA concentrations used have been reported to be within the physiological range of concentrations of the c9, t11 isomer in human phospholipids (Cawood *et al.*, 1983) plasma, bile, duodenal juice (Iversen *et al.*, 1985) and have been previously used in cell culture work (Shultz *et al.*, 1992). The fatty acids were dissolved in ethanol and so control wells were supplemented with equivalent volumes of ethanol (0.25 or 0.8 % v/v). After 24 h and 4 days of incubation, viable cell numbers were quantified using the CellTitre® AQ_{ueous} Non-Radioactive Cell Proliferation Assay kit. The CellTiter 96® AQ_{ueous} Assay was composed of solutions of a novel tetrazolium compound 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt (MTS) and an electron coupling reagent phenazine methosulfate (PMS). After the 24 h or 4 day incubation period the medium of each well was replaced with 100 μL of fresh medium, 200 μL of MTS solution was added and plates were incubated for 4 hours at 37°C. During this time the MTS was bioreduced by cells into a formazan product that was soluble in tissue

culture medium. The conversion of MTS into the aqueous soluble formazan product was accomplished by dehydrogenase enzymes found in metabolically active cells. After the 4 hours the absorbance of the formazan product was measured at 492nm using an Anthos 2010 plate reader. The quantity of formazan product as measured absorbance was directly proportional to the number of living cells in culture.

2.3.5 Lipid extraction and fractionation

Cells were seeded in T-25 cm^2 flasks at a density of 2 x 10^5/flask and grown to 90 % confluency. The MCF-7 and SW480 cells reached 90 % confluency after 4 and 3 days, respectively. The medium was then replaced with medium containing ^{14}C-AA at 0.2 µCi along with either the CLA mixture of isomers, the pure *c*9, *t*11-CLA, the pure *t*10, *c*12-CLA or LA, all at a lipid concentration of 16µg/ml (57 µM). The CLA mixture of isomers at a lipid concentration of 16µg/ml yielded a *c*9, *t*11-CLA and *t*10, *c*12-CLA concentration of approximately 4.7 µg/ml (17 µM) each. Control flasks were supplemented with an equivalent volume of ethanol (0.8 % v/v). After 24 h incubation, cells were harvested using phosphate buffered saline (PBS) containing trypsin (0.25 % v/v). Total lipids were extracted from cell pellet as described (Bligh and Dyer, 1959), dried under nitrogen, redissolved in chloroform and applied to a silica Sep-Pak column to separate the triglyceride (TG), monoglyceride (MG) and phospholipid (PL) fractions as described (Cantwell *et al.*, 1999). An aliquot of each fraction was counted in a Beckman LS6500 scintillation counter before being dried under nitrogen. The PL fraction was separated using normal phase TLC. Samples were co-migrated with authentic standards of phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidylserine PS and

phosphatidylethanolamine (PE). Chloroform/methanol/acetic acid (65:45:4, v/v) was used to separate the phospholipids (Liu and Belury 1997). Iodine vapors were used to identify the position of the phospholipids compared with standards and these bands were removed from TLC plates and placed in vials for counting by liquid scintillation.

2.3.6 Phospholipase C activity.

Inositol triphosphate (IP_3) was used as an index of phospholipase C activity. Cells were treated with the four different fatty acid treatments (all at 16 µg/ml) or ethanol as described above. After 24 h incubation the cells were harvested as described above and IP_3 was extracted from cells using a perchloric acid (10% v/v) extraction method previously described by Palmer (Palmer *et al.*, 1986). A radioreceptor assay kit (Biotrak D-*myo*-Inositol 1,4,5-triphosphate assay system) was used to quantify IP_3 levels in 100 µL of extract according to the manufacturer's instructions. This assay is based on competition between a [^{3}H] IP_3 tracer and unlabelled IP_3 in the standards or samples for binding to a bovine adrenal cortex protein. The bound IP_3 was then separated from the free IP_3 by centrifugation, which brought the binding protein to the bottom of the tube. The free IP_3 in the supernatant was discarded by decantation leaving the bound fraction adherent to the tube. The pellet was resuspended in 1 mL of water which was decanted into 10 mL of scintillation fluid for counting. Measurement of the radioactivity enabled the amount of unlabelled IP_3 in the sample to be determined by interpolation from a standard curve.

2.3.7 Release of ^{14}C-AA derivatives.

Cells were seeded in T-25 cm^2 flasks at a density of 2 x 10^5/flask and grown to 80 % confluency. Medium was replaced with medium containing ^{14}C-AA (0.2 µCi) and incubated for 24 h. After removal of media, cells were washed three times with phosphate-buffered saline (PBS), before addition of medium containing the four different fatty acid treatments (all at 16 µg/ml) or ethanol as described earlier. After 24 h, medium containing the released ^{14}C-AA derivatives was removed and an aliquot was counted by liquid scintillation.

2.3.8 Primary Prostaglandins and 8-epi-$PGF_{2\alpha}$.

Cells were seeded and treated with ^{14}C-AA at 0.2 µCi along with the four different fatty acid treatments (all at 16 µg/ml) or ethanol as described previously. After 24 h incubation the medium were removed from the flasks and eicosanoids were extracted twice with ethyl acetate from medium acidified to pH 3.0 with 0.1 N HCl as described (Liu and Belury, 1998). Eicosanoid extracts were dried under nitrogen, redissolved in ethyl acetate and applied onto normal phase TLC plates. Ethyl acetate/iso-octane/glacial acetic acid/water (55:25:10:50, by vol) was used to separate prostaglandins (PG) (Belury and Kempasteczko, 1997). Samples were co-migrated with authentic standards of PGE_2, $PGF_{2\alpha}$, and PGD_2. Iodine vapors were used to identify the position of each PG compared with the standards. Bands of PGE_2, $PGF_{2\alpha}$, PGD_2 were removed from TLC plates and placed in vials for counting by liquid scintillation. For the 8-epi-$PGF_{2\alpha}$ assay, culture medium was collected after 24 h incubation with the fatty acids treatments described earlier and 8-epi-$PGF_{2\alpha}$ was extracted as described (Watkins *et al.*, 1999). Briefly,

ethanol was added to the medium to a final concentration of 15 % (v/v) and acidified to pH 3.0 with formic acid (98 % v/v). The sample was applied to Supelclean LC-18 SPE columns and washed with water (adjusted to pH 3.0 with formic acid), 15 % (v/v) ethanol in water (pH 3.0) and hexane. Ethyl acetate containing 1 % (v/v) methanol was used to elute 8-epi-$PGF_{2\alpha}$. The eluate was dried under nitrogen, resuspended in assay buffer (BIOXYTECH) and a competitive horseradish peroxidase enzyme-linked immunoassay kit (BIOXYTECH 8-Isoprostane assay system) was used to quantify 8-epi-$PGF_{2\alpha}$ levels according to the manufacturer's instructions. The 8-epi-$PGF_{2\alpha}$ in the sample or standards competed for binding (to the antibody coated on the plate) with 8-epi-$PGF_{2\alpha}$ conjugated to horseradish peroxidase (HRP). The peroxidase activity resulted in colour development. The intensity of colour development was proportional to the amount of 8-epi-$PGF_{2\alpha}$-HRP bound and inversely proportional to the amount of 8-epi-$PGF_{2\alpha}$ in the samples or standards.

2.3.9 5-Hydroperoxyeicosatetraenoate and Leukotriene B_4.

Cells were seeded and treated with the four different fatty acid treatments (all at 16 µg/ml) or ethanol as described earlier. For the 5-HPETE assay, cells were lysed using Triton-X 100 (0.1 % v/v). The assay was initiated by the addition of 50 µL of AA (70 mM prepared in 50 mM Tris-HCl buffer, pH 4) to 50 µL of cell lysate in an ice-cold 96-well plate and incubated at 37^0C for 10 min. The reaction was terminated by the addition of 100µL of the FOX reagent: sulfuric acid (25 mM), xylenol orange (100 µM), iron (II) sulfate (100 µM), methanol:water (9:1 v/v) (Waslidge *et al.*,1995). Absorbance was

measured at 620nm using an Anthos 2010 plate reader. For the LTB_4 assay, eicosanoids were extracted from the medium as described earlier and dried under nitrogen. An enzyme immunoassay kit (Biotrak leukotriene B_4 enzyme immunoassay system) was used to quantify LTB_4 levels according to the manufacturer's instructions. This assay is based on the competition between unlabelled LTB_4 and a fixed quantity of peroxidase labelled LTB_4 for binding sites on a LTB_4 specific antibody.

2.3.10 Statistical analysis.

Three independent experiments were performed in triplicate. The Student's *t* test was used to determine significant differences between treatments.

2.4 Results

2.4.1 Comparison of fatty acid delivery methods

It is important that cell culture conditions mimic the *in vivo* environment as best they can. The presence of albumin, important *in vivo* for binding and transporting PUFA in the blood may have a role as free fatty acids may be more able to enter cells than when bound as large albumin complexes. In this study, the effect of presenting CLA to cells as a sodium salt complexed with BSA (10 mg/ml) was evaluated and compared with presentation of CLA in free fatty acid form dissolved in ethanol (0.1 % v/v). CLA (5, 10, 16 and 20 μg/ml) complexed with bovine serum albumin (10mg/ml) reduced growth of SW480 cells by 33-42 % relative to control but was significantly less toxic ($p<0.05$) than free CLA which inhibited growth by 48-55 % (Figure 2.1). Complexing LA (16 and 20 μg/ml) to BSA reduced growth ($p<0.05$) in SW480 cells relative to incubation with the

free LA, though at lower concentrations (5 and 10 μg/ml) no significant differences were observed. Interestingly, complexing either CLA or LA with BSA produced effects in MCF-7 cells similar to the corresponding free fatty acids (Figure 2.2). The data indicate that the physiological form of presentation of CLA is cytotoxic to both cell lines, the magnitude of which (28-45 %) was lower but not significantly different to the free fatty acid form (47-54 %) in the MCF-7 cell line.

The effect of presenting CLA as a sodium salt to cells instead of free fatty acid was also examined. Sodium salts of CLA (5, 10, 16 and 20 μg/ml) were relatively more toxic ($p<0.05$) to SW480 cells than corresponding free CLA concentrations (Figure 2.1), while MCF-7 cells were equally as sensitive to growth inhibition by the sodium salt and the free fatty acid (Figure 2.2). As expected, complexation with BSA reduced the cytotoxic effect of sodium salt.

In summary, the free fatty acid form of CLA produced cytotoxic effects that were intermediate between those observed following incubation with the sodium salt complexed with BSA and the non-protein bound sodium salt.

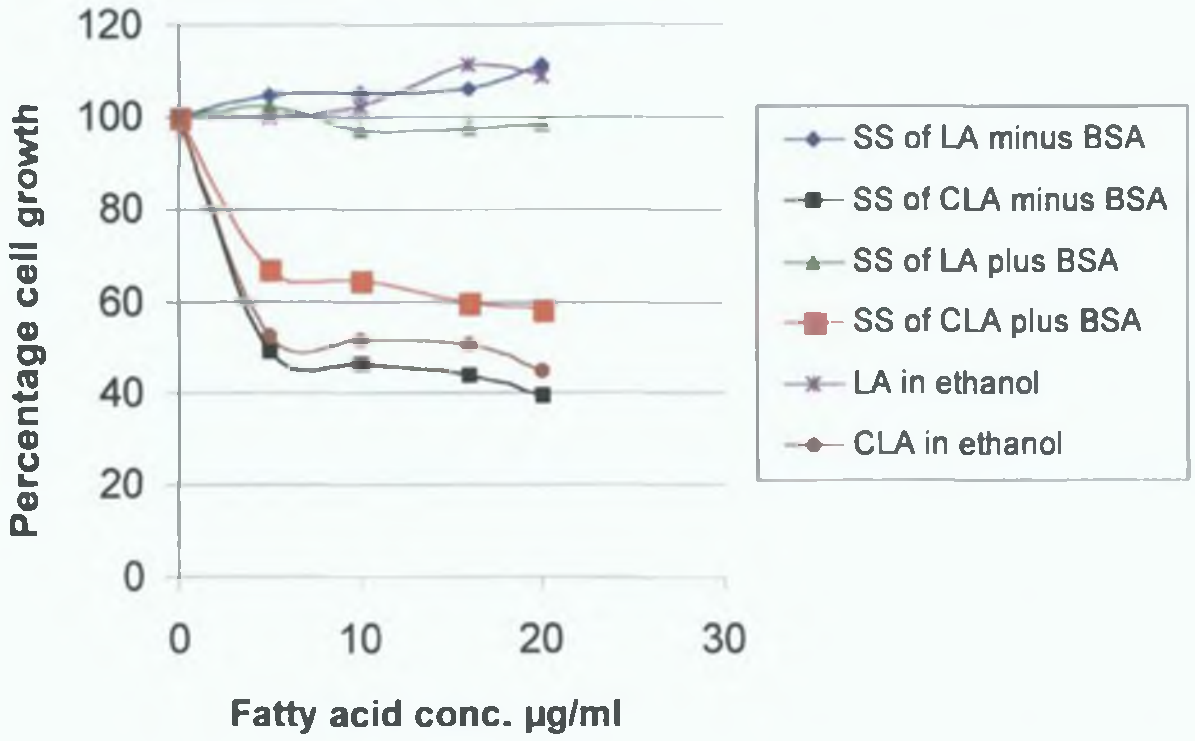


Figure 2.1 The effect of the CLA mixture of isomers (Nu-Chek Prep) or LA at a range of concentrations (5, 10, 16 and 20 µg/ml) presented in three different forms (1. Fatty acids dissolved in ethanol, 2. Sodium salts of fatty acids or 3. Sodium salts of fatty acids complexed to BSA (10mg/ml) on SW480 cell growth after 4 days incubation. Data is expressed as a mean percentage of control for three independent experiments carried out in triplicate. Abbreviations: Sodium Salt, SS; BSA, bovine serum albumin.

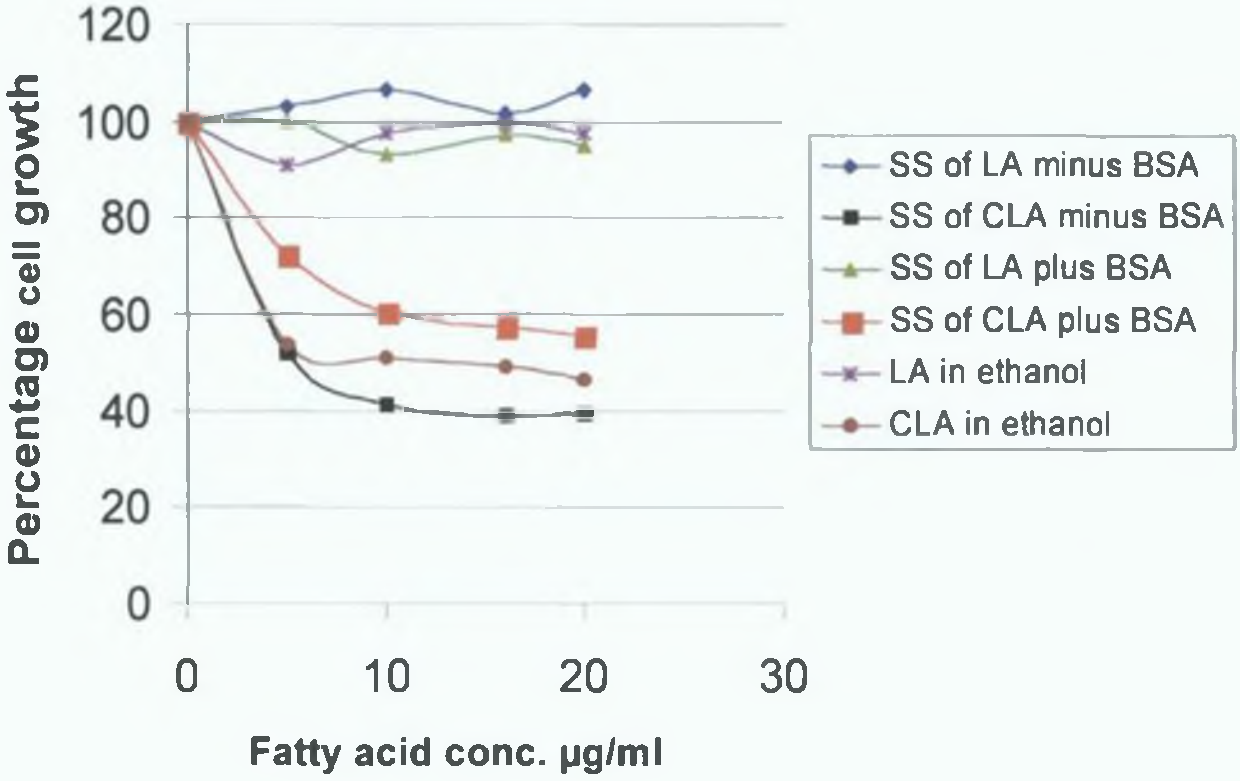


Figure 2.2 The effect of CLA mixture of isomers (Nu-Chek Prep) or LA at a range of concentrations (5, 10, 16 and 20 µg/ml) presented in three different forms (1. Fatty acids dissolved in ethanol, 2. Sodium salts of fatty acids or 3. Sodium salts of fatty acids complexed to BSA (10mg/ml) on MCF-7 cell growth after 4 days incubation. Data is expressed as a mean percentage of control for three independent experiments carried out in triplicate. Abbreviations: Sodium Salt, SS; BSA, bovine serum albumin.

2.4.2 Effect of CLA isomers on cell viability

MCF-7 and SW480 cells were incubated for 24h and 4 days with the CLA mixture of isomers, *c*9, *t*11-CLA, *t*10, *c*12-CLA and LA at two different lipid concentrations (5 and 16 μg/ml corresponding to 17.8 μM and 57 μM, respectively). None of the fatty acids at either 5 μg/ml or 16 μg/ml significantly altered cell viability after 24 h. The CLA mixture of isomers (16 μg/ml) caused a reduction in cell viability after 4 days in both cell lines with a greater reduction noted in MCF-7 cells (58 %) (Figure 2.3) compared with SW480 cells (52 %) (Figure 2.4). The *c*9, *t*11-CLA isomer caused a similar reduction (~50 %) in cell viability to the CLA mixture of isomers following 4 days of incubation at both 5 μg/ml and 16 μg/ml. In both cell lines, the *t*10, *c*12-CLA isomer at 5 μg/ml and 16 μg/ml reduced viability by 38-39 % and 50-60 % respectively following 4 days of incubation. Incubation of SW480 cells with LA (16 μg/ml) for 4 days increased cell viability by 23 % but the lower concentration of 5 μg/ml had no effect at either time point (Figure 2.3). LA (5 and 16 μg/ml) had no effect on the viability of MCF-7 cells following 4 days of incubation in this study.

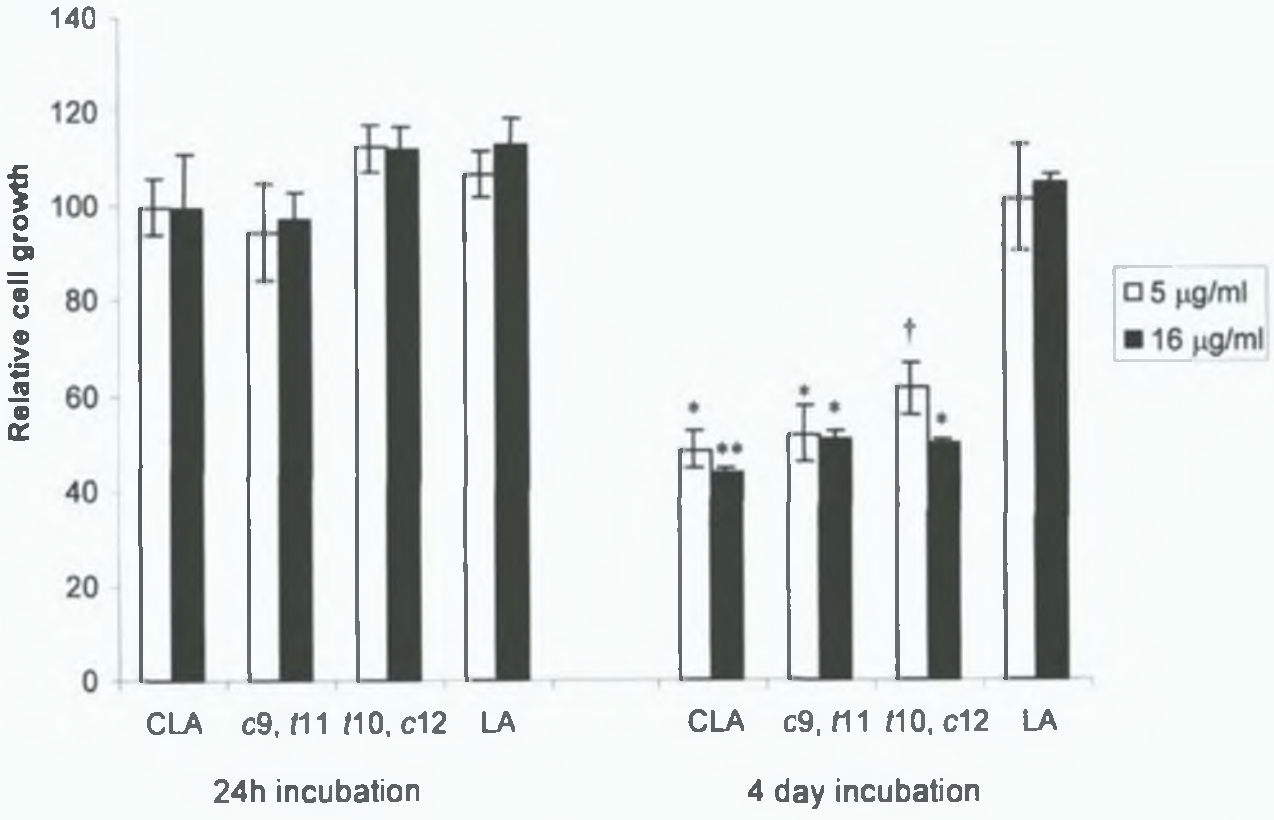


Figure 2.3 Cell viability for MCF-7 cells incubated with 5 or 16 µg/ml CLA mixture, *c*9, *t*11-CLA, *t*10, *c*12-CLA, LA or ethanol control for 24 h and 4 days. Data represents cell viability expressed as a percentage of the control which was taken to be 100 % (** denotes $p<0.001$, * denotes $p<0.02$ and † denotes $p<0.05$). Data is expressed as the percentage mean ± SD for three separate experiments carried out in triplicate.

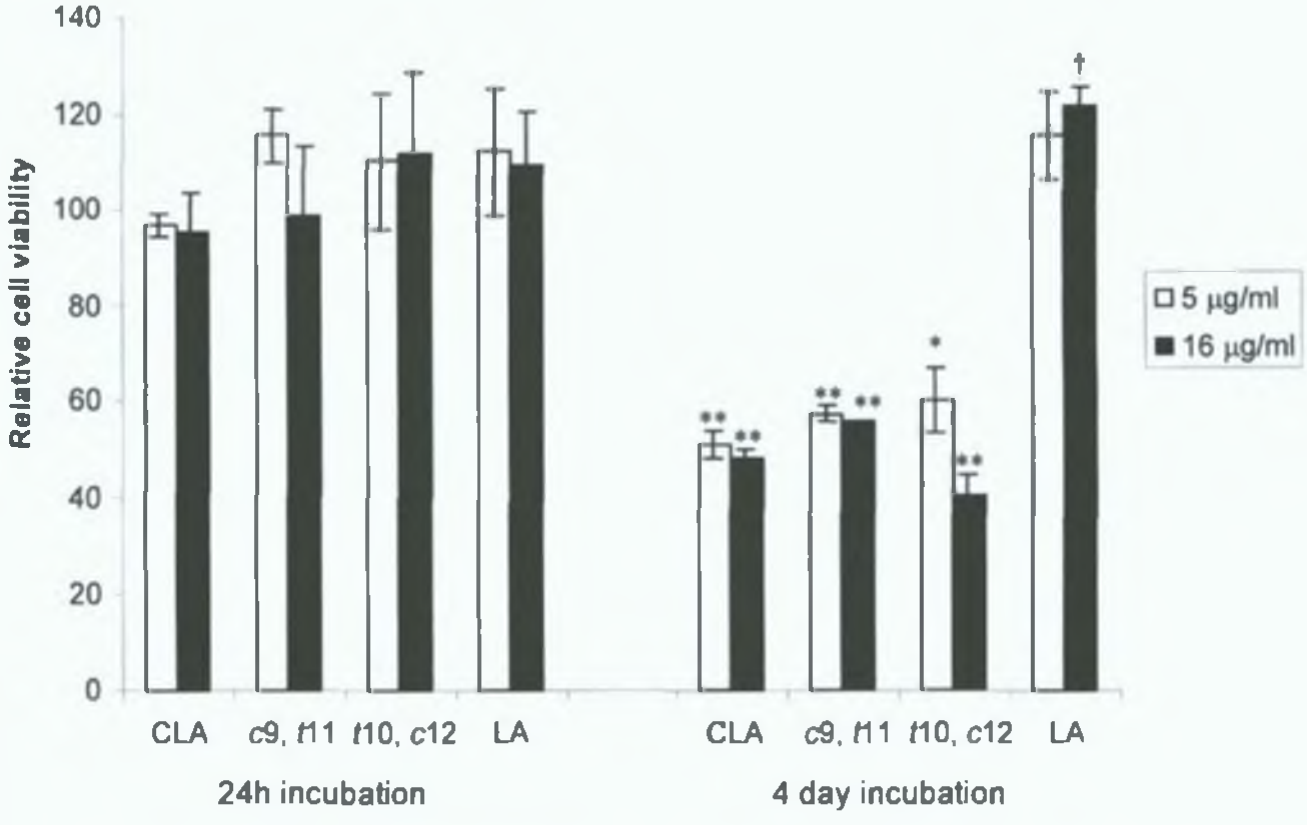


Figure 2.4 Cell viability for SW480 cells incubated with 5 or 16 µg/ml CLA mixture, *c*9, *t*11-CLA, *t*10, *c*12-CLA, LA or ethanol control for 24 h and 4 days. Data represents cell viability expressed as a percentage of the control which was taken to be 100 % (** denotes $p<0.001$, * denotes $p<0.02$ and † denotes $p<0.05$). Data is expressed as the percentage mean ± SD for three separate experiments carried out in triplicate.

2.4.3 Effect of CLA isomers on incorporation of ^{14}C-AA into cellular lipid fractions.

One of the mechanisms involved in growth suppression is an alteration in the AA cascade of events leading to eicosanoid production (Liu and Belury, 1998). In order to examine if cellular AA distribution was altered by CLA, we investigated the effect of CLA isomers on incorporation of ^{14}C-AA into cellular lipid fractions. ^{14}C-AA was preferentially incorporated into the PL fraction in untreated and CLA treated MCF-7 cells and SW480 cells (Table 2.1 and 2.2). Levels of uptake into PL, TG, and MG were 60 %, 33 % and 7 %, respectively, in control MCF-7 cells (Table 2.1). ^{14}C-AA uptake into the MG fractions was increased in MCF-7 cells treated with the CLA mixture $p<(0.05)$ (7.2 %) and the pure *c*9, *t*11-CLA isomer ($p<0.02$) (16.6 %). None of the fatty acid treatments had any effect on uptake of ^{14}C-AA into the TG and PL fractions of the MCF-7 cell line.

Table 2.1 Effect of Fatty Acid Treatments on Incorporation of ^{14}C-AA into Lipid Fractions of MCF-7 cells[1].

Fatty acid treatment	MCF-7		
	MG	TG	PL
Control	7.0 ± 1.3	33.5 ± 5.2	59.5 ± 6.2
CLA mixture	14.8 ± 1.6[b]	28.4 ± 1.2	56.8 ± 2.8
*c*9, *t*11-CLA	23.6 ± 1.0[a]	26.4 ± 4.1	49.9 ± 5.1
*t*12, *c*12-CLA	6.2 ± 0.8	34.4 ± 4.5	59.5 ± 5.1
LA	10.3 ± 2.0	31.2 ± 3.3	58.4 ± 5.2

[1]Data represent the mean percentage of total cellular lipids ± SD for three separate experiments carried out in triplicate. Letters indicate values that are significantly different compared to controls ([a] denotes $p<0.02$ and [b] denotes $p<0.05$). Abbreviations: CLA, conjugated linoleic acid; LA, linoleic acid; MG, monoglyceride; TG, triglyceride; PL, phospholipid.

Levels of uptake into PL, TG and MG were 76 %, 21 %, and 3 %, respectively, in control SW480 cells (Table 2.2). In contrast with MCF-7 cells, uptake of ^{14}C-AA into PL was significantly lowered ($p<0.02$) (~25 %) in the SW480 cells treated with the CLA mixture and *c*9, *t*11-CLA, while both the CLA mixture and *c*9, *t*11-CLA increased AA uptake into TG (25-30 %) ($p<0.05$). These data suggest that ^{14}C-AA uptake into TG occurred at the expense of PL in the SW480 cell line. None of the fatty acid treatments had any effect on uptake of ^{14}C-AA into the MG lipid fraction of the SW480 cells. The *t*10, *c*12-CLA isomer and LA (both at 16µg/ml) had no effect on ^{14}C-AA incorporation into any of the lipid fractions in either cell line.

Table 2.2 Effect of Fatty Acid Treatments on Incorporation of ^{14}C-AA into Lipid Fractions of SW480 cells[1].

Fatty acid treatments	SW480		
	MG	TG	PL
Control	2.9 ± 0.9	21.0 ± 0.6	76.1 ± 1.5
CLA mixture	3.8 ± 1.0	47.2 ± 3.2[a]	48.9 ± 2.2[a]
*c*9, *t*11-CLA	4.7 ± 1.3	45.7 ± 6.1[b]	49.6 ± 7.2[a]
*t*12, *c*12-CLA	4.0 ± 1.9	22.1 ± 4.2	73.4 ± 5.7
LA	3.8 ± 2.0	26.0 ± 9.1	70.1 ± 7.6

[1]Data represent the mean percentage of total cellular lipids ± SD for three separate experiments carried out in triplicate. Letters indicate values that are significantly different compared to controls ([a] denotes $p<0.02$ and [b] denotes $p<0.05$). Abbreviations: CLA, conjugated linoleic acid; LA, linoleic acid; MG, monoglyceride; TG, triglyceride; PL, phospholipid.

2.4.4 Effect of CLA isomers on ^{14}C-AA distribution among phospholipid fractions.

Having shown that ^{14}C-AA was preferentially incorporated into the PL fraction of CLA treated cells, we examined the effect of CLA isomers on ^{14}C-AA distribution among individual PL. PC and PE were the predominant PL classes in which ^{14}C-AA was taken up by control cells. Levels of uptake into PC, PI, PS and PE were 45 %, 8 %, 6 % and 41 %, respectively, in the MCF-7 control cells and 34 %, 3 %, 3 % and 60 %, respectively, in the SW480 control cells (Table 2.3 and 2.4).

Table 2.3 Effect of Fatty Acid Treatments on Incorporation of ^{14}C-AA into MCF-7 Phospholipid Fractions[1].

Fatty Acids treatments	PC	PI	PS	PE
Control	44.4 ± 9.7	8.0 ± 2.8	6.2 ± 4.0	41.4 ± 8.2
CLA mixture	33.5 ± 3.9	6.7 ± 3.7	11.4 ± 5.6	48.4 ± 3.3
*c*9, *t*11-CLA	11.6 ± 2.7[a]	4.3 ± 0.6	1.8 ± 0.5	82.2 ± 3.5[b]
*t*10, *c*12-CLA	27.5 ± 6.9	5.5 ± 1.7	10.8 ± 6.5	56.3 ± 3.0
LA	33.5 ± 1.6	6.8 ± 3.0	3.7 ± 3.1	55.9 ± 7.7

[1]Data represents the mean percentage incorporation of total cellular phoshoplipids ± SD for three separate experiments carried out in triplicate. Letters indicate values that are significantly different compared to controls ([a] denotes $p<0.05$ and [b] denotes $p<0.02$). Abbreviations: CLA, conjugated linoleic acid; LA, linoleic acid; PC, phosphatidycholine; PI, phosphatidylinositol; PS, phosphatidylserine; PE, phosphatidylethanolamine.

Table 2.4 Effect of Fatty Acid Treatments on Incorporation of ^{14}C-AA into SW480 Phospholipid Fractions[1].

Fatty Acids treatments	PC	PI	PS	PE
Control	32.5 ± 8.0	3.5 ± 0.3	2.5 ± 0.7	61.5 ± 8.5
CLA mixture	25.9 ± 1.7	6.7 ± 1.9	14.5 ± 1.2[a]	52.9 ± 0.8
*c*9, *t*11-CLA	8.3 ± 0.2[b]	3.7 ± 0.1	5.5 ± 2.2	82.5 ± 2.1[b]
*t*10, *c*12-CLA	25.8 ± 8.2	10.6 ± 4.4	9.1 ± 3.6[b]	54.4 ± 7.5
LA	36.7 ± 9.6	3.7 ± 1.1	3.2 ± 0.4	56.4 ± 8.5

[1]Data represents the mean percentage incorporation of total cellular phoshoplipids ± SD for three separate experiments carried out in triplicate. Letters indicate values that are significantly different compared to controls ([a] denotes $p<0.01$ and [b] denotes $p<0.05$). Abbreviations: CLA, conjugated linoleic acid; LA, linoleic acid; PC, phosphatidycholine; PI, phosphatidylinositol; PS, phosphatidylserine; PE, phosphatidylethanolamine.

Of all the treatments, only incubation with the pure *c*9, *t*11-CLA isomer altered the distribution of ^{14}C-AA among PL classes in the MCF-7 cells (Table 2.3). The *c*9, *t*11-CLA treatment at 16 μg/ml significantly ($p<0.05$) reduced uptake of ^{14}C-AA into PC (32 %) and increased uptake into PE (41 %). The CLA mixture at 16 μg/ml (which yielded a *c*9, *t*11-CLA isomer concentration of 4.7 μg/ml) had no effect. The *t*10, *c*12-CLA isomer at 16 μg/ml had no effect on the incorporation of ^{14}C-AA into any of the PL fractions in the MCF-7 cells.

Incubation of the SW480 cell line with the *c*9, *t*11-CLA isomer (16 μg/ml) decreased uptake of ^{14}C-AA into PC by 24 % ($p<0.01$) and increased uptake into PE by approximately 20 % ($p<0.01$) (Table 2.4). In contrast with MCF-7 cells, both the CLA mixture at 16 μg/ml (which yielded a *t*10, *c*12 -CLA isomer concentration of 4.7 μg/ml) and the *t*10, *c*12 isomer (16 μg/ml) increased uptake into PS by 12-15 % ($p<0.05$) in the

SW480 cell line. LA treatment had no effect on ^{14}C-AA distribution among PL fractions in either cell line. None of the CLA isomers or LA had any effect on the uptake of ^{14}C-AA in PI.

2.4.5 Effect of CLA isomers on AA release.

AA can be released by two major pathways, the first through the action of PLA_2 which catalyses the hydrolysis of *sn*-2 fatty acyl bond of membrane phospholipids to liberate free AA (Dennis, 1994) and secondly by sequential cleavage of PI by PLC and diacylgyceride lipase (Ballsinde *et al.*, 1991). IP_3 levels were used as an index of PLC activity in this study. The CLA mixture of isomers, the pure *c*9, *t*11- and *t*12, *c*10-CLA isomers and LA did not affect IP_3 in either cell line (Figure 2.5). Total ^{14}C-AA derivatives were increased by 28 % ($p<0.05$) in SW480 cells treated with LA only, while none of the CLA isomers had any effect on the total level of ^{14}C-AA derivatives released by cells (Figure 2.6).

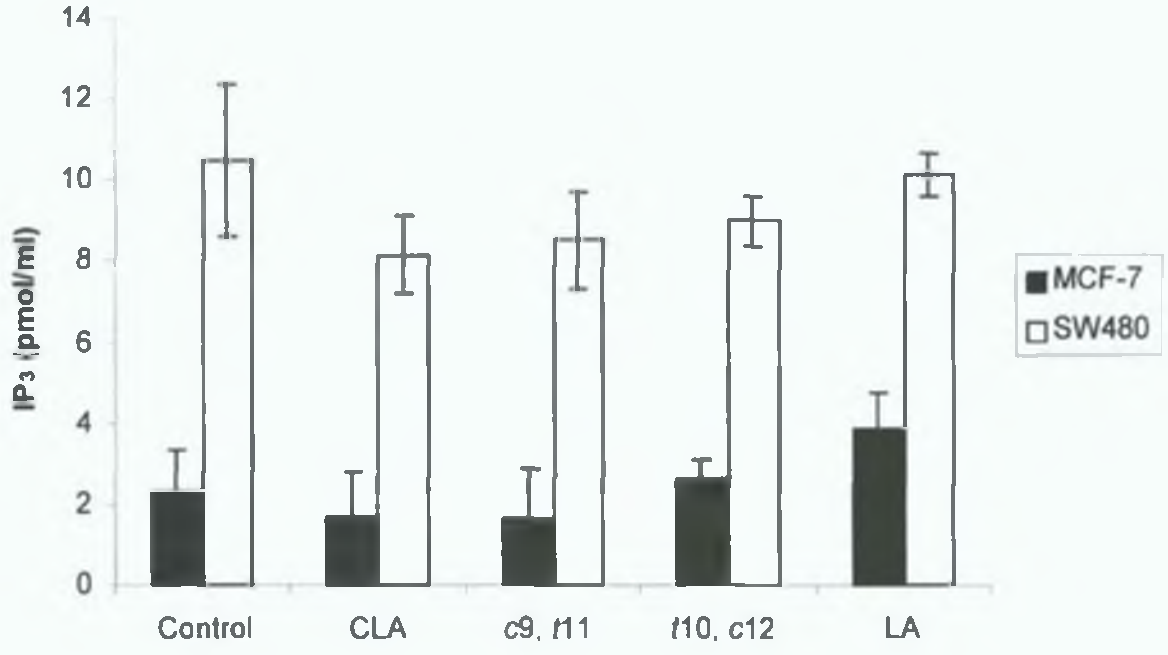


Figure 2.5 Effect of Fatty Acid Treatments on Inositol triphosphate (IP_3) levels in MCF-7 and SW480 cells. Cultures were treated with either the CLA mixture, LA, *c*9, *t*11-CLA, *t*10, *c*12-CLA, (16 µg/ml) or ethanol and then incubated for 24 h. Cells were harvested and IP_3 was extracted and quantifed using a radioreceptor assay kit. Data is expressed as the mean ± SD for three separate experiments carried out in triplicate.

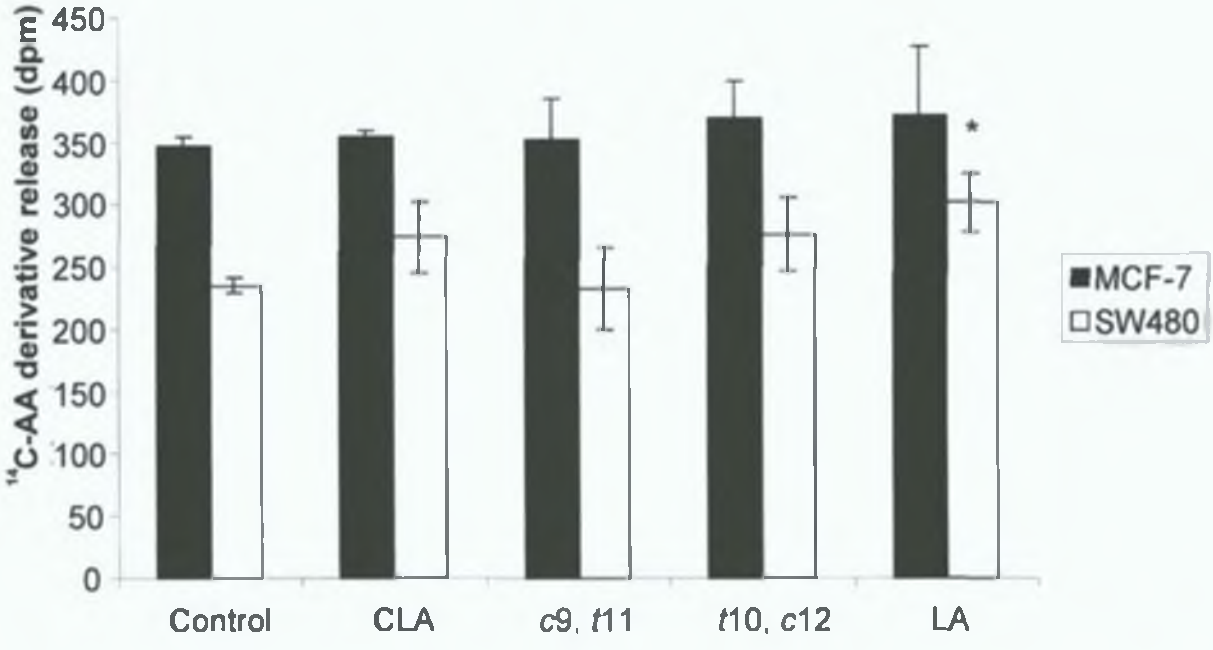


Figure 2.6 The effect of treatments on total ^{14}C-AA release in MCF-7 and SW480 cells. Cultures were treated with ^{14}C-AA at 0.2 μCi for 24 h after which medium was replaced to contain either CLA mixture, LA, *c*9, *t*11-CLA, *t*10, *c*12-CLA, (16 μg/ml) or ethanol and then incubated for 24 h. Medium containing the released ^{14}C-AA was removed and an aliquot was counted by liquid scintillation. Results were expressed as mean ^{14}C-AA released (dpm) ± SD for three separate experiments carried out in triplicate. * Denotes values that are significantly different ($p<0.05$) compared to controls.

2.4.6 Effect of CLA isomers on eicosanoid synthesis.

The effects of various fatty acid treatments on enzymatic conversion of AA to primary eicosanoids (PGD_2, PGE_2, $PGF_{2\alpha}$, LTB_4 and 5-HPETE) and on its non-enzymatic, free radical-catalyzed conversion to 8-epi-$PGF_{2\alpha}$, were examined. Following incubation of MCF-7 and SW480 cells in the presence of the CLA mixture and the pure *c*9, *t*11-CLA isomer at 16 μg/ml, it was found that ^{14}C-AA conversion to ^{14}C-PGE_2 was decreased by 20-30 % ($p<0.05$) while conversion to ^{14}C-$PGF_{2\alpha}$ was increased by 17-44 % relative to control (Figure 2.7a and 2.7b). CLA treatments had a negligible effect on ^{14}C-PGD_2. The *t*10, *c*12-CLA isomer had no effect on the three prostaglandins examined in either cell line. LA significantly ($p<0.05$) increased ^{14}C-PGD_2 by 13-19 % in both cell lines and increased ($p<0.05$) ^{14}C-PGE_2 by 20 % in the SW480 cell line only. Incubation of cells with either the CLA mixture of isomers or the pure *c*9, *t*11- or *t*10, *c*12-CLA isomers did not alter LOX activity or LTB_4 levels in the cells (Figure 2.8 and 2.9) suggesting that CLA may mediate its effect via the cyclooxygenase component of the AA cascade. Linoleic acid significantly increased LOX activity by 27 % ($p<0.05$) in the MCF-7 cell line (Figure 2.7) but had no effect in the SW480 cells compared with untreated controls. The *c*9, *t*11-CLA isomer significantly increased ($p<0.02$) 8-epi-$PGF_{2\alpha}$ in MCF-7 and SW480 cells by 38 % and 48 % respectively (Figure 2.7a and 2.7b). The *t*10, *c*12-CLA isomer increased ($p<0.05$) levels of 8-epi-$PGF_{2\alpha}$ by 30 % in the MCF-7 cell lines but had no effect in the SW480 cells (Figure 2.7b). However treatment of both cell lines with the mixture of CLA isomers and LA treatments had no relative effect in either cell line.

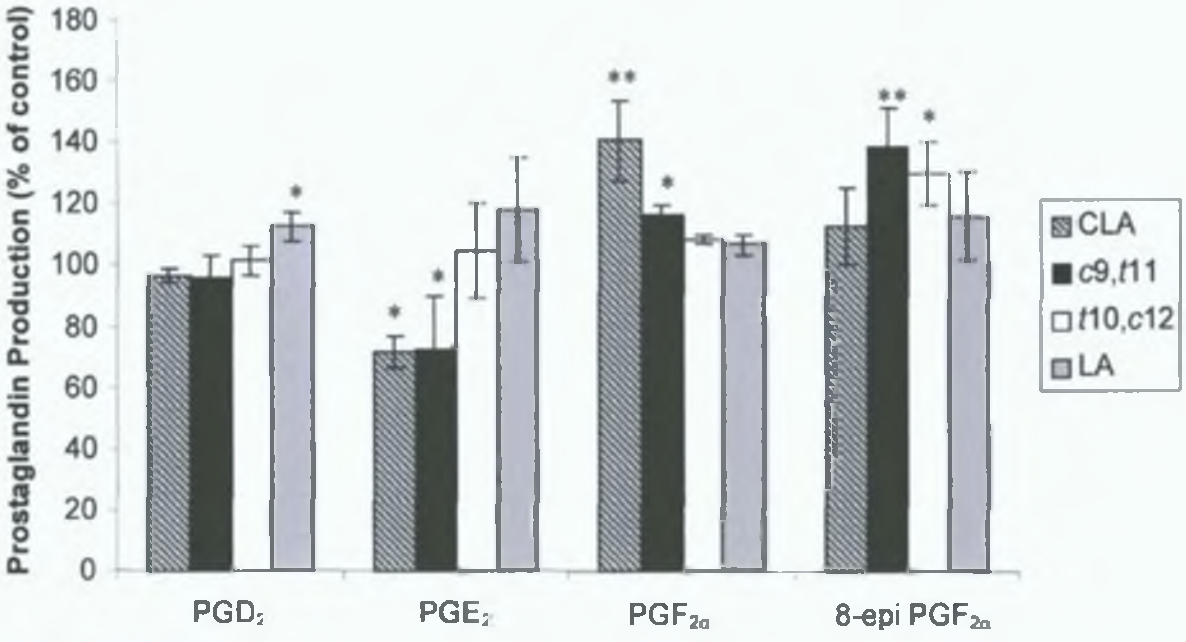


Figure 2.7 (a)

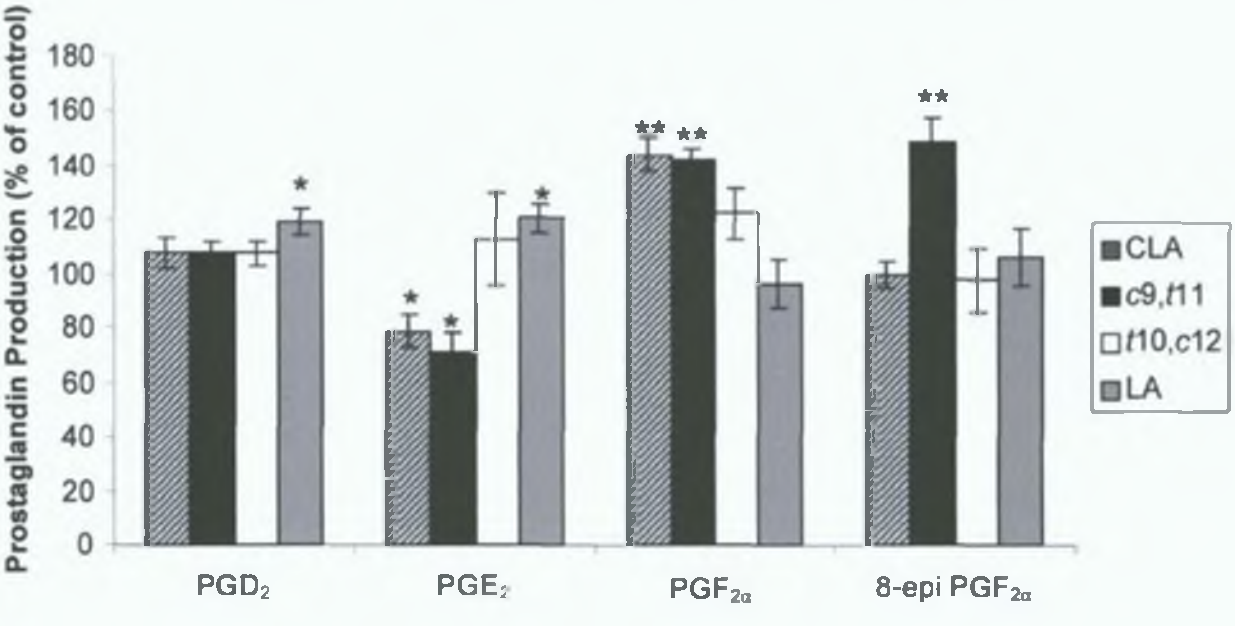


Figure 2.7 (b)

Figure 2.7 Effect of treatments on primary prostaglandins and 8-epi-$PGF_{2\alpha}$ synthesis in (a) MCF-7 and (b) SW480 cells. Cultures were treated with 0.2 μCi/ml ^{14}C-AA along with either the CLA mixture, *c*9, *t*11-CLA, *t*10, *c*12-CLA and LA (16 μg/ml) or ethanol

control for 24 h. Eicosanoids were extracted from medium and prostaglandins were separated using TLC and counted by liquid scintillation. Data represents the mean ^{14}C-Prostaglandin synthesis expressed as a percentage of the control which was taken to be 100 % ± SD for three separate experiments carried out in triplicate. 8-epi-$PGF_{2\alpha}$ levels were quantified using an enzyme immunoassay kit from Bioxytech. Asterisks indicate values that are significantly different compared to controls (**denotes $p<0.02$ and *denotes $p<0.05$).

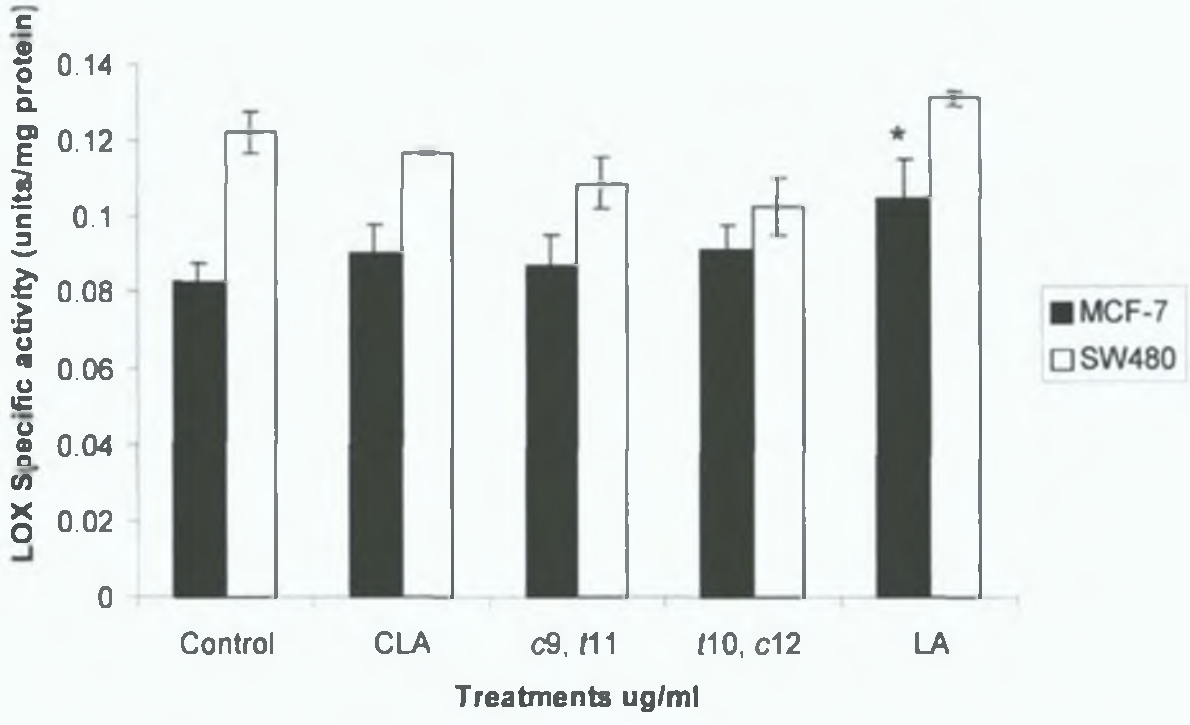


Figure 2.8 Effect of treatments on Lipoxygenase (LOX) activity in MCF-7 and SW480 cells. Cultures were treated with either the CLA mixture, LA, *c*9, *t*11-CLA, *t*10, *c*12-CLA, (16 µg/ml) or ethanol and then incubated for 24 h. Cells were harvested, lysed and analysed for LOX activity using a colorimetric assay. *Denotes values that are significantly different ($p<0.05$) compared to controls. Data is expressed as the mean ± SD for three separate experiments carried out in triplicate.

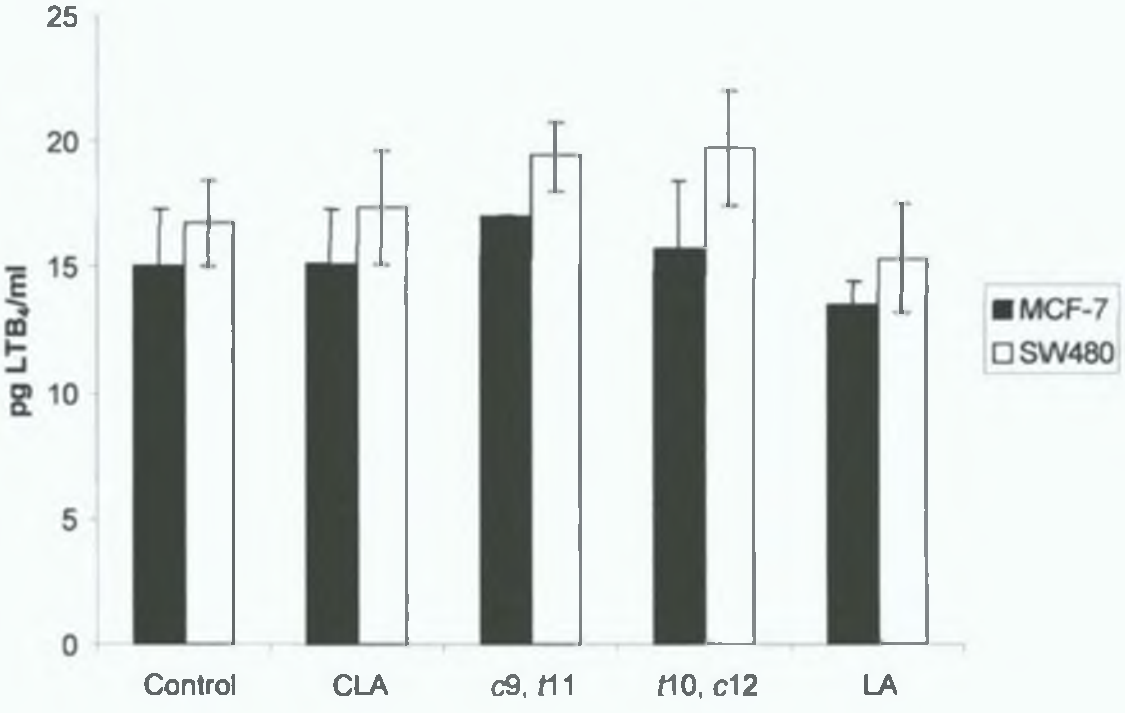


Figure 2.9 Effect of treatments on LTB_4 levels in MCF-7 and SW480 cells. Cultures were treated with either the CLA mixture, LA, *c*9, *t*11-CLA, *t*10, *c*12-CLA, (16 µg/ml) or ethanol and then incubated for 24 h. Cells were harvested and an enzyme immunoassay kit was used to quantify LTB_4 levels. Data is expressed as the mean ± SD for three separate experiments carried out in triplicate.

2.5 Discussion

This study shows that the MCF-7 and SW480 cell lines were sensitive to growth inhibitory effects of not only the CLA mixture but also to both the *t*10, *c*12-CLA and the *c*9, *t*11-CLA isomers following 4 days of incubation with physiological levels of CLA (5-16 μg/ml) (Shultz *et al.*, 1992). The CLA mixture of isomers at 16 μg/ml (yielding a *c*9, *t*11-CLA and *t*10, *c*12-CLA concentration of approximately 4.7 μg/ml each) was equally effective in inhibiting growth of both cell lines as the pure *c*9, *t*11-CLA and *t*10, *c*12-CLA isomer added at 16 μg/ml. This suggests that a plateau effect was reached or that one or more of the other isomer present in the mixture may be capable of altering cell viability. It is imperative however that more basic research be undertaken to determine the specific biological effects of other isomers present in the mixture, particularly *c*11, *t*13 which has recently been detected in natural products (Sehat *et al.*, 1999) and in liver microsomes (Pariza *et al.*, 1998). The growth stimulatory effect of LA previously reported (Shultz *et al.*, 1992, Cunningham *et al.*, 1997) was also seen in this study in the SW480 cell line treated with LA, but no effect was seen in the MCF-7 cells at the concentrations used.

This study provides an insight into the early responses of breast and colon cancer cell lines before growth was altered. Interestingly, the CLA mixture of isomers containing 4.8 μg/ml *c*9, *t*11-CLA was less effective than the pure *c*9, *t*11-CLA isomer (16μg/ml) at redistributing AA among lipid fractions in the MCF-7 cell line and had no effect in altering AA content of individual PL of these cells. Our data demonstrate that the *c*9, *t*11-CLA isomer decreased AA uptake into PC while increasing uptake into PE in both cell

lines. The decrease in uptake into PC is very significant as this is the PL preferentially hydrolysed by PLA_2 to provide AA for eicosanoid synthesis [Hanel *et al.*, 1993). None of the other treatments had any effect on PS in the MCF-7 cell line but in the SW480 cell line the CLA mixture and the *t*10, *c*12-CLA isomer both increased uptake of AA into PS. Although PS is a biosynthetic precursor of PE, it is in itself an important membrane lipid as it is an activator of membrane associated protein kinase C, an enzyme that phosphorylates serine and threonine residues of an extremely diverse group of proteins regulating cell proliferation, activating cellular function, differentiation and even apoptosis (Musashi *et al.*, 2000). It has been postulated that CLA may modulate protein kinase C (Belury, 1995). However, activation of this enzyme is also dependent on DAG, a product of PLC activity and Ca^{++} released from intracellular stores by IP_3. None of the treatments investigated altered the levels of IP_3 in the cells or uptake of AA into PI, suggesting that growth modulatory effects of various treatments in this study were not associated with phospholipase C mediated signal transduction. Other reports also indicate that physiological concentrations of CLA did not mediate changes in either PLC or PKC activity in MCF-7 cells (Park *et al.*, 2000) or in normal rat mammary epithelial cell organoids (Ip *et al.*, 1999).

Interestingly, none of the CLA treatments influenced AA release from cells, yet both the CLA mixture and the *c*9, *t*11-CLA isomer decreased ^{14}C-PGE_2 synthesis and increased ^{14}C-$PGF_{2\alpha}$ in both cell lines suggesting that a modulation of cyclooxygenase and/or downstream isomerase or reductase gene expression may be responsible. By contrast, LA stimulated PGD_2 production in both cell lines while stimulating PGE_2 production in

the SW480 cell line. These changes in prostaglandin synthesis may have been responsible for the differential effects of LA and CLA treatments on growth. Levels of LTB_4 and LOX activity were not altered by any of the CLA treatments suggesting that the anticancer effect of CLA may be mediated independently of the lipoxygenase component of the AA cascade as has been already proposed (Sugano *et al.*, 1998, Truitt *et al.*, 1999).

A differential effect between physiological levels (0.5-5 μg/ml) of *c*9, *t*11 CLA and LA on growth of MCF-7 cells after 4 days has been reported (Park *et al.*, 2000). Growth inhibition by the CLA isomer was not mediated through PLC, PKC or PGE_2-dependent signal transduction pathways suggesting that another inhibitory mechanism may be involved. Because our study did show that PGE_2 synthesis was reduced by higher but near physiological concentrations of CLA it is apparent that there may be a threshold requirement for CLA and LA to affect cellular PGE_2 synthesis. A similar inhibitory effect of CLA on PGE_2 synthesis was observed in keratinocytes (Liu and Belury, 1998) and mouse epidermis (Kavanaugh *et al.*, 1999). More recently CLA has been shown to inhibit prostaglandin H synthase activity in ram seminal vesicle microsomes (Bulgarella *et al.*, 2001).

Basu and co-worker (Basu *et al.*, 2000) reported that CLA induced lipid peroxidation in humans, using urinary 8-iso-$PGF_{2\alpha}$ excretion as a biomarker of non-enzymatic lipid peroxidation. We showed that incubation of both cell lines with the *c*9, *t*11-CLA isomer led to significantly increased 8-epi-$PGF_{2\alpha}$ in both cell lines while incubation with the *t*10, *c*12-CLA led to increases in 8-epi-$PGF_{2\alpha}$ levels in the MCF-7 cell line only. These

isomers may be promoting non-enzymatic oxidation of AA at the expense of the formation of enzymatically derived eicosanoids. The mixture of CLA isomers (at 16 µg/ml) had no effect on 8-epi-$PGF_{2\alpha}$ levels suggesting that a higher concentration of *c*9, *t*11-CLA than 4.7 µg/ml is needed to induce non-enzymatic oxidation of arachidonic acid. A number of studies have now shown that the production of reactive oxygen species serves to trigger an apoptotic signal transduction pathway (reviewed in Rudolph *et al.*, 2001). Further studies to investigate the effects of CLA isomers on the expression of cyclooxygenase isoforms and other signal transduction pathways are warranted to explain the potential inhibitory role of CLA on *in vitro* growth. Intervention studies have shown that increasing CLA intake led to increases in the CLA content in human milk (Park *et al.*, 1999), plasma (Huang *et al.*, 1994), and adipose tissue (Jiang *et al.*, 1999). Although it is attractive to speculate that CLA may be useful in nutritional prevention of cancer in humans, evidence of beneficial effects in cancer patients receiving CLA as dietary supplements is required. To this end, appropriate molecular and biochemical markers of both CLA nutritional status and of tumorigenesis are currently being sought.

2.6 Summary

The relationship between growth and alterations in AA metabolism in human breast (MCF-7) and colon (SW480) cancer cells was studied. Four different fatty acid preparations were evaluated: a mixture of CLA isomers (*c*9, *t*11, *t*10, *c*12, *c*11, *t*13 and minor amounts of other isomers), the pure *c*9, *t*11-CLA isomer, the pure *t*10, *c*12-CLA isomer and LA (all at a lipid concentration of 16 µg/ml). ^{14}C-AA uptake into the MG

fraction of MCF-7 cells was significantly increased following 24 h incubation with the CLA mixture ($p<0.05$) and *c*9, *t*11-CLA ($p<0.02$). In contrast to the MCF-7 cells, ^{14}C-AA uptake into the TG fraction of the SW480 cells was increased while uptake into the PL was reduced following treatment with the CLA mixture ($p<0.02$) and *c*9, *t*11-CLA ($p<0.05$). Distribution of ^{14}C-AA among PL classes was altered by CLA treatments in both cell lines. The *c*9, *t*11-CLA isomer decreased ($p<0.05$) uptake of ^{14}C-AA into PC while increasing ($p<0.05$) uptake into PE in both cell lines. Both the CLA mixture and the *t*10, c12-CLA isomer increased ($p<0.01$) uptake of ^{14}C-AA into PS in the SW480 cells but had no effect on PL in the MCF-7 cells. Release of ^{14}C-AA derivatives was not altered by CLA treatments but was increased ($p<0.05$) by LA in the SW480 cell line. The CLA mixture of isomers and *c*9, *t*11-CLA isomer inhibited ^{14}C-AA conversion to ^{14}C-PGE_2 by 20-30% ($p<0.05$) while increasing ^{14}C-$PGF_{2\alpha}$ by 17-44 % relative to controls in both cell lines. LA significantly ($p<0.05$) increased ^{14}C-PGD_2 by 13-19 % in both cell lines and increased ^{14}C-PGE_2 by 20 % in the SW480 cell line only. LA significantly ($p<0.05$) increased lipoxygenase activity by 27 % in the MCF-7 cell line. Lipid peroxidation, as determined by increased levels of 8-epi-$PGF_{2\alpha}$, was observed following treatment with *c*9, *t*11-CLA isomer in both cell lines ($p<0.02$) and with *t*10, *c*12-CLA isomer in the MCF-7 cell line only ($p<0.05$). These data indicate that the growth promoting effects of LA in the SW480 cell line may be associated with enhanced conversion of AA to PGE_2 but that the growth suppressive effects of CLA isomers in both cell lines may be due to changes in AA distribution among cellular lipids and an altered prostaglandin profile.

CHAPTER 3

Cis 9, *trans* 11- and *trans* 10, *cis* 12-conjugated linoleic acid isomers induce apoptosis in cultured SW480 cells.[1]

[1] Published in Anticancer Research, in press

3 1 Introduction

The majority of human cancers are thought to be the result of various environmental factors with diet being a potent modifying factor (Ames *et al*, 1995) Epidemiological data suggest that diet accounts for approximately one-third of all cancer deaths in affluent populations (Doll, 1992) The human diet contains foods and beverages that may either contribute to induction or prevention of cancer (Wynder, 1997) Although there is a strong correlation between diet and cancer, especially between dietary fat and cancer, differential effects have arisen with respect to individual fatty acids (Guthrie and Carroll, 1999) Conjugated linoleic acid (CLA) is a polyunsaturated fatty acid that has been highly publicised recently as a result of its potent anticarcinogenic properties observed at low dietary levels (< 0 1 % w/w of diet) in animal models of carcinogenesis (Ip *et al*, 1991, Ip *et al*, 1966, Ip *et al*, 1997a, Ip *et al*, 1997b, Ha *et al*, 1990, Belury *et al*, 1996, Liew *et al*, 1995) CLA refers generically to the class of positional and geometric conjugated isomers of linoleic acid, several of which are naturally abundant in food lipids derived from ruminant animals and dairy products (Sehat *et al*, 1999) Increasing the CLA content of certain foods and combining CLA intake with traditional chemotherapy could form a dietary approach to the prevention and treatment of human cancers in the future In addition to its important role in the dietary prevention of cancer in rodent models, CLA exhibits antiatherogenic, antidiabetogenic, antiallergenic, immunomodulating, body composition modulating and bone growth enhancing properties (Pariza *et al*, 2001)

In order to determine the preventive and therapeutic potential of CLA against cancer, its mode of action must first be defined Although the precise mechanisms through which

CLA executes its anticarcinogenicity have yet to be ascertained, several mechanisms have been proposed (reviewed in Belury, 2002) including the initiation of apoptosis Induction of apoptosis in response to CLA was first shown in differentiated rat mammary epithelial colonies as detected by the presence of pyknotic nucleic and double strand breaks in DNA (Ip *et al*, 1999b) In another study, CLA induced apoptosis in cultured mammary tumor cells as determined by DNA fragmentation and also in premahgant lesions of the rat mammary gland via down regulation of the membrane protein bcl-2 (Ip *et al*, 2000) More recently, dietary CLA was shown to stimulate apoptosis in the colon mucosa of 1,2-dimethylhydrazine-treated rats as measured by the terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling technique (Park *et al*, 2001)

It has been demonstrated that cytotoxic effect of CLA isomers in the human SW480 colon tumor cell line was associated with an increased lipid peroxidation leading to activation of cellular antioxidant defence enzymes (O'Shea *et al*, 1999) An increase in superoxide dismutase observed in CLA-treated cells suggests an availability of reactive oxygen species that may initiate cellular death programs leading to apoptosis (O'Shea *et al*, 1999) CLA isomers altered the distribution of arachidonic acid in cellular phospholipids and altered the pattern of eicosanoid production in colon and mammary tumor cells lines (Chapter 2) Both phenomena are indicative of a signaling process regulating apoptosis being initiated at the membrane level

3 2 Objective

The aim of this present study was to elucidate whether apoptosis is the mode of death induced by CLA isomers in the colon SW480 tumor cell line by examining specific markers synonymous with apoptosis The effect of 3 different CLA preparations (the *c*9, *t*11-CLA isomer, the *t*10, *c*12-CLA isomer and the CLA mixture of isomers) on cell morphology, annexin V levels, DNA laddering, the expression of apoptosis regulatory proteins (bcl_2, bax, Apaf-1, cytochrome c and active caspase 3) and on activities of caspases-3 and 9 were evaluated

3 3 Materials and methods

3 3 1 Cell culture and CLA treatments

The SW480 human colon tumor cell line was cultured exactly as outlined in Chapter 2 Three different CLA preparations were evaluated the *c*9, *t*11-CLA isomer, the *t*10, *c*12-CLA isomer and a mixture of CLA isomers All were added at a lipid concentration of 16 μg/ml (57 μM) except in the viability experiment where a range of concentrations were used The concentrations lie within the physiological range of concentrations of the *c*9, *t*11-CLA isomer in human phospholipids (Iversen *et al*, 1985), plasma, bile, duodenal juice (Cawood *et al*, 1983) and has been previously used in cell culture work (Chapter 2 and Shultz *et al*, 1992) Control flasks were supplemented with ethanol at a final concentration of 0 8 % (v/v) as in experimental flasks The CLA mixture of isomers (21 % *c*11, *t*13, 29 0 % *t*10, *c*12, 29 5 % *c*9, *t*11 and 12 3 % *c*8, *t*10) was obtained from Nu Chek Prep (Elysian, MN, USA) Individual CLA isomers, *c*9, *t*11 (95 % pure) and *t*10, *c*12 (95 % pure), were kindly donated by Natural ASA (Hovdebygda, Norway)

3 3 2 Effect of CLA isomers on cell viability

SW480 cells were plated at 5 x 10^4 cells/well in six well plates and cells were cultured for 24 h to allow the cells to attach to the substratum The medium was then replaced with medium supplemented with the CLA mixture of isomers, the *c*9, *t*11-CLA isomer, or the *t*10, *c*12-CLA isomer at a range of concentrations (5, 10, 16 and 20 μg/ml) dissolved in ethanol Control wells were supplemented with an equivalent volume of ethanol Following 4 days of incubation, cells were harvested using phosphate buffered saline (PBS) containing 0 25 % (v/v) trypsin Cell viability was determined using the trypan blue exclusion (0 4 % w/v) method

3 3 3 Morphological analysis using acridine orange staining

To evaluate the effect of the CLA isomers on cell morphology, SW480 cells were plated at 5 x 10^4 cells/well in six well plates containing coverslips The cells were cultured for 24 h to allow the cells to attach to the coverslips The medium was then removed from each well and replaced with fresh medium containing the various treatments or linoleic acid (LA) (Sigma-Aldrich Ireland Ltd , Dublin, Ireland) added at a lipid concentration of 16 μg/mL LA was included as a negative control After 4 days of incubation, the covershps were removed from the wells and fixed in ice cold acetone for 5 min and rehydrated in PBS for 10 min as described previously (Ip *et al* , 2000) Covershps were then stained with acridine orange (Sigma-Aldrich Ireland Ltd , Dublm, Ireland) at a concentration of 10 μg/mL for 5 min, rinsed with PBS, mounted on slides and viewed directly with a flourescent Zeiss Axioskop ultraviolet light microscope (Carl Zeiss Microimaging, Thornwood, NY, USA) Images from a minimum of 6 fields were

photographed using a 3CCD KY-F50 JVC colour video camera (Vitor Companies of Japan Ltd , Japan) and images were captured using Optimus software version 6 5 (Media Cybernetics, Silverspnngs, MD, USA) Cells were scored as healthy or apoptotic using the following criteria viable cells remained adherent and had an oval nuclear morphology while apoptotic cells were rounded up from the coverslip and displayed concentrated acridine orange staining of the condensed chromatin (Figure 3 2, inset) Hard copy pictures of each field were printed out and the % apoptotic cells/field was determined

3 3 4 DNA laddering

Cells were seeded in T-75 cm^2 flasks at a density of 1 x 10^6 cells/flask and were incubated for 24 h The medium was then replaced with fresh media containing the 3 different CLA treatments (all at 16 μg/mL) or ethanol as described above and incubated for 4 days Adherent and floating/loosely-attached cells were collected and processed separately Apoptotic DNA was extracted and precipitated using the Suicide-Track DNA Laddering Isolation Kit (Oncogene Research Products, Boston, MA, USA) according to the manufacturer's instructions This kit had been optimised to separate apoptotic DNA from high molecular weight, intact, genomic DNA In brief, the cell pellet was resuspended in extraction buffer (provided by kit), incubated on ice for 30 min and then centrifuged at 15, 000 xg for 5 min at room temperature The supernatant was removed (containing the apoptotic DNA) and was treated with RNase A and Proteinase K (both provided ny the kit) DNA was precipitated with 3M sodium acetate, pH 5 2 with the aid of pellet paint co-precipitant (provided by the kit) The DNA pellet was rinsed twice with

70 % ethanol and resuspeneded in resuspension buffer (provided by kit) prior to electrophoresis DNA was analysed by electrophoresis in a 1 5 % agarose gel at 50 V (constant volts) for 4 5 h The gel was then stained for 0 5 h with ethidium bromide (0 5 μg/mL) DNA fragments were visualised and photographed using the ImageMaster VDS documentation system from Amersham Biosciences (Little Chadfort, Buckinghamshire, UK)

3 3 5 Western analysis of apoptosis regulatory proteins

Cells were seeded in T-150 cm^2 flasks at a density of 2 x 10^6 cells/flask and allowed to culture for 24 h The medium was then replaced with fresh medium containing the three different CLA treatments (all at 16 μg/mL) as described above The *c*9, *t*11-CLA isomer was also added to the cells at four different concentrations (10, 16, 20, 25 μg/mL) to determine its effect on the bcl-2 protein After 4 days of incubation, both floating cells and adherent cells were collected and pooled Cells were washed twice in ice-cold PBS and resuspended in lysis buffer (10 mM sodium phosphate buffer (pH 7 2), 0 2 mM phenylmethysulfonylfluoride (PMSF) and 100 mM NaCl) containing 0 1 mM leupeptin and 0 2 μg/mL aprotinin as protease inhibitors (both supplied by Sigma-Aldrich Ireland Ltd , Dublin, Ireland) Lysates were somcated using a Vibra Cell VC502 (Sonics, Newtown, CT, USA) on ice and concentrated using Microcons® YM-10 which contain membranes with a 10,000 molecular weight cut off (Millipore, Cork, Ireland) Protein concentrations were determined using the Bio-Rad protein assay (Biorad, Hemel Hempstead, Hertforshire, UK) which is based on the principles of the Bradford assay and used bovine serum albumin as the protein standard SDS-PAGE and Western blots were

carried out essentially by the methods of Laemmli (1970) and Towbin *et al* (1979), respectively Lysates containing approximately 70 μg of protein were solubilized in sample buffer (10 % (w/v) SDS, 600 mM Tris-HCl (pH 6 7) and 50 % (w/v) glycerol) containing β-mercaptoethanol and 50 μg/mL bromophenol blue Samples were boiled for 2 min and resolved by extended electrophoresis Electrophoretically resolved proteins were blotted onto Hybond ECL membrane (Amersham, Little Chadfort, Buckinghamshire, UK) in a Trans-blot Electrophoretic transfer cell (Biorad, Hemel Hempstead, Hertforshire, UK) Blots were stained with Ponceau S Solution (0 2 % w/v) to insure transfer of proteins was complete and to determine if equivalent amount of protein was loaded in each lane The blots were destained with PBS containing 0 1 % (v/v) Tween 20 (PBST) and blocked for 1 h with 5 % nonfat dry milk dissolved in PBST Blots were then incubated with monoclonal antibodies overnight anti-bcl-2 diluted to 1 1000, anti-bax diluted to 1 2000 (both from Sigma-Aldrich Ireland Ltd , Dublin, Ireland), anti-Apaf-1 diluted to 1 250 (Transduction Laboratories, Lexington, KY) or anti-caspase 3 diluted to 1 300 (Alexis Biochemicals, San Diego, CA, USA) in PBST containing 0 5 % nonfat dry milk Blots were washed extensively in PBST and reincubated for 1 h with a horseradish peroxidase-linked secondary antibody (Amersham, Little Chadfort, Buckinghamshire, UK) diluted 1 2000 in PBST containing 0 5 % nonfat dry milk The blots were then thoroughly washed in excess PBST and probed with the Super Signal detection system (Pierce, Rockford, IL, USA) and exposed to autoradiography films (Amersham, Little Chadfort, Buckinghamshire, UK) according to the manufacturer's instructions Densitometry (using NIH Image software) was performed on Ponceau S scans and autoradiographs

3 3 6 Detection of cytochrome c release into cytosol and annexin V levels in membrane preparation

Cells were seeded in T-150 cm^2 flasks at a density of 2 x 10^6 cells/flask and incubated for 24 h The medium was then replaced with fresh medium containing the three different CLA treatments (all at 16 μg/mL) described above The *c*9, *t*11-CLA isomer was also added to the cells at four different concentrations (10, 16, 20, 25 μg/mL) to determine its effect on cytochrome c release After 4 days of incubation floating cells and adherent cells were collected and pooled Cytochrome c release was detected as previously described with minor modification (Tang *et al*, 1998) Briefly, cells were resuspended in a PBS/PMSF buffer (containing 10 mM sodium phosphate buffer (pH 7 2), 100 mM NaCl, 0 2 mM PMSF, 0 1 mM leupeptin, 0 2 μg/ml aprotinin), sonicated on ice and centrifuged at 100,000 x g for 20 min at 4°C The supernatant (cytosolic fraction) was removed and concentrated using Microcons® YM-10 (10,000 molecular weight cut off) (Millipore, Cork, Ireland) The pellet was resuspended in lysis buffer (described earlier) and incubated on ice for 20 min followed by centrifugation at 15,000 x g for 15 min at 4°C The resulting supernatant (the membrane fraction) was analysed for annexin V levels using Annexin V Elisa kit (Alexis Biochemicals, San Diego, CA, USA) according to the manufacturers instructions In brief, annexin V present in the samples or standards bound to an anti-annexin V monoclonal antibody that was adsorbed to the microwells A biotin-conjugated monoclonal anti-annexin V antibody was then added which bound to annexin V captured by the first antibody Streptavidin-HRP was then added and it bound to the biotin-conjugated anti-annexin V A substrate solution reactive with HRP was added to the wells which resulted in the formation of a coloured product The reaction

was terminated by the addition of phosphoric acid and the absorbance was measured at 405 nm The amount of coloured product formed was proportional to the amount of annexin V in the sample Annexin V sample concentration was determined from an annexin V standard curve The cytosolic fractions were analysed by western blot analysis using an anti-cytochrome c monoclonal antibody diluted to 1 300 (Alexis Biochemicals, San Diego, CA)

3 3 7 Measurement of reduced glutathione levels

Cells were seeded in T-75 cm^2 flasks at a density of 1 x 10^6 cells/flask and incubated for 24 h The medium was then replaced with fresh medium containing the three different CLA treatments (all at 16 μg/mL) described earlier and incubated for 4 days after which both floating and adherent cells were collected and pooled Cytosol fractions were prepared as described above The levels of reduced glutathione (GSH) in the cytosol fractions was measured according to the method of Hissen and Hilf (1976) The method takes advantage of the reaction of GSH with *o*-phthaldaldehyde (OPT) at pH 8 Briefly, cytosol fractions (100 μL) were diluted in 1 8 ml phosphate-EDTA buffer (0 1 M sodium phosphate, 0 005 M EDTA, pH 8) and mixed with 100 μL OPT (10 μg/ml) Samples were incubated at 25 °C for 15 min and fluoresence detected at 350 nm excitation and 420 nm emission wavelengths The reduced glutathione concentration of the samples was determined from a standard curve Reduced glutathione levels were expressed relative to the protein content, as determined using the Bio-Rad protein assay

3 3 8 Measurement of caspase 3 and 9 activities

Cells were seeded in T-75 cm^2 flasks at a density of 1 x 10^6 cells/flask and incubated for 24 h The medium was then replaced with fresh medium containing the three different CLA treatments (all at 16 μg/mL) or ethanol described earlier After 4 days incubation, both adherent and floating cells were collected and pooled The levels of caspase 3 and 9 enzyme activities were determined using colorimetric assay kits from R&D Systems Inc , (Minneapolis, MN, USA) Cells were lysed by addition of lysate buffer (provided by the kit) and incubated on ice for 10 min followed by centrifugation at 10,000 x g for 1 min The resulting supernatant was transferred to a fresh tube and kept on ice The protein concentration in the lysate was determined using the Bio-Rad protem assay 50 μl of each cell lysate was placed in a 96-well plate 10 μl dithiothreitol (DTT) was added to 1 mL of reaction buffer (provided by kit) and 50 μl of this combined solution was added to each reaction well 5 μl of the appropriate colorimetric substrate peptide (caspase 3 DEVD-p-nitroanaline or caspase 9 LEHD-p-nitroanaline) was added to reaction wells The cleavage of the peptide by the caspases present in the samples released the chromophore p-mtroanaline which could be quantified spectrophormetrically A control that contained no lysate and another that contained no substrate were included The plate was incubated at 37°C for 2 h and absorbence was read using an Anthos 2010 microplate reader at 405 nm The level of caspase enzymatic activity in the cell lysate was directly proportional to the colour formation

3 3 9 Statistical Analysis

Data represent three independent experiments carried out in triplicate The Student's *t* test was used to determine significance between treatments

3 4 Results

3 4 1 CLA isomers stimulated apoptosis in SW480 human colon cancer cell line

The SW480 cell line was sensitive to the growth inhibitory effect of the CLA isomers as previously reported (Chapter 2) This study evaluated a more extensive range of the different CLA preparations to determine if differences in efficacy occurred The CLA mixture of isomers and the pure *c*9, *t*11-CLA isomer caused a similar reduction (40-52 %) in cell viability over the concentrated range examined (Figure 3 1) The *t*10, *c*12-CLA isomer was the most effective at reducing cell viability, reducing it by 47-61 % The *t*10, *c*12-CLA isomer was more potent than the pure *c*9, *t*11-CLA isomer but was equipotent to the CLA mixture of isomers

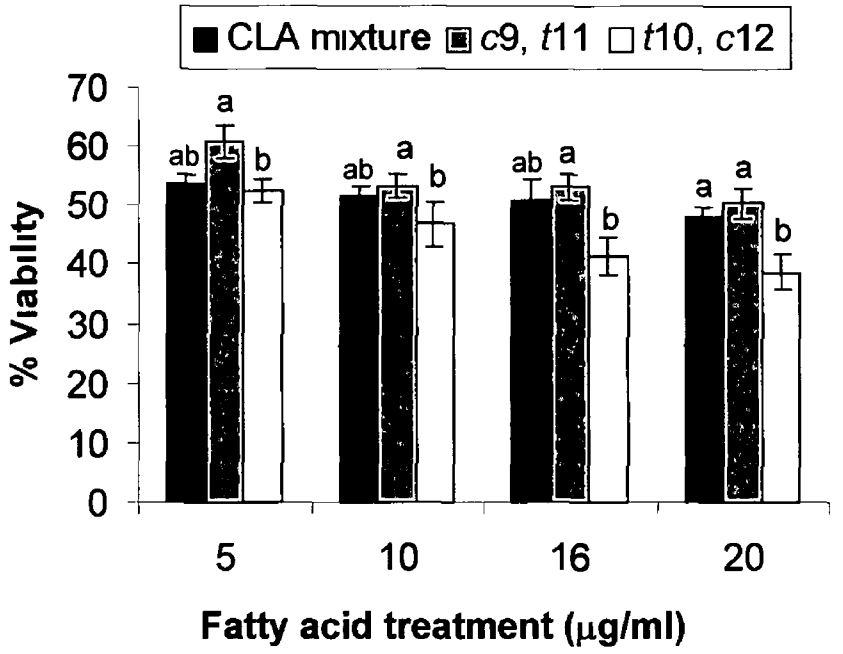


Figure 3 1 Percentage cell viability (relative to control) of SW480 cells incubated with varying levels of CLA isomers for 4 days Within a concentration, the bars not sharing the same letters are significantly different from one another ($p<0.05$) – for example if one bar has the letters 'ab' and another bar has the letter 'a', these two bars are not significantly different from each other as they have a letter in common Data is expressed as the percentage mean ± SD for three separate experiments carried out in triplicate

In order to determine whether this decrease in cell numbers was due to an induction of apoptosis, two markers of apoptosis were initially evaluated in this present study: changes in nuclear morphology and induction of DNA laddering. Nuclear morphology was examined using acridine orange, a fluorescent dye that binds to DNA. Healthy cells remained attached, had an oval nuclear morphology and showed moderate fluoresence, while apoptotic cells were rounded up from the coverslip and fluoresced very brightly due to marked condensation of chromatin. SW480 cells exposed to the CLA mixture of isomers, the *c*9, *t*11-CLA and *t*10, *c*12-CLA isomers but not LA, demonstrated a significant ($p<0.05$) increase (96 %, 107 % and 133 % respectively) in the percentage of apoptotic cells compared with controls (Figure 3.2).

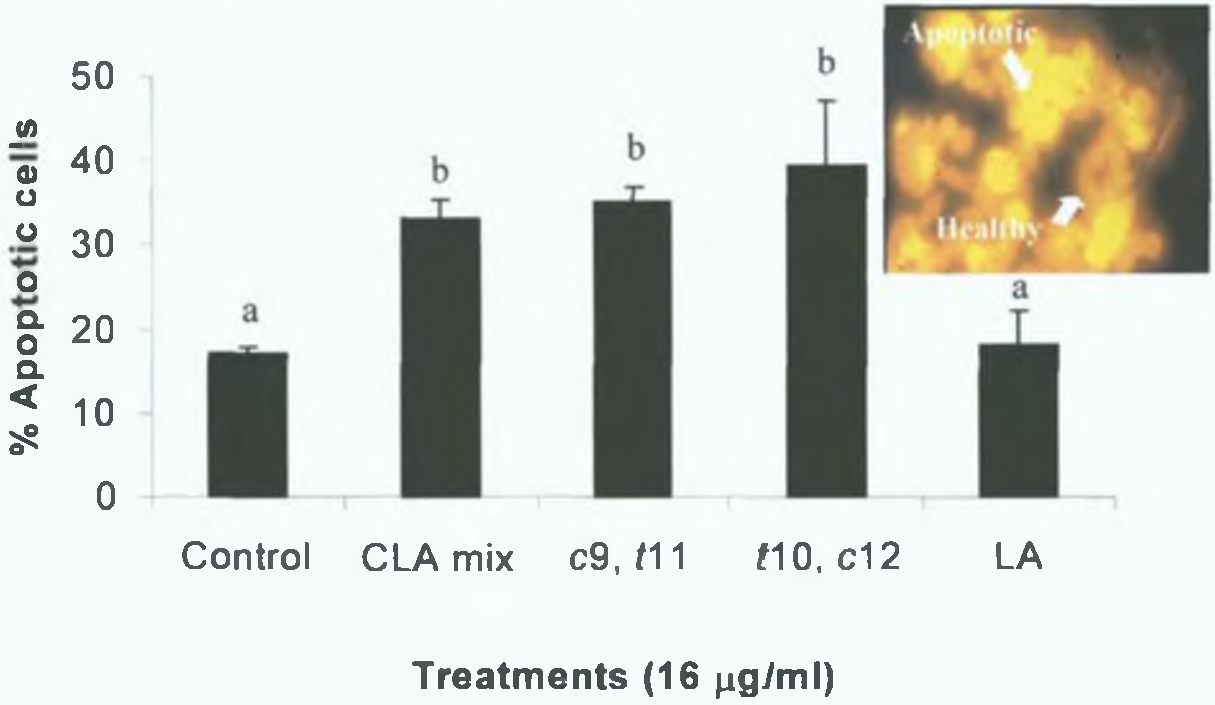


Figure 3.2 Increase in apoptosis of SW480 tumor cells by CLA isomers for 4 days. Bars not sharing the same letters are significantly different from one another ($p<0.05$). Data is expressed as the percentage mean ± SD for three separate experiments carried out in

triplicate Apoptotic cells rounded up from the coverslip and the inset shows acridine orange-stained condensed chromatin Health cells had an oval morphology and remained adherent

The formation of distinct DNA fragments of ohgnucleosomal size (180-200 bp) is a biochemical hallmark of apoptosis in many cells (Bortner *et al*, 1995) and is observed as a DNA ladder in agarose gels Evidence was sought to determine if the cytotoxic action of CLA isomers induced a specific pattern of chromatin cleavage into oligonucleosomes After cells were treated with the CLA isomers for 4 days, the media and PBS wash of the monolayer (both of which may contain apoptotic cells which have floated loose from the monolayer) were collected separately from the attached cells in the monolayer Following CLA treatments, all three sets of cells exhibited the characteristic ladder on electrophoresis of DNA extracted from the floating cells (Figure 3 3a) A small amount of DNA laddering was observed in the control cells which would be expected as a small percentage of cells die normally in culture No DNA laddering was observed in DNA extracted from adherent cells (Figure 3 3b)

A critical stage in the initiation of apoptosis involves surface changes on the cell membranes that include the exposure of phosphatidylserine on the external leaflet of the lipid bilayer Annexin V is a protein present in the extracellular space of cells which binds to phosphatidylserine-exposing apoptotic cells (Cruikshank *et al*, 1987) All CLA treatments significantly ($p<0.05$) increased levels of annexin V (29-32%) in the cell

membrane when compared with concentrations found in the membrane preparations of control cells (Figure 3 4)

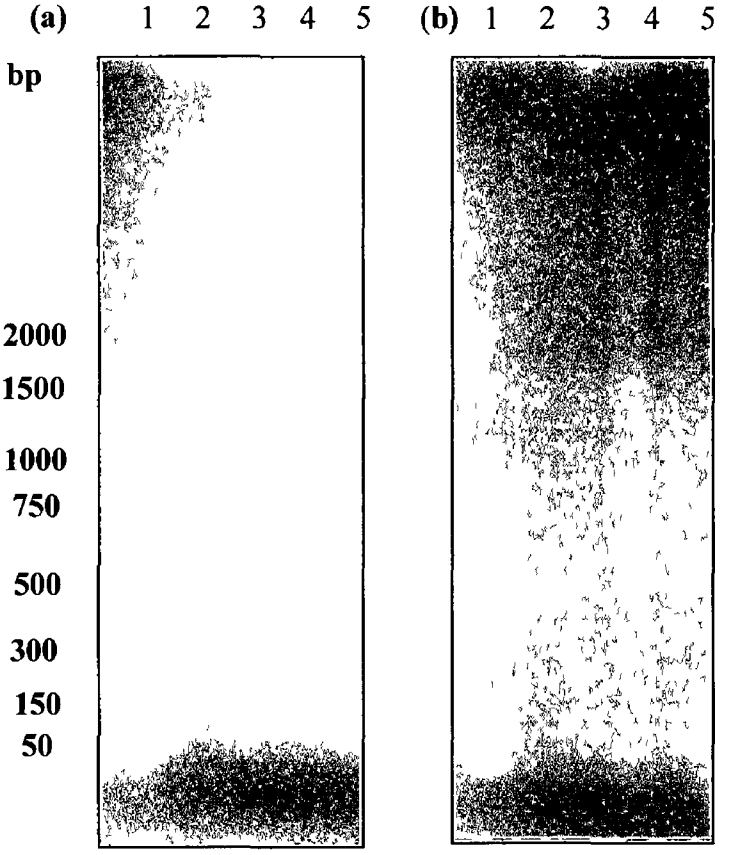


Figure 3.3 Increase in DNA laddering in SW480 tumor cells by CLA (a) Floating SW480 cells collected after 4 days incubation with CLA isomers (all at a lipid concentration of 16 μg/ml) 1 = marker lane containing DNA fragments ranging from 50 to 2000 base pairs, 2 = Control cells treated with ethanol, 3 = CLA mixture of isomers, 4 = the c9, *t*11-CLA isomer, 5 = the *t*10, *c*12-CLA isomer (b) Adherent SW480 cells collected after 4 days incubation with CLA isomers (all at a lipid concentration of 16 μg/ml) Lanes are as described in (a) This figure is representative of three separate experiments

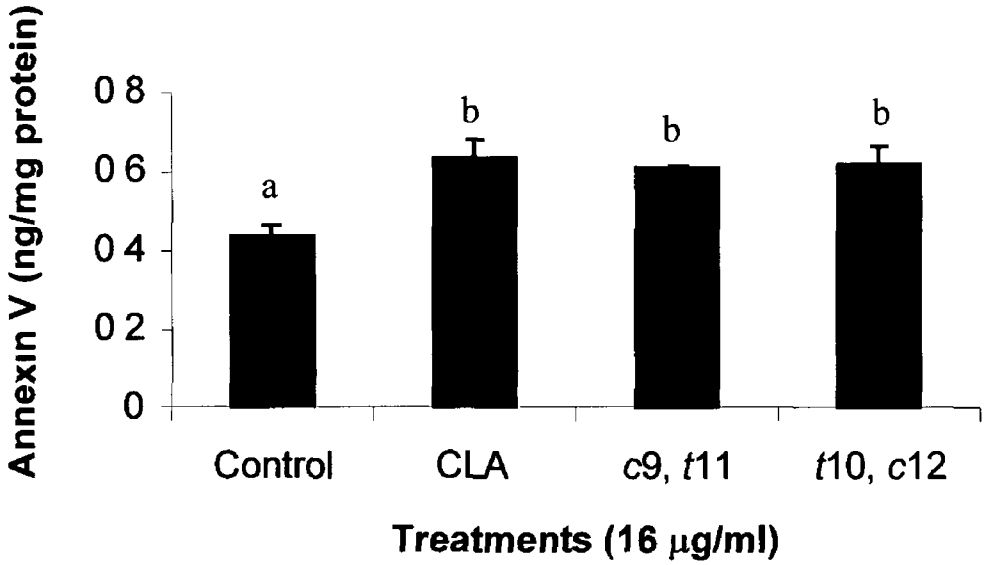


Figure 3 4 CLA isomers increased Annexin V levels in membranes of SW480 cancer cells SW480 cells were cultured for 4 days with either the CLA mixture of isomers, the c9, t11-CLA isomer, the t10, c12-CLA isomers (all at a lipid concentration of 16 μg/ml) or ethanol control After 4 days both floating cells and adherent cells were collected, membrane fractions prepared and analysed for annexin V levels using an ELISA kit Bars not sharing the same letters are significantly different from one another ($p<0\ 05$) Data is expressed as the mean ± SD for three separate experiments carried out in triplicate

3 4 2 CLA isomers reduced the expression bcl-2

The expression of two apoptotic regulatory proteins from the Bcl-2 family were evaluated by immunoblot analysis The Bcl-2 family of proteins consists of anti-apoptotic and pro-apoptotic proteins that regulate apoptosis at the mitochondrial level by controlling the release of cytochrome c and subsequent activation of a caspase cascade resulting in apoptosis Bcl-2 was the first protein to be identified as anti-apoptotic (Vaux *et al*, 1988) and subsequently a large number of bcl-2 related proteins (bcl-x_L, bcl-w, mcl-1, bfl-1 and boo) have been isolated (Tsujimoto and Shimizu, 2000) In contrast, bax is a known inducer of apoptosis along with bak, bad, mtd and diva (Tsujimoto and Shimizu, 2000) The three CLA-treatments (all added at a lipid concentration of 16 μg/ml) were equipotent in significantly ($p<0.05$) reducing the level of the bcl-2 protein after 4 days of incubation (Figure 3 5a) The *c*9, *t*11-CLA isomer is the most abundant isomeric form of CLA found in the diet (Chin *et al*, 1992) and the most physiologically relevant isomer in humans (Iversen *et al*, 1985, Cawood *et al*, 1983, Jiang *et al*, 1999, Park *et al*, 1999, Huang *et al*, 1994) We examined the effect of varying the dose of *c*9, *t*11-CLA on bcl-2 protein levels to determine if the effect of this isomer was concentration dependent The data demonstrated that the *c*9, *t*11-CLA isomer significantly ($p<0.05$) reduced bcl-2 protein expression in a concentration dependent manner from 16-25 μg/ml (Figure 3 5b) In contrast, the pro-apoptotic bax protein was not affected by any of the CLA treatments at the concentration and time point examined (Figure 3 5c)

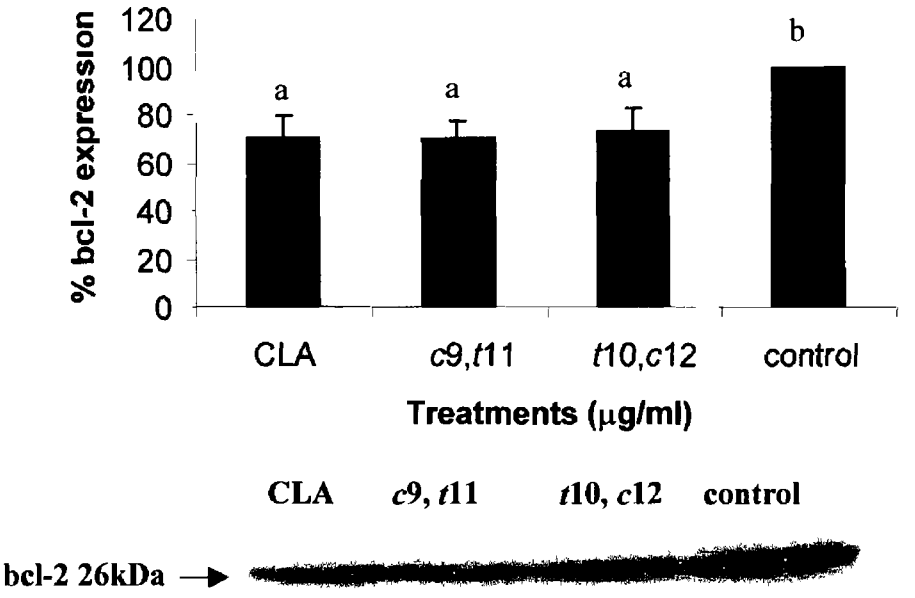


Figure 3 5 (a)

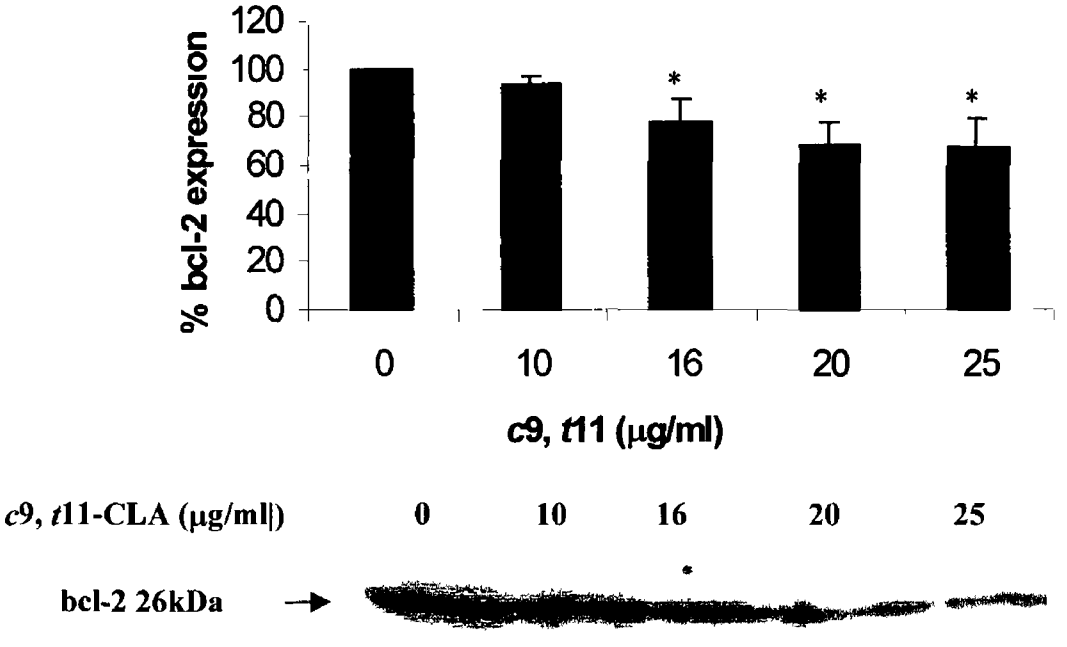


Figure 3 5(b)

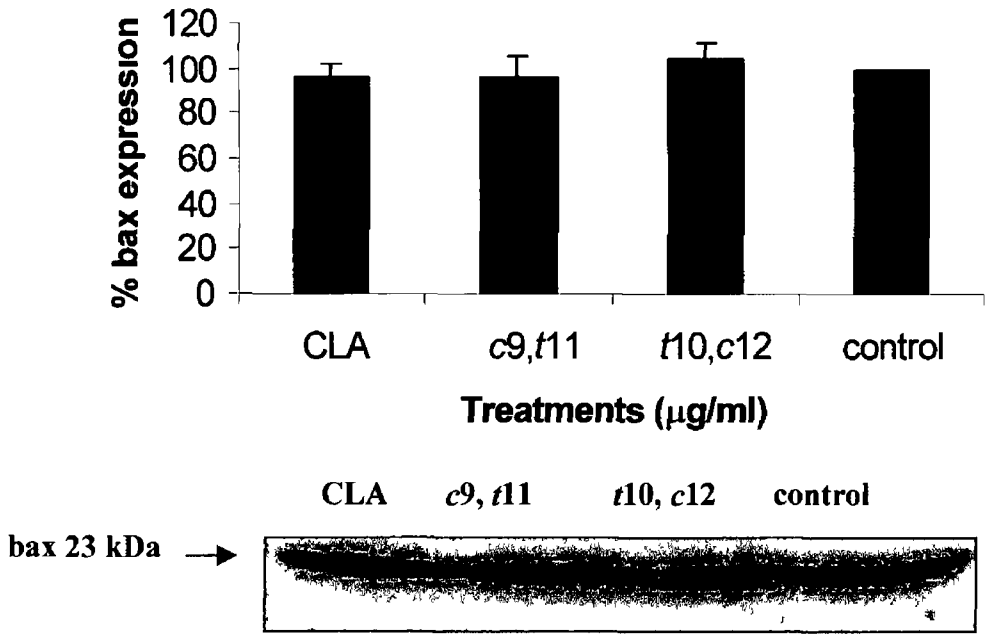


Figure 3 5 (c)

Figure 3 5 Bcl-2 and Bax protein expressions in CLA-treated SW480 cells (a) Western blot and densitometry result for the expression of Bcl-2 in SW480 cells treated with either the CLA mixture, *c*9, *t*11-CLA or *t*10, *c*12-CLA added at a lipid concentration of 16 μg/mL for 4 days Bars not sharing the same letters are significantly different from one another ($p<0.05$) (b) Western blot and densitometry result for the expression of Bcl-2 in SW480 cells treated with 0 – 25 μg/mL *c*9, *t*11-CLA for 4 days (* denotes values significantly ($p<0.05$) different to control) (c) Western blot and densitometry result for the expression of Bax in SW480 cells treated with either the CLA mixture, *c*9, *t*11-CLA or *t*10, *c*12-CLA added at a lipid concentration of 16 μg/mL for 4 days All blots represent one of three independent experiments Values are expressed as a percentage of the control (± SD) which was taken to be 100%

3 4 3 CLA isomers induced cytochrome c release into the cytosol and induced caspase 3 and 9 activation

The effect of the CLA treatments on the levels of cytosolic glutathione and cytochrome c were evaluated Diminished glutathione levels have been observed in apoptotic cells and have been associated with cytochrome c release from the mitochondria (Hall, 1999) SW480 cells were treated with the three different CLA treatments (all at 16 μg/mL) as described earlier and after 4 days all cells were collected and cytosolic proteins were extracted The three CLA-treatments (all added at a lipid concentration of 16 μg/ml) significantly ($p<0.05$) reduced cytosolic glutathione levels (21-29%) with the *t*10, *c*12-CLA isomer having the most potent effect (Figure 3 6) We then examined if this reduction in cytosolic glutathione levels facilitated cytochrome c release from the mitochondria into the cytosol Cytosolic levels of cytochrome c were quantified by densitometric scanning of western blots As demonstrated by the data presented in Figure 3 7(b) all CLA-treatments significantly ($p<0.05$) increased the accumulation of cytosolic cytochrome c when compared with the untreated control The *t*10, *c*12-CLA isomer had a more potent effect on cytochrome c release, increasmg its accumulation in the cytosol by 55% The effect of varying the dose of *c*9, *t*11-CLA on accumulation of cytochrome c in the cytosol was examined to determine if the effect of this isomer was concentration dependent It was found that the *c*9, *t*11-CLA isomer significantly ($p<0.05$) increased the accumulation of cytosolic cytochrome c in a concentration dependent manner from 16-25 μg/ml (Figure 3 7c) The *c*9, *t*11-CLA isomer had no effect on cytochrome c release when added at 10 μg/ml Once released, cytochrome c recruits and activates the adapter protein apoptosis protease activating factor-1 (Apaf-1) which binds downstream to the

initiator caspase 9 and processes it into its proteolytically active form (Zou *et al*, 1997) Apaf-1 expression was not affected by any of the CLA treatments at the concentration and time point examined (Figure 3 7a) Activation of caspase 9 is followed by sequential activation of effector caspases such as caspase 3 (Nunez *et al*, 1998) The proteolytic activities of caspase-3 and 9 were assessed using two commercially available assay systems Caspase activity was directly determined in cell lysates using LEHD-pNA, a synthetic substrate for caspase 9 and DEVD-pNA, a substrate for caspase 3 Compared with control cells, the three CLA treatments exerted increases in both caspase 3 (20-30%) and caspase 9 (36-42%) activities (Figure 3 8a) Western blot analysis for detection of caspase 3 activation was used to confirm caspase 3 processing and it was confirmed that the CLA treatments induced cleavage of pro-caspase 3 into two subunits of 17 and 12 kDa subunits (Figure 3 8b)

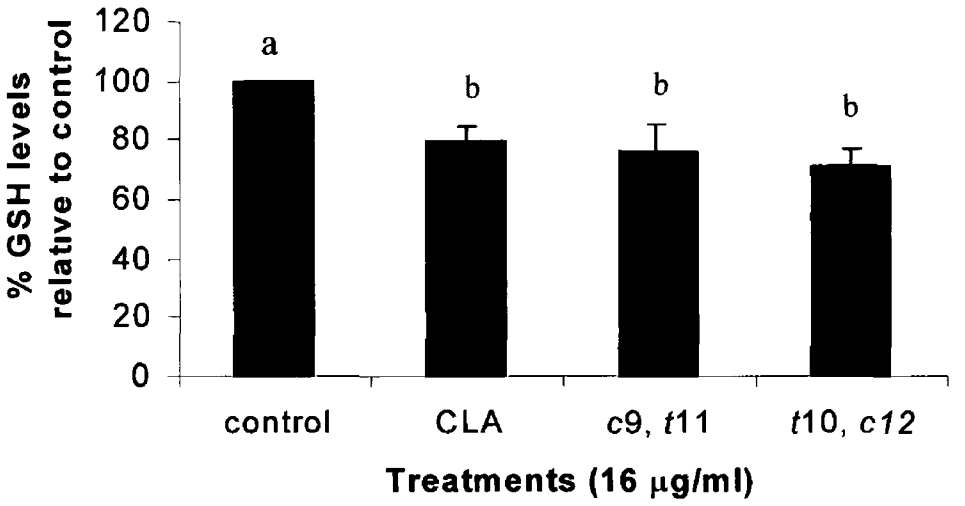


Figure 3.6 Effect of CLA isomers on cytosolic GSH levels in SW480 cancer cells after 4 days Bars not sharing the same letters are significantly different from one another ($p<0.05$) Data is expressed as the percentage mean ± SD for three separate experiments carried out in triplicate

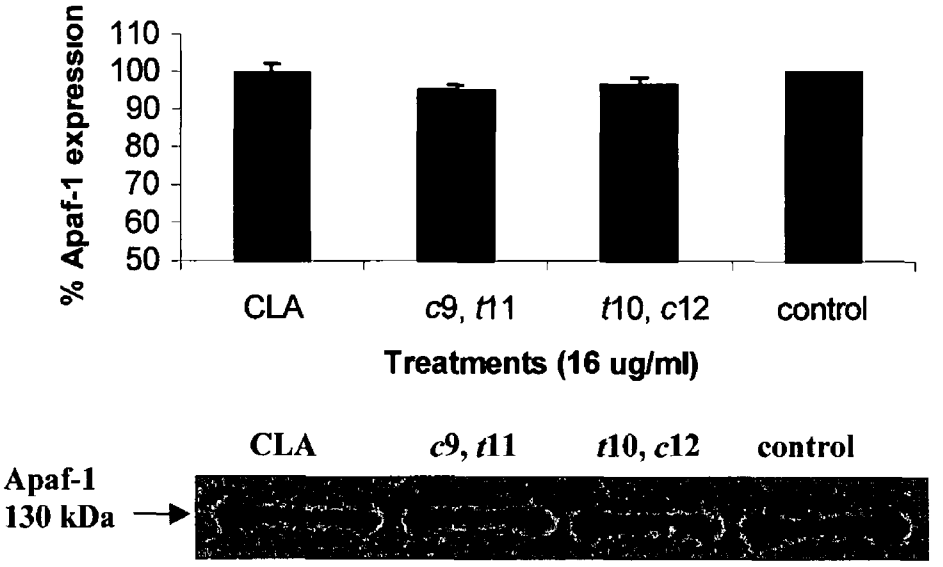


Figure 3 7 (a)

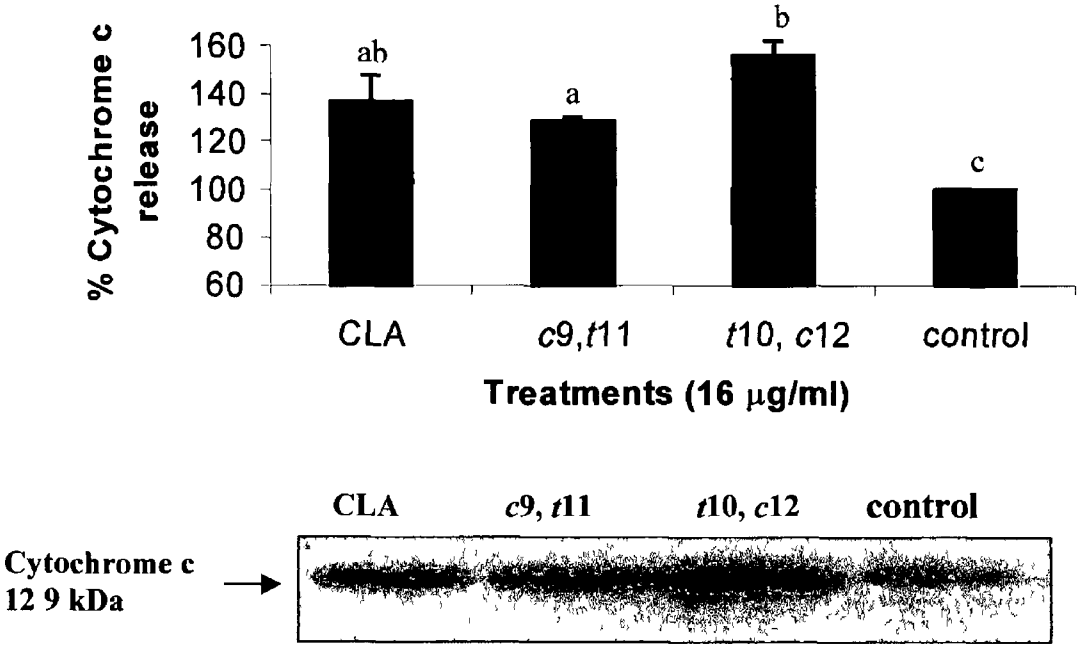


Figure 3 7 (b)

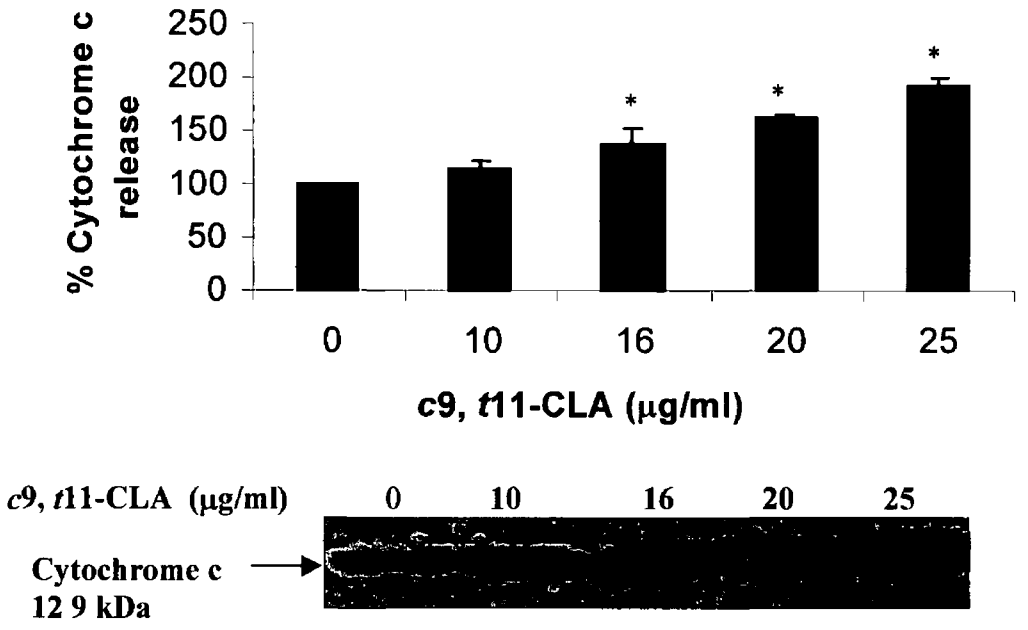


Figure 3 7 (c)

Figure 3 7 Apaf-1 and cytochrome c protein expressions in CLA-treated SW480 cells (a) Western blot and densitometry result for the expression of Apaf-1 in SW480 cells treated with either the CLA mixture, *c*9, *t*11-CLA or *t*10, *c*12-CLA added at a lipid concentration of 16 µg/mL for 4 days (b) Western blot and densitometry result for the release of cytochrome c into the cytosol in SW480 cells treated with CLA as described above Bars not sharing the same letters are significantly different from one another ($p<0.05$) (c) Western blot and densitometry result for the release of cytochrome c into the cytosol in SW480 cells treated with 0 - 25 µg/mL *c*9, *t*11-CLA for 4 days Densitometric quantification was normalized using Ponceau S scans All blots represent one of three independent experiments Values are expressed as a percentage of the control (± SD) which was taken to be 100% (* denotes values significantly different to controls $p<0.05$)

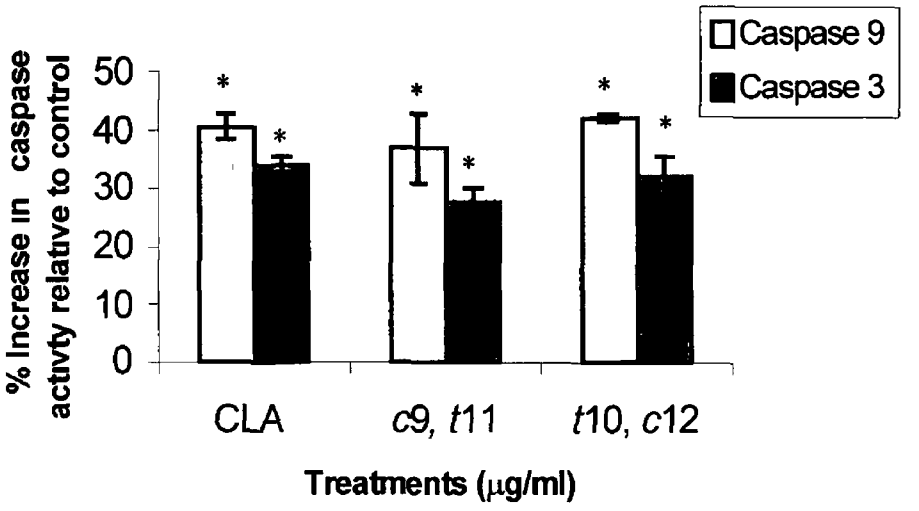


Figure 3 8 (a)

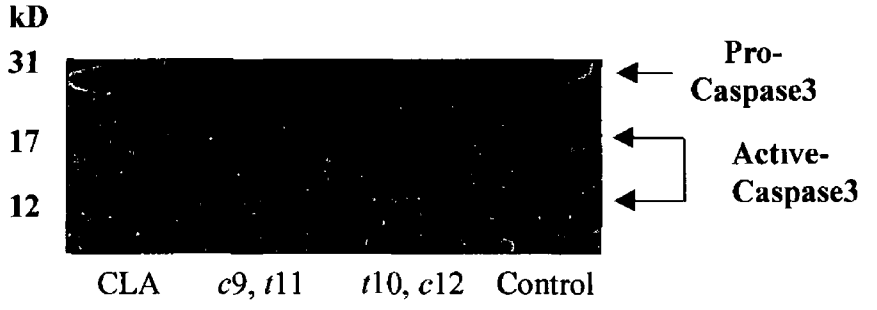


Figure 3 8 (b

Figure 3 8 CLA isomers induce Caspase 3 and 9 activation SW480 cells were cultured for 4 days with either the CLA mixture of isomers, the *c*9, *t*11-CLA isomer, the *t*10, *c*12-CLA isomer (all at a lipid concentration of 16 μg/ml) or ethanol control After 4 days, both floating cells and adherent cells were collected, lysates prepared and (a) analysed for caspase 3 and 9 activities using colormetric assay kits from R&D systems (* denotes values significantly different to controls $p<0.05$) Data is expressed as the percentage mean ± SD for three separate experiments carried out in triplicate (b) The cleavage of caspase 3 to its active form was also analysed by western blot analysis with an anti-caspase 3 antibody that recognized both uncleaved and cleaved caspase 3 The blot is represents one of three independent experiments

3 5 Discussion

While the anticancer activity of CLA isomers has been well documented, there is limited knowledge of the molecular mechanism(s) involved Over the past decade, cell culture studies have provided important clues about specific molecular pathways responsive to CLA Studies with a mammary cancer cell line implicated CLA as a modulator of the cell cycle, blocking entry of cells into S phase of the cell cycle and suppression of c-myc expression (Durgan and Fernandes, 1997) More specifically, CLA has been shown to down regulate the expression of cyclin D1 and cyclin A, key components of cell cycle machinery in rat mammary epithelium (Ip *et al*, 2001) CLA is also a ligand for peroxisome proliferator-activated receptors, a family of nuclear hormone receptors that regulate the expression of immediate early genes which are involved in proliferation, differentiation and apoptosis (Vanden Heuvel, 1999) CLA has been shown to induce apoptosis in a mammary tumor cell line as well as in premalignant lesions of the mammary gland where expression of bcl-2 was reduced (Ip *et al*, 2000) More recently, dietary CLA has been shown to significantly reduce colon tumor incidence in rodents by mechanisms probably involving increased apoptosis (Park *et al*, 2001) It was proposed that apoptosis in colon mucosa could be related to a series of fatty acid-responsive biomarkers such as PGE_2, TXB_2 and DAG all of which were significantly reduced by a CLA mixture of isomers containing predominantly *t*10, *c*12-CLA and *c*9, *t*11-CLA (Park *et al*, 2001) While the exact nature of modulation of apoptosis by eicosanoids needs to be determined, it has been shown that cyclooxygenase-2 derived PGE_2 is a significant inducer of bcl-2 expression and can thereby decrease the percentage of cells undergoing programmed cell death (Sheng *et al*, 1998) Activation of mitogen-activated protein

kinase (MAPK), which preceded induction of bcl-2, indicates a putative signal transduction pathway by which bcl-2 expression and other genes could be induced (Sheng *et al*, 1998)

The present study has clearly shown that a commercial mixture of CLA isomers and the pure isomers *t*10, *c*12- and *c*9, *t*11-CLA all present at physiological level of 16 μg lipid/ml were potent inducers of apoptosis following 4 days incubation with the SW480 human colon tumor cell line Early responses of this cell line before growth was inhibited by CLA implicated a role for arachidonic acid metabolites (PGE_2, 8-epi-PGF_{2a}) and phospholipid signalling in apoptosis (Chapter 2) Specifically, the CLA mixture of isomers and the *c*9, *t*11-CLA isomer decreased uptake of arachidonic acid into the phospholipid fraction of cells and decreased synthesis of PGE_2 The *c*9, *t*11-CLA isomer also stimulated production of 8-epi-PGF_{2a} indicative of non-enzymatic oxidation of unsaturated fatty acyl chains Other effects included an increase in arachidonic acid uptake into phosphatidylserine by the CLA mixture of isomers and by *t*10, *c*12-CLA isomer The exact nature of the mechanism by which oxidation of one or more classes of phospholipid can translate into the recognisable biochemical and morphological stages of apoptosis (chromatin condensation, membrane surface blebbing, ologonucleosomal DNA fragmentation and breakdown of cell into apoptotic bodies for phagocytosis by adjacent cells) remains unclear

There is now strong evidence that oxidative stress, in general and lipid peroxidation in particular are involved in both initiation and mediation of apoptosis (Lopaczynski and

Zeisel, 2001) The bcl-2 protooncogene is unique among cellular genes for its ability to block apoptotic death Expression of the anti-apoptotic bcl-2 protein has been reported to protect membrane lipids from peroxidation during exposure to oxidative stress (Kane *et al*, 1993) and to regulate antioxidant pathways at sites of free radical generation (Hockenbery *et al*, 1993) Hence, this protein appears to have an antioxidant function (Cai and Jones, 1998) The three CLA treatments (all added at a lipid concentration of 16 μg/ml) were equipotent in significantly reducing the level of the anti-apoptotic protein bcl-2 after 4 days of incubation This was consistent with a previous study by Ip *et al* (2000) where an increase in apoptosis in premalignant lesions of the rat mammary gland was associated with a reduction in the expression of bcl-2 in these lesions Bax and other pro-apoptotic proteins show structural similarities with mitochondrial pore-forming proteins and so it is suspected that bax can form ion channels across mitochondrial membranes which result in loss of membrane potential and subsequent cytochrome c release (Basanez *et al*, 1999) Evidence indicates that bcl-2 acts on the mitochondria to counteract the action of the pore-forming pro-apoptotic proteins like bax (Antonsson *et al*, 1997) The expression of the pro-apoptotic bax protein was not affected by any of the CLA treatments at the concentration and incubation times examined This suggests a role for CLA in the localisation of bax from the cytosol to the mitochondria and/or the redistribution of bax on the mitochondrial membrane rather than in its expression

Oxidative stress acts by decreasing intracellular glutathione, the major buffer of the cellular redox status When pumped out of cells, the cytosol changes to an oxidising environment (Vanden Dobbelsteen *et al*, 1996) It is thought that this change in redox

state may play a role in loss of mitochondrial membrane potential seen in apoptotic cells (Hall, 1999) The three CLA treatments significantly reduced cytosolic glutathione levels and this was accompanied by an accumulation of cytosolic cytochrome c Depletion of cellular glutathione, as observed in this study, may be a reflection of the altered redox status of these cells and may serve to initiate release of basic proteins from the mitochondria such as cytochrome c Palombo *et al* (2002) reported significantly higher total caspase activity in MIP-101 colorectal cells treated with the *t*10, *c*12-CLA isomer This study now demonstrates that treatment with not only *t*10, *c*12-CLA isomer but also with the CLA mixture of isomers and the *c*9, *t*11-CLA isomer resulted in a series of events marked by the accumulation of cytochrome c in the cytosol, activation of caspase 9 and subsequent processing and activation of caspase 3 A fundamental mechanism by which apoptotic cells are recognised and eliminated is mediated by the binding of cytochrome c to acidic phosphatidylserine, which triggers phospholipid translocation from the inside to the outside of the membrane The increase in membrane annexin V levels in CLA-treated cells suggests an increased binding of annexin V to externalised phosphatidylserine and serves to illustrate that externalisation of this phospholipid may be the mechanism by which apoptotic colon cells are recognised and eliminated

The apoptosis-inducing effect of the *t*10, *c*12-CLA isomer was more potent than the *c*9, *t*11-CLA isomer and similar to the CLA mixture of isomers as illustrated by cytochrome c release and DNA laddering However no significant differences between any of the CLA treatments were observed with respect to other apoptotic markers examined The potency of the *t*10, *c*12-CLA isomer is consistent with growth inhibition seen in this

study The *t*10, *c*12-CLA isomer was more potent at inhibiting the growth of SW480 cells than the *c*9, *t*11 CLA isomer and this may be explained by its ability to induce greater cytochrome c release The *t*10, *c*12-CLA isomer has also been shown to be more effective in inhibiting the proliferation of HT29 and MIP-101 colorectal tumor cell lines than *c*9, *t*11-CLA isomer (Palombo *et al*, 2002) Together these findings suggest that the *t*10, *c*12-CLA isomer may be the more biologically active isomer for inhibition of colon tumor cell proliferation *in vitro*

This study has identified specific protein targets regulated by CLA isomers within an apoptotic cascade leading to cell death The regulation of apoptosis by means of dietary agents is a novel and promising therapeutic approach for cancer therapy Several genes that regulate the process of apoptosis have been found to be defective in tumor cells The best characterised examples are the bcl-2 family genes which are overexpressed 30-50% in colon cancer cells (Zhang, 2002) Given the importance of apoptosis in cancer development, apoptosis-inducing lipids could conceivably have an important role in adjunct anticancer therapy The potential clinical usefulness of a CLA based approach to cancer therapy requires further study

3 6 Summary

Dietary conjugated linoleic acid (CLA) has been shown to reduce colon tumor incidence in rodents by mechanisms probably involving apoptosis The aim of this study was to evaluate the effects of three commercial CLA preparations (pure *c*9, *t*11-CLA, pure *t*10, *c*12-CLA and a CLA mixture, containing 29 5% *c*9, *t*11 and 29% *t*10, *c*12-CLA) on caspase-dependent apoptosis in colon SW480 tumor cells After 4 days incubation, all CLA-treated cells displayed an increase in caspase 3 (27-34 %) and caspase 9 (37-47 %) activities, cleavage of pro-caspase 3 (32 kDa) to 17 and 12 kDa subunits, increased membrane annexin V levels and reduced expression of bcl-2 compared with untreated controls Cytosolic cytochrome c was increased ($p<0$ 05) by all CLA preparations, with the *t*10, *c*12-CLA isomer being the most potent The data indicate that *t*10, *c*12-CLA may be the more biologically active isomer for inhibition of colon tumor cell proliferation *in vitro*

CHAPTER 4

Trans-vaccenic acid is converted to conjugated linoleic acid (*c*9, *t*11-CLA) in MCF-7 and SW480 cancer Cells.[1]

[1] Submitted for publication to Lipids August 2002.

4 1 Introduction

Cancer cells derive biologically important fatty acids from either *de novo* synthesis or the host circulation (Spector and Burns, 1987) The end products of *de novo* synthesis are palmitoleate and oleate which are synthesised from palmitate and stearic acid respectively by Δ^9-desaturase (Ntambi, 1995) Analysis of the fatty acid composition of cellular lipids clearly shows an altered balance of saturated to monounsaturated fatty acids in tumours compared with non neoplastic cells (Fermor *et al*, 1992) In particular, increased proportions of oleic acid were found in experimental tumours (Cheeseman *et al*, 1986, Zoeller and Wood, 1985, Ruggieri and Fallani, 1979), hepatoma cell lines (Hartz *et al*, 1982) and in virally transformed cell lines (Yau *et al*, 1976, Ruggieri *et al*, 1979) reflecting possible increased expression or activity of Δ^9-desaturase

A large amount of experimental data have shown that tumour cell growth can be modulated by individual fatty acids (Guthrie and Carroll, 1999, Zhou and Blackburn, 1999) Conjugated linoleic acid (CLA) is a group of polyunsaturated fatty acids that have been highly publicised recently as a result of their potent anticarcinogenic properties observed at low dietary levels in animals models of carcinogenesis (reviewed in Scimeca, 1999) The mechanism by which CLA exerts its anticancer activity is attributed in part to a series of events marked primarily by changes in composition of cellular lipids, inhibition of Δ^9-desaturase, modulation of arachidonic acid distribution and metabolism, changes in eicosanoid production, activation of nuclear transcription factors critical for lipid metabolism, induction of apoptosis and modulation of the cell cycle (reviewed in Belury, 2002) Naturally occurring CLA in the diet primarily consists of the *c9, t11*-CLA

isomer with milk fat being the richest natural source of CLA (Chin *et al*, 1992, Parodi, 1977) TVA, the predominant *trans* monounsaturated fatty acid in milk fat, is formed by ruminal biohydrogenation of LA and occurs in the diet at levels up to five times that of CLA (Wolff, 1995, Parodi, 1976) Endogenous synthesis of CLA from TVA represents the primary source of CLA in milk fat of lactating cows (Griinari *et al*, 2000) Santora and co-workers (2000) reported that TVA is desaturated to CLA in mice Rats fed CLA-enriched butter fat accumulated more total CLA in their tissues than those consuming synthetic *c*9, *t*11-CLA suggesting that the availability of TVA in the butterfat served as a precursor for endogenous synthesis of CLA (Ip *et al*, 1999a) This was confirmed in a recent study wherein feeding TVA (2 % of diet w/w) elicited a biological response in a rat mammary tumor model, reducing the total number of premalignant lesions in the rat mammary gland by approximately 50 % in carcinogen-treated rats (Banni *et al*, 2001) Although CLA is present at relatively low concentrations in human adipose tissue, bile, duodenal juice, breast milk and serum lipids (Cawood *et al*, 1983, Harrison *et al*, 1985, Fogerty *et al*, 1985), specific dietary intervention trials markedly increased the CLA content m human milk (Park *et al*, 1999), plasma [Huang *et al*, 1994) and adipose tissue (Jiang *et al*, 1999) Together, these observations suggest that increasing the amount of TVA in the diet may enhance the pool of CLA m human tissues

4 2 Objectives

CLA uptake into MCF-7 human mammary cancer cells was reported to be more proficient from milk fat than from synthetic *c*9, *t11*-CLA suggesting possible formation of CLA from TVA present in the milk fat by a Δ^9 desaturase enzyme (O'Shea *et al*,

1999) The aims of this present study are twofold to evaluate the possible bioconversion of TVA to CLA in the MCF-7 (breast) and SW480 (colon) human cancer cell lines and to determine if CLA was synthesised from TVA in sufficient amounts as to modulate known bio-responsive markers was examined The relative effects of TVA and *c*9, *t*11-CLA on cell growth were compared over a range of concentrations after 4 days incubation Time- and dose-experiments were performed to determine the effects of TVA on fatty acid composition in both cell lines It was reported that the growth suppressive effects of CLA isomers in MCF-7 and SW480 cell lines may be due to alterations in arachidonic acid distribution among cellular lipids, an altered prostaglandin profile, lipid peroxidation (Chapter 2) and stimulation of an apoptotic signal transduction pathway (Chapter 3) In this chapter, we examined the effects of TVA on arachidonic acid uptake and conversion to eicosanoid classes in MCF-7 and SW480 cell lines as well as on its ability to modulate ras expression and induce apoptosis in the SW480 cell line

4 3 Materials and methods

4 3 1 Cell culture

The human breast (MCF-7) and Colon (SW480) cell lines were cultured exactly as outlined in Chapter 2

4 3 2 Bioconversion of TVA to CLA

Cells were seeded in T-25 cm^2 flasks at a density of 5 x 10^5/flask and cultured for 24 h allowing the cells to attach to the substratum The medium was then replaced with medium containing *trans*-vaccenic acid (Sigma-Aldrich Ireland Ltd , Dublin, Ireland) at

5, 10 or 20 μg/ml Control flasks were supplemented with an equivalent volume of ethanol (≤1 % v/v) After 24 h and 4 d of incubation, cells were harvested and total cellular lipids were extracted from cell pellets using the method of Bligh and Dyer (1959), resuspended in 1 ml of chloroform and stored at –20 °C prior to GLC analysis

4 3 3 Fatty acid analysis

Fatty acid methyl esters (FAME) of cellular lipids were prepared using base-catalysed methanolysis, by incubating extracts with tetramethylguamdine as described by Shanta *et al* (1993) FAME were analysed by GLC, using a Varian 3500 GLC (Varian, Harbor City, CA, USA) fitted with a flame ionisation detector (FID) and a Supelcowax-10 capillary GLC column (Supelco Inc, Bellefonte, PA) (60 m x 0 32 mm i d , 0 25μm film thickness) GLC conditions were exactly as previously described (Stanton *et al*, 1997)

4 3 4 Viability experiments

Cells were seeded in 6 well plates and the MCF-7 and SW480 cells were seeded at densities of 1 x 10^5/well and 5 x 10^4/well, respectively Cells were cultured for 24 h to allow the cells attach to the substratum The medium was then replaced with medium containing TVA (99 % pure from Sigma-Aldrich Ireland Ltd , Dublin, Ireland) or the pure *c*9, *t*11-CLA isomer (95 % pure from Natural ASA, Hovdebygda, Norway) at varying concentrations from 5 to 25 μg/ml dissolved in ethanol Control wells were supplemented with equivalent volumes of ethanol After 4 days of incubation, cells were harvested in the presence of phosphate buffered saline (PBS) containing 0 25 % (v/v)

trypsin Viable cell numbers were quantified using the trypan blue exclusion (0 4 % w/v) assay

4 3 5 Uptake up ^{14}C-AA and conversion to eicosanoids

Cells were seeded in T-25 cm^2 flasks at a density of 2 x 10^5/flask and grown to 90% confluency The medium was then replaced with medium containing ^{14}C-AA at 0 2 μCi along with TVA (20μg/ml) or an equivalent volume of ethanol After 24 h incubation, cells were harvested to determine uptake of ^{14}C-AA and the media removed Total cellular lipids were extracted from cell pellets and then separated into triacylglyceride (TG), monoacylglyceride (MG) and phopholipid (PL) fractions as described in (Chapter 2) An aliquot of each fraction was counted in a Beckman LS6500 scintillation counter Eicosanoids were extracted twice with ethyl acetate from medium acidified to pH 3 0 with 0 1 N HCl as described (Chapter 2) Eicosanoid extracts were dried under nitrogen, redissolved in ethyl acetate separated using normal-phase TLC as described previously (Chapter 2) Bands of PGE_2, $PGF_{2\alpha}$, PGD_2 were removed from TLC plates and placed in vials for counting by liquid scintillation The isoprostane, 8-epi-$PGF_{2\alpha}$ was extracted from media as described (Watkins *et al*, 1999) and a competitive horseradish peroxidese (HRP) enzyme-linked immunoassay kit (BIOXYTECH 8-Isoprostane assay system) was used to quantify 8-epi-$PGF_{2\alpha}$ levels according to the manufacturer's instructions

4 3 6 DNA laddering

Cells were seeded in T-75 cm^2 flasks at a density of 1 x 10^6 cells/flask and incubated for 24 h The medium was then replaced with fresh medium containing TVA (20 μg/mL) or ethanol control as described above and incubated for 4 days Adherent and floating/loosely-attached cells were collected and processed separately Apoptotic DNA was extracted and precipitated using the Suicide-Track DNA Laddering Isolation Kit (Oncogene Research Products, Boston, MA) according to the manufacturer's instructions Details of kit described in Chapter 3 DNA was analysed by electrophoresis in a 1 5 % agarose gel at 50 V (constant voltage) for 4 5 h The gel was then stained for 0 5 h with ethidium bromide (0 5 μg/mL) DNA fragments were visualised and photographed using the ImageMaster VDA documentation system from Amersham Biosciences (Little Chadfort, Buckinghamshire, UK)

4 3 7 Measurement of reduced glutathione

Cells were seeded in T-75 cm^2 flasks at a density of 1 x 10^6 cells/flask and incubated for 24 h The medium was then replaced with fresh medium containing TVA (20 μg/mL) and incubated for 4 days after which both floating and adherent cells were collected and pooled Cells were resuspended in a PBS/PMSF buffer containing 10 mM sodium phosphate buffer (pH 7 2), 100 mM NaCl, 0 2 mM phenylmethylsulfonylfluoride (PMSF), 0 1 mM leupeptin, 0 2 μg/ml aprotinin (Sigma-Aldrich Ireland Ltd , Dublin, Ireland), sonicated on ice and centrifuged at 100,000 x g for 1 hour at 4°C Activity of GSH in the cytosolic fraction was measured according to the method of Hissen and Hilf as described in Chapter 3 The glutathione concentration of the samples was determined

from a standard curve and expressed relative to the protein content, as determined using the Bio-Rad protein assay (Biorad, Hemel Hempstead, Hertfordshire, UK)

4 3 8 Total ras expression

SW480 cells were seeded at 5 x 10^6 cells /150cm^2 flask and were cultured for 24 h to allow the cells to attach to the substratum The medium was then replaced with medium containing either *c*9, *t*11-CLA or TVA (20 μg/ml) An equivalent volume of ethanol was added to the control flasks Quercetin was used as a positive control After 24 h incubation the cells were harvested using phosphate buffered saline (PBS) containing 0 25% (w/v) trypsin The pellets were washed twice in ice-cold PBS To assess total ras, a lysis buffer (containing 10mM sodium phosphate buffer (pH 7 2), 100mM NaCl, 10mM sodium deoxycholate, 1mM PMSF, 1% (v/v) Triton-X 100, 0 1mM leupeptin, 0 2 μg/ml aprotinin) was added to the cell pellets which were then sonicated on ice for 10 min The lysates were concentrated using *Microcon* filters The protein content of the lysates was determined using the Biorad protein assay The resulting supernatants were concentrated as described above and electrophoresed using 12% (w/v) polyacrylamide gel (70 μg of protein per well) The separated proteins were transferred onto Hybond ECL membrane (Amersham, Little Chadfort, Buckinghamshire, UK) in a Trans-blot Electrophoretic transfer cell (Biorad, Hemel Hempstead, Hertforshire, UK) Blots were stained with Ponceau S Solution (0 2 % v/v) to ensure transfer of proteins was complete and to determine if an equivalent amount of protein was loaded in each lane The blots were destained with PBS containing 0 1 % (v/v) Tween 20 (PBST) The blots were blocked for 1 h with 5 % (w/v) nonfat dry milk dissolved in PBST Blots were then incubated

overnight with anti-ras monoclonal antibody diluted 1 40 (Oncogene Science, Manhasset, NY) in PBST containing 0 5 % nonfat dry milk Blots were washed extensively in PBST and reincubated for 1 h with a HRP-linked secondary antibody (Amersham, Little Chadfort, Buckinghamshire, UK) diluted 1 2000 in PBST containing 0 5 % (w/v) nonfat dry milk The blots were then thoroughly washed in excess PBST and probed with the Super Signal detection system (Pierce, Rockford, IL) and exposed to autoradiography films (Amersham, Little Chadfort, Buckinghamshire, UK) according to the manufacturer's instructions Densitometry (using NIH Image software) was performed on Ponceau S scans and autoradiographed

4 3 9 Statistical Analysis

Data represent three independent experiments performed in triplicate The Student's *t* test was used to determine significance between treatments

4 4 Results

4 4 1 Effect of TVA uptake on cellular lipids

To determine if TVA was bioconverted to *c*9, *t*11-CLA, MCF-7 and SW480 cells were incubated with increasing concentrations of TVA (5, 10 and 20 μg/ml) and subsequently total cellular lipids were analysed using gas liquid chromatography (GLC) Neither CLA nor TVA were detectable in control untreated SW480 or MCF-7 cells Incubation of the SW480 colon cell line with increasing concentrations of TVA resulted in accumulation of TVA and *c*9, *t*11-CLA in a dose-dependent manner to a maximum of 25 11 and 7 10 g /

100 g FAME respectively following treatment with 20 μg/ml for 24 h (Table 4 1) The percentage bioconversion of TVA to *c*9, *t*11-CLA (g CLA 100 ^{1}g FAME / (g CLA 100 ^{1}g FAME + g TVA 100 ^{1}g FAME) x 100) at 5, 10 and 20 μg/ml was 33 8 %, 28 % and 22 %, respectively As shown in Table 2, there was no further accumulation of TVA after 4 days SW480 cells treated with 20 μg/ml TVA accumulated 20 58 g/100g FAME TVA after 4 days which was lower than the level observed after 24 h Bioconversion to *c*9, *t*11-CLA was increased at this concentration to 29 4 % (Table 4 2) The accumulation of TVA caused perturbations in other fatty acids Treatment with 20 μg/ml TVA for 24 h and 4 days resulted in respective reductions of 32 % and 38 % for palmitic (16 0), 47 % and 41 % for palmitoleic (16 1), 43 % and 44 % for stearic (18 0), 44 % and 40 % for oleic (18 1), 35 % and 29 % for linoleic (18 2), respectively Arachidonic acid (20 4) was reduced by 32 % after 24 h but no effect was observed after 4 days following treatment with 20 μg/ml TVA

In general, the MCF-7 mammary cell data were similar to the SW480 colon cell data As shown in Tables 4 3 and 4 4, the concentrations of TVA and *c*9, *t*11-CLA in cellular lipids increased proportionately with TVA treatment MCF-7 cells treated with 20 μg/ml TVA for 24 h accumulated TVA and *c*9, *t*11-CLA to 18 98 and 12 09 g/100g FAME, respectively The percentage bioconversion of TVA at 5, 10 and 20 μg/ml to *c*9, *t*11-CLA was 38 %, 39 2 % and 38 9 % after 24 h, respectively After 4 days, the levels of TVA had fallen to 15 53 g/100g FAME and this was accompanied by a 44 % level of bioconversion to c9, t11-CLA Even though bioconversion had increased after 4 days, levels of c9, t11-CLA *did not*, suggesting further metabolism by desaturation and

elongase enzymes occured Levels of bioconversion were higher in MCF-7 cells compared with SW480 cells Treatment of MCF-7 cells with 20 μg/ml TVA for 24 h and 4 days resulted in respective reductions of 47 % and 26 % for myristic (14 0), 39 % and 35 % for palmitic (16 0), 33 % and 48 % for palmitoleic (16 1), 39 % and 24 % for stearic (18 0), 14 % and 35 % for oleic (18 1) and 18 % and 27 % for arachidonic acid (20 4), respectively Linoleic acid was decreased by 22 % after 24 but no changes were observed after 4 days

Table 4 1 Fatty acid composition of total cellular lipids from SW480 cells incubated in the presence of *trans*-vaccenic acid (5-20 μg/ml) for 24 h

	SW480 Fatty Acids (g/ 100 g FAME)			
Fatty Acid	Untreated controls	VA 5μg/ml	VA 10 μg/ml	VA 20 μg/ml
$C_{14\,0}$	1 81 ± 0 09	1 74 ± 0 40	2 28 ± 0 24	1 49 ± 0 16
$C_{16\,0}$	19 90 ± 1 15	17 79 ± 2 02	17 03 ± 1 86	13 26 ± 0 89*
$C_{16\,1}$	3 23 ± 0 16	4 04 ± 2 14	2 57 ± 0 41	1 71 ± 0 06*
$C_{18\,0}$	13 67 ± 0 34	10 96 ± 0 41*	10 04 ± 0 82*	7 77 ±0 29*
$C_{18\,1}$	28 04 ± 0 90	21 76 ± 1 86	18 92 ± 0 99*	15 72 ± 0 79*
$C_{18\,1}$ vaccenic	0	7 25 ± 1 24*	12 43 ± 0 71*	25 11 ± 2 86*
$C_{18\,2}$	5 76 ± 0 22	4 46 ± 0 30*	4 50 ± 0 23*	3 74 ± 0 22 *
$C_{18\,2}$ CLA	0	3 71 ± 0*	4 89 ± 0 23*	7 10 ± 0 86*
$C_{20\,4}$	8 544867 ± 0 07	7 03 ± 0 64	6 51 ± 0 37*	5 80 ± 0 11*
Others	19 03 ± 2 5	21 26 ± 5 04	20 83 ± 4 03	18 28 ± 5 40

* Denotes results which are significantly different to untreated cells ($p < 0\,05$) Data is expressed as the mean ± SD for three separate experiments carried out in triplicate

Table 4 2 Fatty acid composition of total cellular lipids from SW480 cells incubated in the presence of *trans*-vaccenic acid (5-20 μg/ml) for 4 d

	SW480 Fatty Acids (g/ 100 g FAME)			
Fatty Acid	Untreated controls	VA 5μg/ml	VA 10 μg/ml	VA 20 μg/ml
$C_{14\,0}$	2 50 ± 0 44	2 25 ± 0 20	1 90 ± 0 02	1 48 ± 0 26
$C_{16\,0}$	23 90 ± 1 13	23 68 ± 0 42	19 50 ± 0 50*	14 78 ± 0 36*
$C_{16\,1}$	2 32 ± 0 26	2 01 ± 0 15	1 70 ± 0 01*	1 38 ± 0 14*
$C_{18\,0}$	18 19 ± 0 30	14 60 ± 1 20*	12 26 ± 0 33*	10 17 ± 0 43*
$C_{18\,1}$	21 26 ± 1 11	17 34 ± 0 29*	15 40 ± 0 39*	12 73 ± 0 42*
$C_{18\,1}$ vaccenic	0	6 75 ± 0 17*	12 69 ± 0 44*	20 58 ± 0 44*
$C_{18\,2}$	5 95 ± 0 16	5 10 ± 0 32 *	4 68 ± 0 28*	4 20 ± 0 09*
$C_{18\,2}$ CLA	0	3 86 ± 0 20*	5 96 ± 0 37 *	8 57 ± 0 48*
$C_{20\,4}$	7 18 ± 0 50	6 21 ± 0 21*	6 42 ± 0 29*	7 13 ± 0 29
Others	18 67 ± 2 90	18 43 ± 0 26	19 49 ± 1 09*	18 95 ± 0 46

* Denotes results which are significantly different to untreated cells ($p < 0\,05$) Data is expressed as the mean ± SD for three separate experiments carried out in triplicate

Table 4 3 Fatty acid composition of total cellular lipids from MCF-7 cells incubated in the presence of *trans*-vaccenic acid (5-20 μg/ml) for 24 h

	MCF-7 Fatty Acids (g/ 100 g FAME)			
Fatty Acid	Untreated controls	VA 5μg/ml	VA 10 μg/ml	VA 20 μg/ml
$C_{14\,0}$	4 43 ± 0 15	3 96 ± 0 30	3 51 ± 0 18*	2 33 ± 0 08*
$C_{16\,0}$	25 78 ± 0 46	25 29 ± 0 49	22 89 ± 0 69*	15 69 ± 0 25*
$C_{16\,1}$	6 85 ± 0 28	6 21 ± 0 24	5 72 ± 0 25*	4 60 ± 0 11
$C_{18\,0}$	18 71 ± 2 87	16 23 ± 1 98	13 28 ± 0 65*	11 49 ± 0 49*
$C_{18\,1}$	20 19 ± 0 74	19 10 ± 0 49	18 76 ± 1 04	17 33 ± 0 44*
$C_{18\,1}$ vaccenic	0	5 87 ± 0 52*	10 77 ± 0 80*	18 98 ± 0 90*
$C_{18\,2}$	3 34 ± 0 27	2 85 ± 0 24*	2 73 ± 0 09*	2 59 ± 0 07*
$C_{18\,2}$ CLA	0	3 63 ± 0 45*	6 95 ± 0 21*	12 09 ± 0 30*
$C_{20\,4}$	4 73 ± 0 28	4 23 ± 0 10	4 13 ± 0 13*	3 89 ± 0 29*
Others	15 98 ± 2 30	12 64 ± 0 17	11 25 ± 0 75	10 99 ± 0 85

* Denotes results which are significantly different to untreated cells ($p < 0\,05$) Data is expressed as the mean ± SD for three separate experiments carried out in triplicate

Table 4 4 Fatty acid composition of total cellular lipids from MCF-7 cells incubated in the presence of *trans*-vaccenic acid (5-20 μg/ml) for 4 d

	MCF-7 Fatty Acids (g/ 100 g FAME)			
Fatty Acid	Untreated controls	VA 5μg/ml	VA 10 μg/ml	VA 20 μg/ml
$C_{14\,0}$	3 17 ± 0 20	2 88 ± 0 10	2 78 ± 0 44	2 34 ± 0 08*
$C_{16\,0}$	26 82 ± 0 80	24 79 ± 0 57*	21 89 ± 0 58*	17 44 ± 1 09*
$C_{16\,1}$	4 37 ± 0 32	2 71 ± 0 13*	2 44 ± 0 14*	2 29 ± 0 33*
$C_{18\,0}$	17 87 ± 0 24	15 91 ± 0 29*	14 93 ± 0 26*	13 51 ± 0 45*
$C_{18\,1}$	19 95 ± 0 60	17 69 ± 0 96	15 25 ± 0 71*	12 92 ± 1 52*
$C_{18\,1}$ vaccenic	0	5 31 ± 0 19*	8 34 ± 0 62*	15 53 ± 2 00*
$C_{18\,2}$	4 08 ± 0 41	4 25 ± 0 35	4 21 ± 0 56	4 06 ± 0 39
$C_{18\,2}$ CLA	0	4 87 ± 0 28*	8 64 ± 2 03*	12 14 ± 1 50*
$C_{20\,4}$	7 09 ± 0 80	6 81 ± 0 18	6 83 ± 0 28	5 18 ± 1 04*
Others	16 63 ± 0 78	14 76 ± 0 82	14 66 ± 1 91	14 58 ± 1 41

* Denotes results which are significantly different to untreated cells ($p < 0\,05$) Data is expressed as the mean ± SD for three separate experiments carried out in triplicate

4 4 2 The effect of TVA and c9, t11-CLA on cell viability

Effects of incubation with TVA and *c*9, *t*11-CLA on growth of MCF-7 and SW480 cells after 4 days incubation were determined Both cell lines were sensitive to the growth inhibitory effect of the *c*9, *t*11-CLA isomer as previously reported (Chapter 2) All *c*9, *t*11-CLA concentrations significantly lowered ($p<0.05$) cell number in both cell lines (Fig 4 1 and 4 2) In the MCF-7 cell line, there were no significant difference between the final cell numbers (27 6 - 26 4 x 10^4) obtained for the 5, 10 and 16 μg/ml *c*9, *t*11-CLA treatments (Fig 4 1) The 20 and 25 μg/ml treatment significantly lowered ($p<0.05$) cell number by 56 and 61 %, respectively, which were not significantly different from each other The two higher *c*9, *t*11-CLA concentrations (20 and 25 μg/ml) had a significantly greater inhibitory effect on cell growth when compared with concentrations of 5 - 16 μg/ml In the MCF-7 cell line, TVA supplementation for 4 days at concentrations less than 20 μg/ml had no effect on cell growth while supplementation with 20 and 25 μg/ml TVA significantly reduced ($p<0.05$) growth by 30 % and 41 % respectively

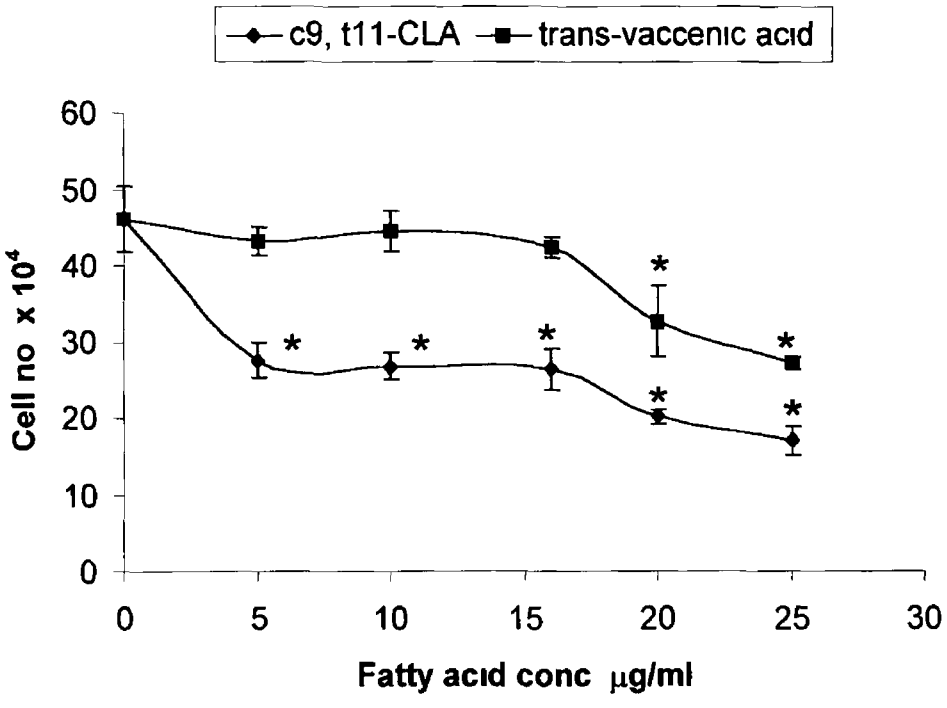


Figure 4 1 MCF-7 cell numbers following treatment with varying concentrations of *c*9, *t*11-CLA and TVA for 4 days * $p<0.05$ relative to control Data is expressed as the mean ± SD for three separate experiments carried out in triplicate

In the SW480 cell line, incubation with of CLA from 5-25 µg/ml decreased cell growth to a similar level (49-52%) (Fig 4 2) Similarly, incubation with a TVA at concentration of 20 µg/ml significantly decreased cell growth in the SW480 cell line The MCF-7 mammary cell line was more sensitive to the growth inhibitory effects of TVA and *c*9, *t*11-CLA

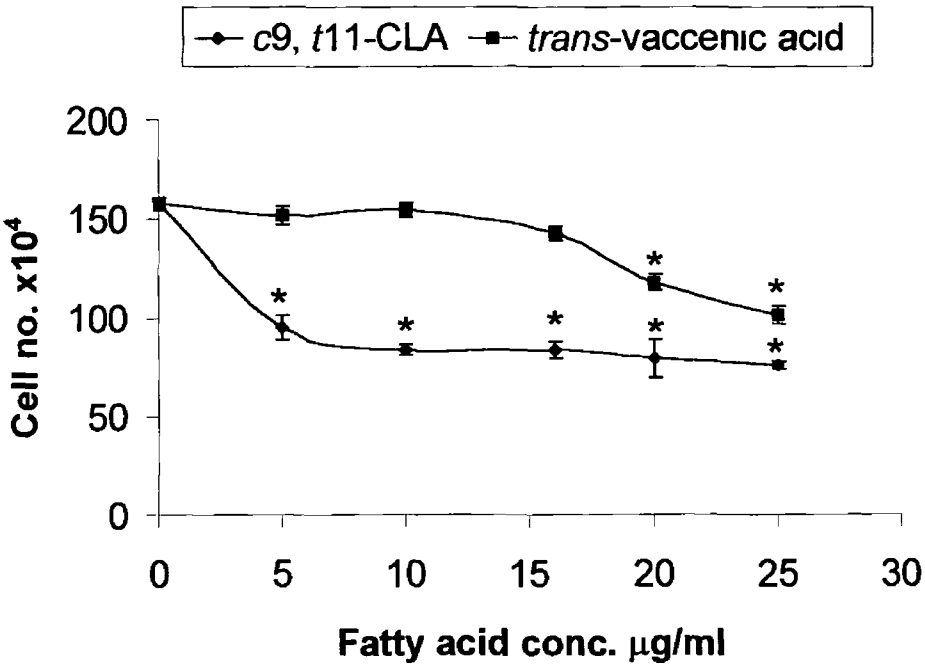


Figure 4 2 SW480 cell numbers following treatment with varying concentrations of *c*9,*t*11-CLA and TVA for 4 days * $p<0.05$ relative to control Data is expressed as the mean ± SD for three separate experiments carried out in triplicate

4 4 3 Effect of TVA on incorporation of ^{14}C-AA into cellular lipid fractions

In order to examine if cellular AA distribution was altered by TVA, we investigated the effect of TVA (20 µg/ml) on incorporation of ^{14}C-AA into cellular lipid fractions Levels of ^{14}C-AA uptake into PL, TG and MG were 64, 27, and 9 %, respectively, in control MCF-7 cells (Figure 4 3 (a)) which are similar to levels of incorporation previously reported (Chapter 2) ^{14}C-AA uptake into the MG fraction was significantly ($p<0.05$) increased by 10% in MCF-7 cells treated with TVA Levels of uptake into PL, TG and MG were 77, 20, and 3 %, respectively, in control SW480 cells (Figure 4 3(b))

which are similar to levels previously reported (Chapter 2) ^{14}C-AA uptake into the MG fraction was significantly ($p<0.05$) increased by 8 % in SW480 cells treated with TVA while uptake into the PL fraction was significantly ($p<0.05$) decreased by 17 %

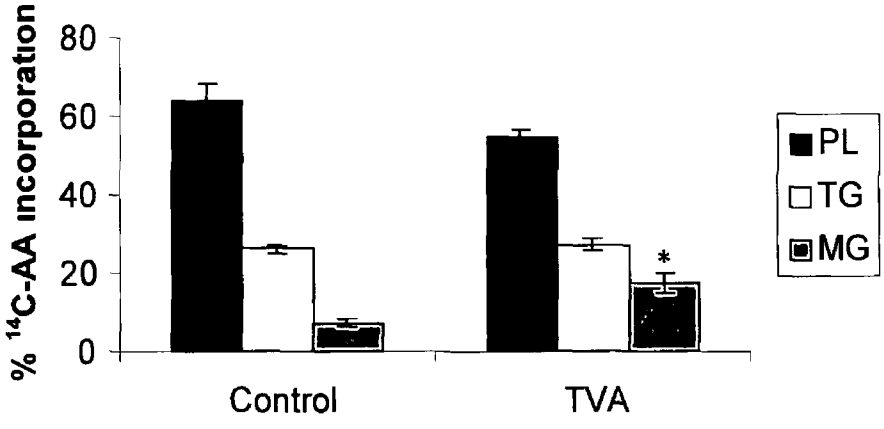


Figure 4.3 (a)

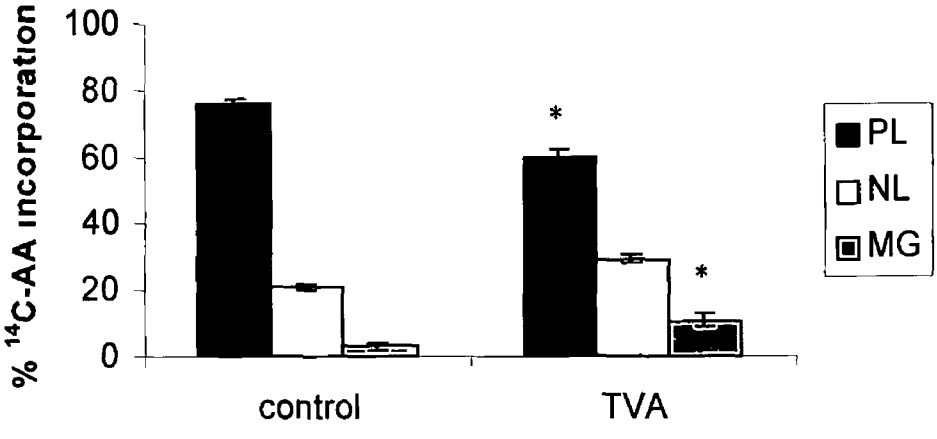


Figure 4.3 (b)

Figure 4.3 Percentage ^{14}C-AA incorporation into phospholipids (PL), triacylglycerol (TG) and monoacylglycerol (MG) following 24h treatment of MCF-7 cells (a) and SW480 cells (b) with TVA (20 μg/ml) * $p<0.05$ relative to control. Data is expressed as the percentage mean ± SD for three separate experiments carried out in triplicate

4 4 4 Effect of TVA on prostaglandin and 8-epi-$PGF_{2\alpha}$ synthesis

The effects of TVA on enzymatic conversion of AA to prostaglandins (PGD_2, PGE_2, and $PGF_{2\alpha}$) and on oxidation to 8-epi- $PGF_{2\alpha}$ were examined Following incubation of both cell lines with TVA (20μg/ml), negligible effects on ^{14}C-AA conversion to ^{14}C-PGD_2, PGE_2, and $PGF_{2\alpha}$ were observed (Figure 4 4) We have previously reported that the *c*9, *t*11-CLA isomer at 16 μg/ml significantly decreased ^{14}C-AA conversion to ^{14}C-PGE_2 while increasing conversion to ^{14}C-$PGF_{2\alpha}$ (Chapter 2) We have also examined the effect of a range of *c*9, *t*11-CLA concentrations (5, 10, 16 and 20 μg/ml) on conversion to ^{14}C-PGE_2 and found that only 16 and 20 μg/ml *c*9, *t*11-CLA significantly decreased ^{14}C-PGE_2 levels (Figure 4 5) Therefore, it is plausible that bioconversion of TVA did not achieve a *c*9, *t*11-CLA concentration high enough to alter prostaglandin synthesis TVA did significantly increase ($p<0$ 05) the levels of the isoprostane 8-epi-$PGF_{2\alpha}$, a biomarker of lipid peroxidation The *c*9, *t*11-CLA isomer has also been shown to increase the levels of 8-epi-$PGF_{2\alpha}$ in both cell lines (Chapter 2)

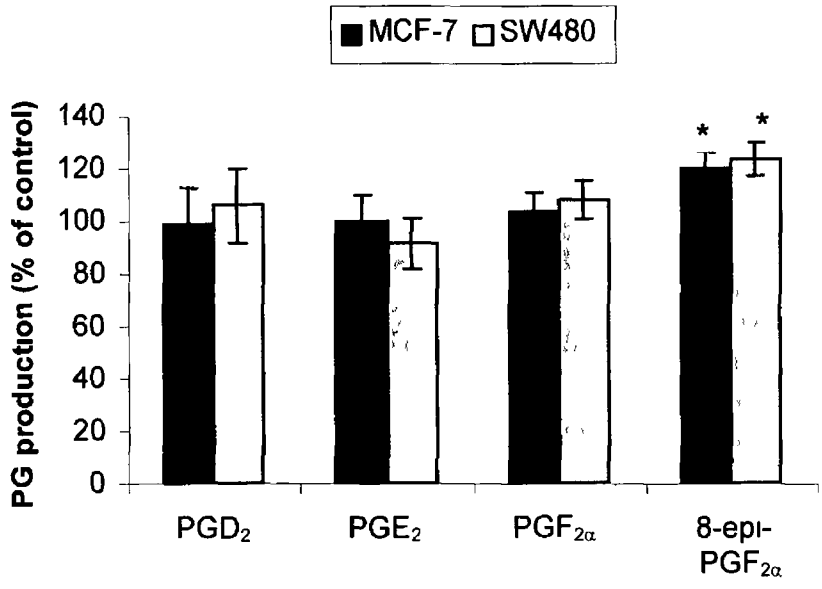


Figure 4 4 Prostaglandin production in MCF-7 cells and SW480 cells following treatment with TVA (20 μg/ml) for 24 hours * $p<0.05$ relative to control Data is expressed as the percentage mean ± SD for three separate experiments carried out in triplicate

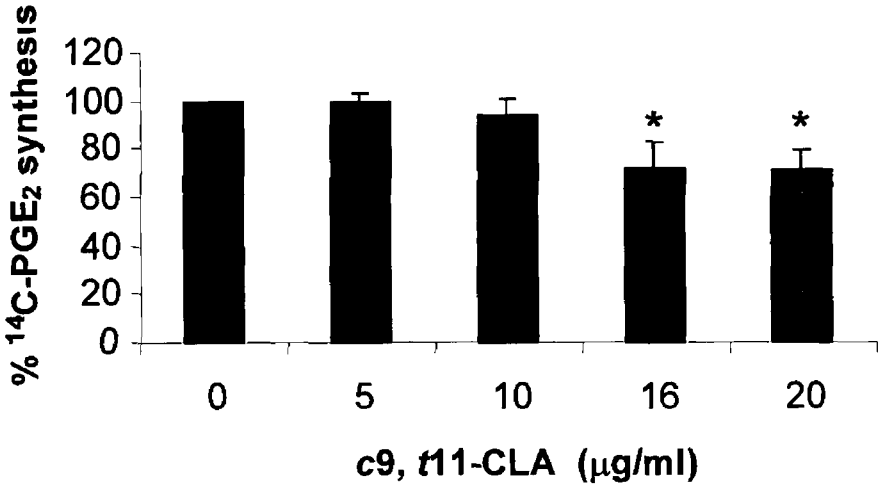


Figure 4 5 Percentage ^{14}C PGE_2 synthesis in SW480 cells following treatment with *c*9, *t*11-CLA (5-25 μg/ml) for 24 h * $p<0.05$ relative to control Data is expressed as the percentage mean ± SD for three separate experiments carried out in triplicate

4 4 5 Effect of TVA on apoptosis in SW480 cells

The formation of distinct DNA fragments of oligonucleosomal size (180-200 bp) is a biochemical hallmark of apoptosis in many cells (Bortner *et al*, 1995) and is observed as a DNA ladder in agarose gels Evidence was sought to determine if the cytotoxic action of TVA induced a specific pattern of chromatin cleavage into oligonucleosomes After cells were treated with TVA for 4 days, the media and PBS wash of the monolayer (both of which may contain apoptotic cells which have floated loose from the monolayer) were collected separately from the attached cells in the monolayer TVA treatment exhibited the characteristic ladder on electrophoresis of DNA extracted from the floating cells (Figure 4 6a) A small amount of DNA laddering was observed in the control cells which would be expected as a small percentage of cells die normally in culture No DNA laddering was observed in DNA extracted from adherent cells (Figure 4 6b) The effect of TVA treatment on levels of cytosolic glutathione was evaluated Diminished glutathione levels have been observed in apoptotic cells and have been associated with cytochrome c release from the mitochondria (Hall, 1999) SW480 cells were treated with TVA (20 µg/mL) as described earlier and after 4 days all cells were collected and cytosolic fraction prepared TVA significantly ($p<0\ 05$) reduced glutathione levels by 15 % (Figure 4 5c)

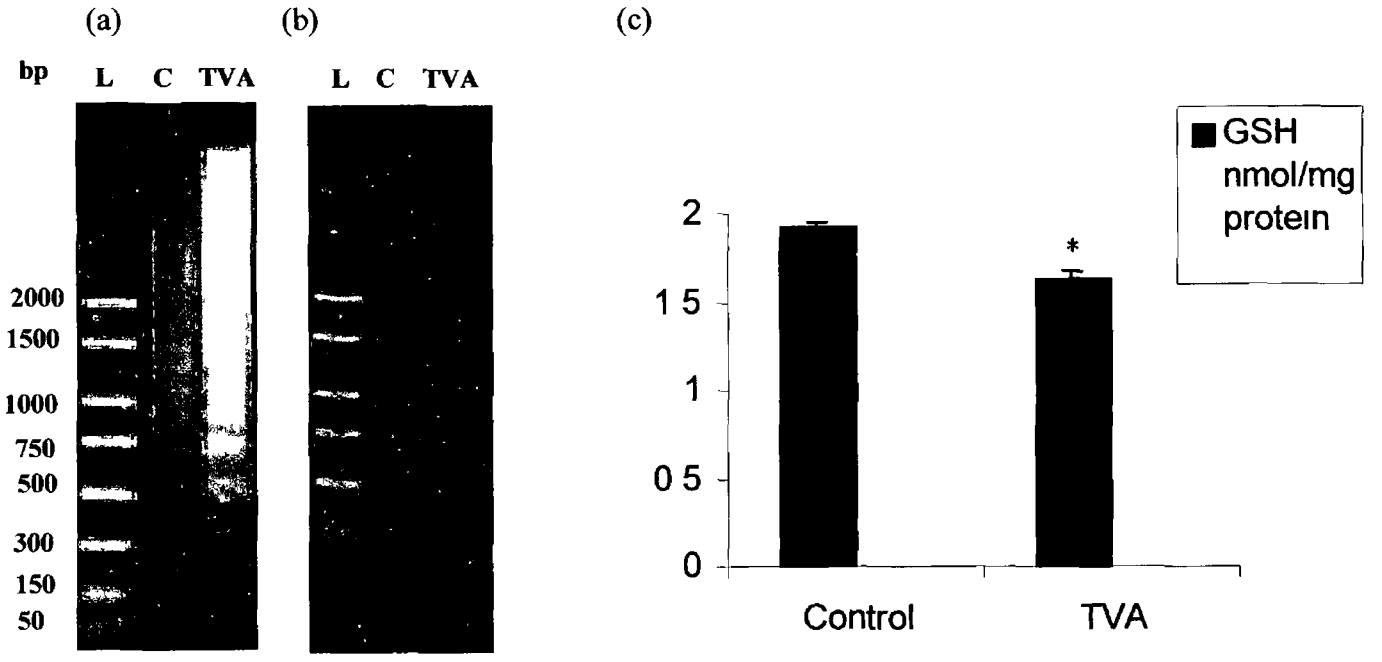


Figure 4 6 Effect of TVA (20 µg/ml) on apoptosis in SW480 cells after 4 days

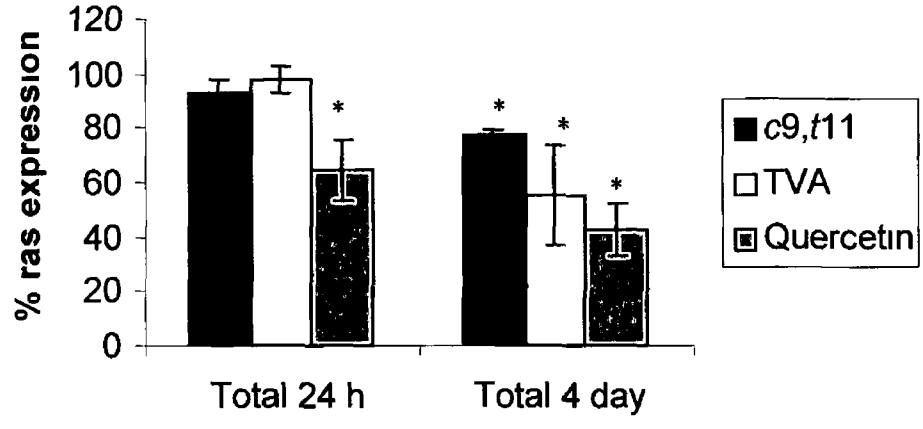
(a) Floating cells collected after 4 days incubation L= marker lane containing DNA fragments ranging from 50 to 2000 base pairs C= Control cells treated with ethanol

(b) Adherent cells collected after 4 days incubation Lanes are as described for (a) and figures represent one of three separate experiments

(c) Effect of TVA on glutathione levels in SW480 cells after 4 days * $p<0.05$ relative to control Data is expressed as the mean ± SD for three separate experiments carried out in triplicate

4 4 6 Effect of TVA and *c*9, *t*11-CLA on ras expression

Molecules that inhibit ras localisation to cell membranes are potential cancer therapeutic agents (Gibbs *et al*, 1994) The *ras* oncogene encodes a protein whose GTPase activity cannot be stimulated and which leaves ras in an active GTP-bound form on the membrane switching on nuclear transcription factors controlling cell proliferation via a cascade of kinase-driven phosphorylation events (Champell et *al*, 1998) Figure 4 7 shows representative examples of Western blot analysis of total ras expression in cells treated with *c*9, *t*11-CLA, TVA and quercetin for 24 h and 4 days, respectively Quercetin was used as a positive control Querectin has been shown to inhibit ras expression in human colon cancer cell lines and in primary colorectal tumors (Ranelletti *et al*, 2000) The doublet present was identified as non-farnesylated ras at 21 kDa and farnesylated *ras* at 23 kDa The upper and lower bands were measured using densitometry Quercetin decreased total ras after 24h and 4 days by 39 and 58% respectively Neither TVA nor *c*9, *t*11-CLA isomer reduced total ras expression after 24 h After 4 days incubation, the *c*9, *t*11-CLA and TVA significantly ($p<0\ 05$) reduced total ras expression by 23 and 45%, respectively



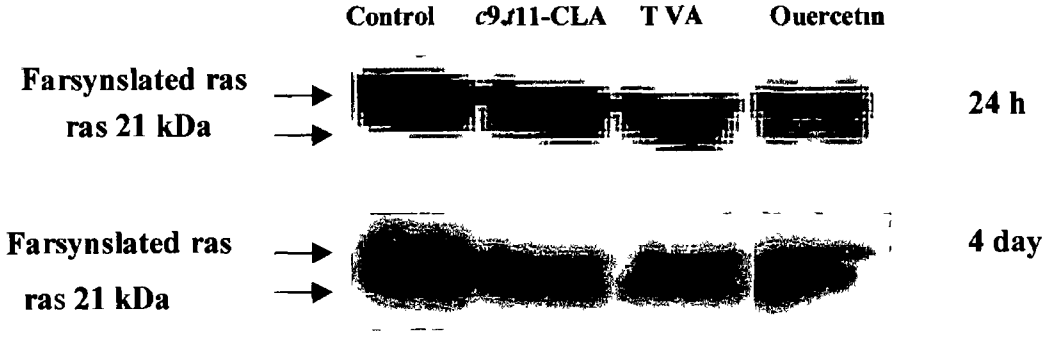


Figure 4 7 Western blot and densitometry result for total ras expression in SW480 cells following treatment with TVA (20 μg/ml) for 24h and 4 days Densitometric quantification was normalised using Ponceau S scans All blots represent one of three independent experiments Values are expressed as percentage of control (± SD) which was taken to be 100% * $p<0.05$ relative to control

4 5 Discussion

It was first proposed by Parodi (1994) that TVA could be converted to CLA in humans based on the observation that a Δ^9 desaturase enzyme from rat liver microsomes has been shown to produce CLA from TVA (Mahfouz *et al*, 1980, Pollard *et al*, 1981) Santora *et al* (2000) reported and quantified the desaturation of TVA to the *c*9, *t*11-CLA isomer in mice fed purified diets Based on concentrations of TVA and *c*9, *t*11-CLA in the total carcass, 11 4 % of dietary TVA and 50 8 % of stored TVA was desaturated The CLA produced from TVA desaturation was found only in triacylglycerols suggesting that bioconversion occurred in the adipose tissue Salminen *et al* (1989) provided evidence to suggest that CLA in human serum was in part derived from the bioconversion of dietary *trans*-fatty acids but provided no quantitative estimate of desaturation Emken *et al* (1986) originally found no evidence for desaturation of TVA in the plasma lipids of men given deuterium-labelled TVA with the limit of detection used in the study However, when one sample from this study was reanalysed, it was demonstrated that TVA was converted to *c*9, *t*11-CLA, at a CLA enrichment of 30 % presumably via the Δ^9 desaturase reaction (Adlof *et al*, 2000) Consistent with this assumption is the recent study showing that the concentrations of CLA and CLA metabolites increased proportionately in the liver and mammary gland of rats fed increasing levels of TVA in the diet (Banni *et al*, 2001)

This study clearly demonstrates that TVA is incorporated into the cellular lipids of MCF-7 and SW480 cancer cells in a dose and time dependent manner and that these cells have the capability to convert a portion of this TVA to *c*9, *t*11-CLA Both cell lines

preferentially synthesised *c*9, *t*11-CLA as indicated by the marked decrease in oleic acid and palmitoleic acid The percentage of TVA (20 μg/ml) bioconverted to *c*9, *t*11-CLA was greater (44 %) in the MCF-7 cell line as compared with the levels observed in the SW480 cell line (29 4 %) after 4 days incubation in the presence of TVA (20 μg/ml) This suggests differences in level of expression and/or activity of Δ^9 desaturase in the two cell lines The MCF-7 cell line has been recently reported to express relatively high levels of Δ^9 desaturase (Choi *et al*, 2002) Unlike the MCF-7 cell line in which the percentage bioconversion to *c*9, *t*11-CLA remained somewhat similar throughout 24 h and 4 days, the dose-dependent decrease in percentage bioconversion in the SW480 cell line suggests product inhibition may have occurred Δ^9 desaturase is regulated by polyunsaturated fatty acids at the level of transcription and mRNA stability (Ntambi, 1995) CLA has been shown to reduce hepatic Δ^9 desaturase mRNA levels in mice, (Lee *et al*, 1998), Δ^9 desaturase mRNA expression and its activity in 3T3-L1 adipocytes (Choi *et al*, 2000) and SCD activity but not expression in the HepG2 human hepatoblastoma cell line (Choi *et al*, 2000) These effects were due to the *t*10, *c*12-CLA isomer However, Choi *et al* (2002) have recently reported that both the *t*10, *c*12- and *c*9, *t*11-CLA isomers have a direct inhibitory effect on SCD enzyme activity in the MCF-7 cell line

TVA has been shown to elicit a biological response *in vivo*, reducing mammary gland premalignant lesions in carcinogen-treated rats (Banni *et al*, 2001) This present study demonstrates that incubation with TVA at a concentration of 25 μg/ml inhibits the growth of human MCF-7 and SW480 cancer cells by up to 41 and 36 %, respectively, after 4 days Another study has also shown that vaccenic acid (8 4 μg/ml), in the form of either

cis or *trans*, significantly reduced growth of HT-29 human colon cancer cells by 17 % when compared with control cells which were supplemented with an equimolar concentration of stearic acid after 9 days (Awad *et al*, 1995) In this present study, TVA treatment induced apoptosis in SW480 cells as indicated by DNA fragmentation Studies suggest that oxidative stress, in general and lipid peroxidation in particular are involved in both initiation and mediation of apoptosis (Lopaczynski and Zeisel, 2001) The isoprostane 8-epi-$PGF_{2\alpha}$, a biomarker of lipid peroxidation was increased while glutathione was reduced following TVA treatment Diminished glutathione levels have been observed in apoptotic cells and are associated with cytochrome c release from the mitochondria (Hall, 1999) We have previously reported that a CLA mixture of isomers, the *t*10, *c*12-CLA and *c*9, *t*11-CLA lowered the expression of the anti-apoptotic bcl-2 protein, decreased cytosolic GSH levels, increased accumulation of cytochrome c in the cytosol, activated caspase 9 and 3 and caused DNA fragmentation (Chapter 3) This study now suggests that growth inhibition by TVA and *c*9, *t*11-CLA in SW480 cells may also be mediated in part by reduced expression of ras oncoprotein The decrease in total ras expression following 4 days treatment of SW480 cells with TVA and *c*9, *t*11-CLA suggests that they may inhibit a *ras* signaling pathway

The growth inhibitory effects of TVA observed in this study were also associated with alterations in AA uptake into cellular lipid fractions In MCF-7 cells, ^{14}C-AA uptake was increased into the MG fraction following treatment with TVA The pattern of incorporation was similar to that previously observed in MCF-7 cells treated with *c*9, *t*11-CLA (Chapter 2) However, a different pattern was observed in SW480 cells, where

TVA treatment, unlike *c*9, *t*11-CLA increased ^{14}C-AA uptake into the MG fraction at the expense of uptake into the PL fraction (Chapter 2) This suggests that TVA alone may specifically influence AA uptake into lipid fractions If only biosynthesised *c*9, *t*11-CLA was influencing AA uptake, it would be expected that ^{14}C-AA uptake would have increased into TG These changes in AA uptake following TVA treatment did not alter prostaglandin profile as was previously observed with c9, t11-CLA This suggests that substrate availability of AA cannot solely account for reduced prostaglandin production CLA isomers have been shown to inhibit the oxygenation of AA by prostaglandin H synthase (Bulgarella *et al*, 2001) CLA and TVA may have different modulatory effects on this enzyme

The human Δ^9 desaturase gene has been isolated, sequenced and shown to be expressed in human skin, adipose, liver and brain tissue (Zhang *et al*, 1999) Expression of the human Δ^9 desaturase gene and enzyme activity was demonstrated recently in MCF-7 cells (Choi *et al*, 2002) While little is known about the expression of desaturase mRNA in normal colon, it is of interest that Δ^9 desaturase mRNA was found to be overexpressed in human colonic tumors (Li *et al*, 1994) Based on the findings in this study, we hypothesis that conversion of TVA to CLA has the potential to increase the levels of CLA in tissues, thereby potentiating the anticancer effect of CLA

4 6 Summary

The aims of this study were to determine if TVA is converted to *c*9, *t*11-CLA in human mammary (MCF-7) and colon (SW480) cancer cell lines and to determine whether TVA

influences cell viability and other CLA-bioresponsive markers When cells were incubated in the presence of TVA from 5 to 20 μg/ml, both TVA and *c*9, *t*11-CLA in cellular lipids increased in a dose-dependent manner After 4 days incubation of SW480 and MCF-7 cells with TVA (20 μg/ml), *c*9, *t*11-CLA increased from undetectable levels to 8 57 and 12 14 g / 100 g FAME in cellular lipids, respectively TVA supplementation for 4 days at concentrations less than 20 μg/ml had no effect on cell growth, while 20 μg/ml significantly ($p<0.05$) reduced cell growth in both cell lines TVA (20 μg/ml) treatment induced DNA fragmentation and significantly ($p<0.05$) depleted cytosolic glutathione levels in the SW480 cell line after 4 days incubation suggesting that apoptosis was the mode of cell death induced by TVA Both TVA and *c*9, *t*11-CLA reduced ($p<0.05$) total *ras* expression in SW480 cells ^{14}C-Arachidonic acid (AA) uptake into the monoacylglycerol fraction was significantly increased ($p<0.05$) in both cell lines while uptake into the phosholipid fraction decreased TVA treatment significantly ($p<0.05$) increased 8-epi-$PGF_{2\alpha}$ in both cell lines The data indicate that growth suppression and cellular responses of both cells lines are likely to be mediated by TVA desaturation to *c*9, *t*11-CLA via Δ^9-desaturase

CHAPTER 5

Conjugated linoleic acid (CLA)-enriched milk fat inhibits growth and modulates CLA-responsive biomarkers in MCF-7 and SW480 human cancer cell lines.[1]

[1] Submitted for publication to International Dairy Journal August 2002

5 1 Introduction

A growing number of dietary components with putative health promoting properties are being identified and a new class of foods, so called functional foods, has emerged (Milner, 1999) Research is unveiling milk fat as a source of bioactive components which include minerals, peptides derived from milk proteins and lipid components (Boland *et al*, 2001) Of particular interest is the fatty acid conjugated linoleic acid (CLA) which has consistently been shown to inhibit chemically induced tumor development in animal models at a number of sites including skin (Belury *et al*, 1996), mammary gland (Ip *et al*, 1994), forestomach (Ha *et al*, 1990) and colon (Liew *et al*, 1995) In addition, cell culture studies have shown CLA to inhibit growth of human cancer cell lines including those of the colon (Chapter 2, Palombo *et al*, 2002), breast (Chapter 2, Park *et al*, 2000) and prostate (Palombo *et al*, 2002) at micromolar concentrations CLA is produced in ruminant animals and as a result milk fat is among the richest natural source of CLA, with the *c*9, *t*11-CLA isomer being the predominant form, accounting for up to 90 % of total milk fat CLA (Chin *et al*, 1992) While CLA is formed in ruminant animals as a first intermediate in the microbial biohydrogenation of linoleic acid (LA) by the action of a bacterial linoleic acid isomerase (Kepler and Tove, 1967), endogenous synthesis from *trans*-vaccemc acid (TVA) represents a more significant source of *c*9, *t*11-CLA in milk fat (Grinari *et al*, 2002)

Because of the health promoting properties associated with CLA, attempts have been made to enrich its content in milk fat Animal diet has a major impact on the CLA content of milk fat and several studies have shown that it can be elevated by modifying the dietary regime of the dairy cow (Lawless *et al*, 1998, Chilhard *et al*, 2000), thus offering

the possibility of producing CLA-enriched dairy products Ip and co-workers reported that feeding CLA enriched butterfat (41 mg/g fat) altered mammary gland morphogenesis and reduced mammary cancer risk in rats by the same magnitude as a synthetic mixture of CLA isomers and a synthetic *c*9, *t*11-CLA preparation (Ip *et al*, 1999a) The group consuming the butterfat consistently accumulated more total CLA in their tissues compared with the group consuming the synthetic CLA preparations and authors suggested that TVA in the butterfat may have been a precursor for endogenous synthesis of CLA In a more recent study, this CLA enriched butterfat effectively suppressed the proliferative activity and the expression of cell cycle regulating proteins (cyclin D1 and A) in the developing rat mammary epithelium (Ip *et al*, 2001)

It was reported in Chapter 2 that the mammary MCF-7 and colon SW480 cancer cell lines were sensitive to the cytotoxic effect of a CLA mixture of isomers and the *c*9, *t*11-CLA isomer The CLA-induced cytotoxicity was related to an increase in lipid peroxidation (Chapter 2,), alterations in the mobilisation and metabolism of arachidonic acid (Chapter 2), a reduction in the level of the anti-apoptotic bcl-2 protein which triggered a cascade of events leading to apoptosis (Chapter 3) and modulation of *ras* expression (Chapter 4) CLA enriched milk fat was previously reported to be more effective than synthetic CLA at decreasing MCF-7 cell numbers and increasing lipid peroxidation after an 8 day incubation period (O'Shea *et al*, 2000) These effects were independent of the variable composition of the milk fat samples, suggesting that CLA may be the active ingredient responsible for the cytotoxic effect in MCF-7 cells

5 2 Objective

The present study investigated whether fatty acids (linoleic, *trans*-vaccenic acid and oleic acid) present in CLA-enriched milk fats could influence growth of MCF-7 and SW480 cells The potency of CLA-enriched milk fats to modulate CLA-responsive biochemical and molecular biomarkers previously observed in the MCF-7 and SW480 cell lines (Chapter 2, 3, 4) were assessed Specifically, the effects of CLA-enriched milk fats on (1) arachidonic acid uptake, distribution and conversion to eicosanoid classes in MCF-7 and SW480 cell lines, (2) lipid peroxidation, (3) apoptotic markers in the SW480 cells line (reduced glutathione levels, membrane annexin V levels and bcl-2 expression) and (4) levels of ras expression in SW480 cells were assessed

5 3 Materials and methods

5 3 1 Cell culture conditions

The MCF-7 and SW480 human colon cancer cell lines were cultured exactly as outlined in Chapter 2

5 3 2 Milk fat samples

The milk fats used in this study were obtained following supplementation of lactating dairy cows on pasture (control) or on pasture supplemented with full fat rapeseeds (FFR) and full fat soybeans (FFS) for 33 and 34 days, respectively (Lawless *et al*, 1998) Table 1 5 summarises the concentrations of CLA, oleic acid, TVA and LA in the milk fat samples The full fatty acid composition is as described (O'Shea *et al*, 2000)

Table 1 5 Fatty acid composition of milk fat samples (g/100g FAME)

Fatty acid	Control milk fat	FFS milk fat	FFR milk fat
CLA	1 69	1 83	2 26
Trans-vaccenic acid	3 14	4 41	4 46
Oleic Acid	20 28	23 37	26 92
Linoleic acid	1 25	4 28	1 69

5 3 3 Quantification of cell numbers

Cells were seeded in 6 well plates and the MCF-7 and SW480 cells were seeded at densities of 1 x 10^5/well and 5 x 10^4/well respectively Cells were cultured for 24 h to allow the cells attach to the substratum The medium was then replaced with medium containing either FFR milk fat, FFS milk fat or control milk fat at a concentration of 1mg/ml to yield CLA concentrations of 22 6, 18 3 and 16 9 μg/ml, respectively Control wells were supplemented with equivalent volumes of ethanol In a separate experiment, the milk fat content of the medium was varied between 0 8 and 1 2 mg/ml to yield a CLA concentration of 20 μg/ml in all milk fat samples Cells were also incubated in the presence of *c*9, *t*11-CLA (95 % pure from Natural ASA, Hovdebygda, Norway), linoleic acid, *trans*-vaccenic acid or oleic acid (all 99 % and obtained from Sigma-Aldrich Ireland Ltd , Dublin, Ireland) at concentrations similar to those found in the milk fat samples For all viability experiments, cells were harvested in the presence of phosphate buffered saline (PBS) containing 0 25 % (v/v) trypsin Viable cell numbers were quantified using the trypan blue exclusion (0 4 % w/v) assay

5 3 4 Uptake up ^{14}C-AA conversion and to eicosanoids

Cells were seeded in T-25 cm^2 flasks at a density of 2 x 10^5/flask and grown to 90% confluency The medium was then replaced with medium containing ^{14}C-AA at 0 2 μCi along with the milk fat samples control, FFS or FFR (all added at milk fat concentration of 1mg/ml yielding CLA concentrations of 16 9, 18 3 and 22 6 μg/ml, respectively) or an equivalent volume of ethanol After 24 h incubation, cells were harvested to determine uptake of ^{14}C-AA and the media was collected Total cellular lipids were extracted from cell pellet and then separated into triglyceride (TG), monoglyceride (MG) and phospholipid (PL) fractions as described (Chapter 2) An aliquot of each fraction was counted in a Beckman LS6500 scintillation counter Eicosanoids were extracted as described (Chapter 2) Eicosanoid extracts were dried under nitrogen, redissolved in ethyl acetate separated using normal-phase TLC as described previously (Chapter 2) Bands of PGE_2, $PGF_{2\alpha}$, PGD_2 were removed from TLC plates and placed in vials for counting by liquid scintillation The isoprostane, 8-epi-$PGF_{2\alpha}$ was extracted from media as described (Watkins *et al*, 1999) and a competitive horseradish peroxidase (HRP) enzyme-linked immunoassay kit (BIOXYTECH 8-Isoprostane assay system) was used to quantify 8-epi-$PGF_{2\alpha}$ levels according to the manufacturer's instructions 5-Hydroperoxyeicosatetraenoate (5-HPETE) was measured using a colorimetric method developed by Washidge and Haynes (1995)

5 3 5 Measurement of reduced glutathione (GSH) and annexin V levels

Cells were seeded in T-75 cm^2 flasks at a density of 1 x 10^6 cells/flask and incubated for 24 h The medium was then replaced with fresh medium containing the milk fat samples,

control, FFS or FFR (1 mg/ml) as described earlier and incubated for 4 days after which both floating and adherent cells were collected and pooled Cells were then resuspended in a PBS/PMSF buffer (10 mM sodium phosphate buffer (pH 7 2), 100 mM NaCl, 0 2 mM phenylmethylsulfonylfluoride (PMSF)) containing 0 1 mM leupeptin, 0 2 µg/ml aprotinin (both from Sigma-Aldrich Ireland Ltd , Dublin, Ireland) as protease inhibitors, sonicated on ice and centrifuged at 100,000 x g for 1 hour at 4°C The supernatant (cytosolic fraction) was analysed for GSH levels according to the method of Hissen and Hilf (1976) The pellet was resuspended in lysis buffer (10 mM sodium phosphate buffer (pH 7 2), 0 2 mM PMSF and 100 mM NaCl) containing 0 1 mM leupeptin and 0 2 µg/mL aprotinin and incubated on ice for 20 min followed by centrifugation at 15,000 x g for 15 min at 4°C The resulting supernatant (membrane fraction) was analysed for annexin V content using Annexin V Elisa kit (Alexis Biochemicals, San Diego, CA, USA) according to the manufacturer's instructions Details of kit are described in Chapter 3 The GSH and annexin V concentrations of the samples were determined from standard curves and expressed relative to the protein content, as determined using the Bio-Rad protein assay (Biorad, Hemel Hempstead, Hertfordshire, UK)

5 3 6 Measurement of bcl-2 and ras expression

SW480 cells were seeded at 5 x 10^6 cells /150cm^2 flask and were cultured for 24 h to allow the cells to attach to the substratum The medium was then replaced with fresh media containing the milk fat samples, control, FFS or FFR (1 mg/ml) After 4 days of incubation, both floating cells and adherent cells were collected and pooled Cells were washed twice in ice-cold PBS and resuspended in lysis buffer (described above) Lysates were sonicated using a Vibra Cell VC502 (Sonics, Newtown, CT, USA) on ice and

concentrated using Microcons® (Millipore, Cork, Ireland) Protein concentrations were determined using the Bio-Rad protein assay (Biorad, Hemel Hempstead, Hertfordshire, UK) SDS-PAGE and Western blots were performed essentially by the methods of Laemmli (1970) and Towbin *et al* (1979), respectively Lysates containing approximately 70 µg of protein were solubilized in sample buffer (10 % (w/v) SDS, 600 mM Tris-HCl (pH 6 7), and 50 % (w/v) glycerol) containing β-mercaptoethanol and 50 µg/mL bromophenol blue Samples were boiled for 2 min and proteins resolved by electrophoresis and blotted onto Hybond ECL membrane (Amersham, Little Chadfort, Buckinghamshire, UK) in a Trans-blot Electrophoretic transfer cell (Biorad, Hemel Hempstead, Hertfordshire, UK) Blots were stained with Ponceau S Solution (0 2 % w/v) to ensure transfer of proteins was complete and to determine if equivalent amount of protein were loaded in each lane The blots were destained with PBS containing 0 1 % (v/v) Tween 20 (PBST) and blocked for 1 h with 5 % (w/v) nonfat dry milk dissolved in PBST Blots were then incubated with monoclonal antibodies overnight anti-bcl-2 diluted to 1 1000 (Sigma-Aldrich Ireland Ltd , Dublin, Ireland) or anti-ras diluted to 1 40 (Oncogene Science, Manhasset, NY) in PBST containing 0 5 % (w/v) nonfat dry milk Blots were washed extensively in PBST and reincubated for 1 h with a HRP-linked secondary antibody (Amersham, Little Chadfort, Buckinghamshire, UK) diluted 1 2000 in PBST containing 0 5 % (w/v) nonfat dry milk The blots were then thoroughly washed in excess PBST and probed with the Super Signal detection system (Pierce, Rockford, IL, USA) and exposed to autoradiography films (Amersham, Little Chadfort, Buckinghamshire, UK) according to the manufacturer's instructions Densitometry (using NIH Image software) was performed on Ponceau S scans and autoradiographs

5 3 7 Statistical analysis

Three independent experiments were performed in triplicate The Student's *t* test was used to determine significance between treatments

5 4 Results

5 4 1 The effects of CLA-enriched milk fat on cell viability

MCF-7 and SW480 cells were incubated for 4 days in the presence of milk fat (1mg/ml) to yield CLA concentrations in the range from 16 9 - 22 6 μg/ml This allowed examination of the effect of increasing milk fat CLA concentration while the milk fat content was kept constant Cell numbers following 4 days of incubation with all three milk fats were significantly ($p<0\ 05$) lower than untreated control cells (Figure 5 1a and 5 1b) A dose-dependent decrease in cell number was observed with increasing CLA content in the milk fats Maximal growth inhibition of 61 % and 58 % occurred in the MCF-7 and SW480 cells, respectively, following treatment with the highest milk fat CLA concentration A significant ($p<0\ 05$) inhibitory effect on cell number was obtained following incubation of both cell lines with *c*9, *t*11-CLA at 16 9, 18 3 and 22 6 μg/ml, representing the concentrations present in Control, FFS and FFR milk fats, respectively (Figure 5 1a and 5 1b) The respective percentages by which cell viability was reduced following addition of *c*9, *t*11-CLA to the medium were 43, 46 and 49 % in the MCF-7 cell line and 41, 44 and 50 % in the SW480 cell line In the MCF-7 cell line, milk fat CLA (16 9 and 18 3 μg/ml) was significantly ($p<0\ 05$) more effective at decreasing cell numbers when compared to the synthetic *c*9, *t*11-CLA at the same concentrations In the

SW480 cell line no significant difference was observed between the milk fat CLA and synthetic *c*9, *t*11-CLA treatments

The milk fat content of the medium was then varied between 0 8 and 1 2 mg/ml to yield a final CLA milk fat concentration of 20 μg/ml All milk fat samples significantly ($p<0$ 05) lowered cell numbers compared with untreated control cells after 4 days incubation by approximately 58 % and 53 % in MCF-7 and SW480 cells respectively (Figure 5 2a and 5 2b) No significant differences in final cell numbers was obtained for all milk fat treatments in either cell line

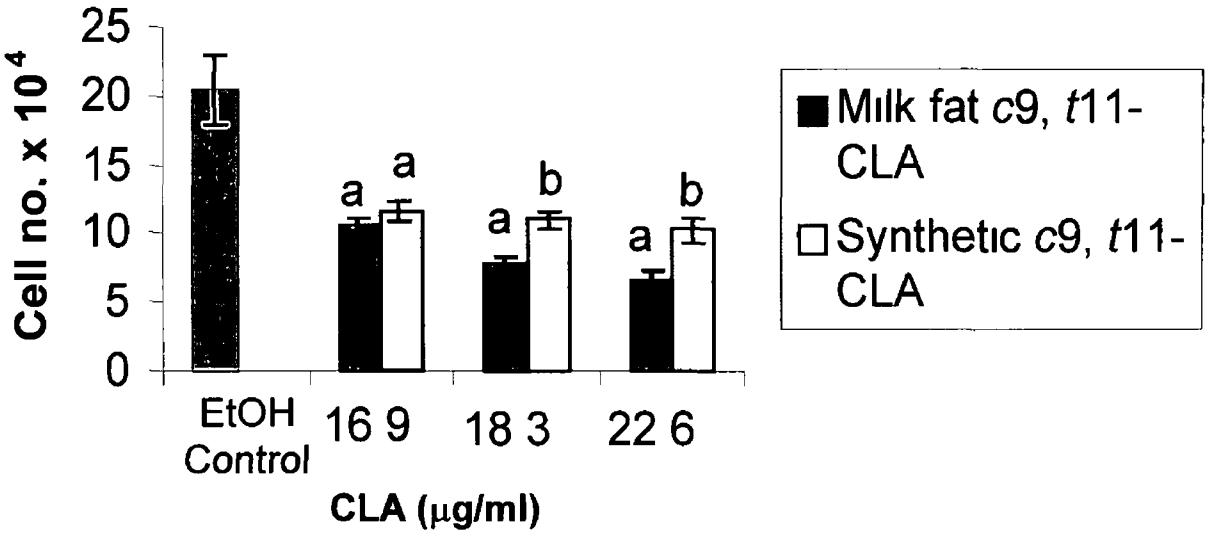


Figure 5 1 (a)

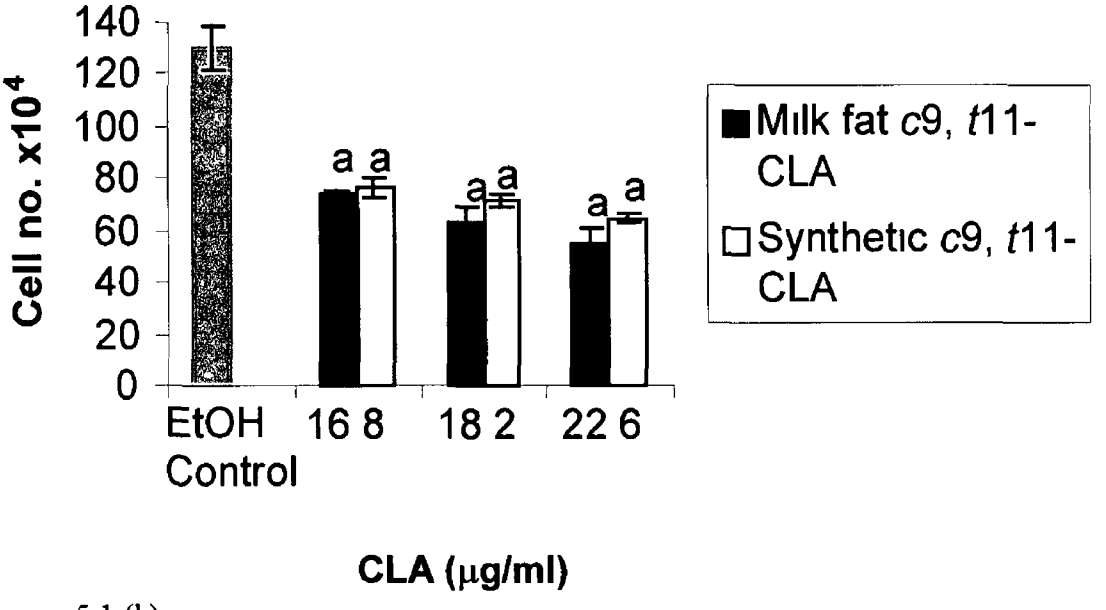


Figure 5 1 (b)

Figure 5 1 Growth of (a) MCF-7 and (b) SW480 cells incubated with milk fat samples and synthetic c9, t11-CLA containing varying levels of CLA for 4 days Within a concentration, the bars not sharing the same letter are significantly different from one another ($p<0.05$) All CLA treatments are significantly different to ethanol controls Data is expressed as the mean ± SD for three separate experiments carried out in triplicate

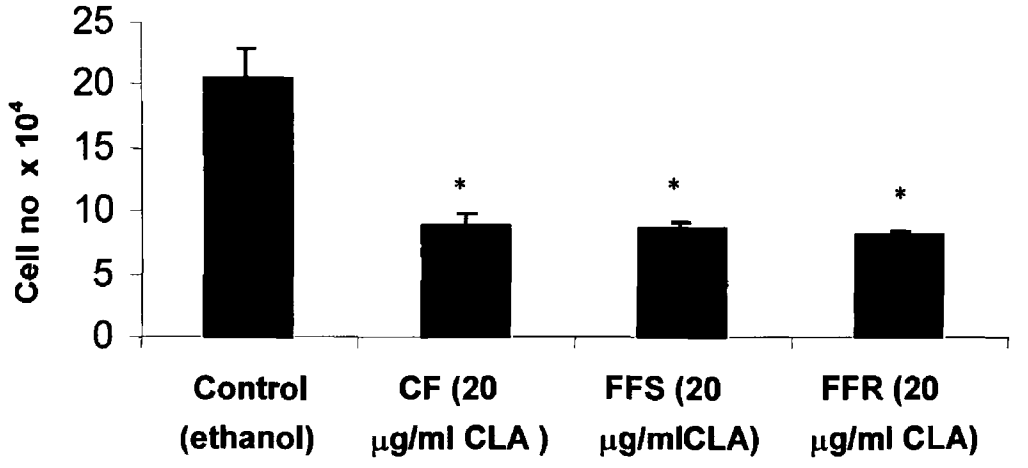


Figure 5 2 (a)

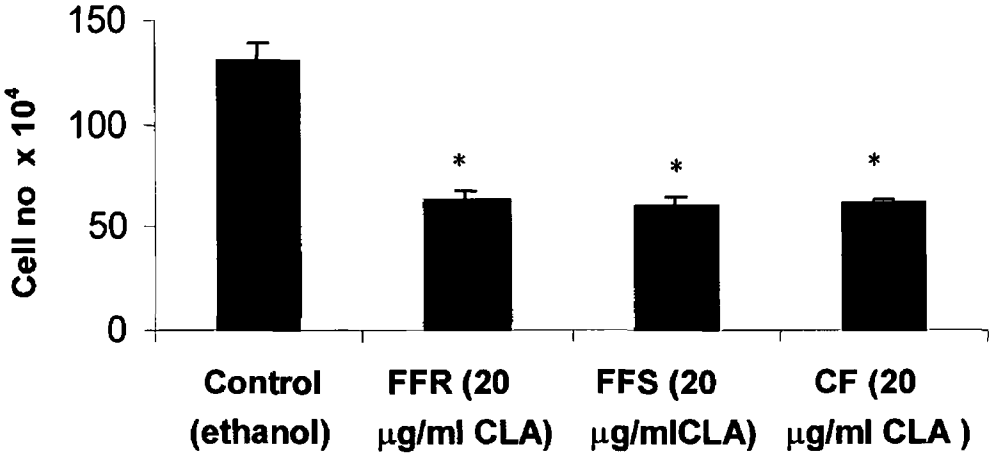


Figure 5 2 (b)

Figure 5 2 Growth of (a) MCF-7 and (b) SW480 cells incubated with milk fat samples containing 20 µg/ml CLA for 4 days Data is expressed as the mean ± SD for three separate experiments carried out in triplicate CF, control milk fat, FFR, full fat rapeseed milk fat, FFS, full fat soyabean milk fat

To elucidate the effects of individual fatty acids present in the milk fat on cell viability, cells were incubated in the presence of either pure linoleic acid (LA), *trans*-vaccenic acid (TVA) or oleic acid at concentrations similar to those found in the milk fat samples and viability was assessed after 4 days incubation TVA significantly decreased ($p<0.05$) cell viability by approximately 22-37 % in both cell lines when added at the concentrations present in the three milk fat samples (31.4, 44.4 and 46.4 μg/ml in the control, FFS and FFR, respectively) The MCF-7 cells were more sensitive to the growth inhibitory effects of TVA When cells were incubated in the presence of LA at the concentrations present in the three milk fat samples (12.5, 42.1 and 16.9 μg/ml in the control, FFS and FFR, respectively) differential effects on growth were observed In the MCF-7 cell line, LA at a concentration of 12.5 μg/ml significantly ($p<0.05$) stimulated cell growth by 26 % but at a concentration of 42.1 μg/ml LA was cytotoxic to the cells inhibiting cell growth by 43 % LA at 16.9 μg/ml had no significant effect on final cell numbers In the SW480 cell line, LA at 12.5 and 16.9 μg/ml stimulated cell growth but the increase was only significant at the higher LA concentration When LA was added at a concentration of 42.1 μg/ml SW480 cell growth was significantly ($p<0.05$) inhibited by 40 % The latter cell line was more sensitive to the growth modulatory effects of oleic acid than the MCF-7 cell line Incubation with oleic acid significantly decreased cell numbers in the MCF-7 only at the highest concentration of 269.2 μg/ml which was representative of the concentration found in the FFR milk fat sample In contrast, incubation of SW480 cells with oleic acid at 202.8, 233.7, 269.2 μg/ml, representing the concentrations present in control, FFS and FFR milk fats respectively, significantly ($p<0.05$) decreased cell viability by 13, 26 and 39 %, respectively

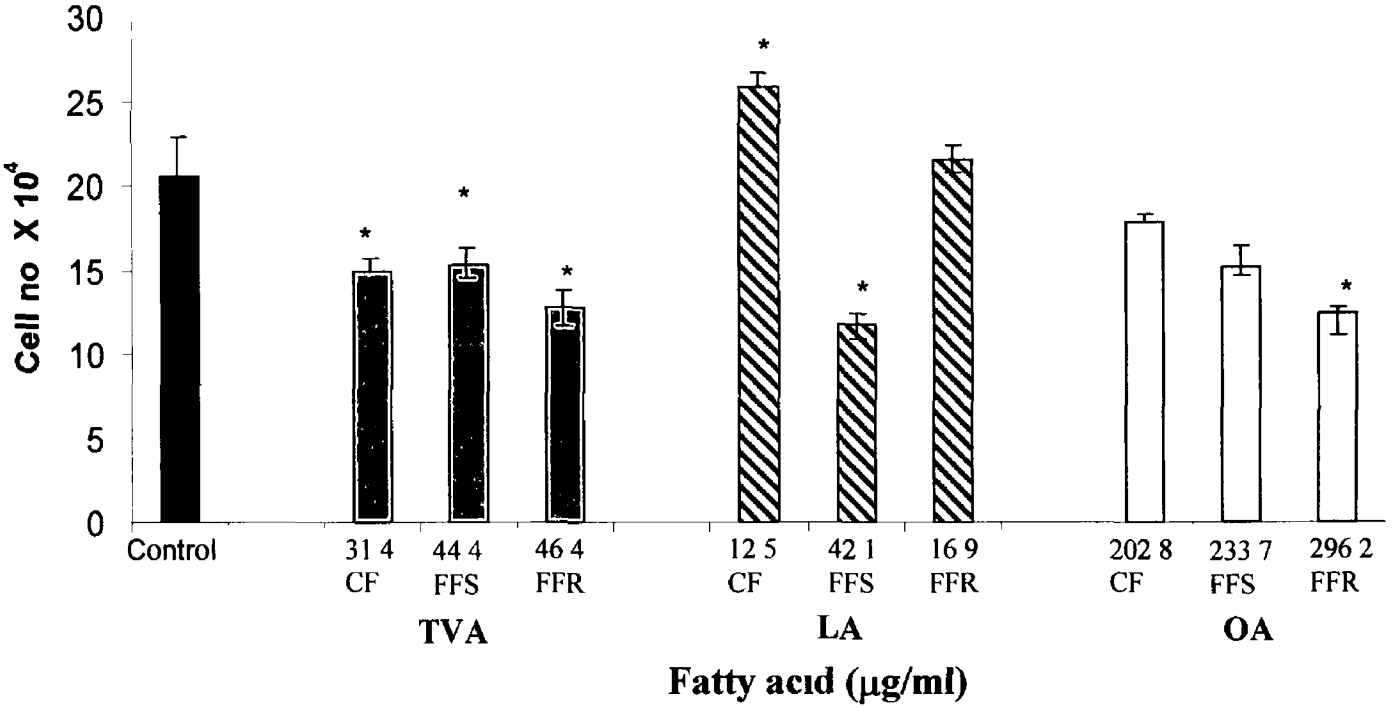


Figure 5 3 (a)

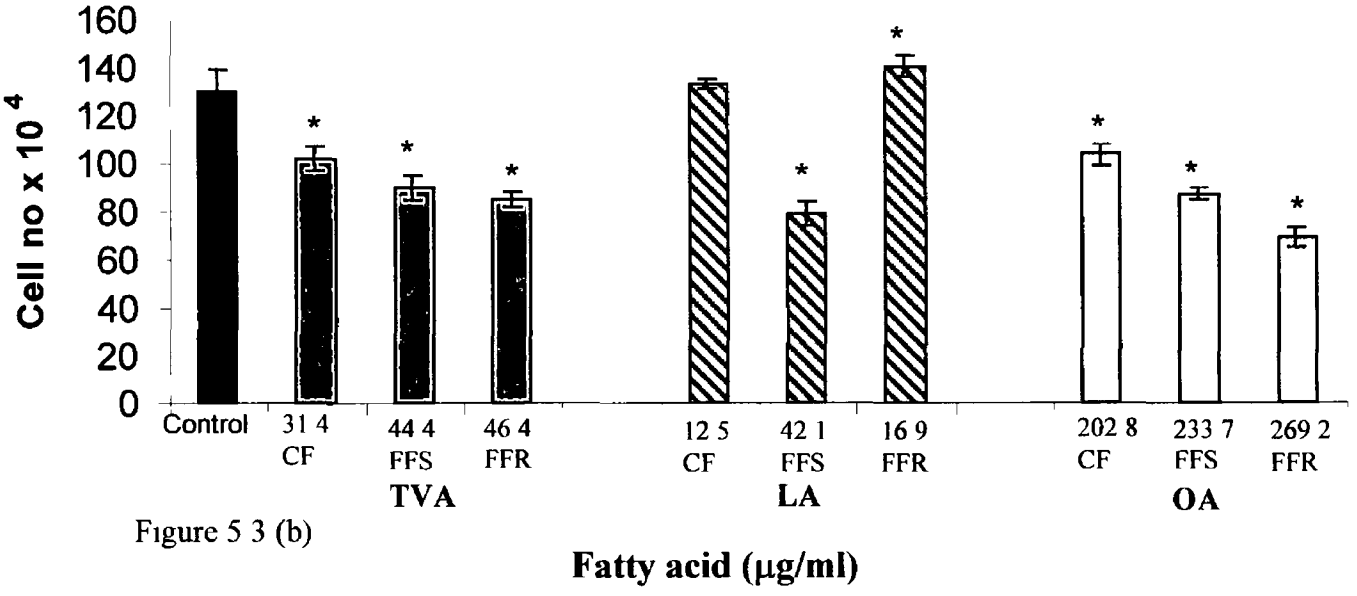


Figure 5 3 (b)

Figure 5 3 Growth of (a) MCF-7 and (b) SW480 cells incubated with TVA, LA and oleic acid (OA) at concentrations similar to those present in the milk fat samples after 4 days

* denotes values significantly different to control cells ($p<0.05$) Data is expressed as the mean ± SD for three separate experiments carried out in triplicate CF, control milk fat, FFR, full fat rapeseed milk fat, FFS, full fat soyabean milk fat

5 4 2 Effect of CLA enriched milk fat on incorporation of ^{14}C-AA into cellular lipid fractions

In order to examine if growth inhibition by milk fat could be attributed to an altered pattern of AA distribution and eicosanoid formation, we investigated the effects of three milk fat samples on incorporation of ^{14} C-AA into cellular lipid fractions of MCF-7 and SW480 cells The control fat, FFS and FFR milk fats were added at 1mg/ml milk fat to yield CLA milk fat concentrations of 16 9, 18 3 and 22 6 μg/ml, respectively Levels of ^{14}C-AA uptake into PL, TG and MG were 64, 27, and 9 %, respectively, in control MCF-7 cells and 74, 22, and 4 %, respectively, in control SW480 cells (Figure 5 4a and 5 4b) These patterns of AA incorporation are similar to those previously reported in these cell lines (Chapter 2) In both cells lines, only incubation with the FFR milk fat, containing the highest CLA levels (22 6 μg/ml), caused perturbations in ^{14}C-AA uptake (Figure 5 4a and 5 4b) In the MCF-7 cell line, treatment with FFR milk fat significantly ($p<0.05$) increased ^{14}C-AA uptake into the MG fraction by 10 % In SW480 cells, FFR milk fat treatment also resulted in a significant ($p<0.05$) increase in ^{14}C-AA uptake into the MG fraction (by 8 %) and this was accompanied by a significant ($p<0.05$) decrease in uptake into the PL fraction (by 12 %)

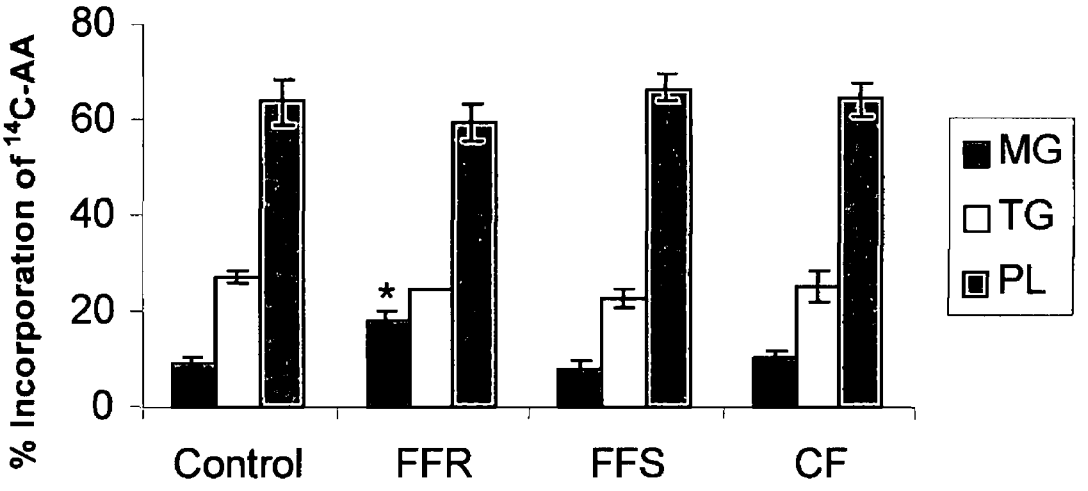


Figure 5 4 (a)

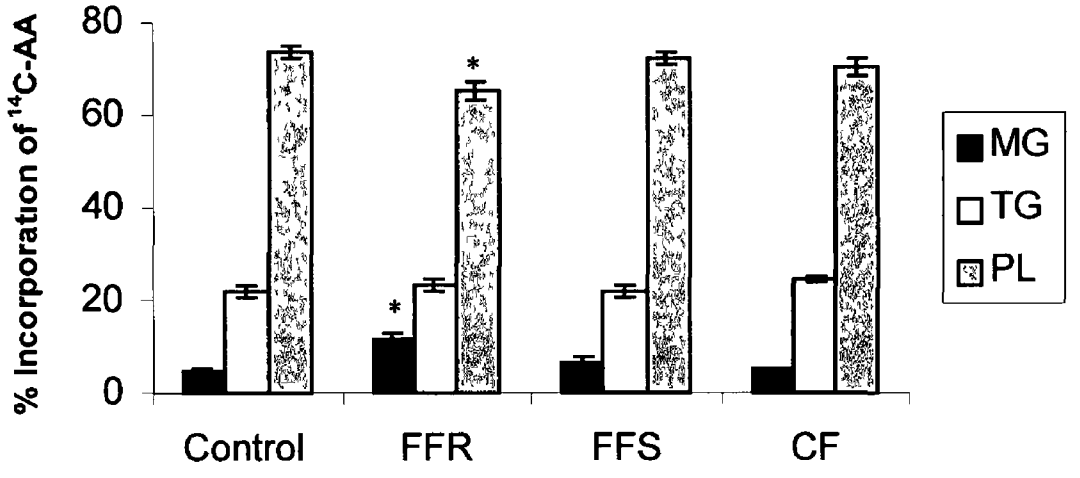


Figure 5 4 (b)

Figure 5 4 Percentage ^{14}C-AA incorporation into phosphlipids (PL), triglyceride (TG) and monoglyceride (MG) following 24 h treatment of MCF-7 (a) and SW480 (b) cells with milk fat samples (1 mg/ml) * denotes values significantly different to control cells ($p<0.05$) Data is expressed as the percentage mean ± SD for three separate experiments carried out in triplicate CF, control milk fat, FFR, full fat rapeseed milk fat, FFS, full fat soyabean milk fat, MG, monoglyceride, TG, triglyceride, PL, phospholipid

5 4 3 Effect of CLA-enriched milk fat on eicosanoid and 8-epi- $PGF_{2\alpha}$ synthesis

The effects of the control, FFS and FFR milk fat treatments (all added at 1mg/ml milk fat) on enzymatic conversion of AA to selected eicosanoids (PGD_2, PGE_2, and $PGF_{2\alpha}$ and 5-HPETE) and on oxidation to 8-epi-$PGF_{2\alpha}$ were examined In both cells lines, only the FFR milk fat treatment altered the eicosanoid profile (Figure 5 5a and 5 5b) Following incubation of both cell lines with FFR milk fat, ^{14}C-AA conversion to PGE_2 was significantly ($p<0\ 05$) decreased (by approximately 21 - 25 %) while conversion to $PGF_{2\alpha}$ was significantly ($p<0\ 05$) increased (by 23 - 27%) A CLA dose-dependent increase in the isoprostane 8-epi-$PGF_{2\alpha}$, a biomarker of lipid peroxidation, was observed in both cell lines following incubation with the three milk fats Maximal stimulation of 8-epi-$PGF_{2\alpha}$ production by 73 and 92 % was observed in MCF-7 and SW480 cells respectively, following treatment with the FFR milk fat which contained the highest CLA concentration None of the milk fat treatments significantly altered the production of 5-HPETE

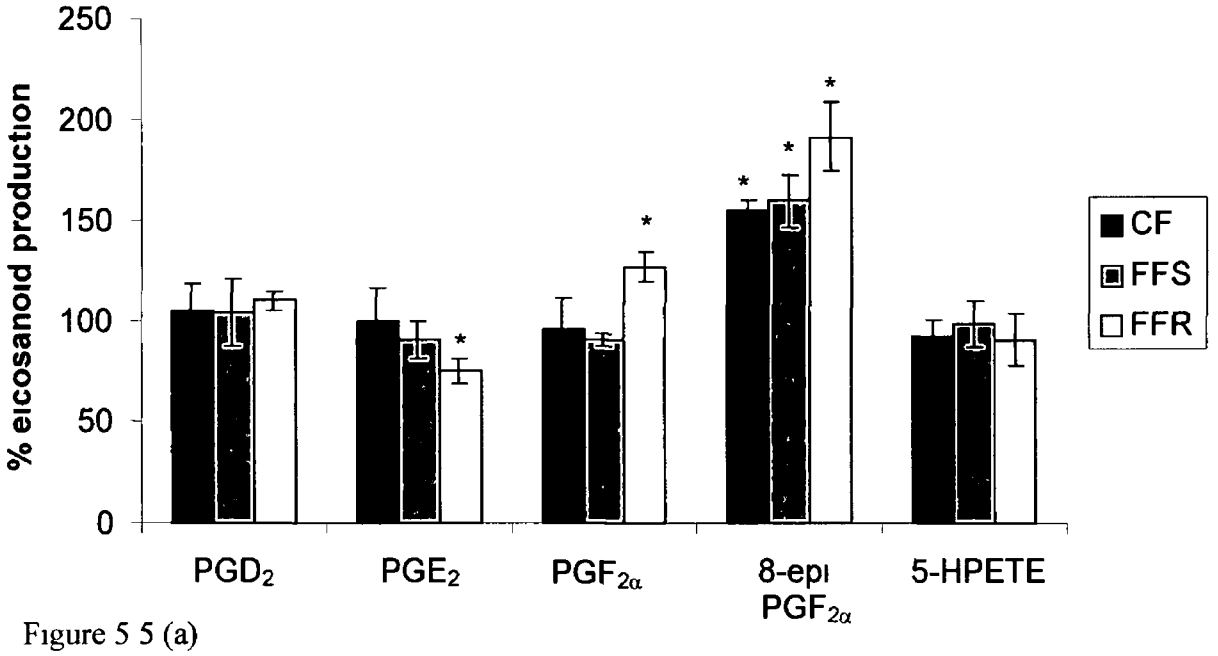


Figure 5 5 (a)

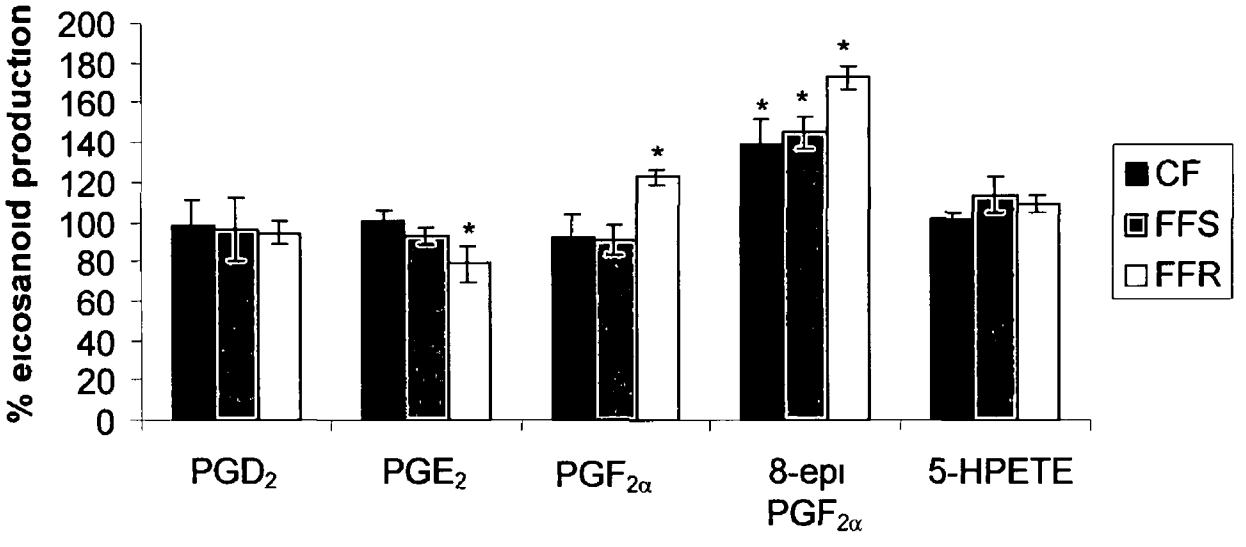


Figure 5 5 (b)

Figure 5 5 Percentage eicosanoid synthesis following 24 h treatment of MCF-7 (a) and SW480 (b) cells with milk fat samples (1 mg/ml) * denotes values significantly different to control cells ($p<0.05$) Data is expressed as the percentage mean ± SD for three separate experiments carried out in triplicate

5 4 4 Effect of CLA-enriched milk fat on apoptotic markers in SW480 cells

To determine if the cytotoxic effect of the milk fat samples was executed via an induction of an apoptotic signalling pathway their effects on cytosolic GSH content, membrane annexin V levels and bcl-2 expression were examined The data demonstrate that treatment of SW480 cells with the milk fat samples significantly ($p<0.05$) reduced bcl-2 protein expression by 23 – 36 % in a milk fat CLA concentration-dependent manner (Figure 5 7) All milk fat samples depleted cytosolic GSH by approximately 21-39% in SW480 cells, with the FFR milk fat exerting the most potent effect (Figure 5 7) All milk fat treatments significantly ($p<0.05$) increased levels of annexin V (29-32%) in the cell membrane when compared with concentrations found in the membrane preparations of untreated SW480 cells (Figure 5 6)

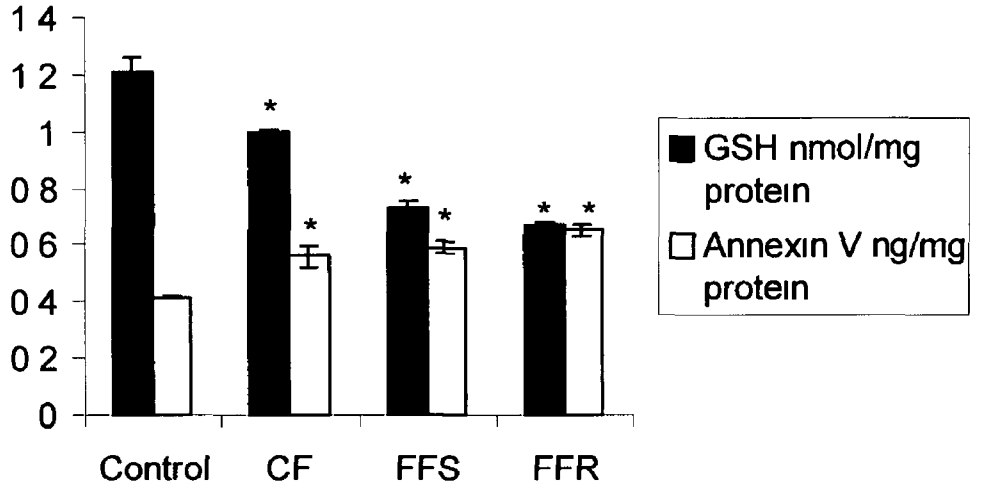


Figure 5 6 Levels of cytosolic GSH and membrane annexin V following 4 day treatment of SW480 cells with milk fat samples (1 mg/ml) * denotes values significantly different to control cells ($p<0.05$) Data is expressed as the mean ± SD for three separate experiments carried out in triplicate

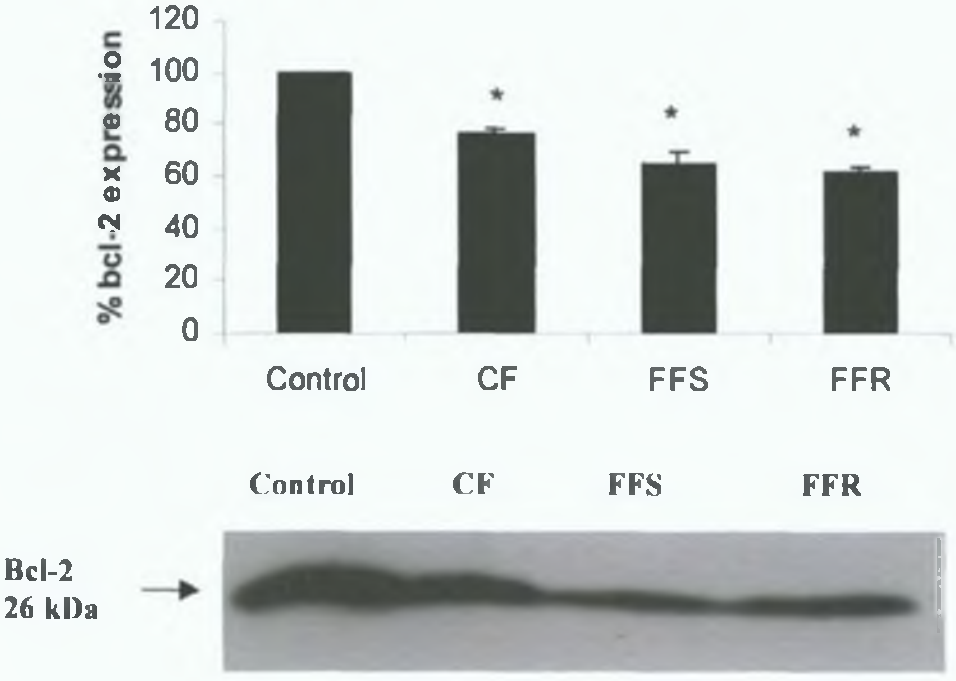


Figure 5.7 Expression of bcl-2 in SW480 cells following 4 day treatment with milk fat samples (1 mg/ml). * denotes values significantly different to control cells ($p<0.05$). Blot represent one of three independent experiments. Values are expressed as a percentage of the control (± SD) which was taken to be 100%

5.4.5 Effect of CLA enriched milk fat on ras expression.

The effect of the milk fat samples on total *ras* expression in the SW480 cell line which overexpresses k ras (Geiser *et al.*, 1989) was examined. Figure 5.8 shows a representative example of Western blot analysis of ras in cells treated with control fat, FFS or FFR milk fat for 4 days. Ras appeared as a doublet with the upper band representing farnesylated membrane-bound ras and the lower band representing non lipid-modified ras p21.

Incubation of SW480 cells with control, FFS and FFR milk fat samples (at 1mg/ml) decreased amounts of total ras by 35, 45 and 52%, respectively, relative to untreated cells

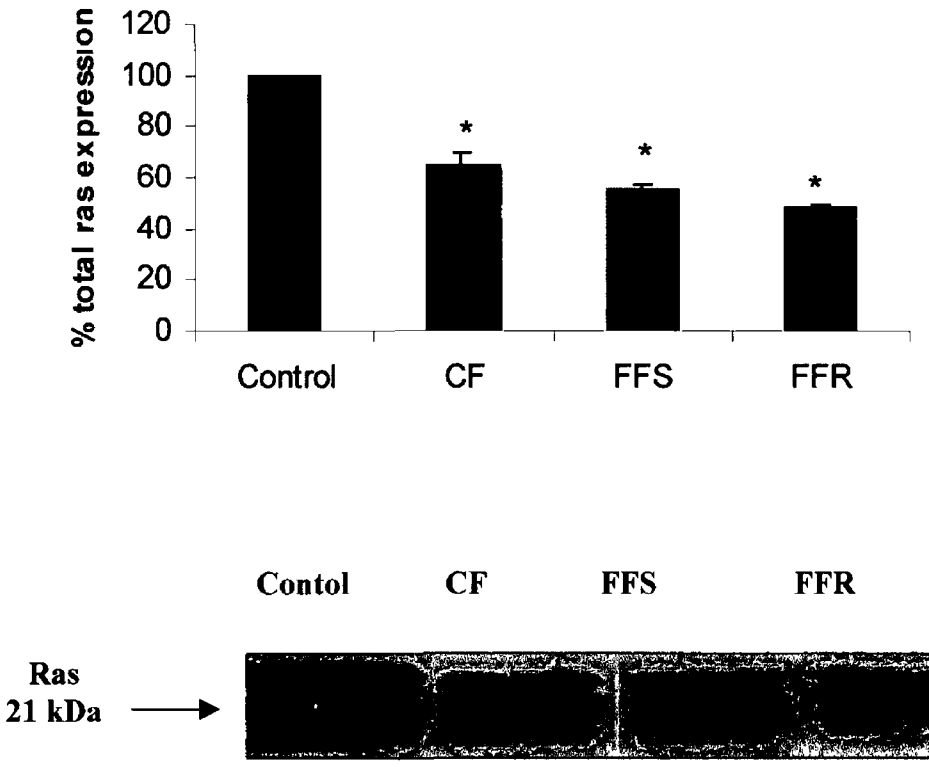


Figure 5 8 Expression of ras in SW480 cells following 4 day treatment with milk fat samples (1 mg/ml) Blot represents one of three independent experiments * denotes values significantly different to control cells ($p<0.05$) Values are expressed as a percentage of the control (± SD) which was taken to be 100%

5 5 Discussion

A prospective cohort study in Finland, revealed that women who developed breast cancer had consumed less milk than cancer-free women and suggested that CLA may be the component in milk providing the protective effect (Knekt *et al*, 1996) Another study revealed an inverse association between dietary intake and serum CLA and risk of breast cancer in postmenopausal women (Aro *et al*, 2000) Using food duplicate methodology, the *c*9, *t*11-CLA intake in the U S was estimated to be approximately 193 and 140 mg/d for men and women, respectively (Ritzenthaler *et al*, 2001) The authors of this study suggest that the *c*9, *t*11-CLA intake must be increased by approximately 3-fold to achieve consumption levels that can exhibit a cancer protective effect (i e , 0 1g/100g diet) A natural approach to enhancing CLA in dairy products is to increase the CLA content of milk fat by modifying the dietary regime of the dairy cow The FFR milk fat used in this study, contained 1 3 fold higher *c*9, *t*11-CLA concentration than control fat

This study confirmed the cytotoxic effect of milk fat CLA in MCF-7 cells previously reported after 8 days incubation (O'Shea *et al*, 2000) and also showed that the SW480 cell line is sensitive to the growth inhibitory effects of milk fat CLA Triglyeride-bound milk fat CLA was as effective an anticancer agent as the free fatty acid form of the *c*9, *t*11-CLA in the SW480 cells and even better in the MCF-7 cells When the milk fats were added to yield a final concentration of 20 μg/ml, similar growth inhibitory effects were observed for all treatments in both cells lines This suggests that CLA may be the active ingredient responsible for the cytotoxic effect in MCF-7 and SW480 cells as already proposed in (O'Shea *et al*, 2000) It is apparent that even at 4 days, (wherein approximately 50% inhibition was observed following all treatments as opposed to 90%

inhibition after 8 days (O'Shea et al., 2000) that the effect of CLA is independent of other components present in milk fat.

Evidence is emerging to suggest that individual fatty acids, even within the same fatty acid type (for example *c*9, *t*11-CLA and LA) may have different effects on carcinogenesis, tumor growth and metastasis (Zhou and Blackburn, 1999) and therefore individual fatty acid-specific effects may be in part responsible for the conflicting results obtained in epidemiological studies of dairy products and tumorigenesis. This study evaluated the individual effect of four fatty acids, at concentrations similar to those found in the three milk fat samples, on the cell growth of SW480 and MCF-7 human cancer cell lines. An inhibitory effect on cell growth was obtained following incubation of both cell lines with the pure *c*9, *t*11-CLA isomer (at concentrations similar to those present in the three milk fat samples), confirming the well established cytotoxic effect of CLA in these two cells lines. Milk fat CLA (FFR and FFS) was more effective at inhibiting the growth of MCF-7 cells while no difference in potency was observed in the SW480 cell line. When media was supplemented with synthetic TVA, at concentrations similar to those present in the three milk fat samples, cell viability significantly decreased in both cell lines. We have previously reported that TVA decreased cell growth and provided evidence to suggest that the growth suppression responses of both cells lines to TVA are likely to be mediated by its desaturation to *c*9, *t*11-CLA (Chapter 4). LA was either stimulatory or had no effect on cell growth when incubated with the cells at the lower concentrations found in the control (12.5µg/ml) and FFR (16.9 µg/ml) milk fats. However, 46.4 µg/ml of LA, the concentration present in the FFS milk fat, had a potent cytotoxic effect and was of similar magnitude to CLA on the growth of both cell lines.

Previous studies have found that incubation of MCF-7 cells with LA at concentration up to 20 μg/ml stimulated growth (O'Shea *et al*, 1999, Shultz *et al*, 1992, Park *et al*, 2000) However, one study showed that LA was more inhibitory to MCF-7 cells than CLA when added at 28 and 140 μg/ml (DesBordes and Lea, 1995) Oleic acid decreased viability of SW480 cells when added at concentrations similar to those present in the three milk fat samples but only decreased cell numbers in MCF-7 cells at the highest concentration of 269 2 μg/ml Oleic acid has been previously shown to be toxic to a range of malignant cells *in vitro*, including the HT29 colon cell line (Fermor *et al*, 1992) If the cytotoxic effects of these fatty acids in milk fat are additive, it would be expected that the accumulative growth suppression effect by milk fats would be much greater than that observed and would vary as milk fat content of the medium varied The observation that final cell numbers were similar when the milk fat content of the medium was varied to yield a final CLA milk fat concentration of 20 μg/ml suggests that the effects of these fatty acids when in triglyceride-bound form in milk fat are lessened Yet, when cells were treated with milk fats containing increasing amounts of CLA they exhibited a dose dependent decrease in cell number These data support the earlier conclusion of Ip *et al* (1996) that CLA is a unique fatty acid with anticancer properties acting independently of other fatty acids

We have previously reported that the growth suppressive effect of the *c*9, *t*11-CLA isomer was associated with changes in AA distribution among cellular lipids and an altered prostaglandin profile (Chapter 2) In this study, we have demonstrated similar effects to AA uptake and conversion to AA using the FFR CLA-enriched milk fat (which contained 22 6 μg/ml CLA) In the MCF-7 cell line, FFR milk fat increased ^{14}C-AA

uptake into the MG fraction The pattern of incorporation was similar to that previously observed in MCF-7 cells treated with pure *c*9, *t*11-CLA isomer (Chapter 2) The FFR milk fat decreased ^{14}C-AA uptake into the PL fraction but in contrast to the pure *c*9, *t*11-CLA isomer which increased uptake in the TG fraction, the FFR milk fat increased its uptake into the MG fraction We have previously reported that TVA (20 μg/ml) had a similar effect on ^{14}C-AA uptake (Chapter 2) The elevated level (51 8 μg/ml) of TVA in the FFR milk may have an influence on AA uptake into lipid fractions The FFR milk fat decreased ^{14}C-AA conversion to PGE_2 while increasing conversion to $PGF_{2\alpha}$ This altered pattern of eicosanoid production is similar to that previously observed following *c*9, *t*11-CLA treatment (Chapter 2) Treatment with the control and FFS milk fats failed to alter ^{14}C-AA uptake into lipid fractions and eicosanoid production even though they contained higher CLA concentrations (16 9 and 18 3 μg/ml, respectively) than that (i e 16 μg/ml as free fatty acid) previously shown to cause perturbations in uptake (Chapter 2) This suggests that other fatty acid present in the milk fats may have different effects to CLA on AA uptake and eicosanoid biosynthesis and therefore their effects together may explain the overall non significant results

We have previously reported that a CLA mixture of isomers (29 5 % *c*9, *t*11, 29 % *t*10, *c*12), the pure *t*10, *c*12-CLA and pure *c*9, *t*11-CLA lowered the expression of the anti-apoptotic bcl-2 protein, decreased cytosolic GSH levels, increased accumulation of cytochrome c in the cytosol, activated caspase 9 and 3 and caused DNA fragmentation (Chapter 3) Treatment of the two cell lines with all three milk fats increased the levels of 8-epi-$PGF_{2\alpha}$, a biomarker of lipid peroxidation The pure *c*9, *t*11-CLA isomer has also

been shown to increase the levels of 8-epi-$PGF_{2\alpha}$ in these cell lines (Chapter 2) Studies suggest that oxidative stress, in general and lipid peroxidation in particular are involved in both initiation and mediation of apoptosis (Lopaczynski and Zeisel, 2001) Depletion of GSH furthers enhances oxidative stress within cells and has been associated with cytochrome c release (Tang *et al*, 1998) Treatment of cells with all milk fat samples depleted cytosolic GSH The milk fat modulated bcl-2 protein levels, reducing its expression in a CLA concentration-dependent manner and increased levels of annexin V in cell membranes in a similar manner to the pure *c*9, *t*11-CLA previously reported (Chapter 3) It can be concluded from these data that incubation of SW480 cells with milk fats resulted in a cellular condition compatible with induction of apoptosis Treatment of SW480 cells with TVA and *c*9, *t*11-CLA was shown to have decreased total ras expression following 4 days of incubation (Chapter 4) Ras is a central player in membrane-to-nucleus signal transduction and has several downstream targets, including the MAP kinase pathway which is involved in cellular proliferation (Campbell *et al*, 1998) Mutations in the dominant oncogene *ras* represent the most commonly found gene mutations in human cancer cells (Gibbs *et al*, 1994) This study now shows that CLA-enriched milk fats may also influence ras signalling by reducing its expression

While preliminary data from human studies have shown CLA to have a number of health effects (Calder, 2002), there is a need for more information on safety, efficacy of isomers and doses required to exert potential benefits in humans To test the efficacy and utility of CLA in cancer prevention, it is necessary to identify CLA bio-responsive markers for use in clinical trials This study has identified two possible protein biomarkers in SW480

cells, bcl-2 and ras expression Further comparative studies with other fatty acids are required before their use as specific biomarkers of CLA exposure can be validated

5 7 Summary

Milk enriched in CLA was obtained from cows on pasture supplemented with full fat rapeseeds (FFR - 2 26 g *c*9, *t*11-CLA / 100 g FAME) and full fat soyabeans (FFS - 1 83 g *c*9, *t*11-CLA / 100 g FAME) (1) A control milk fat (1 69 g *c*9, *t*11-CLA / 100 g FAME) was obtained from cows fed on pasture only This study assessed the potency of the CLA-enriched milk fats to modulate biomarkers that had previously been observed to respond to *c*9, *t*11-CLA in the MCF-7 and SW480 cell lines Cell numbers decreased ($p<0\ 05$) up to 61 % and 58 % following incubation of MCF-7 and SW480 cells, respectively, for 4 days with milk fats (yielding CLA concentrations between 16 9 and 22 6 μg/ml) A comparative study of the effects of synthetic linoleic acid, *trans*-vaccenic acid and oleic acid at concentrations found in the milk fats revealed that individually, they also have modulatory effects on growth, but that the growth-inhibitory effects of milk fats were independent of their variable composition The FFR milk fat, containing the highest CLA content, increased ($p<0\ 05$) ^{14}C-AA uptake into the monoglyceride fraction of MCF-7 and SW480 cells while it decreased ($p<0\ 05$) uptake into the phospholipid fraction of the latter This milk fat also decreased ($p<0\ 05$) ^{14}C-AA conversion to PGE_2 while increasing conversion to $PGF_{2\alpha}$ in both cell lines All milk fat samples increased ($p<0\ 05$) lipid peroxidation as measured by 8-epi-$PGF_{2\alpha}$ in both cell lines In SW480 cells the milk fat samples decreased ($p<0\ 05$) bcl-2 and cytosolic glutathione levels while increasing ($p<0\ 05$) membrane-associated annexin V levels All

milk fat samples decreased ($p<0.05$) expression of ras in SW480 cells. These data suggest that milk fat CLA was effective at modulating synthetic CLA-responsive biomarkers

CHAPTER 6

Final Discussion and Conclusions

It is now known that the process of cancer progression and metastasis may be modified through nutritional intervention. Many food substances traditionally characterised as nutrients affect specific molecular pathways related to cancer and this is the focal point of a new generation of nutritional science known as nutritional oncology. Nutritional oncology recognises that cancer is a chronic disease of the genome that may be influenced at many stages of its natural history by nutritional factors that could impact on both the prevention and treatment of cancer (Heber *et al.*, 1999). Increased knowledge in the nutritional sciences and an improved understanding of the cellular and molecular basis of cancer now make it possible to approach research on nutrient-gene interactions relevant to cancer prevention and treatment. Dietary intervention represents an attractive, non-invasive means of providing anticancer preventative and therapeutic benefits to at-risk individuals.

Among the macronutrients, lipids have a unique property not shared with other nutrients; the type of lipid ingested modulates the chemical composition of cells to a very significant degree. Novel functions for fatty acids and lipid-derived mediators, other than those encompassing membrane structure or provision of energy, have been elucidated. Dietary fat has been shown to have profound effects on gene expression, leading to changes in cell metabolism, growth and cell differentiation (Jump and Clark, 1999, Grimaldi, 2001). As described in Chapter 1, the fatty acid CLA has been shown to be effective at inhibiting carcinogenesis in multiple systems and at several stages including initiation, promotion, progression and metastasis (reviewed in Scimeca 1999 and Belury 2002). By way of comparison, fish oil has been shown to exhibit anticancer properties but

efficacious levels usually exceed 10 % of diet. The ability of CLA to inhibit multiple models of carcinogenesis at much lower dietary levels (0.1 % w/w) appears to be specific for this group of fatty acids and has led to extensive studies being carried out to probe mechanisms and functions that are likely to be unique among PUFAs.

Insight into the relationship between CLA and cancer has come in the main from *in vivo* studies. Experiments which permit the study, in isolation, of the interactions between specific cell types and dietary components are a powerful tool when conducted in conjunction with animal or human studies. The ability to culture epithelial tumor cells *in vitro* has proved very useful in acquiring information on potential mechanisms for the effects of CLA on cancer. Immortalised cell lines have genetic alterations that stabilise them for growth in culture but the ability to culture these cells in the presence of fatty acids and to then measure cell behaviour over a relatively short period of time allows for comprehensive studies with reproducible results that permit insight into the effects of these compounds. Numerous down stream events can be assessed including changes in cellular signalling molecules and gene expression. Considerations in the design of cell culture studies include cell line selection, cell culture condition, the vehicle used to deliver the fatty acid, cell seeding densities, timing of measurements, laboratory procedures and selection of biological endpoints relevant to human cancer.

Most animal and cell culture models used in CLA research to date have employed ill-defined isomer mixtures, thus making mechanistic interpretation difficult. The increasing availability of isomers of CLA with high purity should provide clarity in the future.

Results from this study revealed that the MCF-7 and SW480 cell lines were sensitive to growth inhibitory effects of not only the CLA mixture but also to both the *t*10, *c*12-CLA and the *c*9, *t*11-CLA isomers following 4 days of incubation with physiological levels of CLA. The CLA-induced cytotoxicity was related to an increase in lipid peroxidation, alterations in the distribution of AA among cellular lipids, an altered prostaglandin profile and a reduction in the level of the anti-apoptotic bcl-2 protein which triggered a cascade of events leading to apoptosis. This study identified two possible protein biomarkers, bcl-2 and ras. Further comparative studies with other fatty acids are required however before their use as specific biomarkers of CLA exposure can be validated.

Future cell culture studies involving both tumour and non tumour cell lines should be conducted with CLA isomers that are saponified and complexed with BSA so that physiologically relevant information about cell type specificity of CLA can be obtained. In addition, future studies should be designed so as to show the minimal dose below which no response is observed as well as a clear maximal response. Multi array analysis, a new tool of functional genomics, can be used to identify cell signalling pathways and molecular targets that are relevant to the action of CLA in cancer prevention. While providing a biochemical basis for elucidating the mechanism of action of CLA in cancer prevention, research on CLA-responsive biomarkers also has a practical side because these assays can be applied to human biopsy tissue samples in future CLA intervention trials. A variety of methodologies tissue (e.g. immunohistochemistry, flow cytometry and PCR amplification of cDNA) are available to evaluate multiple biomarkers in a small amount of biopsied tissue.

Metabolism of CLA by desaturases and elongation enzymes has been well documented now. Conjugated metabolites have been identified in numerous tissues. This knowledge opens up a new avenue of research which is related to the question of whether the metabolism of CLA is essential for its anticancer activity. If purified metabolites become available for cell culture studies, it would be important to conduct studies to delineate whether CLA or one of its metabolites is the proximate effector molecule. In the long term, elucidation of the mechanisms by which individual CLA isomers elicit their putative beneficial effects would permit studies to investigate evidence of such effects in cancer patients receiving them as dietary supplements.

Studies with an optimal design and dosing regime will be required to demonstrate whether CLA has real benefits for human patients. The current CLA dietary intake in humans does not seem to be sufficient to exert beneficial effects. Extrapolation of dietary CLA that is effective in animal models indicated that equivalent CLA concentrations in a 70 kg human would be in the order of 3.5 g of CLA per day. Dietary supplements are now widely available commercially as an alternative source. However given that supplements would have to be taken regularly, a more expeditious way could be an enhanced delivery of CLA through the food system. Foods are a relatively inexpensive and effective way to deliver substances with cancer protective properties. The introduction into the food system of CLA-enriched dairy fats and products may afford a chemopreventive effect without the additional cost of oral supplements or the need for dietary changes. In this study we have reported that CLA enriched milk fat was more effective than synthetic *c*9, *t*11-CLA at decreasing the growth of the MCF-7 cell line and

also demonstrated milk fat CLA was effective at modulating synthetic CLA-responsive biomarkers in both cell lines.

Data from this study demonstrate that MCF-7 and SW480 cancer cells have the enzymic capability to convert TVA to *c*9, *t*11-CLA. Furthermore, TVA influenced a number of CLA bio-responsive markers in these cell lines and we postulate that the growth suppression and cellular responses of both cells lines are likely to be mediated via TVA desaturation to *c*9, *t*11-CLA via Δ^9-desaturase. However, it is impossible to rule out the possibility that TVA may have an independent effect itself. Corresponding experiments in which cells are simultaneously treated with cyclopropene fatty acid (an inhibitor of Δ^9-desaturase) may determine whether the anticancer effect of TVA can be negated. The *t*10, *c*12-CLA isomer has been shown to inhibit the activity of Δ^9-desaturase in human cultured hepatoblastoma cells (Choi *et al.*, 2001). It may be useful to treat MCF-7 and SW480 cells with TVA along with a sub-lethal dose of *t*10, *c*12-CLA capable of inhibiting the activity of Δ^9-desaturase (concentration yet to be determined) and to subsequently examine the effect on cell viability and CLA-responsive markers. To establish the importance of TVA as a precursor of endogenous CLA, it is imperative to conduct studies in humans. Both descriptive data (i.e. the activity of Δ^9-desaturase at various tissue sites) and quantitative studies should be undertaken. It may also be useful to determine the levels of Δ^9-desaturase in normal and tumor cell lines and in tumor biopsies using RT-PCR.

Because CLA isomers have been identified as having effects on cellular processes such as proliferation, apoptosis and differentiation, it may be opportune to examine if CLA isomers may be possible chemotherapeutic agents, or potential adjuvants to radio-or chemotherapy. Resistance to chemotherapy drugs is a significant problem in the treatment of cancer. Recent research has indicated that changes in the fluidity of the membranes due to certain lipids can reduce or completely prevent the efflux of cancer drugs out of cells (Schuldes *et al.*, 2000). Plasma membrane fatty acid composition influences how lipophilic drugs diffuse through the membrane. The more soluble the drug is in the membrane the more it can diffuse through. Increased unsaturation decreases lipid molecular packing. Preclinical trials have shown that certain PUFAs may enhance the cytotoxicity of several antineoplastic agents (Conklin, 2002). Polyunsaturated fatty acids such as DHA, eicosapentaenoic (EPA), gamma linolenic acid (GLA) and parinaric acid, have been shown to be cytotoxic to drug-resistant tumour cells by inducing oxidative stress and altering the activity of cell membrane bound enzymes such as sodium-potassium-ATPase and 5'-nucleotidase and the concentratio of protein kinase C, central to reduction of intracellular drug levels (Burns and Spector, 1994, Das *et al.*, 1997, Pallares-Trujillo *et al.*, 2000). Because of enhanced cellular growth rates, certain membrane domains of tumour cells should respond rapidly to circulating fatty acids. Altering the physical and functional properties of tumor cell membranes, by enrichment with CLA alone or in combination with other PUFAs (EPA, DHA and GLA), may increase the response to chemotherapy and may, to some degree reverse the resistance of cancer cells to certain chemotherapeutic agents. Possible synergism in the action of anticancer drugs and CLA to enhance the intracellular concentration of these

drugs warrant investigation A positive outcome from these types of studies could provide a sound scientific basis for combining a lipid based approach with traditional chemotherapy in the treatment of cancer Patients with cancer could ingest defined diets containing CLA and other PUFAs This could be done with formula diets in a clinical research unit or by supplementing their usual diets with CLA enriched dairy products prior to administration of systemic or oral anticancer agents Alternatively, isolated portions of the body could be perfused with triglyceride emulsions or liposomes containing the optimum proportions of these fatty acids followed by systemic or perfused chemotherapy

Abnormal differentiation is thought to be a fundamental defect in the cancer cell (Corn and Et-Deiry, 2002) CLA has been shown to induce markers of differentiation in adipocytes in noncancer models (Houseknecht *et al*, 1998, Satory and Smith, 1998) Whether CLA inhibits carcinogenesis via induction of differentiation has yet to be determined Feeding CLA to rats during the time of mammary gland development and maturation has long-lasting protective effects on mammary carcinogenesis (Ip *et al*, 1995, Thompson *et al*, 1997) More recent data have shown that CLA may inhibit the differentiation of mammary stromal cells to an endothelial cell type (Masso-Welch *et al*, 2002) These data suggest that the role of CLA in protecting against mammary carcinogenesis may be mediated, in part, by modulating tissue differentiation (Belury, 2002) Therefore, it would be prudent to examine the effect of CLA isomers on the rate of cellular differentiation *in vitro* using appropriate measures of differentiation (e g lactalbumin in mammary tumour cell lines, alkaline phosphatase in colon tumour cell

lines) Positive effects of CLA on differentiation may provide a plausible explanation for why diets rich in these fatty acids might slow tumor growth and retard carcinogenesis The most lethal aspect of cancer is the ability of tumor cells to metastasise and form secondary tumors CLA has been shown to be effective at inhibiting carcinogenesis at several levels, including metastasis (Visonneau *et al*, 1997, Cesano *et al*, 1998, Hubbard *et al*, 2000) Neovascularisation, or angiogenesis, is essential for solid tumour growth (Folkman, 1990) and also provides the tumour cells with access to the vascular circulatory system, thus establishing the potential for metastatic disease progression Masso-Welch *et al* (2002) have recently reported that CLA inhibited the formation of functional blood vessels in mice and this was accompanied by decreased serum levels of VEGF and whole mammary gland levels of VEGF and it receptor flk-1 The question remains whether CLA can alter angiogenesis during tumor development The matrix metalloproteinases (MMPs), a multi-gene family of enzymes, degrade components of the extracellular matrix and are implicated as major players in tumor invasion and metastasis (Mc Donnell *et al*, 1999) Feeding CLA to pregnant rats suppressed serum MMP-9 and active MMP-2 (Harris *et al*, 2001) However, no study has yet related the antimetastatic effect of dietary CLA with these enzymes Hence studies to examine the influence of CLA isomers on metalloprotease secretion may be a fruitful area for future research Preliminary data from O'Connor *et al* (2002) has shown that CLA isomers reduced the invasive activity of the highly metastatic 4T1 mammary tumor cell line and this was associated with a reduction in expression of MMP-9 *In vivo* work using CLA and this cell line, for the induction of lung nodules, will be the next step in elucidating the importance of CLA in anti-cancer treatment

Additional epidemiological studies of CLA exposure and cancer risk are imperative An inverse association was observed between dietary and serum CLA and risk of breast cancer in postmenopausal Finnish women (Aro *et al*, 2000) In contrast a French group have found no significant association between CLA levels in breast adipose tissue and breast cancer risk (Chajes *et al*, 2002) Biomarkers of CLA intake need to be identified and validated The physiological consequences of CLA intake throughout the lifespan are currently not understood Evidence suggests that early programming during foetal growth, infancy and childhood might decrease risk for chronic diseases in later life (Lusas, 1991) Thus, a better and more accurate understanding of CLA intakes and factors influencing CLA consumption throughout the lifespan might lend insight into what might be considered appropriate dietary recommendations for this potential nutrient

The goals of future research must therefore be to examine the selectivity of the anti proliferative effect of CLA on a wide variety of cell types including appropriate normal control cells, to examine the selectivity of organ site carcinogenesis intervention by CLA, to carry out epidemiological studies of *c*9, *t*11 CLA exposure and cancer risk and to make use of genomic technology to identify signalling pathways and molecular targets that are relevant to the action of CLA in cancer prevention Cancer-associated surrogate markers may then be investigated in controlled clinical trials to evaluate responsiveness to CLA It is vital that the efficacy of the individual CLA isomers *in vivo* be evaluated and the optimal levels of these isomers required for beneficial effects determined Identification of modulated mechanisms and tangible anti-cancer benefits will give impetus to food

manufacturers to incorporate CLA as a nutraceutical in functional foods which would enhance the health of the general population

CHAPTER 7

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