The effect of Conjugated Linoleic Acid on the proliferation, migration and invasion of a murine mammary cancer cell line

A dissertation submitted for the degree of M.Sc.

by

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Declaration

I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of Master of Science, is entirely my own work and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

Signed: Áine O'Connor I.D. No. 95152784

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Date: 23. September. 2002

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Abstract

Conjugated Linoleic Acid (CLA), a polyunsaturated fatty acid, refers to a group of dienoic derivatives of linoleic acid that can be found in natural food sources, such as milk fat and the meat of ruminant animals. CLA has been shown to have anti-carcinogenic activity in many *in vitro* and *in vivo* studies. Previous studies have focused on the effects of dietary CLA on the prevention of tumour appearance, yet relatively little is known about the actual mechanism of CLA's anti-cancer activity.

The most lethal aspect of cancer is the ability of tumour cells to metastasise and form secondary tumours. The matrix metalloproteinases (MMPs), a multi-gene family of enzymes, which degrade components of the extracellular matrix (ECM) have been implicated as major role players in tumour invasion and metastasis. The aim of this study was to examine the effect of CLA on the proliferation, migration, invasion and MMP-9 expression of a murine mammary cancer cell line, 4T1, which is known to be highly metastatic *in vivo*.

Cells were treated with CLA, which contains a mixture of various isomers, and with the purified predominant isomers present in CLA, 9*c*,11*t*(18:2) and 10*t*,12*c*(18:2). Cytotoxicity of CLA was examined by varying the concentration and incubation time. Sub-lethal as well as lethal doses were determined. Interestingly, it appeared that the 10*t*,12*c*(18:2) isomer had the most lethal effect.

Flow cytometric analysis revealed deregulation of the cell cycle correlating with the observed cytotoxic effects of CLA, especially with the 10*t*,12*c*(18:2) CLA isomer. The effect of CLA and its isomers on apoptosis was also investigated. The results from this assay were inconclusive, although examination of the cell cycle histogram plots indicated that cell death due to CLA was not occurring through an apoptotic pathway as there was no sub-G_0/G_1 peak.

Treatment of the cells with a sub-lethal dose of CLA and its isomers resulted in a reduction of the invasive activity of the 4T1 cell line with the 9*c*,11*t*(18:2) isomer having the greatest effect. CLA and its isomers also resulted in a reduction in the percentage migration of the cells, especially the 9*c*,11*t*(18:2) isomer and the CLA mixture. Substrate zymography gels were used to detect MMP activity and showed that the 4T1 cells expressed significant amounts of murine MMP-9. Following treatment with CLA and its isomers, there was no reduction in MMP expression.

Abbreviations

*	asterisk
9:11	9*c*,11*t*(18:2)
10:12	10*t*,12*c*(18:2)
α	Alpha
AA	arachidonic acid
Abs	Absorbance
ALA	Alpha linolenic acid
ATCC	American tissue culture collection
ATP	Adenosine triphosphate
BRCA1	Breast cancer 1
BRCA2	Breast cancer 2
β	Beta
BCA	Bicinchoninic acid assay
BHK	Baby hamster kidney fibroblasts
BHT	butylated hydroxytoluene
BSA	Bovine serum albumin
c	*Cis*
CAM	Cell adhesion molecule
Col	collagen
COX	cyclooxygenase
DHA	Docosahexaenoic Acid
dH_2O	Distilled water
DMBA	7,12-dimethylbenz(α)anthracene
DMEM	Dulbecco's modified Eagles medium
$DMEM/S_0$	Serum-free Dulbecco's modified Eagles medium
$DMEM/S_5$	5% serum Dulbecco's modified Eagles medium
$DMEM/S_{20}$	20% serum Dulbecco's modified Eagles medium
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
ECM	Extracellular matrix
EDTA	Ethylenediamine tetracetic acid

EFA	Essential fatty acid
EPA	Eicosapentaenoic Acid
ER	Estrogen responsive
EtOH	Ethanol
EtBr	Ethidium bromide
FA	Fatty acids
FBS	Foetal bovine serum
FN	fibronectin
G_0	Gap 0 stage
G_1	Gap 1 stage
G_2	Gap 2 stage
Gel	gelatin
GLA	Gamma Linolenic Acid
gp	Glycoprotein
GPx	Glutathione peroxidase
GTP	Guanosine triphosphate
HCl	Hydrochloric acid
HEPES	N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
HMW	High molecular weight
HRE	Hormone response element
HRP	Horseradish peroxidase
Ig	Immunoglobulin
λ	Lambda
LA	Linoleic acid
LM	laminin
LOX	Lipoxygenase
M	Mitosis
MMP	Matrix metalloproteinase
mRNA	Messenger RNA
MT-MMP	Membrane type MMP
MTS	(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
MW	Molecular weight
OD	Optical density

ω	Omega
%	Percentage
p21	Protein 21
p53	Protein 53
PA	Plasminogen activator
PARP	Poly ADP-ribose polymerase
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PET	Polyethylene terephthalate
PGE_2	Prostaglandin E_2
PI	Propidium iodide
PKC	Protein kinase C
PLA_2	Phospholipase A_2
PLC	Phospholipase C
PG	proteoglycan
PMS	Phenazine methosulphate
PUFA	Polyunsaturated Fatty Acid
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Rotations per minute
ROS	Reactive oxygen species
RT	Room temperature
S	Synthesis stage
SCID	Severe combined immunodeficient
SD	Standard deviation (+/-)
SDS	Sodium dodecyl sulphate
SFA	Saturated Fatty Acids
SOD	super oxide dismutase
t	*Trans*
TAE	Tris-Acetate-EDTA
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substances
TE	Tris EDTA
TEB	Terminal End Bud

TEMED	N N N' N'-Tetramethylethylenediamine
TIMP	Tissue inhibitor of metalloproteinases
Triton-X-100	t-Octylphenoxypolyethoxyethanol
UV	Ultra violet

Units

°C	Degrees Celsius
µg	Microgram
µl	Microlitre
µm	Micrometer
µM	Micromolar
bp	Base pair
cm	Centimetre
cm^2	Centimetre squared
g	Grams
hr	Hour
kDa	KiloDalton
kg	Kilogram
l	Litre
M	Molar
mA	Milliamps
mg	Milligram
min	Minute
ml	Millilitre
mM	Millimolar
ng	Nanogram
nm	Nanometre
s	Second
V	Volt
v/v	Volume per volume
w/v	Weight per volume

Publications

O'Connor, Á (2001) The effect of Conjugated Linoleic Acid (CLA) and its isomers on the proliferation and MMP expression of a metastatic murine mammary cell line and a colon cancer cell line. Poster presentation at the Irish Association of Cancer Research (IACR) Meeting, September, 2001.

Mc Donnell, S., O'Connor, Á., Murray, D. and Lynch, C. (2002) Matrix Metalloproteinases. Chapter 3, pp17-24. Hormone Replacement Therapy and Cancer. The current status of research and practice. Edited by AR Genazzani. Parthenon Publishing.

O'Connor, Á., McDonnell, S., Devery, R. and Stanton, C. (2002) The effect of Conjugated Linoleic Acid (CLA) and its isomers on the proliferation, migration and invasion of a metastatic murine mammary cell line. *British J. Cancer* **86** S82-S83.

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CHAPTER 1

1.1 Cancer

The fundamental abnormality resulting in the development of cancer is the continual unregulated proliferation of cancer cells. Rather than responding appropriately to the signals that control normal cell behaviour, cancer cells grow and divide in an uncontrolled manner, invading normal tissues and organs and eventually spreading throughout the body (Cooper, 1990).

Carcinogenesis, the process by which cancers are generated, is a multistep process resulting from the accumulation of mistakes in vital regulatory pathways. It is initiated in a single cell, which then multiplies and acquires additional changes that give it survival advantage over its neighbours. It takes time and large numbers of cells to generate these errors, and so it follows that the longer one lives the greater the likelihood is to develop cancer. Hence, cancer is a disease associated with old age, although this is not always true, as in the case of childhood leukaemia (King, 2000).

There are many kinds of cancer but only a few occur frequently. The four most common cancers are those of the prostate, breast, lung, and colon/rectum (Parkin *et al*, 2001). Cancer is reported as being the second highest cause of death, behind heart disease, in the western world. In the USA, it is responsible for over 20% of all deaths (King, 2000). Figure 1.1 shows the incidence of, and mortality due to, many cancers.

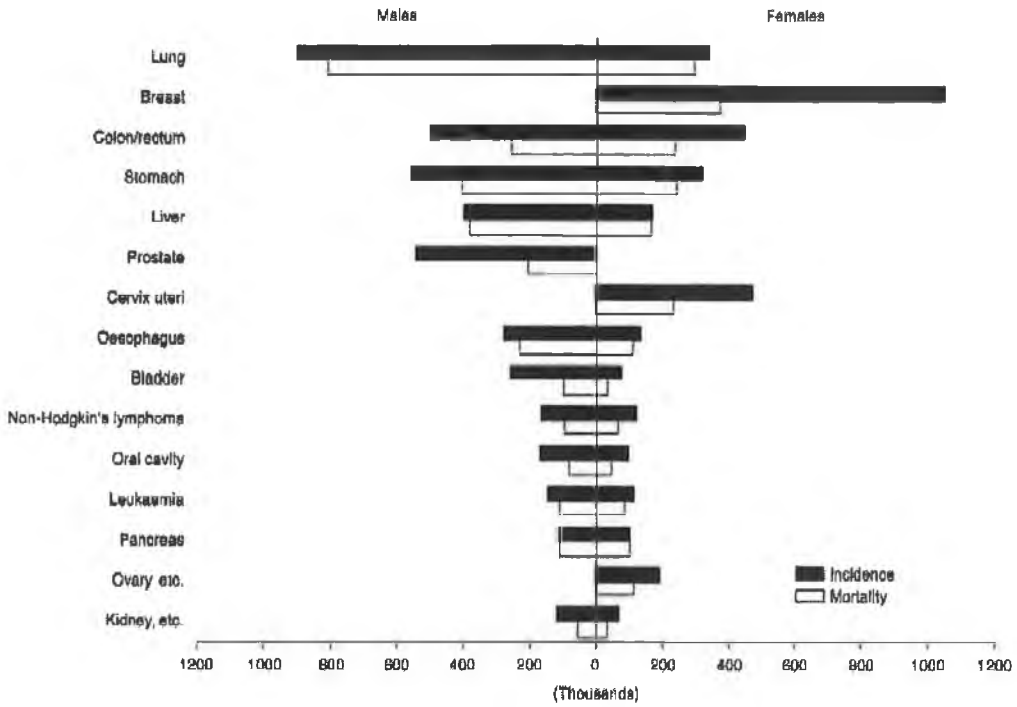


Figure 1.1. Number of new cases (depicted by heavy black line) and deaths (depicted by white line) worldwide for the 15 most common cancers in men and women, 2000 (Parkin *et al*, 2001).

1.2 Breast cancer

Breast cancer is the second most common cancer in the world today, and by far the most common cancer in women, with over 999,000 new cases of breast cancer each year (about 22% of cancers in women) and 375,000 deaths (figure 1.1) (Parkin *et al*, 2001). High rates are observed in the US, Europe, Australia and New Zealand, and in the south of South America, especially Uruguay and Argentina. In contrast, low rates are found in most African and Asian populations, although they are increasing. Survival from breast cancer in Europe is 91% at 1 year and 65% at 5 years (Parkin *et al*, 2001).

The principal causes of death in Ireland in 1997 are outlined in table 1.1. Cancer accounted for almost 24% of all deaths; breast cancer deaths comprised 2% of all deaths and 8.5% of all cancer deaths. Since all these cases occurred in women in this study, this number constitutes 4.2% of all deaths in women and 18.1% of all cancer deaths in women. Breast cancer is confirmed as being a principle cause of premature mortality in women. It constitutes approximately 20% of all female deaths between the ages of 40 and 59 years. Ireland has severely high age-standardised mortality rates due to breast cancer. It lags behind only Denmark and Israel out of 40 countries studied regarding mortality due to breast cancer (Codd *et al*, 1999).

Cause of Death		**Percentage (%)**
Cardiovascular disease		4.0
Cancer	*Lung*	4.0
	Colon	2.0
	Breast	2.0
	Others	15.0
Respiratory		15.0
Other circulatory disease		11.0

Table 1.1. Principle causes of death in Ireland, 1997. Values are rounded to nearest percentage (Codd *et al*, 1999).

Five to ten percent of breast cancer is attributable to the autosomal dominant inheritance of a high-risk susceptibility gene. There are a number of known inherited cancer syndromes that result in a higher risk of breast cancer. Genes, such as the BRCA1 gene, which is responsible for 45% of hereditary early-onset breast cancer, and the

BRCA2 gene that accounts for approximately 40% of hereditary early-onset breast cancer (Miki *et al*, 1994, Futreal *et al*, 1994, Wooster *et al*, 1995, Radford and Zehnbauer, 1996). Mutations in these genes can occur more frequently in certain populations, such as in Ashkenazi Jews (Bertwistle and Ashworth, 1998). Studies of these genes help in not only understanding inherited breast cancer syndromes but also in non-inherited, or sporadic, breast cancer.

Sporadic breast cancer, which constitutes more than 90% of all breast cancers, is a complex and heterogeneous disease at both the clinical and molecular levels. Despite this heterogeneity, the natural history of breast cancers involves a sequential progression through defined clinical and pathological stages starting with atypical epithelial hyperplasia, progressing to carcinoma *in situ* then invasive carcinomas and culminating in metastatic disease (Polyak, 2001). This is depicted in figure 1.2.

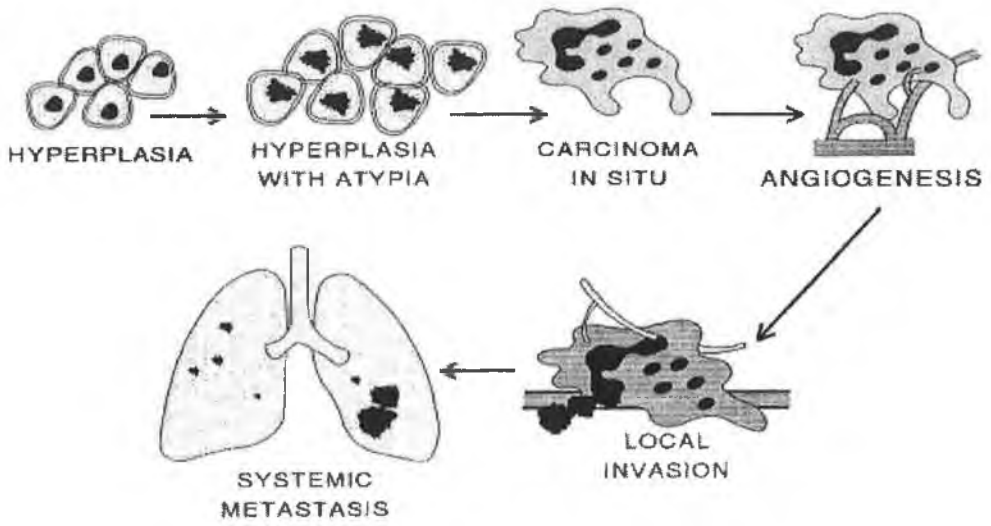


Figure 1.2. The progression of pre-malignant benign breast disease to metastatic carcinoma. The lungs are the major sites for the formation of secondary tumours of breast cancer (Rose & Connolly, 1999).

There is an increase in numbers of women with the disease and this has been largely attributed to mammographic screening programs, which are being practised in many developed countries. Since the establishment of the National Breast Screening Programme in Ireland in 1999, approximately 50% of women aged 50-64 have been targeted for mammographic screening (Codd *et al*, 1999). The stage of the disease at the time of diagnosis, i.e. whether it is localised or has metastasised, is the most important prognostic variable regarding long term survival, and it is here that the importance of the

mammographic screening programmes can be seen. It has been observed that the risk of breast cancer increases with age in women up to 50 years, whereby the onset of the menopause (characterised by lower oestrogen levels) slows down this increase (Parkin *et al*, 2001).

1.2.1 The causes of breast cancer

Many factors are reported as either initiating or promoting cancer through the build-up of a series of mutations in cells, which will lead onto malignant growth. Cigarette smoke, radiation, chemical carcinogens, hormones, and viruses have all been indicated in playing roles in the development of certain cancers, as well as behaviour, lifestyle (which includes diet) and inherited genetic mutations (figure 1.3).

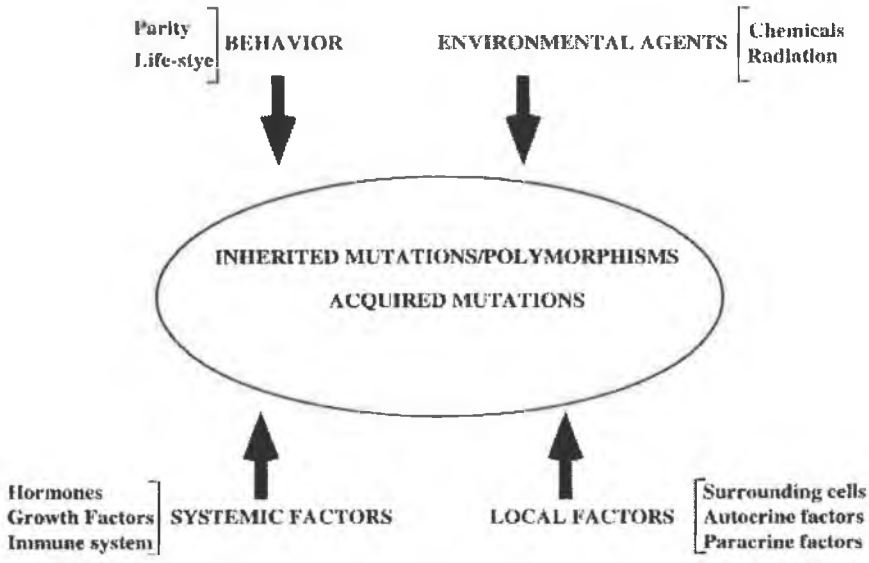


Figure 1.3. Summary of factors influencing breast carcinogenesis.

The development of breast cancer is a long process, comprising a series of biological events that drive a normal mammary cell towards malignant growth. However, it is not known when the initiation of breast cancer occurs. The etiology of breast cancer may be associated with fat, fibre, fruits, vegetables, β-carotene, vitamin C, zinc, phytoestrogens, and alcohol. Variations in diet, found between populations, is a viable possibility to account for the differences in cancer incidence worldwide.

It has been proposed that most women develop subclinical breast cancer at an early premenopausal age and that diet, especially overnutrition, plays an important role in the progression from such putative preneoplastic lesions to clinical disease (Woutersen *et*

al, 1999). Potential carcinogens, as well as cancer preventive agents are all found in foods. There are several dietary factors, which have been implicated, and generally agreed, to play significant roles in cancer. Some examples of these are given in table 1.2 (Cooper, 1990). Although the most important factors influencing the risk of breast cancer are reproductive and hormonal, observation at international level of a strong correlation between mortality from cancer of the breast and *per capita* intake of dietary fat has implicated the role of dietary fat as an important risk factor (Parkin *et al*, 2001).

Dietary Component	**Effect on Cancer Risk**
High fat	Increased risk of colon and possibly breast cancer
High calorie	Obesity resulting in increased risk of endometrial and possibly breast cancer
Cured, smoked, and pickled foods	Increased risk of stomach cancer
Aflatoxin	Increased risk of liver cancer
Vitamin A or β-carotene	Decreased risk of lung and other epithelial cancers
Vitamin C	Decreased risk of stomach cancer
Vitamin E and selenium	Deficiencies are associated with increased cancer risk
Fibre	Decreased risk of colon cancer
Cruciferous vegetables	Decreased cancer risk

Table 1.2. The effect of several common food components on cancer risk.

Saturated Fatty Acids (SFAs) and Polyunsaturated Fatty Acids (PUFAs) have been studied extensively as part of cancer research. Studies carried out by Guthrie and Carroll (1999) showed that SFAs failed to increase the yield of tumours induced by a mammary carcinogen, 7,12-dimethylbenz(α)anthracene (DMBA). However, when SFAs were administered along with PUFAs, the mixture promoted mammary cancer as well as a high level of PUFAs. Fatty acids (FAs) play important roles in cancer growth and proliferation. The fact that PUFAs are present in many foodstuffs, and our intake of dietary fats, in general, is growing with increasing economic development, research into dietary PUFAs, and the effect they have on clinical cancer, is of prime importance in cancer research today.

1.2.2 Epidemiological evidence supporting the relationship between PUFAs and breast cancer incidence

One hypothesis, as to the initiation of breast cancer, is that a high oestrogenic environment, often brought on by a high dietary fat diet, during the perinatal period increases subsequent breast cancer risk. Hilakivi-Clarke *et al* (1999) presented evidence that a high maternal intake of dietary fats increased serum oestrogens during pregnancy and increased breast cancer risk in daughters. High fat diets in women increased greatly the level of circulating oestrogens in the human body. Birth weight was suggested to reflect high foetal oestrogenic environment, and there was a reported higher breast cancer incidence in women with high birth weight. A clear correlation between high fat intake, high oestrogen levels and increased risk of breast cancer has been observed.

Ecological studies show a wide variation in breast cancer incidence and mortality rates between countries and correspondingly large differences in dietary practices. Interestingly, migration studies have shown that when women move from countries such as Japan, in which breast cancer and dietary fat are relatively low, to countries such as the U.S., where the reverse is true, their breast cancer incidence rates increased within one or two generations (Wouterson *et al*, 1999). Although Japanese women continued to have a relatively low incidence of breast cancer, it has risen significantly over the last 30 years accompanied by dietary changes, most notably an increase in total fat consumption, and urbanisation (Rose and Connolly, 1999, Wouterson *et al*, 1999).

It has been widely agreed that epidemiological and ecological studies may not always be accurately relied on. If a survey is being carried out, the researcher must rely on the honesty of the participants. Also, when research is being carried out on sufferers of cancer in the past, recall of important dietary practices may not always be accurate and additional environmental factors may not be reported. This is true for all epidemiological studies, i.e. what other inter-country and inter-population factors may be playing an influential role? Research into the influence of dietary factors on cancer incidence often do not take into account the role played by interacting dietary components (Stoll, 1998). For example, when looking at fatty acids, antioxidant vitamins such as vitamin-E may also be studied (van den berg *et al*, 1995). For these reasons, experimental evidence is required in great quantity and depth. When experimental evidence reflects conclusions drawn from epidemiological studies, the research takes on a more reliable perspective.

1.3 Chemistry of PUFAs

1.3.1 Nomenclature

The customary chemical nomenclature of PUFAs is to begin the systematic numbering of carbons from the carboxyl terminal group (-COOH). The carbons numbered 2 and 3 from the carboxyl group are referred to as the α and β carbons. The last carbon is the ω- (omega-) or *n*- carbon (methyl end, H_3C-). A number following ω- indicates the carbon where the double bond is, when counting from the ω end. The position of the double bond may also be indicated by the symbol Δ, followed by a number. For example, Δ^9 or ω-9 refers to a double bond between carbons 9 and 10.

1.3.2 The metabolism of ω-6 PUFAs

There exist four families of PUFAs, ω-9, ω-7, ω-6 and ω-3. Among these families ω-3 and ω-6 are the essential fatty acids (EFAs). EFAs must be obtained from the diet. All EFAs are PUFAs and contain >two C=C double bonds. Other PUFAs in the ω-6 and ω-3 series are either converted from their parent EFAs or can be obtained directly from the diet (Jiang *et al*, 1998a).

Linoleic acid (LA), an ω-6 fatty acid, is metabolised to longer-chain FAs, largely in the liver. LA is the metabolic precursor of arachidonic acid (AA) and thus eicosanoids (collective name for metabolites of PUFAs). These eicosanoids are known to modulate the interaction of tumour cells with various host components in cancer metastasis. Their synthesis involves the release of AA from cellular phospholipids by phospholipase A_2 (PLA_2), followed by metabolism by cyclooxygenases (COXs) and lipoxygenases (LOXs). Increases in chain length (mediated by elongases) and degrees of unsaturation (mediated by desaturases) are achieved by adding extra double bonds between the existing double bond and the carboxyl group (Jiang *et al*, 1998a). These metabolic events are depicted in figure 1.4.

PUFAs are key elements in our body playing important physiological roles. They serve as metabolic fuel to provide energy, they are inherent parts of cellular membranes, and they serve as building blocks for other lipids and as a source of eicosanoids.

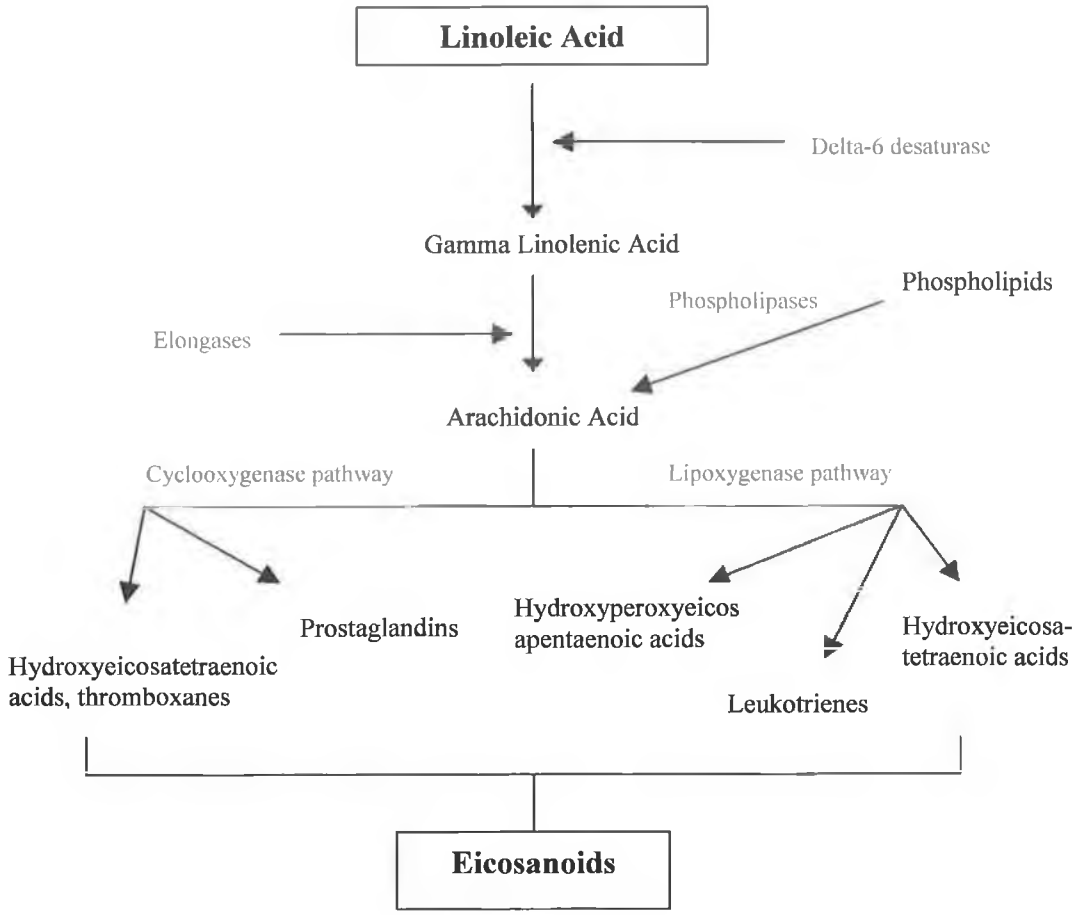


Figure 1.4. The metabolism of LA into the collective group of metabolites known as the eicosanoids. Important enzymes and pathways are highlighted in blue. There are many other pathways that LA and AA may take that are not shown here.

1.3.3 ω-6 PUFAs of Clinical Importance

Due to the fact that the EFAs are PUFAs, and many studies have implicated possible roles they play in cancer development, these PUFAs have become the most widely studied FAs. Table 1.3 shows common PUFAs, including CLA, which have been studied, both *in vitro* and *in vivo*.

Fatty Acid	Tumour Type	Study method	Response
GLA	Breast Cancer	*In vitro*	Decreases invasion
	Prostate cancer	*In vitro*	No significant effect
	Lung cancer	*In vivo*	Reduces tumour growth
	Prostate cancer	*In vitro*	Decreases proliferation
EPA	Breast Cancer	*In vitro*	Inhibits cell growth
	Prostate cancer	*In vitro*	Stimulates growth at low concentrations but inhibits at high concentrations
LA	Breast Cancer	*In vivo*	Promotes metastasis and growth
		In vitro	Increases cell-matrix adhesion and invasion
		In vitro and *In vivo*	Increases cell growth and invasion
	Prostate cancer	*In vitro*	Stimulates proliferation
ALA	Breast cancer	*In vitro*	Inhibits tumour cell growth
	Lung cancer	*In vivo*	Reduces tumour growth
DHA	Breast cancer	*In vivo*	Reduces tumour cell kinetics
CLA	Prostate cancer	*In vivo*	Decreases metastasis and tumour growth.
	Breast cancer	*In vivo*	Inhibits mammary carcinogenesis.

Table 1.3. The effect of several PUFAs on cancer cells as shown by various studies (Jiang *et al*, 1998a, Cesano *et al*, 1998, Ip *et al*, 1997, 1999a, 1999b). GLA-Gamma Linolenic Acid, EPA-Eicosapentaenoic Acid, LA-Linoleic Acid, ALA-Alpha Linolenic Acid, DHA-Docosahexaenoic Acid, CLA-Conjugated Linoleic Acid.

Populations of industrialised Western countries tend to have a high fat diet with a high ω-6 PUFA content, particularly LA, which is found in cheap vegetable oils such as corn and safflower oils. Higher consumption of ω-6 LA is not only a substrate for lipid peroxidation and free radical formation, but in addition, can aggravate insulin resistance and hyperinsulinaemia (Stoll, 1998).

1.4 Conjugated Linoleic Acid (CLA)

Although the role of specific fatty acids in the onset of cancer in humans remains unclear, current evidence cannot discount the association of increased intake of LA with the incidence of colorectal or prostatic cancer in humans (Erickson, 1998; Zock and Katan, 1998). PUFAs are incorporated into cell membrane phospholipids, whereby they can then play a role in cell signalling and metabolism. Due to their increased proliferation, cancer cells have an increased requirement for fatty acids. This opens up a novel avenue for cancer prevention or treatment through the provision of anti-carcinogenic fatty acids to proliferating cancer cells.

CLA, a derivative of LA, has become the focus of much attention recently due to its anti-cancer properties. CLA is a naturally occurring polyunsaturated fatty acid found in small quantities in ruminant meats and diary products. CLA is a collective term, which refers to a mixture of positional and geometric isomers of LA, and like LA it belongs to the ω-6 group of fatty acids. The double bonds in LA are at the 9^{th} and 12^{th} carbon from the carboxyl end in the *cis* configuration, whereby the bonds in CLA are in positions 9 and 11 or 10 and 12 (i.e. conjugated). These bonds can be in the *cis* or *trans* configuration. The 9*c*,11*t*-18:2 and the 10*t*,12*c*-18:2 are believed to be biologically active (Palombo *et al*, 2002). The structures of CLA and LA are shown in figure 1.5.

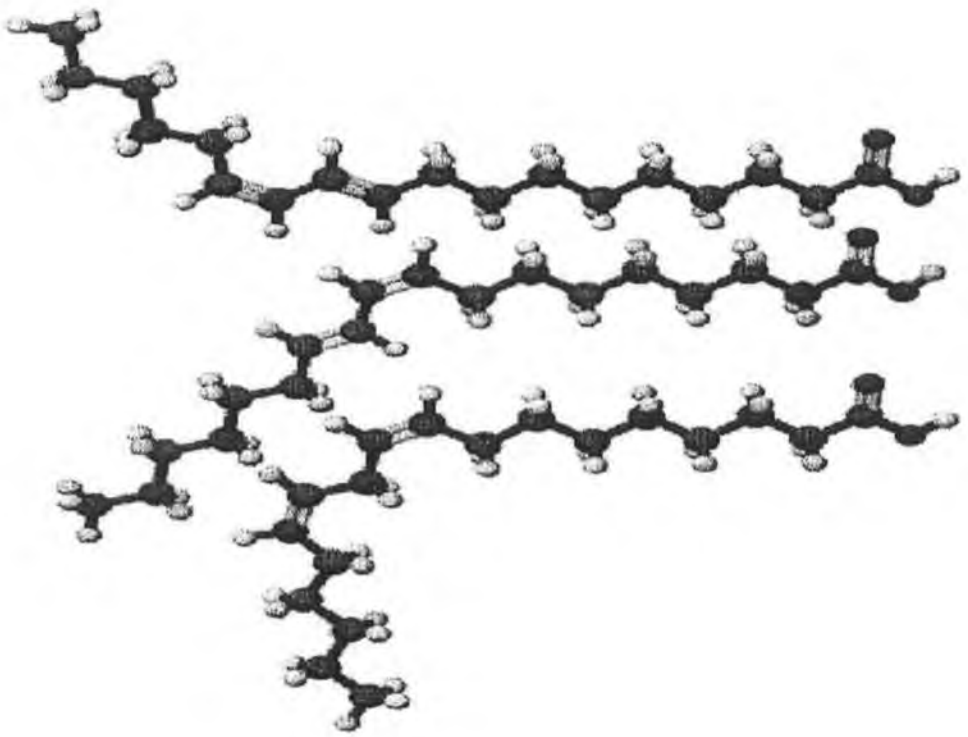


Figure 1.5. Structures of 10*t*,12*c*-18:2 CLA (upper panel), 9*c*,11*t*-18:2 CLA (middle panel) and LA (lower panel) (Pariza *et al*, 2001)

1.4.1 CLA in the diet

CLA is formed as a result of microbial biohydrogenation in the rumen, and therefore is found primarily in ruminant animal and dairy products. The levels of total CLA in various foods have been reported to vary from as low as 0.2mg/g fat in corn and peanut oil to as high as 17mg/g in beef and 30mg/g in milk fat (O'Shea *et al*, 1998). Negligible amounts have been observed in seafoods and vegetable oils. Due to the wide distribution of CLA in common foodstuffs, human dietary intakes of CLA may vary widely depending on food selection.

The CLA content of food products can vary greatly due to numerous reasons. Ha *et al* (1987) demonstrated that the CLA content of ground beef could be increased four-fold after grilling due to free radical-mediated oxidation of LA. Numerous other studies demonstrated several ways in which dairy and meat CLA content can be changed (Garcia-Lopez *et al*, 1994; Shantha *et al*, 1994; Jiang *et al*, 1998b). However, the natural variation of CLA content that occurs in milkfat is by far more significant than those foods mentioned so far. This is due to factors that may affect ruminant animals such as diet, feeding regime, animal breed and seasonal influences (O'Shea *et al*, 1998)

CLA's inhibitory effects on tumourigenesis can be found at intake levels suitable for humans, and therefore CLA may prove to be an important chemopreventive component of our diet. To date, there have been many inconclusive findings regarding the effect of fatty acids on tumourigenicity. This may be due to the use of complex mixtures of dietary fats in experimental studies. By using simple mixtures and individual purified isomers of CLA in experimental research, the principal anti-carcinogenic isomers can be identified. These can then be developed for use in dietary supplements (such as 'functional foods'), especially for patients with an increased risk of developing cancer, or as an adjuvant treatment for patients with cancer (Palombo *et al*, 2002).

1.4.2 Evidence supporting the anti-cancer properties of CLA

1.4.2.1 *In vivo* experimental studies

Cesano *et al* (1998) carried out a study to examine the effects of LA and CLA, both ω-6 FAs, on the local growth and metastatic properties of DU-145, a human prostatic carcinoma cell line, in severe combined immunodeficient (SCID) mice. The mice were fed different diets over a 14-week period (after the second week they were inoculated with the cells). Mice receiving the LA-supplemented diet displayed

significantly higher body weights, lower food intake and increased tumour load as compared to the other groups. Mice fed the CLA-supplemented diet displayed not only smaller local tumours than the regular diet-fed group, but also a drastic reduction in lung metastases. These results support the view that LA and CLA differently influence the prognosis of prostatic cancer patients, thus opening the possibility of new therapeutic options.

Studies were carried out by Hilakivi-Clarke *et al* (1999) where pregnant rats were fed two different isocaloric corn oil diets, one was a high fat diet (high LA), the other was a low fat diet (low LA). The diets were designed to ensure that they contained the same calories so that results obtained could not be attributed to one diet promoting weight gain and the other not, i.e. increased tumour growth could not be attributed to obesity. They concluded that a maternal exposure to these differing diets significantly affected DMBA-induced tumourigenesis in female offspring. Mammary tumour incidence was higher and latency for tumour appearance shorter among the rats that were exposed *in utero via* maternal feeding of an isocaloric diet high in LA, when compared with the offspring of mothers fed a low fat diet. An increase in serum oestrogen was observed for rats fed the high fat diet.

During pregnancy, oestrogen and other placental hormones cause a rapid proliferation of the mother's epithelial structures within the breast, namely the Terminal End Buds (TEBs). TEBs are the primary sites for the chemical induction of mammary carcinomas in rodents (Banni *et al*, 1999). Hilakivi-Clarke *et al*, (1999) concluded that consumption of a high LA diet during pregnancy, possibly through an increase in circulating oestrogen levels and increased growth of transformed TEBs, increased the risk of developing DMBA-induced mammary tumours in female rats.

Ip *et al* (1997, 1999a) reported that CLA had significant activity in inhibiting mammary carcinogenesis. Rats were treated with a single dose of DMBA, and were subsequently given 1% CLA in the diet for 4 weeks, 8 weeks or continuously (20 weeks) (Ip *et al*, 1997). In the 4 and 8 weeks group, no cancer protection was evident, while significant tumour inhibition was observed in the 20 weeks group. In a subsequent experiment (Ip *et al*, 1999a), rats were fed a CLA-enriched butter fat diet during the time of pubescent mammary gland maturation, altered mammary gland morphogenesis was observed. The mass of TEB cells, the target cells for mammary chemical carcinogenesis, was reduced by 30%, and mammary tumour yield was inhibited by 53%. It was

concluded from these experiments that the CLA-enriched butterfat reduced cancer risk in these rats.

Hubbard *et al* (2000) inoculated mice with a metastatic cell line, 4526, which was derived from a mouse mammary adenocarcinoma. They found reduced metastases and tumour burden in the lungs, as well as increased latency in mice fed a CLA diet ranging from 0.1% to 1%. To date, there have been few studies on the effect of CLA on metastasis, and no evidence has been presented to support any mechanisms by which CLA may reduce metastasis.

1.4.2.2 Proposed mechanisms behind CLA's anti-cancer properties

Various factors have been implicated in the mechanism of CLA's anti-tumour action. These include increasing oxidative stress, alterations in the metabolism of fatty acids to the biologically active eicosonoids, and changes in membrane composition, which could affect cell signalling pathways (Diggle, 2002). The first two mechanisms have been the most commonly researched.

Lipid peroxidation is thought to be a major mechanism of CLA's anti-cancer action. Lipid peroxidation begins with the removal of a hydrogen atom from a PUFA double bond, producing a reactive oxygen species (ROS) (figure 1.6), that can propagate further reactions. These ROS can then act on macromolecules, such as DNA, causing damage. The damaged cell can either repair itself, resulting in an anti-proliferative effect, or cell death can occur. The addition of reagents that are able to reverse the cytotoxic effect of PUFAs, such as vitamin E and butylated hydroxytoluene (BHT), is often used to assess if CLA's action is through such oxidative stress and lipid peroxidation (den Berg, 1995, O'Shea, 1999, Igarashi and Miyazawa, 2001, Devery *et al*, 2001).

Figure 1.4 depicts what happens metabolically to AA. The cyclooxygenase and lipoxygenase pathways have been indicated as possible means through which CLA affects the production, notably the inhibition, of eicosanoids (Liu and Belury, 1997, Banni *et al*, 1999, Urquhart *et al*, 2002). The use of inhibitors, some more specific than others, can identify if any of these pathways are important in the anti-proliferative/cytotoxic effect displayed by CLA.

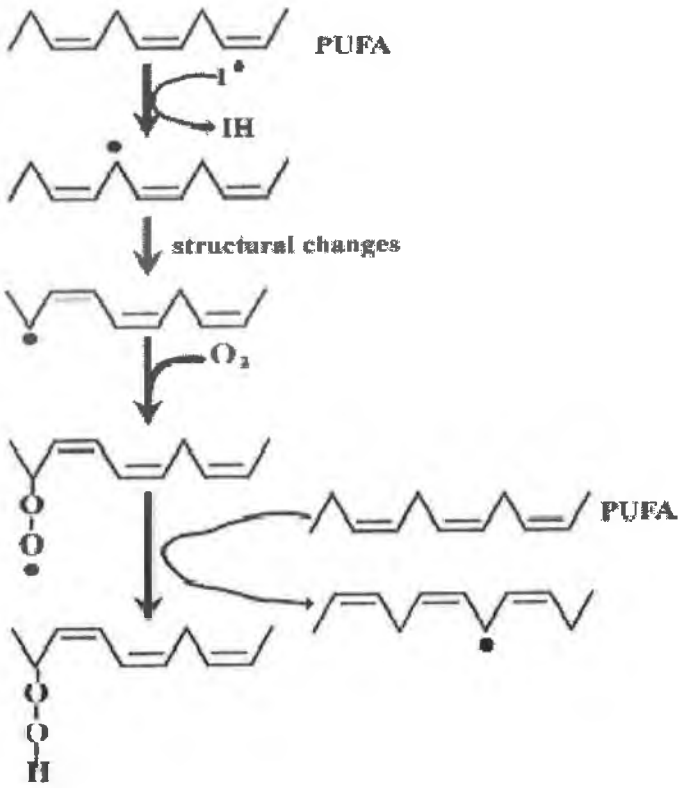


Figure 1.6. Lipid peroxidation of a PUFA. A double bond of the PUFA is being attacked by the reactive oxygen species denoted by a dot (•).

1.4.2.3 *In vitro* research

There has been much *in vitro* research to date into the mechanism behind CLA's antiproliferative effects, but contradictory results have been yielded. As there is little research published to date regarding the differing effects of the numerous individual CLA isomers, when more is known about these effects, a greater understanding of the mechanisms may be established.

One proposed mechanism for CLA's anticancer properties is its relationship to the hormone response system. Durgam and Fernandes (1997) found that CLA selectively inhibited proliferation of estrogen responsive (ER)-positive MCF-7 cells (breast cancer, in origin) as compared with ER-negative MDA-MB-231 cells (breast cancer, in origin). Cell cycle studies using flow cytometry also indicated that a higher percentage of CLA

treated MCF-7 cells remained in the G_0/G_1 phase of the cell cycle as compared to the control and those treated with LA. They concluded that CLA inhibited MCF-7 cell growth by interfering with the hormone-regulated mitogenic pathway of breast cancer.

Lipid peroxidation is another such mechanism, which may account for CLA's anti-cancer activity. O'Shea *et al* (1999) observed a dose dependent decrease in cell numbers and increase in lipid peroxidation, as determined by thiobarbituric acid reactive substances (TBARS) in MCF-7 and SW480 (colon, in origin) cell lines following incubation with CLA. After lipid peroxidation of PUFAs (figure 1.6), ROS, including superoxide and hydroxyl radicals and a complex range of peroxidised and otherwise oxygenated lipids (Horrobin, 1990), are produced. Several of the ROS formed react with thiobarbituric acid (TBA) to produce coloured material that can be detected by spectrophotometry. This measurement of TBARS is a widely used indicator of lipid peroxidation, even though it really only gives a relative indication and is not very accurate. O'Shea *et al* (1999) concluded that the CLA-induced anti-oxidant enzymes (super oxide dismutase [SOD] and glutathione peroxidase [GPx]) failed to protect these cells from cytotoxic lipid peroxidation products.

The effect of CLA on the breast cancer cell line, MCF-7, has been extensively studied. Park *et al* (2000) found that LA stimulated the growth of MCF-7 cells while CLA resulted in inhibition. This data also ruled out the possibility that growth inhibition by CLA was mediated through phospholipase-C (PLC)-, protein kinase-C (PKC)- or Prostaglandin E_2 (PGE_2)-dependent signal transduction pathways.

Research carried out by Attiga *et al* (2000) showed a reduction in both *in vitro* invasion and in matrix metalloproteinase (MMP) levels (these are a group of enzymes that degrade the extra cellular matrix (ECM), and they will be discussed in more detail in chapter 4) in two prostatic cell lines, after treatment with various COX inhibitors. These results indicated a potential role for COX in cancer metastasis, and also provided evidence of a possible link between CLA, a proposed COX inhibitor, and MMPs.

Contrary to the study carried out by O'Shea *et al* (1999), Igarashi and Miyazawa (2001) found that the growth inhibitory effect of CLA on HepG2 (hepatoma cell line) was not due to lipid peroxidation but to a change in fatty acid metabolism. The contrary conclusions drawn by different researchers indicates that the growth inhibitory effect of CLA may be due to more than one mechanism, and may depend on the mixture of CLA or individual isomers used, and also on the cell type used.

The antiproliferative effects of two commercial preparations of CLA and their constituent purified isomers, 9*c*,11*t*(18:2), 9*c*,11*c*(18:2) and 10*t*,12*c*(18:2) were determined *in vitro* by Palombo *et al* (2002), using human colorectal (HT-29, MIP-101) and prostate (PC-3) carcinoma cells. They found that the antiproliferative effects of the preparations were dependent on the type and concentration of the CLA isomer present. The 10*t*,12*c*(18:2) isomer exhibited the greatest potency against colorectal cancer proliferation, and the 9*c*,11*c*(18:2) and 10*t*,12*c*(18:2) isomers were moderately effective against the prostate cancer. The 10*t*,12*c*(18:2) isomer induced caspase-dependent apoptosis in MIP-101 and PC-3 cells.

1.5 Polyunsaturated fatty acids and metastasis

1.5.1 The metastatic cascade

Initiation occurs when a single cell becomes modified to exhibit a growth advantage over the surrounding tissue to form the primary tumour mass, which, at some point, requires neovascularisation to supply nutrients for further growth. As the tumour becomes malignant, it acquires the ability to invade the surrounding normal tissue. The most lethal aspect of cancer is this ability to invade and metastasise.

The first step in metastasis is intravasation, and it occurs when tumour cells cross the basement membrane and enter the lymphatic and/or circulatory system where their presence can be detected using a variety of techniques. Detection of tumour cells at this point is vital in the treatment of breast cancer, and in the prevention of fatalities. Following the tumour cells' survival in the circulation, extravasation occurs when the tumour cells leave the circulation and penetrate the host normal tissue. Metastasis occurs if the tumour cells can establish and grow at this secondary site (McCawley and Matrisian, 2000). One of the primary sites for secondary tumours of breast cancer is the lungs. These events are illustrated in figure 1.7.

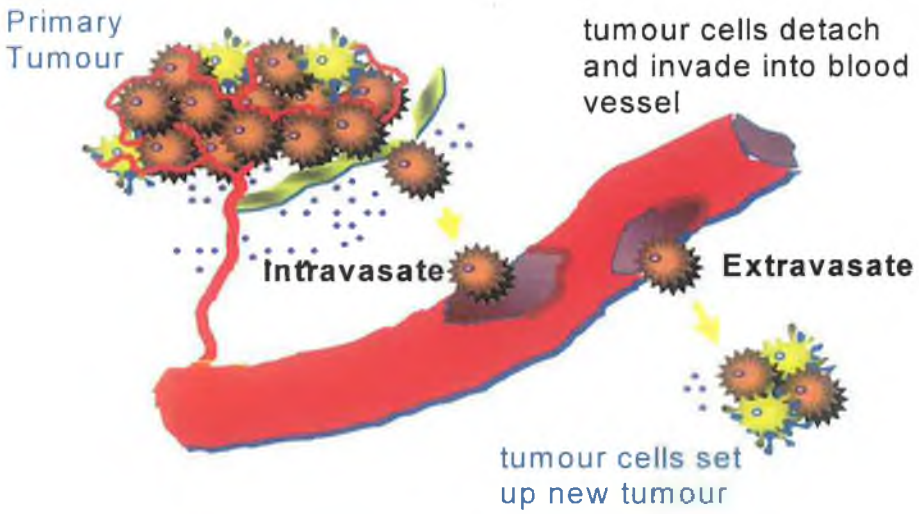


Figure 1.7. Steps involved in tumour migration and metastasis. The primary tumour depicted is in the breast, and the secondary tumour often forms in the lungs. The yellow arrows depict the migration of the cells.

1.5.2 The important roles played by PUFAs in tumour cell migration and metastasis

There has been much research into the effect certain PUFAs have had on tumour cell invasion and metastasis, and a review by Jiang *et al*, 1998a discusses many of these effects. EPA, GLA and AA have all been shown to inhibit the production of factors, involved in metastasis, while LA has been reported as stimulating tumour cell invasion *in vitro*. It has also been demonstrated that EPA and GLA resulted in a time and concentration dependent enhancement of E-cadherin, an adhesion molecule associated with metastatic suppression, in a range of cancer cells. This was also associated with a reduction in invasion. GLA, EPA, DHA and LA have all also been associated with a reduction of tumour adhesion to a range of matrix components (Jiang *et al*, 1998a).

PUFAs also regulate immune cells and host immune response to tumour cells, in order to minimise the motile and invasive behaviour of cancer cells, to influence tumour cell survival in the circulation, and to form mechanisms in normal tissues to combat cancer cells (Jiang *et al*, 1998a). It has been shown that many PUFAs, including LA, play an important role in several steps of the metastatic process. The use of PUFAs in preventing metastasis may be a novel route in breast cancer treatment, which should be investigated.

1.6 Thesis Overview

The research presented in this thesis examines the effect of CLA on the proliferation, invasion and metastasis of the murine mammary cancer cell line, 4T1. It has been divided into two main chapters.

- Chapter 3:

The 4T1 cell line was initially treated with a preparation containing a mixture of CLA isomers and preparations of individual purified isomers, 9*c*,11*t*(18:2) and 10*t*,12*c*(18:2). A variety of concentrations and incubations times were used to investigate cytotoxicity, and to determine if there was a difference in the potency of the isomers.

Using conditions that caused 50% cell death, the effect of the CLA mixture and its purified isomers on the different stages of the cell cycle was investigated using flow cytometry. This same dose and time was used to determine if CLA caused apoptosis to occur.

- Chapter 4:

The 4T1 cell line was treated with a dose and for a time period, which caused minimal cell death to examine the effect of the CLA mixture and its purified isomers on the release of MMP-9 from the cells. This same dose and time was used to look at the effect of CLA on the *in vitro* adhesion, migration and invasion of the cell line.

The thesis is divided into six chapters. Chapter 1 serves as an introduction to breast cancer, CLA and metastasis. There is a common materials and methods section (Chapter 2) and bibliography (Chapter 6). Chapters 3 and 4 each have their own introduction, results and discussion sections, while Chapter 5 provides an overall summary of the thesis.

CHAPTER 2

2.1 Materials

All general purpose chemicals and reagents used in experimental work were of analytical grade and were purchased from Sigma-Aldrich Chemical Company; Dublin, Ireland, BDH Chemicals Ltd., Poole, Dorset, England and Riedal De Haen AG, Seelze, Hannover, Germany.

The CellTiter 96® Aqueous Non-Radioactive Cell Proliferation assay was obtained from Promega Corporation, 2800 Woods Hollow Road, Madison, WI, 53711-5399 USA.

Cell culture medium was obtained from Sigma-Aldrich, Dublin, Ireland.

Foetal calf serum was supplied by Sigma-Aldrich, Dublin, Ireland.

Disposable plastics for cell culture, and 96-well plates were obtained from Sarstedt, Sinnottstown Lane, Drinagh, Co. Wexford, Ireland.

The 4T1 cell line (American Tissue Culture Collection [ATCC] # CRL-2539) was a kind gift from Dr. Judith Harmey, Department of Surgery, Beaumont Hospital, Royal College of Surgeons in Ireland, Dublin 9, Ireland.

The Suicide-Track™ DNA Ladder Isolation Kit was purchased from Oncogene Research Products, 650 Albany Street, Boston, MA 02142

Propidium Iodide (PI) was a kind gift from Claire Condron, Royal College of Surgeons in Ireland research centre, Beaumont, Dublin 9, Ireland

CLA mixture [containing 29.5% 9*c*,11*t*(18:2) and 29% 10*t*,12*c*(18:2) CLA isomers (in addition to minor components of other isomers)] was obtained from Nu Check Prep, Elysian, MN, USA.

Individual purified CLA isomers (99.9% pure), 9*c*,11*t*(18:2) and 10*t*,12*c*(18:2), were a kind gift from Natural ASA, Norway.

PBS tablets were purchased from Oxoid Ltd., Basingstoke, Hampshire, England.

L-glutamine, trypsin, penicillin/streptomycin were purchased from Sigma-Aldrich, Dublin, Ireland.

The BCA reagent for protein determination was obtained from Pierce Chemicals, Rockford, Illinois, USA.

Extra cellular matrix (ECM) gel was obtained from Sigma-Aldrich, Dublin, Ireland.

Falcon® cell culture inserts were obtained from Collaborative Biomedical Products, Becton Dickinson Labware, 2 Oak Park, Bedford, MA 01730, USA.

BD Biocoat™ Matrigel™ Invasion Chambers were obtained from Collaborative Biomedical Products, Becton Dickinson Labware, 2 Oak Park, Bedford, MA 01730, USA.

Equipment used is outlined in the relevant methods section.

2.2 Methods

2.2.1 Preparation and storage of CLA stocks

Three synthetic sources of CLA were used in this study: a mixture of CLA isomers containing 29.5% 9*c*,11*t*(18:2) and 29% 10*t*,12*c*(18:2) CLA isomers (in addition to minor components of other isomers); purified 9*c*,11*t*(18:2) and purified 10*t*,12*c*(18:2) CLA isomers. CLA stocks (2000 and 3000 μg/ml) were made up in ethanol (EtOH), and were stored at –20°C. Further dilutions resulted in stocks of 500, 1000, 1500, 2000, 2500 and 3000μg/ml. 1μl of each stock, when added to 100μl of cell suspension resulted in final concentrations of 5, 10, 15, 20, 25 and 30μg/ml, respectively. These concentrations were used throughout the study.

2.2.2 Cell culture methods

All cell culture techniques were performed in a sterile environment using a Holten HB255 laminar airflow cabinet. Cells were visualised with an Olympus CK2 inverted phase contrast microscope.

2.2.2.1 Culture of adherent cell lines

The 4T1 cell line under study was maintained in Dublecco's modification of Eagles medium (DMEM) supplemented with 5% (v/v) [DMEM/S_5] foetal calf serum (FCS), 2mM L-glutamine, 1mM N-[2-Hydroxyethl]piperazine-N'-[2-ethanesulfonic acid] (HEPES) and 1unit/ml penicillin and 1μg/ml streptomycin. Cells were cultured in 25cm^2 or 75cm^2 tissue culture flasks. As this was a strongly adherent cell line, trypsinisation was required for harvesting cells prior to subculturing. For trypsinisation, the growth medium was aspirated and the flask rinsed with 3ml of phosphate buffered saline (PBS) to remove any residual FCS, which contains a trypsin-inhibitor (α_2-macroglobulin). 2ml of fresh trypsin ethylenediamine tetracetic acid (EDTA) (0.025% (w/v) trypsin with 0.02% (w/v) EDTA in 0.15M PBS, pH 7.4) was then placed in each flask and the flask incubated at 37°C for 5-10min or until all the cells had detached from the surface. The cell suspension was removed to a sterile universal container containing 5ml of growth medium and centrifuged at 2000rpm for 5min. The supernatant was poured off and the cells were resuspended in culture medium, which could then be divided between several flasks depending on what was required of the cells. Typically, 14ml of media in total was

added to a $75cm^2$ flask and 5 ml to a $25cm^2$ flask. The flasks were then labelled with the name of the cell line and the passage number, which denotes the number of times the cell line has been subcultured. The cell line was incubated in a humid, 5% (v/v) CO_2 atmosphere at 37°C in a Heraeus cell culture incubator.

2.2.2.2 Cell counts

Cell counts were performed using a Neubauer haemocytometer slide. Trypan blue exclusion dye was routinely used to determine cell viabilities after the tripsinisation process. 20µl trypan blue was added to 100µl cell suspension, and the mixture left to incubate for 2min. A sample of this mixture was added to the counting chamber of the haemocytometer, and the cells visualised by light microscopy. Viable cells excluded the dye and remained clear while dead cells stained blue. The number of cells was calculated as follows

(Average number of viable cells)*1.2 [dilution factor]*10^4 [volume under the cover slip]
= viable cells/ml of cell suspension.

2.2.2.3 Recovery and storage of cells

Long term storage of cells was achieved by storing the cells in liquid nitrogen and maintaining them in a cryofreezer (supplied and serviced by Cooper Cryoservice Ltd, Dublin, Ireland). Cells to be stored were trypsinised and centrifuged as described in Section 2.2.2.1, and the resulting cell pellet resuspended at a concentration of $1*10^6$ cells/ml in DMEM containing FCS (10% [v/v]) and the cryopreservative, dimethylsulphoxide (DMSO) (10% [v/v]). 1ml aliquots were transferred to sterile cryotubes, and frozen, first at -20°C for 30min, then overnight at -80°C and then immersed in liquid nitrogen. The cryotubes were labelled with the name of the cell line, the passage number, the date of storage and the name of the person storing the cells. Cells were recovered from liquid nitrogen by thawing rapidly at 37°C and the transferred to a sterile universal tube containing 5ml growth media. The cells were centrifuged at 2000rpm for 5min, resuspended in fresh medium, transferred to culture flasks and incubated at 37°C in 5% CO_2.

2.2.3 Cytotoxicty of CLA

The introduction of multiwell plates revolutionised the approach to replicate sampling in cell culture. They are economical, easily automated and diverse in their application. The most popular in cell culture techniques is the 96-well, flat-bottomed microtitration plate. Each well has a growth area of 28-32mm^2, a capacity for 0.1 or 0.2ml of media and up to 10^5 cells.

For every experimental treatment, a standard curve of cells was set up. Ensuring that the curve remained linear, the validity and reproducibility of the assay was monitored. The cells were added to the wells and then allowed to attach to the substratum for 24hr before being treated with the CLA or ethanol. The highest point on the standard curve (2-2.5*10^4 cells/ml, depending on the time period) was used for the treatments. Triplicates were always performed, and cells treated with ethanol, to a final concentration of 1% (v/v), acted as the control. Percentage cell death was calculated as a percentage of the ethanol, whereby the ethanol represents 100% viability. This calculation is shown below.

$$100-[\{(OD\ of\ sample_{average}-OD\ of\ blank_{average})/(OD\ of\ control_{average}-OD\ of\ blank_{average})\}*100]$$

where OD is the optical density/absorbance.

2.2.3.1 Time/Dose Response

In order to study the cytotoxicity of the CLA mixture and the two isomers, several concentrations of the fatty acids and several treatment times were chosen. The 4T1 cell line was incubated in 96-well plates for 1, 2, 3 and 4 days, while being treated with 0, 5, 10, 15, 20, 25 and 30μg/ml of CLA, and 1% (v/v) ethanol.

2.2.3.2 Promega CellTiter 96® AQ_{ueous} Non-Radioactive Cell Proliferation Assay

This is a colourimetric method for determining the number of viable cells in proliferation or chemosensitivity assays. The CellTiter 96® AQ_{ueous} Assay is composed of solutions of a novel tetrazolium compound (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium, inner salt; MTS) and an electron coupling reagent (phenazine methosulphate; PMS). Dehydrogenase enzymes, found in metabolically active cells, convert MTS into a soluble formazan product, whose

absorbance can be read at 490nm. The quantity of formazan product formed is proportional to the number of living cells in culture.

On arrival into the lab the MTS (100ml) and PMS (5ml) were combined and aliquoted into several containers. This reagent was then stored at –20°C in amber containers as it is light sensitive.

When the treatment time was complete, 20µl of MTS/PMS was added to the treated wells, the control and to the standard curve, bringing up the total volume in each well to 120µl. The plate was then incubated for 1-4hr at 37°C in a humidified 5% CO_2 atmosphere, and the absorbance was recorded at 492nm using the multiwell plate reader, Rosys Anthos 2010.

2.2.3.3 Statistical analysis

Using the Microsoft Excel™ Program, 2-tailed T-tests were carried out on all the triplicate values resulting from the MTS assay. A P value of <0.05 was required for significant results.

All results are represented by a value followed by its standard deviation (+/- SD), which was calculated using the Microsoft Excel™ Program.

2.2.4 Treatment and preparation of cells for cell cycle analysis

For cell cycle analysis cells were grown and treated in a $25cm^2$ flask. $8*10^5$ cells were seeded into a flask. Following attachment of the cells to the substratum overnight, they were treated with 30µg/ml CLA for 2 days. This ensured that, while there was cell death, there were also a sufficient number of viable cells to be analysed by flow cytometry.

After treatment for cell cycle analysis, the cells were trypsinised, as described in Section 2.2.2.1, and washed twice in ice-cold PBS. The pellets were then resuspended in ice-cold ethanol-PBS (70-30% v/v). Once prepared, cells could be stored on ice, at 4°C, for up to 7 days or until time of analysis. Before analysis, fixed cells were pelleted and resuspended in 40µg/ml Propidium Iodide (PI) in PBS, and incubated on ice for 15min. Typically, a 70% confluent $25cm^2$ flask would eventually be resuspended in 2ml of PI. Samples were then ready for analysis.

2.2.5 Use of the flow cytometer

Samples were analysed on a FACScan using 488nm argon ion laser measuring forward and orthogonal light scatter with the lysis program to create histograms depicting the cell cycle distribution of the sample.

2.2.6 Treatment and analysis of cells for apoptosis

Cells were grown and treated in a $25cm^2$ flask. $8*10^5$ cells were seeded into a flask. Following attachment of the cells to the substratum overnight, they were treated with 30µg/ml CLA for 2 days. For analysis of apoptosis using the Suicide-Track™ DNA Ladder Isolation Kit from Oncogene Research Products both the cells in the monolayer and the detached cells were examined.

The Oncogene kit (CAT # AM41) provided a fast, nonisotopic method for the detection of DNA laddering in either cell monolayers or suspension (detached) cells. It was possible to separate apoptotic DNA from high molecular weight, intact, genomic DNA. Following the procedure, which could recover even small amounts of DNA, ladder fragments were separated by standard agarose gel electrophoresis and stained with ethidium bromide (EtBr). These were then viewed under ultra violet (UV) light and photographed.

2.2.6.1 Sample preparation and DNA extraction

After treatment of the cells, the media was removed and spun at 2,000rpm for 5min. The supernatant was poured off, the pellet resuspended in 500µl of Extraction Buffer, and left on ice for 30min. In the meantime, 500µl of Extraction Buffer was added to the monolayer, the cell lysate gently transferred to a microfuge tube, and then left on ice for 30min.

Following this the sample was spun at 13,000rpm for 8min at room temperature (RT). The supernatant was removed and transferred to a clean tube, discarding the tube containing the high molecular weight (HMW) DNA. 20µl of Solution # 2 was added to the tube, and it was then incubated at 37°C for 60min. 25µl of Solution # 3 was added, with gentle mixing. The sample was incubated at 50°C overnight.

2.2.6.2 DNA precipitation

2µl of Pellet Paint™ Co-Precipitant, followed by 60µl of 3M Sodium Acetate, pH 5.2, was added to the sample, with brief mixing. 662µl of 2-Propanol was added, and the sample mixed and left for 2min. The sample was centrifuged at 13,000rpm for 8min, whereby a pink pellet was visible.

Following this, the supernatant was removed with a pipette tip and the pellet rinsed with 500µl 70% ethanol. The sample was centrifuged at 13,000rpm for 8min. The supernatant was removed with a pipette tip, the pellet rinsed with 500µl 100% ethanol, and centrifuged at 13,000rpm for 8min. The supernatant was removed with a pipette tip and the sample air-dried by leaving the tube open on the benchtop for a few minutes at RT. The pellet was resuspended in 50µl of Resuspension Buffer.

2.2.6.3 DNA gel electrophoresis

Tris-Acetate-EDTA (TAE) buffer (50X) was made up as follows: 242g Tris base was dissolved in 750ml dH_2O; 100ml 0.5M EDTA (pH 8.0), and 57.1 ml glacial acetic acid, was added; final volume was adjusted to 1 liter with dH_2O.

The 50X TAE was diluted to 1X with dH_2O, and it was used to prepare enough 1.5% agarose to pour a gel approximately 0.75cm thick. After the solution was heated to dissolve the agarose, it was cooled to 60°C and poured into a prepared electrophoresis chamber where the comb was positioned to form sample wells. After the gel was solidified, the comb was removed and the gel was positioned in the gel buffer tank. 1X TAE was added to cover the gel to a depth of 1-2mm.

21µl of DNA ladder sample was added to a clean centrifuge tube, along with 4µl of 6X Gel Loading buffer. The sample was then loaded onto the gel. 5µl of DNA Marker (provided in kit) was also loaded onto the gel.

The lid was assembled onto the electrophoresis chamber and the electrical leads were attached so that the DNA migrated towards the positive (red/anodic) lead. The gel was run at 50 constant volts until the dye front was 1-2cm from the bottom of the gel. This takes about 4-4.5hr. Following electrophoresis, the gel was stained in EtBr (0.5mg/ml) for 0.5-1hr, and viewed by UV illumination.

2.2.7 Treatment and preparation of cells for matrix metalloproteinase (MMP) analysis

For MMP analysis, cells were grown until approximately 60% confluent after which the growth medium was decanted and the cells rinsed with sterile PBS. 8mls of fresh serum-free media (DMEM/S_0) was then added to each 75cm^2 flask. The cells were then incubated in DMEM/S_0 overnight, at 37°C. The following day, fresh DMEM/S_0 was added and the flasks were supplemented with either: 15μg/ml of the CLA mixture; 9*c*,11*t*(18:2) isomer; 10*t*,12*c*(18:2) isomer or 1% (v/v) EtOH. The cells were then returned to the incubator for 24hr, after which the media was collected for protein determination by the BCA assay followed by MMP expression analysis by gelatin zymography. This assay was repeated three times on three separate occasions.

2.2.8 Bicinchoninic acid (BCA) protein microassay

In this assay, Cu^{++} reacts with the protein under alkaline conditions to give Cu^{+}, which in turn reacts with BCA to give a coloured product. Two separate reagents were supplied in the commercially available assay kit (Pierce Chemicals): **A**, an alkaline bicarbonate solution and B, a copper sulphate solution. Working solution was prepared by mixing 1 part reagent **B** with 50 parts reagent **A**. 200μl of this solution was added to 10μl of the treated sample or protein standard in wells of a microtitre plate. The plate was incubated at 37°C for 30min. The absorbance of each well was read at 620nm using a microtitre plate reader (Rosys Anthos 2010). Protein concentrations were determined from a bovine serum albumin (BSA) standard curve in the 0-0.4mg/ml range. Once the protein concentrations of the treated samples were determined, it could be ensured that equal protein was loaded onto the zymography gel.

2.2.9 Zymography

Substrate zymography was performed to localise MMP activity by molecular weight. The Laemmeli discontinuous system was used with alterations concerning the incorporation of a substrate. The gel was prepared by incorporating the protein substrate of interest (gelatin) within the polymerized acrylamide matrix. 10% acrylamide gels were used and the amounts for one gel are given below.

Resolving gel:

2.5 ml Buffer A (1.5 M Tris-HCl, pH 8.8; 0.4% (w/v) SDS)

2.5 ml 3 mg/ml gelatin stock

3.3 ml 30% (w/v) acrylamide stock

1.7 ml distilled water

33 µl 10% (w/v) ammonium persulphate (freshly prepared)

5 µl TEMED

Stacking gel:

0.8 ml Buffer B (0.5 M Tris-HCl, pH 6.8; 0.4% SDS)

0.5 ml 30% acrylamide stock

2 ml distilled water

33µl 10% ammonium persulphate (freshly prepared)

5 µl TEMED

Samples were mixed 3:1 with 4X sample buffer (10% sucrose; 0.25M Tris-HCl, pH 6.8; 0.1% (w/v) bromophenol blue) and loaded. The gels were run at 20mA per gel in running buffer (0.025 M Tris, 0.19M glycine, 0.1% SDS) until the dye front reached the bottom of the gel. Following electrophoresis the gel was soaked in 2.5% Triton-X-100 with gentle shaking for 30min at room temperature (RT) with one change. The gel was then rinsed in substrate buffer (50mM Tris-HCl, pH 8.0; 5 mM $CaCl_2$) and incubated for 24hr in substrate buffer at 37°C. The gel was then stained with Coomassie blue [0.5% (w/v) Coomassie Brilliant Blue in acetic acid:isopropanol:H_2O (1:3:6, v/v/v)] for 2hr with shaking, and destained in water until clear bands were visible against a blue backgound.

To confirm the bands as metalloproteinases, identical gels were run as described above except the substrate buffer contained the MMP inhibitor, 10mM EDTA.

2.2.9.1 Densitometry and statistical analysis

Densitometry of zymography gels was performed using a Pharmacia Amersham Densitometer with Imagemaster software. Two tailed T-tests were performed using Microsoft Excel™, whereby $p = <0.05$.

2.2.10 *In vitro* adhesion assays

For these experiments, 10^6 cells were seeded into 25cm^2 flasks, and allowed to adhere to the substratum overnight. They were then treated with 15µg/ml of CLA mixture or purified isomers, or 1% (v/v) EtOH, for 24hr.

After treatment, 10^4 cells/well were added to a sterile 96-well plate in triplicate, and allowed to adhere to the plastic for 30, 60 and 90min at 37°C. After allowing the cells to adhere for the given time, the media and unattached cells were gently 'flicked' from the wells by inverting the plate. The plate was then spun at 200rpm to gather any media, which may be along the sides of the wells. This media could then be aspirated.

In order to calculate percentage cell adhesion, 100µl of the original cell suspension was added to a well at this stage (note: this sample was not flicked from the plate), thus allowing calculation of 100% attachment. 20µl of MTS/PMS was added to this well.

20µl of MTS/PMS was diluted with 100µl of DMEM, and this was added to each well. The plate was then incubated for 1-4hr at 37°C in a humidified 5% CO_2 atmosphere, and the absorbance was recorded at 492nm using the multiwell plate reader, Rosys Anthos 2010.

2.2.11 *In vitro* migration assays

Falcon® cell culture inserts provided a system that allowed assessment of cells migratory activity *in vitro*. The cell culture inserts contained an 8µm pore size, polyethylene terephthalate (PET) membrane, which allowed cells to migrate from the upper side of the membrane to the lower side, where they can be fixed, stained and visualized. Inserts suitable for a 24-well plate were used.

For these experiments, 10^6 cells were seeded into 25cm^2 flasks, and allowed to adhere to the substratum overnight. They were then treated with 15µg/ml of CLA mixture or purified isomers, or 1% (v/v) EtOH, for 24hr.

The assays was set up by firstly adding 600µl of media containing 20% serum (DMEM/S_{20}), and 15µg/ml CLA, to each well of the plate. The cell culture inserts were then asceptically placed into the wells, and 100µl of cell suspension in DMEM/S_0 (10^4 cells/well) was placed onto the membrane. The DMEM/S_{20} acted as a chemoattractant. The plates were then incubated for 24hr at 37°C in a 5% CO_2 incubator. After incubation, the non-migratory cells are removed from the upper surface of the membrane by cleaning gently with a cotton-tipped swab soaked in PBS. The cells on the lower surface of the membrane were then fixed for 10min in methanol, stained for 5min in Mayers Haematoxylin solution and rinsed in tap water several times to 'blue' the dye. The cells and membrane were then dehydrated by incubation, for 2min each, in a series of

organic solutions (25, 50, 75 & 100% (v/v) ethanol, followed by 100% xylene [mixed isomers]). The membranes were then carefully removed from the insert housing using a scalpel, and mounted on slides using DPX mounting medium (contains xylene). Cells, which had migrated through the membrane, were then viewed under the microscope at 10X and 40X magnification, and counted. The percentage migration for each treatment (CLA mixture and individual purified isomers) was calculated by counting five random fields under 10X magnification. In general, fields that were too close to the edge of the filter were not counted. The number of cells counted for the CLA treated samples were divided by the number cells counted for the ethanol-treated sample to get percentage migration, i.e.

(Number of cells for CLA-treated cells/ Number of cells for ethanol-treated cells)*100

2.2.12 *In vitro* invasion assays

For these experiments, 10^6 cells were seeded into 25cm^2 flasks, and allowed to adhere to the substratum overnight. They were then treated with 15μg/ml of the CLA mixture or purified isomers, or 1% (v/v) EtOH, for 24hr.

BD Biocoat™ Matrigel™ Invasion Chambers provided a system that allowed assessment of cells invasive activity *in vitro*. The cell culture inserts contained an 8μm pore size membrane that was coated with a layer of Matrigel™ basement membrane matrix. The layer of Matrigel™ serves as a reconstituted basement membrane *in vitro*. This layer occludes the pores of the membrane blocking non-invasive cells from migrating through the membrane. In contrast, invasive cells were able to migrate through the ECM-coated membranes.

The 24-well plate invasion chambers were removed from -20°C storage and allowed to come to room temperature. Warm (37°C) DMEM/S_0 was added to the interior of the inserts and allowed to rehydrate the matrix components for 2hr at RT. After rehydration, excess media was removed from the inserts and replaced with 500μl of cell suspension prepared in DMEM/S_0 at a concentration of 10^5 cells/ml. 750μl of chemoattractant (DMEM/ S_{20}) was added to the outside wells of the plate. The plates were then incubated for 24hr at 37°C in 5% CO_2 incubator, after which the membranes were fixed, stained, visualized and counted as described in Section 2.2.11.

CHAPTER 3

3.1 Introduction

3.1.1 Cytotoxicity assay

The use of *in vitro* assay systems for the screening of potential anticancer agents has been common practice almost since the beginning of cancer chemotherapy in 1946, following the discovery of the anti-neoplastic activity of nitrogen mustard (Masters, 2000). The choice of method depends on the context in which the assay is to be used, the type of cells, and the nature of the drug/compound. One important parameter, which may vary between different assays and which may strongly influence the choice of method is the end point used to quantify the drugs' effect. Endpoints often looked at are cytotoxicity/viability and survival (reproductive integrity).

Cytotoxicity assays measure drug-induced alterations in metabolic pathways or structural integrity, which may or may not be related directly to cell death. Cytotoxicity assays, which measure metabolic events, are more accurately quantified and are very sensitive (Masters, 2000).

There are several commercially available cytotoxicity assays. For this project, the one chosen was the CellTiter 96® Aqueous Non-Radioactive Cell Proliferation assay, from Promega Corporation. The MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay, which involves the measurement of the ability of treated cells to metabolise a tetrazolium salt into a coloured product is of this type.

MTS is bioreduced by cells into a formazan product (figure 3.1) that is soluble in tissue culture medium. The absorbance of the formazan at 490nm can be measured directly from 96-well assay plates. The conversion of MTS into the aqueous soluble formazan is carried out by mitochondrial dehydrogenase enzymes, found in metabolically active cells. The quantity of formazan product is directly proportional to the number of living cells in culture (Promega technical bulletin no. 169).

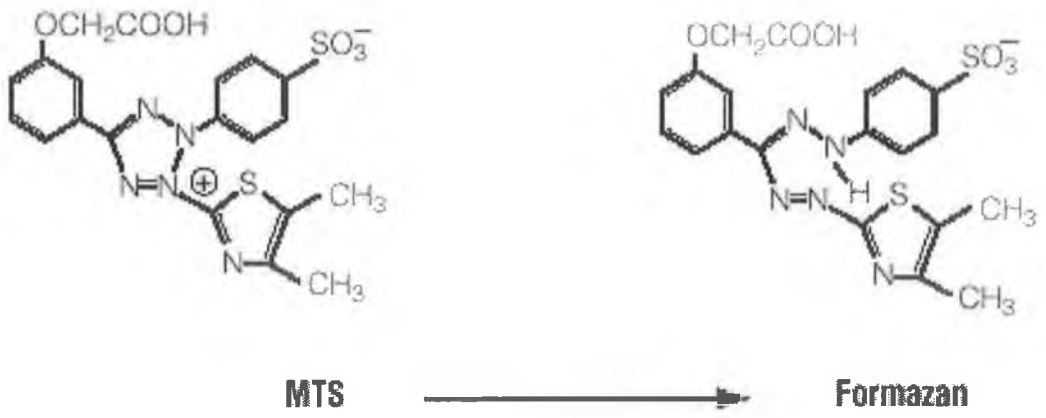


Figure 3.1. Structure of the MTS tetrazolium salt and its formazan product

3.1.2 The cell cycle

For cell division to occur, DNA and all other components of the cell must be replicated and distributed equally to the daughter cells. This occurs during what is known as the cell cycle and it is made up of four main phases, Gap 1 (G_1), Synthesis (S), Gap 2 (G_2) and Mitosis (M) (figure 3.2).

The first phase, G_1, is often divided to include the G_0 phase, in order to distinguish quiescent cells from those continuing in the cell cycle. Cells in G_0/G_1 have a diploid DNA content. Cells in G_0 include cells that have gone into quiescence and senescence. Quiescence is a transient growth arrested state, and it can be reversed, while senescent cells fail to divide even in response to mitotic stimuli, and thus cannot be reversed (Masters, 2000). G_1 is the period in which future commitment to division, differentiation or death is made and the focal point for important regulatory signals (King, 2000).

The second gap phase, G_2 has a tetraploid DNA content, as it lies between the S phase and mitosis (King, 2000). Knowledge of the DNA content of the cells is of importance when cell cycle analysis is carried out and the histogram plot is being interpreted (figure 3.3). Synthesis of DNA and other macromolecules occurs in the S phase.

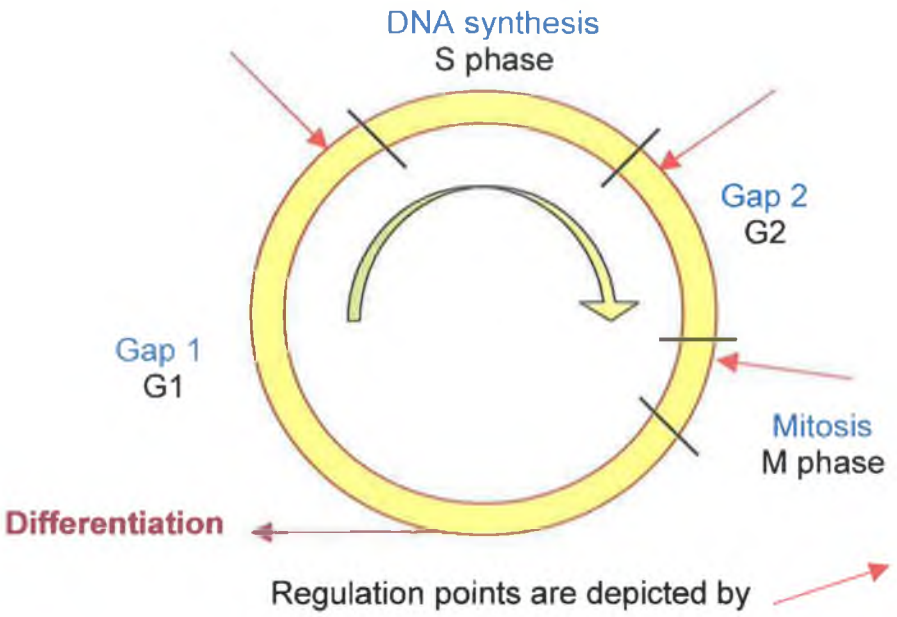


Figure 3.2. The cell cycle and its regulation. In rapidly dividing cells, the different phases have the following durations: M lasts one hour; G_1 lasts 8-30hr; S lasts 8hr; G_2 lasts 3hr, and the whole cycle lasts 20-50hr (King, 2000).

There are three checkpoints within the cycle that allows for careful regulation of events. These are found in G_1, G_2 and M. The checkpoint found at the end of G_1 is of interest to this research. Anti-cancer drugs, such as cisplatin (Lee *et al*, 1999), which may be targeted towards this checkpoint, result in a reduction of the number of cells in the S phase, and an increase in the number of cells in the G_0/G_1 phase. This is referred to as arrest at G_1/S interface.

The number of cells in each phase can be deduced by passing a population of labelled cells through the flow cytometer and measuring the amount of DNA in each cell. Cells in the G_2/M phase will have twice as much DNA as cells in the G_0/G_1 phase (figure 3.3). Results from the cell cycle analysis are presented as a histogram plot whereby the y-axis represents the number of cells, and the x-axis represents the amount of DNA in each cell.

Several dyes may be used to label the DNA, such as Hoechst 33342, and, more commonly, propidium iodide (PI), which intercalates in the DNA helix and fluoresces strongly orange-red. PI was chosen for this research, and it has the advantage that it is excited by 488nm light and can be used on most common flow cytometers. However, it

does require cells to be fixed or permeabilised and therefore non-viable (http://www.icnet.uk/axp/facs/davies/cycle.html#1Col).

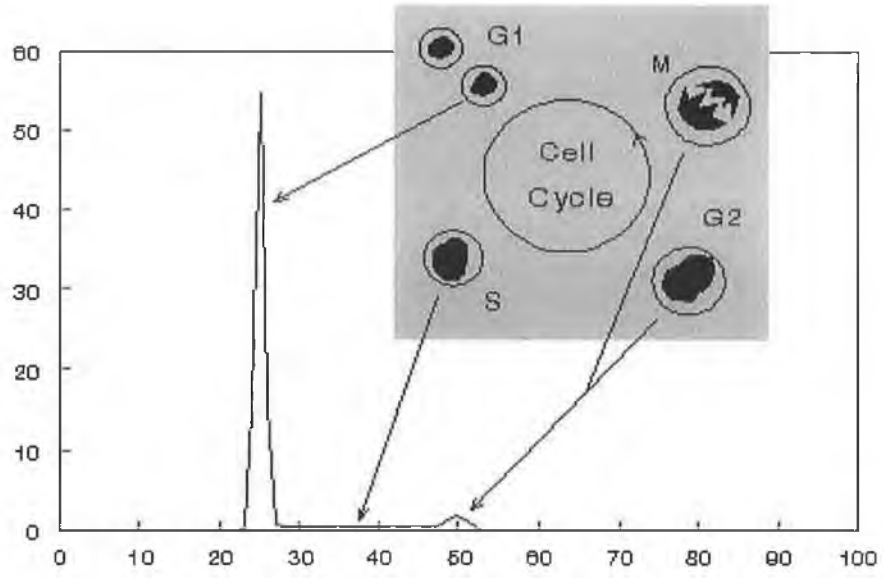


Figure 3.3. Histogram plot depicting numbers of cells on the y-axis versus the amount of DNA per cell (relative units) on the x-axis. It can be seen from the plot that the cells in G_2/M have twice as much DNA per cell than the cells in G_1.

After cells were analysed and histograms were plotted, selection of the peaks was required to ascertain what percentage of cells, of a given population, were in the chosen peak. It was clear which peaks represented which stage of the cell cycle, as there is twice as much DNA in the G_2/M stage as in the G_0/G_1 stage.

3.1.3 Apoptosis

Programmed cell death or apoptosis is initiated by an individual cell in response to a specific stimulus or as a result of an inappropriate set of signals received from the external environment (Masters, 2000). There are several mechanisms resulting in apoptosis but they all share similar phenotypic markers and behaviours, one of which is the removal of unwanted and damaged cells from a population of cells, whether normal or carcinogenic. A regulated balance between apoptosis and cell proliferation exists within a tumour, and one that is increasing in size exhibits increased proliferation and reduced apoptosis. It is the objective of cancer treatments, be they chemical or physical, to regulate proliferation of the cancer cells and increase cell death (King, 2000).

3.1.3.1 Features of apoptosis

Apoptosis may be analysed in a cell population by looking at either molecular or cellular features. Molecular features include activation of caspases and release of cell death signals, such as Bcl2, BAX and Bad. Cellular features include chromatin condensation, changes in cell morphology and DNA laddering. The sequential activation of the caspases results in the major biochemical features of apoptosis, and these are depicted in figure 3.4.

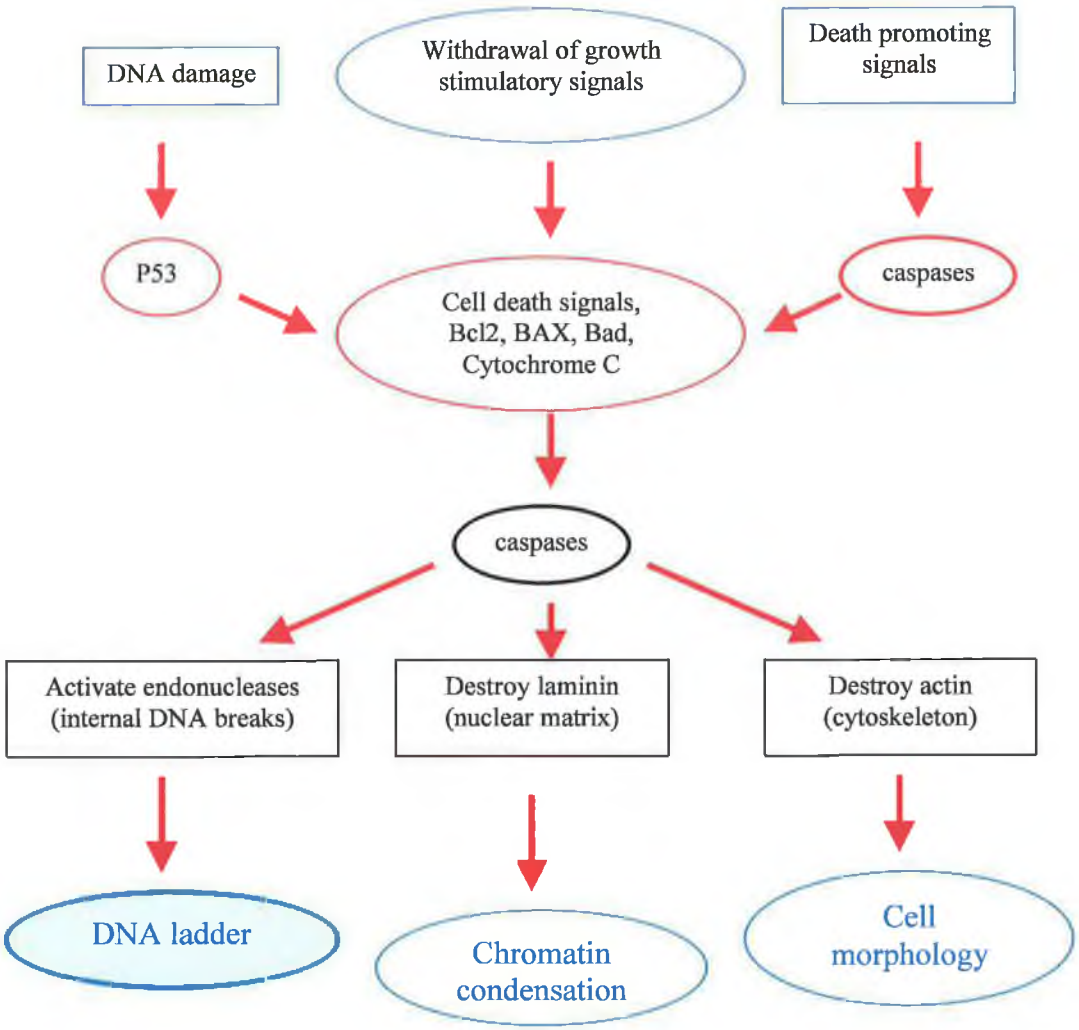


Figure 3.4. Pathways promoting apoptosis (King, 2000). DNA laddering is one of the main cellular features of apoptosis.

During apoptosis, the cytoplasm begins to shrink following the cleavage of laminin and actin filaments by proteases. Nuclear condensation can be observed following the breakdown of chromatin, and often the nuclei of apoptotic cells take on a horseshoe-like appearance. Cells continue to shrink into a form that allows easy clearance by macrophages. These phagocytic cells are responsible for removing apoptotic cells from tissues. Apoptotic cells promote their phagocytosis by undergoing plasma membrane changes, which trigger the macrophage response. An example of this is the transfer of phosphatidylserine from inside the cell to the outer surface. Towards the end of the apoptotic process membrane blebs can be observed. Small vesicles called apoptotic bodies may also be seen.
(http://www.sghms.ac.uk/depts/immunology/%7Edash/apoptosis).

3.1.3.2 DNA laddering

A major biochemical feature of apoptosis is the sequential activation of the caspases, a family of proteases whose substrates include large protein precursors of degradation enzymes. Endonucleases are an example of one such enzyme and they have the ability to degrade DNA. This digestion at internucleosome bridges generates a ladder of DNA fragments, which are multiples of 200bp units. This size is characteristic of a nucleosome (King, 2000).

There are several steps leading towards DNA cleavage. The first is the inactivation of enzymes involved in DNA repair, such as poly ADP-ribose polymerase (PARP). Following this, there is inactivation of enzymes, which are involved in cell replication. For example, DNA topoisomerase II is essential for DNA repair and replication, and it is inactivated by caspases. The third step is the breakdown of structural nuclear proteins, such as laminins. The final step is the fragmentation of DNA, which can be detected through the isolation of this apoptotic DNA and separating it on an agarose gel (http://www.sghms.ac.uk/depts/immunology/%7Edash/apoptosis/). This is easily achieved using a DNA laddering kit such as the Suicide-Track™ DNA Ladder Isolation Kit (Oncogene Research Products).

3.2 Results

3.2.1 4T1 cell line

The 4T1 cell line, which was chosen for this research, was derived from a murine mammary tumour (Aslakson and Miller, 1992). When injected into BALB/c mice, 4T1 cells spontaneously produce highly metastatic tumours that can metastasize to the lung, liver, lymph nodes and brain while the primary tumour is still growing *in situ*. The tumour growth and metastatic spread of 4T1 cells in BALB/c mice very closely mimics that of human breast cancer (www.atcc.org, 2002), which was a major factor in choosing this cell line for this research. Figure 3.5 shows the typical morphology of the 4T1 cell line growing in a tissue culture flask.

Figure 3.5. Inverted phase contrast micrograph of 4T1 cells (10x).

3.2.2 Standardisation of the MTS assay

A range of cell numbers, varying from 0 – $2.5*10^4$ cells, was set up each time a cytotoxicity assay was set up. Depending on the incubation time, i.e. 1 – 4 days, the number of cells plated was either increased or decreased. This allowed the growth of the cells to be monitored, ensuring that they always remained in exponential phase of growth during the assay. Figure 3.7 depicts a typical standard curve of cells, after four days incubation. The absorbance of the cells at 492nm was read after they were treated according to the MTS assay protocol (section 2.2.3.2).

The linear increase in absorbance coincides with increasing cell numbers. The R^2 value was close to 1 thus indicating the linearity of the standard curve. By setting up a range of cell numbers each time the MTS assay was performed, not only was it possible to monitor the exponential growth of the cells, but also the ability to reproduce the assay from day to day was confirmed.

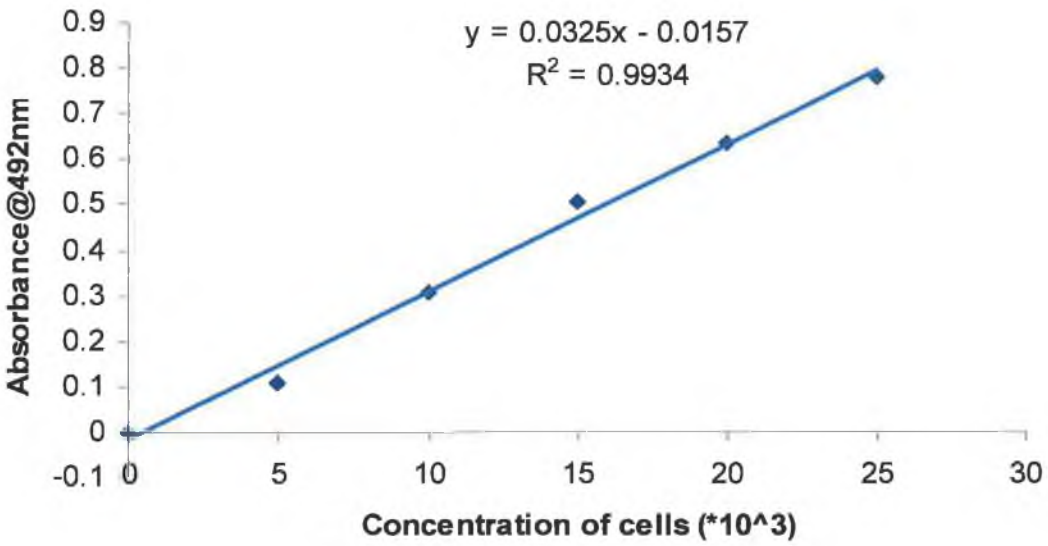


Figure 3.6. The absorbance @ 492nm of the formazan product on the y-axis versus cell number on the x-axis.

3.2.3 Cytotoxicity of ethanol

The CLA mixture and the purified isomers were prepared and diluted in 100% ethanol. Therefore, each time a cytotoxicity assay (section 2.2.3) was set up, the cells were also treated with 1% (v/v) ethanol for the same length of time, i.e. 24, 48, 72 or 96hrs. In this experiment by allowing untreated cells to represent 100% viability, percentage cell death due to 1% (v/v) ethanol could be calculated.

Table 3.1 shows the amount of cell death due to 1% ethanol, for a typical set of assays. As can be seen from table 3.1, percentage cell death due to 1% ethanol was minimal. Following 24hr incubation with ethanol, there was 0.72% cell death, this increased to 0.79% for 48hr, 1.23% for 72hr, and 1.12% for 96hr.

The percentage cell death was very low yet increased for the first three days, and on day four (96hr), the cell death due to 1% ethanol decreased somewhat from 1.23% to 1.12%. It appeared that after this length of time the 4T1 cell line was affected to a lesser extent by treatment with ethanol.

	Incubation time (hr)			
	24	48	72	96
Percentage cell Death (%)	0.72	0.79	1.23	1.12

Table 3.1. Effect of 1% ethanol on percentage cell death of the 4T1 cell line for four different time periods.

3.2.4 Cytotoxicity of CLA

In order to study the cytotoxicity of the CLA mixture and the two individual purified isomers, 9*c*,11*t*(18:2) and 10*t*,12*c*(18:2), several concentrations of the fatty acids and several treatment times were chosen. The 4T1 cell line was incubated for 1, 2, 3 and 4 days, while being treated with 0, 5, 10, 15, 20 and 25 μg/ml of CLA (section 2.2.3). Concentrations up to 20 μg/ml of CLA lie within the physiological range of the 9*c*,11*t*(18:2) isomer in human phospholipids (Cawood *et al*, 1983), plasma, bile, and duodenal juice (Iverson *et al*, 1985) and have been previously used in cell culture work (Shultz *et al*, 1992, Miller *et al*, 2001). The end point of the cytotoxicity assay was measured using the MTS assay.

The highest point on the standard curve of cells (2-4*10^3 cells per well, depending on the time period) was used for the cytotoxicity treatments. Triplicates were always performed, and cells treated with ethanol, to a final concentration of 1% (v/v), acted as the control. Cell death was calculated as a percentage of the ethanol, where percentage cell death due to ethanol was 0%. This calculation is shown below.

$$100-[\{(OD\ of\ sample_{average}-OD\ of\ blank_{average})/(OD\ of\ control_{average}-OD\ of\ blank_{average})\}*100]$$

where OD is the optical density.

The results for the cytotoxicity assays are shown in figures 3.7-3.10. Each graph shows percentage cell death of the 4T1 cell line versus CLA concentration (μg/ml). Ethanol was taken as 100% cell viability, and percentage cell death following treatments calculated accordingly. The assay was carried out in triplicate and on three separate days. Standard deviations were calculated using the average of the replicates. Error bars represent these values on the graph. T-tests were carried out to determine if the values were significant. P values <0.05 were deemed significant.

3.2.4.1 Cytotoxicity of CLA after 24hr incubation

Following 24hr incubation with the CLA mixture and its individual purified isomers, there was significant cell death due to 9*c*,11*t*(18:2) and 10*t*,12*c*(18:2) at concentrations of 20 and 25μg/ml. There was no significant cell death due to the CLA mixture at any concentration (figure 3.7).

At the lower concentrations of 5, 10 and 15μg/ml there was minimal cell death due to the CLA mixture and its isomers. There was no obvious difference in cytotoxicity between either of the individual purified isomers or the CLA mixture for this time point. There was a maximum cell death of 10% with 25μg/ml of the CLA mixture, although statistically this was not significant.

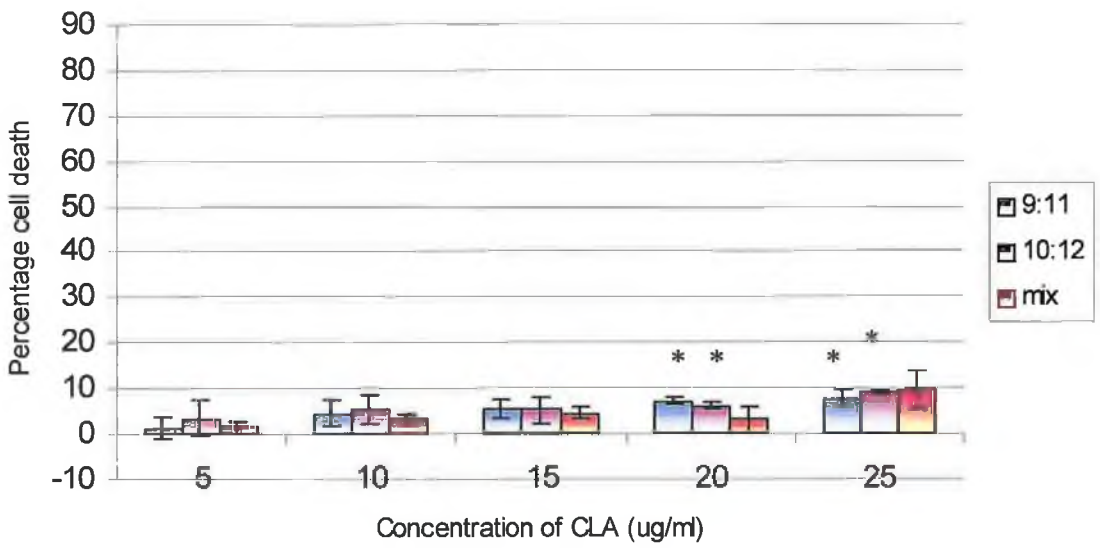


Figure 3.7. Percentage cell death (%) versus concentration of CLA and its purified individual isomers (μg/ml) after 24hr incubation. The asterisk (*) denotes that the p value was <0.05, and therefore significant.

3.2.4.2 Cytotoxicity of CLA after 48hr incubation

Following 48hr incubation with the CLA mixture and its individual purified isomers, there was an overall marked increase in percentage cell death, along with a clear indication of increased potency with the 10*t*,12*c*(18:2) isomer compared to the 9*c*,11*t*(18:2) isomer and the CLA mixture (figure 3.8).

The 10*t*,12*c*(18:2) isomer caused significant cell death at all concentrations, while the 9*c*,11*t*(18:2) isomer caused significant cell death at all concentrations except at 15μg/ml. The 10*t*,12*c*(18:2) isomer displayed increased cytotoxicity when compared to the 9*c*,11*t*(18:2) isomer and the CLA mixture. This was apparent when we examined cytotoxicity at the lower concentration. At 5μg/ml, the 9*c*,11*t*(18:2) isomer killed 6% of cells, while the 10*t*,12*c*(18:2) isomer killed 25%, and the CLA mixture killed only 2%.

The CLA mixture, which was made up of 29.5 and 29% of 9*c*,11*t*(18:2) and 10*t*,12*c*(18:2) respectively, caused significant cell death at all concentrations with the exceptions of 5 and 15 μg/ml.

For this incubation time, there was a maximum cell death of 46% with 25μg/ml of the 10*t*,12*c*(18:2) isomer.

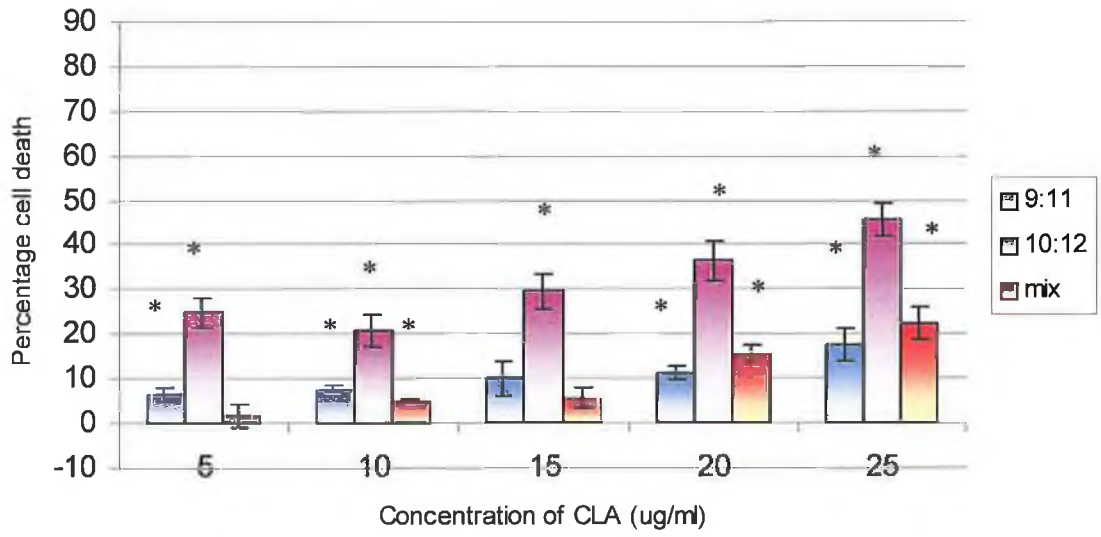


Figure 3.8. Percentage cell death (%) versus concentration of CLA and its purified individual isomers (μg/ml) after 48hr incubation. The asterisk (*) denotes that the p value was <0.05, and therefore significant.

3.2.4.3 Cytotoxicity of CLA after 72hr incubation

Following 72hr incubation with the CLA mixture and its individual purified isomers, there was a significant increase in cell death for all treatments except 5μg/ml of the 9*c*,11*t*(18:2) isomer. Also, at this time point, there was a clear difference in cytotoxicity between the individual isomers and the CLA mixture (figure 3.9).

Even though there was an overall increase in significance for all treatments from 48-72hr incubation, the overall increase in toxicity was not great, especially at the lower concentrations. The increase in cytotoxicity was more apparent at concentrations of 20 and 25μg/ml.

It was clear from the graph that the 10*t*,12*c*(18:2) isomer displayed increased significant cytotoxicity compared to the other treatments. The 9*c*,11*t*(18:2) isomer and the mixture displayed similar toxicity up until this treatment time of 72hr. A maximum toxicity of 61% was seen with 25μg/ml of the 10*t*,12*c*(18:2) isomer.

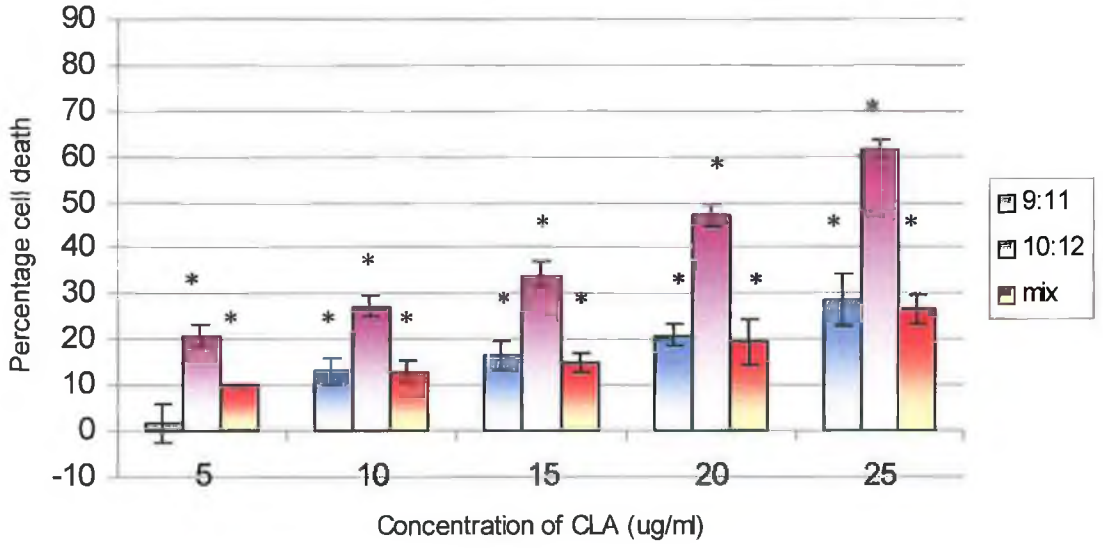


Figure 3.9. Percentage cell death (%) versus concentration of CLA and its purified individual isomers (μg/ml) after 72hr incubation. The asterisk (*) denotes that the p value was <0.05, and therefore significant.

3.2.4.4 Cytotoxicity of CLA after 96hr incubation

Following 96hr incubation with the CLA mixture and its individual purified isomers (figure 3.10), the potency of the 9*c*,11*t*(18:2) isomer approaches that of the 10*t*,12*c*(18:2) isomer for the lower concentrations of 5, 10, 15 and 20μg/ml, whereby the cell death due to the 9*c*,11*t*(18:2) isomer was 17, 36, 43 and 52%, and the cell death due to the 10*t*,12*c*(18:2) isomer was 19, 31, 33 and 58%. After this point, when the concentration was increased to 25μg/ml, the 10*t*,12*c*(18:2) isomer displayed increased cytotoxicity once again. A maximum toxicity of 80% was seen with 25μg/ml of the 10*t*,12*c*(18:2) isomer.

Interestingly, at 96hr an increase in toxicity for the 9*c*,11*t*(18:2) isomer compared to the CLA mixture was seen. For the other incubation times studied, i.e. 24, 48 and 72hr, the cytotoxicity of this isomer and the CLA mixture were similar. For example, at 10μg/ml, the cell death due to the 9*c*,11*t*(18:2) isomer was 36%, and for the CLA mixture it was 14%. This can be compared to the same concentration but for the incubation time of 72hr, whereby 10μg/ml resulted in 13% cell death for both the 9*c*,11*t*(18:2) isomer and the CLA mixture.

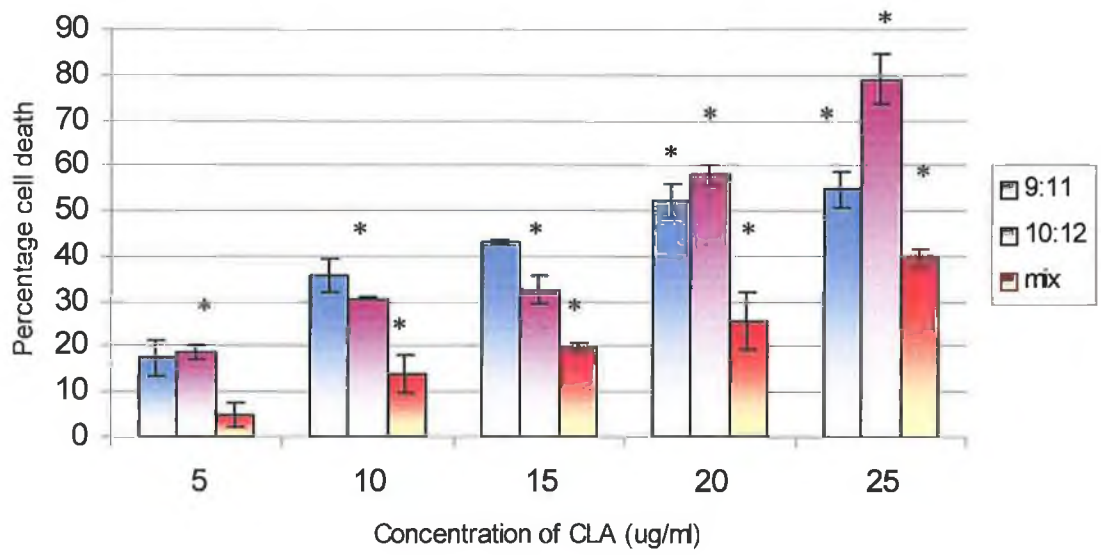


Figure 3.10. Percentage cell death (%) versus concentration of CLA and its purified individual isomers (μg/ml) after 96.hr incubation. The asterisk (*) denotes that the p value was <0.05, and therefore significant.

3.2.5 Treatment of cells for cell cycle and apoptosis analysis

In order to analyse the effect of CLA on the cell cycle of the 4T1 cell line by flow cytometry, a CLA concentration and incubation time that would kill ~50% of cells was needed. This was required so that treatment with CLA and its individual isomers would affect the cell cycle of the remaining viable cells to an extent that they could be analysed (personal communication with Claire Condron, Director of Flow Cytometry Unit, Beaumont Hospital, Dublin).

For the analysis of DNA laddering due to apoptosis, the same CLA concentration and incubation time was used, but this time both the viable (in monolayer) and detached cells were analysed. The 4T1 cell line was therefore treated for 24, 48, 72 and 96hr with 30μg/ml of the CLA mixture and its purified individual isomers (figure 3.11) (section 2.2.4) for both cell cycle and apoptosis analysis.

When the cell line was treated with 30μg/ml of the CLA mixture and its purified individual isomers, the incubation period of 24hr was too short to have a sufficient effect on cell death. The longer times of 72 and 96hr had too great an effect on cell death, for example, the 10*t*,12*c*(18:2) isomer killed 85% of cells at 72hr and 94% at 96hr.

After analysis of the percentage cell death for the different incubation periods, it was decided that 48hr incubation was sufficient to have an effect on the cells for cell cycle and apoptosis analysis. Longer times than this might be too cytotoxic to the cell population, especially as the CLA isomers and CLA mixture have different potencies.

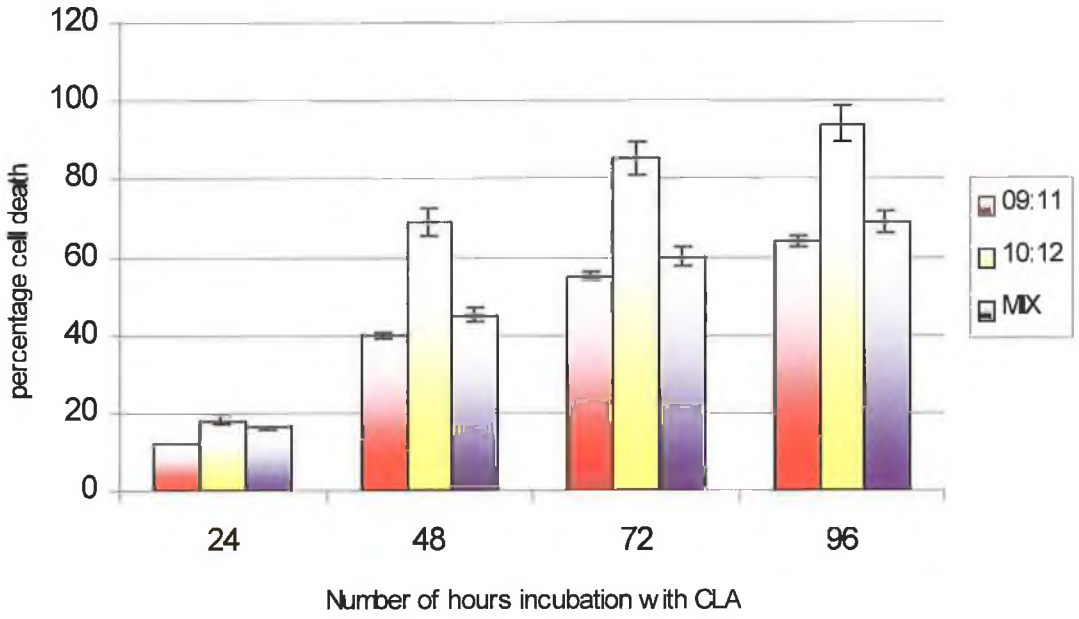


Figure 3.11. The percentage cell death due to 30μg/ml of the 9*c*,11*t*(18:2), 10*t*,12*c*(18:2), isomer, and the CLA mixture. The 4T1 cell line was treated for 24, 48, 72 and 96hr to determine which time point would be chosen for cell cycle and apoptosis analysis.

3.2.6 The effect of CLA on the cell cycle

The 4T1 cell line was treated for 48hr with 30μg/ml of the CLA mixture and its individual purified isomers. The cells were then analysed on a FACScan flow cytometer where the amount of DNA in any given cell population could be determined, thus allowing the stage of the cell cycle in which the cells are in to be determined (section 2.2.4 and 2.2.5). The FL_2 lamp histogram plots display peaks which are representative of the different stages in the cell cycle. The stages in the cell cycle being analysed were G_0/G_1, S and G_2/M. The peaks were selected and the percentages of cells in each peak were calculated. A representative profile of a single experiment is shown and this experiment was repeated three times. Results are shown in both histogram and tabular form.

Figure 3.12 shows the resultant histogram plot of cells, which had previously been treated with 1% ethanol for two days. The first peak represents 4T1 cells in the G_0/G_1 phase in the cell cycle. While, the second peak represents the G_2/M phase. It is possible to recognise what each peak represents because cells in the G_0/G_1 phase have half the amount of DNA than cells in the G_2/M phase. The section of the histogram between the peaks represents the S phase. Table 3.2 shows the percentage of cells in each section of the histogram. The results indicate that 54.01% of cells were in G_0/G_1, 9.35% were in S and 17.83% were in G_2/M.

Figure 3.13 and Table 3.3 show the percentage of cells in each stage of the cell cycle after the 4T1 cell line was treated with 30μg/ml of the purified isomer, 9*c*,11*t*(18:2) for two days. Here we can see a change in the distribution of the cells within the cell cycle. 56.65% of the cells are in the G_0/G_1 phase, compared to 54.01% for the ethanol treated samples. There was also a reduction in the number of cells in the synthesis phase, from 9.35% to 6.82%. This indicates that treatment with this isomer caused a block in synthesis to occur, resulting in a decrease of cells in S and an increase of cells in G_0/G_1.

Figure 3.14 and Table 3.4 show the percentage of cells in each stage of the cell cycle after the 4T1 cell line was treated with 30μg/ml of the purified isomer, 10*t*,12*c*(18:2) for two days. Once again, we see a change in the distribution of the cells within the cell cycle, and this time, to an even greater extent. The number of cells in G_0/G_1 increased from 54.01% for the ethanol-treated samples to 61.78% for the 10*t*,12*c*(18:2)-treated samples. Coinciding with this, the percentage of cells in the synthesis stage reduced even further than it did for the 9*c*,11*t*(18:2) isomer, from 9.35% for the ethanol-treated samples to 5.98% for the 10*t*,12*c*(18:2)-treated samples.

Finally, figure 3.15 and Table 3.5 show the percentage of cells in each stage of the cell cycle following treatment of the 4T1 cell line with 30μg/ml of the CLA mixture for two days. There was a clear difference in the percentage of cells in each stage of the cell cycle, although the difference was similar to that of the 9*c*,11*t*(18:2) isomer rather than the greater difference of the 10*t*,12*c*(18:2) isomer. The percentage of cells in G_0/G_1 increased from 54.01% for the ethanol-treated samples to 59.76% for the CLA mixture-treated samples, while the percentage of cells in S reduced from 9.35% to 6.33%.

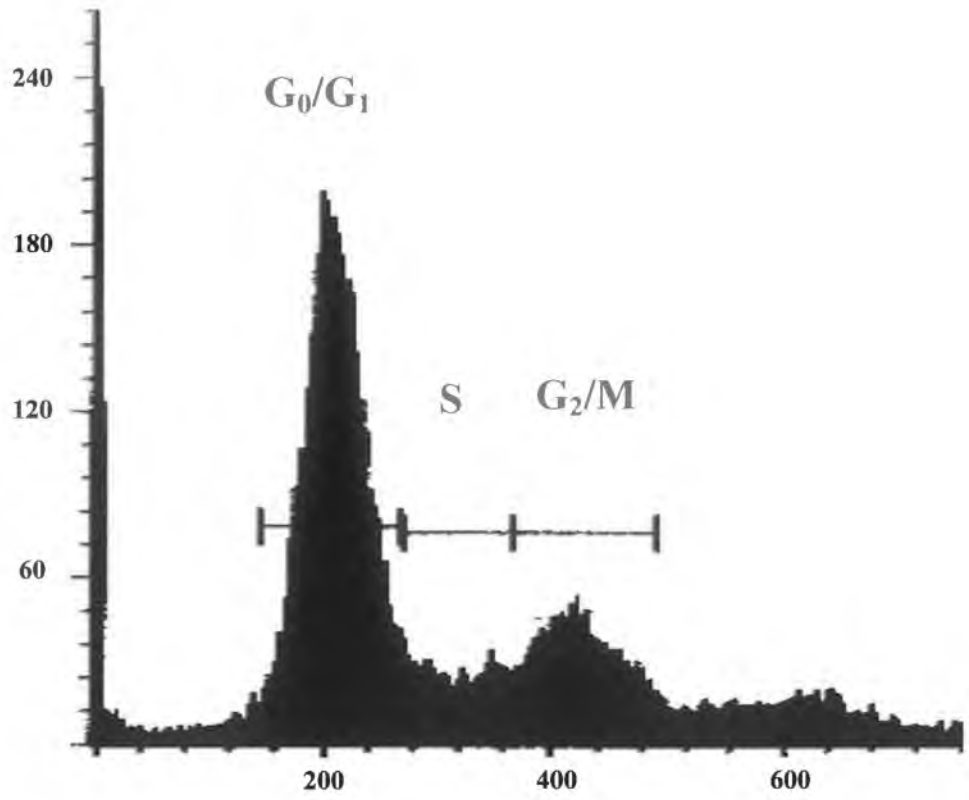


Figure 3.12. The FL_2 lamp histogram plot shows the number of cells, or events, on the y-axis versus the DNA content (arbitrary units) of each cell on the x-axis. Three stages in the cell cycle are depicted, G_0/G_1, S and G_2/M by areas of the plot. This cell population has previously been treated with 1% ethanol for 2 days. A representative profile of a single experiment is shown, and this experiment was repeated three times.

Stage in cell cycle	% of total cell population
G_0/G_1	54.01
S	09.35
G_2/M	17.83

Table 3.2. The percentage of cells in each stage of the cell cycle was calculated from the histogram plot, figure 3.12. This cell population has previously been treated with 1% ethanol for 2 days. A representative profile of a single experiment is shown, and this experiment was repeated three times.

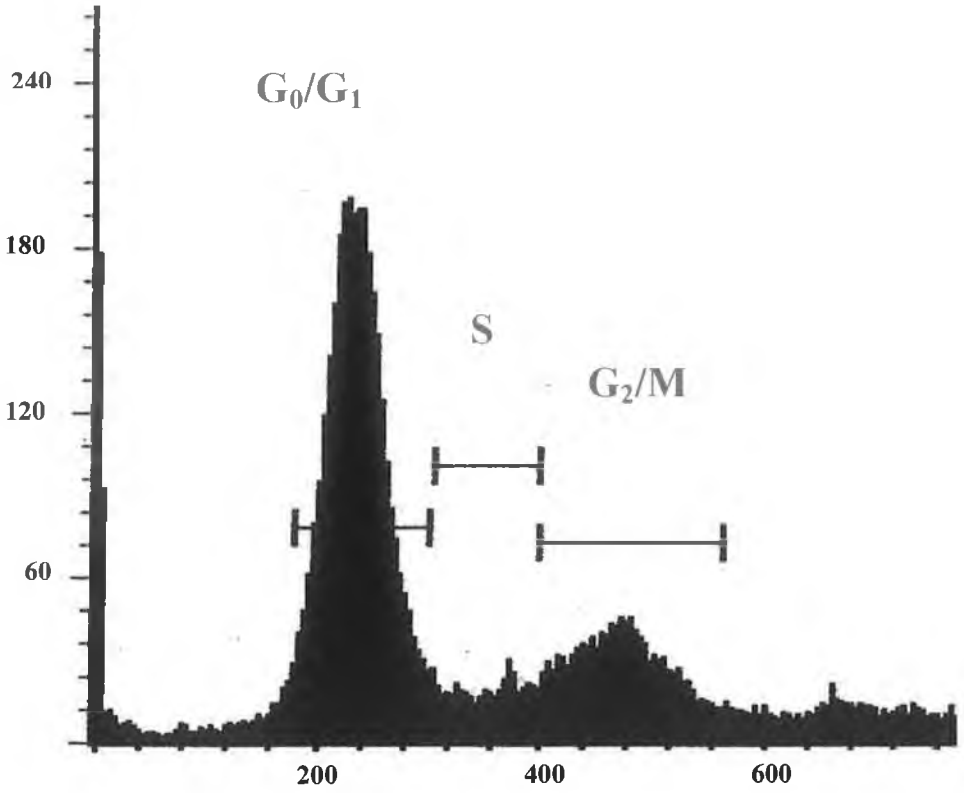


Figure 3.13. The FL_2 lamp histogram plot shows the number of cells, or events, on the y-axis versus the DNA content (arbitrary units) of each cell on the x-axis. Three stages in the cell cycle are depicted, G_0/G_1, S and G_2/M by areas of the plot. This cell population has previously been treated with 30μg/ml of the purified isomer, 9*c*,11*t*(18:2) for 2 days. A representative profile of a single experiment is shown, and this experiment was repeated three times.

Stage in cell cycle	% of total cell population
G_0/G_1	56.65
S	06.82
G_2/M	19.65

Table 3.3. The percentage of cells in each stage of the cell cycle was calculated from the histogram plot, figure 3.13. This cell population has previously been treated with 30μg/ml of the purified isomer, 9*c*,11*t*(18:2) for 2 days. A representative profile of a single experiment is shown, and this experiment was repeated three times.

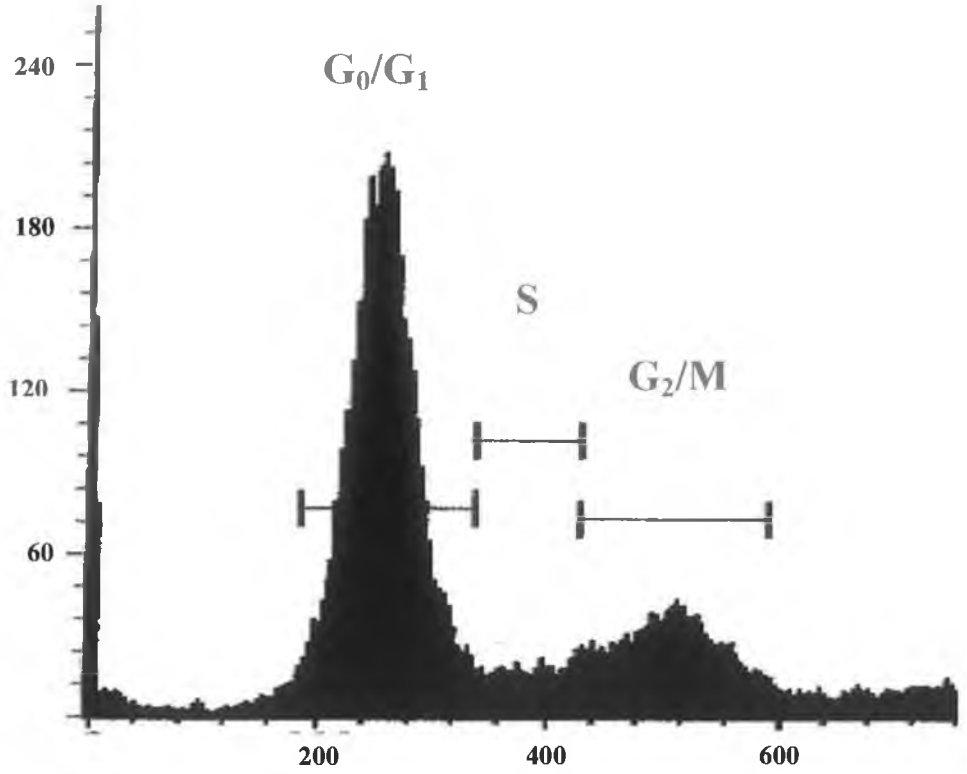


Figure 3.14. The FL_2 lamp histogram plot shows the number of cells, or events, on the y-axis versus the DNA content (arbitrary units) of each cell on the x-axis. Three stages in the cell cycle are depicted, G_0/G_1, S and G_2/M by areas of the plot. This cell population has previously been treated with 30μg/ml of the purified isomer, 10*t*,12*c*(18:2) for 2 days. A representative profile of a single experiment is shown, and this experiment was repeated three times.

Stage in cell cycle	% of total cell population
G_0/G_1	61.78
S	05.98
G_2/M	18.88

Table 3.4. The percentage of cells in each stage of the cell cycle was calculated from the histogram plot, figure 3.14. This cell population has previously been treated with 30μg/ml of the purified isomer, 10*t*,12*c*(18:2) for 2 days. A representative profile of a single experiment is shown, and this experiment was repeated three times.

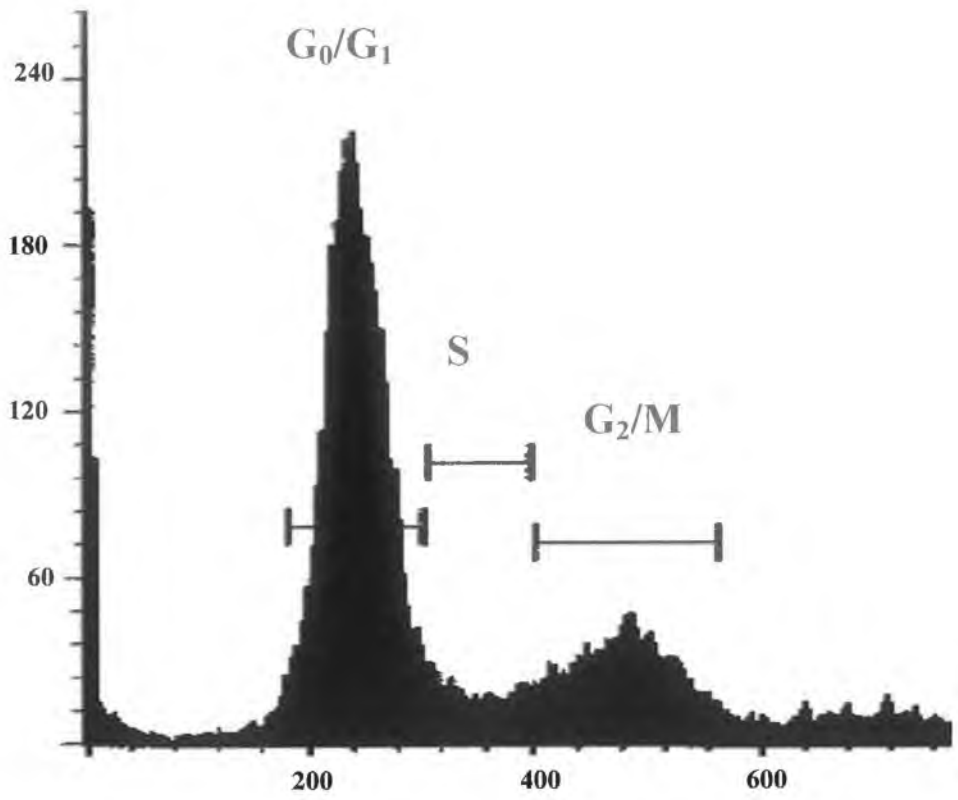


Figure 3.15. The FL_2 lamp histogram plot shows the number of cells, or events, on the y-axis versus the DNA content (arbitrary units) of each cell on the x-axis. Three stages in the cell cycle are depicted, G_0/G_1, S and G_2/M by areas of the plot. This cell population has previously been treated with 30μg/ml of the CLA mixture for 2 days. A representative profile of a single experiment is shown, and this experiment was repeated three times.

Stage in cell cycle	% of total cell population
G_0/G_1	59.76
S	06.33
G_2/M	19.30

Table 3.5. The percentage of cells in each stage of the cell cycle was calculated from the histogram plot, figure 3.15. This cell population has previously been treated with 30μg/ml of the CLA mixture for 2 days. A representative profile of a single experiment is shown, and this experiment was repeated three times.

3.2.7 Examination of 4T1 cell line for apoptosis

The 4T1 cell line was treated for 2 days with either 1% (v/v) ethanol or 30μg/ml of the CLA mixture and the purified individual isomers. Following this, both the cells attached to the flask in the monolayer and the detached cells floating in medium were analysed for DNA laddering using the Suicide-Track™ DNA Ladder Isolation Kit (Oncogene Research Products). Eight samples were examined in total, and ran on an agarose gel where they could then be stained and photographed. The details of the procedure can be found in section 2.2.6.

Examination of the eight samples for apoptosis (figure 3.16) revealed that, as expected, there was no DNA laddering to be found in the monolayer cells (lane 2, 3, 4 and 5), regardless of the treatment. As these cells were still attached to the tissue culture flask, they were most likely still viable and, therefore, were not expected to contain any apoptotic DNA. The kit was designed to isolate just low molecular weight apoptotic DNA, and not high molecular weight chromosomal DNA.

A positive control, which contained apoptotic cells (HL60 cells previously treated with Actinomycin D), was provided with the kit. This was treated in the same way as the samples were. When DNA isolated from the control was ran on the gel (lane 12), there appeared to be a smear in the lane and not discrete bands as expected.

The apoptotic DNA isolated from the detached cells displayed varying degrees of staining, although, similar to the positive control discrete bands were not present. Interestingly, both isomers 9*c*,11*t*(18:2) [lane 8] and 10*t*,12*c*(18:2) [lane 9] resulted in increased isolated DNA compared to the CLA mixture [lane 10].

Although the Suicide-Track™ DNA Ladder Isolation Kit should only isolate low molecular weight apoptotic DNA, it was expected that a laddering effect would be seen with discrete bands representing multiples of 200bp units. This was not seen for either the positive control provided with the kit or the CLA-treated samples. Through examination of the gel in figure 3.16, it was decided that the results for the analysis of apoptosis were inconclusive. Therefore, it was not possible to determine conclusively if apoptosis had taken place.

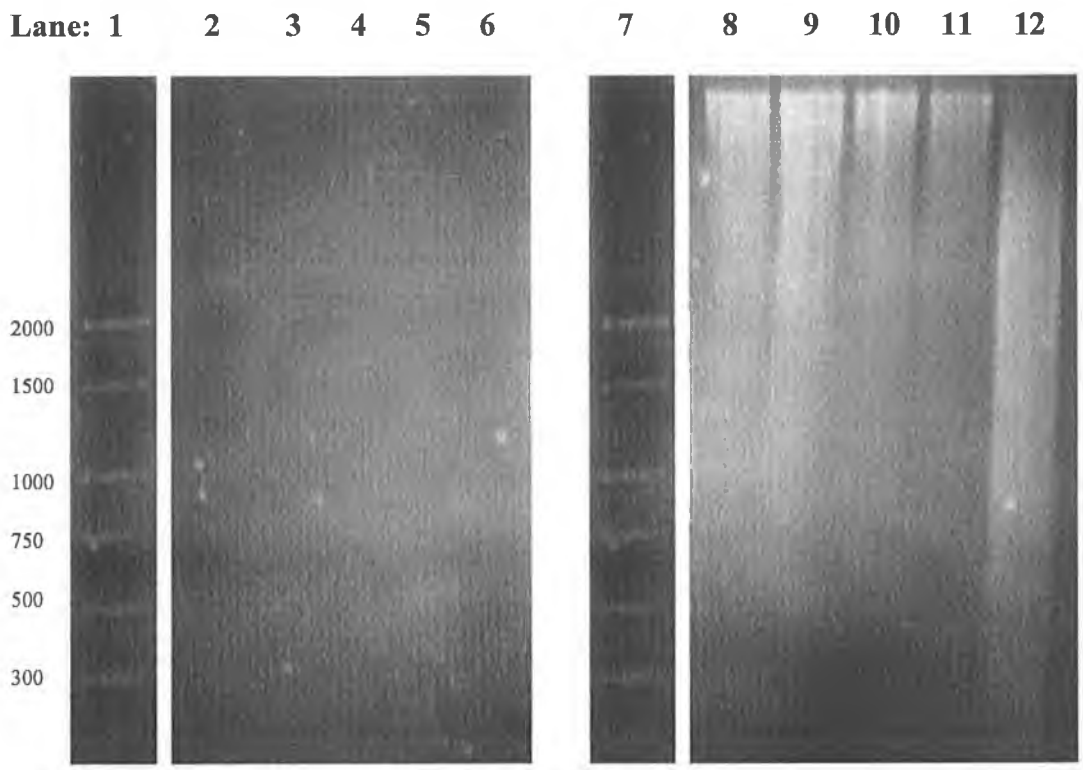


Figure 3.16. Samples were run on a 1.5% agarose gels in TAE, stained with ethidium bromide, and photographed.

- Cells in monolayer (viable) following treatment with: 9c,11t (18:2) [lane 2]; 10t,12c (18:2) [lane 3]; CLA mixture [lane 4]; and ethanol [lane 5].
- Detached cells (non-viable) following treatment with: 9c,11t (18:2) [lane 8]; 10t,12c (18:2) [lane 9]; CLA mixture [lane 10]; and ethanol [lane 11].

Lane 1 and lane 7 contain molecular weight markers, lane 6 contains H_2O and lane 12 contains the positive control provided with the Suicide-Track™ DNA Ladder Isolation Kit.

3.3 Discussion

The anti-carcinogenic properties of CLA have been firmly established by several investigators through animal experiments, which mostly involve rat and mouse models. Many of these have previously been discussed in detail in section 1.4.2.1 (Ip *et al*, 1997, Cesano *et al* 1998, Ip *et al*, 1999a, Hilakivi-Clarke *et al*, 1999, Banni *et al*, 1999, Hubbard *et al*, 2000, Futakuchi *et al*, 2002, Masso-Welch *et al*, 2002). The anti-proliferative properties of CLA in several cell lines, of numerous origins, have also been extensively studied and some of these are discussed in section 1.4.2.3 (Durgam and Fernandes, 1997, Park *et al*, 2000, Igarashi and Miyazawa, 2001, Urquhart *et al*, 2002 and Kim *et al*, 2002).

Breast cancer deaths comprised 2% of all deaths and 8.5% of all cancer deaths in Ireland in 1997. Since all these cases in this study occurred in women, this number constitutes 4.2% of all deaths in women and 18.1% of all cancer deaths in women (Codd *et al*, 1999). The 4T1 cell line has been described as very closely mimicking that of human breast cancer (www.atcc.org, 2002), which was a major factor in choosing it for this research.

A review of the literature reveals that to date, there have been no studies examining the effect of CLA on the 4T1 cell line. In addition, there have only been a few studies published looking at the cytotoxicity of the individual purified CLA isomers 9*c*,11*t*(18:2) and 10*t*,12*c*(18:2). The exact mechanism of action of CLA is still unknown although there are several proposed theories (section 1.4.2.2), which will be dealt with, further in this section. The present study was designed to examine the effect of a CLA mixture and two of its predominant individual purified isomers on the proliferation and metastasis of the 4T1 murine mammary cancer cell line.

One of the earliest anti-cancer studies involving CLA was published by Ha *et al* (1987). In this study, the authors applied a synthetic mixture of CLA to the dorsal area of mouse skin prior to initiation of cancer using the carcinogen, DMBA, and its promotion with 12-O-tetradecanoylphorbal-13- acetate (TPA). Sixteen weeks after treatment, CLA-treated mice consistently exhibited fewer papillomas and a lower tumour incidence than control or LA-treated cells.

In the present study, the anti-proliferative effect of a CLA mixture and its individual purified isomers on the 4T1 cell line was established. The cell line was treated with varying concentrations of a commercially available CLA mixture [29.5% 9*c*,11*t*(18:2) and 29% 10*t*,12*c*(18:2) CLA] and two individual purified isomers,

9*c*,11*t*(18:2) [99.9% pure] and 10*t*,12*c*(18:2) [99.9% pure] for varying times. Cytotoxicity of CLA was studied using an MTS cytotoxicity assay. It can be seen from the results (figures 3.7-3.10) that there was a clear difference in the potency of the different individual isomers and the CLA mixture. For example, after 48hr incubation with 5μg/ml, the 10*t*,12*c*(18:2) isomer displayed greater toxicity (25%) than the 9*c*,11*t*(18:2) isomer (6%) and the CLA mixture (2%).

The effect of CLA on the mammary cell line, MCF-7, has been extensively studied. O'Shea *et al* (1999) observed a dose dependent decrease in cell numbers and increase in lipid peroxidation, as determined by TBARS in MCF-7 and SW480 (colon, in origin) cell lines following incubation with a CLA mixture. Maximum growth suppression occurred in both cell lines following supplementation with 15-30ppm (μg/ml) CLA for 8-10 days. O'Shea *et al* (1999) concluded that the CLA-induced anti-oxidant enzymes (super oxide dismutase [SOD] and glutathione peroxidase [GPx]) failed to protect these cells from cytotoxic lipid peroxidation products.

A study carried out by Park *et al* (2000) on the same breast cancer cell line, MCF-7, found that LA stimulated the growth of the cells while CLA resulted in an inhibition. They concluded that growth inhibition by CLA was not mediated through phospholipase-C (PLC)-, protein kinase-C (PKC)- or Prostaglandin E_2 (PGE_2)-dependent signal transduction pathways.

The antiproliferative effects of two commercial preparations of CLA and their constituent purified isomers, 9*c*,11*t*(18:2), 9*c*,11*c*(18:2) and 10*t*,12*c*(18:2) were determined *in vitro* by Palombo *et al* (2002). Human colorectal (HT-29, MIP-101) and prostate (PC-3) carcinoma cell lines were studied. They found that the 10*t*,12*c*(18:2) isomer exhibited the greatest potency against colorectal cancer proliferation, and the 9*c*,11*c*-18:2 and 10*t*,12*c*-18:2 isomers were moderately effective against the prostate cancer. The 10*t*,12*c*-18:2 isomer also resulted in caspase-dependent apoptosis occurring in the MIP-101 and PC-3 cell lines.

Research carried out by Kim *et al* (2002) also compared the potencies of individual CLA isomers, 10*t*,12*c*(18:2) and 9*c*,11*t*(18:2). The colon cancer cell line, Caco-2, was treated with the isomers and the researchers found that the 10*t*,12*c*(18:2) isomer dose dependently decreased viable cell number (55% reduction at 96hr following the addition of 5μM of the isomer). This same isomer induced apoptosis and decreased DNA synthesis. In, this paper, the 9*c*,11*t*(18:2) isomer was reported as having no significant effect.

Contrary to the research carried out by Kim *et al* (2002), a study carried out by Liu *et al* (2002) on a gastric adenocarcinoma cell line, SGC-7901, concluded that the 9*c*,11*t*(18:2) isomer inhibited cell growth and proliferation (after 8 days, 6% inhibition was found with 25μM [7μg/ml], and 82% with 200μM [56μg/ml]) through blockage of the cell cycle. It must be said, though, that Liu *et al* (2002) only reported results for the 9*c*,11*t*(18:2) isomer.

Following the cytotoxicity assays, it was decided to look at the effect of CLA and its individual isomers on the cell cycle of the 4T1 cell line. Using the cytotoxicity data to establish optimum treatment conditions, a suitable CLA concentration and incubation period was chosen. After analysis of the flow cytometry FL_2 lamp histogram plots for the ethanol and CLA-treated samples (figures 3.12-3.15 and tables 3.2-3.5), it was apparent that treatment of the 4T1 cell line with the CLA mixture, the 9*c*,11*t*(18:2) and the 10*t*,12*c*(18:2) isomer caused both a reduction in the number of cells in the S phase along with an increase in the number of cells in the G_0/G_1 stage. Using figures 3.2 and 3.3 it was possible to conclude that the cytotoxic effect exerted by CLA and its isomers was due to a disruption in the G_1 checkpoint. Anticancer drugs, which may be targeted towards this checkpoint result in a gradual build-up of cells in the G_0/G_1 stage and a reduction of cells in the S stage.

The 10*t*,12*c*(18:2) CLA isomer caused the greatest reduction of cells in S, along with an increase, or arrest, of cells in G_0/G_1. This isomer also caused increased cytotoxicity to this cell line, in comparison to the 9*c*,11*t*(18:2) isomer and the CLA mixture, especially for shorter incubation times. In conclusion, flow cytometric analysis revealed deregulation of the cell cycle correlating with the observed cytotoxic effects of CLA, especially with the 10*t*,12*c*(18:2) CLA isomer.

In a study carried out by Durgam and Fernandes (1997), CLA was found to selectively inhibit proliferation of ER-positive MCF-7 cells compared with ER-negative MDA-MB-231 cells. Cell cycle analysis indicated that a higher percentage of CLA-treated MCF-7 cells remained in the G_0/G_1 phase as compared to the control and those treated with LA, and thus did not progress to the S or G_2/M phase of the cell cycle. These results demonstrated that CLA might inhibit MCF-7 cell growth by interfering with the hormone regulated mitogenic pathway. Other researchers (Liu *et al*, 2002, Kim *et al*, 2002) have also reported a disruption of the cell cycle following treatment with CLA, and/or its individual isomers.

The overall effect on cancer cell proliferation appears to be dependent upon the concentration and type of CLA isomer used, and the type of cancer cells targeted. Palombo *et al* (2002) found that the 10*t*,12*c*(18:2) CLA isomer exhibited the greatest potency against colorectal cancer cell (HT-29) proliferation, while the 10*t*,12*c*(18:2) and 9*c*,11*t*(18:2) isomers were both moderately effective against prostate cancer, PC-3. In this study, the 10*t*,12*c*(18:2) isomer also induced caspase-dependent apoptosis in the colorectal cell line, MIP-101, and in the PC-3 cells.

The underlying mechanisms by which CLA down regulates tumourigenesis and cancer proliferation are not well understood, and because CLA has pleiotropic metabolic effects, individual CLA isomers may compete as ligands for multiple pathways of signal transduction (Liu *et al*, 1997, Banni *et al*, 1999). Belury et al (2002) found that CLA might serve as a ligand for the nuclear hormone receptor, peroxisome proliferator-activated receptors (PPAR). PPAR activation initiates transcription of multiple genes that modulate lipid and carbohydrate metabolism (Palombo *et al*, 2002). Activation of PPARγ in certain cell lines may induce cell differentiation and growth arrest (Kitamura *et al*, 1999).

Also, CLA may down regulate cancer growth by interfering with the metabolism of AA (Liu and Belury, 1998, Banni *et al*, 1999, Urquhart *et al*, 2002). Competition between LA (precursor to AA) and CLA for the same desaturase system would decrease production of AA and alter eicosanoid biosynthesis. Section 1.3.2 and figure 1.4 expand on this metabolic pathway. Other proposed theories to CLA's anti-cancer properties are: CLA-induced lipid peroxidation (O'Shea *et al*, 1999); change in fatty acid metabolism (Igarashi & Miyazawa, 2001); and CLA's relationship to hormone response elements (Durgam and Fernandes, 1997).

Up to this point, it had been established that CLA had significant anticancer effects on the 4T1 cell line through its cytotoxic effects and its disruption of the cell cycle. Following this, apoptotic DNA was isolated from CLA- and ethanol-treated cells, and was separated on an agarose gel (figure 3.16) to see if cell death caused by CLA was occurring by means of an apoptotic pathway. It has been reported by several researchers that either a CLA mixture or individual CLA isomers induced apoptosis in tumour cells (Ip *et al*, 1999b, Ip *et al*, 2000, Kim *et al*, 2002).

Through examination of the gel in figure 3.16, it was possible to observe smears in several lanes, although discrete bands, representing multiples of 200bp units, were not

present. It was decided that these results were inconclusive, and therefore, it was not possible to say that apoptosis had taken place.

Programmed cell death, or apoptosis, can be examined by several methods, such as the TUNEL assay. Flow cytometry may also be used to examine a population of labelled cells for apoptosis. When apoptosis has taken place within a population of cells, the apoptotic cells will appear as a sub-G_0/G_1 peak on an FL_2 lamp histogram plot. Figure 3.17 shows a histogram plot of cells that were previously treated with the apoptosis-inducing drug, SN-38 by Ueno *et al* (2002). There is an apparent sub-G_0/G_1 peak that the researchers report as indicating that apoptosis has taken place. The researchers also carry out a TUNEL assay to confirm that apoptosis has occurred following treatment with the drug.

Careful examination of the FL_2 histogram plots (figures 3.12-3.15) revealed that treatment of the 4T1 cell line with the CLA mixture, and its individual purified isomers caused a disruption in the cell cycle. This resulted in a reduction of the number of cells in S phase coinciding with an increase of the number of cells in G_0/G_1 phase. It was also apparent from these figures that CLA and its individual isomers did not cause apoptosis to occur, as there was no apparent sub- G_0/G_1 peak.

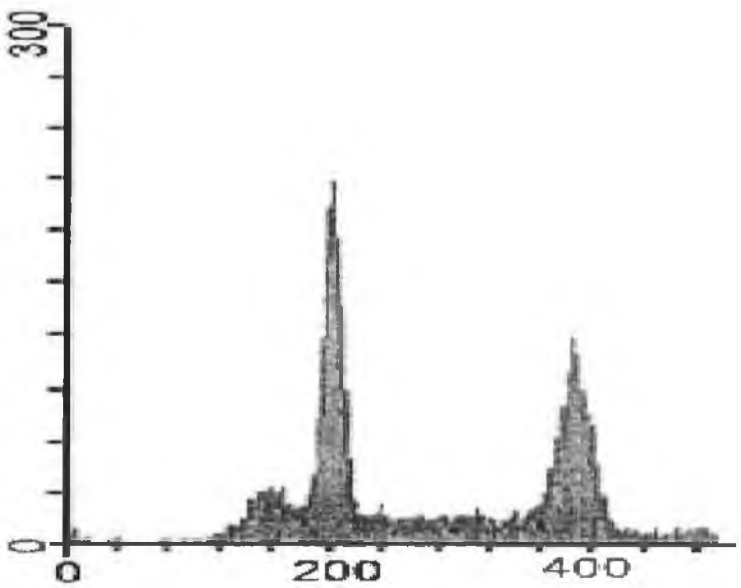


Figure 3.17. A histogram plot, whereby the y-axis represents cell number, and the x-axis represents the amount of DNA per cell. A sub-G_0/G_1 peak (A), indicates that apoptosis had taken place, can clearly be seen.

CHAPTER 4

4.1 Introduction

4.1.1 The extracellular matrix (ECM)

The ECM is a complex and dynamic meshwork that is assembled outside cells from the specialised glycoproteins and proteoglycans secreted by them. The major constituents of the ECM include collagens, non-collagenous glycoproteins, and proteoglycans. As well as providing structural support the ECM also plays an important role in many biological processes during proliferation, migration and differentiation. A balanced interaction between cells and the ECM is essential for these processes (Boudreau and Jones, 1999).

The quality and quantity of the ECM depends not only on structural components but also on the regulated expression of ECM-degrading proteinases and their inhibitors. These proteinases and their inhibitors have, for the most part, been studied in the context of their possible role in tumour invasion and metastasis (McCawley and Matrisian, 2000).

4.1.2 Cancer cell metastasis

Metastasis (the ability to form tumours at a distant site) is a characteristic of malignancy, and is considered to be the most lethal aspect of cancer, having poor clinical outcome (McCawley and Matrisian, 2000). Novel anti-cancer drugs are often aimed at steps in the metastatic process, and failure to pass any step will result in tumour cells being unable to reach a target organ. Cancer metastasis is composed of a number of steps, which a tumour must complete in order to successfully establish a distant metastasis. This is known as the metastatic cascade and is shown in figure 4.1 (McCawley and Matrician, 2000).

Cancer cell initiation occurs when a single cell becomes modified to exhibit a growth advantage over the surrounding tissue, which, at some point, requires neovascularisation to supply nutrients for further growth. As the tumour becomes malignant, it aquires the ability to invade the surrounding normal tissue. Intravasation occurs when tumour cells cross the basement membrane and enter the circulation. Following the tumour cells survival in the circulation, extravasation occurs when the tumour cells leave the circulation and penetrate the host tissue. Metastasis occurs if the tumour cells can establish and grow at this secondary site (figure 4.1) (McCawley and Matrician, 2000).

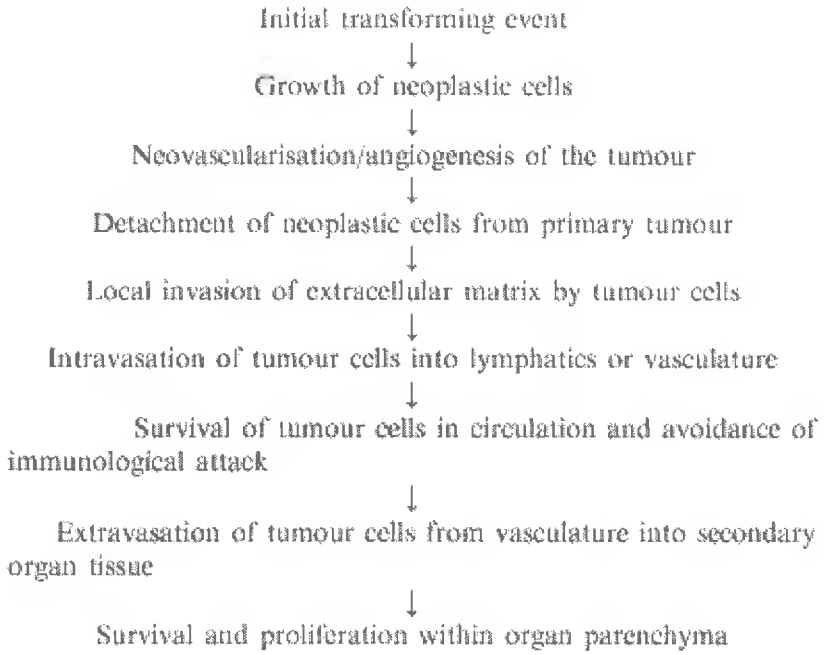


Figure 4.1. Steps involved in the metastatic cascade.

4.1.3. The MMP family

The MMPs are a family of highly conserved zinc dependent endopeptidases, which collectively are capable of degrading the components of the basement membrane and ECM (DeClerck, 2000). They are a continually growing family of enzymes, which currently consist of at least 23 well-defined members. Well characterised members of the MMP family are shown in table 4.1. The MMPs can be defined by the following characteristics:

1. they share common amino acid sequences;
2. their proteolytic activity can be inhibited by tissue inhibitors of metalloproteinases (TIMPs);
3. they are either secreted or exist as transmembrane pro-enzymes that require activation to exert their matrix degrading activity;
4. the active site contains a zinc ion and requires a second metal cofactor such as calcium;
5. enzyme activity is optimal in the physiological pH range

(Nagase and Woessner, 1999).

MMP	Common Name	MW	Substrates
Minimal Domain MMP			
MMP-7	Matrilysin	28	PG, LM, FN, gel, Coll IV
Hemopexin Domain MMPs			
MMP-1	Interstitial Collagenase	55	Fibrillar Collagen
MMP-3	Stromelysin-1	57	Gelatin, Coll III, IV, PG, FN
MMP-8	Neutrophil collagenase	75	Fibrillar Collagen
MMP-10	Stromelysin-2	57	Coll III, IV, Gelatin, PG,FN
MMP-11	Stromelysin-3	51	Laminin, FN
MMP-12	Metalloelastase	53	Elastin, Coll IV, FN
MMP-13	Collagenase-3	65	Fibrillar Collagen
Fibronectin Domain MMPs			
MMP-2	Gelatinase A	72	Gelatins, Collagen IV
MMP-9	Gelatinase B	92	Gelatins, Collagen IV
Transmembrane Domain MMPs			
MMP-14	MT1-MMP	63	Gelatinase A, PG, Coll
MMP-15	MT2-MMP	72	N.D.
MMP-16	MT3-MMP	64	Gelatinase A
MMP-17	MT4-MMP	70	N.D.
MMP-24	MT5-MMP	60	
Miscellaneous			
MMP-19	RASI-1		Gelatins
MMP-20	Enamelysin	54	Amelogenin
MMP-26	Endometase/matrilysin-2	59	Casein

Table 4.1. Properties of the well characterised members of the MMP family. FN-fibronectin, LM-laminin, Gel-gelatin, Col-collagen, PG-proteoglycans, MW-molecular weight (kDa), N.D.-not determined, MT-membrane type.

4.1.3.1 MMPs and cancer

MMPs are believed to promote tumour progression by initiating carcinogenesis, enhancing tumour angiogenesis (the formation of new blood vessels is essential for the secondary tumour to grow beyond minimal size), disrupting local tissue architecture to allow tumour growth, and breaking down basement membrane barriers for metastatic spread. While some MMPs are expressed by tumours cells themselves, MMPs are predominantly produced by surrounding host stromal and inflammatory cells in response to factors released by tumours (Shapiro, 1998, DeClerck, 2000).

4.1.3.2 The relationship between MMPs, PUFAs and cancer metastasis

ECM degradation and invasion by cancer cells represent one of the key events in the metastatic cascade (Ahmad and Hart, 1997). After tumour cells have first adhered to the ECM, proteolytic enzymes bound to the tumour cell membrane or from other sources will degrade the matrix to clear the way for tumour cells to migrate. Key proteolytic enzymes involved in this matrix degradation are the MMPs (collagenases and stromelysins). The balance of these MMPs is maintained by the participation of TIMPs.

There is now substantial *in vitro* and *in vivo* evidence linking MMP expression levels with the ability of tumours to metastasise. Thus, the malignant potential of *in vitro* cultured tumour cells has been correlated with the activity of MMPs. These proteolytic enzymes and their inhibitors are regulated by PUFAs. For example, EPA, GLA and AA have been shown to inhibit the production of the proteinase, collagenase IV (Jiang *et al*, 1998a). It has been reported that LA stimulated tumour cell invasion and MMP-9 production *in vitro*; GLA however inhibited invasion and did not induce activity of the proteolytic enzyme (Liu *et al*, 1996, Begin *et al*, 1989).

Before degradation of the basement membrane and the ECM can take place the cancer cell must first adhere to components within them. It has been demonstrated that EPA and GLA resulted in a time and concentration dependent enhancement of E-cadherin, an adhesion molecule associated with metastatic suppression, in a range of cancer cells. This was also associated with a reduction in invasion. GLA, EPA, DHA and LA have all also been associated with a reduction of tumour adhesion to a range of matrix components (Jiang *et al*, 1998a).

PUFAs also regulate immune cells and host immune response to tumour cells in order to minimise the motile and invasive behaviour of cancer cells, to influence tumour cell survival in the circulation, and to form mechanisms in the tissues to combat cancer cells. It has been demonstrated that many PUFAs, including LA, play an important role in several steps of the metastatic process. The use of PUFAs in preventing metastasis may be a novel route in breast cancer research, which should be followed.

4.1.4 *In vitro* adhesion, migration and invasion

4.1.4.1 The role played by cell adhesion during metastasis

Invasion is an absolute pre-requisite of metastasis and cannot occur while tumour cells are strongly adherent to neighbouring cells within the primary tumour. Therefore for the metastatic process to begin, downregulation of intercellular adhesion must occur. Although adhesion mechanisms must be disrupted to increase the motility of the tumour cells, reattachment of these cells, or increased adhesion, to metastatic sites must also be possible (Ahmad and Hart, 1997). An example of this type of cell interaction is the adhesion of tumour cells to the sub-endothelial basement membrane matrix underlying endothelial cells, which occurs during metastasis formation (Johanning, 1996).

Cell adhesion molecules (CAMs) are vital throughout the step-by-step process of metastasis. They are divided into four categories: cadherins; integrins; selectins and members of the immunoglobulin (Ig) superfamily. Adhesion molecules mediate the process of cell adhesion through either cell-substrate or cell-cell interactions (Johanning, 1996). They also act as key mediators in a variety of processes such as cell motility, tissue integrity and the maintenance of tissue differentiation (Mason *et al*, 2002).

A large family of proteins, known as integrins, mediates adhesion of cells to a variety of ECM components. Integrins are transmembrane proteins, whereby the extracellular domain has binding sites that interact with matrix components. The intracellular domain has binding sites for a range of molecules that will co-ordinate intracellular events (Mason *et al*, 2002).

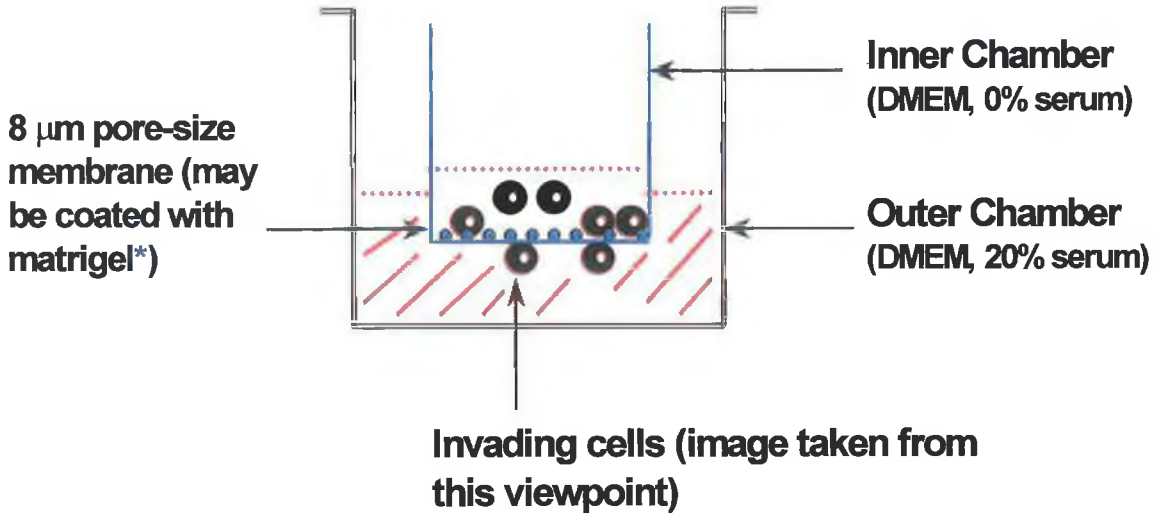
A review carried out by Johanning (1996) on unsaturated fatty acids and breast cancer cell adhesion concluded that unsaturated fatty acids influence the ability of cultured human breast cancer cells to bind to protein components of the basement membrane. The author also showed that unsaturated fatty acids primarily affected the binding of breast cancer cells to type IV collagen, and modulated adhesion differently in tumourigenic and non-tumourigenic cell lines.

4.1.4.2 *In vitro* migration using cell culture inserts

Becton Dickenson Labware/Falcon® products offers a broad line of cell culture inserts incorporating polyethylene terephthalate (PET) track-etched membranes. Perfectly transparent, low pore density PET membranes provide a durable substrate for light microscopy. The membranes are strong and can be easily removed for staining, fixing and other procedures, such as mounting on slides. The membrane has symmetrical, cylindrical pores; both sides of it are tissue culture treated and therefore suitable for cell growth (Becton Dickenson specification sheet, www.bdbiosciences.com).

4.1.4.3 BD Biocoat™ Matrigel™ Invasion Chambers

The BD Biocoat™ Matrigel™ Invasion Chambers (figure 4.2) provided an *in vitro* system for the study of cell invasion through a basement membrane matrix. It consisted of BD Falcon™ Cell Culture Inserts containing an 8μm pore-size PET membrane coated with a uniform layer of BD Matrigel™ Basement Membrane Matrix. This method was discriminating, reproducible and provided an authentic model of *in vivo* basement membrane. Only invasive cells digested the matrix and moved through the insert membrane (Becton Dickenson specification sheet, www.bdbiosciences.com). The method used enabled the study of the metastatic potential of tumour cells and the expression of MMPs on the surface of invasive tumour cells.



* For the migration assay there is no Matrigel™

Figure 4.2. Diagrammatic representation of the *in vitro* migration and invasion assay. The cells above the membrane migrate through the 8μm pores due to the serum gradient. After a given time, they may be stained, viewed and counted. Depending on whether migration or invasion is being studied, the membrane may be previously coated with Matrigel ™.

4.2 Results

4.2.1 The effect of CLA on the *in vitro* attachment of the 4T1 cell line

The 4T1 cell line was treated with 15μg/ml of the CLA mixture, its individual purified isomers, 9*c*,11*t*(18:2) and 10*t*,12*c*(18:2), and 1% ethanol for 24hr (section 2.2.11). This treatment had previously been shown to have minimal toxicity to the cells (section 3.2.4.1). Following this, the cells were trypsinised, counted and diluted to a concentration of 10^5cells/ml. These cells were then added to a 96-well plate in triplicate, and allowed to attach to the plastic for 30, 60 and 90min.

Following this time period, unattached cells were 'flicked' from the plate, the plate was spun to bring down media on the side of the wells, and this media was then aspirated. The MTS assay used in Section 3.2 for cytotoxicity analysis was used here to assess the attachment of the cells to the plastic. MTS (20μl) was added to each well along with DMEM (100μl). At this stage, 100μl of the original untreated cell suspension was added to the plate in triplicate, along with 20μl of MTS. The formazon product, which is formed only in the presence of viable cells, was allowed to develop. After 1 ½ - 2 ½ hours, the absorbance of the coloured product was read at 492nm.

The absorbance of the original untreated cell suspension was taken as 100%. The percentage adhesion of the CLA- and ethanol-treated cells was thus calculated as a percentage of the total number of untreated cells plated. The assay was carried out three times in triplicate, and p values of <0.05 were deemed significant. Standard deviations (+/-) were calculated and are shown following the value in table 4.2.

Figures 4.3, 4.4 and 4.5 illustrate the percentage adhesion of the 4T1 cells following treatment for three different adhesion times. Initially, the assay looked at the adhesion of the 4T1 cells after 90min (figure 4.3). It can be seen that following this adhesion time, 64% of ethanol-treated cells had adhered to the plastic, while 34, 36 and 36% of cells previously treated with the 9*c*,11*t*(18:2) isomer, the 10*t*,12*c*(18:2) isomer and the CLA mixture had attached. There was clearly a reduction in adhesion of the CLA-treated samples by almost 50% when compared to the control, although there was no apparent difference between the CLA mixture and its individual isomers.

Following this, it was decided to carry out the same assay but with one change, the adhesion time was reduced to 60min and then to 30min. By shortening the adhesion time, the conditions became more stringent. It was hoped that a differential effect by either isomer would be seen. When the cells were allowed to adhere for 60min (figure

4.4), no great difference was seen between the CLA mixture and its individual purified isomers, although there was a significant difference for cells treated with the 9*c*,11*t*(18:2) isomer (30%) and the CLA mixture (46%). When the adhesion time was reduced to 30min, there was no difference between the CLA mixture (32%), the 10*t*,12*c*(18:2) isomer (35%) or the 9*c*,11*t*(18:2) isomer (32%).

Attachment Time (min)	Percentage Adhesion (%)			
	Ethanol	**9:11**	**10:12**	**Mix**
30	45 +/-5	32 +/-2	35 +/-3	32 +/-2
60	65 +/-7	30 +/-2	55 +/-6	46 +/-3
90	64 +/-4	34 +/-1	36 +/-2	36 +/-1

Table 4.2. The 4T1 cell line was treated for 24hr with 15μg/ml of the CLA mixture, its individual purified isomers and 1% ethanol. The cells were allowed to adhere to plastic for 30, 60 and 90 min, and the percentage adhesion was calculated.

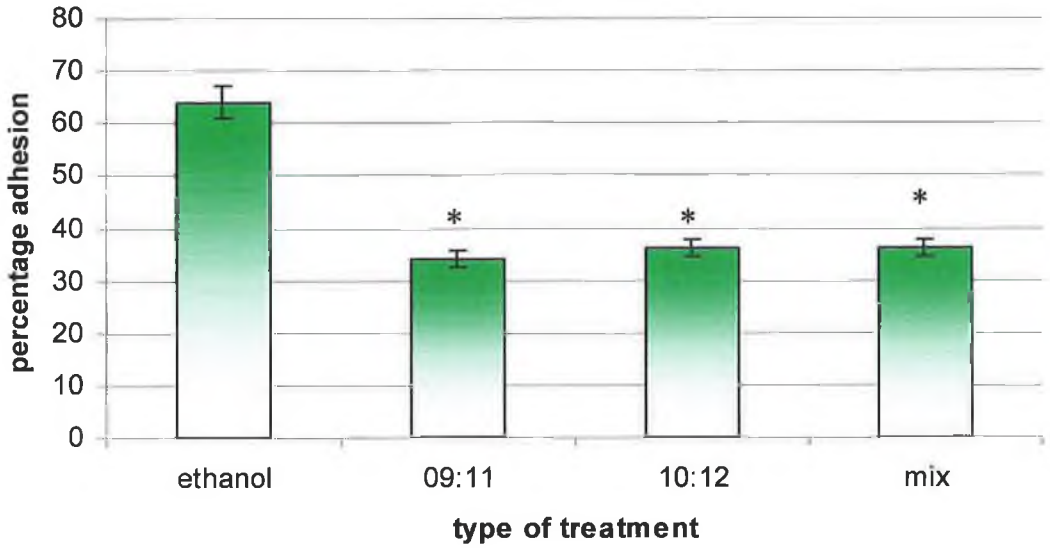


Figure 4.3. Percentage adhesion of the CLA- and ethanol-treated samples from table 4.2 is shown here for the adhesion time of 90min. P values <0.05 are significant and are denoted by the asterisk (*).

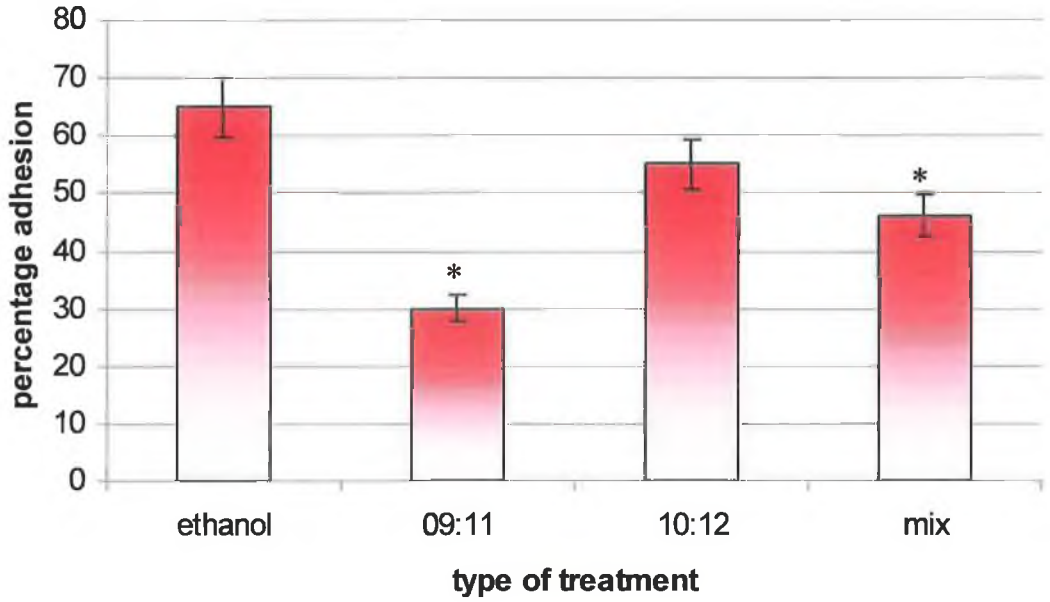


Figure 4.4. Percentage adhesion of the CLA- and ethanol-treated samples from table 4.2 is shown here for the adhesion time of 60min. P values <0.05 are significant and are denoted by the asterisk (*).

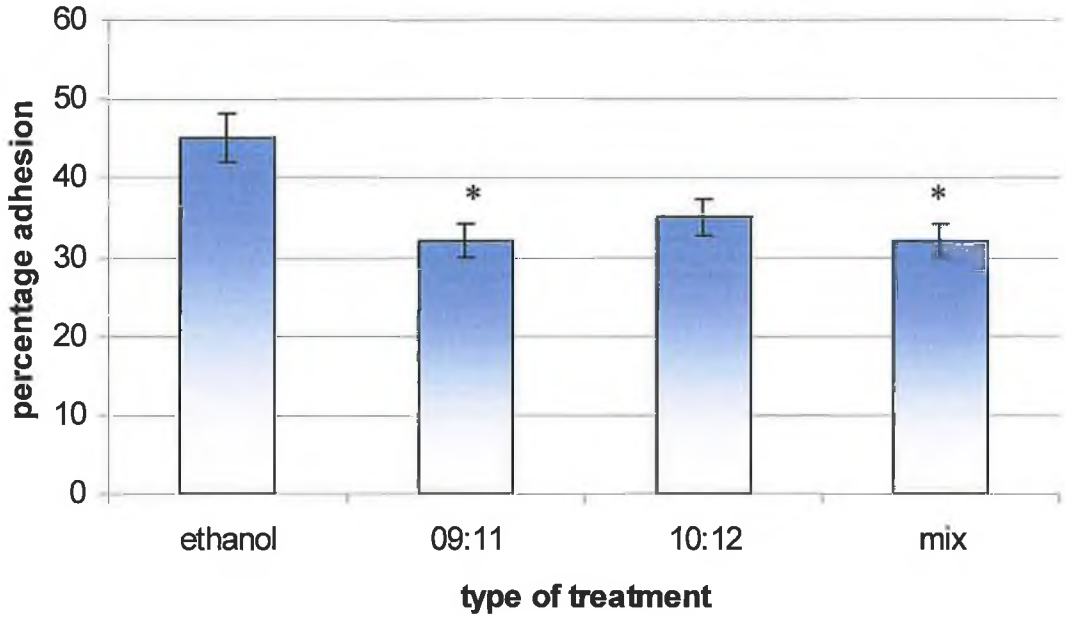


Figure 4.5. Percentage adhesion of the CLA- and ethanol-treated samples from table 4.2 is shown here for the adhesion time of 30min. P values <0.05 are significant and are denoted by the asterisk (*).

4.2.2 The effect of CLA on the *in vitro* migration of the 4T1 cell line.

The 4T1 cell line was treated with 15μg/ml of the CLA mixture, its individual purified isomers, 9*c*,11*t*(18:2) and 10*t*,12*c*(18:2), and 1% (v/v) ethanol for 24hr (section 2.2.12). Following this, the cells were trypsinised, counted and diluted to a concentration of 10^5cells/ml in DMEM/S_0. These cells were then added to the BD Falcon® cell culture inserts along with 15μg/ml of the CLA mixture, its individual purified isomers, 9*c*,11*t*(18:2) and 10*t*,12*c*(18:2), and 1% ethanol. Each of the inserts contained 8μm pore-size membranes through which the cells could migrate towards the chemoattractant (DMEM/S_{20}). After 24hr, the membrane was cut from its housing and the cells on the underside of the membrane were fixed, stained, viewed and counted. In total, five random fields were counted under 10X magnification. In general, fields that were too close to the edge of the filter were not counted.

The percentage migration of the CLA-treated cells was calculated using the ethanol control as 100% migration. The assay was carried out three times in duplicate and a similar trend was observed for each assay. Multiple cell counts were performed, and p values of <0.05 were deemed significant.

Treatment with the CLA mixture and its individual purified isomers resulted in a reduced percentage migration of the cells (table 4.3 and figure 4.6) when compared to the ethanol control, which was assigned a value of 100%. Cells treated with the 10*t*,12*c*(18:2) isomer had a reduced percentage migration of 67%, which was not significant, while those treated with the 9*c*,11*t*(18:2) isomer and the CLA mixture had a significantly reduced percentage migration of 38% and 37%, respectively.

Treatment	Percentage migration
9:11 CLA	38 +/- 5
10:12 CLA	67 +/- 12
Mix CLA	37 +/- 6
Ethanol	100 +/- 8

Table 4.3. The 4T1 cell line was treated for 24hr with 15μg/ml of the CLA mixture, its individual purified isomers, 9*c*,11*t*(18:2) and 10*t*,12*c*(18:2), and 1% ethanol. Following this, the percentage migration of the cells was determined using an *in vitro* migration assay.

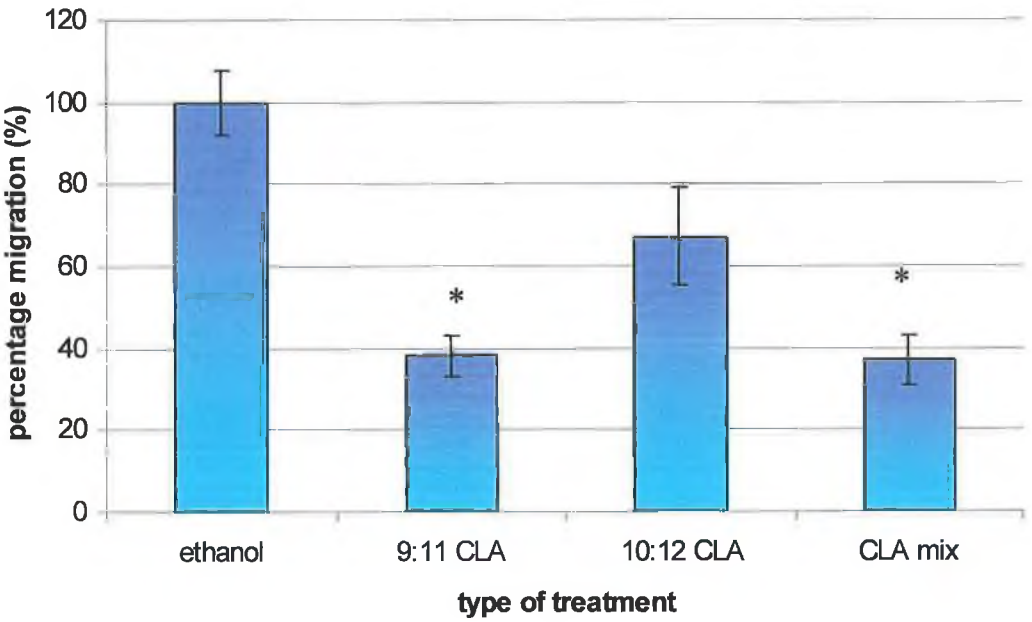
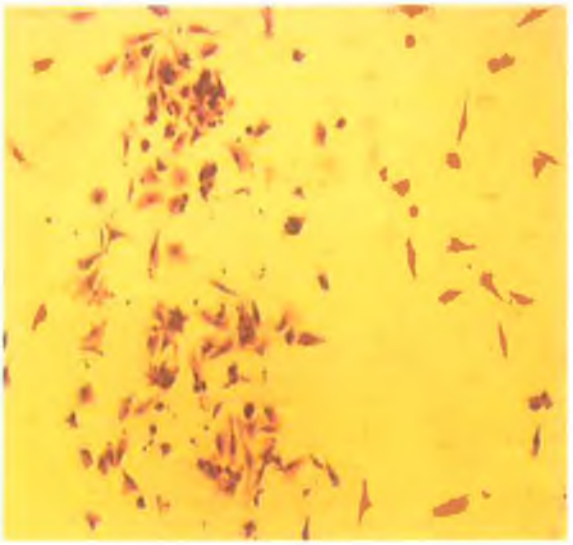


Figure 4.6. The 4T1 cell line was treated for 24hr with 15μg/ml of the CLA mixture, its individual purified isomers and 1% ethanol. Following this, the percentage migration of the cells was determined. The plot represents the migratory ability (%) versus the type of treatment. P values are deemed significant if <0.05 and are denoted by the asterisks (*).

Ethanol

9*c*,11*t*(18:2) isomer



10*t*,12*c*(18:2) isomer

CLA Mixture

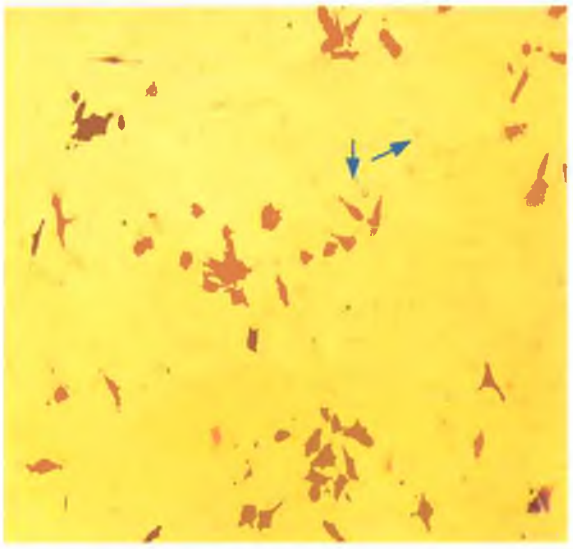


Figure 4.7. The effect of a CLA mixture and its individual purified isomers on the percentage migration of the 4T1 cell line. Membranes were stained and mounted on slides, they were viewed under 10X magnification and five fields of view were counted. The green arrows point at stained cells and the blue arrows point at pores in the membrane.

4.2.3 The effect of CLA on the *in vitro* invasion of the 4T1 cell line

The 4T1 cell line was treated with 15μg/ml of the CLA mixture, its individual purified isomers, 9*c*,11*t*(18:2) and 10*t*,12*c*(18:2), and 1% ethanol for 24hr (section 2.2.13). Following this, the cells were trypsinised, counted and diluted to a concentration of 10^5cells/ml. These cells were then added to the BD Biocoat™ Matrigel™ Invasion Chambers along with 15μg/ml of the CLA mixture, its individual purified isomers, 9*c*,11*t*(18:2) and 10*t*,12*c*(18:2), and 1% ethanol. Only invasive cells could pass through the Matrigel™ and, therefore, the pores in the membrane, where they could then be fixed, stained, viewed and counted. In total, five random fields were counted under 10X magnification. In general, fields that were too close to the edge of the filter were not counted.

Following treatment, the percentage invasion of the CLA-treated cells, when compared to the ethanol control, was calculated. The assay was carried out three times and a similar trend was observed for all assays. Multiple cell counts were performed, and p values of <0.05 were deemed significant.

The percentage invasion of the cells was calculated. These values are shown in table 4.4, and are in graphical form in figure 4.8. The *in vitro* invasive activity of the 4T1 cell line was reduced after treatment with the CLA mixture and its individual isomers, 9*c*,11*t*(18:2) and 10*t*,12*c*(18:2), to 74, 48 and 90%, respectively, although only the value for the 9*c*,11*t*(18:2) isomer was deemed significant. Images were taken of the stained cells, and are shown in figure 4.9, whereby the difference in the invasive ability of the treated cells can be clearly seen.

Type of treatment	Percentage invasion (%)
9:11 CLA	48 +/- 3
10:12 CLA	90 +/-14
Mix CLA	74 +/- 4
Ethanol	100 +/-10

Table 4.4. The 4T1 cell line was treated for 24hr with 15μg/ml of the CLA mixture, its individual purified isomers, 9*c*,11*t*(18:2) and 10*t*,12*c*(18:2), and 1% ethanol. Following this, the percentage invasion of the cells was determined using an *in vitro* invasion assay.

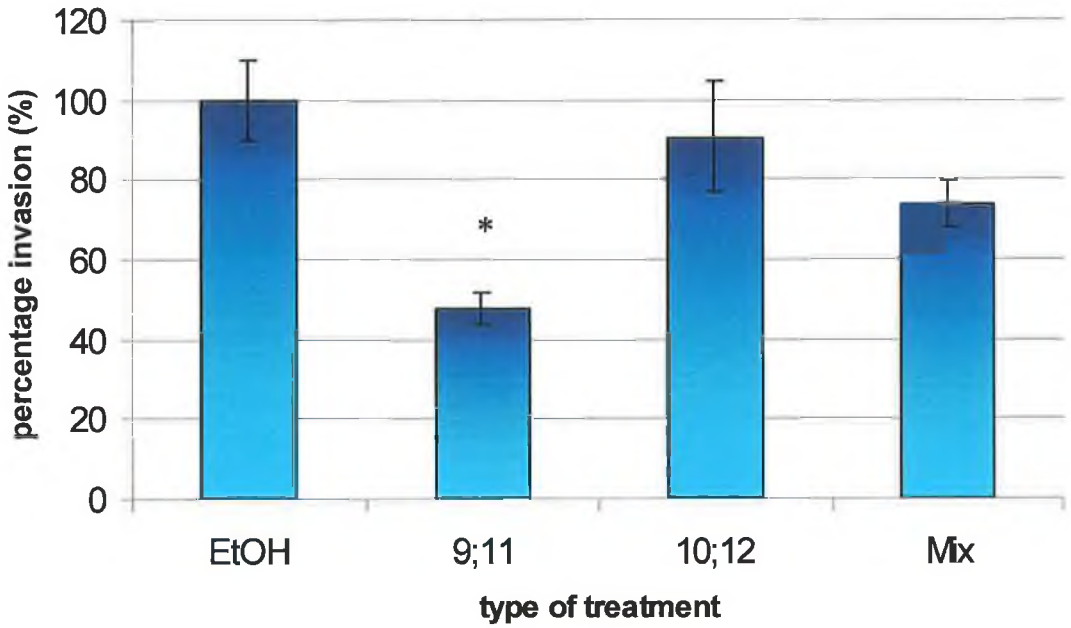


Figure 4.8. The 4T1 cell line was treated for 24hr with 15μg/ml of the CLA mixture, its individual purified isomers, 9*c*,11*t*(18:2) and 10*t*,12*c*(18:2), and 1% ethanol. Following this, the invasive ability of the cells was determined using an *in vitro* invasion assay. The plot represents the invasive ability (%) versus the type of treatment. P values are deemed significant if <0.05 and are denoted by the asterisks (*).

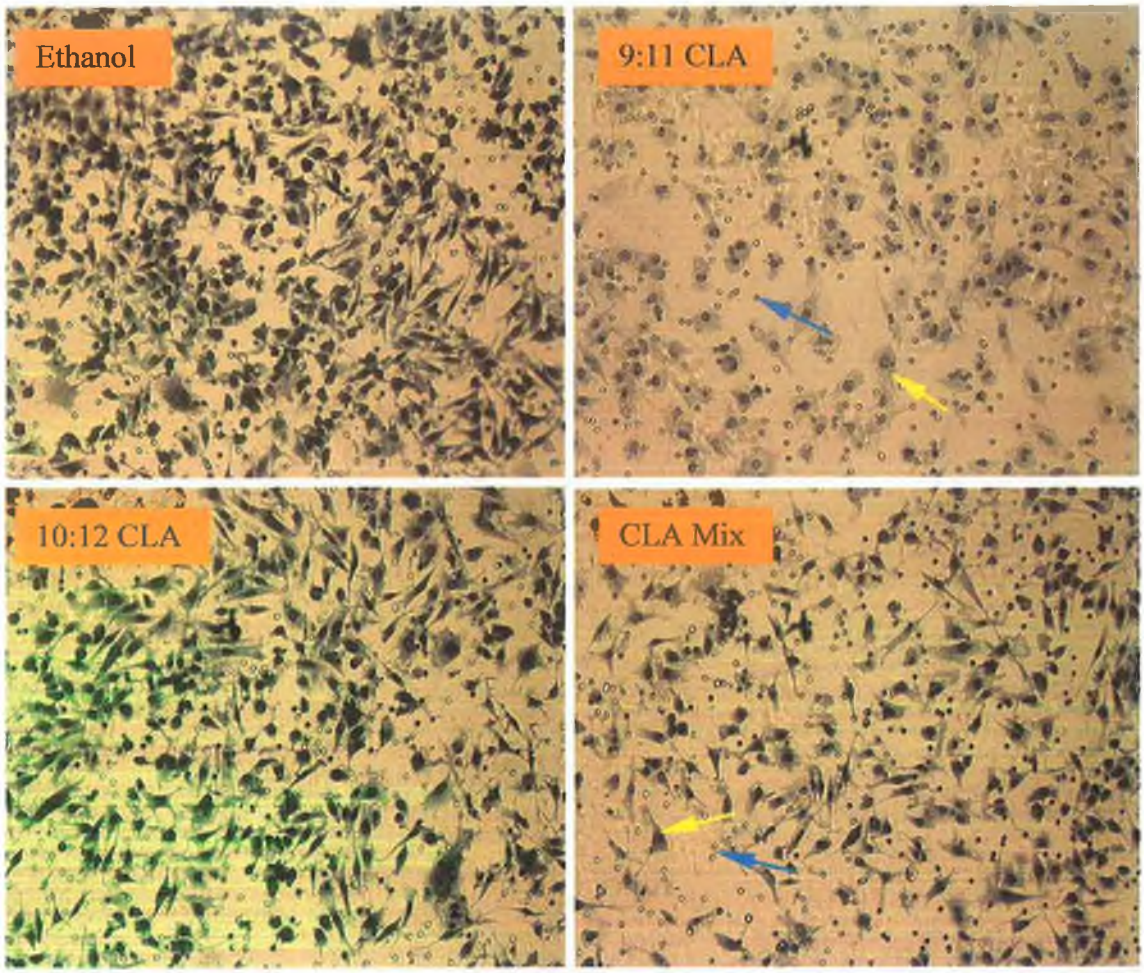


Figure 4.9. The effect of a CLA mixture and its individual purified isomers on the percentage invasion of the 4T1 cell line. Membranes were stained and mounted on slides, they were viewed under 10X magnification and counted. The yellow arrows point at cells and the blue arrows point at pores in the membrane.

4.2.4 Protein (BCA) assay

Four flasks of the 4T1 cell line were incubated in serum-free DMEM (DMEM/S_0) overnight, at 37°C. The following day, fresh DMEM/S_0 was added and the flasks were supplemented with either: 15μg/ml of the CLA mixture; 9*c*,11*t*(18:2) isomer; 10*t*,12*c*(18:2) isomer or 1% ethanol (section 2.2.7). This sub-lethal treatment time and dose did not cause significant cell death to the 4T1 cell line and so was suitable for this assay. The cells were then returned to the incubator for 24hr, after which media was collected from each tissue culture flask for protein determination by the BCA assay (section 2.2.8) followed by MMP expression analysis by gelatin zymography.

It was necessary to carry out a protein assay as the protein concentration of the samples may differ if there was an unequal number of cells seeded into the flask originally. By determining the protein concentration, it was possible to load equal amounts of protein onto the zymography gel, and thus if there was a difference in the amount of MMP released from the cell, it can be accepted as a true difference.

The purple-coloured reaction product of the BCA assay is formed by chelation of two molecules of BCA with one cuprous ion. This water-soluble complex exhibits a strong absorbance between 560-620nm that is nearly linear with increasing protein concentrations over a broad working range (20–2,000 μg/ml).

Each time the BCA assay was performed, a protein standard curve was also set up using bovine serum albumin (BSA) as the standard (figure 4.10). For a standard curve to be acceptable, the R^2 value was always taken into account, and had to be >0.9. Using the equation of the line from the BSA standard curve, the concentration of the CLA- and ethanol-treated samples could be calculated (table 4.5), as follows

$$X = (Y +/- C)/M$$

Where Y is the absorbance of the sample, C is the intercept, M is the slope, and X is the resultant concentration of the sample (mg/ml).

The protein concentrations for the different treatments were very similar. This is an excellent indication that the treatment conditions, i.e. incubation time and concentration, did not cause cell death to occur to any great degree.

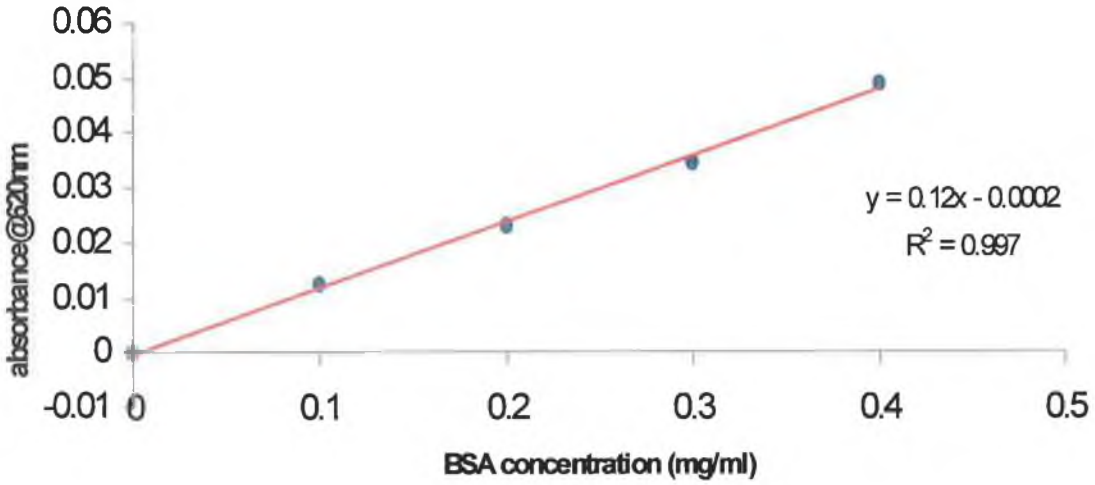


Figure 4.10. The plot shows increasing absorbance read at 620nm on the y-axis versus increasing concentration of the BSA standard (mg/ml) on the x-axis.

Sample	Protein concentration (mg/ml)
A	0.6
B	0.7
C	0.6
D	0.7

Table 4.5. Protein analysis was carried out on the 4T1 samples (A-the CLA mixture, B-9*c*,11*t*(18:2) isomer, C-10*t*,12*c*(18:2) isomer and D-ethanol). The absorbance was read at 680nm. These values were then put into the equation of the line from figure 4.11 to give the protein concentration of the samples.

4.2.5 The effect of CLA on MMP release from the 4T1 cell line

The 4T1 cell line was treated with 15μg/ml of the CLA mixture, its individual purified isomers, 9*c*,11*t*(18:2) and 10*t*,12*c*(18:2), and 1% ethanol for 24hr. Following this, media was collected from each tissue culture flask. A BCA protein assay (section 2.2.8) was carried out on the samples, and equal protein was loaded onto a substrate (gelatin) zymography gel (section 2.2.9). This was to ascertain whether the 4T1 cell line expressed and released any gelatin-degrading MMPs. This experiment was repeated three times on three separate occasions. The zymograph shown in figure 4.11 is a representative gel. Similar results were seen on the other gels.

From figure 4.11, it can be seen that the 4T1 cell line expressed significant quantities of murine gelatinase B, or MMP-9. Previous researchers have also found murine MMP-9 to have a higher molecular weight (105kDa) than human MMP-9 (92kDa) (Canete Soler *et al*, 1995). As a control, medium was collected from a flask of BHK-92 cells (baby hamster kidney cell line, which constitutively expresses MMP-2, and was transfected with the gene for human MMP-9).

MMP-9, like other members of the MMP family, is secreted as a proenzyme, requires zinc for activity, and can be inhibited by naturally occurring inhibitors called tissue inhibitors of metalloproteinases (TIMPs) (McCawley and Matrisian, 2000). MMP-9 contains a fibronectin-like domain (DeClerck, 2000) that confers gelatin-binding properties, hence the use of gelatin as the substrate in the zymography gel. Although the MMP-9 gene is strongly conserved in many species, murine MMP-9 contains additional amino acid inserts in exons 9 and 13 resulting in the higher molecular weight of 105kDa (Canete Soler *et al*, 1995).

To determine the relative amount of MMP-9, densitometry analysis (section 2.2.9.1) of the gel was carried out for each white band against a blue background, and these values are plotted in figure 4.12. It can be seen from the plot that there was no decrease in the amount of MMP-9 released from the 4T1 cell line following treatment. Table 4.5 shows these values again in percentage form, whereby, ethanol was taken as 100%, and the other treatments (CLA and its individual isomers) are calculated as a percentage of it, i.e.

$$\left\{ \frac{\text{densitometry value for CLA-treated samples}}{\text{densitometry value for ethanol-treated samples}} \right\} * 100$$

There was a slight, non-significant, increase in the amount of MMP-9 released from the cells by 21, 12, 13% after treatment with the 9*c*,11*t*(18:2) isomer, the 10*t*,12*c*(18:2) isomer and the CLA mixture, respectively.

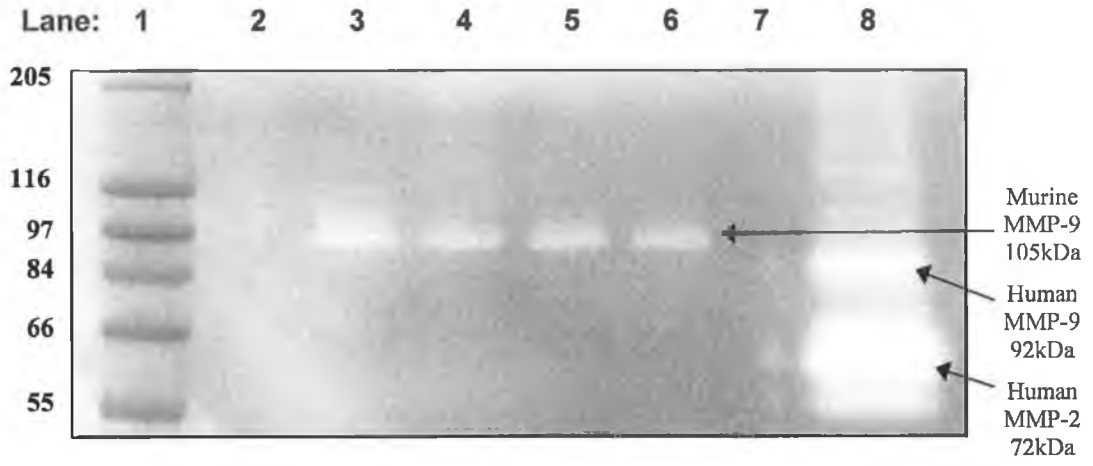


Figure 4.11. Gelatin zymograph of 4T1 cells following treatment with the CLA mixture, its individual purified isomers, and 1% ethanol. Lane 1 is the molecular marker, lane 2 is H_2O, lane 3 is 9*c*,11*t*(18:2), lane 4 is 10*t*,12*c*(18:2), lane 5 is the CLA mixture, lane 6 is ethanol and lane 7 contains no sample. The positive control (lane 8), which is medium collected from the cell line BHK-92, expresses significant quantities of human MMP-2 and MMP-9.

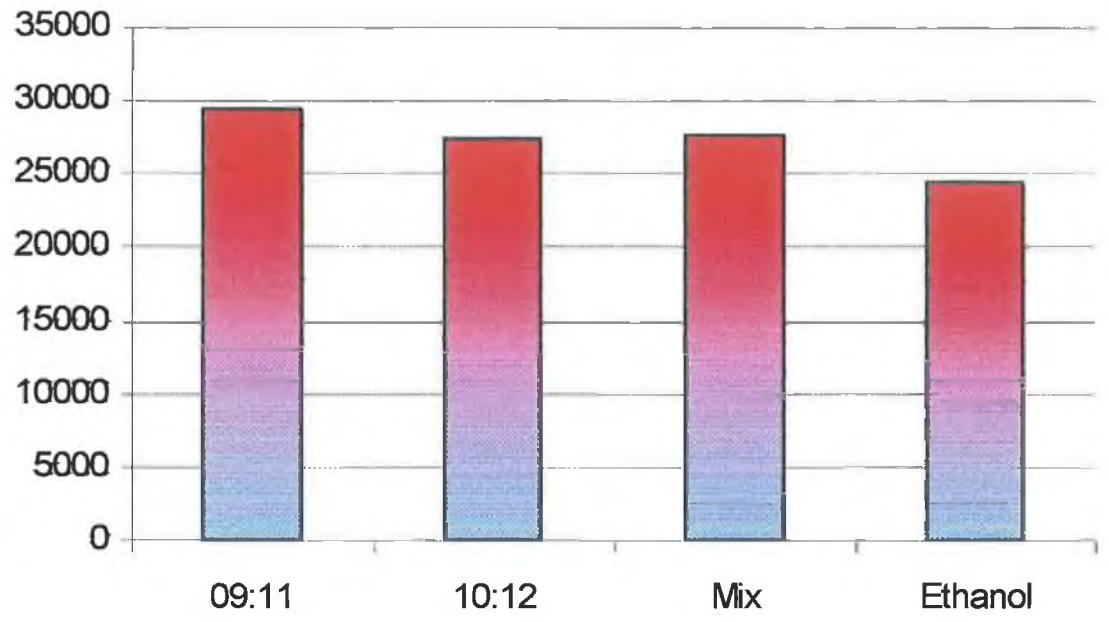


Figure 4.12. Densitometry analysis was carried out on the gel in figure 4.11. The x-axis displays the type of treatment (CLA mixture, its individual purified isomers, 9*c*,11*t*(18:2) and 10*t*,12*c*(18:2), or 1% ethanol), while the y-axis displays arbitrary densitometric values.

Treatment	Densitometry Values (%)
9:11 CLA	121
10:12 CLA	112
Mix CLA	113
Ethanol	100

Table 4.6. Densitometry analysis was carried out on the gel in figure 4.11, and these arbitrary values given for each CLA treatment are shown here as a percentage of ethanol (100%).

4.3 Discussion

The aim of the research carried out in this chapter was to examine the effect of sub-lethal CLA treatments on the *in vitro* migration and invasion of the 4T1 cell line. For invasion and metastasis to begin, downregulation of intercellular adhesion must occur, and increased adhesion to metastatic sites must also be possible (Ahmad and Hart, 1997). The *in vitro* adhesion assay carried out in this research enabled the ability of the 4T1 cells to adhere to plastic to be studied. The multiwell plates, used in this research, had previously been treated by the manufacturers so that they encouraged the attachment and growth of cancer cell lines. By treating the 4T1 cell line with the CLA mixture and its individual purified isomers, it was possible to see if the treatment had any effect on the adhesive ability of the cells.

Figures 4.3, 4.4 and 4.5 illustrate the percentage adhesion of the 4T1 cell line after 30, 60 and 90min, respectively. There did not appear to be any difference in the ability of the cells to adhere in the presence of either the CLA mixture and its individual purified isomers, 9*c*,11*t*(18:2) and 10*t*,12*tc*(18:2), although after 90min all treatments resulted in ~50% reduction in adhesion compared to the ethanol control.

One hypothesis as to CLA's anti-cancer properties is its ability to alter the lipid composition of cell membranes. It is widely agreed that dietary unsaturated fatty acids added to the media of cultured cells or to the diet of animals and humans are capable of modifying the lipid composition of cell membranes after they have been incorporated into the phospholipid fraction (Spector and Yorek, 1985). Changes in phospholipid composition would also be expected to alter interactions among various cell adhesion molecules in the cell membrane, for example, the binding affinity of these molecules might be altered (Johanning, 1996).

Previous researchers have found that ω-6 PUFAs influenced the adhesive ability and metastasis of breast cancer cell lines. Jiang *et al* (1995) found that γ-linoleic acid (GLA) increased expression of E-cadherin, while LA reduced expression of this cell-to-cell adhesion molecule. Since E-cadherin is a tumour/metastasis suppressor, its up-regulation by relatively low concentrations of GLA suggests that this PUFA may be useful in adjuvant therapy for cancer patients.

There has been no published research to date on the effect of CLA on the *in vitro* adhesive ability of the 4T1 cell line, although other cell lines have been studied. Johanning and Lin (1995) found that LA increased human breast cancer (MDA-MB-231) cell adhesion to extracellular matrix components by activating lipoxygnease and/or

protein kinase C pathways. This study used Matrigel™, which was used in the invasion assays from section 4.2.3. The use of ECM components *in vitro* represents more closely what is happening *in vivo*.

Previous work by other researchers such as Hubbard *et al* (2000) found reduced murine mammary tumour metastasis *in vivo* after rodents were fed diets containing CLA. These effects were seen at concentrations as low as 0.1% of the diet. These researchers used a CLA mixture in the diet. There have been no *in vivo* studies to date using individual CLA isomers in the diet.

In the attempt to mimic an *in vivo* system, this present study used *in vitro* migration and invasion assays to study the effect of CLA on the 4T1 cell line. Following treatment with 15μg/ml of CLA and its individual isomers for 24hr, there was a significant decrease in percentage migration of the cells treated with the 9*c*,11*t*(18:2) isomer (38%) and the CLA mixture (37%), while there was also an observed decrease with the 10*t*,12*tc*(18:2) isomer (67%). There was also a decrease following the same treatment conditions in the percentage invasion of this cell line. There was a significant decrease in percentage invasion of cells treated with the 9*c*,11*t*(18:2) isomer (48%), while there was also an observed decrease with the 10*t*,12*c*(18:2) isomer (90%) and the CLA mixture (74%), which was not significant.

It has been proposed that CLA's anti-cancer properties are the result of CLA's ability to reduce the production of eicosanoids (Urquhart *et al*, 2002), which are associated with tumour progression, i.e. CLA may act as a COX inhibitor. Attiga *et al* (2000) showed a reduction in both *in vitro* invasion and in MMP levels in two prostatic cell lines, DU-145 and PC-3, after treatment with various COX inhibitors. These results indicated a potential role for COX in cancer metastasis, and also provided evidence of a possible link between CLA and MMPs.

Results from the *in vitro* invasion assay (section 4.2.3) indicated that at least one isomer of CLA [9*c*,11*t*(18:2)] reduced the percentage invasion of the 4T1 cell line. Following this, it was desirable to see if this alteration of invasion *in vitro* was due to changes in MMP activity of the 4T1 cell line. To date, there have been no publications indicating that the 4T1 cell line expressed high levels of any particular MMP. For this reason, substrate zymographs were set up to study if the cell line did express high levels of MMPs. Gelatin zymographs were used as these allowed potentially MMP-2 and MMP-9 (both gelatinases) to be studied. It was found that the 4T1 cell line did express

significant quantities of murine MMP-9 (105kDa) and that treatment with CLA and its individual isomers had no effect on the expression of this MMP.

Harris *et al* (2001) looked at the effect of diets containing different levels of CLA, DHA and ω-3 fatty acids in pregnant rodents. They found that CLA significantly depressed prostaglandin (PG) $F2_{\alpha}$ synthesis in placenta, uterus and liver of pregnant rats by 50% when the ω-6:ω-3 ratio was 7:1. They also found that CLA and DHA depressed active MMP-2 and serum MMP-9 levels. They suggested supplementation of the maternal diet with CLA and DHA may be effective in the prevention of premature delivery. This was one of the first studies to directly link CLA and MMP activity.

There are many factors involved in cancer cell invasion and ECM degradation, which may result in tumour cell metastasis. TIMPs, the predominant regulators of MMPs, may also be studied in this cell line to see if the reduction in invasion of the 9*c*,11*t*(18:2)-treated cells is related to a possible increase in TIMP levels and, therefore, a stricter regulation of MMP-9 expression. Integrins are a family of transmembrane linker proteins, which provide anchorage for cells to the ECM and are involved in cell adhesion, invasion and motility of cells (Curran and Murray, 2000). Integrins have been reported to affect the transcription of MMP genes (Jones and Walker, 1997 Kossakowska *et al*, 1999). Each of these avenues may be explored in the elucidation of the mechanism of action of CLA's anti-metastatic properties.

CHAPTER 5

5.1 Conclusions

The 4T1 cell line, which was chosen for this research, was derived from a murine mammary tumour (Aslakson and Miller, 1992). When injected into BALB/c mice, 4T1 cells spontaneously produce highly metastatic tumours that can metastasize to the lung, liver, lymph nodes and brain while the primary tumour is still growing *in situ*. The tumour growth and metastatic spread of 4T1 cells in BALB/c mice very closely mimics that of human breast cancer (www.atcc.org, 2002).

A review of the literature reveals that to date, there have been no studies examining the effect of CLA on the 4T1 cell line. In addition, there have only been a few studies published looking at the cytotoxicity of the individual purified CLA isomers, 9*c*,11*t*(18:2) and 10*t*,12*c*(18:2).

Following treatment with CLA and its individual isomers, viability was examined. It was found that CLA had an isomer-specific cytotoxic effect on the 4T1 cell line. It can be seen from the results (figures 3.7-3.10) that there was a clear difference in the potency of the individual isomers and the CLA mixture. The 10*t*,12*c*(18:2) isomer was shown to be the most potent, especially at lower concentrations.

The cell cycle was examined following treatment with CLA and its individual isomers, whereby both a reduction in the number of cells in the S phase along with an increase in the number of cells in the G_0/G_1 phase was observed for the CLA treated samples. These results indicated that the cytotoxic effect exerted by CLA and its isomers was due to a disruption in the G_1 checkpoint. The 10*t*,12*c*(18:2) CLA isomer caused the greatest reduction of cells in S, along with an increase of cells in G_0/G_1. This isomer also caused increased cytotoxicity to this cell line. In conclusion, flow cytometric analysis revealed deregulation of the cell cycle correlating with the observed cytotoxic effects of CLA, especially with the 10*t*,12*c*(18:2) CLA isomer.

DNA was extracted from the 4T1 cell line and was examined for DNA laddering, which is an indication of apoptosis. The results from this experiment (section 3.2.7) were inconclusive. Even so, the flow cytometry histogram plots can also be used to determine whether apoptosis had taken place. It was concluded that CLA and its individual isomers did not cause apoptosis to occur, as there was no apparent sub-G_0/G_1 peak, which is indicative of apoptosis.

Isomer-specific results were also seen for the *in vitro* migration and invasion assays. It was found that the 9*c*,11*t*(18:2) isomer and the CLA mixture significantly

reduced the migration and the invasion of the cells. The 10*t*,12*c*(18:2) isomer had no effect on invasion although it did result in an observed reduction of the migration of the cells.

The expression of MMP was studied as part of this research. It was shown that the 4T1 cell line expressed significant quantities of murine MMP-9. Following treatment with CLA and its individual isomers, there was no apparent reduction in the amount of MMP-9 released from the cells.

There are many directions in which this research could be taken. The next step in the *in vitro* work would be the elucidation of the mechanisms behind CLA's anticancer properties. It has been shown in this research that specific CLA isomers reduced the *in vitro* migratory and invasive ability of the 4T1 cell line. Following the examination of MMP release from the cells it was found that although CLA reduced the invasion of the cell line, it did not effect MMP release. MMPs are only one of many factors involved in cell migration and invasion. The expression of TIMPs, Integrins and CAMs could also be investigated. Integrins and TIMPs are important factors in the regulation of MMP expression.

In vivo work is the natural progression from *in vitro* studies. Using tail vein injections of the 4T1 cells into mice, experimental metastasis could be examined. In addition, injection into the mammary fat pad would allow spontaneous metastasis to also be investigated. The effect of injecting 4T1 cells into mice, which had previously been fed CLA-containing diets could be examined. The effect that CLA had on metastasis, latency, tumour size and tumour numbers could be looked at. The control diet should contain LA in place of the CLA.

The mechanisms by which CLA down regulates tumourigenesis and cancer proliferation are not well understood, and because CLA has pleiotropic metabolic effects, individual CLA isomers may compete as ligands for multiple pathways of signal transduction. It has also been suggested that CLA may down-regulate cancer growth by interfering with the metabolism of AA. Other proposed theories to CLA's anti-cancer properties are: CLA-induced lipid peroxidation; change in fatty acid metabolism; and CLA's relationship to hormone response elements.

It is widely agreed that CLA inhibits tumourigenesis and proliferation of cancer cell lines. However, a better understanding of the anti-carcinogenic properties of CLA preparations and their constituent isomers is required before establishment of intervention trials. It is hoped that this research will contribute to the understanding of the actual

mechanisms by which CLA and its individual isomers function. *In vivo* work, using this fatty acid and this cell line to induce experimental metastasis, should be the next step in the investigation of CLA and its individual purified isomers. It is hoped that a clearer understanding of CLA's anti-cancer properties will result in the use of CLA as a therapeutic agent for primary and metastatic breast cancer. Also, the potential use of CLA in the prevention of primary and recurrent breast cancer is a novel route, which should be explored.

CHAPTER 6

6.1 Bibliography

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