THE EFFECT OF CLAs ON THE EXPRESSION OF PKC ISOFORMS AND CELL VIABILITY IN BREAST CANCER CELL LINES

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Declaration

I declare that the work presented in this thesis is my own, except where otherwise stated, and has not been submitted in any form for another degree or qualification at any other academic institution.

Information derived from the published or unpublished work of others has been acknowledged in the text, and a list of references given.

Rosemary H Crabb-Wyke

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Abstract

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Rosemary H Crabb-Wyke

Breast tumours make up 15% of the total cancer burden in the UK and around 8% of the total number of cancer related deaths. Protein kinase C (PKC) isoforms have been shown to be strongly modulated in many androgen sensitive tumour cell lines. Increased expression of some PKC isoforms has also been linked to immortality in many cancer cell lines. Conjugated linoleic acids (CLAs) are naturally occurring positional and geometric isomers of the fatty acid linoleic acid (LA). CLAs have been shown to reduce growth and increase apoptosis in breast cancer cells, and to alter their PKC expression both in animal models and in various human cell lines.

We investigated the effect of CLA on the oestrogen sensitive MCF-7 cell line, and the oestrogen insensitive MDA-MB-231 cell line for 24 hours at two different concentrations of CLA (25μM and 50μM, optimum concentrations, previously shown in our lab). We measured the variations of three PKC isoforms (α, δ, and ι) in the cytosol (inactive form) and the membrane (active form), as well as the mRNA expression of each in MCF-7 after 12 hours of treatment at 50μM concentrations. The activation of PKC was measured by Western Blot analysis, using PKC isoform specific antibodies and β-actin, or Claudin-3 as the housekeeping protein. Results indicate that the CLA isoforms used (the 911, 1012 and a 50/50 mix of the two) cause a decrease in cell viability in both cell lines.

It was observed that the activation of PKC α was decreased in MCF-7 cells, compared to the control, while the activation of PKC δ and ι was increased. In the MDA-MB-231 cell line PKC ι activation was decreased at both concentrations, while PKC α and δ activation showed no particular pattern. mRNA expression of PKC α and ι was generally decreased after treatment with 50μM of CLA, while PKC δ mRNA expression increased. These effects are similar (but not identical) to the ones seen in prostate cancer cell lines (LNCaP and PC3), which have been previously reported by our lab.

It appears that CLA supplementation influences PKC isoform mRNA expression and protein activation in breast cancer cells. While the mRNA expression occurs in a pattern which appears to fit with that found in previous research the protein activation does not follow the trends suggested by work in other cell lines. It appears that the expression and activation of PKC isoforms are not just influenced by the interactions between CLA isoforms and the PKC genes in breast cancer cells.

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List of Abbreviations Used Throughout This Manuscript.

ΔH	Enthalpy of base stacking interactions.
ΔS	Entropy of base stacking.
ADP	Adenosine-5'-Diphosphate.
ANOVA	Analysis of variance, statistical test.
Apaf-1	Apoptotic protease activating factor-1.
Apc^{Min}	Adenomatosis Polyposis Coli (multiple intestinal neoplasia).
AP	Alkaline phosphatase.
APS	Ammonium Persulfate.
Asp	Aspartic Acid.
ATM	Ataxia Telangiectasia Mutated protein kinase.
Bad	Bcl2 antagonist of cell death.
Bax	Bcl2-associated X protein.
Bcl2	B-cell CLL/lymphoma 2.
Bcr-Abl	Fusion protein, common in chronic myelogenous leukemia.
BRCA1	breast cancer 1, early onset.
BRCA2	breast cancer 2, early onset.
BSA	Bovine Serum Albumin.
c	The molar concentration of primer.
*c*9-*t*11	*cis* 9 – *trans* 11 isomer of CLA.
Caco-2	Human epithelial colorectal adenocarcinoma cell line.
CAPK	Ceramide Activated Protein Kinase.
CCD	Charge-coupled device.
cip1	Cyclin-dependent kinase inhibitor 1A.

CLA	Conjugated Linoleic Acids.
CoC1	Ovarian cancer cell line.
CPE	*Clostridium perfringens* Enterotoxin.
DAG	Di-Acylglycerol.
DEPC	Diethylpyrocarbonate.
DGK	Di-Acylglycerol Kinases.
DHA	Docosahexaenoic Acid.
DMSO	Dimethyl Sulfoxide.
DTT	Dithiothreitol.
EPA	Eicosapentaenoic Acid.
ER	Oestrogen Receptors.
ERα	Oestrogen Receptor Alpha.
ERE_v	Oestrogen Response Element.
ERK	Extracellular Signal-Regulated Kinases.
FA	Fatty Acid.
FAME	Fatty Acid Methyl Ester.
FBS	Foetal Bovine Serum.
FFA	Free Fatty Acid.
FGFR2	Fibroblast growth factor receptor 2.
G_0	Cell quiescence.
G_1	The phase of the cell cycle in which enzymes are synthesised.
G_2	The phase of the cell cycle in which structural proteins are synthesised.
HeLa	Cervical cancer cell line.
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

HRP	Horseradish Peroxidase.
HRT	Hormone Replacement Therapy.
ICAD	DNA fragmentation factor, 45kDa, alpha.
ICE	Interleukin-1 Converting Enzyme.
IL-4	Interleukin 4.
IL-6	Interleukin 6.
IP_3	Inositol-1,4,5-Trisphosphate.
IR	Ionizing Radiation.
JAM	Junctional Adhesion Molecules.
JNK	Jun N-terminal kinase.
LA	Linoleic Acid.
LNCaP	Androgen-Sensitive Human Prostate Adenocarcinoma Cells.
LSP1	Lymphocyte-specific protein 1.
MAPK	Mitogen Activated Protein Kinase.
MAP3K1	Mitogen-activated protein kinase kinase kinase 1.
MCF-7	Oestrogen Receptor Positive Breast Cancer Cell Line.
MD-MBA-231	Oestrogen Receptor Negative Breast Cancer Cell Line.
M-Phase	The Meiotic Phase of the cell cycle.
MRK	Mixed lineage kinase Related Kinase.
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.
NF-κB	Nuclear Factor Kappa B.
NSCLC	Non Small Cell Lung Cancer.
OD	Optical Density.
p21	Cyclin-dependent kinase inhibitor 1A.

p53	Tumour protein 53.
Pak	p21 protein (Cdc42/Rac)-activated kinase 3.
PBS	Phosphate Buffered Saline.
PBS-T	Phosphate Buffered Saline with Tween-20.
PC-3	Androgen independent prostate cancer cell line.
PCR	Polymerase Chain Reaction.
PIP_2	Phosphatidyl Inositol-4,5-Bisphosphate.
PIP_3	3-Phosphoinositide.
PKA	Protein Kinase A.
PKC	Protein Kinase C.
PKD	Protein Kinase D.
PLC	Phospholipase C.
PMA	Phorbol-12-myristate-13-acetate.
PPARγ	Peroxisome Proliferator-Activated Receptor-Gamma.
PPRE	Peroxisome Proliferator Response Elements.
PS	Phosphatidylserine.
PTEN	phosphatase and tensin homolog.
PUFA	PolyUnsaturated Fatty Acid.
PVDF	Polyvinylidene Fluoride.
R	The Gas Constant (1.987).
RA	Rumenic Acid.
Rac1	Ras-related C3 botulinum toxin substrate 1.
RACK	Receptors for Activated C Kinase.
Raf1	v-raf-1 murine leukemia viral oncogene homolog 1.
Ras	Protein responsable for cell proliferation.

ROI	Reactive Oxygen Intermediates.
ROS	Reactive Oxygen Species.
RPMI	Roswell Park Memorial Institute medium.
RT-PCR	Reverse Transcription Polymerase Chain Reaction.
SDS	Sodium Dodecyl Sulphate.
SDS-PAGE	Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis.
S-Phase	The phase of the cell cycle in which DNA is replicated.
*t*10-*c*12	*Trans* 10 – *cis* 12 isomer of CLA.
T_a	Annealing Temperature.
TAG	Triacylglycerol.
TEP	Telomerase Associated Protein.
T_m	Melting Temperature.
TM4	Murine mammary tumour cells.
TM4t	Murine mammary tumour cells, expressing a mutant form of p53.
TNF-α	Tumour Necrosis Factor Alpha.
TNFR1	Tumour necrosis factor receptor 1.
TNFR2	Tumour necrosis factor receptor 2.
TPA	12-o-tetradecanoylphorbol myristic acetate.
TNRC9	TOX high mobility group box family member 3.
VEGF	Vascular endothelial growth factor.
waf1	Cyclin-dependent kinase inhibitor 1A.
WD-40	Short amino acid repeats, culminating in Trp-Asp
Wnt5A	Wingless-type MMTV integration site family, member 5A.

WY14,643	Pirinixic Acid.

Chapter 1.
Introduction

1. Introduction

1.1 Breast Cancer

1.1.1 Cancer and the cell cycle.

1.1.1.1 Cancer.

Cancer is generally described as malignant neoplasia, resulting in “an abnormal mass of tissue, the growth of which exceeds and is uncoordinated with that of the normal tissues, and persists in the same excessive manner after cessation of the stimulus which evoked the change." (Willis, 1952). Breast cancer can be defined as a malignant neoplasm, centered in the mammary tissue.

1.1.1.2 The Cell Cycle.

The cell cycle (Figure 1.1), sometimes called the cell-division cycle, may be described as a sequence of events leading to the replication of an eukaryotic cell. It is generally divided into two separate “phases”, Interphase, and M-Phase. Interphase can be described as the period in which the cell duplicates its DNA and organelles, and increases in size prior to division. M-Phase is the point at which the cell actually splits into two separate “daughter cells”. Interphase may be further subdivided into three distinct phases known as, G_1, S and G_2. Cells which have stopped dividing for any length of time are referred to as being Quiescent, or sometimes as being in G_0 Phase.

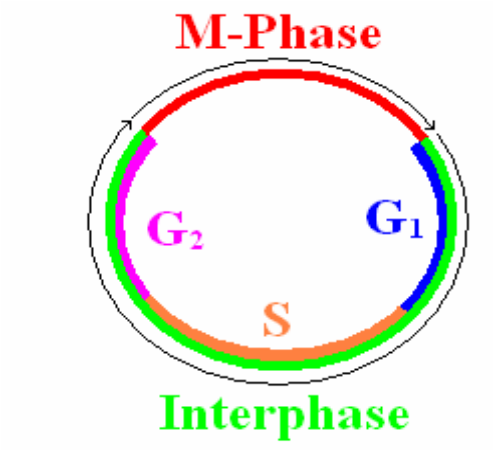


Figure 1.1 A Diagrammatic representation of the Cell Cycle.

G_1 Phase may be defined as the part of the cell cycle during which the cell processes prior to DNA replication occur. These processes include the increased cellular production of the various enzymes required for the replication of the cell.

S Phase is the period during which the DNA of the cell is fully replicated, and histone production is increased. This phase is often aberrant in cancer cells, with DNA not being fully replicated.

G_2 Phase is the point in the cell cycle in which microtubules and other structural proteins are replicated to allow two fully functioning cells to be created after M-Phase.

1.1.2 Breast Cancer Incidence and Patient Survival.

Breast cancer has a total incidence of 15%, the highest of any cancer type in the UK (Cancer Research UK, 2003), despite its relative rarity in males (294 new cases of male breast carcinoma were detected in 2001, compared with 40786 new cases in women). Breast cancer is responsible for around 8% of the total number of deaths from cancer every year (Cancer Research UK, 2007). Given the high incidence, this figure is relatively

low due to the increased understanding of the disease by healthcare professionals and a number of effective treatments available. However the disease may become resistant to the currently available treatments, and when this occurs any remaining treatment is mostly palliative.

1.1.3 Risk Factors for Developing Breast Cancer.

There are a number of risk factors, both biological and environmental, associated with the development of breast cancer which may be used in determining individual risk, these include the reproductive history of the patient, a family history of the disease, their diet, their bodyweight and physical activity as well as class status and socioeconomic factors. In developed countries around three quarters of breast cancer cases are observed in women with no family history of the disease, while only one quarter appear in women with a genetic predisposition (Key *et al.* 2001). Hereditary risk factors, observed in the incidence of cancer clustering within the immediate family, are typified by either the presence of one of 5 known high risk germline mutations (BRCA1, BRCA2, ATM, PTEN and p53), or by a large number of lesser polymorphisms (different phenotypes occurring within a population). The high risk mutations themselves account for only around 5% of the worldwide total of breast cancer, with the lesser polymorphisms contributing to another 20% (Key *et al.* 2001, Peto *et al.* 1999); statistics based on non selective, population based studies.

1.1.4 High Risk Breast Cancer Mutations.

The high risk germline mutations BRCA1 and BRCA2 are known to be responsible for breast and ovarian cancers (Peto *et al.* 1999). Another gene, Rap80, which appears to be required for normal copies of BRCA1 to fulfil their role in DNA repair is currently being

investigated (Wang *et al.* 2007). ATM (ataxia telangiectasia mutated protein kinase) mutations play a role in the inability of the cell to sense and repair double stranded DNA breaks (Prokopcova *et al.* 2006). Mutations of the tumour suppressor gene PTEN are frequent, and known to be involved in Cowden disease, a condition of which breast cancer is a major feature, as well as other heritable syndromes (Eng, 2003). Mutations of the p53 gene, which plays a vital role in repairing DNA damage, and activation programmed cell death, predispose the patient to early onset breast cancer, as well as a spectrum of other cancers. In particular p53 mutations have been linked to Li-Fraumeni syndrome, a multiple cancer syndrome causing many cancers of childhood (Chompret *et al.* 2000). A common feature of these high risk polymorphisms is that they all appear to play a role in the inability of a cell to repair double stranded DNA breaks.

1.1.5 Lower Risk Breast Cancer Mutations.

Research into the cumulative effects of lower risk polymorphisms is still at an early stage (Key *et al.* 2001). Meta analysis of 46 published studies examining the effects of 18 polymorphisms showed only one allele which consistently appeared to have a functional importance (Dunning *et al.* 1999), proving the need for further work in this area. Recently however four of the lower risk polymorphisms have been positively identified, FGFR2, TNRC9, MAP3K1 and LSP1 (Easton *et al.* 2007). With the exception of LSP1, which regulates nutrophil motility, the other low risk polymorphisms are known to all play roles in the regulation of the cell cycle, although they act in very different ways.

1.1.6 Environmental (Non Genetic) Risk Factors for Developing Breast Cancer.

The environmental risk factors, which make up the larger number of cases, are many and varied. Currently there is considerable interest in the effects of endogenous hormones on

the chances of an individual developing breast cancer. In fact, naturally occurring steroid hormones, especially isoforms of oestrogen, are known to influence the normal growth of breast tissue, but they have also been implicated in the transformed growth of breast cancers. In western countries, endogenous hormones are usually found in three forms, as the oral contraceptive pill, in various fertility drugs and as hormone replacement therapy (HRT). Due to the prevalence of these medicines, their safety (or lack thereof) has become a matter of much public interest.

HRT is usually used to alleviate the more unpleasant effects of the menopause in women over 55, although it may also be administered to those who suffer from an early menopause. In a reanalysis of prospective studies on endogenous sex hormones and breast cancer, by the endogenous hormones and breast cancer collaborative group, it was shown that women with non-inherited breast cancer have, in general, a higher serum concentration of sex hormones (included in the study were esterone, testosterone and estradiol) than healthy postmenopausal subjects (Key *et al.* 2002) This correlation across nine separate studies is highly statistically significant. Long-term use of HRT has also been shown to increase nodularity of the breast, as well as the number of fibroadenomas and cysts. Moreover, if the breast already contains established lesions, HRT has been shown to increase the growth rate of these. Breast density in healthy women going through the menopause decreases and studies examining mammograms (which provide the best estimate of breast density) show considerable variation in breast density between women of the same age in industrialised countries. Women with higher breast density are more likely to develop breast cancer according to a large number of studies. Indeed, according to White, in a news article for the Journal of the National Cancer Institute in 2000 this likelihood is of the order of four to six times greater than women with lower

breast densities. White also observes that the only risk factors which have been shown to increase cancer likelihood more are age and mutations of the BRCA1 and 2 genes (White, 2000). The role which diet plays in determining breast density is controversial. This is partially because of the interest which genetic and hormonal factors have excited among the research community, leading to greater exploration, at the expense of research into nutritional causes. It may also be because meta-analysis performed on the data available from both interventional and epidemiological studies fails to support the prevailing hypothesis that increased fat intake leads to increased breast density and higher cancer risk (Hanf and Gonder, 2005). Meta analyses of case control studies have shown that there is a significant and positive association between high saturated fat in the diet and the development of breast cancer (Howe *et al.* 1990).

1.1.7 Oestrogen and Breast Cancer.

Oestrogen is a known promoter of growth and survival (although it is also implicated in the regulation of transport across the epithelia of the reproductive tract) in some forms of breast cancer, binding to the oestrogen receptors (ER's) which, in post-menopausal women, are observed in approximately 75% of breast tumours (Cancer Research UK, 2003). It is the highly proliferative response of ER positive tumours to oestrogen which is the basis for anti-oestrogen therapies to treat breast cancer. In fact, the selective oestrogen receptor modulator Tamoxifen is the current treatment of choice for tumours exhibiting ER's: in the presence of oestrogen, it binds competitively to the receptors, thus preventing the mitogenic effect of oestrogen. Interestingly, in cultured MCF-7 cells, when oestrogen is absent, Tamoxifen exhibits an agonistic effect (Sporn *et al.* 2001).

1.1.8 Oestrogen Receptors and Cell Processes.

It has been shown that ER's, both nuclear and membrane bound, promote the up regulation of genes promoting long term cell survival, the most notable example of which being Bcl2 (Pedram *et al.* 2006). ER's are also found bound to mitochondrial membranes, and it is postulated that, when activated, they have an impact on the function of the mitochondria. The novel functions of these receptors, when in complex with Estrodiol (E_2), are thought to aid the survival of ER positive tumours (Pedram *et al.* 2006). When a cell is "stressed" (usually by ionising radiation), the mitochondria generate ROS, which, being strongly oxidative, can cause damage and mutation to the structures and DNA of the cell. E_2 has been shown to prevent ROS formation by the mitochondria in ER positive cancer cell lines in a dose responsive manner (Pedram *et al.* 2006), although E_2 isoforms, and other steroid hormones (notably progesterone and testosterone) did not exhibit the same effect.

Currently, there are two cell lines which are commonly used as models for ER positive (ER+) and ER negative (ER-) tumours. In their normal state, these are sensitive to the chemotoxic agents used against breast tumours although other lines have been produced from them which exhibit greater or lesser degrees of resistance to various chemicals.

1.1.9 Fatty Acids and Breast Cancer.

Several fatty acids (FAs) have been shown to exert different effects on breast cancer cells, both in culture and on induced mammary tumours in rodents. Of the FAs which appear to exert a beneficial effect by reducing tumour size and enhancing apoptosis in cancerous cells the most studied are the groups known as Omega-3s and Conjugated Linoleic acids (Wahle *et al.* 2004).

1.2 Linoleic Acid, Conjugated Linoleic Acid, and Their Effects on Human Health.

1.2.1 Linoleic Acid.

Linoleic acid (LA) is a common octadecadienoic fatty acid (also called cis-9:cis-12 octadecadienoic acid-18:2n-6) found in a few plant species and in ruminant meat and milk products. LA is required in mammalian systems as the precursor of longer-chain n-6 PUFA (Polyunsaturated fatty acids, containing more than one double bond) that in turn are the more immediate precursors for the biosynthesis of the C-20 eicosanoids, however, it is an essential fatty acid that cannot be synthesised in the human body, its dietary intake is therefore essential. A joint expert report, commissioned by the World Health Organisation, and the UN Food and Agriculture Organisation suggested that an LA intake of between 2-3% of total dietary energy is probably enough to prevent deficiency (Joint Experts. Fats and Oils in Human Nutrition, Report of a Joint Expert Consultation. WHO 1994). LA is also known to be important in the regulation of blood pressure. In addition to this, replacement of saturated fat in the diet with LA has been shown to reduce the concentration of cholesterol in the blood, and also to reduce the risk of coronary heart disease. In a meta-analysis of the literature (Zock and Katan, 1998) it was concluded that there was no increased risk of breast, colorectal or prostate cancer with long term consumption of LA despite the fact that high, long term LA consumption is linked to the development of these cancers in various animal models.

1.2.2 Modification of Linoleic Acid to Conjugated Linoleic Acid.

LA may be modified in many ways to form various positional isomers, including conjugated linoleic acids, commonly known as CLA's (see figure 1.2).

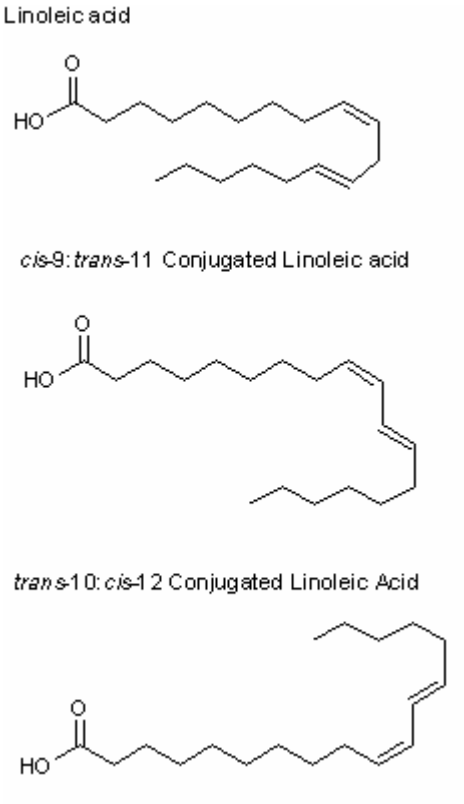


Figure 1.2: Structures of the two most naturally abundant CLA isoforms (*t*10-*c*12 and *c*9-*t*11) compared with linoleic acid.

CLA's are a group of around 28 isomers of LA with conjugated double bonds at various positions along the fatty acid chain, the main form being the *cis*-9:*trans*-11 isomer which is found in the greatest abundance in ruminant fatty tissue and milk (90% of the total CLA content). Other isomers, of LA, including the *trans*-10:*cis*-12 form are also observed, but make up a much lower portion of the total CLA compliment (Jensen, 2002). The isomers of CLA are found naturally in some plants or formed in ruminant animals by a process of bio-hydrogenation, which is facilitated by microbial anaerobic fermentation. This process turns polyunsaturated fatty acids (PUFA) from the animal's food, in particular plant matter, into saturated fats, but incomplete hydrogenation results in positional and geometric isomers of LA, a process which takes place in the rumen. CLA can also be produced commercially by partial hydrogenation of LA or by heating LA under alkaline conditions. The first step in the process of bio-hydrogenation is the transposition of the

delta 12 double bond of PUFA to form the *cis*-9:*trans*-11 isomer, often called rumenic acid (RA). RA can also be formed by the oxidation (introduction of a double bond) of *trans*-11vaccenic acid by delta-9 desaturase; these two routes of RA formation probably contribute to its natural abundance. Further isomerisation and transposition of RA leads to the formation of other, less abundant isomers, such as the *trans*-10:*cis*-12 isomer, which exhibits some anticancer effects (Parodi, 2003).

1.2.3 CLA Production by Ruminants.

The way in which ruminants are fed has a large impact on their CLA production and tissue concentration. It has been shown that cattle fed with oil rich corn or soy bean supplements have a higher concentration of CLA found in the fatty tissue than cattle fed on silage without any supplements (Beaulieu *et al.* 2002, Duckett *et al.* 2002). It has also been observed that the fatty tissue and milk of pasture fed cattle have higher concentrations of muscle- based CLA than feedlot (silage fed) cattle, although the pattern of CLA distribution in the muscle fat is very different between the pasture fed, and silage fed groups (Rule *et al.* 2002).

1.2.4 CLA in Plants.

CLA is found in some natural seed oils, including the aforementioned soy bean oil and pomegranate seed oil (as well as Safflower oil, from which most available over the counter preparations are derived), but is not present in the commercially produced vegetable oils used in cooking and the production of margarine products. However, the catalytic hydrogenation and refining process that these oils go through causes some limited CLA production (0.1-0.7mg/g), quantities which are negligible. It is also worth

noting that in making these “low fat” products much of the CLA present is removed (Parodi *et al.* 2003).

1.3 CLA intake in Human populations

1.3.1 CLA Intake in Food.

Edible oils, ruminant milk and ruminant meat products make up the main intake of CLA by humans, although consumption of these varies greatly within different populations. For example, Australian populations show estimated daily intakes of CLA in the region of 1500mg although it is possible that this value is due to the consumption of wallaby meat, which contains the highest natural concentration of CLA observed so far. In comparison, German people have an estimated intake of between 246 and 430mg/g per day although the methods used to assess daily intake were different for both populations (Parodi *et al.* 2003). In humans, dietary CLA can be detected in the plasma and lymph fluid and is known to have a positive effect on various conditions, including type II diabetes, obesity, arthrosclerosis and some cancers. These effects are usually protective, and appear to prevent, or delay the more serious problems associated with these conditions.

Clinical trials involving CLA in humans have so far been limited to looking at the effects of the abundant *c*9:*t*11 and *t*10:*c*12 isomers on body fat and metabolism, insulin production and atherosclerosis. Thus far, some show results similar to those seen in rats and mice fed with a high concentration of CLA (Doyle *et al.* 2005). Results can vary within the population of one species, depending on age, gender, fitness and the genotype of the subject. However, in animal models it is much easier to control these variables, which may account for the differences between the human and animal studies of CLA. One human study which has yielded positive results looked at the effect of a mix of predominantly *c*9: *t*11 and *t*10: *c*12 CLA isomers had on the body weight of patients with type II diabetes (Belury *et al.* 2003). A significant number of the subjects taking a CLA

supplement rather than the placebo showed an inverse correlation between both body weight and blood plasma levels of CLA, was observed.

1.3.2 CLA Absorption.

The absorption of CLA, both in the form of Free Fatty Acids (FFAs) and Triacylglycerols (TAGs) has been shown to be similar in human and animal studies. The most common form taken by dietary supplements is that of FFAs. However, in studies carried out on young male Wistar rats, which are, to some extent duplicated in humans, there appears to be no significant advantage of one form over the other with equal absorption and incorporation into tissues. Similar results have been seen in studies using fish oils, with no significant difference in uptake between the isomers EPA and DHA (Eicosapentaenoic Acid, and Docosahexaenoic Acid). It is however worthy of mention that CLA in the form of Fatty Acid Methyl Esters (FAMEs) are less readily absorbed by the tissues (Plourde *et al.* 2006).

1.4 CLA and disease states

1.4.1 Chronic Diseases.

Chronic diseases such as diabetes, obesity, cardiovascular disease and cancer are becoming an important socio-economic problem in many industrialised countries. In the UK, this problem is exacerbated by an ageing workforce, leading to many man hours being lost due to these diseases. The mechanisms underlying these problems have not yet been fully elucidated, however it is known that those with a sedentary lifestyle and a calorie rich diet are at most risk of developing one or more of these problems at some point during their lifetime. It has been suggested that dietary fatty acids could have an effect in protecting the body against the onset of some of these diseases. This would obviously provide many benefits to society and is a large and intensive area of research.

1.4.2 Obesity and Cancer Risk.

There is a strong link between obesity and the risk of developing certain types of cancer, in particular colorectal and premenopausal breast cancer. Obesity is commonly connected with high saturated fat consumption, leading to a higher energy intake than energy used by the body. However, animal model studies have shown that increased CLA intake actually decreases adiposity in rats, mice, hamsters and pigs (Wang and Jones, 2004). The effect of CLA on carcinogenesis has been similarly demonstrated, both in animal models and *in vitro*, although there have been no experiments proving its efficacy in humans *in vivo* (Ip *et.al* 1999, Song *et.al* 2004).

1.4.3 Animal Studies on CLA Treatment and Tumour Size.

In animal models, especially rats, mammary tumours are either induced chemically or human xenografts are implanted surgically and allowed to grow and spread. The diet of

the animals is either an unsupplemented control, or enriched with CLA or other fatty acids from various sources. The animals are humanely killed and the tumours surgically excised and compared (Dauchy *et al.* 2004, Belury, 2002, Kohno *et al.* 2004, and Kohno *et al.* 2004). Animal studies show that not only did those rats fed a diet high in CLA have a smaller tumour weight and less metastasis, but they were also less likely to develop tumours in the first place.

1.4.3.1 CLA in Animal Models

It is well reported that supplementation with CLA in animal models decreases tumorogenisis. It appears not only to decrease mammary tumour size and weight in severe combined immunodeficient mice (SCID mice) inoculated with MDA-MB-468 cells, but also it reduces the metastatic spread of breast cancer cells to other parts of the body (Visonneau *et.al* 1997). The MDA-MB-468 cell line is synonymous with the MDA-MB-231 cell line used throughout this research. It is likely that SCID mice inoculated with the MDA-MB-231 cell line would exhibit a similar decrease in tumourogenisis. Feed including 20% CLA enriched butter fat has been shown to down-regulate the morphologic maturation of mammary epithelium in female Sprague-Dawley rats after a treatment period of one month (Ip *et.al* 1999). This study showed no impact on the overall growth of the rats fed with the high CLA feed compared with control, but did show a less complex network of ductal-alveolar branching within the mammary glands. Treated rats had, on average a 25% lower mammary epithelial mass, than the control group, but no difference in the total area of the mammary fat pad. After one month feeding with the enriched food the rats were given a single dose of methylnitrosourea (MNU, a known inducer of mammary carcinogenesis) and the incidence and yield of the resultant tumours were measured. In this incidence the overall reduction in mammary carcinoma risk

decreased by 50%, and the protection conferred appeared to be long lasting as no additional CLA was supplied after treatment with MNU. In addition the rats were fed with CLA enriched butter from both naturally occurring and artificial sources, the effect of the different types of CLA appeared to be negligible with regards to mammary epithelial mass, and modulation of mammary cancer risk, although some differences were observed in CLA uptake into tissues. In 1997 Thompson *et al.* observed similar results in female Sprague-Dawley rats fed with a diet supplemented with 1% CLA and treated with the carcinogen 7,12-dimethylbenz(α)anthracene suggesting a more universal role for chemoprotection by CLA.

1.4.4 CLA studies on Cells In Vitro.

In vitro studies on various cell lines, both from breast cancers and other tumour types support the evidence presented by animal studies. Cancer cells, most notably prostate, breast and colonic, incubated with various concentrations of CLA showed decreased cell viability, cellular proliferation and increased apoptosis (Dugram and Fernandes, 1997, Majumda *et al.* 2002, Song *et al.* 2004, Field and Schley, 2004, and Bocca *et al.* 2006). Moreover, leukaemia cells show evidence of CLA incorporation into the cell membrane, possibly leading to increased apoptosis (Agatha *et al.* 2004). CLA is also shown to differentially regulate the expression of various proto-oncogenes, including p53, p21 and Bcl-2 (Majumder *et al.* 2002). However in some animal models it has been shown to have no effect on carcinogenesis, and some isoforms may even act as a promoter of tumour growth, especially in the Apc^{Min} mouse (Mandir and Goodlad, 2008).

1.4.5 Fatty Acids and Tumours, Potential Mechanisms of Action.

The mechanism by which fatty acids act to inhibit the growth of tumour cells is, as yet, still unknown although it has been hypothesised that the effect occurs because the fatty acids interfere with components of the cell cycle controlling cell replication (although some authors disagree, notably Pedram *et al.* 2006). It is possible that the tumour cells slow down in the S-Phase of the cell cycle, as it appears that treatment with fish oil at physiologically active concentrations (5% w/w of the total fat content in the diet) in rats causes an increase in the time taken for DNA replication in implanted tumour cells (Robinson *et al.* 2001). The work done in this area so far suggests that this temporary cell cycle arrest may be caused by the activation/inactivation of key proteins, including the aforementioned p53, p21 cip1/waf1 and cyclins (Field and Schley, 2004). How this regulation is actually effected, however, remains a mystery. Other proposed mechanisms of action include: changes to the function and structure of the cell membranes, altering their affinity for protein binding, immune development, increasing oxidative stress on the cells and changes in gene expression (Field and Schley, 2004).

1.4.6 CLA and Cell Death.

1.4.6.1 Programmed Cell Death (Apoptosis).

Apoptosis may be defined as a process leading to specific morphology and death in eukaryotic cells. The principal of apoptosis was first described in 1842 by Carl Vogt, and described more precisely in 1885 by William Flemming. In 1965 John Kerr described a process which at the time he called “Shrinkage necrosis”, in work looking at liver damage in rats. Kerr subsequently described a set of unique morphological changes linked to this form of cell death. Further research, with increasingly complex tools suggests that

apoptosis is the result of a carefully balanced interplay between the various cell signalling pathways (O'Rourke and Ellem, 2000).

Apoptosis occurs when cells are undergoing programmed remodelling, for example the differentiation of fingers and toes during embryo development, or after catastrophic DNA or cellular damage caused by mutagenic chemicals or radiation, or by viral infection or starvation. In healthy subjects, cell proliferation and apoptosis form a careful balance, however, too much cell death leads to ischemic disorders, while cells gaining immortality leads to cancers.

Apoptosis is controlled by a range of external and internal cell signals (inducers). Internal inducers are triggered by cellular stress, including, but not limited to starvation, extreme temperature fluctuations, and hypoxia. External inducers may be in the form of toxins (including those used as chemotherapeutic drugs), hormones, or growth factors, and require either internalisation, or signal transduction to effect a response.

Once apoptosis has been triggered the cell goes through several distinct stages before being engulfed by phagocytes to ensure that no potentially harmful intracellular substances are released into the surrounding tissue (see Fig 1.3, page 19). In the first stage the apoptotic signal is transmitted throughout the cell by a series of regulatory proteins, which allow termination of the signal should the death of the cell no longer be required.
The two main methods of apoptotic regulation so far discovered are transduction via adaptor proteins, and mitochondrial targeting. One method of direct signal transduction is via Tumour Necrosis Factor (TNF) receptors. The cytokine TNF is a major factor in apoptotic regulation in eukaryotes, and it is known to bind to two separate receptors in

human cells. Tumour necrosis factor receptor 1 (TNFR1) binding by TNF induces apoptosis, while binding to TNFR2 (tumour necrosis factor receptor 2) prevents apoptosis. The binding of these apoptotic regulators causes changes in the relative levels of the apoptotic regulatory proteins, Bcl2, Bax and Bad.

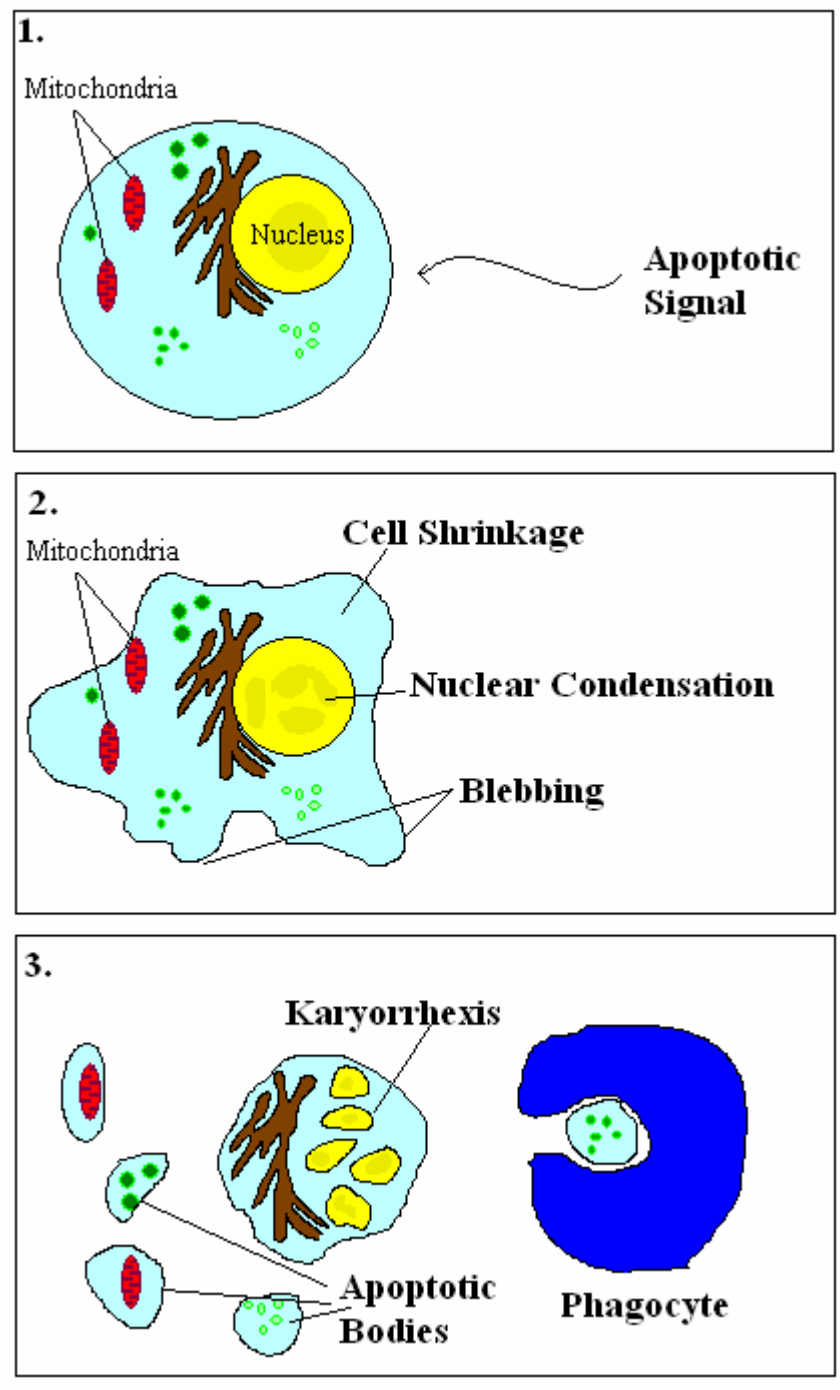


Figure 1.3. The three main stages of Apoptosis, 1. Initiation, 2. Cell Shrinkage, 3. Cell Breaking, and Karyorrhexis.

The second method also involves changes in the Bcl2/Bax/Bad balance within the cell. Cellular stresses decrease Bcl2, and increase Bax and Bad production, it is possible to measure how stressed a group of cells are by the relative amounts of these three proteins. Bcl2 is an inhibitor of apoptosis and is localised to the outer membrane of mitochondria, where it blocks caspase activation. When Bcl2 is inactivated by Bax and Bad the mitochondrial membrane becomes permeable, forming mitochondrial apoptosis induced channels, allowing cytochrome c, and other apoptotic proteins from the mitochondria to escape into the cytosol. This cytochrome c forms a complex with the protein apoptotic protease activating factor-1 (Apaf-1), which in turn bind to other Apaf-1/cytochrome c complexes to form structures sometimes known as "apoptosomes". In turn the apoptosomes bind to caspase-9, causing activation and triggering a caspase signalling cascade. The caspases triggered by this process degrade structural proteins within the cell, leading to the characteristic shrinking and "blebbing" as the lamin and actin filiments in the cytoskeleton are degraded, and the cell loses integrity. Furthermore, the DNA within the nucleus condenses, and the nuclear membrane degrades, a process known as Karyorrhexis. Finally the cell breaks down further into apoptotic bodies, surrounded by a membrane envelope, and tagged with phagocytotic molecules (for example Phosphatidyl Serene) allowing digestion by phagocytes (Potten and Wilson, 2004).

1.4.6.2 Cell Death and CLA Supplimentation.

The suggestion that CLA and other n-3 fatty acids cause tumour cells to undergo necrotic cell death due to increased oxidative stress is based on the idea that tumour cells have insufficient antioxidant defence systems compared to healthy cells. During lipid peroxidation, the main substrates within the cell are the Polyunsaturated Fatty Acids (PUFAs). Therefore, reactive compounds derived from PUFAs could cause intracellular

changes to the cell leading to necrosis. It does appear that spermatozoa enriched with unesterified PUFA have a higher incidence of lipid peroxidation and induction of reactive oxygen species (ROS) (Aitken *et al.* 2006).

1.4.7 CLA and Cell Viability.

In the human colorectal cancer cell line Caco-2, concentrations of CLA above 1μM had some effect on the viability of cells after up to 72 hours incubation (Bocca *et al.* 2006). Repeated doses of CLA also appeared to reduce the total cell viability compared to single doses for the same duration (74% reduction of growth for multiple doses over 72 hours, compared to 66% reduction of growth from a single dose) indicating a potential for repeated treatments leading to tumour shrinkage in animal and human models.

1.5 CLA and Breast Cancer

It is interesting to note that the advice commonly given to women undergoing treatment for, or recovering from, breast tumours is to avoid both dairy products and red meat, although it is still unclear how this helps recovery and reduces the likelihood of recurrence. Diet, as a factor influencing breast cancer development, is currently under intense scrutiny, as is discovering the specific dietary components that increase or decrease the chances of an individual developing a tumour. These approaches could be significant, both from a health and an economic perspective.

Different patterns of breast cancer incidence across different populations with different dietary habits provide the best current evidence for a diet based component in the risk of developing breast tumours. CLA is known to promote cell death in the weakly metastatic and ER+ MCF-7 cell line (>60% inhibition of growth), even at low concentrations (25μM) although it appears to have little or no effect on the strongly metastatic and ER- MDA-MB-231 line (<5% inhibition of growth) (Tanmahasamut *et al.* 2004). The addition of CLA also has a direct action on oestrogen signalling, both by decreasing ERα receptor expression and by suppressing the active binding of the oestrogen response element (ERE_v) to the ERα receptor. The decrease in receptor expression appears to be linked to a decrease in ERα mRNA, linked to a decrease in the protein itself, as confirmed by Western Blot analysis (Tanmahasamut *et al.* 2004).

1.6 Protein Kinase C isoforms

1.6.1 The PKC Family.

The protein kinase family are implicated in many intracellular signalling pathways, sometimes exhibiting opposing functions, including the signalling cascades, which trigger cell division. PKC is a subfamily of serine/threonine kinases comprising 12 known isoforms. These isoforms can, in turn, be split into three categories, typical PKC's (α, βI, βII, and γ), novel PKC's (δ, ε, η, θ, and μ) and atypical PKC's (ζ, and ι/λ) based on the biochemical properties of each isoform. The hallmark of the activation of all the PKC isoforms is the translocation of the protein from the cytoplasm to the cellular membrane. The typical and novel isoforms of PKC are known to be dependant on the presence of phosphatidylserine (PS) for activation, these isoforms also require allosteric activation, either by diacylglycerol (DAG, a lipid-derived second messenger, generated in the plasma membrane), or by negatively charged phospholipids. The atypical PKC isoforms, however, require neither PS nor DAG for activation; PS is still capable of activating these isoforms, but it is not a necessary step in the activation process and can be replicated by the addition of phosphatidylethanolamine. The atypical PKCs may be regulated by 3-phosphoinositides (PIP_3, Nakanishi *et al.* 1993). If prolonged PKC activity is being observed *in vitro,* the extensively studied group of tumour promoters known as phorbol esters (2 orders of magnitude more potent than DAG) also act as activators of PKC (Newton AC, 1995) for recruitment to the plasma membrane. DAG exerts its effect by increasing the affinity between the PKC isoforms and the PS enriched areas of the cell membrane; this causes a conformational change which serves to remove the pseudosubstrate domain from the binding site, thus activating PKC (Carter, 2000). The typical PKCs also require calcium ions to induce maximal activation, C standing for calcium in PKC and the first isoforms to be differentiated was named PKC α. If no

calcium or DAG is present within the cell then the typical PKCs are still capable of interacting with the plasma membrane, but these interactions are, at best, weak and transitory (Steinberg, 2004). DAG is produced by the turnover of phosphatidylinositol by various cellular receptors, including p21, Wnt5A (*via* G-protein coupled receptors, also leading to a rise in intracellular Ca^{2+}, Dissanayake *et al.* 2007), and receptors in the *ras* pathway, and upon activation by extracellular factors. Anderson and Welsh (1990) suggested that phospholipase C (PLC), bradykinin, isoproteranol and adenosine 3',5'-cyclic monophosphate (cAMP) are the main ones, with PLC and bradykinin stimulating membrane bound phosphatidylinositol hydrolysis, and cAMP and isoproteranol acting on a separate, unknown source. For this reason, PKC is believed to play an important role in signal transduction. It was observed by Newton in 1997 that when PLC hydrolyses phosphatidylinositol, specifically phosphatidylinositol-4,5-bisphosphate, it produces intracellular inositol-1,4,5-trisphosphate in addition to DAG. It is this inositol-1,4,5-trisphosphate which binds to intracellular IP_3 receptors to cause the increase in intracellular Ca^{2+} ions which facilitate the preferential binding of typical PKC isoforms to the plasma membrane (Newton, 1997).

1.6.2 The PKC Isoforms.

In 1995, Ford *et al.* looked at differential expression of PKC isoforms in various tissue types of the horseshoe crab, *Limulus polyphemus*. These experiments appeared to suggest that certain isoforms are abundantly present in different cell types: this, therefore, suggests that a unique function is performed by each isoenzyme (Ford *et al.* 1995, See Table 1.1).

Table 1.1 : The PKC isoforms and their effects

PKC isoform	**Effect on the cell**
PKC α	Enhances cell proliferation, anti apoptotic, increased multi drug resistance (Leszczynski *et al.* 1996)
PKC βI	Increased blood vessel growth and leakage, enhances abnormal VEGF signalling (Pass *et al.* 2001)
PKC βII	Increased blood vessel growth and leakage, transmits hypertrophic signals in the myocardium (Pass *et al.* 2001)
PKC γ	Only expressed in brain and neuronal tissues, implicated in neuronal function (Saito and Shirai, 2002)
PKC δ	Reduced cell growth, reduced cell motility, reduced cell morphology, pro apoptotic (Mischak *et al.* 1993)
PKC ε	Enhances cell proliferation, anti apoptotic, desensitisation to TNFα (Mischak *et al.* 1993)
PKC η	Aberrant morphology, increased tumour metastasis, anti apoptotic (Masso-Welch *et al.* 2001)
PKC μ	Pro-apoptotic (Nakajima, 2006)
PKC θ	Pro-apoptotic (Nakajima, 2006)
PKC ι	Anti-apoptotic, up-regulates NF-κB and activates pathways essential for transformed cell growth, linked to determining cellular polarity (Lu *et al.* 2001)
PKC λ	Anti-apoptotic, up-regulates NF-κB and activates pathways essential for transformed cell growth, linked to determining cellular polarity (Lu *et al.* 2001)
PKC ζ	Anti–apoptotic and implicated in multi drug resistance (Jiang *et al.* 2000)

For example, over expression of PKC α and ε appear to be involved in enhanced cell proliferation (Leszczynski *et al.* 1996, Mischak *et al.* 1993) whereas over expression of PKC δ causes a slowing of cell growth and a reduction of cell density, as well as significant changes in morphology (Mischak *et al.* 1993). These effects are isoform and cell type specific, but also appear to depend on context, with isoforms exhibiting both pro- and anti-apoptotic properties in response to different cellular events or insults. It has been postulated that the presence of multiple PKC isoforms in a single cell type is indicative of a unique, rather than overlapping, function assigned to each isoform (Carter, 2000). The downstream signalling pathways of PKC are less well characterised although it is known that the main signal cascade activated by PKC is the MERK-ERK. PKCα has

been shown to activate Raf1, which is another Serine-Threonine kinase. Raf1 is also an activator of MERK-ERK. The activation of Raf1 by the PKCα in turn activates MRK1 and 2 by phosphorylation and triggers the mitogen activated protein kinase (MAPK) cascade. Once activated, the MAPK cascade induces the transcription of genes involved in cell division and precipitates the cell into S-phase (Mackay and Twelves, 2003). PKC ζ is activated by the second messenger protein ceramide and may also be implicated, along with a host of other ceramide activated protein kinases (CAPKs) in cellular stress response pathways. Specifically PKC ζ appears to activate Jun N-terminal kinase (JNK) proteins, involved in cell response to cellular stress (see figure 1.4 for a diagrammatic representation of the PKC pathway) (Herr and Debatin, 2001).

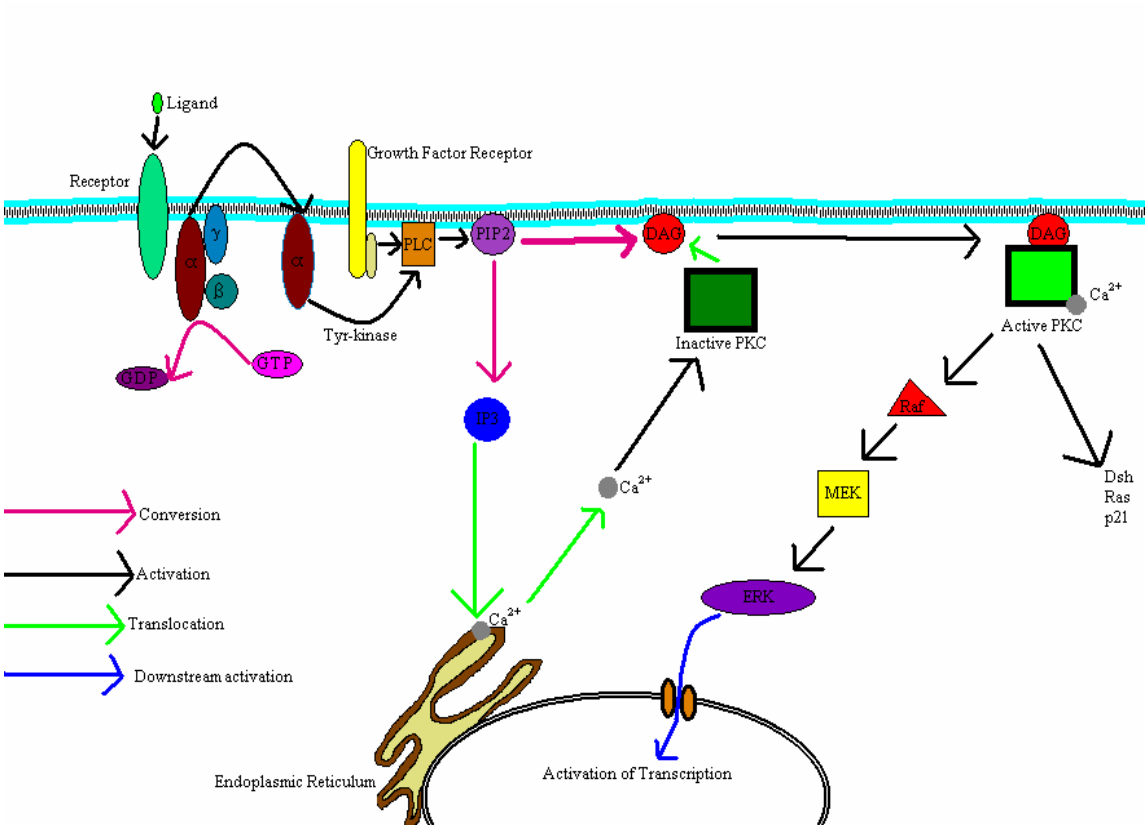


Figure 1.4 A diagrammatic view of general PKC activation and a few downstream effects of activated PKC.
Key: GDP- guanosine-5'-diphosphate; GTP- guanosine-5'-triphosphate; PLC- Phospholipase C; PIP2- Phosphatidyl inositol-4,5-bisphosphate; IP3- inositol-1,4,5-trisphosphate; DAG- Diacylglycerol.

There is also evidence that PKC signalling cascades are implicated in early and late stage of fertilization events, at least in mammalian egg cells, probably due to DAG production *via* oscillations in cellular Ca^{+} although direct evidence of this is lacking (Halet, 2004).

1.6.3 PKC in Breast Cancer Cells.

In the oestrogen positive MCF-7 breast cancer cell line, three universally expressed PKC isoforms have been detected, (α, δ, and ζ) as well as PKC ε and η (Basu *et al.* 2002, de Vente *et al.* 1995, Soh *et al.* 2003, Lavie *et al.* 1998, Kambhampati *et al.* 2003 and Masso-Welch *et al.* 2001). Cells over-expressing PKC α and ε are shown to proliferate at a higher rate than the parental cells (de Vente *et al.* 1995), to have an increased multi-drug resistance (greater expression of PKCα) and, where PKCε is over-expressed, to have a desensitisation to tumour necrosis factor (TNF). Nuclear localisation of PKC δ is required for apoptosis (DeVries *et al.* 2002), also cells which over-express PKC ζ are expected to be apoptotic (Jiang *et al.* 2000), while cells which over express PKC η are shown to have an aberrant morphology and, it is hypothesised, that PKC η is implicated in tumour metastasis (Masso-Welch *et al.* 2001) (see table 1.1, page 25, for the effects of different PKC isoforms).

1.6.4 Structure of PKC.

1.6.4.1 The PKC Domains.

PKC has been shown to be a single polypeptide chain formed of two structurally distinct regions. The 45kDa C-terminus contains the highly conserved catalytic domain and shows, mostly, an *α*-helical conformation. On the other hand, the 20-70kDa N-terminus region contains the regulatory, and co-factor binding domain and a *β*-pleated sheet structure. These two domains are separated by a hinge region which, during activation of the enzyme, becomes proteolytically labile (Newton, 1995). In the 1980's, the cloning of

the α, β, and γ PKC isoforms identified in human and bovine tissue revealed four highly conserved domains of the protein sequence interspersed with variable regions (Coussens *et al.* 1986). These domains are now referred to as C1-C4. The C1, C3 and C4 domains are found in all identified PKC isoforms, although the atypical PKCs have a slightly differing structure (Newton, 1995, Figure 1.5).

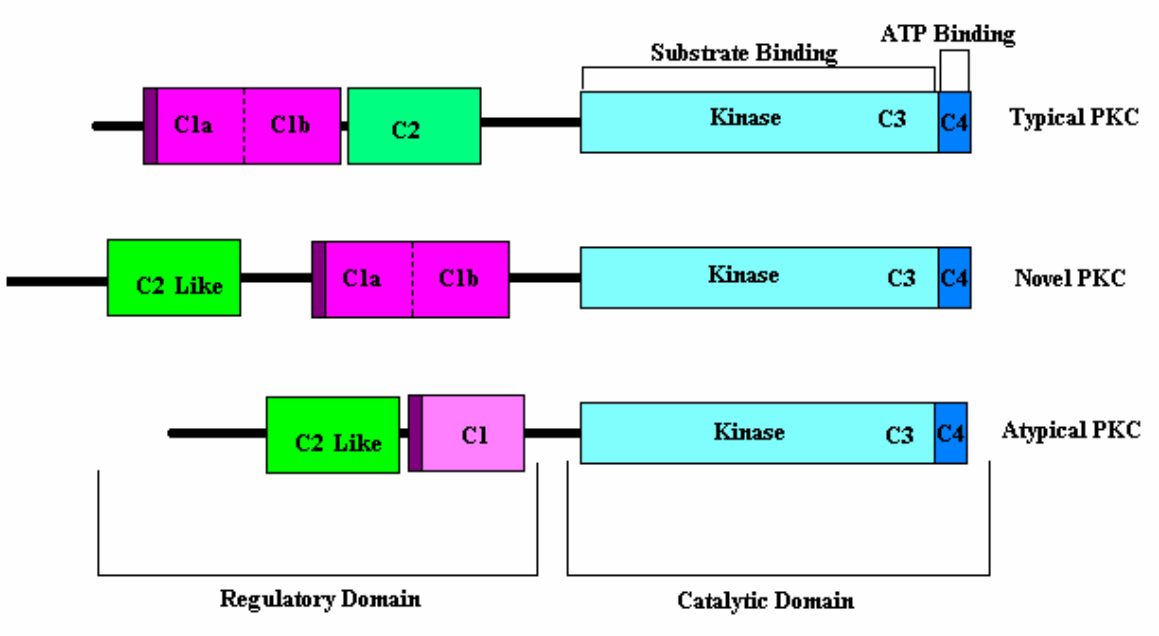


Figure 1.5. Diagrammatic representations of the three classes of PKC isoform showing the various domains.

Typical and Novel PKCs contain two tandem C1 domains (commonly referred to as C1a and C1b), while atypical PKCs only contain one which lacks the ability to bind to DAG. The C2 domain, which appears to confer the calcium, and acidic phospholipid binding properties to the molecule, is only found in the typical and novel PKC isoforms (Burns and Bell, 1991), although, in novel PKCs, it lacks the residues which allow it to bind to Ca^{2+} ions. All these domains have a specific function, established by mutational and biochemical analysis. Moreover, also found in the C2 domain, are areas for interaction with receptors for activated C kinase (RACK). It is believed that RACKs play a role in the translocation of the activated PKC from the cytosol to the plasma membrane. RACK may

also be important modulators of PKC activity, probably through alterations to the regulatory sites by phosphorylation (da Rocha *et al. 2002*).

1.6.4.2 The C1 Domain.

The C1 domain is prefaced by an auto inhibitory pseudo substrate sequence between amino acids 19 and 36 which resembles a substrate phosphorylation site, thought to be responsible for maintaining the isoform in its inactive state (House and Kemp, 1987). The C1 domain itself is a region of approximately 50 amino acids approximately 8kDa in weight, and contains a double cystine rich motif (in atypical PKCs, there is only a single one of these motifs) containing a pair of Zn^{2+} ions which are interleaved and integral to the structure (Burns and Bell, 1991). It has been shown to be the lipid binding site for DAG and for phorbol esters such as PMA and TPA. This was first determined by the use of radio-ligands (specifically [^{3}H] phorbol 12, 13-dibutyrate) in the 1970's (this method being the gold standard for determining ligand and lipid affinity in protein/membrane interactions) (Colon-Gonzalez and Kazanietz, 2006). C1 domains with a single repeat of the cystine motif, identified as:

$$HX_{12}CX_2CX_nCX_2CX_4HX_2CX_7C$$

(C being Cystine, H being Histidine and n being either 13 or 14)

C1 domains are also found in related proteins, including diacylglycerol kinases (DGK) and Protein Kinase D (PKD). This domain was originally believed to contain binding regions for DNA, as it is structurally similar to the zinc finger domain of various transcription factors. It must be noted however, that the biochemistry of this site is quite different and cannot bind to DNA (Newton, 1995).

Dries *et al.* in 2007 observed that the 22nd residue of the C1 (C1a in typical PKCs) domain in both the typical and the novel PKC isoforms is always Tryptophan (Trp), and it is thought that this is the best candidate for modulating ligand dependant affinity with cellular membranes. The mechanism of action is believed to lie within the ability of the residue, (sited at the surface of the protein, along one of the two loops of the C1 domain known to be responsible for ligand binding), to interact with the membrane, due to the indole ring which makes up the functional group of the amino acid. The experiment done by Dries *et al* show that mutation of the C1b domain of the typical PKCs, PKC βI and II from a Tyrosine (Tyr) to a Trp residue causes a 31 fold increase in the ability of the isoforms to bind to lipid vesicles containing 5 mol % DAG and 30 mol % Phosphyltidylserine (PS). It is noteworthy, however, that neither the mutant, nor the wild type isoforms bound to the vesicles which contained 0 mol % DAG and 30 mol % of PS, showing that the presence of 1,2-DAG is crucial to the membrane binding mechanism of the isoform. They use this fact to suggest that the C1 domain is a potential target for isoform specific compounds, designed to mimic the structure of DAG. A ring of positive charges, at the middle of the C1 domain, is believed to facilitate membrane binding, possible by interaction with the PS, or other lipids with anionic properties (Hurley and Meyer, 2001). The DAG sensitive C1 domain has also been shown to have a role in the redistribution of PKC isoforms in response to ATP competitive inhibitors. Takahashi and Namiki showed that the endogenous isoforms PKC α and δ are translocated from the membrane to the cytoplasm of HeLa cells when the cells are treated with the ATP-competitive inhibitor GF 109203X. Translocation occurs at a concentration of 1μM for PKC δ (preferentially to the perinuclear membrane region), and a slightly higher concentration (10μM) for PKC α (causing translocation mainly to the endothelial, and plasma membranes). PKC ζ translocation remained unaffected by any concentration of

GF 109203X. Other known ATP-competitive inhibitors, including the antiproliferative Staurosporine have also been shown to induce PKC α, and δ translocation.

1.6.4.3 The C2 Domain.

The C2 domain contains a 70-residue β-pleated sheet structure which, with loops at the N and C termini, and five aspartate residues (different on the non Ca^{2+} binding novel PKCs), forms the recognition site for Ca^{2+}. The negatively charged (acidic) phospholipids are believed to be bound by aromatic amino acids and Ca^{2+} increases the enzyme affinity for the phospholipids (missing in novel PKCs), possibly by causing a conformational change in the N terminus region (Newton, 1995). The engagement of the C1 and C2 domains with the plasma membrane is thought to lead to the conformational change responsible for expelling the pseudosubstrate domain from the substrate binding site, leading to the activation of PKC as an enzyme capable of phosphorylating substrates bound to the membrane (Steinberg, 2004).

1.6.4.4 The C3 and C4 Domains.

The C3 and C4 domains are implicated in the binding of ATP and of the enzyme substrate MgATP, a property which it shares with the archetypal kinase, protein kinase A (PKA) (Newton, 1995). The C3 and 4 domains are also the target of the ATP-competitive inhibitors mentioned previously. Although this model provides an adequate explanation of how the enzyme is activated, and anchored to the membrane (by bringing it into closer proximity with its various membrane bound substrates, peptides with sequences similar to the pseudosubstrate domain of the enzyme), it fails to show how PKC isoforms exert their activity on non-membrane bound proteins.

1.6.5 Enzymology of PKC

The structure of the catalytic core of the archetypal PKC is homologous with protein kinase A (PKA), which uses ATP atoms bound to Mg^{2+} (MgATP). Typical PKCs and PKA are thought to show a 40% homology in the structure of their catalytic domain. It is the substrate binding cavity of PKC however which, unlike PKA exhibits a cluster of acidic residues which appear to hold the pseudosubstrate in place (Newton, 1995). PKA is highly stereospecific and phosphorylates serine and threonine residues with high specificity, requiring specific charges at the site of phosphorylation (Newton, 1995). However, PKC has apparently no requirement for positively charged residues at the phosphorylation site (Newton, 1995, Kishimoto *et al.* 1985). PKC phosphorylates L-amino acids as well as D-serine and D-threonine: however, the phosphorylation of the D-residues is a whole order of magnitude smaller than for an L-peptide. On the other hand, the Km (the Michaelis constant, which assumes that the enzyme and substrate are in equilibrium with the complex, and that the complex dissociates to leave the free enzyme, and its product.) value for PKC when one D-residue is introduced is close to the biological norm, and if two or more D-residues are substituted the Km increases (Eller *et al.* 1993). In addition to this, PKC isoforms also exhibits ATPase activity, and, in the presence of excess ADP, they will exhibit phosphatase activity as well (Newton, 1995).

1.6.6 PKC isoforms.

1.6.6.1 PKC α

PKC α has been characterised as being generally anti-apoptotic after radiation induced damage to the cell. However, the persistent translocation of the 82kD PKC α isoform to the cellular membrane has been implicated in apoptosis in LNCaP (androgen sensitive prostate cancer cells) (Powell *et al.* 1996). This translocation appears to be driven by

ATP-induced Ca^+ influx into the cells, as opposed to Ca^+ from intracellular stores (Marin-Vicente *et al.* 2005). Other evidence suggests that all PKC isoforms translocate to the membrane in the same manner. MCF-7 cells, over-expressing PKC α, show a higher growth rate and more cells in S-phase at any time point (Ways *et al.* 1996). These cells also exhibit anchorage independent proliferation and a loss of ER, possibly leading to decreased tamoxifen sensitivity as well as multi-drug resistance. Potentially, the inhibition of PKC α will, in cells which over-express the isoform, trigger the signalling cascade leading to apoptosis and cell death. However, in cell lines under-expressing PKC α (for example in colon cancer), the isoform has the potential to act either as a growth inhibitor, or as a promoter of apoptosis, although the activation of other PKC isoforms, especially PKC δ, may be required (da Rocha *et al.* 2002).

PKC α is also a known modulator of the Bax (sometimes referred to as Bax ζ) and Bcl2 genes. Bax is a part of the Bcl2 gene family which are known for their effects on apoptosis, both pro- and anti-apoptotic *via* the mitochondrial pathway.

Bax and Bcl2 form a heterodimer which functions as an activator of apoptosis. It is reported that the heterodimeric protein associates with the mitochondria and activates voltage-dependant anion channels, causing the release of cytochrome c (NCBI Entrez Gene Database, Herr and Debatin, 2001). Members of the Bcl family can act in an agonistic or antagonistic manner in relation to each other, directly influencing the response of the cell to cellular stress. It is interesting to note that increased levels of Bax appear to increase the susceptibility of tumours to chemotherapeutic drugs, while increased PKC α has quite the opposite effect. There is also some evidence that PKC α affects the regulation of the telomeric protection enzyme telomerase by binding to the telomerase associated protein 1 (TEP-1), a 300kD peptide with a structure similar to that

of RACK-1 (which also contains multiple WD-40 repeats in the C-terminal region). The telomerase is responsible for the synthesis and maintenance of the DNA at the telomeres (ends) of eukaryotic chromosomes. The telomere stability is essential as DNA shortening can affect chromosome pairing and genetic stability. The lack of enough functional telomerase and the resulting chromosome degradation usually leads to cell cycle arrest and potentially fusion of chromosomes (as seen in genetic defects such as Down Syndrome). However, it appears that the telomerase is over-expressed in many human cancers, suggesting its role in cell immortalisation (Li *et al.* 1998).

PKC α is also implicated in cell adhesion, transformation and the control of cell volume as well as functioning as a regulator of the cell cycle checkpoint. Studies in PKC α "knockout" mice suggest that the protein also plays a role in regulating cardiac contractility (Braz *et al.* 2004). For its activation and to exert its enzymatic effects, PKC α requires phosphorylation at threonine 497. It may be assumed that the anti-apoptotic properties and actions of PKC α differ depending on where, within the cell, it is located (cytosol or membrane). Its effects are also a function of the cell type. This can also be applied to the different PKC isoforms.

1.6.6.2 PKC δ.

The 78 kD PKC δ was the first isoform discovered which did not require calcium for activation, hence its "novelty". Being the first to be discovered, it is also the most widely researched of the novel PKC sub-family. PKC δ is usually characterised as being pro-apoptotic based on its reaction after radiation damage to the cell, it is also known to enhance differentiation in a number of undifferentiated cell lines (Steinberg, 2004). Phorbol esters such as 12-o-tetradecanoylphorbol myristic acetate (TPA) bind with high

affinity to PKC δ, causing activation and cell cycle arrest even without the presence of DAG (Watanabe *et al.* 1992). However, long term exposure to phorbol esters causes a down regulation of PKC δ. PKC δ was the first PKC isoenzyme to be identified as a substrate for the group of apoptotic proteases known as interleukin-1 converting enzyme (ICE) like proteases, also known as the "Executioner caspases" (Basu, 2003). Subsequently, PKC δ was specifically identified as a substrate for caspase-3. Caspase-3 cleaves PKC δ from its auto inhibitory regulatory domain, leading to the activation of its catalytic domain. In cells lacking a functional caspase-3, like MCF-7, PKC δ remains uncleaved, and, due to the different intercellular localisation of the full length and cleaved proteins, PKC δ exerts different effects (Basu, 2003). Upon activation (i.e. response to cellular stress such as H_2O_2) it has been observed that PKC δ translocates from the cytoplasm to the cell membrane. Moreover, it has been demonstrated that it can also translocate to the mitochondrial membrane (Basu, 2003), therefore initiating cellular apoptosis.

Activation of PKC δ is believed to inhibit cell cycle progression and its down regulation has been linked to tumour progression. However, some studies have shown that the up-regulation of mutant forms of PKC δ in MCF-7 causes increased tamoxifen resistance and decreased the number of oestrogen receptors at the cell surface both *in vivo* and *in vitro* (other studies ascribe similar properties to PKC α, Mackay and Twelves, 2003). This suggests that, in the presence of tamoxifen, the PKC δ pathway is a major alternative pathway for cell growth (Nabha *et al.* 2005). The ability of PKC δ mutant forms to stimulate cell growth is linked to the activation in transformed cells of the ERK/MAPK cascade independent of the *ras* pathway, especially by the constitutively active PKC δ mutant (Steinberg, 2004). It has also been observed that, when cells are exposed to a death

stimulus (either radiation or chemical), the proteolytic activation of PKC δ is increased. This activation correlates with an increased rate of apoptosis, which suggests a link between the two.

There is some evidence that PKC δ can act in a lipid independent fashion if phosphorylated at specific tyrosine residues by the Src family Kinases (SFKs). They also accumulate in the cytosol of cells subjected to oxidative stress. It is, therefore, postulated that it may have widely differing properties to the membrane bound form as they are in a position to phosphorylate substrates throughout the cell (Steinberg, 2004).

Finally, PKC δ is also known to regulate the co-transport of potassium, sodium and chloride ions across the pulmonary epithelia.

1.6.6.3 PKC ι.

PKC ι shares 72% amino acid similarity with the other atypical PKC λ and ζ, with the highest degree of conservation being observed in the catalytic domain. In humans, the tissue distribution of PKC ι (observed using Northern Blot analysis) shows the strongest expression in the brain and then in lungs and kidneys (Selbie *et al.* 1993). In addition to its activities as an oncogene in its own right in non small cell lung cancer (NSCLC, Regela *et al.* 2005) PKC ι is implicated in the signalling pathways of several oncogenes, including NF-κB, Bcr-Abl and Ras/MEK. In particular, PKC ι is a critical regulator of the cell survival factor NF-κB in chronic myeloid leukaemia cells, and prostate cancer cells (Lu *et al.* 2001, Win and Acevedo-Duncan, 2008). In NSCLC, it is indispensable for transformed, but not adherent growth both *in vivo*, and *in vitro* (Regala *et al.* 2005), and it

activates a Rac1/Pak/Mek1,2/Erk1,2 pathway responsible for transformed growth (figure 1.6).

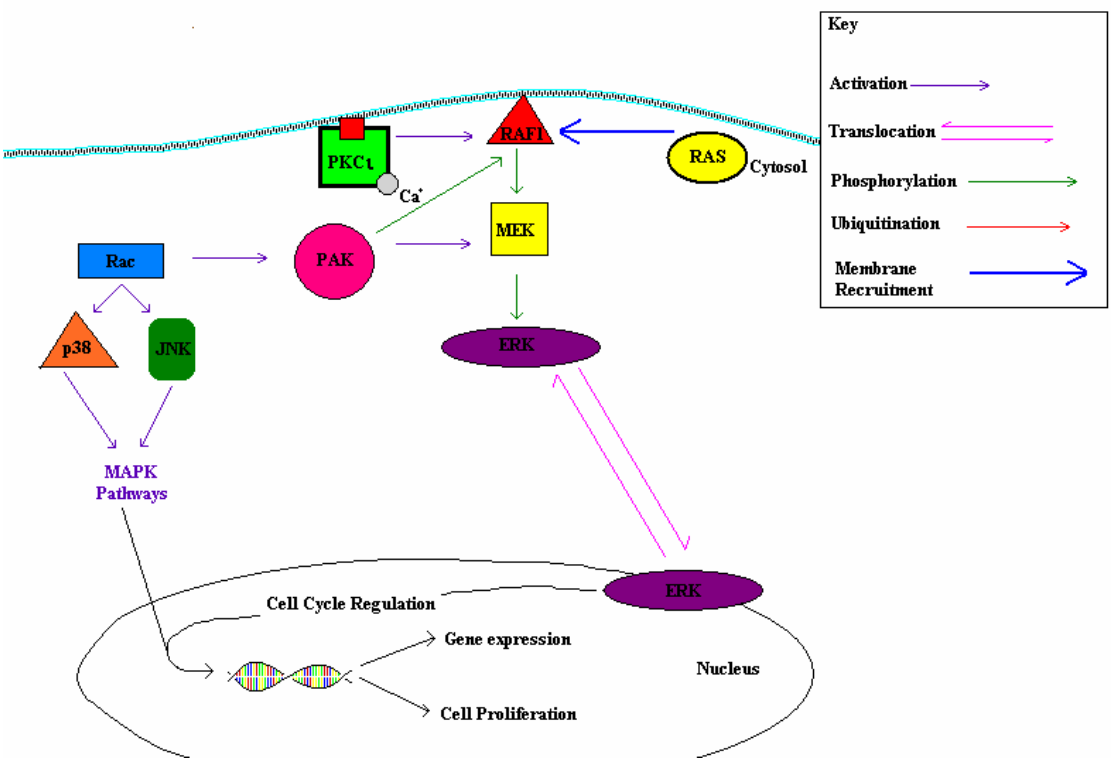


Figure 1.6. A Diagrammatic view of the Rac1/PAK/MEK/ERK cancer pathway. Showing activation of RAF1 by PKC ι, leading to phosphorylation of MEK, the dissociation of the MEK/ERK complex, and translocation of ERK to the nuclear membrane (Eblen *et al.* 2002).

High levels of PKC ι have been shown to contribute to poor prognosis in patients with ovarian cancer (Eder *et al.* 2005), leading to speculation that it may prove to be a useful biomarker for aggressive ovarian carcinoma. It has also been shown to increase nicotine stimulated cell migration and invasion (Xu and Deng, 2006), as well as being implicated in colon carcinogenesis (Murray *et al.* 2004). This has lead to speculation that increased PKC ι levels may also exert similar effects in breast cancer cells.

1.6.6.3.1 PKC ι and Cellular Polarity.

It has been observed that PKC ι is involved in the polarisation of *Drosophila* cells: abnormal cellular polarity may be linked to increased chance of metastasis in cancer cells.

It has been observed that cancerous tissues containing high levels of PKC ι, such as lung carcinoma, are more likely to be metastatic than other cancers, possibly due to improper polarity causing the cells to fail to adhere to their neighbours. Activation of PKC isoforms in fertilized mammalian oocytes is concentrated around the polar body (daughter cells generated by asymmetrical cell division which appear to play a role in early embryo polarity), again suggesting a role for PKC in determining cell polarity. However, in this case, the specific isoforms were not distinguished (Halet, 2004). The atypical PKC isoforms appear to have highly specific roles *in vivo* in mammalian cells. Genetic targeting has shown that in the early embryonic stages of mouse embryos a deficiency of PKC ι is fatal (Bandyopadhyay *et al.* 2004). However, embryos deficient in PKC ζ are still viable but they lack proper IL-4 and NF-κB signalling ability (Martin *et al.* 2002).

1.7 PKC and apoptosis

As previously discussed, it is known that the PKC isoforms are intercellular signalling molecules and have been shown to be involved in a number of cellular processes by the phosphorylation of key components of various pathways, including those regulating cytoskeletal functions, transcription of genes and apoptosis. In particular, there are definite links between cellular apoptosis and the presence of the various PKC isoforms, in particular PKC δ, although most cells co-express different isoforms, making the PKC family a potential target for chemotherapeutic drugs.

The three key features of tissue homeostasis are cell proliferation, differentiation and apoptosis. Apoptosis is the programmed death of a cell characterised by cell shrinkage, release of cytochrome-c from the mitochondria, and continued metabolic processes until the later stages. It is opposed to necrosis, which is an un-programmed death involving leakage of cell components and therefore inflammation of the surrounding tissues. Many apoptotic processes, in different cell types, appear to be mediated, to some extent, by the activation of the cystiene protease family known as caspases. Caspases have also been shown to be cleaved by PKC isoforms (however, see section 1.6.6.2 for an alternative), which suggests that they are acting downstream of PKC in the signalling cascade (Adayev *et al.* 2003). At least two groups of caspases have been identified, the ones initiating apoptosis (sometimes referred to as "initiator caspases") and those which actually cleave specific substrates (for example the cytoskeletal proteins or the DNA protector protein ICAD), called "effector caspases". After activation, the caspases are known to cleave their substrate proteins at the aspartic acid residues in the tetrapeptide recognition motif (Herr and Debatin, 2001).

Caspase-3 production can be induced by UV radiation (Matsumura *et al.* 2003). After DNA damage, the mitochondria releases, among other compounds, the cytochrome-c into the cytosol which triggers the activation of caspase-9 by the interaction between procaspase-9 and Apaf-1 (=a "caspase adaptor protein"). The caspase-9 then activates a number of downstream caspases, including caspase-3 (Herr and Debatin, 2001). The ability of caspase-3 to cause apoptosis can be reduced by over-expression of some PKC, in particular PKC η (Matsumura *et al.* 2003).

1.8 PKC and Cancer

The PKC isoforms can be activated by phorbol esters (tumour promoting agents) in the same way as DAG. The action of phorbol esters is twice as potent as that of DAG. In fact, they induce conformational changes in the protein surface increasing its hydrophobicity and, therefore, its affinity for membrane lipid groups. However, it has been observed *in vivo* that the long-term activation of PKC by phorbol esters causes the cell to down-regulate PKC isoform production.

Elevated PKC isoform levels have been observed in many different types of tumour, including prostate (Song *et al.* 2004), breast (Soh *et al.* 2003, de Vente *et al.* 1995) and lung (Regala *et al.* 2005). They are also associated with malignant transformation of cells. The different isoforms are under-, or over-expressed at different stages in different cancer types (Mackay and Twelves, 2003). “Mislocalization” of PKC isoforms may also be implicated in the creation of abnormal cells, potentially by causing changes in cell polarity and therefore altering the cells adhesion properties (Eder *et al.* 2005).

1.9 PKC as a target for anti-cancer therapies

1.9.1 PKC Isoform Expression in Breast Cancer.

A study, comparing PKC expression in cancerous and normal tissues of mastectomy patients (9 patients), showed elevated PKC levels in the cancerous tissues compared to the healthy tissue. Therefore, it has been hypothesised that PKC inhibitors are strong candidates to be the next generation of chemotherapeutic drugs (Mackay and Twelves, 2003). Agents targeting specific isoforms, classified according to the PKC site where they exert their effect, could be used to decrease cell viability, increase androgen receptors (linked to good prognosis in breast and prostate cancers) and increase the cytotoxicity of existing chemotherapeutic drugs. On the other hand, the current state of knowledge about the downstream signalling pathways of PKC may prove a bar to the use of PKC inhibitors to treat cancer. Indeed, their effects on healthy cells are likely to be diverse and unpredictable. Moreover, currently, there are only a small number of isoform specific PKC inhibitors, which therefore limits the scope of action.

Some bacteria have the ability to prevent DNA damage by ionizing radiation (IR) *via* the production of superoxide anions and reactive oxygen intermediates (ROI). Moreover, eukaryotic cells up regulate PKC δ expression in response to DNA damage induced by IR (Basu, 2003, Limoli and Ward, 1993). Following IR damage, PKC δ activation appears to be induced by ICE-like proteases. However, no PKC δ activation has been detected in response to stress caused by H_2O_2 (Limoli and Ward, 1993). Staurosporine is a known antiproliferative agent, a microbial alkaloid produced by *Streptomyces* bacteria, with physical properties similar to ICE-like proteases. Staurosporine is not specific and binds indiscriminately to all typical and novel, but not atypical isoforms of PKC (Newton, 1997), which causes their inhibition. However, it is this property that enabled the

discovery of potential isoform specific PKC inhibitors. More selective, but less potent than staurosporine are the calphostins, extracted from *Cladosporium cladosporoides*, a fungus. Calphostins are part of a group of chemicals known as perylenequinones. Their mechanism of action on PKC isoforms is twofold: competitive binding at the DAG binding site and covalent modification of the lipid binding regulatory domain. However, they also interfere with intracellular transport, inhibit cell growth and induce apoptosis in normal cells. Therefore, more research and structural modifications are required before they can be considered for trial as anticancer agents (da Rocha *et al.* 2002).

Bryostatins, which are derived from the *Bugula neritina* species of marine bryozoan (colonial marine animals, superficially similar to corals), are a class of up to 20 macrocyclic lactone compounds. They have been shown to have an effect in activating the isoforms of typical and novel PKC. However, in the presence of DAG (or, in cancer cells, phorbol esters) the bryostatin compounds have been shown to act as antagonists (Mackay and Twelves, 2003). *In vivo*, bryostatin treatment shows differences in PKC isoform expression and increases in TNF-α and IL-6, both known to decrease tumour size. In phase I trials, bryostatin showed some beneficial clinical effects. However, in phase II trials these effects could not be replicated (Mackay and Twelves, 2003).

1.9.2 Development of PKC Isoform Specific Inhibitors.

The process of trying to develop PKC isoform specific inhibitors is being investigated and some success has been found with agents binding to the ATP binding site. Of these compounds Rottlerin (Mallotoxin) which is derived from the Kamala plant (*Mallotus philippinesis*) is probably the best investigated (Way *et al.* 2000). Rottlerin is a compound

which exhibits some selectivity in inhibiting PKC δ, however, more experiments are required to examine the extent of its usefulness.

Studies in breast cancer have shown that tamoxifen, a triphenylethylene with antioestrogenic properties, inhibits the kinase activity of various PKC isoforms. Tamoxifen exerts this effect essentially in oestrogen receptor negative cells (da Rocha *et al.* 2002).

A more specific approach is to design antisense oligonucleotides and peptides that will promote, or inhibit the translocation to the cell membrane of specific PKC isoforms to specific anchoring sites. This method has already been tested, with marked success, in animal models, where PKC α expression was decreased by the antisense oligonucleotide ISIS 3521 (da Rocha *et al.* 2002). However, in human trials, there appeared to be no dose limiting toxicity and clinical activity was observed in 99 cycles of treatment. However, the half-life of the drug was very short (18-92 minutes). Moreover, there was a dose dependant increase in the maximum concentration of the drug in the plasma (up to levels seen in primate models) and a dose dependant decrease in the drug clearance. Therefore, further studies, including prolonged schedules of treatment, are required to take this potential treatment further (Nemunaitis *et al.* 1999).

1.9.3 PKC Isoforms and Cancer Prevention.

With the high incidence of breast cancer in Western populations some focus of research has moved, almost inevitably, into its prevention. CLA is known to confer a chemoprotective effect on tumour initiation/development in rats (Field and Schley, 2004), but its mechanism of action can still only be surmised. From its effect on various different

PKC isoforms in the LNCaP cell line (Song *et al.* 2004), this unusual fatty acid shows several potential targets, including modulation of membrane composition and gene expression. It is the objective of this study to determine the effect that treatment with various concentrations of the two most abundant isoforms of CLA over different time periods have on PKC isoform expression and activation in MCF-7 and MDA-MB-231 breast cancer cells.

1.10 Structural Proteins in the Cell, Claudin-3 and β Actin.

1.10.1 Cell Junctions

In normal, healthy human cells direct cell to cell contacts are essential for maintaining the normal structure, function and homeostasis of tissues. There are three basic forms of cell junction, each with a specific protein complex and function.

1.10.1.1 Desmosomes.

Desmosomes, also known as macular adhesions, are found on the lateral sides of the cell, especially in epithelial tissues. Desmosomes follow no fixed pattern in their adhesion, making them more resistant to shearing forces than other junctional protein complexes. The cadherin super family of proteins form the basis for desmosome complexes, usually by homophillic binding with linking proteins to form a dense "attachment plaque" between cells.

1.10.1.2 Adherens Junctions.

Adherens junctions are usually found at the basal membrane of the cell, either forming localised adhesion plaques or as zonula adhesion bands depending on the tissue type. Similar to desmosomes, the adherens junctions are based on a complex of cadherins, although they form homodimers and are anchored to actin filaments rather than keratin by complexes of α and β catenin. This leads to a much smaller intercellular space between cells than with desmosomes.

1.10.1.3 Tight Junctions.

Tight junctions are an essential mechanism if the body is to correctly control paracellular ion flux across tissue sheets at a cellular level, if this is not controlled then normal tissue

homeostasis is disturbed and cells can become prematurely apoptotic (Morin, 2005). The tight junction itself is thought to be composed of microdomains, spatially organised within the plasma membrane by the presence of sphingolipids and cholesterol (Miyoshi and Takai, 2005). Electron microscopy shows multiple strands forming at tight junctions, probably allowing structural adhesion between cells. Tight junctions prevent too much lateral migration by membrane bound proteins, thereby aiding the cell in its maintenance of polarity as well as keeping different tissue types separated. It is known that tight junctions are composed of three major protein families, all of which are integral to the structure and function of the cellular membrane; these are the claudins, the occludins and the junctional adhesion molecules (JAMs). Of these, the claudins are of the most interest to this work.

1.10.2 The Claudin Super Family.

The claudin super family comprises of 23 different isoforms (first identified in chicken liver by Furuse *et al* in 1998) which are known to be tissue specific, although most tissues express multiple isoforms. It is believed that the claudins form the backbone for the strands at the tight junction protein complex, the different isoforms combine in a tissue specific conformation to form homo or hetro dimers. It is thought that the combination of claudins within a tissue is what determines the strength of the tight junction (Morin, 2005).

Claudins are integral to the plasma membrane and comprise four membrane spanning domains and two extracellular loops, with the C and N terminus of the protein being found intracellularly. The extracellular loop which is closest to the N terminus is significantly larger than the C terminal loop. The larger loop also expresses a conserved

WWCC motif (with the structure $WX_{(17-22)}WX_{(2)}CX_{(8-10)}C$ (Turksen and Troy, 2004, Figure 1.7).

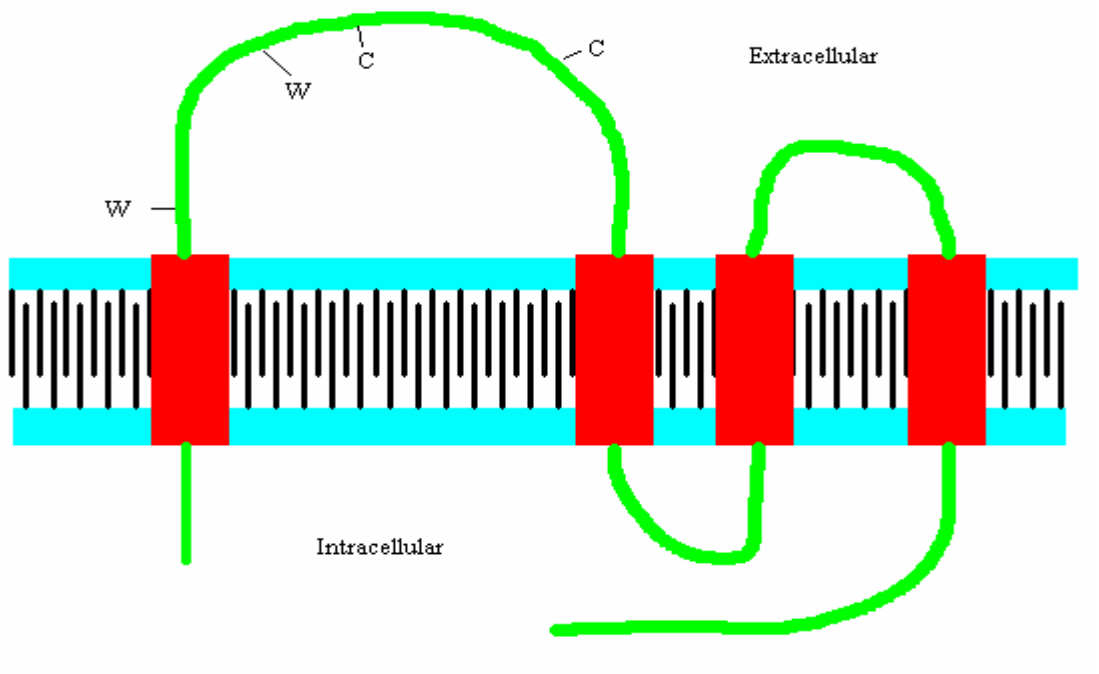


Figure 1.7. Model Claudin showing conserved domains in extracellular loops (Morin, 2005).

It is thought that this conserved domain is what permits claudins to bind both homo- and heterotypically.

1.10.3 Claudin-3.

Claudin-3, along with the highly similar Claudin-4, are known to be a natural receptors for the *Clostridium perfringens* enterotoxin (CPE) (Katahira *et al.* 1997). CPE is a known causative agent of diarrhoea, by altering the permeability of the membrane of epithelial cells in the intestinal tract. This altered permeability leads to fluid accumulation within the intestine and bowels. Sensitivity to CPE is not limited to single species, suggesting that it has an effect on membrane proteins with a high degree of structural similarity between species (Katahira *et al.* 1997). Katahira *et al.* in 1997 showed that the genes

encoding the functional CPE receptor (now referred to as Claudin-3) in both human and mouse cell lines exhibited 96.7% similarity.

1.10.4 Claudin-3 and Cancer.

Studies have shown that expression of Claudin-3 is elevated in some common tumour types, including bladder, ovarian, colon, prostate, uterine and breast tumours.

Beta-actin is a 40kD protein found ubiquitously in the cytoplasm of all cells in the body. Because of its high expression and relative resistance to changes caused by various drug treatments, it is commonly used as a loading control for immunoblotting and other protein assays.

1.10.5 Actin.

Actin is a highly conserved protein between species, with approximately 95% of the protein being conserved between the primary structures of actin in *Saccharomyces cerevisiae* and *Homo sapiens*. This conservation is mostly due to the use of actin filaments in binding to a large number of disparate proteins within the cell. Mammals express 6 different isoforms of actin, designated α, β, and γ, which are involved in cell integrity, structure and motility. It is known that ß-actin mutations are responsible for a number of developmental midline malformations, including harelips and cleft palates, as well as problems like hearing loss and the generalised movement disorder dystonia (Procaccio *et al.* 2006).

1.10.6 ß-actin as a Loading Control for Western Blot Analysis.

Currently, ß-actin is the loading control of choice for many different cell treatments and proteins for researchers carrying out Western blot analysis and enzyme linked

immunosorbent assays (ELISA). As a loading control, ß-actin is almost ideal as it is mostly uninfluenced by a wide variety of cell treatments, and is expressed abundantly and with very little change in many of the different cell lines currently available. It is worth noting, however, that in 2006 Dittmer and Dittmer suggested that at higher protein loads, and over longer incubation times it is much less sensitive to changes in protein loading, due to its high cellular abundance. ß-actin is also only found in the soluble protein fraction; not in the insoluble fraction meaning that it is also unsuitable for detecting protein loading for membrane bound proteins. It is for this reason that an important developmental part of this study will be to determine whether or not Claudin-3 can be considered as an effective loading control for membrane- bound proteins extracted from MCF-7 and MDA-MB-231 cells previously treated with different isomers of CLA.

1.11 Experimental Aims of the Current Research.

Since the discovery of the PKC isoforms, their known regulation by tumour promoters like phorbol esters and their impact on tumorogenisis in the 1980's, much work has been done to elucidate their mechanism of action and to discover compounds which act specifically on individual isoforms to enhance or decrease their individual properties.

Studies in prostate cancer cells suggest that CLA isoforms may be an effective means of causing tumour shrinkage and apoptosis (Song *et al.* 2004), due, at least in part, to their apparent ability to enhance the activation of the pro-apoptotic PKC isoforms and to decrease the activation of the anti-apoptotic ones. It is notable that similar experiments have not yet been performed using breast cancer cells. It is, therefore, the focus of this research to study whether CLA's have similar effects in breast cancer cells to those observed in prostate cancer cells and, if so, which of the selected isoforms of PKC show activation or inactivation in the MCF-7 and MDA-MB-231 lines of cancer cells (by translocation of PKC to the cellular membrane, or sequestering within the cytosol) after treatment with individual isoforms or a mix of the two main isoforms.

The mechanism of PKC activation by CLA isoforms in breast cancer cell lines is also of interest, as future work to develop CLAs as either a defence against cancer (preventative), or as an adjunct to conventional cancer therapy (treatment) must hinge on how the isoforms affect the cell as a whole.

These studies are part of a larger study that is investigating the role of various nutrients and nutraceuticals as possible chemopreventive and adjunct chemotherapeutic agents.

These aims will be realised by a variety of techniques, including Western Blotting, and protein analysis, RT-PCR, and cell culture.

Chapter 2.
Materials and Methods

2. Materials and Methods

2.1 Materials

Foetal Bovine Serum (FBS) was purchased from Perbio, UK, 1mM Sodium Pyruvate and 2% Penicillin/Streptomycin from Invitrogen, UK. RPMI 1640 medium containing GlutaMAX™ 1 and 25mM HEPES buffer, from Fisher, UK.

Pure Conjugated Linoleic Acid, Linoleic acid isomers and 98% pure Bovine Serum Albumin (BSA) was purchased from Sigma. Calbiochem ProteoExtract™ Native Protein Extraction kit was from Calbiochem. 10k centrifugal filters were from Pall. Alkaline copper tartrate solution (Reagent A), dilute Folin reagent (Reagent B) were from Bio-Rad. Pre-stained Protein Marker, Broad Range (6-175 kDa) was bought from New England Biosciences, USA. PVDF membrane was bought from GE Healthcare, UK and 3mm chromatography paper from Fisher, UK. X-Ray Photographic Film, Developing solution and Fixing Solution were all purchased from Kodak, UK. SuperSignal West Pico Chemiluminescent kit; Luminol Enhancer, and Stable Peroxide, and Restore Plus Western Blot Stripping Buffer, both bought from Pierce, USA. Gel Electrophoresis, and Western Blot equipment were all supplied by Bio-Rad. Trizol (phenol based solution), and DEPC treated water were purchased from Sigma, chloroform, ethanol, and Isopropyl alcohol, were all supplied by Fisher. iCycle, Thermocycler unit supplied by Bio-Rad, Spectrophotometer supplied by Heλios.

The following buffers were made up; SDS Loading Buffer (190mM Tris, pH 6.8, 30% Glycerol, 6% SDS, 150mM DTT, 0.15M bromophenol blue. Running Buffer (250mM glycine, 25mM Tris, 0.1% SDS). Wet Transfer Buffer (10% running buffer, 20% Methanol, 70% water). Tris Buffered Saline with Tween-20 (TBS-T) (20mM Tris, 0.13M

Sodium Chloride, 0.1% Tween-20, pH 7.6). Blocking Buffer (50mM TBS-T, 5% Skimmed, dried milk, (Marvel®)). Ponceau Red Stain (0.1% Ponceau R, 0.5% Acetic acid). Acridine Orange/Ethidium Bromide (100µg/ml Acridine Orange, and 100µg/ml Ethidium Bromide in PBS). Trypan Blue (0.4 % Trypan blue in PBS).

All plasticware from Fisher, UK, unless otherwise stated. All chemicals were Sigma, UK, unless otherwise stated.

2.2 Cell Culture

2.2.1 Cell Lines

Breast cancer cell lines MCF-7 (oestrogen receptor positive) and MDA-MB-231 (oestrogen receptor negative) were a gift from Professor Steve Heys (Department of Surgery, University of Aberdeen, UK), and routinely cultured in RPMI 1640 medium, supplemented with 10% FBS, 100µg/ml Sodium Pyruvate and 100µg/ml Penicillin/ Streptomycin. Cells were incubated at 37˚C in a 5% CO_2 atmosphere and passaged every 3 to 4 days at approximately 80% confluence.

2.2.2 Recovering Cells from Liquid Nitrogen

The cell vial was removed from the liquid nitrogen storage and incubated for 10 minutes at 37˚C; cell suspension (1ml) was decanted into a culture flask containing 19ml of supplemented culture medium. The cells were allowed to settle overnight at 37˚C after which the medium was removed and the cells gently washed with PBS, followed by the addition of fresh supplemented medium (20ml).

2.2.3 Procedure for Cell Subculture

Cells were grown in standard 75cm^2 tissue culture flasks. Once confluence had been reached the medium was discarded and the cells washed twice with sterile PBS. Trypsin (2ml) was added and the cells were incubated at 37˚C for 1-2 minutes. Once the cells were detached, the trypsin was inactivated by the addition of 18ml of the supplemented medium and the cells aspirated through a 19G hypodermic needle to give a uniform cell suspension. Fresh supplemented medium (20ml) was added to the flask and the suspended cells were divided equally between two flasks which were incubated as previously stated until confluence.

2.2.4 Freezing Cells for Storage

Cells at confluence were washed twice with PBS and detached from the flask as described above. After trypsinisation 10 ml of the unsupplemented RPMI 1640 medium was added, the cells transferred into a 50ml Falcon tube and centrifuged for 8mins at 380g. The supernatant was discarded, 100µl of unsupplemented medium, 1.8ml FBS added along with 100µl of DMSO were added to the cells, which were then resuspended and the mixture aliquoted into two Cryovials (1ml/cryovial). The cryovials were placed in an isopropanol bath ("Mr Frosty") to insure a steady rate of freezing at 1˚C per minute when placed at -80˚C for 24 hours. The vials were then transferred from the freezer into Liquid nitrogen for long term storage.

2.3 Staining Cells for Counting

2.3.1 Supplementation with Conjugated Linoleic Acid

100mM stock solutions of pure Conjugated Linoleic Acid isomers (*c*9:*t*11, *t*10:*c*12 and a 50:50 mix of the two isomers), and Linoleic acid dissolved in 100% ethanol (Fisher) were

diluted to give test concentrations of 25 and 50μM. Stock solutions were stored at -20˚C under gaseous nitrogen.

2.3.2 Acridine Orange/ Ethidium Bromide Uptake

Cells were plated onto 6 well plates at an approximate density of 100,000 cells per well. 0.5ml of 100% confluent cell suspension from a standard T80 tissue culture flask was diluted in 2.5ml of medium. Plates were incubated at 37˚C for 24 hours to ensure adequate cell adherence to the plate. Cells were then treated with either 25 or 50μM of Conjugated Linoleic acids, as detailed in section 2.3.1. Plates were incubated at 37˚C for a further 24 hours, and the medium removed. Cells were washed twice with PBS and 40μl Acridine Orange/Ethidium Bromide dye solution, diluted in 1ml PBS was added to each of the wells. Plates were incubated at room temperature for 2 minutes and cells viewed under a Leica DC 200 Fluorescence Microscope.

2.3.3 Trypan Blue Dye Exclusion

Cells in T80 flasks were brought to confluence and supplemented with Conjugated Linoleic Acids for 24 hours as stated in section 2.3.1. Cells were detached from the flask using Trypsin as detailed in section 2.1.4. Trypsinised cells were suspended in the medium, and 0.5ml of cell suspension was mixed with 0.5ml of Trypan Blue. 20μl of the suspension was added to the counting chamber of a haemocytometer and viewed under a 10X objective microscope. Both total cells and non viable cells were counted.

2.4 Protein Extraction

2.4.1 Supplementation with Conjugated Linoleic Acid

Supplementation with Conjugated Linoleic Acid was carried out exactly as stated in section 2.3.2.

2.4.2 Protein Extraction

Cells at confluence were incubated with fatty acids at the required concentrations (25 or 50µM) for 24hr. After incubation cells were washed once with cold PBS, scraped from the flask and transferred into 15ml centrifuge tubes. Cells were then centrifuged at 1000g for 5 minutes at 4˚C. The proteins were extracted using the Calbiochem ProteoExtract™ Native Protein Extraction kit following the manufacturer's protocol: the PBS was discarded and cells re-suspended in ice-cold Wash Buffer (2ml), the cells were then centrifuged at 1000g for 5 minutes at 4˚C. This process was repeated once more with fresh Wash Buffer. The excess buffer was carefully aspirated from the cell pellet and Protease Inhibitor Cocktail (10µl) and ice-cold Extraction Buffer I (2ml) were added to the cells. The cells were then incubated at 4˚C for 10 mins under constant agitation to prevent the formation of clumps. The insoluble material was centrifuged at 16,000g and 4˚C for 15 mins: the supernatant contained the soluble cytosolic proteins. The supernatant was collected and stored at -80˚C until further analysis. Protease Inhibitor Cocktail (5ml) and ice-cold Extraction Buffer II (1ml) was added to resuspend the pellet (i.e. the insoluble material). The cells were then incubated at 4˚C for 30 mins, as before and centrifuged at 16,000g and 4˚C for 15 mins. The supernatant containing all the insoluble membrane bound proteins was collected for storage at -80˚C until used for protein concentration determination before Western Blot analysis.

2.4.3 Concentration of cytosolic protein fraction

The cytosolic protein fraction obtained from the MDA-MB-231 cells was centrifuged through a 10k centrifugal filter to remove excess buffer at 3000g and 4˚C for 140 mins.

2.4.4 Protein assay

Protein sample concentration was determined using an adapted method from the standard one set out by Lowry *et al.* in 1951. Protein concentrations of the samples obtained above were determined against a standard curve of the following protein concentrations 0, 0.5, 1, 1.25, and 1.5mg/ml read at 620nm. 5µl duplicates of each protein fraction were used, and made up to 25µl with distilled water. 25µl of Reagent A and 200µl of Reagent B were added to each sample and incubated for 15mins. Absorbance was read using a Dynex Technologies, MRX II microplate reader.

2.5 Gel Electrophoresis and Western Blotting

Table 2.1. Make up of resolving polyacrylamide gels (4 gels)

Gel concentration	7%	9%	12%	15%
Acyle:Bis (29:1)	3.3ml	4.4ml	5.7ml	7.1ml
Tris 1.5M pH 8.8	4.6ml	4.6ml	4.6ml	4.6ml
Distilled H_2O	10.9ml	9.7ml	8.4ml	7.0ml
APS 10%	150µl	150µl	150µl	150µl
TEMED	15µl	15µl	15µl	15µl

2.5.1 Gel Electrophoresis of Protein Samples

4% polyacrylamide stacking gels and 12% separating gels were used to perform Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) based on the method described in 1970 by Laemmli *et al.*. The following protocol was used:

The resolving gel was cast in a glass mould (see table 2.1 for gel make up), and allowed to set under a layer of water saturated buthanol for 20mins, the buthanol was removed, and a layer of stacking gel was added along with a comb used to create the wells. Initially 40µg of protein sample (determined by Lowry microassay) was denatured by boiling in 3µl of SDS loading buffer for 7mins. The samples were loaded onto the gel with a protein marker in the first, and final lanes and the gels run in the running buffer at 200 volts. Samples were run for 90mins.

Table 2.2 Antibody origin, specificity, and dilution.

Antibody	Raised in	Primary/Secondary	Working Dilution
PKC α (Abcam)	Mouse	Primary	1:1000
PKC δ (Abcam)	Rabbit	Primary	1:1000
PKC ι (Santa Cruz)	Rabbit	Primary	1:1000 (cytosolic sample) 1:2000 (membrane sample)
Beta-Actin (Sigma)	Mouse	Primary	1:30000
Claudin-3 (Abcam)	Goat	Primary	1:500
Goat anti Mouse IgG (Santa Cruz)	Goat	Secondary	1:2000
Goat anti Rabbit IgG (Santa Cruz)	Goat	Secondary	1:2000 (cytosolic sample) 1:5000 (PKC ι membrane sample)
Rabbit anti Goat IgG (Santa Cruz)	Rabbit	Secondary	1:10000

2.5.2 Western Blotting

After the proteins had fully separated within the gel matrix they were transferred from the polyacrylamide gel to the PVDF membrane using a wet electro-blotting protocol. The stacking gel was removed and discarded. The separating gel soaked in wet transfer buffer solution. The membrane was soaked in methanol for approximately 10secs and then washed in deionised water. The cassette was stacked from the positive side upwards (See Fig 2.1); a piece of sponge, well soaked in buffer was placed on the positive surface. 3 pieces of 3mm chromatography paper (5x9cm) were stacked on the sponge after being briefly soaked in the buffer and air bubbles removed by rolling a Pasteur pipette across

the surface. The membrane was placed on the paper and the gel on the membrane with a further three soaked pieces of chromatography paper on top. Any air bubbles were carefully removed and a further sponge soaked in buffer placed on top of the pile, the apparatus was then assembled and the proteins transferred for 45mins at 300 volts.

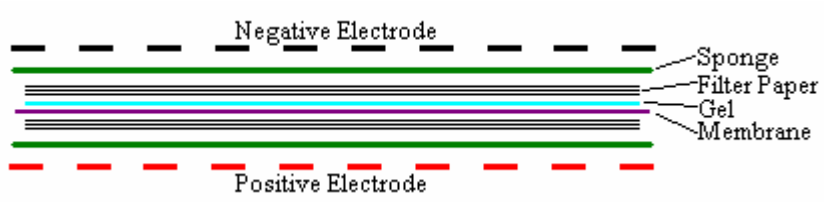


Figure 2.1 Protein transfer stack for wet electro blotting protocol.

After the transfer was completed, the membrane was incubated in blocking buffer (as detailed previously) for up to two hours. The membrane was incubated overnight at 4˚C, on a roller shaker, with 5ml of antibody solution (where the antibody was diluted in blocking buffer; see table 2.2 for antibody and dilutions). The membrane was washed 3 times for 10mins each in TBS-T, and incubated for one hour at room temperature with the secondary antibody diluted as before (see table 2.2 for dilutions), rotating constantly. The membrane was then washed 3 times for 10mins each in TBS-T.

The bands were visualised using X-ray film. The membrane was covered with 2ml of stable peroxide solution and 2ml of luminol / enhancer solution and allowed to incubate at room temperature for 5 mins. The membrane pieces were blotted to remove excess enhancer solution, and wrapped in a single layer of colourless Saran food wrap. The light sensitive photographic film was exposed to the treated membrane in for a period of between 30secs and 10mins, the film was then treated with developing solution for 2mins,

briefly rinsed in water, immersed in fixer solution (1min) and washed once more in water. The film was allowed to dry overnight.

Membranes on which the cytosolic protein samples were run were stripped using 10ml of Restore Plus Western Blot Stripping Buffer at 37˚C for one hour, rotating constantly. Membranes were blocked in blocking buffer for a further 1 hour, and re-probed with either Beta-Actin (for proteins in the cytosolic fraction) or Claudin-3 (for proteins in the membrane bound fraction) antibodies (See table 2.2)

2.5.3 Staining membranes with Ponceau Red.

After transfer or detection membranes were stained for 5 minutes in Ponceau Red solution. Then, the membranes were de-stained for 30 seconds in 50ml deionised water, then blotted to remove excess moisture and allowed to air dry at room temperature.

2.6 RT-PCR

2.6.1 Total RNA extraction

Cells at confluence were lysed with 1ml of Trizol, and incubated for 2 minutes at room temperature. The cell lysate was passed through a pipette tip several times to ensure proper mixing, then transferred to an RNAse free microcentrifuge tube. The cell lysate was then incubated for a further 5 minutes at room temperature. Chloroform (200µl) was added to the cell lysate, and shaken for 15 seconds; the mixture was again incubated at room temperature for 2 minutes. Tubes were centrifuged at 15300g and 4˚C for 15 minutes, and the aqueous phase of the supernatant was transferred to a new tube. The aqueous phase had 500µl of isopropyl alcohol added and was mixed gently, then incubated at room temperature again for 10 minutes. The mixture was then centrifuged at 15300g at 4˚C for 10 minutes to precipitate the RNA. The supernatant was removed, and

the pellet washed with ethanol (75%) and DEPC treated water. The pellet was mixed and centrifuged at 15300g and 4˚C for 5 minutes. The pellet was allowed to air dry and then covered with DEPC treated water. The pellet was frozen at -20˚C over night, then dissolved by incubating at 65˚C for 10 minutes, and mixing gently.

2.6.2. RNA assay

RNA was assayed for concentration and purity using a Heλios γ Spectrophotometer (Thermo Spectronic). The extracted RNA was diluted to a ratio of 1:200 in DEPC treated water, and placed in a glass curvette. The OD_{260}, and OD_{280} readings were measured, and the RNA concentration calculated using the formula:

$$\text{Concentration } (\mu g/\mu l) = \frac{OD_{260} \times 40 \times \text{Dilution factor}}{1000}$$

Sample purity was obtained by the formula:

$$\text{Purity} = \frac{OD_{260}}{OD_{280}}$$

With a purity of 2 being very pure, and a purity of less than 1.3 being of poor quality, and causing the samples to be discarded.

2.6.3 Poly-dT two step PCR

For the RT reaction step, 1µl total RNA, with 4µl 5x first strand buffer, 2µl DTT, 2µl dNTP's, 1µl RNAse out, 2µl BSA, 1.5µl poly-dT primers, 0.4µl Superscript II RNAse H RT and DEPC treated water was placed in 0.5ml eppindorf tubes, and placed in a BioRad Thermal Cycler at 37˚C for 60 minutes, then at 95˚C for a further 10 minutes. A PCR

master mix was made up, comprising; 8.8µl DEPC treated water, 1.6 µl of pre mixed buffer without magnesium chloride ($MgCl_2$), 1.4µl of $MgCl_2$, 2µl each of the forward and reverse PCR primers (see table 2.3), and 0.2µl of Taq polymerase. After removel from the Thermal Cycler the RT mix was allowed to cool on ice for 1 minute, then 4µl per tube added to 16µl of the PCR master mix, along with an equal volume of distilled water for the blank samples. Samples were mixed and transferred to the Thermal Cycler, for 35 cycles (25 cycles for ß-actin, see table 2.4)

Table 2.3 Make up of PCR primer pairs

Protein	**Forward Primer (5' to 3')**	**Reverse Primer (5' to 3')**
ß-Actin	CAT GTA CGT TGC TAT CCA GGC	CTC CTT AAT GTC ACG CAC GAT
PKC α	CTG CTT CCA GAC AGT GGA TCG	GCC GCA TAG AAT ACT GCT TGT
PKC δ	GAG GCC AAG TTC CCA ACG AT	TGC ATT TGT AGC CTT GCT TGT
PKC ι	CAC CAG GTC CGG GTG AAA G	TGA AGA GCT GTT CGT TGT CAA A

Table 2.4 Cycles of Hot-Start PCR

Cycle (repeats)	Temperature (˚C)	Time (Mins)
1 (1)	94	4:00
2 (35)	94	1:00
	59	2:00
	72	2:00
3 (1)	72	8:00
4 (∞)	4	∞

2.6.4 DNA Electrophoresis on Agarose Gel

50ml, 1% Agarose gels were made up using pre-mixed 1xTBE buffer, and 2.5µl 10mg/ml Ethidium bromide solution. Gels were cast in a plastic mould with two 20 well combs. Samples and 100bp DNA ladder were loaded with 3µl of pre-mixed loading buffer and run in pre-mixed 1xTBE buffer for 30 minutes at 80 volts. Gels were photographed, and analysed using a Bio-Rad ChemiDoc™ EQ imaging device.

2.7 Analysis of Results.

2.7.1 Protein Quantification

After detection the protein was quantified using a Bio-Rad ChemiDoc™ EQ imaging device, and the associated Quantity One 1D Analysis software v.4.5.0 (Microsoft Windows compatible version). The software measures the signal intensity of each band in units of optical density (OD). Protein quantity (%) was expressed relative to an ethanol control (100%) of equivalent volume.

2.7.2 RNA Quantification

After detection the RNA was quantified using a Bio-Rad ChemiDoc™ EQ imaging device, and the associated Quantity One 1D Analysis software v.4.5.0 (Microsoft Windows compatible version). The software measures the signal intensity of each band in units of optical density (OD). RNA quantity (%) was expressed relative to an ethanol control (100%) of equivalent volume.

2.7.3 Statistics

All data is expressed as a mean value of n=6 samples from 3 independent experiments, all data is shown with the standard errors of 6 observations. The results of the protein, and RNA quantity were all treated with a standard Mann-Whitney paired t-Test to determine statistical significance, considered at a *P* value of <0.05, of each treatment against the mean control value.

Chapter 3.
Optimisation and Development of the Various Techniques Utilised in the Current Research

3. Optimisation and Development of the Various Techniques Utilised in the Current Research

Every method requires optimisation to ensure it works to its optimal efficiency. Even a method which works perfectly with one piece of equipment may not work as well with another.

3.1 Protein Extraction

3.1.1 Initial methods used.

On starting this research there were no readily available and reliable kits for extracting both membrane bound, and cytosolic protein separately. It was possible to purchase kits to extract the total protein (cytosolic and membrane bound together), or to extract either separately, however this separate extraction usually resulted in the contamination of the other protein fraction in some way that rendered it unsuitable for use in a Western Blot.

Initially the method which was used involved ultracentrifugation, which provided some problems, one of the biggest was the initial lack of a suitable ultracentrifuge rotor capable of accepting 1.5ml ultracentrifuge tubes, required due to the small sample volumes being collected. The second problem was that this method was not particularly efficient; either at separating the cytosolic and membrane bound protein fractions, or at extracting the majority of membrane bound proteins from the membrane. It was only after considerable, detailed investigation of the localisation of ß-actin within the cell that it was accepted that the presence of ß-actin within the membrane bound protein fraction (Fig 3.1) was indicative of poor separation.

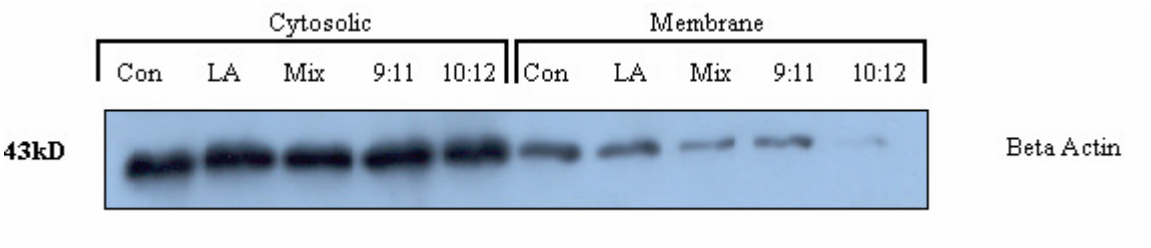


Fig 3.1 ß-actin presence in the Membrane Bound protein fraction. ß-actin as observed in the cytosolic and the membrane bound protein fractions after extraction by ultracentrifugation.

In 2006, new membrane bound protein extraction kit had become available (Calbiochem ProteoExtract™ Native Protein Extraction kit), which produced a much better separation (Fig 3.2).

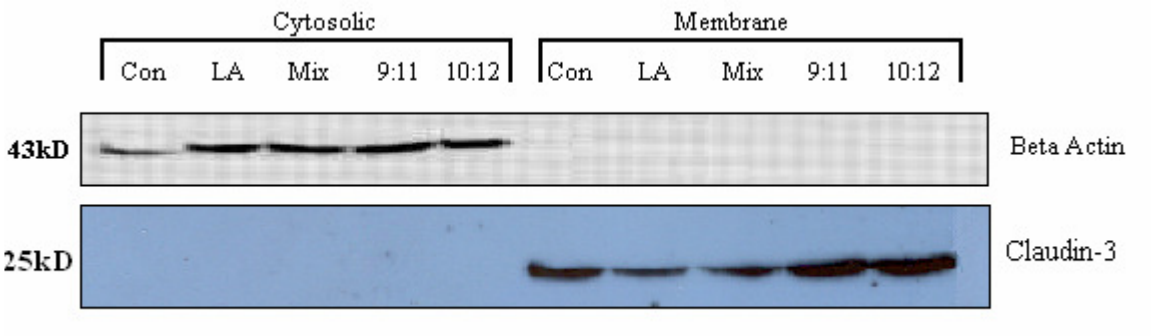


Fig 3.2 Separation of ß-actin and Claudin-3. ß-actin and Claudin-3 from samples extracted using the Calbiochem ProteoExtract™ Native Protein Extraction kit (initial results, not fully optimised, but indicative of seperation).

3.1.2 Optimising the Calbiochem ProteoExtract™ Native Protein Extraction Kit.

The extraction kit produced a new challenge; that the cytosolic protein fraction was very dilute, sometimes so much so that the volume of extract required to load 40µg of protein was greater than the actual capacity of the well. There are several methods of concentrating down protein; it was decided to investigate the use of commercially available centrifugal filters as they appeared to offer the best chance of retaining all the proteins of interest.

To concentrate the protein to an acceptable level several centrifugal filters, from several different companies were investigated. It was determined that the optimal filter would have a molecular weight exclusion point of 10kD or less, this would ensure retention of all the proteins of interest, while removing the excess buffer. The filter was also required to be capable of filtering volumes of samples between 1 and 5 ml, filters only capable of handling smaller amounts would not be any use with a sample volume of 2ml, and filters with a greater capacity run the risk of either not filtering enough buffer, or spinning to dryness, leaving the proteins stuck to the filter membrane. Only one filter was considered to meet the criteria, the Pall, Gelman Microsep 10K Omega. The filter was first rinsed by spinning at 3000g for 15 minutes with distilled water (as per manufacturers recommendations). The cytosolic fraction collected using the Calbiochem ProteoExtract™ Native Protein Extraction kit was then spun, again at 3000g. The manufacturer recommended that the first spin should be of no more than 80 minutes at the lowest effective speed (3000g). If an acceptable solute concentration was not achieved then it was recommended that either the speed of centrifugation (up to 7500g), or the spin time be increased. The first trial proved very successful, with 140 minutes of spin at 3000g and 4˚C leading to an eightfold increase in protein concentration (Fig 3.3).

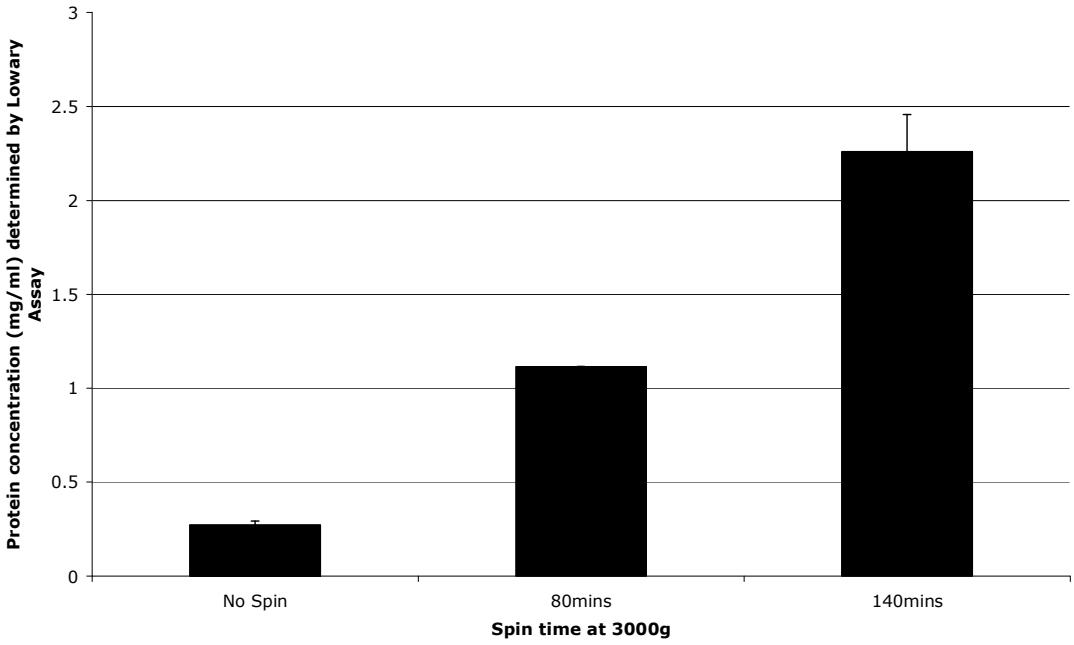


Fig 3.3: Centrifugal Concentration of cytosolic protein. Concentration of proteins by Pall, Gelman Microsep 10K Omega filters. Protein concentrations determined by Lowry Assay after 0, 80, and 140 minutes at 3000g.

3.2 Western Blotting

Western Blotting and gel electrophoresis appear to be particularly sensitive to a myriad of small changes, including ambient temperature, and humidity. The two methods for Western Blotting, currently in use are known as “wet”, and “semi dry”. Both have a theoretically equal chance of success, and opinion appears to be divided as to which works most consistently. During the course of this work both the wet, and the semi dry method have been used, and refined in an attempt to obtain reliable results.

3.2.1 Semi Dry Western Blotting

The semi dry method has the advantage of requiring much less buffer, which helps to keep costs down. The system required a constant pressure to ensure that the current was being passed equally through the whole surface of the gel. If the pressure was unequal then the proteins nearest the top of the machine (where the electrodes plugged into the power pack) were transferred with greater fidelity due to a greater pressure at one end, due to this a large amount of protein was found to be left within the gel (Fig 3.4) after transfer.

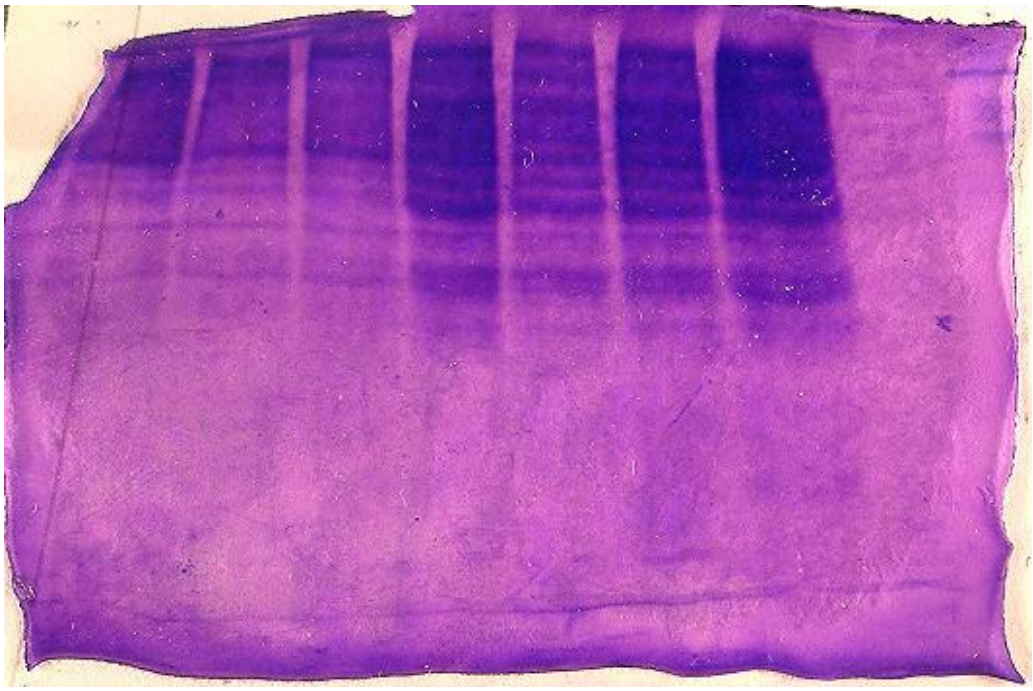


Fig 3.4 Comassie Blue staining of a Gel. Showing poor protein transfer.

Results gained using this equipment were inconsistent, and frequently of poor quality (Fig 3.5).

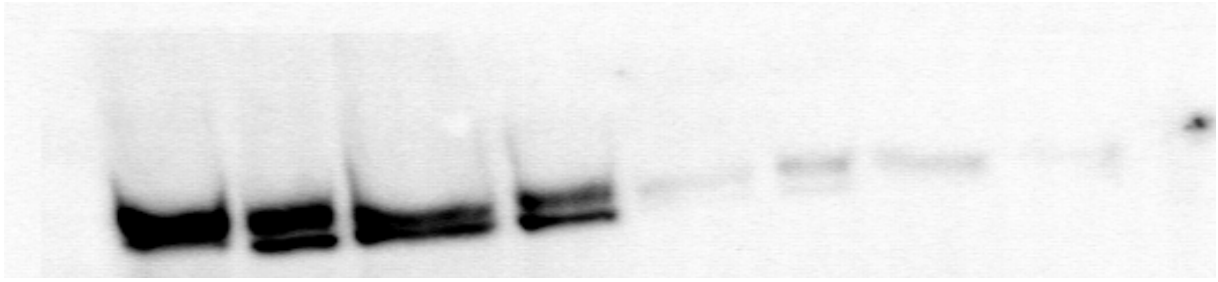


Fig 3.5 Poor transfer of protein. An example of results gained using the Biometra Semi-Dry Electroblotter. The protein being observed in this example is PKC α.

3.2.2 Wet Western Blotting

Wet Western blotting has been carried out throughout using a Bio-Rad Mini Proteon-3 transfer kit. The wet method has, so far, during the course of this research proved to be the most reliable, with approximately 80% of the experiments performed working first time. For proteins of up to 100kD it was found that this system produced clear, consistent results, however for larger proteins (>150kD) transfer is still of poor quality. Fortunately

in this work the various PKC isoforms all have a molecular weight of less than 100kD, as do the Claudin-3 and ß-actin used as loading controls.

3.2.3 Western Blot Buffer

Wet and Semi-Dry blotting both require the same buffer for correct transfer, this buffer composition is very important as it provides the electrolytes required to carry an electric charge, and the SDS which imparts a negative charge to the proteins, allowing them to migrate towards the positive electrode. Originally buffer without SDS was used with the Semi-Dry blotting, but this was found not to work at all. The pH of the buffer is also important, but it was found that adding sodium hydroxide to increase the pH was affecting the migration of the protein. The ideal pH for the buffer should be 8.8, however the actual pH is more usually around 8.3 – 8.4, which is still well within the acceptable range. Too high (or too low) a pH often causes overheating of the buffer, and consequently overheating of the gel, as well as causing poor mobilisation of the proteins.

3.2.4 Western Blot Antibodies

Each specific antibody used during these experiments required careful optimisation. Because not all the antibodies were ordered from the same source there was a mixture of mono- and poly- clonal types, as well as different species, this necessitated using several different blocking agents and strengths. Monoclonal antibodies often exhibit a higher background when blocked with skimmed dried milk, compared to BSA, while polyclonal antibodies appear to give better results with skimmed dried milk. The extreme specificity of monoclonal antibodies also leads to masking at higher concentrations, while polyclonal antibodies exhibit excessive banding if the concentration is too low.

From the optimisation experiments performed it was determined that a buffer made up of 3% BSA in TBS-T worked best for the monoclonal antibodies used, while 5% skimmed dried milk was better for the polyclonal antibodies. The only real exception to this was the Claudin-3 antibody, which was from a monoclonal culture raised in goat rather than in rabbit or mouse. Goat antibodies show a tendency to bind very strongly to BSA, leading to a very high background, however, this was reduced considerably by blocking in 5% non fat milk. Initially high background dogged all the antibodies used, with the exception of ß-actin which had been previously optimised, changes to the concentration and type of blocking buffer either resulted in masking, (no, or very little signal seen), or high background. All membranes were being washed for 3x10 minutes in a 0.1% solution of TBS-T, and increasing the washing time was only marginally effective, however, it was eventually determined that washing with a 0.5% solution of TBS-T almost completely eliminated the background, with the higher concentration of detergent acting to remove loosely bound antibody.

3.3 Development of an Elisa Assay for PKC α

The Enzyme-Linked Immunosorbent Assay (ELISA) is a similar technique to the Western Blot, involving, as it does, an unknown amount of antigen being affixed to a surface, usually a 96 well plate, the plate is then incubated with a specific antibody (in this case PKC α). This antibody is tagged with an enzyme, usually either Horseradish Peroxidase (HRP), or alkaline phosphatase (AP). After incubation the specific substrate is added, and a fluorescent or colorimetric reading is taken, this reading is then compared to standards of a known antigen amount. This method is known as an "indirect" ELISA (Fig 3.6), but there are other variations, including the "Competitive" ELISA (Fig 3.7), and the "Sandwich" ELISA (Fig 3.8). As there were no commercially available kits for detection of the separate PKC isoforms development of one was considered a viable option.

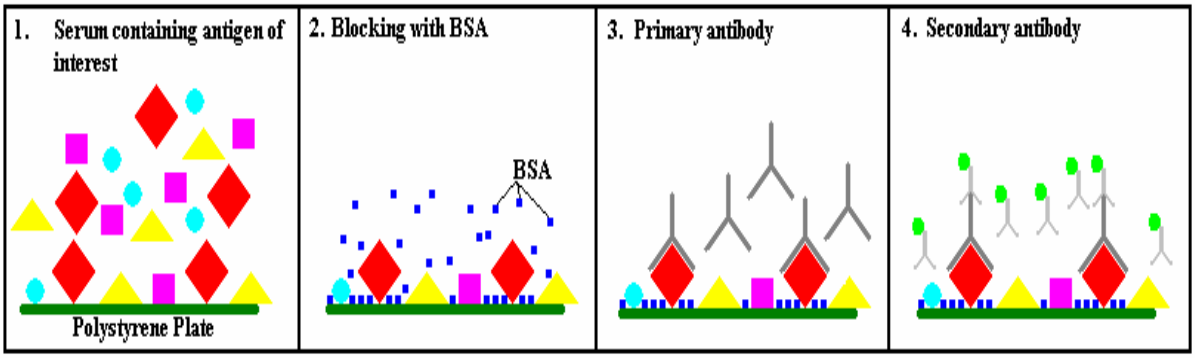


Figure 3.6 Indirect ELISA Method

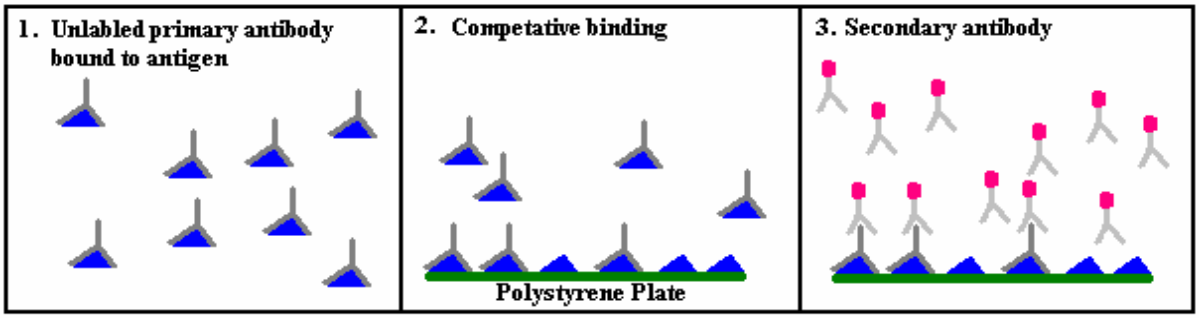


Figure 3.7 Competitive ELISA Method

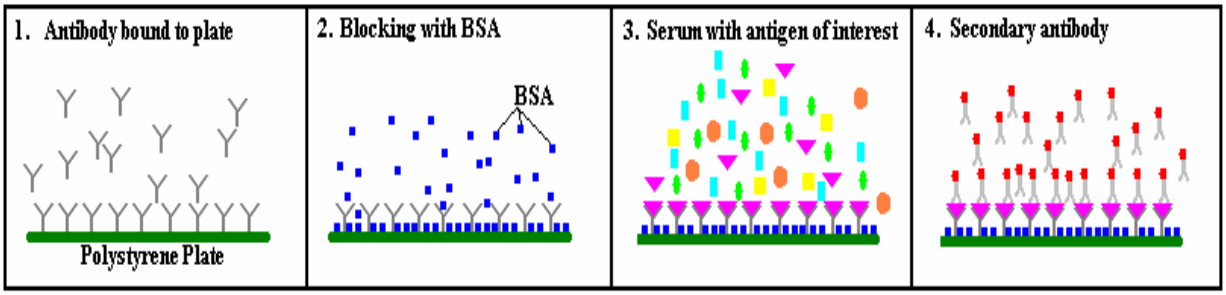


Figure 3.8 Sandwich ELISA Method

Competitive ELISAs involve the incubation of unlabeled antibody with its specific antigen. The antibody/antigen complexes are then added to an antigen coated well. After washing to remove any unbound antibody a specific secondary antibody is added which binds to the primary, this secondary antibody is tagged with either HRP or AP and the signal intensity is again read. The Sandwich ELISA was considered to be the most effective type of ELISA assay for the purpose of this experiment. A known quantity of monoclonal primary antibody was bound to the wells, and all non specific binding sites were blocked using a BSA based blocking buffer. The plate was incubated with the protein sample, and a second, tagged primary antibody. As in other ELISA assays the signal intensity was read, and compared to the standard curve.

The Sandwich ELISA was the assay type of choice as it picks up a small concentration of antigen with greater fidelity than either the Indirect or the Competitive ELISAs. This was especially important as the PKC isoforms are not present in any great quantity within the cell, and as such the indirect and competitive ELISA assays would be less effective.

There were several complications involved with developing a Sandwich ELISA specific to PKC α. Development of an assay for the specific PKC isoforms was considered to be

desirable from a commercial viewpoint. Unfortunately due to budgetary and time constraints the project had to be abandoned.

3.4 Development of an “In-Gel” detection method.

“In-Gel Technology provides a more accurate representation of relative protein levels than traditional Western Blotting.” *Pierce In-Gel Chemiluminescent Detection;* www.piercenet.com.

This is the claim about the only in-gel protein detection system currently available, manufactured by Thermo Fisher Scientific Inc (Rockford Il). The theory suggests that by detecting proteins within the Polyacrylamide gel, rather than transferring them to a membrane a more accurate picture of protein expression can be gained as there should be minimal protein loss through the electrophoresis stage. There is also the possibility that this method can detect proteins in their native form.

The method is comparatively simple, the protein sample is run on a Polyacrylamide gel (Pre-cast or homemade), along with any blanks and standards required. The gel is then pre-treated with either 50% Isopropanol or Methanol to allow antibody penetration. The gel is incubated with primary antibody, then washed with PBS-T, and then incubated with the secondary antibody and washed again. The secondary antibody is tagged with Horseradish Peroxidase, so chemiluminescence with stable peroxide solution and enhancer is performed as in Western blotting. The gel can then be photographed using either light sensitive film, or a specialized CCD camera (for example a Bio-Rad Gel Doc system). The gel may be stripped and re-probed with another primary antibody as required.

The advantages of this system are obvious, cost wise it is more advantageous than the traditional Western Blotting after optimization, because, in theory less can go wrong, and

fewer experiments have to be performed, the quality of the results is also theoretically much better. In terms of time saved it is also very good. Where a traditional Western Blot takes two days to perform, as the primary antibody needs to be incubated over night, the In-Gel system takes less than a day, time being saved by not performing the Transfer step or the blocking step, and a much shorter time required to incubate the gel with the antibody.

The disadvantages to this system are perhaps less obvious. First, and most importantly, the amount of protein required for detection is in the region of one nanogram, a relatively easy amount to load for a highly expressed and abundant protein like ß-actin, a much more difficult prospect when dealing with a less abundant protein like any of the PKC isoforms, especially PKC ι. For this reason it was not possible to utilise, or fully optimise this method.

Chapter 4.
Viability of Breast Cancer Cells After 24h Treatment with CLA and LA.

4. Cancer Cell Viability After Treatment with 25 or 50µM of CLA for 24 Hours.

4.1 Introduction

Measuring the viability of cells in culture after the addition of a pro-, or anti-apoptotic agent is an important method for determining how the agent in question may affect cancer cells *in vivo.* There are several methods for determining cell viability, most relying on dyes, to measure the proportion of cells undergoing apoptosis and necrosis.

For the purpose of this experiment Trypan Blue dye was used to stain the cells prior to manual counting using a haemocytometer and light microscope.

4.2 Results

Viability of MCF-7 and MDA-MB-231 Cells After Treatment

4.2.1 MCF-7 Cells treated with 25µM of CLA for 24 hours.

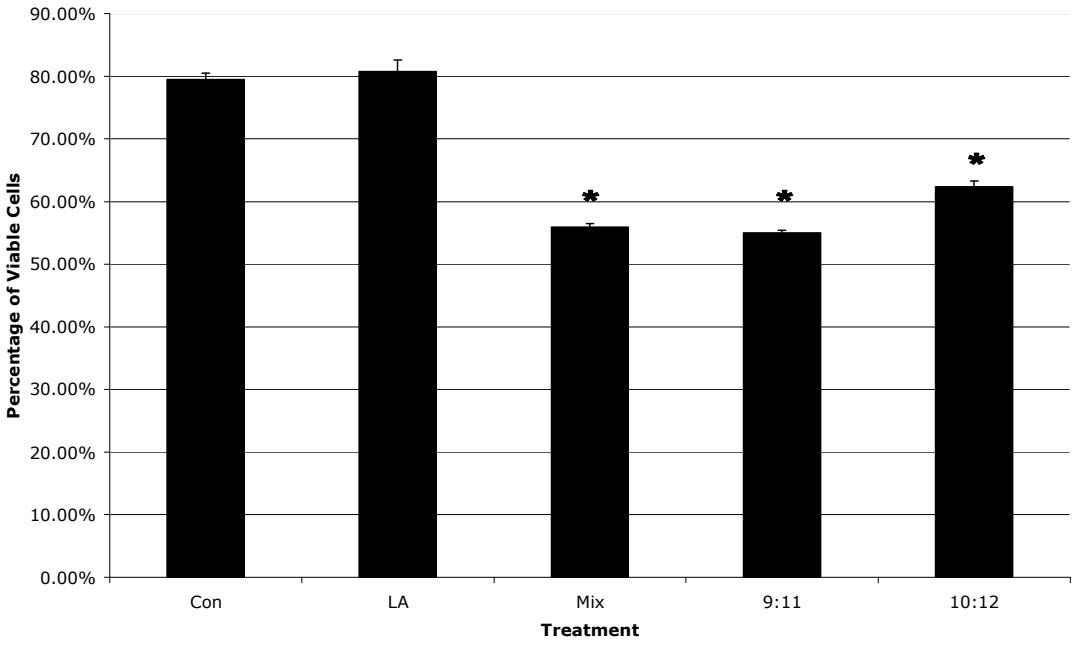


Fig 4.1: MCF-7 Cell viability after treatment with 25µM CLA. Percentage of viable cells after 24 hours treatment with 25µM CLA. Average of three experiments, cells incubated with Trypan Blue.
Key: Control – 100% ethanol (equivalent volume); LA – 25µM Linoleic Acid; Mix – 25µM 50:50 mixture of the *c*9:*t*11 and *t*10:*c*12 isomers; 9:11 - 25µM *c*9:*t*11 isomer alone; 10:12 - 25µM *t*10:*c*12 isomer alone. P<0.05 (*)

MCF-7 cells treated with Linoleic acid (25µM) showed no significant cell death compared to the control. The cells treated with the 50:50 mix of isomers were, on average, 23.6% less viable than the control. Cells treated with the 9:11 isomer alone were, on average 24.5% less viable compared to the control, and cells treated with the 10:12 isomer alone showed the smallest decrease in viability, with a drop of 17.1% (Fig 4.1). A standard one way ANOVA test applied to these results showed that there were no significant differences between the control and Linoleic acid values or between the Mix and 9:11 values. There were significant differences however, between the Mix and 9:11

values when compared to the 10:12 value, and between the Mix, 9:11 and 10:12 values compared to the control (Table 4.1, Appendix B).

4.2.2 MCF-7 Cells treated with 50µM of CLA for 24 hours.

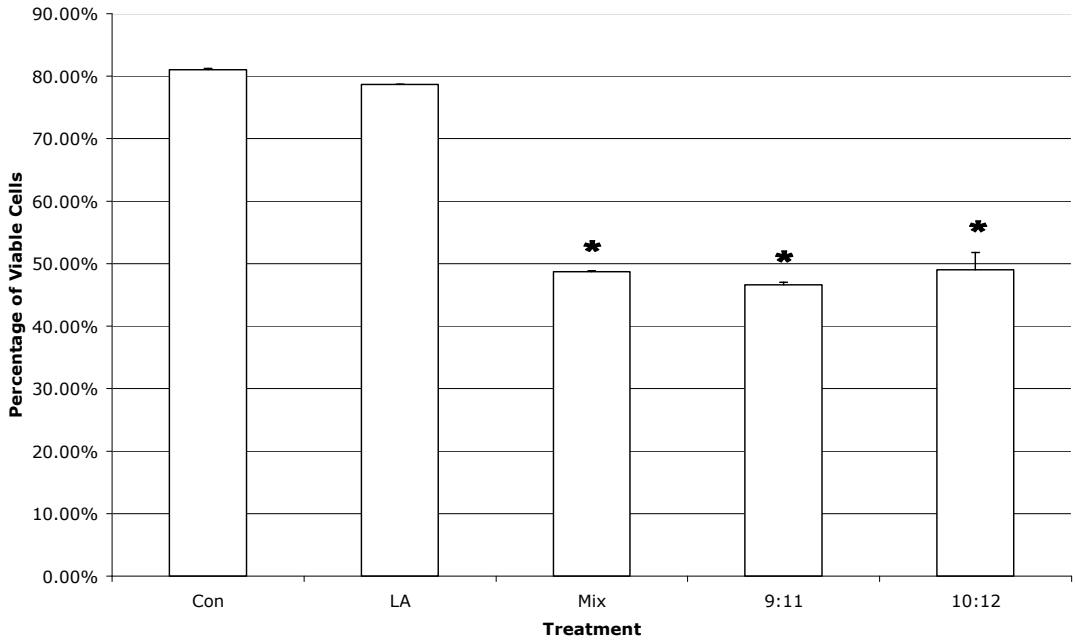


Fig 4.2: MCF-7 Cell viability after treatment with 50µM CLA. Percentage of viable cells after 24 hours treatment with 50µM CLA. Average of three experiments, cells incubated with Trypan Blue.
Key: Control – 100% ethanol (equivalent volume); LA – 50µM Linoleic Acid; Mix – 50µM 50:50 mixture of the *c*9:*t*11 and *t*10:*c*12 isomers; 9:11 - 50µM *c*9:*t*11 isomer alone; 10:12 - 50µM *t*10:*c*12 isomer alone. $P<0.05$

MCF-7 cells treated with 50µM of Linoleic acid showed no significant cell death compared to the control. The cells which were treated with the mix of isomers were on average 32.4% less viable than the control, cells treated with the 9:11 isomer alone were on average 39.4% less viable compared to the control, and the cells treated with the 10:12 isomer alone again showed the smallest decrease in viability, with a drop of 32.1% (Fig 4.2). An ANOVA test showed no significance between the values for the Mix, 9:11 and 10:12 isomers, or between the control and Linoleic acid values. There was however, a

significant difference between the Mix, 9:11 and 10:12 values and the control (P<0.05) (Table 4.2, Appendix B).

4.2.3 MDA-MB-231 Cells treated with 25µM of CLA for 24 hours.

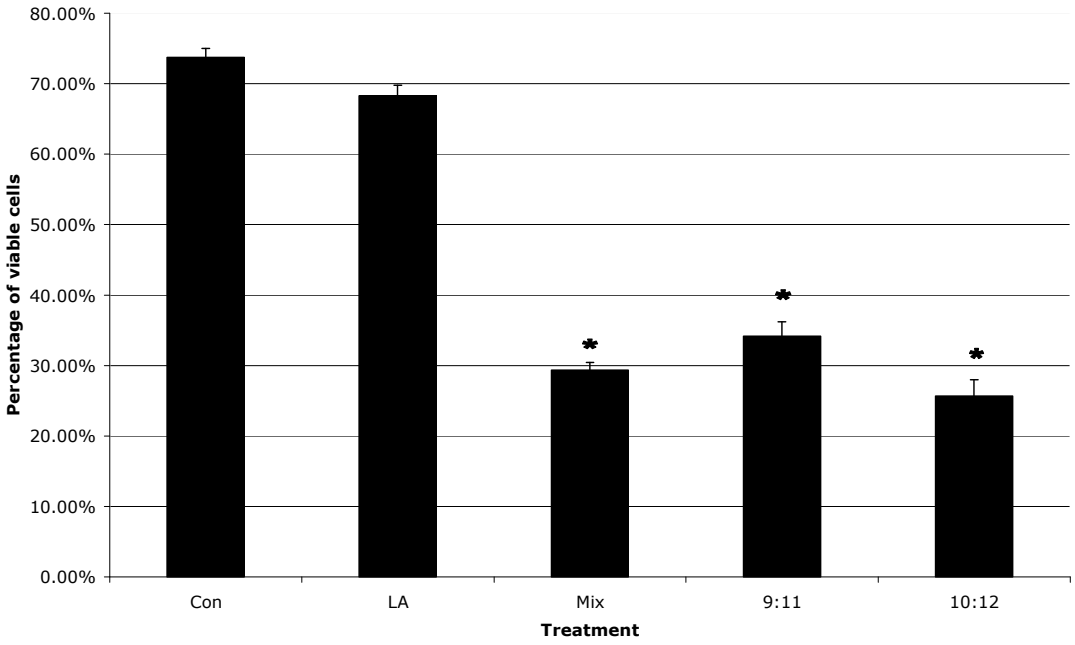


Fig 4.3: MDA-MB-231 Cell viability after treatment with 25µM CLA. Percentage of viable cells after 24 hours treatment with 25µM CLA. Average of three experiments, cells incubated with Trypan Blue.
Key: Control – 100% ethanol (equivalent volume); LA – 25µM Linoleic Acid; Mix – 25µM 50:50 mixture of the *c*9:*t*11 and *t*10:*c*12 isomers; 9:11 - 25µM *c*9:*t*11 isomer alone; 10:12 - 25µM *t*10:*c*12 isomer alone. P<0.05

MDA-MB-231 cells treated with 25µM of Linoleic acid showed no significant cell death compared to the control. The cells which were treated with the mix of isomers were on average 44.4% less viable than the control, cells treated with the 9:11 isomer alone were on average 39.6% less viable compared to the control, and the cells treated with the 10:12 isomer alone showed the largest decrease in viability, with a drop of 48.1% (Fig 4.3). An ANOVA test applied to these results showed that there were no significant differences between the control and Linoleic acid values or between the Mix and 9:11 values. There

was, however, a significant difference between the 9:11 and the 10:12 value, and between the Mix, 9:11 and 10:12 values compared to the control ($P<0.05$) (Table 4.3 Appendix B).

4.2.4 MDA-MB-231 Cells treated with 50µM of CLA for 24 hours.

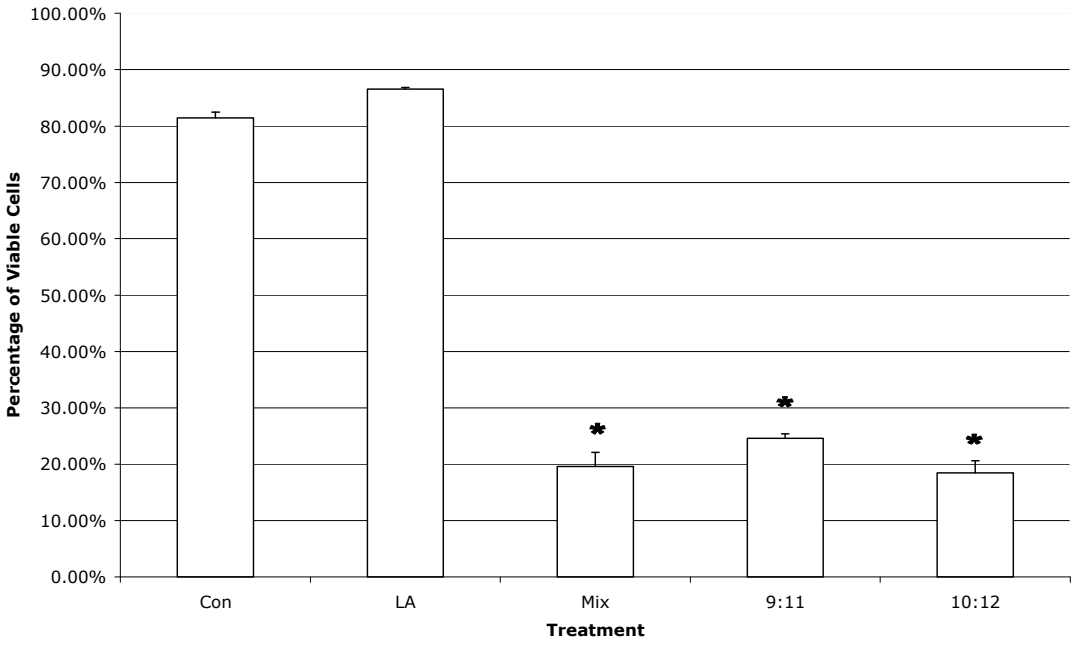


Fig 4.4: MDA-MB-231 Cell viability after treatment with 50µM CLA. Percentage of viable cells after 24 hours treatment with 50µM CLA. Average of three experiments, cells incubated with Trypan Blue.
Key: Control – 100% ethanol (equivalent volume); LA – 50µM Linoleic Acid; Mix – 50µM 50:50 mixture of the *c*9:*t*11 and *t*10:*c*12 isomers; 9:11 - 50µM *c*9:*t*11 isomer alone; 10:12 - 50µM *t*10:*c*12 isomer alone. $P<0.05$

MDA-MB-231 cells treated with 50µM of Linoleic acid showed a very small increase in cell viability (5.1%) compared to the control, this was, however, not significant. The cells which were treated with the mix of isomers were on average 61.9% less viable than the control, cells treated with the 9:11 isomer alone were on average 57.2% less viable compared to the control, and the cells treated with the 10:12 isomer alone showed the largest decrease in viability, with a drop of 63.0% (Fig 4.4). Again an ANOVA test showed no significance between the Mix, 9:11 and 10:12 values or between the control

and Linoleic acid values. There was however a significant difference between the Mix, 9:11 and 10:12 values and the control ($P<0.05$) (Table 4.4, Appendix B).

4.3: Discussion

Effect of Treatment with CLA on Cell Viability.

The viability of both MCF-7 and MDA-MB-231 cells changes significantly with the addition of CLA at both 25 and 50µM concentrations. As previously noted ruminant meat and milk products contain predominantly the *c*9:*t*11 isomer (90%), with the rest being made up of the other, less abundant, CLA isomers, including *t*10:*c*12.

It is notable that both concentrations of CLA have a greater apoptotic effect on the MDA-MB-231 cells (oestrogen receptor negative) after 24 hours than on the MCF-7 (oestrogen receptor positive) cells. This effect can also be observed with the extraction of protein, the MDA-MB-231cells providing, on average, a lower protein yield that the MCF-7 cells.

The cytotoxic effects of CLA's demonstrated here are somewhat at variance with recently published results. In particular Fite *et al.* showed in 2007 that when MDA-MB-231 cells were treated for 48 hours with 40µM of CLA (*c*9:*t*11, *t*10:*c*12 and a 50:50 mix of the two) they exhibited significant cell death (especially in cells treated with the mix and *t*10:*c*12 isomers), but did not show as great a decrease in cell viability as exhibited by the results above. The work of Fite *et al* (2007) showed 30% growth inhibition in the MDA-MB-231 cells treated with 40µM of the isomer mix, and 35% growth inhibition in cells treated with the *t*10:*c*12 isomer alone. This compares to the decreases of 61.9%, and 63.0% for cells treated with 50µM of the same isomers for 24 hours. Fite showed no significant decrease in cell viability for MCF-7 cells treated with CLA, although lower cell viability was noted. The greater effect by the CLA on the viability of the MDA-MB-231 cells, compared with the MCF-7 cells can also be clearly seen in the current research. There are, however, some significant differences between the methods used here and in the work by Fite *et al.* Most importantly Fite *et al.* used an MTT assay to measure cell viability,

compared to the Trypan Blue assay used here. MTT assays are considered to be considerably more accurate as they take a reading of optical density, corresponding to cell viability. The Trypan Blue assay effectively involves manual counting of viable (white) and non viable (blue) cells and coming up with a value for cell viability according to the proportion of white to blue cells. As this involves considerable time spent looking down a light microscope (with ensuing eye tiredness etc) it is highly prone to human error.

The two CLA isoforms used here are also known to exert a dose dependant effect on the cell survival of rat hepatic stellate cells, with the *t*10:*c*12 isomer exerting the strongest effect. In this case the mix of the two isomers (44%:41% *c*9:*t*11: *t*10:*c*12) exerted a rather smaller effect (80% cell survival compared to around 40% at the highest dose of 180µM) that the two isomers did alone, indicating that isomer concentration also has an effect (Yun *et al.* 2008).

Chapter 5.
Investigating a Potential Novel Loading Control for Western Blotting.

5. Investigating a Potential Novel Loading Control for Western Blotting.

5.1 Introduction

5.1.1 The history of Western Blotting.

The method for Western Blotting, as set out by Towbin *et al.* in 1979 (based on a method first detailed by Reiser *et al.* for transferring DNA fragments to a membrane in 1978), is still a widely used tool by researchers wishing to detect and quantify proteins of interest in cellular extracts. Although it has been slightly revised, and specialised equipment is now available, the method has remained largely unchanged in over 20 years. The term "Western Blotting" was coined by W. Neal Burnette in 1981.

5.1.2 Western Blotting Techniques.

The technique involves separating an extract of cellular proteins by sodium dodecyl sulphate-Polyacrylamide gel electrophoresis (SDS-PAGE), allowing separation of proteins by size by passing a current through the gel matrix or mesh. The separated proteins are then transferred to a protein absorbing membrane (PVDF or nitrocelulose) by passing a current through the gel, causing the positively charged proteins to move out of the gel matrix. Once on the membrane all potential non-specific protein binding sites are blocked by a purified, non-reactive protein (often BSA, dehydrated milk preparations, or Casein) and a primary antibody to the protein of interest is added. A generalised fluorescently labelled secondary antibody, usually from the immunoglobulin super-family, is allowed to attach to the primary antibody, and the fluorescence produced measured. The secondary antibody is usually tagged with the Horseradish Peroxidase enzyme (HRP), which allows fluorescence to be produced. The enzyme is extracted from the roots of the Horseradish plant (*Armoracia rusticana*, syn. *Cochlearia armoracia*) by affinity chromotography, and its high specificity and versatility are the main reason it is

used as a popular research tool. HRP exerts its effect by cleavage of various substrates to produce either a colorimetric, or a fluorescent signal. In the case of Western Blotting the HRP enzyme is used to catalyse, and enhance the natural oxidation of luminol in the presence of Hydrogen Peroxide. The areas of enhanced chemiluminescence around the high concentration of the HRP enzyme show up as dark bands on the final picture. Measurements are usually calculated as the intensity of signal per mm^2, the theory being that the more protein bound to the membrane, the greater the amount of antibody bound, and therefore the greater the signal. The intensity of the bands is measured using a sensitive computer software package, capable of measuring the density of individual pixels in a selected area. Using this software the band of interest is selected manually, and the computer works out an average density according to the size of the area selected, and the intensity of the pixels. This average intensity per mm^2 can then be compared to the value for the control.

5.1.3 The Use of Loading Controls in Western Blotting.

Usually a second protein is also selected to act as a loading control or housekeeping protein in order to allow any results to be standardised and analysed with a degree of confidence as to their relevance having eliminated errors due to differences in loading between wells. Several popular antibodies are sold by a number of different specialist antibody suppliers as “loading controls”, the most notable of these being ß-actin. ß-actin is very widely used as a loading control, however in 2006 Dittmer and Dittmer suggested that at higher protein loads, and over longer incubation times it is much less sensitive to changes in protein loading, due to its high cellular abundance. ß-actin is also only found in the soluble protein fraction; not in the insoluble membrane fraction meaning that it is unsuitable for detecting protein loading for membrane bound proteins. Currently there is

no ubiquitously expressed, non-modifiable membrane- bound protein which is considered suitable for general use as a loading control.

5.1.4 Claudin-3

Claudin-3 is a member of the Claudin super family, first identified by Furuse *et al* in 1998. The Claudins are a family of 23 membrane bound proteins, which are found in tissue specific conformations throughout the body. They are part of an important tight junction complex (Miyoshi *et al.* 2005), forming the backbone on which the complex is based. Claudin-3 is found in cells throughout the body (Katahira *et al.* 1997), and its increased expression has been observed in a number of different cancer types (Morin, 2005). Claudin-3 has been shown to be present in a number of different cancer cell lines, including MCF-7 and MDA-MB-231, and to be unaffected by the addition of conjugated linoleic acid isomers (Murphy *et al.* 2006). As yet no research has been done into the effect which the omega-3 fatty acids EPA and DHA have on Claudin-3 in any cell line.

5.1.5 Linoleic acid and CLA.

As previously stated (Chapter 1, page 8) Linoleic acid (LA) is a common fatty acid found in a few plant species and in ruminant meat and milk products, LA may be modified to form various different isomers, including conjugated linoleic acids (CLA's). CLA's are a group of isomers of LA with conjugated double bonds along the fatty acid chain, the main form being the *cis*-9:*trans*-11 isomer (Ha *et al*. in 1987). As with many other fatty acids CLA's are of great interest to researchers due to their apparent chemo-protective and anti-adiposity qualities (Wang *et al.* 2004, Dauchy *et al.* 2004, Belury, 2002, Kohno *et al.* 2004, Kohno *et al.* 2004).

In MCF-7 breast cancer cells, CLA isomers are known to induce apoptosis, and to slow growth and metastasis of implanted mammary tumours (Majumder *et al.* 2002, Liu *et al.* 2004, Chujo *et al.* 2003). CLA's are also known to up- or down- regulate a wide spectrum of proteins, including PPAR and PKC, within many different cancer cell lines.

5.1.6 Experimental Aims

The aims of this experiment are to determine whether Claudin-3 can be considered as an effective loading control for membrane bound proteins extracted from CLA treated MCF-7 cells, and to determine if Claudin-3 shows a greater degree of sensitivity to changes in loading than Ponceau Red staining.

5.2 Results
Claudin-3, ß-Actin and Ponceau Red, a Comparison.

5.2.1 ß-actin in cytosolic and membrane bound protein fractions.

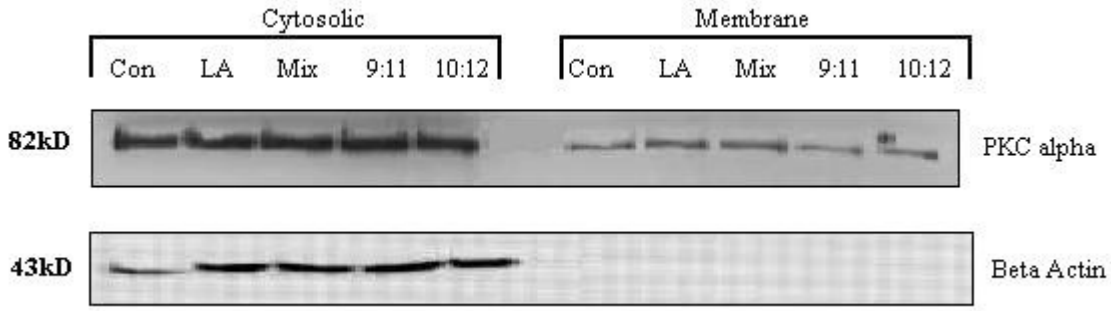


Figure 5.1: ß-actin in MCF-7 cytosolic and membrane bound protein fractions.
MCF-7 cells were treated with 25µM CLA isoforms.
Key: Control – 100% ethanol (equivalent volume); LA – 25µM Linoleic Acid; Mix – 25µM 50:50 mixture of the *c*9:*t*11 and *t*10:*c*12 isomers; 9:11 - 25µM *c*9:*t*11 isomer alone; 10:12 - 25µM *t*10:*c*12 isomer alone. PKC alpha is included to verify loading.

Protein extracted using the Calbiochem ProteoExtract Native Protein Extraction kit exhibited detectable levels of ß-actin expression only in the cytosolic protein fraction of MCF-7 cells (Figure 5.1). PKC α, a protein known to be present in both fractions was used as a control. Optimisation experiments showed that the PKC isoforms were detected with enough strength and clarity to enable accurate measurement of band density at 40µg of protein; this was determined by a standard Lowry assay.

5.2.2 Claudin-3 expression in membrane bound protein fractions.

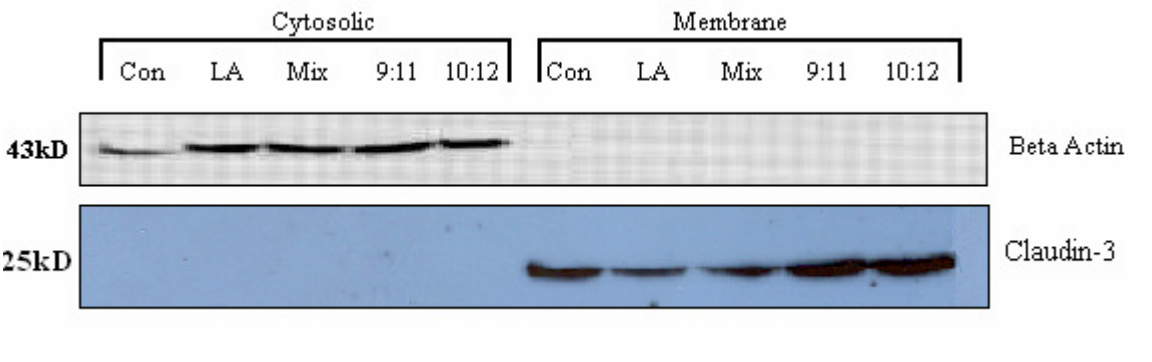
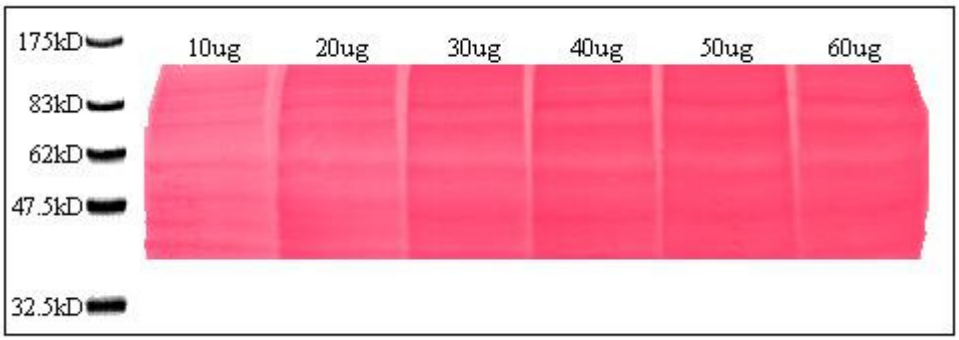


Figure 5.2: Expression of Claudin-3 and ß-actin in total protein extracts. MCF-7 cells, treated for 48 hours with 25µM CLA.
Key: Control – 100% ethanol (equivalent volume); LA – 25µM Linoleic Acid; Mix – 25µM 50:50 mixture of the *c*9:*t*11 and *t*10:*c*12 isomers; 9:11 - 25µM *c*9:*t*11 isomer alone; 10:12 - 25µM *t*10:*c*12 isomer alone (initial results, not fully optimised, but indicative of separation).

Initial experiments showed that Claudin-3 was well expressed in the membrane fraction of MCF-7 cells (Figure 5.2). Total protein from MCF-7 cells treated with CLA and LA showed detectable levels of Claudin-3 and Beta-actin with sufficient band strength and clarity to enable an accurate measurement of band density at 40µg of protein (determined by a standard Lowry assay).

5.2.3 Ponceau Red staining of membrane bound protein.

A.



B.

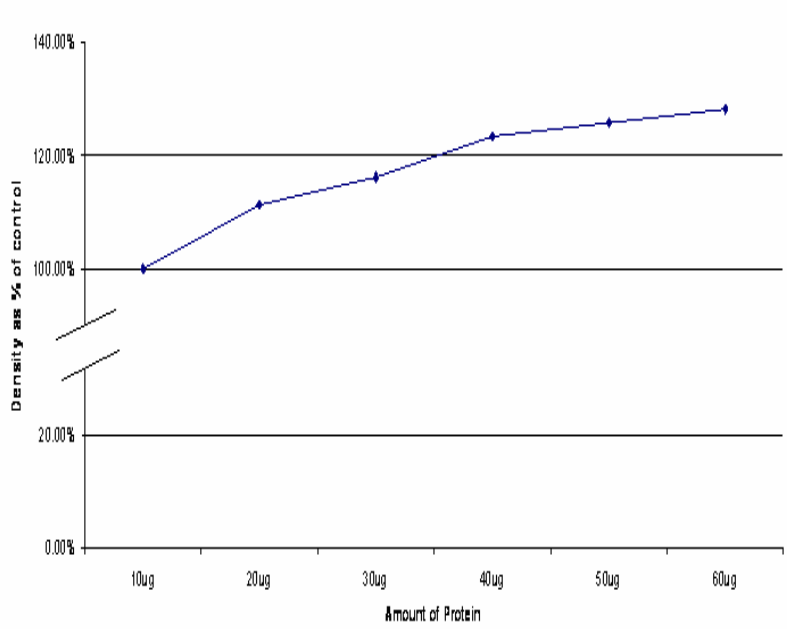


Figure 5.3: Determining protein loading by Ponceau R staining. A: Staining pattern of 10µg, 20µg, 30µg, 40µg, 50µg and 60µg of protein, blocked in 5% non- fat milk and TBS-T and stained with 0.1% Ponceau red solution for 5 minutes. **B:** Density of the 83kD to 47.5kD area expressed as a percentage of the 10µg lane, data obtained is for three independent experiments (one sample per experiment). $P<0.05$

Ponceau red stained all proteins adhering to a PVDF membrane (Figure 5.3.A). The density of the stained band can be read from anywhere on the membrane, the area between 83 and 47.5 KD being an arbitrary choice. Lanes loaded with 10- 60µg of total

protein and stained with Ponceau Red showed increasing band density, although the higher protein amounts showed a decreased sensitivity to changes into the amount of protein loaded (Figure 5.3.B).

5.2.4 Comparing Claudin-3 and ß-actin.

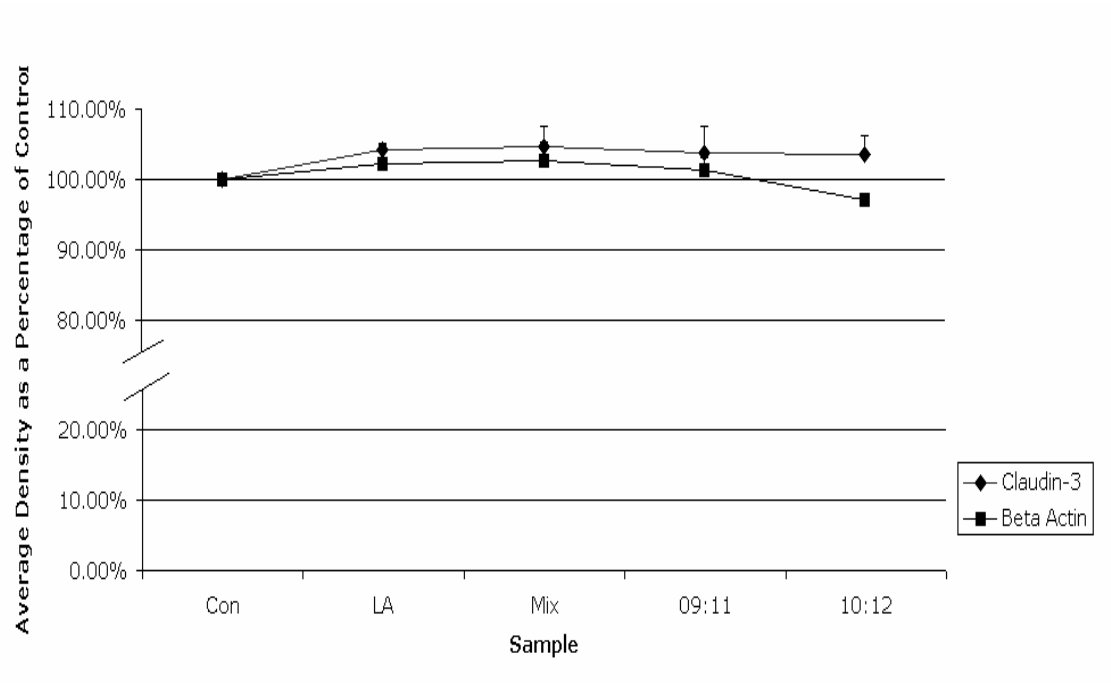


Figure 5.4: Analysis of band density for ß-actin and Claudin-3. As a percentage of the density of the control band. Data represents the results obtained from three independent experiments (two samples per treatment). P<0.05

Neither the Claudin-3, nor the ß-actin exhibited an increase or decrease of more than 4.7% of the control value for any treatment. The difference between the unadjusted Claudin-3 and ß-actin values for all five treatments did not exceed 6.3% of the control (treatment with the *t*10:*c*12 isomer alone), and showed an average of 3.2% across both samples (figure 5.4). Apart from the *t*10:*c*12 isomer treatment the difference between the values for Claudin-3 and ß-actin is largely constant, although Claudin-3 exhibited slightly elevated levels (around 2.2% more than ß-actin). None of these figures showed any statistical significance when treated with a standard Mann-Whitney paired t test.

5.2.5 Comparing Ponceau Red and ß-actin.

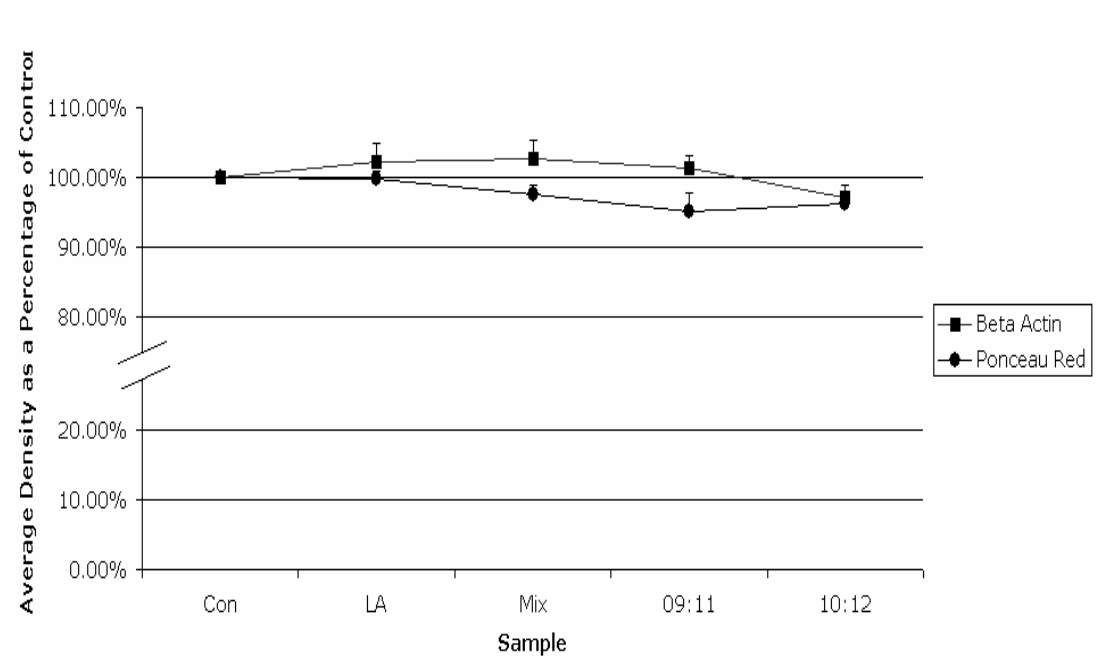


Figure 5.5: Band density for ß-actin and Ponceau Red staining. As a percentage of the density of the control band. Data represents the results obtained from three independent experiments (two samples per treatment). $P<0.05$

The average difference in un-adjusted density as a percentage of the control for the Ponceau Red and ß-actin was 3.7%, with the value not exceeding 6.3% (treatment with the 9:11 isomer alone, figure 5.5). Neither the Ponceau Red nor the ß-actin densities exhibited an increase or decrease of more than 4.8% of the control value and none of the figures showed any statistical significance. The values showed very little constancy although the Ponceau Red values are further from the control than the values for the ß-actin.

5.2.6 Comparing Claudin-3 and Ponceau Red.

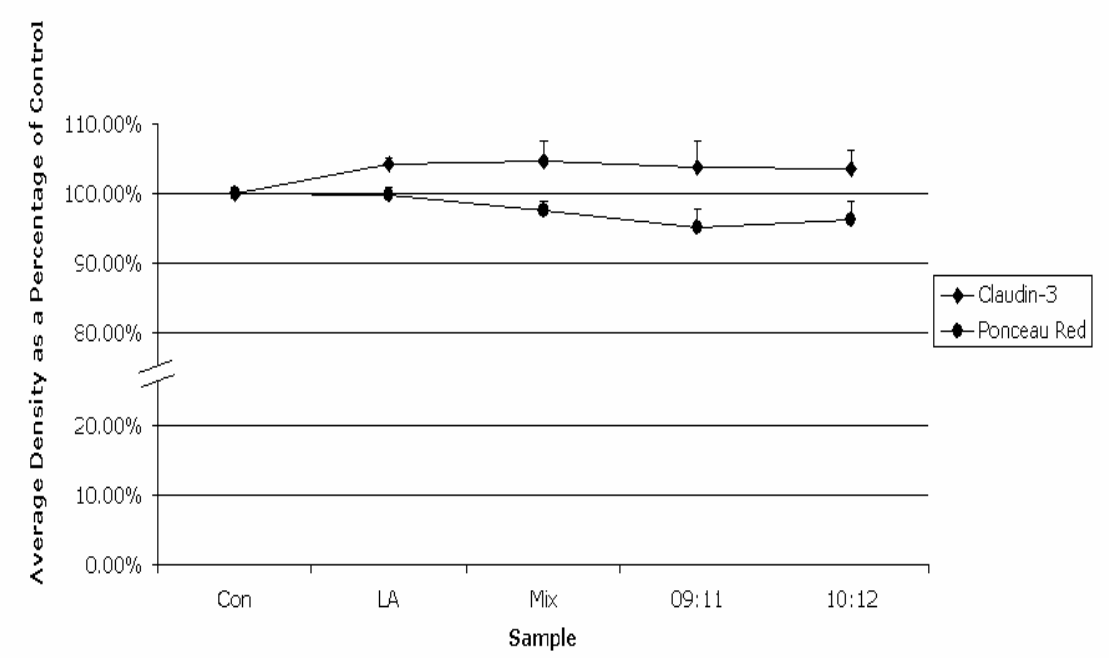


Figure 5.6: Band density for Claudin-3 and Ponceau Red staining. As a percentage of the density of the control band. Data represents the results obtained from three independent experiments. $P<0.05$

The average difference in un-adjusted density as a percentage of the control for the Ponceau Red and Claudin-3 was 6.9% (figure 5.6), almost double the equivalent values for either density compared to the ß-actin. The largest single difference, for the treatment with the 9:11 isomer alone was 8.7%, again greater than the difference in values for either method compared to ß-actin. None of the figures show any statistical significance. Despite the differences neither the Claudin nor the Red Ponceau exhibited an increase or decrease of more than 4.8% of the control value for any treatment. Apart from the values for the linoleic acid treatment the difference between the values was reasonably constant.

5.3. Discussion

Claudin-3 and its suitability as a loading control.

The expression of the Claudin-3 protein does not appear to change significantly, if at all, with the five different treatments used. As the two CLA isomers used are those most commonly explored, due to their relative natural abundance or known beneficial effects on disease processes. It is, therefore, likely that the relative expression of this protein would remain unchanged in cells treated with a more natural mix of isomers. This is because milk and ruminant meat products contain a predominance of the *c*9:*t*11 isomer (90%). The *c*9:*t*11 isomer is the most efficiently produced positional isomer of LA by microbial anaerobic fermentation and bio-hydrogenation in the ruminant gut. The range of other isomers of CLA produced in this way make up the remaining 10%, although this may be altered by a change to the diet of the ruminant. It is also noteworthy that the *c*9:*t*11 and the *t*10:*c*12 isomers generally have the greatest impact on the growth and apoptosis of cells and tumours. Therefore, they are in much greater use, and exhibit much greater popularity as commercial supplements and as research tools.

The expression of ß-actin in the total protein extracts, but not in the membrane fraction extracts, was expected, with beta actin being found only in the cytosol in most cell types. This suggests that ß-actin is not a suitable loading control for all experiments, especially those looking only at active or inactive proteins in the cell membrane.

The Ponceau Red staining pattern on the PVDF membranes, as expected, showed differences only where different amounts of protein were loaded. Two issues must be addressed when considering Ponceau Red as a loading control; the first is that the dye has a relatively small effective range since protein loading of greater than 30µg resulted in reduced sensitivity of the stain. According to experiments performed by Yonan *et al.* at

protein concentrations <0.5µg Ponceau Red does not detect proteins on PVDF membranes, although the stain is slightly more sensitive on Nitrocellulose membranes. The second factor is the relative difficulty in getting a constant staining pattern across the full surface of the membrane, and the highly water soluble properties of the dye are issues which must be addressed when using Ponceau Red as a quantitave stain. The current, and highly sensitive computer hardware and software used to measure quantative staining uses only a small section of the area on which the protein is loaded, therefore any spots, over rinsed areas or accidental watermarks can cause a false positive or false negative result. As a qualitative stain to check for transfer of protein it has remained a popular technique due to its reversibility and versatility.

As a reliable general loading control for membrane bound proteins, Claudin-3 may still be said to be unproven as there has been no other data in the literature regarding its usage in this manner. The present results show that, as a loading control for CLA treated MCF-7 breast cancer cells, Claudin-3 may provide a higher degree of accuracy as a housekeeping protein (as examined in section 5.1.3, P94) in the study of membrane bound proteins than Ponceau red staining.

Chapter 6

Modulation of Protein Kinase C (PKC) isoforms in MCF-7 Cells, when treated with Conjugated Linoleic acid (CLA) and Linoleic Acid (LA)

6. Modulation of Protein Kinase C (PKC) isoforms in MCF-7 Cells, when treated with Conjugated Linoleic acid (CLA) and Linoleic Acid (LA)

6.1 Introduction

6.1.1 Cancer incidence and survival

Breast cancer accounts for 15% of the total cancer burden in the UK, this relates to 30% of all new cancers detected in women. The five year survival rates for women with breast cancer currently stand at circa 77%, due largely to improvements in chemotherapeutic drugs (Cancer Research UK, 2001). Hormone receptor positive breast cancer is preferentially treated with Tamoxifen (Taylor and Taguchi, 2005). Within the past few years, however, many people have been turning towards complimentary therapies, in particular nutritional supplements, to help treat breast cancer in tandem with conventional medical techniques. There is some evidence to suggest that some of these nutritional therapies, in particular the well publicized "five-a-day" fruit and vegetable initiative, do actually increase the survival rates and reduce the risk of recurrence of breast cancer (Joshipura *et al.* 2001).

6.1.2. Fatty acids and cancer

Linoleic acid is found in a few plant species and in ruminant meat and milk products, as previously noted LA may be modified in many ways to form various positional isomers, including conjugated linoleic acids (CLA's). Edible oils and ruminant milk and meat products make up the main intake of CLA by humans, although consumption of these varies. In humans, dietary CLA can be detected in the plasma and lymph fluid and is known to have a positive effect on various conditions, including diabetes, obesity, arthrosclerosis and some cancers (Wang and Jones, 2004, Belury, 2002). It has already been established that CLA has an anti proliferative effect on androgen sensitive prostate

cancer cells, and both oestrogen receptor positive and negative breast cancer cell lines respond in a similar manner (Song *et.al* 2004, Wang *et.al* 2008 and Miglietta *et.al* 2006).

6.1.3 Mechanisms affected by CLA

Various cancer cell lines exhibit cellular apoptosis when treated with isomers of CLA *in vitro*, similarly tumours, either induced or grafted in animal models, especially rats, show a decrease in size and weight when the diet is supplemented with CLAs (Dugram and Fernandes, 1997, Song *et al.* 2004, Field and Schley, 2004, and Bocca *et al.* 2006). The reasons for increased apoptosis are unclear. However, CLA has been shown to influence the expression of various proto-oncogenes, including p53, p21 and Bcl-2 (Majumder *et al.* 2002), all of which have pro-apoptotic properties.

6.1.4. PKC-isoforms and cancer

There are definite links between cellular apoptosis and the presence of the various Protein Kinase C (PKC) isoforms. Differential expression of PKC isoforms in various tissue types has been investigated, with certain isoforms appearing abundantly in different cell types, this suggests unique functions are performed by each isoenzyme (Ford *et al.* 1995, Ryer *et al.* 2005, and Mischak *et al.* 1993).

6.1.5 Experimental Aims

As yet very little or nothing has been reported about the effect of CLA, on the PKC profile of MCF-7 cells. The current research aims to study the effect of CLA (25 and 50μM) on the three PKC isoforms α, δ, and ι using protein extraction from cytosolic and membrane fractions and western blotting techniques.

6.2 Results
PKC Activation in MCF-7 Cells After Treatment With CLA.

6.2.1 PKC α

6.2.1.1 Treatment of MCF-7 Cells With 25μM CLA.

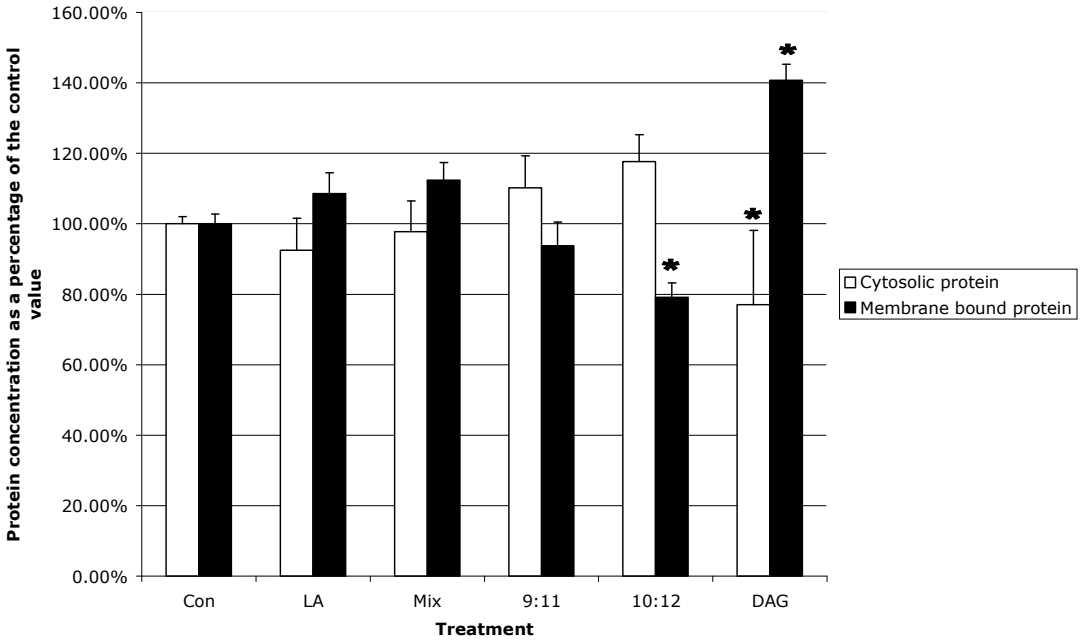


Figure 6.1: Effect of CLA's on PKC α. MCF-7 cells treated with 25μM CLA isoforms or 25μM DAG. ß-actin was used to verify loading in cytosolic protein samples, Claudin-3 was used to verify loading in membrane bound protein samples. Data are from three independent experiments.
Key: Control – 100% ethanol (equivalent volume); LA – 25μM Linoleic Acid; Mix – 25μM 50:50 mixture of the *c*9:*t*11 and *t*10:*c*12 isomers; 9:11 - 25μM *c*9:*t*11 isomer alone; 10:12 - 25μM *t*10:*c*12 isomer alone. P<0.05 (*)

Membrane abundance of active PKC α showed an increase of 8.6% and 12.4% (24 hours, 25μM LA or 50:50 mix treatment, Fig 6.1). With LA treatment the inactive cytosolic PKC α was reduced by 7.5%, while with the mix of isomers PKC α was decreased by 2.2%. Cells treated with the individual *c*9:*t*11 and *t*10:*c*12 isomers at 25μM concentrations showed a decrease in membrane bound PKC α by 6.3% and 20.9% respectively. A corresponding increase in the amount of inactive PKC α was detected in the cytosol. Only the result for the membrane bound *t*10:*c*12 treatment alone shows any statistical significance when treated with a standard Mann-Whitney paired t test. The membrane

abundance of active PKC α exhibited an increase of 40.7% in cells treated with 25μM DAG, the comparative decrease in cytosolic PKC α was 23.0%, both of these results show statistical significance.

6.2.1.2 Treatment of MCF-7 Cells With 50μM CLA.

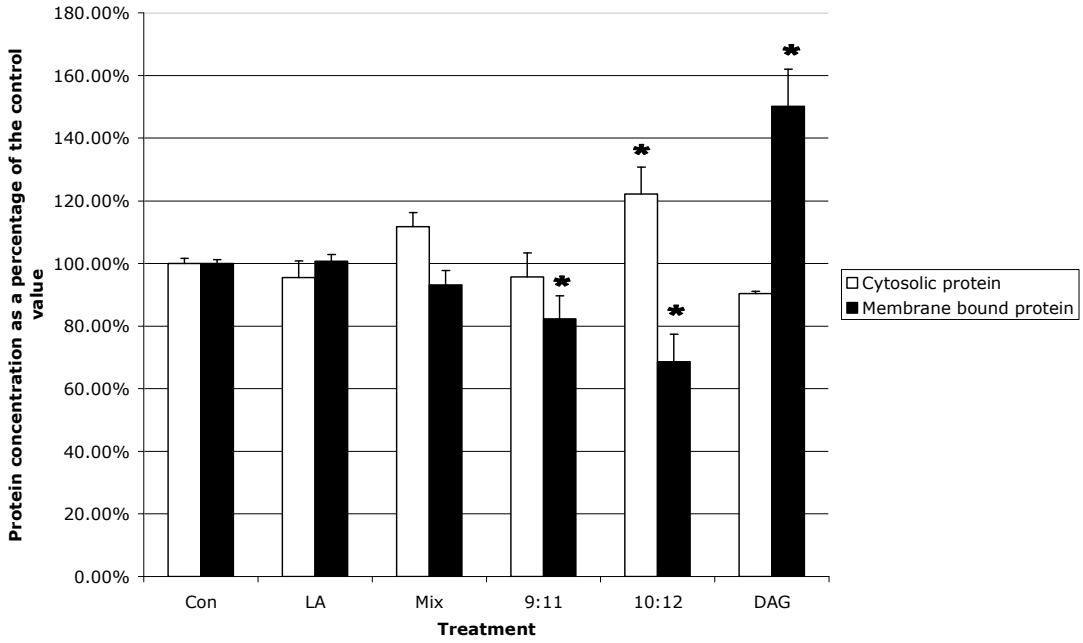


Figure 6.2: Effect of CLA's on PKC α. MCF-7 cells treated with 50μM CLA isoforms. ß-actin was used to verify loading in cytosolic protein samples. Claudin-3 was used to verify loading in membrane bound protein samples. Data are from three independent experiments.
Key: Control – 100% ethanol (equivalent volume); LA – 50μM Linoleic Acid; Mix – 50μM 50:50 mixture of the *c*9:*t*11 and *t*10:*c*12 isomers; 9:11 - 50μM *c*9:*t*11 isomer alone; 10:12 - 50μM *t*10:*c*12 isomer alone. $P<0.05$

Membrane abundance of active PKC α was decreased after treatment for 24 hours with 50μM of the individual *c*9:*t*11 and *t*10:*c*12 isomers and the 50:50 mix of isomers (Fig 6.2). The 50:50 mix showed a non significant decrease of the active PKC α (6.8%) while causing a slight increase of the inactive cytosolic PKC α (11.7%). The *c*9:*t*11 and *t*10:*c*12 isomers both caused significant decreases of active PKC α by 17.7% and 31.4% respectively. A significant increase (22.2%) of cytosolic PKC α was observed in cells

treated with the *t*10:*c*12 isomer (50 µM) alone, however with the *c*9:*t*11 isomer a non significant decrease of 4.2% in inactive PKC α was obtained. The membrane abundance of PKC α in cells treated for 24 hours with 50µM of LA showed a very small increase (0.8%), while the cytosolic PKC α was decreased by 4.6%, neither of these results are statistically significant. Treatment with 50µM DAG however exhibited significant increase (50.2%) in the membrane bound protein PKC α. The abundance of cytosolic PKC α on the other hand, showed an insignificant decrease (9.6%)

6.2.2 PKC δ

6.2.2.1 Treatment of MCF-7 Cells With 25µM CLA.

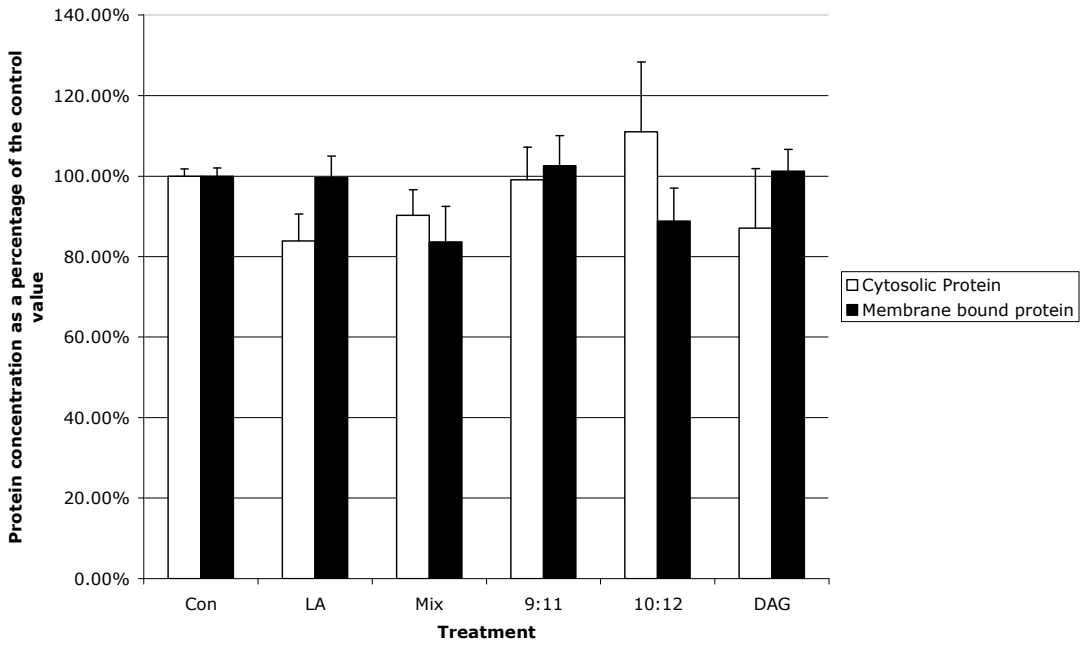


Figure 6.3: Effect of CLA's on PKC δ. MCF-7 cells treated with 25µM CLA isoforms or 25µM DAG. ß-actin was used to verify loading in cytosolic protein samples, Claudin-3 was used to verify loading in membrane bound protein samples. Data are from three independent experiments.
Key: Control – 100% ethanol (equivalent volume); LA – 25µM Linoleic Acid; Mix – 25µM 50:50 mixture of the *c*9:*t*11 and *t*10:*c*12 isomers; 9:11 - 25µM *c*9:*t*11 isomer alone; 10:12 - 25µM *t*10:*c*12 isomer alone. $P<0.05$

No statistical significance was observed for any of the four 25μM treatments used when PKC δ was detected in either its inactive or active forms. Both the isomer mix and the *t*10:*c*12 treatment alone showed a slight decrease (16.4% and 11.2% respectively, Fig 6.3) in the amount of membrane bound PKC δ, and the inactive protein in cells treated with the mix of isomers exhibited a decrease of 9.8%. An even smaller change in membrane bound PKC δ, this time an increase, was observed in the cells treated with the *c*9:*t*11 isomer. 2.6% more active protein was seen, the decrease in the cytosolic PKC δ was only 0.9%. The cells treated with LA exhibited a 0.4% decrease in PKC δ concentration; however, the cytosolic fraction showed a larger decrease of 16.0%. In cells treated with 25μM DAG these experiments exhibited a small, but not significant increase in the membrane abundance of active PKC δ (1.2%), equally the decrease in cytosolic PKC δ was not significant (12.9%).

6.2.2.2 Treatment of MCF-7 Cells With 50µM CLA.

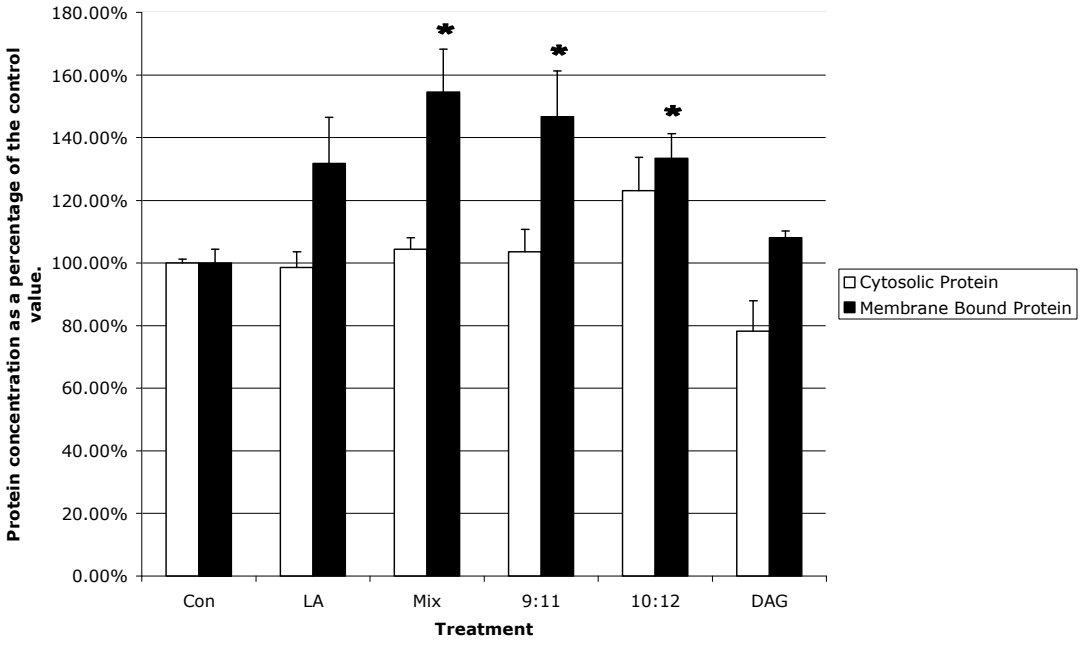


Figure 6.4: Effect of CLA's on PKC δ. MCF-7 cells treated with 50µM CLA isoforms. ß-actin was used to verify loading in cytosolic protein samples. Claudin-3 was used to verify loading in membrane bound protein samples. Data are from three independent experiments.
Key: Control – 100% ethanol (equivalent volume); LA – 25µM Linoleic Acid; Mix – 25µM 50:50 mixture of the *c*9:*t*11 and *t*10:*c*12 isomers; 9:11 - 25µM *c*9:*t*11 isomer alone; 10:12 - 25µM *t*10:*c*12 isomer alone. $P<0.05$

Membrane abundance of active PKC δ was increased in cells treated for 24 hours with 50µM LA, the 50:50 mix of the isomers, and the *c*9:*t*11 and *t*10:*c*12 isomers alone (Fig 6.4). With the LA treatment the active PKC δ increased by 31.8%, while the inactive cytosolic PKC δ decreased by 1.5%. The cells treated with the 50:50 mix of isomers showed a significant increase in the active, but not the inactive PKC δ, 54.7% and 4.5% respectively. The individual *c*9:*t*11 and *t*10:*c*12 isomers at 50µM concentrations showed a significant increase of 46.8% (*c*9:*t*11) and 33.5% (*t*10:*c*12) of active PKC δ, there was also a corresponding increase in the amount of inactive PKC α detected in the cytosol of 3.6% (*c*9:*t*11) and 23.1% (*t*10:*c*12). The membrane abundance of active PKC δ exhibited

an increase of 8.1% in cells treated with 50µM DAG, the comparative decrease in cytosolic PKC δ was 21.8%, neither of these results showed statistical significance.

6.2.3 PKC ι

6.2.3.1 Treatment of MCF-7 Cells With 25µM CLA.

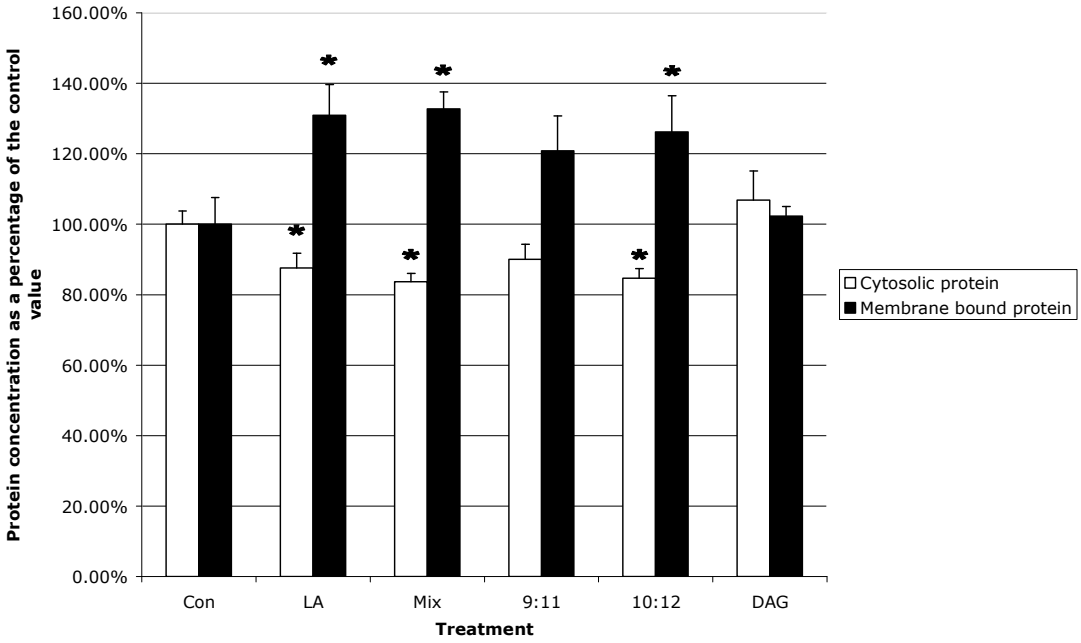


Figure 6.5: Effect of CLA's on PKC ι. MCF-7 cells treated with 25µM CLA isoforms or 25µM DAG. ß-actin was used to verify loading in cytosolic protein samples. Claudin-3 used to verify loading in membrane bound protein samples. Data are from three independent experiments.
Key: Control – 100% ethanol (equivalent volume); LA – 25µM Linoleic Acid; Mix – 25µM 50:50 mixture of the *c*9:*t*11 and *t*10:*c*12 isomers; 9:11 - 25µM *c*9:*t*11 isomer alone; 10:12 - 25µM *t*10:*c*12 isomer alone. $P<0.05$

The concentration of membrane bound PKC ι was increased in all four treatments (Figure 6.5), with this increase showing statistical significance in the cells treated with the LA, isomer mix and *t*10:*c*12 alone (30.9%, 32.8% and 26.2%). The cells treated with the *c*9:*t*11 isomer alone also exhibited an increase of 20.9%, but this was not found to be significant. The corresponding concentration of inactive PKC ι in the cytosol showed a reduction in all four treatments, again the LA, isomer mix and *t*10:*c*12 alone showed

statistical significance. The results for PKC ι in membrane and cytosolic protein fractions when treated with 25µM DAG are not statistically significant, showing an increase of 2.3% and 6.8% respectively.

6.2.3.2 Treatment of MCF-7 Cells With 50µM CLA.

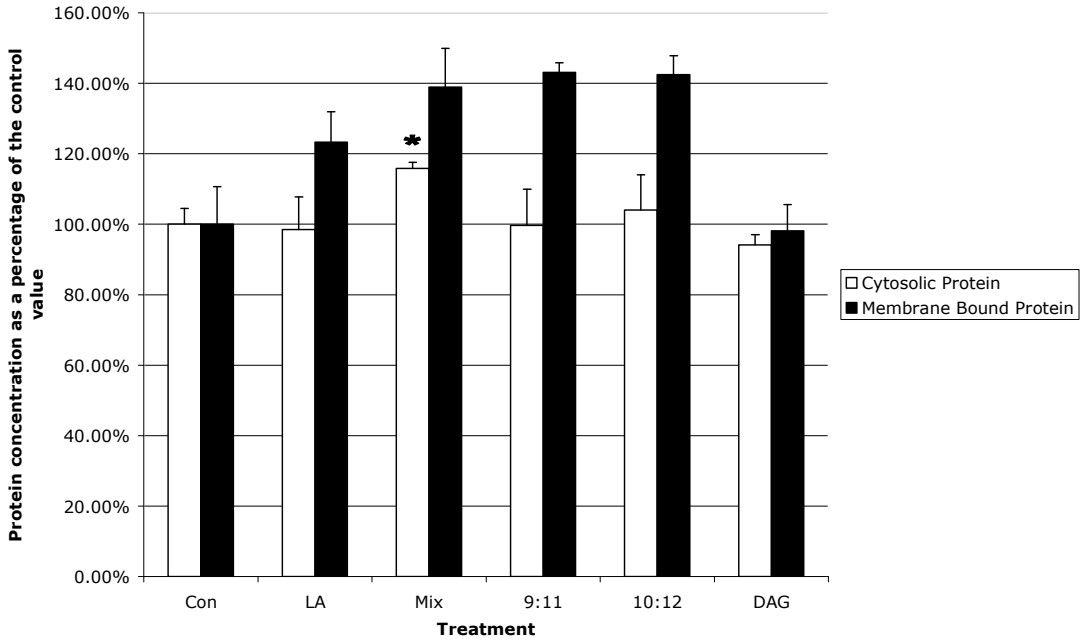


Figure 6.6: Effect of CLA's on PKC ι. MCF-7 cells treated with 50µM CLA isoforms or 25µM DAG. ß-actin was used to verify loading in cytosolic protein samples, Claudin-3 used to verify loading in membrane bound protein samples. Data are from three independent experiments.
Key: Control – 100% ethanol (equivalent volume); LA – 25µM Linoleic Acid; Mix – 25µM 50:50 mixture of the *c*9:*t*11 and *t*10:*c*12 isomers; 9:11 - 25µM *c*9:*t*11 isomer alone; 10:12 - 25µM *t*10:*c*12 isomer alone. $P<0.05$

Membrane abundance of active PKC ι was increased in cells that had been treated for 24 hours with 50µM of the LA, 50:50 mix, *c*9:*t*11 and *t*10:*c*12 isomers respectively (Fig 6.6). With LA treatment the inactive cytosolic PKC ι was reduced by 1.5% while the active PKC ι showed the smallest increase (23.4%). The cells treated with the 50:50 mix at the 50µM concentration showed a significant increase of 16.0% in the amount of inactive PKC ι, a corresponding increase of 39.0% in the membrane bound PKC ι was also

observed. Cells treated with the *c*9:*t*11 isomer alone demonstrated a 0.2% decrease in inactive PKC ι and a 43.1% increase in active PKC ι. Cells treated with the *t*10:*c*12 isomer alone showed a 4.0% increase in inactive PKC ι and a 42.5% increase in active PKC ι. None of these results showed any statistical significance. The membrane abundance of active PKC ι exhibited a decrease of 1.8% in cells treated with 50μM DAG, the comparative decrease in cytosolic PKC ι was 5.2%, neither of these results show statistical significance.

6.3 Discussion

Effects of Treatment With CLA in Oestrogen Receptor Positive Cells on Abundance of Active and Inactive PKC.

There have been several studies, both *in vivo,* and *in vitro* which appear to indicate that CLA has the potential to suppress the development and proliferation of various types of cancer cells, including breast cancer (most recently, Wang *et al.* 2008). While some steps have been made to elucidate the mechanism by which CLA exerts these effects much of its action upon cell signalling pathways is still unclear. In this study however the effects of CLA upon the regulation of various PKC isoforms in the oestrogen receptor positive breast cancer cell line MCF-7 have been clearly shown.

It has already been demonstrated that CLA has a pro-apoptotic effect upon prostate cancer LNCaP cells (Song *et. al* 2004), and this study shows evidence of a similar effect by CLA in MCF-7 cells.

Membrane association of PKC is indicative of activation, through allosteric activation, by diacylglycerol (DAG) or by negatively charged phospholipids. The presence of phosphatidylserine (PS) is required by the typical and novel isoforms, and phosphatidylethanolamine or 3-phosphoinositides (PIP_3) by the atypical PKCs.

The incubation of 25 and 50µM of CLA with MCF-7 cells *in vitro* has a significant pro-apoptotic effect, with 50µM of the *cis*-9:*trans*-11 isomer alone causing a 39.4% decrease in cell viability, as measured by Trypan blue assay. Thus it would be expected that these changes would be associated with changes to activation of the three PKC isoforms studied so far.

6.3.1 Effect of CLA on PKC α Expression

Active PKC α has been implicated in both anti-apoptotic and anti-proliferative effects, in various cell lines (Dempsey *et. al* 2000), however there is also strong evidence that it exerts a pro-apoptotic effect in LNCaP cells, due to persistent activation (Powell *et al.* 1996). Generally PKC α activation is associated with increased cell growth and survival in several cell lines, including MCF-7 (Ways *et al.* 1996). In this study it was shown that PKC α activation was decreased with both 25 and 50μM *cis*-9:*trans*-11 and *trans*-10:*cis*-12 isomers of CLA treatment. This concurs with the findings of Song *et al.* (2004), who observed a similar effect, although the decrease was more pronounced in PKC α activation after 24 hours treatment with 25 or 50μM of the individual isomers in the LNCaP prostate cancer cell line. It is noteworthy that, while the decrease in PKC α activation in the MCF-7 cells lines used in this experiment is greater after treatment with 50μM of CLA, in the LNCaP cells this decrease was greater after treatment with 25μM. In comparison to the behaviour of the LNCaP cells the changes in PKC α activation in MCF-7 cells are much less dramatic, and much less significant. After 24 hours treatment with the 50:50 mix of the two isomers, at a concentration of 25μM, the activated PKC α being expressed by the cells appeared to increase. The fact that the two isomers separately at the same concentration both cause a significant decrease in active PKC α raises speculation that this result, with the Mix, is an anomaly, especially given that at 50μM of CLA the isomer mix causes a non significant decrease in PKC α activation. In LNCaP cells however this pattern is repeated, although there is a slight decrease in active PKC α rather than a slight increase (Song *et al.* 2004).

6.3.2 Effect of CLA on PKC δ Expression

PKC δ activation is believed to mediate opposing effects to PKC α activation (Dempsey *et. al* 2000). Many of these effects are believed to be due to the action of activated PKC δ as a substrate for interleukin-1 converting enzyme (ICE) like proteases; also known as the "Executioner caspases", specifically caspase-3 (Basu, 2003). In general, PKC δ activation increases pro-apoptotic events, by cleavage from its auto-inhibitory domain, and subsequent migration to cell and mitochondrial membranes (Basu, 2003). Down-regulation of PKC δ has been linked to tumour progression, while up-regulation is known to inhibit the progression of the cell cycle (Mischak *et al.* 1993). There is however a suggestion that, in the presence of tamoxifen, the PKC δ pathway is a major alternative pathway for cell growth in MCF-7 cells (Nabha *et al.* 2005).

In this study a significant increase in PKC δ activation was observed after treatment with 50μM of the 50:50 isoform mix, the *cis*-9:*trans*-11 and the *trans*-10:*cis*-12 isomers alone. Equally an increase in the amount of cytosolic PKC δ was also seen, suggesting that any increase may not be due solely to translocation to the cell membrane, but may in fact have an altered gene expression or a genetic component as well. This increase is similar to the activity pattern observed by Song *et al.,* in the LNCaP cell line. However, with the PKC α the increase is several orders of magnitude smaller in the LNCaP. In LNCaP cells the *cis*-9:*trans*-11 isomer alone appears to exert the greatest effect on PKC δ activation, with the *trans*-10:*cis*-12 isomer and the 50:50 mix of the two exerting a smaller effect. In the MCF-7 cell line we see what appears to be the opposite happening, the 50:50 mix is exerting the greatest effect on PKC δ activation, while the *cis*-9:*trans*-11 and the *trans*-10:*cis*-12 isomers individually exert smaller effects.

At concentrations of 25μM, the situation is different, rather than increasing the activation of PKC δ it appears that, unlike the LNCaP cells, lower concentrations of the 50:50 mix, and the *trans*-10:*cis*-12 isomer alone CLA actually decrease active PKC δ. A small increase in active PKC δ is observed after treatment with 25μM of the *cis*-9:*trans*-11 isomer, but this was not significant.

6.3.3 Effect of CLA on PKC ι Expression

PKC ι is a marker of poor prognosis, and increased cell survival in many different types of cancer, including, non small cell lung cancer, ovarian cancer and prostate cancer (Lu *et al.* 2001, Eder *et al.* 2005; Win and Acevedo-Duncan, 2008). PKC ι resides on chromosome 3q26; amplification of this region is common in non small cell lung cancer (predominantly squamous cell carcinoma), and ovarian tumours. 3q26 amplification has also been observed in cervical, and oesophageal tumours, suggesting that PKC ι may play an even more important role in cancer biology than previously thought (Fields *et al.* 2007). It is actually the first of the PKC isoforms to be shown to possess the three singular characteristics of a cancer gene (i. critical to cancer cell biology, ii. activated in primary tumours and, iii. targets for tumour specific genetic alterations, Regala *et al.* 2005). In breast cancer cells, recent research suggests that the over expression of PKC ι increases the invasiveness of breast cancer cells (progression from ductal carcinoma in stitu to invasive ductal carcinoma), although no correlation with other prognostic indicators (i.e oestrogen receptor status, progesterone receptor status, HER2 etc) was found (Kojima *et al.* 2008). Results from immunohistochemistry staining for PKC ι showed an increase corresponding to the severity of the pathological type of tumour, although there was no apparent correlation between PKC ι presence and tumour grade. This suggests that the active protein expression for PKC ι should decrease after treatment with the various CLA

isoforms. The results gained for both the 25 and the 50μM treatments indicate that this is not the case; although neither do they display any real significance. The work of Song *et al.* in LNCaP cells showed a clear decrease in active, membrane bound PKC ι after treatment with CLA, and significantly increased cytosolic abundance of inactive PKC ι. This data, from Song *et al.* is consistent with the increased apoptosis observed after treatment with CLA, as PKC ι expression is a potent factor in cell survival. Explanations for the increased active PKC ι levels observed in MCF-7 cells in the present study are unclear, but would indicate an adverse effect by CLA on MCF-7 cells. This is country to the cytotoxic effects of CLA on MCF-7 cells demonstrated in chapter 4. The aspect of marked differences in PKC isoform expression pattern in different cell lines on exposure to CLA requires further evaluation.

Chapter 7

Modulation of Protein Kinase C (PKC) isoforms in MDA-MB-231 Cells, when treated With Conjugated Linoleic acid (CLA) and Linoleic Acid (LA)

7. Modulation of Protein Kinase C (PKC) isoforms in MDA-MB-231 Cells, when treated with Conjugated Linoleic acid (CLA) and Linoleic Acid (LA)

7.1 Introduction

Oestrogen receptor negative breast cancers make up only 25% of the total number of new breast cancers detected every year. The MDA-MB-231 breast cancer cell line is an accepted model for oestrogen, and progesterone receptor negative breast cancers. The cell line was first obtained in 1973 from a pleural effusion of a tumour for which the patient had already undergone a mastectomy and radiotherapy. MDA-MB-231 cells display an aberrant morphology, forming spindle shaped epithelial like cells.

Oestrogen and its various isoforms are best known for the effects they have upon the female reproductive system, among other functions they play a large part in controlling; ovulation, pregnancy, childbirth and lactation. Oestrogen is also a known promoter of cell growth and survival, binding to oestrogen receptors (ER's). The presence of oestrogen receptors is known to confer an increased likelihood of disease free survival, and increased 5 year survival on patients, while oestrogen receptor negative tumours have a higher rate of recurrence, regardless of the many factors which have been found to change risk of recurrence in oestrogen receptor positive tumours (Knight *et al.* 1977). There are several potential mechanisms by which previously ER positive cells may loose the ability to express ER receptors, these include long term oestrogen withdrawal (as caused by menarche, especially early menarche, or long term use of hormonal contraceptives, Santen *et al.* 2002) and hypoxia (Stoner *et al.* 2002). ER expression may also be suppressed by the up regulation of Mitogen Activated Protein Kinase (MAPK) (Creighton *et al.* 2006). It has also been shown that cells which have lost the ability to express ER receptors can be persuaded to re-express them by inhibition of hyperactive MAPK expression. This inhibition leads to restoration of the ER expression and the associated sensitivity of the

cells to anti-oestrogen therapies, without this anti-oestrogen therapies, used to treat oestrogen receptor positive breast cancer (for which the MCF-7 cell line is a model), are mostly ineffective against oestrogen receptor negative tumours (Bayliss *et al.* 2007).

7.2 Results
PKC Activation in MDA-MB-231 Cells after Treatment with CLA

7.2.1 PKC α

7.2.1.1 Treatment of MDA-MB-231 Cells With 25μM CLA.

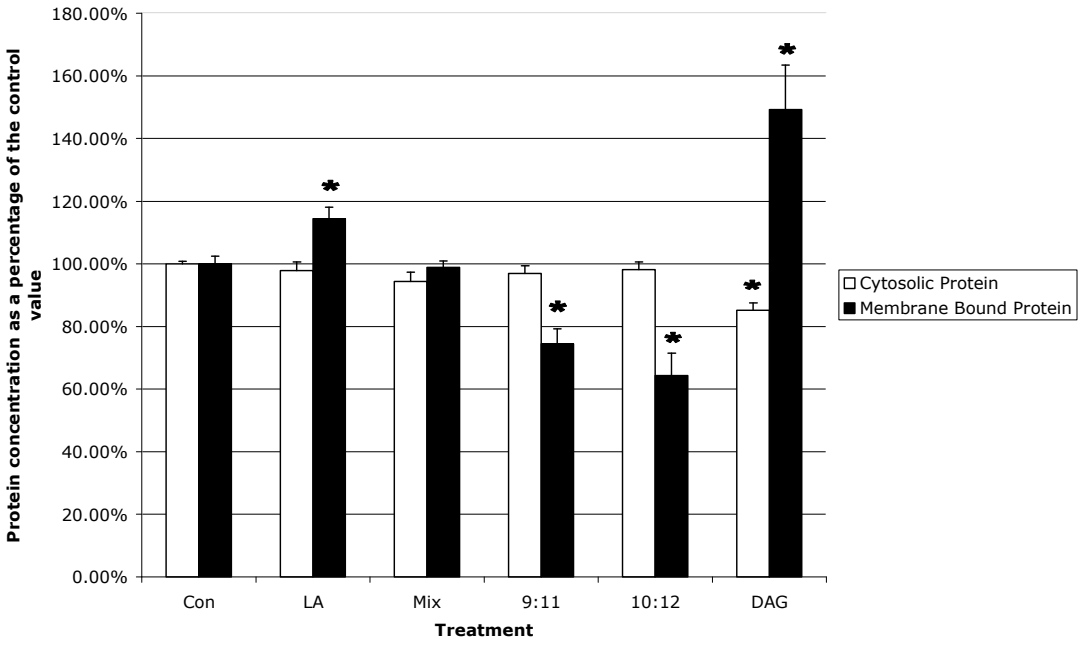


Figure 7.1: Effect of CLA's on PKC α. MDA-MB-231 cells treated with 25μM CLA isoforms or 25μM DAG. ß-actin was used to verify loading in cytosolic protein samples, Claudin-3 was used to verify loading in membrane bound protein samples. Data are from three independent experiments.
Key: Control – 100% ethanol (equivalent volume); LA – 25μM Linoleic Acid; Mix – 25μM 50:50 mixture of the *c*9:*t*11 and *t*10:*c*12 isomers; 9:11 - 25μM *c*9:*t*11 isomer alone; 10:12 - 25μM *t*10:*c*12 isomer alone. P<0.05 (*)

The abundance of active PKC α in cells treated for 24 hours with 25μM of the 50:50 isomer mix, the *c*9:*t*11 isomer alone and the *t*10:*c*12 isomer alone showed a general decrease (Figure 7.1). Treatment with the isomer mix produced only a small decrease in active PKC α (1.2%), however treatment with the *c*9:*t*11 and the *t*10:*c*12 isomers individually produced a much greater, and highly significant decrease (25.3% and 35.7% respectively). All four treatments showed a small, and non significant decrease in the amount of inactive PKC α present in the cytosol. Treatment with LA produced a

significant increase in the amount of active PKC α in the membrane bound fraction, 14.5%.

Treatment with 25μM DAG for 24 hours showed a significant increase in active PKC α (49.3%) and also a significant decrease in inactive PKC α (14.8%).

7.2.1.2 Treatment of MDA-MB-231 Cells With 50μM CLA.

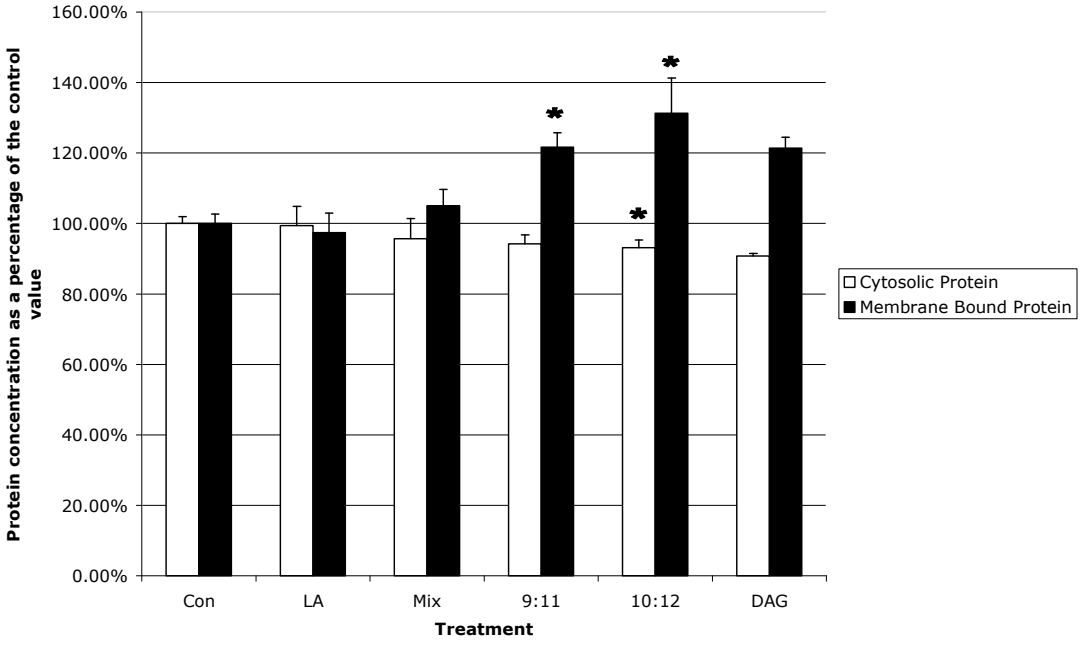


Figure 7.2: Effect of CLA's on PKC α. MDA-MB-231 cells treated with 50μM CLA isoforms. ß-actin was used to verify loading in cytosolic protein samples. Data are from three independent experiments.
Key: Control – 100% ethanol (equivalent volume); LA – 25μM Linoleic Acid; Mix – 25μM 50:50 mixture of the *c*9:*t*11 and *t*10:*c*12 isomers; 9:11 - 25μM *c*9:*t*11 isomer alone; 10:12 - 25μM *t*10:*c*12 isomer alone. $P<0.05$

Membrane abundance of active PKC α was increased by 5.1%, 21.7% and 31.3% in cells treated for 24 hours with 50μM of the 50:50 mix and the *c*9:*t*11 and *t*10:*c*12 isomers separately (Figure 7.2). With the LA treatment the active PKC α was slightly decreased by 2.6%. The cells treated with the mix and the *c*9:*t*11 isomer at 50μM concentrations showed a non significant decrease in inactive PKC α, 4.2% (*mix*) and 5.7% (*c*9:*t*11)

respectively. The *t*10:*c*12 treated cells showed a significant decrease in the amount of inactive PKC α (6.9%). The results for the active PKC α in cells treated with the *c*9:*t*11, and *t*10:*c*12 isomers alone showed statistical significance. Treatment with 50μM DAG for 24 hours produced an increase in active PKC α by 21.4% and a decrease in inactive PKC α by 9.3%. Neither of which is significant.

7.2.2 PKC δ

7.2.2.1 Treatment of MDA-MB-231 Cells With 25μM CLA.

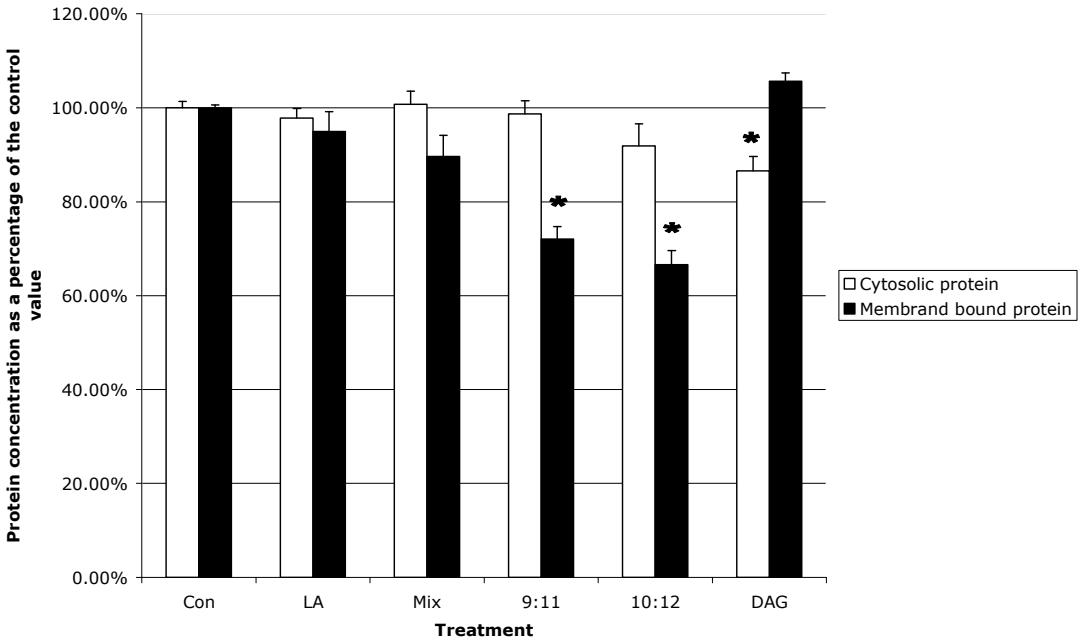


Figure 7.3: Effect of CLA's on PKC δ. MDA-MB-231 cells treated with 25μM CLA isoforms or 25μM DAG. ß-actin was used to verify loading in cytosolic protein samples, Claudin-3 was used to verify loading in membrane bound protein samples. Data are from three independent experiments.
Key: Control – 100% ethanol (equivalent volume); LA – 25μM Linoleic Acid; Mix – 25μM 50:50 mixture of the *c*9:*t*11 and *t*10:*c*12 isomers; 9:11 - 25μM *c*9:*t*11 isomer alone; 10:12 - 25μM *t*10:*c*12 isomer alone. $P<0.05$

Treatment with 25μM of CLA produced a general decrease in active PKC δ across all the isoforms (Figure 7.3). 25μM of LA caused a decrease of 5.0% and treatment with the 50:50 isomer mix caused a decrease of 10.4%, neither of which is significant. Treatment

with the *c*9:*t*11 and *t*10:*c*12 isomers alone showed significant decreases in active PKC δ, 28.0% for the *c*9:*t*11 isoform, and 33.4% for the *t*10:*c*12 isoform. Inactive PKC δ was largely unchanged across all the treatments, with the LA, 50:50 mix and *c*9:*t*11 isomer exhibiting no significant change in inactive PKC δ (2.9% decrease, 0.8% increase and a 1.3% decrease respectively). The *t*10:*c*12 isoform exhibited a significant, although comparatively small decrease of 8.2%.

Treatment with 25μM DAG for 24 hours produced an increase, although not a significant one of 5.6% in active PKC δ. The decrease in inactive PKC δ (13.5%) however is statistically significant.

7.2.2.2 Treatment of MDA-MB-231 Cells With 50μM CLA.

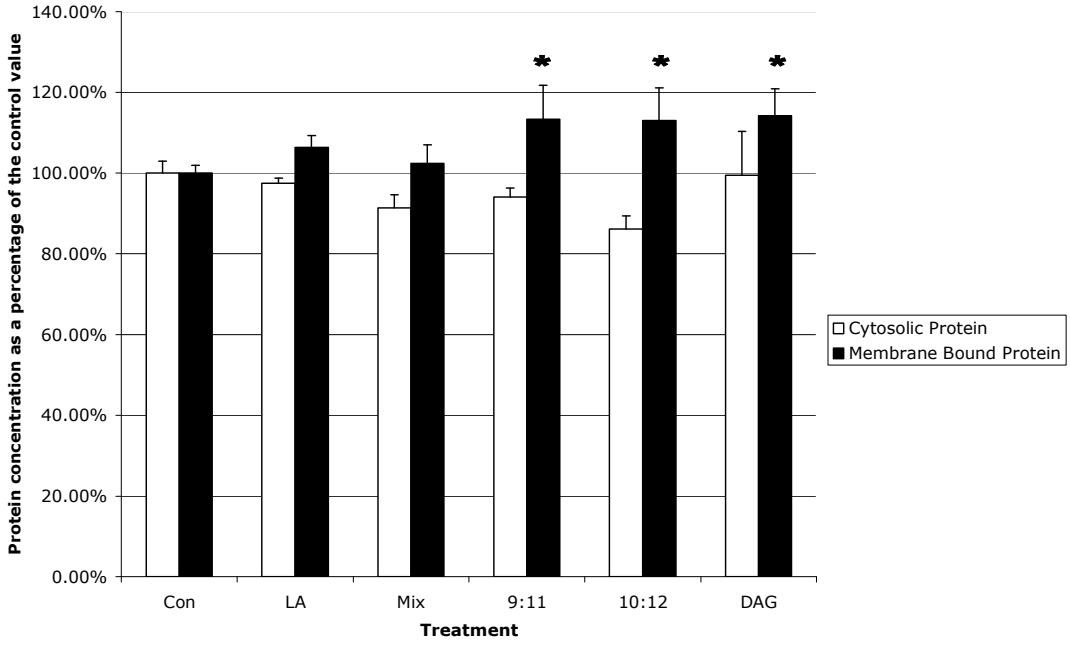


Figure 7.4: Effect of CLA's on PKC δ. MDA-MB-231 cells treated with 50μM CLA isoforms. ß-actin was used to verify loading in cytosolic protein samples. Data are from three independent experiments.
Key: Control – 100% ethanol (equivalent volume); LA – 25μM Linoleic Acid; Mix – 25μM 50:50 mixture of the *c*9:*t*11 and *t*10:*c*12 isomers; 9:11 - 25μM *c*9:*t*11 isomer alone; 10:12 - 25μM *t*10:*c*12 isomer alone. P<0.05

Cells treated for 24 hours with 50μM of the *c*9:*t*11 and *t*10:*c*12 isomers alone exhibited a significant increase in membrane bound PKC δ, of 13.4% and 13.1% respectively. Cells treated with 50μM of the 50:50 isomer mix showed only a small increase (2.4%) in active PKC δ which fails to be statistically significant (Figure 7.4). Similarly, treatment with LA also led to a small, insignificant increase in active PKC δ (6.3%).

Decreases in inactive PKC δ also fail to exhibit statistical significance, it was notable that inactive PKC δ decreased for all the treatments, but was not proportional to active PKC δ. Treatment with 50μM DAG for 24 hours produced a significant increase in active PKC δ (14.2%), but no correspondingly significant decrease in inactive PKC δ (0.6%).

7.2.3 PKC ι

7.2.3.1 Treatment of MDA-MB-231 Cells With 25μM CLA.

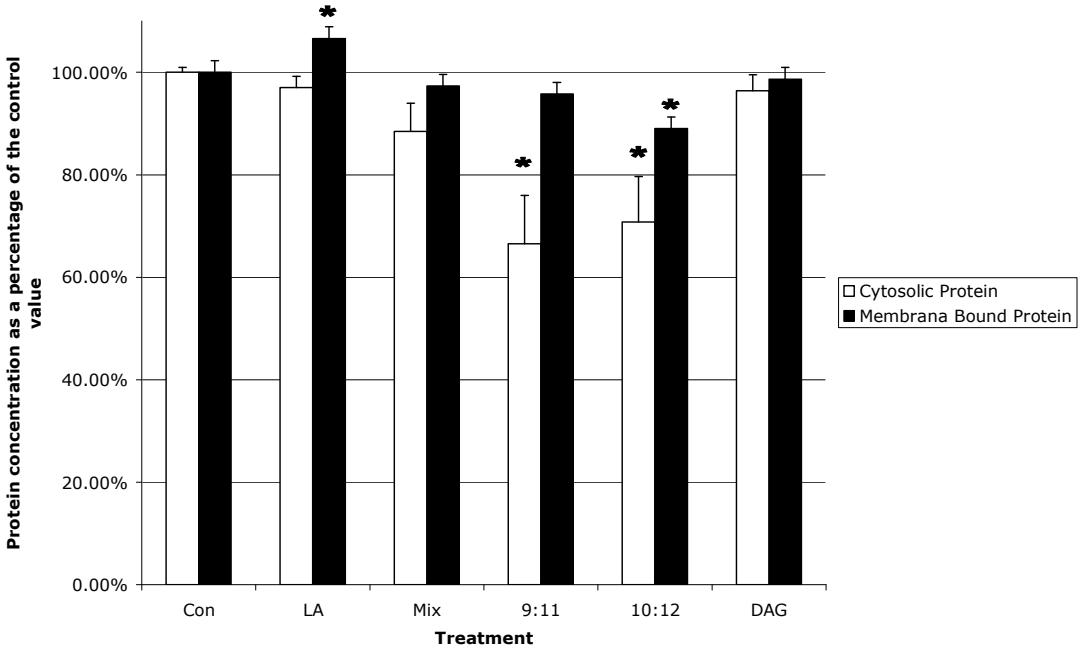


Figure 7.5: Effect of CLA's on PKC ι. MDA-MB-231 cells treated with 25μM CLA isoforms or 25μM DAG. ß-actin was used to verify loading in cytosolic protein samples, Claudin-3 was used to verify loading in membrane bound protein samples. Data are from three independent experiments.
Key: Control – 100% ethanol (equivalent volume); LA – 25μM Linoleic Acid; Mix – 25μM 50:50 mixture of the *c*9:*t*11 and *t*10:*c*12 isomers; 9:11 - 25μM *c*9:*t*11 isomer alone; 10:12 - 25μM *t*10:*c*12 isomer alone. P<0.05

Statistical significance was observed in cells treated with the *t*10:*c*12 isomer alone for both the active and the inactive PKC ι, a decrease of 11.0% is observed in the active form, and a decrease of 29.2% for the inactive form (Figure 7.5). The LA treatment caused a significant increase (6.6%) in the amount of membrane bound PKC ι, with a corresponding, but non significant decrease of 2.9% in the cytosolic protein. Small, non significant changes were observed in the membrane bound PKC ι of cells treated with the 50:50 isomer mix, and the *c*9:*t*11 isomer. A non significant decrease of 2.7% in active PKC ι was observed in cells treated with the isomer mix and a decrease of 4.27% in cells treated with the *c*9:*t*11. Cytosolic PKC ι was decreased by 11.6% after treatment with the

isomer mix, and by 33.5% after treatment with the *c*9:*t*11 isomer alone, however only the latter is statistically significant.

Treatment with 25µM DAG for 24 hours produced a small decrease of 1.4% in active PKC ι, and a similar decrease of 3.6% in inactive PKC ι. Neither of these was statistically significant.

7.2.3.2 Treatment of MDA-MB-231 Cells With 50µM CLA.

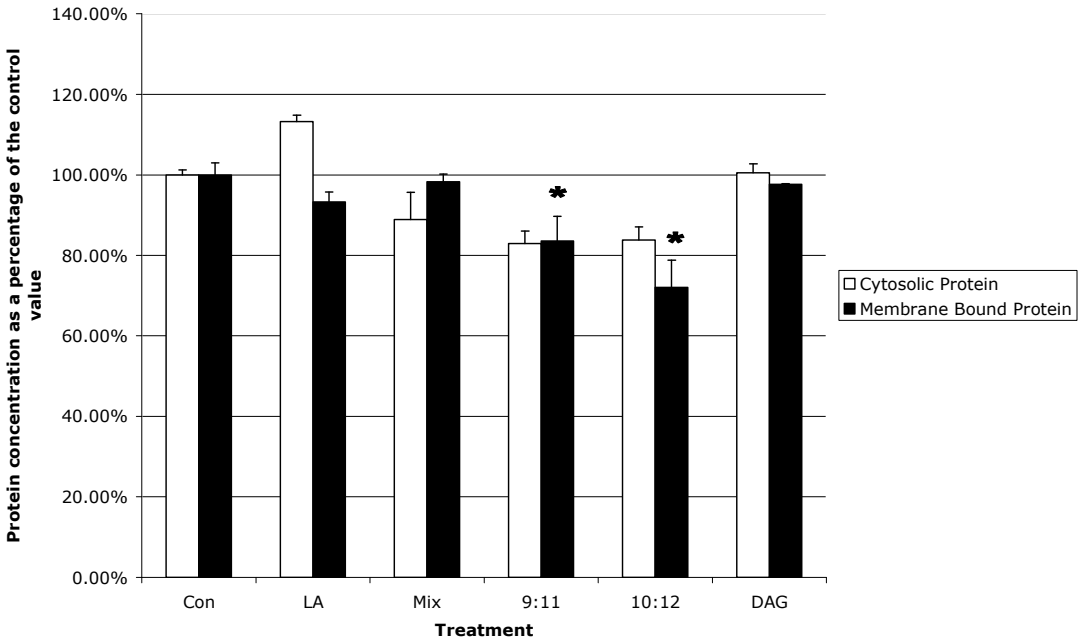


Figure 7.6: Effect of CLA's on PKC ι. MDA-MB-231 cells treated with 50µM CLA isoforms. ß-actin was used to verify loading in cytosolic protein samples. Data from three independent experiments.
Key: Control – 100% ethanol (equivalent volume); LA – 25µM Linoleic Acid; Mix – 25µM 50:50 mixture of the *c*9:*t*11 and *t*10:*c*12 isomers; 9:11 - 25µM *c*9:*t*11 isomer alone; 10:12 - 25µM *t*10:*c*12 isomer alone. $P<0.05$

Membrane abundance of active PKC ι was significantly decreased in cells treated with 50µM of the *c*9:*t*11 or *t*10:*c*12 isomers respectively (16.4% and 28.0%, Fig 7.6). With the *c*9:*t*11 treatment the inactive cytosolic PKC ι was decreased by 17.0 % and by 16.2% in cells treated with the *t*10:*c*12 isomer. The cells treated with LA at the 50µM concentration

showed a non significant decrease of 6.7% in the amount of active PKC ι, and a corresponding increase of 13.2% in the cytosolic PKC ι. Cells treated with the 50:50 mix of isomers showed a 1.7% decrease in active PKC ι and an 11.1% decrease in inactive PKC ι. The membrane abundance of active PKC ι exhibits a decrease of 2.4% in cells treated with 50μM DAG, the comparative decrease in cytosolic PKC ι was 0.5%, neither of these results show statistical significance.

7.3 Discussion

Effects of Treatment With CLA in Oestrogen Receptor Negative Cells on Abundance of Active and Inactive PKC.

The androgen independent prostate cancer cell line, known as PC-3 may be considered to be somewhat analogous with MDA-MB-231 breast cancer cell line. Both cell lines fail to express the hormone receptors which are the targets for several different chemotherapeutic drugs. CLA is known to decrease levels of the anti-apoptotic protein Bcl-2 in PC-3 cells, although at 25 and 50μM concentrations this is not significant (Ochoa *et al.* 2004). It has been suggested that CLA acts through the ER receptors in breast cancer cells (Wang *et al.* 2008), however the results in this study show a greater increase in cell death after CLA treatment in the MDA-MB-231 cell line.

Since PKC δ and PKC α are known modulators of Bax, and Bad it is tempting to assume that an increase in PKC δ, and a decrease in PKC α will lead to apoptosis. In the MDA-MB-231 cell line this does not appear to happen. At 25μM concentrations of CLA the levels of active PKC α and PKC δ both decrease significantly ($P<0.05$), while at 50μM concentrations they both increase. When the large increase in apoptosis observed in chapter 4 is taken into account this may suggest that the idea of the traditional Bcl-2 pathway being modulated by the PKC isoforms does not tell the whole story. Similarly, the decrease of active PKC ι at 25μM of CLA, and its increase at 50μM does not fit the traditional role of apoptotic inhibitor assigned to this protein. The reasons for this result are currently unclear, and require further investigation.

It does not appear, given the activation pattern of PKC after treatment with 50μM CLA in the current research, that the PKC/Bcl-2 pathway is the mechanism by which CLA causes apoptosis in MDA-MB-231 cells. If that was the case then the increases in PKC α after

treatment with the *cis*-9:*trans*-11, and *trans*-10:*cis*-12 isomers alone would be expected to decrease apoptosis. The results from chapter four clearly show that this is not occurring. It appears likely that the PKC isoforms are acting through a different pathway, possibly by the translocation of active PKC δ directly to the mitochondrial membrane. Given the decrease in PKC α at the 25μM treatments it is possible that apoptosis is being controlled through two different pathways, depending on the concentration of CLA used to treat the cells. The Bcl-2 pathway may be a candidate at lower concentrations, and membrane incorporation of CLA may be assisting apoptosis at higher concentrations. Previous research into the action of CLA on MDA-MB-231, and androgen insensitive prostate cancer cell lines, is somewhat limited. It does, however, suggest that CLA causes apoptosis, especially at high concentrations of CLA. Migiletta *et al.* suggested in 2006 that CLA caused apoptosis in MDA-MB-231 cells by inducing cytosolic release of cytochrome c from the mitochondria. This was in direct contrast to previous research which had suggested that CLA was acting to cause apoptosis via oestrogen response elements (Tanmahasamut *et al.* 2004).

Chapter 8

The Effect of CLA (50μM) on the RNA Expression in MCF-7 Cells

8. The Effect of CLA (50µM) on the RNA Expression in MCF-7 Cells.

8.1 Introduction

The Reverse Transcription Polymerase Chain Reaction (RT-PCR) is a semi-quantative technique which allows amplification of a defined piece of RNA. RT-PCR can be performed as either a one step, or two step process. Generally RT-PCR is performed as follows:

1. RNA is extracted from treated cells using a Trizol based process (see Chapter2, page 57 for more detail).
2. RNA is reverse transcribed into complimentary DNA (cDNA).
3. Complimentary DNA is amplified using primers specific for the gene of interest (see also Fig 8.1)

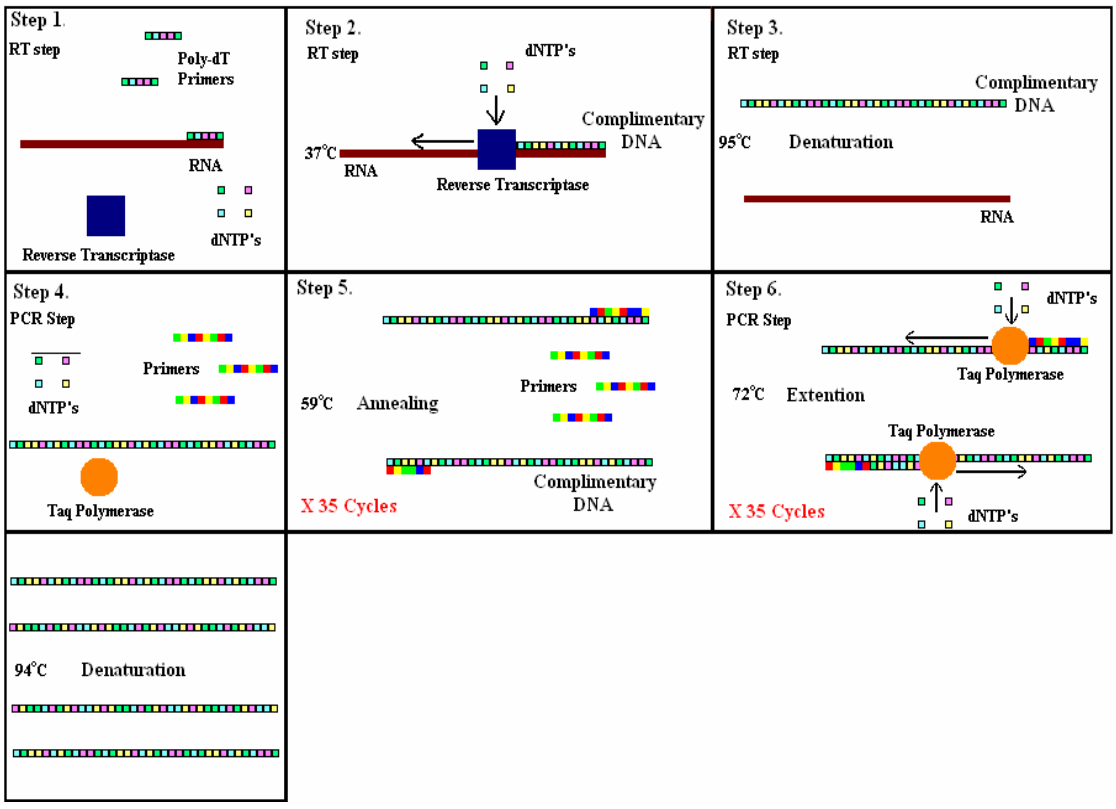


Fig 8.1 RT-PCR

8.1.1 Primer design

Primers are required for the amplification of the correct gene sequence during the PCR step. Typically primers are "paired", with one reading from the 5' end of the cDNA to the 3' end, and the other starting at the 3' end, and reading to the 5'end. Paired primers ensure that the cDNA produced is double stranded, allowing binding by DNA polymerases (usually Taq from the *Thermus aquaticus* bacterium).

Primer design must follow several criteria to maximize the chance of amplifying the correct genomic sequence. Primers must be specific to the sequence being amplified. To achieve reasonable fidelity in this most primers are designed to be between 18 and 25 bases long. This length is optimal, as it is short enough to allow binding at the annealing temperature, yet long enough to ensure adequate specificity. Primers which are amplifying a piece of cDNA of transcriptional origin must furthermore contain no untranscribed sequences. The area of cDNA being amplified (the amplicon) may be of variable length, but 100-500 base pairs is generally considered to be optimum. Because a single strand of cDNA is a naturally unstable construct in that it preferentially binds to another strand, it is vitally important to avoid the presence of secondary structures within the amplicon itself. If the cDNA is folded back on itself it is much more difficult for the primers to bind faithfully. Equally the primers themselves must be designed to avoid either the formation of primer dimers, or secondary structures within the primer, as again this makes binding to the cDNA almost impossible. Long repeated sequences, and single base repeats in the primer region should also be avoided as they have a higher chance of mispriming. The maximum number of repeats is thought be four (e.g ATATATAT). Finally, the temperatures at which the primers anneal (T_a) and melt (T_m, defined as the temperature where the number of paired nucleotides are equal to the number of unpaired

nucleotides, (Wetmur, 1991) is very important, for maximal PCR product yield; the two primers should have a very closely matched T_a and T_m.

There are three methods used for calculating T_m, known as the Arbitrary Method, the Nearest Neighbour Method, and the Long Probe method.

1. The Arbitrary Method was first mentioned by Wallace *et al.* in 1979, and is worked out as:

$T_m=2(A + T) + 4(G + C)$

Where A is the number of Adenine bases in the Primer, G the number of Guanine bases, T the number of Thymine bases, and C the number of Cytosine bases.

2. The Nearest Neighbour method was first described by Borer *et al.* in 1974, and is generally considered to be the most accurate calculation of T_m:

$T_m=(-1000 \times \Delta H) / (-10.8 - \Delta S + R \times \ln(2.5\text{^}c/4)) - 273.15 - 16.6(\log10\ M)$

In this case ΔH is the enthalpy of base stacking interactions (adjusted for helix initiation factors), ΔS is the entropy of base stacking (adjusted for helix initiation factors, and for the contributions of salts), R is the gas constant (1.987), c is the molar concentration of primer (250 pM), and M is the molar concentration of Na+ (50 mM).

3. The Long Probe method first appeared in a paper by Meinkoth and Wahl in 1984 and may be described as:

$T_m=81.5 + 16.6(\log10\ M) + 0.41(\%\ GC) - 0.61(\%\ form) - 500 / Length\ in\ bp$

M is again the molar concentration of Na+ (set at 0.75 M) and % form is the percentage of formamide (set to 50%).

8.1.2 Experimental Aims

The current research shows significant changes in the activation of the three PKC isoforms in cells treated with CLA. RNA extraction techniques and RT-PCR, using specifically designed primers will help to indicate whether these changes are the result of increased transcription of the PKC genes.

8.2 RT-PCR Results

8.2.1 Developing a Time Course

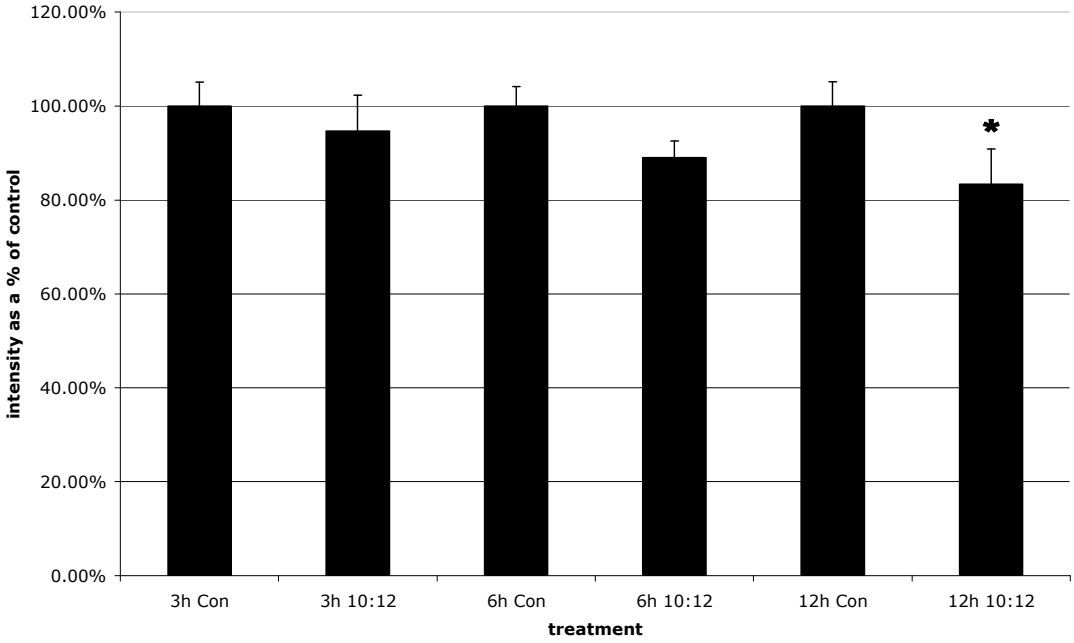


Fig 8.2 Time course study. PKC α RNA expression in MCF-7 cells treated with 50μM CLA isoforms or equivalent volume of ethanol, for 3, 6, and 12 hours. ß-actin was used as a housekeeping gene.
Key: Control – 100% ethanol (equivalent volume); 10:12 - 50μM *t*10:*c*12 isomer alone. $P<0.05$ (*)

Expression of PKC α RNA (Fig 8.2) showed a general decrease at all time points studied, with only the 12 h treatment exhibiting a significant decrease (16.6%).

8.2.2 Expression of PKC α after 12 hours treatment with 50μM CLA

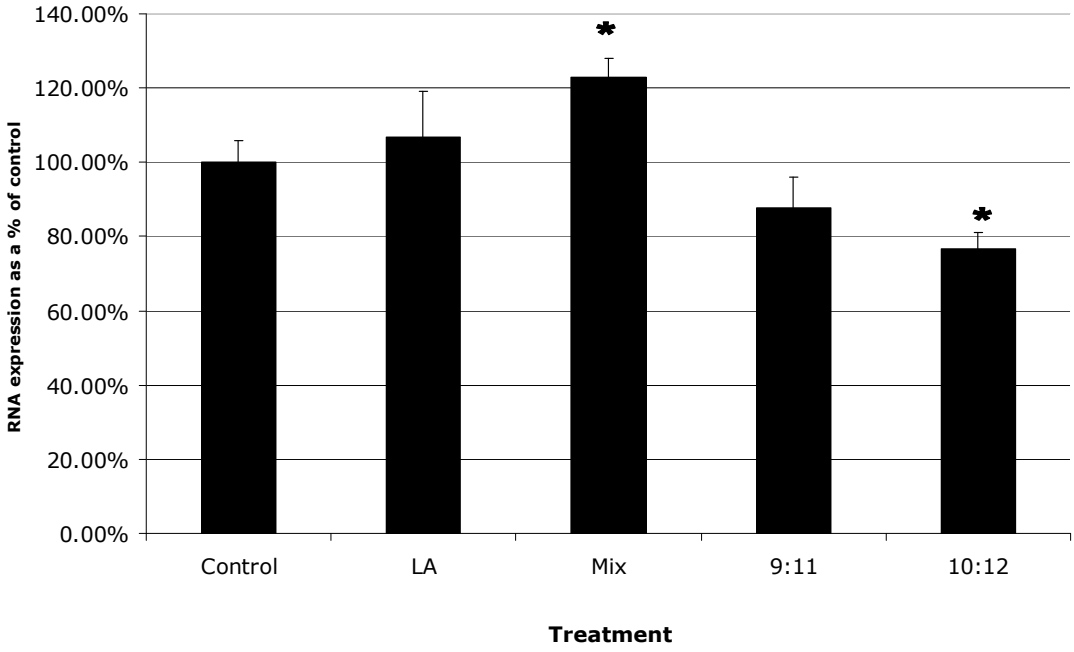


Fig 8.3 PKC α RNA expression. PKC α RNA expression in MCF-7 cells treated with 50μM CLA isoforms or equivalent volume of ethanol. ß-actin was used as a housekeeping gene.
Key: Control – 100% ethanol (equivalent volume); LA – 50μM Linoleic Acid; Mix – 50μM 50:50 mixture of the *c*9:*t*11 and *t*10:*c*12 isomers; 9:11 - 50μM *c*9:*t*11 isomer alone; 10:12 - 50μM *t*10:*c*12 isomer alone. P<0.05

Transcription of PKC α RNA can be seen to increase (6.6%) after 12 hours treatment with 50μM LA (Fig 8.3). With the 50:50 isomer mix PKC α RNA transcription also increased by 23.0%. However treatment with the *c*9:*t*11 and *t*10:*c*12 isomers alone showed a decrease in PKC α transcription by 12.1% and 23.3% respectively. Only the result for the 50:50 mix and the *t*10:*c*12 treatment alone showed any statistical significance.

8.2.3 Expression of PKC δ RNA after 12 hours treatment with 50μM CLA

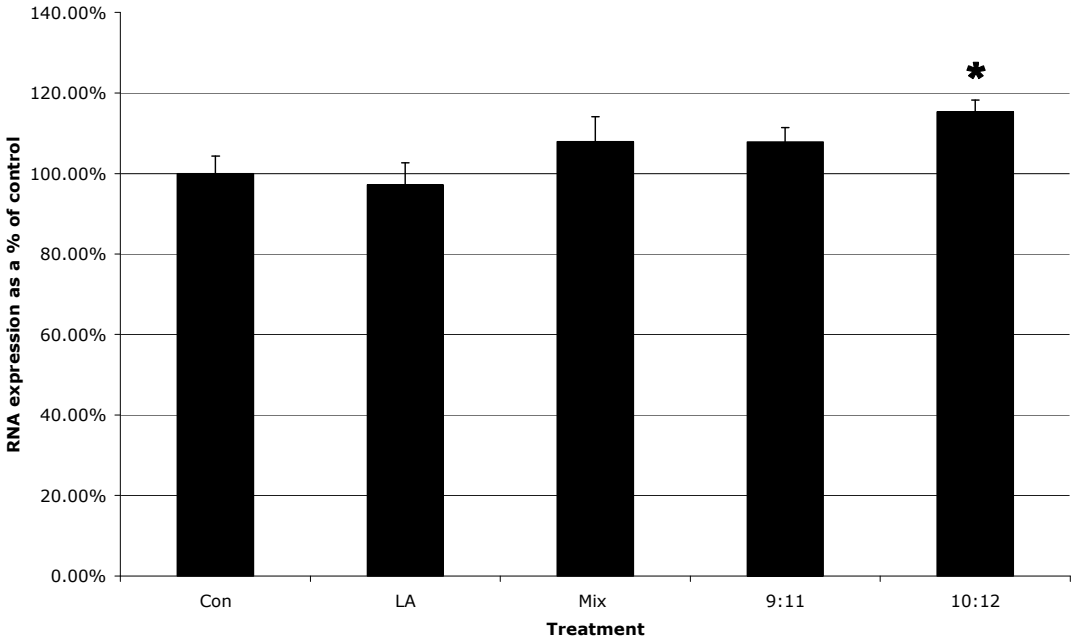


Fig 8.4 PKC δ RNA expression. PKC δ RNA expression in MCF-7 cells treated with 50μM CLA isoforms or equivalent volume of ethanol. ß-actin was used as a housekeeping gene.
Key: Control – 100% ethanol (equivalent volume); LA – 50μM Linoleic Acid; Mix – 50μM 50:50 mixture of the c9:*t*11 and *t*10:*c*12 isomers; 9:11 - 50μM *c*9:*t*11 isomer alone; 10:12 - 50μM *t*10:*c*12 isomer alone. P<0.05

Transcription of PKC δ RNA showed a 2.9% decrease with 12 hours treatment of 50μM LA (Fig 8.4). With the 50:50 isomer mix and the *c*9:*t*11 isomer alone PKC δ RNA transcription was increased by 7.9% and 7.8% respectively. Treatment with the *t*10:*c*12 isomer alone caused a significant (15.3%) increase in PKC δ transcription.

8.2.4 Expression of PKC ι after 12 hours treatment with 50μM CLA

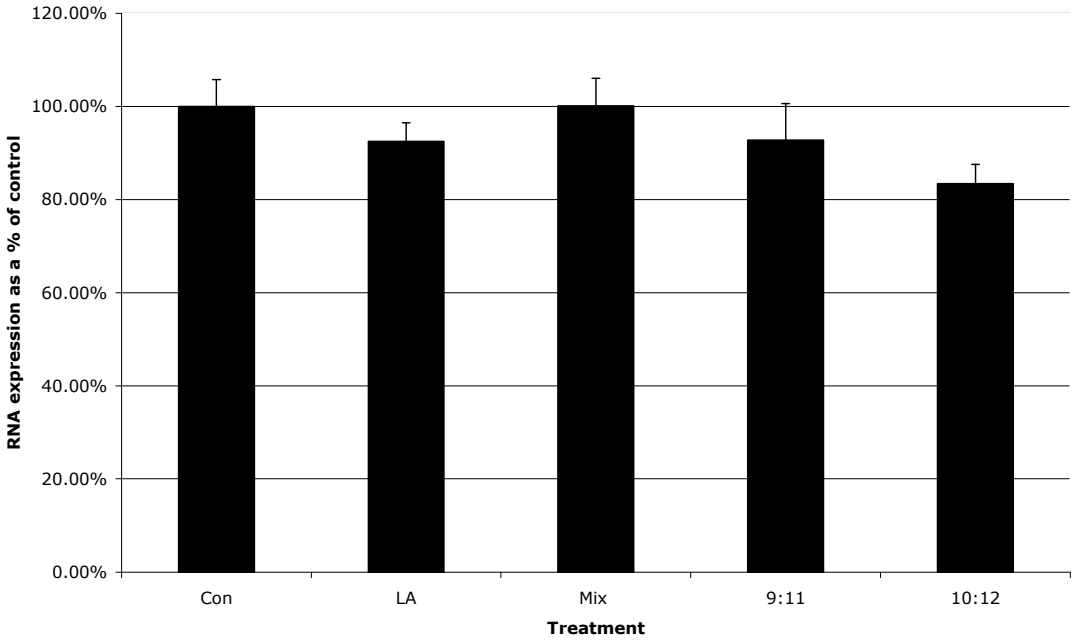


Fig 8.5 PKC ι RNA expression . PKC ι RNA expression in MCF-7 cells treated with 50μM CLA isoforms or equivalent volume of ethanol. ß-actin was used as a housekeeping gene.
Key: Control – 100% ethanol (equivalent volume); LA – 50μM Linoleic Acid; Mix – 50μM 50:50 mixture of the *c*9:*t*11 and *t*10:*c*12 isomers; 9:11 - 50μM *c*9:*t*11 isomer alone; 10:12 - 50μM *t*10:*c*12 isomer alone. $P<0.05$

Transcription of PKC ι RNA showed a decrease by 7.5% in cells treated with 50μM LA (Fig 8.5). With the 50:50 isomer mix PKC ι RNA transcription increased by 0.2%. Treatment with the *c*9:*t*11 isomer and *t*10:*c*12 isomers each caused a decrease in PKC ι transcription by 7.2% and 16.6% respectively. None of these results showed any statistical significance.

8.3 Discussion

8.3.1 Effects of CLA on the expression PKC RNA in MCF-7 Cells

The above results clearly show a correlation between treatment with CLA and the regulation of PKC α, δ, and ι transcription.

8.3.1.1 Time Course Experiment

The time course experiments were performed at 3, 6, and 12 hours. It was decided to look at MCF-7 cells, partly due to time, and cost constraints, and partly because MCF-7 is the model for the far more common type of breast cancer, so the results from these experiments would have a much greater practical impact.

Based on the results from the protein analysis as described in chapters 6 and 7, and also the work of Song *et al.* it is clear that the *t*10:*c*12 isomer alone had the greatest single impact on all three PKC isoforms. In general, treatments with 50μM of CLA had a greater impact than treatments with 25μM. From this it was decided to use the *t*10:*c*12 at 50μM for the whole RT-PCR time course. PKC α was the obvious choice of isoform for the time course because the primers had already been optimised for use with RNA extracted from MCF-7 cells.

The time course experiment confirmed that significant effect occurred only after 12 hours. This was a somewhat longer period than was first expected, however some effect was apparent even after three hours. The decrease in the amount of PKC α RNA appears to decrease very steadily, then tail off over the time course. The decrease between 0 and 3 hours is around 5%, as is the decrease between 3 and 6 hours. Between 6 and 12 hours this steady decrease can be seen to tail off, as the decrease is around 6%, correlating to a

decrease of around 1% an hour, compared with approximately 1.7% per hour seen previously. A fourth time point at 9 hours would show more precisely how this decline occurs.

8.3.1.2 Effect of CLA on the expression of PKC α mRNA

The expression pattern of the PKC α RNA almost matches the expression pattern of the PKC α protein in MCF-7 Cells treated with 50μM CLA. Actual and precise correlation is difficult to assess as the method of measuring both involves measuring intensity of signal as a percentage of the control, rather than an actual amount of protein. It is further complicated by the fact that no total protein data for PKC α has been done. As it stands it appears that levels of PKC α protein in cells treated with 50μM of LA, *c*9:*t*11 alone, and *t*10:*c*12 alone all decrease slightly, while PKC α protein in cells treated with 50μM of the 50:50 isomer mix increase slightly. This is a very inaccurate measure as it assumes that active and inactive isomers of PKC α are found in the same levels in the cytosol and the membrane. The increase in PKC α RNA, observed after treatment with the isomer mix, and the decreases seen after treatment with the *c*9:*t*11, and *t*10:*c*12 isomers alone mirrors this, although there are several anomalies. While both the active and inactive PKC α protein appears to decrease in cells treated with the *c*9:*t*11 isomer alone, inactive PKC α increases by a significant amount in cells treated with the *t*10:*c*12 isomer alone. This may be partly due to the equally significant decrease in active PKC α leaving a greater amount of inactive PKC α in the cytosol. The apparent differences in activation as well as PKC α protein production suggest that the CLA isoforms may be exerting a dual effect, both on the cell membranes, making them more, or less likely to allow PKC α activation, and on the transcription of RNA in the cell.

8.3.1.3 Effect of CLA on the expression of PKC δ mRNA

The expression pattern of both the active, and the inactive PKC δ protein shows a very high degree of correlation with the expression of PKC δ RNA in MCF-7 cells treated with 50μM of CLA. The only anomaly is in the cells treated with LA, where the active PKC δ protein increases, while the inactive PKC δ protein decreases slightly. However, the RNA shows a slight decrease in transcription, although again this may be explained by the difficulty in assessing actual relative amounts of protein. It is also worth noting that all the protein assessments were done after 24 hours treatment with CLA, it may be that the results after 12 hours would reflect the RNA expression pattern more closely.

In cells treated with 50μM the 50:50 mix, the *c*9:*t*11 alone, and the *t*10:*c*12 alone there are increases in both the active and inactive PKC δ, this is similar to the pattern which is observed in the RNA expression after treatment with the same isoforms. The dual increase suggests that, in the case of PKC δ, the increased level of PKC δ RNA transcription after treatment with CLA causes greater translation of PKC δ protein. There may of course be differences if the experiment was repeated using total protein extracts, but these may be caused by the differences in isoform activation, as PKC δ localisation is affected by caspase activity, or by an, as yet un-looked for effect of CLA on the cell membrane.

8.3.1.4 Effect of CLA on the expression of PKC ι mRNA

There appears to be no correlation between the expression of PKC ι RNA and PKC ι protein in MCF-7 cells treated with 50μM of CLA. RNA expression shows no significant decrease after treatment, but does show a decrease, while the protein activation increases dramatically with every treatment. Protein expression appears to be increased in all the samples, despite the difficulty in assessing actual amounts of protein. To assess actual

total PKC ι protein levels after treatment would require further work involving total protein extraction. It may be that if the cells from which the RNA was extracted were treated for 24 hours in the way that the cells from which the protein was extracted were a different result, possibly involving an increase in mRNA expression, would be observed.

Chapter 9
Summary, Conclusions, and Future Work

9. Summary, Conclusions, and Future Work

9.1 Summary

The current research shows increases and decreases in the abundance of active PKC isoforms after treatment with different concentrations of CLA. The *t*10:*c*12 isomer of CLA appears to show the greatest, and most consistent, effect in breast cell lines (MCF-7 and MDA-MB-231) studied, both at the level of an mRNA transcription, and protein activation (as measured by translocation of protein to the membrane throughout).

9.1.1 Summary of the effects of the t*10:*c*12 Isomer on MCF-7 and MDA-MB-231 Cells*

In MCF-7 cells the *t*10:*c*12 isomer (at 25 and 50μM concentrations) causes a decrease in PKC α protein activation, and a significant reduction in cell viability. Furthermore at 50μM, a significant decrease in PKC α mRNA transcription is also observed. In the MDA-MB-231 cells PKC α protein activation is significantly decreased at 25μM concentrations, while the reverse is observed at 50μM. It must be noted that cell viability is decreased at both concentrations in MDA-MB-231 cells.

PKC δ protein activation is slightly less consistent, with reductions occurring at 25μM concentrations in both cell lines while increases occur at 50μM. Transcription of PKC δ mRNA in MCF-7 cells is significantly increased after treatment with 50μM of the *t*10:*c*12 isomer, suggesting that the decrease seen at 25μM may be due to other factors.

Here we show for the first time that PKC ι is present in detectable quantities in both the MCF-7 and MDA-MB-231 breast cancer cell lines (Nutrition Society Abstract, 2004). However, previous work had only detected the presence of PKC ι protein in serum starved MCF-7 cells (Muscella *et al.* 2005). Recent work by Kojima *et al.* (2008) reported that

PKC ι is over expressed in 88 out of the 100 cases of mammary tumours studied. PKC ι mRNA transcription decreases after treatment with the *t*10:*c*12 isomer (50μM). However, in the same cell line (MCF-7) both 25 and 50μM cause an increase in protein activation of PKC ι, while in MDA-MB-231 cells a decrease in protein activation is seen.

9.1.2 Summary of the effects of the c9:t*11 Isomer on MCF-7 and MDA-MB-231 Cells*

In MCF-7 cells the *c*9:*t*11 isomer causes the greatest reduction in cell viability, although this is not significant when compared with the decrease caused by the mix and *t*10:*c*12 isomer. In MDA-MB-231 cells however the *c*9:*t*11 isomer causes the smallest decrease in cell viability. Similarly this is not significant when compared with the mix and *t*10:*c*12.

PKC α protein activation is decreased after treatment (25 and 50μM concentrations) with the *c*9:*t*11 isomer in MCF-7 cells. A similar reduction is observed with 25μM of *c*9:*t*11 in the MDA-MB-231 cells. However, at 50μM in the MDA-MB-231 cells the activation of PKC α protein increases in a similar manner to *t*10:*c*12 isomer. Expression of PKC α mRNA in MCF-7 cells treated with 50μM of *c*9:*t*11 is decreased, although not significantly.

PKC δ protein activation exhibits a similar pattern in cells treated with the *c*9:*t*11 isomer to the one seen in cells treated with the *t*10:*c*12 isomer. Only one point of difference actually exists, and that is a non-significant increase in protein activation in MCF-7 cells treated with 25μM. Generally cells treated with the *t*10:*c*12 isomer exhibit a greater increase or decrease in protein activation than the *c*9:*t*11 isomer. The exception to this, are MCF-7 cells treated with 50μM of *c*9:*t*11 which exhibit greater activation of PKC δ protein.

In MCF-7 cells treated with the *c*9:*t*11 isomer, PKC ι protein activation increases, significantly at 25μM, and not significantly at 50μM. This difference in significance may be due to the statistical test used (Mann-Whitney paired t-test). Expression of PKC ι mRNA in the same cell line (MCF-7) also decreases without significance. MDA-MB-231 cells treated with either 25 or 50μM of the *c*9:*t*11 isomer exhibit a decrease in the active PKC ι protein, although, as with PKC δ, not to the same extent as in cells treated with the *t*10:*c*12 isomer.

9.1.3 Summary of the effects of the Isomer Mix on MCF-7 and MDA-MB-231 Cells

At both concentrations (25 and 50μM) and in both cell lines the isomer mix generally provokes either the most minimal effect, or a totally opposing effect, on the mRNA expression and protein activation of all three isoforms of PKC. In MCF-7 cells viability is generally lower when treated with the mix, than with the *t*10:*c*12 isomer. In MDA-MB-231 cells viability is lower with the mix than with the *c*9:*t*11 isomer.

Expression of PKC α mRNA is significantly increased after treatment with the isomer mix when compared with individual *c*9:*t*11, and *t*10:*c*12 isomers. After treatment with 25μM of the isomer mix a small increase in PKC α protein activation is seen in MCF-7 cells. This is compared with the decrease observed after treatment with the same concentration of individual isomers. In MCF-7 cells (50μM isomer mix), PKC α protein activation is decreased. In MDA-MB-231 cells a decrease in the activation of PKC α protein is observed with 25μM of the isomer mix, while an increase is recorded with the 50μM concentration, neither of these are significant.

PKC δ expression and activation are both increased in MCF-7 cells after treatment with 50μM of the isomer mix. Treatment with 25μM however produces a non-significant decrease, similar to that produced by treatment with the *t*10:*c*12 isomer alone. Similar observations are observed in MDA-MB-231 cells.

Increases in PKC ι protein activation are observed in MCF-7 cells after both treatments (25 and 50μM). An insignificant increase in PKC ι mRNA expression is also seen in this cell line after treatment with 50μM of the isomer mix. In MDA-MB-231 cells only a small decrease of PKC ι activation after both treatments is observed.

9.1.4 Summary of the effects of Linoleic acid on MCF-7 and MDA-MB-231 Cells

Treatment with LA caused no significant change in cell viability compared with the control at either concentration (25 and 50μM).

There was a small increase in PKC α protein activation in MCF-7 cells treated with 25μM of LA, and in cells treated with 50μM. PKC α mRNA expression exhibited a small increase in the same cell line. A similar increase in PKC α protein activation was exhibited by MDA-MB-231 cells treated with 25μM; however, a decrease was seen in those cells treated with 50μM.

PKC δ mRNA expression was non-significantly decreased in MCF-7 cells treated with 50μM of LA, however, PKC δ protein activation was increased in the same cells. In cells treated with the 25μM concentration activation was almost unchanged compared to the control. Activation of PKC δ protein in MDA-MB-231 cells followed a similar pattern to

that observed after treatment with the isomer mix, although activation was slightly greater in cells treated with 50μM of LA.

PKC ι protein activation was significantly increased in MCF-7 cells treated with a concentration of 25μM of LA. Similarly cells treated with 50μM of LA also showed increased PKC ι protein activation, however PKC ι mRNA expression was slightly decreased. In MDA-MB-231 cells the activation also increased (25μM concentration), again significantly, and in contrast to the decrease in activation caused by every other treatment at the same concentration. PKC ι protein activation in the same cell line treated with 50μM of LA exhibited a non-significant decrease.

9.2 Conclusions

9.2.1 Effects of Treatment with CLA Isoforms on MCF-7, and MDA-MB-231 Cells.

Table 1.1 (Chapter 1, page 24) summarised the general effects of the various PKC isoforms. It would therefore be expected, given that treatment with CLA caused significant apoptosis (as demonstrated in chapter 4), that both cell lines would exhibit an increase in activation of the pro-apoptotic isoforms, and a decrease in activation of the anti-apoptotic isoforms. This does not appear to be the case for all treatments. PKC α, a known promoter of cell survival, exhibits significant increase in its activation in MDA-MB-231 cells treated with both the *c*9:*t*11, and *t*10:*c*12 isomers at 50μM. These two CLA isomers definitely cause a reduction in PKC α protein activation in MCF-7 cells, and at lower concentration (25μM) the same effect is observed in MDA-MB-231 cells. This anomaly in the cells which have exhibited the greatest loss in viability (MDA-MB-231, 50μM) strongly suggests that CLA is acting through multiple pathways. Equally the dramatic increase in activation of PKC ι protein in treated MCF-7 cells is in direct contrast to the considerable recent research which implicates it as a potent cell survival factor and oncogene (Eder *et al.* 2005, Fields *et al.* 2007). The activation pattern of PKC ι protein in MDA-MB-231 cells shows a decrease which would be expected in cells undergoing apoptosis. This is borne out by the fact that MCF-7 cells treated with CLA are apparently more viable. Possibly PKC ι is activated in these cells in response to cellular stress.

PKC δ is considered to be highly pro-apoptotic, and the significant increase in its activation in MCF-7 cells treated with 50μM of CLA is entirely consistent with this. However at 25μM of CLA the effect observed was not consistent. In MDA-MB-231 all

CLAs (25μM) cause a decrease in the activation of PKC δ protein. At 50μM of CLA PKC δ protein activation is increased as found in the MCF-7 cells.

Because the results collected use transcriptional and activation data, it is difficult to conclude whether CLA is acting entirely at a genetic level. This, and previous research, would appear to suggest that both genetic and physiological factors are playing a part in the chemoprotective, and chemotheraputic effects of CLA in breast cancer. CLA, as a fatty acid may well be affecting cellular signalling, by incorporation into cellular membranes.

9.2.2 CLA as a Cancer Treatment.

Currently the *in vivo* and *in vitro* results available from studies into the effect of CLAs on breast cancer cells do not correlate well. The strongly chemotherapeutic, and chemoprotective effect observed *in vitro* is not as pronounced in *in vivo* studies. Some *in vivo* studies either do not show any effect at all, or even show a totally opposing effect.

9.3 Future Work

9.3.1 CLA Incorporation into Cellular Membranes

Given the somewhat unexpected results of the current research it now appears that CLA is involved in more than just altering genetic expression. It is important to look, not just at the up and down regulation of the active form of these isomers, but also at their enzymic activity. This has a profound effect on how the isoforms exert their effects within the cell. The incorporation of CLA into cellular membranes, and its effects on protein activation when there, is an area of research which may prove fruitful in the future. Incorporation of radio labelled CLA isomers into the cell membranes, coupled with tagging of the various PKC isoforms to measure activation would help in bringing this about. An understanding of how CLA affects the physiology of the cell will assist our understanding of how it exerts its multifarious effects.

9.3.2 CLA as a Chemoprotective Agent

The use of CLA as a chemoprotective agent has not been explored in the current research. Based on its effect on the PKC α isoform, which is known to affect the resistance of breast cancer cells to chemotherapeutic drugs, it may be that the decrease in the active form of this isomer after treatment with the *c*9:*t*11 and *t*10:*c*12 isomers alone plays a role in chemoprotection. This decrease may begin in normal cells, and prevent levels of PKC α reaching the peaks required to cause significant apoptotic resistance. The significant increases in the abundance of active PKC δ caused by treatment with 50μM of the *c*9:*t*11 and *t*10:*c*12 isomers alone in both MCF-7 and MDA-MB-231 cells may also be a significant factor in the chemoprotective effect of CLA. PKC δ is known to promote apoptosis, and significantly increased levels may persist even after treatment has been stopped.

Data relating to the decline of active PKC isoforms in cells after prolonged treatment with CLA, as well as the IC_{50} value of CLA (measured by competition binding assays) in animal models has not, as yet, been published. Also required are tests on serum concentrations of CLA to measure how fast it is cleared from the body, both in animals and humans. The results from these experiments would provide a clearer picture of how the CLA exerts its effects on cells, at a physiological level. They would also provide an idea of what CLA intake in grams per day is required to exert an effect on cells in the body.

9.3.3 The Chemoprotective Effect of CLA in Human Studies

The evidence for a chemoprotective effect by CLA is primarily based on studies done on animal models and with cancer cells *in vitro*, potentially warranting more studies on CLA intake, and chemo-protectiveness in humans. The limited studies of CLA performed in human populations so far, have been somewhat inconclusive. The section below details the methods and findings of the largest of these.

9.3.3.1 The Netherlands Cohort Study

The Netherlands Cohort Study on Diet and Cancer (Voorrips *et al.* 2002), looked at the intake of CLA, as well as other fatty acids in 62,573 postmenopausal women (between 55-69 years old). The study took the form of self completed questionnaires, looking at personal and family cancer incidence, lifestyle and dietary habits. The food frequency section of the study was semi quantative, comprising 150 items, and concentrating on the habitual consumption of food and beverages. Observer bias was minimised by use of a blinding system to mask the sub-cohort status of the subjects. Specific fatty acid intake

was calculated using a database derived from the TRANSFAIR study of 1999 (Hulshof *et al.* 1999), involving the sampling and analysis of foods from 14 European countries (a maximum of 100 foods per country). The mean CLA consumption by volunteers was 0.2g/d (± 0.1), a sub cohort, made up of 941 women who went on to develop breast cancer, and 1598 control women (without breast cancer) was left for analysis after 6.3 years. A multivariate analysis showed no statistical significance between the incidence of breast cancer, and fat, or energy intake, including separate analyses for intake of vegetable fats, animal fats, total fatty acids, and total polyunsaturated fatty acids. There was however a higher incidence of breast cancer among volunteers who consumed the higher amounts of *trans* unsaturated fatty acids, and a weakly positive trend with regards to breast cancer incidence was observed in those who consumed a higher amount of CLA, although this did not appear to be related to consumption of milk, milk products, fresh, or processed meat.

9.3.3.2 The Finnish Study

Between 1992 and 1995 levels of dietary and serum CLA were looked at in 195 Finnish patients with either pre, or postmenopausal breast cancer, and compared to 208 control subjects without cancer. The results by Aro *et al.* in 2000 showed that women suffering from postmenopausal breast cancer had significantly lower levels of both serum, and dietary CLA, as well as lower levels of myristic, and trans-vaccenic acid. As in the Netherlands study, validated food frequency questionnaires were used to determine dietary levels of the various fatty acids.

9.3.3.3 The WEB study

The WEB study of 2004 (McCann *et.al*) looked at intake of dietary CLA by 1122 women with breast cancer, and 2036 healthy control subjects in Western New York (USA). As in previous studies the majority of subjects were of Caucasian origin, although it was the first of its kind to take place in the USA. In this study intake of both total CLA, and of the *c*9:*t*11 isomer alone was measured using a food frequency questionnaire, which, as in the other studies was self administered. The WEB study observed no association between CLA intake, and breast cancer risk, either pre, or post menopausal. There was however, an indication that increased consumption of the *c*9:*t*11 isomer alone may be linked to a reduced risk of premenopausal subjects presenting with ER- tumours. This was apparently not statistically significant. This final result may be linked to the results of chapter 4 in the present study, which showed a definite decrease in cell viability for the MDA-MB-231 cells which had been treated with 50μM of CLA for 24 hours.

9.3.3.4 Limitations of These Studies

It is worth noting that only one of these studies actually looked at serum concentrations of CLA, which are a more accurate assessment of CLA uptake by the body (although not as accurate as assessments of levels of CLA in red blood cell membranes), and this was the study which showed a correlation between breast cancer risk and CLA intake. Questionnaires are a much cruder method of measuring intake of any food type, with so much food being bought pre-packaged, and pre-prepared it is more difficult to be accurate. Because different companies will rely on slightly different recipes for their products it is difficult to get a true representation of the intake of a particular nutrient, also people will have differing ideas as to what constitutes a "portion". Food questionnaires also rely on the respondent remembering to note down the exact contents of every meal,

and do not usually take in to account any change in dietary habits bought about by a diagnosis of cancer.

What is really required in this field is a study focussing on CLA supplementation and serum concentrations, and how it affects breast cancer incidence, survival and tumour size.

9.3.3.5 Future Studies into CLA Intake and Breast Cancer.

Because CLA is already a recognised supplement, and sold as such, both in the US and the UK, it would be possible to measure its intake with a much higher degree of success in human trials. One possible downside to this is the fact that many of the over the counter preparations of CLA are derived from Safflower Oil, rather than animal fats so before any clinical trials could go ahead an initial study into the efficacy of these preparations would need to be carried out. Once the fitness of these supplements was proved a possible human based study would involve patients who had undergone surgery to remove tumours. Patients could be asked to take either the supplement, or a placebo (possibly Olive Oil) after surgery, and their likelihood of recurrence measured. It would be hoped that supplementation with the CLA would reduce the recurrence of breast tumours, and reduce the mean size of tumours in women who did suffer a recurrence. Other possibilities would involve asking patients to take CLA or a placebo as an adjunct to Tamoxifen therapy, or between diagnosis and surgery to look at their effects on tumour size. Such studies would be much more controlled than previous ones, and could potentially give a more accurate picture of the benefits of CLA to human health.

Chapter 10.
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Appendix A.

Names and Addresses of Companies Named as Supplying Materials for this Work.

Company	Address	Country
Thermo Fisher Scientific	Unit 5, The Ringway Centre Edison Road Basingstoke Hants RG21 6YH	UK
Perbio Science UK Ltd. (also Pierce Biotechnology)	Unit 9 Atley Way, North Nelson Industrial Estate Cramlington, Northumberland NE23 1WA	USA
Merck Chemicals Limited (Calbiochem)	Padge Road, Nottingham, NG9 2JR	UK
GE Healthcare Bio-Sciences AB	SE-751 84 Uppsala Sweden	Sweden
Bio-Rad Laboratories	1000 Alfred Nobel Drive Hercules, CA 94547	USA
Thermo Spectronic	5225 Verona Rd., Madison, WI	USA
New England Biosciences	New England Biolabs (UK) Ltd. 75/77 Knowl Piece, Wilbury Way, Hitchin, Herts, SG4 0TY	UK
Dynex Technologies	Church Farm Business Park, Corston, Bath, BA2 9AP	UK
Sigma-Aldrich	PO Box 14508 St. Louis, MO 63178	USA
Abcam	1 Kendell Square, Ste 341, Cambridge, MA 02139-1517	USA
Santa Cruz	Holly Ditch Farm, Mile Elm, Calne, Wiltshire, SN11 0PY	UK
Pall	2200 Northern Boulevard East Hills, NY 11548	USA

Company	Address	Country
Microsoft Corporation	Microsoft Campus, Thames Valley Park, Reading, Berkshire, RG6 1WG	USA
Invitrogen	1600 Faraday Avenue PO Box 6482 Carlsbad, California 92008	USA
Kodak	343 State Street Rochester, NY 14650-0505	USA

Appendix B

ANOVA Analysis Performed on Cell Viability Data (Chapter 4).

Multiple Comparisons

Dependent Variable: % Viable cells

Tukey HSD

(I) Treatment	(J) Treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Con	LA	-1.27333%	1.49584%	.908	-6.1963%	3.6496%
	Mix	23.58333%(*)	1.49584%	.000	18.6604%	28.5063%
	9:11	24.51667%(*)	1.49584%	.000	19.5937%	29.4396%
	10:12	17.13667%(*)	1.49584%	.000	12.2137%	22.0596%
LA	Con	1.27333%	1.49584%	.908	-3.6496%	6.1963%
	Mix	24.85667%(*)	1.49584%	.000	19.9337%	29.7796%
	9:11	25.79000%(*)	1.49584%	.000	20.8671%	30.7129%
	10:12	18.41000%(*)	1.49584%	.000	13.4871%	23.3329%
Mix	Con	-23.58333%(*)	1.49584%	.000	-28.5063%	-18.6604%
	LA	-24.85667%(*)	1.49584%	.000	-29.7796%	-19.9337%
	9:11	.93333%	1.49584%	.968	-3.9896%	5.8563%
	10:12	-6.44667%(*)	1.49584%	.010	-11.3696%	-1.5237%
9:11	Con	-24.51667%(*)	1.49584%	.000	-29.4396%	-19.5937%
	LA	-25.79000%(*)	1.49584%	.000	-30.7129%	-20.8671%
	Mix	-.93333%	1.49584%	.968	-5.8563%	3.9896%
	10:12	-7.38000%(*)	1.49584%	.004	-12.3029%	-2.4571%
10:12	Con	-17.13667%(*)	1.49584%	.000	-22.0596%	-12.2137%
	LA	-18.41000%(*)	1.49584%	.000	-23.3329%	-13.4871%
	Mix	6.44667%(*)	1.49584%	.010	1.5237%	11.3696%
	9:11	7.38000%(*)	1.49584%	.004	2.4571%	12.3029%

* The mean difference is significant at the .05 level.

Table 4.1: ANOVA test of values from MCF-7 Cells treated with 25µM CLA.
Percentage of viable cells after 24 hours treatment with 25µM CLA. Average of three experiments, cells incubated with Tryphan Blue.
Key: Control – 100% ethanol (equivalent volume); LA – 25µM Linoleic Acid; Mix – 25µM 50:50 mixture of the *c*9:*t*11 and *t*10:*c*12 isomers; 9:11 - 25µM *c*9:*t*11 isomer alone; 10:12 - 25µM *t*10:*c*12 isomer alone.

Multiple Comparisons
Dependent Variable: % viable cells

Tukey HSD

(I) Treatment	(J) Treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Con	LA	2.3733333%	1.8168985%	.694	-3.606229%	8.352895%
	Mix	32.3600000%(*)	1.8168985%	.000	26.380438%	38.339562%
	9:11	34.4000000%(*)	1.8168985%	.000	28.420438%	40.379562%
	10:12	32.0733333%(*)	1.8168985%	.000	26.093771%	38.052895%
LA	Con	-2.3733333%	1.8168985%	.694	-8.352895%	3.606229%
	Mix	29.9866667%(*)	1.8168985%	.000	24.007105%	35.966229%
	9:11	32.0266667%(*)	1.8168985%	.000	26.047105%	38.006229%
	10:12	29.7000000%(*)	1.8168985%	.000	23.720438%	35.679562%
Mix	Con	-32.3600000%(*)	1.8168985%	.000	-38.339562%	-26.380438%
	LA	-29.9866667%(*)	1.8168985%	.000	-35.966229%	-24.007105%
	9:11	2.0400000%	1.8168985%	.792	-3.939562%	8.019562%
	10:12	-.2866667%	1.8168985%	1.000	-6.266229%	5.692895%
9:11	Con	-34.4000000%(*)	1.8168985%	.000	-40.379562%	-28.420438%
	LA	-32.0266667%(*)	1.8168985%	.000	-38.006229%	-26.047105%
	Mix	-2.0400000%	1.8168985%	.792	-8.019562%	3.939562%
	10:12	-2.3266667%	1.8168985%	.708	-8.306229%	3.652895%
10:12	Con	-32.0733333%(*)	1.8168985%	.000	-38.052895%	-26.093771%
	LA	-29.7000000%(*)	1.8168985%	.000	-35.679562%	-23.720438%
	Mix	.2866667%	1.8168985%	1.000	-5.692895%	6.266229%
	9:11	2.3266667%	1.8168985%	.708	-3.652895%	8.306229%

* The mean difference is significant at the .05 level.

Table 4.2: ANOVA test of values from MCF-7 Cells treated with 50µM CLA
Percentage of viable cells after 24 hours treatment with 50µM CLA. Average of three experiments, cells incubated with Tryphan Blue.
Key: Control – 100% ethanol (equivalent volume); LA – 50µM Linoleic Acid; Mix – 50µM 50:50 mixture of the *c*9:*t*11 and *t*10:*c*12 isomers; 9:11 - 50µM *c*9:*t*11 isomer alone; 10:12 - 50µM *t*10:*c*12 isomer alone.

Multiple Comparisons

Dependent Variable: % viable cells

Tukey HSD

(I) Treatment	(J) Treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Con	LA	5.4566667%	2.4409898%	.242	-2.576831%	13.490165%
	Mix	44.3700000%(*)	2.4409898%	.000	36.336502%	52.403498%
	9:11	39.6100000%(*)	2.4409898%	.000	31.576502%	47.643498%
	10:12	48.0733333%(*)	2.4409898%	.000	40.039835%	56.106831%
LA	Con	-5.4566667%	2.4409898%	.242	-13.490165%	2.576831%
	Mix	38.9133333%(*)	2.4409898%	.000	30.879835%	46.946831%
	9:11	34.1533333%(*)	2.4409898%	.000	26.119835%	42.186831%
	10:12	42.6166667%(*)	2.4409898%	.000	34.583169%	50.650165%
Mix	Con	-44.3700000%(*)	2.4409898%	.000	-52.403498%	-36.336502%
	LA	-38.9133333%(*)	2.4409898%	.000	-46.946831%	-30.879835%
	9:11	-4.7600000%	2.4409898%	.353	-12.793498%	3.273498%
	10:12	3.7033333%	2.4409898%	.575	-4.330165%	11.736831%
9:11	Con	-39.6100000%(*)	2.4409898%	.000	-47.643498%	-31.576502%
	LA	-34.1533333%(*)	2.4409898%	.000	-42.186831%	-26.119835%
	Mix	4.7600000%	2.4409898%	.353	-3.273498%	12.793498%
	10:12	8.4633333%(*)	2.4409898%	.038	.429835%	16.496831%
10:12	Con	-48.0733333%(*)	2.4409898%	.000	-56.106831%	-40.039835%
	LA	-42.6166667%(*)	2.4409898%	.000	-50.650165%	-34.583169%
	Mix	-3.7033333%	2.4409898%	.575	-11.736831%	4.330165%
	9:11	-8.4633333%(*)	2.4409898%	.038	-16.496831%	-.429835%

* The mean difference is significant at the .05 level.

Table 4.3: ANOVA test of values from MD-MBA-231 Cells treated with 25µM CLA. Percentage of viable cells after 24 hours treatment with 25µM CLA. Average of three experiments, cells incubated with Tryphan Blue.
Key: Control – 100% ethanol (equivalent volume); LA – 25µM Linoleic Acid; Mix – 25µM 50:50 mixture of the *c*9:*t*11 and *t*10:*c*12 isomers; 9:11 - 25µM *c*9:*t*11 isomer alone; 10:12 - 25µM *t*10:*c*12 isomer alone.

Multiple Comparisons

Dependent Variable: % viable cells

Tukey HSD

(I) Treatment	(J) Treatment	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Con	LA	-5.0533333%	2.2376535%	.235	-12.417635%	2.310968%
	Mix	61.9166667%(*)	2.2376535%	.000	54.552365%	69.280968%
	9:11	56.8933333%(*)	2.2376535%	.000	49.529032%	64.257635%
	10:12	62.9700000%(*)	2.2376535%	.000	55.605698%	70.334302%
LA	Con	5.0533333%	2.2376535%	.235	-2.310968%	12.417635%
	Mix	66.9700000%(*)	2.2376535%	.000	59.605698%	74.334302%
	9:11	61.9466667%(*)	2.2376535%	.000	54.582365%	69.310968%
	10:12	68.0233333%(*)	2.2376535%	.000	60.659032%	75.387635%
Mix	Con	-61.9166667%(*)	2.2376535%	.000	-69.280968%	-54.552365%
	LA	-66.9700000%(*)	2.2376535%	.000	-74.334302%	-59.605698%
	9:11	-5.0233333%	2.2376535%	.239	-12.387635%	2.340968%
	10:12	1.0533333%	2.2376535%	.988	-6.310968%	8.417635%
9:11	Con	-56.8933333%(*)	2.2376535%	.000	-64.257635%	-49.529032%
	LA	-61.9466667%(*)	2.2376535%	.000	-69.310968%	-54.582365%
	Mix	5.0233333%	2.2376535%	.239	-2.340968%	12.387635%
	10:12	6.0766667%	2.2376535%	.121	-1.287635%	13.440968%
10:12	Con	-62.9700000%(*)	2.2376535%	.000	-70.334302%	-55.605698%
	LA	-68.0233333%(*)	2.2376535%	.000	-75.387635%	-60.659032%
	Mix	-1.0533333%	2.2376535%	.988	-8.417635%	6.310968%
	9:11	-6.0766667%	2.2376535%	.121	-13.440968%	1.287635%

* The mean difference is significant at the .05 level.

Table 4.4: ANOVA test of values from MD-MBA-231 Cells treated with 50µM CLA. Percentage of viable cells after 24 hours treatment with 50µM CLA. Average of three experiments, cells incubated with Tryphan Blue.
Key: Control – 100% ethanol (equivalent volume); LA – 50µM Linoleic Acid; Mix – 50µM 50:50 mixture of the *c*9:*t*11 and *t*10:*c*12 isomers; 9:11 - 50µM *c*9:*t*11 isomer alone; 10:12 - 50µM *t*10:*c*12 isomer alone.