University of Alberta

Mechanism of cytotoxicity of conjugated linoleic acid in human breast cancer cells

By ©

Danielle Lise Amarù

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

in

Nutrition and Metabolism

Department of Agricultural, Food and Nutritional Science

Edmonton, Alberta

Spring 2008

Abstract

Conjugated linoleic acid (CLA) consists of a group of positional and geometric stereoisomers and has been shown in animal models to inhibit mammary tumour initiation, promotion and progression. (1) The two major dietary isomers c9,t11 and t10,c12 have been the most studied and although both inhibit the growth of mammary tumours they may work through different mechanisms of action and have different possible degrees of cytotoxicity The objective of this research was to establish the effectiveness of both c9,t11 and t10,c12 CLA isomers and the mechanism by which they inhibit the growth of breast cancer cells. MCF-7 breast cancer cells were chosen as a model of estrogen-receptor positive breast cancer. T10,c12 CLA reduced viability and increased cell death to a greater extent than c9,t11 ($p<0.05$) as compared to linoleic acid (LA) and oleic acid (OA). The inhibitory effect of t10,c12 was observed both in the presence and absence of LA, an essential fatty acid. All of the treatment fatty acids were significantly incorporated into cell phospholipids ($p<0.05$). OA and untreated cells demonstrated significantly greater incorporation of monounsaturated fatty acids, particularly OA into cell phospholipids ($p<0.05$) as compared to LA and both CLA isomers. The greater OA appeared to be related to an improved cell survival.

IGF-I is a growth factor that promotes cell proliferation and prevents apoptosis or cell death. It is a ligand for a membrane-bound receptor tyrosine kinase, IGFIR. IGFIR has been shown to be up-regulated in estrogen-positive breast cancer.

It was hypothesized that CLA would incorporate into the cell's membrane phospholipids and decrease total IGFIR resulting in a reduction in the cell's ability to respond to IGF-I. t10,c12 CLA incorporated into MCF-7 cells phospholipids to a greater extent than c9,t11

(32% vs. 22% of total phospholipids) and reduced the total amount of IGFIR greater than all the other fatty acid treatments (p<0.05).

In summary the results of this thesis support the evidence for a cytotoxic effect of t10,c12 CLA on estrogen-receptor positive breast cancer cells. Further follow up studies are warranted to confirm t10,c12's interference with IGF-I signaling as possibly mediated through interference with membrane phospholipids. This will be useful for future animal and human studies in determining CLA's potential use as an adjuvant treatment for breast cancer.

Acknowledgments

I would like to thank my supervisor Dr. Catherine Field for her guidance, support and optimism throughout my experience.

I would also like to thank my committee members Dr. Vickie Baracos and Dr. Vera Mazurak for their input and initiation of journal club. I have enjoyed the weekly discussions and only wish it had started sooner.

To my husband Tony Amarù, I appreciate that you moved to this winter wonderland so that I could complete my degree. I am very excited for our future together.

This degree would not have been as much fun or as rewarding without all of my fellow graduate students. I have a special thank you for those who had a direct impact on my project. Specifically, Trish Biondo who taught me the basics to cell culture work, I miss our tennis matches and grueling post-game bike rides. Erin O'Connell who helped me overcome a significant road block in my research and got me hooked on so you think you can dance and homemade poppycock. Finally, Megan Ruth whose patience with my wide-ranging questions, to which she always had an answer, is greatly appreciated. I always enjoyed our gym sessions even if I talked more than actually exercised.

Thank you to Sue Goruk, Marnie Newell, Dr. David Wright, Joan Turchinsky and Dr. Goh for your technical support. I will miss our lunches Dr. Goh.

I would also like to acknowledge my parents for their unconditional support and encouragement throughout my academic endeavors. Thank you to my brother Matthew for inspiring me to return to school.

TABLE OF CONTENTS

List of Tables

List of Figures

List of Abbreviations

AA	arachidonic acid
BCA	Bicinchoninic acid
bFGF	basic fibroblast growth factor
CLA	conjugated linoleic acid
DHA	docosahexanoic acid
ECM	extracellular matrix
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
Erk	extracellular signal-related kinase
FBS	fetal bovine serum
IGF-I	insulin like growth factor 1
IGF-2	insulin like growth factor 2
IGFIR	insuling like growth factor receptor
IRS-1	insulin receptor substrate 1
LA	linoleic acid
LDH	lactate dehydrogenase
mg	milligram
MUFA	monounsaturated fatty acid
OA	oleic acid
PI3K	phosphatidylinositol 3-kinase
PL	phospholipid
PPAR	peroxisome proliferator-activated receptors
PUFA	polyunsaturated fatty acid
SFA	saturated fatty acid
TVA	trans vaccenic acid
μM	micromolar
VEGF	vascular endothelial growth factors
WHEL	Women's Healthy Eating and Living Study
WINS	Women's Intervention Nutrition Study
Wt/wt	weight per weight

1. Introduction and Literature Review

1.1 Cancer

Over their lifetime 44% of Canadian men or 39% of Canadian women are expected to develop cancer (1). It is estimated that 1 in 4 Canadians will die of cancer (1). Cancer is defined as uncontrolled growth of abnormal cells that are not responsive to the body's normal regulatory mechanisms. This definition oversimplifies the complexity of a disease of which there are 100 different types, in addition to subsets within each cancer type (2). Due to the great variability and constantly evolving nature of tumours a singular solution for the prevention and eradication of cancer remains elusive and possibly unrealistic. However as an understanding of the complex underlying nature of cancer emerges there appears to be a few common traits that characterize cancer, which may help in developing a multi-approach solution.

The key underlying attribute behind the multi-step development of cancer is genomic instability and changes to the genetic material of a cell. Both epigenetic & genetic changes that either activate proto-oncogenes (promote growth) or suppress tumour suppressor genes (block growth) of a normal cell gradually transform it into one considered tumourgenic (3). The six acquired capabilities termed "hallmarks of cancer" include the ability to evade apoptosis, self-sufficiency in growth signals, insensitivity to anti-growth signals, limitless replicative potential (immortalization), sustained angiogenesis, and tissue invasion and metastasis (3). Overall 90% of cancer deaths are due to metastasis (4).

1.1.1 Breast Cancer

In Canada it is estimated in 2006 that 28.9% of all new cases of cancer in women will be attributed to breast cancer, which translates to 1 in 9 Canadian women being afflicted during their lifetime (1). At the same time, the mortality rate for breast cancer has been decreasing. The current five year survival rate for women diagnosed with breast cancer in Canada is 86%, which is greater than the overall cancer average of 62%. In spite of the

improving survival rate, breast cancer continues to be a major health concern for women. Even though breast cancer does occur in males, it is at a much lower frequency, less than 1% of breast cancer cases are in males (1).

There is information to support several possible risk factors associated with breast cancer. Increasing age shows the strongest correlation with increased risk (5). Numerous risk factors that have shown a consistent relationship with breast cancer appear to be intimately related to endogenous or exogenous hormones (estrogen, progesterone, testosterone and possibly prolactin) (5) (6). See Table 1-1

Table 1.1: **Risk factors for breast cancer linked to endogenous or exogenous hormones** (5).

Risk Factor	Effect on breast cancer risk	Theoretical mechanism
Early menarche (<12yrs old) and/or Late menopause (>54yrs)	↑ risk	Longer lifetime exposure to ovulatory cycles and corresponding hormonal environment
Young age at first birth (<20yrs old vs. >30yrs old)	↓ risk	Reduced ovulatory cycles
Increasing parity	↓ risk	Reduced ovulatory cycles
Lactation	↓ risk	Reduced ovulatory cycles
Increased Height	↑ risk	Height related to growth hormones
Obesity	↑ risk in postmenopausal women, ↓ risk in premenopausal women	Postmenopausal – fat stores primary source of estrogen post menopause Premenopausal – irregular ovulatory cycles due to obesity results in reduced exposure to hormone surges
Hormone Replacement Therapy or Combined Birth Control Pill	Modest ↑ risk (dependant on time)	Exogenous hormones

Other risk factors that have been identified include family history and genetic predisposition such as, BRCA1 (breast cancer gene 1) and BRCA2, benign breast disease (atypical hyperplasia), and exposure to ionizing radiation during puberty (5;7). Although there is considerable interest in possible lifestyle factors the only one for which there is consistently strong evidence at this time is alcohol consumption, which increases risk. Other factors such as physical activity, the intake of folate, vitamin D, dietary fat,

vegetables and fruit continue to be investigated, yet there is not sufficient evidence at this time to conclude a strong relationship (7).

Current primary and adjuvant treatment options for people with breast cancer include surgery, radiation, chemotherapy, endocrine and/or biological therapies (7). As with the variability of cancer types even within breast cancer there are phenotypic differences. Some adjuvant treatments that are available are effective only in certain subgroups whose tumours display specific phenotypes. For instance in tumours that express the estrogen receptor, endocrine therapies such as selective estrogen receptor modulators (Tamoxifen) or aromatase inhibitors (Letrozole) are prescribed (7). A more recent biological therapy, Trastuzumab is a monoclonal antibody for the epidermal growth factor receptor HER2/neu, which is over expressed in 25-30% of breast cancers (8).

As the profiling and comprehension of breast cancer advances there continues to be a strong interest in discovering treatments or factors that will complement or improve upon current treatment options able to interfere with the development and spread of cancer.

1.2   Dietary Fat and Breast cancer

In 1981 Doll et al. estimated that 30% of cancers could be prevented by dietary factors (9). Epidemiology and animal studies have supported the hypothesis that nutritional factors may play an important role in the etiology of breast cancer (10;11). Ecological and migration studies on breast cancer risk have shown a strong correlation between fat consumption and breast cancer incidence (12). In 2003 a meta-analysis of cohort, and case-control trials supported the direct association between high fat intake and increased risk of breast cancer with an odds ratio of 1.13 (C.I. 1.03-1.25) (13).

Three intervention studies were designed to test the hypothesis that a reduction in dietary fat intake would reduce the incidence or recurrence of breast cancer. The Women's Health Initiative was a large prevention trial in the United States that recruited 48,835 participants (14). They did not find a significant difference between the intervention (low fat group) and control group. However the authors argued that the non-significant trend

for reduced breast cancer risk with a reduction in dietary fat intake may have been significant if follow-up was longer than 8.1 years (14). Both the Women's Intervention Nutrition Study (WINS) (15) and the Women's Healthy Eating and Living Study (WHEL) (16), were in breast cancer patients previously treated by surgery/adjuvant chemotherapy, at risk for relapse. WINS implemented a low fat diet (15% energy from fat) and observed a modest reduction on body weight and reduced risk of relapse in the intervention group (15). In comparison WHEL focused on increased vegetables, fruit, fibre and reduced fat and did not observe any significant weight change or reduction in risk in the intervention group. Of note the intake of fat in the intervention group in WHEL had returned to the initial 28.9% of total energy by the end of the 6 years of the study, thereby impairing the ability to detect differences between the intervention and control group (16).

These inconsistent findings from intervention studies continue to be debated in the academic community with arguments including; measurement error of the diet (food frequency vs. food records) (17), identification of the appropriate life stage to study the effect of dietary fat (i.e. adolescence vs. menopause), and the failure to focus on obesity rather than dietary fat (18).

Studies on the role of total dietary fat intake during breast cancer treatment on treatment outcome or prognosis in humans have not been preformed. Observational data has suggested that a high dietary fat intake is related to poorer survival in premenopausal breast cancer patients (19). Goodwin noted in breast cancer patients that dietary fat intake had more of a u-shaped rather than linear relationship for treatment outcomes, which indicates both extremes in dietary fat intake were associated with decreased survival (20). Although the connection between total dietary fat intake and breast cancer is not clear at this time, researchers speculate that the association between dietary fat and breast cancer may be more complex than simply total fat intake and are exploring the differential effect of various types of dietary fat.

Dietary fatty acids differ in their structure and physiological function. Three main classes of fatty acids are saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), and polyunsaturated fatty acids (PUFA). A meta-analysis of rodent mammary cancer studies concluded that SFA and n-6 PUFA (linoleic acid) promote mammary tumours (21). The studies performed in humans are not as strong, although the large European Prospective Investigation into Cancer observed an increased risk for breast cancer with a high saturated fat intake as measured by food records (17). There is evidence in vivo (animal studies) and in vitro (human cancer cells) that the long chain n-3 polyunsaturated fatty acids, eicosapentaenoic acid and docosahexaenoic acid, protect against the initiation, promotion and/or progression of breast cancer (22). However, as with the studies on total fat intake, human studies examining the association between long chain n-3 fatty acids have reported inconsistent findings (23).

In 1979 Pariza et al. discovered an anti-carcinogenic property of fried ground beef, (24) by 1987 the compound was isolated and identified as a polyunsaturated fatty acid called conjugated linoleic acid (CLA) (25). Since then, numerous animal and cell culture studies have demonstrated that CLA may be protective against breast cancer. The remainder of this review will review what is currently known about the anti-carcinogenic properties of CLA in breast cancer and potential gaps in the literature.

1.3   Conjugated Linoleic Acid (CLA)

More recently, there has been considerable interest in the anti-carcinogenic properties of a unique PUFA. The polyunsaturated fatty acid CLA consists of a group of positional and geometric (cis-trans) stereoisomers (same atomic connectivity with different atomic arrangement in space). CLA is predominantly found in the meat and milk of ruminant animals. They are intermediate products of the biohydrogenation of linoleic acid to stearic acid that occurs in ruminant animals (Figure 1) (26).

In the Rumen

Diet source   18:2n6 (Linoleic Acid)

$\downarrow$ Isomerization

18:2 (CLA – most often c9,t11 isomer)

$\downarrow$ Hydrogenation

Trans-11 18:1 (TVA)

$\downarrow$ Hydrogenation

18:0 (Stearic Acid)
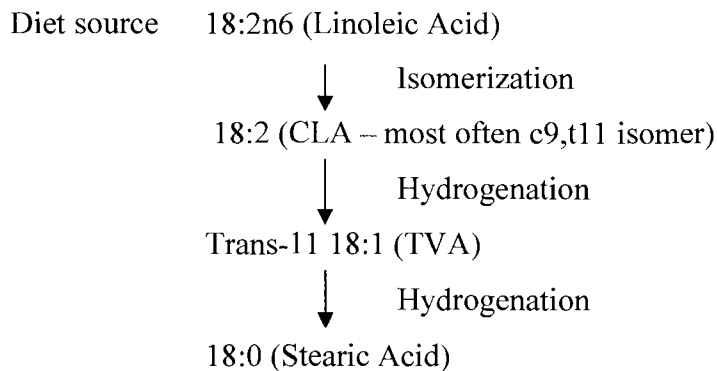
In the mammary gland or adipose tissue (endogenous synthesis)

18:2 (CLA)

$\uparrow$ Δ9-desaturase

Trans-11 18:1 (TVA)
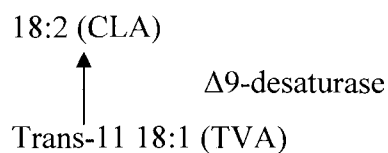
Figure 1-1: **Pathways for the natural formation of CLA in ruminant animals** (26).

There are numerous isomers of CLA that are included in Table 1-2. The major isomer, c9,t11, on average accounts for 73-94 % of the total CLA isomers in ruminant products (26). Commercial production of CLA, most commonly via alkali isomerization of LA results in a mix of some of the more minor isomers (Table 1), with c9,t11 and t10,c12 accounting for around 40 % each (26).

Table 1-2: **CLA isomers in found in food and synthetic sources** (26)

CLA isomers found in Ruminant foods	t12,t14; t11,t13; t10,t12;t9,t11; t8,t10; t7,c9; t6,t8; c12,t14; t11,c13; c11,t13; c10,t12; c9,t11; c8,t10;c7,t9; c9,c11; c11,c13
CLA isomers in synthetic mixture	c9,t11 (41%), t10,c12 (44%), t9,t11/t10,t12 (4-10%).

The initial research and strongest evidence to date for a health effect of CLA involved its' anti-carcinogenic properties. However the CLA isomers have also being researched and linked to potentially beneficial effects in adiposity, insulin sensitivity, immunity (27). The research in these areas is beneficial to consider when determining possible mechanisms of action.

1.3.1 CLA content in foods and average intake

There are varying estimates in the literature as to the current average intake of CLA by the general population in westernized countries ranging from 95 to 430 mg per day (Table 1-3). These estimates are complicated by the variability of CLA content in the aforementioned food sources that occur because of differences in feeding practices and inter-animal differences which affect the production of CLA in the rumen and mammary gland (28). This variability in the food supply makes it challenging to study the intake of CLA in the general population and its effect on health.

Table 1-3: **Estimated average human intake of CLA**

Study	Data collection	Men	Women
Fritsche1998 – Germany (29)	Shopping basket survey	430 mg/day	350 mg/day
Herbel1998 (30)	3-4 day food records	139mg/d	
Jiang1999 – Sweden (31)	Food records & 24hr recalls	160mg/d	
Park1999 (32)	FFQ	20-290mg/day (lactating women)	
Ens 2001 – Canada (33)	2 7-day food records	94.9 mg/d (15-174mg/d)	
Ritzenthaler2001 – U.S. (34)	Food frequency	197mg/d	93mg/d
	Diet record	176mg/d	104mg/d
	Food duplicate	212 mg/d	151 mg/d
Fremann 2002 – Germany (35)	FFQ		246 mg/d
	7-day food record		323 mg/d

1.4 CLA and Cancer

1.4.1 Human Studies in CLA and Breast Cancer

To date there have been no clinical trials in humans to test the effect of CLA on cancer prevention or treatment. The only human data available is from epidemiological studies.

Four studies have examined the correlation between CLA intake and risk of breast cancer (Table 1-4). The results between the four studies are conflicting, both increased and decreased risk have been reported and do not clarify if there is a beneficial role of CLA in breast cancer in humans.

Table 1-4: **Summary of epidemiological studies of CLA & breast cancer risk.**

Type of Study	Results	Notes
Case Control - Finland (36)	0.4 odds ratio for breast cancer in highest quintile of CLA intake (~200mg/d)	Reduced risk in postmenopausal women
Cohort Study- Netherlands (37)	Positive trend in highest quintile of c9,t11 intake (~290mg/d)	Possible increased risk
Case Control- U.S.A. (38)	Slight protective effect at highest intake of c9,t11 in premenopausal women	Protection only observed with estrogen-receptor negative breast cancer
Case Control – France (39)	No change in risk for metastasis	CLA content in breast adipose tissue samples taken during breast cancer surgery

In summary there is a lack of consistent and clear support for a protective effect of CLA from the epidemiological data to date. Some limitations with the current evidence available including the variability of CLA in the food supply, the difficulty of assessing long term intake of this minor dietary component and the highest consumption of CLA identified in these studies appears to be less than what would be predicted to be the 'protective dose' observed in animal models. There are different reports on how to convert animal study dosages to human intake levels, estimates range from 600mg/day to 3g/day required for minimal protection in humans (40) (41). Either way both the minimal effective dose in animals continues to be greater than the highest intake dose currently estimated in humans.

Although there is no data on the effect of CLA in the treatment of breast cancer in humans, there is support for a protective effect in both animal and in vitro models. An extensive review of the literature that has examined the effect of dietary CLA on breast cancer was conducted. All papers published between 1991 and 2007 were gathered and reviewed (Table 1-5 & 1-6). The two isomers c9,t11 and t10,c12 have been the most studied and there appears to be differences between the isomers as to their individual effectiveness and most likely mechanisms of action. The rest of this review will cover the in vivo and in vitro studies examining the effect of CLA on breast cancer and potential mechanisms of action.

1.4.2 Animal studies of CLA and mammary cancer

The animal studies (Table 1-5) were examined and critically reviewed by looking at the animal model of cancer used, the manner the tumour was induced, the diet that was fed, the CLA content and isomer mixture and the key results. The overall conclusions from this review were that both synthetic and naturally enriched sources of CLA added to the diet have been shown to inhibit mammary tumour initiation by exogenous carcinogens (40;42-47) (48-54) (55) (56) (57), promotion and progression (58-64) in rodents. A range between 0.1% - 1% wt/wt of CLA independent of the level and type of fat in the diet has shown to be effective, with no further benefits beyond 1% wt/wt. (40;47). The stage at which CLA is introduced in the diet appears to impact its effectiveness. When Ip et al (43) provided CLA solely during mammary development prior to the injection of the carcinogen, the protection against mammary cancer continued for the duration of the study even though CLA was no longer in the diet. In contrast if CLA was introduced in the diet after mammary development, its intake had to be continuous for the remainder of the experiment to confer a protective effect (43). In contrast to the majority of the animal studies, no effect on tumour growth was observed with the injection of WAZ-2T mammary cancer cells into a mouse model and the t10,c12 isomer was shown to increase initiation rate and lung metastasis although not survival time in a transgenic mouse over expressing the growth receptor erbB2 (65;66). CLA has been shown to dramatically alter fat deposition and body weight in mice, yet similar results have not been observed for humans. (67) The transgenic mouse model effects appeared to be due to changes to the stroma (which consists partly of adipocytes) surrounding the cells rather than the epithelial cells themselves (66) therefore translation to humans is questionable due to CLAs' differential effects on adipocytes in mice and humans. The role of the stroma in tumour development will be discussed later in this literature review.

Table 1-5: CLA and mammary tumours in animal models

Reference	Animal Model	Tumour	Diet & form of CLA	CLA content	Results	Mechanism tested
Ip1991 (42)	Female Sprague Dawley Rats	High dose DMBA	AIN-76A synthetic CLA: c9,t11 & t9,c11 43%, t10,c12 45%	CLA 0.5%, 1%, 1.5% by wt. Diet started 2 weeks prior to DMBA	↓ # and size of tumours	No change to estrus cycle Only c9,t11 isomer incorporated into PL mammary tumour > liver ↓lipid peroxidation in mammary gland not liver, max antiox. activity 0.25% cla
Ip1994 (40)	Weanling female Sprague-dawley rats	DMBA or MNU	AIN-76A Synthetic CLA from LA same as above	CLA 0.05, 0.1, 0.25, 0.5% by wt 2 weeks prior to DMBA to term. or 1% CLA weaning to 1 week past DMBA, then back to basal diet	Dose-dependant ↓ in tumour incidence & yield. ≥0.1% CLA significant	1% CLA small ↓ proliferation of lobuloalveolar compartment, not ductal compartment of mammary tree CLA did not directly interfere with DMBA activation
Ip1995 (43)	Female Sprague-Dawley rats	MNU	Mod. AIN 76A Synthetic FFA CLA vs TG CLA	1% CLA Wean → 55 d then MNU or MNU then CLA for 1, 2 mo. or continuous	FFA & TG CLA both ↓ Tumour # & incidence	Continuous feeding required post MNU injection to derive the same benefit as CLA feeding at weaning.
Ip1996 (44)	Sprague-Dawley rats	DMBA	AIN76 – 10-20% wt/wt of Veg fat blend similar to N.America fat, or 20% corn oil, or 8% corn + 12% lard	1% CLA or one study with 20% corn oil and 0.5, 1 or 1.5% CLA	CLA ↓ tumour incidence by 50% independent of level or type of fat in diet Maximal inhibition at 1% of CLA	CLA ↓ MDA (lipid peroxidation) in mammary gland homogenate 8-OHdG (cell oxidative stress) unaffected by CLA
Visonneau 1997 (58)	SCID mice	MDA-MB468 cells injected	Synthetic CLA: c9,t11 42%, t10,c12 44%	1% CLA mix 2 weeks prior to MDA injection → termin.	↓ tumour mass & ↓ metastasis	CLA works independently of the immune system
Wong1997 (65)	BALB/c mice	WAZ-2T mammary ca cells	5% (4.1% corn oil, rest CLA/safflower)	0.1, 0.3, 0.9% corn oil, (derived from safflower oil – 35% c9,t11, 39% t10,c12)	No effect on tumour volume	↑lymphocyte maturity (blastogenesis) ↑IL-2 production, No change to lipid peroxidation
Ip1997A (45)	Female Sprague-Dawley	DMBA	20% corn oil with 1% CLA	CLA after DMBA for 4 or 8 weeks or continuous or none	Significant inhibition only in continuously-fed group	Inhibition not dependant on 'ras' CLA retained faster and in greater amount in neutral lipids vs. phospholipids

Reference	Animal Model	Tumour	Diet & form of CLA	CLA content	Results	Mechanism tested
Thompson 1997 (46)	Female Sprague Dawley Rats	DMBA-induced	AIN-76A	1% CLA b/n wean → 50 d., or from 55d → termination Or from Wean → termination	CLA ↓ total # of tumours by 50% (Overall same % inhibition with all 3 CLA tx)	No effect on fat deposition in mammary gland ↓ density of mammary epithelium branching in CLA tx ↓ DNA synthesis in terminal end buds & lobuloalveolar buds ↑ CLA metabolites CD 18:3 & 20:4
Josyula 1998 (68)	Female Fischer F344 rats	Dietary mutagen PhIP	AIN 76A minus antioxidants Synthetic CLA	0.1, 0.5 or 1% CLA wt/wt.	Not reported	Cla inhibits PhIP-DNA adduct formation in the liver & white blood cells but not in mammary epithelial or colon
Banni1999 (47)	Female Sprague-Dawley Rats	Subgroup MNU-induced	AIN-76A	O.5, 1, 1.5 or 2% CLA until MNU injection	↓ # & incidence of tumours after MNU admin. (sig. up to 1% CLA)	Not given MNU ≤1% CLA ↓ terminal end bud density ↑ CLA storage, CLA metabolites (18:3 & 20:3), & ↓ LA metabolites in particular AA in mammary gland Difference b/n CLA & LA in rate of uptake or utilization
Ip1999A (48)	Female Sprague Dawley Rats	subgroup MNU -induced	Butter fat until MNU, then corn oil	Control: 0.1% CLA butter fat 0.8% CLA b.f., Matreya CLA (81% c9,t11 or Nuchek CLA (36.5% t10,c12, 25.3% c9,t11)	All CLA tx ↓ # & incidence of tumours	Those not tx with MNU: CLA ↓ mammary epithelial branching, ↓ terminal end bud density & ↓ proliferative activity of TEB > CLA accumulation in mammary fat pad from butter CLA than synthetic CLA
Ip2000 (49)	Female Sprague Dawley	NMU- induced	AIN 76A	1% c9,t11 or 1% CLA mix (36.5% 10,12, 25.3% 9,11, 17.6% 11,13, 15.3% 8,10)	C9,t11 & CLA mix ↓ # of premalignant IDP lesions.	C9,t11 & mix ↑apoptosis & ↓bcl-2 in IDP lesions, No effect on bak or bax
Hubbard 2000 {Hubbard 2000}	Female BALB/cAnN mice	Mouse mammary tumour cell line 4526	Semi purified exp. Diet with 20% w/w fat (major corn oil)	0.1, 05, 1% w/w CLA mix	0.5% & 1% CLA ↑ latency time to tumour, no sig. inhibition of growth, ↓ metastasis	Indomethacin more effective at inhibition than CLA

Reference	Animal Model	Tumour	Diet & form of CLA	CLA content	Results	Mechanism tested
Kimoto 2001 (50)	Female F344 rats	DMH, DMBA, BBN, DHPN	Oriental MF basal diet	0.1 or 1% CFA from safflower (71.3 % CLA)	CFA-S ↓ mammary ca incidence (0.1%>1%)	1% CFA-S ↑ papillary or nodular hyperplasia in bladder but not tumours
Futakuchi 2002 (51)	Female Sprague Dawley	PhIP-induced	Oriental MF basal diet	0.1% CFA-safflower (CLA) or CFA-perilla (CLnA)	↓ mammary adenocarcinomas	Both ↓PhIP adduct formation
Banni2001 (52)	Female Sprague-Dawley Rats	MNU -induced	AIN-76 basal diet with 5% butter fat in place of corn oil	1, 2, or 3% TVA for lipid incorporation 2% TVA vs. 1% c9,t11 for MNU tx	TVA & c9,t11 ↓ premalignant Intradutal proliferation lesions	≥2% TVA = ↓ LA & LA metabolites in the liver, whereas only 3% TVA ↓ oleic and palmitoleic in the mammary gland
Masso-Welch 2002 (60)	Cd2/F1 mice	EHS-RBM in vivo angiogenesis model	AIN-76A, 5% corn oil	+/- 1 or 2% CLA 50-50 mix of c9,t11 & t10,c12 In vitro – 0,25,50, 75, 100, 125µM c9,t11 & t10,c12 isomer	CLA ↓formation of functional blood vessels	CLA ↓ serum & mammary gland VEGF-A, ↓ Flk-1 (marker for VEGF-A receptor 2) In vitro: stromal cells with EHS-RBM: CLA mix & t10,c12 ≥75µM & c9,t11 ≥100µM ↓ angiogenesis
Ip2002 (53)	Female Sprague-Dawley Rats	MNU – induced	AIN-76 90% pure CLA isomers	0.5% c9,t11 or t10,c12	c9,t11 & t10,c12 ↓ premalignant intraductal prolif. lesions & ↓ tumour incidence	c9,t11 > t10,c12 accumulated in mammary fat pad c9,t11, minimal changes to PUFA [] in MFP T10,c12 – sig. ↓ 20:2, 20:3, 20:4, 22:4, 22:6 & ↑ 16:1, 16:2
Hubbard 2003 (61)	BALB/cAnN mice	Mouse mammary tumour cell line 4526	Diet: Casein, cellulose, 20% w/w corn oil	0.1% or .25% of c9,t11, t10,c12 or mix	No sig. difference in latency to tumour, CLA ↓ tumour burden & size of pulmonary nodules (mets)	No mechanism tested
Masso-Welch 2004 (62)	CD2FICr Mice TNFα (+/+) or (-/-)	Matrigel pellet angiogenesis assay	AIN-76A Purified isomers	0, 5, or 10g/kg c9,t11 or t10c12 CLA	Both isomers ↓ serum VEGF, formation of functional blood vessels Both ↓ size of unilocular adipocytes (>in t10,c12 & reversible in c9,t11).	T10,c12 & mix ablated BAT T10,c12 ↓ serum leptin & ↑ apoptosis of adipose blood vessels and adipocytes C9,t11 ↑ BAT in mammary gland
Cheng 2003 (54)	Sprague-Dawley Rats	DMH & DMBA-induced	Oriental MF diet	0.01, 0.05, 0.01, 1 or 2% CFA rich in CLA from safflower oil	↓ # of adenocarcinomas with 1% CFA-S	None tested

Reference	Animal Model	Tumour	Diet & form of CLA	CLA content	Results	Mechanism tested
Lavillonniere 2003 (55)	Female Sprague-Dawley Rats	MNU	APEA diet with 5% w/w sunflower oil	1% CLA mix or c9,t11	CLA ↓ in tumour mass, initial ↓ in incidence that didn't last	Cla > in mammary fat pads vs. tumour tissues,
Corl2003 (56)	Rats	MNU-induced	AIN 76, ↑↓ TVA butter +/- synthetic C9,t11	0.13, 0.73, 1, 1.6% TVA 0.05-37% c9,t11	↓ tumour # & incidence with ↑ c9,t11, > ↓ with TVA	↑ amounts of c9,t11 in mammary fat pad with ↑ c9,t11 & TVA
Dauchy 2004 (63)	Adult in-bred ovary intact, non-estrogenized nude rats	MCF-7 xenografts	Infused donor blood from Male Sprague Dawley Rats	CLA isomers (c9,t11, t9,t11, t10,c12) 0-360uM in serum	T10, c12 & t9, t11 ↓ tumour H3-Thymidine uptake. No effect with c9,t11	T10,c12 > t9,t11 Inhibit LA uptake, cAMP, ERK ½, 13-HODE formation
Lock2004 (57)	Sprague Dawley Rats	MNU-induced	AIN 76 ↑↓ TVA Butter +/- synthetic c9,t11	After MNU: 0.13% or 1.6% TVA butter +/- Sterculic oil	↑VA without SO ↓ # & activity of premalignant lesions compared to other txs	SO inhibits Δ9-desaturase, anticarcinogenic activity of TVA ↓ when conversion to c9,t11 blocked
Hubbard 2006 (64)	Female BALB/cANN mice	Mouse mammary tumour cell line 4526	20%wt/wt fat diet either Veg fat blend &/or Beef tallow or corn oil	0.05%, 0.1% CLA mix (33% c9,t11, 33% t10,c12)	CLA no effect on tumour growth rate	CLA in VFB:BT group ↓ lung mets compared to other groups, especially VFB:CO
Ip2007 (66)	FVB/N-Tg (MMTVneu)2 02Mul/J & FVB/J female mice	Transgenic mice over express Neu onocogene in mamm epithel.	AIN-76A >90% pure CLA isomers	0.5% c9,t11 or t10,c12 CLA Started diet at 24 days of age or 68-72 days of age or for 10 days at age 70 days	C9,t11 no effect T10,c12 ↓ latency, ↑ lung metastasis, no effect on # or size of primary tumours	T10,c12 slight ↑ survival time compared to control T10,12 ↓ in wt. gain, modified mammary gland development, ↓ adipocytes, ↑ fibrocellular stroma, ↑ size of lymph nodes, no change to erb2 T10,c12 ↑ wt of spleen, heart, & liver (fatty liver)

DMBA – requires metabolic activation
MNU – direct alkylating agent

1.4.3 Cell culture studies of CLA and breast cancer cell lines

Breast cancer cell lines isolated from patients with breast cancer are utilized to investigate mechanisms of action for anti-carcinogenic substances at the cellular and molecular level to aid in the development of hypothesis for animal and clinical studies (69). There are a limited number of cell lines available due to the difficulty in culturing them from primary breast cancers (69). Two of the most commonly utilized breast cancer cell lines, MCF-7 and MDA-MB-231 were isolated from pleural effusions of clients with metastatic breast cancer. Based on the various characteristics of the established cell lines they appear to correlate with those of breast cancer cells in vivo and therefore are considered to be a relevant model (69).

The MCF-7 cell line the most extensively utilized cell line, was established in 1970 and expresses estrogen and progesterone receptors. It is commonly used as a model for hormone-responsive breast cancer. MCF-7 cells are less aggressive then other breast cancer cells and are poorly metastatic in animals (69). They highly express the insulin-like growth factor receptor, but not that of other growth factors erb/B2 or EGFR (70;71). Twenty five of the thirty published cell culture studies reviewed in Table 1-6 used MCF-7 as their model.

Similar to the animal research, a critical review of the cell culture literature in CLA and breast cancer was undertaken. Consistent with the in vivo results, CLA provided as individual isomers, a 50:50 mixture of c9,t11 and t10,c12, or as CLA enhanced milk fat decreased the growth, viability and/or increased death in a variety of breast cancer cells as reviewed in Table 1-6. Effectiveness ranged from 10-200μM, which falls within the observed levels in human serum, 10-350μM, including those taking long-term CLA supplementation (72). Some of the earlier studies provided CLA as a free fatty acid in ethanol, whereas others conjugated CLA to the carrier protein albumin. Free fatty acids are toxic to cells and desBordes (73) showed that by increasing the albumin to fatty acid ratio there was a decrease in the inhibitory effect of CLA. This difference in supply of

fatty acids may explain why some studies were able to see inhibition at lower concentrations of CLA than others.

As previously mentioned multi-targeted approaches are of interest for the prevention and treatment of cancer therefore it is important to elucidate mechanisms by which CLA is interfering with breast cancer to develop a 'multi-targeted' approach possibly in combination with other dietary factors or pharmaceutical agents.

Different mechanisms by which CLA exerts it's anti-carcinogenic potential that have been studied include: interference with the cell cycle, induction of apoptosis, modification to PPAR activity, oxidation, essential fatty acid metabolism, angiogenesis, modification of the surrounding stroma and interference with growth factor signaling.

Table 1-6: CLA and breast cancer in cell lines

Reference	Breast Cancer Cell Line used	Fatty Acids tested	Control	Culture Conditions	Result on growth/death	Mechanism tested
Shultz1992 (74)	MCF-7, breast ca	CLA mix	Media no added f.a.	Conc. range = 0, 17.8uM, 35.7 µM, 71.4 µM	CLA ↓ growth of 3 cell lines MCF-7 most sensitive	MCF-7 ↓ thymidine, uridine, & leucine incorp. B-carotene & CLA not as effective as CLA alone
Shultz1992 A (75)	MCF-7	CLA mix LA	Media no added f.a.	Conc. range = 0, 17.8uM, 35.7uM, 71.4uM 0, 5, 10, 20 µg/ml	LA ↑ growth @ day 4, but ↓ growth @ day 8-12. CLA ↓ growth starting at 4 days on incubation,	cytostatic @ 17.8µM, cytotoxic @ 35.7 & 71.4 µM CLA > cytotoxicity than LA
desBordes 1995 (73)	T47D & MCF-7 breast ca	CLA mix OA, LA & Elaidic acid, linoelaidic	No added f.a.	(100, 200, 500uM)	LA inhibit H thymidine incorp more than CLA mix LA>linoelaidic>oleic>elaidic	When albumin [] ↑ed (1 → 38mg/ml) inhibitory effects of fatty acid ↓ed
Cunningham 1997 (76)	HMEC norm. human mammary epithel.cells MCF-7	LA CLA mix	Media – no added f.a.	Serum-free media 1.8, 3.6, 17.8, 35.7 µM (0.5-10µg/ml)	HMEC- LA ↑ growth & H-thymidine CLA ↓ growth & H-thymidine (greater than MCF-7) LA ↓ growth MCF7 @ high [], CLA ↓ growth @ all	LA ↑ LPOxide, CLA no effect Eicosanoid inhibitors ↑ growth of CLA tx. HMEC Cells, CLA & indomethacin in MCF-7= ↑ growth, CLA & NDGA in MCF-7 = ↓ growth
Durgam 1997 (77)	MCF-7 MDA-MB-231	LA CLA mix	Media – no added f.a.	17, 35, 71 µM	CLA/LA co-culture ↓ growth and H-thym in MCF-7, no ↓ in MDA-MB231 compared to cells with no fatty acid tx.	removal of CLA tx = regrowth of MCF-7 CLA tx = ↑ cells in G0/G1, ↓ c-myc expression compared to LA or no-tx cells 35µM optimal dose with no cytotoxicity
O'Shea1999 (78)	MCF-7 SW480-colon ca	CLA mix from Nu-check	Ethanol – no added f.a.	0, 5, 10, 20 ppm counted days 4, 8, 12	dose and time dep. ↓ in both cell lines --> cytotoxic influence on MCF-7	↑ lipid peroxidation start @ 15ppm, > in MCF7, SOD activity ↑, > in MCF7 Catalase activity ↑ days 8-12, > in MCF7 Glutathione Peroxidase activity ↑ 8-12 days, > MCF7
Ip1999 (79)	Female Sprague-Dawley rats Female CD2F1 mice	AIN-76 with 5,10,20% corn oil	Serum-free media no added f.a.	with or w/out 1% FFA CLA from weaning	CLA [] ↓ growth of mammary epithelial cell – most noticeable @ 14 day of culture	LA ↓ casein accumulation (functional differentiation) CLA ↓ DNA synthesis in pre-differentiated cells and ↑ apoptosis in differentiated MEC PKC activity in MEC not altered by CLA PKC activity changed in adipocyte stroma

Reference	Breast Cancer Cell Line used	Fatty Acids tested	Control	Culture Conditions	Result on growth/death	Mechanism tested
Park2000 (80)	MCF-7	LA C9,t11 CLA	Ethanol no added f.a.	Serum-free, mitogen EGF F.A. complexed to BSA 1.8, 3.6, 17.8 µM	CLA↓ growth (3.6 & 17.8µM) LA slight ↑ growth (max at 3.6µM)	LA ↑18:2, 14:0, 16:1, 20:2 membrane uptake, CLA ↑ membrane CLA LA ↑ PLC activity, CLA no PKC not effected PGE_2 [] not influenced
O'Shea2000 (81)	MCF-7	Milk fat from cow: Pasture: full fat rapeseed or f.f. soybean CLA mix, LA c9,t11 t10,c12,	Ethanol no added f.a.	F.A. in ethanol 20ppm	a) Milk fat was as effective as synthetic CLA in inhibiting cell growth b) c9,t11 > effectiveness than t10,c12, LA ↑ growth	CLA ↑ SOD, catalase and GPx activity ↑ CLA uptake into phospholipids from M.F. vs. isomers CLA ↑ malondialdehyde (marker of lipid peroxidation CLA and LA ↑ superoxide dismutase, catalase, and glutathione peroxidase activity
Miller2001 (82)	MCF-7 SW480 (colon)	CLA mix (29% t10,c12, 30% c9,t11) C9,t11; t10,c12 CLA LA	Ethanol no added f.a.	5% FBS 5-16µg/ml (17.8-57 µM)	CLA txs ↓ viability after 4 days. ↓@ 4 days with CLA mix and c9,t11 for both cell lines. Less ↓ with lower [17.8µM] t10,c12, but inhibition similar with ↑ [57µM] of t10,c12 LA no effect MCF-7, ↑ growth SW480 @ 16ug/ml	AA uptake in PL (SW480) ↓ed by mix & c9,t11, & they also ↑ed TG uptake, No effect seen in either cell line with t10,c12 MCF-7 –only c9,t11 altered AA distribution (↓ed uptake into PC & ↑ed uptake into PE) SW480 – c9,t11 ↓ AA into PC ↑ AA into PE CLA mix & t10,c12 ↑ AA in PS ↑ AA release with LA SW 480, no effect from CLA Mix & c9,t11 modified PG synthesis - cyclooxygenase route not lipooxygenase, poss. threshold effect on PGE2 synthesis Nonenzymatic oxid. of AA to PGF2α by CLA
Choi2002 (83)	MDA-MB-231 MCF-7	CLA mix (c9,t11 48%, t10,c12 46%) C9,t11 CLA T10,c12 LA	Albumin no added f.a.	Serum free media (45&100uM) LA – 100uM F.A. complexed to BSA	No measures of growth were completed	CLA no ↓ in mRNA for SCD, but did ↓ stearoyl-CoA desaturase (SCD) protein levels in MDA-MB-231, not in MCF-7, ↓ SCD activity in both cell lines. LA ↓ SCD mRNA & SCD ptn in both cell lines CLA tx cells ↓ palmitoleic (16:1) and oleic (18:1), ↓ desaturation indices in both cell lines

Reference	Breast Cancer Cell Line used	Fatty Acids tested	Control	Culture Conditions	Result on growth/death	Mechanism tested
Majumder 2002 (84)	Benign MCF-10A MCF-7 MDA-MB-231	CLA – 50:50 mix C9,t11, t10,c12 LA Stored in ethanol	Ethanol no added f.a.	10% FCS 0-200µM bound to BSA	CLA Mix ↓ MCF7 & MDA-MBA-231 > effect indiv isoforms or LA. Did not specify data on indiv. Isomers. MCF10A inhibit at lower doses	CLA ↑ expression of wild-type p53 mRNA in MCF-7 & MCF-10A ↑ expression of p21WAF1/CIP1 in MCF7 & MDA-MB-231 ↑ expression of bcl-2 in MDA, ↓ trend in MCF7 ↑ expression of bax in MDA – balanced ↑ in bcl-2 = apoptosis –this paper misidentified bcl-x as proapoptotic
Ma2002 (41)	MDA-MB-231	C9,t11, t10,c12, LA & mix	Media no added f.a.	5%FBS 10, 30, 60µM All had LA (60µM)	C9,t11, t10,c12 & mix co-incubated with LA inhibited H-thymidine uptake – no dose response observed	Preferential incorporation of LA in phospholipids (means no competitive inhibition of incorp with CLA) ↓ AA in phospholipids of cells tx with mix & t10 (also c9 but not as great a ↓) T10 interfered with conversion of LA → AA LA↑PGE2, CLA tx ↓PGE2 (except when cells tx with AA not LA)
Oku2003 (85)	Rat liver homogenate SKBR3	C9,t11 FFA form Myristic acid	Media no added f.a.	Not defined 0.25mM	Not reported	C9,t11 ↓ incorporation of C14-acetate into phospholipids of SKBR-3 cells C9,t11, t10c12 inhibited fatty acid synthase activity (c9 more potent), not FAS expression (mRNA)
Kemp2003 (86)	MCF-7 HCT-116 (colon) [wild-type & p53 deficient]	CLA mix C9,t11 T10,c12	Media alone & with ethanol no added f.a.	10% FCS 10, 20,40,80, 160µM	CLA mix ↓ cell growth (started at 40µM with trypan blue and 10µM with MTT assay)	160µM CLA mix = Cell cycle ↑ G0/G1 arrest Accumulation of p53, p21, p27 and hypophosphor. Rb, ↓ cyclin D1 & E T10,c12 more effective than c9,t11
Chujo2003 (87)	MCF-7	C9,t11 & t10,c12 CLA mixture (c9,t11 49%, t10,c12 41%)	Media no added f.a.	5% FCS or Charcoal tx FCS 10µM CLA	C9,t11 more potent inhibitor of proliferation of MCF-7 than t10,c12 & mix in FCS	T10,c12 strongest inhibitor of estrogen stim. growth T10,c12 strongest inhibitor of insulin stim. growth & cell viability Neither isomer effected EGF stim growth

Reference	Breast Cancer Cell Line used	Fatty Acids tested	Control	Culture Conditions	Result on growth/death	Mechanism tested
Miller2003 (88)	MCF-7 SW480	Milk fat CLA C9,t11, LA, Oleic, TVA isomers	Ethanol no added f.a. or milk fat	5% FCS 1mg/ml M.F. = 60.2, 65.2, 80.6 µM CLA or control M.F. for 71.3µM Isomers same []	Milk fat with ↑CLA ↓ cell #, > reduction than c9,t11 isomer. LA ↑ cell # at 44uM, ↓cell # at 156uM, Oleic ↓ cell # at 952uM, TVA ↓ cell #	Oxidation & eicosanoids ↓ AA conversion to PGE2, ↑ conversion to PGF2α Bcl-2
Tanmahasa mut2004 (89)	MCF-7, MDA-MB-231 RPMI 1640, 10% FCS	CLA mix (sigma) C9,t11, t9,t11, c9,c12, t10,c12, c11,t13	Solvent that f.a. stored in no added f.a.	10%FCS CLA concentration 25-200µmol/L	Order of growth inhibition potency on MCF-7: c9,c11 > t10,c12 > t9,t11 > c11,t13 & c9,t11 No inhibition of MDA-MB-231	↓ERα mRNA, ↓ ERα ptn ↓ nuclear binding of ERE & activation of ERE Inhibition in presence of exogenous ER – CLA still inhibited ERE therefore CLA ↓ in ER is not the mechanism by which it ↓ERE. ↑CLA isomer-dependent activation of PPRE-PPAR response element (c9,c11, > c9,t11) – believed that PPAR/RXR may compete with activated ER for binding to the ERE.
Maggiora 2004 (90)	Breast ones: MCF-7, MDA-MB-231	CLA LA	Serum free media no added f.a.	5% FCS 100mM CLA or LA in FCS	Results for MCF-7 and MDAs CLA ↓ growth time and dose dependant (though 50uM > effective than 200uM) Less ↓ effect of CLA on MDAs	MCF-7 - PPARγ ↑ by CLA, & ↓PPARβ/δ MDA - ↑PPARβ/δ, no affect on PPARα, ↑PPARγ2 isomer
Kim2005 (91)	Mouse mammary tumour cell line 4526	LA, c9,t11, t10,c12	Ethanol no added f.a.	5% FCS, serum free, insulin, transferrin, BSA 10, 50 or 100µM	t10,c12 ↓ viability at 10, 50 & 100µM @48hr, c9,t11 only 10 & 100µM ↓ viability. LA no effect	10uM t10c12 ↓ 5-HETE production Adding 5-HETE into media = ↓ t10,c12 effects T10,c12 & c9,t11 ↑apoptosis, ↓ cell prolif.
Albright 2005 (92)	MCF-7 MCF10A (non-ca)	CLA mix not defined	DMSO or hydrogen peroxide no added f.a.	10% FCS, insulin 0-50µM	CLA ↓ growth of MCF-7 cells but not MCF-10A cells (measured by BrdU incorporation)	CLA ↑4HNE (oxidation), CLA & HPO ↑ nuclear translocation of phosphorylated p53 ↓ phosphorylation of transcription factor FKHRser256 ↓ phos histone H3 (= cell cycle arrest), ↓mitosis
Liu 2005 (93)	MCF-7	CLA mix (50% c9..t11, 40% t10,c12, 10% c10,c12)	Solvent no added f.a.	10%FCS 25-200umol/L	Not reported	↓ PR mRNA, ↓ ERα phosphorylation, ↑ ptn phosphatase 2A (which dephosphorylates serine 118 on ERα)

Reference	Breast Cancer Cell Line used	Fatty Acids tested	Control	Culture Conditions	Result on growth/death	Mechanism tested
Wang 2005 (72)	MCF-7 co-cultured with Human breast stromal cells	C9,t11 T10,c12	No added f.a.	5% FCS dextran-charcoal tx media 40μM	Both isomers ↓ cell proliferation of MCF-7, t10,c12 was more effective when MCF-7 co-incubated with stromal cells	Both ↓ VEGF-A mRNA & protein expression. T10,c12 > than c9,t11
Wang 2006 (94)	1° Breast epithel cells & stromal cells, MCF-7, MDA-MB231	C9,t11 T10,c12	No added f.a.	5% FCS phenol red free ↑ calcium charcoal treated DMEM/F12 40μM CLA	Not reported	↑ expression of estrogen-regulated tumour suppressor gene, Protein tyrosine phosphatase gamma (not in breast ca stromal cells), t10,c12 > c9,t11, especially ER+ve epithelial cells
Degner 2006 (95)	MCF-7, MDA-MB-231	CLA mix, C9,t11 & t10,c12	Media no added f.a.	DMEM 0.5% FBS 20,40,80 μmol/L	Not reported	CLA mix & isomers ↓ cox-2 transcription, by interfering with the recruitment of AP-1
De la Torre 2006 (96)	MCF-7	Beef F.A., CLA enrich from beef, synth. mix = to beef CLA	Ethanol no added f.a.	100μmol/L, 175μmol/L for CLA enriched (to = 100μM CLA)	Greatest ↓ in cell growth seen in mixtures with cis,trans> c,c or t,t Total f.a. from beef > ↓ than CLA mix	Not studied
Miglietta 2006 (97)	MDA-MB-231	CLA from Sigma not defined	serum-free media with no added f.a.	10% FCS 60μM CLA Tx: serum free DMEM, insulin, transferrin, selenite, CLA, glutamine, antibiotic, albumin	Cell growth and viability – decreased after prolonged exposure to CLA (48-72 hours), % of apoptotic cells ↑ @48hrs & @ 72hr	Cell cycle 72 hr - ↑ accumulation in S phase condensed chromatin observed @ 72 hr. 2 fold ↑ Bak & 4 fold ↓ Bcl-xl @72 hr. – 4.21x cytochrome c with CLA @ 72 procapsase 9 and 3 were cleaved after 72 hr with CLA. Procapsase 8 not. MAPK: Raf kinase activity ↓ @ 72 hr ↓ Phosphorylated ERK
Miglietta 2006A (98)	MCF-7	CLA mix	No added f.a.	10% FCS Tx: same as Miglietta 2006	5, 10, 20,40,60, 100uM, inhibition at 72hr, 60uM most potent [ ], LDH release ↑ at 72hr	↓Raf-1 ptn rates, ↓pErk ½, ↓ c-myc
Bocca 2007 (99)	MCF-7	CLA mix	No added f.a.	Same as Miglietta 2006 - 60μM	Referred to Maggiora2004 & Miglietta2006	↑PPAR γ levels and translocation to nucleus ↑ & redistributed β-catenin & E-cadherin
Guo 2007 (100)	MCF-7	CLA mix Conj. CLA to polymeric carrier ↑ solub	No added f.a.	50, 100, 200 μM	↓ cell viability with plu-conjugated CLA than unconjugated CLA	Plu-CLA ↑p53, ↓Bcl-2, ↑Bax

1.5   Potential Mechanisms of Cytotoxicity of CLA Isomers to Breast Cancer Cells

1.5.1   Cell cycle

Stimulation by growth factors, hormones or cytokines induces a cell to proliferate by passing through the cell cycle. The cell cycle consists of four stages; G1 the initial growth stage followed by S phase whereby DNA synthesis and chromosomal duplication occurs, G2 phase then ensures the DNA has replicated and the cell is ready for M (mitosis) where the chromosomes separate and the cell divides in two (101). Sometimes after mitosis the cell will exit the cell cycle to a resting state termed G0. Cells that are terminally differentiated stay quiescent in the G0 phase. The different phases of the cell cycle are closely regulated by cyclin-dependant kinases (cdk) and their binding partners, cyclins. The levels of the specific cyclins fluctuate as they are needed during the cell cycle.
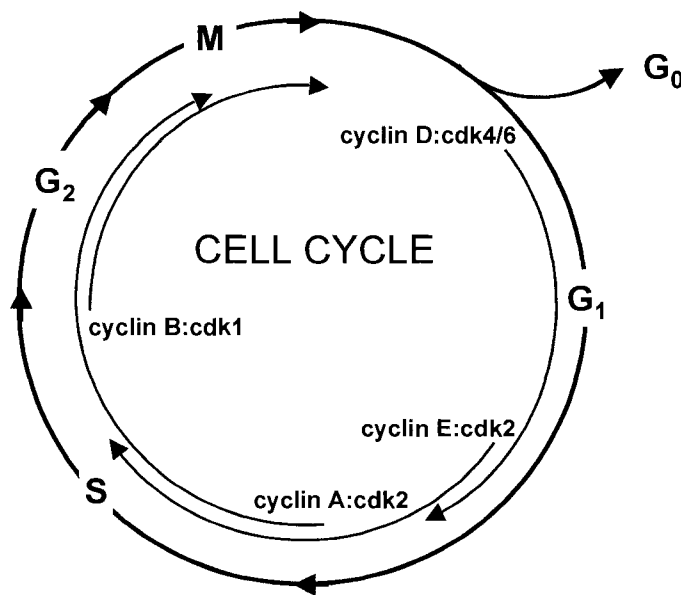
Figure 1-2: Cell cycle, used with permission from P. Schley (102).

Cyclin D (D1, D2, D3) is induced by growth factor signaling and stimulates the cell cycle progression through the G1 phase. It associates with and activates cdk4 and cdk6, which activate the E2F family of transcription factors involved in DNA synthesis and cell cycle progression. Cyclin E associates with cdk2 and is required for the progression from G1 to S. Accumulation of Cyclin A occurs at the end of G1 and during the S phase. It is required for the transition into S, the completion of the S phase and the transition through to Mitosis. Finally, Cyclins B1 and B2 promote the entry into and the progression through Mitosis, however they need to be degraded before the completion of the cell cycle (103).

Negative regulators of the cell cycle include retinoblastoma protein (Rb), p53 transcription factor, and the family of cyclin-dependant kinase inhibitors (p21$^{Cip1/WAF1}$, p27, p57, and the INK4 proteins). Hypophosphorylated Rb binds to and inactivates the E2F family of transcription factors, which are required for cell cycle progression. The cdk/cyclinD complex reverses this inhibition by phosphorylating Rb, which then disassociates from the transcription factors allowing them to promote progression through the cell cycle (103). p53 is a transcription factor that accumulates in response to a stress stimuli. Depending on the stimuli or extent of damage to the cell p53 may regulate the production of factors for cell cycle arrest or apoptosis (104). Cyclin-dependant kinase inhibitors (CDKI) interfere with the activity of the cyclin/cdk complexes and cause cell cycle arrest (103). The gene for p21$^{Cip1/WAF1}$ a CDKI which inhibits the cells transition from the G1 phase to the S phase is induced by p53. In cancer cells numerous of the regulators are either up regulated or down regulated to support a continued cycling through the cell cycle, leading to uncontrolled cell proliferation (105).

Different measures for cell viability or cell proliferation.
Cell viability measures do not distinguish between cells that are actively dividing or in the quiescent state (G0). Two ways to measure cell viability include cell counting using a hemocytometer and measuring metabolic activity using a tetrazolium salt that is cleaved by active metabolic enzymes to release a colored product (MTT and Wst-1 assay). The most common measure of cell proliferation, where cells are actively passing through the

cell cycle and dividing, is of DNA synthesis (^{3}H-thymidine or Bromodeoxyuridine uptake) (106). As summarized in Table 1-6, CLA isomers have been shown to decrease both cell viability and cell proliferation in breast cancer cell lines.

CLA treatment of breast cancer cell lines has been reported to interfer with progression of the cell cycle. Studies in human breast cancer cell lines have shown an accumulation of cells in the G0/G1 phase along with a corresponding decrease in cell cycle promoters (transcription factor c-myc expression, cyclin D1) and an increase in those that cause cell cycle arrest (p53, p21$^{Cip1/WAF1}$, p27). (77)(86)(84;92) The isomer t10,c12 was more effective at inhibiting proliferation than c9,t11 (86).

1.5.2 Cell Death: Apoptosis and Necrosis

Cell death has traditionally been designated as two processes, necrosis or apoptosis. Necrosis is a response to cellular stress or insult and does not utilize ATP. Signs of necrosis include mitochondrial and cytoplasm swelling, leakage of small molecules, random digestion of DNA and at the final stage the plasma membrane bursts releasing cytosolic enzymes like lactate dehydrogenase into the surrounding environment. The debris left by the dead cell promotes an inflammatory response (107).

In contrast to necrosis, apoptosis, also known as programmed cell death, requires ATP and is characterized by cell shrinkage, chromatin condensation, nuclear fragmentation, and plasma membrane blebbing. The cell is broken apart to separate bodies with intact membranes and signals the surrounding cells and macrophages to engulf and/or absorb them. As there is no debris in the surrounding environment, the stimulation of inflammation is less (107).

There are two different pathways to apoptosis, the extrinsic and intrinsic. Both pathways lead to the activation of cysteine proteases termed caspases. The caspases belong to two groups, the initiators, caspase-8, caspase-10 and caspase-9 or the effectors, caspase-3, caspase-6, caspase-7. Once activated by apoptotic stimuli the initiators activate the effectors by cleaving their pro-caspase form to their active caspase form. The effector

caspases proceed to cleave and activate specific proteins involved in the execution of apoptosis (108). The extrinsic pathway is mediated by death receptors located in the plasma membrane. Ligands that bind to these receptors stimulate the activation of initiator caspases. In contrast the intrinsic pathway is mediated by the mitochondria and is regulated by members of the bcl-2/bax family. Those with close homology to bcl-2 are considered anti-apoptotic and help stabilize the mitochondrial membrane. Alternatively the members bax, bid, and bad are proapoptotic and upon activation create pores in the mitochondrial membrane leading to the release of effector pro-caspases, cytochrome c and other proteins involved in apoptosis (109). There is recent evidence that necrosis and apoptosis are not mutually exclusive and the route in which the cell dies may be dependant on the presence or absence of ATP (107).

If a cell has sustained significant damage or nuclear errors, wild type p53 will initiate apoptosis rather than cell cycle arrest. p53 is often mutated in cancer rendering it ineffective in responding to cellular damage (110).

CLA treatment has been shown to increase apoptosis in mammary human and rodent cancer cells (79;84;111). Some of the changes observed that indicated increased susceptibility to apoptosis include; increased wild-type p53, cells with condensed chromatin, increased proapototic bax and bak, reduced antiapoptotic bcl, increased cytochrome c in the cytosol and increased cleavage of initiator and effector caspases (79;84;111).

1.5.3   Perxoisome proliferator-activated receptors

Peroxisome proliferator-activated receptors (PPAR) are nuclear hormone receptors that have been implicated in multiple cell processes including regulation of the cell cycle and apoptosis. They consist of three isoforms (PPAR α, PPAR β/δ and PPAR γ) that are distributed in various tissues of the body and appear to have different, yet also overlapping roles (112). They regulate gene expression by forming a heterodimer with retinoid x receptors, then binding to specific response elements (PPREs) in the promoter

regions of target genes. Although PPAR γ appears to be highly involved in adipocyte regulation it also appears as though it's agonists target multiple hallmarks of cancer including, cell cycle arrest, induced differentiation, apoptosis, and angiogenesis (112). See table 1-7 for a list of factors influenced by PPAR γ agonists. PPAR γ is expressed in human breast adenocarcinomas,(113) and it's induced up regulation decreases the proliferation of MCF-7 cells (114) The role of PPAR β/δ ligands in tumourigenesis continues to be debated in the research literature, (115) there is evidence to support that they stimulate the growth of breast cancer cell lines (116). There is limited data on the role of PPAR α agonists in cancer, however a PPAR α ligand has been shown to induce apoptosis in human breast cancer cell lines (117).

Polyunsaturated fatty acids and their metabolites are ligands for PPARs. CLA treatment of MCF-7 breast cancer cells was shown to increase the anti-proliferative PPAR γ and decrease the anti-apoptotic PPAR β/δ (90;99). It also increased the localization of PPAR γ from the cytosol to the nucleus, and increased PPAR response element activation (89;99). Use of a PPAR γ antagonist abolished the growth inhibitory effects of CLA (99). CLA exerted different effects on the estrogen receptor negative MDA-MB-231 cell line. It increased the isoform of PPARγ2, yet it also increased PPAR β/δ, which may explain why CLA did not induce apoptosis in the MDA-MB-231 cells. Of note neither of these two studies compared the effect of CLA on PPARs to that of other known PPAR agonists, such as other PUFAs Linoleic, Oleic and Linolenic.

Table 1-7: **Some of the factors affected by PPAR γ agonists** (112).

Function promoted	Molecular markers
Anti-proliferation	↑ CDK inhibitors, ↓ CDK activity, ↓PI3K/Akt activity
Apoptosis	↓ bcl-2, ↑BAX & BAD, ↑caspase activity, ↓c-myc
Inhibit angiogenesis	↓ VEGF, ↓ Leptin, ↓ Flik-1

1.5.4 The tumour microenvironment

The tumour microenvironment plays an active role in tumourigenesis. The tissue that surrounds and intertwines between cancer cells is termed the tumour stroma and it consists of the extracellular matrix (ECM), immune and inflammatory cells, fibroblasts, adipocytes and endothelial cells. The cross-talk between the cancer cells and stromal cells has shown to be important for the creation of a microenvironment supportive of malignant tumour growth as well as for the promotion and progression of the tumour (118). Cancer cells release growth factors such as basic fibroblast growth factor (bFGF) and the family of vascular endothelial growth factors (VEGF) to stimulate changes in the stroma to support the tumours' development. They also release proteases which remodel the surrounding ECM to allow the spreading of the cancer cells. Once activated, stromal fibroblasts produce further growth factors to stimulate the continued growth of the cancer cells (118). The formation of blood vessels (angiogenesis) is critical to support tumour growth and progression. Cross talk between cancer cells that release VEGF and stromal endothelial cells that express the VEGF-2 receptor (Flk1) results in local angiogenesis. Blocking VEGF2/Flk1 has been shown to reverse invasive carcinoma to a pre-malignant non-invasive tumour phenotype (118).

Including CLA up to 1% wt/wt in the diet has been shown to alter mammary gland development during puberty in rodents (47). CLA decreased epithelial branching and the density of terminal end buds, which are highly susceptible to carcinogenesis (47;79). The epithelial branching and terminal end bud formation of mammary gland development are dependant on the presence of stromal fibroblasts, (119) which suggests CLA may interfere with signaling between the stroma and epithelial cells. There is evidence to support that the decreased epithelial branching could be due to CLA's direct inhibition of epithelial growth, however changes in adipocyte stroma due to incubation with CLA have been observed and could also contribute (48;79).

Both c9,t11 & t10,c12 have been shown to decrease the formation of microcapillary networks (angiogenesis) in mice and with a stromal cell assay, although t10,c12 was more potent (60;62). Changes to pathways related to angiogenesis included ↓VEGF-A

26

serum levels, ↓ local production of VEGF in mammary gland, ↓ Flk-1 protein, and ↓ serum leptin (promotes angiogenesis through endothelial cells) (60;62) (72). Studying the effect of CLA on the stroma in the mouse model is a concern due to t10,c12's complete ablation of brown adipose tissue in the mammary gland, which thereby would change the composition of the surrounding stroma.

1.5.5   Oxidation

Tumour cells demonstrate an increased susceptibility to oxidative stress and current chemotherapy and radiation treatments derive their cytotoxic effects from increasing oxidative stress (92). Due to their double bond structure, PUFAs and their metabolic products have an increased susceptibility to oxidation. Lipid peroxidation products have been shown to cause cell cycle arrest and induce tumour cell death (120).

There is conflicting research to show that CLA may augment or reduce oxidative stress in breast cancer cells. Albright et al. reported a preferential increase in oxidative stress and a resulting inhibition of the cell cycle with 50μM of CLA mix in MCF-7 cancer cells as compared to normal mammary epithelial ductal cells (MCF-10A) (92). CLA treatment has also been shown to increase the activity of the cells' protective enzymes against oxidative stress, superoxide dismutase, catalase and glutathione peroxidase, (81) as well as increase the lipid peroxidation product 8-epi-PGF2α (88). These findings support the argument that CLA may induce an upregulation of oxidative stress. In contrast a one month CLA feeding trial in Sprague-Dawley rats showed that CLA mix reduced the presence of lipid peroxidation products in the mammary gland (42). Research into other models of cancer also showed conflicting results regarding CLAs potential role in oxidative stress (25;121). Some believe that CLAs interference with the metabolism of other fatty acids, such as LA, which may be more susceptible to peroxidation, is the pathway by which it has an effect on oxidation (47). This potential interference will be discussed later in this chapter.

1.5.6 Changes to cell membrane structure and function

PUFAs with cis-double bonds are believed to impact the physical properties of the cellular membrane, due to their more 'curved/kinked' structure, which allows for less tight packing of surrounding fatty acyl groups (122). Whereas the incorporation of the straighter trans fatty acids into membranes has been shown to decrease membrane fluidity and interfere with the function of a membrane receptor (123-125). Changes to the dietary fatty acid composition of the plasma membrane and subsequently the physical structure has been shown to alter lipid-protein interactions, affect ion transporters, receptors, signal transducers and enzymes (122;126-128). CLA is a group of polyunsaturated fatty acids, whereby the two most commonly investigated isomers c9,t11 and t10,c12 contain both a cis and a trans bond. When provided in the diet or cell culture media CLA isomers are readily incorporated into the phospholipids of cell membranes (41). The trans-bond in the major isomers has the potential to alter the physical structure of the membrane and this could then alter the function of important proteins and signals that are located or generated from the plasma membrane. Few studies have examined the effect of CLA-induced membrane changes on membrane bound receptors (proteins).

The impact of CLA incorporation into membranes on essential fatty acid metabolism and the subsequent production of eicosanoids has been studied. Due to the chemical similarities between LA and CLA, it was initially hypothesized that CLA may compete with the incorporation or metabolism of LA in membrane phospholipids and thereby interfere with the synthesis of the essential fatty acid arachidonic acid (C20:4n-6, AA) (129). AA is a substrate for eicosanoids, prostaglandins via cycloxygenase enzymes and leukotrienes via lipoxygenase enzymes (see figure 1-3). Eicosanoids are hormone-like compounds that exert many cellular functions and have been demonstrated to be involved in cell growth and apoptosis in human breast cancer (130).

n-6 PUFA (LA)

LA (18:2n6)

⟵ $\Delta6$ – Desaturase

(18:3n6)

⟵ Elongase

(20:3n6)

⟵ $\Delta5$ – Desaturase

AA (20:4n6)

COX ⟶   ⟵ LOX

PGE2,, TXA2   LTB4, HETEs
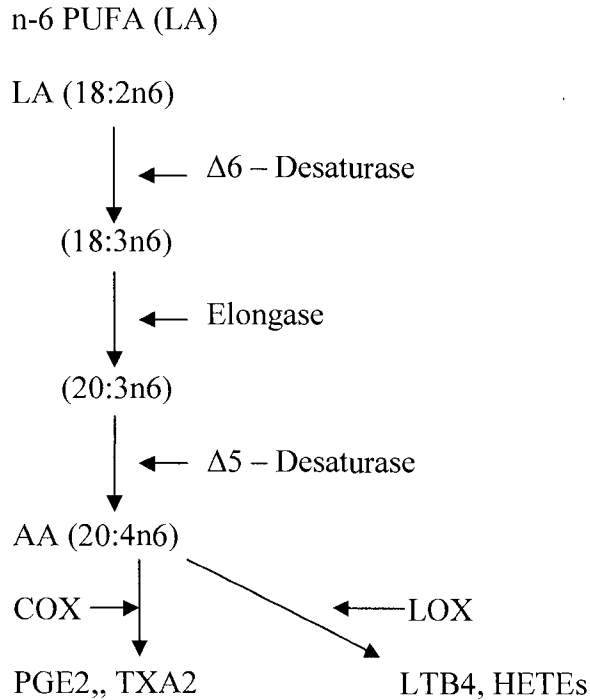
Figure 1-3: **The metabolism of the essential fatty acid Linoleic Acid.** Abbreviations are: LA, linoleic acid, AA, Arachidonic Acid, COX, cyclooxygenase, LOX, Lipoxygenase. (131)

Neither major isomer of CLA or CLA mix appears to decrease the incorporation of LA in membrane phospholipids (44) (132) (41;47), however, CLA has been reported to interfere with essential fatty acid metabolism. Both CLA mix (c9,t11 & t10,c12) and/or t10,c12 alone reduce the elongation and desaturation products of LA, most importantly that of AA (41;47). Consistent with this, CLA has been reported to interfere with the production of PGE_2 as well as 5-HETE, an important substrate of the lipoxygenase pathway (41) (91;133). The distribution of fatty acids among the different phospholipid classes of the plasma membrane influences eicosanoid production. Phospholipase A2 preferentially detaches fatty acids from phosphatidylcholine (PC) making PC the preferential source of AA for eicosanoid synthesis. Miller et al. showed that CLA decreases the amount of AA stored in PC and increases it to PE (phosphatidylethanolamine) along with a subsequent decrease in the production of PGE2 (82). Although both Miller and Ma reported that CLA interfered with essential fatty acid metabolism, they attributed their results as more effective by opposite isomers, c9,t11 (82) or t10,c12 (41).

1.5.7 Growth Factor receptors/pathways

Growth factors, estrogen, insulin-like growth factor (IGF) and epidermal growth factor (EGF) are proteins that stimulate cell proliferation and inhibit apoptosis. Their receptors and/or signaling pathways are often up-regulated in cancer (134). Currently there are chemotherapy treatments designed or in the process of being designed to interfere with their promotion of cell growth. For example, aromatase inhibitors used in breast cancer therapy interferes with the endogenous synthesis of estrogen thereby reducing the amount of ligand available to promote tumour cell growth (135). In addition, Trastuzumab (herceptin), blocks the overexpressed Her2/neu receptor, which is part of the EGF family (134).

There is significant cross-talk between the cellular signaling pathways of estrogen, IGF and EGF (136) and resistance to both Tamoxifen and Trastuzumab have been linked to a corresponding up-regulation of growth factor receptors like that for IGF (136) (137).

In vitro CLA has been reported to interfere with both estrogen and insulin stimulated proliferation of MCF-7 cells (77;87;89), with t10,c12 showing greater inhibition that c9,t11 or CLA mix (87). Interference with EGF or IGF has not been researched in CLA treated breast cancer models.

1.5.8 IGF-I receptor and signaling pathway

The IGF pathway has been shown to stimulate both growth promoting and anti-apoptotic signals (138). The IGF group consists of two ligands, IGF-I and IGF-2, two receptors, IGF-IR and IGF-IIR, and six IGF binding proteins (134). IGF-IIR does not have tyrosine kinase activity and is believed to be involved primarily in the sequestering of IGF-2. IGF-IR is a transmembrane tyrosine kinase receptor and shares 70% homology with the insulin receptor (139). IGF-IR consists of two alpha subunits and two intercellular beta subunits which autophosphorylate upon binding of the ligand. Both IGF-I and IGF-2 are

ligands for this receptor. The tyrosine phosphorylation of the IGFIR leads to numerous downstream signaling events. Figure 1-4 highlights some of the key pathways IGFIR activates or inhibits and some of the factors that have been shown to be affected by CLA treatment. High circulating levels of IGF-1 have been linked with an increased risk of premenopausal breast cancer (140). There is ongoing interest in developing or discovering a therapy for breast cancer clients that targets this signaling pathway (138;141).
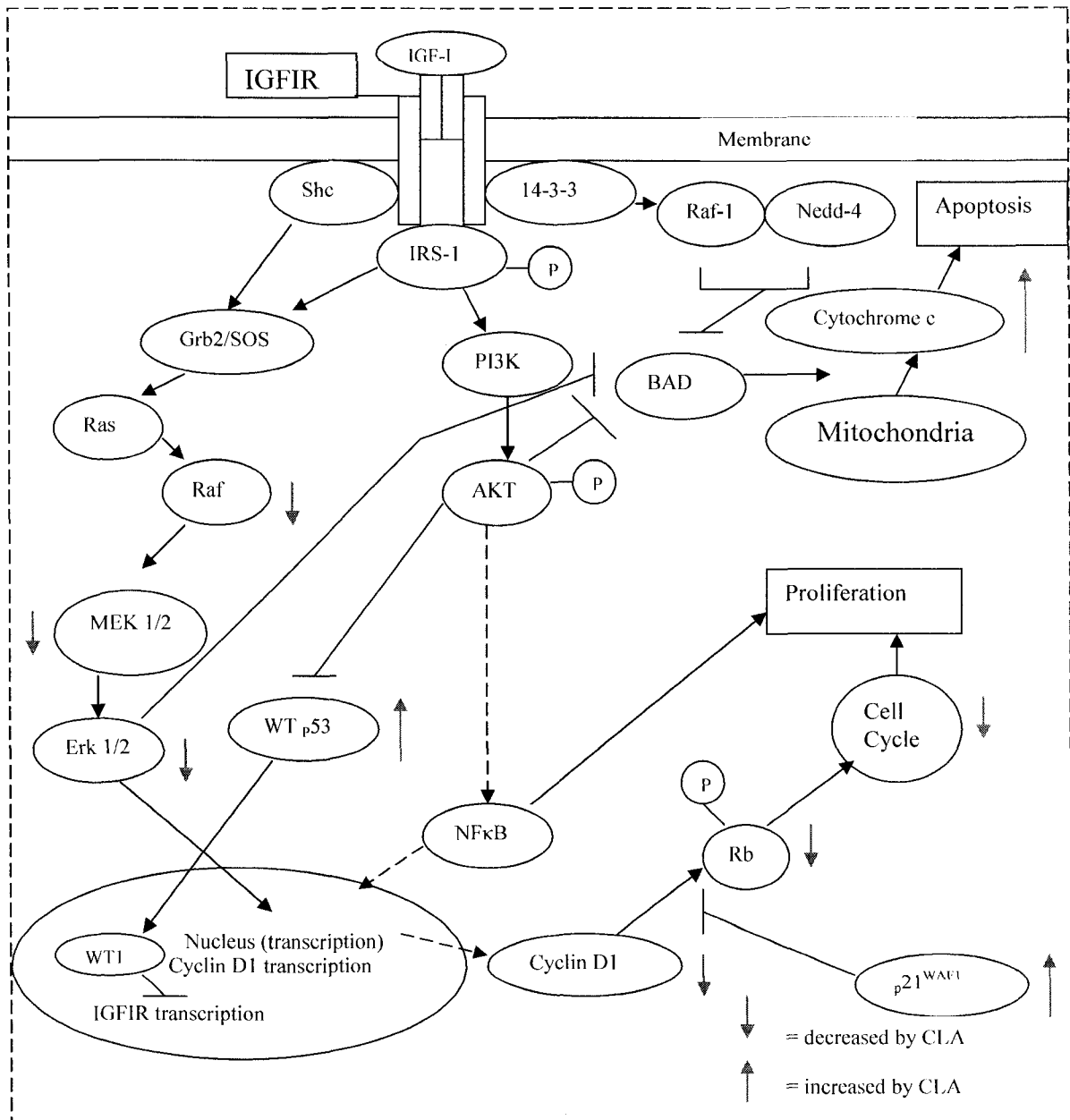
Figure 1-4: **IGFIR signaling pathways**. (77;84;86;97;98;100;138)

The possibility that some of the anti-cancer effects of CLA isomers could be mediated through the IGF signaling pathway has not been explored in breast cancer models, however studies by Kim et al. have shown that CLA interferes with IGF signaling in the HT-29 human colon cancer cell line (107). The effect of CLA on colon cancer in animal and cell culture studies has yielded some conflicting results (142), as compared to the more consistent inhibition observed in mammary cancer models. Therefore the mechanism of action of CLA in these two types of cancers may not be the same and confirmation that CLA interferes with IGF signaling needs to be explored in mammary cancer models. Support for the hypothesis that CLA interferes with IGF in mammary cancer includes the finding that CLA interferes with estrogen signaling (89). Estrogen has been shown to stimulate the expression of IGFIR (143). Other indirect evidence in the literature to support this hypothesis includes CLA modifying mammary gland development, PPAR γ activation, and interference with insulin sensitivity, as discussed below (144) (145) (146).

IGF-I is essential for pubertal mammary development of the terminal end buds (119;147). Rodent mammary cancer models have shown that CLA interferes with terminal end bud development, (144) thereby supporting the argument that CLA may interfere with the IGF signaling. PPAR gamma agonist ligands have been shown to decrease IGF-I concentrations (145). MCF-7 breast cancer cells produce IGF-I, (148) which in turn may activate IGF signaling in an autocrine manner. Finally the insulin like growth factor receptor and insulin receptor share 70% homology and IGF-I has been shown to be able to stimulate the insulin receptor (149). CLA supplement trials in obese humans have shown that both isomers c9,t11 and t10,c12 when provided individually decreased insulin sensitivity (150) (151). If CLA is reducing the body's sensitivity to insulin it could hypothetically also be interfering with the closely related IGF signaling.

1.6   Summary

Basic research into the mechanisms behind CLA's anti-carcinogenic effect offer insight into its modifications to cellular function and the surrounding stromal environment. From

32

a review of the current literature, it can be concluded that CLA has the potential to target multiple characteristics or hallmarks of cancer such as apoptosis, angiogenesis and sensitivity to growth signals. The convincing anticancer effects of both major CLA isomers that have been observed in human tumour cell lines and animal models of cancer provide preliminary evidence to suggest an application in humans. However, before trials can be proposed it is necessary to elucidate the biological mechanism(s) that might explain the anti-tumour effects of the CLA isomers. This possible mechanism would need to be novel and/or complementary to current therapies available for the treatment of breast cancer. Animal studies would be required before the progression to human clinical trials to further establish the specificity of CLA to mammary cancer and effectiveness of a plausible intake of CLA for human trials. A greater understanding of CLAs' mechanism of action will support the development of clinical trials to evaluate the potential effectiveness of CLA in the treatment of breast cancer.

1.7 Literature Cited

1. Cdn Cancer Society Statistics 2007. www.cancer.ca

2. Weinberg RA. How cancer arises. Sci Am 1996;275:62-70.

3. Hanahan D, Weinberg RA. The hallmarks of cancer. Cell 2000;100:57-70.

4. Weigelt B, Peterse JL, van ', V. Breast cancer metastasis: markers and models. Nat Rev Cancer 2005;5:591-602.

5. Hankinson SE, Colditz GA, Willett WC. Towards an integrated model for breast cancer etiology: the lifelong interplay of genes, lifestyle, and hormones. Breast Cancer Res 2004;6:213-8.

6. Hankinson SE. Endogenous hormones and risk of breast cancer in postmenopausal women. Breast Dis 2005;24:3-15.

7. Veronesi U, Boyle P, Goldhirsch A, Orecchia R, Viale G. Breast cancer. Lancet 2005;365:1727-41.

8. Kaklamani VG, Gradishar WJ. Adjuvant therapy of breast cancer. Cancer Invest 2005;23:548-60.

9. Doll R, Peto R. The causes of cancer: quantitative estimates of avoidable risks of cancer in the United States today. J Natl Cancer Inst 1981;66:1191-308.

10. Holmes MD, Willett WC. Does diet affect breast cancer risk? Breast Cancer Res 2004;6:170-8.

11. Buell P. Changing incidence of breast cancer in Japanese-American women. J Natl Cancer Inst 1973;51:1479-83.

12. Prentice R, Thompson D, Clifford C, Gorbach S, Goldin B, Byar D. Dietary fat reduction and plasma estradiol concentration in healthy postmenopausal women. The Women's Health Trial Study Group. J Natl Cancer Inst 1990;82:129-34.

13. Boyd NF, Stone J, Vogt KN, Connelly BS, Martin LJ, Minkin S. Dietary fat and breast cancer risk revisited: a meta-analysis of the published literature. Br J Cancer 2003;89:1672-85.

14. Prentice RL, Caan B, Chlebowski RT et al. Low-fat dietary pattern and risk of invasive breast cancer: the Women's Health Initiative Randomized Controlled Dietary Modification Trial. JAMA 2006;295:629-42.

15. Chlebowski RT, Blackburn GL, Thomson CA et al. Dietary fat reduction and breast cancer outcome: interim efficacy results from the Women's Intervention Nutrition Study. J Natl Cancer Inst 2006;98:1767-76.

16. Pierce JP, Natarajan L, Caan BJ et al. Influence of a diet very high in vegetables, fruit, and fiber and low in fat on prognosis following treatment for breast cancer: the Women's Healthy Eating and Living (WHEL) randomized trial. JAMA 2007;298:289-98.

17. Gonzalez CA. The European Prospective Investigation into Cancer and Nutrition (EPIC). Public Health Nutr 2006;9:124-6.

18. Smith-Warner SA, Stampfer MJ. Fat intake and breast cancer revisited. J Natl Cancer Inst 2007;99:418-9.

19. Borugian MJ, Sheps SB, Kim-Sing C et al. Insulin, macronutrient intake, and physical activity: are potential indicators of insulin resistance associated with mortality from breast cancer? Cancer Epidemiol Biomarkers Prev 2004;13:1163-72.

20. Goodwin PJ, Ennis M, Pritchard KI, Koo J, Trudeau ME, Hood N. Diet and breast cancer: evidence that extremes in diet are associated with poor survival. J Clin Oncol 2003;21:2500-7.

21. Fay MP, Freedman LS. Meta-analyses of dietary fats and mammary neoplasms in rodent experiments. Breast Cancer Res Treat 1997;46:215-23.

22. Rose DP, Connolly JM. Omega-3 fatty acids as cancer chemopreventive agents. Pharmacol Ther 1999;83:217-44.

23. MacLean CH, Newberry SJ, Mojica WA et al. Effects of omega-3 fatty acids on cancer risk: a systematic review. JAMA 2006;295:403-15.

24. Pariza MW, Ashoor SH, Chu FS, Lund DB. Effects of temperature and time on mutagen formation in pan-fried hamburger. Cancer Lett 1979;7:63-9.

25. Ha YL, Grimm NK, Pariza MW. Anticarcinogens from fried ground beef: heat-altered derivatives of linoleic acid. Carcinogenesis 1987;8:1881-7.

26. Dhiman TR, Nam SH, Ure AL. Factors affecting conjugated linoleic Acid content in milk and meat. Crit Rev Food Sci Nutr 2005;45:463-82.

27. Bhattacharya A, Banu J, Rahman M, Causey J, Fernandes G. Biological effects of conjugated linoleic acids in health and disease. J Nutr Biochem 2006;17:789-810.

28. Lock AL, Bauman DE. Modifying milk fat composition of dairy cows to enhance fatty acids beneficial to human health. Lipids 2004;39:1197-206.

29. Fritsche and Steinhart H. Amounts of conjugated linoleic acid (CLA) in German Foods and evaluation of daily intake. A Lebensm Unters Forsch A 206, 77-82. 1998.
Ref Type: Generic

30. Herbel BK, McGuire MK, McGuire MA, Shultz TD. Safflower oil consumption does not increase plasma conjugated linoleic acid concentrations in humans. Am J Clin Nutr 1998;67:332-7.

31. Jiang J, Wolk A, Vessby B. Relation between the intake of milk fat and the occurrence of conjugated linoleic acid in human adipose tissue. Am J Clin Nutr 1999;70:21-7.

32. Park Y, McGuire MK, Behr R, McGuire MA, Evans MA, Shultz TD. High-fat dairy product consumption increases delta 9c,11t-18:2 (rumenic acid) and total lipid concentrations of human milk. Lipids 1999;34:543-9.

33. Ens JG, Ma DW, Cole KS, Field CJ, Clandinin MT. An assessment of c9,t11 linoleic acid intake in a small group of young Canadians. Nutr Res 2001;21:955-60.

34. Ritzenthaler KL, McGuire MK, Falen R, Shultz TD, Dasgupta N, McGuire MA. Estimation of conjugated linoleic acid intake by written dietary assessment methodologies underestimates actual intake evaluated by food duplicate methodology. J Nutr 2001;131:1548-54.

35. Fremann D, Linseisen J, Wolfram G. Dietary conjugated linoleic acid (CLA) intake assessment and possible biomarkers of CLA intake in young women. Public Health Nutr 2002;5:73-80.

36. Aro A, Mannisto S, Salminen I, Ovaskainen ML, Kataja V, Uusitupa M. Inverse association between dietary and serum conjugated linoleic acid and risk of breast cancer in postmenopausal women. Nutr Cancer 2000;38:151-7.

37. Voorrips LE, Brants HA, Kardinaal AF, Hiddink GJ, van den Brandt PA, Goldbohm RA. Intake of conjugated linoleic acid, fat, and other fatty acids in relation to postmenopausal breast cancer: the Netherlands Cohort Study on Diet and Cancer. Am J Clin Nutr 2002;76:873-82.

38. McCann SE, Ip C, Ip MM et al. Dietary intake of conjugated linoleic acids and risk of premenopausal and postmenopausal breast cancer, Western New York Exposures and Breast Cancer Study (WEB Study). Cancer Epidemiol Biomarkers Prev 2004;13:1480-4.

39. Chajes V, Lavillonniere F, Maillard V et al. Conjugated linoleic acid content in breast adipose tissue of breast cancer patients and the risk of metastasis. Nutr Cancer 2003;45:17-23.

40. Ip C, Scimeca JA, Thompson HJ. Conjugated linoleic acid. A powerful anticarcinogen from animal fat sources. Cancer 1994;74:1050-4.

41. Ma DW, Field CJ, Clandinin MT. An enriched mixture of trans-10,cis-12-CLA inhibits linoleic acid metabolism and PGE2 synthesis in MDA-MB-231 cells. Nutr Cancer 2002;44:203-12.

42. Ip C, Chin SF, Scimeca JA, Pariza MW. Mammary cancer prevention by conjugated dienoic derivative of linoleic acid. Cancer Res 1991;51:6118-24.

43. Ip C, Scimeca JA, Thompson H. Effect of timing and duration of dietary conjugated linoleic acid on mammary cancer prevention. Nutr Cancer 1995;24:241-7.

44. Ip C, Briggs SP, Haegele AD, Thompson HJ, Storkson J, Scimeca JA. The efficacy of conjugated linoleic acid in mammary cancer prevention is independent of the level or type of fat in the diet. Carcinogenesis 1996;17:1045-50.

45. Ip C, Jiang C, Thompson HJ, Scimeca JA. Retention of conjugated linoleic acid in the mammary gland is associated with tumour inhibition during the post-initiation phase of carcinogenesis. Carcinogenesis 1997;18:755-9.

46. Thompson H, Zhu Z, Banni S, Darcy K, Loftus T, Ip C. Morphological and biochemical status of the mammary gland as influenced by conjugated linoleic acid: implication for a reduction in mammary cancer risk. Cancer Res 1997;57:5067-72.

47. Banni S, Angioni E, Casu V et al. Decrease in linoleic acid metabolites as a potential mechanism in cancer risk reduction by conjugated linoleic acid. Carcinogenesis 1999;20:1019-24.

48. Ip MM, Masso-Welch PA, Shoemaker SF, Shea-Eaton WK, Ip C. Conjugated linoleic acid inhibits proliferation and induces apoptosis of normal rat mammary epithelial cells in primary culture. Exp Cell Res 1999;250:22-34.

49. Ip C, Ip MM, Loftus T, Shoemaker S, Shea-Eaton W. Induction of apoptosis by conjugated linoleic acid in cultured mammary tumour cells and premalignant lesions of the rat mammary gland. Cancer Epidemiol Biomarkers Prev 2000;9:689-96.

50. Kimoto N, Hirose M, Futakuchi M, Iwata T, Kasai M, Shirai T. Site-dependent modulating effects of conjugated fatty acids from safflower oil in a rat two-stage carcinogenesis model in female Sprague-Dawley rats. Cancer Lett 2001;168:15-21.

51. Futakuchi M, Cheng JL, Hirose M et al. Inhibition of conjugated fatty acids derived from safflower or perilla oil of induction and development of mammary

tumours in rats induced by 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP). Cancer Lett 2002;178:131-9.

52. Banni S, Angioni E, Murru E et al. Vaccenic acid feeding increases tissue levels of conjugated linoleic acid and suppresses development of premalignant lesions in rat mammary gland. Nutr Cancer 2001;41:91-7.

53. Ip C, Dong Y, Ip MM et al. Conjugated linoleic acid isomers and mammary cancer prevention. Nutr Cancer 2002;43:52-8.

54. Cheng JL, Futakuchi M, Ogawa K et al. Dose response study of conjugated fatty acid derived from safflower oil on mammary and colon carcinogenesis pretreated with 7,12-dimethylbenz[a]anthracene (DMBA) and 1,2-dimethylhydrazine (DMH) in female Sprague-Dawley rats. Cancer Lett 2003;196:161-8.

55. Lavillonniere F, Chajes V, Martin JC, Sebedio JL, Lhuillery C, Bougnoux P. Dietary purified cis-9,trans-11 conjugated linoleic acid isomer has anticarcinogenic properties in chemically induced mammary tumours in rats. Nutr Cancer 2003;45:190-4.

56. Corl BA, Barbano DM, Bauman DE, Ip C. cis-9, trans-11 CLA derived endogenously from trans-11 18:1 reduces cancer risk in rats. J Nutr 2003;133:2893-900.

57. Lock AL, Corl BA, Barbano DM, Bauman DE, Ip C. The anticarcinogenic effect of trans-11 18:1 is dependent on its conversion to cis-9, trans-11 CLA by delta9-desaturase in rats. J Nutr 2004;134:2698-704.

58. Visonneau S, Cesano A, Tepper SA, Scimeca JA, Santoli D, Kritchevsky D. Conjugated linoleic acid suppresses the growth of human breast adenocarcinoma cells in SCID mice. Anticancer Res 1997;17:969-73.

59. Hubbard NE, Lim D, Summers L, Erickson KL. Reduction of murine mammary tumour metastasis by conjugated linoleic acid. Cancer Lett 2000;150:93-100.

60. Masso-Welch PA, Zangani D, Ip C et al. Inhibition of angiogenesis by the cancer chemopreventive agent conjugated linoleic acid. Cancer Res 2002;62:4383-9.

61. Hubbard NE, Lim D, Erickson KL. Effect of separate conjugated linoleic acid isomers on murine mammary tumourigenesis. Cancer Lett 2003;190:13-9.

62. Masso-Welch PA, Zangani D, Ip C et al. Isomers of conjugated linoleic acid differ in their effects on angiogenesis and survival of mouse mammary adipose vasculature. J Nutr 2004;134:299-307.

63. Dauchy RT, Dauchy EM, Sauer LA et al. Differential inhibition of fatty acid transport in tissue-isolated steroid receptor negative human breast cancer

xenografts perfused in situ with isomers of conjugated linoleic acid. Cancer Lett 2004;209:7-15.

64. Hubbard NE, Lim D, Erickson KL. Beef tallow increases the potency of conjugated linoleic acid in the reduction of mouse mammary tumour metastasis. J Nutr 2006;136:88-93.

65. Wong MW, Chew BP, Wong TS, Hosick HL, Boylston TD, Shultz TD. Effects of dietary conjugated linoleic acid on lymphocyte function and growth of mammary tumours in mice. Anticancer Res 1997;17:987-93.

66. Ip MM, McGee SO, Masso-Welch PA et al. The t10,c12 isomer of conjugated linoleic acid stimulates mammary tumourigenesis in transgenic mice over-expressing erbB2 in the mammary epithelium. Carcinogenesis 2007;28:1269-76.

67. House RL, Cassady JP, Eisen EJ, McIntosh MK, Odle J. Conjugated linoleic acid evokes de-lipidation through the regulation of genes controlling lipid metabolism in adipose and liver tissue. Obes Rev 2005;6:247-58.

68. Josyula S, He YH, Ruch RJ, Schut HA. Inhibition of DNA adduct formation of PhIP in female F344 rats by dietary conjugated linoleic acid. Nutr Cancer 1998;32:132-8.

69. Lacroix M, Leclercq G. Relevance of breast cancer cell lines as models for breast tumours: an update. Breast Cancer Res Treat 2004;83:249-89.

70. Bartucci M, Morelli C, Mauro L, Ando S, Surmacz E. Differential insulin-like growth factor I receptor signaling and function in estrogen receptor (ER)-positive MCF-7 and ER-negative MDA-MB-231 breast cancer cells. Cancer Res 2001;61:6747-54.

71. Anderson NG, Ahmad T, Chan K, Dobson R, Bundred NJ. ZD1839 (Iressa), a novel epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor, potently inhibits the growth of EGFR-positive cancer cell lines with or without erbB2 overexpression. Int J Cancer 2001;94:774-82.

72. Wang LS, Huang YW, Sugimoto Y et al. Effects of human breast stromal cells on conjugated linoleic acid (CLA) modulated vascular endothelial growth factor-A (VEGF-A) expression in MCF-7 cells. Anticancer Res 2005;25:4061-8.

73. sBordes C, Lea MA. Effects of C18 fatty acid isomers on DNA synthesis in hepatoma and breast cancer cells. Anticancer Res 1995;15:2017-21.

74. Shultz TD, Chew BP, Seaman WR, Luedecke LO. Inhibitory effect of conjugated dienoic derivatives of linoleic acid and beta-carotene on the in vitro growth of human cancer cells. Cancer Lett 1992;63:125-33.

75. Shultz TD, Chew BP, Seaman WR. Differential stimulatory and inhibitory responses of human MCF-7 breast cancer cells to linoleic acid and conjugated linoleic acid in culture. Anticancer Res 1992;12:2143-5.

76. Cunningham DC, Harrison LY, Shultz TD. Proliferative responses of normal human mammary and MCF-7 breast cancer cells to linoleic acid, conjugated linoleic acid and eicosanoid synthesis inhibitors in culture. Anticancer Res 1997;17:197-203.

77. Durgam VR, Fernandes G. The growth inhibitory effect of conjugated linoleic acid on MCF-7 cells is related to estrogen response system. Cancer Lett 1997;116:121-30.

78. O'Shea M, Stanton C, Devery R. Antioxidant enzyme defence responses of human MCF-7 and SW480 cancer cells to conjugated linoleic acid. Anticancer Res 1999;19:1953-9.

79. Ip C, Banni S, Angioni E et al. Conjugated linoleic acid-enriched butter fat alters mammary gland morphogenesis and reduces cancer risk in rats. J Nutr 1999;129:2135-42.

80. Park Y, Allen KG, Shultz TD. Modulation of MCF-7 breast cancer cell signal transduction by linoleic acid and conjugated linoleic acid in culture. Anticancer Res 2000;20:669-76.

81. O'Shea M, Devery R, Lawless F, Murphy J, Stanton C. Milk fat conjugated linoleic acid (CLA) inhibits growth of human mammary MCF-7 cancer cells. Anticancer Res 2000;20:3591-601.

82. Miller A, Stanton C, Devery R. Modulation of arachidonic acid distribution by conjugated linoleic acid isomers and linoleic acid in MCF-7 and SW480 cancer cells. Lipids 2001;36:1161-8.

83. Choi Y, Park Y, Storkson JM, Pariza MW, Ntambi JM. Inhibition of stearoyl-CoA desaturase activity by the cis-9,trans-11 isomer and the trans-10,cis-12 isomer of conjugated linoleic acid in MDA-MB-231 and MCF-7 human breast cancer cells. Biochem Biophys Res Commun 2002;294:785-90.

84. Majumder B, Wahle KW, Moir S et al. Conjugated linoleic acids (CLAs) regulate the expression of key apoptotic genes in human breast cancer cells. FASEB J 2002;16:1447-9.

85. Oku H, Wongtangtintharn S, Iwasaki H, Toda T. Conjugated linoleic acid (CLA) inhibits fatty acid synthetase activity in vitro. Biosci Biotechnol Biochem 2003;67:1584-6.

86. Kemp MQ, Jeffy BD, Romagnolo DF. Conjugated linoleic acid inhibits cell proliferation through a p53-dependent mechanism: effects on the expression of G1-restriction points in breast and colon cancer cells. J Nutr 2003;133:3670-7.

87. Chujo H, Yamasaki M, Nou S, Koyanagi N, Tachibana H, Yamada K. Effect of conjugated linoleic acid isomers on growth factor-induced proliferation of human breast cancer cells. Cancer Lett 2003;202:81-7.

88. Miller A, Stanton C, Murphy J, Devery R. Conjugated linoleic acid (CLA)-enriched milk fat inhibits growth and modulates CLA-responsive biomarkers in MCF-7 and SW480 human cancer cell lines. Br J Nutr 2003;90:877-85.

89. Tanmahasamut P, Liu J, Hendry LB, Sidell N. Conjugated linoleic acid blocks estrogen signaling in human breast cancer cells. J Nutr 2004;134:674-80.

90. Maggiora M, Bologna M, Ceru MP et al. An overview of the effect of linoleic and conjugated-linoleic acids on the growth of several human tumour cell lines. Int J Cancer 2004;112:909-19.

91. Kim JH, Hubbard NE, Ziboh V, Erickson KL. Attenuation of breast tumour cell growth by conjugated linoleic acid via inhibition of 5-lipoxygenase activating protein. Biochim Biophys Acta 2005;1736:244-50.

92. Albright CD, Klem E, Shah AA, Gallagher P. Breast cancer cell-targeted oxidative stress: enhancement of cancer cell uptake of conjugated linoleic acid, activation of p53, and inhibition of proliferation. Exp Mol Pathol 2005;79:118-25.

93. Liu J, Sidell N. Anti-estrogenic Effects of Conjugated Linoleic Acid through Modulation of Estrogen Receptor Phosphorylation. Breast Cancer Res Treat 2005;94:161-9.

94. Wang LS, Huang YW, Sugimoto Y et al. Conjugated linoleic acid (CLA) up-regulates the estrogen-regulated cancer suppressor gene, protein tyrosine phosphatase gamma (PTPgama), in human breast cells. Anticancer Res 2006;26:27-34.

95. Degner SC, Kemp MQ, Bowden GT, Romagnolo DF. Conjugated linoleic acid attenuates cyclooxygenase-2 transcriptional activity via an anti-AP-1 mechanism in MCF-7 breast cancer cells. J Nutr 2006;136:421-7.

96. De La TA, Debiton E, Juaneda P et al. Beef conjugated linoleic acid isomers reduce human cancer cell growth even when associated with other beef fatty acids. Br J Nutr 2006;95:346-52.

97. Miglietta A, Bozzo F, Gabriel L, Bocca C, Canuto RA. Extracellular signal-regulated kinase 1/2 and protein phosphatase 2A are involved in the antiproliferative activity of conjugated linoleic acid in MCF-7 cells. Br J Nutr 2006;96:22-7.

98. Miglietta A, Bozzo F, Bocca C et al. Conjugated linoleic acid induces apoptosis in MDA-MB-231 breast cancer cells through ERK/MAPK signalling and mitochondrial pathway. Cancer Lett 2006;234:149-57.

99. Bocca C, Bozzo F, Francica S, Colombatto S, Miglietta A. Involvement of PPAR gamma and E-cadherin/beta-catenin pathway in the antiproliferative effect of conjugated linoleic acid in MCF-7 cells. Int J Cancer 2007;121:248-56.

100. Guo DD, Moon HS, Arote R et al. Enhanced anticancer effect of conjugated linoleic acid by conjugation with Pluronic F127 on MCF-7 breast cancer cells. Cancer Lett 2007.

101. Malumbres M, Barbacid M. To cycle or not to cycle: a critical decision in cancer. Nat Rev Cancer 2001;1:222-31.

102. Field CJ, Schley PD. Evidence for potential mechanisms for the effect of conjugated linoleic acid on tumour metabolism and immune function: lessons from n-3 fatty acids. Am J Clin Nutr 2004;79:1190S-8S.

103. Johnson DG, Walker CL. Cyclins and cell cycle checkpoints. Annu Rev Pharmacol Toxicol 1999;39:295-312.

104. Maddika S, Ande SR, Panigrahi S et al. Cell survival, cell death and cell cycle pathways are interconnected: implications for cancer therapy. Drug Resist Updat 2007;10:13-29.

105. Schwartz GK, Shah MA. Targeting the cell cycle: a new approach to cancer therapy. J Clin Oncol 2005;23:9408-21.

106. Rode HJ, Eisel D, and Frost I. Roche Applied Sciences Apoptosis, Cell Death and Proliferation. 3rd Edition. 2007.
Ref Type: Generic

107. Kim EJ, Kang IJ, Cho HJ, Kim WK, Ha YL, Park JH. Conjugated linoleic acid downregulates insulin-like growth factor-I receptor levels in HT-29 human colon cancer cells. J Nutr 2003;133:2675-81.

108. Igney FH, Krammer PH. Death and anti-death: tumour resistance to apoptosis. Nat Rev Cancer 2002;2:277-88.

109. Zamzami N, Kroemer G. The mitochondrion in apoptosis: how Pandora's box opens. Nat Rev Mol Cell Biol 2001;2:67-71.

110. Kumar S, Walia V, Ray M, Elble RC. p53 in breast cancer: mutation and countermeasures. Front Biosci 2007;12:4168-78.

111. Miglietta A, Bozzo F, Bocca C et al. Conjugated linoleic acid induces apoptosis in MDA-MB-231 breast cancer cells through ERK/MAPK signalling and mitochondrial pathway. Cancer Lett 2005.

112. Wang T, Xu J, Yu X, Yang R, Han ZC. Peroxisome proliferator-activated receptor gamma in malignant diseases. Crit Rev Oncol Hematol 2006;58:1-14.

113. Mueller E, Sarraf P, Tontonoz P et al. Terminal differentiation of human breast cancer through PPAR gamma. Mol Cell 1998;1:465-70.

114. Kim KY, Kim SS, Cheon HG. Differential anti-proliferative actions of peroxisome proliferator-activated receptor-gamma agonists in MCF-7 breast cancer cells. Biochem Pharmacol 2006;72:530-40.

115. Hollingshead HE, Killins RL, Borland MG et al. Peroxisome proliferator-activated receptor-{beta}/{delta} (PPAR{beta}/{delta}) ligands do not potentiate growth of human cancer cell lines. Carcinogenesis 2007.

116. Stephen RL, Gustafsson MC, Jarvis M et al. Activation of peroxisome proliferator-activated receptor delta stimulates the proliferation of human breast and prostate cancer cell lines. Cancer Res 2004;64:3162-70.

117. Crowe DL, Chandraratna RA. A retinoid X receptor (RXR)-selective retinoid reveals that RXR-alpha is potentially a therapeutic target in breast cancer cell lines, and that it potentiates antiproliferative and apoptotic responses to peroxisome proliferator-activated receptor ligands. Breast Cancer Res 2004;6:R546-R555.

118. Mueller MM, Fusenig NE. Friends or foes - bipolar effects of the tumour stroma in cancer. Nat Rev Cancer 2004;4:839-49.

119. Sternlicht MD. Key stages in mammary gland development: the cues that regulate ductal branching morphogenesis. Breast Cancer Res 2006;8:201.

120. Tang DG, La E, Kern J, Kehrer JP. Fatty acid oxidation and signaling in apoptosis. Biol Chem 2002;383:425-42.

121. Igarashi M, Miyazawa T. The growth inhibitory effect of conjugated linoleic acid on a human hepatoma cell line, HepG2, is induced by a change in fatty acid metabolism, but not the facilitation of lipid peroxidation in the cells. Biochim Biophys Acta 2001;1530:162-71.

122. Stubbs CD, Smith AD. The modification of mammalian membrane polyunsaturated fatty acid composition in relation to membrane fluidity and function. Biochim Biophys Acta 1984;779:89-137.

123. Niu SL, Mitchell DC, Litman BJ. Trans fatty acid derived phospholipids show increased membrane cholesterol and reduced receptor activation as compared to their cis analogs. Biochemistry 2005;44:4458-65.

124. Ibrahim A, Natrajan S, Ghafoorunissa R. Dietary trans-fatty acids alter adipocyte plasma membrane fatty acid composition and insulin sensitivity in rats. Metabolism 2005;54:240-6.

125. Roach C, Feller SE, Ward JA, Shaikh SR, Zerouga M, Stillwell W. Comparison of cis and trans fatty acid containing phosphatidylcholines on membrane properties. Biochemistry 2004;43:6344-51.

126. Grimble RF, Tappia PS. Modulatory influence of unsaturated fatty acids on the biology of tumour necrosis factor-alpha. Biochem Soc Trans 1995;23:282-7.

127. Jolly CA, Jiang YH, Chapkin RS, McMurray DN. Dietary (n-3) polyunsaturated fatty acids suppress murine lymphoproliferation, interleukin-2 secretion, and the formation of diacylglycerol and ceramide. J Nutr 1997;127:37-43.

128. de Pablo MA, varez de CG. Modulatory effects of dietary lipids on immune system functions. Immunol Cell Biol 2000;78:31-9.

129. Ha YL, Storkson J, Pariza MW. Inhibition of benzo(a)pyrene-induced mouse forestomach neoplasia by conjugated dienoic derivatives of linoleic acid. Cancer Res 1990;50:1097-101.

130. Tong WG, Ding XZ, Adrian TE. The mechanisms of lipoxygenase inhibitor-induced apoptosis in human breast cancer cells. Biochem Biophys Res Commun 2002;296:942-8.

131. Jones R, del-Alvarez LA, Alvarez OR, Broaddus R, Das S. Arachidonic acid and colorectal carcinogenesis. Mol Cell Biochem 2003;253:141-9.

132. Ip C. Review of the effects of trans fatty acids, oleic acid, n-3 polyunsaturated fatty acids, and conjugated linoleic acid on mammary carcinogenesis in animals. Am J Clin Nutr 1997;66:1523S-9S.

133. Kavanaugh CJ, Liu KL, Belury MA. Effect of dietary conjugated linoleic acid on phorbol ester-induced PGE2 production and hyperplasia in mouse epidermis. Nutr Cancer 1999;33:132-8.

134. Nahta R, Hortobagyi GN, Esteva FJ. Growth factor receptors in breast cancer: potential for therapeutic intervention. Oncologist 2003;8:5-17.

135. Goss P, Wu M. Application of aromatase inhibitors in endocrine responsive breast cancers. Breast 2007.

136. Song RX, Zhang Z, Chen Y, Bao Y, Santen RJ. Estrogen signaling via a linear pathway involving insulin-like growth factor I receptor, matrix metalloproteinases, and epidermal growth factor receptor to activate mitogen-activated protein kinase in MCF-7 breast cancer cells. Endocrinology 2007;148:4091-101.

137. Lu Y, Zi X, Zhao Y, Mascarenhas D, Pollak M. Insulin-like growth factor-I receptor signaling and resistance to trastuzumab (Herceptin). J Natl Cancer Inst 2001;93:1852-7.

138. Pollak MN. Insulin-like growth factors and neoplasia. Novartis Found Symp 2004;262:84-98.

139. Ullrich A, Gray A, Tam AW et al. Insulin-like growth factor I receptor primary structure: comparison with insulin receptor suggests structural determinants that define functional specificity. EMBO J 1986;5:2503-12.

140. Schernhammer ES, Holly JM, Pollak MN, Hankinson SE. Circulating levels of insulin-like growth factors, their binding proteins, and breast cancer risk. Cancer Epidemiol Biomarkers Prev 2005;14:699-704.

141. Tao Y, Pinzi V, Bourhis J, Deutsch E. Mechanisms of disease: signaling of the insulin-like growth factor 1 receptor pathway--therapeutic perspectives in cancer. Nat Clin Pract Oncol 2007;4:591-602.

142. Rajakangas J, Basu S, Salminen I, Mutanen M. Adenoma growth stimulation by the trans-10, cis-12 isomer of conjugated linoleic acid (CLA) is associated with changes in mucosal NF-kappaB and cyclin D1 protein levels in the Min mouse. J Nutr 2003;133:1943-8.

143. Kahlert S, Nuedling S, van EM, Vetter H, Meyer R, Grohe C. Estrogen receptor alpha rapidly activates the IGF-1 receptor pathway. J Biol Chem 2000;275:18447-53.

144. Ip C, Singh M, Thompson HJ, Scimeca JA. Conjugated linoleic acid suppresses mammary carcinogenesis and proliferative activity of the mammary gland in the rat. Cancer Res 1994;54:1212-5.

145. Stoll BA. Linkage between retinoid and fatty acid receptors: implications for breast cancer prevention. Eur J Cancer Prev 2002;11:319-25.

146. Tricon S, Burdge GC, Williams CM, Calder PC, Yaqoob P. The effects of conjugated linoleic acid on human health-related outcomes. Proc Nutr Soc 2005;64:171-82.

147. Kleinberg DL, Feldman M, Ruan W. IGF-I: an essential factor in terminal end bud formation and ductal morphogenesis. J Mammary Gland Biol Neoplasia 2000;5:7-17.

148. Huff KK, Kaufman D, Gabbay KH, Spencer EM, Lippman ME, Dickson RB. Secretion of an insulin-like growth factor-I-related protein by human breast cancer cells. Cancer Res 1986;46:4613-9.

149. Denley A, Carroll JM, Brierley GV et al. Differential activation of insulin receptor substrates 1 and 2 by insulin-like growth factor-activated insulin receptors. Mol Cell Biol 2007;27:3569-77.

150. Riserus U, Arner P, Brismar K, Vessby B. Treatment with dietary trans10cis12 conjugated linoleic acid causes isomer-specific insulin resistance in obese men with the metabolic syndrome. Diabetes Care 2002;25:1516-21.

151. Riserus U, Vessby B, Arnlov J, Basu S. Effects of cis-9,trans-11 conjugated linoleic acid supplementation on insulin sensitivity, lipid peroxidation, and proinflammatory markers in obese men. Am J Clin Nutr 2004;80:279-83.

2. Study Rationale

2.1 Rationale

The cytotoxicity (growth inhibition and induction of cell death) by conjugated linoleic acid (CLA) isomers in various animal and cell culture models of mammary cancer is well established (see tables 1-3 and 1-4). Most of the research in animal models has been done using mixtures of CLA isomers, however in vitro work suggests that both major isomers (c9,t11 and t10,c12) have cytotoxic affects against cancer cells. The promising results on potential anti-carcinogenic and other health effects of CLA has prompted a strong interest in modifying current agricultural practices to optimize the amount of CLA in animal products and supplements (1), as well as interest in utilizing CLA the treatment of cancer (2). Before human and animal trials on the efficacy of using CLA to treat cancer, an understanding of cellular and/or molecular mechanisms by which it exerts anti-carcinogenic activity needs to be established.

A variety of hypotheses to explain how CLA kills and/or reduces the growth of cancer cells have been proposed as reviewed in Chapter 1. Although the evidence for being a nuclear hormone receptor PPAR agonist may explain some of CLA's anti-carcinogenic activity, the studies conducted thus far have not compared CLA's effect on PPAR to that of other PUFAs such as linoleic, oleic and the n-3 fatty acids, which also have been shown to display PPAR agonist activity (2). The membrane is important in mediating cell growth and death (3) and other dietary fats have been demonstrated to alter tumour cell function by changes in membrane mediated events and receptor functions (4). The possible disruptive effect of the unique conjugated double bond structure of CLA when incorporated into tumour cell membrane phospholipids and resulting changes to membrane function has not been studied in human breast cancer cells and may offer a possible mechanism for anti-cancer effects of CLA isomers.

The purpose of this thesis research was to determine the ability of the two main CLA isomers c9,t11 and t10,c12 to be incorporated into the cellular membrane phospholipids of MCF-7 tumour cells and determine if their incorporation alters responsiveness of

47

MCF-7 cells to IGF-I signaling. Feeding CLA has been demonstrated to alter mammary gland development in rodents, (5) which is dependant on IGF-I signaling (6;7). The incubation of cells with CLA isomers has been shown to reduce estrogen signaling. Cross-talk between IGF-I and estrogen signaling has been documented (8), therefore interference with estrogen signaling may also reduce IGF-I signaling. Furthermore the receptor for IGF-I shares a close homology with the insulin receptor and IGF-I has been shown to stimulate the insulin receptor (9). CLA supplement trials in humans have shown that CLA isomers may reduce the body's sensitivity to insulin. (10;11) These findings suggest that CLA may be acting via the growth factor pathway mediated by IGF-I.

2.2   Selection of tumour model

To answer a question of mechanism of action and the direct impact a fatty acid may have on the cancer cell, cell culture models are a useful starting point. The specificity that cell culture models allow for is helpful in the generation of hypothesis for animal and clinical studies. The current breast cancer cell lines available have been shown to display similar characteristics to tumour cells in vivo and are considered a relevant model to gain an understanding of how a particular nutrient/drug/hormone might affect a transformed cell (12).

The MCF-7 breast cancer cell line was isolated from a pleural effusion of a woman with metastatic breast cancer in 1970. Due to its estrogen receptor positive status it has become the preferred cell line to study hormone-responsive breast cancer (12;13). As reviewed in Table 1-4 the majority of in vitro studies for CLA and breast cancer have used the MCF-7 cell line. MCF-7's are poorly metastatic in animal models, and have greater levels of IGFIR than the estrogen receptor negative MDA-MB231 cell line (14). These cells are reported to have reduced activity of Δ6-desaturase and 12-lipoxygenase, enzymes that are involved in essential fatty acid metabolism (16). For this reason MCF-7 cells are not considered a good model to study the desaturation and oxidation of LA and arachidonic acid to downstream eicosanoids. This thesis does not plan to investigate possible interference with essential fatty acid metabolism, therefore the reduced activity

of these enzymes in this model were kept in consideration however were not believed to alter the results of our experiments.

A range of concentrations of CLA (50-256µM) was selected for this study, all which fell within the range of 10-350µM that has been observed in human serum, including that of subjects who have been on CLA supplements (15). Although serum levels do not necessarily account for true availability to tumour cells it is the current best estimate in the literature for cell culture studies.

2.3　Objective and Hypothesis

The objective of this research was to determine the mechanism by which CLA induces cytotoxicity in hormone responsive breast cancer cells. Specifically, the following hypotheses were tested. It was hypothesized that:

A: incubating MCF-7 cells with CLA isomers will inhibit IGF-I –stimulated proliferation. This inhibition will be independent of the closely related essential fatty acid linoleic acid.

B: after incubation with CLA isomers, MCF-7 cells will readily incorporate these isomers into their membrane lipid (phospholipids) and changes in membrane lipid will help explain differences in cell proliferation.

C. alterations of the phospholipids as a result of incubation with CLA isomers will reduce the amount of the membrane bound receptor IGFIR and the ability of IGF-I to stimulate MCF-7 proliferation (or growth)

2.4　Chapter format

Experiments were conducted to test the above hypotheses. The results are reviewed in two separate chapters written in manuscript form.

Chapter 3 examines the effect of incubating cells with two CLA isomers, c9,t11 and t10,c12, on the proliferation of MCF-7 cells in the presence of fetal calf serum. The effect of these two isomers on cell proliferation and cell death are also investigated in the

absence of serum after stimulation with IGF-I. It was hypothesized that incubating MCF-7 cells with CLA isomers will inhibit IGF-I –stimulated proliferation and this inhibition will be independent of the closely related essential fatty acid linoleic acid.

Chapter 4 investigates the incorporation of the CLA isomers into cellular phospholipids and the effect on other fatty acids. It was hypothesized that alterations of the phospholipids as a result of incubation with CLA isomers will reduce the sensitivity of the IGF-I membrane bound receptor, IGFIR, to IGF-I signaling.

Chapter 5 summaries the thesis research and suggests future directions for determining the action by which CLA isomers exert their anti-carcinogenicity.

2.5   Literature Cited

1. Dhiman TR, Nam SH, Ure AL. Factors affecting conjugated linoleic Acid content in milk and meat. Crit Rev Food Sci Nutr 2005;45:463-82.

2. Belury MA. Inhibition of carcinogenesis by conjugated linoleic acid: potential mechanisms of action. J Nutr 2002;132:2995-8.

3. Grunicke HH. The cell membrane as a target for cancer chemotherapy. Eur J Cancer 1991;27:281-4.

4. Schley PD, Brindley DN, Field CJ. (n-3) PUFA alter raft lipid composition and decrease epidermal growth factor receptor levels in lipid rafts of human breast cancer cells. J Nutr 2007;137:548-53.

5. Ip C, Singh M, Thompson HJ, Scimeca JA. Conjugated linoleic acid suppresses mammary carcinogenesis and proliferative activity of the mammary gland in the rat. Cancer Res 1994;54:1212-5.

6. Kleinberg DL, Feldman M, Ruan W. IGF-I: an essential factor in terminal end bud formation and ductal morphogenesis. J Mammary Gland Biol Neoplasia 2000;5:7-17.

7. Sternlicht MD. Key stages in mammary gland development: the cues that regulate ductal branching morphogenesis. Breast Cancer Res 2006;8:201.

8. Kahlert S, Nuedling S, van EM, Vetter H, Meyer R, Grohe C. Estrogen receptor alpha rapidly activates the IGF-1 receptor pathway. J Biol Chem 2000;275:18447-53.

9. Denley A, Carroll JM, Brierley GV et al. Differential activation of insulin receptor substrates 1 and 2 by insulin-like growth factor-activated insulin receptors. Mol Cell Biol 2007;27:3569-77.

10. Riserus U, Arner P, Brismar K, Vessby B. Treatment with dietary trans10cis12 conjugated linoleic acid causes isomer-specific insulin resistance in obese men with the metabolic syndrome. Diabetes Care 2002;25:1516-21.

11. Riserus U, Vessby B, Arnlov J, Basu S. Effects of cis-9,trans-11 conjugated linoleic acid supplementation on insulin sensitivity, lipid peroxidation, and proinflammatory markers in obese men. Am J Clin Nutr 2004;80:279-83.

12. Lacroix M, Leclercq G. Relevance of breast cancer cell lines as models for breast tumours: an update. Breast Cancer Res Treat 2004;83:249-89.

13. Simstein R, Burow M, Parker A, Weldon C, Beckman B. Apoptosis, chemoresistance, and breast cancer: insights from the MCF-7 cell model system. Exp Biol Med (Maywood) 2003;228:995-1003.

14. Peyrat JP, Bonneterre J, Dusanter-Fourt I, Leroy-Martin B, Djiane J, Demaille A. Characterization of insulin-like growth factor 1 receptors (IGF1-R) in human breast cancer cell lines. Bull Cancer 1989;76:311-9.

15. Wang LS, Huang YW, Sugimoto Y et al. Effects of human breast stromal cells on conjugated linoleic acid (CLA) modulated vascular endothelial growth factor-A (VEGF-A) expression in MCF-7 cells. Anticancer Res 2005;25:4061-8.

16. Rose DP. Effects of dietary fatty acids on breast and prostate cancers: evidence from in vitro experiments and animal studies. Am J Clin Nutr 1997;66:1513S-22S.

3.   Cytotoxicity of CLA isomers on MCF-7 Breast Cancer Cells

3.1   Introduction

The role of dietary fat in the prevention or treatment of breast cancer is controversial. Although the relationship between total dietary fat intake and breast cancer risk or treatment is not clear at this time, it is suggested that some of the controversy may be due to different dietary fats exerting varying effects on tumour growth. The anti-carcinogenic effect of long-chain polyunsaturated n-3 fatty acids has been clearly established on tumour cell lines (1) and in animal models (2). Recently, there has been considerable interest in the anti-carcinogenic properties of another polyunsaturated fat (PUFA), conjugated linoleic acid (CLA). CLA consists of a group of positional and geometric stereoisomers and has been shown in animal models to inhibit mammary tumour initiation, promotion and progression (as reviewed by Ip) (3). The two major dietary isomers c9,t11 and t10,c12 have been the most studied and although both inhibit the growth of mammary tumours (3;4) they may work through different mechanisms of action and have different possible degrees of cytotoxicity (3;5-7).

C9,t11 CLA is the most prevalent isomer in the fat from ruminant-derived foods (8). The t10,c12 CLA isomer is present only in trace quantities in animal foods but is found in a approximately a 50:50 ratio with c9,t11 CLA in commercial preparations of CLA, such as that produced by the alkali isomerization of safflower oil (8). The original cancer research on CLA utilized a mix that contained equal amounts of both of these two major isomers, however, due to the availability of purified or enriched isomer preparations, recent studies have been able to compare the anti-carcinogenic effects of both isomers separately. Animal feeding studies have shown that although both CLA isomers appear to be protective against breast cancer (3;9;10), the t10,c12 isomer of CLA may be slightly more effective than c9,t11 isomer (10;11). How the CLA isomers reduce breast cancer viability remains to be elucidated.

There are many factors that stimulate the growth of breast tumours. Insulin like growth factor 1 (IGF-I) is a growth factor that has been shown in vitro to have both growth

stimulatory and anti-apoptotic properties (12). High circulating level of IGF-I have been associated with an increased risk of premenopausal breast cancer (13;14). The formation of terminal end buds during puberty is dependant on IGF-I signaling. (15;16). Currently there are no breast cancer treatments available that specifically target IGF signaling, although possible pharmaceutical antagonists are under development (17). There is some indirect evidence that CLA might influence IGF-1 mediated effects. CLA fed during puberty to rodents was shown to reduce the development of mammary tumours by altering the development of the mammary gland, specifically by reducing the number of terminal end buds, which are the primary sites where breast cancer develops (18). Additionally, Kim et al. (19) reported that CLA reduced HT-29 colon cancer cell responsiveness to IGF-I signaling. This possible effect of CLA on IGF-I has not been examined in breast cancer models.

Traditionally cell culture studies have compared the effect of a fatty acid to a group of cells that have not been treated with any exogenous fatty acid. Due to the possibility of a toxic effect of exogenously provided fatty acids, without a comparison fatty acid this comparison is not particularly useful. In the present study we compared the inhibitory effect of the two major CLA isomers to breast cancer cells that had been exposed to two other carbon 18 fatty acids, linoleic acid (LA), which has growth promoting properties in tumour cells (20) and oleic acid (OA), which had not, to our knowledge, been shown to definatively affect tumour growth (21).

The purposes of the experiments in this chapter were to establish whether the CLA isomers, c9,t11 or t10,c12, inhibit IGF-I induced proliferation of breast cancer cells and if their inhibition was greater than that of other carbon 18 fatty acids, LA and OA. To address this purpose we compared the anti-carcinogenic effects of the CLA isomers to that of equal concentrations of LA and OA. We chose to include the essential fatty acid LA in each of the treatments to eliminate the possible explanation that decreased growth with the CLA treatment was due to an essential fatty acid deficiency. Specifically we hypothesized that the CLA isomers would be cytotoxic to IGF-I stimulated breast cancer cells as compared to cells treated with LA or OA.

3.2   Materials and Methods

Maintenance of cell line

MCF-7 estrogen receptor positive breast cancer cells were obtained from American Type Culture Centre (Manassas, VA, USA) and were cultured in phenol red Dulbecco's Modified Eagle Medium: Nutrient Mix F-12 (Ham) 1;1 (DMEM:F12) with 15mM Hepes and 365mg/L of L-glutamine containing 5% v/v fetal bovine serum and 1% v/v antibiotic/antimycotic (all purchased from Invitrogen/Gibco, Burlington, Ontario, Canada). Cells were maintained in culture in 75cm^2 tissue culture flasks (Fisher Scientific, Ottawa, Ont., Canada) at 37°C in a humidified atmosphere of 5% v/v CO_2 and 95% v/v air. The media was changed every 2 days and cells were routinely passaged at 80-90% confluence. They were detached from the flasks with 0.25% v/v Trypsin EDTA (4ml) (Invitrogen/Gibco, Burlington, Ont., Canada) and centrifuged at 460 x g for 5min in a 15ml sterile centrifuge tube (Fisher Scientific, Ottawa, Ont., Canada). The pelleted cells were counted using a hemocytometer (Brightline, Horsham PA, USA), resuspended in media and utilized for the following described experiments with an additional flask to maintain the cell line.

Fatty acids

To establish a model to test for growth promotion or inhibition with CLA isomers, four different fatty acids were utilized: LA, OA, CLA isomer c9,t11, and CLA isomer t10,c12 (all purchased from Matreya, Pleasant Gap, PA, USA). Stock solutions of fatty acids were prepared by mixing in ethanol or hexane at a concentration of 10 mg/ml. Aliquots of the stock solution were stored at -80°C until needed.

Complexing fatty acids to BSA

Prior to using in culture, the fatty acids were complexed to essentially fatty acid free bovine serum albumin (#A7030, Sigma, St. Louis, MD, USA) at a 4:1 ratio using the following method (22). A 12mg aliquot of the fatty acid stock (10mg/ml in ethanol or hexane) was added to a 15ml acid washed glass tubes and the ethanol or hexane evaporated under nitrogen. One mL KOH (0.1M) was added to the fatty acids and they

were incubated for 10 minutes in a shaking waterbath at 50°C. After 10 minutes, 9 ml of sterile filtered 7.5% v/v BSA in doubly distilled water was added. The solution was vortexed then kept at room temperature for 3 hours, after which it was stored at 4°C overnight. The final fatty acid-BSA suspension was stored in sterile eppendorf microcentrifuge tubes (Axygen Scientific Inc. Union City, CA, USA) at -30°C until used.

Determination of growth inhibition in the presence of fetal bovine serum

Cells were detached from 75 cm^2 flask, as described above, and plated in triplicate at a density of 3000 cells per well of a 96 well flat bottomed cell culture plate (Corning Inc. Corning, NY, USA) with 200μl of 5% v/v FBS supplemented DMEM:F12 media (see Figure 3-1). The plates were kept at 37°C in a humidified atmosphere containing 5% v/v CO$_2$. Forty eight hours later, the media was replaced with fresh media containing the various concentrations of BSA-complexed test fatty acids. Three wells contained media without additional fatty acids and the cells in these wells were labeled as untreated. Every 24 hours for 72 hours the media was replaced with the fatty acid treatments. After 72 hours in culture the test media was replaced with 5% v/v FCS in DMEM/F12 without additional fatty acids for a 48 hour washout period. The washout period was included for consistency with other lab protocols in our lab at the time. At 168 hours fresh media was added (100μl/well) with 10 μl/well of Wst-1 cell proliferation reagent (Roche, Indianapolis, IN, USA) and placed in the humidified atmosphere (37°C) for 2 hours. The plates were then premixed for 60 seconds and the absorbance was read at 440 nm on a microplate spectrophotometer (Spectramax plus by Molecular Devices, Sunnyvale, CA, USA).

Determination of growth inhibition in the presence of IGF-I and the absence of serum

Cells were plated in duplicate 96-well flat bottomed cell culture plates (Corning Inc. Corning, NY, USA) at a density of 3000 cells/200μl/well following the same procedure as described above. After culture for 48 hrs the media was replaced with serum-free phenol-red free DMEM-F12 (Invitrogen/Gibco, Burlington, Ontario, Canada) containing 0.5mg/ml BSA (Sigma, St. Louis, Missouri, USA) [Serum-free media] and incubated for an additional 24 hours, as illustrated in Figure 3-2 (23) (24). Following this 50 nM IGF-1

(Sigma, human, recombinant expressed in E-coli, St. Louis, Missouri, USA) in serum-free media with either 128 μM or 256 μM LA, c9,t11 CLA or t10,c12 CLA was added and the cells were incubated for 48 hours (Table 3.1). The media was then removed and fresh serum free media was added (100 μl/well) with 10 μl/well of Wst-1 cell proliferation reagent and placed in a humidified atmosphere (37°C) for 2 hours (Figure 3-2). The plates were then premixed for 60 seconds and the absorbance was read at 440nm on a microplate spectrophotometer (Spectramax plus by Molecular Devices, Sunnyvale, CA, USA).

After the single fatty acids were studied this experiment was repeated with the addition of a base of 128 μM LA to each fatty acid treatment as described in (Table 3-1). This was to ensure that any effect seen on growth were not due to interference with LA metabolism or a deficiency of LA in the media. An OA group was added to represent a fatty acid that is not reported to have affects on tumour cell growth. Cell metabolic activity was measured after 24 h or 48 h of fatty acid incubation using the Wst-1 assay described above.

Table 3-1: Different fatty acid treatments in IGF-I stimulation experiments

	Untreated	C9,t11	LA	T10,c12	Oleic
48hr incubation no suppl. LA	No exogenous fatty acids	128 μM or 256 μM c9,t11	128μM or 256μM LA	128μM or 256 μM t10,c12	Not studied
24 or 48hr incubation with suppl. LA	No exogenous fatty acids	128μM c9,t11 + 128μM LA	256μM LA	128μM t10,c12 + 128μM LA	128μM Oleic + 128μM LA

Determining cell cytotoxicity during IGF-I stimulation

Lactate dehydrogenase (LDH) release was measured using the CytoTox 96 Nonradioactive Cytotoxicity Assay Kit (Promega Corporation, Madison, WI) according to the manufacturer's instructions. MCF-7 cells were seeded and grown in duplicate 96 well flat bottomed plates (Corning Incorporated, Corning, NY) using the same protocol and serum free conditions used for the Wst-1 experiment, including the 48 hr wash out period. After 24 hr or 36 hr of fatty acid treatment, the media was removed and centrifuged at 250 x g. The supernatant was collected and (frozen at -30°C until analysis or immediately analyzed for LDH). To determine maximum LDH release cells were

lysed by adding 200 µl of 1.5% v/v Triton-X 100 (BDH Chemicals, Toronto, Ont., Canada) to the wells, incubated for 60 min at 37°C. The plates where then centrifuged at 250 x g. Matching aliquots (50 µL) of media were added to a 96 well flat bottomed plate. A 50 µL aliquot of the reaction mixture was added to each well and the plate was stored for 30 minutes at room temperature in the dark. After that time 50 µL of 1M acetic acid (stop solution) was added to each well and the colour change was read at 492 nm on the Molecular Devices SpectraMax 190 spectrophotometer (Sunnyvale, CA).

Statistical analysis

Statistical analyses were conducted using SAS, version 9 (SAS Institute, Cary, NC) to compare the effects of fatty acid treatment on MCF-7 breast cancer growth and death. A One-way analysis of variance (ANOVA) was used blocking for passage number. When a significant effect of treatment ($P<0.05$) was found, least squared means was used for post hoc analysis. Data is presented as mean ± SEM.
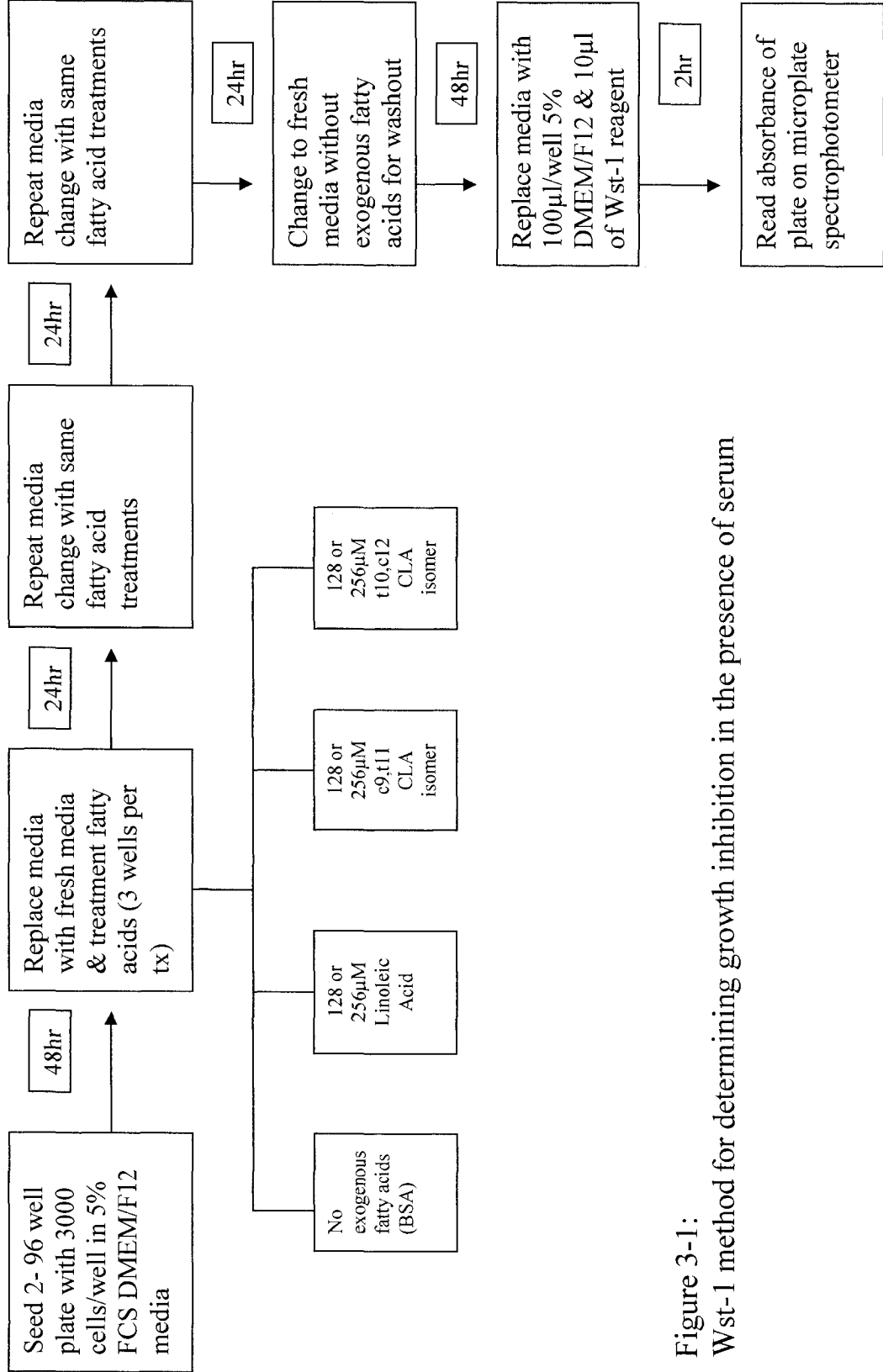
Figure 3-1:

Wst-1 method for determining growth inhibition in the presence of serum

59

Figure 3-2:

Wst-1 method for determining growth inhibition in the absence of serum with IGF-I

Seed 2- 96 well plates with 3000 cells/well in 5% FCS DMEM/F12 media

48hr

Replace media with serum free(sf) phenol red free(pf) DMEM/F12 media with 0.5mg/ml BSA

24hr

Replace media with sf pf DMEM/F12 with 0.5mg/ml BSA, 50nM IGF-I & fatty acids (3 wells/tx)

No exogenous fatty acids (BSA)

128μM LA

Or

256μM LA

128μM c9,t11 CLA isomer

or

128μM c9,t11 CLA isomer with 128μM LA

128μM t10,c12 CLA isomer

or

128μM t10,c12 CLA isomer with 128μM LA

128μM Oleic Acid isomer with 128μM LA

24hr

Repeat media change with same fatty acid treatments

24hr

Replace media with 100μl/well sf/pf DMEM/F12 with BSA & 10μl of Wst-1 reagent

2hr

Replace media with 100μl/well sf/pf DMEM/F12 with BSA & 10μl of Wst-1 reagent

2hr

Read absorbance of plate on microplate spectrophotometer

60

3.3   Results

The t10,c12 CLA isomer inhibited the growth of MCF-7 breast cancer cells grown in fetal bovine serum.

As exogenous fatty acids are reported to exhibit some cytotoxic effects on cultured cells, we selected LA, rather than media alone, as a more appropriate comparison for the CLA isomers. We confirmed this for LA at concentrations at and above 150 μM in Figure 1. The CLA isomer, t10,c12, significantly inhibited the growth of the MCF-7 cells as compared to LA ($p \leq 0.01$, Figure 3-3) under our culture media conditions at concentrations of 100 μM or greater. The rate of growth (defined by the Wst-1 assay) for cells incubated with CLA c9,t11 did not significantly differ from that of cells incubated with LA at any of the concentrations studied ($p > 0.05$, Figure 1). The same data is shown in Appendix A expressed as raw values rather than as a ratio to LA.

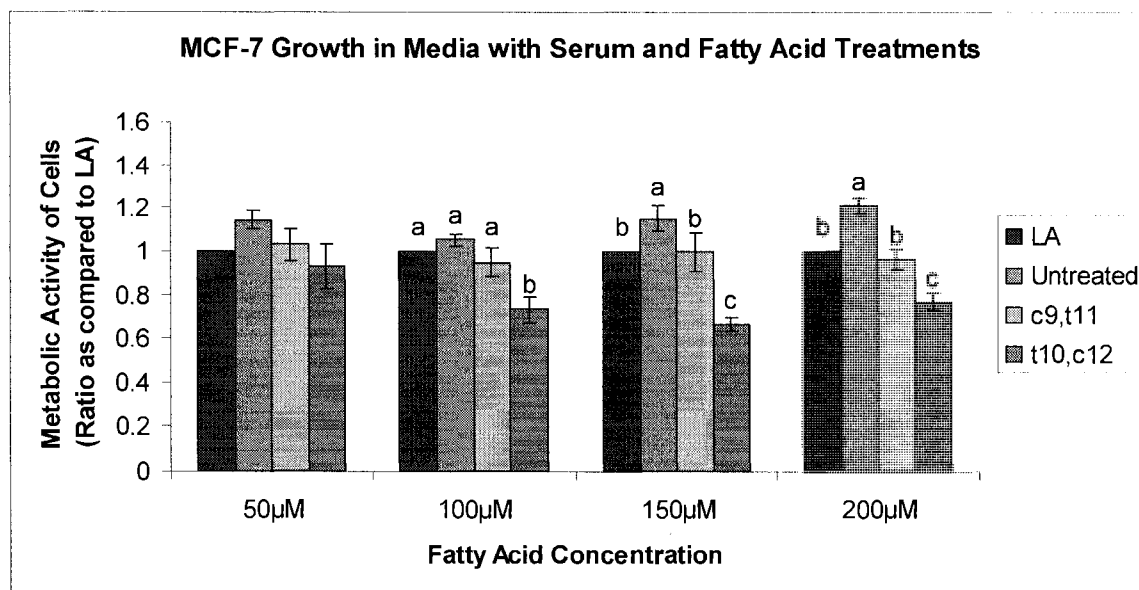Figure 3-3: **Metabolic activity (mitochondrial enzyme activity) of MCF-7 breast cancer cells grown in serum treated with fatty acids for 48hr at various concentrations** as labeled on the X axis. Values are expressed as a ratio to LA. Bars represent the means ± SEM of 3 experiments. Within each fatty acid concentration group, bars that do not share a letter are significantly different (p<0.05).

CLA inhibits growth of MCF-7 breast cancer cells stimulated by IGF-I.

MCF-7 cells were grown in serum free media for 48 hr with or without 128 μM or 256 μM of the experimental fatty acids and stimulated with IGF-1. At both fatty acid concentrations studied there was a significantly lower growth rate (defined as metabolic activity) by cells incubated with CLA t10,c12 ($P<0.5$, Figure 3-4). There was no difference in growth among the other treatments.

To determine if growth inhibition by CLA t10, c12 was due to interference with LA metabolism, MCF-7 cells were incubated in the serum free conditions with IGF-1 as untreated, with LA (256 μM), c9,t11 (128 μM LA + 128 μM c9,t11) or t10,c12 (128μM LA + 128μM t10,c12). An oleic acid treatment (128 μM LA+ 128 μM oleic) was added in this experiment to represent a fatty acid treatment that has not been reported to alter tumour growth. At 24 hr LA showed greater inhibition compared to OA although there were no differences with the CLA isomers and LA or OA. All fatty acid treated cells grew to a greater extent than the untreated cells for the 24 or 48 hours that were studied in this experiment ($P<0.05$, Figure 3-5). After 48 hr of treatment clear differences were seen between groups. Both CLA isomers significantly inhibited growth compared to the LA group ($p<0.01$, Figure 3-3) and the OA treatment increased growth compared to the LA treatment ($p<0.01$, Figure 3-5). The CLA t10,c12 treatment resulted in a significantly lower growth than the C9,t11 group ($p<0.01$, Figure 3-3). The same results are shown in Appendix A expressed as raw values rather than as a ratio to LA.

Growth of MCF-7 cells when incubated with fatty acids & IGF-I for 48h
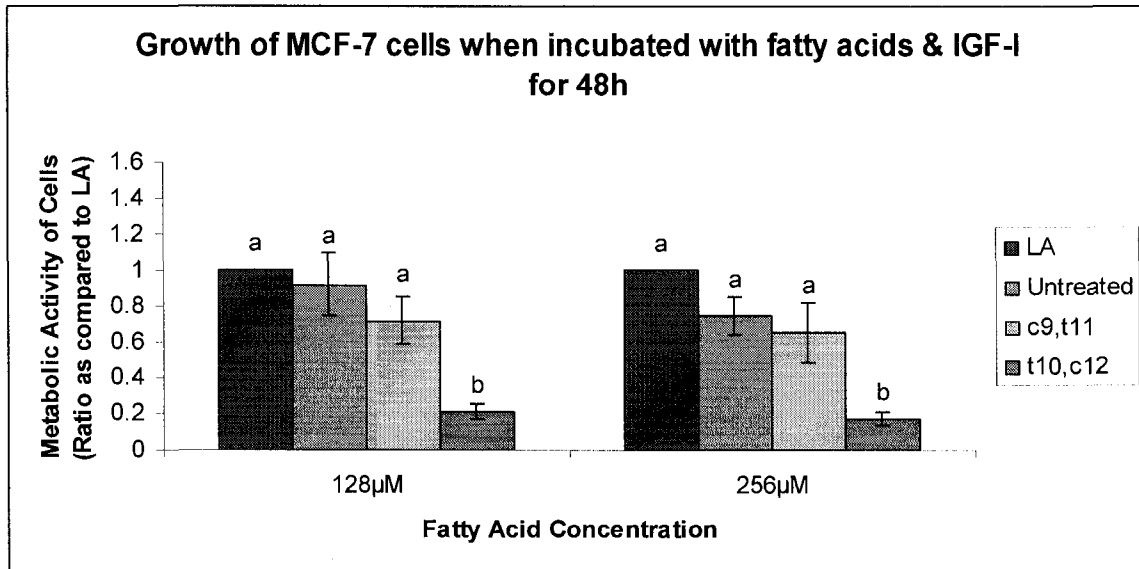
Figure 3-4: **Metabolic activity (mitochondrial enzyme activity) of MCF-7 breast cancer cells grown in the absence of serum treated with fatty acids and IGF-I for 48hr at various concentrations** as described in the Materials and Methods section. Values expressed as a ratio to LA. Bars represent the means ± SE of 3 experiments. Within each fatty acid concentration group, bars that do not share a letter are significantly different (p<0.05).

Growth of MCF-7 cells when incubated with fatty acids & IGF-I with a supplemental base of LA
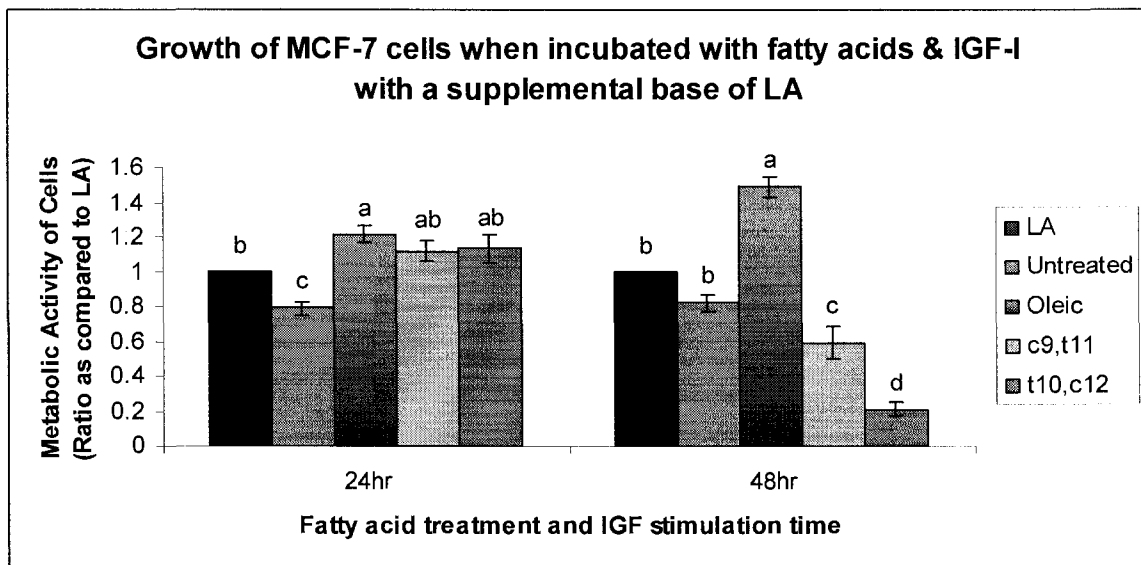
Figure 3-5: **Metabolic activity (mitochondrial enzyme activity) of MCF-7 breast cancer cells grown in the absence of serum treated with IGF-I**, 128 μM base of LA and 128 μM of specified fatty acid for 24 or 48hr as described in the Materials and Methods section. Values expressed as a ratio to LA. Bars represent the means ± SE of 3-4 experiments. Within each fatty acid concentration group, bars that do not share a letter are significantly different (p<0.05).

T10,c12 increases cell death in MCF-7 cells treated with IGF-I

LDH release was used to assess cell death/necrosis. Despite not detecting differences in cell growth, at 24hr of incubation, the CLA isomer t10,c12 (128 µM t10,c12 & 128 µM LA) induced significantly greater LDH release than the other experimental treatments and the untreated group (p<0.01, Figure 3-4). However, the t10,c12 (128 µM t10,c12 & 128 µM LA), LA (256 µM), and c9,t11 (128 µM c9,t11 & 128 µM LA) groups all had significantly greater LDH release than the untreated or OA treated (128 µM oleic & 128 µM LA) groups (p<0.01, Figure 3-6).
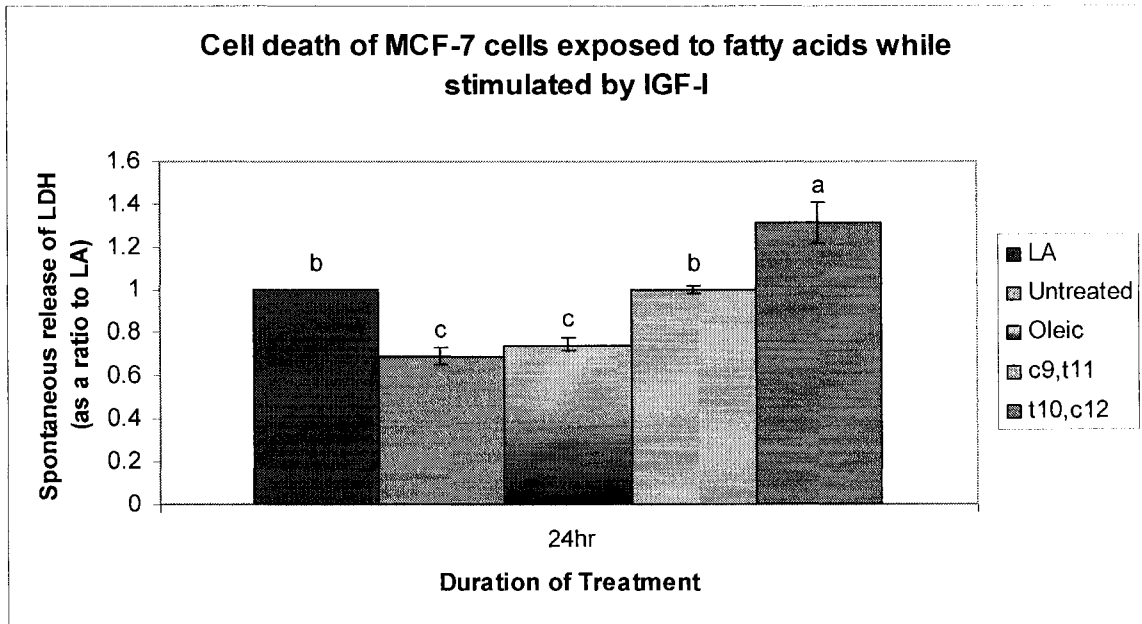
Figure 3-6: **LDH release from MCF-7 breast cancer cells grown in the absence of serum treated with IGF-I**, 128uM base of LA and 128uM of specified fatty acid for 24h as described in the Materials and Methods section. Results expressed as a ratio to that released by cells treated with 256uM LA. Bars represent the means ± SE of 3-4 experiments. Within each fatty acid concentration group, bars that do not share a letter are significantly different (p<0.05).

3.4   Discussion

The two most common CLA isomers, c9,t11 and t10,c12 used in the present study, have been reported to reduce the incidence and size of mammary tumours in rodent models when they were fed together or individually (3;25). Consistent with the animal models, the anticarcinogenic activity of these two isomers has been demonstrated in human breast cell lines, (26;27) Despite both isomers being reported to have anti-cancer activity there continues to be questions as to the level of activity of, c9,t11 or t10,c12, and the mechanism as to how they mediate this cytotoxic activity against tumour cells.   Our results clearly demonstrate that on a gram per gram basis t10,c12 is the more inhibitory of the two isomers. This is consistent with other reports in the literature, also performed on estrogen-receptor positive MCF-7 cells (4;6;28).   Our work extends these earlier reports by confirming that this occurs in the presence of LA.

The effect of the essential fatty acid LA on the growth of various breast cancer cell lines has not been clearly demonstrated in the literature (29). Half of the studies that compared the supplementation of breast cancer cells with LA versus no exogenous fatty acids reported an increased growth (30),(31),(32),(26),(20) whereas the others noted either no effect (27),(33),(34;35) or an inhibitory effect of LA (20;36). In the current study LA did not stimulate or inhibit growth as compared to the untreated cells in conditions where serum was present and the concentration of LA was lower than 150uM. When the concentration of LA reached or exceeded 150μM, growth was inhibited. This finding is consistent with Miller (20) who reported that LA did not inhibit growth of MCF-7 cells, compared to cells grown in the absence of exogenous fatty acids, until concentrations were greater than 156μM.

In contrast, when serum was removed for the IGF-1 experiments LA did not inhibit growth below a concentration of 256μM, as compared to the untreated group. This apparent lack of inhibition by LA at high concentrations may be due to an essential fatty acid deficiency in the untreated cells. This deficiency might have been exaggerated by providing t10,c12 CLA. To rule out this possibility the next set of experiments were

performed by ensuring that LA was added to all the fatty acid treatments. Due to LA's lack of stimulatory activity, the monounsaturated fatty acid, Oleic Acid (OA), was added as a positive control.

When OA is complexed to BSA, similar to this study's protocol, it has been shown to stimulate the proliferation of MCF-7 cells as compared to untreated cells (37). For consistency with the other treatment groups we co-incubated OA with LA. To our knowledge there are no published studies that have examined the supplementation of LA and OA together on the viability of breast cancer cells. This study showed that the mixture of OA and LA in the presence of IGF-I led to a doubling of cell viability as compared to both LA alone and untreated cells after 48hr of treatment. Together they also appeared to protect against cell death as compared to the other fatty acid treated groups. In comparison the increased cell death in cells treated with LA alone at $256\mu M$ compared to OA or untreated cells indicates that LA at high doses is indeed cytotoxic to breast cancer cells. Confirmation that this effect is due to the high dose of LA or the absence of other fatty acids such as OA in the serum-free environment needs to be explored further.

We did not examine the effect of the 18C saturated fatty acid stearic acid, in the present study. To our knowledge the effect of albumin-bound stearic acid on breast cancer cell viability has not been established, although free stearic acid has been shown to inhibit the growth of rat mammary tumour cells as compared to LA or OA (38). It may be of benefit to include this fatty acid as a comparison in future studies.

The CLA isomer t10,c12 decreased viability at 48hr of treatment compared to the untreated group and all other fatty acids. The addition of LA in the serum-free conditions did not change the effect of t10,c12 on the metabolic activity of MCF-7 cells. This suggests that t10,c12's inhibitory effect on MCF-7 cells is not dependant on the presence of LA. The greater decrease in viability in the conditions where IGF-I was the sole exogenous source of growth stimulation requires further exploration as it suggests that the mechanism for the growth effects of t10,c12's might be mediated through interference of IGF-I signaling.

Interestingly, incubation with t10,c12 CLA did not significantly decrease the metabolic activity of cells at 24hr, but it did increase our measure of cytotoxicity (LDH release) compared to the other treatments. This might be the result of the ways that these two assays are measured. LDH release assess the amount of LDH released into the media at 24 hr while the viability assay looks at the remaining live cells and assessed the increase in mitochondrial reductase activity. Our results suggest that at 24hr, a significant number of t10c12 CLA treated cells had died but the remaining whole cells had a similar mitochondrial activity to the other groups.

When provided at the same concentrations the effects of c9,t11 CLA did not differ from LA in both serum and serum free conditions. However in serum-free conditions where c9,t11 CLA was provided with LA for 48 hr it resulted in a lower viability of MCF-7 cells, although it was not as inhibitory as t10,c12 CLA. The differential growth inhibitory effect dependant on the presence of LA indicates that the growth inhibitor effects of c9,t11 CLA may be through the interference of LA metabolism. MCF-7 cells are not considered a good model to study essential fatty acid metabolism due to their reported deficiencies of Δ6-desaturase and 12-lipoxygenase, key enzymes in the desaturation and oxidation of LA and arachidonic acid to downstream eicosanoids (29). The only other published study that examined the effect of CLA isomers co-incubated with LA was in MDA-MB-231 estrogen receptor negative cells (5). Unlike in MCF-7 cells the enzymes involved in essential fatty acid metabolism are completely functional in MDA-MB-231 cells, and that study reported a growth stimulatory effect of LA. They also observed that the inhibitory effect of c9,t11 when co-incubated with LA was not strongly related to a reduction in phospholipid amounts of arachidonic acid and it was hypothesized that the inhibition by c9,t11 in the presence of LA could occur further downstream of the initial production of essential fatty acid metabolites.

The orientation of double bonds in fatty acids, such as a cis or trans bond, or the length of a fatty acid impacts the positioning and spacing of neighboring fatty acids in the plasma membrane (40). Changes to the fatty acid composition of the plasma membrane and

subsequently the physical structure have been shown to alter lipid-protein interactions, and ultimately the function of the membrane (40;41). Due to it's conjugated cis and trans double bonds, the cytotoxic mechanism of t10,c12 CLA in the MCF-7 breast cancer cells may be attributable to it's incorporation into and subsequent disruption of the cellular membrane functions. Based on the findings that t10,c12 reduced cell viability when IGF-I was the sole exogenous source of growth stimulation, suggests that further investigation to determine if t10,c12 interferes with the cell's responsiveness to IGF-I is warranted.

3.5　Literature Cited

1. Schley PD, Jijon HB, Robinson LE, Field CJ. Mechanisms of omega-3 fatty acid-induced growth inhibition in MDA-MB-231 human breast cancer cells. Breast Cancer Res Treat 2005;92:187-95.

2. Hardman WE. (n-3) fatty acids and cancer therapy. J Nutr 2004;134:3427S-30S.

3. Ip C, Dong Y, Ip MM et al. Conjugated linoleic acid isomers and mammary cancer prevention. Nutr Cancer 2002;43:52-8.

4. Wang LS, Huang YW, Sugimoto Y et al. Effects of human breast stromal cells on conjugated linoleic acid (CLA) modulated vascular endothelial growth factor-A (VEGF-A) expression in MCF-7 cells. Anticancer Res 2005;25:4061-8.

5. Ma DW, Field CJ, Clandinin MT. An enriched mixture of trans-10,cis-12-CLA inhibits linoleic acid metabolism and PGE2 synthesis in MDA-MB-231 cells. Nutr Cancer 2002;44:203-12.

6. Tanmahasamut P, Liu J, Hendry LB, Sidell N. Conjugated linoleic acid blocks estrogen signaling in human breast cancer cells. J Nutr 2004;134:674-80.

7. Chujo H, Yamasaki M, Nou S, Koyanagi N, Tachibana H, Yamada K. Effect of conjugated linoleic acid isomers on growth factor-induced proliferation of human breast cancer cells. Cancer Lett 2003;202:81-7.

8. Dhiman TR, Nam SH, Ure AL. Factors affecting conjugated linoleic Acid content in milk and meat. Crit Rev Food Sci Nutr 2005;45:463-82.

9. Hubbard NE, Lim D, Erickson KL. Effect of separate conjugated linoleic acid isomers on murine mammary tumourigenesis. Cancer Lett 2003;190:13-9.

10. Masso-Welch PA, Zangani D, Ip C et al. Isomers of conjugated linoleic acid differ in their effects on angiogenesis and survival of mouse mammary adipose vasculature. J Nutr 2004;134:299-307.

11. Dauchy RT, Dauchy EM, Sauer LA et al. Differential inhibition of fatty acid transport in tissue-isolated steroid receptor negative human breast cancer xenografts perfused in situ with isomers of conjugated linoleic acid. Cancer Lett 2004;209:7-15.

12. Pollak MN. Insulin-like growth factors and neoplasia. Novartis Found Symp 2004;262:84-98.

13. Schernhammer ES, Holly JM, Pollak MN, Hankinson SE. Circulating levels of insulin-like growth factors, their binding proteins, and breast cancer risk. Cancer Epidemiol Biomarkers Prev 2005;14:699-704.

14. Renehan AG, Zwahlen M, Minder C, O'dwyer ST, Shalet SM, Egger M. Insulin-like growth factor (IGF)-I, IGF binding protein-3, and cancer risk: systematic review and meta-regression analysis. Lancet 2004;363:1346-53.

15. Kleinberg DL, Feldman M, Ruan W. IGF-I: an essential factor in terminal end bud formation and ductal morphogenesis. J Mammary Gland Biol Neoplasia 2000;5:7-17.

16. Sternlicht MD. Key stages in mammary gland development: the cues that regulate ductal branching morphogenesis. Breast Cancer Res 2006;8:201.

17. Ibrahim A, Natrajan S, Ghafoorunissa R. Dietary trans-fatty acids alter adipocyte plasma membrane fatty acid composition and insulin sensitivity in rats. Metabolism 2005;54:240-6.

18. Ip C, Singh M, Thompson HJ, Scimeca JA. Conjugated linoleic acid suppresses mammary carcinogenesis and proliferative activity of the mammary gland in the rat. Cancer Res 1994;54:1212-5.

19. Kim EJ, Kang IJ, Cho HJ, Kim WK, Ha YL, Park JH. Conjugated linoleic acid downregulates insulin-like growth factor-I receptor levels in HT-29 human colon cancer cells. J Nutr 2003;133:2675-81.

20. Miller A, Stanton C, Murphy J, Devery R. Conjugated linoleic acid (CLA)-enriched milk fat inhibits growth and modulates CLA-responsive biomarkers in MCF-7 and SW480 human cancer cell lines. Br J Nutr 2003;90:877-85.

21. Ip C. Review of the effects of trans fatty acids, oleic acid, n-3 polyunsaturated fatty acids, and conjugated linoleic acid on mammary carcinogenesis in animals. Am J Clin Nutr 1997;66:1523S-9S.

22. Keating AF, Kennelly JJ, Zhao FQ. Characterization and regulation of the bovine stearoyl-CoA desaturase gene promoter. Biochem Biophys Res Commun 2006;344:233-40.

23. Guvakova MA, Adams JC, Boettiger D. Functional role of alpha-actinin, PI 3-kinase and MEK1/2 in insulin-like growth factor I receptor kinase regulated motility of human breast carcinoma cells. J Cell Sci 2002;115:4149-65.

24. Lynch L, Vodyanik PI, Boettiger D, Guvakova MA. Insulin-like growth factor I controls adhesion strength mediated by alpha5beta1 integrins in motile carcinoma cells. Mol Biol Cell 2005;16:51-63.

25. Ip C, Chin SF, Scimeca JA, Pariza MW. Mammary cancer prevention by conjugated dienoic derivative of linoleic acid. Cancer Res 1991;51:6118-24.

26. Shultz TD, Chew BP, Seaman WR. Differential stimulatory and inhibitory responses of human MCF-7 breast cancer cells to linoleic acid and conjugated linoleic acid in culture. Anticancer Res 1992;12:2143-5.

27. Miller A, Stanton C, Devery R. Modulation of arachidonic acid distribution by conjugated linoleic acid isomers and linoleic acid in MCF-7 and SW480 cancer cells. Lipids 2001;36:1161-8.

28. Kemp MQ, Jeffy BD, Romagnolo DF. Conjugated linoleic acid inhibits cell proliferation through a p53-dependent mechanism: effects on the expression of G1-restriction points in breast and colon cancer cells. J Nutr 2003;133:3670-7.

29. Rose DP. Effects of dietary fatty acids on breast and prostate cancers: evidence from in vitro experiments and animal studies. Am J Clin Nutr 1997;66:1513S-22S.

30. O'Shea M, Devery R, Lawless F, Murphy J, Stanton C. Milk fat conjugated linoleic acid (CLA) inhibits growth of human mammary MCF-7 cancer cells. Anticancer Res 2000;20:3591-601.

31. Park Y, Allen KG, Shultz TD. Modulation of MCF-7 breast cancer cell signal transduction by linoleic acid and conjugated linoleic acid in culture. Anticancer Res 2000;20:669-76.

32. Cunningham DC, Harrison LY, Shultz TD. Proliferative responses of normal human mammary and MCF-7 breast cancer cells to linoleic acid, conjugated linoleic acid and eicosanoid synthesis inhibitors in culture. Anticancer Res 1997;17:197-203.

33. Majumder B, Wahle KW, Moir S et al. Conjugated linoleic acids (CLAs) regulate the expression of key apoptotic genes in human breast cancer cells. FASEB J 2002;16:1447-9.

34. Maggiora M, Bologna M, Ceru MP et al. An overview of the effect of linoleic and conjugated-linoleic acids on the growth of several human tumour cell lines. Int J Cancer 2004;112:909-19.

35. Kim JH, Hubbard NE, Ziboh V, Erickson KL. Attenuation of breast tumour cell growth by conjugated linoleic acid via inhibition of 5-lipoxygenase activating protein. Biochim Biophys Acta 2005;1736:244-50.

36. sBordes C, Lea MA. Effects of C18 fatty acid isomers on DNA synthesis in hepatoma and breast cancer cells. Anticancer Res 1995;15:2017-21.

37. Hardy S, Langelier Y, Prentki M. Oleate activates phosphatidylinositol 3-kinase and promotes proliferation and reduces apoptosis of MDA-MB-231 breast cancer cells, whereas palmitate has opposite effects. Cancer Res 2000;60:6353-8.

38. Wicha MS, Liotta LA, Kidwell WR. Effects of free fatty acids on the growth of normal and neoplastic rat mammary epithelial cells. Cancer Res 1979;39:426-35.

39. Chajes V, Hulten K, Van Kappel AL et al. Fatty-acid composition in serum phospholipids and risk of breast cancer: an incident case-control study in Sweden. Int J Cancer 1999;83:585-90.

40. Stubbs CD, Smith AD. The modification of mammalian membrane polyunsaturated fatty acid composition in relation to membrane fluidity and function. Biochim Biophys Acta 1984;779:89-137.

41. Grimble RF, Tappia PS. Modulatory influence of unsaturated fatty acids on the biology of tumour necrosis factor-alpha. Biochem Soc Trans 1995;23:282-7.

4. CLA incorporates into cell phospholipids and reduces total IGFIR protein.

4.1   Introduction

The polyunsaturated fatty acid conjugated linoleic acid (CLA) is formed during the biohydrogenation of linoleic acid (LA) by ruminant animals. The two main isomers of CLA, c9,t11 and t10,c12 have been shown to exert anti-tumourgenic effects on mammary cancer in animal and human breast cancer cell line models. (1) Although they are both conjugated fatty acids with a cis and a trans double bond, findings in the literature suggest that they may function by different mechanisms of action (2).   The isomer t10,c12 appears to have a greater level of direct cytotoxicity than that of c9,t11 against MCF-7 cells (chapter 3). The method by which t10,c12 CLA exerts it's direct cytotoxicity on breast cancer cells has not yet been established.

Modifications of membrane phospholipid composition due to the incorporation of different fatty acids can alter the function of membrane-bound receptors (3;4). Incorporation of n-3 fatty acids into breast cancer cell phospholipids has been shown to alter the phosphorylation and location of the epidermal growth factor receptor, in the cell membrane (5). Similar to n-3 fatty acids, CLA has been shown to be incorporated into MDA-MB-231 breast cancer cellular membrane phospholipids (6). However, to our knowledge there has been no study to examine the incorporation of CLA into membrane phospholipids and resulting modifications to the activity of membrane bound receptors.

The receptor for insulin-like growth factor (IGF) is located in the outer membrane of the cell. Upon binding of the ligand, IGF-I, the receptor IGFIR tyrosine phosphorylates, which initiates various signaling cascades resulting in an increase in cellular proliferation and an inhibition of apoptosis (7). IGFIR levels and IGF signaling has been shown to be upregulated in breast cancer tumours and MCF-7 breast cancer cells (8) (9).

In this chapter we establish that t10,c12 CLA is incorporated into and modifies the fatty acid profile of phospholipids of MCF-7 breast cancer cells in the presence of IGF-I. We examine these changes in relation to the reduction in the breast cancer cells' responsiveness to IGF-I and link it possibly to modifications to the IGFIR.

4.2 Materials and Methods

Phospholipid fatty acid composition

Lipids were extracted from whole cell pellets after 24hr treatment with IGF-I and fatty acids explained in chapter 3 using a modified Folch Procedure (10) as previously described (11). Total phospholipids were isolated on silica gel 'g' thin layer chromatography plate (Whatman Inc. Clifton NJ USA) using an 80:20:1 v/v/v ratio of solvents (petroleum ether: diethyl ether: glacial acetic acid) for 1 hour. The phospholipid band was identified using 8-anilino-1-naphthalene-sulfonic acid under long wave ultraviolet light. Methyl esters of the phospholipids were prepared by adding sodium methoxide and benzene (12) and heated at 50°C for 10 minutes. Samples were then dissolved in hexane and the fatty acids were then separated by automated gas liquid chromatography using hydrogen gas on 100m CP-Sil 88 fused capillary column (Varian Inc, Mississauga, Ont) as previously described in detail (13).

Western blot analysis for total IGFIR

Lysates of whole cells were prepared using lysis buffer (20 mM TrisHCl pH 7.4, 137 mM NaCl, 10 % v/v glycerol, 1 % v/v Nonidet P-40, 2 mM EDTA) in the presence of protease and phosphatase inhibitor cocktails (P8340 & P2850 Sigma-Aldrich Canada Ltd., Oakville, Ontario, Canada). Protein content was determined using the Bicinchoninic acid (BCA) protein assay (Sigma-Aldrich Canada Ltd., Oakville, Ontario, Canada) according to manufacturer's instructions. Equal amounts of protein from each treatment were separated by SDS-PAGE on a 10% polyacrylamide gel, then transferred electrophoretically onto Nitrocellulose membrane (Amersham Biosciences, Baie D'Urfe, Quebec, Canada) at 200mAmps for 3 hours. Precision Plus All Blue marker (BioRad Laboratories, Hercules, CA) was utilized to mark molecular weight. Even protein loading

between treatments was confirmed by use of Ponceau Red S staining (Sigma-Aldrich Canada Ltd., Oakville, Ontario, Canada). Membranes were blocked by 5% w/v powdered milk in TBST (0.01M TrisHCL pH 6.8, 0.15 M NaCl, 0.1% Tween-20) for one hour. After rinsing with TBST, membranes were incubated overnight at 4°C in the presence of IGFIRβ C-20 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:10000 in 1% w/v milk in TBST. A 1:10000 dilution of the Secondary antibody HRP conjugated donkey anti-rabbit IgG (Jackson Immunoresearch Labs, Burlington, Ont. Canada) in 1% w/v milk in TBST was incubated with the membrane for 1 hour at room temperature. Membranes were developed using Amersham ECL Plus Western Blotting Detection Reagents (GE Healthcare Life Sciences, Buckinghamshire UK) then read on Typhoon trio + variable mode imager (Amersham Biosciences, GE Healthcare Life Sciences), using fluorescence, ECL+ setting. The intensity of target bands was quantified using Image Quant TL software (Amersham Biosciences).

4.3   Results

Fatty acid composition of phospholipids

The CLA Isomers c9,t11 and t10,c12 were incorporated respectively into membrane phospholipids but the incorporation of t10,c12 appeared to occur to a greater extent than C9,t11 CLA (32% vs 22%). The higher incorporation in the t10c12 groups occurred at the expense of total SFA as the relative content of total n-6 and n-3 fatty acids and the proportion of 20:4(6) did not differ between the two CLA groups.

Similarly incubation with LA and with oleic acid significantly increased the incorporation of these fatty acids into membrane phospholipids. Compared to the OA (the group that had the greatest cell growth), PL from all of the treatment groups had significantly less C18:1(9). There were no other major differences with the CLA groups (except the difference in CLA content). Compared to the LA group, PL from all of the treatment groups had significantly less LA, and C20:2(6). There was significantly more SFA in the PL of LA incubated cells compared to those incubated in T10c12 CLA.

The untreated group had significantly less total n-6 PUFA (% of total phospholipids), specifically LA, as compared to all the fatty acid treatments, however the content of 20:6(6) was higher than all the treatment groups. As a result the untreated group had a greater proportion of SFA compared to OA and t10,c12 treated cells as well as a greater proportion of MUFA as compared to LA, c9,t11 and t10,c12 treated cells.

Fatty Acid	Untreated	LA	Oleic	c9,t11	t10,c12	significance
C 14:0	3.5 ± 1.0^a	0.8 ± 0.2^b	1.1 ± 0.1^b	1.1 ± 0.2^b	1.1 ± 0.2^b	P< 0.05
C 16:0	14 ± 1.0	10.2 ± 0.4	8.3 ± 0.7	14.9 ± 6.8	7.4 ± 0.4	NS
C 17:0	0.3 ± 0.0	0.1 ± 0.1	0.1 ± 0.1	0.2 ± 0.0	0.2 ± 0.0	NS
C 18:0	14.0 ± 3.0^b	17.6 ± 3.3^a	13.0 ± 2.8^b	11.0 ± 1.5^b	11.3 ± 2.0^b	P < 0.01
C 20:0	0.3 ± 0.0	0.2 ± 0.1	0.2 ± 0.1	0.1 ± 0.0	0.2 ± 0.0	NS
C 22:0	0.2 ± 0.0	0.1 ± 0.1	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	NS
Total SFA	33 ± 2^a	29.1 ± 3.4^{ab}	23.6 ± 3.1^{bc}	28.8 ± 7.8^{ab}	20.3 ± 2.6^c	P < 0.01
c 14:1	2.8 ± 1.6^a	1.2 ± 0.7^{ab}	0.0 ± 0.0^b	0.0 ± 0.0^b	0.0 ± 0.0^b	p < 0.05
C 16:1 (t9)	1.9 ± 1.0	0.5 ± 0.0	$0.8 \pm.0.3$	0.8 ± 0.6	0.8 ± 0.4	NS
C 16:1 (c9)	11.7 ± 2.3^a	1.4 ± 0.4^b	1.5 ± 0.4^b	2.1 ± 0.3^b	1.7 ± 0.5^b	P < 0.01
C 18:1 (t11)	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.1	0.2 ± 0.1	0.2 ± 0.0	NS
C 18:1 (c9)	26.1 ± 0.4^b	12.1 ± 1.5^c	38.3 ± 3.3^a	13.5 ± 4.2^c	10.0 ± 1.0^c	P < 0.01
c 18:1 (c11)	5.6 ± 0.7^a	1.6 ± 0.2^b	1.2 ± 0.2^b	1.6 ± 0.3^b	1.3 ± 0.2^b	P < 0.01
C 20:1 (c9)	0.01 ± 0.0^b	0.1 ± 0.1^b	0.1 ± 0.1^b	1.3 ± 0.5^a	1.3 ± 0.3^a	P < 0.01
Total MUFA	48.9 ± 4.4^a	16.9 ± 0.7^b	42.1 ± 3.7^a	19.6 ± 4.0^b	15.5 ± 1.2^b	P < 0.01
C 18:2 (6)	8.5 ± 3.5^c	47.8 ± 4.1^a	29.1 ± 2.7^b	25.8 ± 6.2^b	27.9 ± 1.8^b	P < 0.01
C 20:2 (6)	0.1 ± 0.0^b	2.8 ± 0.5^a	1.0 ± 0.1^b	1.2 ± 0.5^b	0.4 ± 0.1^b	P < 0.01
C 20:3 (6)	0.6 ± 0.1^a	0.1 ± 0.1^c	0.3 ± 0.0^b	0.2 ± 0.1^{bc}	0.2 ± 0.0^{bc}	P < 0.01
C 20:4 (6)	4.3 ± 0.6^a	1.2 ± 0.2^b	1.4 ± 0.3^b	1.2 ± 0.3^b	1.3 ± 0.2^b	P < 0.01
C 22:2 (6)	ND	0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	NS
C 22:4 (6)	0.2 ± 0.0	0.0 ± 0.0	0.5 ± 0.5	0.0 ± 0.0	0.1 ± 0.0	NS
C 22:5 (6)	0.4 ± 0.1	0.3 ± 0.2	0.2 ± 0.1	0.2 ± 0.0	0.2 ± 0.0	NS
Total n-6	13.9 ± 2.8^c	52.4 ± 3.9^a	32.5 ± 2.0^b	28.7 ± 6.7^b	30.1 ± 1.6^b	P < 0.01
C 18:3 (3)	0.4 ± 0.1^b	0.7 ± 0.3^{ab}	1.0 ± 0.2^a	0.3 ± 0.1^b	0.5 ± 0.2^b	p < 0.01
C 20:5 (3)	1.4 ± 0.9	0.1 ± 0.0	0.00 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	NS
C 22:5 (3)	1.2 ± 1.0	0.2 ± 0.1	0.2 ± 0.2	0.2 ± 0.1	0.4 ± 0.3	NS
C 22:6 (3)	1.1 ± 0.2^a	0.7 ± 0.3^b	0.6 ± 0.2^{bc}	0.3 ± 0.0^c	0.5 ± 0.2^c	p < 0.01
Total n-3	4.1 ± 1.9^a	1.6 ± 0.5^{ab}	1.8 ± 0.4^{ab}	0.9 ± 0.1^b	1.4 ± 0.5^b	P < 0.05
c9,t11 CLA	ND	ND	ND	22.0 ± 5.4	ND	P < 0.01
t10,c12 CLA	ND	ND	ND	0.1 ± 0.5	32.0 ± 4.0	P < 0.01
Total PUFA (incl. CLA)	16.8 ± 2.9^c	51.3 ± 2.4^{ab}	32.6 ± 1.3^{bc}	48.9 ± 10.9^{bc}	60.4 ± 2.4^a	P < 0.05

Table 4:1: **Fatty acid composition of whole cell phospholipids.** MCF-7 breast cancer cells were exposed to fatty acids for 24hr in serum free conditions. Values are percentages of total fatty acids and are expressed as a mean ± standard error they were determined from 3-4 experiments. Values within a row that do not share a common superscript letter were significantly different. Values within a row with no superscripts are not significantly different. Both CLA isomers contain two double bonds therefore they are included in total PUFA. ND, not detectable; NS, not significant; SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; n-6, fatty acids whose first double bond is six carbons from the methyl end of the fatty acid; n-3, fatty acids whose first double bond is three carbons from the methyl end of the fatty acid; CLA, conjugated linoleic acid; PUFA, polyunsaturated fatty acid, LA, linoleic acid.

Results of western blot analysis for total IGFIR

Treatment with the CLA isomer t10,c12 resulted in reduced total cellular IGFIR protein levels in MCF-7 cells as compared to all other groups. Both LA and c9,t11 reduced IGFIR protein as compared to OA and untreated cells, but were not significantly different from each other.
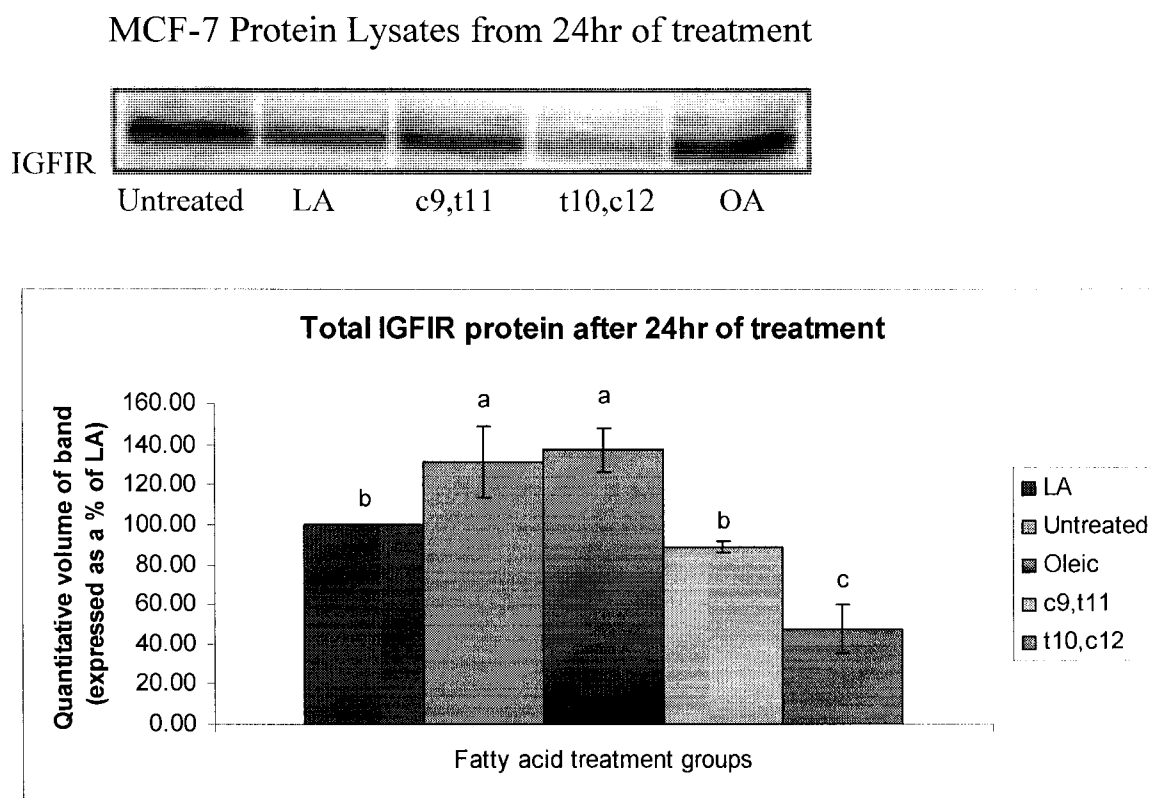
MCF-7 Protein Lysates from 24hr of treatment

IGFIR

Untreated   LA   c9,t11   t10,c12   OA

Figure 3-1: **Effect of fatty acids on total IGFIR protein in MCF-7 breast cancer cells after 24hr treatment in the presence of IGF-I.** Results are representative of 3 independent experiments. Treatments that do not share a letter are significantly different, based on quantification of band volume and intensity ($p<0.05$).

4.4   Discussion

The phospholipid fatty acid composition of untreated cells was significantly different from all the fatty acid treated cells, therefore a decision was made that it was not an appropriate 'control' group to compare the effects of fatty acid incubation on membrane composition and function. Although we hypothesized the untreated cells would experience an essential fatty acid deficiency in the serum-free conditions, the observed

greater proportion of arachidonic acid (AA) as compared to the other groups, does not support this hypothesis. This lower depletion of AA is consistent with the reduced growth rate that was observed in the untreated MCF-7 group.

Both CLA isomers, c9,t11 and t10,c12 are structurally similar in that they contain two conjugated double bonds, one in the cis formation and the other in the trans position. Using synthetic model membranes Yin et al. showed that incorporation of c9,t11 CLA perturbed membrane structure at the location of the conjugated double bonds and increased oxygen permeability, as compared to LA, MUFA and SFA (14). The perturbation was defined using electron spin resonance technology and they suggested that this could result in an alteration in lipid-protein interactions in the cell membrane (15). Although, this study only used the c9,t11 isomer, it is likely that their results are relevant to the incorporation of t10,c12 as it also contains a conjugated double bond. As t10,c12 was incorporated into the cell membrane to a greater extent than c9,t11 (33% vs. 22%), this suggests that the membrane might be more disrupted in this group.

The greater incorporation of t10,c12 was at the expense of saturated fatty acids as compared to the LA and c9,t11 CLA treated groups. However it is unlikely that the change in SFA concentration in the membrane accounts for the differences in growth as the concentration of SFA in the t10,c12 CLA treated group was not different from that of the OA treated group. Our results suggest that the high incorporation of the t10,c12 isomer might be responsible for the difference in biological function, rather than the displacement of a specific saturated or unsaturated fatty acid.

Cells from the LA and c9,t11 treatments did not differ significantly in their relative content of total PUFA, SFA, MUFA and n-3 fatty acids, except for the content of LA and c9,t11 CLA, respectively. These similarities in fatty acid concentration in cellular PL are consistent with the similar effects on cellular viability and death.

OA treated and untreated cells had a higher relative content of MUFA than the other three treatments. The greater proportion of MUFA in the OA treated group was due to the high incorporation of OA, whereas for the untreated group 16:1, 18:1 (c9), and 18:1 (c11)

all were greater than the CLA or LA groups. The increased MUFA in the OA group occurred without a proportional replacement of any one specific fatty acid or fatty acid group, as compared to the LA, c9,t11 or t10,c12 treated groups. As both the untreated and OA treated group had reduced rates of cell death as compared to the other groups, this may indicate that 18:1 incorporation into cellular phospholipids is important for cell survival. Similar to our results, incubation with OA was reported to promote cell survival in the MDA-MB-231, MDA-MB-468 and T47D breast cancer cell lines in serum-free conditions (16;17) as compared to LA, AA, DHA and palmitate (17). However they did not report any survival benefit of OA in the cell line used in this study, MCF-7 (16).

Changes in the fatty acid composition of membrane PL are well established to alter the functional activity of cell membranes, including the activity of membrane associated receptors (3) (4). Addition of long chain polyunsaturated fatty acids (DHA but not AA) was reported to increase the number of insulin receptors in retinoblastoma cells, although this same study did not note a change in the binding ability of IGF-I receptors, nor did they study the addition of LA or trans fatty acids (18). The current study used total cell phospholipids to evaluate the association with changes in the total amount of the IGF-I receptor in MCF-7 cells. Although changes to the fatty acid profile of the total phospholipids has been linked with disruptions to membrane function there are other more specific changes to the membrane lipids that have also been linked with alterations in membrane function. Phospholipids consist of different phospholipid classes defined by the structure attached to their polar end, such as choline in phosphatidylcholine. Each phospholipid class has various and unique roles in membrane structure and alterations to their fatty acid composition have been shown to alter the function of membrane receptors (4;19). Therefore it may be of benefit to examine alterations to the fatty acid profile of different phospholipid classes in future experiments. In addition the plasma membrane contains microdomains of saturated lipids that have clustered together to form lipid "rafts" (5). Receptors and proteins involved in cellular signaling have been shown to aggregate in these lipid rafts (20). Our group has shown that incubation of estrogen-receptor negative MDA-MB-231 breast cancer cells with n-3 fatty acids can alter the fatty acid composition of lipid rafts, which resulted in a reduction of epidermal growth factor

receptors (EGFR) located in lipid rafts. This displacement of EGFR led to an increase in whole cell tyrosine phosphorylated EGFR, which was hypothesized to be associated with an increase in apoptosis (5). Since IGFIR is reported to also be located in lipid rafts (21), the disruption of the lipid composition of these microdomains due to CLA is a future area of investigation to pursue.

The observed change in total amount of IGFIR in the different treatments is consistent with the LDH results, presented in the previous chapter. The untreated and OA treated groups demonstrated the least amount of LDH release/cell death and had the greatest amount of IGFIR. In contrast the t10,c12 CLA treated group had the greatest cell death with the lowest amount of total IGFIR protein. As changes to the total protein level of the transmembrane signaling molecule is most likely due to changes at the genetic or transcription level, in the current study it is not possible to directly link the changes in total IGFIR to specific lipid changes in membrane PL. However the dramatic incorporation of t10,c12 into cell phospholipids indicates a probable disruption of the function of membrane associated proteins, such as IGFIR. Furthermore the observed reduction in total IGFIR protein levels in the t10,c12 treated cells supports possible t10,c12 CLA induced interference with IGF signaling.

In order to confirm t10,c12 CLA interference with this pathway in breast cancer cells further exploration of the activation of the IGF-IR and the possible link to the membrane perturbations is warranted. In addition to our findings that the total amount of IGFIR is decreased, it would be of benefit to examine whether the provision of t10,c12 reduced the activation or responsiveness of the IGFIR through a reduction in phosphorylation of the receptor. It may also be of benefit to examine the activation of downstream signaling mediators of IGFIR such as insulin receptor substrate 1, IRS-I, phosphatidylinositol 3-kinase, PI3K, or extracellular signal-related kinase (ERK). Kim et al (22) showed that a mixture of CLA isomers decreased IGFIR levels and the activation of downstream signals in HT-29 colon cancer cells. Our findings with reduced IGFIR levels in breast cancer cells incubated with the t10,c12 CLA isomer suggest that this is the active isomer in the mixture.

4.5   Literature Cited

1. Ip MM, Masso-Welch PA, Ip C. Prevention of mammary cancer with conjugated linoleic acid: role of the stroma and the epithelium. J Mammary Gland Biol Neoplasia 2003;8:103-18.

2. Ip C, Dong Y, Ip MM et al. Conjugated linoleic acid isomers and mammary cancer prevention. Nutr Cancer 2002;43:52-8.

3. Hulbert AJ, Turner N, Storlien LH, Else PL. Dietary fats and membrane function: implications for metabolism and disease. Biol Rev Camb Philos Soc 2005;80:155-69.

4. Spector AA, Burns CP. Biological and therapeutic potential of membrane lipid modification in tumours. Cancer Res 1987;47:4529-37.

5. Schley PD, Brindley DN, Field CJ. (n-3) PUFA alter raft lipid composition and decrease epidermal growth factor receptor levels in lipid rafts of human breast cancer cells. J Nutr 2007;137:548-53.

6. Ma DW, Field CJ, Clandinin MT. An enriched mixture of trans-10,cis-12-CLA inhibits linoleic acid metabolism and PGE2 synthesis in MDA-MB-231 cells. Nutr Cancer 2002;44:203-12.

7. Pollak MN. Insulin-like growth factors and neoplasia. Novartis Found Symp 2004;262:84-98.

8. Nahta R, Hortobagyi GN, Esteva FJ. Growth factor receptors in breast cancer: potential for therapeutic intervention. Oncologist 2003;8:5-17.

9. Peyrat JP, Bonneterre J, Dusanter-Fourt I, Leroy-Martin B, Djiane J, Demaille A. Characterization of insulin-like growth factor 1 receptors (IGF1-R) in human breast cancer cell lines. Bull Cancer 1989;76:311-9.

10. FOLCH J, LEES M, SLOANE STANLEY GH. A simple method for the isolation and purification of total lipides from animal tissues. J Biol Chem 1957;226:497-509.

11. Field CJ, Ryan EA, Thomson AB, Clandinin MT. Dietary fat and the diabetic state alter insulin binding and the fatty acyl composition of the adipocyte plasma membrane. Biochem J 1988;253:417-24.

12. Kramer JK, Fellner V, Dugan ME, Sauer FD, Mossoba MM, Yurawecz MP. Evaluating acid and base catalysts in the methylation of milk and rumen fatty acids with special emphasis on conjugated dienes and total trans fatty acids. Lipids 1997;32:1219-28.

13. Cruz-Hernandez C, Kramer JK, Kennelly JJ et al. Evaluating the conjugated linoleic acid and trans 18:1 isomers in milk fat of dairy cows fed increasing amounts of sunflower oil and a constant level of fish oil. J Dairy Sci 2007;90:3786-801.

14. Yin JJ, Mossoba MM, Kramer JK et al. Effects of conjugated linoleic acid on oxygen diffusion-concentration product and depletion in membranes by using electron spin resonance spin-label oximetry. Lipids 1999;34:1017-23.

15. Wiedmann TS, Pates RD, Beach JM, Salmon A, Brown MF. Lipid-protein interactions mediate the photochemical function of rhodopsin. Biochemistry 1988;27:6469-74.

16. Przybytkowski E, Joly E, Nolan CJ et al. Upregulation of cellular triacylglycerol - free fatty acid cycling by oleate is associated with long-term serum-free survival of human breast cancer cells. Biochem Cell Biol 2007;85:301-10.

17. Hardy S, El-Assaad W, Przybytkowski E, Joly E, Prentki M, Langelier Y. Saturated fatty acid-induced apoptosis in MDA-MB-231 breast cancer cells. A role for cardiolipin. J Biol Chem 2003;278:31861-70.

18. Yorek M, Leeney E, Dunlap J, Ginsberg B. Effect of fatty acid composition on insulin and IGF-I binding in retinoblastoma cells. Invest Ophthalmol Vis Sci 1989;30:2087-92.

19. Lewis RE, Czech MP. Phospholipid environment alters hormone-sensitivity of the purified insulin receptor kinase. Biochem J 1987;248:829-36.

20. Brown DA, London E. Functions of lipid rafts in biological membranes. Annu Rev Cell Dev Biol 1998;14:111-36.

21. Huo H, Guo X, Hong S, Jiang M, Liu X, Liao K. Lipid rafts/caveolae are essential for insulin-like growth factor-1 receptor signaling during 3T3-L1 preadipocyte differentiation induction. J Biol Chem 2003;278:11561-9.

22. Kim EJ, Kang IJ, Cho HJ, Kim WK, Ha YL, Park JH. Conjugated linoleic acid downregulates insulin-like growth factor-I receptor levels in HT-29 human colon cancer cells. J Nutr 2003;133:2675-81.

5. General Summary and Discussion

5.1 Summary of results

A: incubating MCF-7 cells with CLA isomers will inhibit IGF-I –stimulated proliferation. This inhibition will be independent of the closely related essential fatty acid linoleic acid.

This hypothesis was supported by the results reported in chapter 3. We observed cytotoxicity with the t10,c12 CLA isomer but not the c9,t11 isomer when provided at the same concentration for the same length of time. The t10,c12 significantly reduced the viability of MCF-7 breast cancer cells as measured by Wst-1, a measure of mitochondrial reductase activity. This isomer also increased cell death as measured by lactate dehydrogenase release compared to the other fatty acid treated groups.

Effects of t10,c12 is unlikely due to a deficiency of LA as co-treatment with LA still resulted in a cytotoxic effect of this isomer.

B: after incubation with CLA isomers, MCF-7 cells will readily incorporate these isomers into their membrane lipid (phospholipids) and changes in membrane lipid will help explain differences in cell proliferation.

The results in chapter 4 support this hypothesis. All of the treatment fatty acids were incorporated into the MCF-7 phospholipids. The t10,c12 CLA isomer was incorporated into the phospholipids at greater concentration than c9,t11 CLA. The greater oleic acid (OA) in both the untreated and OA treated groups suggested a possible role of OA in cell survival.

C. alterations of the phospholipids as a result of incubation with CLA isomers will reduce the amount of the membrane bound receptor IGFIR and the ability of IGF-I to stimulate MCF-7 proliferation (or growth)

This hypothesis was supported in chapter 3 by the reduced viability of cells treated with t10,c12 in the presence of IGF-I and in chapter 4 by the observed decreased levels of total IGFIR.

5.2   Discussion

The overall objective of this research was to explore a logical mechanism by which CLA isomers exerted direct cytotoxicity on breast cancer cells. Some studies to date have attempted to address this question however this question remains unanswered. Prior to addressing the question we established a cell culture model whereby CLA isomers reduced the viability or increased cell death in MCF-7 breast cancer cells

Past research initially utilized a synthetically derived CLA mix containing a 43%/45% mix of two isomers, c9,t11 CLA, which is the primary isomer found in food sources and t10,c12 CLA, which is a minor isomer in food sources. Once the two isomers were available to be studied individually, it became apparent that they may be different in their effectiveness and mechanism of action (1). Ip et al. (1) showed that although there was 40% less accumulation of t10,c12 CLA than c9,t11 CLA in the mammary fat pad of rodents they both reduced the incidence of mammary cancer to the same extent. The results of our current research support previous cell culture studies that have shown t10,c12 CLA to be the more effective isomer of the two when provided at the same concentration (2;3). This may be related to it's higher incorporation when provided at the same concentration as the c9,t11 isomer.

Before investigating CLA's possible interference with IGF function in the cell, which was our proposed mechanism of action, it was important to establish inhibition of either or both CLA isomers in conditions whereby IGF-I was the sole source of growth stimulation. The isomer t10,c12 CLA has been shown to inhibit growth factor (estrogen and insulin) stimulated growth of MCF-7 cells (4). Similarly Kim et al. showed a CLA mix interfered with IGF-I signaling in colon cancer cells, but did not compare this effect to other fatty acids (5). In support of these findings our results demonstrated that t10,c12 CLA interfered with the ability of MCF-7 breast cancer cells to respond to IGF-I.

Changes to the fatty acid profile of a cell's membrane phospholipids has been shown to modify the activity of membrane bound proteins and ultimately cell function (6;7). We confirmed that CLA is incorporated into cell phospholipids. The CLA isomer t10,c12 appeared to be incorporated at a greater concentration than c9,t11 CLA and this greater incorporation may explain the difference in effectiveness between the two.

Interestingly the fatty acid OA appeared to promote cell survival in the cells compared to the other fatty acid treatments. The greater amounts of OA in the phospholipids in the untreated and OA groups were linked with reduced cell death as measured by LDH assay. A possible explanation for this protective effect of OA as compared to LA may be due to a previously reported reduction in the activity of Δ-6 desaturase enzyme in MCF-7 breast cancer cells (8). Δ-6 desaturase is the rate limiting enzyme in the conversion/metabolism of LA to arachidonic acid, a precursor for eicosanoids that are integral to LA's growth promotion of cells (8;9). In addition both CLA isomers c9,t11 and t10,c12 have been shown to reduce the activity of Stearoyl-CoA desaturase (SCD) (Δ-9 desaturase) in MCF-7 breast cancer cells (10). OA is not considered an essential fatty acid due to the cell's ability to endogenously produce OA through the activity of SCD. Because exogenous OA is not present in our serum free protocol a reduction in SCD due to CLA treatment could have resulted in a conditional essentiality of OA. There is limited and conflicting information as to OA's role in breast cancer. OA has been shown to reduce expression of Her-2/neu in SKBR-3 cells and synergistically reduce the viability of SKBr-3 and BT-474 breast cancer cells treated with trastuzumab (11). In contrast OA has been shown to stimulate growth in MDA-MB-231, MCF-7 & ZR-75-1 breast cancer cells but not T47-D (12). Animal studies have reported that the inhibition of mammary cancer by OA is dependant on the amount of LA that is present in the diet (13). The interaction between fatty acids is an important consideration for in vitro studies examining the effect of CLA on breast cancer cells. Fetal calf serum which was not utlized in the serum-free or IGF-I stimulation protocol in this study contains negligible amounts of CLA and the essential n-3 fatty acids along with low doses of LA (3μl if using 5% serum v/v) and OA (10μl) (25), therefore the simple addition of a base of fatty acids to match the fatty acid profile seen in

fetal calf serum may not be adequate to overcome any potential fatty acid conditional essentiality in our model. A future study may incorporate both LA and OA as supplemental bases with the treatment of CLA.

Taken together the results of this thesis support inhibition of breast cancer cells through the incorporation of t10,c12 CLA into cell phospholipids, however it does not exclude other possible mechanisms such as modifications to gene expression through PPAR agonists.

IGF-I signaling promotes cellular proliferation and is anti-apoptotic. It has been shown to be up-regulated in hormone-responsive breast cancer (14). IGF-I signaling in cells occurs through the activation of the membrane-bound receptor IGFIR. One possible way to determine if CLA's anticarcinogenic activity is primarily through interference with IGF-I signaling would be to incubate breast cancer cells with an IGFIR inhibitor, such as Tyrphostin AG1024, a monoclonal antibody (mab) for the receptor (15) or the new h7C10 a mab for both IGFIR and the hybrid IGFIR/insulin (16).

Changes to membrane phospholipid fatty acid content can alter the function of receptors located in the cell membrane (6;7). Although we were able to show that treatment with t10,c12 greatly reduced the amount of total IGFIR in breast cancer cells, with the data available in this thesis it is difficult to make a direct link between this and the disruption to the fatty acid composition of the phospholipids. It would be of benefit in future studies to examine the phosphorylation/activation of IGFIR as this occurs at the membrane level, or the activation of further downstream signals of IGFIR such as IRS-1, PI3K, or MAPK/erk and compare these changes to the fatty acid profile of the membrane phospholipids or lipid rafts.

Lipid rafts are membrane microdomains high in cholesterol, involved in the clustering of signaling proteins (17). Our group has shown that n-3 fatty acids were able to disrupt the epidermal growth factor receptor membrane location and overall activation, by altering the lipid content of breast cancer cell lipid rafts (18). IGFIR has been shown to be present

in lipid rafts, (19) and the possible disruption of lipid rafts by the incorporation of t10,c12 into membrane phospholipids remains a possible route of investigation.

Further potential hypotheses to explain the decrease in total IGFIR due to CLA treatment independent of membrane changes include, possible PPAR agonist activity and interference with estrogen signaling, both of which have been shown to be involved in the expression of IGFIR (15;20;21).

Research in rodents reported that CLA taken during puberty modified the development of the mammary gland and as a result offered lifelong protection from mammary cancer (22). These findings suggest the potential that CLA may also exert anti-carcinogenic activity through changes to the surrounding stroma in addition to direct cytotoxicity. Using MCF-7 cells Wang (23) showed that t10,c12 CLA was more effective at reducing cell proliferation when they were co-incubated in the presence of human breast stromal cells. IGF-I signaling is both paracrine and autrocrine and IGF-I growth factors are produced by stromal cells. The incorporation of CLA isomers in stromal cells and interference with IGF-I signaling in mammary cancer is another possible future area of investigation.

If further studies are to confirm CLA's interference with IGF signaling there is the potential to investigate CLA's ability to synergistically improve the effectiveness of certain chemotherapy treatments for breast cancer, such as Trastuzumab (herceptin). Trastuzumab targets the her2/neu receptor and resistance to it has been linked with up-regulated IGF-I signaling (24).

The findings in this thesis supports the anti-carcinogenic activity of the t10,c12 isomer of CLA. It demonstrates that this cytotoxicity may be through interference with the cell's ability to respond to IGF-I. Further investigation is required to definitively link this interference with modifications to the membrane of breast cancer cells.

5.3 Literature Cited

1. Ip C, Dong Y, Ip MM et al. Conjugated linoleic acid isomers and mammary cancer prevention. Nutr Cancer 2002;43:52-8.

2. Kemp MQ, Jeffy BD, Romagnolo DF. Conjugated linoleic acid inhibits cell proliferation through a p53-dependent mechanism: effects on the expression of G1-restriction points in breast and colon cancer cells. J Nutr 2003;133:3670-7.

3. Tanmahasamut P, Liu J, Hendry LB, Sidell N. Conjugated linoleic acid blocks estrogen signaling in human breast cancer cells. J Nutr 2004;134:674-80.

4. Chujo H, Yamasaki M, Nou S, Koyanagi N, Tachibana H, Yamada K. Effect of conjugated linoleic acid isomers on growth factor-induced proliferation of human breast cancer cells. Cancer Lett 2003;202:81-7.

5. Kim EJ, Kang IJ, Cho HJ, Kim WK, Ha YL, Park JH. Conjugated linoleic acid downregulates insulin-like growth factor-I receptor levels in HT-29 human colon cancer cells. J Nutr 2003;133:2675-81.

6. Hulbert AJ, Turner N, Storlien LH, Else PL. Dietary fats and membrane function: implications for metabolism and disease. Biol Rev Camb Philos Soc 2005;80:155-69.

7. Spector AA, Burns CP. Biological and therapeutic potential of membrane lipid modification in tumours. Cancer Res 1987;47:4529-37.

8. Grammatikos SI, Subbaiah PV, Victor TA, Miller WM. n-3 and n-6 fatty acid processing and growth effects in neoplastic and non-cancerous human mammary epithelial cell lines. Br J Cancer 1994;70:219-27.

9. Rose DP. Effects of dietary fatty acids on breast and prostate cancers: evidence from in vitro experiments and animal studies. Am J Clin Nutr 1997;66:1513S-22S.

10. Choi Y, Park Y, Storkson JM, Pariza MW, Ntambi JM. Inhibition of stearoyl-CoA desaturase activity by the cis-9,trans-11 isomer and the trans-10,cis-12 isomer of conjugated linoleic acid in MDA-MB-231 and MCF-7 human breast cancer cells. Biochem Biophys Res Commun 2002;294:785-90.

11. Menendez JA, Vellon L, Colomer R, Lupu R. Oleic acid, the main monounsaturated fatty acid of olive oil, suppresses Her-2/neu (erbB-2) expression and synergistically enhances the growth inhibitory effects of trastuzumab (Herceptin) in breast cancer cells with Her-2/neu oncogene amplification. Ann Oncol 2005;16:359-71.

12. Hardy S, Langelier Y, Prentki M. Oleate activates phosphatidylinositol 3-kinase and promotes proliferation and reduces apoptosis of MDA-MB-231 breast cancer cells, whereas palmitate has opposite effects. Cancer Res 2000;60:6353-8.

13. Ip C. Review of the effects of trans fatty acids, oleic acid, n-3 polyunsaturated fatty acids, and conjugated linoleic acid on mammary carcinogenesis in animals. Am J Clin Nutr 1997;66:1523S-9S.

14. Pollak MN. Insulin-like growth factors and neoplasia. Novartis Found Symp 2004;262:84-98.

15. Chong YM, Colston K, Jiang WG, Sharma AK, Mokbel K. The relationship between the insulin-like growth factor-1 system and the oestrogen metabolising enzymes in breast cancer tissue and its adjacent non-cancerous tissue. Breast Cancer Res Treat 2006;99:275-88.

16. Pandini G, Wurch T, Akla B, Corvaia N, Belfiore A, Goetsch L. Functional responses and in vivo anti-tumour activity of h7C10: a humanised monoclonal antibody with neutralising activity against the insulin-like growth factor-1 (IGF-1) receptor and insulin/IGF-1 hybrid receptors. Eur J Cancer 2007;43:1318-27.

17. Field CJ, Schley PD. Evidence for potential mechanisms for the effect of conjugated linoleic acid on tumour metabolism and immune function: lessons from n-3 fatty acids. Am J Clin Nutr 2004;79:1190S-8S.

18. Schley PD, Brindley DN, Field CJ. (n-3) PUFA alter raft lipid composition and decrease epidermal growth factor receptor levels in lipid rafts of human breast cancer cells. J Nutr 2007;137:548-53.

19. Huo H, Guo X, Hong S, Jiang M, Liu X, Liao K. Lipid rafts/caveolae are essential for insulin-like growth factor-1 receptor signaling during 3T3-L1 preadipocyte differentiation induction. J Biol Chem 2003;278:11561-9.

20. Lecka-Czernik B, ckert-Bicknell C, Adamo ML et al. Activation of peroxisome proliferator-activated receptor gamma (PPARgamma) by rosiglitazone suppresses components of the insulin-like growth factor regulatory system in vitro and in vivo. Endocrinology 2007;148:903-11.

21. Maor S, Mayer D, Yarden RI et al. Estrogen receptor regulates insulin-like growth factor-I receptor gene expression in breast tumour cells: involvement of transcription factor Sp1. J Endocrinol 2006;191:605-12.

22. Ip C, Scimeca JA, Thompson HJ. Conjugated linoleic acid. A powerful anticarcinogen from animal fat sources. Cancer 1994;74:1050-4.

23. Wang LS, Huang YW, Sugimoto Y et al. Effects of human breast stromal cells on conjugated linoleic acid (CLA) modulated vascular endothelial growth factor-A (VEGF-A) expression in MCF-7 cells. Anticancer Res 2005;25:4061-8.

24. Lu Y, Zi X, Zhao Y, Mascarenhas D, Pollak M. Insulin-like growth factor-I receptor signaling and resistance to trastuzumab (Herceptin). J Natl Cancer Inst 2001;93:1852-7.

25. Ma. DW. Identification of the conjugated linoleic acid isomer responsible for inhibiting essential fatty acid metabolism and tumor cell growth. PhD Thesis 2001.

Appendix A:

Reduction in viability of MCF-7 cells when treated with t10,c12 CLA in the presence of serum or IGF-I stimulation.

These results are from the same experiments discussed in Chapter 3 with the exception that the data is expressed as absorbance as read by the microplate spectrophotometer rather than as a ratio to the LA results.
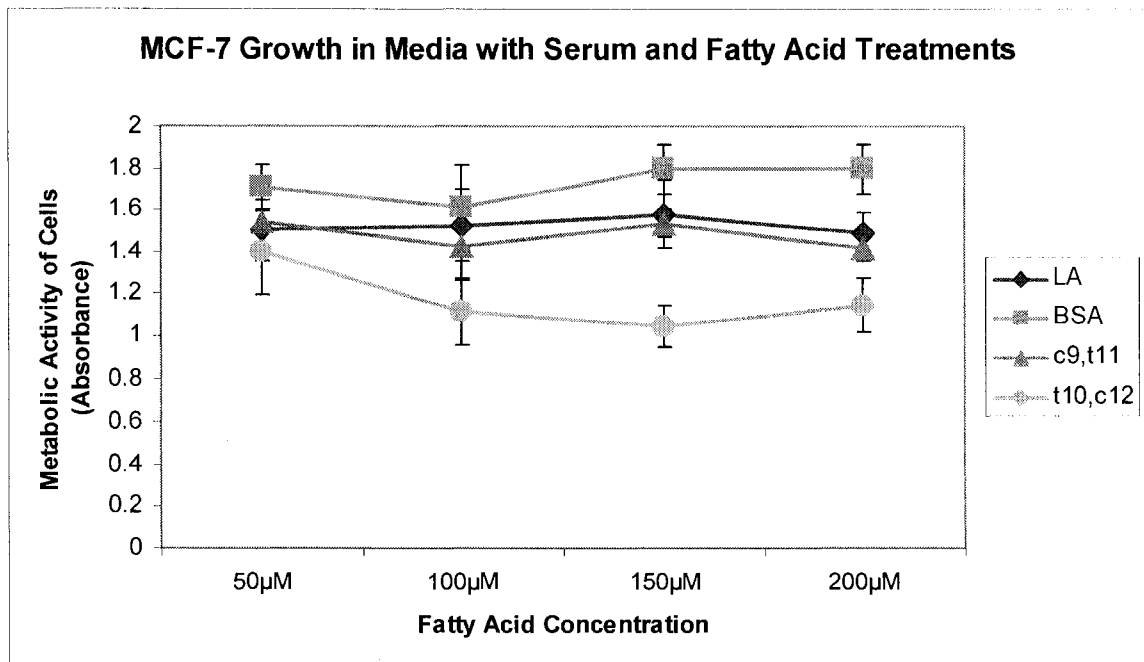

Figure A-1: **Metabolic activity (mitochondrial enzyme activity) of MCF-7 breast cancer cells grown in serum treated with fatty acids for 48hr at various concentrations** as labeled on the X axis. Values are expressed as absorbance measured on a microplate spectrophotometer. Bars represent the means ± SEM of 3 experiments.
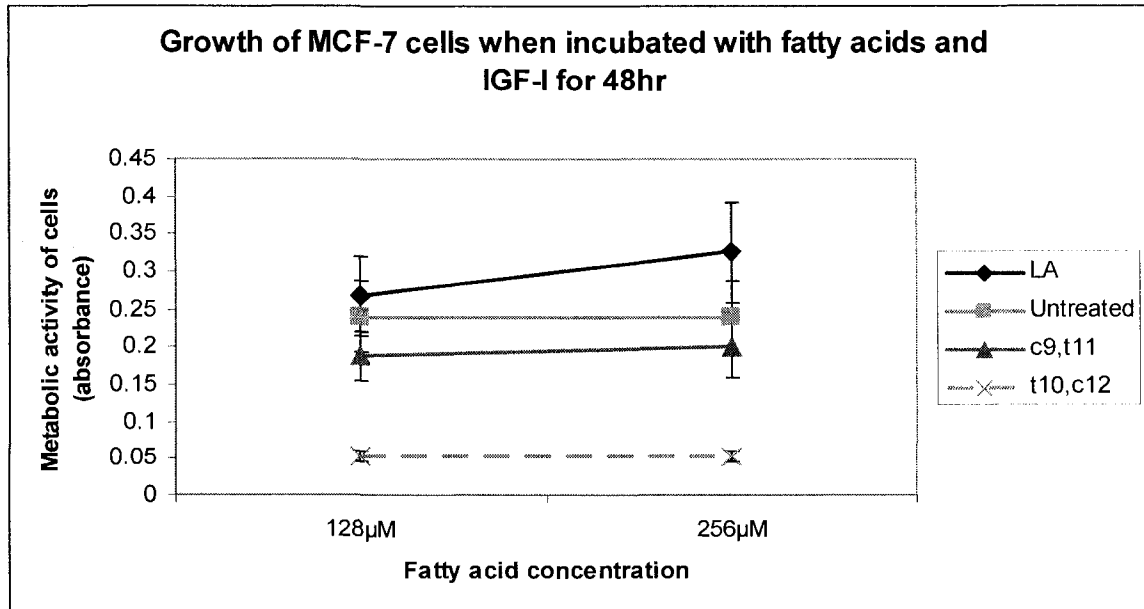
Figure A-2: **Metabolic activity (mitochondrial enzyme activity) of MCF-7 breast cancer cells grown in the absence of serum treated with fatty acids and IGF-I for 48hr at various concentrations** as described in the Materials and Methods section. Values expressed as absorbance measured on a microplate spectrophotometer. Bars represent the means ± SE of 3 experiments. Within each fatty acid concentration group, bars that do not share a letter are significantly different (p<0.05).
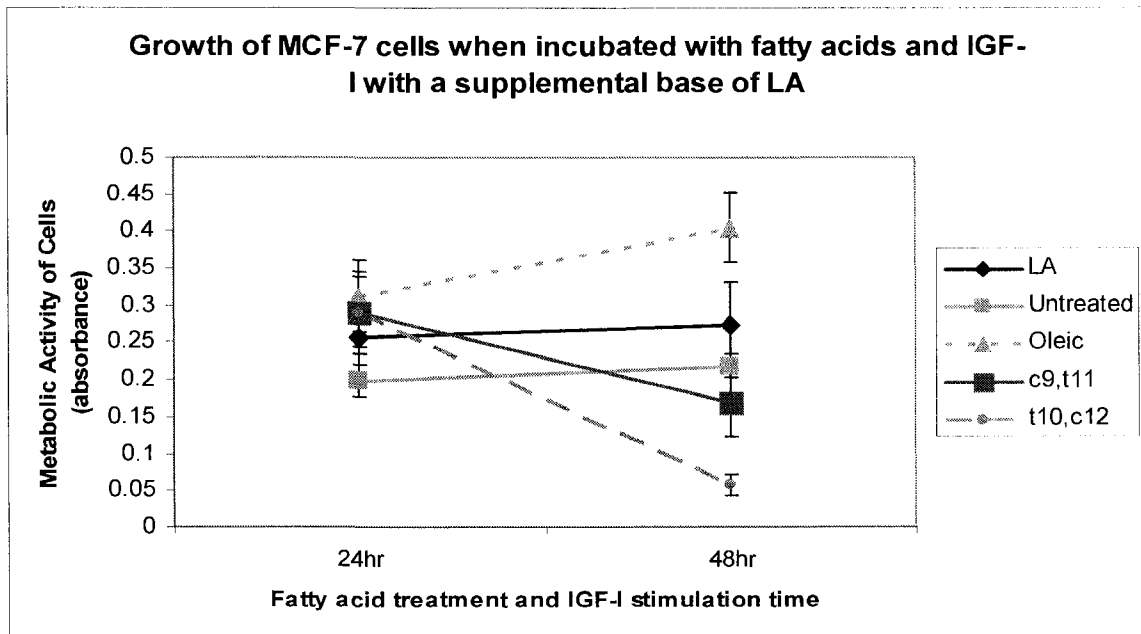

Figure A-3: **Metabolic activity (mitochondrial enzyme activity) of MCF-7 breast cancer cells grown in the absence of serum treated with IGF-I,** 128 µM base of LA and 128 µM of specified fatty acid for 24 or 48hr as described in the Materials and Methods section. Values expressed as absorbance measured on a microplate spectrophotometer. Bars represent the means ± SE of 3-4 experiments. Within each fatty acid concentration group, bars that do not share a letter are significantly different (p<0.05).