The Role of Conjugated Linoleic Acid in Breast Cancer Growth and Development

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Abstract: Conjugated linoleic acid (CLA) consists of a group of naturally occurring and synthetic positional and geometric (*cis-trans*) stereoisomers of the polyunsaturated fatty acid linoleic acid. The *cis*-9,*trans*-11 (c9,t11) CLA isomer (the most prevalent form found in ruminant-derived foods) and the *trans*-10,*cis*-12 (t10,c12) CLA isomer (present in commercial preparations) are the two most widely studied CLA isomers in breast cancer. Studies using both animal and cell culture models indicate that these CLA isomers, when added to the diet or included in the cell culture medium, inhibit mammary tumour initiation, promotion and progression in rodents, and alter tumour cell viability *in vitro*. The mechanism of CLA's anticancer effect is not well understood, but may involve interference with the cell cycle, induction of apoptosis, modulation of gene expression *via* the activation of peroxisome proliferator-activated receptors, lipid peroxidation, modulation of the tumour microenvironment, changes to the structure and/or function of the cell membrane, and interference with growth factor receptor signaling. A greater understanding of the mechanism of action of CLA will support the development of clinical trials to evaluate the potential effectiveness of CLA in the treatment of breast cancer.

Keywords: Breast cancer, conjugated linoleic acid, mammary, mechanisms, tumour.

INTRODUCTION

The pioneering work of Dr. T.K. Basu at the University of Alberta, Edmonton, Canada has demonstrated that both classical nutrients such Vitamin B-6 [1], niacin [2] and fiber [3] and isolated phytochemicals such as echinacea [4] and fenugreek [5] can have health benefits in the treatment and prevention of chronic and acute diseases. In 1981, Doll *et al*. estimated that 30% of all cancers could be prevented by dietary factors [6]. Epidemiological and animal studies support the hypothesis that nutritional factors play an important role in the etiology of breast cancer [7, 8]. There is a growing interest in the potential role of neutraceuticals as these 'nutritional factors' that might have efficacy in both the prevention and treatment of cancer. In 1979, Pariza *et al*. discovered an anti-carcinogenic property of fried ground beef [9]. By 1987, the compound was isolated and identified as a polyunsaturated fatty acid (PUFA) called conjugated linoleic acid (CLA) [10]. Since then, numerous animal and cell culture studies have demonstrated that CLA may protect against breast cancer. The remainder of this review will describe what is currently known about the anti-carcinogenic properties of CLA in breast cancer and potential mechanisms for its anti-cancer properties.

Conjugated Linoleic Acid (CLA)

CLA consists of a group of positional and geometric (*cis-trans*) stereoisomers of linoleic acid (LA) that are commercially synthesized from plant oils [11] or formed during the biohydrogenation of LA to stearic acid by ruminant animals [12]. The *cis*-9,*trans*-11 (c9,t11) CLA isomer is the most prevalent form found in ruminant-derived foods (e.g. milk and meat) [12]. The *trans*-10,*cis*-12 (t10,c12) CLA isomer is present only in trace amounts in animal foods but is found in an approximate 50:50 ratio with c9,t11 CLA in commercial CLA preparations [12].

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 [13-16]. These estimates are complicated by the variability of CLA content in food sources due to differences in feeding practices and inter-animal differences which affect the production of CLA in the rumen and mammary gland [17]. This variability in the food supply has made it challenging to study the intake of CLA in the general population and its effect on health.

Human Studies Of CLA and Breast Cancer

To date there have been no clinical trials in humans to test the effects of CLA on breast cancer prevention or treatment. The only human data available is from epidemiological studies. Five such studies have examined the relationship between CLA intake and risk of breast cancer (Table **1**). The results of these studies are conflicting: both an increased and decreased risk of breast cancer have been reported for CLA consumption. The epidemiological studies may be limited by the variability of CLA in the food supply as well as the difficulty of assessing the intake of this minor dietary component.

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Table 1. Epidemiological Studies of CLA and Breast Cancer Risk

Reference	Type of Study	Results
[108]	Case control (Finland)	0.4 odds ratio for breast cancer for highest quintile of CLA intake (~200 mg/d) in postmenopausal women
[109]	Cohort (Netherlands)	Positive trend for highest quintile of c9,t11 CLA intake (~290 mg/d)
[110]	Case control (USA)	Slight protective effect at highest intake of c9,t11 CLA in estrogen receptor negative breast cancer in premenopausal women
[111]	Case control (France)	No change in risk for metastasis using CLA content in breast adipose tissue obtained during surgery
[112]	Cohort (Sweden)	No significant association between dietary CLA intake and risk of breast cancer

Animal Studies of CLA and Mammary Cancer

Numerous studies using various animal models support the hypothesis that both synthetic and naturally enriched sources of CLA added to the diet inhibit mammary tumour initiation [18-34], promotion and progression [35-41] in rodents (Table **2**). A range of 0.1% to 1% w/w of CLA, independent of the level and type of fat in the diet, has shown to be effective, with no further benefits beyond 1% w/w [19,24]. The stage at which CLA is introduced in the diet appears to impact its effectiveness. When Ip *et al*. [20] provided CLA solely during mammary development prior to the injection of 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 [20]. In contrast to the majority of animal studies, no effect on tumour growth was observed with the injection of WAZ-2T mammary cancer cells into a mouse model. Furthermore, the t10,c12 CLA isomer was shown to increase initiation rate and lung metastasis, although not survival time, in a transgenic mouse overexpressing the growth receptor ErbB2 [42, 43]. CLA has been shown to dramatically alter fat deposition and body weight in mice [44]. Thus, the transgenic mouse model effects may have been due to changes to the stroma (which consists partly of adipocytes) surrounding the cells rather than the epithelial cells themselves [43].

Cell Culture Studies of CLA and Breast Cancer

Consistent with the *in vivo* results, CLA provided as individual isomers, a 50:50 mixture of c9,t11 CLA and t10,c12 CLA, or as CLA-enhanced milk fat decreases the growth, viability and/or increases death in a variety of breast cancer cell lines as reviewed in Table **3**. Effectiveness has ranged from 10-200 μM, which falls within the concentrations of CLA that have been observed in human serum (10-350 μM), including among people taking CLA supplements long-term [45].

Some studies have 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 and Lea [46] 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.

POTENTIAL MECHANISMS OF CYTOTOXICITY OF CLA ISOMERS TO BREAST CANCER CELLS

Interference with the Cell Cycle

CLA treatment of breast cancer cell lines has been reported to interfere with progression of the cell cycle. Studies in human breast cancer cell lines have shown an accumulation of cells in the G0/G1 (resting/growth) phase along with a corresponding decrease in the mRNA or protein expression of cell cycle promoters (e.g. c-myc, cyclin D1) and increased mRNA or protein expression of negative cell cycle regulators (p53, $p21^{Cip1/WAF1}$, p27) with CLA treatment [47-51]. The t10,c12 CLA isomer was more effective at inhibiting proliferation than c9,t11 CLA in the MCF-7 breast cancer cell line [48].

Induction of Apoptosis

CLA treatment is reported to increase apoptosis in human and rodent mammary cancer cells [25, 51-56]. Some of the observed changes consistent with the induction of apoptosis include: increased wild-type p53 protein levels, chromatin condensation, increased pro-apoptotic bax and bak protein levels, reduced anti-apoptotic bcl-2 protein levels, increased cytochrome c in the cytosol, and increased cleavage of initiator and effector caspases, reduced COX-2 activity and the generation of PGE_2 [25, 51, 52, 54-57]. A recent report indicated that t10,c12 CLA induces apoptosis in TM4t mammary tumour cells using certain components of the endoplasmic reticulum (ER) stress response, an alternative pathway to apoptosis in addition to the classical death receptor ("extrinsic") or mitochondrial ("intrinsic") pathways [53]. The mechanism of CLA-induced ER stress was proposed to involve increased lipid peroxidation products among CLA-treated cells [53].

Activation of Peroxisome Proliferator-Activated Receptors

Peroxisome proliferator-activated receptors (PPARs) are nuclear hormone receptors that have been implicated in multiple cell processes including regulation of the cell cycle

Table 2. Animal Studies of CLA and Mammary Cancer

Reference	Animal Model	Tumour	Basal Diet	CLA Content	Results	Mechanism Tested
[18]	Female Sprague-Dawley rats	DMBA-induced	AIN-76A	0.25, 0.5, 1, 1.5% w/w CLA (synthetic source: 43% c9,t11 and t9,c11, 45% t10,c12), fed 2 wks prior to DMBA administration until termination	CLA ↓ number and size of tumours	Only c9,t11 CLA was incorporated into mammary tumour and liver PL CLA ↓ lipid peroxidation in the mammary gland but not the liver; maximal antioxidant activity occurred with 0.25% CLA, whereas maximal tumour suppression occurred with 1% CLA, suggesting another mechanism besides lipid peroxidation No change to estrus cycle
[19]	Weanling female Sprague-Dawley rats	Experiment 1: DMBA-induced Experiment 2: DMBA- or NMU-induced	AIN-76A	Experiment 1: 0.05, 0.1, 0.25, 0.5% w/w CLA (synthetic source: 43% c9,t11 and t9,c11, 45% t10,c12), fed 2 wks prior to DMBA administration until termination Experiment 2: 1% w/w CLA fed for 5 wks (from weaning until 1 wk post-DMBA administration)	Experiment 1: CLA ↓ total mammary tumour yield in a dose-dependent manner from 0.05 to 0.5% CLA; as little as 0.1% CLA significantly ↓ tumour number Experiment 2: 1% CLA significantly ↓ mammary tumour yield in both DMBA and NMU tumour models	1% CLA ↓ proliferation of the lobuloalveolar compartment of the mammary tree
[20]	Female Sprague-Dawley rats	NMU-induced	Modified AIN-76A	1% w/w CLA (synthetic FFA vs. TG) fed from 21 to 42 d of age, or 1% CLA fed post-NMU (injected at 56 d of age) for 1 month, 2 months, or continuously	Both FFA and TG forms of CLA ↓ tumour number and incidence	Continuous feeding was required for maximal inhibition of tumourigenesis when CLA feeding was started after NMU injection to derive the same benefit as CLA feeding during the early post-weaning and pubertal period only
[21]	Sprague-Dawley rats	DMBA-induced	AIN-76: 10-20% w/w vegetable fat blend, or 20% corn oil, or 8% corn oil + 12% lard	1% w/w CLA, or 20% corn oil and 0.5, 1.0 or 1.5% w/w CLA	CLA ↓ tumour incidence by 50%, independent of level or type of fat in diet Maximal inhibition was seen at 1% w/w CLA	CLA ↓ lipid peroxidation (malondialdehyde) in mammary gland homogenate Cell oxidative stress (8-hydroxydeoxy-guanosine) was unaffected by CLA
[35]	SCID mice	MDA-MB-468 cells	N/A	1% w/w CLA mix (synthetic source: 42% c9,t11, 44% t10,c12), fed 2 wks prior to tumour cell injection until termination	CLA ↓ tumour mass and metastases	CLA works independently of the immune system
[42]	Eight wk-old female BALB/c mice	WAZ-2T cells	5% fat (4.1% corn oil)	0.1, 0.3, 0.9% CLA (derived from safflower oil: 35% c9,t11, 39% t10,c12), fed 2 wks prior to tumour injection until termination	CLA did not affect tumour volume, incidence or latency	CLA ↑ lymphocyte maturity (blastogenesis) and ↑ IL-2 production, but did not affect mammary tumour lipid peroxidation activity
[22]	Female Sprague-Dawley rats	DMBA-induced	20% corn oil	1% CLA, fed post-DMBA for 4 or 8 wks or continuously	Only CLA fed continuously significantly ↓ tumour growth	Inhibition was not dependent on ras CLA was retained faster and in greater amounts in neutral lipids vs. PL in the mammary gland

Table 2. contd….

Reference	Animal Model	Tumour	Basal Diet	CLA Content	Results	Mechanism Tested
[23]	Female Sprague Dawley rats	DMBA-induced	AIN-76A	1% w/w CLA fed from weaning to 50 d, or from 55 d to termination, or from weaning to termination	CLA ↓ the total number of tumours by 50%	CLA ↓ the density of mammary epithelium branching, ↓ DNA synthesis in terminal end buds and lobuloalveolar buds, and ↑ CLA metabolites (18:3 and 20:3) in the mammary gland CLA had no effect on fat deposition in the mammary gland
[113]	Female Fischer F344 rats	PhIP-induced	AIN-76A minus antioxidants	0.1, 0.5 or 1% w/w CLA (synthetic source), fed from 4 wks of age for 8 wks	Not reported	CLA inhibited PhIP-DNA adduct formation in the liver and white blood cells but not in mammary or colon epithelial cells CLA did not affect cytochrome P450 1A1 or 1A2 mRNA levels in the liver
[24]	Female Sprague-Dawley rats	NMU-induced	AIN-76A	0.5, 1, 1.5% or 2% w/w CLA fed from weaning until NMU injection, then fed basal diet until termination	CLA ↓ the number and incidence of tumours in a dose-dependent manner from 0.5 to 1% CLA; no further benefit detected above 1% CLA	CLA ↓ terminal end bud density in the mammary gland in a dose-dependent manner from 0.5 to 1% CLA; no further benefit detected above 1% CLA CLA and CLA metabolites accumulated in mammary tissue dose-dependently from 0.5% to 2% CLA CLA did not affect LA accumulation but did ↓ LA metabolites, in particular AA, in mammary gland
[25]	Female Sprague-Dawley rats	NMU-induced	AIN-76-based with 20% w/w control butter fat	20% w/w high CLA butter fat (providing 0.8% w/w CLA), or 20% w/w control butter fat + 0.7% Matreya CLA (81% c9,t11), or 20% w/w control butter fat + 0.7% Nu-chek CLA (36.5% t10,c12, 25.3% c9,t11), fed from weaning until NMU injection, then fed a 5% corn oil diet without CLA	All CLA treatments ↓ the number and incidence of tumours by ~50%	CLA ↓ mammary epithelial branching, ↓ terminal end bud density and ↓ proliferative activity of terminal end buds Rats consuming the CLA-enriched butter fat consistently accumulated more CLA in the mammary gland and other tissues than those animals consuming synthetic free fatty acid CLA
[26]	Female Sprague-Dawley rats	NMU-induced	AIN-76A	1% c9,t11 CLA or 1% CLA mix (36.5% t10,c12, 25.3% c9,t11, 17.6% 11,13, 15.3% 8,10)	c9,t11 CLA and CLA mix ↓ the number of premalignant intraductal proliferations by 50%	c9,t11 CLA and CLA mix ↑ apoptosis and ↓ bcl-2 expression in premalignant lesions, but did not affect bak or bax CLA did not induce apoptosis in normal mammary gland alveoli or terminal end buds
[36]	Female BALB/cAnN mice	Mouse mammary tumour cell line 4526	Semi purified diet with 20% w/w fat (mostly corn oil)	0.1%, 0.5%, or 1% w/w CLA mix	0.5% and 1% CLA ↑ tumour latency time and ↓ lung metastases No significant inhibition of growth	Indomethacin was more effective at inhibition than CLA
[27]	Female F344 rats	DMH-, DMBA-, BBN-, DHPN-induced	Oriental MF basal diet	0.1% or 1% CLA from safflower oil (71.3% CLA)	0.1% CLA ↓ mammary cancer incidence more than 1% CLA	1% CLA ↑ papillary or nodular hyperplasia in bladder but not tumours

Table 2. contd….

Reference	Animal Model	Tumour	Basal Diet	CLA Content	Results	Mechanism Tested
[28]	Female Sprague-Dawley rats	PhIP-induced	Oriental MF basal diet	0.1% CLA-rich safflower oil, fed during or after PhIP treatment until termination	CLA ↓ mammary adeno-carcinoma incidence when fed in the post-initiation period	CLA ↓ proliferation of mammary adenocarcinoma cells when fed in the post-initiation period CLA ↓ PhIP-DNA adduct formation in mammary gland epithelial cells
[29]	Female Sprague-Dawley rats	NMU-induced	AIN-76 basal diet with 5% w/w butter fat in place of corn oil	2% VA or 1% c9,t11 CLA, fed from NMU-injection for 6 wks	VA and c9,t11 CLA ↓ premalignant lesions in the mammary gland	Dietary VA ↑ CLA concentration in mammary gland 2% VA ↓ LA and LA metabolites in the liver but not mammary gland
[37]	Cd2/F1 mice	EHS-RBM *in vivo* angiogenesis model	AIN-76A with 5% corn oil	0, 1 or 2% CLA (50:50 mix of c9,t11 and t10,c12), fed 6 wks prior to angiogenic challenge	Both CLA diets ↓ forma-tion of functional blood vessels	CLA ↓ serum and mammary gland levels of VEGF and its receptor, Flk-1
[30]	Female Sprague-Dawley rats	NMU-induced	AIN-76	0.5% c9,t11 or t10,c12 CLA (90% pure)	c9,t11 and t10,c12 CLA ↓ premalignant intraductal proliferations and ↓ tumour incidence	c9,t11 CLA accumulated in mammary fat pad to a greater extent than t10,c12 CLA t10,c12 CLA significantly ↓ 20:2, 20:3, 20:4, 22:4, 22:6 and ↑ 16:1, 16:2 in mammary fat pad, whereas c9,t11 CLA had minimal effect on polyunsaturated fatty acid concentrations
[38]	BALB/cAnN mice	Mouse mammary tumour cell line 4526	20% w/w total fat	0, 0.1, or 0.25% w/w of c9,t11 CLA, t10,c12 CLA, or 0.125% c9,t11 CLA + 0.125% t10,c12 CLA (CLA mix)	Neither CLA isomer nor the mix affected tumour latency or growth All CLA diets ↓ tumour burden in lungs and size of pulmonary nodules	No mechanism tested
[39]	CD2FICr mice TNFα (+/+) or (-/-)	Matrigel pellet angiogenesis assay	AIN-76A	0, 5, or 10 g/kg c9,t11 or t10,c12 CLA (purified isomers)	Both isomers ↓ serum VEGF, formation of functional blood vessels, and ↓ size of unilocular adipocytes (effect was greater with t10,c12 CLA and reversible with c9,t11 CLA)	t10,c12 and CLA mix ablated BAT t10,c12 CLA ↓ serum leptin and ↑ apoptosis of adipose blood vessels and adipocytes c9,t11 CLA ↑ BAT in mammary gland
[31]	Sprague-Dawley rats	DMH- and DMBA-induced	Oriental MF diet	0.01, 0.05, 0.1, 1 or 2% CLA-rich safflower oil, fed following tumour initiation until termination	1% safflower oil ↓ adenocarcinoma incidence	No mechanism tested
[32]	Female Sprague-Dawley rats	NMU-induced	5% w/w sunflower oil	1% w/w CLA mix or 1% w/w c9,t11 CLA	Both CLA diets ↓ tumour mass, with no effect on tumour incidence or latency	CLA levels were higher in mammary fat neutral lipids than tumour PL
[33]	Female Sprague-Dawley rats	NMU-induced	AIN-76 with 10% w/w butter fat	0.13, 0.73, 1.0, or 1.6% VA, or 0.05, 0.18, 0.24 or 0.37 c9,t11 CLA, fed post-NMU injection for 24 wks	VA ↓ tumour number and incidence to a greater extent than c9,t11 CLA	VA and c9,t11 CLA ↑ amounts of c9,t11 CLA in mammary fat pad
[40]	Adult in-bred ovary-intact, non-estrogenized nude rats	MCF-7 xenografts	Essential FA-replete diet	Mammary tumours were perfused *in situ* with donor blood spiked with 0-360 µM c9,t11, t9,t11, or t10,c12 CLA	t10,c12 CLA and t9,t11 CLA, but not c9,t11 CLA, ↓ tumour ^{3}H-thymidine uptake	t10,c12 CLA inhibited LA uptake, cAMP content, ERK 1/2 activity, and 13-hydroxyoctadecadienoic acid formation in mammary tu-mours to a greater extent than t9,t11 CLA

Table 2. contd….

Reference	Animal Model	Tumour	Basal Diet	CLA Content	Results	Mechanism Tested
[34]	Female Sprague-Dawley rats	NMU-induced	AIN-76	0.13% w/w VA or 1.6% w/w VA +/ – sterculic oil, fed post-NMU injection for 6 wks	1.6% VA without sterculic oil ↓ the number of premalignant lesions in the mammary gland	1.6% VA ↓ the proliferative activity of premalignant cells in the mammary gland Treatment with sterculic oil reversed the effects of VA by inhibiting Δ9-desaturase activity, suggesting that the anticarcinogenic effects of VA are mediated through conversion to c9,t11 CLA
[41]	Female BALB/cAnN mice	Mouse mammary tumour cell line 4526	20%w/w fat (vegetable fat blend and/or beef tallow or corn oil)	0.05 or 0.1% CLA mix (33% c9,t11 and 33% t10,c12)	CLA had no effect on tumour growth rate	CLA in vegetable fat blend/beef tallow group ↓ lung metastases compared to other groups, especially vegetable fat blend/corn oil
[43]	FVB/N-Tg (MMTVneu) 202Mul/J and FVB/J female mice	ErbB2-overexpressing mammary tumours	AIN-76A	0.5% c9,t11 or t10,c12 CLA (>90% pure isomers), fed from weaning or fed from ~70 d of age	t10,c12 CLA fed from weaning or after puberty ↓ tumour latency and ↑ lung metastases, but had no effect on the number or size of primary tumours c9,t11 CLA had no effect	t10,c12 CLA slightly ↑ survival time compared to control t10,c12 CLA ↓ weight gain, modified mammary gland development, ↓ adipocytes, ↑ fibrocellular stroma, ↑ size of mammary lymph nodes, with no change to ErbB2 expression or localization t10,c12 CLA ↑ weight of spleen, heart, and liver (fatty liver) t10,c12 CLA ↑ the number of terminal end buds in the mammary glands of wild-type mice
[74]	Female BALB/cAnN mice	Mouse mammary tumour cell line 4526	20% w/w total fat from corn oil	0.1, 0.5, or 1% w/w CLA (33% c9,t11 CLA and 34% t10,c12 CLA)	Effects of CLA on tumour growth not reported	CLA ↑ mRNA levels of MMP-2 and MMP-9 in tumour cells but significantly ↓ MMP-9 activity levels CLA ↑ mRNA levels of tissue inhibitors of metalloproteinases
[102]	FVB/N-Tg (MMTVneu) 202Mul/J (‘ErbB2 transgenic’) female mice	ErbB2-overexpressing mammary tumours	AIN-76A	0.5% c9,t11 or t10,c12 CLA (>90% pure isomers), started at 6-10 wks of age and fed for 10 days, 4 wks, or until death, depending on the experiment	t10,c12 CLA ↑ mammary tumour growth, ↑ the number of new tumours, ↓ latency, and ↓ survival t10,c12 CLA ↑ mammary gland branching, ductal budding, and lobular development t10,c12 CLA ↑ polymorphonuclear lymphocyte infiltration and decreased adipocytes in mammary stroma no effect of c9,t11 CLA	t10,c12 CLA ↑ phosphorylation of PI3K, Akt, MEK, ERK and IGF-IR/IR, with no effect on the phosphorylation of ErbB2 no effect of c9,t11 CLA

Abbreviations: AIN, American Institute of Nutrition; BBN, *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine; cAMP, cyclic adenosine monophosphate; DHPN, dihydroxy-di-*n*-propylnitrosamine; DMBA, 7,12-dimethylbenz (a)anthracine; DMH, 1,2-dimethylhydrazine; EHS-RBM, Engelbreth-Holm-Swarm sarcoma-derived reconstituted basement membrane; ERK, extracellular signal-regulated kinase; FFA, free fatty acid; IGF-IR, insulin-like growth factor I receptor; IR, insulin receptor; MMP, matrix metalloproteinase; NMU, N-nitroso, N-methylurea; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine; PI3K, phosphatidylinositol 3-kinase; PL, phospholipids; TG, triglyceride; VA, vaccenic acid; VEGF, vascular endothelial growth factor.

Table 3. Cell Culture Studies of CLA and Breast Cancer

Reference	Cell Line	Fatty Acids Tested	Control	Fatty Acid Concentration and Culture Conditions	Result on Growth/Death	Mechanism Tested
[114]	MCF-7	CLA mix	No added FA	0, 17.8, 35.7, 71.4 μM	CLA ↓ growth of MCF-7 cells	CLA ↓ thymidine, uridine, and leucine incorporation β-carotene + CLA was not as effective as CLA alone
[115]	MCF-7	CLA mix, LA	No added FA	0, 17.8, 35.7, 71.4 μM	LA ↑ growth after 4 d, but ↓ growth after 8-12 d CLA ↓ growth after 4 d	CLA was cytostatic at 17.8 μM, and cytotoxic at 35.7 and 71.4 μM CLA was more cytotoxic than LA
[46]	T47D, MCF-7	CLA mix, LA, OA, linoelaidic acid, elaidic acid	No added FA	100, 200, 500 μM	LA ↓ ^{3}H-thymidine uptake more than CLA mix LA>linoelaidic>OA>elaidic	When [albumin] ↑ (from 1 to 38 mg/ml), the inhibitory effects of the fatty acids were decreased
[116]	MCF-7, HMEC (benign)	CLA mix, LA	No added FA	1.8, 3.6, 17.8, 35.7 μM Serum-free medium	All [CLA] and high [LA] ↓ growth of MCF-7 cells CLA ↓ growth and ^{3}H-thymidine uptake (to a greater extent than in MCF-7 cells) and LA ↑ growth and ^{3}H-thymidine uptake of HMEC cells	LA ↑ lipid peroxidation, CLA had no effect Eicosanoid inhibitors ↑ growth of CLA-treated HMEC cells CLA and indomethacin ↑ growth of MCF-7 cells, whereas CLA and NDGA ↓ growth of MCF-7 cells
[47]	MCF-7, MDA-MB-231	CLA mix, LA	No added FA	17, 35, 71 μM	CLA/LA co-culture ↓ growth and ^{3}H-thymidine uptake in MCF-7 cells; no effect on MDA-MB-231 cells	CLA treatment ↑ cells in G0/G1, and ↓ c-myc expression compared to LA or untreated cells
[117]	MCF-7	CLA mix	No added FA (ethanol vehicle)	0, 5, 10, 20 ppm	CLA ↓ MCF-7 cell number and ^{3}H-thymidine uptake in a dose- and time-dependent manner	CLA ↑ lipid peroxidation CLA induced activity of superoxide dismutase, catalase and glutathione peroxidase
[71]	Normal rat mammary epithelial cell organoids	LA, CLA mix	No added FA (serum-free medium vehicle)	4-128 μM CLA FA complexed to BSA	CLA ↓ growth and survival of mammary epithelial cells compared to LA treatment	LA, but not CLA, ↓ casein accumulation (functional differentiation) CLA, but not LA, ↓ DNA synthesis in pre-differentiated cells and ↑ apoptosis in differentiated mammary epithelial cells PKC activity in mammary epithelial cells was not altered by CLA, but PKC expression was upregulated in mammary adipocytes
[118]	MCF-7	c9,t11 CLA, LA	No added FA (ethanol vehicle)	1.8, 3.6, 17.8 μM FA complexed to BSA Serum-free medium, EGF added	CLA ↓ growth (3.6 and 17.8 μM) LA slightly ↑ growth (maximally at 3.6 μM)	LA ↑ 18:2, 14:0, 16:1, 20:2 membrane uptake, whereas CLA ↑ membrane CLA uptake LA ↑ PLC activity, CLA had no effect PKC activity was not affected PGE_2 secretion was not influenced
[77]	MCF-7	CLA mix, c9,t11 CLA, t10,c12 CLA, LA	No added FA (ethanol vehicle)	20 ppm FA in ethanol	Bovine milk fat enriched with CLA was as effective as synthetic CLA in inhibiting cell growth c9,t11 CLA was more effective than t10,c12 CLA LA ↑ growth	CLA and LA ↑ superoxide dismutase, catalase, and glutathione peroxidase activity CLA from milk fat had a greater uptake into PL than individual CLA isomers CLA ↑ lipid peroxidation (malondialdehyde)

Table 3. contd….

Reference	Cell Line	Fatty Acids Tested	Control	Fatty Acid Concentration and Culture Conditions	Result on Growth/Death	Mechanism Tested
[94]	MCF-7	CLA mix (29% t10,c12 and 30% c9,t11), c9,t11 CLA, t10,c12 CLA, LA	No added FA (ethanol vehicle)	17.8-57 μM 5% FBS	CLA mix and c9,t11 CLA ↓ viability after 4 d LA had no effect on MCF-7 cells	c9,t11 CLA, but not t10,c12 CLA, ↓ AA uptake into phosphatidylcholine and ↑ AA uptake into phosphatidylethanolamine CLA mix and c9,t11 CLA ↓ AA conversion to PGE_2 c9,t11 CLA and t10,c12 CLA ↑ lipid peroxidation
[119]	MDA-MB-231, MCF-7	CLA mix (48% c9,t11 and 46% t10,c12), c9,t11 CLA, t10,c12 CLA, LA	No added FA	45 and 100 μM FA complexed to BSA Serum-free medium	No measures of growth were completed	c9,t11 CLA and t10,c12 CLA ↓ stearoyl-CoA desaturase protein levels in MDA-MB-231 but not MCF-7 cells c9,t11 CLA and t10,c12 CLA ↓ stearoyl-CoA desaturase activity in both cell lines No effect on stearoyl CoA desaturase mRNA levels in either cell line LA ↓ stearoyl-CoA desaturase mRNA and protein levels in both cell lines CLA ↓ palmitoleic (16:1) and oleic (18:1) acid concentrations and ↓ desaturation indices in both cell lines
[49]	MCF-10A (benign), MCF-7, MDA-MB-231	CLA mix (50:50 c9,t11 and t10,c12), c9,t11 CLA, t10,c12 CLA, LA	No added FA (ethanol vehicle)	0-200 μM FA bound to BSA 10% FBS	CLA mix ↓ MCF-7 and MDA-MBA-231 growth more than individual isomers or LA MCF-10A growth was inhibited at lower doses	CLA ↑ expression of wild-type p53 mRNA in MCF-7 and MCF-10A cells, ↑ expression of p21WAF1/CIP1 in MCF-7 and MDA-MB-231 cells, and ↑ expression of bax and bcl-2 in MDA-MB-231 cells
[92]	MDA-MB-231	CLA mix, c9,t11 CLA, t10,c12 CLA, LA	No added FA	10, 30, 60 μM, co-cultured with 60 μM LA 5% FBS	c9,t11 CLA, t10,c12 CLA and CLA mix co-incubated with LA inhibited ^{3}H-thymidine uptake No dose response observed	CLA did not interfere with LA incorporation into membrane PL (LA was preferentially incorporated into PL despite equal molar concentrations of CLA) CLA mix or t10,c12 CLA ↓ AA in PL, and interfered with conversion of LA → AA LA ↑ and CLA ↓ PGE_2 synthesis
[120]	SKBR-3	c9,t11 CLA, myristic acid	No added FA	0.25 mM	Not reported	c9,t11 CLA ↓ incorporation of ^{14}C-acetate into PL of SKBR-3 cells c9,t11 CLA inhibited fatty acid synthase activity, but not expression (mRNA), to a greater extent than t10,c12 CLA
[48]	MCF-7	CLA mix, c9,t11 CLA, t10,c12 CLA	No added FA (ethanol vehicle or medium alone)	10, 20, 40, 80, 160 μM 10% FBS	40 μM CLA mix ↓ cell viability with trypan blue assay 10 μM CLA mix ↓ cell viability as measured by MTT assay	160 μM CLA mix induced a cell cycle arrest in G0/G1 160 μM CLA ↑ p53, p21, p27 and hypo-phosphorylated Rb proteins, and ↓ cyclin D1 and E protein levels Effects of CLA on p21 and cyclin E levels were mediated by p53 t10,c12 CLA was more effective than c9,t11 CLA at reducing cell viability and affecting p53 and Rb levels
[101]	MCF-7	CLA mix (49% c9,t11 and 41% t10,c12)	No added FA	10 μM 1% FBS or charcoal-treated FBS	c9,t11 CLA was a more potent inhibitor of proliferation of MCF-7 cells grown in the absence of growth factors than t10,c12 CLA or CLA mix	t10,c12 CLA was the strongest inhibitor of estrogen-stimulated growth t10,c12 CLA was the strongest inhibitor of insulin-stimulated growth and cell viability Neither isomer affected EGF-stimulated growth

Table 3. contd….

Reference	Cell Line	Fatty Acids Tested	Control	Fatty Acid Concentration and Culture Conditions	Result on Growth/Death	Mechanism Tested
[78]	MCF-7	Milk fat CLA, c9,t11 CLA, LA, OA, VA	No added FA (ethanol vehicle or milk fat)	60.2, 65.2, 80.6 μM CLA provided in 1 mg/mL milk fat samples 5% FBS	All 3 milk fat samples ↓ cell number in a dose-dependent manner compared to untreated cells Milk fat CLA was more effective at ↓ cell number than c9,t11 CLA provided at the same concentrations LA ↑ cell number at 44 μM, but ↓ cell number at 156 μM OA ↓ cell number at 952 μM VA ↓ cell number at 111-164 μM	80.6 μM CLA ↑ AA uptake into the monoacylglycerol fraction 80.6 μM CLA ↓ AA conversion to PGE_2 and ↑ conversion to $PGF_{2\alpha}$, and ↑ lipid peroxidation (as measured by levels of 8-epi- $PGF_{2\alpha}$)
[69]	MCF-7, MDA-MB-231	CLA mix, c9,t11 CLA, t9,t11 CLA, c9,c12 CLA, t10,c12 CLA, c11,t13 CLA	No added FA (solvent vehicle)	25-200 μM 10% FBS	c9,c11 CLA inhibited MCF-7 cell growth > t10,c12 > t9,t11 > c11,t13 > c9,t11 CLA CLA did not inhibit MDA-MB-231 cell growth	CLA ↓ ERα mRNA and protein levels CLA ↓ nuclear binding of ERα to the estrogen response element (ERE), as well as inhibition of ERE promoter activity CLA inhibition of ERE was not entirely accounted for by ↓ ERα levels CLA isomers differentially activated a PPAR response element (c9,c11 > c9,t11), suggesting that PPAR complexes might compete with activated ER for binding to the ERE
[67]	MCF-7, MDA-MB-231	CLA, LA	No added FA (serum-free medium vehicle)	100 μM 5% FBS	CLA ↓ growth of both cell lines At the same concentration, CLA was less effective in MDA-MB-231 cells	CLA ↑ PPARγ and ↓ PPARβ/δ protein levels in MCF-7 cells CLA ↑ PPARβ/δ, ↑ PPARγ2, and had no affect on PPARα protein levels in MDA-MB-231 cells
[93]	4526 mouse mammary tumour cells	c9,t11 CLA, t10,c12 CLA, LA	No added FA (ethanol vehicle)	10, 50, 100 μM 5% FBS in serum-free medium Insulin, transferrin, BSA	t10,c12 CLA ↓ viability at 10, 50 and 100 μM after 48 hr LA had no effect	t10,c12 CLA ↑ apoptosis and ↓ cell proliferation t10,c12 CLA ↓ 5-HETE production Adding 5-HETE into media ↓ the effects of t10,c12 CLA on apoptosis and proliferation
[50]	MCF-7, MCF-10A (benign)	CLA mix	No added FA (DMSO or hydrogen peroxide vehicle)	0-50 μM 10% FBS Insulin	CLA ↓ growth of MCF-7 cells but not MCF-10A cells	CLA ↑ lipid peroxidation in MCF-7 cells CLA ↑ nuclear translocation of phosphorylated p53 and ↓ phosphorylation of transcription factor FKHRser256 in MCF-7 cells CLA ↓ phosphorylated histone H3, blocking entry into mitosis in MCF-7 cells
[121]	MCF-7	CLA mix (50% c9,t11, 40% t10,c12, and 10% c10,c12)	No added FA (solvent vehicle)	25-200 μM 10% FBS	Not reported	CLA ↓ progesterone receptor mRNA levels, ERα protein levels, ERα phosphorylation, and ERα-ERE binding CLA ↑ protein phosphatase 2A activity (which dephosphorylates ERα and thus inhibits its transactivating potential)
[45]	MCF-7 cells co-cultured with human breast stromal cells	c9,t11 CLA, t10,c12 CLA	No added FA	40 μM 5% FBS in dextran-charcoal-treated medium	Both isomers ↓ proliferation of MCF-7 cells t10,c12 CLA was more effective when MCF-7 cells were co-cultured with stromal cells	t10,c12 CLA ↓ VEGF-A mRNA and protein levels to a greater extent than c9,t11 CLA

Table 3. contd….

Reference	Cell Line	Fatty Acids Tested	Control	Fatty Acid Concentration and Culture Conditions	Result on Growth/Death	Mechanism Tested
[122]	Primary breast epithelial cells and stromal cells, MCF-7, MDA-MB-231	c9,t11 CLA, t10,c12 CLA	No added FA	40 μM 5% FBS in phenol red-free, high-calcium, charcoal-treated DMEM/F12 medium	Not reported	Both CLA isomers ↑ expression of the estrogen-regulated tumour suppressor gene, protein tyrosine phosphatase gamma, in primary cultured normal breast epithelial cells, normal breast stromal cells and breast cancer epithelial cells, but not in breast cancer stromal cells t10,c12 CLA appeared more effective than c9,t11 CLA, especially in ERα-positive breast cancer epithelial cells
[123]	MCF-7, MDA-MB-231	CLA mix, c9,t11 CLA, t10,c12 CLA	No added FA	20, 40, 80 μM 0.5% FBS in DMEM	Not reported	CLA ↓ COX-2 transcription by interfering with the recruitment of activator protein-1
[124]	MCF-7	FA extracts prepared from beef lipid and varying in CLA content, purified CLA-enriched fractions, and mixtures of pure synthetic CLA isomers	No added FA (ethanol vehicle)	100 μM	Beef total FA↓ cancer cell growth to a greater extent than the corresponding CLA-enriched fractions The greatest ↓ in cell growth was seen in mixtures with *cis-trans* isomers (vs. *cis-cis* or *trans-trans* isomers)	Not studied
[126]	MDA-MB-231	CLA (not defined)	No added FA (serum-free medium vehicle)	60 μM 10% FBS in serum-free DMEM Insulin, transferrin, selenite, glutamine, antibiotic, albumin	CLA ↓ cell growth and viability after prolonged exposure (48-72 h) CLA ↑ the % of apoptotic cells at 48 and 72 h	CLA ↑ the accumulation of cells in S phase and ↑ markers of apoptosis (condensed chromatin, ↑ bak protein levels, ↓ Bcl_{XL} protein levels, ↑ translocation of cytochrome c to the nucleus, ↑ pro-caspase 3 and 9 cleavage) CLA ↓ Raf-1 and phosphorylated ERK 1/2 protein levels
[125]	MCF-7	CLA mix (50:50 c9,t11 and t10,c12)	No added FA	5, 10, 20, 40, 60, 100 μM 10% FBS in serum-free DMEM Insulin, transferrin, selenite, glutamine, antibiotic, albumin	CLA ↓ cell growth time- and dose-dependently, which was maximal with 60 μM and at 72 h CLA ↑ lactate dehydrogenase release at 72 h	CLA ↓ Raf-1 protein levels, phosphorylated ERK 1/2 levels, and c-myc protein levels CLA ↑ protein phosphatase 2A protein levels CLA did not affect levels of proteins involved in apoptosis signaling (bak, Bcl_{XL}, caspases)
[68]	MCF-7	CLA mix	No added FA	60 μM 10% FBS in serum-free DMEM Insulin, transferrin, selenite, glutamine, antibiotic, albumin	CLA effects on tumour cell growth have been reported in [67] and [125]	CLA ↑ PPARγ protein levels and caused PPARγ translocation to nucleus CLA ↑ protein levels of β-catenin and E-cadherin and ↑ membrane association of β-catenin
[127]	MCF-7	CLA mix (CLA conjugated to polymeric carrier)	No added FA	50, 100, 200 μM	Plu-conjugated CLA ↓ cell viability more than unconjugated CLA	Plu-CLA ↑ p53, ↓ bcl-2, ↑ bax

Table 3. contd….

Reference	Cell Line	Fatty Acids Tested	Control	Fatty Acid Concentration and Culture Conditions	Result on Growth/Death	Mechanism Tested
[95]	MCF-7	c9, t11 CLA, t10,c12 CLA, CLA mix (50% c9,t11 and t9,c11, 40% t10,c12, 10% c10,c12)	No added FA	20, 40, 80, 160 µM	Not reported	Transcription activity of the COX-2 promoter was equally repressed by the CLA mix and t10,c12 CLA at all concentrations; c9,t11 CLA was less effective t10,c12 CLA ↓ COX-2 protein expression to a greater extent than c9,t11 CLA
[128]	MCF-7, MDA-MB-231	c9, t11 CLA, t10,c12 CLA, CLA mix (50:50 c9,t11 and t10, c12)	No added FA, LA	40 µM	t10,c12 CLA and CLA mix ↓ MDA-MB-231 cell viability compared to no added FA after 48 and 72 h t10,c12 CLA ↓ MCF-7 cell viability only after 72 h c9,t11 CLA and LA had no effects on viability of either cell line	Not studied
[74]	4526 mouse mammary tumour cells	CLA (not defined)	No added FA, LA, AA, OA	10, 100, 1000 nM	Not reported	CLA at all concentrations ↓ the number of invasive cells (migration of tumour cells through an extracellular matrix) compared to no added FA, LA, AA and OA
[57]	p53-mutant TM4t mouse mammary tumour cells	t10,c12 CLA	No added FA	10-40 µM	t10,c12 CLA ↓ cell viability in a concentration-dependent manner compared to the control	t10,c12 CLA induced apoptosis as indicated by cell morphology (cell shrinkage and membrane blebbing), cleavage of caspases 3 and 9, release of cytochrome c into the cytosol, and ↓ bcl-2 protein levels No effect of CLA on bak or bax protein levels
[51]	MCF-7	Polyethylene glycol-conjugated CLA mix (PCLA)	CLA mix (46% c9,t11 and 50% t10,c12)	50-200 µM	PCLA and CLA reduced cell viability equally at 50, 100 and 200 µM	PCLA and CLA delayed cell entry into S phase of the cell cycle and ↑ the number of cells in sub G1 phase PCLA and CLA ↑ p53 and bax protein levels and ↓ bcl-2 protein levels
[54]	MCF-7	t,t CLA (44% t9,t11 CLA, 42% t10,t12 CLA), c9,t11 CLA, t10,c12 CLA	LA	5-60 µM	40 µM t,t CLA inhibited cell growth to a greater extent than 40 µM c9,t11 or t10,c12 CLA	t,t CLA ↑ p53 and bax protein levels, ↓ bcl-2 protein levels, ↑ cytochrome c release from the mitochondria, ↑ caspase-3 activation and PARP cleavage compared to c9,t11 CLA, t10,c12 CLA and LA
[53]	p53-mutant TM4t mouse mammary tumour cells	c9,t11 CLA, t10,c12 CLA	No added FA	10-40 µM	t10,c12 CLA ↓ cell viability in a concentration-dependent manner compared to the control	t10,c12 CLA induced apoptosis *via* endoplasmic reticulum stress, as indicated by dilatation of the endoplasmic reticulum, ↑ expression and splicing of X-box binding protein-1 mRNA, ↑ phosphorylation of eukaryotic initiation factor 2α, ↑ expression of the CHOP/GADD153 proapoptotic transcription factor, PARP cleavage, and ↑ caspase-12 cleavage
[56]	ERα(+) transfected and wild type MDA-MB-231 cells	Not stated, assumed mixture of c9,t11 CLA, t10,c12 CLA	No added FA	10 and 80 µM	CLA ↑ apoptosis in ERα(+) transfected MDA-MB-231 cells but not in the wild type MDA-MB-231 cells	CLA ↓ estrogen-stimulated bcl-2 protein expression in ERα(+) breast cells

Table 3. contd….

Reference	Cell Line	Fatty Acids Tested	Control	Fatty Acid Concentration and Culture Conditions	Result on Growth/Death	Mechanism Tested
[55]	SKBr3	t10,c12 CLA	No added FA	40, 80 μM	t10,c12 CLA ↑ TNFα-induced apoptosis	CLA ↓ HER2 protein expression, nuclear protein levels of NF-κb, and ↓ PGE_2 production when AA was provided, suggesting lower COX-2 activity
[88]	MCF-7	c9,t11 CLA, t10,c12 CLA	No added FA, LA, OA	128 μM CLA co-cultured with μM LA	Both CLA isomers ↓ cell growth at 48 h (t10,c12 > c9,t11) compared to LA-treated cells t10,c12 CLA ↑ lactate dehydrogenase release at 48 h	t10,c12 CLA ↓ total and phosphorylated IGF-IR protein levels Both isomers ↓ insulin receptor substrate-1 protein levels compared to untreated cells
[96]	MDA-MB-231, T47D	c9,t11 CLA, t10,c12 CLA, mixture of isomers	LA and palmitic acid	8-64 μM	Both isomers alone and in combination caused a dose-related ↓ growth of both cell lines	CLA mixture ↓ S14 and FAS mRNA levels (involved in fatty acid synthesis) in T47D cells

Abbreviations: AA, arachidonic acid; BSA, bovine serum albumin; COX, cyclooxygenase; DMSO, dimethyl sulfoxide; EGF, epidermal growth factor; ER, estrogen receptor; ERE, estrogen response element; ERK, extracellular signal-regulated kinase; FA, fatty acids; FBS, fetal bovine serum; 5-HETE, 5-hydroxyeicosatetraenoic acid; HMEC, human mammary epithelial cells; IGF-IR, insulin-like growth factor I receptor; LA, linoleic acid; MTT, methylthiazolyldiphenyl-tetrazolium bromide; NDGA, nordihydroguaiaretic acid; OA, oleic acid; PGE_2, prostaglandin E2;$PGF_{2\alpha}$, prostaglandin F 2α; PKC, protein kinase C; PL, phospholipids; PLC, phospholipase C; PPAR, peroxisome proliferator activated receptor; ppm, parts per million; VA, vaccenic acid.

and apoptosis. They consist of three isoforms (PPARα, PPARβ/δ and PPARγ) that are distributed in various tissues of the body and appear to have different yet also overlapping roles [58]. PPARs regulate gene expression by forming a heterodimer with retinoid x receptors, then binding to specific response elements in the promoter regions of target genes. PPARγ appears to be highly involved in adipocyte regulation, but it also appears as though its agonists target multiple hallmarks of cancer, including cell cycle arrest, differentiation, apoptosis, and angiogenesis [58]. PPARγ is expressed in human breast adenocarcinomas [59] and its upregulation decreases the proliferation of MCF-7 cells [60]. The role of PPARβ/δ ligands in tumourigenesis continues to be debated in the research literature [61]; there is evidence to support that they stimulate the growth of breast cancer cell lines [62]. There is limited data on the role of PPARα agonists in cancer, however a PPARα ligand has been shown to induce apoptosis in human breast cancer cell lines [63].

PUFAs and their metabolites are ligands for PPARs [64-66]. CLA treatment of MCF-7 breast cancer cells was shown to increase protein levels of the anti-proliferative PPARγ and decrease protein levels of the anti-apoptotic PPARβ/δ [67, 68]. CLA also increased the localization of PPARγ from the cytosol to the nucleus, and increased PPAR response element activation [68,69]. Use of a PPARγ antagonist abolished the growth inhibitory effects of CLA [68]. CLA exerted different effects on the estrogen receptor-negative MDA-MB-231 cell line [67]. It increased protein levels of the PPARγ2 isoform, yet it also increased PPARβ/δ levels, which may explain why CLA did not induce apoptosis in the MDA-MB-231 cells. Of note, none of these studies compared the effect of CLA on PPARs to that of other known PPAR agonists, such as linoleic, oleic or linolenic acids.

Effects on the Tumour Microenvironment

The tumour microenvironment plays an active role in tumourigenesis. The tissue that surrounds and intercalates 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. Cross-talk between cancer and stromal cells is important for the creation of a microenvironment supportive of malignant tumour growth as well as for the promotion and progression of the tumour [70]. Cancer cells release growth factors such as basic fibroblast growth factor and the family of vascular endothelial growth factors (VEGF) to stimulate changes in the stroma to support the tumour's development. Proteases are also released, which remodel the surrounding ECM to allow the spreading of the cancer cells. Once activated, stromal fibroblasts produce more growth factors to stimulate the continued growth of the cancer cells [70]. 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 VEGF2 receptor results in local angiogenesis. Blocking VEGF2 has been shown to reverse invasive carcinoma to a pre-malignant non-invasive tumour phenotype [70].

Including CLA (up to 1% w/w) in the diet has been shown to alter mammary gland development during puberty in rodents [24]. CLA decreased epithelial branching and the density of terminal end buds, which are highly susceptible to carcinogenesis [24, 71]. The epithelial branching and terminal end bud formation of mammary gland development are dependent on the presence of stromal fibroblasts [72], 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 [25,71].

Both c9,t11 CLA and t10,c12 CLA have been shown to decrease the formation of microcapillary networks in mice and *in vitro*, although t10,c12 was more potent [37, 39]. Changes to pathways related to angiogenesis included: decreased VEGF-A serum levels, decreased local production of VEGF in the mammary gland, decreased VEGF2 receptor protein, and decreased serum leptin (which promotes angiogenesis through endothelial cells) [37, 39, 45]. Studying the effect of CLA on the stroma in the mouse model has been hampered by t10,c12 CLA's complete ablation of brown adipose tissue in the mammary gland, which thereby would change the composition of the surrounding stroma. However, in other tissues, the ability of CLA to reduce angiogenesis is well characterized [73]. Recent studies also report a reduction in the activity of matrix metalloproteinases in both mammary tumours [74] and colon cancer cells [75] with CLA treatment, suggesting an additional mechanism by which CLA may reduce tumour invasion and spread.

Lipid Oxidation

Tumour cells demonstrate an increased susceptibility to oxidative stress, and current chemotherapy and radiation treatments derive their cytotoxic effects from increasing oxidative stress [50]. 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 [76]. There is conflicting research suggesting that CLA either augments or reduces 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 50 μM of a CLA mixture in MCF-7 cells as compared to normal mammary epithelial ductal cells (MCF-10A) [50]. CLA treatment has also been shown to increase the activity of superoxide dismutase, catalase and glutathione peroxidase (protective enzymes against oxidative stress) [77], as well as increase the lipid peroxidation product 8-epi-PG (prostaglandin)F2α [78]. These findings support the argument that CLA induces oxidative stress. In contrast, a one month CLA feeding trial in Sprague-Dawley rats showed that a CLA mixture reduced the presence of lipid peroxidation products in the mammary gland [18]. Research into other models of cancer also report conflicting results regarding CLA's potential role in oxidative stress [10, 79]. Some believe that CLA's interference with the metabolism of other fatty acids, such as linoleic acid (LA, which may be more susceptible to peroxidation) is the pathway by which it has an effect on oxidation [24].

Changes to Cell Membrane Structure and Function

PUFAs with *cis*-double bonds are believed to impact the physical properties of the cell membrane due to their 'kinked' structure, which allows for less tight packing of surrounding fatty acyl groups [80]. In contrast, the incorporation of the straighter *trans* fatty acids into membranes has been shown to decrease membrane fluidity and interfere with the function of membrane receptors [81-83]. Changes to the dietary fatty acid composition of the plasma membrane and subsequently the physical structure have been shown to alter lipid-protein interactions, affect ion transporters, receptors, signal transducers and enzymes [80, 84-86]. The two most commonly investigated CLA isomers, c9,t11 and t10,c12, contain both a *cis* and a *trans* bond. When provided in the diet or cell culture medium, CLA isomers are readily incorporated into the phospholipids of tumour cell membranes [87, 88]. The *trans*-bond in the major CLA 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. Recently it was demonstrated that incubation with CLA was associated with a reduction in the expression of the membrane receptor HER2 in the HER-2 expressing breast cancer cell line SKBr3 [55] and incorporation of both of the major isomers of CLA in the membrane phospholipids was associated with a reduction in the amount of the IGF-1 receptor in MCF-7 cells [88].

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 (AA, C20:4n-6) [89]. AA is a substrate for eicosanoids: prostaglandins are produced from AA *via* cycloxygenase enzymes and leukotrienes are produced *via* lipoxygenase enzymes. 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 [90]. Neither major isomer of CLA, nor a CLA mixture, appears to decrease the incorporation of LA in membrane phospholipids [21, 24, 91, 92]. However, CLA has been reported to interfere with essential fatty acid metabolism. Both a CLA mixture (c9,t11 and t10,c12) and/or t10,c12 CLA alone reduce the elongation and desaturation products of LA, most importantly AA [24, 92]. 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 [55, 87, 93]. 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*. [94] showed that CLA decreases the amount of AA stored in PC and increases it in phosphatidylethanolamine (PE) along with a subsequent decrease in the production of PGE_2. Although these researchers have reported that CLA may interfere with essential fatty acid metabolism, the more effective isomer is still controversial [92, 94].

Interestingly, a recent study reported that either a mixture of CLA isomers or t10,c12 CLA was effective at reducing the transcriptional activity of the cyclooxygenase-2 (COX-2) promoter in MCF-7 cells (the c9,t11 CLA isomer was less effective), suggesting an alternative mechanism by which CLA may interfere with eicosanoid metabolism [95]. Additionally, a recent study suggests that a mixture of CLA isomers decreased S14 and fatty acid synthetase in T47D breast

cancer cells [96], suggesting that CLA also interferes with fatty acid synthesis in these cancer cells.

Interference with Growth Factor Receptors/Signaling Pathways

The 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 [97]. Some current chemotherapy treatments are designed, or are in the process of being designed, to interfere with growth factor promotion of cell growth. For example, Trastuzumab (Herceptin), blocks the function of the Her2/neu/ErbB2 receptor (part of the EGF family), which is often overexpressed in breast cancers [97]. Recently, it was demonstrated that incubation with t10,c12 CLA reduced HER2 expression in SKBr3 cells [55].

At this time, little is known about the effects of CLA on EGF signaling. Recently we demonstrated that incubation with either major isomer of CLA decreased the cellular concentrations of IGF-1 in MCF-7 cells [88]. Only a small number of studies have investigated the effects of CLA on IGF-I signaling. *In vitro*, CLA interferes with IGF signaling in the HT-29 human colon cancer cell line [98]. IGF-1 is essential for pubertal mammary development of the terminal end buds [72, 99]. Studies using rodent mammary cancer models have shown that CLA interferes with terminal end bud development [100], providing preliminary support for an inhibitory effect of CLA on IGF signaling. Since then, CLA has been reported to interfere with insulin-stimulated proliferation of MCF-7 cells *in vitro*, with t10,c12 CLA showing greater inhibition than c9,t11 CLA or a CLA mixture [101]. Studies from our own laboratory showed that t10,c12 CLA (but not c9,t11 CLA) reduced the levels of phosphorylated IGF-1 receptor in MCF-7 cells [88]. In contrast to the cell culture studies, Meng *et al*. recently reported the results of an animal feeding study in which t10,c12 CLA, when fed to female transgenic mice bearing ErbB2-overexpressing mammary tumours, increased mammary tumour growth and increased phosphorylation of the IGF-IR/IR and its downstream targets PI3K, Akt, MEK, and ERK [102]. The CLA-induced stimulation of tumour growth in this model is consistent with a previous report by this group [43]. It is not clear whether the conflicting reports between *in vitro* and *in vivo* studies are related to differences in tumour model or to the contribution of the tumour microenvironment *in vivo*, but clearly further research is warranted.

SUMMARY

The interest and evidence that CLA may become a future neutraceutical treatment for the treatment and prevention of cancer is rapidly growing. The evidence is summarized in Tables **1**-**3**. Basic research into the mechanisms behind the anti-carcinogenic effect of CLA offers insight into its modifications to cellular function and the surrounding stromal environment. From 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. Dr. T.K. Basu so elegantly demonstrated during his career that one needs to demonstrate an effect first *in vitro*/in situ [103,104], then in the appropriate animal models [3-5] and finally demonstrate efficacy in well designed human trials [105-107]. Thus, before trials can be proposed to test efficacy of CLA, 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. More animal studies are 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 CLA's mechanism of action will support the development of clinical trials to evaluate the potential effectiveness of CLA in the treatment of breast cancer.

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CONFLICT OF INTEREST

There is no conflict of interest by any of the authors

ABBREVIATIONS

AA	=	Arachidonic acid
CLA	=	Conjugated linoleic acid
COX-2	=	Cyclooxygenase-2
ECM	=	Extracellular matrix
EGF	=	Epidermal growth factor
IGF	=	Insulin-like growth factor
LA	=	Linoleic acid
PC	=	Phosphatidylcholine
PE	=	Phosphatidylethanolamine
PG	=	Prostaglandin
PPAR	=	Peroxisome proliferator-activated receptor
PUFA	=	Polyunsaturated fatty acid
VEGF	=	Vascular endothelial growth factors

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