Chapter 1.3

The Health Promoting Properties of the Conjugated Isomers of α-linolenic Acid (CALA)

1.3.1 Introduction

Numerous investigations have attributed functional properties to a range of conjugated fatty acids of which the conjugated linoleic acid (CLA) isomers are best characterized (Bhattacharya *et al.*, 2006; Igarashi & Miyazawa, 2005; Wahle *et al.*, 2004). The health promoting attributes of the CLA isomers have been reported in detail, however, there are currently few reviews which address the other group of natural conjugates, the conjugated α-linolenic acid (CALA) isomers, which will be addressed here. These CALA isomers combine the conjugated double bond system of CLA with the octadecatrienoic fatty acid structure of α-linolenic acid, conferring these CALA isomers with a high bioactive potential. CALA isomers are the positional and geometric isomers of α-linolenic acid, and are characterized by having one or more double bonds in the *cis* (*c*) or *trans* (*t*) conformation, which are separated by simple carbon-carbon linkage as opposed to being separated by a methylene group similar to the CLA isomers **(Figure 1.3.1)**.

These CALA isomers are readily found in nature, with the most common sources being pomegranate seed (*c*9, *t*11, *c*13 CALA), tung seed, bitter gourd seed, snake gourd seed and parwal seed (*c*9, *t*11, *t*13 CALA), catalpa seed (*t*9, *t*11, *c*13 CALA), and pot marigold seed (*t*8, *t*10, *c*12 CALA) **(Table 1.3.1)**. The presence of these conjugated fatty acids in these seed oils is primarily as a result of the action of divergent forms of the enzyme, fatty acid conjugase on linoleic acid or α-linolenic acid (Cahoon *et al.*, 2001; Cahoon *et al.*, 2006). Additionally, the *c*9, *t*11, *c*15 CALA and *t*9, *t*11, *c*15 CALA isomers may also be produced via the isomerisation of α-linolenic acid by intestinal and ruminal bacteria via the action of the enzyme

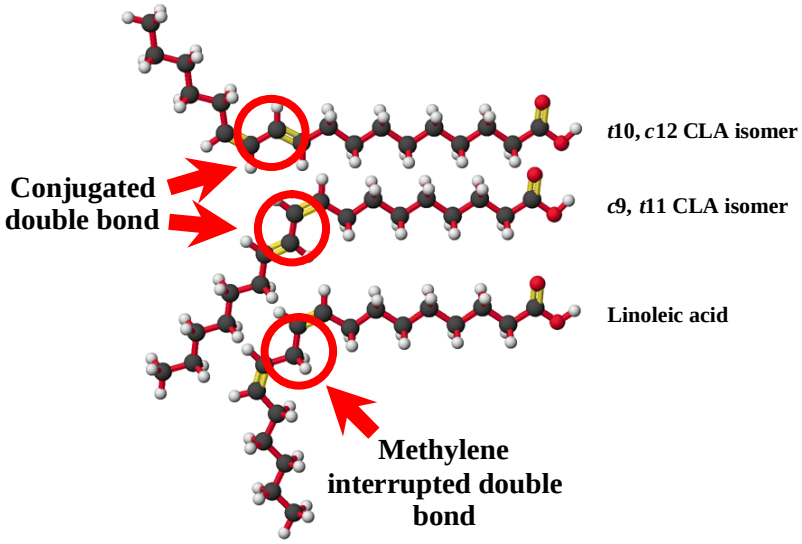

Figure 1.3.1 Structure of conjugated double bonds

linoleic acid isomerase (Coakley *et al.*, 2009; Destaillats *et al.*, 2005b; Ogawa *et al.*, 2005).

CALA isomers have been associated with potent anti-carcinogenic, anti-inflammatory and anti-atherosclerotic properties both *in vitro* and *in vivo*. These studies have demonstrated how the efficacy of these conjugated fatty acids against a particular condition may vary substantially between the individual isomers. Thus, the conjugation process can result in the production of a range of fatty acids with diverse biogenic profiles (Bhattacharya *et al.*, 2006; Igarashi & Miyazawa, 2005; Tsuzuki *et al.*, 2007; Wahle *et al.*, 2004). The mechanisms behind the health promoting properties of the conjugates range from the ability of these fatty acids to modulate the expression of genes associated with disease pathogenesis, to the ability of these fatty acids to compete with pro-inflammatory ω-6 fatty acids such as linoleic and arachidonic acids for incorporation into the cell membrane phospholipids. In addition, there is evidence to suggest that CALA isomers may undergo elongation and desaturation reactions similar to α-linolenic acid (Destaillats *et al.*, 2005a; Plourde *et al.*, 2006). This process results in the production of conjugated derivatives of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which may also possess potent biogenic properties (Tsujita-Kyutoku *et al.*, 2004; Tsuzuki *et al.*, 2007). Indeed, synthetically produced conjugated EPA isomers have displayed potent anti-carcinogenic and anti-adipogenic properties (Igarashi & Miyazawa, 2000a; Tsuzuki *et al.*, 2004a; Tsuzuki *et al.*, 2007; Yonezawa *et al.*, 2005), while conjugated DHA isomers have shown potent anti-carcinogenic properties (Danbara *et al.*, 2004).

Table 1.3.1 The principal conjugated α-linolenic acid isomers (CALA) and their sources

Conjugate	Source	Conc.	Reference	Comments
C18:3 CALA				
c 9, *t* 11, *c* 13	Pomegranate seed	83%	(Takagi & Itabashi, 1981)	
	Milk fat	≤0.03%	(Destaillats *et al.*, 2005)	Canadian milk fat
	Rapeseed oil	2.50%	(Koba *et al.*, 2007)	GMO rapeseed
c 9, *t* 11, *t* 13	Tung seed	67.7%	(Takagi & Itabashi, 1981)	
	Bitter gourd seed	56.2%	(Takagi & Itabashi, 1981)	
	Snake gourd seed	30-50%	(Dhar & Bhattacharyya, 1998)	
	Parwal seed	30-50%	(Dhar & Bhattacharyya, 1998)	
	Sugihiratake mushroom	N.S.	(Amakura *et al.*, 2006)	Edible Japenese mushroom
t 9, *t* 11, *c* 13	Catalpa seed	42.3%	(Takagi & Itabashi, 1981)	
t 9, *t* 11, *t* 13	Chemosynthesized	>97%	(Yasui *et al.*, 2006)	Larodan Fine Chemicals, Sweden
t 8, *t* 10, *c* 12	Pot marigold seed	52.2%	(Takagi & Itabashi, 1981)	
t 8, *t* 10, *t* 12	Pot marigold seed	4.7%	(Nagao & Yanagita, 2005)	
c 9, *t* 11, *c* 15	*Lactobacillius plantarum* AKU 1009a	67%	(Kishino *et al.*, 2003)	At an α-linolenic acid conc. of 63 mg/ml
	Milk fat	≤0.03%	(Destaillats *et al.*, 2005)	Canadian milk fat
t 9, *t* 11, *c* 15	*Lactobacillius plantarum* AKU 1009a	33%	(Kishino *et al.*, 2003)	At an α-linolenic acid conc. of 63 mg/ml

1.3.2 The role CALA in inflammatory response and immune function

Both ω-3 fatty acids such as α-linolenic acid and conjugated fatty acids such as the CLA isomers (*c*9, *t*11 and *t*10, *c*12 CLA isomers) have been directly associated with anti-inflammatory and immune enhancing properties. Investigations into the mechanisms through which this activity is mediated have highlighted a) the down-regulation of eicosanoid production (prostaglandins & leukotrienes) (Belury, 2002; Chang *et al.*, 2008); b) increased peroxisome proliferator-activated receptor (PPAR) mediated anti-inflammatory response (Yang *et al.*, 2000); c) suppression of inflammatory response through the regulation of the cell transcription factor NF-kB (Cheng *et al.*, 2004; Ren & Chung, 2007); and d) the reduced expression of pro-inflammatory proteins such as TNF-α, IL-6, and IL-1 beta, (Jiang *et al.*, 1998; Nelson & Hickey, 2004; Yang *et al.*, 2000), as important factors. Furthermore, both α-linolenic acid and the CLA isomers have also been associated with improving the immune response in both animals and humans. Indeed, both the *t*10, *c*12 and *c*9, *t*11 CLA isomers have been associated with reducing mitogen-induced T lymphocyte activation in humans (Tricon *et al.*, 2004) and improving immunoglobulin profiles in both humans and animals (O'Shea *et al.*, 2004; Turpeinen *et al.*, 2008). Similarly, α-linolenic acid has also been associated with improving immune response, reducing the proliferation of peripheral blood mononuclear cells without impacting on the concentration of helper and suppressor cells or T and B lymphocytes (Bjerve *et al.*, 1989; Kelley *et al.*, 1991).

The extent and range of the anti-inflammatory and immune enhancing activities of both α-linolenic acid and the CLA isomers have prompted

investigations into the impact if any the CALA isomers have on inflammation and immune response **(Table 1.3.2)**. It was found that pomegranate seed oil (83% *c*9, *t*11, *c*13 CALA) enhanced the function of B-cells, which play a prominent role in the humoral immune response (Yamasaki *et al.*, 2006). In the study, increased production of IgG and IgM, two key immunoglobulins involved in the body's antigenic response and produced by B-cells, was observed. During investigations into the effect of a range of vegetables on interferon-gamma and interleukin-4, Ike *et al* (2005) discovered the ability of bitter gourd to induce interferon-gamma production in mice treated with heat inactivated *Propionibacterium acnes*. Interferon-gamma is directly associated with Th1 T-helper cells, which play a crucial role in the cellular immune response, maximizing the killing efficacy of the macrophages and the proliferation of cytotoxic CD8+ T cells (Ike *et al*., 2005). Further investigations into the activity of bitter gourd demonstrated that while the pulp yielded the highest increases in interferon-gamma, the peel and seed (sources of the *c*9, *t*11, *t*13 CALA isomer) also resulted in increased interferon-gamma production. Given these observations, more detailed investigations into the effect of the CALA isomers on inflammation and immune response perhaps merit further study, particularly in light of the potent activity displayed by α-linolenic acid and the CLA isomers.

1.3.3 The role of CALA in obesity

As one of the major health concerns facing the Western world, obesity and strategies to combat the condition have received substantial attention. In a number of studies, both α-linolenic acid and in particular the *t*10, *c*12 CLA isomer have

Table 1.3.2 Assessing the role of the conjugated isomers of α-linolenic acid (CALA) on immune function and growth.

Function	Isomer	Mechanism of action	Compared to	References
Immune Function				
↑ production of IgG and IgM	Pomegranate seed oil (*c*9, *t* 11, *c* 13 CALA)	Potential ↑ B-cell function	N.S.	(Yamasaki *et al,* 2006)
↑ production of interferon-gamma	Bitter gourd seed	Potentially through increased production of Th1 T-helper cells	N.S.	(Ike *et al,* 2005)
Growth Promotion				
May promote growth of OLETF obese rats, displays characteristics similar to CLA	Pomegranate seed oil (*c*9, *t* 11, *c* 13 CALA)	↑ food effiency	N.S.	(Arao *et al.,* 2004)

(N.S. = Not Stated)

proven themselves to be effective anti-adipogenic agents (Bhattacharya *et al.*, 2006; Ikemoto *et al.*, 1996; Javadi *et al.*, 2004; Keim, 2003). The anti-adipogenic activity of α-linolenic acid has been attributed to factors such as the high proportion of the fatty acid which undergoes β-oxidation (Cunnane & Anderson, 1997; Li *et al.*, 2003; Sinclair *et al.*, 2002), the less efficient storage of α-linolenic acid in adipose tissue (Lin *et al.*, 1993; Yeom *et al.*, 2002), the higher mobilization of stored α-linolenic acid from adipose tissue relative to linoleic acid (Raclot *et al.*, 1997) and the ability of α-linolenic acid to regulate genes associated with fatty acid metabolism (Ide *et al.*, 2000; Ikeda *et al.*, 1998; Iritani *et al.*, 1998; Kim *et al.*, 2004; Kim & Choi, 2005; Takahashi & Ide, 2000). Similarly, the anti-adipogenic activity of the CLA has also been attributed to increased cellular β-oxidation (Keim, 2003), to the ability of the fatty acid to modulate the production of enzymes involved in fatty acid metabolism (particularly lipoprotein lipase and carnitine palmitoyltransferase) (Park *et al.*, 1997; Park *et al.*, 1999), and to reduce proliferation and differentiation of preadipocytes (Brodie *et al.*, 1999; Satory & Smith, 1999).

The promise shown by α-linolenic acid and the CLA isomers in the treatment of obesity has prompted investigations into the effect of the various CALA isomers on the condition (Bhattacharya *et al.*, 2006; Navarro *et al.*, 2006; Wahle *et al.*, 2004) **(Table 1.3.3)**. Koba *et al.* (2002) found that the dietary intake of CALA, prepared from α-linolenic acid via alkaline isomerisation, resulted in reductions in perirenal and epididymal adipose tissue and increased mitochondrial and peroxisomal β-oxidation. Dietary pomegranate seed oil, rich in the *c*9, *t*11, *c*13

CALA isomer, has also been shown to reduce omental white adipose tissue weights in lean Long–Evans Tokusima Otsuka (LETO) rats but not abdominal white adipose tissue weights in obese, hyperlipidemic Otsuka Long Evans Tokushima Fatty (OLETF) rats (Arao *et al*., 2004a). Further studies into the activity of the *c*9, *t*11, *c*13 CALA isomer, using genetically modified rapeseed and ICR CD-1 mice, suggest that this isomer reduced leptin production and increased carnitine palmitoyl-transferase activity (Koba *et al*., 2007). There is also evidence that the *t*8, *t*10, *c*12 CALA isomer may decrease the body fat content of male mice (Chardigny *et al*., 2003). Indeed, mice fed the isomer had a significantly lower percentage body fat than animals on the control diet. However, the *t*10, *c*12 CLA isomer resulted in significantly higher body fat reductions than the *t*8, *t*10, *c*12 CALA isomer. There is some evidence to suggest that the anti-adipogenic activity of CALA may stem from its ability to activate the nuclear receptor proteins, PPARs, and in particular PPARα. PPARα plays a key role in the activation of enzymes involved in lipid catabolism, and both the *c*9, *t*11, *t*13 CALA isomer and *t*9, *t*11, *c*13 CALA isomer have been directly associated with its activation (Hontecillas *et al*., 2008). Moreover, these CALA isomers have been shown to increase the activity of acetyl-CoA carboxylase, a key enzyme involved in the peroxisomal β-oxidation of lipids under the control of PPARα.

1.3.4 The role of CALA in cardio-vascular health

The dietary intake of α-linolenic acid, the non-conjugated parent form of the CALA isomers, has been associated with improving arterial elasticity, hypertension,

Table 1.3.3 Assessing the role of the conjugated isomers of α-linolenic acid (CALA) in the treatment of obesity.

Function	Isomer	Mechanism of action	Compared to	References
Obesity & Diabetes				
↓ perirenal and epididymal adipose tissue weight in Sprague Dawley rats	N.S.	↑ mitrocondrial and peroxisomal β-oxidation, ↑ carnitine palmitoyl-transferase activity, ↓ leptin conc.	Compared to α-linolenic acid and CLA	(Kobo *et al.,* 2002)
↓ omental white adipose tissue weight (27%), ← on abdominal white adipose tissue in OLETF obese rats	Pomegranate seed oil (*c*9, *t* 11, *c* 13 CALA)	↓ conc of MUFA in plasma, ↓ activity of Δ9-desaturase	N.S.	(Arao *et al.,* 2004)
↓ perirenal and epididymal adipose tissue weight, lower hepatic triglyceride conc in ICR CD-1 mice	Transgenic rapeseed oil (2.5% *c* 9, *t* 11, *c* 13 CALA)	↑ carnitine palmitoyl-transferase activity, ↓ serum leptin concentration	Compared to rapeseed oil	(Kobo *et al.,* 2007)
↓ percentage body fat	Calendic acid oil (*t* 8, *t* 10, *c* 12 CALA)	N.S.	Both the *t* 10, *c* 12 and *c* 9, *t*11 CLA isomers resulted in greater ↓ in percentage body mass	(Chardigny *et al,* 2003)
Activated PPARα, ↑ acetyl-CoA oxidase activity in H4IIEC3 murine hepatoma cell line	Bitter gourd seed oil (*c* 9, *t*11, *t* 13 CALA)	N.S.	Activation of PPAR α by the CALA isomer was similar to that achieved with CLA	(Chuang *et al,* 2006)

(N.S. = Not Stated)

platelet function, cardiac arrhythmia, and atherosclerosis, all of which lend themselves to improved cardiovascular health (Djousse *et al.*, 2001; Djousse *et al.*, 2005; Li *et al.*, 2003; Mozaffarian, 2005; Sinclair *et al.*, 2002). These activities have been attributed to the impact of the fatty acid on eicosanoid production, sodium ion channels and low density lipoprotein (LDL) receptor activity (Ander *et al.*, 2007; Bierenbaum *et al.*, 1993; Cintra *et al.*, 2006; Dupasquier *et al.*, 2007; Ferretti & Flanagan, 1996; London *et al.*, 2007; Mandasescu *et al.*, 2005; Munoz *et al.*, 2001; Rupp *et al.*, 1996). The CLA isomers have also displayed potent anti-atherosclerotic activity. The existing evidence would suggest that this anti-atherosclerotic activity is mediated through their effect on the expression of genes such as the LDL receptor gene and on acyl-coenzyme A:Cholesterol acyltransferase (Lam *et al.*, 2008; Ringseis *et al.*, 2006; Yu-Poth *et al.*, 2004) or by their impact on the production of pro-inflammatory eicosanoids (Bassaganya-Riera *et al.*, 2002; Belury, 2002; Nakamura *et al.*, 2008).

The strong anti-atherosclerotic activity displayed by both α-linolenic acid and the CLA isomers have prompted a number of investigations into the activity of a range of CALA isomers against the condition and in particular to determine if they possess any anti-hypercholesterolemic activity. In a recent study, the *c*9, *t*11, *t*13 CALA isomer has been associated with significantly lowering total and non-high density lipoprotein (HDL) cholesterol in diabetic rats (Dhar *et al.*, 2006). This activity may be mediated through the impact of the CALA isomers on the secretion of apo-lipoprotein B100 and on PPARα **(Table 1.3.4)**. Apo-lipoprotein B100 is an essential component of both very low density lipoprotein (VLDL) and LDL

cholesterol types, which are associated with the increased risk of coronary artery disease. Human hepatoma cells treated with the *c*9, *t*11, *c*13 CALA isomer have been shown to produce less apo-lipoprotein B100 than cells treated with an equivalent concentration of α-linolenic acid (Arao *et al.*, 2004b). Perhaps more significant is the increased activation of PPARα by CALA given its role in lipid uptake and metabolism (Berger & Moller, 2002; Chuang *et al.*, 2006). Indeed activators of PPARα such as fibrates have been directly associated with lowering serum cholesterol levels (Stahlberg *et al.*, 1995). Regardless of this evidence other studies such as that of Dhar *et al.* (1999) have found no differences in plasma total, HDL, and non-HDL cholesterol when rats were fed *c*9, *t*11, *t*13 CALA, relative to animals on the control diet. These results may suggest that the positive impact of CALA on plasma cholesterol is limited to diabetic subjects.

Lipoprotein oxidation *in vivo* has been increasingly associated with the development and progression of atherosclerosis (Esterbauer *et al*., 1992). A number of natural compounds such as garlic oil, fenugreek, ferulic and importantly CLA have been shown to possess antioxidant properties which may combat the oxidation of these lipoproteins. These observations have prompted investigations into the potential of CALA to reduce lipoprotein oxidation during *in vivo* and *in vitro* studies **(Table 1.3.4)**. In one such study male albino rats fed a diet containing 0.5% by weight *c*9, *t*11, *t*13 CALA were found to be significantly less susceptible to lipoprotein peroxidation and peroxidation of erythrocyte membrane lipids (Dhar *et al*., 1999). In rats with alloxan-induced diabetes mellitus the *c*9, *t*11, *t*13 CALA isomer has also proved effective in reducing the oxidation of LDL cholesterol and

Table 1.3.4 Assessing the role of the conjugated isomers of α-linolenic acid (CALA) in the treatment of cardio-vascular disease.

Function	Isomer	Mechanism of action	Compared to	References
Cardio-vascular disease				
↓ plasma total and non-HDL cholesterol in albino rats with alloxan induced diabetes, ↓ LDL and erythrocyte lipid peroxidation	Bitter gourd seed oil (*c* 9, *t* 11, *t* 13 CALA)	Free radical scavaging	N.S.	(Dhar *et al,* 2006)
↑ plasma total cholesterol, significantly ↑ plasma TAG	Pomegranate seed oil (*c*9, *t* 11, *c* 13 CALA)	N.S.	Compared to control	(Yamasaki *et al,* 2006)
↓ apolipoprotein B100 secretion, ↓ TAG synthesis in HepG2 human hepatoma cells	Bitter gourd seed oil (*c* 9, *t* 11, *t* 13 CALA)	Potentially reduces VLDL production	Compared to α-linolenic acid	(Arao *et al,* 2004)
↓ lipoprotein peroxidation, ↓ erythrocyte lipid peroxidation in albino rats	Bitter gourd seed oil (*c* 9, *t* 11, *t* 13 CALA)	Free radical scavaging	Compared to sunflower oil	(Dhar *et al,* 1999)
↓ plasma lipid peroxidation, lipoprotein peroxidation and erythrocyte membrane lipid peroxidation in both diabetic and non diabetic blood samples	Bitter gourd seed oil (*c* 9, *t* 11, *t* 13 CALA)	Free radical scavaging	Compared to control	(Dhar *et al,* 2007)
↓ serum triglyceride conc in F344 rats suffering from azoxymethane induced colonic aberrant crypt	Catalpa seed oil (*t* 9, *t* 11, *c* 13 CALA)	N.S.	N.S.	(Suzuki *et al.,* 2006)

(N.S. = Not Stated)

of erythrocyte membrane lipids (Dhar *et al*., 2006). The antioxidant effect of *c*9, *t*11, *t*13 CALA in relation to plasma lipoprotein is not exclusive to murine models. In a recent study, the *in vitro* antioxidant activity of the isomer in the blood of diabetic and non-diabetic humans was assessed (Dhar *et al.*, 2007). The results showed that *c*9, *t*11, *t*13 CALA significantly reduced plasma lipid peroxidation, lipoprotein peroxidation and erythrocyte membrane lipid peroxidation in both diabetic and non-diabetic blood samples.

1.3.5 The role of CALA in cancer

Cancer cell lines exposed to α-linolenic acid have shown that the fatty acid displays a potent inhibitory effect against colon cancer (Dwivedi *et al.*, 2005; Oikarinen *et al*., 2005), mammary cancer (Chen *et al*., 2002; Fritsche & Johnston, 1990; Hardman, 2007; Numata, 1995), melanoma (Yan *et al.*, 1998) and hepatoma (Vecchini *et al*., 2004) **(Table 1.3.5)**. While reductions in the expression or cellular concentrations of cycloxygenase-2 (COX-2) and prostaglandins synthesis were reported as contributing factors in the anti-carcinogenic activity of α-linolenic acid against most tumor types, other various tissue specific mechanisms were also identified (Fritsche & Johnston, 1990; Horia & Watkins, 2005; Oikarinen *et al.*, 2005; Vecchini *et al.*, 2004). Reductions in the incidence of colon cancer in mice as a result of the dietary inclusion of α-linolenic acid have been inversely associated with the concentration of β-catenin and protein kinase C-ζ (Oikarinen *et al*., 2005). Redistribution of β-catenin to its more 'normal' location in the membrane impairs activation of the nuclear Tcf/Lef transcription factor targeting proliferative genes.

Table 1.3.5 Assessing the role of the conjugated isomers of α-linolenic acid (CALA) in the treatment of cancers.

Function	Isomer	Mechanism of action	Compared to	References
Cancer				
Cytotoxic to the normal A31 and transformed SV-T2 mouse fibroblast cell lines at conc ≥ 25 μM, cytotoxic to the human monocytic leukemia cell line U-937 at conc ≥5 μM	Pomegranate seed oil (*c*9, *t* 11, *c* 13 CALA)	↑ lipid peroxidation, supported by the ↓ in cytoxicity on addition of BHT and high susceptibility of the oil to lipid peroxidation	Similar cytotoxicity to tung seed oil, substantially more cytotoxic than catalpa seed oil or pot marigold	(Suzuki *et al.,* 2001)
↓ incidence (38%-56 %) and multiplicity (0.50± 0.73 to 0.88±0.96) of azoxymethane induced colonic aberrant crypt foci (control diet: 81% and 1.88±1.54, respectively)	Pomegranate seed oil (*c*9, *t* 11, *c* 13 CALA) (0.01-1.0 %)	↑ *c* 9, *t*11 CLA conc and PPARγ expression in the non-lesional colonic mucosa	1% CLA had no effect on the incidence and multiplicity of azoxymethane induced colonic aberrant crypt foci	(Kohno *et al.,* 2004a)
90% ↓ in the proliferation of MCF-7 breast cancer cells at 100 μg/m, 75 %↓ in the invasiveness of MCF-7 line at conc 10 μg/ml. Induced 54% apoptosis of the MDA-MB-435 cell line	Pomegranate seed oil (*c*9, *t* 11, *c* 13 CALA)	N.S.	N.S.	(Kim *et al.,* 2002)
↓ angiogenesis	Pomegranate seed oil (*c*9, *t* 11, *c* 13 CALA)	Downregulation of the angiogenic promoter "vascular endothelial growth factor" in MCF-7 and breast cancer cells and MCF-10A immortalised breast epithelial cells, upregulation of the angiogenic suppressors "migration inhibitory factor" in MDA-MB-231 breast cancer cells	N.S.	(Toi *et al.,* 2003)
↓ incidence and multiplicity of 7,12-dimethylbenzanthracene induced skin tumour in CD1 mice	Pomegranate seed oil (*c*9, *t* 11, *c* 13 CALA) (5%)	17% reduction in 12-O-tetradecanoylphorbol 13-acetate induced ornithine decarboxylase activity	N.S.	(Hora *et al.,* 2003)
↓ proliferation of LNCaP, PC-3 and DU145 human prostate cancer cell lines, ↓ in PC-3 invasion	Pomegranate seed oil (*c*9, *t* 11, *c* 13 CALA)	↑ in G2/M cells from 11 % to 22% , (2.3± 0.001-fold) upregulation of cyclin-dependent kinase inhibitor p21 and (0.6±0.14-fold) down-regulation of c-myc, in the DU145 cell line	N.S.	(Albrecht *et al.,* 2004)

(N.S. = Not Stated)

Table 1.3.5 Assessing the role of the conjugated isomers of α-linolenic acid (CALA) in the treatment of cancers (contd).

Function	Isomer	Mechanism of action	Compared to	References
↓ invasion of PC-3 prostate cancer	Pomegranate seed oil (*c*9, *t* 11, *c* 13 CALA)	N.S.	N.S.	(Lansky *et al.,* 2005)
Cytotoxic to DLD-1 colorectal cancer, HepG2 heptoma, A549 lung cancer, MCF-7 breast cancer, and MKN-7 stomach cancer, cell lines at conc ≥ 25 μM	Tung seed oil (*c* 9, *t*11, *t* 13 CALA)	N.S.	CLA up to a conc of 100 μM was not cytotoxic towards the cancer cell lines assayed	(Igarashi & Miyazawa, 2000)
↓ incidence (47%) and multiplicity (64%) of azoxymethane induced colonic aberrant crypt foci at a conc of 0.01%	Bitter gourd seed oil (*c* 9, *t*11, *t* 13 CALA)	↑ *c* 9, *t*11 CLA conc and PPARγ expression in the non-lesional colonic mucosa	N.S.	(Kohno *et al.,* 2004)
↑ cell apoptosis in transplanted human DLD-1 colon cancer cells	Tung seed oil (*c* 9, *t*11, *t* 13 CALA)	↑ lipid peroxidation, ↑ DNA fragmentation	Supplementation with *c* 9, *t*11 and *t* 10, *c* 12 CLA resulted in lower DNA fragmentation than tung seed oil	(Tsuzuki *et al.,* 2004)
↑ apoptosis of Caco-2 colon cancer cells	Bitter gourd seed oil (*c* 9, *t*11, *t* 13 CALA)	↑ expression of GADD45, p53 and PPARγ. ↓ expression of Bcl-2	Supplementation with *c* 9, *t*11 CLA resulted in lower DNA fragmentation and higher cell viability than bitter gourd seed oil or pure *c* 9, *t* 11, *t*13 CALA	(Yasui *et al.,* 2005)
↑ apoptosis of Caco-2 colon cancer cells	*c* 9, *t*11, *t* 13 CALA	↓ expression of Bcl-2, ↑ DNA fragmentation, ↑ lipid peroxidation, Activity lost on addition of 5 μM α-tocopherol	N.S.	(Yasui *et al.,* 2006b)
Cytotoxic to the normal A31 and transformed SV-T2 mouse fibroblast cell lines at conc ≥50 μM and ≥ 25 μM, respectively, cytotoxic to the human monocytic leukemia cell line U-937 at conc ≥5 μM	Tung seed oil (*c* 9, *t*11, *t* 13 CALA)	↑ lipid peroxidation, supported by the ↓ in cytoxicity on addition of BHT and high susceptibility of the oil to lipid peroxidation	Similar cytotoxicity to pomegranate seed oil, substantially more cytotoxic than catalpa seed oil or pot marigold	(Suzuki *et al.,* 2001)

(N.S. = Not Stated)

Table 1.3.5 Assessing the role of the conjugated isomers of α-linolenic acid (CALA) in the treatment of cancers (contd).

Function	Isomer	Mechanism of action	Compared to	References
Small non-significant ↓ in the incidence, multiplicity and volume of mammary and colon cancers induced by	70.7% pure *c* 9, *t*11, *t* 13 CALA	N.S.	N.S.	(Kitamura *et al.,* 2006)
Cytotoxic to the normal A31 and transformed SV-T2 mouse fibroblast cell lines at conc ≥ 210 μM and ≥25 μM, respectively, cytotoxic to the human monocytic leukemia cell line U-937 at conc ≥10 μM	Catalpa seed oil (*t* 9, *t* 11, *c* 13 CALA)	↑ lipid peroxidation, supported by the ↓ in cytoxicity on addition of BHT and high susceptibility of the oil to lipid peroxidation	Less cytotoxic than tung seed oil or pomegranate seed oil, substantially more cytotoxic than pot marigold	(Suzuki *et al.,* 2001)
↓ incidence of azoxymethane induced colonic aberrant crypt foci in F344 rats from 99±28 in the control to 35±18 at conc of 0.1%, significantly ↑ indices of apoptosis and ↓ indices of proliferation at conc of 1%	Catalpa seed oil (*t* 9, *t* 11, *c* 13 CALA)	↑ *t* 9, *t* 11 CLA conc in the liver and non-lesional colonic mucosa, ↓ expression of COX-2	N.S.	(Suzuki *et al.,* 2006)
↑ apoptosis of Caco-2 colon cancer cells	>97% pure (*t* 9, *t*11, *t*13 CALA)	↓ expression of Bcl-2, ↑ expression of bax, ↑ DNA fragmentation, ↑ lipid peroxidation	Compared to the *c* 9, *t* 11, *t*13 CALA isomer, this isomer retained activity at α-tocopherol conc ≥ 5 μM	(Yasui *et al.,* 2006b)
Cytotoxic to the human monocytic leukemia cell line U-937 at conc >45 μM	Pot Marigold (*t* 8, *t* 10, *c* 12 CALA)	↑ lipid peroxidation, supported by the ↓ in cytoxicity on addition of BHT and high susceptibility of the oil to lipid peroxidation	Less cytotoxic than tung seed oil, pomegranate seed oil, or catalpa seed oil	(Suzuki *et al.,* 2001)
↑ apoptosis of Caco-2 colon cancer cells	>98% pure (*t* 8, *t*10, *c* 12 CALA)	↑ DNA fragmentation, ↑ lipid peroxidation, Activity lost on addition of 50 μM α-tocopherol	Higher DNA fragmentation than either the *c* 9, *t* 11, *t* 13 or *t*9, *t* 11, *t*13 CALA isomers	(Yasui *et al.,* 2006b)
↑ apoptosis of Caco-2 colon cancer cells	>97% pure (*t* 8, *t*10, *t*12 CALA)	↑ DNA fragmentation, ↑ lipid peroxidation	Compared to the *t* 8, *t* 10, *c* 12 CALA isomer, this isomer retained activity at α-tocopherol conc ≥50 μM	(Yasui *et al.,* 2006b)

(N.S. = Not Stated)

The anti-carcinogenic activity of α-linolenic acid against mammary cancer has been associated with reductions in expression of the onocogenes fatty acid synthase, and HER2 (erbB-2) (Menendez *et al*., 2004; Menendez *et al*., 2006) and with the down regulation of insulin-like growth factor 1 and human epidermal growth receptor 2 (Chen *et al*., 2002; Chen *et al.*, 2007a; Chen *et al.*, 2007b). Vecchini *et al.* (2004) associated the apoptotic activity of α-linolenic acid against hepatoma cells with reductions in the expression of sterol regulatory element binding proteins (SREBPs), nuclear transcription factors which regulate lipid metabolism and lipogenic enzymes including fatty acid synthase. The CLA isomers have also been strongly linked with anti-carcinogenic properties against a range of tumors including those of the mammary gland, colon, skin and liver (Banni *et al*., 2003; Bhattacharya *et al*., 2006; Wahle *et al.*, 2004). Much work has been conducted with regard to elucidating the mechanisms behind this anti-carcinogenic activity. The results have identified the CLA isomers as effective modulators of the expression of pro and anti-apoptotic oncogenes such as bcl-2, bax, bak, bad, p53, and p21, of eicosanoid synthesis (via their impact on COX-2 and cellular membranes composition), and on the activity of cell transcription factors such as NF-kB and PPAR (Banni *et al*., 2003; Bhattacharya *et al*., 2006; Wahle *et al.*, 2004).

In light of the potent activity which both α-linolenic acid and the CLA isomers have displayed against a range of cancers types much research has been directed towards identifying the effect that the various CALA isomers have on cancer, both *in vitro* and *in vivo*. These investigations indicate that the various CALA isomers differ substantially in their anti-carcinogenic properties and in the

mechanisms through which this anti-carcinogenic activity is mediated **(Table 1.2.5)**.

Pomegranate seed oil, rich in *c*9, *t*11, *c*13 CALA, has been associated with inhibiting the incidence and multiplicity of chemically induced colonic aberrant crypt foci in male F344 rats. This anti-carcinogenic activity was attributed to the increased concentration of *c*9, *t*11 CLA isomer and expression of PPARγ in the colonic mucosa (Kohno *et al.*, 2004a). Pomegranate seed oil has also been associated with reducing the proliferation and invasion of the MCF-7 mammary cancer cell line and increasing apoptosis of the MDA-MB-435 mammary cancer cell line (Kim *et al.*, 2002). Potential reasons for this anti-carcinogenic activity include the anti-angiogenic properties of pomegranate seed oil and its ability to inhibit prostaglandin synthesis (Nugteren & Christ-Hazelhof, 1987; Toi *et al.*, 2003). Hora *et al.* (2003) suggested that pomegranate seed oil could be a safe and effective chemopreventive agent against skin cancer. During their investigations, the oil was found to significantly reduce the incidence and multiplicity of chemically induced skin cancer, potentially through reduced ornithine decarboxylase activity. Interestingly, pomegranate oil has also been associated with suppressing the proliferation and invasion of human prostate cancer despite the association of α-linolenic acid with the condition (Brouwer *et al.*, 2004; Brouwer, 2008) **(Table 1.2.5)**. In one such study, pomegranate seed oil rich in *c*9, *t*11, *c*13-CALA was found to possess anti-proliferative activity against a range of prostate cancers *in vivo*, while in another, *c*9, *t*11, *c*13-CALA was found to significantly

reduce the invasiveness of the PC-3 prostate cancer cell line (Albrecht *et al.*, 2004; Lansky *et al*., 2005).

One of the first investigations into the anti-carcinogenic activity of CALA found that tung seed oil (67.7 % *c*9, *t*11, *t*13 CALA) was cytotoxic to a range of cancer cell lines at concentrations greater than 25 μM (Igarashi & Miyazawa, 2000b) **(Table 1.2.5)**. Since that time a range of further investigations have demonstrated that oils rich in *c*9, *t*11, *t*13 CALA have anti-proliferative and apoptosis inducing activity against a range of cancers and in particular those of the colon (Tsuzuki *et al.*, 2004b; Yasui *et al*., 2005; Yasui *et al.*, 2006a; Yasui *et al.*, 2006b). A number of mechanisms have been suggested for this activity including the increased expression of PPARγ and the cell cycle arrest genes GADD45 and p53, along with decreased expression of the apoptosis suppressor Bcl-2 (Kohno *et al*., 2004b; Yasui *et al*., 2005; Yasui *et al*., 2006a; Yasui *et al*., 2006b). In addition, increased lipid peroxidation within cancer cells, as a result of the uptake of *c*9, *t*11, *t*13 CALA, has also been suggested as a reason for the anti-carcinogenic effect of the isomer (Suzuki *et al.*, 2001; Tsuzuki *et al.*, 2004b). A number of these studies compared the anti-carcinogenic properties of the *c*9, *t*11, *t*13 CALA isomer or oils rich in the *c*9, *t*11, *t*13 CALA isomer with that of CLA or certain anti-cancer drugs. When *c*9, *t*11, *t*13 CALA was compared with both the *c*9, *t*11 and *t*10, *c*12 CLA isomers, the CALA isomer was found to have stronger anti-carcinogenic activity against the DLD-1 colon cancer cell line than the CLA isomers (Tsuzuki *et al.*, 2004b). Similarly, the *c*9, *t*11, *t*13 CALA isomer was found to have a higher anti-carcinogenic activity than the PPARγ ligand, troglitazone (Yasui *et al*., 2005; Yasui

et al., 2006b). However, not all studies have observed the anti-carcinogenic properties of the *c*9, *t*11, *t*13 CALA isomer. Kitamura *et al.* (2006) assessed the impact of the isomer on chemically induced mammary and colon carcinogenesis in female Sprague-Dawley rats. The results indicated that treatment with the isomer only slightly reduced the incidence, multiplicity and volume of tumors.

The *t*9, *t*11, *c*13 CALA isomer, the predominant conjugated fatty acid in catalpa seed oil, has also been shown to possess anti-carcinogenic properties **(Table 1.2.5)**. One of the first studies to comment on this anti-carcinogenic activity displayed the cytotoxicity of the isomer on transformed mouse fibroblast cell lines and on the human monocytic leukemia cell line U-937 (Suzuki *et al.*, 2001). Further investigations by the same group into this anti-carcinogenic activity showed the isomer reduced the incidence of chemically induced colonic aberrant crypt foci in rats, increasing apoptosis and reducing proliferation of cancer cells (Suzuki *et al.*, 2006). These studies have suggested increased lipid peroxidation and reduced expression of the enzyme COX-2 as reasons for this anti-carcinogenic activity. Supplementation of the animals diet with catalpa seed oil was also observed to increase the concentration of *t*9, *t*11 CLA isomer in the colonic mucosa and liver. The *t*9, *t*11 isomer has previously been shown to decrease expression of Bcl-2, the anti-apoptotic onocone, and may play a role in the apoptotic effect seen with catalpa seed oil (Beppu *et al.*, 2006). Yasui *et al.* (2006b) compared the anti-proliferative and pro-apoptotic properties of the *t*9, *t*11, *t*13 CALA isomer with that of the *c*9, *t*11, *c*13 CALA isomer using the Caco-2 colon cancer cell line. Comparatively, the *t*9, *t*11, *t*13 CALA isomer was significantly more cytotoxic to the Caco-2 colon

cancer line than the *c*9, *t*11, *c*13 CALA isomer. The isomer caused a high level of DNA fragmentation, increased expression of the pro-apoptotic onocogene bax, and decreased expression of Bcl-2 suggesting increased cellular apoptosis as the reason for the reduction in cancer cell numbers. Increased lipid peroxidation was also shown to play a role in this anti-carcinogenic activity. However, the *t*9, *t*11, *t*13 CALA isomer remained active even in the presence of elevated concentrations of α-tocopherol, suggesting that cancer cell apoptosis triggered by increased cellular lipid peroxidation is not the mechanism behind the isomers anti-carcinogenic activity.

The *t*8, *t*10, *c*12 and *t*8, *t*10, *t*12 CALA isomers derived from pot marigold have also been shown to possess some anti-carcinogenic properties **(Table 1.2.5)**. The *t*8, *t*10, *c*12 CALA isomer has been shown to possess apoptotic activity against a range of cancers including the human monocytic leukaemia cell line U-937 and the Caco-2 colon cancer cell line (Suzuki *et al.*, 2001; Yasui *et al.*, 2006a). Investigations into the oxidative stability of the fatty acid and the impact of the antioxidants BHT and α-tocopherol led the authors to conclude that the anti-carcinogenic activity of the isomer was related to lipid peroxidation **(Table 1.2.5)**. The anti-carcinogenic activity of the *t*8, *t*10, *t*12 CALA isomer has also been investigated using the Caco-2 cell line (Yasui *et al.*, 2006a). In this study, the isomer exhibited substantial cytotoxicity to the Caco-2 cell line and causing a substantial increase in DNA fragmentation. However, the mechanism behind this anti-carcinogenic activity remained unclear and could only partially be attributed to

increased cellular lipid peroxidation, as the *t*8, *t*10, *t*12 CALA isomer remained active even in the presence of α-tocopherol.

Comparatively, the anti-carcinogenic activity of the CALA isomers varies substantially. When the cytotoxicity of the *c*9, *t*11, *c*13, the *c*9, *t*11, *t*13, and the *t*9, *t*11, *c*13 CALA isomers were compared to that of the *t*8, *t*10, *c*12 CALA isomer using the U-937 monocytic leukemia cell line and the SV-T2 transformed mouse fibroblast cell line, the 9, 11, 13-CALA isomers displayed much greater activity (Suzuki *et al*., 2001). Yasui *et al.* (2006b) assessed the impact that the *trans* content of CALA had on its apoptotic activity using the Caco-2 cancer cell line. The results showed that the all *trans* CALA isomers (*t*9, *t*11, *t*13 and *t*8, *t*10, *t*12) were more inhibitory to Caco-2 growth than their partial *trans* counterparts (*t*9, *t*11, *c*13 and *t*8, *t*10, *c*12). When compared to CLA isomers (*c*9, *t*11 and/or *t*10, *c*12 CLA), tung and bitter gourd seed oils (rich in the *c*9, *t*11, *t*13 CALA isomer) and pomegranate seed oil (rich in the *c*9, *t*11, *c*13 CALA isomer) display a higher anti-carcinogenic activity against colon cancers than the CLA isomers (Kohno *et al.*, 2004a; Tsuzuki *et al*., 2004b; Yasui *et al*., 2005). These investigations highlight the impact of bond position, bond number and bond conformation on the properties of these conjugated fatty acids.

Investigations into the metabolism of CALA have suggested that the body rapidly converts 9, 11, 13-CALA isomers to CLA via a Δ^{13}-saturation reaction catalyzed by a NADPH dependent enzyme (Kohno *et al.*, 2004a; Kohno *et al.*, 2004b; Suzuki *et al*., 2006; Tsuzuki *et al*., 2003; Tsuzuki *et al*., 2004c). Hence, it may be CLA rather than CALA which exerts the anti-carcinogenic activity *in vivo*.

Interestingly, when rats were supplied with equivalent concentrations of CLA or CALA, it was CALA which caused the highest increase in CLA concentrations in both the non-lesional mucosa and liver, potentially explaining the higher anti-carcinogenic activity seen with CALA. All studies have not witnessed the high conversion of CALA to CLA *in vivo*. Plourde *et al.* (2006) found that when fed a mixture of CALA isomers (i.e. *c*9, *t*11, *c*15 and *c*9, *t*13, *c*15 isomers), CALA could be detected in the liver, blood plasma and adipose tissue of Wistar rats. This may suggest that the 9, 11, 13-CALA isomers typically found in plants and seed oils, and the 9, 11, 15-CALA isomers produced by bacteria may have very different metabolic fates and hence, their activity on disease and health may differ substantially.

1.3.6 Conclusions

Investigations into the health promoting activity of the CALA isomers suggest they possess a similar bioactive range to both α-linolenic acid and the CLA isomers, being effective in the treatment of cancer, obesity, and cardiovascular disease. When compared with α-linolenic acid, CALA isomers often appear more active than the parent fatty acid. Moreover, certain CALA isomers appear more active than the other well characterized conjugates, the CLA isomers. One of the most interesting aspects of the CALA is how the activity differs among the different isomers. Observations have shown that the activity of these conjugates against diseases such as cancer differs considerably with the position of the double bonds, the number of conjugated double bonds present, and the conformation of these double bonds. Given our relatively poor knowledge of the overall physiological impact of CALA isomers, the effect of their widespread use as therapeutics or in functional foods is currently unknown and warrants further investigation.

1.3.7 References

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