Chapter 4

Assessing the Anti-carcinogenic Properties of Conjugated Fatty Acids against the SW480 Colon Cancer Cell Line

Abstract

In this study we assessed three novel bifidobacterially produced conjugated fatty acids for inhibitory activity against the SW480 colon cancer cell line. These conjugated fatty acids were prepared via the action of the enzyme linoleic acid isomerase on α-linolenic acid, γ-linolenic acid and stearidonic acid, resulting in the production of *c*9, *t*11, *c*15 CALA, *c*6, *c*9, *t*11 CGLA and *c*6, *c*9, *t*11, *c*15 CSA, respectively. All three novel conjugated fatty acids displayed potent, selective, inhibitory against the SW480 cell line relative to the normal fetal human colonic epithelial cell line (FHC). On a cellular level exposure of the SW480 cell line to CSA and CGLA resulted in an increase in the saturated fatty acid content of the cell membrane, and improvements in the ratio of the ω-6 to ω-3 PUFA, both of which are associated reducing the inflammatory status of the cell and the risk of cancer. The inhibitory activity of both CGLA and CSA against the SW480 cell line was substantially diminished in the presence of the antioxidant α-tocopherol, suggesting the prominent role of lipid peroxidation in the inhibitory activity of both, while CALA remained unaffected. At a protein level CALA, CSA, α-linolenic acid and stearidonic acid also impacted on the expression of the anti-apoptotic protein, Bcl-2 in the SW480 cell line significantly reducing its cellular concentration. In conclusion, the study has demonstrated the selective anti-carcinogenic properties of CALA, CGLA and CSA against the SW480 cell line. Increased cellular lipid peroxidation was found to play a major role in the anti-carcinogenic activity of CGLA and CSA, while reduced cellular concentrations of the anti-apoptotic protein Bcl-2 were associated with the anti-carcinogenic activity of CALA and CSA.

4.1 Introduction

Conjugated fatty acids are the positional, geometric isomers of several unsaturated fatty acids, which possess one or more conjugated double bonds. These conjugated isomers are commonly found in nature, with the greatest concentrations found in the milkfat and tallow of ruminant animals (Dhiman *et al.*, 2005; Stanton *et al.*, 2003), in plant seed oils (Chisholm & Hopkins, 1967; Kohno *et al.*, 2004b; Liu *et al.*, 1997; Yasui *et al.*, 2005) and in marine algae (Burgess *et al.*, 1991; Lopez & Gerwick, 1987; Mikhailova *et al.*, 1995; Wise *et al.*, 1994). In addition, a range of conjugated fatty acids may be formed chemically via the alkaline isomerisation of unsaturated fatty acids (Park *et al.*, 2005; Yonezawa *et al.*, 2005). Of these conjugated fatty acids, it is the conjugated linoleic acid (CLA) and conjugated α-linolenic acid (CALA) isomers, which are the best characterised thus far. These isomers have frequently displayed potent anti-atherosclerotic, anti-obesogenic and anti-diabetogenic activities, however, it is their anti-carcinogenic activities which are probably best characterised (Bhattacharya *et al.*, 2006).

Cancers of the mammary tissue, prostate, liver, stomach, and lungs have all increased in incidence in recent decades, however, it is cancers of the colon which are of particular interest to this study due to their strong association with diet. Colon cancer, the third most common malignant neoplasm in the world, has been associated with the increased "Westernisation" of the human diet and in particular increased fat intake (Shike *et al.*, 1990; Tanaka, 1997). In an attempt to combat this disease, diets rich in fatty acids with known anti-carcinogenic properties such as CLA and CALA have received particular attention (Beppu *et al.*, 2006; Kohno *et al.*, 2004a; Wahle *et al.*, 2004; Yasui *et al.*, 2005; Yasui *et al.*, 2006a). The potency of these conjugated fatty acids in the treatment of colon cancer has prompted increased research into the identification and economic production of other novel

conjugated fatty acids (Coakley *et al.*, 2006; Ogawa *et al.*, 2005). These investigations have in particular highlighted the ability of a range of microbes to conjugate the *c*9, *c*12 double bond system of certain unsaturated fatty acids via the action of the enzyme linoleic acid isomerase (Hennessy *et al.*, 2007; Sieber *et al.*, 2004). This activity is most frequently reported to yield the *c*9, *t*11-C18:2 CLA isomer from linoleic acid (Barrett *et al.*, 2007; Coakley *et al.*, 2003; Jiang *et al.*, 1998). However, recent reports have also highlighted the ability of strains of bifidobacteria to produce the *c*9, *t*11, *c*15-C18:3 CALA isomer from α-linolenic acid, the *c*6, *c*9, *t*11-C18:3 conjugated γ-linolenic acid (CGLA) isomer from γ-linolenic acid and the *c*6, *c*9, *t*11, *c*15-C18:4 conjugated stearidonic acid (CSA) isomer from stearidonic acid (Coakley *et al.*, 2009) (Chapter 3).

The aim of the current study was to assess the ability of the bifidobacterially produced conjugated fatty acids, *c*9, *t*11, *c*15-C18:3 CALA, *c*6, *c*9, *t*11-C18:3 CGLA, and *c*6, *c*9, *t*11, *c*15-C18:4 CSA, to inhibit the growth of the SW480 colon cancer cell line relative to their respective parent unsaturated fatty acids. Furthermore, to determine if this inhibitory activity was selective for colon cancer the activity of the conjugates against the SW480 cell line was compared with that of the normal human fetal epithelial cell line (FHC). Once the selective inhibitory activity against of the conjugated fatty acids against the SW480 colon cancer cell line was confirmed, we attempted to elucidate the mechanisms through which this anti-carcinogenic activity was mediated.

4.2 Materials and methods

4.2.1 Production of conjugated fatty acids

Conjugated fatty acids were produced using the intestinally-isolated bifidobacterial strain *Bifidobacterium breve* DPC6330 and the substrate fatty acids α-linolenic acid (Sigma Aldrich, St Louis, Mo), γ-linolenic acid (Nu-chek Prep, Elysian, MN, U.S.A.) and stearidonic acid (Cayman Europe, Akadeemia tee, Tallinn, Estonia). The conjugated fatty acids were isolated and identified as previously described in Chapter 3. All fatty acids were purified to a concentration of >95% by reverse phase high performance liquid chromatography (RP-HPLC) and were stored as a 100 mg/ml stock solution in chloroform, at -20^{o}C under nitrogen.

4.2.2 Cells and Cell cultures

The SW480 colon cancer cell line was obtained from the European Collection of Cell Cultures (ECACC, Porton Down, Salisbury, UK). Culture media and supplements were supplied by Sigma Aldrich. SW480 colon cancer cells were maintained in Dulbecco's Minimum Essential Medium (DMEM) supplemented with 5% (v/v) fetal bovine serum (FBS), 0.2 mmol/L L-glutamine, 1 mmol/L HEPES and 1 unit/mL penicillin and streptomycin (Sigma Aldrich). The normal human fetal colonic epithelial FHC cells (CRL-1831; ATCC, Rockville, MD) were cultured in Ham's F12 and Dulbecco's modified Eagle's mediums (1:1) containing HEPES (25 mM), cholera toxin (10 ng/ml), insulin (5 μg/ml), transferrin (5 μg/ml) and hydrocortisone (100 ng/ml), 10% (v/v) FBS and 1 unit/mL penicillin and streptomycin (Sigma-Aldrich). Both cell lines were maintained at 37°C in a humidified atmosphere. The pH of the media was maintained by a required flow of 95% air and 5% CO_2. When required SW480 cells were grown in 96 well plates and

maintained at 37°C in a humidified atmosphere. Initially, 1 x 10^4 SW480 cells were seeded in wells and cultured for 24 h at 37°C, allowing the cells to attach to the substratum. Similarly, when required the FHC cells were grown in 96 well plates and maintained at 37°C in a humidified atmosphere. Initially, 2.5 x 10^3 FHC cells were seeded in wells and cultured for 48 h at 37°C, allowing the cells to attach to the substratum.

To assess the effect that exposure to conjugated fatty acids or their unsaturated parent fatty acids had on the viability of the SW480 and FHC cell lines, the medium from both was replaced with medium containing the respective fatty acids delivered in the form of a 3 mg/ml stock solution in ethanol. Investigations into the effect of fatty acid concentration on the inhibitory activity were conducted at concentrations ranging from 0 μM - 200 μM over 24 h, 48 h and 72 h exposure. Similarly, comparative studies between the inhibitory activity of the conjugated fatty acids and their respective parent unsaturated fatty acids were also conducted at concentrations ranging from 0 μM - 200 μM over 24 h, 48 h and 72 h. Studies comparing the inhibitory activity of the conjugated fatty acids against the SW480 and FHC cell lines were conducted at fatty acid concentrations of 200 μM in the case of CALA and CGLA and at a concentration of 100 μM for CSA following 24 h exposure. To assess the role of lipid peroxidation in the inhibitory activity of the conjugates the SW480 cells were exposed to 0 μM, 50 μM and 200 μM of CALA, CGLA or CSA for 24 h in the presence or absence of 50 μM of the antioxidant α-tocopherol which was delivered in the form of a 100 mg/ml stock solution in ethanol as described by Igarashi and Miyazawa, (2000). In each study, cell viability was determined using the MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium), method (Promega Corporation, Madison, WI, USA) as described by Coakley *et al*, (2009). Cell

viability (%) after treatment was expressed relative to the ethanol control, which alone represents 100%.

4.2.3 Gas Liquid Chromatography (GLC)

The fatty acid composition of the pure oils was determined by gas liquid chromatography (GLC) using the method described by Stanton *et al.* (1997). The fatty acid methyl esters (FAME) were separated using a CP Sil 88 column (100 m x 30.25 mm i.d., 0.20 mm film thickness; Chrompack, Middelburg, The Netherlands) and quantified using a gas liquid chromatograph (3400; Varian, Harbor City, CA, USA). The GLC instrument was calibrated using a range of commercial fatty acid standards (Sigma-Aldrich). The GLC instrument was fitted with a flame ionisation detector (FID) and helium (37 psi) was used as the carrier gas. The injector temperature was held isothermally at 225^{o}C for 10 min and the detector temperature was 250^{o}C. The column oven was held at an initial temperature of 140^{o}C for 8 min and then programmed to increase at a rate of 8.5^{o}C/min to a final temperature of 200^{o}C, which was held for 41 min. Data were recorded and analysed on a Minichrom PC system (VG Data System, Manchester, UK).

4.2.4 Determination of cellular fatty acid composition

SW480 colon cancer cells were exposed to 0 or 50 μM of CALA, CGLA or CSA for 24 h. Cells were harvested by scraping and washed twice with phosphate buffer saline (PBS). Total cellular lipids were extracted from the SW480 cells using the method of Folch *et al.* (1957). The impact of CALA, CGLA and CSA on the fatty acid composition of cellular phospholipids was determined using solid phase extraction (SPE) with amino-propyl cartridges as described by Bondia-Pons *et al*, (2006). Methylation of total cellular lipids and the SPE isolated cellular

phospholipids was carried out by *in situ* transesterification with 0.5 N methanolic NaOH followed by 14% boron trifluoride in methanol as described by Park and Goins (1994). The FAME were separated by GLC as previously described.

4.2.5 Quantitative determination of cellular p53, COX-2 and Bcl-2 concentrations

The SW480 colon cancer cells were cultured as previously described. The effects of the incubation of C18 unsaturated fatty acids linoleic acid, α-linolenic acid, γ-linolenic acid and stearidonic acid or their respective conjugated isomers CLA, CALA, CGLA and CSA for 72 h on the expression of p53, cycloxygenase-2 (COX-2) and Bcl-2 were compared. Initially 1 x 10^6 cells were seeded in 25 cm^2 flasks and cultured for 24 h in 5 mls of medium allowing the cells to attach to the substratum. The medium was then replaced with fresh medium containing the respective fatty acids at a concentration of 25 μM, delivered in the form of a 3 mg/ml stock solution in ethanol, while an equivalent amount of ethanol was added to a flask to serve as a control. Following 72 h incubation cells were harvested by scraping and the cellular lysis and enzyme-linked immunosorbent assay (ELISA) performed as specified by the kit manufacturer (Calbiochem, Merck KGaA, Darmstadt, Germany). To determine the concentration of p53 in the cell lysates a human total p53 ELISA kit was employed (Calbiochem), COX-2 concentrations in cell lysates were measured using a human COX-2 ELISA kit (Calbiochem) following stimulation of the cells using lipopolysaccharide (LPS) derived from *Salmonella typhimurium*, while Bcl-2 concentrations in the cell lysates were measured using a human Bcl-2 ELISA kit (Calbiochem).

4.2.6 Statistical analysis

Three independent experiments were performed in triplicate for each treatment when the MTS assay was employed. Two independent experiments were performed in triplicate for each treatment for both the fatty acid analysis and the ELISA assays. The Student's *t* test was used to determine significant differences between treatments. Statistically significant differences were represented as follows (* = $P \leq 0.05$; ** = $P \leq 0.01$; *** = $P \leq 0.001$)

4.3 Results

SW480 cells were incubated for 24 h, 48 h and 72 h in the presence of varying concentrations of the conjugated fatty acids CALA, CGLA, or CSA or their respective parent unsaturated fatty acid, α-linolenic, γ-linolenic and stearidonic acid. This enabled an assessment of the inhibitory activity of both the conjugated fatty acids and their respective parent unsaturated fatty acids against the SW480 cell line over time and at a range of fatty acid concentrations. Exposure of the SW480 cell line to CALA reduced cell viability in a dose-dependent manner **(Figure 4.1)**. The viability of the SW480 colon cancer cells treated with 25 to 100 μM CALA for 24 h ranged from 91-97%, while at a CALA concentration of 200 μM, viability was reduced to 77%. Exposure to 200 μM CALA for 48 h resulted in a significant decrease in cancer cell viability, which declined from 77% at 24 h exposure to 55% after 48 h exposure ($P \leq 0.01$) **(Figure 4.2)**. This trend was also evident following 72 h exposure to CALA with cell viability declining from 55% at 48 h to 41% at 72 h ($P \leq 0.01$) **(Figure 4.2)**. When the viability of the SW480 colon cancer cells exposed to equivalent concentrations of CALA or α-linolenic acid for 24 h, 48 h and 72 h was compared, no significant differences in cell viability were observed **(Figure 4.3)**. This was further emphasised on determination of the IC50 for both CALA and α-linolenic acid which were calculated to be 180 ± 4.0 μM and 178 ± 3.4 μM, respectively, following 72 h. After 24 h exposure to CGLA, a significant reduction in the viability of the SW480 cell line was observed at concentrations of 100 μM (10%) ($P \leq 0.05$) and 200 μM (72%) ($P \leq 0.001$) **(Figure 4.4)**. Following 48 h, significant decreases in cell viability were observed at CGLA concentrations of 50 μM, 100 μM and 200 μM, with cell viability determined to be 93%, 72% and 3%, respectively. Comparatively, the reductions in the viability of the SW480 cell line at CGLA concentrations of 100 μM ($P \leq 0.05$) and 200 μM ($P \leq 0.001$) after

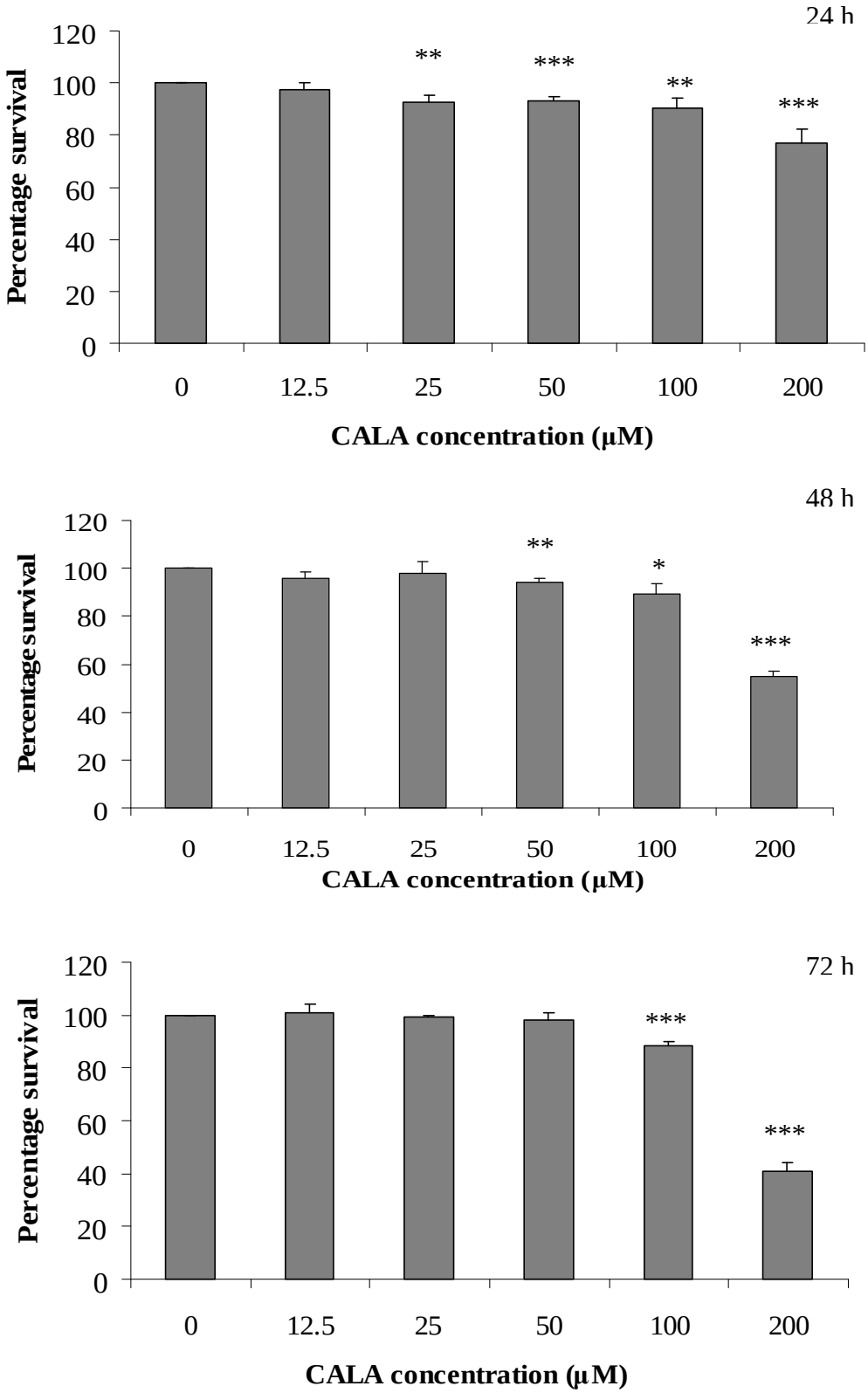


Figure 4.1 Effect of fatty acid concentration on the viability of SW480 colon cancer cells following 24 h, 48 h and 72 h exposure to CALA. Asterisks highlight concentrations which are significantly different from 0 µM. (* = $P \leq 0.05$; ** = $P \leq 0.01$; *** = $P \leq 0.001$)

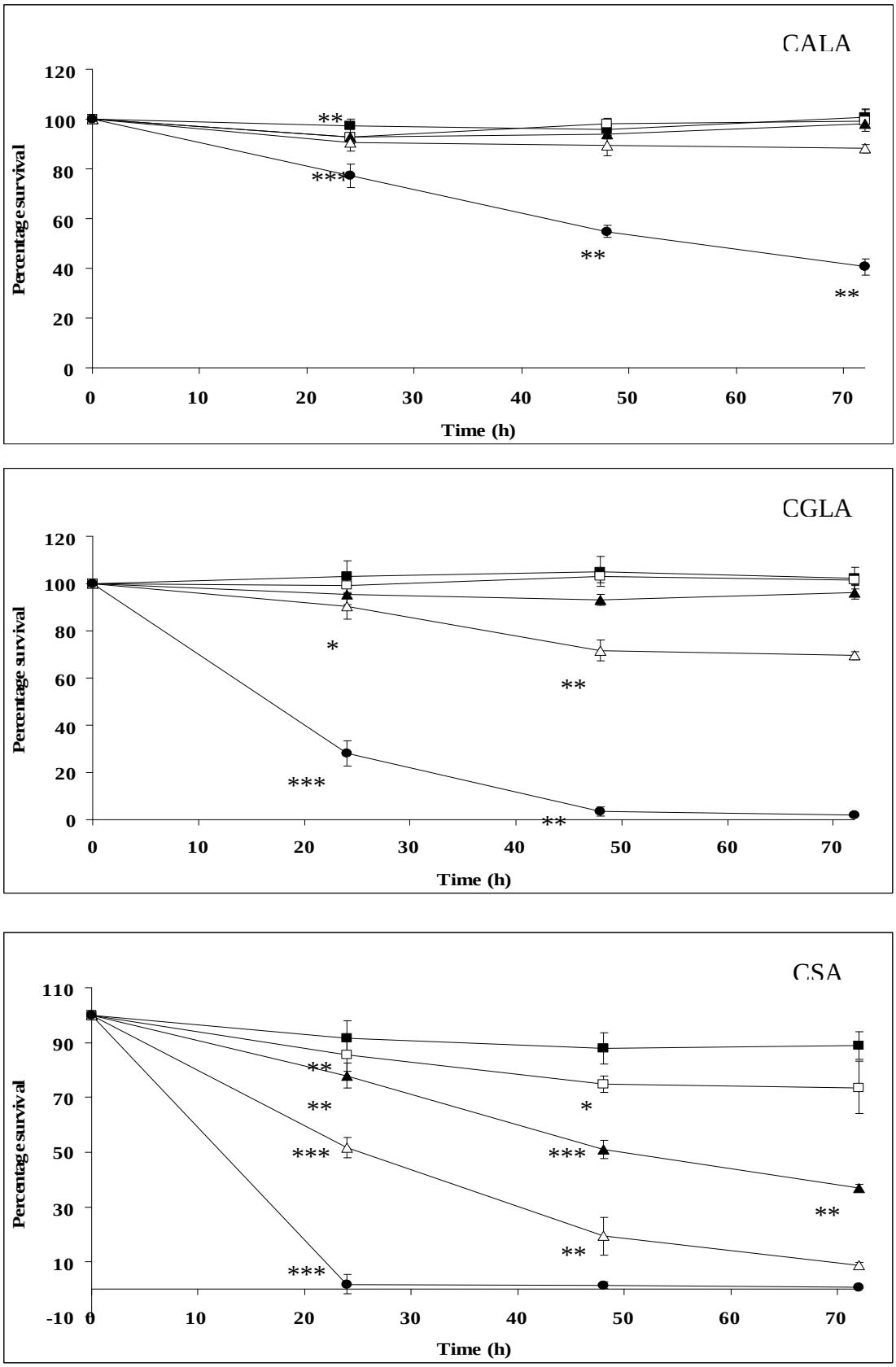


Figure 4.2 Effect of duration of exposure to a) CALA, b) CGLA and c) CSA on the viability of SW480 cell line. (■) 12.5 μM, (□) 25 μM, (▲) 50 μM, (Δ) 100 μM and (●) 200 μM. Asterisks highlight time points which are significantly different from the previous. (* = $P \leq 0.05$; ** = $P \leq 0.01$; *** = $P \leq 0.001$)

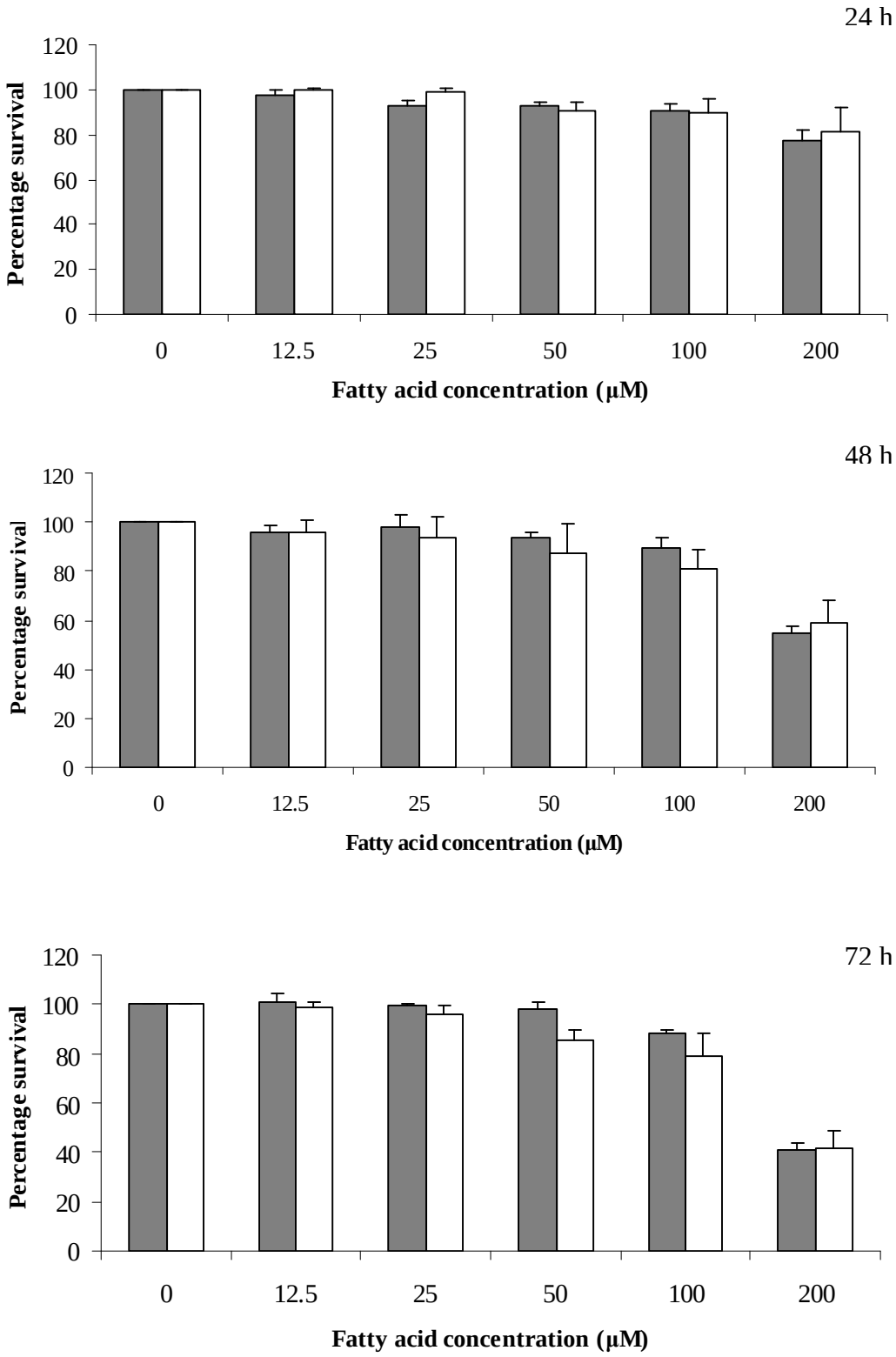


Figure 4.3 Comparing the inhibitory effect of exposure to CALA (■) on the viability of the SW480 colon cancer cells relative to that of α-linolenic acid (□) following 24 h, 48 h and 72 h. (Statistical analysis: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$)

48 h exposure marked a significant reduction relative to those observed following 24 h exposure to equivalent concentrations of CGLA **(Figure 4.4)**. Following 72 h exposure, no significant reductions in cell viability were observed relative to those observed following 48 h exposure. Of the fatty acid concentrations assayed following 24 h exposure, only a concentration of 200 μM CGLA resulted in a reduction in cell viability which was significantly greater than that of an equivalent concentration of its parent unsaturated fatty acid, γ-linolenic acid. The reductions in cell viability observed at this fatty acid concentration were 28% and 67% for γ-linolenic acid and CGLA, respectively ($P \leq 0.01$) **(Figure 4.5)**. After 48 h exposure, significant differences in the inhibitory activity of CGLA (28% reduction in viability) and γ-linolenic acid (16% reduction in viability) could also be observed at a fatty acid concentration of 100 μM, ($P \leq 0.05$) **(Figure 4.5)**. The inhibitory activity of CGLA reached its maximum following 48 h exposure to a fatty acid concentration of 200 μM, with a 97% reduction in cell viability observed relative to the ethanol control **(Figure 4.5)**. Indeed, the greatest difference in the inhibitory activity of CGLA and γ-linolenic acid was also found at this concentration and time point, with exposure to CGLA resulting in a 40% greater reduction in cell viability than an equivalent concentration of γ-linolenic acid **(Figure 4.5)**. Following 72 h exposure, the extent of the difference in inhibitory activity between CGLA and γ-linolenic acid was greatly reduced. The IC50 for CGLA following 24 h was determined to be 165 ± 5.1 μM, however, an equivalent for γ-linolenic acid could not be calculated as the inhibitory activity of the isomer did not reach 50% at this time point. After 48 h exposure the IC50 for CGLA had fallen to 132 ± 3.2 μM, while the IC50 for γ-linolenic acid was determined to be 192 ± 1.7 μM. Following 72 h exposure, the IC50 for CGLA was determined to be 129 ± 3.3 μM, while that

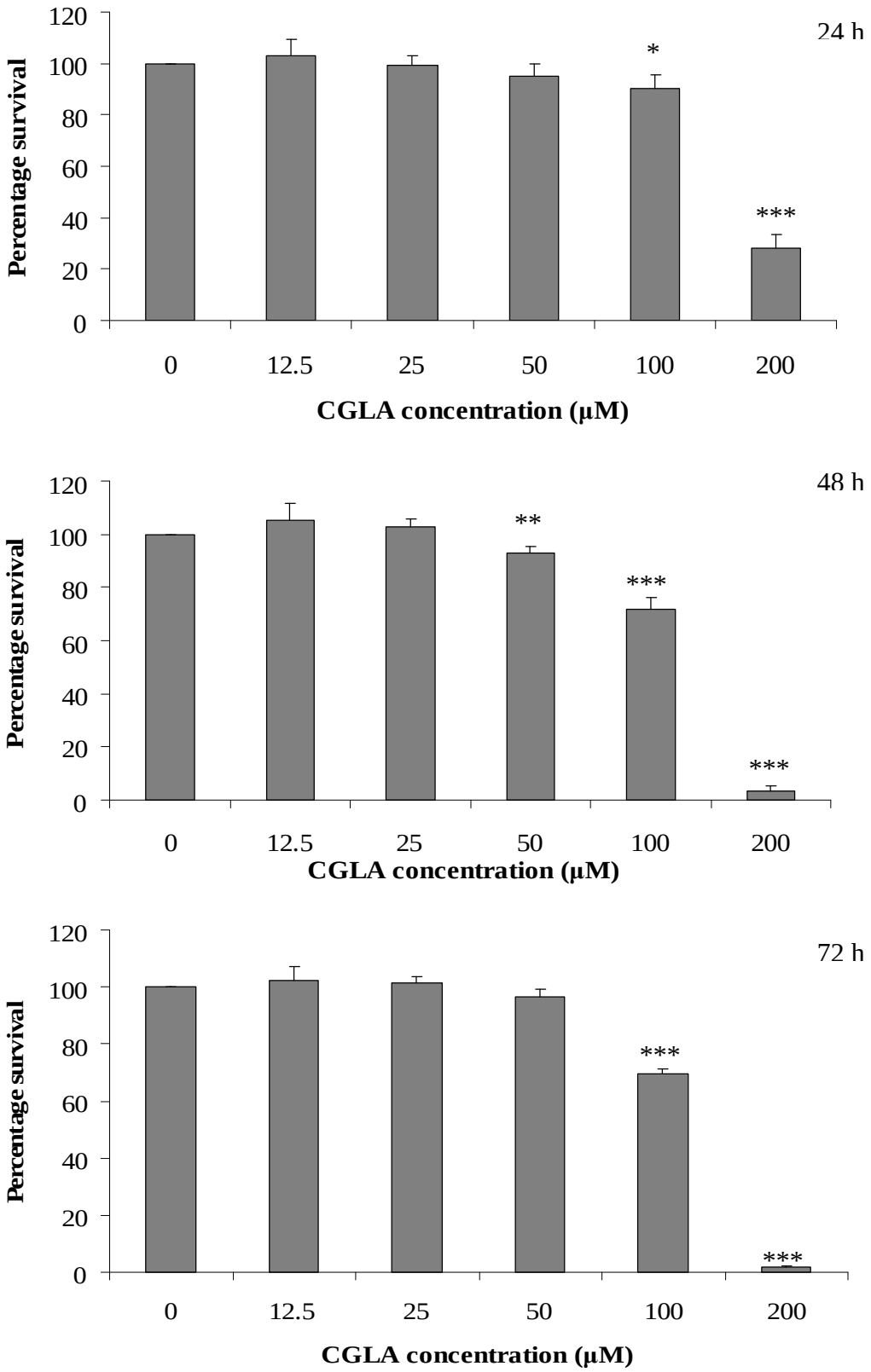
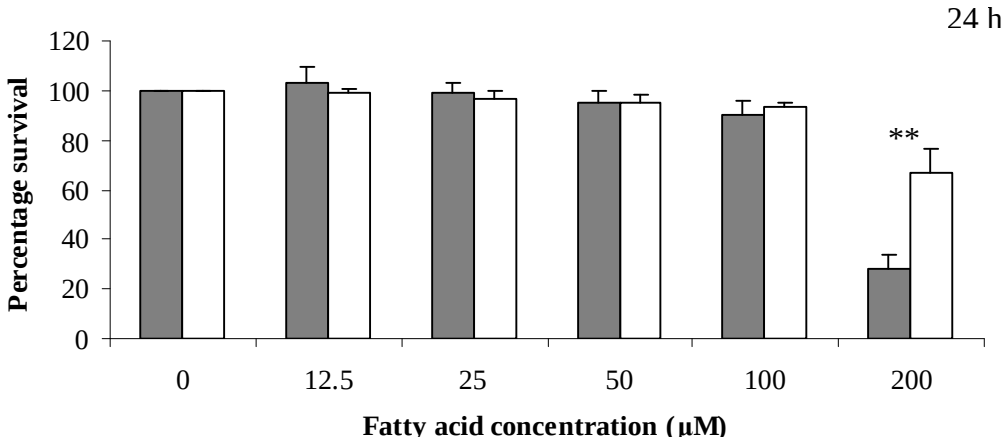
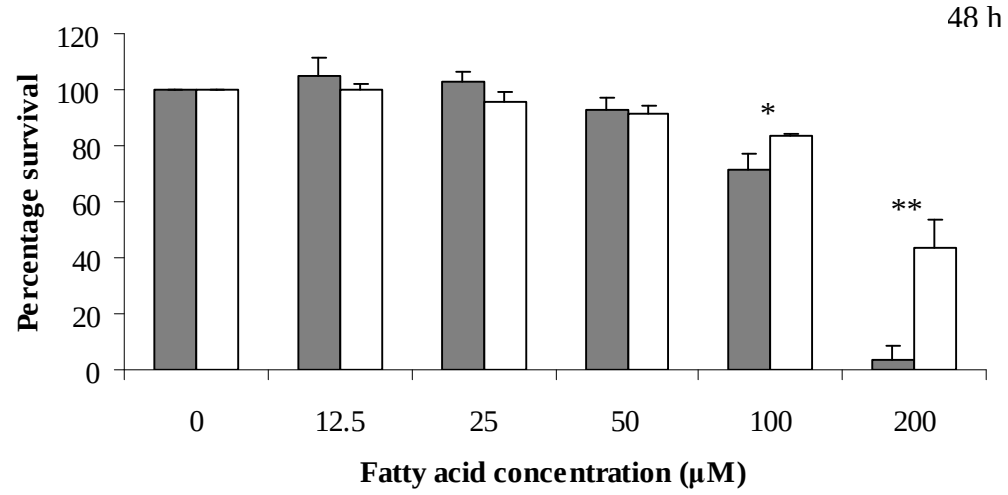


Figure 4.4 Effect of fatty acid concentration on the viability of SW480 colon cancer cells following 24 h, 48 h and 72 h exposure to CGLA. Asterisks highlight concentrations which are significantly different from 0 µM. (* = $P \leq 0.05$; ** = $P \leq 0.01$; *** = $P \leq 0.001$)





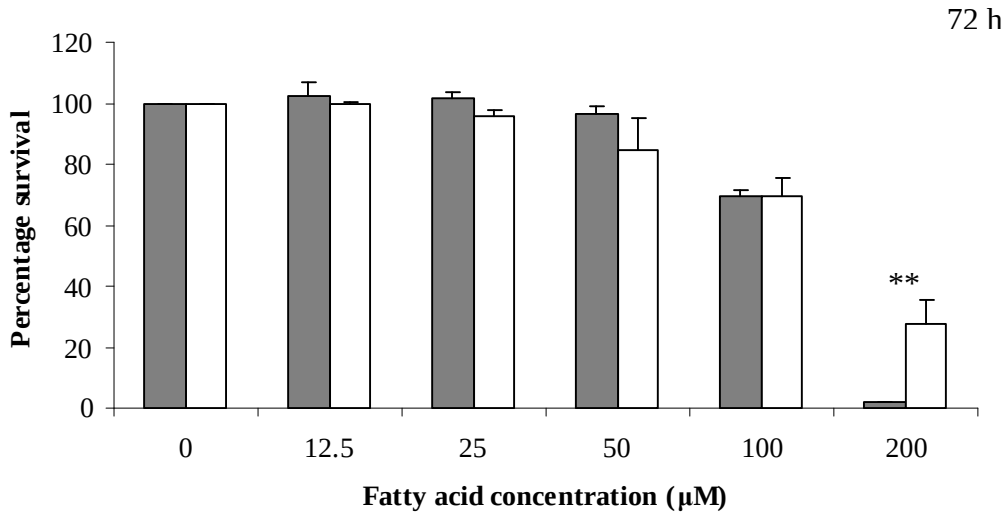


Figure 4.5 Comparing the inhibitory effect of exposure to CGLA (■) on the viability of the SW480 colon cancer cells relative to that of γ-linolenic acid (□) following 24 h, 48 h and 72 h. (Statistical analysis: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$).

of γ-linolenic acid was determined to be 147 ± 2.1 μM. Of the conjugated C18 fatty acids assayed, CSA displayed the greatest inhibitory activity against the SW480 cell line. Exposure of the SW480 cell line to CSA concentrations of between 25 μM and 200 μM resulted in significant reductions in cell viability, ranging from 15% to 98% in a dose dependent manner **(Figure 4.6)**. This significant dose response was also evident following 48 h and 72 h exposure to CSA at concentrations ranging from 12.5 μM to 200 μM **(Figure 4.6)**. The reductions in the viability of the SW480 cell line at CSA concentrations of 50 μM and 100 μM after 48 h marked a significant reduction in cell viability relative to those observed after 24 h exposure **(Figure 4.2)**. Following 72 h, only exposure to 50 μM CSA resulted in a significantly greater reduction in cell viability than was observed following 48 h **(Figure 4.2)**. CSA was found to have a greater inhibitory activity against the SW480 cell line than stearidonic acid at all concentrations assayed. Following 24 h and 48 h exposure, the differences in the inhibitory activity of CSA and stearidonic acid reached significance at fatty acid concentrations ranging from 50-200 μM, while following 72 h exposure significant differences in inhibitory activity were only observed at concentrations of 50-100 μM **(Figure 4.7)**. The greater potency of CSA relative to stearidonic acid in the inhibition of the SW480 cell line was reflected in the IC50 of both fatty acids. After 24 h exposure, the IC50 for CSA was determined to be 104 ± 2.9 μM, while the IC50 for stearidonic acid was determined to be 162 ± 3.7 μM. At 48 h exposure, the IC50 for both fatty acids declined, with the IC50 for CSA determined to be 56 ± 5.2 μM, while the IC50 of stearidonic acid was determined to be 120 ± 2.7 μM. This trend continued following 72 h exposure, with the IC50 for CSA determined to be 46 ± 8.1 μM, while for stearidonic the IC50 was determined to be 101 ± 4.0 μM.

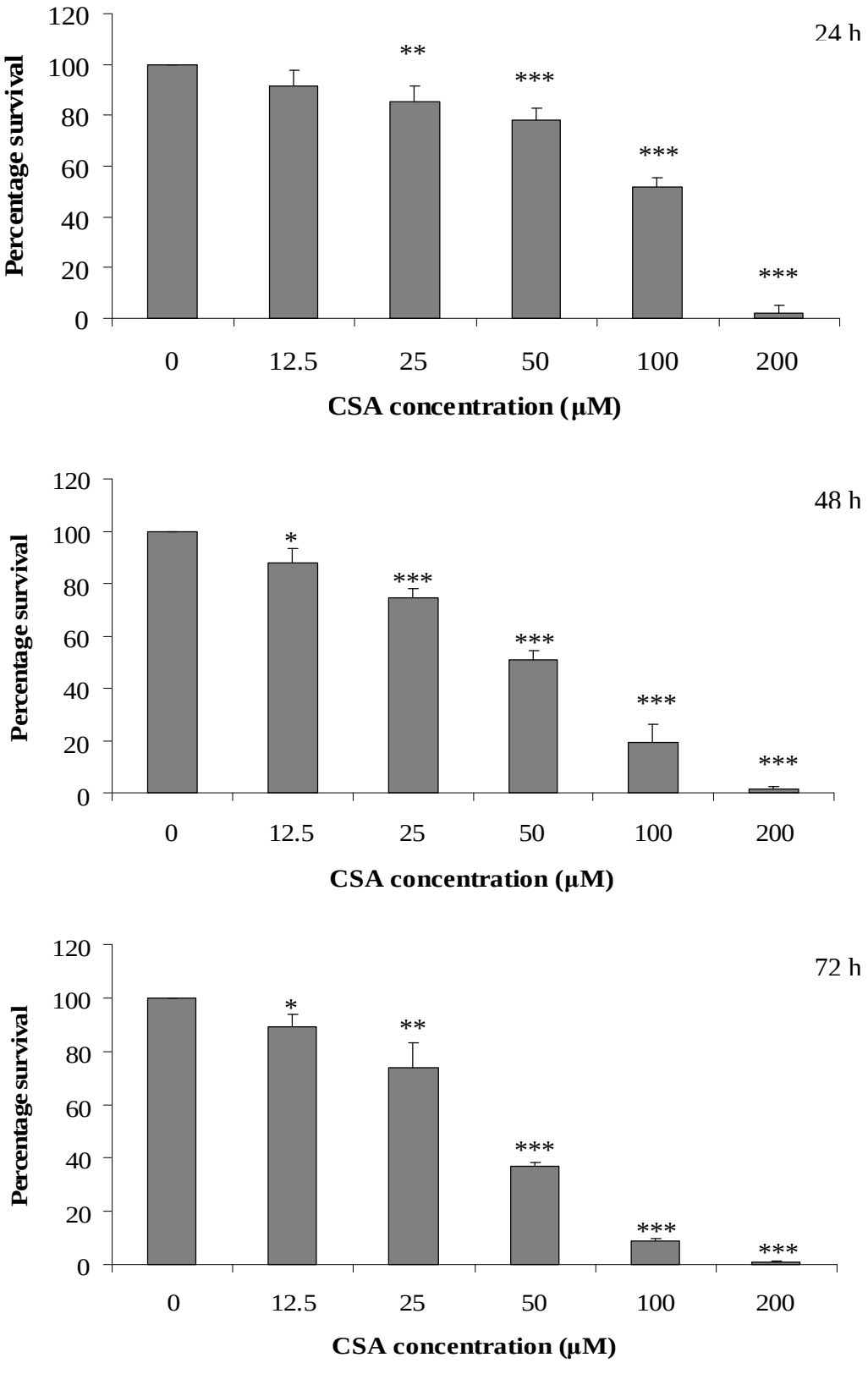


Figure 4.6 Effect of fatty acid concentration on the viability of SW480 colon cancer cells following 24 h, 48 h and 72 h exposure to CSA. Asterisks highlight concentrations which are significantly different from 0 µM. (* = $P \leq 0.05$; ** = $P \leq 0.01$; *** = $P \leq 0.001$).

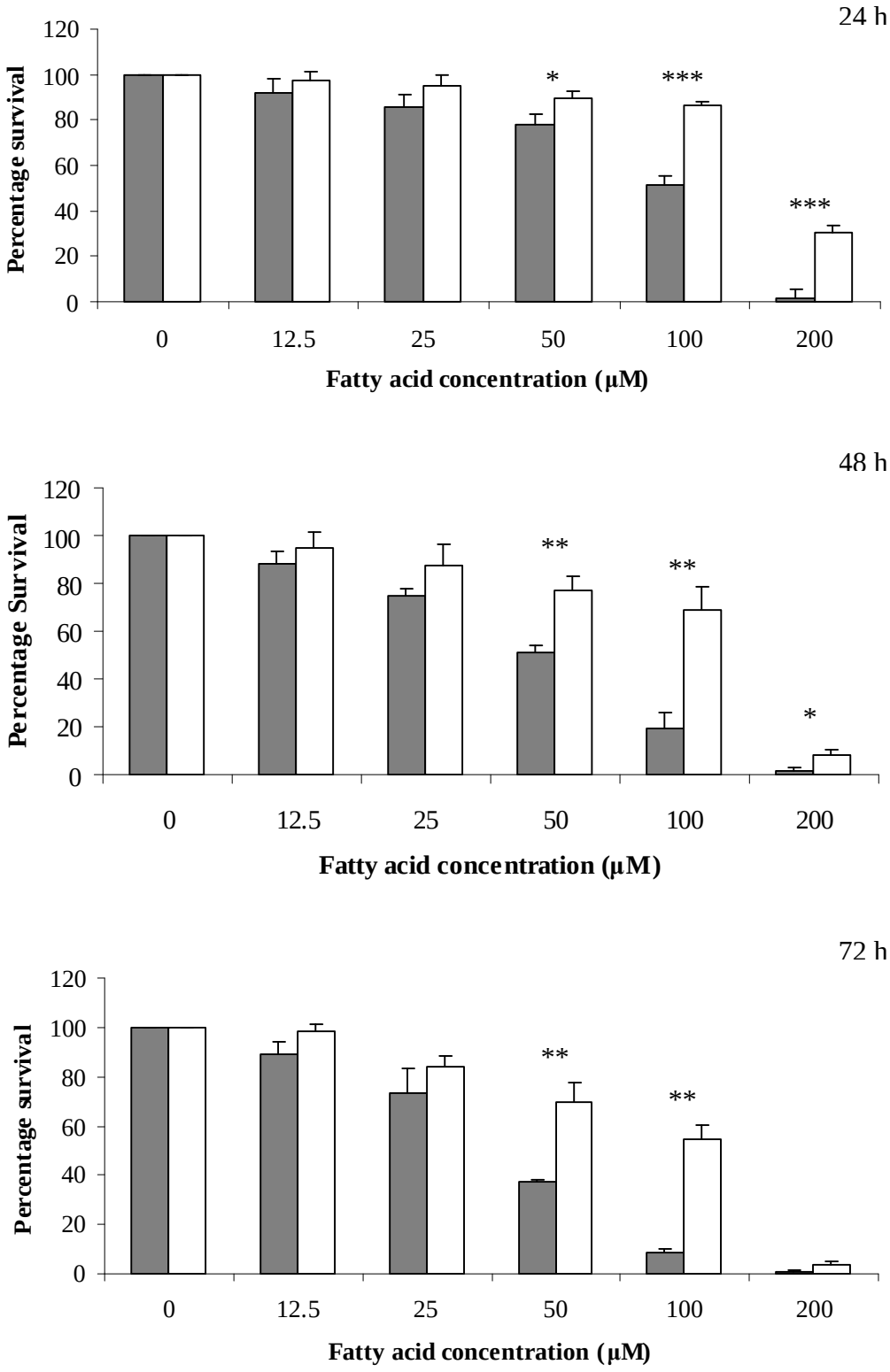


Figure 4.7 Comparing the inhibitory effect of exposure to CSA (■)on the viability of the SW480 colon cancer cells relative to that of stearidonic acid (□) following 24 h, 48 h and 72 h. (Statistical analysis: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$).

In this study, the inhibitory effect of three microbially produced conjugated fatty acids, CALA, CGLA and CSA against the SW480 colon cancer cell line and on the normal colonic epithelial FHC cell line were compared **(Figure 4.8)**. The results demonstrated that following 24 h exposure to the respective conjugated fatty acids, the viability of the SW480 cell line was inhibited significantly more than that of the normal FHC cell line. Indeed, exposure to 200 μM CALA resulted in a 24.2% greater reduction in the viability of the SW480 cell line relative to the FHC cell line ($P \leq 0.01$), while exposure to 200 μM CGLA resulted in a 45.2% greater reduction in the viability of the SW480 colon cancer cell line relative to the FHC cell line ($P \leq 0.001$). At a concentration of 200 μM, CSA inhibited the growth of both the SW480 and FHC cell lines by approximately 95%. This inhibitory activity made it difficult to determine if the conjugate was more active against the SW480 cell line than the normal FHC cell line. As a result of the strong inhibitory activity of CSA against both the SW480 cell line and the normal fetal colonic epithelial FHC cell line, it was decided to reduce the concentration of the fatty acid to which both cell lines were exposed to 100 μM. The results showed that exposure to CSA caused a 19% greater reduction in the viability of the SW480 cell line than in the FHC cell line ($P \leq 0.01$). These results would suggest that the SW480 colon cancer cells have a greater susceptibility to the inhibitory activity of the conjugated fatty acids than the normal FHC cell line.

In our study, the inhibitory effect of CALA, CGLA and CSA at concentrations of 50 μM and 200 μM were assessed in the presence and absence of the natural antioxidant α-tocopherol **(Figure 4.9)**. Following 48 h exposure, the inhibitory activity of 50 μM CALA against the SW480 cell line was reduced by 10.21% in the presence of α-tocopherol, while following 72 h exposure, the

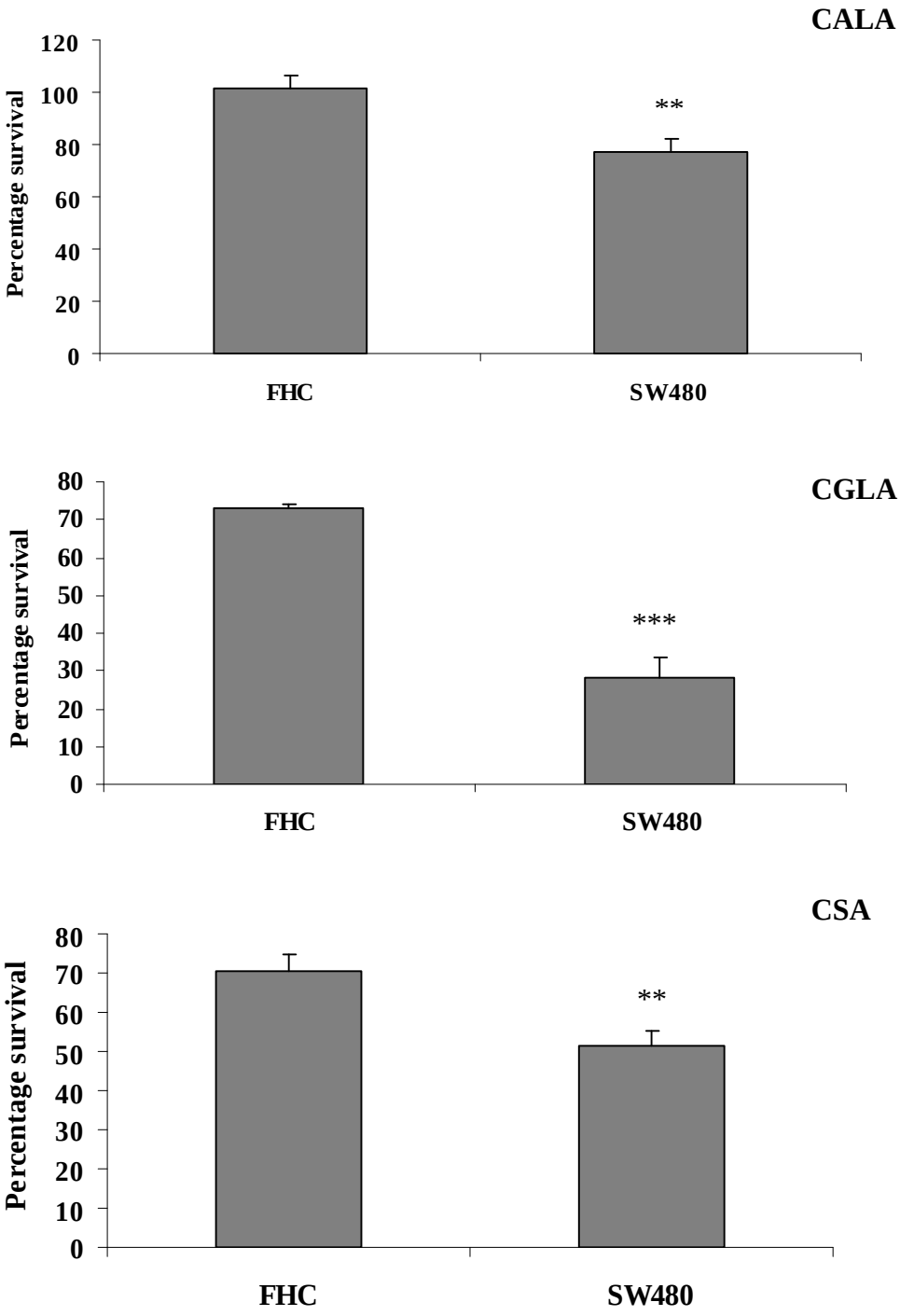


Figure 4.8 Comparing the inhibitory activity of 200 μM CALA, 200 μM CGLA or 100 μM CSA against the SW480 colon cancer relative to that of the normal colonic FHC cell line after 24 h. (Statistical analysis: * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$)

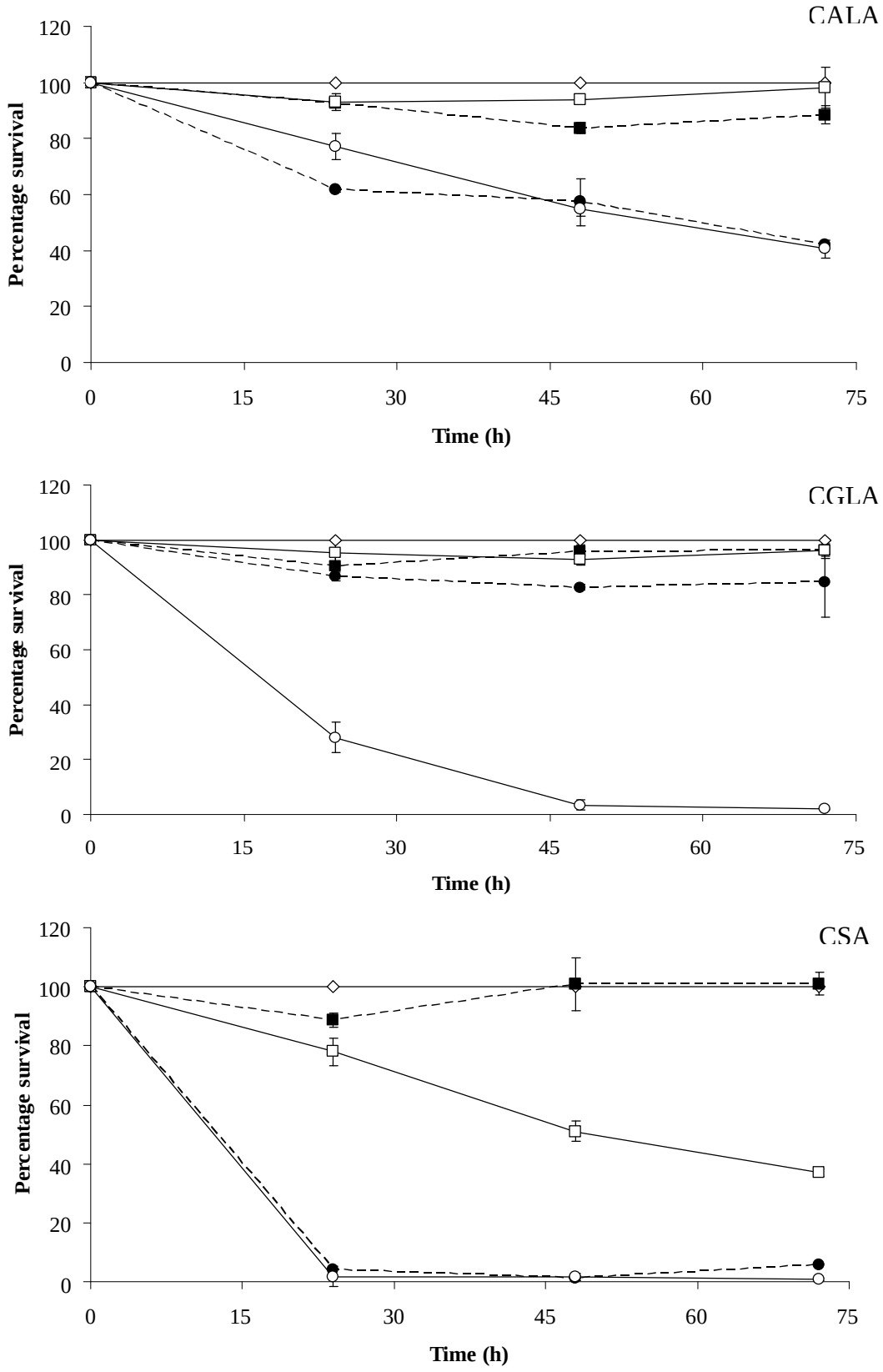


Figure 4.9 Assessing the impact of α-tocopherol on the inhibitory activity of CALA, CGLA and CSA over 72 h. Control ◇, 50 μM conjugate □, 50 μM conjugate & α-tocopherol ■ 200 μM conjugate ○, 200 μM conjugate & α-tocopherol ●

inhibitory activity of 50 μM CALA was reduced by 9.61% in the presence of α-tocopherol. These observations suggest that at these time points, lipid peroxidation plays a minor role in the inhibitory activity of 50 μM CALA against the SW480 cell line. In the presence of α-tocopherol, the inhibitory activity of 200 μM CALA was relatively unaffected, however, following 24 h exposure a 15.62% reduction in the inhibitory activity of the fatty acid against the SW480 cell line was observed. The inhibitory activity of 50 μM CGLA in the presence of α-tocopherol did not significantly differ from that of 50 μM CGLA alone over the 72 h assayed. However, at a CGLA concentration of 200 μM, 50 μM α-tocopherol was found to substantially reduce the inhibitory activity of the fatty acid against the SW480 cell line relative to 200 μM CGLA alone. Indeed, following 24 h exposure, the inhibitory activity of 200 μM CGLA was reduced by 58.77% in the presence of α-tocopherol and subsequently to 79.37% following 48 h exposure and to 82.75% following 72 h exposure. Comparatively, the inhibitory activity of 50 μM CSA in the presence of α-tocopherol was lower than that of 50 μM CSA alone. The reduction in the inhibitory activity of CSA against the SW480 cell line ranged from 10.58% following 24 h, through 49.96% following 48 h and to 64.07% following 72 h exposure. A difference in the inhibitory activity of CSA alone or CSA in the presence of α-tocopherol was not observed at a CSA concentration of 200 μM following 24 h and 48 h exposure. However, given the potent effect of α-tocopherol on the inhibitory activity of 50 μM CSA, it is likely that the lack of effect seen here is a result of the capacity of CSA to be oxidised, exceeding the antioxidant capacity of α-tocopherol. A small difference in inhibitory activity was observed at a CSA concentration of 200 μM following 72 h, with α-tocopherol exposure reducing the activity of CSA by 5.19%. These results indicate that lipid peroxidation plays a

major role in the inhibitory activity of CGLA and CSA against the SW480 cell line. Moreover, lipid peroxidation would appear to play a minor role, in the inhibitory activity of CALA against the SW480 cell line. Thus, the inhibitory activity of CALA is more likely related to the action of the fatty acid on internal cellular circuitry involved in the regulation of cell proliferation and apoptosis.

In this study, we investigated the impact of the exposure of the SW480 colon cancer cell line to 50 μM CALA, CGLA or CSA for 24 h on both the total cellular fatty acid composition and on that of the cellular phospholipids. Exposure of the SW480 cells to CALA for 24 h resulted in significant increases in the cellular concentrations of tridecanoic acid ($P \leq 0.05$), vaccenic acid ($P \leq 0.05$) and CALA ($P \leq 0.01$) relative to cells exposed to the ethanol control **(Table 4.1)**. Statistically significant reductions in the cellular concentrations of palmitoleic acid ($P \leq 0.001$), heptadecenoic acid ($P \leq 0.05$) and vaccenic acid ($P \leq 0.05$) were also observed on exposure of the cells to CALA relative to cells exposed to the ethanol control. Using SPE the impact that exposure to 50 μM CALA had on the fatty acid composition of the cellular phospholipids of the SW480 cell line was elucidated **(Table 4.2)**. Significant increases, in the concentrations of tridecanoic acid ($P \leq 0.01$), pentadecanoic acid ($P \leq 0.001$), vaccenic acid ($P \leq 0.01$) and CALA ($P \leq 0.001$) were detected in the cellular phospholipids, while significant decreases in the concentrations of palmitic acid ($P \leq 0.01$), palmitoleic acid ($P \leq 0.001$), stearic acid ($P \leq 0.01$) and linoleic acid ($P \leq 0.05$) were also observed. Overall, exposure to CALA resulted in a small (0.88 g/100g FAME) increase in the cellular concentration of saturated fatty acids **(Table 4.1)**. However importantly, the concentration of saturated fatty acids found in the cellular phospholipids was reduced by 3.1 g/100g FAME relative to the ethanol control ($P \leq 0.05$) **(Table 4.2)**.

Table 4.1 Total cellular fatty acid composition of SW480 cells treated with 50 μM CALA, CGLA or CSA for 24 h (g/100g FAME; Mean ± Std. dev.)

Fatty acid	Control	CALA	CGLA	CSA
Caproic (C6:0)	2.92 ± 0.13	3.23 ± 0.19	3.53 ± 0.10	3.25 ± 0.21
Caprylic (C8:0)	3.36 ± 0.13	3.36 ± 0.31	3.58 ± 0.23	3.45 ± 0.02
Decanoic (C10:0)	1.68 ± 0.09	1.69 ± 0.00	2.24 ± 0.00	2.25 ± 0.15
Lauric (C12:0)	0.39 ± 0.02	0.39 ± 0.02	0.52 ± 0.03	0.41 ± 0.01
Tridecanoic (C13:0)	0.28 ± 0.02	0.31 ± 0.01	0.32 ± 0.03	0.28 ± 0.11
Myristic (C14:0)	2.46 ± 0.11	2.64 ± 0.07	3.23 ± 0.08	3.74 ± 0.09
Myristoleic (C14:1)	0.15 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.20 ± 0.01
Pentadecanoic (C15:0)	0.75 ± 0.10	0.65 ± 0.03	0.58 ± 0.02	0.78 ± 0.05
Palmitic (C16:0)	51.59 ± 1.26	50.93 ± 2.43	54.74 ± 1.83	54.41 ± 0.42
Palmiteladic (C16:1 *t*)	0.65 ± 0.09	0.70 ± 0.02	0.65 ± 0.00	0.78 ± 0.00
Palmitoleic (C16:1 *c*)	4.30 ± 0.02	2.99 ± 0.23	3.84 ± 0.10	3.92 ± 0.07
Heptadecanoic (C17:0)	0.66 ± 0.12	0.54 ± 0.05	0.60 ± 0.03	0.57 ± 0.03
Heptadecenoic (C17:1)	0.85 ± 0.30	0.34 ± 0.04	0.37 ± 0.03	0.38 ± 0.03
Stearic (C18:0)	13.66 ± 0.51	14.90 ± 0.25	13.75 ± 0.27	12.96 ± 0.06
Vaccenic (C18:1 *t*-11)	0.12 ± 0.12	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Oleic (C18:1)	9.63 ± 0.23	8.86 ± 0.22	7.61 ± 0.06	6.20 ± 0.02
(C18:1 *c*-11)	2.65 ± 0.12	4.27 ± 1.81	1.84 ± 0.15	1.40 ± 0.02
Linoleic (C18:2)	0.42 ± 0.02	0.34 ± 0.08	0.41 ± 0.01	0.38 ± 0.02
CALA (C18:3)	0.00 ± 0.00	0.98 ± 0.24	0.00 ± 0.00	0.00 ± 0.00
CGLA (C18:3)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
CSA (C18:4)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.29 ± 0.04
Arachidonic (C20:4)	0.28 ± 0.00	0.38 ± 0.06	0.25 ± 0.01	0.32 ± 0.01
EPA (20:5)	0.09 ± 0.09	0.23 ± 0.02	0.00 ± 0.00	0.10 ± 0.10
Total saturated fatty acids	77.76 ± 1.07	78.64 ± 0.54	83.09 ± 0.82	82.09 ± 1.01
ω-3 family[a]	0.09 ± 0.00	1.21 ± 0.06	0.00 ± 0.00	0.39 ± 0.00
ω-6 family[b]	0.70 ± 0.03	0.72 ± 0.08	0.66 ± 0.01	0.70 ± 0.11
ω-6 to ω-3 Ratio	7.78	0.6	-	1.79

[a] (CALA + EPA)

[b] (Linoleic acid + Arachidonic acid)

Table 4.2 Fatty acid compositions of cellular phospholipids extracted from SW480 cells treated with 50 μM CALA, CGLA or CSA for 24 h. (g/100g FAME; Mean ± Std. dev.)

Fatty acid	Control	CALA	CGLA	CSA
Lauric (C12:0)	0.79 ± 0.03	0.65 ± 0.10	1.42 ± 0.00	0.58 ± 0.10
Tridecanoic (C13:0)	1.32 ± 0.06	1.85 ± 0.17	1.88 ± 0.00	1.42 ± 0.16
Myristic (C14:0)	3.59 ± 0.41	3.15 ± 0.12	4.30 ± 0.30	3.96 ± 0.30
Pentadecanoic (C15:0)	0.86 ± 0.02	1.22 ± 0.06	1.74 ± 0.01	1.55 ± 0.22
Palmitic (C16:0)	45.06 ± 0.13	42.51 ± 0.89	44.20 ± 0.34	46.41 ± 1.31
Palmiteladic (C16:1 *t*)	1.32 ± 0.02	1.42 ± 0.15	1.41 ± 0.31	1.95 ± 0.32
Palmitoleic (C16:1 *c*)	9.31 ± 0.35	5.23 ± 0.14	6.58 ± 0.91	6.01 ± 0.17
Heptadecanoic (C17:0)	0.76 ± 0.07	0.80 ± 0.00	0.90 ± 0.06	0.90 ± 0.05
Stearic (C18:0)	1.10 ± 0.01	0.72 ± 0.09	0.90 ± 0.09	0.94 ± 0.30
Oleic (C18:1)	8.87 ± 0.27	10.22 ± 0.21	11.40 ± 0.97	11.98 ± 0.28
(Vaccenic C18:1 *t*-11)	15.96 ± 0.39	18.40 ± 0.71	16.89 ± 1.02	15.27 ± 0.07
Linoleic (C18:2)	4.16 ± 0.04	4.87 ± 0.25	3.56 ± 0.51	3.70 ± 0.13
Arachidic (C20:0)	1.33 ± 0.22	0.81 ± 0.07	0.76 ± 0.07	0.77 ± 0.02
CALA (C18:3)	0.00 ± 0.00	2.49 ± 0.35	0.00 ± 0.00	0.00 ± 0.00
CGLA (C18:3)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
CSA (C18:4)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.42 ± 0.01
Arachidonic (C20:4)	1.00 ± 0.06	0.92 ± 0.03	0.78 ± 0.04	1.08 ± 0.02
Total saturated fatty acids	54.81 ± 0.49	51.71 ± 1.01	56.10 ± 0.39	56.55 ± 0.55
ω-3 family[a]	0.00 ± 0.00	2.49 ± 0.38	0.00 ± 0.00	0.42 ± 0.09
ω-6 family[b]	5.16 ± 0.11	5.79 ± 0.29	4.35 ± 0.22	4.78 ± 0.31
ω-6 to ω-3 Ratio	-	2.32	-	11.36

[a] (CALA + CSA + EPA)

[b] (Linoleic acid + CGLA + Arachidonic acid)

As an ω-3 unsaturated fatty acid, exposure to CALA would be expected to alter the ratio of ω-6 to ω-3 fatty acids. Reductions in the ratio of ω-6 to ω-3 fatty acids have been regularly associated with the reduced pathogenesis of diseases, including cardiovascular disease, cancer and inflammatory and autoimmune diseases, thus changes in the ratio of these fatty acids may play an important role in any inhibitory activity of fatty acids against the SW480 cell line (Simopoulos, 2006; Simopoulos, 2008). As expected exposure to 50 μM CALA reduced the ratio of ω-6 to ω-3 fatty acids found in the total cellular fatty acids from 7.78 to 0.6 **(Table 4.1)** and from the absence of ω-3 fatty acids in the ethanol control to a ω-6 to ω-3 fatty acid ratio of 2.32 in the 50 μM CALA in the cellular phospholipids **(Table 4.2)**.

Exposure of the SW480 cell line to 50 μM CGLA for 24 h resulted in significant increases in the cellular concentrations of decanoic acid ($P \leq 0.001$), lauric acid ($P \leq 0.01$) and myristic acid ($P \leq 0.01$) relative to the ethanol control **(Table 4.1)**. Interestingly, an increase in the concentration of cellular CGLA was not detected, suggesting the fatty acid was not incorporated into the cell although it is possible the conjugate was rapidly metabolised. Significant decreases in the cellular concentrations of pentadecanoic acid ($P \leq 0.05$), palmitoleic acid ($P \leq 0.01$), heptadecenoic acid ($P \leq 0.05$), vaccenic acid ($P \leq 0.001$) and arachidonic acid ($P \leq 0.05$) were also observed on exposure of the SW480 cell line to CGLA relative to the ethanol control **(Table 4.1)**. Changes in the fatty acid composition of the cellular phospholipids were also observed when the SW480 cancer cell line was exposed to 50 μM CGLA **(Table 4.2)**. Indeed, significant increases in the concentration of lauric acid ($P \leq 0.001$), tridecanoic acid ($P \leq 0.001$), and pentadecanoic acid ($P \leq 0.001$) within the cellular phospholipids of the SW480 cell

line were observed relative to the ethanol control. Furthermore, decreases in the concentrations of palmitic acid ($P \leq 0.05$), palmitoleic acid ($P \leq 0.01$), stearic acid ($P \leq 0.05$), heptadecenoic acid ($P \leq 0.05$), linoleic acid ($P \leq 0.05$) and arachidonic acid ($P \leq 0.01$) were observed in the phospholipids of cells exposed to CGLA relative to the ethanol control. Unsurprisingly, given its absence from the fatty acid profile of the total cellular lipid, CGLA was not detected in the fatty acid profile of the cellular phospholipids either. Exposure to 50 μM CGLA resulted in an increase in both the total cellular and cellular phospholipid saturated fatty acid concentrations, of 5.33 and 1.29 g/100g FAME, respectively, relative to the ethanol control ($P \leq 0.05$) **(Table 4.1 & 4.2)**. These increases in saturated fatty acids suggest the higher cytotoxicity of CGLA towards the SW480 cell line relative to fatty acids such as CALA. As CGLA is not an ω-6 unsaturated fatty acid, due to the movement of the first double bond from the sixth to the seventh carbon from the methyl end of the carbon chain, its impact on the ratio of ω-6 to ω-3 fatty acids in the cell would not be expected to be as profound as an ω-3 fatty acid such as CALA. In our study, exposure to CGLA resulted in a reduction in the overall ω-6 fatty acid concentration relative to that of the ethanol control. These included reductions in the concentration of the pro-inflammatory fatty acid, arachidonic acid, and its precursor linoleic acid found in the cellular phospholipids. The presence of detectable concentrations of ω-3 fatty acids were not found in either the total cellular lipids or cellular phospholipids **(Table 4.1 & 4.2)**.

In this study exposure of the SW480 colon cancer cells to CSA for 24 h resulted in significant increases in the cellular concentrations of decanoic acid ($P \leq 0.01$), myristic acid ($P \leq 0.001$), myristoleic acid ($P \leq 0.01$), palmitic acid ($P \leq 0.05$), CSA ($P \leq 0.001$) and arachidonic acid ($P \leq 0.001$) relative to the ethanol

control **(Table 4.1)**. Decreases in the cellular concentrations of palmitoleic acid, ($P \leq 0.001$) and vaccenic acid ($P \leq 0.001$) were found in the SW480 colon cancer cell line when exposed to 50 μM CSA relative to the ethanol control. Changes in the fatty acid composition of the cellular phospholipids observed when the SW480 cancer cell line was exposed to 50 μM CSA **(Table 4.2)**. Statistically significant increases in the concentrations of pentadecanoic acid ($P \leq 0.01$), palmitoleic acid ($P \leq 0.01$) and CSA ($P \leq 0.001$) were observed in the cellular phospholipids of the SW480 colon cancer cell line when exposed to CSA for 24 h. The concentrations of lauric acid ($P \leq 0.01$), palmitoleic acid ($P \leq 0.001$), vaccenic acid ($P \leq 0.05$) and linoleic acid ($P \leq 0.05$) in the cellular phospholipids were also significantly decreased when the SW480 colon cancer cell line was exposed to CSA. Similarly to CGLA, exposure to 50 μM CSA resulted in an increase in the saturated fatty acid content of both the total cellular and cellular phospholipid fatty acids. In the total cellular fatty acids, a 4.33 g/100g FAME increase in the concentration of saturated fatty acids was detected ($P \leq 0.05$) **(Table 4.1)** while a 1.74 g/100g FAME increase in the concentration of saturated fatty acids in the cellular phospholipids was also observed ($P \leq 0.05$) **(Table 4.2).** In a similar manner as exposure to CALA, exposure to the ω-3 conjugated fatty acid CSA would be expected to alter the ratio of ω-6 to ω-3 fatty acids. In this study, exposure of the SW480 cell line to 50 μM CSA resulted in a decrease in the ratio of ω-6 to ω-3 fatty acids in the cellular fatty acids from 7.78 in the ethanol control to 1.79 in the cells exposed to CSA **(Table 4.1)**. An increase in the concentration of ω-3 fatty acids and decrease in the concentration of ω-6 fatty acids was also observed in the cellular phospholipids of SW480 cells exposed to CSA relative to those exposed to the ethanol control **(Table 4.2)**. Despite this observation, comparisons between the cellular

phospholipid ω-6 to ω-3 fatty acid ratio of cells exposed to CSA or the ethanol control could not be determined due to the absence of detectable concentrations of ω-3 fatty acids in the ethanol control. However, the trend of increased concentrations of ω-3 fatty acids and decreased concentrations ω-6 fatty acids in the cellular phospholipids of SW480 cells exposed to CSA is considered to be indicative of a reduced risk of carcinogenesis (Deschner *et al.*, 1990; Galli & Butrum, 1991; Simopoulos, 2008).

Of the conjugated fatty acids assayed, the cellular uptake of CALA was found to be approximately 3.38-fold greater than that of CSA, while the incorporation of CALA into the cellular phospholipids was found to be 5.93-fold greater than CSA. The other conjugated fatty acid isomer, CGLA, was not detected in either the total cellular fatty acids or in the phospholipid fraction. These results suggest the SW480 colon cancer cell line has a preference for incorporating conjugated fatty acids in the order of CALA > CSA > CGLA. Furthermore, the absence of intercellular concentrations of CGLA would suggest than any inhibitory activity displayed by the fatty acid is mediated extracellularly.

The impact of exposure to the conjugated fatty acids CLA, CALA, CGLA and CSA and their respective parent unsaturated fatty acids linoleic acid, α-linolenic acid, γ-linolenic acid and stearidonic acid on the cellular concentration of the p53 protein was investigated using a p53 ELISA (Calbiochem). At a fatty acid concentration of 25 μM, both linoleic acid and CLA supplemented SW480 cells significantly reduced the concentration of cellular p53 relative to the ethanol control ($P \leq 0.001$ and $P \leq 0.05$, respectively) **(Figure 4.10)**. Exposure of the SW480 cell line to 25 μM of γ-linolenic acid or its conjugated isomer CGLA resulted in a small reduction in the concentration of cellular p53 detected relative to the ethanol control

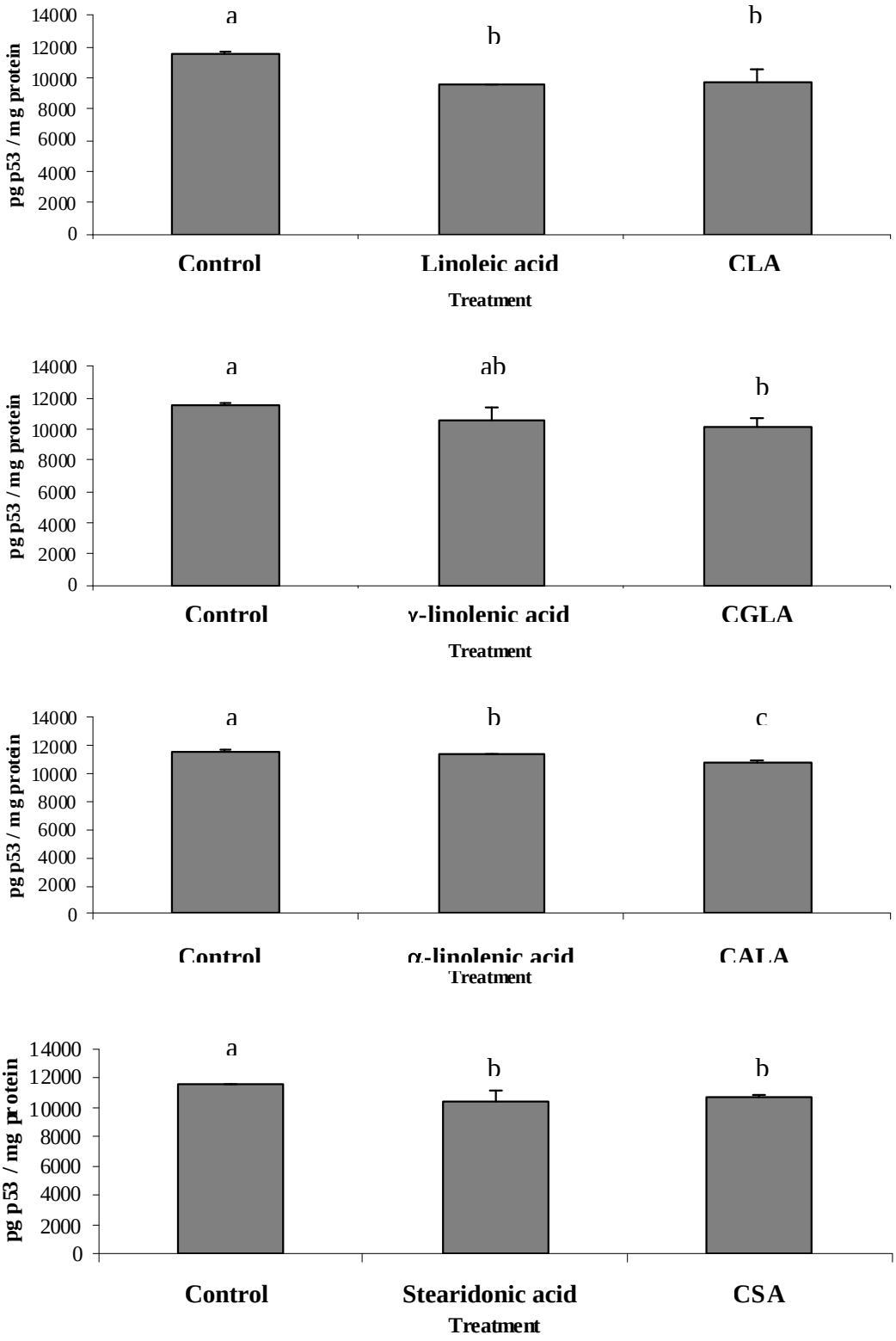


Figure 4.10 Effect of 25 μM CLA, CALA, CGLA or CSA, on the cellular concentration of the p53 protein relative to that observed in the presence of 25 μM linoleic acid, γ-linolenic acid, α-linolenic acid, stearidonic acid, or an ethanol control following 72 h. [a,b,c]. Values without a common subscript are significantly different ($p \leq 0.05$).

The reduction in cellular p53 reached significance upon exposure of the cells to 25 μM CGLA ($P \leq 0.01$) **(Figure 4.10)**. Exposure of the SW480 cell line to 25 μM both α-linolenic acid or its conjugated isomer CALA resulted in reductions in the concentration of p53 detected in the SW480 cell line **(Figure 4.10)**. Relative to the ethanol control, the reductions in cellular p53 concentrations reached significance in the case of both α-linolenic acid and CALA ($P \leq 0.05$ and $P \leq 0.001$, respectively), however, the reductions in cellular p53 observed on exposure of the cells to CALA were significantly greater than those seen with α-linolenic acid ($P \leq 0.001$). Exposure of SW480 cell line to either 25 μM of stearidonic acid or CSA resulted in significant reductions in the production of the p53 protein relative to the ethanol control ($P \leq 0.05$ and $P \leq 0.001$, respectively). Comparatively however, the reductions in cellular p53 detected on exposure of the cells to 25 μM stearidonic acid or CSA were not found to be significantly different.

At a fatty acid concentration of 25 μM, both linoleic acid and CLA reduced COX-2 expression relative to the ethanol control **(Figure 4.11)**. On exposure to 25 μM CLA, the concentration of cellular COX-2 was found to be significantly lower than that of the ethanol control ($P \leq 0.05$). However, in this study CLA did not have a significantly greater effect on the concentration of COX-2 than its parent unsaturated fatty acid, linoleic acid. When the SW480 colon cancer cell line was exposed to 25 μM γ-linolenic acid or CGLA, significant reductions in the concentration of cellular COX-2 were observed ($P \leq 0.05$ and $P \leq 0.01$, respectively). Of these fatty acids exposure to 25 μM γ-linolenic acid resulted in the greatest mean reduction in the concentration of COX-2, however, this reduction was not found to be significantly greater than that observed with 25 μM CGLA **(Figure 4.11)**. Exposure of the SW480 cell line to both 25 μM CALA or to 25 μM

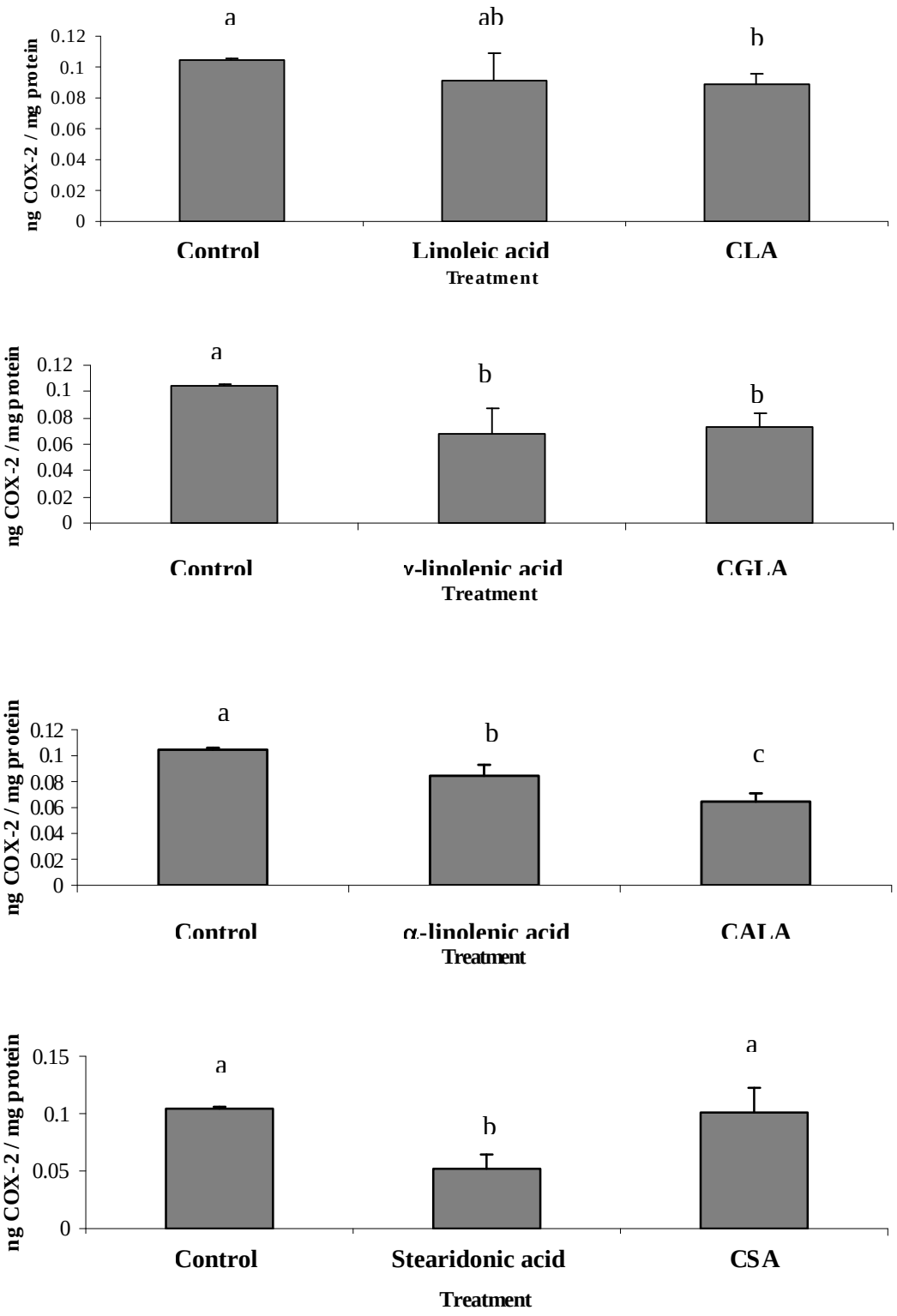
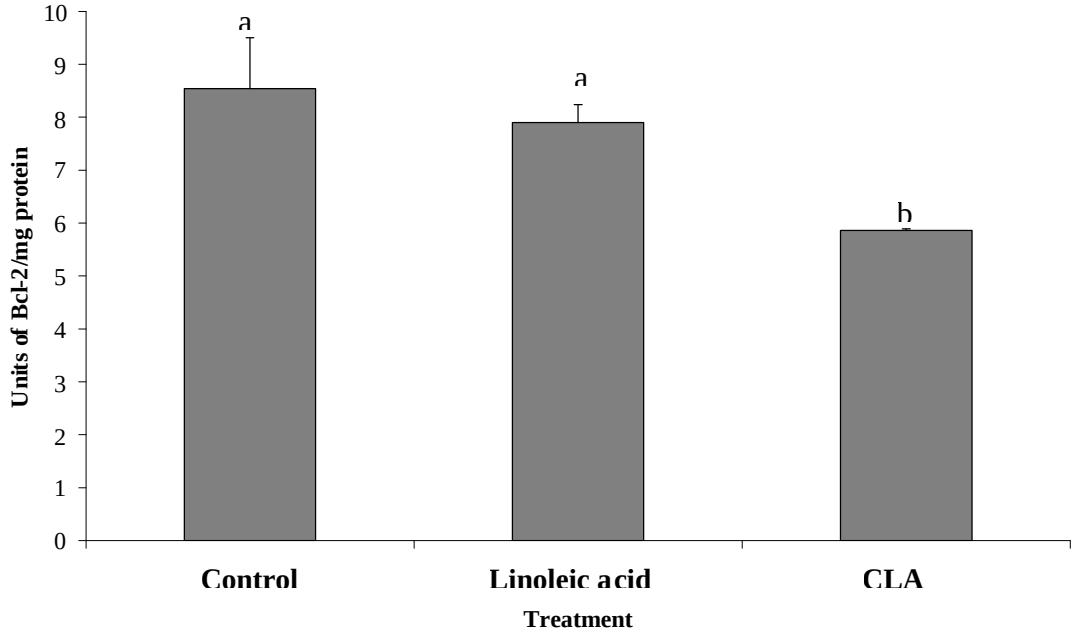


Figure 4.11 Effect of 25 μM CLA, CALA, CGLA or CSA, on the cellular concentration of the enzyme COX-2, relative to that observed in the presence of 25 μM linoleic acid, γ-linolenic acid, α-linolenic acid and stearidonic acid, or an ethanol control following 72 h. [a,b,c] Values without a common subscript are significantly different ($p \leq 0.05$).

of its parent fatty acid, α-linolenic acid resulted in reductions in the cellular concentration of COX-2 relative to the ethanol control **(Figure 4.11)**. While exposure to both 25 μM CALA or 25 μM α-linolenic acid resulted in significant reductions in the concentration of cellular COX-2 ($P \leq 0.001$ and $P \leq 0.05$, respectively), the reductions observed with 25 μM CALA were determined to be significantly greater than those observed with 25 μM α-linolenic acid ($P \leq 0.001$). Exposure of the SW480 cells to 25 μM stearidonic acid resulted in a significant reduction in the expression of COX-2 when compared to the ethanol control ($P \leq 0.01$) **(Figure 4.11)**. Interestingly, despite the activity of the other conjugates in reducing COX-2 expression, exposure to 25 μM CSA did not affect the concentration of cellular COX-2 detected relative to the ethanol control. Comparatively, the conjugated fatty acids reduced cellular COX-2 concentrations in the order of CALA > CGLA > CLA > CSA, while the unsaturated parent fatty acids reduced cellular COX-2 concentrations in the order of stearidonic acid > γ-linolenic acid > α-linolenic acid > linoleic acid.

In an attempt to explore the effects of the conjugated fatty acids CLA, CGLA, CALA and CSA on cellular apoptosis relative to their parent unsaturated fatty acids after 72 h exposure, we examined the impact that exposure to the conjugated fatty acids CLA, CALA, CGLA or CSA, or their respective unsaturated parent fatty acids, linoleic acid, α-linolenic acid, γ-linolenic acid or stearidonic acid, had on the concentration of cellular Bcl-2 using a Bcl-2 ELISA (Calbiochem). **Figures 4.12 and 4.13** show the results of the exposure of the SW480 cell line to 25 μM of the respective fatty acids relative to the control cells which were treated with ethanol alone. In the study, we observed that exposure to both 25 μM linoleic acid and 25 μM CLA resulted in a reduction in the concentration of cellular Bcl-2

detected **(Figure 4.12)**. Of the two fatty acids, the reduction in the concentration of cellular Bcl-2 observed with 25 μM CLA was found to be significantly greater than that observed with 25 μM linoleic acid ($P \leq 0.001$) or the ethanol control ($P \leq 0.01$). Similarly, to linoleic acid or CLA, exposure to 25 μM γ-linolenic acid or to 25 μM of its conjugated isomer CGLA, also resulted in reduction in the amount of cellular Bcl-2 detected **(Figure 4.12)**. Of the two fatty acids, CGLA resulted in the greatest reduction in cellular Bcl-2. However, this reduction was not statistically significant when compared to γ-linolenic acid or the ethanol control. Of the conjugated fatty acids assayed, only 25 μM CALA was found to be significantly less active than its parent unsaturated fatty acid in reducing the concentration of Bcl-2 ($P \leq 0.001$). Regardless both CALA and its unsaturated parent fatty acid α-linolenic acid, both significantly reduced the cellular concentration of Bcl-2, relative to the ethanol control ($P \leq 0.05$ and $P \leq 0.01$, respectively) **(Figure 4.13)**. Stearidonic acid and its conjugated isomer CSA, were both observed to significantly reduce the concentration of cellular Bcl-2 when compared with the ethanol control ($P \leq 0.05$ and $P \leq 0.05$, respectively) **(Figure 4.13)**. The reduction in cellular Bcl-2 was found to be significantly greater in the presence of 25 μM CSA than 25 μM stearidonic acid ($P \leq 0.05$). Comparatively, the conjugated fatty acids reduced Bcl-2 in the order of CLA > CGLA > CSA > CALA, while the parent unsaturated acids reduced the cellular concentration of Bcl-2 in the order of α-linolenic acid > stearidonic acid > γ-linolenic acid > linoleic acid.



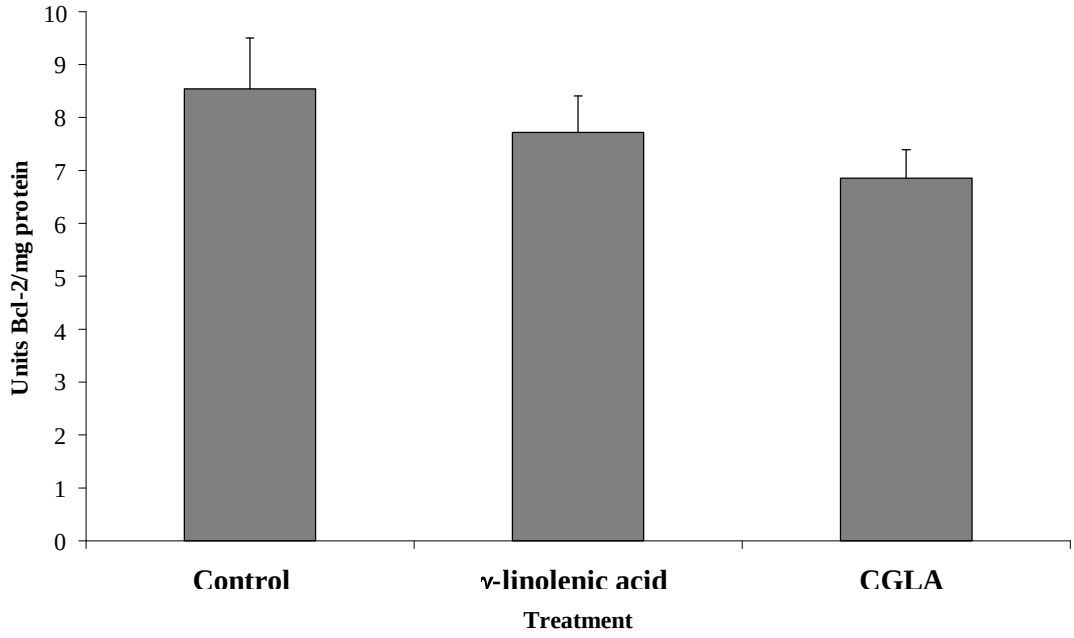
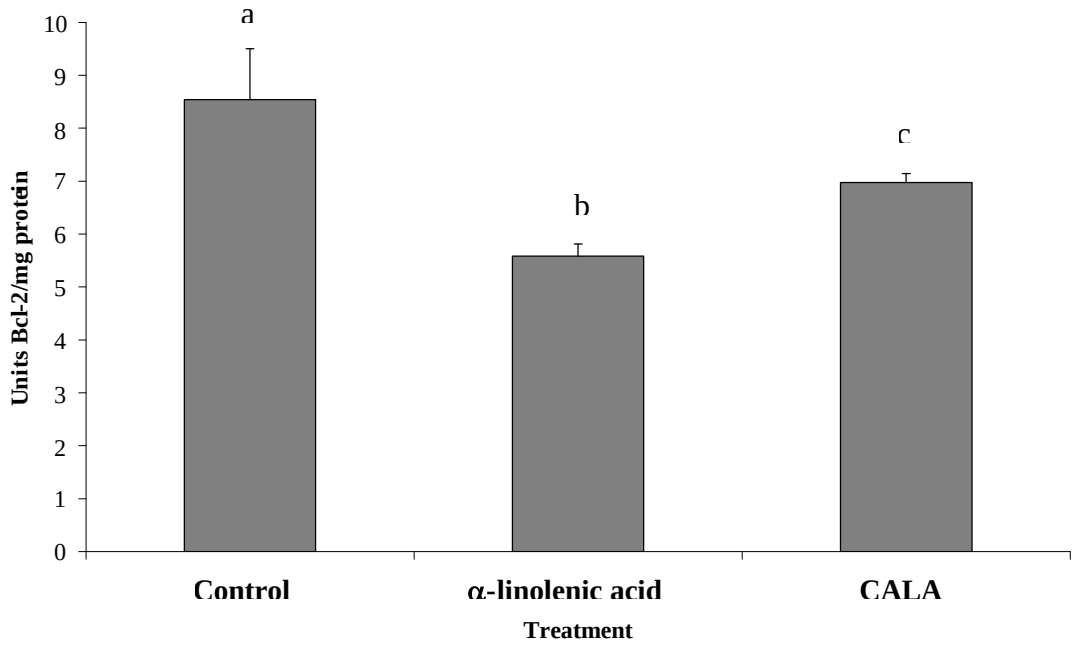


Figure 4.12 Effect of 25 μM CLA or CGLA, on the cellular concentration of the anti-apoptotic oncoprotein Bcl-2 relative to that observed in the presence of 25 μM linoleic acid, γ-linolenic acid, or an ethanol control following 72 h. [a,b,c] Values without a common subscript are significantly different ($p \leq 0.05$).



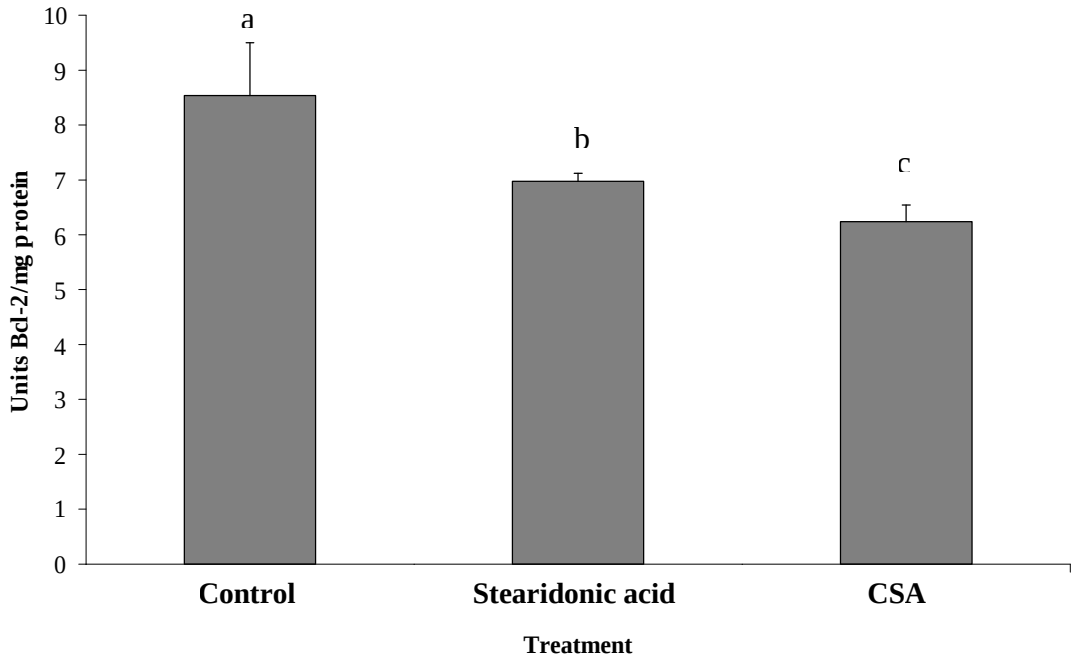


Figure 4.13 Effect of 25 μM CALA or CSA, on the cellular concentration of the anti-apoptotic oncoprotein Bcl-2 relative to that observed in the presence of 25 μM α-linolenic acid, stearidonic acid, or an ethanol control following 72 h. [a,b,c] Values without a common subscript are significantly different ($p \leq 0.05$).

4.4 Discussion

One of the most widely studied characteristics of conjugated fatty acids is their inhibitory activity against cancer cells and in particular those of the colon (Bhattacharya *et al.*, 2006; Tsuzuki *et al.*, 2007) (Chapter 1.3). Given the significant presence of bifidobacteria in the colon and reports of the ability of these bacteria to conjugate dietary unsaturated fatty acids *in vivo* and *ex vivo,* we aimed to assess the impact of three novel bifidobacterially produced conjugated fatty acids on the viability of the SW480 colon cancer cell line (Barrett *et al.*, 2007; Ewaschuk *et al.*, 2006; Wall *et al.*, 2009). Evaluation of the inhibitory activity of CALA against the SW480 colon cancer cell line demonstrated that the isomer did indeed exert a suppressive effect on tumor growth. Despite the positive impact of CALA on cancer cell proliferation its potency was not shown to be significantly greater than that of its parent unsaturated fatty acid, α-linolenic acid. A recent study employing the same CALA isomer, in the form of a crude oil, demonstrated that this isomer possessed a greater inhibitory activity than α-linolenic acid (Coakley *et al.*, 2009). In this study SW480 cells were exposed to the crude oil for 120 h at which stage the oil displayed an inhibitory activity which was 1.7 fold greater than α-linolenic acid. Thus, it is possible that in our study we may have missed the window in which CALA exhibits its greatest inhibitory activity or as a result of the purification of the conjugate removed other fatty acids which have a synergistic effect on the activity of the isomer.

Like CALA, an evaluation of the inhibitory activity of CGLA on the SW480 colon cancer cell line indicated that the isomer also exerts a suppressive effect on tumor growth. This inhibitory activity manifested itself at CGLA concentrations of 100 μM and 200 μM and increased with exposure time up to 48 h. While CGLA displayed inhibitory properties against the SW480 cell line at concentrations of 100

μM and 200 μM, exposure to concentrations below this resulted in increased cancer cell proliferation. Indeed, similar observations have been witnessed with γ-linolenic acid where a sub-inhibitory concentration of the fatty acid was observed to have a stimulatory effect on the growth of cancer cells (Hrelia *et al.*, 1999). Unlike CALA, the inhibitory activity of CGLA at the higher concentrations was observed to be greater than that of γ-linolenic acid, suggesting that the conjugation process increases the inhibitory activity of γ-linolenic acid.

Of the conjugated fatty acids assayed CSA proved the most potent in terms of its inhibitory activity against the SW480 colon cancer line. This inhibitory activity manifested itself across a range of concentrations in a dose dependent manner. The inhibitory activity of CSA proved greater than that of its parent unsaturated fatty acid, stearidonic acid, at the concentrations assayed, reaching significance at concentrations of 50 μM, 100 μM and 200 μM following 24 h, 48 h and 72 h exposure.

In addition to demonstrating the inhibitory properties of CALA, CGLA and CSA we have to our knowledge also revealed the potent and selective inhibitory activity of these bifidobacterially produced conjugated fatty acids against the SW480 colon cancer cell line relative to a normal colonic epithelial cell line, namely the FHC cell line. CSA displayed its most potent inhibitory activity against the SW480 cell line at a concentration of 200 μM CSA, however, at this concentration CSA was observed to display substantial inhibitory activity against the normal FHC colonic cell line. The inhibition of the FHC cell line by CSA suggests that the inhibitory activity of the fatty acid *in vitro* may partially be due to its cytotoxic properties. Indeed, this observation is substantiated by the higher concentration of saturated fatty acids found in the cellular phospholipids of SW480 cells treated with CSA, as such increases in cellular saturated fatty acids are

traditionally associated with increases in cellular stress (Ramos *et al.*, 2002). When exposed to a CSA concentration of 100 μM for 24 h a greater reduction in the viability of the SW480 cell line than in the viability of the FHC was observed. These results suggest that while at elevated concentration CSA displays inhibitory activity against both the SW480 and FHC cell lines at lower concentrations the fatty acids displays a higher inhibitory activity against the SW480 colon cancer cell line. The higher inhibitory activity which CSA displayed against the SW480 cell line relative to the FHC cell line correlate well with similar studies where anti-carcinogenic compounds such as (-)-epigallocatechin gallate and polyphenon E (both catechins) were found to have higher inhibitory activity against common cancer cell lines such as the Caco-2, HCT116, HT29 and SW480 cell lines than was exhibited by the normal fetal epithelial FHC cell line (Shimizu *et al.*, 2005). At a concentration of 200 μM exposure to CGLA resulted in reductions in the viability of both the SW480 (72%) and FHC (26.8%) cell lines. The inhibitory activity displayed by the fatty acid against the FHC cell line along with an increase in the concentration of saturated fatty acids found in the cellular phospholipids of SW480 cells suggest the fatty acid exhibits some cytotoxicity towards both cell lines. Of the conjugated fatty acids assayed, CALA alone exerted its inhibitory activity solely against the SW480 cell line. Indeed, while a 22.8% reduction in the viability of the SW480 cell line was observed a 1.4% increase in the viability of the normal FHC cell line was observed. Interestingly, a decrease in the concentration of saturated fatty acids in the cellular phospholipids of the SW480 cell line was observed reflecting the lower cytotoxicity of CALA against eukaryotic cells relative to the other conjugates assayed.

In addition to their impact on the saturated fatty acid concentration of cellular phospholipids, exposure of the SW480 cell line to conjugated fatty acids

was also observed to alter the profile of cellular ω-6 / ω-3 unsaturated fatty acids. The profile of ω-6 / ω-3 unsaturated fatty acids in tissues is deemed to play an important role in the development of a number of cancers (Aronson *et al.*, 2001; Bagga *et al.*, 1997). This is thought to stem from the competition of ω-3 unsaturated fatty acids with their ω-6 counterparts as substrates for the enzymes COX-2 and lipoxygenase (Karmali, 1987). The products of the action of these enzymes on ω-6 unsaturated fatty acids are inflammatory prostanoids, which have been associated with increased cancer risk, thus the competition provided by the ω-3 unsaturated fatty acids may reduce the risk of carcinogenesis (Lupulescu, 1996). As ω-3 fatty acids, exposure of the SW480 cell line to both CALA and CSA created a membrane phospholipid ω-6 / ω-3 fatty acid profile which is more favourable for the reduced risk of carcinogenesis. This was as a result of the incorporation of these fatty acids into the cellular phospholipids of the SW480 cell line, which increased the concentration of ω-3 fatty acids present.

Unlike CALA or CSA, CGLA was not detected in either the total cellular fatty acids or in the cellular phospholipids. Despite its absence from the phospholipid fraction, CGLA did alter the ω-6 / ω-3 profile of the phospholipid fraction of the SW480 cell line. This change was observed in the concentration of ω-6 unsaturated fatty acids detected in the phospholipid fraction, with reductions in the concentration of pro-inflammatory arachidonic acid and in its precursor linoleic acid. Similar results have been observed with CGLA's parent unsaturated fatty acid, γ-linolenic acid, where a reduction in the uptake of arachidonic acid into the cellular phospholipid fraction was detected when cells were exposed to the fatty acid (Graham *et al.*, 1994). This decrease in arachidonic acid is generally attributed to the competition of γ-linolenic acid with arachidonic acid for incorporation into the

membrane phospholipids. However, given the absence of CGLA from the cellular lipid fraction it is difficult to extrapolate the reasons for the reductions in arachidonic acid and linoleic acid seen here.

A number of studies have attributed the inhibitory effect of conjugated fatty acids against cancer cells to their apparent ability to induce cellular lipid peroxidation (Igarashi & Miyazawa, 2000; Yasui *et al.*, 2006b). Confirmation of this has been achieved via the detection of increased concentrations of cellular phospholipid hydroperoxides and thiobarbituric acid reactive substances in cells exposed to conjugated fatty acids and the subsequent loss of inhibitory activity in the presence of the hydrophobic radical scavenger, α-tocopherol (Igarashi & Miyazawa, 2000). Indeed, such increases in cellular lipid peroxidation are deemed desirable given that cancer cells are often void of antioxidant defence systems (Cheeseman *et al.*, 1986; Tisdale & Mahmoud, 1983). In terms of inhibitory activity against the SW480 cell line, both CSA (50 μM) and in particular CGLA (200 μM), were substantially inactivated in the presence of α-tocopherol suggesting a prominent role for increased oxidative stress in the activity of these fatty acids. Contrastingly, the inhibitory activity of CALA against the SW480 cell line remained relatively unaffected by the presence of α-tocopherol. The reasons for this lack of effect are unclear as a number of other conjugated isomers of α-linolenic acid such as the *c*9, *t*11, *c*13 CALA isomer, *c*9, *t*11, *t*13 CALA isomer and the *t*8, *t*10, *c*12 CALA isomer have all been observed to displayed reduced cytotoxicity in the presence of α-tocopherol (Suzuki *et al.*, 2001; Yasui *et al.*, 2006b). However, as many of these CALA isomers contain two conjugated double bonds it may be that the CALA isomer assayed here, with is single conjugated double bond, has a greater oxidative stability.

In addition to the ability of conjugated fatty acids to induce changes in the cancer cell at the cellular level, a number of studies have reported the ability of these molecules to induce changes in cancer cells at the genetic level, which result in both the suppression of growth and increased cellular apoptosis (Tsuzuki *et al.*, 2004a; Tsuzuki *et al.*, 2004b; Tsuzuki *et al.*, 2007). On the genetic level conjugated fatty acids have been associated with the up regulation of a number of genes involved in apoptosis, cell cycle arrest and down regulation of certain anti-apoptotic genes. Genes which are known to be affected include p53 and GADD45, which are involved in cell cycle arrest (Nagamine *et al.*, 2003; Tsuzuki *et al.*, 2007), PPARs, known ligand dependent transcription factors, Bcl-2 the anti-apoptotic onocone (Beppu *et al.*, 2006; Yasui *et al*., 2006a), Bax the pro-apoptotic oncogene (Yasui *et al*., 2006a) and COX-2 involved in the conversion of arachidonic acid to prostaglandins (Fujimura *et al*., 2006; Suzuki *et al.*, 2006). In our study, we looked at the impact of both the conjugates and their respective parent unsaturated fatty acids on the expression of three proteins thought to be intimately involved in the induction of cancer cell apoptosis and proliferation, namely p53, Bcl-2 and COX-2.

In the cell p53 serves as a guardian of the genome scanning the DNA for mutations and implementing repairs, initiating cell cycle arrest or cellular apoptosis as necessary. Given its prominence in the control of cancer, p53 activation by conjugates can make it difficult to assess what impact these compounds have on other proteins involved in the inhibition of cancer. As it is considered to be essentially deficient in the active p53 protein, the SW480 cell line is the perfect model for assessing impact of anti-carcinogenic agents independently of the activity of p53 (Rochette *et al.*, 2005). In our study, SW480 cells exposed to both linoleic acid and CLA displayed reduced cellular concentration of p53. This result corresponds well with the investigations of Liu *et al*, (2002) who observed that the

expression of mutant p53 in the SGC-7901 gastric cancer cell line is reduced in the presence of the *c*9, *t*11 CLA isomer. In addition to CLA, exposure of the SW480 cell line to the conjugated fatty acids CGLA, CALA and CSA also induced reductions in cellular p53 concentrations. Thus, it would appear that in cell lines which contain mutant p53, exposure to conjugated fatty acids induces a reduction in the cellular concentration of p53 as opposed to cells which contain wild type p53 (Kemp *et al.*, 2003; Yasui *et al.*, 2005). In addition to the reductions in the concentration of cellular p53 observed with the conjugated fatty acids, both α-linolenic acid and stearidonic acid also reduced the production of p53 in the SW480 cell line relative to the ethanol control. Despite being essentially deficient in p53, the mutant p53 protein found in the SW480 cell line can mediate some anti-carcinogenic activities such as nucleotide excision repair (Rochette *et al.*, 2005). Thus, the decreases in p53 found in the SW480 cells in this study are important, ensuring that the concentration of cellular mutant p53, with its limited anti-carcinogenic activity, is lower in the samples treated with the conjugated or unsaturated fatty acids, than in the ethanol control. What impact a reduction in the concentration of cellular p53 in the SW480 cell line might have on the viability of the cancer line is unclear. However, what is certain is that the p53 protein is most unlikely to have had an influence on the inhibitory activity of these unsaturated fatty acids and their conjugates. Importantly, the reduced concentrations of cellular p53 detected in the presence of the parent unsaturated fatty acids or conjugated fatty acids permit us to evaluate with greater certainty the impact of Bcl-2 on the viability of the SW480 cell line.

Like p53, COX-2 has been shown to play an important role in the development and proliferation of cancer due to its role in the metabolism of arachidonic acid to pro-inflammatory prostanoids. Indeed, a number of studies have

associated the increased expression of COX-2 with increased cancer cell proliferation and the inhibition of cancer cell apoptosis (Brown & DuBois, 2005; Dempke *et al.*, 2001). Conjugated fatty acids have been shown to be potent inhibitors of COX-2 production both at the genetic and protein level (Degner *et al.*, 2006; Ochoa *et al.*, 2004; Suzuki *et al.*, 2006). The inhibition of COX-2 production by conjugated fatty acids makes it difficult to assess the role of proteins such as Bcl-2 in the inhibitory activity of conjugated fatty acids against colon cancer. As the SW480 cell line is considered to be essentially COX-2 free it proves a perfect model for assessment of the role of proteins such as Bcl-2 independently of the effect of COX-2 (Nath *et al.*, 2003; Sakoguchi-Okada *et al.*, 2007). The relative absence of COX-2 is a result of a mutation on both alleles of the gene coding for the adenomatous polyposis coli gene which plays an important role in regulating the production of the COX-2 protein. In the current study, exposure of the SW480 cell line to both the conjugated fatty acids (with the exception of CSA) and their parent unsaturated fatty acids (with the exception of γ-linolenic acid) resulted in significant reductions in the concentration of cellular COX-2. This result is surprising given that the SW480 cell line is considered to be essentially free of COX-2 (Campbell *et al.*, 2006; Smith *et al.*, 2000). However, in a study by Li *et al*, (2006), surfactant protein immunochemistry was successfully used to detect the COX-2 protein in the SW480 cell line (Li *et al.*, 2006). Thus, it may be likely that rather than being COX-2 free the SW480 cell line expresses the protein at extremely low concentrations. Unsaturated fatty acids such as α-linolenic acid and stearidonic acid which have been used in this study have regularly been associated with reducing COX-2 expression in a range of cancer cell lines (Horia & Watkins, 2005; Vecchini *et al.*, 2004). Similarly, the conjugated isomers of linoleic acid (*c*9, *t*11 CLA) and of α-linolenic acid (*t*9, *t*11, *c*13 CALA) have also proved potent inhibitors of COX-2

production in cancer cells (Degner *et al.*, 2006; Suzuki *et al.*, 2006). Thus, the inhibition of COX-2 production by the conjugated fatty acids and their respective parent unsaturated fatty acids observed in the current study comply with previous findings.

Reductions in the concentration of the anti-apoptotic oncoprotein, Bcl-2 are commonly observed when cancer cells are exposed to conjugated fatty acids (Beppu *et al.*, 2006; Miller *et al.*, 2002; Yasui *et al.*, 2006b). As Bcl-2 is thought to prevent the induction of cancer cell apoptosis by forming stable heterodimers with the pro-apoptotic oncogene Bax, neutralising the latter's activity, any reduction in its cellular concentration is considered beneficial (Reed, 1994). In the current study, CLA, CGLA, CALA and CSA and their respective parent unsaturated fatty acids, were observed to reduce the cellular concentrations of Bcl-2 in SW480 cells exposed to the fatty acids. With the exception of CALA, in almost all instances exposure to the conjugated fatty acids resulted in a lower concentration of cellular Bcl-2 than an equivalent concentration of their respective parent unsaturated fatty acids. Indeed, while exposure to CALA did result in a significant reduction in cellular Bcl-2, its parent unsaturated fatty acid, α-linolenic acid, was found to be more potent in reducing cellular Bcl-2 concentrations. The ability of unsaturated fatty acids to reduce cellular Bcl-2 is not a novel observation with both exposure to linoleic acid and γ-linolenic acid reported to reduce cellular concentrations of the Bcl-2 protein (Das, 2007; Kwon *et al.*, 2008). However, it would appear that the conjugation process significantly increases the activity of unsaturated fatty acids in reducing cellular Bcl-2.

4.5 Conclusions

The bifidobacterially produced conjugated fatty acids CALA, CGLA and CSA display potent inhibitory activity against the SW480 cell line, which in the case of CGLA and CSA were significantly greater than that of their parent unsaturated fatty acids. Importantly, all three conjugated fatty acids displayed a higher inhibitory activity against the SW480 cell line than the normal colonic FHC cell line suggesting their activity is highly selective for cancer cells. The inhibitory activity of both CGLA and CSA appear to stem largely from their ability to increase lipid peroxidation, while the activity of CALA would appear to be mediated internally. All three conjugated fatty acids were observed to modulate the fatty acid composition of the membrane phospholipids found in the SW480 cell line. Exposure to CALA and CSA resulted in favourable changes in ω-3 fatty acid profile of the membrane phospholipids while exposure to CGLA resulted in reductions in the concentration of ω-6 fatty acids. At a mechanistic level the conjugated fatty acids were observed to induce significant reductions in the concentration of cellular COX-2 (with the exception of CSA) and in the concentration of the anti-apoptotic oncoprotein Bcl-2 (with the exception of CGLA). Overall the production of conjugated fatty acids active against colon cancer by bifidobacteria, a microbe normally abundant in the human colon, presents a valuable opportunity for the *in situ* production of a bioactive at its target site. As the substrate fatty acids from which CALA, CGLA and CSA are produced (α-linolenic acid, γ-linolenic acid and stearidonic acid) can be found in some abundance in the human diet their *in vivo* production is a realistic possibility (Fan & Chapkin, 1998; Li *et al*., 2003; Whelan, 2009). Indeed, further credence is given to this theory in light of the recent evidence pertaining to the *in vivo* production of CLA from dietary linoleic acid (Wall *et al.*, 2009).

4.6 References

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