



Title	Potent inhibitory effect of trans9, trans11 isomer of conjugated linoleic acid on the growth of human colon cancer cells
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Abstract: This study compared the growth inhibitory effects of pure conjugated linoleic acid (CLA) isomers (cis(c)9,c11-CLA, c9,trans(t)11-CLA, t9,t11-CLA, and t10,c12-CLA) on human colon cancer cell lines (Caco-2, HT-29, and DLD-1). When Caco-2 cells were incubated up to 72 h with 200 μ M each isomer even in the presence of 10% fetal bovine serum (FBS), cell proliferation was inhibited by all CLA isomers in a time dependent manner. The strongest inhibitory effect was shown by t9,t11-CLA, followed by t10,c12-CLA, c9,c11-CLA, and c9,t11-CLA, respectively. The strongest effect of t9,t11-CLA was also observed in other colon cancer cell lines (HT-29 and DLD-1). The order of the inhibitory effect of CLA isomer was confirmed in the presence of 1% FBS. CLA isomers supplemented in the culture medium were readily incorporated into the cellular lipids of Caco-2 and changed their fatty acid composition. The CLA contents in cellular lipids were 26.2 \pm 2.7% for t9,t11-CLA, 35.9 \pm 0.3% for c9,t11-CLA, and 46.3 \pm 0.8% for t10,c12-CLA, respectively.

DNA fragmentation was clearly recognized in Caco-2 cells treated with t9,t11-CLA. This apoptotic effect of t9,t11-CLA was dose- and time-dependent. DNA fragmentation was also induced by 9c,11t-CLA and t10,c12-CLA. However, fragmentation levels with both isomers were much lower than that with t9,t11-CLA. t9t11-CLA treatment of Caco-2 cells decreased Bcl-2 levels in association with apoptosis, whereas Bax levels remained unchanged. These results suggest that decreased expression of Bcl-2 by t9t11-CLA might increase the sensitivity of cells to lipid peroxidation and to programmed cell death, apoptosis.

Potent inhibitory effect of *trans*⁹, *trans*¹¹ isomer of conjugated linoleic acid on the growth of human colon cancer cells

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Abstract

This study compared the growth inhibitory effects of pure conjugated linoleic acid (CLA) isomers (*cis*(c)⁹,c¹¹-CLA, c⁹,*trans*(t)¹¹-CLA, t⁹,t¹¹-CLA, and t¹⁰,c¹²-CLA) on human colon cancer cell lines (Caco-2, HT-29, and DLD-1). When Caco-2 cells were incubated up to 72 h with 200 μM each isomer even in the presence of 10% fetal bovine serum (FBS), cell proliferation was inhibited by all CLA isomers in a time dependent manner. The strongest inhibitory effect was shown by t⁹,t¹¹-CLA, followed by t¹⁰,c¹²-CLA, c⁹,c¹¹-CLA, and c⁹,t¹¹-CLA, respectively. The strongest effect of t⁹,t¹¹-CLA was also observed in other colon cancer cell lines (HT-29 and DLD-1). The order of the inhibitory effect of CLA isomer was confirmed in the presence of 1% FBS.

CLA isomers supplemented in the culture medium were readily incorporated into the cellular lipids of Caco-2 and changed their fatty acid composition. The CLA contents in cellular lipids were $26.2 \pm 2.7\%$ for t9,t11-CLA, $35.9 \pm 0.3\%$ for c9,t11-CLA, and $46.3 \pm 0.8\%$ for t10,c12-CLA, respectively. DNA fragmentation was clearly recognized in Caco-2 cells treated with t9,t11-CLA. This apoptotic effect of t9,t11-CLA was dose- and time-dependent. DNA fragmentation was also induced by c9,t11-CLA and t10,c12-CLA. However, fragmentation levels with both isomers were much lower than that with t9,t11-CLA. t9,t11-CLA treatment of Caco-2 cells decreased Bcl-2 levels in association with apoptosis, whereas Bax levels remained unchanged. These results suggest that decreased expression of Bcl-2 by t9,t11-CLA might increase the sensitivity of cells to lipid peroxidation and to programmed cell death, apoptosis.

Keywords: Conjugated linoleic acid; isomer specificity; colon cancer cell; apoptosis

1. Introduction

Conjugated linoleic acid (CLA) is a general term for the geometrical and positional isomers of octadecadienoic (18:2) acid with a conjugated double bond system. CLA is a naturally occurring substance in food sources, such as milk fat and the meat of ruminant animals. It occurs as mixtures of positional (ranging from 7,9- to 13,15-CLA) and geometrical (*cis(c),c*; *c,trans(t)*; *t,c*; *t,t*) isomers and the major isomer is c9,t11-CLA [1]. A biologically important role of CLA originated from beef was first reported as an anticarcinogen by Pariza and Hargraves [2]. It was later found that synthetic CLA had several beneficial effects such as anticarcinogenesis [3,4], antiatherogenic [5,6], decrease of body fat [7,8], and regulation of immunological reactions [9,10].

Synthetic CLA predominantly consists of c9,t11- and t10,c12-isomers. Although

both isomers are known to possess biological activities, there is evidence indicating that more than one biological activity is involved in the specific effects of t10,c12-CLA. Data from animal models revealed that t10,c12-CLA reduces body fat and enhances lean body mass in mice [11]. Choi et al. [12] reported that t10,c12-CLA downregulated stearoyl-CoA desaturase gene expression in 3T3-L1 adipocytes, while c9,t11-CLA did not alter adipocyte gene expression. Furthermore, Yamasaki et al. [13] reported that t10,c12-CLA but not c9,t11-CLA showed a potent cytotoxic effect on the rat hepatoma cells by activating the apoptotic pathway. The higher anticarcinogenic effect of t10,c12-CLA than c9,t11-CLA has been also found in breast cancer cells [14] and in min mouse model [15].

The isomer-specific effects of c9,t11- and t10,c12-CLA suggest the different biological activity of each CLA isomer. However, there has been no report on the physiological effects of other CLA isomers such as t,t-isomers. In this study, we compared the inhibitory effect of four kinds of CLA isomers (c9,c11; c9,t11; t9,t11; t10,c12) on the growth of human colon cancer cells and found the stronger effect of t9,t11-CLA than other two common CLA isomers.

2. Methods and materials

2.1. Materials

Colon cancer cells line, Caco-2 (HTB-37), HT-29 (HTB-38) and DLD-1 (CCL-221) were obtained from the American Type Culture Collection. c9,c11-CLA, c9,t11-CLA, t9,t11-CLA and t10,c12-CLA were purchased from Matreya, Inc. (State College, PA, USA). Each CLA was converted to corresponding methyl ester with H₂SO₄-methanol solution [16]. Each methyl ester was analyzed by capillary gas chromatography (GC). GC analysis showed that the purity of CLA used was more than 99%. GC analysis was

done on a Shimadzu GC-14B equipped with a flame-ionization detector and a capillary column [SP-2560 (100 m×0.32 mm i.d.); Supelco, Bellefonte, PA, USA]. The column temperature was held at 150°C for 47 min, raised to 200°C at the rate of 1°C/min, then to 215°C at the rate of 5°C/min, and finally held at 215°C for 20 min. Helium gas was used as the carrier at a flow rate of 1.2 ml/min. The injector and detector were held at 250°C and 260°C, respectively.

2.2. Cell culture conditions

Caco-2 cells were cultured in minimum essential medium (MEM) supplemented with 10 % or 1% fetal bovine serum (FBS), 1% nonessential amino acid, 100 U/mL penicillin and 100 µg/ml streptomycin. HT-29 and DLD-1 were cultured in Dulbecco's modified Eagle's medium (DMEM) and RPMI 1640 medium supplemented with 10 % FBS, 100 U/ml penicillin and 100 µg/ml streptomycin. Cell cultures were maintained in a humidified atmosphere of 95% air and 5% CO₂ at 37 °C.

2.3. Cell viability

Cell viability was assessed with 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium sodium salt (WST-1, Wako Pure Chemical, Osaka, Japan), which is based on cleavage of the WST-1 reagent by mitochondrial dehydrogenase of viable cells to formazan dye [17]. Human colon cancer cells preincubated as described above were seeded at a density of 2x10³ cells/well in 96-well microplates and cultured in 100 µL medium/well for 24 h. Each CLA was dissolved in 10 µl of 5% ethanol solution and then added to the culture. After 21 h of incubation, 10 µl of WST-1 solution was added to each well, and then the plate was incubated for a further 3 h. Cell viability was then measured spectrophotometrically at 450 nm (Microplate reader, Emax; Molecular Devices, Sunnyvale, CA, USA) and

was expressed as a percentage of the viability obtained in control cultures, which were incubated 10 μ l of 5% ethanol solution without the addition of CLA.

2.4. Measurement of DNA fragmentation

Quantitative measurement of apoptotic cells was performed using a commercial kit (Cell Death Detection ELISA^{PLUS}, Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. The assay is based on a quantitative sandwich enzyme immunoassay to detect the histone-associated DNA fragments produced during apoptosis. Cell culture conditions were the same as in cell proliferation assay.

2.5. Western blot analysis

Caco-2 cells (1.5×10^6 cells) were cultivated in 150-mm tissue cultured dish for 24 h and 9,11-CLA was then added into culture medium as ethanol solution. The final ethanol solution was below 0.1% (v/v). After incubation for 96 h, adherent cells were trypsinized and washed three times with phosphate buffered saline (PBS). Pellet was then scraped in a cold RIPA buffer (pH 7.4) containing 20 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 0.1 mg/ml phenylmethylsulfonyl fluoride, 50 μ g/ml aprotinin and 1 mM Na_3VO_4 . Further, cell lysates were centrifuged at 4 °C, 15,000 rpm for 20 min. The supernatants (40 μ g protein/lane) were separated by 10% SDS-polyacrylamide gel electrophoresis. Proteins were transferred to polyvinylidene difluoride membrane, and membrane was then blocked with TBS-T (20 mM Tris-HCl (pH 7.6), 137 mM NaCl, and 0.1% Tween 20) containing 5% nonfat dry milk for 1 h at room temperature. The membrane was incubated with anti Bcl-2 (Trevigen, MD) and anti Bax (Trevigen) for 1 h. After washing, the membranes were incubated with a secondary antibody, anti-mouse IgG-HRP (Santa Cruz Biochemistry, Santa Cruz, CA, USA), for 1 h at room

temperature. Finally, the membrane was treated with the reagents in the chemiluminescence detection kit (ECL system, Amersham Pharmacia Biotech, NJ, USA) according to the manufacturer's instructions. Actin was used as the control with human actin antibody (Santa Cruz Biotechnology). Densitometric analysis of the protein bands was performed with the software Scion Image (NIH Image, MD, USA).

4.6. Lipid extraction and analysis of the fatty acid composition

Caoco-2 cells were preincubated as described above in 30 ml medium/dish for 48 h. Each CLA in ethanol was added to the culture at concentration of 50 μ M. After 24 h incubation, cells were washed twice with PBS and total lipids (TL) were extracted with chloroform/methanol (2:1,v/v). TL was transmethylated with 0.5 M CH_3ONa in MeOH by heating in a sealed tube at 60-70°C for 30 min under nitrogen. The fatty acid methyl esters were extracted with hexane. The extract was washed with water, dried over anhydrous sodium sulfate, concentrated *in vacuo*, purified by silicic acid column chromatography, and then put through to GC. A capillary column [SP-2560 (100 m \times 0.32 mm i.d.); Supelco] was used for the identification of each CLA isomer in the methyl esters. The GC condition was the same as described above. Fatty acid profile was analyzed by a Shimadzu GC-14B equipped with a flame-ionization detector and a capillary column [Omegawax 320 (30 m \times 0.32 mm i.d.); Supelco]. The column temperature was held at 200°C. The injection port and flame ionization detector were operated at 250°C and 260°C, respectively. Helium was used as carrier gas and its flow was 4.1 ml/min. The fatty acid methyl esters were identified by comparison of retention times with authentic standards and with the equivalent chain length (ECL) values. The analysis was triplicate and there was little difference in the oxygen consumption and polymer formation rates for each determination.

The analysis was done three times for the same sample and calculated ECL values

are the mean values of several GC runs.

2.7. Statistical analysis

Data are expressed as means \pm SD. Statistic analyses between multiple groups were determined by ANOVA. Statistical comparisons were made by Scheffe's *F*-test.

3. Results

When Caco-2 cells were incubated with 200 μ M each CLA isomer for 24 h, all isomers caused a reduction in cell viability (Fig. 1). This reduction occurred in a time-dependent manner in all isomers. Among them, t9,t11-CLA showed the strongest inhibitory effect on the cell growth during the whole culture period, followed by c10,t12-CLA, c9,t11-CLA and c9,t11-CLA, respectively. The strong activity of t9,t11-CLA was confirmed by dose- and time-dependent growth inhibition on Caco-2 cells (Fig. 2). The same tendency was observed in other kinds of colon cancer cells. As shown in Fig. 3, t9,t11-CLA (100 μ M) significantly reduced the viability of DLD-1 and HT-29 colon cancer cells as compared with the control, while other CLA isomers showed no inhibitory effect on both colon cancer cells at 100 μ M. Fatty acid can form complex with bovine serum albumin. In the present experimental conditions, a part of CLA would form complex with the albumin in the medium. Fig. 4 shows the effect of CLA on Caco-2 cell viability in 1% FBS concentration. In the presence of 10% FBS any CLA had no inhibitory effect on cell growth under 25 μ M within 48 h incubation. On the other hand t9,t11-CLA and t10,c12-CLA significantly inhibited the cell growth at 10 and 25 μ M by the reduction of FBS to 1%. The effect of t9,t11-CLA was higher than that of t10,t12-CLA.

To characterize the mechanism of cell death by CLA, cytological alterations and DNA degradation of Caco-2 cells were analyzed. Caco-2 cells killed by t9,t11-CLA

were accompanied by nuclear condensation and brightness occurred as observed by fluorescent microscope of the stained cells. The measurement of cytoplasmic histone-associated DNA fragments as an indicator of apoptosis showed that t9,t11-CLA (200 μ M) significantly induced DNA fragmentation in Caco-2 cells after 48 h incubation (Fig. 5). However, there were no significant effects of c9,t11-CLA and t10,c12-CLA (200 μ M) on DNA fragmentation after 48 h incubation. The DNA fragmentation by c9,t11-CLA and t10,c12-CLA was found after 72 h incubation, but the levels were lower than that by t9,t11-CLA. Fig. 6 shows the DNA fragmentation in Caco-2 cells incubated with different concentration of t9,t11-CLA for 72 h. Significant induction of DNA fragmentation was observed at 50 μ M and this effect was dose-dependent manner.

In an attempt to explore the effects of t9,t11-CLA on apoptosis regulating proteins, we examined expression of Bcl-2 and Bcl-xL, which suppress programmed cell death, and that of Bax, which appears to promote it. Fig. 7 shows the results of immunohistochemical analysis of Bcl-2 expression (Fig. 7A) in Caco-2 cells incubated with varying t9,t11-CLA concentrations (0, 100 and 200 μ M) for 96 h. Treatment with the CLA reduced the percentage of Bcl-2 protein expression in a dose-dependent manner. In contrast, no changes in the percentage of Bax expression (Fig. 7B) were found following a 96 h t9,t11-CLA treatment.

When Caco-2 cells were incubated with CLA for 24 h, significant amount of each isomer was incorporated into the cancer cells. On the other hand, these CLA were not detected in control cells (Table 1). Main fatty acid of control cells were 18:1n-9, 16:0, 18:1n-7, 20:4n-6, 16:1n-7, and 18:0. The levels of these fatty acids decreased with the incorporation of CLA into the cells. Furthermore, two peaks (<1%) were detected between peaks of 20:4n-3 and 22:6n-3, which were not observed in the methyl esters

from TL of control cells. Judging from ECL values, both peaks are suggested to be elongated metabolites of CLA isomers. Moreover, a new peak appeared between peaks of 16:1n-7 and 18:1n-9 in the methyl esters from TL of CLA supplemented cells. The content of this new peak were $3.3\pm 0.2\%$ for t9,t11-CLA ($3.3\pm 0.2\%$), for c9,t11-CLA ($0.5\pm 0.1\%$), and for t10,c12-CLA ($1.7\pm 0.0\%$). Judging from the ECL of each peak and absence of this peak in control cells, these peaks would be derived from each CLA isomer with less degree of unsaturation.

4. Discussion

It has been considered that both c9,t11-CLA, which is the CLA isomer present in highest amounts in milk fat, as well as t10,c12-CLA, which is present at considerable lower levels naturally, are equipotent in terms of their ability to inhibit mammary carcinogenesis. However, recent in vitro studies [13,18,19] showed that t10,c12-CLA is somewhat more efficacious than c9,t11-CLA for many parameters, suggesting the possibility that lower doses of t10,c12-CLA can be utilized in vivo, with good efficacy and low toxicity. The results in Fig. 1 confirmed the higher activity of t10,c12-CLA than c9,t11-CLA.

There have been many papers on the anti-carcinogenic activity of c9,t11-CLA and t10,c12-CLA. However, the effect of other naturally occurring CLA isomer has not been investigated. In the present study, inhibitory effect of t9,t11-CLA on the growth of colon cancer cells was compared with those of c9,c11-CLA, c9,t11-CLA and t10,c12-CLA. As shown in Fig. 1, Fig. 3, and Fig.4, t9,t11-CLA showed the strongest cytotoxic effect on colon cancer cells. The high activity of t9,t11-CLA was consistent with the level of apoptosis induced by this CLA isomer in Caco-2 cells (Fig. 5). In apoptotic pathway, the death signal triggers a cascade of various molecular actions resulting in the cleavage of

genomic DNA and specific proteins. It has been reported that CLA induces apoptosis in cancer cells such as mammary adenocarcinoma [20], normal rat mammary epithelial cells [21], and human hepatoma [22]. However, the data on isomer specificity has been limited [13]. The present study demonstrated the higher inhibitory effect of t9,t11-CLA on the growth of human colon cancer cells with apoptotic pathway than those of t10,c12-CLA, c9,t11-CLA, and c9,c11-CLA.

CLA mixture (predominantly c9,t11-CLA and t10,c12-CLA) is obtained by the chemical isomerization of linoleic acid. Industrial separation of t10,c12-CLA can be done by chemical procedure from this CLA mixture. On the other hand, t9,t11-CLA is a minor component in the CLA mixture and a large scale separation of t9,t11-isomer would be impossible. t9,t11-CLA is known to be contained in milk fat and dairy products. In this case, t9,t11-CLA would be biologically synthesized. Kishino et al. reported the bacterium production of two CLA isomers (c9,t11- and t9,t11-CLA) from ricinoleic acid [23] and linoleic acid [24]. Among both isomers, t9,t11-CLA was the main component and this isomer was produced at more than 97% purity by lactic acid bacteria, if the reaction is done long enough with a low linoleic acid concentration [25]. This biological system for t9,t11-CLA production may promise the large scale and selective preparation of t9,t11-CLA.

Agatha et al. [26] reported that c9,t11-CLA and c9,c11-CLA supplemented in the culture medium was readily incorporated and esterified into a human leukemia cellular phospholipids in a concentration- and time-dependent manner. The cellular phospholipids contained high level of CLA (range: 32-63 g/100g total phospholipids). As shown in Table 1, CLA was also easily incorporated into human colon cancer cells (Caco-2) leading to an extensive alteration of the fatty acid profile of the cellular lipids. In the human leukemia cells, the supplementation with CLA resulted in the

accumulation of the corresponding fatty acid with conjugated double bonds by desaturation/elongation system [26]. However, a little amount of metabolites of CLA with long-chain and/or higher degree of unsaturation was detected in the total lipids of human colon cancer cells (Caco-2) supplemented with CLA (200 μ M) for 72 h incubation.

The mechanism of action of CLA is still unknown. However, different mechanisms of the anticarcinogenic activity of CLA have been hypothesized. Studies show that the growth inhibitory effect of CLA on cancer cell lines is related to the increase in lipid peroxidation, alteration of cellular fatty acid composition and regulation of some gene expressions [27]. The present study showed the changes in the fatty acid composition of cellular lipid by each CLA supplementation (Table 1). However, the change in fatty acid composition by t9,t11-CLA, which showed the most potent inhibitory effect on colon cancer cells (Fig. 1, Fig. 3 and Fig. 4), was relatively smaller than those by t10,c12-CLA and c9,t11-CLA (Table 1), suggesting no relation of the activity of t9,t11-CLA with the change in fatty acid composition. It has been reported that overexpression of Bcl-2 in transgenic models leads to protection of many cell types against apoptosis induced by a variety of oxidative stresses, including lipid peroxidation, suggesting that Bcl-2 exerts an antioxidative function [28-31]. In addition, anti-apoptotic activity of Bcl-2 is found to be antagonized by a homologous Bax protein which is able to form heterodimers with Bcl-2. Thus, the ratio of Bax to Bcl-2 within the cell is the critical determining factor for the propensity of a cell to undergo apoptosis [32]. In this study we showed that t9,t11-CLA treatment led to a decrease in Bcl-2 expression but not the level of Bax. The decreased Bcl-2 levels along with normal levels of Bax may be sufficient to shift the balance toward apoptosis in Caco-2 cells.

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Figure legend

Figure 1. Cell viability for Caco-2 cells incubated with four kinds of CLA (200 μ M) up to 72 h. Data represent cell viability expressed as a percentage of the control, which was taken to be 100%. Incubation was carried out in the presence of 10% FBS. Data were means \pm SD for three samples. Values with different roman letters are significantly

different ($P<0.01$) at the same incubation time.

Figure 2. Dose- and time dependent effect of t9,t11-CLA on the growth of Caco-2 cells. Incubation was carried out in the presence of 10% FBS. Data represent cell viability expressed as a percentage of the control, which was taken to be 100%. Data were means \pm SD for three samples.

Figure 3. Cell viability for DLD and HT cells incubated with four kinds of CLA (100 μ M) for 72 h. Incubation was carried out in the presence of 10% FBS. Data represent cell viability expressed as a percentage of the control, which was taken to be 100%. Data were means \pm SD for three samples. Values with different roman letters are significantly different ($P<0.01$). Values with asterisk mark denote significant difference from the control ($P<0.01$).

Figure 4. Cell viability for Caco-2 cells incubated with four kinds of CLA (10 and 25 μ M) up to 48 h. Data represent cell viability expressed as a percentage of the control, which was taken to be 100%. Incubation was carried out in the presence of 1% FBS. Data were means \pm SD for three samples. Values with different roman letters are significantly different ($P<0.01$) at the same incubation time.

Figure 5. DNA fragmentation in Caco-2 cells treated with t9,t11-, c9,t11-, and t10,c12-CLA (200 μ M). Incubation was carried out in the presence of 10% FBS. Relative DNA fragmentation was assigned the control to a value of 1.0. Data were means \pm SD for three samples. Values with different roman letters are significantly different ($P<0.01$) at the same incubation time.

Figure 6. Dose-dependent apoptic effect of t9,t11-CLA on Caco-2 cells. Caco-2 cells were incubated with different concentrations of each CLA for 72 h. Incubation was carried out in the presence of 10% FBS. Relative DNA fragmentation was assigned the control (0 μ M of t9,t11-CLA) to a value of 1.0. Data were means \pm SD for three samples. Values with asterisk mark denote significant difference from the control ($P<0.01$).

Figure 7. Effect of t9,t11-CLA treatment on Bcl-2 (A) and Bax (B) protein levels in Caco-2 cells. Incubation was carried out in the presence of 10% FBS. Reactive protein bands were analyzed using chemiluminescence detection kit (ECL system) with specific antibodies. Expressed proteins were represented as the percentage to the control β -actin. Each bar represents the approximate amount of protein corresponding to the banding pattern that was measured by densitometric analysis performed with the software Scion Image (NIH Image, MD).

Table 1
Incorporation of CLA into the cellular total lipids after incubation for 24hr^a

Fatty acid (wt%)	Cell			
	Control	+t9,t11-CLA	+c9,t11-CLA	+t10,c12-CLA
14:0	2.5±0.2	2.1±0.5	1.7±0.1	1.2±0.0
16:0	22.2±0.2	13.4±2.1	15.0±0.5	10.8±0.0
18:0	7.6±0.0	4.0±0.1	5.2±0.2	5.5±0.1
16:1n-7	8.2±0.5	6.5±1.3	4.3±0.1	2.5±0.1
18:1n-7	10.8±0.0	6.6±0.2	6.4±0.1	4.8±0.0
18:1n-9	24.2±0.1	18.3±0.8	14.2±0.3	10.7±0.0
18:2n-6	3.1±0.0	2.6±0.1	2.0±0.1	1.9±0.0
20:4n-6	8.6±0.4	6.6±0.1	5.7±0.1	5.6±0.1
22:6n-3	2.6±0.5	1.9±0.7	1.9±0.0	1.9±0.1
t9,t11-CLA	ND ^b	26.2±2.7	ND ^b	ND ^b
c9,t11-CLA	ND ^b	ND ^b	35.9±0.3	ND ^b
t12,c12-CLA	ND ^b	ND ^b	ND ^b	46.3±0.8

^a Data are expressed as mean±SD (n=3).

^b Not detected.

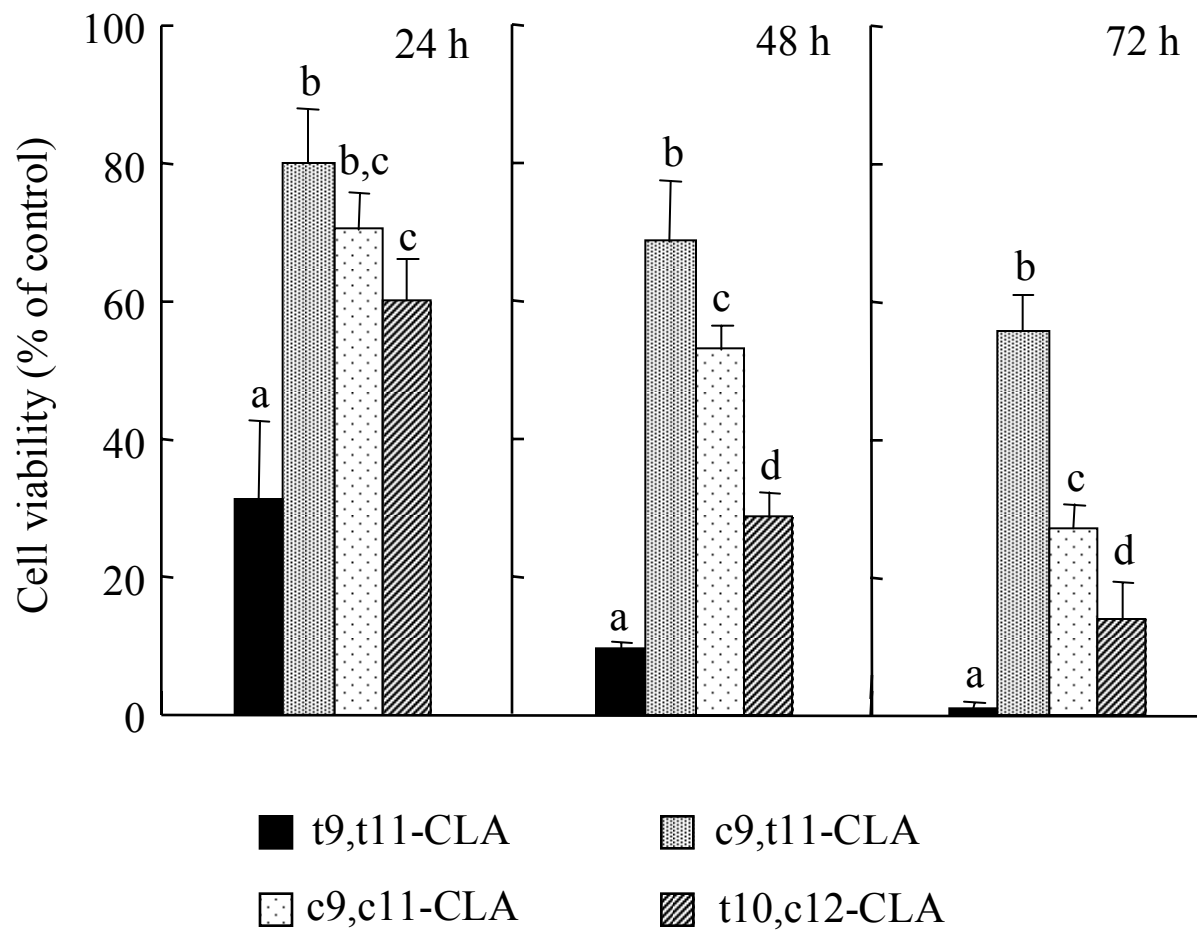


Fig. 1

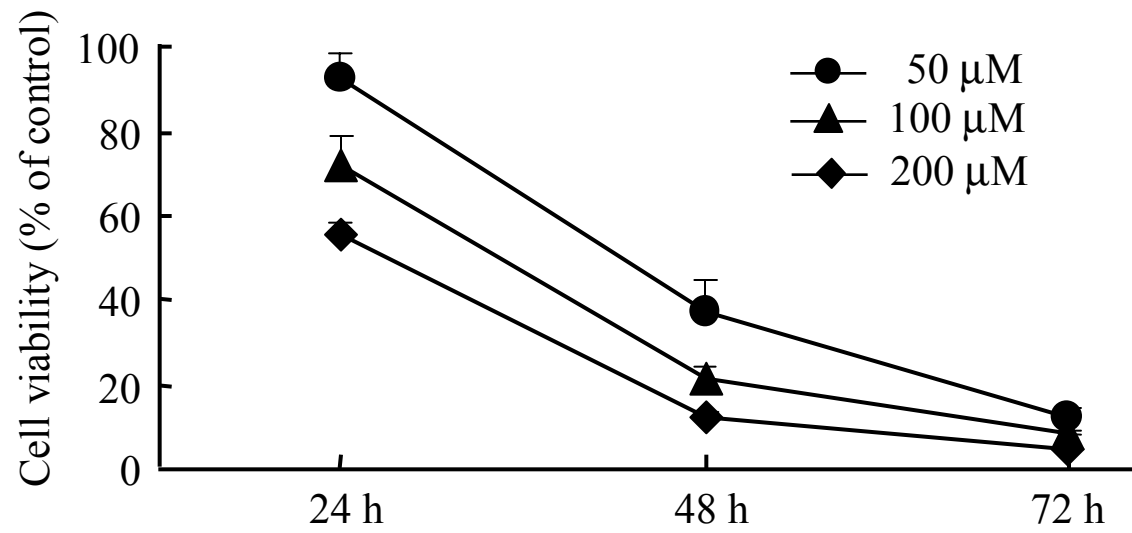


Fig. 2

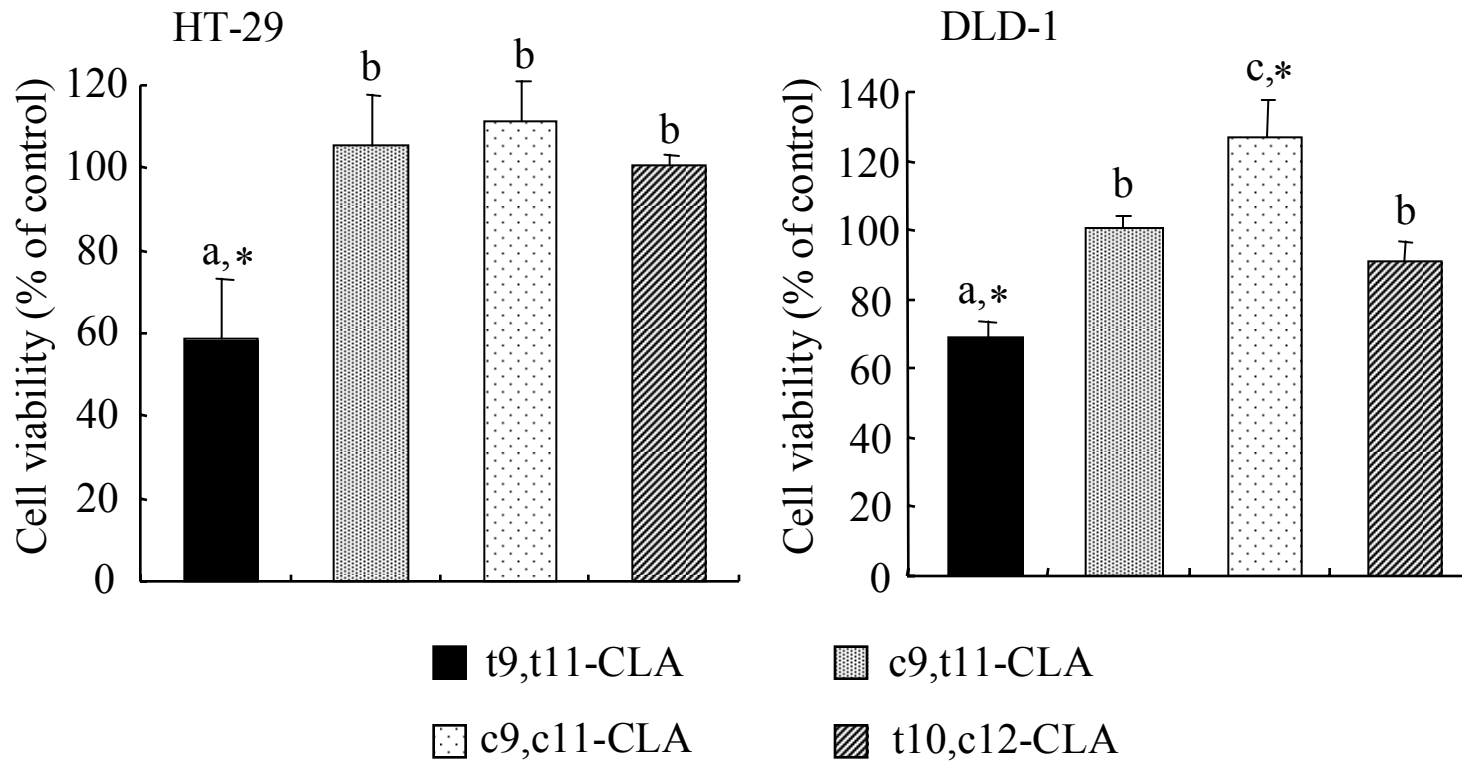


Fig. 3

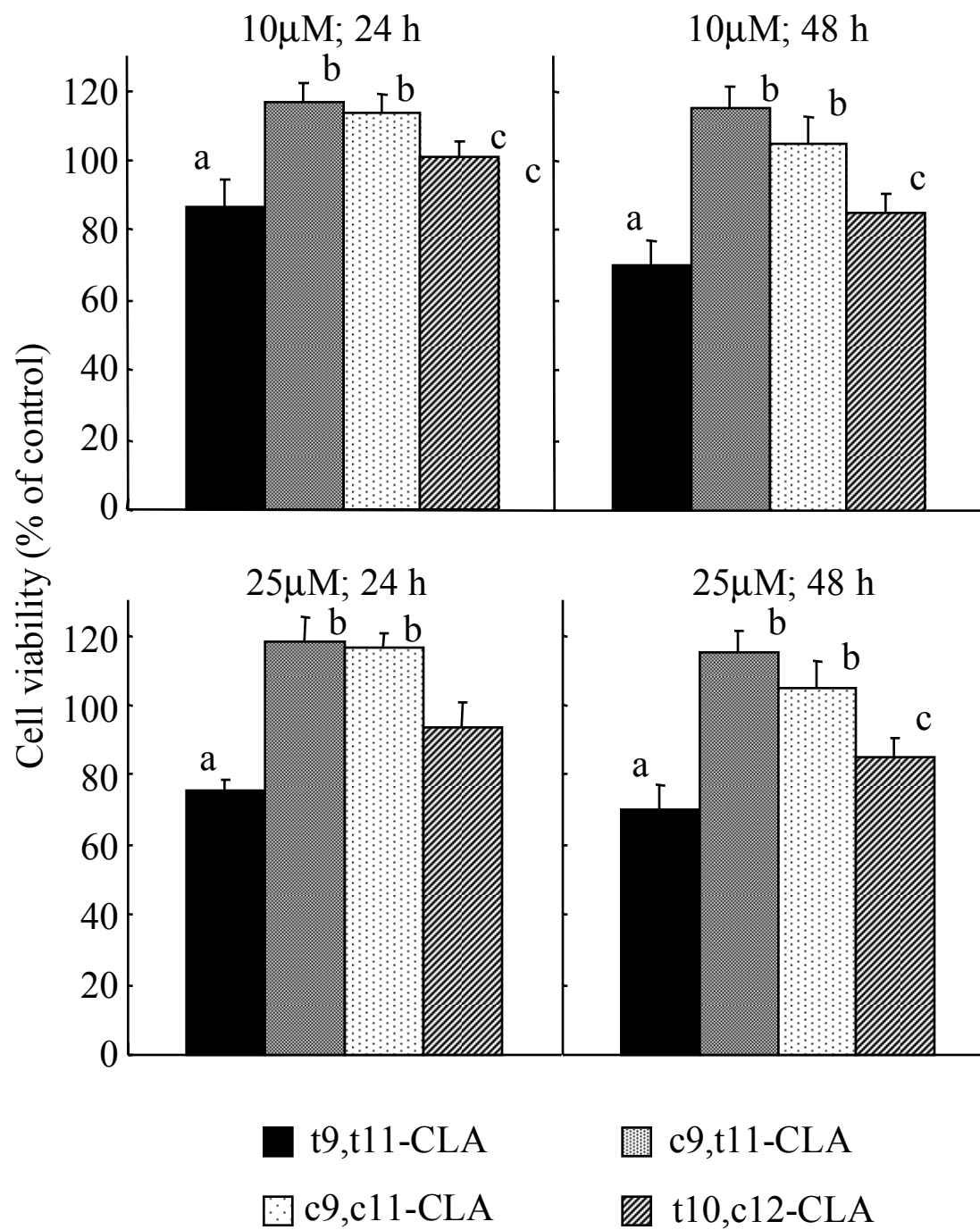


Fig. 4

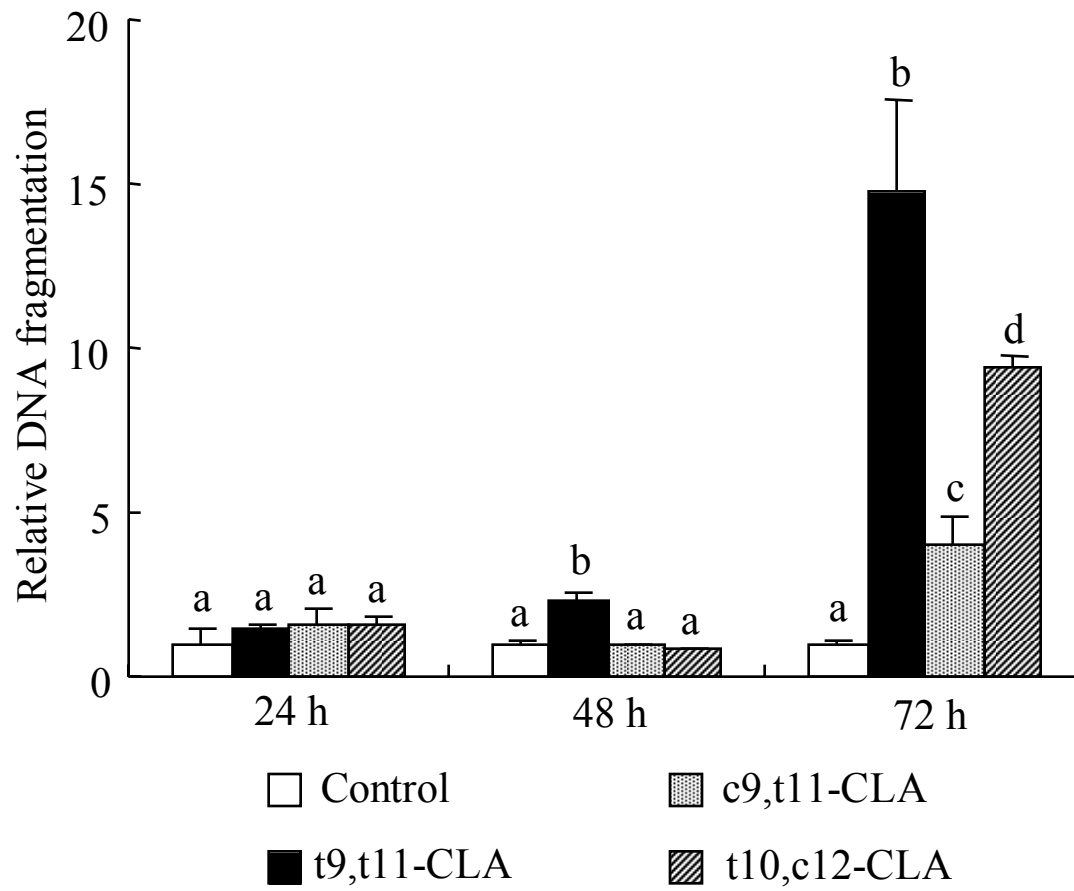


Fig. 5

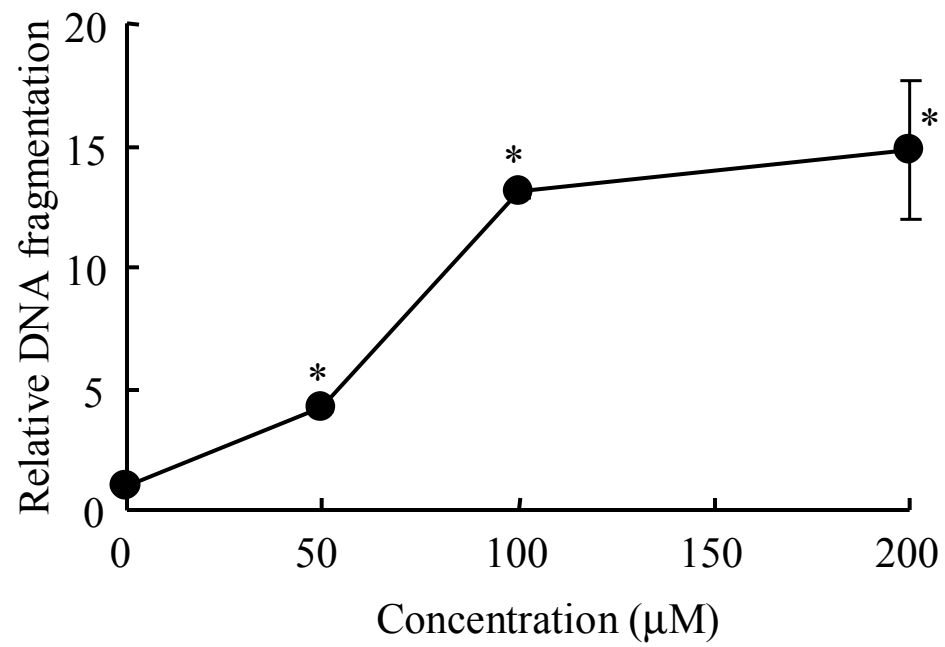


Fig. 6

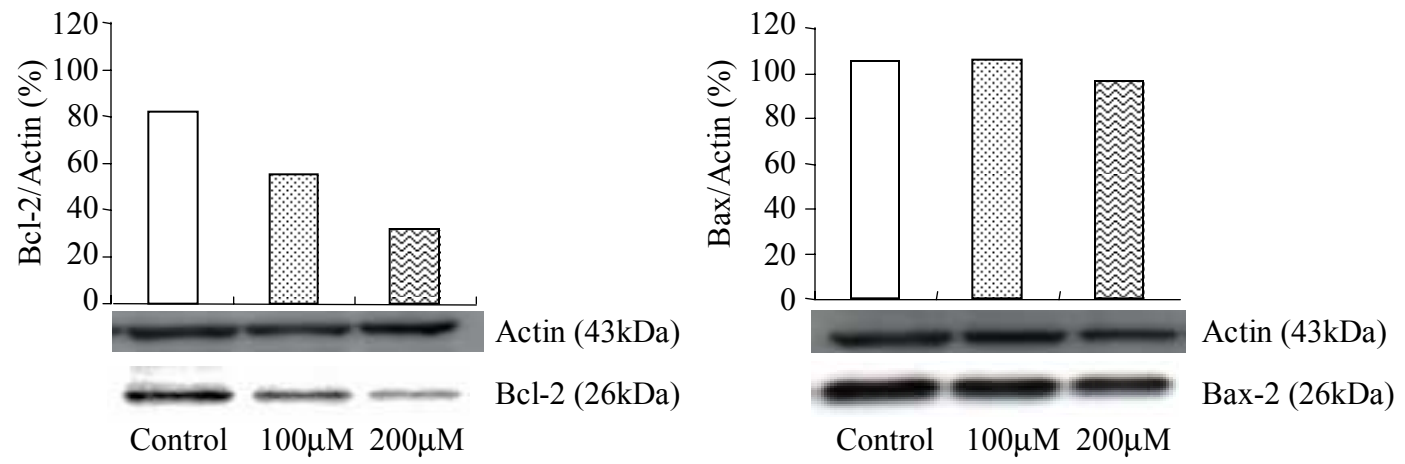


Fig. 7

Reviewer suggestions

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