

# Regulatory Activities from Conjugated Linoleic Acid of p38 Protein in Melanoma Cells

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**Abstract** This study investigated the activities of *trans*<sub>10</sub>,*cis*<sub>12</sub>-conjugated linoleic acid (CLA) were then passed through the p38 in *in vitro* model. Cell viability, apoptosis, expression of p38 was evaluated using B16 melanoma cells. Our result showed that the *trans*<sub>10</sub>,*cis*<sub>12</sub>-CLA increased cell apoptosis in melanoma cells. In addition, CLA stimulated the expression of p38 and phosphorylation p38 (p-p38) level using western blotting. The p38 was presumed that the CLA were involved in regulation of apoptosis-related pathway in the melanoma cells signaling pathway. Considering the results obtained, the present study finding that the regulatory activities of *trans*<sub>10</sub>,*cis*<sub>12</sub>-CLA may be important as a therapeutic target in ameliorating melanoma cells mediated cancer or/and tumor on *in vitro* model.

**Keywords:** conjugated linoleic acid, p38, melanoma cells, cell apoptosis

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## 1. Introduction

Melanoma is one of the most aggressive forms of skin cancer, with high metastatic potential and extraordinary resistance to cytotoxic agents [1,2]. Recently, about 3 million of non-melanoma skin cancers and over the hundred thousand melanoma skin cancers occur globally each year [3]. Despite extensive research and partial successes gained by use of various anti-cancer drug and immune research, currently there is no effective chemotherapy against invasive melanoma [4,5]. Therefore, it is necessary to develop new pharmaceuticals with potent activity against melanoma.

p38 mitogen-activated protein kinase (MAPK) is typically activated after cellular damage such as osmotic shock, stress or ionizing radiation, however, it also has an important role in a number of physiological processes, as well as being implicated in a number of different pathologies [6]. Typically, three kinds of MAPK family are the extracellularly responsive kinase (ERK), the c-Jun N-terminal kinase (JNK) and the p38 [8,9]. Early reported that p38 is a major intracellular signaling molecule critical to pigmentation, and p38 pathway activation has been reported to be related to an increase in melanin synthesis [10,11].

CLA refers to a group of geometric and positional isomers of linoleic acid, which was originally identified as an anti-cancer component from ground beef extract [11,12]. A number of CLA isomers that differ in the positions of the double bond are possible [13].

Additional differences can exist in the configuration of the double bond so that *cis-trans*, *trans-cis*, *cis-cis*, or *trans-trans* configurations are all possible. Early reported that the CLA have been shown to have variety physiological activities such as antioxidant [14,15] anti-inflammation [16,17], anti-cancer [18,19,20], anti-obesity [15], anti-carcinogenic [21], anti-hypertension [12] effects and reduction in the development of atherosclerosis [22,23]. Among them, CLA effect on anti-cancer or anti-tumor has extensively been studied. However, studies on the mechanism of melanoma cells are insufficient information. Therefore, our studies have used a mixture of CLA isomers with a major forms, *trans*<sub>10</sub>,*cis*<sub>12</sub>-CLA. Also, we were to determine the effect of *trans*<sub>10</sub>,*cis*<sub>12</sub>-CLA on the regulatory activity of p38 signal pathway in B16 melanoma *in vitro* model.

## 2. Materials and Methods

### 2.1. Materials

CLA was purchased from Matreya Inc. (State College, PA). B16 cells, the melanoma model for skin function studies, were obtained from the American Type Culture Collection (ATCC CRL-6475™, Manassas, VA, USA). Dulbecco's Modified Eagle's Medium (DMEM) was obtained from COSMO BIO Co., Ltd., (Tokyo, Japan) and supplemented with 5% fetal bovine serum (FBS; Biological Industries, Kibbutz Beit Kaemek, Israel). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Logan, UT, USA). A propidium iodide (PI)

Apoptosis Detection Kit I was purchased from BD Science (San Diego, CA, USA). Tween-20 was supplied by Novagen (Madison, WI, USA). Antibodies were purchased from Santa Cruz Biotechnology (Delaware, CA, USA). Also, an Enhanced Chemiluminescence (ECL) Advanced Detection Kit was obtained from Amersham Bioscience (Uppsala, Sweden). All other reagents were of the highest grade commercially available.

## 2.2. Cell Culture

The B16 cells were grown at 37°C in a used in 5% CO<sub>2</sub>, 95% air humidified atmosphere in growth medium consisted of DMEM supplemented with 5% heat-inactivated FBS and antibiotics (i.e., 100 U/ml of penicillin, 100 µg/ml of streptomycin) this study were used in no more than 10-13 passages.

## 2.3. Cell Proliferation Assay

Cell viability was determined by using counting the number of cells. In brief, B16 cells were seeded at  $1.0 \times 10^4$  cells per well in 24-well plates in complete medium (i.e., DMEM with 5% FBS) and incubated at 37°C for 24 hr. At the end of incubation period, and then the cells were treated with 1, 5, 10 and 20 µM of CLA in DMEM at 37°C for 72 hr. Cell proliferation was evaluated by counting the number of cells (ZI; Coulter Electronics, Hialeah, FL, USA).

## 2.4. Measurement of Cell Apoptosis

For sub-G1 and cell cycle analysis, B16 cells were suspended in ethanol with 0.5% Tween-20 and left for 24 h at 4°C. The cells were harvested by centrifugation and resuspended in 1.0 ml of PBS with 0.05 mg/ml of PI and 10 µg/ml of RNase A, and incubated at 37°C for 30 min. Analysis of apoptotic cell death was performed by measuring the hypodiploid DNA contents using a flow cytometer (FACS-caliber; Becton Dickinson, Franklin Lakes, NJ, USA). The cells in the sub-G1 population were considered apoptotic cells, and the percentage of each phase of the cell cycle was determined.

## 2.5. Western Blot

To obtain the total cell lysate, 50 µL of lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 50 mM NaF, 30 mM Na<sub>2</sub>P<sub>2</sub>O<sub>7</sub>, and 1 mM phenylmethylsulfonyl fluoride 2 µg/mL aprotinin) was added to the B16 cells ( $3 \times 10^5$  cells/mL) cultured in six-well plates. The cells were harvested, incubated for 10 min on ice and centrifuged at 12,000 rpm for 10 min at 4°C. The protein concentration was determined through the use the Bio-Rad DC protein assay, and 20 µg of whole cell lysate was separated on 8% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE). Electrophoresis was performed and the proteins were transferred to PVDF membranes using electro-blotting apparatus. These membranes were blocked with bovine serum albumin (Roche, Penzberg, Germany) for 1 h and then incubated overnight with primary antibodies diluted by 1:1000-5000 and immune complexes were incubated for an hour at room temperature with horseradish

peroxidase-conjugated antibody diluted by 1:10000. After application of the secondary antibody, triplicate washes were followed with TBS-T, and developed for visualization using an ECL detection kit (Amersham Pharmacia Biotech, UK). The optical densities of the antibody-specific bands were analyzed through the use of NIH Image-J software (Bethesda, MD).

## 2.6. Statistical Analysis

Study data are expressed as mean ± standard error of mean (SEM). Statistical analyses of differences between treatment groups were conducted using Student's *t*-test for paired data, and  $p < 0.05$  was considered to have statistical significance. All analyses were carried out in triplicate using Graph Pad Prism software version 4.00 (Graph Pad Software Inc., San Diego, CA).

## 3. Results

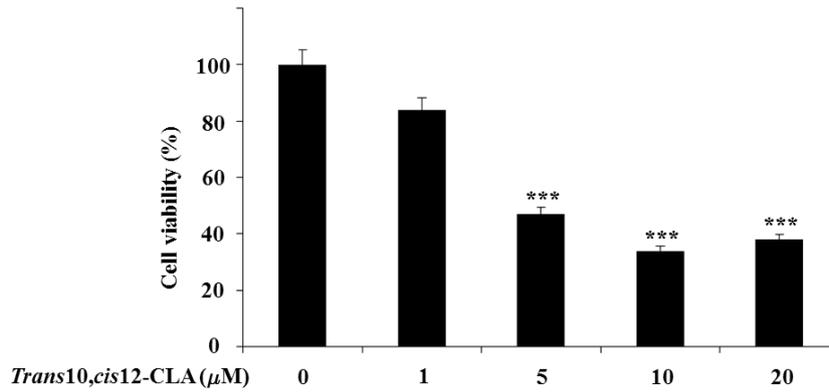
The effects of two kinds of CLA that the viability of melanoma cells were examined by counting the number of cells. B16 cells were treated with *trans*10,*cis*12-CLA at various concentrations (0, 1, 5, 10 and 20 µM) for 72 h. As shown [Figure 1](#), cell proliferation was cytostatic activities by *trans*10,*cis*12-CLA up to 5 µM on B16 cells. Data regarding the effects of CLA on cell proliferation B16 cells have not been reported previously and the mode of activity of CLA remains unknown. We were 5 µM CLA selected for further study.

We further investigated the underlying basis of inhibit proliferation effects of CLA. Cell apoptosis analysis showed distribution in cell cycle, as shown in [Figure 2](#), in the presence of non-treated CLA, the percentage of apoptotic cells observed were 2.49%. The percentages of apoptotic cells observed was 10.91% for treated *trans*10,*cis*12-CLA ([Figure 2](#)). Therefore, these results indicate that cell apoptosis increased the treated *trans*10,*cis*12-CLA group. Cancer or/and tumor may be reduced by inhibiting cells growth or increasing apoptosis. CLA has anti-cancer or tumor activity such as MCF-7, MDA-MB-231, T47D, A-549, DLD-1, M4beu, PC-3, HT-29, Caco-2, MIP-101, SGC-7901 cell line [19]. However, melanoma inhibitory effect of CLA has a little information. Therefore, we showed *trans*10,*cis*12-CLA induced apoptosis in B16 cells.

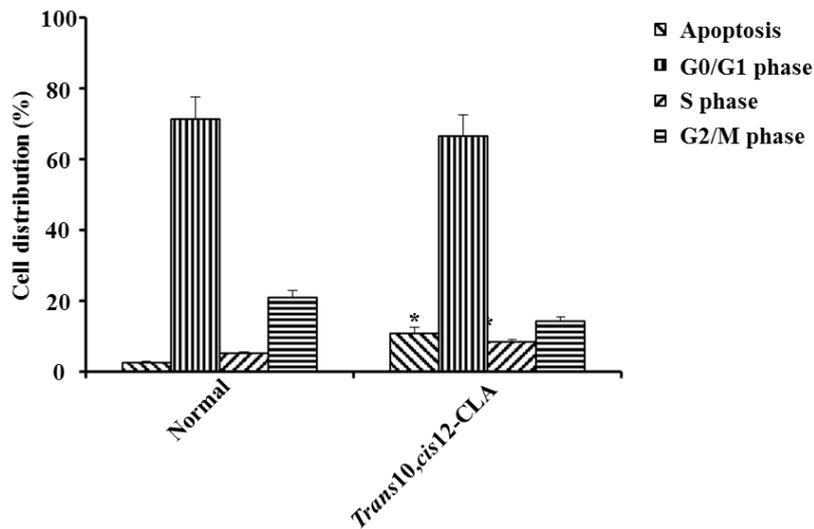
MAPKs have been implicated in regulating the proliferation and differentiation of a variety of cells [24,25,26,27]. To determine whether MAPKs are involved in *trans*10,*cis*12-CLA, the impact of *trans*10,*cis*12-CLA on p-p38 was examined by western blotting, we canvassed the p38 following the treatment of these cells with 12 and 24 h on B16 cells ([Figure 3](#)). At the concentration of 5 µM, *trans*10,*cis*12-CLA for 12 and 24 hr significantly reduced the p-p38 expression level by 37%. However, *cis*9,*trans*11-CLA was without significant influence on p38 expressions in B16 cells (data not shown). Previous research reported that CLA are able to modulate the proliferation and differentiation of skeletal muscle cells through MAPK pathway [28]. Ye et al [29] reported that p-p38 was dose-dependently decreased by Chinese traditional herbal in B16 cells. In addition, Huang et al [30]

research was show the p38 pathway in B16 cells and suggests that regulatory effect of melanoma. Consequentially, CLA are required in order to clarify this statement, the

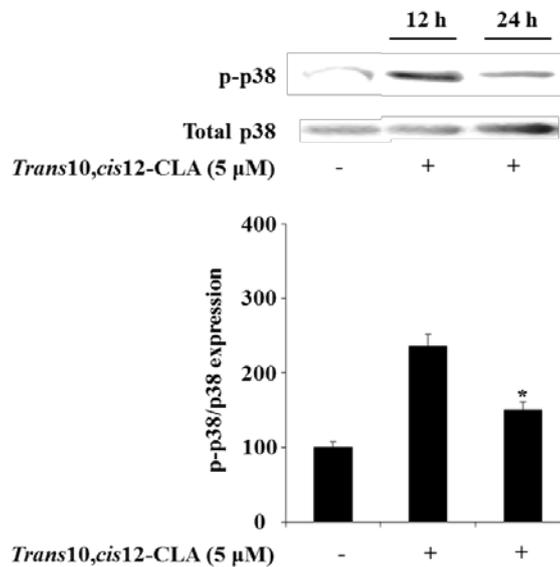
molecular mechanism responsible for the specific receptor of action and the bioavailability of *trans10,cis12*-CLA in melanoma cells.



**Figure 1.** Effect of *trans10,cis12*-CLA on cell viability in B16 cells. Cell viability of B16 cells incubated with 0, 1, 5, 10 and 20 μM *trans10,cis12*-CLA for 72 h determined. Data are presented as mean ± S.E.M. (n = 3) for three independent experiments. Significance was determined by Student’s *t*-test. \**p* < 0.05, \*\* *p* < 0.01 and \*\*\* *p* < 0.001.



**Figure 2.** Cell death and cell cycle of B16 cells after treatment with *trans10,cis12*-CLA treatment. Means±SD of determinations were made in triplicate experiments. \*(*p*<0.05) and \*\*(*p*<0.01) are significantly different as analyzed by paired *t*-test that compared the non-treated group



**Figure 3.** Phosphorylation levels of p38 by *trans10,cis12*-CLA during B16 cells. B16 cells were treated with CLA (5 μM) for 12 and 24 hr, and phosphorylation level of p38 was examined by western blot analysis. All data were expressed as the mean ± SD (n = 3). Statistical significance was analyzed by Student *t* test. \**p* < 0.05.

## 4. Conclusions

The major research study finding is that regulation of the p38 levels of MAPKs may be involved in the CLA-induced cytostatic of melanoma cells. As important, this CLA was analyzed for cell proliferation assay and protein expression by western blotting. It showed the highest melanoma B16 cells cytostatic activity compared with that of anti-cancer drug. In addition, *trans*10,*cis*12-CLA inhibit B16 cell proliferation and induce apoptosis. Based on these results, this *trans*10,*cis*12-CLA has the potential to be developed into new health foods and pharmaceuticals with potent activity against melanoma.

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