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Original Research Article

Cyclohexadione-aniline conjugate inhibits proliferation of melanoma cells via upregulation of Mek 1/2 kinase activity

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Abstract

Purpose: *To investigate the antiproliferative effect of cyclohexadione-aniline conjugate (CHAC) on melanoma cells, and the mechanism of action involved.*
Methods: *Human melanoma cell lines (B16 F1 and A375) were used in this study. The cells were cultured in RPMI 1640 medium supplemented with 10 % fetal bovine serum (FBS) and 1 % penicillin/streptomycin at 37 °C in a humidified atmosphere of 5 % CO_2 and 95 % air. After attaining 70 - 80 % confluency, the cells were treated with serum-free medium and graded concentrations of CHAC (10 – 60 µM) for 24 h. Normal cell culture without CHAC served as control group. B16 F1 and A375 cells were used in logarithmic growth phase in this study. Cell viability and apoptosis were assessed using 3-(4, 5-dimethylthiazol-2-yl) 2, 5-diphe¬nyltetrazolium bromide (MTT) and flow cytometric assays, respectively. Western blotting was used to assess the levels of protein expression of X linked inhibitor of apoptosis (XIAP), survivin, p-Erk 1/2, and p-Mek 1/2.*
Results: *Treatment of B16 F1 and A375 cells with CHAC led to significant and concentration-dependent reductions in their viability ($p < 0.05$). The proliferation of B16 F1 cells decreased from 93.41 to 32.87 %, while that of A375 cells was reduced from 95.23 to 36.50 %. Treatment of B16 F1 cells with CHAC significantly and concentration-dependently increased the population of cells in G0/G1 phase, and significantly reduced cell proportion in S and G2/M phases ($p < 0.05$). It also significantly and concentration-dependently promoted apoptosis in B16 F1 cells ($p < 0.05$). CHAC treatment significantly and concentration-dependently down-regulated the expressions of XIAP and survivin proteins ($p < 0.05$). Exposure of B16 F1 cells to CHAC significantly and concentration-dependently upregulated the expression of p-Mek 1/2, but down-regulated p-Erk 1/2 protein expression ($p < 0.05$). Densitometric analysis revealed that the expression of p-Mek 1/2 was increased from 12 to 91 %.*
Conclusion: *The results of this study indicate that CHAC inhibits the proliferation of melanoma cells via upregulation of Mek 1/2 kinase activity, and therefore may find application in the management of melanoma.*

Keywords: *Melanoma cells, Apoptosis, Proliferation, Mek 1/2 kinase activity*

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INTRODUCTION

Melanoma refers to cancer of melanocytes [1]. It is common among women aged 25 to 29 years [2]. Patients with metastatic melanoma have mean survival period of about 7 months [3,4]. Chemotherapy and surgical resection are strategies usually employed for the treatment of

melanoma [3,4]. However, their use in clinical practice is limited by chemo-resistance and rapid recurrence [5,6]. Products containing cosmeceuticals are used to treat symptoms of melanoma such as darkening of skin and formation of wrinkles [7-10]. The development of novel compounds that can effectively treat melanoma remains a global challenge. Apoptosis, a controlled and programmed cell death, is a cellular mechanism for discarding unwanted or diseased cells [11]. It is characterized by blebbing, cell shrinkage, nuclear fragmentation, chromatin condensation, chromosomal DNA fragmentation, and global mRNA decay [12]. Therefore, the induction of apoptosis in carcinoma cells is key in cancer treatment. Apoptosis is regulated by the phosphorylation of various factors which act as mediators [13]. Extracellular signal-regulated kinase (Erk)-1/2 belongs to the family of mitogen-activated protein kinases (MAPKs), and plays an important role in the proliferation and survival of cells. Phosphorylation of Erk 1/2 transmits various extracellular stimuli from cell surface to the nucleus, thereby stimulating the transcription of genes associated with cell cycle progression from G0/G1 to S phase [14].

Coumarins, which are bi-cyclic compounds in plants, possess a wide range of pharmacological effects such as antioxidant and antitumor properties [15-17]. The present study investigated the antiproliferative effect of CHAC on melanoma cells, and the mechanism involved.

EXPERIMENTAL

Materials

Human melanoma cell lines (B16 F1 and A375) were obtained from American Type Culture Collection (ATCC) (USA). Fetal bovine serum (FBS) was a product of Biological Industries (Israel), while RPMI 1640 medium was purchased from Gibco (USA). Phosphate-buffered saline (PBS) and MTT assay kit were products of Solarbio (China). Flow cytometer was purchased from BD Biosciences (USA). Bicinchoninic acid (BCA) assay kit was obtained from Beyotime (China). Enhanced Chemiluminescence (ECL) kit was obtained from Thermo Fisher Scientific, Inc. (USA). Polyclonal anti-p-Mek 1/2, p-Erk 1/2, XIAP, survivin, and β-actin were products of Santa Cruz Biotechnology, Inc (USA).

Cell culture

B16 F1 and A375 cells were cultured in RPMI 1640 medium supplemented with 10 % FBS and 1 % penicillin/streptomycin at 37 °C in a humidified atmosphere of 5 % CO_2 and 95 % air. After attaining 70 - 80 % confluency, the cells were treated with serum-free medium and graded concentrations of CHAC (10 – 60 µM) for 24 h. Normal cell culture without CHAC served as control group. Cells in logarithmic growth phase were selected and used in this study.

Cell viability assay

The effect of CHAC on the viability of B16 F1 and A375 cells was assessed using MTT assay. The cells (3×10^5 cells/well) were seeded in 96-well plates and cultured in Dulbecco's modified Eagle's medium (DMEM) for 24 h. Then CHAC (10 - 60 µM) was added to the cells and incubated for 72 h. At the end of the third day, 20 µL of 5 g/L MTT solution was added to the wells, followed by incubation for another 4 h. The medium was finally replaced with 150 mL of 0.1 % dimethyl sulfoxide (DMSO) solution, agitated at 50 oscillations/min for 10 min to completely dissolve the formazan crystals formed, and absorbance of the samples was read in a microplate reader at 565 nm. The assay was performed in triplicate. Cell viability (V) was calculated as shown in Eq 1.

$$V (\%) = (At/Ac)100 \quad \ldots\ldots\ldots\ldots (1)$$

where At and Ac are the absorbance of the test and control samples, respectively.

Apoptosis assay

The B16 F1 cells (3×10^6 cells/well) were seeded in 6-well plates and cultured for 24 h. Then, CHAC (10 – 60 µM) was added to the medium and incubated for another 48 h at 37 °C. The cells were thereafter washed with PBS, and thoroughly mixed with 300 µL binding buffer. The cells were subsequently stained with 5 µL each of Annexin V-fluorescein isothiocyanate and propidium iodide within 25 min at room temperature in the dark. Cell apoptosis was assessed using a flow cytometer fitted with argon laser operated at 485 nm, and apoptotic cell population was counted.

Cell cycle analysis

The effect of CHAC on distribution of B16 F1 cells among different phases of the cell cycle was determined using a flow cytometer. The cells were seeded in 6-well plates at a density of 3×10^6 cells/well. They were treated with graded concentrations of CHAC (10 – 60 µM) and incubated for 72 h. The cells were then washed with PBS, and fixed with 70 % ethyl alcohol at 4

^{o}C overnight. Tris-hydrochloride buffer (pH 7.6) containing 1 % RNase A was thereafter added to the plates for 30 min at 37 °C in the dark. The cells were subsequently stained with propidium iodide and injected into the flow cytometer for analysis.

Western blotting

The B16 F1 cells at a density of 2 x 10^5 cells/well were incubated with graded concentrations of CHAC (10 – 60 µM) for 72 h. The cells were then washed twice with PBS, trypsinized, and lysed with 250 µL of ice-cold radio-immunoprecipitation assay (RIPA) buffer containing protease and phosphatase inhibitors for 1 h. The resultant lysate was centrifuged at 14,000 rpm for 30 min at 4 °C, and the protein concentration of the supernatant was determined using Bradford assay [18]. A portion of total cell protein (30 µg) from each sample was separated on 12 % sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis and transferred to a fixed polyvinylidene fluoride membrane at 110 V and 90 °C for 120 min.

Subsequently, non-fat milk powder (5 %) in Tris-buffered saline containing 0.2 % Tween-20 (TBS-T) was added with gentle shaking at 37 ^{o}C and incubated to block non-specific binding of the blot. Incubation of the blots was performed overnight at 4 ◦C with primary antibodies of rabbit polyclonal anti-p-Mek 1/2, p-Erk 1/2, XIAP, survivin, and β-actin, each at a dilution of 1 to 1000. Then, the membrane was washed thrice with PBS and further incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody for 1h at room temperature. The blot was developed using an X-ray film. Grayscale analysis of the bands was performed using ECL. The respective protein expression levels were normalized to that of β-actin which was used as a standard.

Statistical analysis

Data are expressed as mean ± SD, and statistical analysis was performed using SPSS (20.0). Groups were compared using Student *t*-test. Values of $p < 0.05$ were considered statistically significant.

RESULTS

Effect of CHAC on the proliferation of B16 F1 and A375 cells

As shown in Figure 1, treatment of B16 F1 and A375 cells with CHAC led to significant and concentration-dependent reductions in their viabilities ($p < 0.05$). The proliferation of B16 F1 cells decreased from 93.41 to 32.87 %, while that of A375 cells was reduced from 95.23 to 36.50 %.

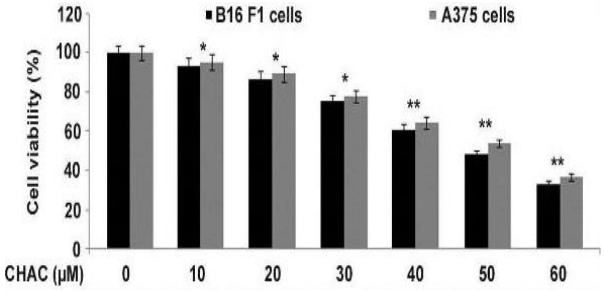

Figure 1: Effect of CHAC on the proliferation of B16 F1 and A375 cells; $^{*}p < 0.05$; $^{**}p < 0.01$, when compared with control cells

Effect of CHAC on cell cycle

Treatment of B16 F1 cells with CHAC led to significant and concentration-dependent increases in the population of cells in G0/G1 phase, and significant reduction of cell proportion in S and G2/M phases ($p < 0.05$; Figure 2).

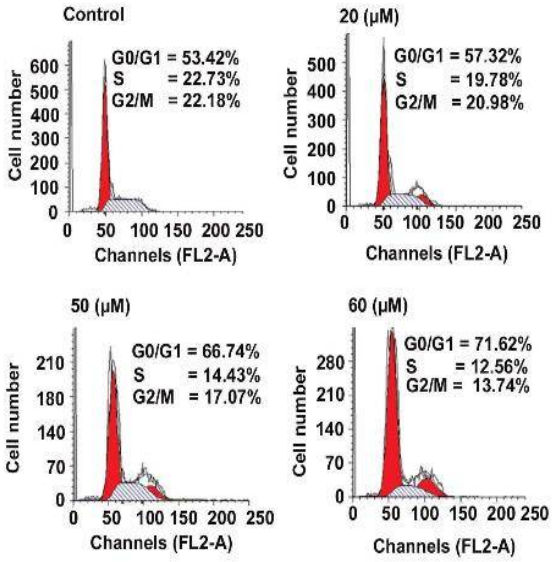

Figure 2: Effect of CHAC on the distribution of B16 F1 cells among phases of the cell cycle; $^{*}p < 0.05$ and $^{**}p < 0.01$, when compared with control cells

Effect of CHAC on apoptosis of B16 F1 cells

As shown in Figure 3, treatment with CHAC significantly and concentration-dependently promoted apoptosis in B16 F1 cells ($p < 0.05$). The percentage of early and late apoptotic cells were significantly and concentration-dependently increased by CHAC treatment ($p < 0.05$).

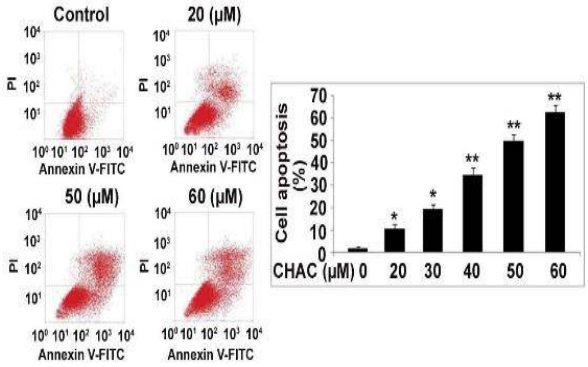

Figure 3: Effect of CHAC on apoptosis of B16 F1 and A375 cells; **(A)**: Flow cytometric analysis of B16 F1 cells after staining with Annexin V/PI; and **(B):** Quantified data from flow cytometric analysis; $^{*}p < 0.05$; $^{**}p < 0.01$, when compared with control cells

Effect of CHAC on expressions of survivin and XIAP in B16 F1 cells

Treatment of B16 F1 cells with CHAC significantly and concentration-dependently down-regulated the expressions of XIAP and survivin proteins ($p < 0.05$; Figure 4).

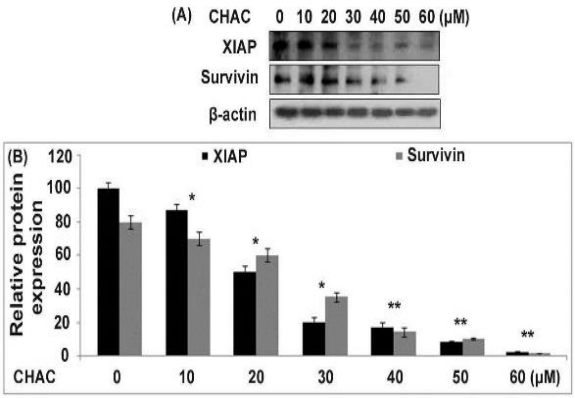

Figure 4: Effect of CHAC treatment on XIAP and survivin expressions in B16 F1 cells. **(A):** Expressions of XIAP and survivin as measured using Western blotting; and **(B):** Densitometric analysis of the levels of expression of XIAP and survivin. $^{*}p < 0.05$; $^{**}p < 0.01$, when compared with control cells

Effect of CHAC on the expressions of Erk 1/2 and Mek 1/2 in B16 F1 cells

Treatment of B16 F1 cells with CHAC significantly and concentration-dependently upregulated the expression of p-Mek 1/2, but down-regulated p-Erk 1/2 protein expression ($p < 0.05$). Densitometric analysis revealed that the expression of p-Mek 1/2 was increased from 12 to 91 %. These results are shown in Figure 5.

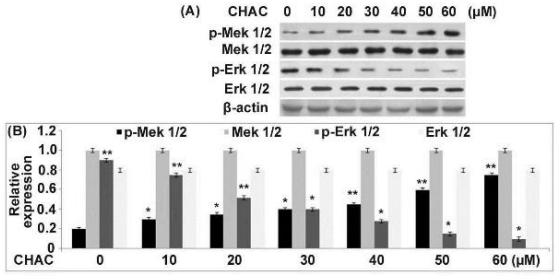

Figure 5: Effect of CHAC on expressions of p- Erk 1/2 and p-Mek 1/2 in B16 F1 cells. **(A):** Expressions of p-Erk 1/2 and p-Mek 1/2 in B16 F1 cells as measured using Western blotting; and **(B):** Densitometric analysis of the levels of expression of p-Erk 1/2 and p-Mek 1/2 in B16 F1 cells; $^{*}p < 0.05$, $^{**}p < 0.01$, when compared with control cells

DISCUSSION

This study investigated the antiproliferative effect of CHAC on melanoma cells, and the mechanism of action involved.

Apoptosis is a physiological process of autonomous, regulated cell death in response to disease and exogenous stress. It is regulated by two major pathways: receptor-mediated pathway (extrinsic pathway) and mitochondrial-dependent pathway (intrinsic pathway) [12]. Effective chemotherapeutic agents are drugs that can induce apoptosis in cancer cells in a bid to reduce their viability and proliferation.

The results obtained from MTT assay showed that CHAC significantly and concentration-dependently reduced the viability of B16 F1 and A375 cells, an indication that it may exert antiproliferative effect on melanoma cells. Treatment of B16 F1 cells with CHAC significantly and concentration-dependently increased the number of apoptotic cells, and arrested the cells in G0/G1 phase of the cell cycle. These results suggest that CHAC may inhibit the proliferation of melanoma cells via induction of apoptosis.

Survivin functions to inhibit caspase activation, thereby leading to downregulation of apoptosis. It promotes cell cycle progression, and it is overexpressed in carcinoma cells [19]. It has been reported that survivin expression is markedly higher in melanoma cells, a phenomenon which aids their survival [19]. The relationship between survivin and pathogenesis of melanoma has been supported *in vitro* and *in vivo* studies [20]. High expression of survivin in melanoma patients correlates with poor prognosis [21,22]. In this study, treatment of B16 F1 cells with CHAC significantly and concentration-dependently down-regulated the

expressions of XIAP and survivin proteins. These results suggest that CHAC may induce apoptosis in melanoma cells via suppression of survivin and XIAP protein expressions.

Extracellular signal-regulated kinase-1/2 plays an important role in the regulation of cell survival and promotion of proliferation [14]. It transmits signals from cell surface to the nucleus, thereby influencing the transcription of genes required for progression of the cell through G0/G1 phase of the cell cycle [14]. The phosphorylated form of Mek 1/2 (p-Mek 1/2) regulates the MAPK pathway via Erk 1/2 activation [23]. The results of Western blotting showed that treatment of B16 F1 cells with CHAC significantly and concentration-dependently upregulated the expression of p-Mek 1/2, but down-regulated p-Erk 1/2 protein expression. Thus, it is likely that CHAC promotes Mek 1/2 activation.

CONCLUSION

The results of this study indicate that CHAC inhibits the proliferation of melanoma cells via upregulation of Mek 1/2 kinase activity. Therefore, cyclohexadione-aniline conjugate has a potential for use in the development of anti-melanoma drugs.

DECLARATIONS

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Yunpeng Wen designed the study and wrote the manuscript. Nan Hu and Shiquan Pang performed the experimental work Zhiping Xiao carried out the literature study and compiled the data. CuiE Kuang and Nan Hu performed literature survey, analyzed the data and compiled the data. The research article was thoroughly read by all the authors prior to submission for consideration of publication.

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