Original Research Article

Cyclopentadione-aniline conjugate suppresses proliferation and induces apoptosis in liver cancer cells via up-regulation of p38 phosphorylation

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Abstract

Purpose: To investigate the effect of cyclopentadione-aniline conjugate (CAC) on proliferation of liver cancer cells.

Methods: MTT assay and flow cytometry were used for the determination of the effect of CAC on cell proliferation and apoptosis. Western blotting was used to measure the influence of CAC on the expressions of various proteins, while Matrigel-coated Transwell assay was used for assessment of cell invasion.

Results: CAC inhibited proliferation of liver cancer cells in a concentration-dependent manner. The degree of proliferation of HepG2 cells was 98, 89, 76, 66, 51, 42 or 36 %, on treatment with 0.25, 0.5, 1.0, 1.5, 2.0, 2.5 or 3.0 µM CAC, respectively. In H4TG cells, treatment with 0.25, 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 µM CAC decreased proliferation of cells to 99, 91, 79, 70, 54, 46 and 40 %, respectively. Apoptosis was induced in 34.56, 37.37 and 52.98 % cells, on treatment with 2.0, 2.5 and 3.0 µM CAC, respectively. The invasive potential of HepG2 cells was significantly decreased by CAC (p < 0.05). Marked decreases were observed in Bcl-2, MMP-2, MMP-9, c-ERK1/2 and phospho-Akt levels in CAC-treated HepG2 cells. CAC treatment markedly upregulated Bax and phospho ERK1/2, but significantly downregulated phospho PI3K, phospho mTOR and phospho Akt in HepG2 cells (p < 0.05). However, the level of phospho p38 was decreased in CAC-treated cells.

Conclusion: These results demonstrate that CAC inhibits proliferation of liver cancer cells via apoptosis induction. Thus, CAC can potentially be used for the treatment of liver cancer in humans.

Keywords: Phosphoinositide 3 kinase, Phospho p38, Apoptosis, Mitochondria, Collagen

INTRODUCTION

New cases of hepatocellular carcinoma (HCC) diagnosed annually worldwide number about 600,000 [1,2]. Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related deaths globally [1,2]. Although surgical intervention has improved the five-year survival of some patients, the prognosis still depends on the stage at which HCC is detected [3]. It has been reported that...
HCC is resistant to available chemo- and radio therapeutic strategies, and it readily metastasizes to other tissues [4]. Thus, most of the HCC patients do not get any benefit from traditional chemotherapeutic agents [3]. The common target tissues of HCC are lymph nodes, pulmonary tissues, brain, bone and adrenal glands [4]. Thus, there is a dire need for development of novel chemo- and radio therapeutic strategies for HCC.

Tumorigenesis is characterised by the inhibition of cell apoptosis, a process which plays a vital role in the development of carcinoma [5]. Induction of apoptosis is regulated by the expression of proteins belonging to the Bcl-2 family [6]. These proteins are involved in the regulation of mitochondrial membrane permeability and activation of caspases [6]. Imbalance in the ratio of Bcl-2/Bax initiates apoptotic signalling through the mitochondrial pathway by the activation of caspases [7]. Metastasis of cancer cells to distant organs is facilitated by the degradation of the extracellular matrix due to increase in the expression of MMPs which catalyse the process [8]. Higher expression of MMPs has been found to enhance the migration potential of cancer cells [9, 10]. In tumor tissues, the levels of MMP-2 and MMP-9 have been found to be markedly higher than their corresponding levels in normal tissues [11,12].

Plant-derived natural products exhibit potent anti-cancer activities either by inducing apoptosis or autophagy or both [13,14]. For instance, in ovarian cancer cells and glioma cells, resveratrol treatment inhibits proliferation by induction of apoptosis [15]. In addition, curcumin treatment has been reported to inhibit cancer cell growth through up-regulation of Akt/mTOR (mammalian target of rapamycin) as well as extracellular signal-regulated kinase (ERK)1/2 signalling pathways [16]. In HCC cells, arenobufagin induces apoptosis through suppression of phosphoinositide-3 kinase (PI3K)/Akt/mTOR pathway [17]. Various cellular processes such as proliferation, apoptosis and growth are regulated by PI3K/Akt/mTOR signalling pathway [18]. In the present study, the effect of CAC on the proliferation of hepatic cancer cells was investigated. The mechanism of CAC action was also studied.

**EXPERIMENTAL**

**Cell culture**

HepG2 and H4TG cancer cell lines were purchased from the Shanghai Institute of Biochemistry and Cellular Biology, Chinese Academy of Sciences (Shanghai, China). The culture of the cells was performed in Dulbecco’s modified Eagle’s medium (DMEM; Gibco Life Technologies, Carlsbad, CA, USA) containing 10 % fetal bovine serum. The medium was supplemented with antibiotics, penicillin (100 U/ml) and streptomycin (100 U/mL). The temperature in the incubator was maintained at 37 °C and atmosphere contained 5 % CO₂ and 95 % air.

**Determination of cell proliferation**

HepG2 and H4TG cells in DMEM were plated at a density of 2 x 10⁶ cells per well in 96-well plates and incubated at 37 °C overnight. The medium was replaced with new medium containing 0.25, 0.5, 1.0, 1.5, 2.0, 2.5 or 3.0 µM of CAC or DMSO (control) and the plates were incubated for 48 h. After incubation, 20 µl of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; 5 mg/ml) was added to the plates and the cells were incubated at 37 °C for 2 h. Then DMSO (100 µL) was added to the plates to dissolve the purple-blue colored precipitate of formazan formed. The absorbance measurement for each plate was performed in triplicates at 585 nm wavelength.

**Flow cytometry**

After treatment with 2.0, 2.5 or 3.0 µM of CAC or DMSO (control) for 48 h, HepG2 cells were subjected to trypsinization. The cells were washed twice with phosphate-buffered saline and subsequently re-suspended at a density of 2 x 10⁶ cells per ml in binding buffer. The cells were then treated with annexin-V-fluorescein isothiocyanate (FITC; 5 µL) and propidium iodide (PI; 5 µL) followed by incubation for 15 min under dark at room temperature. Measurement of the apoptotic cell percentage was performed using a FACScalibur Flow Cytometer (BD Biosciences, San Jose, CA, USA).

**Invasion assay**

The invasive potential of HepG2 cells after CAC treatment was determined by Transwell chamber (Corning Costar; Corning Incorporated, Tewksbury, MA, USA). The chamber contained 10.0-µm pore bearing membrane filter made up of polycarbonate and pre-coated with Matrigel (BD Biosciences). The cells were subjected to incubation with 2.0, 2.5 or 3.0 µM of CAC or DMSO (control) for 48 h, harvested and subsequently put at a density of 3 x 10⁶ cells per ml into the upper chamber in serum-free DMEM. Into the lower chamber was placed DMEM
supplemented with fetal bovine serum (10 %). The cells were cultured under an atmosphere of 5 % CO₂ at 37 °C temperature. The inverted Transwell chambers were subjected to hematoxylin staining and the cell invasion potential was determined by calculation of cells migrating to the lower membrane side. The cells were calculated in five randomly selected fields at x400 magnification in Transwell chamber.

**Western blot analysis**

After treatment with CAC HepG2 cells were washed two times with PBS and subsequently treated with 1 X RIPA lysis buffer. The cells were collected and then agitated at 4 °C for 45 min. The lysate was subjected to centrifugation for 15 min at 13,000 x g at a temperature 4 °C. The Bicinchoninic Acid Protein Assay kit (Thermo Fisher Scientific, Waltham, MA, USA) was used for the determination of concentration of proteins in the lysates. The protein samples were separated using 10 % sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently transferred onto nitrocellulose membrane. The membrane incubation was performed for 1 h at room temperature in blocking buffer (PBS with 0.05 % Tween 20 and 5 % non-fat dry-milk) at room temperature. The membranes were incubated overnight with primary antibodies at 4 °C. The primary antibodies used were: anti-Beclin-1 (catalog number 3738); anti-p-PI3K (catalog number 4228); anti-PI3K (catalog number 4292); anti-p-mTOR (catalog number 2971); anti-mTOR (catalog number 2983); anti-p-Akt (catalog number 9275); anti-Akt (catalog number 9272); anti-p-ERK1/2 (catalog number 4370); anti-ERK1/2 (catalog number 4695); anti-JNK (catalog number 4668); anti-p-p38 (catalog number 4511); anti-p38 (catalog number 9212). All the antibodies were obtained from Cell Signaling Technology, Inc., Danvers, MA, USA). After incubation, membranes were washed with PBS and then incubated for 1 h at room temperature with HRP-conjugated goat anti-rabbit (catalog number AP132P) secondary antibodies (Merck Millipore). The enhanced chemiluminescence reagent was used for the visualization of the blots after washing with 1X PBS-Tween 20 solution.

**Statistical analysis**

The presented data are the mean of three experiments performed independently. Data processing was performed using the Statistical Package for Social Sciences (SPSS for Windows, version 17.0; SPSS, Inc., Chicago, IL, USA) and analysed by monofactorial analysis of variance. Differences were considered statistically significant at p < 0.05.

**RESULTS**

**CAC decreased proliferation of HepG2 and H4TG cells**

HepG2 and H4TG cell cultures were separately incubated with various concentrations of CAC (0.25, 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 µM), and cultured for 48 h. Thereafter, cell proliferation was determined by MTT assay. The degree of HepG2 cell proliferation was 98, 89, 76, 66, 51, 42 and 36 %, on treatment with 0.25, 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 µM CAC, respectively. In H4TG cells, treatment with 0.25, 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 µM CAC decreased proliferation of cells to 99, 91, 79, 70, 54, 46 and 40 %, respectively. These results are shown in Figure 1.

![Figure 1: Effect of CAC on proliferation of liver cancer cells. The values presented are the mean of triplicate experiments; *p < 0.005, *p < 0.001, *p < 0.001, compared to control cells](image)

**CAC treatment induced apoptosis in HepG2 cells**

In HepG2 cells, apoptosis induction was measured on treatment with 2.0, 2.5 or 3.0 µM CAC at 48 h, using flow cytometry. The degrees of apoptosis were 34.56, 37.37 and 52.98 %, on treatment with 2.0, 2.5 and 3.0 µM CAC, respectively. In control cells, the apoptotic cell population was only 0.98 % (Figure 2).

![Figure 2: CAC treatment induced apoptosis in HepG2 cells](image)

**Invasion of HepG2 cells was inhibited by CAC**

Treatment of HepG2 cells with 1.5, 2.0, 2.5 and 3.0 µM CAC for 48 h was followed by determination of invasion using Transwell cell invasion assay. A significant decrease was observed in invasive potential of HepG2 cells on treatment with CAC (Figure 3). Increase in the concentration of CAC from 2.0 to 3.0 µM caused a significant decrease in the invasion potential of HepG2 cells (p < 0.05).
Figure 2: Effect of CAC on apoptosis in HepG2 cells. The cells were incubated with 2.0, 2.5 and 3.0 µM CAC for 48 h and then stained with Annexin V and propidium iodide. Flow cytometry was used for determination of apoptosis.

Figure 3: Effect of CAC on HepG2 cell invasion. The cells, after treatment with 1.5, 2.0, 2.5 or 3.0 µM of CAC, were subjected to Matrigel-coated Transwell assay. A significant decrease in cell penetration through Transwell chamber was observed on increasing the CAC concentration. The values are expressed as mean of three measurements (n = 3). *p < 0.05, **p < 0.02, ***p < 0.02, relative to control cells.

CAC changed protein expressions in HepG2 cells

Following treatment of HepG2 cells with 1.5, 2.0, 2.5 and 3.0 µM of CAC for 48 h, the cells were subjected to western blotting. Marked decreases were observed in the expressions of Bcl-2, MMP-2, MMP-9, c-ERK1/2 and phospho-Akt in HepG2 cells on treatment with CAC. The expression of Bax was increased by CAC treatment in HepG2 cells at 48 h. However, the expressions of t-ERK1/2 and t-Akt showed no marked changes in the cells treated with CAC (Figure 4).

Figure 4: Effect of CAC on protein expressions in HepG2 cells. Western blotting was used for determination of changes in protein expressions caused by CAC treatment. The cells were incubated with varying doses of CAC for 48 h, after which the expressions of Bcl-2, Bax, phospho-Akt, phospho-ERK1/2, MMP-2 and MMP-9 were measured using immunoblotting.

CAC activated PI3K/Akt/mTOR pathway in HepG2 cells

The expressions of PI3K, phospho-PI3K, mTOR and phospho-mTOR in HepG2 cells were determined using western blot assay. When compared to the control cells, the expressions of phospho-PI3K and phospho-mTOR were markedly decreased in CAC-treated HepG2 cells (Figure 5). The decreases in expressions of phospho-PI3K and phospho-mTOR were accentuated with increase in concentration of CAC.

Figure 5: Effect of CAC treatment on phospho-PI3K and phospho-mTOR expressions. Following incubation for 48 h with 1.5, 2.0, 2.5 and 3.0 µM CAC, western blotting was used to determine the expressions of PI3K, phospho-PI3K, mTOR and phospho-mTOR in the cells, with β-actin as loading control. (PI3K = phosphoinositide-3 kinase)

CAC activated JNK1/2MAPK pathways in HepG2 cells

After incubation of HepG2 cells for 48 h with 2.0, 2.5 and 3.0 µM CAC, the expressions of JNK, phospho-JNK, p38 and phospho-p38 were assayed using western blot analysis. As shown in Figure 6, CAC treatment for 48 h did not
significantly affect the expression of JNK, relative to control cells. However, the expression of phospho-p38 was decreased by CAC treatment in HepG2 cells.

Figure 6: Effect of CAC on JNK, phospho-JNK, p38 and phospho-p38 expressions. HepG2 cells were treated with CAC for 48 h, and were analysed for the expressions of JNK, phospho-JNK, p38 and phospho-p38 using western blot analysis. The internal loading control was β-actin. (JNK, c-Jun N-terminal kinase)

DISCUSSION

The present study investigated the effect of CAC on liver cancer proliferation, and the mechanism involved. The CAC treatment inhibited proliferation of liver cancer cells through induction of apoptosis. Apoptosis inhibition is the characteristic feature of tumorigenesis because apoptosis plays an important role in the removal of unwanted cells and puts a check on cell proliferation [5]. In the present study the effect of CAC on apoptosis induction was investigated in liver cancer cells. Flow cytometric analysis revealed that treatment of HepG2 cells with CAC led to the induction of apoptosis. The effect of CAC on the expression of proteins related to apoptosis was investigated using western blotting. The members of Bcl-2 protein family play a vital role in the regulation of cellular apoptosis [6]. The pro-apoptotic protein, Bax and anti-apoptotic protein, Bcl-2 regulate mitochondrial membrane permeability and activation of caspases [6]. The apoptotic signalling pathway is mediated by Bcl-2/Bax protein ratio [7].

In the present study, the expressions of Bcl-2, MMP-2, MMP-9, c-ERK1/2 and phospho-Akt in HepG2 cells were decreased by treatment with CAC. However, the Bax expression was increased in liver cancer cells on exposure to CAC. Thus, CAC treatment decreases the protein ratio of Bcl-2/Bax in liver cancer cells. These findings suggest that CAC induces apoptosis in liver cancer cells through activation of the mitochondrial pathway. The most lethal characteristic feature of cancer cells is the tendency to undergo metastasis and invasion. In the current study, CAC treatment decreased metastasis and invasion potential of HepG2 liver cancer cells.

Up-regulation of MMP expression causes increase in cancer cell metastasis due to the fact that MMPs catalyse the degradation of the extracellular matrix [8]. In addition, the MMP-catalysed breakdown of extracellular matrix enhances the migration potential of cancer cells [9,10]. Degradation of membrane collagen by MMP-2 and MMP-9 increases migration and invasion potential of cancer cells [19, 20]. Studies have shown that MMP-2 and MMP-9 levels are higher in carcinoma tissues than in normal tissues [11,12]. In the present study, CAC treatment of liver cancer cells caused marked decreases in the expressions of MMP-2 and MMP-9, when compared to normal cells.

The activation of PI3K/Akt and MAPK/ERK pathways plays important role in controlling the gene expressions involved in regulation of cell proliferation, invasion and apoptosis [21]. In the present study, the activation of PI3K/Akt was markedly inhibited in liver cancer cells by treatment with CAC. Activation of MAPK/ERK pathway increases cancer cell proliferation, promotes angiogenesis, induces drug resistance and decreases apoptosis [22,23]. The results revealed that CAC treatment inhibits activation of ERK1/2 in liver cancer cells. Cancer cell death is also be inhibited by the activation of PI3K, Akt and mTOR [24]. In the current study, CAC treatment caused down-regulation of PI3K, Akt and mTOR in liver cancer cells. It has been reported that activation of JNK is associated with the regulation of cell apoptosis [25]. In the present study, CAC treatment led to markedly increased expression of phospho-ERK1/2, relative to control cells.

CONCLUSION

The findings of the present study demonstrate that CAC decreases liver cancer cell proliferation and induces apoptosis. The inhibition of liver cancer cell proliferation by CAC partly involves activation of mitochondrial and ERK1/2/JNK/p38 pathways. Thus, CAC may be suitable for the treatment of liver cancer.
DECLARATIONS

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Conflict of interests

The authors declare that no conflict of interest is associated with this work.

Authors’ contribution

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. Heze Yu and Gao Liang performed the experimental work. Bo Dou, carried out the literature study and compiled the data. Jiangdong Ni designed the study and wrote the paper. All the authors wrote the paper before communication.

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