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Original Research Article

Trichlorophenyl-benzoxime induces apoptosis in human colon carcinoma cells via activation of mitochondria-dependent pathway

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Abstract

Purpose: *To determine the apoptotic effect of trichlorophenyl-benzoxime (TCPB) on colorectal cancer (CRC) cells, and to elucidate the mechanism of action.*
Methods: *Colon carcinoma cell lines (DLD-1 and HT-29) were used in this study. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal bovine serum (FBS) and 1 % penicillin/streptomycin at 37 ˚C in an atmosphere of 5 % CO_2 and 95 % air. When the cells attained 60 - 70 % confluency, they were treated with serum-free medium and graded concentrations of TCPB (1.0 – 6.0 µM) for 24 h. Cell viability and apoptosis were assessed using 3-(4, 5-dimethylthiazol-2-yl) 2, 5-diphenyltetrazolium bromide (MTT) and flow cytometric assays, respectively. Western blotting and 2', 7' dichlorofluorescein diacetate (DCFH DA) assays were used for the determination of expression levels of apoptotic proteins, and levels of reactive oxygen species (ROS), respectively.*
Results: *Treatment of DLD-1 and HT-29 cells with TCPB led to significant and dose-dependent reductions in their viability, as well as significant and dose-dependent increases in the number of apoptotic cells ($p < 0.05$). Treatment of HT-29 cells with TCPB led to significant increases in the population of cells in the G0/G1 phase, but significant reduction of cell proportion in S and G2/M phases ($p < 0.05$). It also significantly and dose-dependently upregulated the expressions of caspase-3 and bax, down-regulation of the expression of bcl-2 ($p < 0.05$). TCPB treatment upregulated the expressions of p53, cytochrome c (cyt c), procaspase-3, and procaspase-9, but down-regulated the expression of p-Akt dose-dependently ($p < 0.05$). The expression of Akt in HT-29 cells was not significantly affected by TCPB ($p > 0.05$). However, TCPB significantly enhanced the cleavage of PARP1, and significantly and dose-dependently increased the levels of ROS in HT-29 cells ($p < 0.05$).*
Conclusion: *These results suggest that TCPB exerts apoptotic effect on CRC cells via activation of mitochondria-dependent pathway, and thus can be suitably developed for the management of colon cancer.*

Keywords: *Colorectal cancer, Trichlorophenyl-benzoxime, Mitochondrial pathway, Apoptosis*

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INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer of the gastrointestinal tract (GIT), and one of the most frequently diagnosed malignancies of the intestines [1,2]. It is one of the leading causes of early mortality in developed nations [1,2]. In Asia, the incidence of CRC has greatly increased due to a shift to Western-type diets, changing environment, and lifestyle modification [3]. The disease is characterised by insidious onset which makes its diagnosis difficult [3]. In more than 35 % of patients with CRC, diagnosis is made at the late stage of the disease [4]. Colorectal cancer metastasises very quickly and its recurrence usually occurs within 5 years post-surgery [4]. Surgery and adjuvant chemotherapy are the two common strategies for treating CRC. Development of resistance remains the major factor responsible for poor prognosis of CRC [5].

Apoptosis is programmed cell death which occurs in multicellular organisms [6,7]. The apoptotic process is characterised by changes in cell morphology such as blebbing of the plasma membrane, cell shrinkage, altered mitochondrial membrane potential, and condensation and cleavage of chromatin [6,7]. Cell apoptosis is generally regulated by two pathways: extrinsic and intrinsic pathways [8]. Death receptors on surfaces of cells are involved in the induction of apoptosis via the extrinsic pathway [9]. These surface receptors elicit their effects by activating and upregulating the expression of caspase-8 [9]. In contrast, the intrinsic pathway of apoptosis involves several stress stimuli which change the level of bcl-2 protein resulting in the release of cytochrome *c* from mitochondria. In addition, Smac and Apaf-1 are also released from the mitochondria under the influence of bcl-2 protein overexpression, which together with cytochrome c catalyse the activation of caspases 3 and 9 [9]. The anticancer activity of most chemotherapeutic agents is exerted via induction of apoptosis [10].

Compounds of natural origin have been investigated for their cytotoxicity against different types of carcinoma cells [11]. Indeed, compounds containing flavonoid scaffold have been shown to effectively inhibit the proliferation of various types of carcinoma such as pulmonary, breast and prostate cancers [12]. It has been reported that flavonoid-bearing compounds are generally less toxic, and this makes them suitable candidates for the development of potential anticancer drugs [12]. The present study investigated the apoptotic effect of TCPB on CRC cells, and the underlying mechanism.

EXPERIMENTAL

Materials

Dulbecco's modified Eagle's medium (DMEM) and MTT solution were products of Sigma-Aldrich (USA). Fetal bovine serum (FBS) and Applied Biosystems 7300 RT- PCR machine were products of Life Technologies (USA). Polyvinylidene fluoride membranes, chemiluminescence (ECL) kit and autoradiography film were purchased from Millipore Co. Ltd (USA), while RNase A was a product of Nanjing KeyGen Biotech Co., Ltd. Flow cytometer was obtained from BD Biosciences (USA).

Cell lines and culture

Colon carcinoma cell lines (DLD-1 and HT-29) were obtained from the Academia Sinica (China). The cells were cultured in DMEM supplemented with 10 % FBS and 1 % penicillin/streptomycin at 37 ˚C in an atmosphere of 5 % CO_2 and 95 % air. When the cells attained 60 - 70 % confluency, they were treated with serum-free medium and graded concentrations of TCPB for 24 h. Cells in logarithmic growth phase were selected and used in this study.

Assessment of cell viability

The viability of the cells in the presence of TCPB was assessed using MTT assay. The cells (3 x 10^5 cells/well) were seeded in 96-well plates and cultured in DMEM for 24 h. Then, TCPB (1.0 – 6.0 µM) was added to the cells and incubated for 72 h. At the end of the third day, 20 µL of 5 mg/mL MTT solution was added to the wells, followed by incubation for another 4 h. The medium was finally replaced with 150 µL of 0.1 % dimethyl sulfoxide (DMSO), agitated at 50 oscillations/min for 10 min to completely dissolve the resultant formazan crystals. The absorbance of the samples was read in a microplate reader at 563 nm. The assay was performed in triplicates. Cell viability (V) was calculated as shown in Eq 1.

V (%) = (As/Ac)100 …….. (1)

where As and Ac are the absorbance of experimental and control samples, respectively.

Western blotting

HT-29 cells (5 x 10^8/L) were incubated with TCPB (1.0 – 6.0 µM) for 72 h. The cells were then washed twice with phosphate-buffered saline (PBS) and 250 µL of ice-cold radio-immunoprecipitation assay buffer (RIPA) containing protease and phosphatase inhibitor

was used to lyse them. The resultant lysate was centrifuged at 14, 000 rpm for 20 min at 4 °C, and the protein concentration of the supernatant was determined using Bradford method. A portion of total cell protein (10 µg) from each sample was separated on 8 % 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- Akt, p-Akt, bcl-2, bax, p53, cyt-c, procasapse-3, procaspase-9, PARP1 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 Enhanced chemiluminescence (ECL). Respective protein expression levels were normalized to that of β-actin which was used as a standard.

Cell cycle analysis

The effect of TCPB on cell cycle distribution in HT-29 cells was determined using a flow cytometer. The HT-29 cells treated with varied concentrations of TCPB (1.0 – 6.0 µM) were seeded into 6-well plates and incubated for 72 h. The cells were then washed with PBS, and fixed with 70 % ethyl alcohol at 4°C overnight. Tris-hydrochloride buffer (pH 7.5) containing 1 % RNase A was then added to the plates. The cells were subsequently stained with propidium iodide and injected into the flow cytometer for analysis.

Apoptosis assay

The cells were seeded at a density of 2.5 x 10^6 cells/well into 6-well plates and cultured for 24 h. Then, TCPB (1.0 – 6.0 µM) was added to the medium and incubated for another 72 h, and thereafter washed with PBS, and thoroughly mixed with 300 µL binding buffer. The cells were then 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.

Determination of ROS

The levels of ROS in HT-29 cells were determined using DCFH-DA assay. The cells treated with TCPB (1.0 – 6.0 µM) were washed with PBS after an initial incubation for 72 h. Then, 10 µM solution of DCFH-DA was added to the plates and incubated for another 35 min at 37°C. Thereafter, the cells were washed with PBS and injected into the flow cytometer for analysis.

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 TCPB on the viability of DLD-1 and HT-29 cells

As shown in Figure 1, treatment of DLD-1 and HT-29 cells with TCPB significantly and dose-dependently reduced their viability ($p < 0.05$). The viability of DLD-1 cells decreased progressively from 98 to 25 %, while the viability of HT-29 cells decreased from 99 to 19 %.

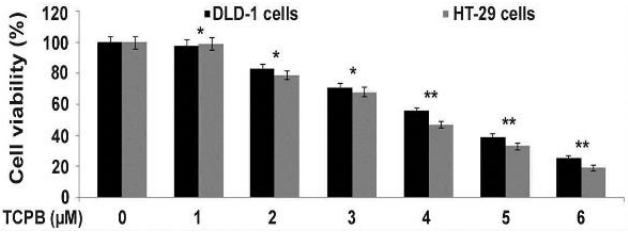

Figure 1: Effect of TCPB on DLD-1 and HT-29 cell viability. Incubation of cells with 1 - 6 µM of TCPB for 72 h was followed by MTT assay. The values shown are mean ± SD of the triplicate experiments; *$p < 0.05$ and **$p < 0.01$ versus untreated DLD-1 and HT-29 cells

Effect of TCPB on HT-29 cells apoptosis

Treatment of HT-29 cells with TCPB significantly and dose-dependently increased the number of apoptotic cells ($p < 0.05$). The population of apoptotic cells increased from 11.16 to 68.34 % (Figure 2).

Effect of TCPB on cell cycle progression in HT-29 cells

Treatment of HT-29 cells with TCPB significantly increased cell population in the G0/G1 phase, and significantly reduced cell proportion in S and G2/M phases ($p < 0.05$). In control cultures, the popula

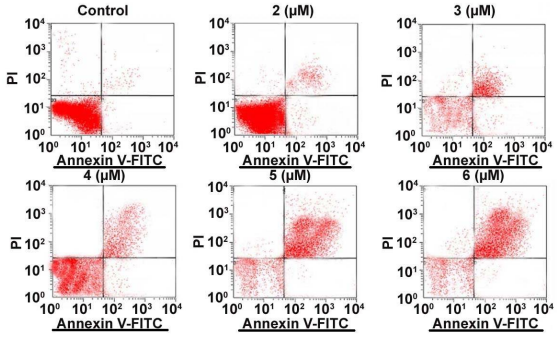

Figure 2: Effect of TCPB on HT-29 cell apoptosis. (A) HT-29 cells after exposure to 2, 3, 4, 5 and 6 μM of TCPB for 72 h were analysed by flow cytometry for apoptosis. (B) The population of apoptotic HT-29 cells after 72 h of exposure to various concentrations of TCPB; *p < 0.05 and **p < 0.02 versus untreated HT-29 cells

tion of cells in G0/G1 phase was progressively increased from 52.63 to 68.33 % on treatment with varied concentrations of TCPB. Treatment with TCPB decreased HT-29 cell population in G2/M phase from 23.67 to 17.36 %. These results are shown in Figure 3.

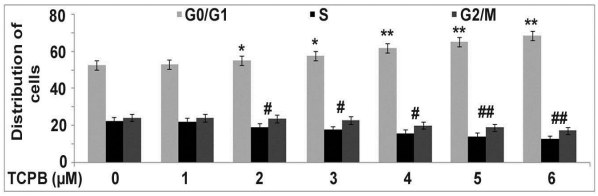

Figure 3: Effect of TCPB on cell cycle distribution in HT-29 cells; *p < 0.05 and **p < 0.01, when compared with control cells

Effect of TCPB on the expression levels of apoptotic proteins in HT-29 cells

Treatment of HT-29 cells with TCPB significantly and dose-dependently upregulated the expressions of caspase-3 and bax, and significantly and dose-dependently down-regulated the expression of bcl-2 (p < 0.05; Figure 4).

Effect of TCPB on levels of expression of mitochondrial proteins in HT-29 cells

Treatment of HT-29 cells with TCPB significantly and dose-dependently upregulated the expressions of p53, cyt-c, procaspase-3, and procaspase-9 (p < 0.05). However, it significantly and dose-dependently down-regulated the expression of p-Akt (p < 0.05), but did not significantly alter the level of expression of Akt in HT-29 cells (p > 0.05). However, it promoted the cleavage of PARP1 (p < 0.05). These results are shown in Figure 5.

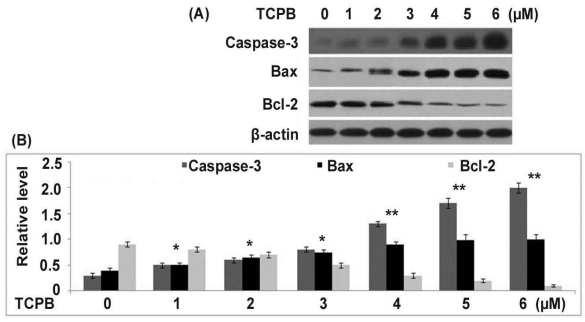

Figure 4: Effect of TCPB on the expression levels of apoptotic proteins in HT-29 cells treated with TCPB. **(A):** Expressions of apoptotic proteins as determined using Western blotting; and **(B):** Densitometric analysis of Western blot; *p < 0.05 and **p < 0.01, when compared with control cells

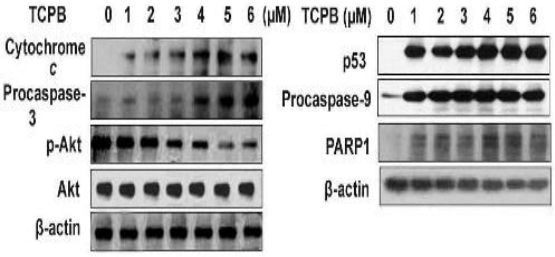

Figure 5: Levels of expression of mitochondrial proteins in HT-29 cells treated with TCPB

Effect of TCPB on ROS production in HT-29 cells

As shown in Figure 6, treatment of HT-29 cells with TCPB significantly and dose-dependently increased the levels of ROS (p < 0.05).

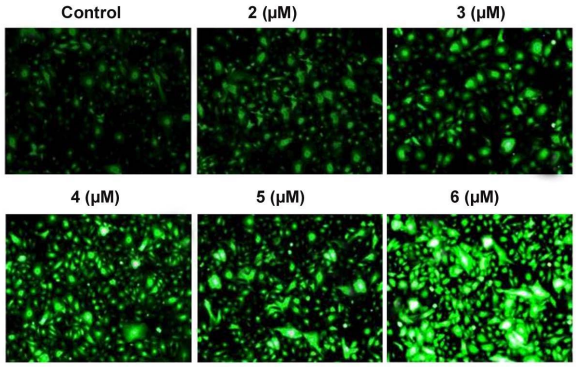

Figure 6: ROS levels in HT-29 cells treated with TCPB

DISCUSSION

Colorectal cancer (CRC), also known as bowel or colon cancer, is cancer of the colon or rectum. Its symptoms depend on the size and location of the cancer, and they include changes in bowel habits and stool consistency, presence of blood in the stool (haematochezia) and abdominal

discomfort. The present study investigated the apoptotic effect of TCPB on CRC cells, and the underlying mechanism.

The results of MTT assay showed that treatment of DLD-1 and HT-29 cells with TCPB significantly and dose-dependently reduced their viability. Moreover, TCPB promoted apoptosis and ROS generation, and upregulated the expressions of pro-apoptotic proteins, while down-regulating the expression of anti-apoptotic protein in CRC cells. Generally, mammalian cells undergo apoptosis via the mitochondrial pathway [13]. Most anticancer drugs inhibit proliferation of cancer cells via activation of caspases and alteration in established potential of the mitochondrial membrane [13]. The disruption of mitochondrial membrane potential is as indication of early stage of cell apoptosis which is characterized by release of cytochrome c from the mitochondria [14,15]. Efflux of cytochrome c from the mitochondria is accompanied by increased expression of caspases 3 and 9 [14,15]. The results of this study suggest that TCPB may trigger caspase-3 activation in HT-29 cells.

In this study, treatment of HT-29 cells with TCPB significantly arrested cell cycle progression to S phase (cell cycle arrest at G0/G1 phase), an indication that TCPB may reduce viability of HT-29 cells by induction of apoptosis in these cells. Treatment of HT-29 cells with TCPB significantly and dose-dependently upregulated the expressions of caspase-3 and bax, and significantly and dose-dependently down-regulated the expression of bcl-2.

Treatment of HT-29 cells with TCPB significantly and dose-dependently upregulated the expressions of p53, cyt-c, procaspase-3, and procaspase-9. However, it significantly and dose-dependently down-regulated the expression of p-Akt, but did not significantly alter the level of expression of Akt in HT-29 cells. However, it promoted the cleavage of PARP1.

The activation of Akt plays a vital role in inhibiting cell apoptosis [16]. It has been reported that phosphorylation of Akt is closely associated with the inhibition of caspase-8 activity through increased expression of FLICE inhibitory protein [17]. Overexpression of bax protein plays is vital in cellular apoptosis [18]. In cells, bcl-2 is highly expressed at sites of ROS production such as mitochondria and nuclear membranes [19]. It promotes the scavenging of ROS, thereby preventing apoptosis [19]. Anticancer drugs that increase bax/bcl-2 protein ratio have been shown to exert apoptosis-inducing potential [20]. It is likely that TCPB induces apoptosis in CRC cells by increasing the bax/bcl-2 ratio. Excess production of ROS leads to oxidative damage of mitochondria and this serves as an apoptotic signal [21]. In this study, treatment of HT-29 cells with TCPB significantly and dose-dependently increased the levels of ROS, an indication that TCPB may promote ROS production in HT-29 cells.

CONCLUSION

The results obtained in this study suggest that TCPB exerts apoptotic effect on CRC cells via activation of mitochondria-dependent pathway. Thus, this compound has a potential for further development as an agent for the treatment of colorectal cancer in humans.

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. Kuan Hu designed the study and wrote the paper. Zhongfu Xiao, Ke Zhang and Yun Huang performed the experimental work, Yufan Zhou carried out the literature study and compiled the data. Zhongfu Xiao, Ke Zhang and Yun Huang performed literature survey, analyzed the data and compiled the data. The research article was thoroughly read by all the authors before commination for the consideration of publication.

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