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Original Research Article

Chlorophenyl-benzoxime inhibits pancreatic cancer cell proliferation, invasion and migration by down-regulating the expressions of interleukin-8 and cyclooxygenase-2

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Abstract

Purpose: *To investigate the effects of chlorophenyl-benzoxime (CPBZX) on pancreatic cancer (PC) cell proliferation, invasion and migration, and the underlying mechanism of action.*
Methods: *Pancreatic carcinoma cell lines (HuP-T4, HuP-T3 and BxPC-3) were cultured in Dulbecco's Modified Eagle medium (DMEM) containing 10 % fetal bovine serum (FBS), penicillin (100 U/mL) and streptomycin (10 μg/mL) at 37 ˚C in a humidified atmosphere containing 5 % CO_2 and 95 % air. Cell proliferation was assessed using MTT assay. Real-time quantitative polymerase chain reaction (qRT-PCR) and Western blotting were employed for the determination of changes in the levels of expression of carcinoembryonic antigen (CEA), interleukin-8 (IL-8) and cyclooxygenase-2 (COX 2). Cell invasion and migration were determined using Transwell and wound healing assays, respectively.*
Results: *The results of MTT assay showed that CPBZX significantly and dose-dependently inhibited the proliferation of PC cells ($p < 0.05$). Incubation of HuP-T4 cells with CPBZX significantly and dose-dependently reduced the invasive ability of the cells ($p < 0.05$). The migratory ability of HuP-T4 cells was also significantly and dose-dependently inhibited by CPBZX ($p < 0.05$). The results of Western blotting and qRT PCR showed that CPBZX treatment significantly and dose-dependently upregulated CEA mRNA expression ($p < 0.05$). On the other hand, the expressions of IL-8 and COX-2 were significantly and dose-dependently down-regulated by CPBZX. Treatment of pancreatic tumor mice with CPBZX significantly decreased tumor growth and metastasis of tumor cells to the pulmonary tissues, liver and lymph nodes ($p < 0.05$).*
Conclusion: *The results of this study suggest that CPBZX inhibits the development and metastasis of PC via the down-regulation of IL-8 and COX 2 expressions, and therefore may find application in pancreatic cancer therapy.*

Keywords: *Pancreatic cancer, Chlorophenyl-benzoxime, Cyclooxygenase, Interleukin-8, Proliferation*

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INTRODUCTION

The incidence of PC is about 12 cases per 100,000 people in the United States of America, and approximately 6 patients in every 100,000 people throughout the world [1,2]. Pancreatic cancer is ranked fourth in the hierarchy of tumors and among the leading causes of cancer-related deaths in the United States of America, and the eighth leading cause of deaths due to carcinoma

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worldwide [2,3]. Pancreatic ductal adenocarcinoma (PDAC) alone accounts for more than 80 % of all diagnosed cases of PC [4]. This disease is common among the people aged 70 years and above [4,5]. Advancement in treatment strategies, especially combination therapy has led to improvement in the survival of PC patients [4].

Combination therapy has greatly increased the chances of survival of patients who are at the metastatic or advanced stage of PC [4]. Adjuvant therapy is usually preferred to surgery in that it lengthens patients' survival [4-6]. However, despite advancements in treatment methods, the disease is still characterized by high mortality: the percentage of patients that survive up to 5 years ranges from 6 - 7 % [1]. Malignant PC is characterized by uncontrolled proliferation, increased potential for metastasis and inhibition of differentiation of pancreatic acinar cells into normal duct-like cells [7].

Efforts aimed at developing new anticancer drugs are targeted at the inhibition of COX-2 and suppression of cell proliferation [8]. The overexpression of COX-2 has been observed in early stage tumors of stromal cells and larger tumors of dysplastic epithelium [9, 10]. The development of PC from inflammation is facilitated by the aggregation of inflammatory cells and secretion of cytokines [11]. The present study investigated the effects of *CPBZX* on PC cell proliferation, invasion and migration, and the underlying mechanism.

EXPERIMENTAL

Materials

Matrigel was purchased from BD Biosciences. Light microscope was obtained from Nikon (Japan). Olympus-CX31 microscope was a product of Olympus Co., Ltd. Trizol reagent was purchased from Thermo Fisher Scientific (USA), while SYBR Premix Ex Taq was bought from Takara Bio Inc. (Japan). UltraSYBR Mixture was purchased from Cwbiotech, (China), and BALB/c mice were obtained from Beijing HFK Bioscience Co., Ltd.

Cell lines and culture

Pancreatic carcinoma cell lines (HuP-T4, HuP-T3 and BxPC-3) were purchased from Chinese Academy of Preventive Medicine (China). The cells were cultured in Dulbecco's Modified Eagle medium (DMEM) containing 10 % fetal bovine serum (FBS), penicillin (100 U/mL) and streptomycin (10 µg/mL) at 37 ˚C in a humidified atmosphere containing 5 % CO_2 and 95 % air.

MTT assay

This was performed to determine the proliferative ability of the cells and their viability in the presence of *CPBZX*. The cells (3×10^5 cells/well) were seeded into 96-well plates and cultured in DMEM for 24 h. Then, *CPBZX* (2 – 10 µmol/L) was added to the cells and incubated for 2 days at 37 °C. At the end of the third day, 20 µL of 1.0 mg/mL 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, and absorbance of the sample was read in a microplate reader at 485 nm. The control cell cultures were incubated in a medium containing DMSO alone.

Cell invasion assay

The invasive ability of HuP-T4 cells was determined using Transwell assay. The cells (2×10^6 cells/mL) were placed in Transwell chamber coated with Matrigel (1:2 dilution) and cultured in 300 µL serum-free DMEM. The cells were incubated for 48 h with varied doses of *CPBZX* (2 – 10 µmol/L). Medium containing 10 % FBS was added to the lower chamber. After 24 h, the cells that passed through the matrix gel membrane were stained with crystal violet after fixation, photographed and counted using an inverted microscope.

Wound healing assay

This was used to assess the migratory ability of the cells. The HuP-T4 cells in logarithmic growth phase (2×10^6 cells/well) were seeded into 6-well plates until they attained 60 - 70 % confluency in DMEM supplemented with 10 % FBS, and scratches were made on cell monolayers with plastic tip (1 mm) of the pipette. The cells were then incubated in DMEM containing 2 % FBS and treated with varied concentrations of *CPBZX* (2 - 10 µmol/L). The cells were further incubated for 48 h, after which they were observed and photographed. The photomicrographs were analysed using an Olympus-CX31 microscope.

Western blotting

Changes in the expression levels of CEA, IL-8 and COX-2 were determined using Western blotting. HuP-T4 cells (5×10^8/L) were incubated with *CPBZX* (2 - 10 µmol/L) for 48 h. The cells were then washed twice with phosphate-buffered saline (PBS) and ice-cold radio-

immunoprecipitation assay buffer (RIPA) containing protease inhibitor was used to lyse them. The resultant lysate was centrifuged at 12, 000 rpm for 20 min at 4 °C, and the protein concentration of the supernatant was determined using BCA assay kit. A portion of total cell protein (30 µg) from each sample was separated on a 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 (3 %) 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-CEA, mouse monoclonal anti-IL-8 and mouse monoclonal anti-COX-2 and GAPDH at a dilution of 1 to 1000. Then, the membrane was washed thrice with TBS-T and further incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody for 1.5 h 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 GAPDH which was used as a standard.

qRT-PCR

Trizol RNA extraction reagent was used to extract total RNA from HuP-T4 cells after 48 h of incubation with varied doses of *CPBZX* (2 - 10 µmol/L). Trizol reagent (1 mL) was added to the cells, followed by chloroform treatment. The resultant cell suspension was then centrifuged at 12,000 rpm for 20 min at 4 ˚C to obtain supernatant. The supernatant was treated with isopropyl alcohol and subsequently centrifuged at 12,000 rpm for 15 min at 4 ˚C. The RNA pellet was dissolved in ethyl alcohol, and centrifuged as at other times and the products obtained were suspended in aqueous solution of diethyl pyrocarbonate. The concentration of RNA was measured using a UV-spectrophotometer. Reverse transcription of RNA into cDNA was carried out using 5X All-In-One RT MasterMix system for 20 min at 37 ˚C, and for 10 s at 85 ˚C. The RNA samples were maintained at a temperature of -20 ˚C. The SYBR Premix Ex Taq was used for the measurement of the levels of expression of CEA, IL-8 and COX-2. The cycling process consisted of 92 ˚C for 25 s; 92 ˚C for 5 s and 58 ˚C for 25 s over a total of 40 cycles. The average threshold value for each cycle was normalized to the expression of GAPDH.

Experimental mice

BALB/c male mice (n = 20) aged 6 weeks were used for this study. The mice were housed in plastic cages under standard laboratory conditions: 12 h light/dark cycles, 25 ˚C and 50 % humidity. They had free access to feed and clean drinking water.

Preparation of pancreatic xenograft mice model

HuP-T4 cells (2 x 10^6 cells/mL) in logarithmic growth phase were harvested using trypsin EDTA, and then washed thrice with RPMI medium, and re-suspended in the medium. Following anesthesia, the mice were placed in the supine position and inoculated with HuP-T4 cells. A small incision of about 6 - 8 mm diameter was made in the anorectal region of the mice through anorectal wall in the anterior end. This incision was made to prevent the obstruction of colon due to the development of rectal tumor. A 27-gauge (20 mm) needle was used for subcutaneous injection of HuP-T4 cells (2 x 10^6 cells) suspended in RPMI into the posterior wall of the mice. The mice were then randomly assigned to three groups: normal control group, negative control group and treatment group. Mice in the treatment group were further divided into two subgroups on day 2 of tumor injection: 5 mg/kg body weight, bwt, *CPBZX* group and 10 mg/kg bwt *CPBZX* group. Mice in normal control and negative control groups received equivalent volumes of physiological saline in place of drug. The mice were sacrificed on the 31st day of tumor inoculation after pentobarbital sodium anaesthesia.

Assessment of tumor development and metastasis

The tumors in peri-anorectal region were excised and weighed to determine the effect of *CPBZX* on tumor growth in the treatment groups. The lungs, liver and lymph nodes were also excised and examined for tumor metastasis. Total DNA was isolated from the tissue sections using proteinase K-phenol and chloroform extraction kit according to the instructions of the manufacturer. A portion of the DNA (1 µg) was subjected to amplification with the primers using ABI Prism 7700 Sequence Detector (TaqMan). The number of tumor cells metastasized into the organs was determined using qRT-PCR.

Statistical analysis

Data are expressed as mean ± SD, and the statistical analysis was performed using SPSS

(13.0). Groups were compared using Student's *t*-test. Values of $p < 0.05$ were considered statistically significant.

RESULTS

Effect of *CPBZX* on PC cell proliferation

As shown in Figure 1, *CPBZX* significantly and dose-dependently inhibited the proliferation of PC cells ($p < 0.05$). However, it had no significant effect on the proliferation of normal pancreatic cells (CRL-4039) ($p > 0.05$).

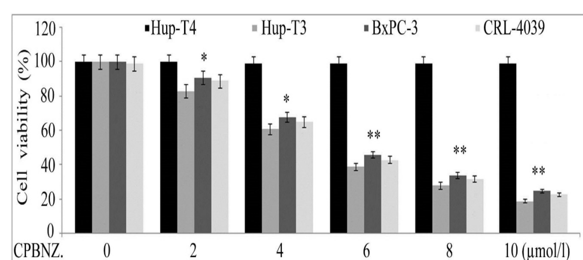

Figure 1: Inhibition of PC cell proliferation by *CPBZX*. $^{*}p < 0.05$; $^{**}p < 0.01$, when compared with control group (0 μmol/L *CPBZX* group)

Effect of *CPBZX* on *HuP-T4* cell invasion

Treatment of *HuP-T4* cells with *CPBZX* significantly and dose-dependently reduced the invasive ability of the cells ($p < 0.05$; Figure 2).

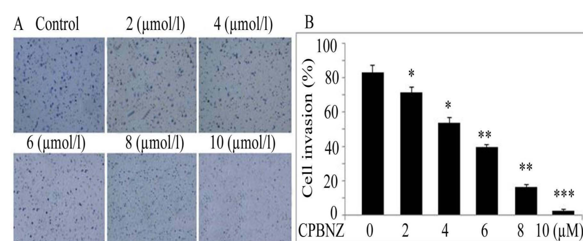

Figure 2: Effect of *CPBZX* on invasion of HuP-T4 cells *in vitro*. (A): Cell invasion as measured after fixing and staining the cells on bottom side of the filter with crystal violet (x200); and **(B):** The percentages of cells invaded to the bottom filter, expressed in relation to the control (0 μmol/L). $^{*}p < 0.05$; $^{*}p < 0.01$; $^{**}p < 0.001$, when compared with control group (0 μmol/L *CPBZX)*

Effect of *CPBZX* on *HuP-T4* cell migration

The migratory ability of HuP-T4 cells was significantly and dose-dependently inhibited by *CPBZX* ($p < 0.05$; Figure 3).

Effect of *CPBZX* on CEA, IL-8 and COX-2 mRNAs expression in HuP-T4 cells

The results of Western blotting and qRT-PCR showed that *CPBZX* treatment significantly and dose-dependently upregulated CEA mRNA expression ($p < 0.05$). However, the levels of expression of IL-8 and COX-2 were significantly and dose-dependently down-regulated by *CPBZX* ($p < 0.05$). These results are shown in Figure 4.

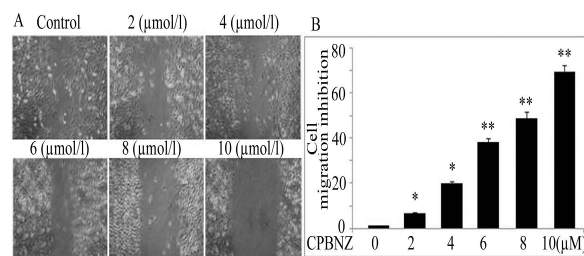

Figure 3: Inhibitory effect of *CPBZX* on HuP-T4 cell migration *in vitro*. **(A):** Photomicrographs of the cells 48 h after treatment (x 200); and **(B):** Cell migration expressed in percentages. $^{*}p < 0.05$; $^{**}p < 0.02$, when compared with control cells (0 μmol/L *CPBZX*)

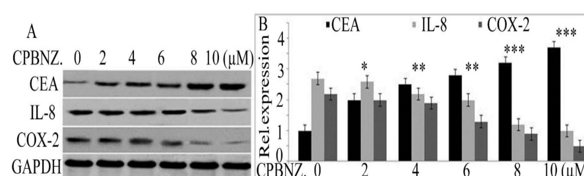

Figure 4: Changes in the expression patterns of CEA, IL-8 and COX-2 in HuP-T4 cells. **(A):** Expressions of CEA, IL-8 and COX-2 as measured using Western blotting; and **(B):** Levels of expression of CEA, IL-8 and COX-2 as measured using qRT-PCR. $^{*}p < 0.05$, $^{**}p < 0.01$ & $^{***}p < 0.001$, when compared with control cells (0 μmol/L *CPBZX*)

Effect of *CPBZX* on tumor development and metastasis

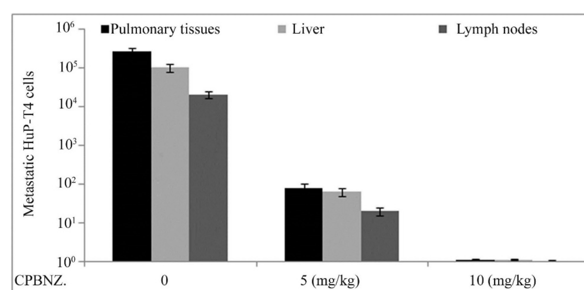

Figure 5: Suppression of tumor growth and metastasis by *CPBZX*. $^{*}P < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$, when compared with negative control group (0 mg/kg *CPBZX*)

Treatment with 10 mg/kg bwt *CPBZX* significantly reduced tumor growth ($p < 0.05$). The mean wet tumor weights excised from the control, 5 mg/kg *CPBZX* and 10 mg/kg *CPBZX* were 3.56, 1.43 and 0.21 g, respectively. Treatment of pancreatic tumor mice with *CPBZX* significantly decreased the metastasis of tumor

cells to the pulmonary tissues, liver and lymph nodes ($p < 0.05$). These results are shown in Figure 5.

DISCUSSION

The pancreas secretes enzymes that aid digestion and hormones that regulate the metabolism of sugars. Pancreatic cancer (PC) has sudden onset, spreads rapidly, and is accompanied by poor prognosis. There are usually no symptoms in the early stages of the disease. The later stages are associated with symptoms, but these can be non-specific, such as lack of appetite and weight loss. Treatments for PC include surgery, radiation and chemotherapy [2]. The present study investigated the effects of *CPBZX* on PC cell proliferation, invasion and migration, and the underlying mechanism.

The results of MTT assay showed that *CPBZX* significantly and dose-dependently inhibited the proliferation of PC cells. Incubation of *HuP-T4* cells with *CPBZX* significantly and dose-dependently reduced the invasive ability of the cells. The migratory ability of the cells was also significantly and dose-dependently inhibited by *CPBZX.* The results of Western blotting and qRT-PCR showed that *CPBZX* treatment significantly and dose-dependently upregulated CEA mRNA expression. However, the levels of expression of IL-8 and COX-2 were significantly and dose-dependently down-regulated by *CPBZX.* These results suggest that *CPBZX* may inhibit the proliferation of HuP-T4 cells by promoting their differentiation. It has been reported that expression of CEA promotes differentiation of pancreatic cells [12].

The expression of IL-8 has been reported to be significantly higher in PC tissues than in normal cells [13,14]. The expression of IL-8 in carcinoma tissues is associated with tumor growth and metastasis [15,16]. This cytokine is a mediator of carcinoma cell angiogenesis and it is responsible for promoting the growth and development of pancreatic tumors [13,17]. In animal models of cancer, *in vivo* studies have shown that the expression of IL-8 in carcinoma tissues is directly linked to the extent of angiogenesis and tumorigenicity [15]. Under normal physiological conditions, the expression of COX-2 in most tissues is almost negligible [18]. The expression of COX-2 is usually upregulated in cancer cells and it is responsible for the uncontrolled cell proliferation and oxidative damage [19,20].

In animal models of cancer, growth and metastasis of tumors are facilitated by increased expressions of COX-2 and related prostaglandins via induction of new blood vessel formation (angiogenesis) [21]. Studies have also shown that inhibition of COX-2 expression suppresses tumor angiogenesis and release of prostaglandin E2 [22]. Metastasis of tumor cells is responsible for the ineffectiveness of most therapies currently used for cancers. Therefore, inhibition of PC metastasis is an important therapeutic strategy for improving patients' survival.

In this study, *CPBZX* significantly inhibited the development of tumor and its metastasis to pulmonary tissues, liver and lymph nodes. However, metastasis of tumor cells to lymph nodes was almost completely absent in mice treated with 10 mg/kg *CPBZX*.

CONCLUSION

The results of this study suggest that *CPBZX* inhibits the development and metastasis of PC via the down-regulation of IL-8 and COX-2 expressions. Therefore, further studies are required to ascertain its potential application in humans.

DECLARATIONS

Acknowledgement

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Conflict of interest

No conflict of interest is associated with this study.

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. Xiao Yu designed the study, compiled the data, performed literature survey and wrote the paper. Pinyan Wang and Yanan Xue performed the experimental work. All the authors read the paper thoroughly before communicating it for publication.

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