

Original Article

Anti-tumor activity of CrTX in human lung adenocarcinoma cell line A549

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Aim: To assess the cytotoxic effect of crotoxin (CrTX), a potent neurotoxin extracted from the venom of the pit viper *Crotalus durissus terrificus*, in human lung adenocarcinoma A549 cells and investigated the underlying mechanisms.

Methods: A549 cells were treated with gradient concentrations of CrTX, and the cell cycle and apoptosis were analyzed using a flow cytometric assay. The changes of cellular effectors p53, caspase-3 and cleaved caspase-3, total P38MAPK and pP38MAPK were investigated using Western blot assays. A549 xenograft model was used to examine the inhibition of CrTX on tumor growth *in vivo*.

Results: Treatment of A549 cells with CrTX (25–200 µg/mL) for 48 h significantly inhibited the cell growth in a dose-dependent manner (IC₅₀=78 µg/mL). Treatment with CrTX (25 µg/mL) for 24 h caused G₁ arrest and induced cell apoptosis. CrTX (25 µg/mL) significantly increased the expression of wt p53, cleaved caspase-3 and phospho-P38MAPK. Pretreatment with the specific P38MAPK inhibitor SB203580 (5 µmol/L) significantly reduced CrTX-induced apoptosis and cleaved caspase-3 level, but G₁ arrest remained unchanged and highly expressed p53 sustained. Intraperitoneal injection of CrTX (10 µg/kg, twice a week for 4 weeks) significantly inhibited A549 tumor xenograft growth, and decreased MVD and VEGF levels.

Conclusion: CrTX produced significant anti-tumor effects by inducing cell apoptosis probably due to activation of P38MAPK and caspase-3, and by cell cycle arrest mediated by increased wt p53 expression. In addition, CrTX displayed anti-angiogenic effects *in vivo*.

Keywords: crotoxin (CrTX); human lung adenocarcinoma; apoptosis, P38MAPK; caspase-3; cell cycle; p53; angiogenesis

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Introduction

Crotoxin (CrTX) is a potent neurotoxin that is extracted from the venom of the pit viper *Crotalus durissus terrificus*^[1]. CrTX possesses phospholipase A2 activity and inhibits neuromuscular transmission. Recently, some studies have reported that CrTX has anti-tumor effects. However, the potential mechanism is unclear.^[2–5]

Lung cancer is the most common cancer worldwide with the highest mortality rate among cancers. Current drugs cannot meet the therapeutic needs^[6]. Previous study demonstrated that CrTX has cytotoxic effects on A549 cells, which are human lung adenocarcinoma cells with the wild type p53 gene, and shows synergistic effects when combined with Iressa, which is currently a widely-used drug for lung cancer therapy^[7, 8]. In this study, we further investigated the anti-tumor effects of CrTX on A549 cells and tried to elucidate the mechanisms of CrTX-mediated cell growth inhibition and cell apoptosis.

Some evidence has indicated that the P38MAPK pathway plays an important role in the process of apoptosis and the cell cycle. Therefore, we detected the expression of apoptotic proteins and cell cycle-related factors, such as p53, cleaved caspase-3 and phospho-P38MAPK, to discover the potential molecular mechanisms that mediate the anti-tumor activities of CrTX. The anti-tumor activity of CrTX was also analyzed in the A549 xenograft model.

Materials and methods

Cells and reagents

A549 cells, which are human lung adenocarcinoma cells with wt p53, purchased from Shanghai Institutes of Biological Science), were cultured in RPMI-1640 media containing 10% fetal bovine serum and maintained in an incubator (5% CO₂, 37 °C). Crotoxin (CrTX) was provided by Celtic Biotechnology (Dublin, Ireland). The anti-phospho-P38MAPK, anti-p53 and anti-cleaved caspase-3 antibodies were purchased from Cell Signaling Technology (Woburn, MA, USA). SB203580, which is a specific inhibitor of P38MAPK, was purchased from Calbiochem, Inc (Madison, WI, USA).

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Cell growth viability assay

Based on the results of preliminary experiments, we selected four different concentrations of crotoxin (25 $\mu\text{g}/\text{mL}$, 50 $\mu\text{g}/\text{mL}$, 100 $\mu\text{g}/\text{mL}$ and 200 $\mu\text{g}/\text{mL}$) in the study. After collecting A549 cells in the logarithmic growth period, we prepared 1.0×10^5 cells/mL of cell suspension after 0.25% trypsin treatment and seeded them into 96-well plates. Each well contained 100 μL of cell suspension. The control (cell suspension) and blank groups (RPMI-1640 medium) were included in the study. Once cells were allowed to adhere to the plate for 24 h, we removed the medium and applied different concentrations of CrTX to treat the cells for 48 h. Then, 20 μL of MTT (5 mg/mL) was added, and the cells were incubated at 37 $^{\circ}\text{C}$ for 4 h. The supernatant in each well was replaced with 150 μL DMSO. After 30 min of incubation at room temperature and gentle agitation for 10 min, the optical density (OD) was measured with an automatic multiwell spectrophotometer at 570 nm. Three independent experiments were performed to generate averaged values. The cytostatic rate was calculated using the following equation: cytostatic rate (%) = $(1 - \text{average OD value of experimental group} / \text{average OD value of control group}) \times 100$.

Flow cytometry analysis of CrTX-induced cell cycle arrest and apoptosis

Four experimental groups were included in the study: control, CrTX (25 $\mu\text{g}/\text{mL}$), SB203580+CrTX and SB203580 only (5 $\mu\text{mol}/\text{L}$). SB203580 was added to the culture medium 1 h before CrTX. All groups of cells were digested with 0.25% trypsin and centrifuged at 1000 r/min. The collected cells were rinsed in PBS (phosphate buffered saline) twice, fixed with 70% pre-cooled ethanol and stored at 4 $^{\circ}\text{C}$. The cells were centrifuged to remove the fixation reagent and suspended in PBS. The cells were incubated at 37 $^{\circ}\text{C}$ for 30 min after adding 200 μL RNaseA (1 $\mu\text{g}/\mu\text{L}$). PI (propidium iodide) was added to the cells and incubated for 30 min in the dark. Finally, the cell cycle and apoptosis were analyzed using flow cytometry.

Western blot analysis of p53, caspase-3 and cleaved caspase-3, total P38MAPK and pP38MAPK

Three experimental groups were included in the study as follows: control, CrTX and SB203580 + CrTX groups. The dose response and time course studies were performed as mentioned previously. Cells were washed twice with pre-cooled PBS. The pellets were lysed on ice with lysis buffer. The lysates were centrifuged at 12000 r/min for 10 min at 4 $^{\circ}\text{C}$, and the supernatants were collected. Protein concentrations were determined using the BCA protein assay. For Western blot analysis, proteins were denatured for 5 min by boiling. Protein extracts were subjected to SDS-PAGE with 10% gels and electroblotted onto nitrocellulose membranes. Membranes were incubated overnight at 4 $^{\circ}\text{C}$ in 5% non-fat milk with primary monoclonal antibodies of p53, caspase-3, cleaved caspase-3, total P38MAPK, phospho-P38MAPK and β -actin (Santa Cruz Biotechnology, Inc, Santa Cruz, CA). After incubating for 2 h at room temperature with a secondary HRP-

conjugated antibody, antigens were visualized using enhanced chemiluminescence using ECL (enhanced chemiluminescence) according to the manufacturer's instructions.

In vivo anti-tumor efficacy and mechanism study

Female Balb/C nude mice with average body weights of 18–20 g (4–6 weeks) were purchased from a local commercial vendor and housed in a SPF (specific pathogen free) animal facility. All manipulations (ie, handling, invasive procedures and tumor volume measurements) were performed in a laminar flow hood under strict sterile conditions. Mice were injected into the right axillary space tissue with 5×10^7 cells/mL A549 cells that were suspended in 0.2 mL of PBS. Treatment with CrTX was initiated 8 d after xenotransplantation, and the tumor reached approximately 5–6 mm in diameter. With the exception of two no-tumor mice, sixteen remaining mice were randomly assigned to one of two experimental groups ($n=8$ per group): the control or CrTX (10 $\mu\text{g}/\text{kg}$, ip, twice a week) group. After 4 weeks of treatment, the mice were sacrificed, and the tumor xenografts were removed, sectioned and analyzed using TEM. The data for tumor growth inhibition were expressed as the mean tumor weight \pm standard deviation. Tumor xenografts were further analyzed using a microscope, and microvascular density (MVD) was counted using Weidner methods. Briefly, a microscopic field was defined by a grid that was placed on the eyepiece. Endothelial cells in distinct cell clusters showing CD31 staining were considered as a single, countable microvessel. MVD was determined by calculating the mean of the obtained vascular counts in 15 random fields across the tissue section. The MVD data were expressed as the mean \pm standard deviation. In addition, 0.5 mL of blood was harvested from each mouse, and 0.2 mL of serum was collected after centrifuging for 10 min at 3000 r/min. VEGF levels were analyzed in each serum sample using ELISA. VEGF levels were expressed as the mean \pm standard deviation.

Statistical analysis

The data were expressed as the mean \pm standard deviation and analyzed using the one-way analysis of variance (ANOVA) or *t*-test. Differences were statistically significant when $P < 0.05$.

Results

CrTX inhibited A549 cell proliferation in vitro

A549 cells were treated with CrTX 25, 50, 100, or 200 $\mu\text{g}/\text{mL}$ for 48 h, and the viability of the cells was determined using the MTT assay. The results show that CrTX significantly inhibits the growth of A549 cells with an IC_{50} value of 78 $\mu\text{g}/\text{mL}$ in a concentration-dependent manner. The CrTX-induced inhibition rates were 87.5% (200 $\mu\text{g}/\text{mL}$), 53.2% (100 $\mu\text{g}/\text{mL}$), 24.1% (50 $\mu\text{g}/\text{mL}$), and 15.7% (25 $\mu\text{g}/\text{mL}$) (Table 1).

CrTX induced cell apoptosis and arrested cells in G₁ phase

To further understand the mechanism of CrTX in cell growth inhibition, flow cytometric assays were used to analyze cell apoptosis and cell cycle arrest. After treatment with CrTX (25 $\mu\text{g}/\text{mL}$) for 24 h, a significantly increased population

Table 1. The effect of CrTX on A549 cell proliferation. ^bP<0.05 compared with control group.

CrTX concentration (μg/mL)	Inhibition rate (%)
Control	
25	15.7±3.4 ^b
50	24.1±1.0 ^b
100	53.2±12.0 ^b
200	87.5±9.2 ^b

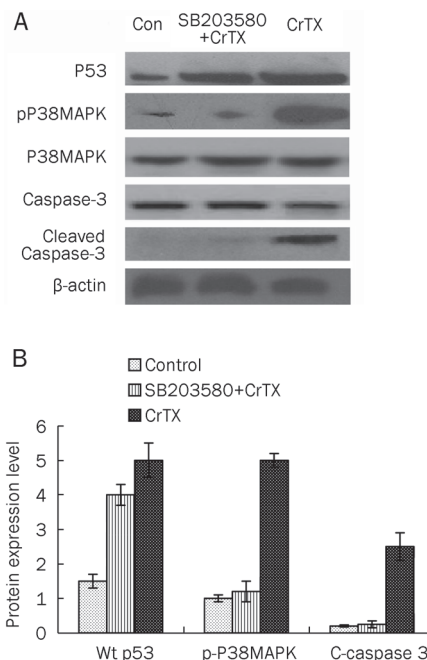
of cells was halted at the sub-G₀ phase, which is an index of cell apoptosis, compared to the control group (Table 2). The results suggest that CrTX potently induces apoptosis in A549 cells, which suggests a potential mechanism for growth inhibition. To assess this mechanism for apoptosis, A549 cells were pretreated with SB203580, which is a specific P38MAPK inhibitor, and then treated with CrTX. No significant differences were observed in the sub-G₀ phase cell population (Table 2). The results show that SB203580 protects A549 cells from apoptosis, indicating that P38MAPK plays an important role in apoptosis, suggesting that P38MAPK plays an important role in CrTX-induced cell apoptosis. In addition, CrTX significantly increased A549 cells in the G₁ phase compared to the control group (Table 2). SB203580 failed to alter CrTX-induced arrest of A549 cells in the G₁ phase, suggesting that inhibition of P38MAPK has no effect on CrTX-induced cell cycle arrest (Table 2).

Table 2. Analysis of CrTX-induced apoptosis and cell cycle arrest of A549. ^bP<0.05 compared with control group.

Groups	Sub-G ₀ (%)	G ₀ /G ₁ (%)	S (%)	G ₂ /M (%)
Control	0.70±0.06	55.82±2.15	30.15±1.32	13.95±0.85
CrTX	11.42±1.02 ^b	76.85±2.30 ^b	16.04±0.52	6.80±0.30
SB203580	0.516±0.03	54.71±1.64	29.45±0.86	14.68±0.92
SB203580+CrTX	0.87±0.08	75.06±1.83 ^b	15.36±0.71	9.08±0.52

Effects of CrTX on p53, caspase-3 and cleaved caspase-3, total P38MAPK and pP38MAPK expression

A549 cells were treated with 25 μg/mL of CrTX for 24 h and harvested for Western blot analysis for pro-apoptotic proteins. As shown in Figure 1, CrTX significantly increased cleaved caspase-3 in A549 cells. CrTX also increased the wild type p53 and phospho-P38MAPK levels without affecting the total P38MAPK levels in A549 cells. After pre-treating cells with SB203580, phospho-P38MAPK levels were reduced to control levels. Meanwhile, CrTX-induced production of cleaved caspase 3 was also inhibited, suggesting that CrTX-induced apoptosis was regulated by P38MAPK. However, the expression of wild type p53 remained high in the presence of SB203580 (Figure 1).

**Figure 1.** Expression of wild-type p53, caspase-3 and cleaved caspase-3, total P38MAPK and pP38MAPK protein in A549 cells. Cells were treated with 25 μg/mL of CrTX or 5 μmol/L of SB203580 plus 25 μg/mL of CrTX for 24 h. (A) Cell lysates were then collected for Western blot analysis on p53, caspase-3 and cleaved caspase-3, total P38MAPK and pP38MAPK. β-actin was used as loading control. (B) Protein levels of p53, phospho-P38MAPK and cleaved caspase-3 were analyzed and shown in bar graph.

CrTX inhibited A549 tumor growth *in vivo*

The anti-tumor activity of CrTX was tested in the A549 lung tumor xenograft model. As shown in Figure 2A, CrTX at a biweekly dosage of 10 μg/kg for 4 weeks had an efficacy effect on the A549 xenograft with 36.3% tumor growth inhibition (TGI) and no significant body weight loss. The observed *in vivo* activity of CrTX was consistent with its *in vitro* anti-proliferation effect on A549 cells. Further analysis showed that CrTX destroyed the blood vessels surrounding the tumor xenograft, which was indicated by the decrease in microvascular density (MVD) from 34.29±8.64 to 13.56±3.27 (Figure 2B). To understand the potential mechanism of CrTX in tumor angiogenesis, the VEGF level was analyzed using ELISAs with mouse sera from both groups. Interestingly, the VEGF level was also decreased in the CrTX treatment group (32.57±10.35 pg/mL) compared to the control group (46.83±13.62 pg/mL) (Figure 2C), suggesting that anti-angiogenesis might also contribute to the anti-tumor activity of CrTX *in vivo*.

Discussion

In this study, the anti-tumor effects of CrTX on A549 cells were verified and appeared to be dose-dependent. The results of flow cytometry show that CrTX significantly increased the cell population in the sub-G₀ and G₀/G₁ phases, which indicates that the anti-tumor effects of CrTX were closely related to the

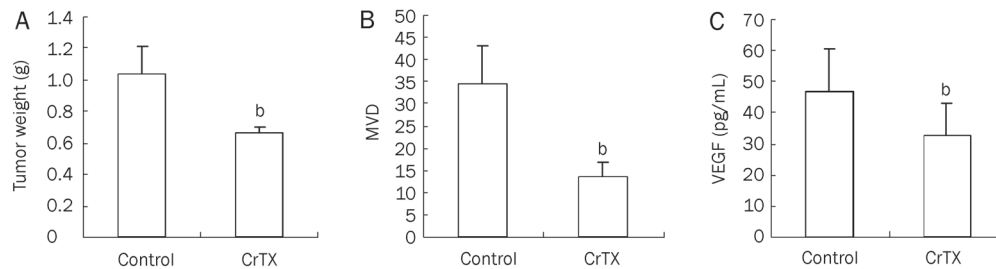


Figure 2. *In vivo* study of CrTX in A549 tumor xenograft model. Five million A549 cells were implanted into female nude mice subcutaneously. When the tumor reached about 5–6 mm in diameter, the tumor-bearing mice were randomized into 2 groups with 8 mice in each group and treated with vehicle control and CrTX (10 µg/kg, ip, twice a week). (A) After 4 weeks treatment, the mice were sacrificed, and the tumor xenografts were removed, sectioned and weighted. (B) Tumor xenografts were observed under microscope and microvascular density (MVD) was counted by Weidner methods. (C) In the meanwhile, 0.5 mL of blood was harvested from each mouse and 0.2 mL of serum was collected after centrifuging for 10 min at 3000 r/min. VEGF level was thus analyzed in each serum sample by ELISA. Data from each mouse sample were analyzed and shown in bar graph with statistic analysis as mean±SD. ^b*P*<0.05.

induction of apoptosis and cell cycle arrest.

Many studies have investigated the signaling pathways that are mediated by P38MAPK, which is one of the main signal transduction systems regulating apoptosis^[9, 10]. Cells transmit extracellular signals to the nucleus using P38MAPK^[11]. Ultra-violet radiation, TNF or anti-tumor drugs induce pathway activation, which leads to P38MAPK phosphorylation. Therefore, these data suggest that P38MAPK plays an important role in cell apoptosis and cell cycle arrest^[12, 13].

Caspase 3, which is a proteolytic enzyme, exists in an inactive proenzyme form in cells. As a central apoptotic effector, it plays a key role in promoting apoptosis^[14]. After cleaving caspase-3, the active form of caspase 3 is released, indicating that the caspase 3 proenzyme is activated and has begun to execute its apoptotic function^[15].

Previous studies have suggested that CrTX induces autophagy in human breast cancer MCF7 cells as a main mechanism for CrTX-induced cytotoxic effects^[16]. In this study, we determined that CrTX induced cell apoptosis and cell cycle arrest in lung cancer A549 cells, suggesting distinct mechanisms for various types of cells. During the process of CrTX-induced apoptosis of A549 cells, we found that phospho-P38MAPK was upregulated and that the expression of wt p53 and cleaved caspase-3 increased. These results indicate that the anti-tumor effects of CrTX are strongly correlated with the activation of P38MAPK, the upregulation of wt p53 and the activation of caspase 3^[17–19]. We investigated whether CrTX-induced apoptosis and G₁ arrest were mediated by the P38MAPK pathway by pretreating cells with SB203580, which is a pyridine glyoxaline compound and specific inhibitor of the P38MAPK pathway that has been verified using both *in vitro* and *in vivo* models^[20]. CrTX-induced apoptosis was attenuated in A549 cells following pretreatment with SB203580. The sub-G₀ peak decreased from 11.42% to 0.87%. However, G₁ arrest did not change. Western blot analysis revealed that SB203580 pretreatment suppressed the CrTX-induced expression of active caspase 3, but not wt p53. These results suggest that the mechanism of CrTX to induce apoptosis is mediated

by P38MAPK activation, which probably mediates caspase-3 activation.

A549 cells are human lung adenocarcinoma cells, which express wild type p53. The CrTX-induced upregulation of wild type p53 was confirmed using Western blot analysis. We propose that the effects of CrTX on G₁ arrest are probably due to the upregulation of wt p53. Meanwhile, SB203580 had no effect on CrTX-induced G₁ arrest and p53 induction. Therefore, these results suggest that the P38MAPK pathway is not involved in p53 upregulation.

A close and complex relationship is evident between tumor cell apoptosis and cell cycle arrest^[21]. Although apoptosis and cell cycle arrest complement each other by inhibiting tumor progression, the effects of CrTX on tumor cell apoptosis and G₁ arrest are achieved via distinct intracellular pathways. Our results demonstrate that the induction of apoptosis by CrTX is due to the activation of P38MAPK and caspase 3, whereas G₁ arrest is possibly due to the upregulation of wt p53.

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Author contribution

Jing-kang HE and Zheng-hong QIN designed research; Bin YE performed research; Bin YE and Yan XIE wrote the paper; Zheng-hong QIN and Jun-chao WU polished the paper; Jun-chao WU and Rong HAN analyzed data.

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