

Bangladesh Journal of Pharmacology

Research Article

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## Eriocalyxin B inhibits proliferation and induces apoptosis through down-regulation of Bcl-2 and activation of caspase-3 in human bladder cancer cells

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### Article Info

Received: 14 February 2013

Accepted: 23 February 2013

Available Online: 8 March 2013

DOI: 10.3329/bjp.v8i2.13797

### Cite this article:

Rasul A, Liu J, Liu Y, Millimouno FM, Wang X, Tsuji I, Yamamura T, Li J, Li X. Eriocalyxin B inhibits proliferation and induces apoptosis through down-regulation of Bcl-2 and activation of caspase-3 in human bladder cancer cells. Bangladesh J Pharmacol. 2013; 8: 116-123.

### Abstract

Eriocalyxin B (EriB) has been shown to possess promising anti-cancer potential against various cancer cells. However, its effect against bladder cancer cells remained unexplored. In this study, for the first time, we investigated the effects of EriB on cell proliferation, cell cycle, and apoptosis in bladder cancer T24 cells. In order to examine the effects of EriB on cell proliferation, cell cycle, and apoptosis, we performed MTT assay, flow cytometric analysis and Western blot. The results revealed that EriB decreased the cell viability of T24 cells. Flow cytometric analysis demonstrated that EriB markedly induced apoptosis of T24 cells and arrested cell cycle at G2/M phase in a dose-dependent manner. Further characterization showed that EriB involved in the down-regulation of Bcl-2 and activation of caspase-3 before culminating in apoptosis in EriB-treated T24 cells. These *in vitro* results suggested that EriB should be further examined for *in vivo* activity in human bladder cancer.

## Introduction

Natural products provide many promising sources of potential anti-cancer agents and several lead structures in the past decades, which were originally isolated from plants such as paclitaxel, camptothecin, *vinca* alkaloids, and etoposide have potential application in cancer chemotherapy, therefore, plants are considered as one of the most vital sources for the development of novel anti-cancer drugs (Amin et al., 2009; Cragg and Newman, 2005). Diterpenoids constitute a vast class of isoprenoid natural products, which are found mainly in plants and fungi and also been found in marine organisms and insects as well (Garcia et al., 2007; Hanson, 2004). Eriocalyxin B (EriB) is a natural entkaurene diterpene compound isolated from *Isodon eriocalyx* var. *laxiflora*, a herb of the Labiatae family

distributed in the South-West China and has been reported to have wide spectrum of biological effects, including anti-inflammatory and antibacterial agent in local folk medicine (Ikezoe et al., 2003; Wang et al., 2007). Furthermore, EriB has anti-proliferative effect and induced apoptosis in cancer cells such as ovarian cancer (Leizer et al., 2010), pancreatic adenocarcinoma (Li et al., 2012) and leukemia (Wang et al., 2007; Zhang et al., 2010). However, the cytotoxic effects of EriB on bladder cancer and its mechanism were still unknown.

Bladder cancer is an increasingly common and potentially lethal malignancy (Ploeg et al., 2009). In 2012, approximately 37,510 new urinary bladder cancer cases will be diagnosed and 14,880 will die in the United States (Siegel et al., 2012). Although, many new strategies have been applied to treat bladder cancer but



still further work is required in order to improve the lives of patients with this disease (Clark, 2007). In the last few decades, several natural compounds have shown a great promise in treatment of cancer and prevention of metastasis; most of the researchers are interested in and are focusing on finding a cure for cancer and several published reports have identified numerous dietary and botanical natural compounds that have chemopreventive potential (Rasul et al., 2013; Rasul et al., 2012a; Rasul et al., 2012d). Therefore, novel therapeutic agents and treatment approaches are desired to improve the clinical outcome. For this purpose, natural compounds were screened against human bladder cancer T24 cells. EriB was one of them which showed potent anti-proliferative effect against the malignant bladder cancer cells. Further characterization showed that EriB effectively inhibited the proliferation of T24 cells through arresting cell cycle at G2/M phase and induction of apoptosis, which is regulated by down-regulation of Bcl-2 and activation of caspase-3.

## Materials and Methods

### Chemical and reagents

Eriocalyxin B (Figure 1) was purchased from the BioBioPha Co., Ltd. of Pharmaceutical and Biological Products (Kunming, China). Fetal bovine serum was purchased from Hangzhou Sijiqing Biological Engineering Materials Co., Ltd. DMEM, MTT [3'-(4,5dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide], propidium iodide (PI) and dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical Company (St. Louis, USA). Annexin V-FITC Apoptosis Detection Kit and cleaved caspase-3 antibody were purchased from Beyotime Institute of Biotechnology (Shanghai, China). Rabbit polyclonal anti-human Bcl-2 antibody was purchased from Wuhan Boster Biological Technology Co., Ltd. Mouse anti-β-

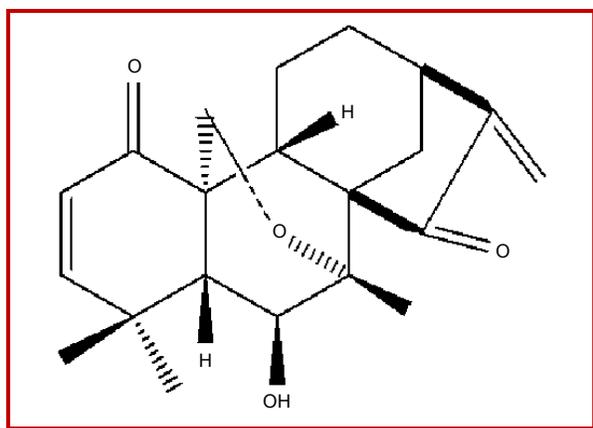


Figure 1: Structure of Eriocalyxin B (EriB)

actin and anti-rabbit antibodies were purchased from Santa Cruz Biotechnology. Ponceou and cell lysis buffer for Western and IP were purchased from Bio SS Beijing. Rho-123 was purchased from Eugene Co. (Oregon, U.S.A.).

### Cell culture

Human bladder cancer T24 cells were propagated in DMEM nutrients mixture supplemented with 10% FBS and antibiotics at 37°C in a humidified atmosphere with 5% CO<sub>2</sub> and 95% air. Cells were seeded in 10 cm culture dish and allowed to grow to approximately 70% confluence before experimentation.

### Cell proliferation assay

The cytotoxic effects of the EriB on the cells were determined by MTT assay as described previously (Rasul et al., 2011a; Rasul et al., 2011b). Briefly, T24 cells were seeded at a density of  $1 \times 10^4$  cells per well in 96-well plates and were allowed to grow overnight. Cells were incubated with 100 μL of complete culture medium containing 0.1, 1, 3, 5, 10, and 30 μM of EriB. After incubation for 24 hours, growth of cells was determined by adding 10 μL MTT (5 mg/mL in phosphate buffered saline) to each well and incubated for 4 hours. After removal of the medium, 150 μL DMSO was added to each well and shaken carefully. The absorbance was read at a wavelength of 570 nm in a plate reader (ELX 800, BIO-TEK Instruments Inc.). The growth curve was plotted against mean values which were calculated using the following equation:

$$I\% = [A_{570}(\text{control}) - A_{570}(\text{treated})] / A_{570}(\text{control}) \times 100$$

### Flow cytometric analysis of cell cycle

For cell analysis, T24 cells were seeded in 12-well plates and then treated with 3 and 5 μM of EriB for 24 hours. After treatments, the percentages of cells in the different phases of cell cycle were evaluated by determining the DNA content after propidium iodide (PI) staining (Rasul et al., 2012b). Briefly, cells were washed with PBS, trypsinized and centrifuged at 1,000 rpm at 4°C for 5 min. Pellets were fixed overnight in 70% cold ethanol. After fixation, cells were washed twice with PBS and incubated in PBS containing RNase (1 mg/mL) for 10 min at room temperature. Finally, samples were stained with propidium iodide (1 mg/mL) for 30 min at 4°C. Data acquisition was done by flow cytometry (EPICSXL-MCL, Beckman Coulter, US) using Cell Quest software.

### Flow cytometric determination of apoptosis

The apoptotic rate of T24 cells was examined by flow cytometry using annexin V-FITC/PI staining. Briefly, T24 cells were cultured in 6-well plates and allowed to attach overnight. Cells were treated with 3 and 5 μM of EriB for 24 hours. Then cells were collected, washed and resuspended in PBS. Apoptotic cell death was

measured by double staining annexin V-FITC and PI using the Annexin V-FITC apoptosis detection kit (Beyotime Biotechnology Shanghai, China) according to the manufacturer's instructions. Flow cytometric analysis was performed immediately after staining. Data acquisition and analysis were performed by flow cytometry using Cell Quest software.

#### Western blotting

To reveal the mechanism of the apoptotic effect of EriB, Western blotting was done for apoptotic related proteins as previously described (Rasul et al., 2012c; Rasul et al., 2012d). Briefly, T24 cells were incubated with 3 and 5  $\mu\text{M}$  of EriB for indicated time. Cells were trypsinized, collected in 1.5 mL centrifuge tube and washed with PBS. The cell pellets were resuspended in lysis buffer and were lysed on ice for 30 min. After centrifugation for 15 min, the supernatant fluids were collected and the protein content of the supernatant was measured by the NanoDrop 1000 spectrophotometer (Thermo scientific, USA). The protein lysates were separated by electro-phoresis on 12% SDS-polyacrylamide gel and transferred to a PVDF membrane (Amersham Biosciences, Piscataway, NJ). The membranes were soaked in blocking buffer (5% skimmed milk) for 2 hours. To probe for Bcl-2, Caspase-3, and  $\beta$ -actin; membranes were incubated overnight at 4°C with relevant antibodies, followed by appropriate HRP conjugated secondary antibodies and ECL detection.

#### Statistical analysis of data

For the statistical analysis of data, comparisons between results from different groups were analyzed with SPSS for Window Version 15.0. Student's t-test was employed to determine the statistical significance of the difference between different experimental groups and control group.  $p < 0.05$  value was defined as statistically significant. All experiments were repeated at least three times. Data were presented as mean  $\pm$  standard deviation (SD).

## Results and Discussion

The investigation was initiated with screening of natural compounds against T24 human bladder cancer cells. We found that EriB exhibited cytotoxic effects on the growth of T24 cells. EriB is a natural compound that belongs to a diterpene family. We determined the effects of EriB on the growth of T24 human bladder cancer cells by quantifying the viable cells using MTT assay. EriB inhibited the growth of T24 human bladder cancer cells in a dose-dependent manner (Figure 2). Morphological changes were observed under phase contrast microscopy after treating cells with 3 and 5  $\mu\text{M}$  of EriB. There was a significant decrease in the number of T24 cells treated with EriB as compared to the control group. Furthermore, the cells become round-shaped and poorly adhered to the cultured plates while the control group cells showed a typical polygonal and

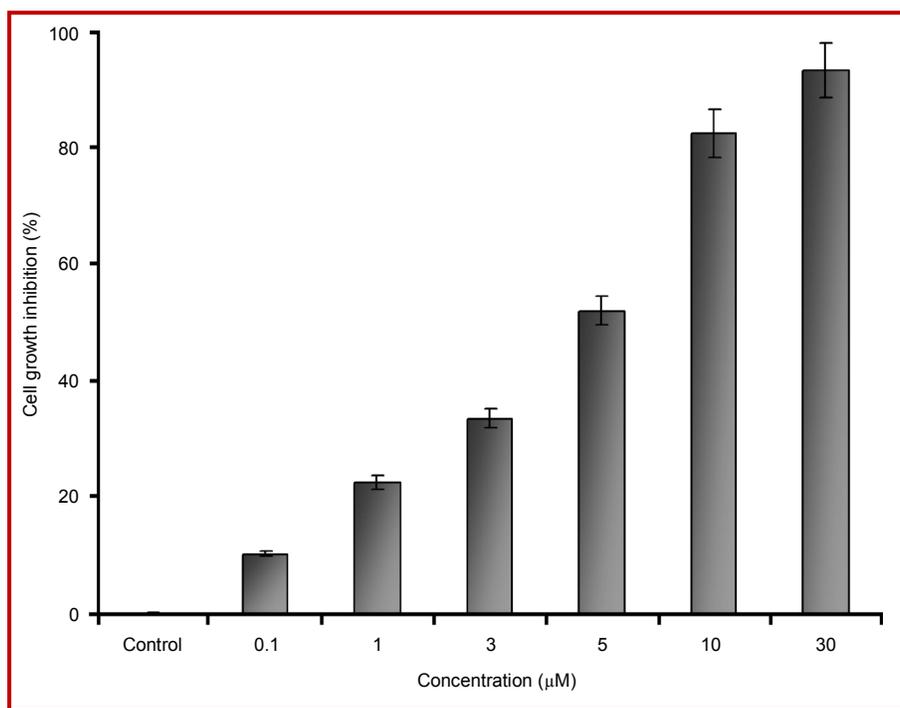


Figure 2: The effects of EriB on viability of T24 cells. T24 cells were treated with various concentrations of EriB for 24 hours. Cell death was determined by using MTT assay. Data shown are means  $\pm$  SD (n = 3)

cobblestone monolayer appearance and remained firmly attached to cultured plates (data not shown). The results indicated that EriB induced growth inhibition of T24 cells, in addition to other type of cancer cells previously reported including ovarian cancer (Leizer et al., 2010), pancreatic adenocarcinoma (Li et al., 2012) and leukemia (Wang et al., 2007; Zhang et al., 2010).

There are several mechanisms which control the cell cycle to ensure the correct cell division. It is well known that progression of cell cycle is maintained by different check points in normal cells and the transition from one cell cycle phase to another occurs in an orderly fashion. In cancerous cells, some basic modifications occurred in the genetic control of cell division, resulting in an uncontrolled cell proliferation. As the deregulation of cell cycle progression is the hallmark of cancer; thereby

cell cycle regulation could be a potential and effective strategy for the treatment of cancer (Grana and Reddy, 1995; Vermeulen et al., 2003). Therefore, we analyzed effect of EriB on cell cycle progression of T24 cells. It was found that EriB arrested cell cycle at G2/M phase, which supported the previous reported results in pancreatic adenocarcinoma (Li et al., 2012). The percentage of accumulation of cells in the G2/M phase was increased from 15.97% in control group to 25.64%, and 38.48% in the cells treated with 3 and 5  $\mu\text{M}$  of EriB respectively for 24 hours (Figure 3). These findings revealed that G2/M phase cell cycle arrest was one of the mechanisms through which EriB induces cytotoxicity in T24 cells. A number of recent studies have shown that by arresting the cell division at certain checkpoints in the cell cycle, several chemotherapeutic

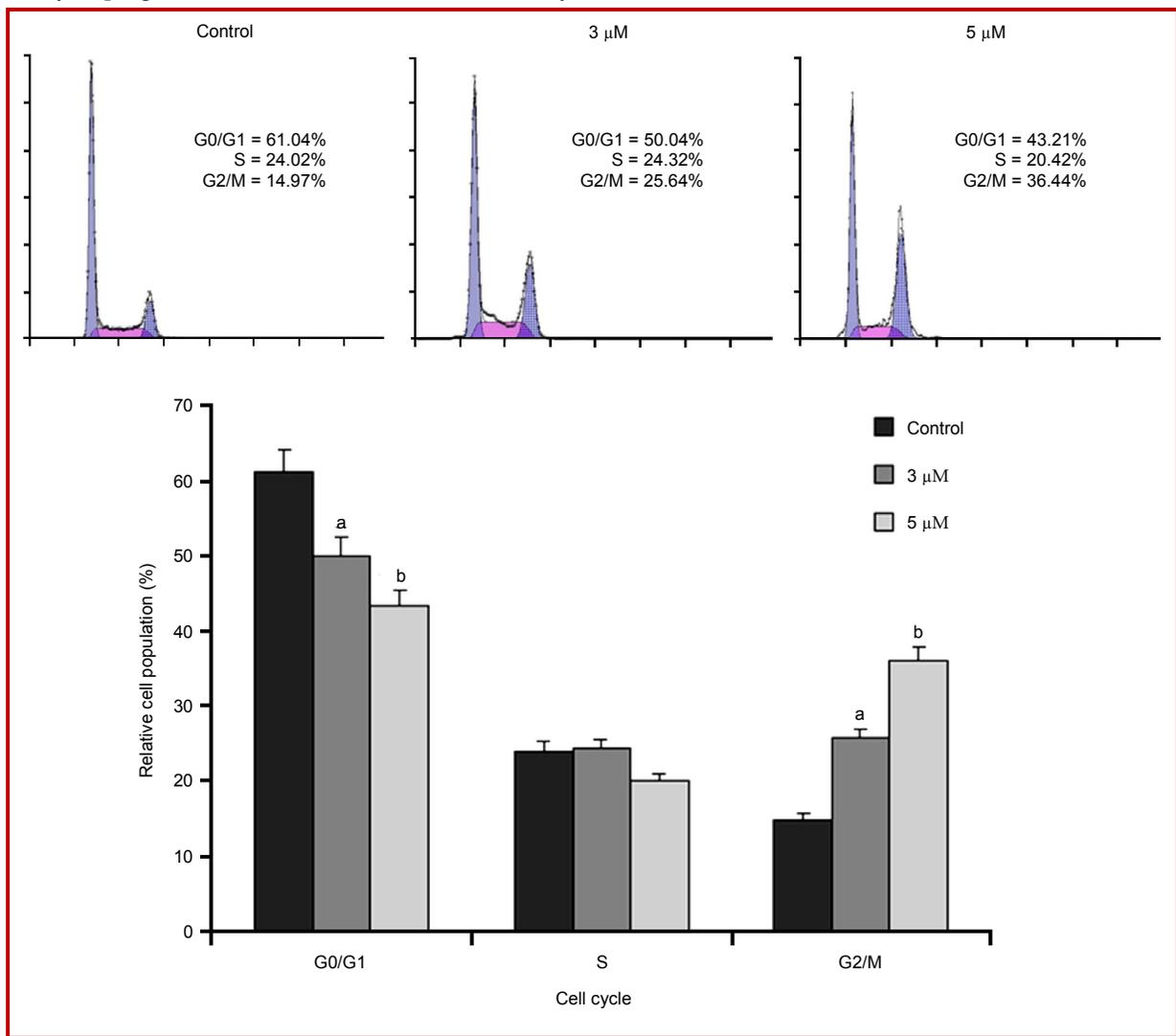


Figure 3: Effect of EriB on the cell cycle distribution. T24 cells were treated with 0, 3 and 5  $\mu\text{M}$  of EriB for 24 hours and then they were stained with PI for flow cytometric analysis. Histograms show number of cells/channel (*y*-axis) vs. DNA content (*x*-axis) and the values indicate the percentage of cells in the indicated phases of cell cycle. The data shown are representative of three independent experiments with the similar results. <sup>a</sup> $p < 0.05$  and <sup>b</sup> $p < 0.01$  compared with the control

and chemopreventive agents have demonstrated potential anti-proliferative effects (Rasul et al., 2013; Rasul et al., 2012b).

There are various modes of cell death such as apoptosis, autophagy and necrosis (Leist and Jaattela, 2001). Apoptosis is most organized, well fashioned, and systematic mode of cell death, in which cells themselves play an active role in their own death (Elmore, 2007; Hengartner, 2000). The normal cellular signals for regulation of their growth are lost in the cancerous cells due to various mutations, preventing the cells from apoptosis and cell growth to uncontrolled status (Hanahan and Weinberg, 2000). The regulation of apoptosis is, therefore, most important in the treatment of cancer (Fulda, 2010; Lawen, 2003; Reed, 2002). The chemopreventive agents, which can treat the cancer

effectively, have potential to restore the natural signaling apoptotic pathway (Reed, 1999). It is well known that various chemopreventive agents cause cell death through induction of apoptosis in different cancer cells (Srivastava and Gupta, 2006; Xu et al., 2009). We studied whether EriB inhibits cell growth in T24 cells through the induction of apoptosis. EriB-induced apoptosis was determined by flow cytometric analysis.

For flow cytometric analysis, cells were seeded in the 12 well plates. After incubation of cells without (control) or with EriB for 24 hours, cells were collected in centrifuged tubes and stained with annexin V-FITC and PI double staining as described in material and methods part. The results of flow cytometric analysis showed that rates of apoptosis were  $17.6 \pm 2.1$  and  $24.9 \pm 2.5\%$  in the cells treated with 3 and 5  $\mu\text{M}$  of EriB

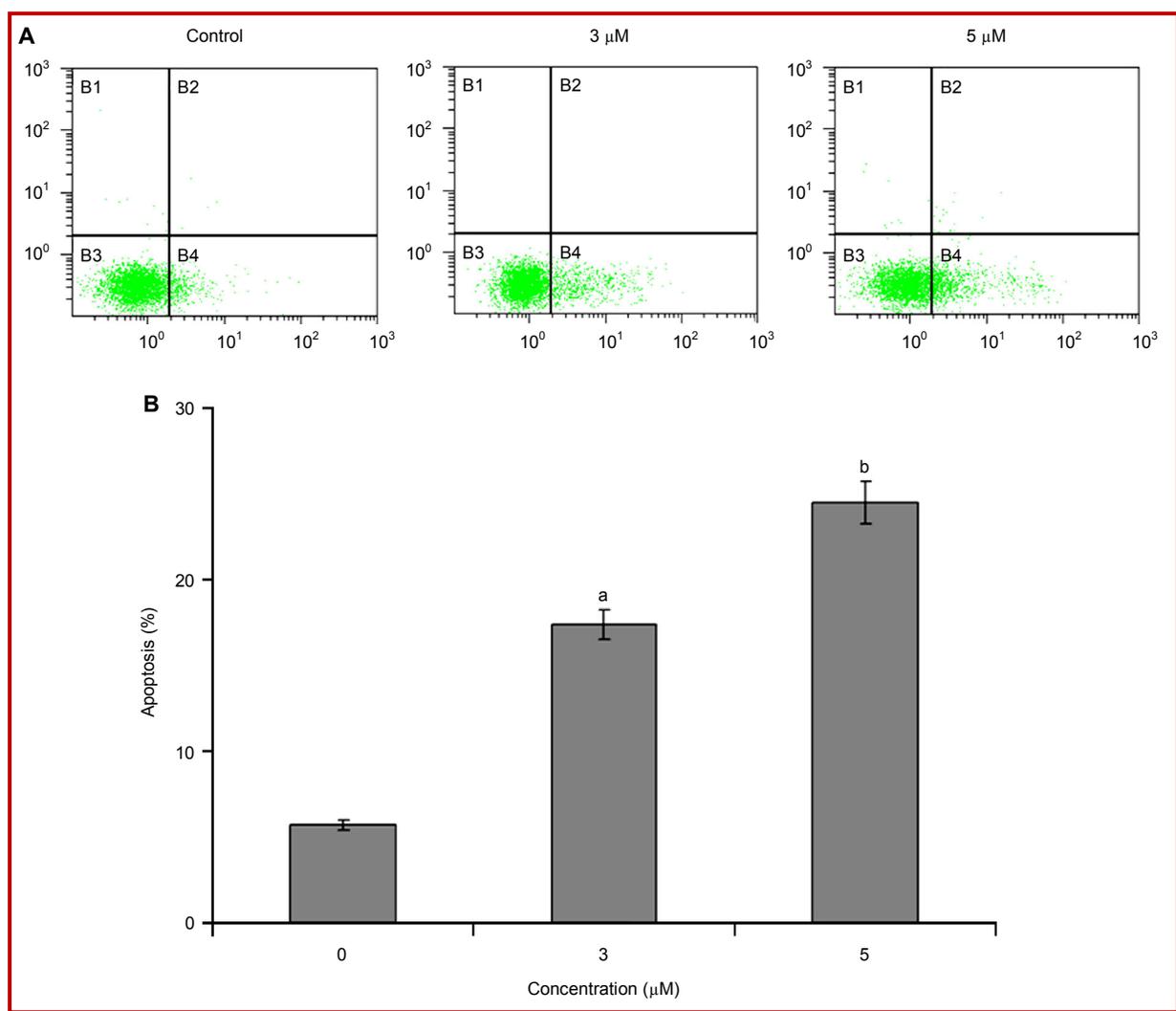


Figure 4: Effects of EriB on apoptosis in T24 cells. (A) T24 cells were treated with 0, 3 and 5  $\mu\text{M}$  of EriB for 24 hours. Then they were stained with Annexin V-FITC/PI and were analyzed in flow cytometry. Cell populations shown in the lower right (Annexin V<sup>+</sup>/PI<sup>-</sup>) represents early apoptotic cells, upper right (Annexin V<sup>+</sup>/PI<sup>+</sup>) represents late apoptotic cells. (B) The values indicate the percentage of apoptotic cells treated without (control) and with (3 and 5  $\mu\text{M}$ ) EriB for 24 hours. The data shown are representative of three independent experiments with the similar results. <sup>a</sup> $p < 0.05$  and <sup>b</sup> $p < 0.01$  compared with the control

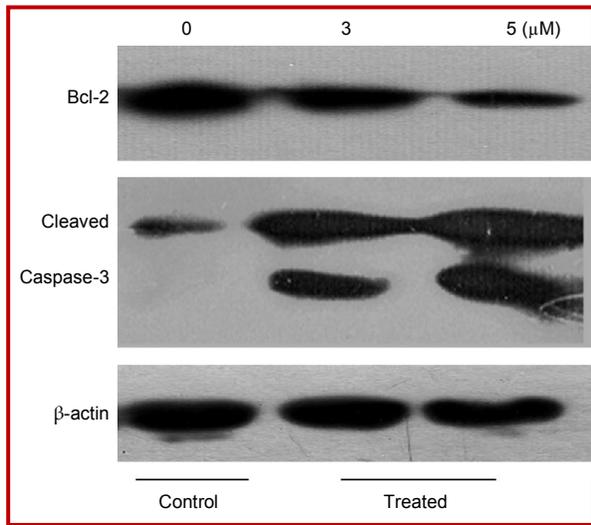


Figure 5: The effects of EriB on expression levels of apoptosis related proteins. Expression levels of Bcl-2 and Caspase-3 in T24 cells treated without (control) and with (3 and 5  $\mu\text{M}$ ) EriB for 24 hours were monitored by Western blot assay.  $\beta$ -actin was used as loading control. Western blots are representative of three independent experiments

respectively for 24 hours as compared to  $5.9 \pm 1.0\%$  in the control cells (Figure 4). EriB-induced apoptosis in T24 cells was consistent with previously reported studies in ovarian cancer (Leizer et al., 2010), pancreatic adenocarcinoma (Li et al., 2012) and leukemia (Wang et al., 2007; Zhang et al., 2010).

Mitochondria play a fundamental role in the regulation of apoptotic cell death and consist of various pro-apoptotic proteins and cytochrome C. As highlighted earlier, apoptosis involves a dysfunction of mitochondrial membrane integrity, which leads to cell death (Jeong and Seol, 2008). Previously it has been documented that disintegration of the mitochondrial membrane potential and the redistribution of cytochrome C are crucial actions in the apoptotic cascade (Kluck et al., 1997; Wang, 2001). Cytochrome C plays central role in mitochondrial mediated apoptosis. Upon the attenuation of mitochondrial transmembrane potential, Cytochrome C releases from the mitochondria into the cytosol (Kluck et al., 1997). Once released into the cytosol, cytochrome C binds to form an "apoptosome" of Apaf-1, cytochrome C, and caspase-9, which subsequently cleaves the effector caspase-3 (Ricci and Zong, 2006).

The caspases are a family of proteins related to cysteine proteases that is one of the focal executors of the apoptotic process via triggering the death receptors and mitochondrial pathways to accomplish the programmed cell death (Cohen, 1997). Caspases are present in the form of inactive zymogens those are activated during apoptosis. Among them, caspase-3 is a frequently activated death protease, catalyzing the specific clea-

vage of many key cellular proteins (Adams, 2003; Porter and Janicke, 1999). In order to determine effects of EriB on expression of caspase-3, Western blotting was done. The results showed that procaspase-3 was cleaved to its active fragments in treated cells with 3 and 5  $\mu\text{M}$  of EriB after 24 hours as compared to that of control cells (Figure 5). These findings are supported by previous studies (Kanno et al., 2008; Lee et al., 2001; Park et al., 2001). These results markedly showed that EriB induced caspase-dependent cell death in T24 cells.

The release of cytochrome *c* from the mitochondria into cytosol is coupled with the ratio of Bax/Bcl-2 proteins (Kluck et al., 1997). Bcl-2 family proteins are mostly involved in the mitochondrial apoptotic pathway (Reed, 1998). Furthermore, Bcl-2 family plays a central role in activation of Caspases (Burlacu, 2003). Bcl-2 and Bax work in an antagonistic manner, Bcl-2 is anti-apoptotic while Bax is proapoptotic member of Bcl-2 family and Bcl-2 forms heterodimers with Bax. Bax inserts into outer membrane of mitochondria under stress conditions, as a results transmembrane permeability become higher which facilitate the release of cytochrome C due to formation of pores on the mitochondrial outer membrane (Adams and Cory, 2007; Danial, 2007). The balance between these two groups is critical in the cell decision to undergo apoptosis or not (Mallat and Tedgui, 2000). We performed Western blotting to examine the effect of EriB on the expression of Bcl-2. EriB was observed to be involved in the down regulation of Bcl-2 in dose-dependent manner (Figure 5). These results are similar with previously reported studies in pancreatic adenocarcinoma (Li et al., 2012).

To conclude, EriB induced apoptosis of T24 human bladder cancer cells accompanied by a G2/M phase cell cycle arrest. Further characterization showed that EriB involved in the down-regulation of Bcl-2 and activation of caspase-3 before culminating in apoptosis in EriB-treated T24 cells. These *in vitro* results suggested that EriB should be further examined for *in vivo* activity and molecular mechanism in human bladder cancer.

## Acknowledgments

This study was supported by Ministry of Science and Technology (No. 2010DFA31430); Ministry of Education of China (NCET-10-0316); Jilin Provincial Science & Technology Department (No. YYZX201241, 20070719, 200905116); Changchun Science & Technology Department (No. 2011114-11GH29) and National Natural Science Foundation of China (No. 30871301).

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