Eriocalyxin B induces apoptosis and cell cycle arrest in pancreatic adenocarcinoma cells through caspase- and p53-dependent pathways

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A B S T R A C T
Pancreatic cancer is difficult to detect early and responds poorly to chemotherapy. A breakthrough in the development of new therapeutic agents is urgently needed. Eriocalyxin B (EriB), isolated from the Isodon eriocalyx plant, is an ent-kaurane diterpenoid with promise as a broad-spectrum anti-cancer agent. The anti-leukemic activity of EriB, including the underlying mechanisms involved, has been particularly well documented. In this study, we demonstrated for the first time EriB's potent cytotoxicity against four pancreatic adenocarcinoma cell lines, namely PANC-1, SW1990, CAPAN-1, and CAPAN-2. The effects were comparable to that of the chemotherapeutic camptothecin (CAM), but with much lower toxicity against normal human liver WRL68 cells. EriB's cytotoxicity against CAPAN-2 cells was found to involve caspase-dependent apoptosis and cell cycle arrest at the G2/M phase. Moreover, the p53 pathway was found to be activated by EriB in these cells. Furthermore, in vivo studies showed that EriB inhibited the growth of human pancreatic tumor xenografts in BALB/c nude mice without significant secondary adverse effects. These results suggest that EriB should be considered a candidate for pancreatic cancer treatment.

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Introduction

Pancreatic cancer is the fourth and eighth leading cause of cancer related death for both genders in the U.S. and worldwide, respectively (Bayraktar et al., 2010). In the U.S. in 2010, more than 43,000 individuals were diagnosed with pancreatic cancer and 36,800 patients died from it (Jemal et al., 2010). This form of cancer is associated with a poor prognosis and a 5-year survival rate below 5% (Bayraktar et al., 2010). There are number of factors contributing to its high mortality rate. Firstly, it metastasizes easily to other tissues and organs; secondly, although surgery is the only potentially curative treatment option, only about 20% of all pancreatic cancer patients have resectable tumors; additionally, pancreatic cancers are strongly resistant to conventional radio- and chemo-therapies (Li et al., 2010).

In spite of great advances in the treatment of commonly seen cancers being made, there has been little progress in the development of new treatments for pancreatic cancer due to its high resistance to radio- and chemo-therapies. Gemcitabine has replaced 5-fluorouracil (5-FU) as the first-line chemotherapeutic agent for locally advanced and metastatic pancreatic cancer (Bayraktar et al., 2010; Li et al., 2010), but offers only a moderate 1.5-month increase in median survival time (Burris et al., 1997). Moreover, gemcitabine can cause severe adverse secondary effects including compromised bone marrow function, breathing problems, and kidney damage (Aapro et al., 1998; Dunsford et al., 1999; Geffen and Horowitz, 2000; Pavlakis et al., 1997). Better cancer treatment and prevention options are desperately needed. Indeed, the lack of effective treatments remains one of the greatest challenges in clinical oncology.

Herbal medicines have been used for thousands of years, including some with anticancer activity and low toxicity. In recent years, herbal medicines have been investigated extensively as potential alternative therapies for the treatment of various cancers (in both clinical and pre-clinical studies), including liver, lung, ovarian, breast, prostate, and pancreatic cancers as well as leukemia (Harinantenaina et al., 2010; Lucas et al., 2010; McCulloch et al., 2006; Motoo and Sawabu, 1994; Szliszka et al., 2011; Thoppil and Bishayee, 2011). Herbal treatments for pancreatic cancer, alone or in combination with conventional anticancer agents, have been examined with beneficial effects (Aghdassi et al., 2007; Chen et al., 2009; Patil et al., 2009; Sahu et al., 2009). There is a growing interest in exploring herbal medicines to overcome the resistance of pancreatic cancer to clinical treatments. In this regard, we have been investigating the cytotoxic effects of several commonly used anticancer herbs since 2005. Among them, we have revealed Brucine D (BD), a quassinoid found abundantly in Brucia javanica fruit and its associated mechanism of action on pancreatic cancer;
however, one of the potential disadvantages of BD may be its relatively toxic effects on the animal model used (Lau et al., 2008, 2009, 2010).

The Chinese plant *Ison an eriocalyx* (Dunn.) Hara (family Lamiaceae) is a well-known source of anticancer diterpenoids (Chen et al., 1999). The diterpenoid with the best recognized anticancer activity is an ent-kauranoid known as Eriocalyxin B (EriB) (Sun et al., 2006). EriB has been reported to induce apoptosis of leukemia cells *in vitro* and *in vivo* (Wang et al., 2007; Zhang et al., 2010). Additionally, EriB has been shown to regulate inflammatory processes in ovarian cancer stem cells through the nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) pathway (Leizer et al., 2011); it has also been shown to exhibit broad-spectrum cytotoxicity against HL-60, A549, MKN-28, and HCT116 cancer cell lines with IC50 values in the range of 0.22–2.15 μM (Sun et al., 2001). Our recent bioassay-guided fractionation of *I. eriocalyx* extract revealed that EriB was the most potent apoptosis inducer isolated from *I. eriocalyx* (Han et al., 2010).

In the present study, we investigated the cytotoxic effects of EriB on four pancreatic adenocarcinoma cell lines, namely PANC-1, SW1990, CAPAN-1 and CAPAN-2. We examined EriB’s ability to induce apoptosis and cell cycle arrest, by quantifying expression of apoptotic factors and *p53* (Gupta et al., 2010; Sipos et al., 2003). Finally, we tested whether EriB can inhibit tumor growth with low toxicity in a xenograft animal model of pancreatic cancer.

**Materials and methods**

**Materials.** EriB was isolated from *I. eriocalyx* in our laboratory, and its structure (Fig. 1A) was determined by spectroscopic analysis (Han et al., 2010; Wang and Xun, 1982). High performance liquid chromatography was used for cytotoxicity assay. SW1990 cells were cultured in L-15 medium, supplemented with 10% v/v FBS, 100 U/ml penicillin, and 10 U/ml streptomycin, and incubated at 37 °C without 5% CO2, in a 95% humidified atmosphere. CAPAN-1 cells were cultured in IMDM medium supplemented with 20% v/v FBS, 100 U/ml penicillin, and 10 U/ml streptomycin, and incubated at 37 °C without 5% CO2, in a 95% humidified atmosphere. SW1990 cells were cultured in L-15 medium, supplemented with 10% v/v FBS, 100 U/ml penicillin, and 10 U/ml streptomycin, and incubated at 37 °C without 5% CO2, in a 95% humidified atmosphere. CAPAN-1 cells were cultured in IM DM medium supplemented with 20% v/v FBS, 100 U/ml penicillin, and 10 U/ml streptomycin, and incubated at 37 °C in a 5% CO2, 95% humidified atmosphere.

**Preparation of human peripheral blood mononuclear cells (PBMC).** Fresh human buffy coat was obtained from the Hong Kong Red Cross Blood Transfusion Service. Preparation of PBMC was performed as described previously (Yue et al., 2011). The viability of cells was checked by trypan blue staining. The isolated cells with 95% or above viability were used for cytotoxicity assay. PBMC were cultured in RPMI-1640 medium supplemented with 10% v/v FBS, 100 U/ml penicillin, and 10 U/ml streptomycin, and incubated at 37 °C without 5% CO2, in a 95% humidified atmosphere.

All cell culture reagents were purchased from Invitrogen, USA. 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide (MTT), propidium iodide (PI), and Pluronic F68 were obtained from Sigma-Aldrich, USA. Monoclonal antibodies against pERK1/2, ERK1/2, pp38, p38, caspases 3, 8, 7, 9, cleaved-caspases 3, 7, 9, poly (ADP-ribose) polymerase (PARP), cleaved-PARP, and bak were obtained from Cell Signaling Technology, USA. Monoclonal antibodies against p53, p21, and cyclin B1 were obtained from BD Biosciences, USA. Antibody against cdk1 was purchased from Oncogen, USA and antibody against bcl-2 was purchased from Santa Cruz Biotechnology, USA. Annexin-V-FUOS was purchased from Roche Diagnostics, Swiss.

**Cell line culture.** PANC-1, CAPAN-2, and WRL68 cells were cultured in DMEM media, supplemented with 10% v/v fetal bovine serum (FBS), 100 U/ml penicillin, and 10 U/ml streptomycin, and incubated at 37 °C in a 5% CO2, 95% humidified atmosphere. SW1990 cells were cultured in L-15 medium, supplemented with 10% v/v FBS, 100 U/ml penicillin, and 10 U/ml streptomycin, and incubated at 37 °C without 5% CO2, in a 95% humidified atmosphere. CAPAN-1 cells were cultured in IMDM medium supplemented with 20% v/v FBS, 100 U/ml penicillin, and 10 U/ml streptomycin, and incubated at 37 °C in a 5% CO2, 95% humidified atmosphere.

![Fig. 1.](image-url) (A) Chemical structure of EriB. Cytotoxic effects of (B) EriB, (C) CAM, and (D) 5-FLU on the cell viability of human pancreatic adenocarcinoma cell lines (PANC-1, CAPAN-2, CAPAN-1, SW1990), and of a normal human liver cell line (WRL68) and human peripheral blood mononuclear cells (PBMC). Drug treatment was initiated 24 h after seeding the cells in 96-well plates. Cell viability was assessed by the MTT assay (XTT assay for PBMC) after 72 h of drug treatment. Results were expressed as percentages of MTT absorbance with respect to the untreated vehicle control wells (mean ± S.D. of 4–5 independent experiments with five wells each). IC50 values were shown in parentheses in the graphic legends.
Cytotoxicity assay. Colorimetric MTT or XTT assays were used to determine cell viability (Lewis et al., 2001; Yue et al., 2010, 2011). The chemotherapeutic agents, camptothecin (CAM, Wako, Osaka, Japan) and 5-FU (Sigma Aldrich, USA) were used as positive controls. They were dissolved in DMSO to produce a stock solution. To minimize potential solvent effects on cell growth, the final concentrations of DMSO in all culture wells were less than 0.05%. Pancreatic cancer cells PANC-1 and CAPAN-2 (both at 5 × 10^5 cells/well), CAPAN-1 and SW1990 (both at 1 × 10^5 cells/well), and WRL68 normal liver cells (at 5 × 10^4 cells/well) were seeded in 96-well microplates. Drugs were diluted with culture medium to various concentrations and added to the cells. After 72 h treatment, 0.15 mg MTT was added to each well. The samples were incubated at 37 °C for 3 h; the medium was discarded and the formazan blue that formed in the cells was dissolved with 100 μl DMSO, and the absorbance was measured at 540 nm by a microplate spectrophotometer (μQuant, Biotek, USA). Absorbance readings were blanked relative to DMSO and cell viability was expressed relative to vehicle control data. The cytotoxicity effects on the human peripheral blood mononuclear cells PBMC were determined by MTT assay. PBMC (at 3 × 10^6 cells/well) were seeded in DMSO-premitter microplates. Drugs were diluted with culture medium to various concentrations and added to the cells. After 72 h treatment, 0.15 mg MTT was added to each well. The samples were incubated at 37 °C for 3 h; the medium was discarded and the formazan blue that formed in the cells was dissolved with 100 μl DMSO, and the absorbance was measured at 540 nm by a microplate spectrophotometer. The IC50 values were calculated using GraphPad Prism 5.0 software (GraphPad Software Inc., San Diego, CA). Because CAPAN-2 cells showed high sensitivity to EriB (see below), they were used for the subsequent mechanistic studies.

Hoehst 33258 staining for morphological evaluation (Lau et al., 2009). EriB-treated CAPAN-2 cells were stained with Hoechst 33258 (Invitrogen, CA, USA). Approximately 5 × 10^4 cells/well were seeded in 6-well plates, and cells were incubated with or without 1.4 μM EriB for 24 h. After the incubation, the cells were washed with phosphate buffered saline (PBS), fixed in 4% paraformaldehyde for 30 min, and then stained with 20 μg/ml Hoechst 33258 for 15 min at room temperature in the dark. Morphological changes of EriB-treated cells were assessed using a fluorescence microscope (Olympus IX71, Japan).

DNA fragmentation detection by DNA ladder. Detection of DNA fragmentation by agarose gel electrophoresis was performed as described previously with minor modifications (Yue et al., 2010). In brief, CAPAN-2 cells were seeded in culture dishes (100 mm^2, 1 × 10^6 cells/dish). EriB treatment was initiated 24 h after seeding. All cells were harvested from dishes by gentle scraping after a 24-h, 48-h, or 72-h treatment. DNA lysis buffer (1% NP-40 in 20 mM/L EDTA, 50 mM/L Tris–HCl, pH 7.5) was added after washing the cells with PBS. Supernatant containing apoptotic DNA fragments was obtained by centrifugation (6000 × g, 5 min), then 50 μl of 5% SDS solution and 10 μl of RNase A (0.4 mg/ml) were added at 56 °C for 90 min. After incubation, the mixture was incubated with 20 μl proteinase K (1.5 mg/ml) at 56 °C for a further 90 min. The DNA was precipitated with 30 μl of 3 M sodium acetate and 750 μl of cold absolute ethanol. After centrifugation at 20,000 × g for 30 min, the supernatant was aspirated. The DNA pellets were washed with 1 ml of 70% ethanol and then with 1 ml of absolute ethanol. The washed samples were left to air dry. The dried DNA pellets were incubated in 30 μl TE buffer at 37 °C for 30 min. The isolated DNA was size-fractionated by 1.5% agarose gel electrophoresis. The DNA bands were visualized with ethidium bromide under ultraviolet illumination.

Cell death detection ELISA. The amount of cleaved DNA/histone complexes (nucleosomes) in cells was quantified using a Cell Death Detection ELISAPlus kit in accordance with the manufacturer’s instructions (Roche Applied Science, Basel, Switzerland). Briefly, CAPAN-2 cells were seeded at 3 × 10^4 cells/well in 24-well plate and incubated with EriB or CAM. After a 24-h treatment, the cells were collected by trypsin and pelleted by centrifugation of the plate at 200 × g for 10 min and the supernatant was then aspirated. The cells were resuspended and incubated in 200 μl lysis buffer for 30 min at room temperature. After lysis, the intact nuclei were pelleted by centrifugation at 200 × g for 10 min. Then, 20-μl aliquots of the supernatant (lysate) were transferred to streptavidin-coated wells in a microtitre plate and incubated for 2 h with an immunoreagent containing monoclonal antibodies against histone (biotinlabeled) and DNA (peroxidase-conjugated). Each well of the plates was washed three times with 250 μl lysis buffer to remove cell components that were not immunoreactive. ABTS solution (100 μl) was added to each well and the plates were incubated at room temperature on a plate shaker for 15 min; ABTS stop solution (100 μl) was then added immediately to each well. Finally, the amount of colored product (i.e. the immobilized antibody–histone complexes (DNA fragments)) in the plate was measured at 405 nm on a microplate spectrophotometer using ABTS solution as a blank control.

Apoptosis detection by flow cytometry. EriB-induced apoptosis, the translocation of phosphatidylserine (PS) was assessed using Annexin V and PI double staining followed by quantitative flow cytometry analysis (Yue et al., 2010). CAPAN-2 cells were seeded at 1.5 × 10^5 cells/well in 6-well culture plates. EriB was added, and incubated for 24 h or 48 h at 37 °C under 5% CO2. Adherent and floating cells were collected after EriB treatment, washed in cold PBS, and centrifuged. Incubation reagent was added to each cell pellet in the dark for 15 min at room temperature. Finally, 400 μl of binding buffer was added to each sample and the samples were analyzed by flow cytometry (Becton Dickinson FACS Canto II, USA) within 1 h.

Cell cycle analysis by flow cytometry. CAPAN-2 cells were cultured in 6-well plates at 1.5 × 10^5 cells/well, and collected after 48 h of exposure to EriB (1.4, 2.1 or 2.8 μM). Cells were washed with PBS, and permeabilized with ice-cold 70% v/v ethanol at 4 °C overnight. The permeabilized cells were resuspended in PBS containing PI (20 μg/ml) and RNase A (10 μg/ml) and incubated in the dark at 37 °C for 30 min. After incubation, the cells were analyzed by flow cytometry. Data from 10,000 cells per sample were collected and calculated using Modfit LT (Verity Software House, ME, USA).

Western blotting analysis. CAPAN-2 cells were seeded in culture dishes (100 mm^2, 1 × 10^5 cells/dish). EriB treatment was initiated 24 h after seeding. All cells were harvested from dishes by gently scraping after 24 h, 36 h or 48 h of treatment. Cells were lysed with radioimmunoprecipitation assay buffer [1% Triton X-100, 0.25% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 5 mM EDTA, and 50 mM Tris–HCl (pH 7.5)] containing protease inhibitor cocktail (Roche Molecular Biochemicals, Swiss). Then protein concentration was determined using a BCA assay (Bicinchoninic acid kit, Sigma-Aldrich, USA). Twenty-five microgram protein aliquots (5 μg for caspases 8 and 7) were placed in each lane and electrophoresed on 7.5%, 10%, or 12% SDS-PAGE gels for 2 h at 100 V. Separated proteins were transferred electrophoretically for 1–1.5 h at 90 V to 0.45 μm polyvinylidene fluoride (PVDF) membrane (Immobilon, Millipore, USA). The membrane was blocked with 5% (w/v) non-fat dry milk in tris buffered saline (TBS) for 1 h and subsequently incubated overnight at 4 °C with primary antibodies at the following concentrations: rabbit anti-pERK1/2, ERK1/2 [1:1000], rabbit anti-p38, p38 [1:1000], mouse anti-p53 [1:1000], mouse anti-p21 [1:2000], mouse anti-ckd1 [1:1000], mouse anti-cyclinB1 [1:500], rabbit anti-caspase 8 [1:2000], rabbit anti-caspases 3, 7, 9 [1:1000], rabbit anti-cleaved-caspase 3 [1:500], rabbit anti-cleaved-caspases 7, 9 [1:1000], rabbit anti-bak [1:1000], mouse anti-bcl-2 [1:1000], rabbit anti-cleaved-parp, parp [1:1000], then washed with TBS (0.1% v/v Tween20 in TBS) and probed for 1 h with an appropriate secondary antibody (1:2000). To confirm equal protein loading, the membranes were
subsequently stripped and incubated with monoclonal antibody against β-actin (1:10,000), and followed by a horseradish peroxidase conjugated goat anti-mouse IgG (1:5000). Protein bands were visualized by reaction with enhanced chemiluminescence assay kit (GE Healthcare, USA).

In vivo evaluation of tumor inhibition. Male BALB/c nude mice (6–8 wks of age) were supplied by the Laboratory Animal Services Centre of The Chinese University of Hong Kong. Animals were bred and maintained in pathogen-free conditions (sterile food and water ad libitum) in specifically designed air-controlled rooms with a 12-h light/dark cycle. The care and use of the animals were in compliance with the institutional guidelines, and the experimental procedures were approved by the Animal Experimentation Ethics Committee of the CUHK.

An established murine xenograft pancreatic cancer model was applied to test the in vivo efficiency of EriB [Lau et al., 2010]. Briefly, 5×10⁶ CAPAN-2 cells in PBS were injected subcutaneously into the back of mouse. Once the tumors reached a mean diameter of 7–8 mm, animals were randomized into the control group (14 mice) or one of two different EriB dose treatment groups (16 mice each). EriB was dissolved in 1% Pluronic F68 in PBS [Wang et al., 2007] at 1.25 or 2.5 mg/kg, or control diluent, were administered once daily intraperitoneally for 20 consecutive days. Tumor volume was measured every other day with a caliper and calculated using the formula (a×b×c)/2, where a indicates the length, b the width, and c the depth of the tumor. At the same time, each animal was weighed.

At the end of the treatment period, the mice were anesthetized and whole blood was obtained by cardiac puncture. The animals were then sacrificed by cervical dislocation. Plasma was collected by centrifugation (1700 x g, 10 min, 4 °C) and stored at −20 °C. Concentrations of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and creatine kinase (CK) for assessment of heart damage were analyzed according to the manufacturer's instructions (Stanbio Laboratory, USA). Enzyme concentrations (U/L) were calculated using the following equation U/L = (AA/Min×Vs) / (Absorptivity×Vs), where Vs = total volume; Vs = sample volume.

Terminal deoxynucleotidyl transferase-catalyzed DNA nick-end labeling (TUNEL) assay. In vivo cell apoptosis was detected on deparaffinized 5 μm thick tumor or organ (heart, liver and kidney) sections by using In Situ Cell Death Detection Kit, POD (Roche Applied Science, Basel, Switzerland) according to manufacturer’s instruction. Briefly, tumor, heart, liver, and kidney of the mice were dissected out after cervical dissection, fixed in formaldehyde, processed for paraffin embedding, and by Shando Pathcentre Tissue Processor (Thermo scientist, MA, USA). The paraffin-embedded tissue blocks were sectioned at 5 μm using a biocut rotary microtome machine (Shandon Finesse 325, Thermo Scientific, USA) onto gelatin-coated slides. The sections were heated at 43 °C for 45 min, then de-waxed by xylene, and subsequently rehydrated by ethanol gradient (absolute to 70% ethanol, each for 1 min). After several washing, the sections were pretreated with proteinase K (20 μg/ml in 10 mMTris/HCi, pH 7.6) for 45 min, and blocked with 3% hydrogen peroxide in methanol for 10 min to eliminate endogenous POD. The sections were then rinsed with PBS for 5 min twice. The slide was incubated with 50 μl TUNEL reaction mixture (In Situ Cell Death Detection Kit, POD, Roche) for 60 min at 37 °C in a humidified chamber. After incubation, the slides were rinsed with PBS three times. Each sample was added 50 μl Converter-POD solution, and incubated for 30 min at 37 °C. The sections were incubated with 100 μl diaminobenzidine substrate solution for 15 min at room temperature. The slides were finally mounted onto microscopic rapid-mounting media (Richard Allan Scientific, Thermo Scientific, USA) under glass coverslip. The DNA strand breaks during apoptosis were analyzed under a light microscope (Olympus IX71, Japan).

Results
EriB induces cytotoxic effect in human pancreatic cancer cells

As shown in Fig. 1B, C and D, MITT and XTT assays employing CAM and 5-FU as positive controls showed that EriB suppressed cell proliferation dose-dependently in all four pancreatic cancer cell lines, PANC-1, CAPAN-2, CAPAN-1, SW1990, yielding IC₅₀ values in the range of 0.73–1.40 μM. Meanwhile, EriB slightly suppressed growth of WRL68 cells and PBMC. The potency of EriB was comparable to that of CAM (IC₅₀ values in the range of 0.16–1.66 μM) and higher than that of 5-FU (IC₅₀ values in the range of 59.1–238 μM). By contrast, CAM was more toxic to WRL68 cells than EriB, with an IC₅₀ value of 1.92 μM. Importantly, the action of EriB was cell type specific. Pancreatic cancer CAPAN-2 cells appeared to be more sensitive to the treatment of EriB, and therefore were used for the subsequent mechanistic studies.

EriB induces apoptosis in CAPAN-2 cells

Morphological evaluation of Hoechst 33258 stained cells revealed nuclear clearing of untreated cells without nuclear condensation (Fig. 2A). In contrast, EriB treatment at the IC₅₀ value (1.4 μM, using MITT assay, data not shown) for 24 h dramatically altered CAPAN-2 cell morphology. Most of the EriB-exposed cells were shrunken and detached from the substratum of the culture plate. Following EriB treatment, Hoechst-stained CAPAN-2 cells displayed fewer intercellular connections and their nuclei exhibited typical apoptotic morphology as characterized by chromatin condensation and DNA fragmentation (Fig. 2B).

To further determine whether the cell growth inhibitory effect of EriB was associated with induction of apoptosis, DNA fragmentation was evaluated using a DNA ladder assay. Agarose gel electrophoresis revealed a typical internucleosomal DNA fragmentation ladder pattern for cells subjected to EriB treatment for 24 h, 48 h, or 72 h (Fig. 2C). Quantification of DNA fragmentation using the Cell Death Detection ELISA Kit revealed an EriB concentration-dependent induction of cellular apoptosis. Notably, EriB induced significantly (P<0.001) more DNA fragmentation than CAM after 24 h of exposure (Fig. 2D).

Quantitative flow cytometry analysis of cells subjected to Annexin V and PI double staining showed that EriB exerted a concentration- and time-dependent apoptotic effect on CAPAN-2 cells (Fig. 2E). When exposed to 2.1, 2.8, and 3.5 μM EriB for 24 h, 13.2%, 33.9%, 44.4% of CAPAN-2 cancer cells, respectively, were observed to be undergoing apoptosis. Extension of the EriB treatment time to 48 h slightly augmented cellular apoptosis (2.1 μM EriB, 14.2%; 2.8 μM EriB, 44.5%; 3.5 μM EriB, 66.1%).

Activation of pro-apoptotic caspases and modulation of bcl-2/bak ratio in EriB-treated CAPAN-2 cells

Expression of two bcl-2 family members, namely the anti-apoptotic protein bcl-2 and the pro-apoptotic protein bak, was detected by western blotting after 24 h or 48 h of EriB treatment (Fig. 3). EriB markedly decreased bcl-2 expression (Fig. 3B), while increasing bak expression (Fig. 3C). Therefore, EriB-induced apoptosis may be attributed, at least
Fig. 2. Evidence of EriB-induced apoptosis in CAPAN-2 cells. CAPAN-2 cell morphological alterations after 24-h EriB treatment as revealed by DNA-specific Hoechst 33258 stain and visualized by fluorescence microscopy. CAPAN-2 cells were treated with (A) DMEM medium (control) or (B) 1.4 μM EriB. Arrowheads indicate cells exhibiting chromatin condensation consistent with the occurrence of apoptosis. Images are representative of three independent experiments. (C) A typical ladder pattern of internucleosomal DNA fragmentation was observed after EriB treatment for 24 h, 48 h, and 72 h. (D) Concentration-dependent DNA fragmentation induced by EriB or CAM was revealed by Cell Death Detection ELISA™ Kit. Data were expressed as means±S.D. from three independent experiments. *P<0.05, **P<0.01, ***P<0.001 vs. medium control; ###P<0.001 1.4 μM EriB vs. CAM (one-way ANOVA followed by post-hoc Tukey’s multiple comparison test). (E) Quantitative analysis of EriB-induced apoptosis by Annexin V-PI staining assay. Cells treated with 2.1, 2.8 or 3.5 μM EriB for 24 h or 48 h were double-stained with annexin V and PI and analyzed by flow cytometry. Criteria were set to distinguish between viable (bottom left), early apoptotic (bottom right), late apoptotic (top right), and necrotic (top left) cells.
activation of the p53 pathway

EriB modulates expression of G2/M cell cycle regulatory proteins through p38 MAPK were observed after treatment with EriB for 24 h (Fig. 5A). On the other hand, exposure of CAPAN-2 cells to EriB for 36 h or 48 h significantly increased expression of the cyclin-dependent kinase inhibitor p21. This p53 activation was related to increased expression of G2/M cell cycle regulatory proteins (mean fold of control ± S.D. of three independent experiments). Differences between the treated and untreated control groups were determined by one-way ANOVA followed by post-hoc Dunnett's test. 

EriB causes G2/M cell cycle arrest

To gain further insight into the mechanism of the growth inhibitory effects of EriB, CAPAN-2 cells were treated with different concentrations of EriB for 48 h and analyzed for cell cycle distribution by flow cytometry. As shown in Supplementary Fig. 1, EriB treatment significantly increased the percentage of cells in the G2/M phase from 13.25 ± 1.50% to 22.77 ± 4.29% (2.1 μM EriB) or 29.04 ± 6.75% (2.8 μM EriB), and decreased the percentage of cells in G0/G1 phase from 58.35 ± 2.14% to 22.77 ± 4.29% (2.1 μM EriB) or 29.04 ± 6.75% (2.8 μM EriB).

EriB modulates expression of G2/M cell cycle regulatory proteins through activation of the p53 pathway

As shown in Fig. 5D, 3.5 μM EriB induced strong p53 protein expression in CAPAN-2 cells, 5.2-fold of control levels at 36 h and 3.0-fold of control levels at 48 h. This p53 activation was related to increased expression of the cyclin-dependent kinase inhibitor p21. On the other hand, exposure of CAPAN-2 cells to EriB for 36 h or 48 h significantly (P<0.01) reduced expression of cdk1 relative to medium-treated control cells. In contrast, expression of cyclinB1 increased with EriB exposure after 36 h of treatment. These results suggest that p53/p21/cdk1-cyclinB1 signaling may be involved in EriB-mediated G2/M cell cycle arrest. In addition, EriB also provoked a dose-dependent proteolytic cleavage of caspase 3, caspase 7, caspase 9, and PARP was detected.

EriB inhibits the growth of CAPAN-2 human pancreatic tumor xenografts and induces in vivo cell apoptosis without toxicity

Pancreatic tumor xenografts developed successfully in nude mice after subcutaneous injection with human pancreatic adenocarcinoma CAPAN-2 cells. After daily injection of EriB (1.25 mg/kg or 2.5 mg/kg) for 20 consecutive days, the tumor sizes of EriB-treated mice were, in general, smaller than those of the control group (injected with 1% Pluronic F68 in PBS vehicle). Tumor growth was inhibited by EriB in a dose-dependent manner (Fig. 6A). Tumor volume was significantly decreased in the high dose group (2.5 mg/kg) after 20 days of treatment (P<0.01); the inhibitory effect on tumor growth became significant by day 8 of EriB treatment (Fig. 6A). Nude mice treated with EriB intraperitoneally at the high dose (2.5 mg/kg) for 20 days did not show any drug-related side effects.

Discussion

Discussion

Diterpenoids isolated from Isodon species have previously been shown to exert significant cytotoxicity against solid tumor cells and hematological malignant cells (Chen et al., 2005; Li et al., 2011; Liu et al., 2004; Shen et al., 2005; Sun et al., 2006). Among these diterpenoids, EriB has been reported to exhibit significant anti-proliferative action on a number of cancer cell types including leukemia, liver and ovarian cancers (Leizer et al., 2011; Sun et al., 2001, 2006; Wang et al., 2007; Zang et al., 2010). However, EriB’s anti-tumor effect against pancreatic cancer had not been explored. This is the first report to demonstrate an anti-proliferative effect of EriB on pancreatic adenocarcinoma cells and further to provide information regarding the mechanism.

This study employed two anti-tumor positive control substances for comparison: CAM and 5-FU. CAM is a plant-derived alkaloid with broad-spectrum anti-tumor activities in vitro and in vivo that targets DNA topoisomerase I (Ulukan and Swaan, 2002). 5-FU is the most common drug used to treat pancreatic cancer (Stathis and Moore, 2010). The presently reported cytotoxic effects of EriB on PANC-1, SW1990, CAPAN-1, and CAPAN-2 cells were comparable to...
those of CAM, and more potent than those of 5-FU. Importantly, EriB was less cytotoxic to normal human liver cells (WRL68 cell line) as well as human peripheral blood mononuclear cells (PBMC). Therefore, EriB should be considered a potential lead compound for chemotherapy of pancreatic cancer.

Subsequent mechanistic experiments in this study indicated that EriB was capable of inducing apoptosis in CAPAN-2 cells. Apoptotic pathways can be triggered by numerous stimuli. These stimuli induce cell death via intrinsic and extrinsic pathways which interact at different levels. The intrinsic (or mitochondrial) pathway is initiated by a...
loss of mitochondrial membrane potential and a consequent release of cytochrome c from mitochondria into the cytosol. Cytochrome c binds to apoptosis inducing factor (AIF) to form the apoptosome complex, which activates pro-caspase 9. The extrinsic pathway involves activation of death receptors on the cell surface, such as Fas and tumor necrosis factor (TNF) receptor, which leads to subsequent activation of pro-caspase 8. The two pathways converge at caspase 3. Upon activation, pro-caspase 3 undergoes autaproteolytic cleavage, forming active cleaved-caspase 3, which then activates other pro-caspases, such as pro-caspase 7 and PARP (Elmore, 2007; Fulda et al., 2010; Igney and Krammer, 2002; Kroemer et al., 2007; Westphal and Kalthoff, 2003). Our results indicated that EriB induced apoptosis in pancreatic cancer cells through both the extrinsic pathway, mediated by caspase 8 activation, and the intrinsic pathway, mediated by caspase 9 activation. Both pathways activate...

![Western blot analysis of the protein expression involved in EriB-induced activation MAPKs and p53 pathway. CAPAN-2 cells were treated with 1.4, 2.8, or 3.5 μM EriB for 24 h, 36 h or 48 h. Immunoblotting was performed three times using independently prepared whole cell lysates. (A) Representative immunoblots showing the effects of EriB treatment in CAPAN-2 cells on the expression of pERK1/2, ERK1/2, pp38, and p38. (D) Representative immunoblots showing the effects of EriB treatment in CAPAN-2 cells on the expression of p53, p21, cdk1, and cyclin B1. Actin expression was determined to confirm equal protein loading. (B, C, E-H) The histograms showing quantified results of protein levels, which were adjusted relative to corresponding β-actin protein level and expressed as fold of control (mean fold of control ± S.D. of three independent experiments). Differences between the treated and untreated control groups were determined by one-way ANOVA followed by post-hoc Dunnett’s test. *P<0.05, **P<0.01 vs. medium control at the same time point.](Image)
downstream executor caspases—mainly caspase 3, caspase 7, and PARP—eventually leading to apoptosis.

EriB induction of CAPAN-2 cell apoptosis occurred in conjunction with regulation of bcl-2 and bak proteins. Decreased ratios of the anti-apoptotic to pro-apoptotic bcl-2 family members elicit cytochrome c release, which is associated with the intrinsic apoptosis pathway (Fulda, 2009; Igney and Krammer, 2002). Some studies have shown that high levels of bcl-2 expression conferred poor prognosis and resistance to chemotherapy and radiotherapy in pancreatic cancer patients (Lee et al., 1999; Rückert et al., 2010; Vogler et al., 2008). Therefore, therapies that target bcl-2 family proteins may enhance treatment efficacy.

Both cyclin-dependent protein kinases (cdks) and cyclins play critical roles in cell cycle regulation. Cdk activity is dependent on conjunction with their activating subunits known as cyclins. In most circumstances, specific cyclin/cdk complexes are activated during different cell cycle phases, such as cyclin D/cdk4 or cdk6 during early G1 phase, cyclin A/cdk2 during S phase, and cyclin B/cdk1 during G2/M phase. Thus, phosphorylation of particular proteins permits the cell cycle processes to continue. Specific cyclins accumulate within the phases in which they are required, and degrade in the phases in which they are not needed. Cyclin/cdk complexes can be bound by cdk inhibitor proteins, such as p21, which inhibit kinase activity and prevent cell cycle progression (King and Cidlowski, 1998; Malumbres and Barbacid, 2009; Senderowicz, 2003; Vermeulen et al., 2003).

The results of flow cytometry suggested that 48-h EriB treatment can cause cell cycle arrest at G2/M phase. Western blotting results fit with the possibility that cell cycle arrest may occur later than induction of apoptosis in EriB-treated cells because expression of p53 and p21CIP1/WAF1 not only remained after 24 h (data not shown), but showed further increases after 36 to 48 h of treatment. Increased expression of p53 and p21 is associated with decreased expression of cdk1 and subsequent inactivation of the cdk1-cyclinB1 complex, ultimately inducing G2-M arrest (DiPaola, 2002). On the other hand, anaphase promoting complex (APC)-mediated cyclin B1 degrades as mitosis reaches completion (Farhana et al., 2002; Visanji et al., 2006), hence, it is reasonable to suppose that accumulation of cyclin B1 induced by EriB prevented the cells from completing mitosis.

In cells not exposed to stress, p53 is kept at low levels by its negative regulator Mdm2. Upon introduction of a stressor, such as ionizing...
radiation, UV light, growth factor deprivation, reactive oxygen species, and DNA damage induced by various cytotoxic agents. p53’s transcription factor activities are induced, resulting in the regulation of downstream target genes, such as apoptosis-promoting factor, pro-apoptotic bcl-2 family proteins (i.e., bax and bak), which lead to different cellular outcomes such as cell cycle arrest and apoptosis (Zhang et al., 2011). In addition, the wild-type p53 product can also inhibit bcl-2 expression (Ferreira et al., 1999). The negative modulation of bcl-2/bak or bak ratios can induce mitochondria to release cytochrome c, thereby activating the intrinsic apoptotic pathway. This whole process is known as p53-mediated caspase activation (Schuler and Green, 2001). The resultant p53-mediated cell cycle arrest allows time for DNA damage repair. If the repair is not successful, the cells will undergo apoptosis, necrosis, or permanent suppression of proliferation (Cmielova et al., 2011). The mitogen activated protein kinases (MAPKs), such as p38 MAPK, c-Jun N-terminal kinases (JNK) and extracellular-signal-related kinases (ERK) are known to be the signaling molecules involved in cell proliferation, differentiation, transformation survival and death (Dhillon et al., 2007). These signaling pathways are also considered the upstream mediator of p53 pathway (Li et al., 2008). Activation p38 MARK could activate the p53 signaling pathway through direct phosphorylation of p53 (Huang et al., 2008). In view of this, our data support the notion that MAPKs (ERK and p38) and the downstream transcription factor p53 are the major mediators of CAPAN-2 cells during apoptosis and cell cycle arrest. Previous studies have demonstrated that activation of p38 and ERK1/2 kinases may serve as a death signal via the mediation of caspase cascade activation (Habiro et al., 2004; Ki et al., 2012; Lau et al., 2009). We thus believe that MAPK activation followed by caspase activation is involved in regulating ErbB-induced apoptosis.

The convergent data presented here indicated that ErbB-mediated CAPAN-2 cell apoptosis and cell cycle arrest were mediated mechanistically through activation of the p53 pathway. A working mechanistic model was developed based on these findings and was summarized in a schematic diagram (Fig. 7).

Our findings of p53 wild-type CAPAN-2 cells that were more susceptible to ErbB than p53 mutant cells (PANC-1, SW1990, and CAPAN-1) suggesting that p53 phenotype may be an important factor in determining cellular responsiveness to ErbB. There is accumulating evidence showing that p53 status has a significant impact on drug sensitivity. Cells expressing wild-type p53 were more sensitive to chemotherapy or radiotherapy than p53-mutant cells (Fojo, 2002; Ji et al., 2011; Mohiuddin et al., 2002). However, the fact that the p53 mutant cell lines were also inhibited, albeit to a lesser degree, by ErbB suggested that there were other determinants of human pancreatic adenocarcinoma cells sensitively to ErbB. Other protein targets, such as Nfkb, have been implicated in ErbB-mediated malignancy inhibition (Leiser et al., 2011; Wang et al., 2007; Zhang et al., 2010). Thus, ErbB may hit multiple targets in human pancreatic cancer cells. In addition, the signaling pathway upstream of ErbB treatment could be mediated through reactive oxygen species. Given the important downstream mediators of ROS-induced signaling pathways of the MAPKs (Huang et al., 2008; Torres and Forman, 2003), it is plausible that ErbB leads to apoptosis via the mediation of ROS-induced MAPK activation, thus regulating p53 pathway. In view of this, the cell type specific action of ErbB may be due, at least in part, to ROS threshold concept whereby cancer cells produce higher levels of ROS than normal cells, and thus they reach the death threshold earlier (Kirschner et al., 2008; Wang and Yi, 2008).

The anti-tumor efficacy of ErbB on pancreatic cancer growth was further examined using a nude mice model in which mice were xenografted with human pancreatic adenocarcinoma CAPAN-2 cells. Daily ErbB administration dose-dependently suppressed tumor growth in the absence of toxicity in vivo. These observations were in agreement with our in vitro data showing that ErbB had preferential toxicity toward pancreatic cancer cells relative to normal human liver cells and human peripheral blood mononuclear cells. At the 2.5 mg/kg/day dose, ErbB treatment significantly reduced tumor size relative to the untreated control group. The selection of drug concentration was based on a previous study (Wang et al., 2007). An earlier pilot study that tested ErbB at concentrations of 1.25, 2.5, and 3.75 mg/kg/day showed similar anti-tumor efficacy for the 2.5 and 3.75 mg/kg/day doses, with no toxicity either at the 2.5 or 3.75 mg/kg/day dose (data not shown).

In conclusion, the present study demonstrated that ErbB, a diterpenoid isolated from I. eriocalyx, possessed anti-proliferative activity in cultured pancreatic cancer cell lines. ErbB's anti-proliferative effects can be attributed, at least in part, to regulation of cellular apoptosis and cell cycle arrest through activation of caspase and p53 pathways. An in vivo study provided confirmation of ErbB's low/negligible toxicity and anti-tumor properties, suggesting that ErbB should be considered a good candidate for further development as a chemotherapy agent in human patients with pancreatic cancer.

Conflict of interest statement

None of the authors has any potential financial interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.taap.2012.04.021.

References


