Involvement of the mitochondrial pathway in bruceine D-induced apoptosis in Capan-2 human pancreatic adenocarcinoma cells

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Abstract. The fruit of Brueca javanica L. is a common herb used in Chinese medicine for the treatment of a variety of cancers. Our research group has previously identified bruceine D (BD), a quassinoid found abundantly in B. javanica, to have potent cytotoxic effect on a number of pancreatic cancer cell lines, including Panc-1, SW1990 and Capan-1 cells. In the present study, we showed that BD was also able to inhibit the growth of the Capan-2 human pancreatic adenocarcinoma cell line, but it exerted only modest cytotoxicity on the WRL68 human hepatocyte cell line and a human pancreatic progenitor cell line. The antiproliferative effects of BD were comparable to those exhibited by camptothecin and gemcitabine in our culture system. We found a dose-dependent decrease of the mitochondrial membrane potential in BD-treated Capan-2 cells as measured by the JC-1 assay. BD exposure was able to attenuate the expression of Bcl-2 protein in Capan-2 cells as detected by western blot analysis. In addition, the expression of both caspase 9 and caspase 3 in BD-treated Capan-2 cells was significantly accentuated. Moreover, BD was capable of inducing the fragmentation of genomic DNA in Capan-2 cells as evidenced by Hoechst staining. Cell cycle analysis demonstrated that BD could increase the percentage of Capan-2 cells in the subG phase in a dose-related manner. An increase in the apoptosis of Capan-2 cells was also observed by Annexin V and PI staining. These results unequivocally indicate that BD induces cytotoxicity in Capan-2 cells via the induction of cellular apoptosis involving the mitochondrial pathway.

Introduction

Human pancreatic cancer is a gastrointestinal malignancy with extremely poor prognosis owing to a lack of early clinical diagnosis and its intrinsic resistance to radiotherapy and chemotherapy (1,2). According to the Hong Kong Cancer Registry in 2009 pancreatic cancer was the 8th and 6th major cause of cancer-related deaths in males and females, respectively. As for the management of pancreatic cancer, surgical resection remains the only potentially curative treatment of pancreatic cancer. However, only 5-25% of the patients diagnosed with pancreatic cancer were operable, and chemotherapy thus remains the mainstay of palliative management for the locally advanced and metastatic stage of this cancer. The median disease-free survival following complete resection of the pancreatic tumor and adjuvant chemotherapy with the first line chemotherapeutic agent gemcitabine is 13.4 and 6.9 months, respectively (3-6). Although camptothecin has a positive effect on arresting pancreatic tumor growth, many unwanted side-effects limit its clinical application (5,7). All these dismal data highlight an urgent need for new therapeutic agents that could produce better clinical outcomes for this deadly disease.

Our previous studies have shown that the fruit of Brueca javanica L., a Chinese medicinal plant commonly used for cancer treatment, has potent in vitro anti-pancreatic cancer activity (8). Furthermore, bruceine D (BD), a quassinoid found in abundance in B. javanica, was identified as the principal active ingredient of this cancer-treating Chinese herb (9). In order to evaluate the potential of BD as a chemotherapeutic agent for pancreatic cancer, in the present study we investigated the anti-pancreatic cancer activity of BD on human pancreatic adenocarcinoma Capan-2 cells and elucidated the underlying mechanisms of action. In addition, the cytotoxic selectivity of BD on different cell lines was also determined.

Materials and methods

Chemicals. BD was purified from the fruit of Brueca javanica in our laboratory and its identity was confirmed based on nuclear magnetic resonance (NMR) and mass spectrometry (MS) data. The purity of BD, which was in white powder form, was >95% based on high performance liquid chromatography (HPLC) analysis. The chemical structure of BD is shown in Fig. 1.

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General cell culture. Capan-2, a human pancreatic adenocarcinoma cell line and WRL68, a human hepatocyte cell line, were purchased from American Type Culture Collection (ATCC, VA, USA). For general cell culture, Capan-2 and WRL68 cells were routinely maintained in DMEM medium supplemented with 10% FBS, 4 mM L-glutamine, 4.5 g/l glucose, 1.5 g/l sodium bicarbonate, 100 U/ml penicillin and 10 U/ml streptomycin and incubated at 37°C in a 5% CO₂, 95% air humidified atmosphere.

Human fetal pancreas tissue, obtained by extraction after termination of pregnancy between the 9th and 15th weeks of gestation, was provided by the Department of Obstetrics and Gynecology, Prince of Wales Hospital, The Chinese University of Hong Kong. Maternal consent and ethical approval from the Clinical Research Ethics Committee (CREC-2005.461) were obtained before the procedures. The experiments were performed using the preparations derived from a single fetal pancreas. To establish the primary culture of human pancreas, the fetal pancreas was minced and digested with 3 mg/ml collagenase P (Roche, Mannheim, Germany). Digested cell clusters were resuspended in modified RPMI-1640 medium supplemented with 10% FBS, 1 mM sodium pyruvate, 1% penicillin and streptomycin, 10 mM HEPEs buffer (Invitrogen, Carlsbad, CA, USA) and 71.5 μM β-mercaptoethanol (Sigma Aldrich, St. Louis, MO, USA) in 60 mm diameter non-treated culture dishes (Corning Incorporated, Lowell, MA, USA). Rounded, non-adherent cell clusters were formed within 48 h of incubation. Pancreatic progenitor cell (PPC) outgrowth from the cell clusters was induced by further addition of 20 ng/ml each of basic fibroblast growth factor (Sigma Aldrich) and epidermal growth factor (Invitrogen) in the medium. A monolayer of PPCs from the cell clusters was established within 3-4 days, and the cells were harvested with 0.05% trypsin-EDTA (Invitrogen) upon reaching confluence.

Proliferation assay. The proliferation rates of Capan-2, WRL68 and PPC were determined by the SRB assay, a colorimetric end-point microplate assay which quantifies viable cells by staining their cellular protein content (10). BD was reconstituted in DMSO to produce a stock solution and then diluted with culture medium to various concentrations for cell culture experiments. As the first-line chemotherapeutic agent for locally advanced and metastatic pancreatic cancer, camptothecin (WAM, Osaka, Japan) and gemcitabine (Lilly France, Fegersheim, France) were included in the experiment as positive controls. Like BD, camptothecin was dissolved in DMSO (Sigma Aldrich, Fegersheim, France) upon reaching confluence.

Apoptosis detection with Annexin V and PI staining. Both adherent and floating cells were collected after 0.55, 1.10 and 2.20 mM BD treatment for 24 and 48 h. The collected cells were washed with ice-cold PBS followed by permeabilization with ice-cold 7% ethanol for at least 1 h. The permeabilized cells (10⁶ cells) were suspended in 0.5 ml PI/RNase solution (BD Pharmingen, San Diego, CA, USA) and incubated at room temperature for 15 min in the dark to apply the PI stain to cellular DNA. The stained cells were analyzed by a Cytomics FC500 flow cytometer (BioTek, Gene Co. Limited, VT, USA). The growth curves of different cell types were then constructed and the IC₅₀ values were calculated using GraphPad Prism 5.01 computer software (GraphPad Software, Inc., San Diego, CA, USA).
binding buffer. After adjusting the cell density to 2.5x10^5 cells/ml, 10 ml of Annexin V-FITC (Invitrogen) was added to 190 ml of cell suspension, followed by incubating the cells for 10 min at room temperature. Next, the cells were washed with 1X binding buffer and spun down followed by resuspension in 190 ml binding buffer. Ten microliters of 20 mg/ml propidium iodide stock solution was finally added to stain the cells. The stained cells were then analyzed by flow cytometry.

Estimation of the changes of mitochondrial membrane potential (MMP) by the JC-1 assay. Capan-2 cells were seeded at 0.3x10^5 cells/well in 6-well plates and incubated with BD at 0.55, 1.10 and 2.20 µM for 24 h. The treated cells were collected and washed twice with ice-cold PBS, followed by addition of 10 ml of 200 mM JC-1 (Invitrogen) to each tube and incubation at 37°C, 5% CO_2 for 15-30 min. The change of MMP was evaluated by flow cytometry at 488 nm excitation and 590 nm emission wavelengths.

Western blot analysis of Bcl-2, caspase 9 and caspase 3. Capan-2 cells at 2.2x10^6 cells/plate were seeded in 100-mm plates. After 4-h incubation, the cells were exposed to BD at 2.20 and 11.0 µM for 72 h. The cells were then collected and lysed with CytoBluster protein extraction reagent (Novgen, Darmstadt, Germany). The protein concentrations were measured with the Micro BCA™ Protein Assay kit (Thermo Scientific, Rockford, IL, USA). Equal amount of proteins were then resolved by SDS-PAGE for 2 h at 100 V and transferred electrophoretically to PVDF membranes for 30 min at 15 V. Membranes were blocked with 5% (w/v) non-fat milk in PBS-T (0.1% v/v Tween-20 in PBS) for 2 h and subsequently incubated overnight at 4°C with primary anti-Bcl-2, primary anti-caspase 9, primary anti-caspase 3 and primary anti-β-actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at concentrations of mouse anti-Bcl-2 (1:1000), mouse anti-caspase 9 (1:1000), rabbit anti-caspase 3 (1:500) and mouse anti-β-actin (1:2000), respectively. The membrane was then incubated for 1 h at room temperature with HRP-conjugated sheep anti-mouse IgG (1:2000) and horseradish peroxidise (HRP)-conjugated donkey anti-rabbit IgG (1:1000), respectively. The protein bands were visualised by ECL western blotting detection reagents (Amersham Biosciences, Sweden). Rainbow molecular weight markers were used as size markers for the determination of protein size.

Statistical analysis. Multiple comparisons between data sets were performed using one-way analysis of variance (ANOVA) followed by the Dunnett’s post-hoc test, in which the nontreated group served as the controls. Statistical analyses were conducted using a GraphPad Prism 5.01 software package (GraphPad Software, Inc.). A P-value of <0.05 was considered statistically significant.

Results

Effects of BD, camptothecin and gemcitabine on Capan-2 cell viability. As shown in Fig. 2A, both BD and camptothecin significantly inhibited the proliferation of Capan-2 cells, with IC_{50} values of 1.10 and 1.29 mM, respectively, as assessed by the SRB assay. As the first-line chemotherapeutic agent for locally advanced and metastatic pancreatic cancer, gemcitabine exhibited a steady but modest cytotoxic effect on Capan-2 cells, with an IC_{50} value of 23.54 mM. On the other hand, BD showed a much less potent cytotoxic effect on cultured non-tumorigenic hepatocyte WRL68 and PPC cells. Drug treatment was initiated 4 h after seeding the cells in 96-well plates. Cell viability was assessed by the SRB assay after 72 h of drug treatment.

Figure 2. Effects of brucein D, gemcitabine and camptothecin on the viability of human pancreatic adenocarcinoma Capan-2 cells (A), human hepatocyte WRL68 (B) and human pancreatic progenitor cells PPC (C). Drug treatment was initiated 4 h after seeding the cells in 96-well plates. Cell viability was assessed by the SRB assay after 72 h of drug treatment.
Hoechst fluorescence staining for morphological changes of Capan-2 cells. Hoechst staining followed by observation under a fluorescence microscope revealed only slight Hoechst nuclear staining in non-treated (medium only) cells, and no apoptotic nuclei were observed (Fig. 3A). In contrast, 0.55, 1.10 and 2.20 µM BD treatment for 32 h dramatically altered Capan-2 cell morphology, in that Hoechst-stained Capan-2 cells displayed fewer intercellular connections and exhibited typical apoptotic morphology characterized by chromatin condensation and DNA fragmentation (Fig. 3B-D).

BD on cell cycle progression. As shown in Fig. 4, flow cytometric analysis revealed that treatment with BD induced an increase in the percentage of subG1 phase (apoptotic cells) in Capan-2 cells in a dose-dependent manner. When treated with BD at 0.55 µM for 32 h, the percentage of Capan-2 cells at subG1 phase was only 4.9%. When treated with BD at 1.10 µM for 32 h, the percentage of cells in the subG1 phase was 19.8%. An increase of BD to 2.20 mM resulted in 26.0% of subG1 cells in the total cell population. In contrast, non-treated cells demonstrated normal cell viability with very few cells in the subG1 phase.

Quantitation of apoptotic cells by Annexin V and PI staining. It has been observed that phosphatidylserine (PS) is translocated from the inner to the outer surface of the plasma membrane in the early stage of apoptosis. Therefore, PS exposure represents a useful target for evaluating apoptosis (11,12). To assess plasma membrane changes, cells were stained with Annexin V (which binds preferentially to PS in the presence of Ca2+) and PI simultaneously. As shown in Fig. 5, the viable cells were localized in the lower left quadrant and the apoptotic cells were localized in the lower right quadrant. When treated with BD at 0, 0.55, 1.10, 2.20 µM for 32 h, the percentages of apoptotic cells in the total cell population were 0.1, 2.3, 7.7 and 25.4%, respectively, suggesting BD dose-dependently induced cellular apoptosis to Capan-2 cells.

BD-induced alteration of MMP by the JC-1 assay. It is clear from Fig. 6 that MMP of Capan-2 cells was significantly attenuated by BD treatment. When treated with BD at 0, 0.55, 1.10, 2.20 µM for 24 h, the number of cells with decreased MMP were 5.9, 65.1, 78.1 and 86.7%, respectively, indicative of the ability of BD to induce mitochondrial membrane depolarization, a distinct event leading to cellular apoptosis.

Western blot analysis. As shown in Fig. 7, exposure of Capan-2 cells to BD significantly attenuated the expression of Bcl-2 as evidenced by the appearance of a 27-30 kDa fragment (A). On the other hand, BD treatment markedly augmented caspase 9 and caspase 3 expression, as evidenced by the appearance of a 36-40 kDa (B) and 19-21 kDa (C) fragment respectively. These results unambiguously demonstrated that the underlying mechanism of BD-induced apoptosis in Capan-2 cells at the protein level involves the reduced expression of anti-apoptotic protein Bcl-2 and augmented activation of the pro-apoptotic proteins caspase 9 and caspase 3.

| Table I. IC50 values of Brucein D, gemcitabine and camptothecin of the growth of Capan-2, WRL68 and PPC cells. |
|---|---|---|
| Cell line | Bruceine D | Gemcitabine | Camptothecin |
| Capan-2 | 1.10 | 23.54 | 1.29 |
| WRL68 | 276.56 | 742.28 | 118.39 |
| PPC | 162.48 | >759.87 | 38.46 |

*The IC50 values of the three compounds on the growth of Capan-2, WRL68 and PPC cells were determined by the SRB assay. *IC50 values were calculated using a GraphPad Prism computer program.

Figure 3. Morphological alteration in BD-treated Capan-2 as revealed by Hoechst 33342 fluorescence staining. Capan-2 cells were treated for 32 h with medium control (A); 0.55 µM BD (B); 1.1 µM BD (C); 2.2 µM BD (D). Note that nuclear condensation as indicated by arrows was observed in BD-treated cells.
Figure 4. Cell cycle analysis of brucein D (BD)-induced apoptosis by PI/RNase staining assay. Cells were collected after 0, 0.55, 1.1 and 2.2 µM BD treatment for 32 h, followed by PI staining and flow cytometric analysis. M, subG1 phase; P, S phase; N, G0+G1 phase; Q, G2+M phase.

Figure 5. Quantitative analysis of brucein D (BD)-induced apoptosis by Annexin V-PI staining assay. Cells treated with 0, 0.55, 1.1 and 2.2 µM BD treatment for 32 h were double stained with Annexin V and PI, followed by flow cytometric analysis. G1, G2, G3 and G4 quadrants represent necrotic, secondary necrotic, viable and apoptotic cells, respectively.
Discussion

The fruit of *Brucea javanica* as a Chinese herb was first recorded in a Chinese material medical book called ‘Supplementations to the Compendium of Chinese Materia Medica’ by Zhao Xue-Ming some 250 years ago. One of the main functions of...
this herb is to clear heat and remove toxicity, and it is commonly prescribed by traditional Chinese medicine practitioners to treat a variety of cancers including esophageal carcinoma, small cell lung cancer, stomach cancer and pancreatic cancer (9,13-15). However, active chemical ingredients responsible for the clinical anticancer efficacy of this herb have so far not been thoroughly investigated.

Our previous studies focusing on *B. javanica* for pancreatic cancer have identified bruceine D (BD), a quassinoid found in relatively large quantity in the fruits of *B. javanica*, as a major active chemical ingredient possessing potent anti-pancreatic cancer activity. BD was able to inhibit the growth of a number of pancreatic cancer cell lines including Panc-1, SW1990 and Capan-1 cells. Panc-1 cells were established from a well-differentiated pancreatic adenocarcinoma with duct metastasis, while SW1990 cells were derived from metastasis of the spleen and Capan-1 from a well-differentiated pancreatic adenocarcinoma with liver metastasis. It is worth noting that the Capan-2 cell line used in the present study has an origin of a primary pancreatic tumor.

It is important to examine the anti-pancreatic cancer activities of BD in various cell types of different tissue origins so that the cellular selectivity of this compound can be elucidated. The selectivity data is of significance should BD is to be developed into clinical treatment for pancreatic cancer. Our results showed that BD significantly inhibited the growth of Capan-2 cells with an IC₅₀ value of 1.10 μM, whereas it only exerted modestly cytotoxic towards other non-tumorigenic cells including WRL68 and PPC. These results add further evidence to suggest that BD possesses good selectivity on pancreatic cancer cells without inflicting marked cytotoxicity to other cells of non-tumorigenic origin.

Since cellular apoptosis is a major pathway leading to cell growth inhibition, the present study elucidated whether cellular apoptosis is the underlying mechanism of action responsible for BD-induced Capan-2 growth inhibition using a variety of morphological and biochemical assays.

In mammalian cells, signalling cascades leading to apoptosis can be divided into two broad groups, the intrinsic pathway and extrinsic pathway. The intrinsic pathway, also called mitochondrial pathway, is characterized by the central role of mitochondria in the initiation of the caspase cascade executing the apoptosis program (16). As depicted in Fig. 6, we found a dose-dependent decrease of the mitochondrial membrane potential in BD-treated Capan-2 cells as detected by the JC-1 assay. Meanwhile, western blot analysis also showed that BD exposure attenuated the expression of Bcl-2 protein in Capan-2 cells. In addition, in the BD-treated Capan-2 cells both caspase 9 and caspase 3 protein expression were significantly accentuated. Moreover, BD was capable of inducing the fragmentation of genomic DNA in Capan-2 cells as evidenced by Hoechst staining. Cell cycle analysis revealed that BD dose-dependently increased the occurrence of subG1 phase (apoptosis cells) Capan-2 cells. Concomitant Annexin V and PI staining also revealed that BD was able to cause cellular apoptosis in Capan-2 cells. Taken together, our results demonstrate that the underlying mechanism of action responsible for the BD-induced cell growth inhibition on cultured Capan-2 cells is the cellular apoptosis involving the mitochondrial pathway.

In conclusion, the present study provides further evidence demonstrating that bruceine D is a promising anti-pancreatic cancer chemical agent. The results offer scientific support for the use of the fruit of *Brucea javanica*, from which BD is derived, for treatment of pancreatic cancer. Further experiments to evaluate the anti-pancreatic cancer effects of BD using an orthotopic transplantation animal model of pancreatic cancer are ongoing in our laboratory.

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**References**