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Original Article

Bruceine D induces apoptosis in human chronic myeloid leukemia K562 cells via mitochondrial pathway

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Abstract: Chronic myeloid leukemia (CML), an acquired malignant myeloproliferative disorder of hematopoietic stem cells, is one of the three most common forms of leukemia. In this study, we investigated the effects of bruceine D, which have been isolated from *Brucea javanica* (L.) Merr. on human chronic myeloid leukemia K562 cells. MTT assay was used to evaluate cell growth inhibition. Flow cytometry was performed to analyze mitochondrial membrane potential (ΔΨm). Western blot was applied to detect expression of cytochrome *c*, caspases-9, -3, PARP and other proteins. Bruceine D exhibited potent cytotoxicity to K562 cells with IC50 of 6.37 ± 0.39 μM. It led to loss of ΔΨm, release of cytochrome *c*, activation of caspases-9, -3 and cleavage of PARP, which suggested that bruceine D induced apoptosis of K562 cells through mitochondrial pathway. In addition, bruceine D inhibited the phosphorylation of AKT and ERK. It's indicative that the potent anticancer activity of bruceine D be related to MAPK and PI3K pathways.

Keywords: Bruceine D, apoptosis, mitochondrial pathway, AKT, ERK, phosphorylation

Introduction

The continuing global demographic and epidemiologic transitions signal an ever-increasing cancer burden in future. Chronic myeloid leukemia (CML), an acquired malignant myeloproliferative disorder of hematopoietic stem cells, is one of the three most common forms of leukemia. Leukemia accounted for some 352,000 new cases (2.5% of all new cancer cases) and for 265,000 deaths (3.2% of all deaths) in 2012 worldwide [1]. These depressing statistics highlight the urgent need for discovering new, effective agents capable of arresting tumor growth and producing better clinical outcomes.

Natural products, with increasing scientific attention, have been studied in various anticancer studies and clinical trials recently [2]. They not only have useful therapeutic and pharmacological effects, but also provide further leads for anticancer drug development with stronger antitumor activity and fewer side effects [3]. The seeds of *Brucea javanica* (L.) Merr. have been employed for the treatment of malaria, dysentery and cancer for centuries. Photochemical studies reveal that quassinoids are the predominant constituents of this plant [4, 5]. Bruceine D (**Figure 1**) is a quassinoid isolated from *B. javanica* and has exhibited cytotoxic effects on various cancer cell lines [6]. However, mechanism investigation of bruceine D mainly focused on pancreatic cancer [7-9].

Therefore, in order to evaluate the potential of bruceine D as a chemotherapeutic agent for chronic myeloid leukemia, the present study was designed to evaluate bruceine D-induced cell growth inhibition in human chronic myeloid leukemia K562 cells and to explore the potential mechanisms.

Material and methods

Chemicals and reagents

Bruceine D with a purity of more than 98% was isolated from the seeds of *B. javani-*

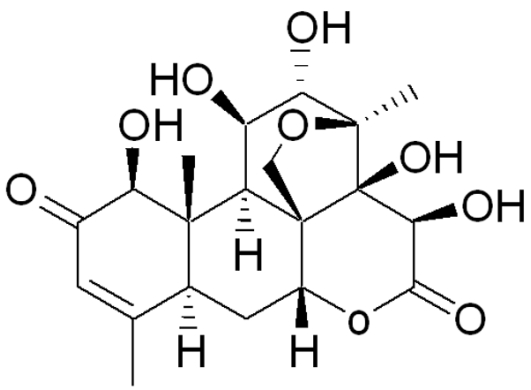

Figure 1. Chemical structure of bruceine D.

ca. 3-(4,5-Dimethyl-2-thiazolyl)2,5-diphenyl-2H-tetrazolium bromide (MTT) and 3,3'-dihexyloxacarbocyanine iodide (DiOC6(3)) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Annexin V-FITC/PI Apoptosis Detection Kit was purchased from KeyGEN Biotech (Nanjing, China). Primary antibodies against caspase-3, caspase-9, PARP, cytochrome *c*, AKT, p-AKT, ERK, p-ERK and GAPDH were obtained from Signalway Antibody Co. (College Park, MD, USA) or Santa Cruz Co. (Dallas, TX, USA). Secondary anti-mouse and anti-rabbit IgG-HRP were products of KangChen Biotechnology Co. (Shanghai, China). All culture media and growth supplements were acquired from Gibco-BRL Co. (Gaithersburg, MD, USA). Other routine reagents applied in research were of analytical or HPLC grade and purchased from commercial sources.

Cell culture

K562 cells are human cell line of chronic myeloid leukemia. Cells were cultured in RPMI 1640 medium containing 100 U/mL penicillin, 100 μg/mL streptomycin and 10% FBS in a humidified atmosphere incubator of 5% CO_2 at 37°C.

Cell viability assay

MTT assay was used to measure cell viability as previously described [10]. K562 cells were seeded in 96-well plates. After 24 h, cells were treated with bruceine D at various concentrations (3.0, 6.0, 12.0 μM) for another 68 h. 10 μl MTT was then added to each well and cells were further cultured for 4 h at 37°C. The absorbance of each sample was read at 540 nm with 655 nm as reference filter on a microplate reader (BIO-RAD Model 550). The growth inhibitory effect of bruceine D was expressed as the IC50, which was estimated by the concentration-response curve (Bliss's software). Cell survival ratio was calculated by the following formula: survival (%) = (mean experimental absorbance/mean control absorbance) × 100%.

Annexin V-FITC/PI apoptosis detection

Annexin V-FITC/PI Apoptosis Detection Kit was performed following the manufacturer's instruction [11]. After treatment with bruceine D (12.0 μM) for 12, 24 and 36 h, the cultured K562 cells were harvested and resuspended in 0.5 mL binding buffer containing Annexin-V (1:50) and 40 ng/sample of PI for 30 min at 37°C in the dark. The stained samples were analyzed by flow cytometer (BD FASCanto) and CellQuest software. Apoptosis rate (%) = (the number of apoptotic cells/the number of total cells observed) × 100%.

Mitochondrial membrane potential (ΔΨm) measurement

ΔΨm were estimated using cationic lipophilic fluorochrome DiOC6(3) as previously described [12]. K562 cells were incubated in the absence and presence (3.0, 6.0, 12.0 μM) of bruceine D for 24 h. Cells were subsequently harvested and stained with 40 nM DiOC6 at 37°C for 20 min in the dark. Fluorescence intensity was measured by FACS caliber flow cytometer (Beckman-coulter, Elite; excitation/emission at 484/501 nm) and analyzed by CellQuest software. Results were expressed as mean fluorescence intensity (MFI). At least 10000 events of each sample were analyzed.

Western blot analysis

Expression of caspase-9, -3, PARP, cytochrome *c*, AKT, p-AKT, ERK and p-ERK was detected by Western blot as previously described [13, 14]. 3.5-4.0 × 10^6 per well were seeded into culture dishes, incubated with 0-12 μM bruceine D for different time periods and then washed with ice-cold PBS.

For whole cell lysate, the pellet was lysed in 1 × loading buffer (50 mM Tris-Cl (pH 6.8), 10% glycerol, 2% sodium dodecylsulphate, 0.25‰

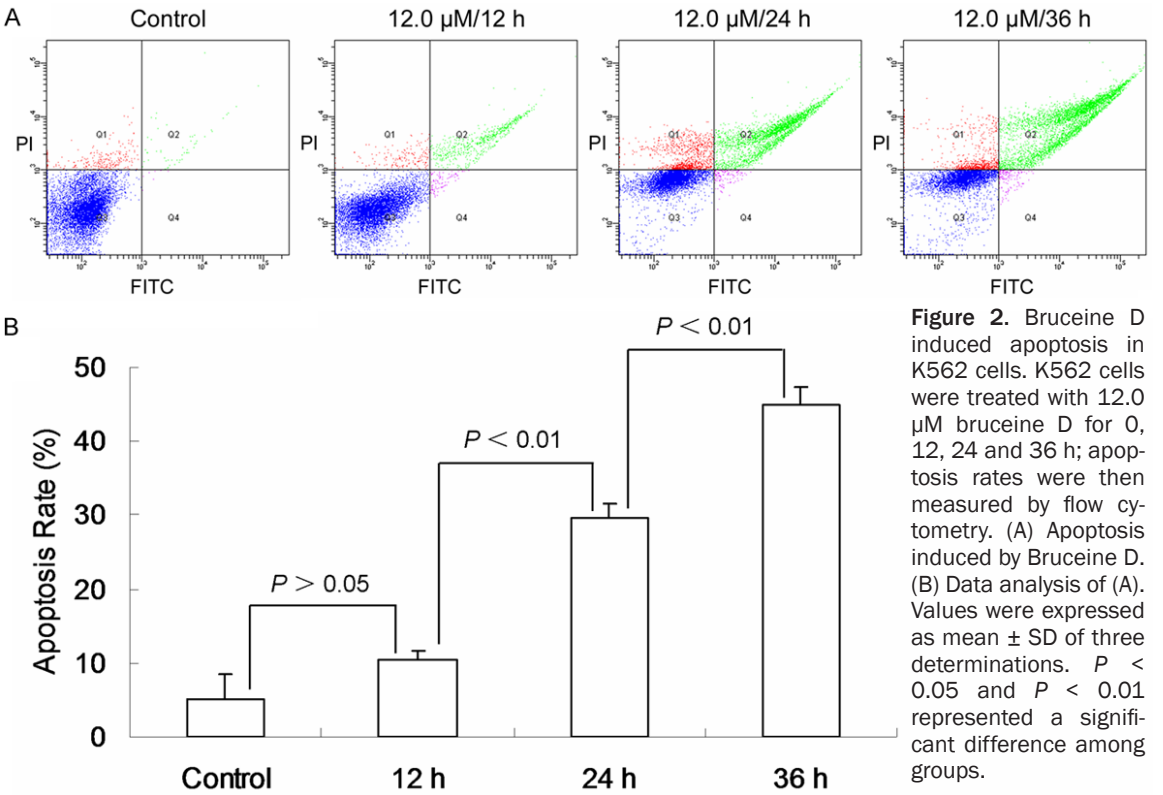

Figure 2. Bruceine D induced apoptosis in K562 cells. K562 cells were treated with 12.0 μM bruceine D for 0, 12, 24 and 36 h; apoptosis rates were then measured by flow cytometry. (A) Apoptosis induced by Bruceine D. (B) Data analysis of (A). Values were expressed as mean ± SD of three determinations. $P < 0.05$ and $P < 0.01$ represented a significant difference among groups.

bromphenol blue, 0.1 M dithiothreitol). After being boiled at 100°C for 20 min, the lysates in the eppendorff were centrifuged and the supernatant was collected.

For subcellular fractionation, the pellet was resuspended with 5-fold volume of ice-cold cell extract buffer (20 mM Hepes-KOH (pH 7.5), 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 250 mM sucrose, 0.1 mM PMSF and 0.02 mM aprotinin) for 40 min at 4°C. The supernatant was collected after repeated centrifugation and lysed in 5 × loading buffer (250 mM Tris-Cl (pH 6.8), 50% glycerol, 10% sodium dodecylsulphate, 1.25‰ bromphenol blue, 0.5 M dithiothreitol). The mixture was then heated at 100°C for 15 min and centrifuged.

Equal amounts of protein samples were loaded onto 8-12% sodium SDS-PAGE and transferred to PVDF membranes (Millipore, USA) and the membranes were incubated with specific primary antibodies. Proteins were then visualized using HRP-conjugated secondary antibody and a Phototope™-HRP Detection Kit (Cell Signaling, USA) on Kodak medical X-ray processor (Kodak, USA).

Statistical analysis

All values were expressed as mean ± SD on the basis of three or more replicates of each experiment. Statistical analysis was conducted by either t-test or one-way ANOVA with SPSS 13.0 software (SPSS Inc., USA). Statistical significance was adopted at $p < 0.05$ and $p < 0.01$.

Results

Effect of bruceine D on K562 cell viability

Cell growth inhibition of bruceine D was assessed by MTT assay. Bruceine D inhibited the proliferation of human chronic myeloid leukemia K562 cells potently, with IC50 values of 6.37 ± 0.39 μM.

Bruceine D induced apoptosis in K562 cells

To investigate whether apoptosis induction is involved in the mechanism of action, Annexin V-FITC/PI staining was carried out. When treated with bruceine D at 12.0 μM for 0, 12, 24 and 36 h, the percentages of apoptotic cells in the total cell population were 5.1 ± 3.3%, 10.4 ± 1.3%, 29.5 ± 2.1% and 45.0 ± 2.4%, respec-

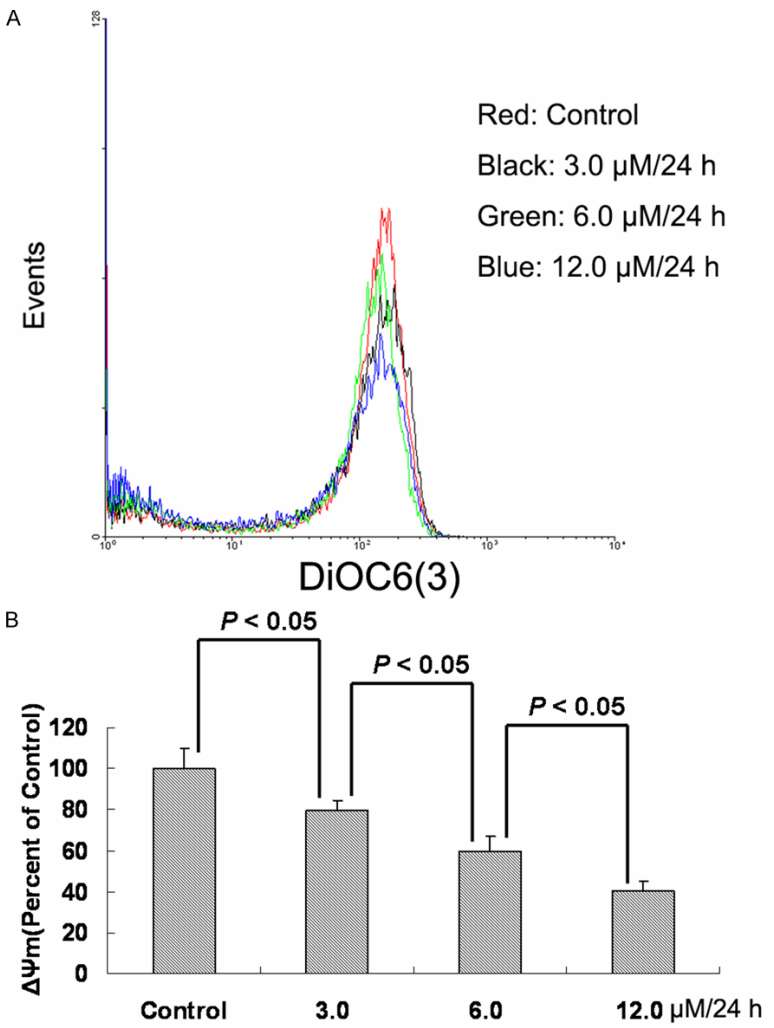

Figure 3. Loss of ΔΨm induced by bruceine D. After bruceine D (0, 3.0, 6.0 and 12.0 μM) treatment for 24 h, cells were incubated with DiOC6(3) and then measured by flow cytometry. A. Loss of ΔΨm was observed in K562 cells. B. ΔΨm levels were exhibited as percentage of MFI compared to control group. Data were expressed as mean ± SD of at least three determinations. $P < 0.05$ and $P < 0.01$ represented a significant difference among groups.

tively, suggesting bruceine D time-dependently induced cellular apoptosis to K562 cells (**Figure 2**).

Loss of ΔΨm induced by bruceine D

ΔΨm was measured after K562 cells were exposed to bruceine D at different concentrations (3.0, 6.0, 12.0 μM) for 24 h. ΔΨm of control were 79.84 ± 4.46%, 59.74 ± 7.48% and 40.66 ± 4.37%, respectively ($P<0.05$). It is clear from **Figure 3** that ΔΨm of K562 cells was significantly attenuated in concentration-dependent manner by bruceine D treatment.

Bruceine D-induced release of cytochrome c

Cytochrome c in cytosol was detected by Western blot. As presented in **Figure 4**, release of cytochrome c increased in dose dependent manner after treatment of bruceine D. Relative gray values (cytochrosome c/GAPDH) for control, 3.0, 6.0 and 12.0 μM bruceine D were 8.58 ± 0.86%, 69.09 ± 5.76%, 123.88 ± 11.63% and 166.04 ± 13.96% in K562 cells, respectively ($P<0.05$).

Bruceine D-induced caspases-9, -3 activation and PARP cleavage

After K562 cells were exposed to tested compounds at 0, 3.0, 6.0, 12.0 μM for 48 h, relative gray values of 37/35 KD caspase-9/GAPDH were 4.68 ± 0.73%, 21.79 ± 0.95%, 33.45 ± 4.07% and 35.24 ± 3.69%, respectively; relative gray values of 17 KD caspase-3/GAPDH were 5.77 ± 0.72%, 41.56 ± 3.88%, 56.92 ± 4.01% and 66.98 ± 2.96%; relative gray values for 89 KD PARP/GAPDH were 6.77 ± 0.54%, 28.28 ± 4.01%, 35.42 ± 2.69% and 47.46 ± 7.80%. The results above imply that BD is able to dose-dependently augment activation of the pro-apoptotic proteins caspase-9,-3 and cleavage of PARP (**Figure 5**).

Bruceine D inhibited phosphorylation of AKT and ERK

To determine whether MAPK and PI3K pathways are related to the anticancer activity of bruceine D, changes of total and phosphorylation forms of AKT and ERK were investigated in K562 cells. The results showed that bruceine D decreased the expression of p-AKT and p-ERK in dose-corresponding and time-dependent manner. On the other hand, the expression of

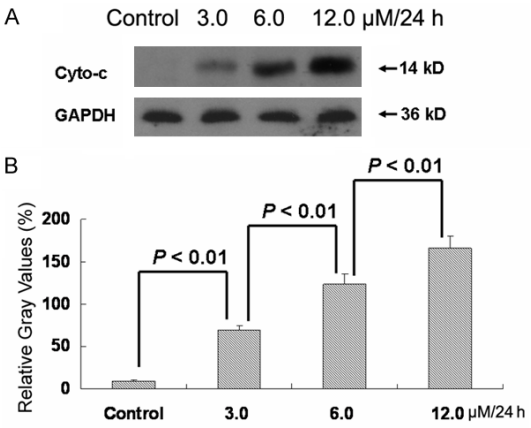

Figure 4. Bruceine D-induced release of cytochrome c. K562 cells were treated with 0-12.0 μM bruceine D for 24 h, then the protein expression of cytochrome *c* was measured by Western Blot. (A) Expression of cytochrome *c* in K562 cells. (B) Gray intensity analysis of Western blot results of (A). GAPDH (36 kDa) was used to ensure equal protein loading. Data were expressed as mean ± SD of at least three determinations. P < 0.05 and P < 0.01 represented a significant difference among groups.

total AKT and ERK didn't change after exposure to bruceine D. When K562 cells were treated with 0, 3.0, 6.0 and 12.0 μM bruceine D for 6 h, relative gray values of p-AKT/GAPDH were 65.90 ± 1.58%, 44.71 ± 3.12%, 26.89 ± 3.40% and 9.76 ± 2.05%, respectively; relative gray values of p-ERK/GAPDH were 86.56 ± 1.79%, 51.07 ± 1.44%, 21.90 ± 1.93% and 3.06 ± 1.01%, respectively (**Figure 6**). When K562 cells were treated with 6.0 μM bruceine D for 0, 2, 4, 6 h, relative gray values of p-AKT/GAPDH were 191.30 ± 8.32%, 111.11 ± 4.33%, 49.90 ± 7.55% and 32.42 ± 6.27%, respectively; relative gray values of p-ERK/GAPDH were 85.47 ± 6.57%, 69.32 ± 2.36%, 53.80 ± 8.19% and 27.79 ± 3.63%, respectively (**Figure 7**).

Discussion

Recent research on different cancer has revealed that herbal medicines and natural products isolated from plants could provide additional strategies for monotherapy or combination treatments [15]. As shown in Newman *et al*'s research, 73% of all approved antitumor drugs worldwide are other than synthetic, with 47% actually being either natural products or directly derived therefrom [16]. In this context, natural products possesses great promise for development and application. Bruceine D is a quassinoid found in abundance in B. *javanica*. Our study showed that it was able to inhibit the growth of human chronic myeloid leukemia K562 cells potently with an IC50 value of 6.37 ± 0.39 μM. Herein, we carried out further investigations to elucidate the possible mechanisms involved.

Under our current understanding, apoptosis plays a critical role in different kinds of physiological processes and pathological conditions. Its deregulation can result in cancer, autoimmune and degenerative diseases [17]. Meanwhile, anticancer agents take their effects mainly by inducing apoptosis. Annexin V-FITC/PI assay confirmed the fact that bruceine D induced apoptosis in K562 cell in time-dependent manner (**Figure 2**).

Two principal signal transduction pathways constitute the basic machinery for triggering apoptosis, death receptor pathway and mitochondrial pathway. Once mitochondrial pathway activated, mitochondrial permeability transition pore opens, ΔΨm decreases and sequestered pro-apoptotic proteins releases from the intermembrane space into the cytosol. After release into the cytoplasm, cytochrome *c* stimulates apoptosome formation (a complex composed of apoptotic protease-activating factor [Apaf-1], dATP, cytochrome *c* and caspase-9) followed by activation of caspase-9. The "initiator" caspase-9 then causes the activation of the "executioner" caspase-3, which cleaves vital substrates PARP, resulting in cellular death [18, 19].

As depicted in **Figure 3**, ΔΨm were dose-dependently decreased in bruceine D-treated K562 cells. Western blot analysis also showed that bruceine D exposure augmented the release of cytochrome *c* in concentration corresponding manner (**Figure 4**). Moreover, activation of caspase-9, -3 and cleavage of PARP were observed in the bruceine D-treated K562 cells (**Figure 5**). Taken together, our results demonstrate that the underlying mechanism of action responsible for the bruceine D-induced cell growth inhibition on K562 cells is the cellular apoptosis via the mitochondrial pathway.

The RAS/RAF/MAP kinase-ERK kinase (MEK)/extracellular-signal-regulated kinase (ERK) (MAPK) pathway and the PI3K/protein kinase B (PKB/AKT)/mammalian target of the rapamycin (mTOR) (PI3K) pathway play important roles in

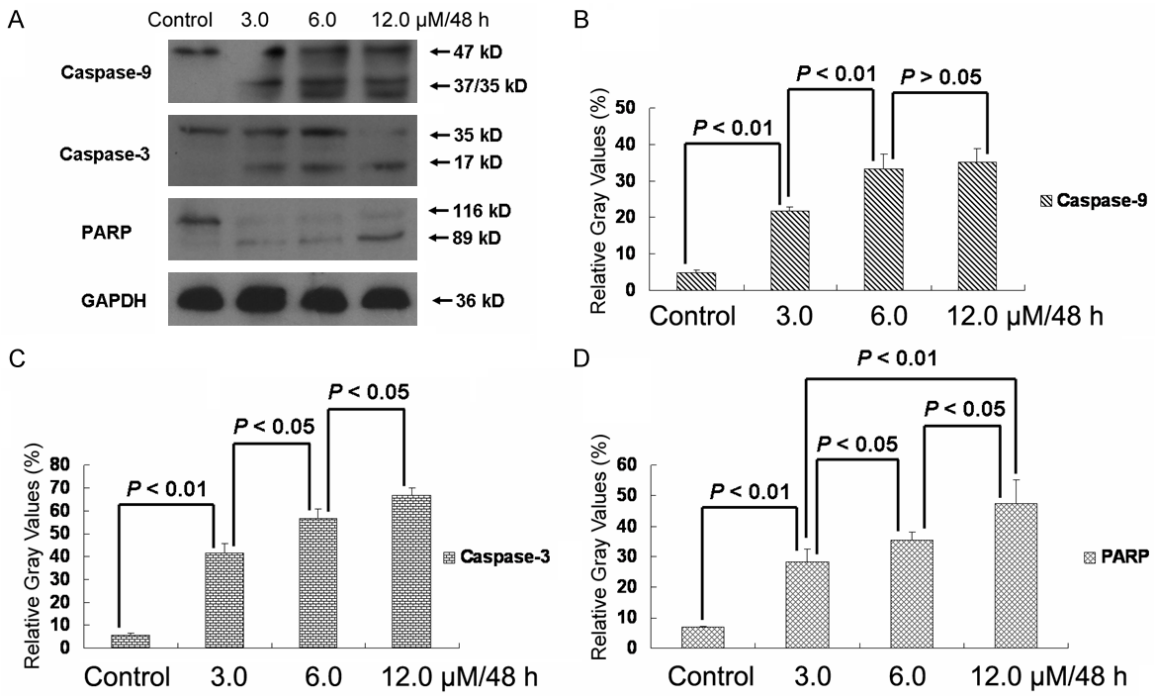

Figure 5. Bruceine D-induced caspases-9, -3 activation and PARP cleavage. K562 cells were treated with 0-12.0 μM bruceine D for 48 h, then the protein expression of caspases-9, -3 and PARP was measured by Western blot. (A) Expression of caspases-9, -3 and PARP in K562 cells (B), (C, D) Gray intensity analysis of Western blot results of caspases-9, -3 and PARP in (A), respectively. GAPDH (36 kDa) was used to ensure equal protein loading. Data were expressed as mean ± SD of at least three determinations. P < 0.05 and P < 0.01 represented a significant difference among groups.

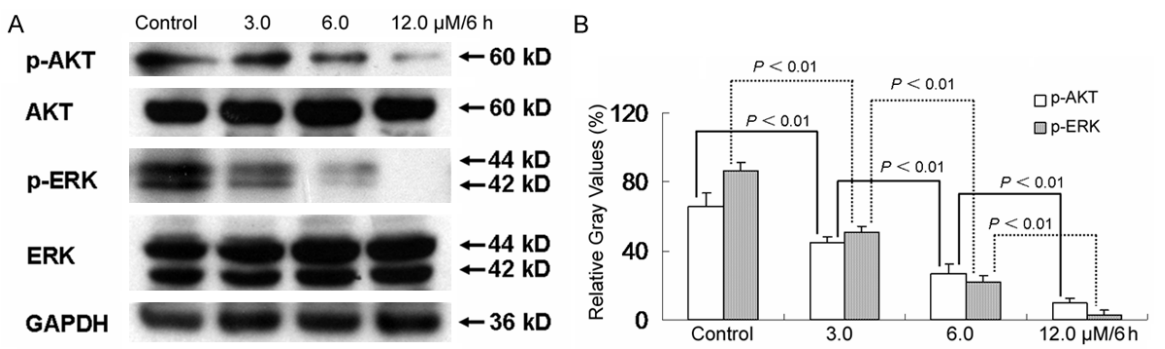

Figure 6. Bruceine D dose-dependently inhibited phosphorylation of AKT and ERK. K562 cells were treated with 0-12.0 μM bruceine D for 6 h, the protein expression of AKT, p-AKT, ERK and p-ERK was then measured by Western blot. (A) Expression of AKT, p-AKT, ERK and p-ERK in K562 cells. (B) Gray intensity analysis of Western blot results of p-AKT and p-ERK in (A). GAPDH (36 kDa) was used to ensure equal protein loading. Data were expressed as mean ± SD of at least three determinations. *P* < 0.05 and *P* < 0.01 represented a significant difference among groups.

cell proliferation, survival, migration, differentiation and metabolism. Deregulation of these two pathways can lead to uncontrolled cell growth and survival, ultimately resulting in oncogenic transformation and progression. Several studies demonstrated that blockade of one pathway may lead to the activation of the other signaling cascade. Blockade of both pathways with combinations of signaling inhibitors might result in a more efficient antitumor effect, as compared with treatment with a single agent [20]. AKT and ERK, which are activated by phosphorylation, are novel molecules in MAPK and PI3K pathways. As shown in **Figures 6** and **7**, phosphorylation of both AKT and ERK were inhibited by bruceine D while the

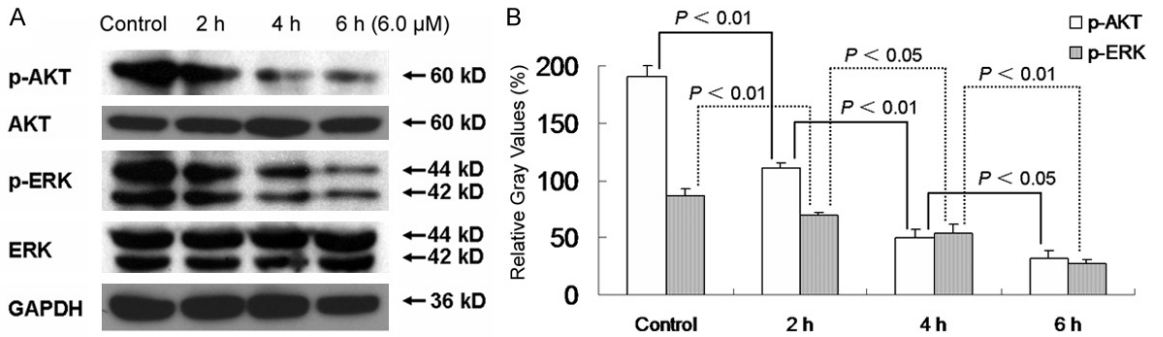

Figure 7. Bruceine D time-dependently inhibited phosphorylation of AKT and ERK. K562 cells were treated with 6.0 μM bruceine D for 0, 2, 4, 6 h, the protein expression of AKT, p-AKT, ERK and p-ERK was then measured by Western blot. (A) Expression of AKT, p-AKT, ERK and p-ERK in K562 cells. (B) Gray intensity analysis of Western blot results of p-AKT and p-ERK in (A). GAPDH (36 kDa) was used to ensure equal protein loading. Data were expressed as mean ± SD of at least three determinations. $P < 0.05$ and $P < 0.01$ represented a significant difference among groups.

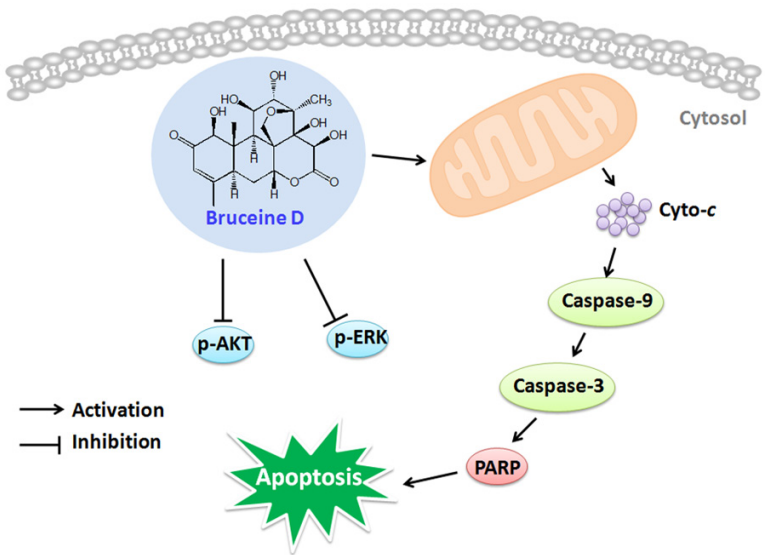

Figure 8. Mechanisms involved in Bruceine D-induced cell growth inhibition on human chronic myeloid leukemia K562 cells.

expression of total AKT and ERK didn't change. It suggests that the MAPK and the PI3K pathways are also responsible for bruceine D-induced cell growth inhibition.

In recent years, majority of human tumors display startling heterogeneity in many morphological and physiological features, such as expression of cell surface receptors, proliferative and angiogenic potential [21]. Treatments with a single targeted agent are not sufficient to treat a genetically heterogeneous tumor, because of the existence of acquired resistance [22]. Herein, treatments with multi-targeted agents capture increasing attention. Bruceine D, in this study, was found to possess anticancer activity through mitochondrial pathway in apoptosis, MAPK signaling and PI3K pathways (summarized in **Figure 8**). It has the potential to be a multi-targeted agent and improve chemotherapeutic effect. However, further experiments to evaluate the bruceine D-induced interaction among these three pathways are still needed.

Conclusion

To conclude, the present study provides evidence demonstrating that bruceine D has potent anti-proliferative activity against human chronic myeloid leukemia K562 cells. Bruceine D induces apoptosis in K562 cells via the mitochondrial pathway. Bruceine D also inhibits phosphorylation of AKT and ERK which involved in MAPK and PI3K pathways respectively.

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Disclosure of conflict of interest

None.

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