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Full Length Research Paper

Brazilein overcame ABCB1-mediated multidrug resistance in human leukaemia K562/AO2 cells

Liyang Tao[1]*, Jianying Li[2] and Jianye Zhang[3]

[1]Department of Pathology, Guangzhou Medical College, Guangzhou, 510182, China.
[2]Department of Nephrology, the First Affiliated Hospital, Sun Yat-sen University, Guangzhou, 510080, China.
[3]Department of Pharmaceutical Sciences, Guangzhou Medical College, Guangzhou, 510182, China.

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Multidrug resistance (MDR) was still a major obstacle to the success of chemotherapy in cancer treatment. One of the underlying mechanisms of MDR was cellular overexpression of ABCB1 transporter which pumped various anticancer drugs out of the cells. Here, we investigated the anticancer activity of brazilein (a compound isolated from *Caesalpinia sappan* Linn.) against human leukaemia K562 and ABCB1 overexpression K562/AO2 cells. Cytotoxicity of brazilein was examined using 3-(4, 5-dimethylthiazol-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. FITC-Annexin V/PI staining was used to detect apoptotic cells. The expression of ABCB1 was determined by western blotting and RT-PCR assays, respectively. Flow cytometry was used to determine the intracellular accumulation of doxorubicin. The ATPase activity of ABCB1 was estimated using Pgp-Glo™ Assay Systems. MTT and apoptotic analysis showed that brazilein exerted similar cytotoxicity against K562 and K562/AO2 cells. The IC_{50} values were 5.45 ± 0.36 and 5.62 ± 0.43 µmol/L for K562 and K562/AO2 cells, respectively. Western blotting and RT-PCR assays showed that brazilein did not affect the expression of ABCB1 in K562/AO2 cells. The fluorescence intensity assay demonstrated that brazilein did not promote the intracellular accumulation of doxorubicin. Luminescent ATPase assays proved that brazilein did not interfere with the ATPase of ABCB1. Our results showed that brazilein was not a substrate of ABCB1 and escaped the excretion of ABCB1 transporter to overcome ABCB1-mediated MDR. The present data suggested that brazilein would be promising to develop as an anticancer candidate for circumventing multidrug resistance.

Key words: Brazilein, multidrug resistance (MDR), ABCB1 transporter, K562/AO2 cells.

INTRODUCTION

Cancer is a major public health problem in the world and is the first cause of death in persons younger than 85 years (Jemal et al., 2010). Chemotherapy is the popular usage to treat cancer, because about 50% of total cancer cases do not respond to local excision or radiation. Although, progress has been made in chemotherapy, the development of multidrug resistance (MDR) is still a significant obstacle to the success of chemotherapy in many cancers (Perez-Tomas, 2006). The most common cause of MDR is the overexpression of ATP binding cassette (ABC) transporters, which actively extrude a broad range of anticancer drugs out of the cancer cells (Lee, 2010). Forty-nine members in this ABC transporter superfamily have been identified and were divided into seven subfamilies (A to G) based on sequence similarities (Liu et al., 2005). The ABC transporter subfamily B member 1 (ABCB1), also named P-glycoprotein (Pgp) or MDR1, is the most prevalent and important cause of MDR (Baguley, 2010). Most published work has concentrated on the drug transporters to overcome MDR. *C. sappan* Linn. has long been used in traditional medicine in China to promote blood circulation, treat thrombosis or antagonize inflammation (Jeong et al., 2009; Washiyama et al., 2009). Brazilein, a compound isolated from *C. sappan*, has exhibited some pharmacological activities,

*Corresponding author. E-mail: sohutly@163.com. Tel: +86-(20)-813-403-47.

such as cardiotonic effect, immunosuppressive activity, neuroprotective effect (Ye et al., 2006; Zhao et al., 2006; Shen et al., 2007). However, in a recent research, brazilein showed anticancer activity in human hepato-cellular carcinoma HepG2 cells (Zhong et al., 2009). Here, we showed that brazilein exhibited the potent anticancer activity against ABCB1-overexpression human leukaemia K562/AO2 cells. It was deserved to explore the action mechanisms of brazilein on overcoming ABCB1-mediated MDR.

MATERIALS AND METHODS

Chemicals and reagents

PRMI 1640 media was obtained from Gibco BRL. (Gaithersburg, MD). FITC-Annexin V/PI apoptosis assay kit was purchased from Molecular Probes, Lnc. (Eugene, OR). ABCB1 and GAPDH antibodies were products of Santa Cruz Biotechnology. Trizol Reagent and the First-Strand cDNA Synthesis Kit were bought from Molecular Research Center. The Pgp-Glo™ Assay Systems were acquired from Promega Biotech Co., Ltd (Madison, WI). 3-(4, 5-dimethylthiazol-yl)-2, 5-diphenyltetrazolium bromide (MTT), doxorubicin (DOX), verapamil (VRP) and other chemicals were purchased from Sigma Chemical Co. Brazilein (purity >98% according to high performance liquid chromatography (HPLC)) was provided by PhD. Xu ZL (Sun Yat-Sen University, Guangzhou).

Cell lines

The human drug-resistant leukaemia cell line K562/AO2 (ABCB1-overexpression) and its parent drug-sensitive cell line K562 were cultured in PRMI 1640 medium containing 100 U/ml penicillin, 100 µg/ml streptomycin and 10% fetal bovine serum (FBS). The cells were maintained in a humidified atmosphere incubator of 5% CO_2 at 37℃ (Liu et al., 2010).

Cell proliferation assay

Cells were distributed evenly into 96-well microtiter plates at a density of 3×10^3 cells/well in a final volume of 190 µl/well. After 12 h incubation, 10 µl medium containing various concentrations of brazilein was added into the wells. After 68 h treatment, 20 µl MTT (5 mg/ml) was added to each well and the plate was additionally incubated for 4 h. Subsequently, the plate was centrifugated at 1, 5000 rpm for 10 min and the supernatant was removed. Thereafter, 100 µl of dimethylsulfoxide (DMSO) were added into each well to dissolve the formazan crystals. Finally, the cell viability was measured by Model 550 microplate reader (BIO-RAD, USA) at 540 nm with 655 nm as reference filter. The concentrations required to inhibit growth by 50% (IC_{50}) were calculated from survival curves. Experiments were performed at least three times. The percent of survival was calculated by the following formula: (mean experimental absorbance/mean control absorbance) × 100%.

Cell apoptosis assay

K562 and K562/AO2 cells were treated with brazilein at various concentrations for 48 h. Then, the cells were harvested and washed with ice-cold PBS at 4℃. After stained cells with FITC-Annexin V and PI for 30 min, apoptotic cells were detected from 10,000 cells with Coulter Epics XL-MCL™ Flow Cytometer (Beckman Coulter, USA), according to manufacturer instruction. Finally, the percent of apoptotic cells was calculated by CXP software.

Western immunoblotting

Cells were lysed by 1 × SDS sample buffer and equal amount of total lysate protein was separated on 8 to 10% SDS-PAGE gel. The protein was electrotransfered to PVDF membrane (Pall, USA). After transfer, the membrane was incubated in Tris-Buffered Saline with Tween-20 (TBST) buffer containing 5% nonfat milk for 1 h to block the nonspecific binding sites. Then the specific primary antibody was added at the appropriate dilution with gentle agitation overnight at 4℃. Thereafter, the HRP-conjugated secondary antibody was added for additional 1 h incubation at room temperature. Finally, the immunoblots were visualized with 10 ml LumiGLO (Cell Signaling, USA) and exposed to x-ray film. GAPDH was used as a loading control.

Reverse transcription-PCR

Total cellular RNA was isolated by Trizol Reagent (Molecular Research Center, USA) and the First-Strand cDNA was reversely transcribed by Oligo dT primers, according to the manufacturer instruction. PCR primers were 5'-ccc atc att gca ata gca gg-3' (forward) and 5'-gtt caa act tct gct cct ga-3'(reverse) for ABCB1 and 5'-ctt tgg tat cgt gga agg a-3' (forward) and 5'-cac cct gtt gct gta gcc-3' (reverse) for GAPDH, respectively. The cDNA was amplified in a 35-cycle PCR reaction using the GeneAmp PCR system 9700 thermal cycler (PE Applied Biosystems, USA). The reaction was carry out at 94℃ for 2 min; then repeat 35 cycles of 30 s at 94℃, 30 s at 58℃ and 1 min at 72℃; finally, extend at 72℃ for 10 min. Products were resolved using 1.5% agarose gel electrophoresis. Expected PCR products were 157 bp for ABCB1 and 475 bp for GAPDH, respectively.

DOX accumulation assay

The effect of brazilein on the intracellular accumulation of DOX was measured by flow cytometry as previously described (Tao et al., 2009). Briefly, 3×10^3 cells were seeded in 6-well plate and treated with vehicle or brazilein of desired concentration for 3 h at room temperature. Then, DOX at a final concentration of 10 µmol/L was added for additional 3 h incubation. Finally, the cells were collected and prepared for Cytomics FC500 flow cytometric analysis (Beckman Coulter, USA). The X-mean values of FL-2 were used to indicate intracellular concentration of DOX. The relative values were calculated by dividing the fluorescene intensity of treated cells by that of untreated control cells. Verapamil (VRP) was used as a positive control.

ABCB1 ATPase activity assay

The effect of brazilein on the ABCB1 ATPase activity was estimated by Pgp-Glo™ Assay Systems as previously described (Tao et al., 2009). Briefly, various concentrations of brazilein was incubated in assay buffer containing 5 mmol/L MgATP and 25 µg recombinant human Pgp membranes for 40 min at 37℃. Then, ATP detection buffer was added to stop reaction and initiate luminescence. After reaction for 20 min, the white opaque 96-well plate (corning, USA) was read on spectraMax M5 luminometer (molecular devices, USA). Sodium orthovanadate (Na_3VO_4) and verapamil (VRP) were used as control to demonstrate drug-inhibited and drug-stimulated activity of Pgp ATPase, respectively (Ambudkar et al., 1999).

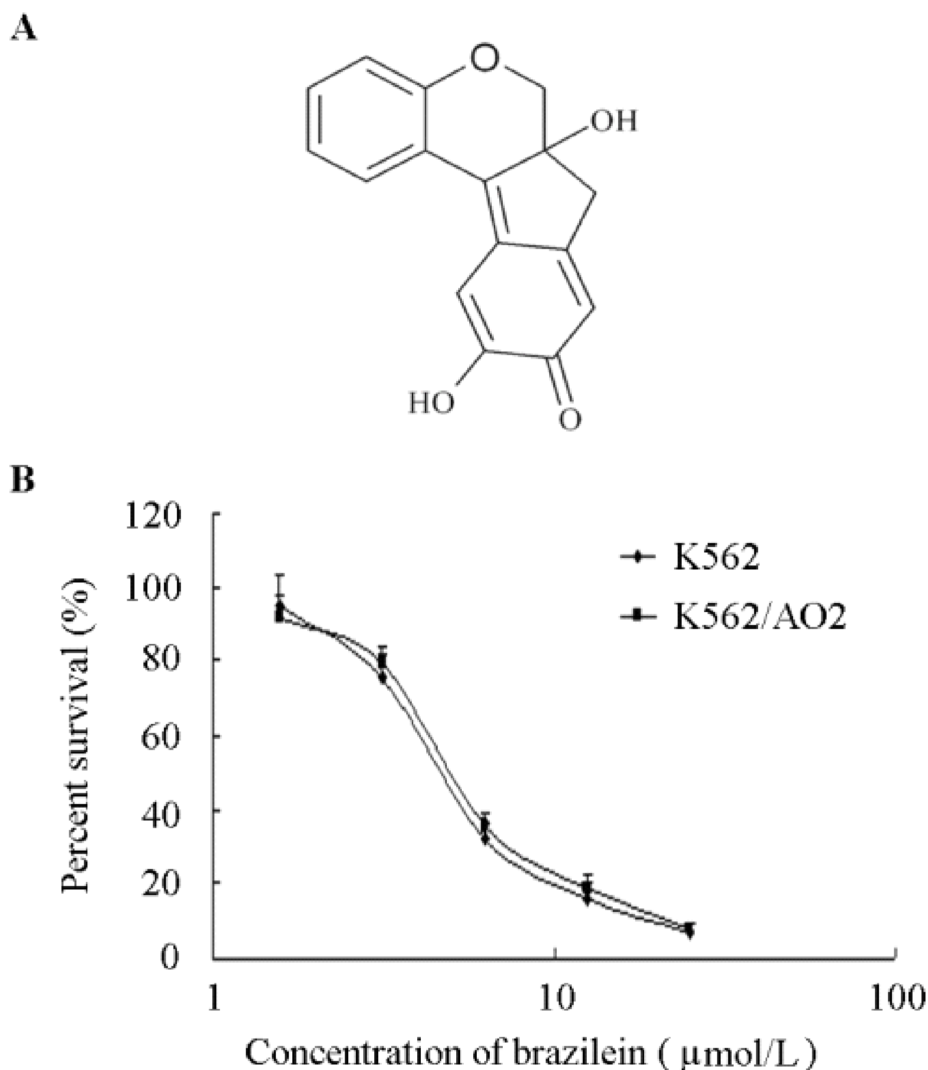

Figure 1. similar cytotoxic activity exerted by brazilein against human leukaemia drug-sensitive K562 and drug-resistant ABCB1-overexpression K562/AO2 cells. (A) The chemical structure of brazilein. (B) Brazilein showed potent cytotoxicity to K562 and K562/AO2 cells. The cells were exposed to the indicated concentrations of brazilein for 72 h. Cytotoxicity was measured by MTT assays as described in methods. Each point represents the mean ± standard deviations (SDs) for three determinations.

RESULTS

Brazilein exerted similar cytotoxicity against human leukaemia cells K562 and K562/AO2

The cytotoxicity of brazilein in drug-sensitive K562 cells and its ABCB1-overexpression drug-resistant K562/AO2 cells was determined by MTT assay. The IC_{50} values were 5.45 ± 0.36 and 5.62 ± 0.43 μmol/L for K562 and K562/AO2 cells, respectively (Figure 1). The statistical results showed that the antitumor activity of brazilein had no significant difference between K562 and K562/AO2 cells by Student's *t* test ($P > 0.05$). The MTT results demonstrated that brazilein overcame ABCB1-mediated MDR in human leukaemia cells.

Brazilein induced significant apoptotic death in human leukaemia K562 and K562/AO2 cells

In order to further investigate whether brazilein exerted potent cytotoxicity against ABCB1-overexpression K562/AO2 cells, the cell apoptosis was detected by FITC-Annexin V/PI staining and flow cytometry analysis.

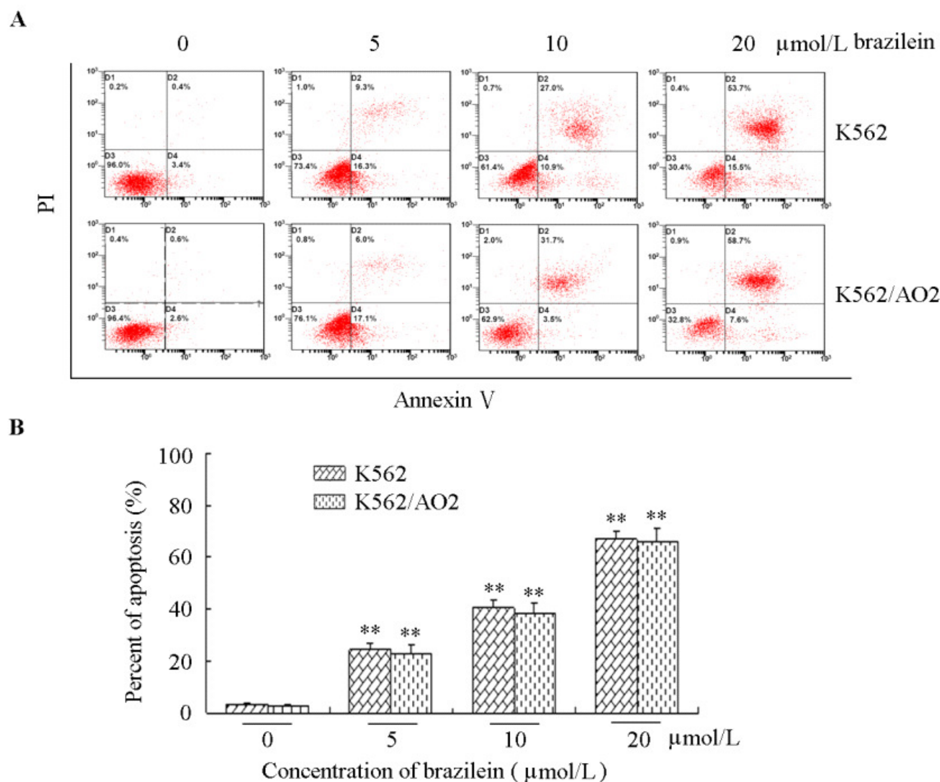

Figure 2. Brazilein induced apoptosis in human leukaemia K562 and ABCB1-overexpression K562/AO2 cells. (A) Brazilein induced apoptosis in K562 and K562/AO2 cells. The cells were treated with 0, 5, 10 and 20 μmol/L brazilein for 48 h, respectively. The harvest cells were stained by FITC-Annexin V and PI, and apoptotic cells were determined by flow cytometric analysis as described in methods. All these experiments were repeated at least thrice, and a representative example was shown. (B) Brazilein increased the proportion of apoptotic cells in a dose-dependent manner in K562 and K562/AO2 cells. The percentage of apoptotic cells was evaluated by CXP software. After treatment 48 h with 0, 5, 10 and 20 μmol/L brazilein, the percentage of apoptotic cells was increased from 3.41 ± 0.55 to 24.67 ± 2.08, 40.33 ± 3.51 and 66.67 ± 3.64% in K562 cells, and 2.82 ± 0.57 to 23.01 ± 3.60, 38.23 ± 4.06 and 65.77 ± 5.51% in K562/AO2 cells, respectively. Columns, means of triplicate determinations; bars, SDs. **P < 0.01, compared with the untreated control in K562 and K562/AO2 group, respectively.

Treated with 0, 5, 10 and 20 μmol/L brazilein for 48 h, the proportion of apoptotic cells was increased from 3.41 ± 0.55 to 24.67 ± 2.08, 40.33 ± 3.51 and 66.67 ± 3.64% in K562 cells, and 2.82 ± 0.57 to 23.01 ± 3.60, 38.23 ± 4.06 and 65.77 ± 5.51% in K562/AO2 cells, respectively (Figure 2). Compared with K562 and K562/AO2 cells, no significant difference was found in apoptotic analysis. The present data demonstrated that brazilein was not resistant to ABCB-mediated MDR.

Brazilein did not alter the mRNA and protein expression of ABCB1 in K562/AO2 cells

Inhibiting transporters function or decreasing their expression was the main strategy to reverse ABC transporter-mediated MDR (Nobili et al., 2006; Tao et al., 2009). Therefore, we first determined the effect of brazilein on the expression of ABCB1. As showed in Figure 3, brazilein at 5, 10 and 20 μmol/L, did not significantly alter the protein and mRNA expression of ABCB1 by western blotting and RT-PCR assay, respectively. These data showed that brazilein did not mediate the expression of ABCB1 to exert potent cytotoxicity against human leukaemia MDR cells.

Brazilein did not enhance the accumulation of DOX in ABCB1-overexpression K562/AO2 cells

Previous western blotting and RT-PCR results showed that brazilein did not affect the expression of ABCB1. Thus, we next explored whether brazilein inhibited the function of ABCB1. Doxorubicin (DOX) was a substrate of

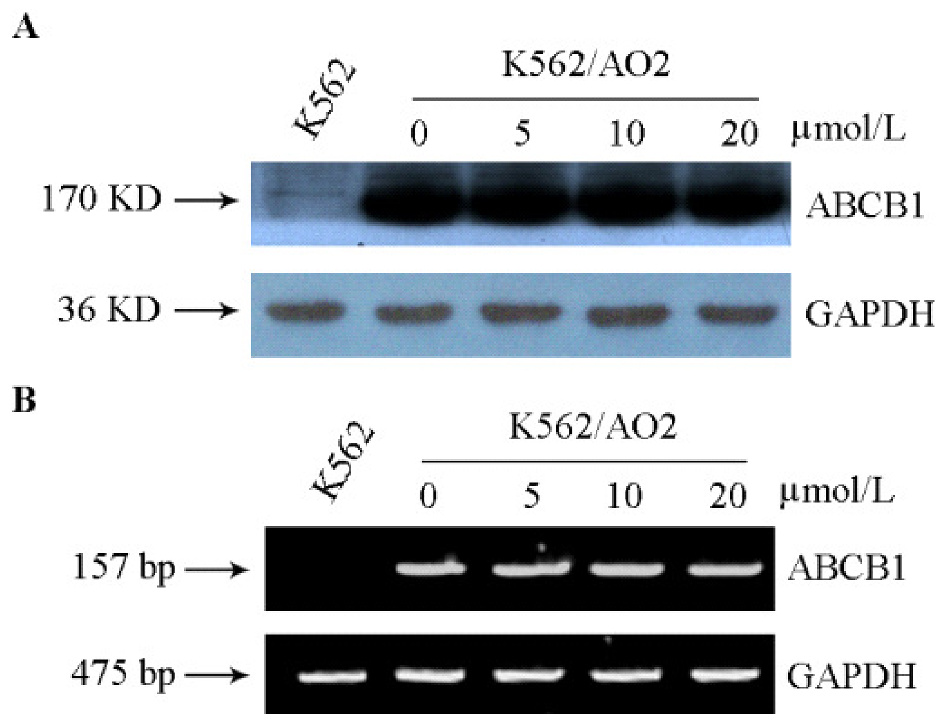

Figure 3. Brazilein had no effects on the protein and mRNA expression of ABCB1 in K562/AO2 cells. (A) Brazilein did not alter the expression of ABCB1 in the protein level. K562/AO2 cells were treated with brazilein at 0, 5, 10 and 20 μmol/L for 48 h, respectively. Equal amounts of total cell lysates were used for loading and detected with western blotting. K562 cells were used as a negative control to demonstrate the specific immunoblotting of ABCB1. (B) Brazilein did not alter the expression of ABCB1 in the mRNA level. After treated K562/AO2 cells at the indicated concentration for 48 h, the mRNA level of ABCB1 was determined by RT-PCR as described in methods. All these experiments were repeated at least thrice, and a representative experiment is shown in each panel.

ABCB1 and is usually used to indicate the pump function of ABCB1 transporter. We treated K562/AO2 cells with 5, 10 and 20 μmol/L brazilein, and the fluorescence of DOX was detected by flow cytometer. The results showed that brazilein at various concentrations did not significantly elevate the intracellular DOX accumulation (Figure 4). However, verapamil (VRP), an ABCB1 reversal agent, significantly increased the intracellular level of DOX (Figure 4). These results suggested that brazilein did not inhibit ABCB1-mediated transport in K562/AO2 cells.

Brazilein did not affect the ATPase activity of ABCB1

ABCB1 was an ATP-dependent drug efflux pump and compounds could interact with its ATPase to reverse MDR. To investigate whether brazilein stimulated or inhibited the ATPase activity of ABCB1, we performed luminescent ABCB1 ATPase assay. ATP was first incubated with ABCB1 and compounds. Then, the remaining unmetabolized ATP was detected using light-generating reaction of firefly luciferase to reflect ATP consumption by ABCB1. Thus, the luciferase-generated luminescent signal would be lower or higher than that of untreated sample. Figure 5 showed that brazilein had no significant effect on the ATPase activity of ABCB1 when compared with the untreated control. Na_3VO_4 and VRP were used as controls to show the inhibition or stimulation activity of ABCB1 ATPase, respectively (Figure 5).

DISCUSSION

Nearly one-third of the cancers were diagnosed as leukemia's in children ages birth to 14 years (Liu et al., 2010). Chemotherapy was the main measure to treat cancer patients of leukemia. However, multidrug resistance was still a major obstacle to the effective treatment of cancer. Generally, MDR was the result of over expression of certain transporters involved B in pumping drugs out of the cells, after exposure to chemotherapeutic drugs over a period of time (Gottesman et al., 2002). Expression of the ABC transporter ABCB1 (P-glycoprotein (Pgp)) has generally been reported to correlate with MDR in leukemia (Ross, 2000; Mahadevan and List, 2004). ABCB1 was implicated as a major factor

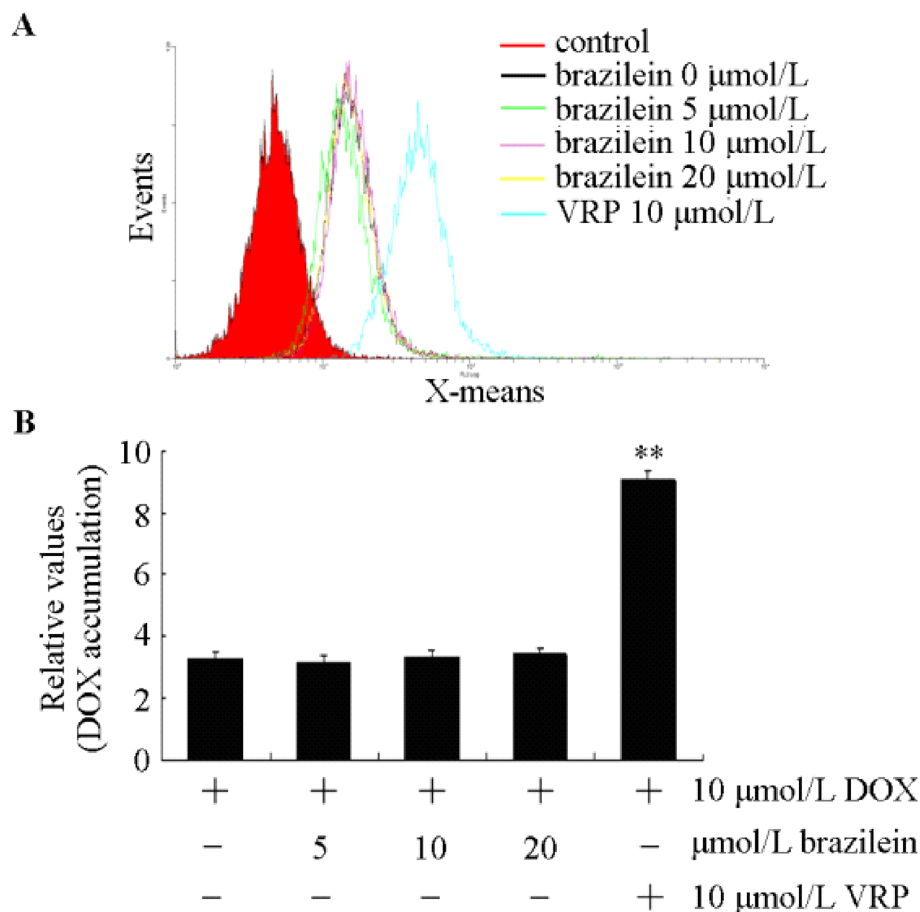

Figure 4. Brazilein did not affect the intracellular accumulation of DOX in K562/AO2 cells. (A) Brazilein did not increase the intracellular concentration of doxorubicin (DOX) in K562/AO2 cells. K562/AO2 cells were incubated with brazilein (0, 5, 10 and 20 μmol/L) at 37°C for 3 h, then 10 μmol/L DOX was added for another 3 h incubation. The fluorescene intensity of DOX was detected by flow cytometer with an excitation wavelength of 488 nm. Verapamil (VRP, 10 μmol/L) was used as a positive control. Independent experiments were performed at least three times, and a representative experiment was shown. (B) Effects of brazilein on the accumulation of DOX. The accumulation of DOX was measured by flow cytometric analysis as described in methods. X-mean values were used to indicate the intracellular fluorescene intensity of DOX. The results were presented as fold change in fluorescence intensity relative to untreated control MDR cells. The untreated control MDR cells did not receive brazilein or DOX treatment. Columns, means of triplicate determinations; bars, SD. **P < 0.01, versus the only DOX treatment group.

factor in MDR and could efflux a wide substrate drugs from the cells, such as the anthracyclines, vinca alkaloids, taxanes, epipodophyllotoxins and imatinib mesylate (Ambudkar et al., 2003; Dulucq et al., 2008). Therefore, the development of new chemotherapeutic agents that were not substrates of ABCB1 transporter showed significant therapeutic benefit in cancer treatment.

C. sappan Linn. has been used as traditional Chinese medicine for centuries and exhibited various pharmacological activities (Kim et al., 2005; Jeong et al., 2009; Washiyama et al., 2009). Brazilein was a compound isolated from *C. sappan* Linn., and which also exhibited significantly pharmacological activities, including antitumor activity (Ye et al., 2006; Zhao et al., 2006; Shen et al., 2007; Shen et al., 2008; Zhong et al., 2009). In this study, we reported that brazilein exerted potent anticancer activity against human leukaemia K562 and K562/AO2 (ABCB1-overexpression) cells (Figure 1). FITC-Annexin V/PI staining was an effective approach to detect apoptosis. Using this method, we found that brazilein induced apoptotic cell death in a dose-dependent manner in K562 and K562/AO2 cells (Figure 2). MTT and apoptosis assays proved that brazilein overcame ABCB1-mediated MDR in human leukaemia cells. The inhibition or transcriptional regulation of ABC

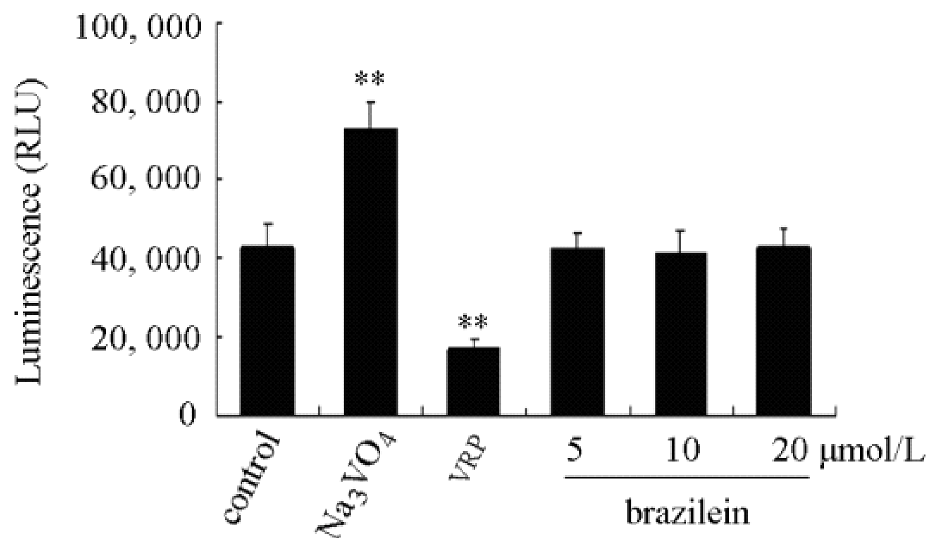

Figure 5. Brazilein had no effects on the ATPase activity of ABCB1. Luminescent ABCB1 ATPase activity assays were performed according to Pgp-Glo™ Assay Systems instruction. Na_3VO_4 and VRP were used as controls to inhibite and stimulate the ATPase activity of ABCB1, respectively. Columns, means of triplicate determinations; bars, SD. **P < 0.01, versus the untreated control group.

transporter, or development nonsubstrate drugs of ABC transporter were the three mainly approaches to overcome MDR in cancer cells (Gillet et al., 2007). Therefore, we used western blotting and RT-PCR assays to determine the expressed changes of ABCB1, and DOX accumulation and ABCB1-ATPase activity assay were used to detect the targeted inhibition of ABCB1 transporter. Figure 3 showed that brazilein had no effect on the protein and mRNA expression of ABCB1. Subsequent studies demonstrated that brazilein did not elevate the intracellular DOX concentration in ABCB1-overexpression K562/AO2 cells or did not affect the ATPase activity of ABCB1 transporter (Figures 4 and 5). The collective data suggested that brazilein was not a substrate of ABCB1 transporter.

Conclusions

In conclusion, our results showed that brazilein overcame ABCB1-mediated MDR in human leukaemia K562/AO2 cells. Further studies showed that the inhibition or transcriptional regulation of ABCB1 was not involved in its action mechanisms. The results of the present study suggested that brazilein was not a substrate of ABCB1 transporter. In this regard, brazilein would be a promising anticancer candidate to develop for circumventing ABCB1-mediated MDR.

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