

***In Vitro* and *In Vivo* Characterizations of Tetrandrine on the Reversal of P-Glycoprotein-mediated Drug Resistance to Paclitaxel**

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Abstract. *Background:* Multidrug resistance (MDR) is one of the major obstacles limiting the efficacy of cancer chemotherapy. Through screening a series of natural products, we have previously identified six naturally occurring bisbenzylisoquinoline alkaloids (BBIs) that possess potent activity to reverse P-glycoprotein (gp)-mediated drug resistance. *In this study, we characterized one of these compounds, termed tetrandrine, and evaluated its reversal activity on P-gp-mediated drug resistance to paclitaxel in vitro and in vivo. Materials and Methods:* Using the human MDR tumor cell line KBv200 and its drug sensitive parental cell line, the reversal activity of tetrandrine on P-gp-mediated resistance to paclitaxel and docetaxel were determined by MTT and other in vitro drug evaluation assays. Further, through establishment of xenograft models bearing the intrinsically resistant KBv200 tumor, we also examined the effect of tetrandrine on potentiating the antitumor activity of paclitaxel in vivo. *Results:* In vitro studies showed that co-administration of tetrandrine at 2.5 μ M, which has little cytotoxicity alone, reversed the sensitivity of KBv200 cells to paclitaxel and docetaxel around 10-fold. In vivo

experiments also demonstrated that tetrandrine significantly potentiated the antitumor activity of paclitaxel in xenograft models bearing the intrinsically resistant KBv200 tumors. In addition, accumulation and efflux studies with [3 H]-paclitaxel indicated that tetrandrine increases the intracellular accumulation of [3 H]-paclitaxel in MDR cells through inhibition of P-gp-mediated drug efflux. *Conclusion:* The present in vitro and in vivo studies demonstrated that tetrandrine possesses potent and specific activity in reversing P-gp-mediated drug resistance. This naturally occurring compound may be used as a chemosensitizer in the treatment of P-gp-mediated MDR cancers.

Multidrug resistance (MDR) is one of the most common causes of failure of cancer chemotherapy. Many important anticancer drugs, such as *vinca* alkaloids (vinblastine, vincristine, vinorelbine), anthracyclines (doxorubicin, daunorubicin, epirubicin, idarubicin) and taxanes (paclitaxel and docetaxel) are involved in the so-called MDR (1, 2). The phenomenon of MDR can be intrinsic or acquired, depending on the time of its occurrence. Although there are several different mechanisms associated with the development of MDR, the overexpression of a M_r 170,000 trans-membrane glycoprotein (P-gp) is believed to be the major cause. P-gp is the product of the *mdr1* gene and belongs to the ABC superfamily of transporter proteins. It acts as an energy-dependent drug efflux pump that can prevent the intracellular accumulation of drugs by expelling them from cells before they are able to interact with their cellular targets (3, 4). Thus, the development of resistance to various classes of anticancer drugs is mainly due to the increased efflux of drugs, which in turn results in their decreased intracellular accumulation.

A promising strategy to conquer MDR is to develop functional MDR modulators, also called chemosensitizers, that can inhibit P-gp and/or MRP activities (5). In recent years, a broad range of compounds have been reported to possess potential activities to reverse the MDR phenotype.

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Abbreviations: MDR, multidrug resistance; P-gp, P-glycoprotein; TET, tetrandrine; PTX, paclitaxel; DTX, docetaxel; VCR, vincristine; Dox, doxorubicin; BBIs, bisbenzylisoquinoline alkaloids; MTT, 3-(4,5-dimethylthiazol-yl)-2,5-diphenyltetrazolium bromide.

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These compounds can be obtained either from natural products or by chemical synthesis. Based on their different structures and biological functions, these potential modulators include calcium channel blockers (*e.g.*, verapamil, nifedipine) (6), calmodulin antagonists (*e.g.*, trifluoperazine, chlorpromazine) (7), various steroids (*e.g.*, progesterone, tamoxifen) (8), quinolines (*e.g.*, MS-209, chloroquine, quinidine) (9), immunosuppressive drugs (*e.g.*, cyclosporine, rapamycin) (10), antibiotics (*e.g.*, rifapicin, racyclines) (11), surfactants (*e.g.*, Tween 80, Cremophor EL) (12) and yohimbine alkaloids (*e.g.*, reserpine, yohimbine) (13). Most of these compounds have shown some reversal activity of P-gp-mediated drug resistance *in vitro*, and some have also exhibited effectiveness in animal experiments (3, 14). Combined therapy with MDR-related anticancer drugs and MDR modulators could shrink tumor size and prolong the lifespan in animal models. For example, calcium channel blockers such as verapamil have been extensively studied and shown to have some effect on the inhibition of P-gp function. However, these agents are relatively weak P-gp inhibitors, and data regarding their clinical efficacy are not yet available. Moreover, these MDR reversal agents may also possess unacceptable side-effects or toxicity at doses required for effectiveness (15). These limitations have spurred efforts to search for new, more effective compounds to specifically reverse MDR through inhibition of P-gp activity.

Through screening a series of compounds isolated from natural plants, we have recently identified six naturally occurring bisbenzylisoquinoline alkaloids (BBIs) that possess potent activities to reverse P-gp-mediated drug resistance (16). When compared with verapamil, one of the best-known MDR modifiers (6), these BBIs not only showed much stronger activities in the reversal of P-gp-mediated drug resistance, but also exhibited little cytotoxicity by themselves (16). These findings suggest that these compounds possess great promise as a novel class of MDR modifiers. Through examining their chemical structures, one of these BBIs was identified as tetrandrine, that has been used as herbal medicine for the treatment of silica-induced pulmonary fibrosis in China in the 1970-1980s (17, 18). Considering its previous application and relatively low toxicity in humans, tetrandrine was selected in the present study to evaluate its reversal activity on P-gp-mediated drug resistance *in vitro* with cultured MDR tumor cells and *in vivo* with tumor-bearing animal models and also its potential mechanisms.

Materials and Methods

Drugs and chemicals. Tetrandrine (TET) was obtained from Conba Pharmaceutical Co. (Jinhua, PRC) and dissolved in 0.05N HCl and neutralized with NaOH. Paclitaxel (PTX), docetaxel (DTX) and vincristine (VCR) were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and dissolved in dimethyl sulfoxide (DMSO).

Doxorubicin (DOX) were also purchased from Sigma and dissolved in water. [³H]-Paclitaxel (1.0 mCi/ml) was purchased from Moravек Biochemicals, Inc. (Brea, CA, USA).

Cell lines and cell culture. The MDR cell line KBv200 and its drug-sensitive parental cell line KB are human epidermoid carcinoma cell lines obtained from the Chinese Academy of Medical Sciences (Beijing, China). KBv200 is a MDR cell line with a high expression of P-gp, being about 70-fold resistant to vincristine (19) in comparison to its drug-sensitive parental KB cell. Both KBv200 and KB cells were cultured in RPMI 1640 medium with 10% fetal bovine serum and 1% antibiotic-antimycotic solution (Gibco Life Technologies Inc., Rockville, MD, USA). To keep the feature of resistance, KBv200 cells were maintained in the medium containing 100 nM of vincristine. The vincristine was removed by several washes with fresh drug-free media before experimental treatments.

In vitro cytotoxicity assays. Cell viability was determined by modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylazolum bromide dye reduction method (the MTT assay), as described previously (20, 21). Briefly, cells were harvested with trypsin and resuspended to a final density of 4×10^4 cells/ml. Aliquots of 200 μ l from each cell suspension were distributed evenly into 96-well tissue culture plates (Falcon, Oxnard, CA, USA). After the cells had been incubated for 24 h, designated wells were treated with different concentrations of paclitaxel or other antineoplastic agents with or without the combination of tetrandrine. After 72 h of drug treatment, the plates were centrifuged to collect all the detached cells and the medium was carefully removed. Then, 100 μ l of 1 mg/ml MTT solution, diluted in culture medium, was added to each well. The plates were incubated at 37°C in a 5% CO₂ atmosphere for 4 h, allowing viable cells to reduce the yellow tetrazolium salt (MTT) into dark blue formazan crystals. Then the MTT solution was removed and 100 μ l of DMSO was added to each well to dissolve the formazan crystals. The absorbance in individual wells was determined at 560 nm using a microplate reader (Molecular Devices, Sunnyvale, CA, USA). The cell growth inhibition was determined by triplicate assays. IC₅₀ values were calculated from cytotoxicity curves. The degree of resistance was calculated by dividing the IC₅₀ for MDR cells by that for parental sensitive cells. The fold-reversal of MDR was calculated by dividing the IC₅₀ for cells to paclitaxel or other drugs in the absence of tetrandrine by that in the presence of tetrandrine.

Determination of internucleosomal DNA fragmentation. After tumor cells were treated with various concentrations of paclitaxel, tetrandrine or their combinations for 48 h, the cells were harvested and suspended in lysis solution (5 mM Tris-HCl, 20 mM EDTA, and 0.5% (v/v) Triton X-100) for 30 min on ice. The crude DNA samples were extracted twice with phenol/chloroform/isoamyl alcohol (25:24:1). The remaining steps for detection of DNA fragmentation were performed as described previously (22, 23). DNA samples were analyzed by electrophoresis in a 1.5% agarose slab gel containing 0.3 μ g/ml ethidium bromide, and visualized under UV illumination.

Flow cytometric analysis. Cell sample preparation, including propidium iodide staining for flow cytometric analysis, were performed according to the method described by Nicoletti *et al.* (24). Briefly, at the end of each time-point, both attached and detached cells were harvested and washed twice with PBS. Cells

were then fixed with 70% ethanol diluted in PBS on ice for at least 30 min. After fixation, the cells were incubated in PBS containing 100 µg/ml RNase and 40 µg/ml propidium iodide at room temperature in complete darkness for 30 min prior to flow cytometric analysis. Cell cycle distribution and DNA content were determined by using a Coulter Epics V instrument (Beckman Coulter, Inc., Fullerton, CA, USA) with an argon laser set to excite at 488 nm. The results were analyzed using Elite 4.0 software (Phoenix Flow System, San Diego, CA, USA).

Western blotting. After the treatments of paclitaxel, tetrandrine or their combinations, both KB and KBv200 cells were harvested by trypsinization and washed with PBS. Cellular protein was isolated using the protein extraction buffer containing 150 mM NaCl, 50 mM Tris-HCl (pH 7.2), 5 mM EDTA, 1% Triton X-100 and 0.1% SDS. Protein concentrations were determined with the Bio-Rad DC Protein Assay (Bio-Rad, Hercules, CA, USA). Equal amounts (50 µg/lane) of proteins were fractionated on 10% SDS-PAGE gel and transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were incubated with anti-MDR1 (Santa Cruz Biotechnology), bcl-2 (DAKO Corp.) and β-actin (Sigma) primary antibodies, respectively. After washing with PBST (PBS containing 0.1% v/v Tween-20), the membranes were incubated with peroxidase-conjugated goat anti-rabbit or goat anti-mouse secondary antibodies (Jackson ImmunoResearch Laboratories) followed by enhanced chemiluminescent staining using the ECL system (Amersham Biosciences).

Measurement of accumulation and efflux of [³H]-paclitaxel. The drug accumulation and efflux was determined mainly according to the method described by Sumizawa *et al.* (25). Briefly, for measurement of the intracellular accumulation of [³H]-paclitaxel, confluent monolayers of KB and KBv200 cells in 24-well plates were incubated with complete culture medium containing 54 nM mixed paclitaxel ([³H]-paclitaxel:paclitaxel = 1:12), with or without the combination of 2.5 µM tetrandrine. After three washes with ice-cold PBS at the end of each time-point, the cells were lysed in 0.1 N NaOH and the radioactivity was determined. To determine drug efflux in both cell lines, after incubation with the paclitaxel mixture only or in combination with 2.5 µM tetrandrine for 3 h, the cells were washed three times using fresh medium with or without tetrandrine. The cells were then incubated in the washing medium for various time-points at 37°C, followed by the determination of radioactivity.

Establishment of KB and KBv200 xenograft models. Female, 6 to 8-week-old homozygous nude athymic mice were purchased from the National Cancer Institute (USA). They were maintained under specific pathogen-free conditions in the animal center of the Medical University of South Carolina, USA. To develop the tumor xenograft models, *in vitro* growing KB and KBv200 cells were harvested by exposure to trypsin-EDTA, washed three times with PBS, and implanted subcutaneously into the right flanks of the mice (1 × 10⁶ cells in 0.2 ml). The animals were treated with drugs when the tumors had reached a mean diameter of 0.4-0.6 cm, usually after 4-5 days of tumor implantation.

In vivo efficacy evaluation. The antitumor activity of paclitaxel, either alone or in combination with tetrandrine, was evaluated *in vivo* in nude mice bearing KB or KBv200 xenografts. When the tumors had reached a mean diameter of about 0.4-0.6 cm, the animals were

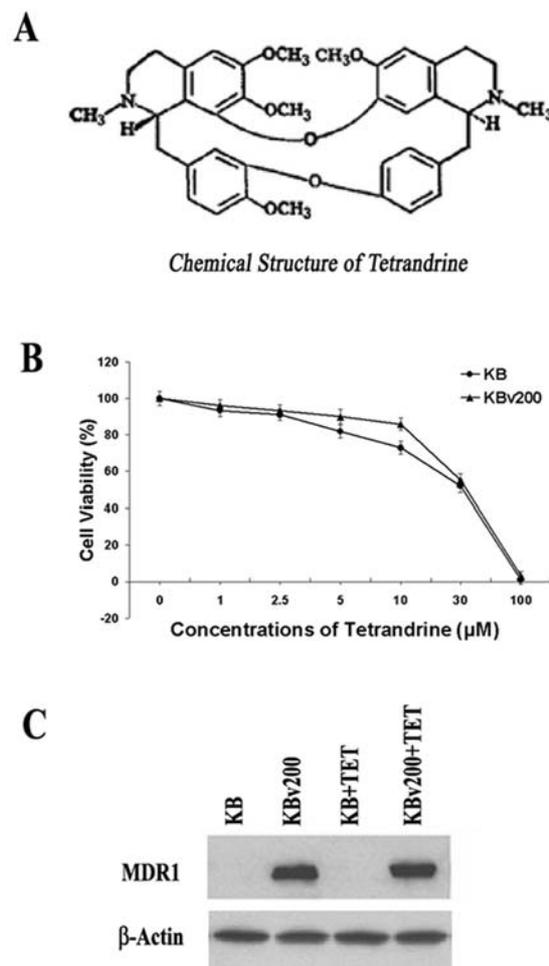


Figure 1. Structure and the intrinsic cytotoxicity of tetrandrine. (A) Chemical structure of tetrandrine; (B) Intrinsic cytotoxicity of tetrandrine on KB and KBv200 cells by MTT assay; (C) Expression of MDR1 protein in KB and KBv200 cells with or without tetrandrine treatment.

divided randomly into four groups (6 mice per group) and treated with various regimens on day 0. i) Saline; ii) paclitaxel alone at 20 mg/kg, *i.p.*; iii) tetrandrine alone at 35 mg/kg, *i.p.*; iv) paclitaxel at 20 mg/kg, *i.p.* plus tetrandrine at 35 mg/kg, *i.p.*. Paclitaxel was purchased from Mead Johnson Co. (Princeton, NJ, USA). Saline or drugs were administered every 3 days started from day 0 for 5 injections. Each animal was earmarked and followed individually throughout the experiments. Two perpendicular diameters (A and B) of the tumors were measured every 3 days up to day 18 when the animals were sacrificed. Tumor volumes (V) were calculated and body weights were recorded as described previously (26, 27). The reversing activity of tetrandrine was evaluated by comparing the antitumor efficacy of the combination of paclitaxel and tetrandrine with that of paclitaxel treatment alone. Statistical analysis was performed using the Student's *t*-test and the differences were considered significant at $p < 0.05$. The curve of tumor growth was drawn according to tumor volume and time after drug treatment. When the animals were sacrificed, the tumor tissues were removed

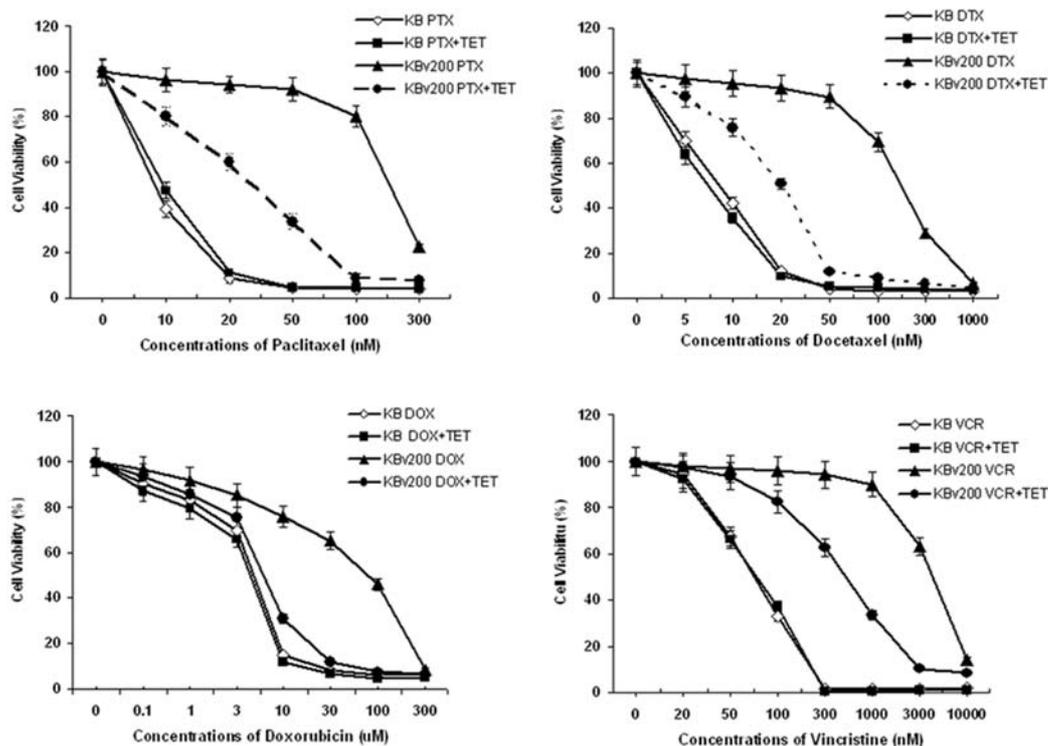


Figure 2. Reversal of multidrug resistance by tetrandrine. KB and KBv200 cells were exposed to a series of concentrations of indicated drugs with or without co-treatment of 2.5 μM tetrandrine. After 72 h of treatment, the sensitivities of KB and KBv200 cells to paclitaxel or other drugs were determined by measurement of their survival rate. Data presented in the bar graph are Mean ± S.D., based on three independent experiments. TET, tetrandrine; PTX, paclitaxel; DTX, docetaxel; DOX, doxorubicin; VCR, vincristine.

and weighed. The rate of inhibition of tumor growth (IR) was calculated according to the following formula:

$$IR = 1 - \frac{\text{Mean tumor weight of experimental group}}{\text{Mean tumor weight of control group}} \times 100\%$$

Results

Modulation of drug resistance by tetrandrine in vitro. To evaluate the reversal activity of tetrandrine on P-gp-mediated drug resistance *in vitro*, we first examined the cytotoxicity of tetrandrine itself on the MDR cell line KBv200 and its parental KB cell line. The intrinsic cytotoxicity of tetrandrine on both cell lines is shown in Figure 1B. The IC₅₀ of tetrandrine in both cell lines was approximately 30~32 μM. Cell survival rates were over 90% in both KB and KBv200 cells when exposed to 2.5 μM or lower concentrations of tetrandrine. Therefore, the concentration of 2.5 μM tetrandrine was used for the following experiments to test its reversal activity on P-gp-mediated drug resistance. In addition, immunoblotting showed that tetrandrine has no effect on the expression of P-gp protein (Figure 1C).

Next, KB and KBv200 cells were exposed to a series of concentrations of paclitaxel and docetaxel with or without co-administration of 2.5 μM tetrandrine for 72 h. The reversal ability of tetrandrine to MDR was then assessed by MTT assay. As shown in Figure 2, in the absence of tetrandrine, KBv200 cells exhibited high resistance to both paclitaxel and docetaxel compared with KB cells. The IC₅₀ of these drugs for KBv200 was about 20 to 25-fold higher than those of its parental KB cell. Co-treatment of tetrandrine had little effect on its drug-sensitive parental KB cell, but significantly enhanced the cytotoxicity of paclitaxel and docetaxel in KBv200 cells. Co-administration with 2.5 μM of tetrandrine reversed the sensitivity of KBv200 cells to paclitaxel and docetaxel around 10-fold. In addition, we also examined the reversal activity of tetrandrine to several other antineoplastic agents and found that co-administration of tetrandrine also significantly enhanced the cytotoxicity of doxorubicin and vincristine. In particular, tetrandrine almost fully restored the sensitivity of KBv200 cells to doxorubicin. These results indicate that tetrandrine possesses potent activity in reversing P-gp-mediated multidrug resistance in MDR cells *in vitro*.

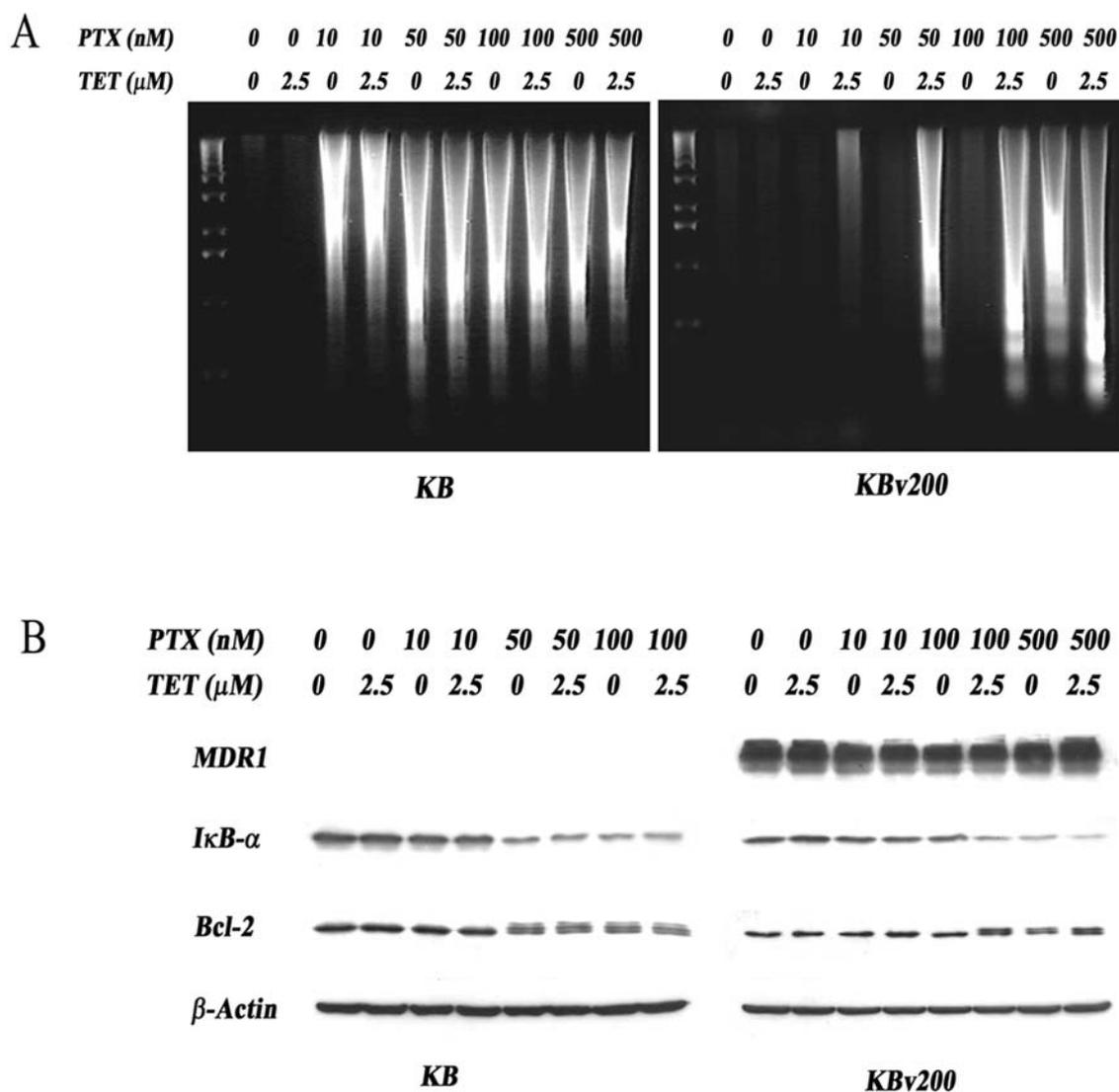


Figure 3. (A) Tetrandrine enhances paclitaxel-induced DNA fragmentation. KB and KBv200 cells were treated with paclitaxel, tetrandrine or their combinations at different concentrations for 48 h. Cells were then harvested for DNA extraction. Fragmented DNA was analyzed by electrophoresis in 1.5% agarose gel containing 0.3 μ g/ml ethidium bromide. (B) Western blot analyses. Total proteins were extracted from KB and KBv200 cells treated with tetrandrine, paclitaxel or their combinations for 24 h. Equal amounts (50 μ g/lane) of cellular protein were fractionated on a 10% SDS-polyacrylamide gel and transferred to PVDF membranes, followed by immunoblotting with anti-MDR1, I κ B α , bcl-2 and β -actin antibodies. PTX, paclitaxel; TET, tetrandrine.

Enhancement of paclitaxel-induced apoptosis by tetrandrine. Paclitaxel can cause mitotic arrest and apoptosis in tumor cells (22, 28). An important feature of apoptotic cell death is the fragmentation of genomic DNA, producing a characteristic ladder on agarose gel electrophoresis. First, we performed a DNA fragmentation assay to determine the apoptotic susceptibility of the KB and KBv200 cells in response to paclitaxel-induced apoptosis by using different drug regimes. As shown in Figure 3A, the characteristic DNA fragmentation ladders were clearly observed following treatment of KB cells

with 10 nM or higher concentrations of paclitaxel for 48 h. However, this paclitaxel-induced DNA fragmentation was not detected in KBv200 cells until the concentration of paclitaxel reached 500 nM. In combination with 2.5 μ M tetrandrine, the paclitaxel-induced DNA fragmentation was observed in KBv200 cells with 50 nM paclitaxel or higher concentrations. Further, we analyzed the paclitaxel-induced apoptotic cell death by using flow cytometric assay. As depicted in Figure 4, paclitaxel concentrations at 10 nM or higher in KB cells and 500 nM in KBv200 cells, respectively, induced a distinct sub-

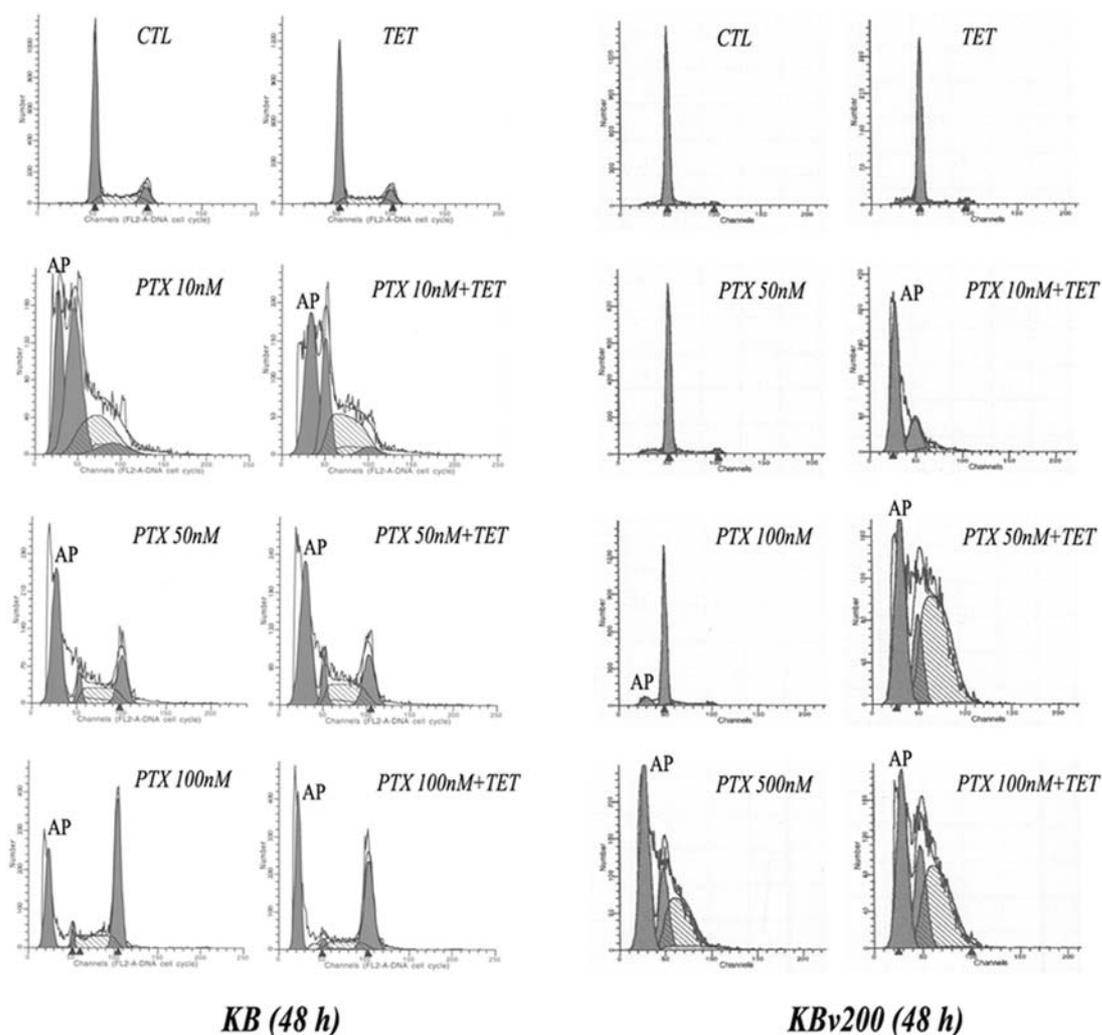


Figure 4. Flow cytometric assay. KB and KBv200 cells were treated with the indicated concentrations of tetrandrine, paclitaxel or their combinations. After 48-h incubation, the cells were harvested and analyzed by flow cytometry. The AP (sub-G1) peaks represent the population of apoptotic cells. PTX, paclitaxel; TET, tetrandrine.

G1 peak (AP peak), which represented an apoptotic cell population in the tumor cells. In combination with 2.5 μ M tetrandrine, the AP peak was seen in KBv200 cells treated with only 10 nM paclitaxel. These findings indicate that 2.5 μ M tetrandrine can significantly enhance the paclitaxel-induced apoptotic cell death.

Further, we examined the possible influence of a combination with tetrandrine on $\hat{I}\hat{B}\alpha$ and bcl-2 proteins, whose expressions or alterations were reported to be involved in paclitaxel-induced apoptosis and cell cycle arrest (22, 29). The results depicted in Figure 3B show paclitaxel-induced bcl-2 phosphorylation and degradation of $\hat{I}\hat{B}\alpha$, which occurred in KB cells, did not occur in KBv200 cells, unless the cells were also treated with 2.5 μ M tetrandrine.

Inhibition of [³H]-paclitaxel efflux by tetrandrine. The above studies demonstrate that tetrandrine is a potent and selective MDR modulator. To examine the mechanism of action of tetrandrine in the reversal of P-gp-mediated drug resistance in MDR cells, we compared the kinetics of [³H]-paclitaxel in KBv200 and KB cells in the presence or absence of tetrandrine. Figure 5A illustrates the effect of tetrandrine on the intracellular accumulation of [³H]-paclitaxel in KBv200 and KB cells. In the absence of tetrandrine, the intracellular concentration of [³H]-paclitaxel in KBv200 cells was much lower than that in KB cells. However, when KBv200 cells were co-treated with 2.5 μ M tetrandrine, [³H]-paclitaxel in the cells was significantly increased. Tetrandrine exhibited little influence

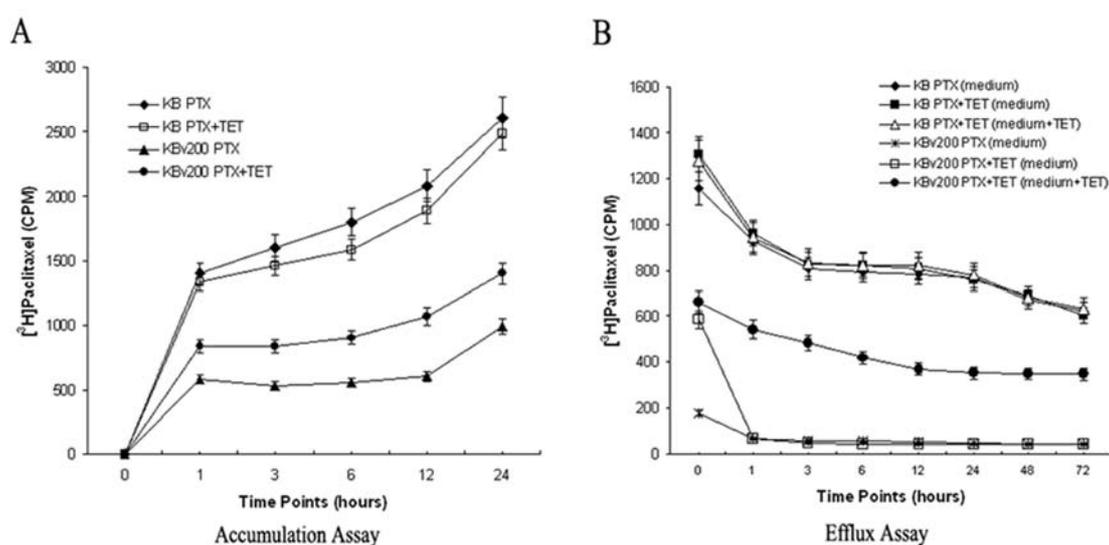


Figure 5. Effect of tetrandrine on $[^3\text{H}]$ -paclitaxel accumulation and efflux in KB and KBv200 cells. Tumor cells cultured in 24-well plates were incubated with medium containing 54 nM mixed paclitaxel with or without tetrandrine. At the end of each time point, cells were harvested and the radioactivity of $[^3\text{H}]$ -paclitaxel was determined as described in Materials and Methods. (A) Accumulation of $[^3\text{H}]$ -paclitaxel in KB and KBv200 cells; (B) After 3 h incubation with the mixed paclitaxel with or without tetrandrine, the cells were washed three times with drug free medium or medium containing tetrandrine and the efflux of $[^3\text{H}]$ -paclitaxel was determined. Values are the average of three independent experiments. PTX, paclitaxel; TET, tetrandrine.

on the drug-sensitivity of KB cells. Further, we examined whether the increased accumulation of paclitaxel in KBv200 cells by tetrandrine was due to inhibition of the $[^3\text{H}]$ -paclitaxel efflux. The time-course of $[^3\text{H}]$ -paclitaxel release shown in Figure 5B indicated that tetrandrine had little effect on the drug efflux in KB cells, but it significantly inhibited the efflux of $[^3\text{H}]$ -paclitaxel in KBv200 cells. At 1 h, almost all of the accumulated $[^3\text{H}]$ -paclitaxel was extruded from the KBv200 cells in the absence of tetrandrine, but in the presence of 2.5 μM of tetrandrine, only around 20% accumulated $[^3\text{H}]$ -paclitaxel was extruded from the KBv200 cells. Interestingly, we further found that, on using tetrandrine-free medium to wash the KBv200 cells that had formerly been treated with the combination of paclitaxel and tetrandrine, the accumulated $[^3\text{H}]$ -paclitaxel decreased significantly to the same level as that in KBv200 cells formerly treated with paclitaxel alone. This result indicates that tetrandrine possesses significant inhibitory effects on the efflux of $[^3\text{H}]$ -paclitaxel in MDR KBv200 cells, which result in the increase of cellular accumulation of the drug.

Tetrandrine increases the therapeutic efficacy of paclitaxel in vivo. The efficacy of tetrandrine in reversal of MDR *in vivo* was evaluated using human tumor xenografts. As described in Materials and Methods, both KBv200 and drug-sensitive KB cells were used for the development of xenograft models. Mice bearing KBv200 or KB tumors were randomly divided into four groups (n=6) and treated with the

following regimens: saline; tetrandrine only (35 mg/kg, q3d x 5, *i.p.*); paclitaxel only (20 mg/kg, q3d x 5, *i.p.*); combination of paclitaxel and tetrandrine, respectively. The experimental results showed that tetrandrine alone did not show any effect on the tumor growth in mice bearing KB or KBv200 tumors. In the mice bearing KB xenografts, the inhibitory rates of tumor growth were 93.38% and 96.05%, respectively, in the groups treated with paclitaxel alone and in combination with tetrandrine (Figure 6A). The treatment with paclitaxel resulted in slight inhibition of KBv200 tumor growth (IR=13.04%). However, co-administration of paclitaxel with tetrandrine significantly reduced the tumor growth (IR=76.09%) (Figure 6B). The average weight of KBv200 tumors in the group co-administered with paclitaxel and tetrandrine was much smaller compared to other groups ($p < 0.01$) (Figure 6C). The effective combination treatments of paclitaxel and tetrandrine were well-tolerated, as indicated by the changes in body weights compared with those in the drug alone group (Figure 6D). These results indicate that co-administration of tetrandrine potentiates the antitumor activity of paclitaxel to MDR tumor *in vivo*.

Discussion

P-gp-mediated drug resistance is a major cause of chemotherapy failure in cancer treatment. During the last two decades, a broad range of MDR modulators have been

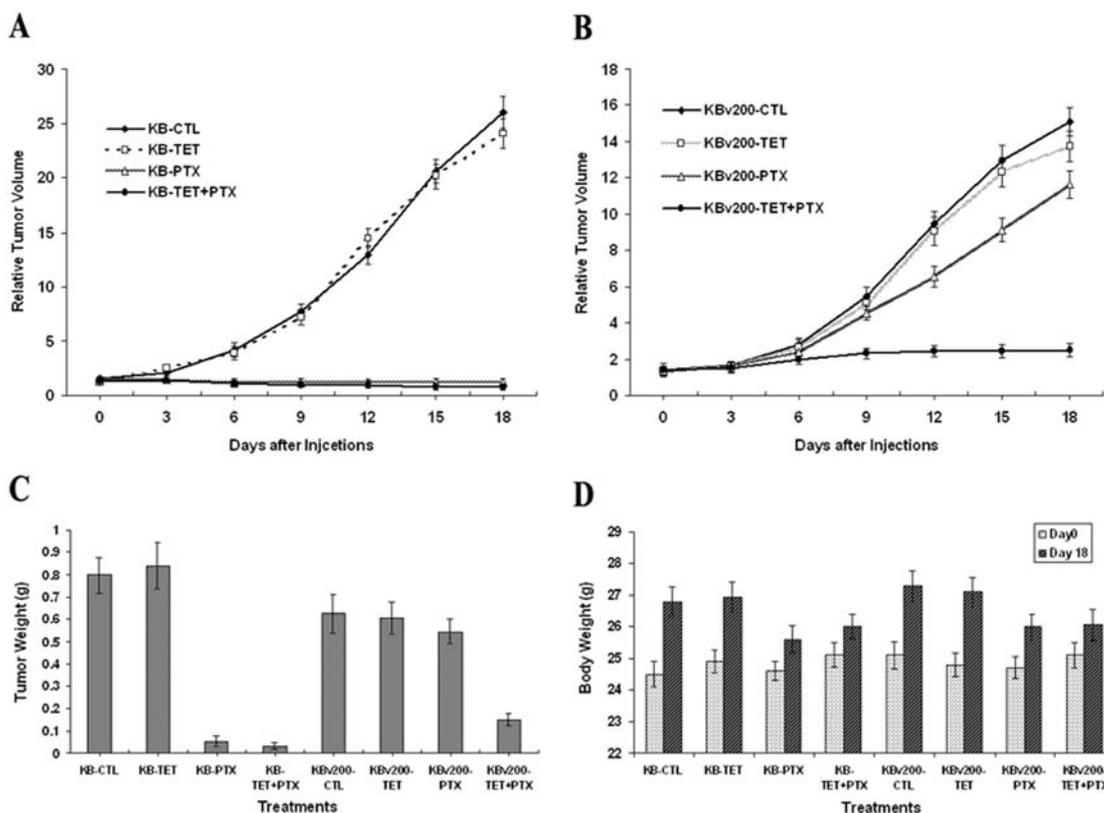


Figure 6. Tetrandrine increases the therapeutic efficacy of paclitaxel in KBv200 xenografts. Nude mice bearing KB and KBv200 tumors were treated with paclitaxel (20 mg/kg, q3d x 5) with or without co-administration of tetrandrine (35 mg/kg). The tumor volume, tumor weight and mice body weight were measured as described in Materials and Methods. (A) Relative tumor volume of mice bearing KB tumors exposed to various treatments. (B) Relative tumor volume of mice bearing KBv200 tumors exposed to various treatments. (C) Average tumor weight for each group of mice bearing KB or KBv200 xenografts. (D) Changes of average body weight for each group of mice bearing KB or KBv200 xenografts. Data represents Mean \pm S.D. of each group based on three independent experiments. CTL, control; TET, tetrandrine; PTX, paclitaxel.

reported to reverse P-gp-mediated drug resistance. The first generation of MDR modifiers used included calcium channel blockers such as verapamil, calmodulin inhibitors, and the immunosuppressive agent cyclosporin A (7, 8, 11). Although having some efficacy, these agents are relatively weak P-gp inhibitors. In addition, the dose-limiting side-effects restricted their clinical utility (15, 30, 31). The requirement for more potent agents has led to the development of a second generation of MDR modifiers such as PSC833, VX-710 and the acridone carboxamide derivative GF120918 *etc.* (11, 32-34). *In vitro* and preclinical studies have demonstrated that many of these modifiers usually possess higher affinity for P-gp and exhibit stronger activity in the reversal of MDR. However, an additional problem with most second-generation P-gp inhibitors is that they may alter the cytotoxicity or pharmacokinetics of many antitumor agents. For example, it was reported that PSC833 and VX-710 caused a significant change in the pharmacokinetics and enhancement of toxicity of anticancer

drugs such as paclitaxel (32, 33), which has necessitated the reduction of drug doses when administered with these MDR modulators. Due to these problems and limitations, there has been considerable interest in the development of more potent and selective MDR modifiers. In recent years, several novel MDR modifiers, such as S9788 (35), OC144-093 (36) and XR9576 (26), have been described. These P-gp inhibitors usually exhibit more potent and specific action in the reversal of MDR.

More recently, through screening a series of compounds isolated from natural plants, we discovered several naturally occurring BBIs that possess potent activity in the reversal of P-gp-mediated drug resistance (16). In the present study, we characterized one of these compounds, tetrandrine, and evaluated its activity in the reversal of P-gp-mediated drug resistance to paclitaxel *in vitro* and *in vivo*. First, by utilizing the human MDR tumor cell line KBv200, which expresses high levels of P-gp, and its drug-sensitive parental KB cell line, we demonstrated that tetrandrine is a very potent

modulator in the reversal of P-gp-mediated MDR. In the *in vitro* cytotoxicity assay, tetrandrine exhibited significant reversal activity of drug resistance to paclitaxel and docetaxel in KBv200 cells, but there was little effect on the drug-sensitive KB cell line (see Figure 2). This result suggests that the reversal activity of tetrandrine for taxane resistance is selective and only acts in the MDR cells with high expression of P-gp. Further, the promising activity of tetrandrine demonstrated *in vitro* was confirmed in *in vivo* efficacy studies. Through development of human tumor xenografts implanted with either KBv200 or KB cells, we demonstrated that tetrandrine also possesses potent activity in restoring the antitumor activity of paclitaxel in the xenograft models without an apparent increase in toxicity (see Figure 6). The inhibition of tumor growth in xenograft models by co-administration of tetrandrine with paclitaxel was over 60-70% in comparison to the groups treated with paclitaxel only ($p < 0.01$). Another important feature of this naturally occurring MDR modifier is its low cytotoxicity. At concentrations below 2.5 μM , tetrandrine may achieve dramatic reversal of MDR in KBv200 cells, but has little cytotoxicity by itself (see Figure 1). These findings suggest that tetrandrine may hold great promise as a selective and potent MDR modulator with potential clinical value.

Additionally, this study also examined the potential mechanism of action by which tetrandrine reverses P-gp-mediated drug resistance to paclitaxel in MDR cells. The ability of tetrandrine to inhibit P-gp-mediated drug transport was evaluated by comparing the kinetics of [^3H]-paclitaxel in KBv200 cells and its parental KB cells. The results indicate that tetrandrine significantly enhanced the intracellular accumulation of [^3H]-paclitaxel in MDR cells. As shown in Figure 5, in the absence of tetrandrine, the intracellular concentration of [^3H]-paclitaxel in KBv200 cells was much lower than that in KB cells, but it was significantly increased in the presence of tetrandrine. Further, analyses of drug efflux showed that tetrandrine specifically inhibited the efflux of [^3H]-paclitaxel in KBv200 cells. These results indicate that tetrandrine, similar to many other MDR modifiers (13, 24, 37), may increase the intracellular accumulation of anticancer drugs through inhibition of P-gp-mediated drug transport.

In summary, tetrandrine is a naturally occurring MDR inhibitor. The present *in vitro* and *in vivo* studies demonstrated that it possesses highly potent and specific activity in the reversal of P-gp-mediated drug resistance. Like many other MDR modifiers, tetrandrine is able to directly interact with P-gp, through which it inhibits drug efflux and increases the intracellular drug accumulation in MDR cells. Considering its potent reversal activity and low toxicity, tetrandrine, and probably other identified naturally occurring BBIs (16), may hold great promise for development as a novel class of modifier for the treatment of P-gp-mediated MDR cancers.

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