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ORIGINAL ARTICLE

Jing Jin · Feng-Peng Wang · Huailing Wei
Gengtao Liu

Reversal of multidrug resistance of cancer through inhibition of P-glycoprotein by 5-bromotetrandrine

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Abstract *Purpose*: The present study aimed to evaluate the MDR reversal activity of bromotetrandrine (BrTet), a bromized derivative of tetrandrine (Tet), in vitro and in vivo. *Methods*: Drug sensitivity was determined using the MTT assay. The in vivo effect of Tet was investigated using nude mice grafted with sensitive and resistant KB human epidermoid cancer cells. Doxorubicin (Dox) accumulation was analyzed by fluorospectrophotometry and the protein and mRNA levels of P-glycoprotein (P-gp) were determined by immunocytochemistry and RT-PCR, respectively. *Results*: BrTet at 0.25, 0.5 and 1 μ*M* reversed Dox resistance in MDR human breast cancer MCF-7/Dox cells dose-dependently and its potency was greater than that of Tet at the same concentrations. BrTet reversed vincristine (VCR), Dox and paclitaxel resistance in MDR human oral epidermoid carcinoma KBv200 cells as well as innate VCR and Dox resistance in human hepatocellular carcinoma Bel_{7402} cells. However, BrTet showed no effect on the IC_{50} values of the above-mentioned anticancer drugs in sensitive MCF-7 and KB cells. No reversal effect of BrTet on the cytotoxicity of 5-fluorouracil and cisplatin, non-P-gp substrates, was observed. In nude mice bearing KBv200 xenografts on the left flank and KB xenografts on the right flank, i.p. injection of 5 mg/kg and 10 mg/kg BrTet significantly enhanced the antitumor activity of Dox against KBv200 xenografts with inhibitory rates of 33.0% and 39.2%, while Dox alone inhibited the growth of KBv200 xenografts by only 11.6%. No enhancement by BrTet was seen in KB xenografts. Moreover, BrTet at 5 mg/kg reversed paclitaxel resistance in KBv200 xenografts. Fluorospectrophotometric assay showed that BrTet significantly increased the intracellular accumulation of Dox in MCF-7/Dox cells in a dose-dependent manner. BrTet also inhibited the overexpression of P-gp in MCF-7/Dox cells, but had no effect on mdr1 expression. *Conclusions*: BrTet showed significant MDR reversal activity in vitro and in vivo. Its activity may be related to the inhibition of P-gp overexpression and the increase in intracellular accumulation of anticancer drugs. BrTet may be a promising MDR modulator for eventual assessment in the clinic.

Keywords Multidrug resistance · Modulator · Bromotetrandrine

Abbreviations BrTet: Bromotetrandrine · Dox: Doxorubicin · EDTA: Ethylenediamine tetraacetic acid · 5-FU: 5-Fluorouracil · GADPH: Glyceraldehyde-3-phosphate dehydrogenase · MDR: Multidrug resistance · MTT: Dimethyl thiazolyl-2,5-diphenyltetrazolium bromide · PBS: Phosphate-buffered saline · P-gp: P-glycoprotein · Tet: Tetrandrine · VCR: Vincristine · VPM: Verapamil

Present address: J. Jin
Department of Pharmacology,
University of Cambridge,
Cambridge, UK

F.-P. Wang
Department of Medicinal Natural Products,
West China College of Pharmacy, Sichuan University,
Chengdu, People's Republic of China

H. Wei
Department of Pharmacology,
Institute of Materia Medica,
Chinese Academy of Medical Sciences, Beijing,
People's Republic of China

G. Liu (✉)
Department of Pharmacology,
Institute of Materia Medica,
Peking Union Medical College and Chinese Academy
of Medical Sciences, 1 Xian Nong Tan Street,
Beijing, People's Republic of China
E-mail: liugt@imm.ac.cn
Tel.: +86-010-63165178
Fax: +86-010-63017757

Introduction

During past years, significant advances in the treatment of solid tumors and hematological malignancies have

been made. However, multidrug resistance (MDR) of cancer cells to anticancer therapy is a primary cause of failure to cure cancer. MDR is particularly a problem for those drugs including anthracyclines, taxanes, vinca alkaloids, epipodophyllotoxins and mitomycin C [1]. Although there are several different mechanisms associated with the development of MDR, the common cause of MDR is believed to be the enhanced expression of a transmembrane glycoprotein termed P-glycoprotein (P-gp) in cancer cells.

P-gp belongs to the ABC superfamily of transporter proteins and functions as an ATP-dependent drug efflux pump, which rapidly extrudes a variety of hydrophobic anticancer drugs from the target cancer cells and hence reduces intracellular drug accumulation. P-gp is expressed in a cell-specific and tissue-specific manner, with high levels detectable in the colon, pancreas, kidney and liver [2]. So solid tumors originating from these organs generally show a poor therapeutic response to most chemotherapeutic agents because of intrinsic resistance [1, 3]. There is growing evidence that P-gp plays an important role in clinically acquired MDR in several human malignancies. Some tumors, either initially sensitive (such as acute leukemia [4], non-Hodgkin's lymphoma [5], small-cell lung cancer, ovarian cancer [6, 7]) or less sensitive (such as neuroblastoma [8] and breast carcinoma [9]) to chemotherapy, express no P-gp before treatment, but high levels of P-gp are detected at relapse. It is now generally accepted that the presence of P-gp correlates well with a poor response to anticancer drug therapy and subsequent shorter survival time in neuroblastoma [8], soft tissue sarcoma, colon cancer, locally advanced breast cancer, hematological malignancies and osteosarcoma [10].

The recognition that P-gp-mediated MDR is frequently encountered in the clinic has provided the impetus for discovery of effective reversing agents to overcome MDR. Tsuruo et al. [11] first reported that a noncytotoxic concentration of verapamil (VPM), a calcium channel blocker, enhanced the cytotoxicity of vincristine (VCR) and vinblastine in P388 leukemia cells and P388 leukemia VCR-resistant cells. Since then, various classes of compounds including calcium channel blockers, cyclosporins, steroid hormones, calmodulin inhibitors, antiarrhythmics and other compounds have been found to enhance the intracellular accumulation of P-gp-transported anticancer drugs, which results in an increase in the cytotoxicity of these drugs. However, most of these compounds show serious toxicity at the concentrations required to inhibit P-gp function in cancer patients, so they are not used in the clinic [12, 13]. Some agents with higher affinity for P-gp, such as the less cardiotoxic *R*-enantiomer of VPM (dexverapamil) [14], and a non-immunosuppressive analogue of cyclosporin A, PSC-833 (valspodar), have been developed. PSC-833 is now undergoing phase II and III trials [15, 16]. An encouraging result with PSC 833 was its MDR-modulatory effect in human hematological malignancies. However, PSC-833 has profound effects on the pharmacokinetics of doxorubicin (Dox), etoposide, and other anticancer drugs, which lead to an increase in the toxicity of these drugs [17]. So, novel and more potent modulators of MDR are still needed.

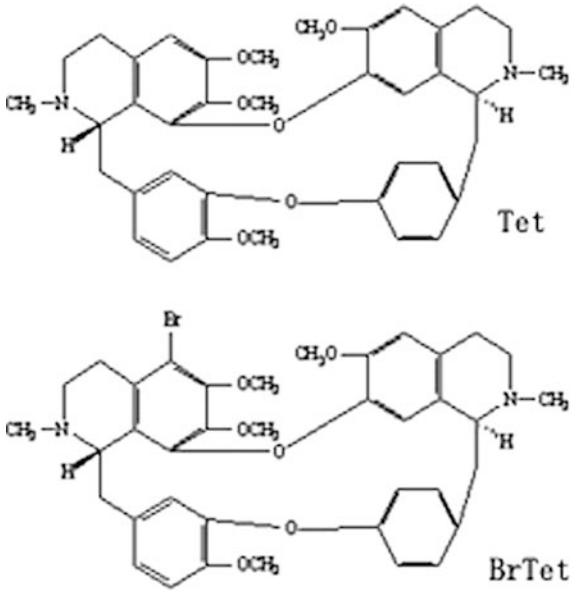

Fig. 1 Chemical structures of tetrandrine (Tet) and bromotetrandrine (BrTet)

Tetrandrine (Tet) is a bis-benzylisoquinoline alkaloid which is the main active component in the root of *Stephania tetrandra* S. Moore (or Fenfangji) of the Menispermaceae family. Tet has been used in the treatment of hypertension, cardiac arrhythmia and angina pectoris in China since the 1950s. Tet has been shown to be a Ca^{2+} channel antagonist and to interact with the voltage-activated L-type and T-type Ca^{2+} channels and the slowly gating $K_{(Ca)}$ channel with varying degree of specificity and affinity [18]. Recent studies have shown that Tet has a reversal effect on P-gp-mediated MDR [19, 20]. 5-Bromotetrandrine (BrTet) is a bromized derivative of Tet (Fig. 1). In preliminary screening, BrTet was shown to be more potent than Tet in modulating MDR in vitro. The present study was undertaken to further evaluate the potency of BrTet in sensitizing several MDR cell lines to various anticancer drugs in vitro and in vivo along with its ability to inhibit P-gp expression.

Material and methods

Cell culture

The tumor cell lines used, their origin and the orders of drug resistance are listed in Table 1. All cells were grown in RPMI1640 medium (GibcoBRL) supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO_2 and 95% air, and passaged by 0.25% trypsin plus 0.02% EDTA twice a week. The resistant cell lines were cultured in the presence of anticancer drugs until at least

Table 1 Characteristics of tumor cell lines used in this study and the cytotoxicity of BrTet in these cell lines. The cytotoxicity of BrTet to various tumor cell lines was determined by the MTT assay. Cells were exposed continuously to different concentration of BrTet for 72 h. The IC_{50} values of BrTet were calculated by a computer program based on the median-effect plot, and the values presented are means ± SD of at least three independent experiments, with each concentration tested in triplicate

Cell line	Origin/derivation	Level of resistance[a]	IC_{50} of BrTet (μ*M*)
MCF-7	Metastatic breast cancer	–	9.90 ± 1.01
MCF-7/Dox	Continuous exposure to 0.5 μ*M* Dox	110 to Dox; 40 to VCR	9.32 ± 2.00
KB	Human oral epidermoid carcinoma	–	8.55 ± 0.46
KBv200	Continuous exposure to VCR	110 to VCR; 15 to Dox; 10.4 to etoposide; 205 to paclitaxel	9.86 ± 1.54
Bel_{7402}	Human hepatocellular carcinoma	Intrinsic resistance to Dox, VCR, paclitaxel compared to KB cells	3.05 ± 0.12
Bel_{7402}/5-FU	Continuous exposure to 10 μg/ml 5-FU	160 to 5-FU	7.14 ± 1.82
L1210	Mouse lymphoid leukemia	–	2.07 ± 0.76
L1210/CDDP	Continuous exposure to cisplatin	16 to cisplatin	2.69 ± 0.13

[a]Relative resistance: IC_{50} of a drug in the resistant cell line divided by that of the drug in the parental drug-sensitive cell line

3 days before starting of the experiments. The resistance of all resistant sublines was found to be stable during the course of all experiments in the absence of continuous drug exposure. BrTet and Tet with a purity ≥99% were synthesized by Prof. Fengpeng Wang of Sichuan University.

Cytotoxicity assay

The in vitro chemosensitivity was measured using the dimethyl thiazolyl-2,5-diphenyltetrazolium bromide (MTT) assay [21]. Briefly, tumor cells were inoculated into 96-well plates (Costar) in 100 μl medium per well and allowed to attach and grow for 24 h. To determine the antiproliferative effect of BrTet in various cell lines, various concentrations of BrTet in 100 μl diluted with the medium were added into the wells. For determining the reversal effect of BrTet, graded concentrations of the anticancer drugs with or without BrTet 100 μl volumes were added. Then the cells were exposed to drugs continuously for 3 days at 37°C, after which MTT (Sigma; 0.5 mg/ml diluted in RPMI1640) was added to each well and cultured for an additional 4 h. The formed formazan was dissolved in 150 μl dimethyl sulfoxide after aspiration of the culture medium. The plates were shaken mechanically for 5 min and the optical density of each well was immediately read on a microplate reader (Bio-Rad Model 450) at a wavelength of 570 nm. The results are expressed as IC_{50} values which were determined by a computer program based on the median-effect plot. The reversal activity of BrTet on drug resistance is expressed as the fold reversal (FR) calculated according to the following equation: $FR = IC_{50\ \text{anticancer drug alone}}/IC_{50\ \text{anticancer drug + modulator}}$.

Cellular accumulation of Dox

Cellular Dox accumulation was determined as described previously [22]. In brief, MCF-7/Dox cells in exponential growth were exposed to 10 μ*M* Dox (Shenzhen Main Luck Pharmaceuticals, Guangzhou, China) in the absence or presence of BrTet at 0.25, 0.5 and 1 μ*M* or VPM (Sigma) at 10 μ*M* for 3 h. Then the cells were harvested by centrifugation. Cell pellets were resuspended in 0.3 *M* HCl in 50% ethanol and sonicated in an ultrasonic disintegrator for 30 s. Following centrifugation at 10,000 rpm for 15 min, the supernatant was removed and assayed spectrofluorometrically for Dox content at excitation and emission wavelengths of 470 nm and 585 nm. The intracellular Dox content was calibrated with a standard curve of Dox and is expressed in picomoles per 10^6 cells.

Immunocytochemical assay of P-gp

For the detection of P-gp, proliferating MCF-7 and MCF-7/Dox cells were grown on glass coverslips placed in a sterile dish overnight. The cells were exposed to BrTet or VPM for 24 h, and then the cells grown on the coverslips were washed twice with ice-cold phosphate-buffered saline (PBS), and fixed in 10% ice-cold acetone for 5 min. After treating with 10% newborn calf serum in PBS for 20 min, the cells were incubated with monoclonal antibody JSB-1 (Biogenes, diluted in PBS/2% BSA, 1:20) at 37°C for 1.5 h, washed three times with PBS, incubated with Goat anti-mouse IgG-FITC at 37°C for another 1 h, and washed with PBS twice again. The P-gp-associated fluorescence in MCF-7 and MCF-7/Dox cells was examined in a confocal laser scanning microscope (MERIDIAN Ultima 212).

RT-PCR analysis of mdr1 expression

Total RNA was isolated from MCF-7 cells using TRIzol (GibcoBRL) and RT-PCR was performed using an Access RT-PCR Introductory System (Promega) as described in the manufacturer's manual. Primers for mdr1 amplification were for TCG TAG GAG TGT CCG TGG AT sense strand and CAT TGG CGA GCC TGG TAG for antisense strand. The reaction mixture (50 μl)

contained 1–2 μg of total RNA as a template, 0.1 U/μl AMV reverse transcriptase, 0.1 U/μl *Tfl* DNA polymerase, 0.2 m *M* of each dNTP, 1 m *M* $MgSO_4$ and 1 μ*M* primers. First strand cDNA synthesis was obtained after 45 min at 48°C. After 2 min at 94°C, 28 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 1 min and extension at 68°C for 2 min were performed. The estimated product size was 458 bp. PCR products were analyzed on 2% agarose gels in Tris/borate/EDTA buffer and visualized by staining with ethidium bromide. As a control for semiquantitative evaluation of PCR, glyceraldehyde-3-phosphate dehydrogenase (GADPH) primers (sense ACG GAT TTG GTC GTA TTG GG, antisense 5′-CGC TCC TGG AAG ATG GTG AT) were used to amplify a 214 bp fragment. Finally, 28 cycles of PCR at 94°C for 30 s, 55.5°C for 1 min and 68°C for 2 min were performed.

Xenografts in nude mice

KB and KBv200 xenografts were initially established in female BALB/c nude mice (Center of Experimental Animals, Chinese Academy of Medical College) at 5–7 weeks of age and body weight of 17–20 g. The mice were implanted with 10^7 parental KB and 10^8 resistant KBv200 cells, respectively, by subcutaneous injection into the interscapular area. Xenografts were maintained for several generations by subcutaneous implantation of about 50 mg non-necrotic tumor tissue using a trocar. The animal experimentation was approved by the Animal Ethics Committee at Peking Union Medical College and performed following the Animal Care and Use Guidelines of the US National Institutes of Health.

Reversal activity of BrTet in vivo

Each nude mouse received two xenografts: a drug-sensitive (KB) xenograft in the left flank and a resistant (KBv200) xenograft in the right flank. Drug treatment was started when the tumor size reached to 75–150 mm^3. The nude mice with xenografts were divided into groups randomly. Each group contained seven to ten mice and was treated with various regimens on day 1. A dose of 2 mg/kg Dox was administered on day 2 after grouping the mice and then every other day for five times. Paclitaxel (Union Pharmaceutical Factory, Beijing) 10 mg/kg was administered on day 3 then every other day for five times. Solution A (0.02% EDTA, 0.05% thiourea, in pH 3–4 sterile deionized water) was given form day 1, once daily for 12 days as the vehicle control. Three dosages of 2.5, 5 and 10 mg/kg BrTet dissolved in Solution A or VPM 10 mg/kg dissolved in normal saline was given from day 1, once daily for 12 days. BrTet and VPM were given 3 h before the injection of Dox or paclitaxel. A group of nude mice was treated only with sterile normal saline as normal controls. All agents were injected intraperitoneally (i.p.) in a volume of 0.2 ml/20 g body weight.

Tumor growth was monitored by measuring two perpendicular diameters with a caliper. Tumor volume was calculated from the following expression: $1/2(L \times W_2)$, where L is the length and W is the width in millimeters. This is considered a valid estimation of volume [23]. The tumor sizes were standardized in different groups by using the relative tumor volumes (RTV) calculated from the expression: $RTV = V_x/V_1$, where V_x represents the tumor volume on day x and V_1 represents the initial tumor volume at the onset of treatment. Tumor measurements and body weight of the mice were recorded every other day. The mice were killed humanely when the total volume of both xenografts reached 2000 mm^3, i.e., about 10% of the animal body weight. The antitumor activity was assessed in terms of the inhibitory rate (IR), which was defined as: IR(%) = (1−mean RTV of the treated group/mean RTV of the control group)×100%

Statistical analysis

The significance of differences in the mean values between groups was analyzed using the unpaired two-tailed Student's *t*-test; *P* values < 0.05 were considered statistically significant.

Results

Evaluation of the cytotoxic effect of BrTet

The cytotoxicity of BrTet alone in each cell line was assayed by the MTT method. The data in Table 1 clearly indicate that BrTet at 2–10 μ*M* exerted considerable cytotoxicity. The resistant cells showed the same sensitivity to BrTet as their parental cells.

Effect of BrTet on susceptibility of tumor cells to anticancer drugs

The VCR-selected resistant subline KBv200 and the Dox-selected resistant subline MCF-7/Dox were used to represent the acquired MDR phenotype, and Bel_{7402} to represent the intrinsic MDR phenotype [22] because of P-gp overexpression. The cytotoxicity-enhancing effects of BrTet and Tet were investigated using the MTT assay. VPM at 10 μ*M* was used as a positive control in the MTT assay and also in the following in vitro assays. VPM at 10 μ*M* showed little cytotoxicity in KB cells which showed a mean percentage viability of 83%, but no cytotoxicity to other cells.

The ability of BrTet and Tet to reverse Dox resistance was first compared in MCF-7/Dox cells. BrTet showed a significant reversal effect on Dox resistance in MCF-7/Dox, and its potency was greater than that of Tet (Table 2). BrTet also actively reversed Dox resistance in KBv200 and Bel_{7402} cells. The results in Table 2 also show that BrTet exhibited appreciable reversal of vincristine and paclitaxel resistance in KBv200 cells and of VCR

Table 2 Effect of BrTet on the cytotoxicities of Dox, VCR and paclitaxel expressed as IC_{50} values. Tumor cells were treated with graded concentrations of Dox, VCR or paclitaxel in the absence or presence of BrTet and Tet. After 72 h of continuous drug exposure, the cell survival rate was measured by MTT assay. The data presented are the mean ± SD IC_{50} values (μ*M*) from at least three independent experiments. The values in parentheses are fold reversal (FR) calculated from the expression: $FR = IC_{50\ \text{anticancer drug alone}}/IC_{50\ \text{anticancer drug + modulator}}$ (*ND* not determined)

	Cell lines				
	KB	KBv200	MCF-7	MCF-7/Dox	Bel_{7402}
Dox (μ*M*)	0.04 ± 0.06	0.60 ± 0.27	0.56 ± 0.21	62.45 ± 18.89	2.05 ± 0.28
+ BrTet (1 μ*M*)	0.04 ± 0.02 (1.0)	0.11 ± 0.07 (5.5)	0.63 ± 0.55 (0.9)	3.20 ± 0.81 (22.9)	0.16 ± 0.10 (12.9)
+ BrTet (0.5 μ*M*)	0.06 ± 0.04 (0.7)	0.17 ± 0.12 (3.6)	0.44 ± 0.16 (1.3)	4.17 ± 0.28 (17.6)	1.46 ± 0.21 (1.4)
+ BrTet (0.25 μ*M*)	0.11 ± 0.03 (0.4)	0.17 ± 0.02 (3.6)	0.46 ± 0.07 (1.2)	20.30 ± 4.15 (3.6)	1.46 ± 0.39 (1.4)
+ Tet (1 μ*M*)	ND	ND	ND	7.00 ± 1.45 (10.5)	ND
+ Tet (0.5 μ*M*)	ND	ND	ND	15.30 ± 4.76 (4.8)	ND
+ Tet (0.25 μ*M*)	ND	ND	ND	24.46 ± 2.60 (3.0)	ND
+ VPM (10 μ*M*)	0.02 ± 0.01 (2.0)	0.03 ± 0.01 (21.5)	ND	1.10 ± 0.57	0.09 ± 0.05 (17.2)
VCR (μ*M*)	0.04 ± 0.02	4.43 ± 1.19	ND	ND	1.52 ± 0.10
+ BrTet (1 μ*M*)	0.02 ± 0.01 (2.0)	0.58 ± 0.33 (7.6)	ND	ND	0.14 ± 0.11 (10.9)
+ BrTet (0.5 μ*M*)	0.04 ± 0.01 (1.0)	2.37 ± 0.66 (1.9)	ND	ND	0.23 ± 0.21 (6.7)
+ BrTet (0.25 μ*M*)	0.05 ± 0.01 (0.8)	2.52 ± 0.01 (1.8)	ND	ND	1.05 ± 0.51 (1.5)
+ VPM (10 μ*M*)	0.006 ± 0.0001 (7.0)	0.08 ± 0.01 (55.4)	ND	ND	0.35 ± 0.12 (5.8)
Paclitaxel (ng/ml)	8.50 ± 1.02	1744 ± 943	ND	ND	ND
+ BrTet (1 μ*M*)	6.06 ± 0.77 (1.4)	197 ± 100 (8.9)	ND	ND	ND
+ BrTet (0.5 μ*M*)	9.53 ± 1.30 (0.9)	258 ± 67 (6.8)	ND	ND	ND
+ BrTet (0.25 μ*M*)	5.03 ± 2.07 (1.7)	328 ± 39 (5.3)	ND	ND	ND
+ VPM (10 μ*M*)	5.69 ± 3.49 (1.5)	58 ± 5 (30.1)	ND	ND	ND

resistance in Bel_{7402} cells. BrTet was marginally more effective when combined with Dox than when combined with VCR in Bel_{7402} cells. On the contrary, VPM proved more effective in association with VCR (FR 17.2) than in association with Dox (FR 5.8).

In the experiments with other two resistant cell lines, Bel_{7402}/5-FU cells selected by 5-FU and L1210/cisplatin cells selected by cisplatin, no reversal effect of BrTet on the cytotoxicities of 5-FU and cisplatin in the test cells was observed (Table 3). Taken together, BrTet markedly reversed resistance to Dox, VCR and paclitaxel in both acquired and intrinsic tumor cell lines, and the activity of BrTet in reversing resistance to Dox was greater than its activity in reversing resistance to VCR and paclitaxel.

Effect of BrTet on cellular accumulation of Dox in MCF-7 cells

MDR tumor cells accumulate and retain less anticancer drug than their drug-sensitive cells. The ability of BrTet to inhibit P-gp-mediated transport of anticancer drug was evaluated by examining its effect on the accumulation of Dox in MCF-7/Dox cells by fluorospectrometry. Accumulation of Dox in MCF-7/Dox cells was much lower than in sensitive MCF-7 cells (Fig. 2). When the cells were preincubated with various concentrations of BrTet for 3 h, BrTet significantly increased Dox accumulation in MCF-7/Dox cells in a dose-dependent manner, but not in sensitive MCF-7 cells. BrTet at concentration of 1 μ*M* enhanced the accumulation of Dox to a level of 8.3-fold higher than the level in untreated control cells, indicating a potency greater than that of 10 μ*M* VPM.

Immunocytochemical analysis of P-gp expression

P-gp expression in sensitive and resistance MCF-7 cells was evaluated using immunocytochemical analysis. As expected, the fluorescence intensity of P-gp was upregulated in MCF-7/Dox cells as compared with parental

Table 3 Effect of BrTet on the cytotoxicities of 5-FU and cisplatin, expressed as IC_{50} values, in Bel_{7402} and L1210 cells. Tumor cells were treated with graded concentrations of 5-FU or cisplatin in the absence or presence of BrTet at the indicated concentrations. After 72 h of continuous drug exposure, cell survival was measured using the MTT assay. Values are the means ± SD of three independent experiments. The values in parentheses are fold reversal calculated from the expression: $FR = IC_{50\ \text{anticancer drug alone}}/IC_{50\ \text{anticancer drug + modulator}}$

	IC_{50} of 5-FU (μ*M*)		IC_{50} of cisplatin (ng/ml)	
	Bel_{7402}	Bel_{7402}/5-FU	L1210	L1210/CDDP
Control	4.6 ± 2.1	837.6 ± 85.3	0.19 ± 0.01	3.02 ± 1.6
BrTet 1 μ*M*	3.8 ± 1.1 (1.2)	1360.3 ± 471.0 (0.6)	0.15 ± 0.03 (1.2)	1.62 ± 1.59 (1.9)
BrTet 0.5 μ*M*	3.2 ± 2.5 (1.4)	861.6 ± 63.2 (1.0)	0.14 ± 0.05 (1.3)	1.26 ± 1.45 (1.3)
BrTet 0.25 μ*M*	2.5 ± 0.2 (1.8)	896.8 ± 74.4 (0.9)	0.18 ± 0.02 (1.1)	1.35 ± 1.02 (2.2)

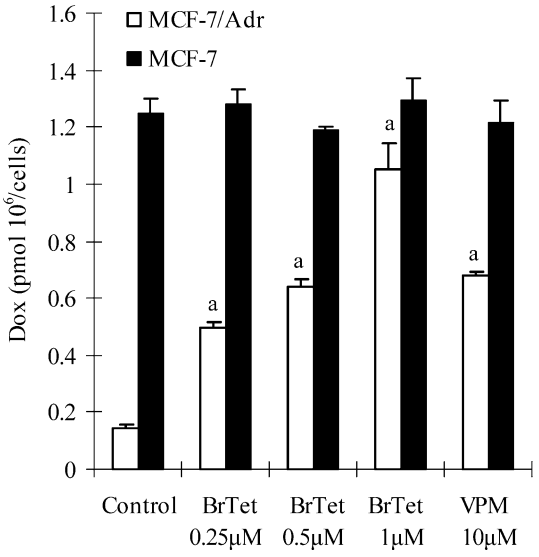

Fig. 2 Enhanced accumulation of Dox in MCF-7/Dox cells by BrTet. MCF-7/Dox cells were incubated with Dox (10 μ*M*) with or without the indicated concentrations of BrTet for 3 h. Intracellular Dox content was measured fluorospectrophotometrically and is expressed as picomoles per 10^6 cells. *Bars* present means ± SD from triplicate determinations. $^{a}P < 0.001$ compared with the control MCF-7/Dox (MCF-7/Adr) cells

MCF-7 cells. When MCF-7/Dox cells were exposed to 0.5 μ*M* and 1 μ*M* of BrTet for 24 h, the fluorescence intensity of P-gp in the cells decreased markedly, indicating that BrTet inhibited the level of P-gp expression (Fig. 3).

Fig. 3 Immunocytochemical analysis for P-gp expression in MCF-7 cells. The relative P-gp overexpression was determined by confocal laser scanning microscopy after labeling with JSB-1 monoclonal antibody and FITC-labeled anti-IgG. **A** Sensitive MCF-7 cells; **B**–**D** resistant MCF-7/Dox cells (**B** control; **C**, **D** incubated with 0.5 μ*M* and 1 μ*M* BrTet, respectively, for 24 h)

Effect of BrTet on mdr1 expression

The mdr1 gene encodes the drug efflux pump P-gp, whose overexpression is associated with the development of MDR, and the overexpression of P-gp is primarily regulated at the transcriptional level. MCF-7 and MCF-7/Dox cells were assessed using semiquantitative RT-PCR. As expected, there was no apparent mdr1 expression in MCF-7 cells, whereas mdr1 expression was clearly observed in MCF-7/Dox cells with acquired MDR (Fig. 4a). The treatment of MCF-7/Dox cells with BrTet or VPM for 24 h did not alter the mdr1 expression level significantly (Fig. 4B).

Antitumor activity in nude mice bearing xenografts

In the experiments with nude mice bearing two xenografts, a KB xenograft in the left flank and a KBv200 xenograft in the right flank, i.p. injection of Dox alone 2 mg/kg every 2 days for five times, inhibited the growth of KB xenografts by about 44% (Fig. 5a), while it had slight antitumor action on KBv200 xenografts with an inhibitory rate of 11.6% (Fig. 5b). When BrTet was coadministered with the same schedule of Dox, no enhancement of inhibition of tumor growth was observed in KB xenografts. However, coadministration of BrTet with Dox potentiated the antitumor activity of Dox in KBv200 xenografts, and the enhancement was

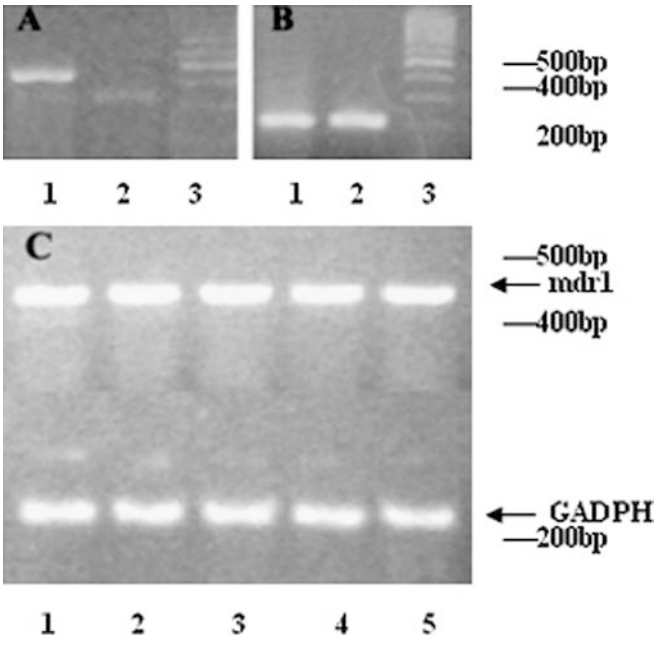

Fig. 4 RT-PCR analysis of mdr1 expression. **A** mdr1 expression in MCF-7 and MCF-7/Dox cells. **B** GADPH expression in MCF-7 and MCF-7/Dox cells (*lane 1* MCF-7/Dox cells, *lane 2* MCF-7 cells, *lane 3* 100 bp DNA marker. **C** MCF-7/Dox cells were treated with 0.25, 0.5 or 1 μ*M* BrTet for 24 h. For details of the experimental protocols see "Material and methods" (*lane 1* control, *lane 2* solution A, *lane 3* 0.25 μ*M* BrTet, *lane 4* 0.5 μ*M* BrTet, *lane 5* 1 μ*M* BrTet)

clearly detectable on day 7 and was dose-dependent. The action of BrTet at doses of 5 and 10 mg/kg in the mice treated for 15 days was statistically significant in comparison with the vehicle-treated control mice ($P < 0.001$).

Paclitaxel injected alone significantly reduced the growth rate of KB xenografts by 70% (Fig. 6a), and coadministration of BrTet did not enhance the antitumor activity of paclitaxel (Fig. 6b). However, coadministration of BrTet at 5 mg/kg with paclitaxel potentiated the antitumor activity of paclitaxel in KBv200 xenografts. The difference between the cotreated group and the vehicle-treated control group was significant ($P < 0.05$). But the difference in tumor growth between mice treated with paclitaxel alone and combined with BrTet at 2.5 mg/kg was not statistically significant ($P > 0.05$). This enhancing effect of BrTet on paclitaxel was less than that obtained with the combination treatment of BrTet with Dox. A daily dose of 10 mg/kg VPM combined with Dox or paclitaxel had little effect on the growth of both KB and KBv200 xenografts. During treatment, no death or signs of clinical intoxication were observed following i.p. injection of 10 mg/kg BrTet alone. The maximum tolerated dose of BrTet combined with Dox was 10 mg/kg with a maximum body weight loss of 13.6%.

Discussion

It is well known that VPM and other calcium channel blockers such as nifedipine, azidopine, tetrandrine can

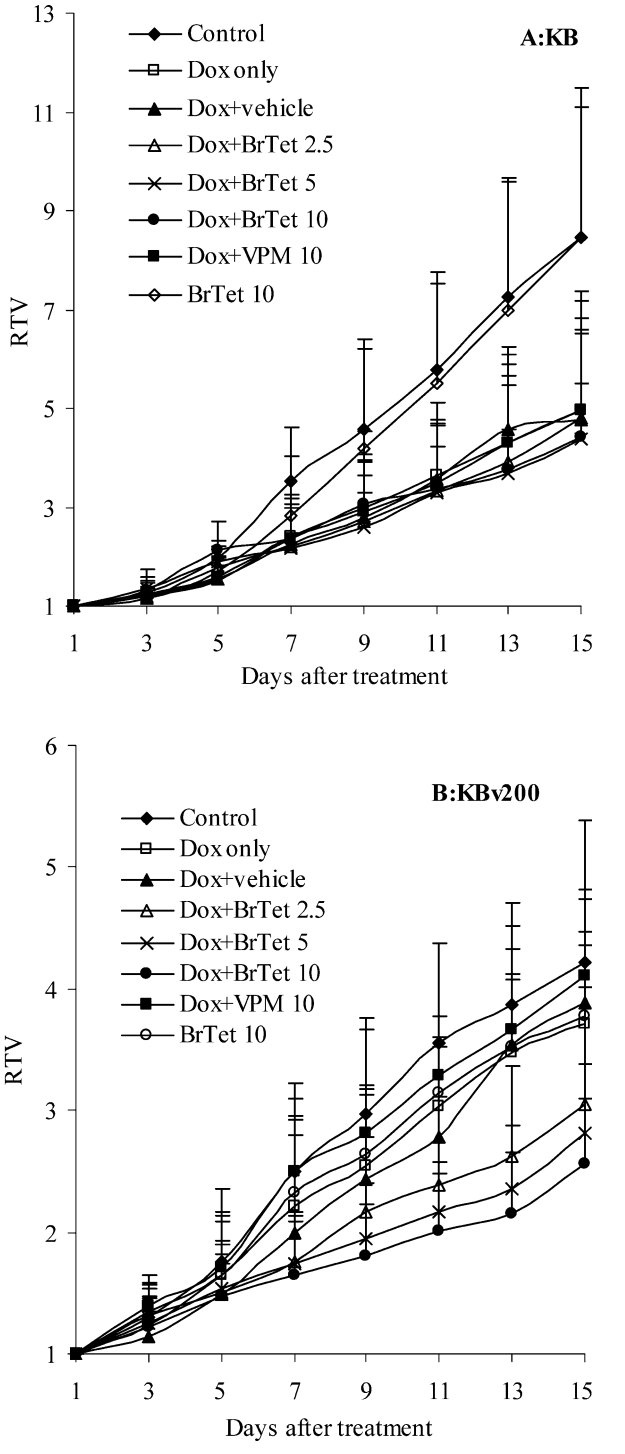

Fig. 5 Effect of BrTet on the antitumor activity of Dox in (**A**) KB-bearing and (**B**) KBv200-bearing nude mice. Each group of seven to ten BALB/c mice was implanted subcutaneously with KB xenografts in the left flank and KBv200 xenografts in the right flank. The animals were injected i.p. with sterile normal saline (*Control*), Dox 2 mg/kg (*Dox only*), BrTet 10 mg/kg (*BrTet 10*), Dox plus solution A (*Dox + vehicle*), Dox plus VPM 10 mg/kg (*Dox + VPM 10*), and Dox plus BrTet 2.5, 5 and 10 mg/kg (*Dox + BrTet 2.5*, *Dox + BrTet 5*, *Dox + BrTet 10*, respectively). Solution A, VPM and BrTet were injected i.p. from day 1 to day 12 when the tumor size reached to 75–150 mm^3. Dox was injected i.p. on days 2, 4, 6, 8 and 10. The data are presented as means ± SD of the relative tumor volume (RTV) as described in "Materials and methods"

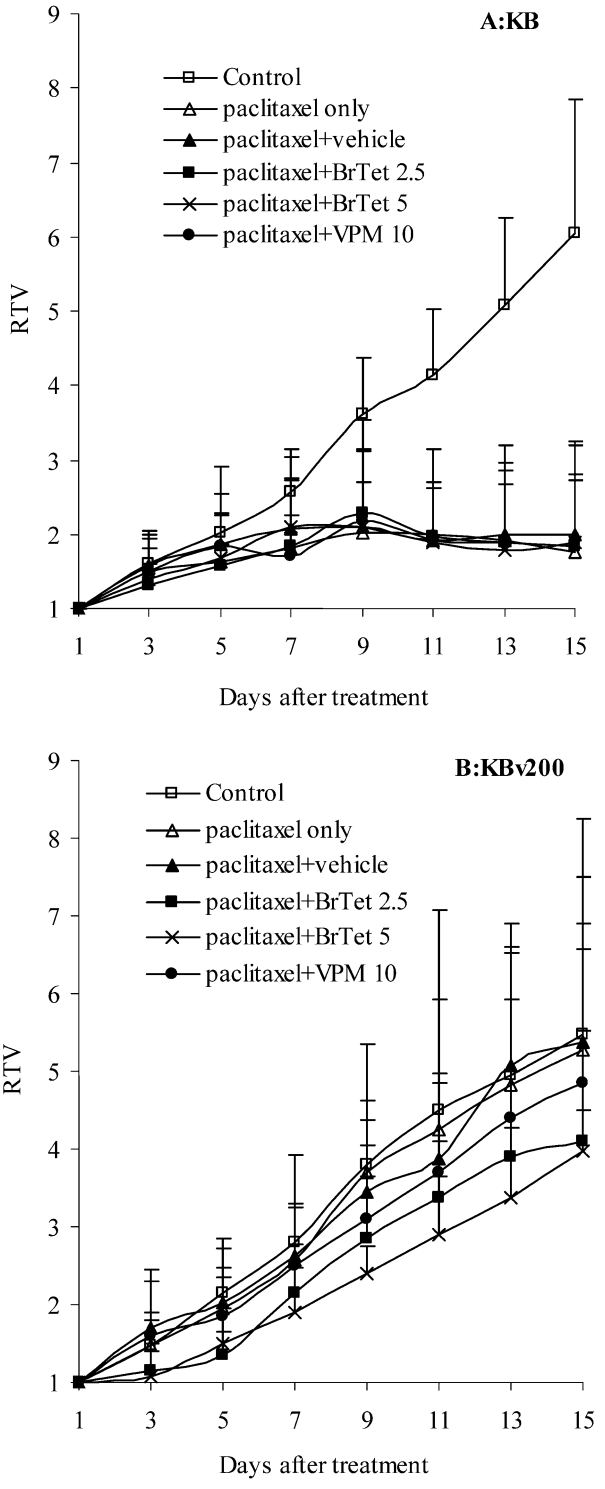

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Fig. 6 Effect of BrTet on antitumor activity of paclitaxel in (**A**) KB-bearing and (**B**) KBv200-bearing nude mice. Each group of eight to ten BALB/c nude mice was implanted subcutaneously with KB xenografts in the left flank and KBv200 xenografts in the right flank. The animals were treated with sterile normal saline (*Control*), paclitaxel 10 mg/kg (*paclitaxel only*), paclitaxel plus solution A (*paclitaxel + vehicle*), paclitaxel plus VPM 10 mg/kg (*paclitaxel + VPM 10*), and paclitaxel plus BrTet 2.5 and 5 mg/kg (*paclitaxel + BrTet 2.5*, *paclitaxel + BrTet 5*). Solution A, VPM and BrTet were injected i.p. from day 1 to day 12 when the tumor size reached to 75–150 mm^3. Paclitaxel was injected i.p. on days 3, 5, 7, 9 and 11. The data are presented as mean ± SD of the relative tumor volume (RTV) as described in "Materials and methods"

inhibit the efflux of antitumor agents from tumor cells, thus overcoming MDR [24, 25]. Combination chemotherapy with VPM is theoretically useful, but induces serious side effects such as reversible hypotension and arrhythmias. Tetrandrine is also a Ca^{2+} channel inhibitor, which has been used traditionally for the treatment of congestive circulatory disorders and inflammatory diseases in China. He et al. [19] in 1996 reported that tetrandrine is able to reduce Dox resistance in harringtonine-resistant human leukemia cells. Since then, other authors have reported the MDR-modulating activity of Tet in vitro and in vivo [26, 27, 28]. The structure difference between Tet and BrTet is the hydrogen at position C5 substituted by a bromine (Fig. 1). In this study, the results clearly indicated that the reversal activity of BrTet is greater than that of Tet at the same doses in MCF-7/Dox cells. Tet at the maximal nontoxic dose of 2.5 μ*M* lowered the IC_{50} of Dox in MCF-7/Dox cells from 11.02 μ*M* to 0.63 μ*M* with a fold reversal of 17.4 [20], whereas BrTet at 1 μ*M* lowered the IC_{50} of Dox in MCF-7/Dox cells from 62.45 μ*M* to 3.20 μ*M* with a fold reversal of 22.9. In the in vivo experiments, combination treatment with BrTet 5 mg/kg and Dox showed significantly greater antitumor activity than Dox alone. With regard to Tet, it has been reported that the dose of Tet to obtain a marked P-gp-modulating activity in nude mice is 20 or 30 mg/kg [27].

Besides MCF-7/Dox cells, VCR-selected MDR KBv200 cells and intrinsically resistant Bel_{7402} cells were used to investigate the ability of BrTet to modulate Dox, VCR and paclitaxel resistance in vitro. All of the above three cell lines overexpressed mdr1-encoded P-gp detected at both the protein level and the RNA transcript level. The results of the MTT assay indicated that BrTet itself exerted significant and similar cytotoxicity in both sensitive and resistant cell lines, indicating that BrTet itself may not be a substrate of P-gp. In an attempt to discriminate the synergic cytotoxicity from the chemosensitivity-enhancing effect of BrTet, noncytotoxic concentrations of BrTet were chosen. BrTet at 0.25, 0.5 and 1 μ*M* showed no cytotoxic effects in parental cell lines lacking P-gp expression or in the resistant cells, while BrTet at the above concentrations potentiated the sensitivities of both acquired and intrinsically resistant cells to Dox, VCR or paclitaxel. However, no similar effects were observed in non-P-gp-overexpressing parental cell lines. Furthermore, no potentiating effect of BrTet on the cytotoxicity of non-P-gp substrates such as cisplatin and 5-FU were seen. It appears that BrTet may be more effective in reversing the MDR activity of Dox, at least in Bel_{7402} and MCF-7/Dox cell lines. The reversal activity of BrTet in Bel_{7402} cells is particularly interesting since a large proportion of hepatic carcinoma samples taken from untreated patients are found to be P-gp-positive and there was a close association between P-gp

overexpression and resistance to chemotherapeutic drugs with MDR activity.

The overexpression of P-gp is a major cause of MDR development. Suppressing the expression of P-gp at either the transcriptional or protein level is a key approach to reversing MDR. In order to clarify whether the reversal effect of BrTet on MDR is mediated by modulating P-gp or not, the effect of BrTet on mdr1/P-gp expression in MCF-7/Dox cells was studied. Incubation of MCF-7/Dox cells with 0.5 μ*M* and 1 μ*M* BrTet for 24 h induced significant inhibition of P-gp overexpression, whereas BrTet showed no effect on P-gp mRNA expression. As to whether BrTet affects the mRNA transcription or modifies P-gp function, further investigation is required. The fluorospectrophotometric assay found that BrTet strongly enhanced Dox accumulation in resistant MCF-7/Dox cells but not in sensitive MCF-7 cells, and the potency of 1 μ*M* BrTet in the enhancement of Dox accumulation was stronger than that of 10 μ*M* VPM. The results suggest the possibility that the reversal effect of BrTet on Dox, VCR and paclitaxel resistance is attributable to its inhibition of P-gp function.

Although pharmacological reversal of MDR by some compounds has been demonstrated frequently in cell or tissue culture, there is minimal evidence of therapeutic effectiveness in clinical trials or against solid tumors in animals. One main cause is that the concentration of these compounds in serum required for MDR reversal effectiveness cannot be achieved. In addition, the effect of reversal agents on uptake and cytotoxicity of MDR substrates decreases markedly in solid tumors [29]. So, we studied the MDR reversal effect of BrTet in combination with Dox or paclitaxel in nude mice bearing sensitive KB xenografts and resistant KBv200 xenografts, one in each flank. BrTet at 10 mg/kg given alone exhibited no therapeutic effect on KB and KBv200 xenografts, and neither Dox nor paclitaxel given alone had an antitumor effect in KBv200 xenografts. However, the combination of BrTet at 10 mg/kg and Dox showed a significantly greater antitumor effect in KBv200 xenografts than Dox alone. Coadministration of 5 mg/kg BrTet also effective potentiated the antitumor activity of Dox and paclitaxel. The increase in antitumor activity of paclitaxel by BrTet was slightly less than that of Dox, which was consistent with the in vitro data. BrTet at 2.5 mg/kg increased the antitumor activity of Dox in KBv200 xenografts only slightly. Xiao et al. [30] have reported that after an i.v. injection of BrTet at 10 mg/kg, the plasma level of BrTet reaches 2 μ*M* which is greater than the concentration required to inhibit P-gp and reverse MDR in vitro, suggesting the possibility that BrTet is a MDR-reversal agent in vivo. In our experiments in nude mice, 10 mg/kg BrTet alone induced no death or signs of clinical intoxication. Although a body weight loss of 13.6% was observed following treatment with 10 mg/kg BrTet combined with Dox, coadministration of 5 mg/kg BrTet and 2.5 mg/kg with Dox showed no significant effects on body weight in comparison with the vehicle control group.

It has been reported that P-gp has several physiological roles including protection against toxic xenobiotics by excretion into bile and urine, maintenance of the blood–brain barrier, and transport of steroid hormones and cholesterol [1, 31]. P-gp is not essential for normal function but is required for protection against the toxicity of xenobiotics because P-gp can influence their pharmacokinetics and pharmacodynamics. For example, clinical trials of dexverapamil have demonstrated significant enhancement of the pharmacokinetics and toxicity of anticancer drugs such as paclitaxel, which has necessitated reduction of paclitaxel dose when coadministered with dexverapamil [32]. Thus, compounds that inhibit P-gp are likely to alter the pharmacokinetics and enhance the toxicity of a cytotoxic drug. Xiao et al. [30] investigated the pharmacokinetic interaction of a single i.v. injection of BrTet (5 mg/kg) and Dox (2 mg/kg) in rats. The results showed that BrTet does not affect the pharmacokinetics of Dox. However, Dox slows the elimination of BrTet from blood as indicated in a prolonged elimination half-life ($t_{1/2}\beta$). The cytotoxicity of BrTet itself should be born in mind when combination therapy is used. Zuo et al. at the Beijing Zhao Yan New Drug Research Center completed toxicity testing of BrTet in rats. They found that diffusely necrotic peritonitis was the main toxicity caused by i.p. injection of 10 mg/kg BrTet six times a week for 12 weeks (data not published). However, the general appearance of the rats improved markedly, and abdominal lumps disappeared gradually after cessation of the injection of BrTet. The appearance of diffusely necrotic peritonitis after repeated injection of BrTet might be due to the irritation by the compound.

In conclusion, BrTet is a potent inhibitor of P-gp-mediated MDR in vitro and in vivo, and its active mechanism to reverse MDR is associated with an increase in the intracellular drug accumulation through inhibiting the overexpression of P-gp. Our study provides pharmacodynamic evidence to support the clinical evaluation of BrTet in combination with antitumor drugs especially with Dox in patients whose tumors are identified as P-gp-positive.

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