Reversal of P-gp-mediated Multidrug Resistance by Bromotetrandrine *In Vivo* Is Associated with Enhanced Accumulation of Chemotherapeutical Drug in Tumor Tissue

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Abstract. *Background: Our previous studies have shown that tetrandrine (Tet) reverses the effect of P-glycoprotein (P-gp)-mediated multidrug resistance (MDR) both in vitro and in vivo. 5-Bromotetrandrine (Br-Tet) is a newly synthesized brominated derivative of Tet. In this study, we investigated the MDR reversal activity of Br-Tet in vitro and in vivo and the mechanism involved in this reversal. Materials and methods: The ability of Br-Tet to reverse drug resistance in vitro was evaluated by MTT assay in human MDR KBv200 cells and the parental drug-sensitive KB cells. A KBv200 cell xenograft model was established to investigate the MDR reversal activity of Br-Tet in vivo. Doxorubicin (Dox) accumulation in KBv200 and KB cell lines was determined by flow cytometry and Dox accumulation in KBv200 xenografts tissue was examined by spectrofluorometer. The effect of Br-Tet on the expression of P-glycoprotein was detected by flow cytometry and Western blot, respectively. Results: Br-Tet significantly enhanced the cytotoxicity of Dox, paclitaxel, taxotere, vincristine and epirubicin in KBv200 cells but not in KB cells. Co-administration of 10 mg/kg Br-Tet and 2 mg/kg epirubicin significantly enhanced the antitumor activity of epirubicin without increasing the toxicity. Br-Tet increased the Dox accumulation in the MDR KBv200 cell line and in KBv200 xenograft tissue in a time- and dose-dependent manner. However, it did not reduce the expression of P-gp in KBv200 cells. Conclusion: Br-Tet caused a significant reversal of P-gp-mediated MDR, not only in vitro but also in vivo. The MDR reversal activity of Br-Tet in vivo was associated with the enhancement of accumulation of chemotherapeutical drugs in tumor tissue.*

Multidrug resistance (MDR) remains a major clinical challenge for successful cancer chemotherapy. The etiology of MDR is complex. However, the overexpression of P-glycoprotein (P-gp) is a major contributor to MDR.

To date, the strategy of reversing MDR has principally focused on the inhibition or modulation of P-gp activity. Reports in the early 1980s demonstrating reversal of resistance *in vitro* by verapamil raised hopes that drug resistance could be reversed by inhibiting drug efflux and resources and efforts were focused on this endeavor. However, attempts in several clinical studies to reverse MDR by using MDR modulators have not yet generated promising results.

Tetrandrine (Tet) is a *bis*-benzylisoquinoline alkaloid that 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 (1). Our previous studies have shown that Tet has a reversal effect on P-gp-mediated MDR both *in vitro* and *in vivo* (2, 3). For better activity and lower toxicity, Tet was modified on the basis of structure-activity relationship of MDR modulators. 5-Bromotetrandrine (Br-Tet) is a synthesized brominated derivative of Tet (Figure 1). In the present study, we evaluated the MDR reversal activity of Br-Tet *in vitro* and *in vivo* and the mechanism involved in this reversal.

Abbreviations: MDR, Multidrug resistance; P-gp, P-glycoprotein; Tet, tetrandrine; Br-Tet, bromotetrandrine; VCR, vincristine; Dox, doxorubicin; TAX, paclitaxel; TXT, taxotere; EADM, epirubicin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

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Key Words: Multidrug resistance, xenograft, nude mice, drug accumulation, bromotetrandrine.

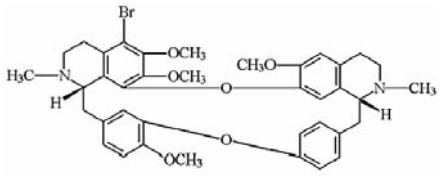

Figure 1. *Chemical structure of bromotetrandrine.*

Materials and Methods

Materials. Br-Tet (chemical structure is shown in Figure 1) was provided by West-China School of Pharmary, Si-Chuan University. Epirubicin (EADM) and doxorubicin (Dox) were purchased from HISUN Pharmaceutical Co (P. R. China). Vincrinstine (VCR) was purchased from ShenZhen (China) Main Luck Pharmaceuticals Inc. Taxotere (TXT) was from Rhone-poulenc Rorer (Vitry-sur-Seine, France). Paclitaxel (TAX) was from Bristol-Myers Squibb Co. (Princeton, New Jersey, USA).

Cell lines and cell culture. KB cells and KBv200 cells (P-gp overexpression) are human epidermoid carcinoma drug-sensitive and MDR cell lines, respectively. Both cell lines were cultured in RPMI 1640 medium with 10% newborn calf serum, benzylpenicillin (100 kU/l), and streptomycin (100 kU/l) at 37˚C in a humidified atmosphere of 5% CO_2 and 95% air. KBv200 cells were cloned from parental KB cells by stepwise exposure to increasing doses of VCR and ethylmethane sulfonate (EMS) mutagenesis. P-gp overexpression is a main reason to induce MDR in KBv200 cells. Both cells were obtained from the Chinese Academy of Medical Sciences, Beijing. For more details, see our earlier publication (4). Cells were cultured under the condition mentioned above when used in the following experiments.

Animals. BALB/C nude mice, 6 to 7 weeks of age, weighing 18-25 g were used for the KBv200 cell xenografts. Mice were obtained from the Center of Experimental Animals, Sun Yat-sen University and maintained in the Center.

MTT cytotoxicity assay. Cells were harvested during logarithmic growth phase and seeded in 96-well plates at 1.0×10^3 cells/well in RPMI 1640 medium. After 24 h incubation, 10 μl Br-Tet and 10 μl cytotoxic agent, or vehicle were added to duplicate plates. After 68 h, 10 μl of MTT solution (5 mg/ml in saline) were added to each well and the incubation was continued for an additional 4 h, then 100 μl of dimethyl sulphoxide (DMSO) were added to each well. Absorbance was measured by microplate reader (Bio-Rad Model 550; Hercules, CA, USA) at a primary wavelength of 570 nm and a second wavelength of 630 nm. The concentrations required to inhibit growth by 50% (IC_{50} value) were calculated from the cytotoxicity curves (Bliss's method) (4). The degree of resistance was calculated by dividing the IC_{50} for KB cells by that for KBv200 cells (5). The fold-reversal of MDR was the ratio of the IC50 in the absence and presence of modulator.

In vivo evaluation. To evaluate the optimal regimen of co-administration of Br-Tet and anticancer drugs such as epirubicin, experiments of MDR reversal *in vivo* were performed. The KBv200 cell xenograft model was established as described by Liang and colleagues (6). In detail, KBv200 cells grown *in vitro* were harvested and re-suspended to a final density of 1.0×10^7 cells/ml. A volume of 0.2 ml was implanted subcutaneously (*s.c.*) under the shoulder in each nude mice. When the tumors had reached the diameter of 0.3-0.6 cm, the animals were randomized into 12 groups according to body weight, with 10 to 14 mice in each group, and treated with different regimens (Table I). The control group was given saline at a dose of 10 ml/kg (*qd*×7, *i.p.*). The indicated dose of Br-Tet and EADM was given from the grouping day (*qd*×7, *i.p.*) and from the day after grouping (*q2d*×3, *i.p.*), respectively.

Table I. *In vivo reversal activity of Br-Tet for EADM in MDR KBv200 tumor-bearing mice.*

Group	Dose (mg/kg)		Animal numbers		Weight loss (%)	RTV[c]	IR (%)
	Br-Tet[a]	EADM[b]	pre-	post-			
01			14	14	+1.9	9.11±2.78	
02	5		10	9	+4.2	9.21±3.56	–1.1
03	7.5		10	10	+1.6	7.76±3.27	14.8
04	10		10	10	+1.2	6.97±2.32	23.5
05		1	10	10	+1.7	7.58±3.39	16.9
06	5	1	10	10	+1.0	6.96±1.71	23.6*
07	7.5	1	10	10	–5.7	6.72±1.46	26.2*
08	10	1	10	10	–3.1	6.07±2.78	33.4*
09		2	10	10	–0.5	6.47±2.66	29.0*
10	5	2	10	10	–13.4	5.96±2.30	34.6**
11	7.5	2	10	9	–12.1	5.59±2.02	38.6**
12	10	2	10	10	–13.6	4.74±1.65	48.0**

[a]Br-Tet was given from the grouping day, *qd*×7, *i.p.*; [b]EADM was given from the day after grouping, *q2d*×3, *i.p.*; [c]RTV (mean relative tumor volume±s.d.), the ratio of the tumor volume on the last day to that on the grouping day. *$p<0.05$ and **$p<0.01$ *versus* control (01) group, respectively.

The animal's body weight was measured every 3 days for modulation of the drug dosage. The short diameter (A) and long diameter (B) of tumors were recorded every 3 days and tumor volume (V) was estimated according to equation 1:

$$V = A^2 \times \frac{B}{2}$$

The relative tumor volume was the ratio of the tumor volume on the last day to that on the grouping day. The curve of tumor growth was drawn according to the relative tumor volume and time of implantation. The mice were ethically anaesthetized and killed on the 13th day after grouping. Tumor tissue was excised from the mice and the tumor weight was measured. The inhibitory rate (IR) was calculated according to equation 2:

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

Table II. *Experimental schedule of Br-Tet in Dox accumulation in MDR tumor tissue.*

Group	Dosage regimen			Sampling period (hours)*
	Br-Tet			
	Time point A[a]	Time point B[b]		
1			8 ml/kg NS	3
2	10 mg/kg	10 mg/kg		3
3			8 mg/kg Dox	1
4			8 mg/kg Dox	2
5			8 mg/kg Dox	3
6	10 mg/kg	10 mg/kg	8 mg/kg Dox	1
7	10 mg/kg	10 mg/kg	8 mg/kg Dox	2
8	10 mg/kg	10 mg/kg	8 mg/kg Dox	3
9			2 mg/kg Dox	3
10			4 mg/kg Dox	3
11	10 mg/kg	10 mg/kg	2 mg/kg Dox	3
12	10 mg/kg	10 mg/kg	4 mg/kg Dox	3

[a]24 h before injection of Dox; [b]2 h before injection of Dox; NS, normal saline; *sampling period indicates time period after Dox injection, after which the mice were anesthetized and killed and tumor samples were excised.

DOX accumulation in MDR human KBv200 cell xenograft tissue. The nude mouse xenograft model was established as described by Liang YJ and colleagues (6). When the tumors had reached approximately 1 g, the animals were randomized into 12 groups according to body weight, with 9 mice in each group, and treated with the regimens shown in Table II.

The groups given Dox alone were given a single *i.v.* injection of the different doses of Dox. In the groups given Br-Tet + Dox, to ensure the inhibition of functional P-gp by Br-Tet, the desired dosage of Br-Tet was injected *i.p.* one day before Dox injection (*i.v.*) and the same dose of Br-Tet was given once again *i.p.* two hours before the Dox injection. A group treated with saline was used as control. The mice were anaesthetized and killed and tumor samples were excised from mice after the desired period. Tumor tissue of 150-200 mg was resuspended in 1 ml/100 mg tumor lysis solution which contained 0.3 mol/l HCl and 60% ethanol (1:1) and was homogenized completely at 4˚C and then centrifuged at 12,000×g. The supernatant was removed and its fluorescence value was read by a spectrofluorometer at λ_{ex} 470 nm and λ_{em} 590 nm. Simultaneously, the standard curve of Dox was made at the same condition. The Dox accumulation in tumor tissue (μg/g) was calculated according to the fluorescence value using a standard curve.

DOX accumulation in vitro. The intracellular Dox accumulation was examined by flow cytometry. In detail, logarithmically growing cells cultured in RPMI 1640 medium were treated with Br-Tet of 0.5, 1.0, 1.5 μmol/l or vehicle, respectively, at 37˚C for 2 h. Dox at 5 μmol/l was added to the medium and the incubation continued for another 3 h. The cells were then collected and washed twice with cold PBS containing 10 μg/ml verapamil. Cells were resuspended in 200 μl PBS and then analyzed by flow cytometry (Beckman-Coulter, Elite; Fullerton, CA, USA), using excitation 488 nm (argon laser) to detect the mean fluorescence intensity (MFI) of intracellular Dox (7).

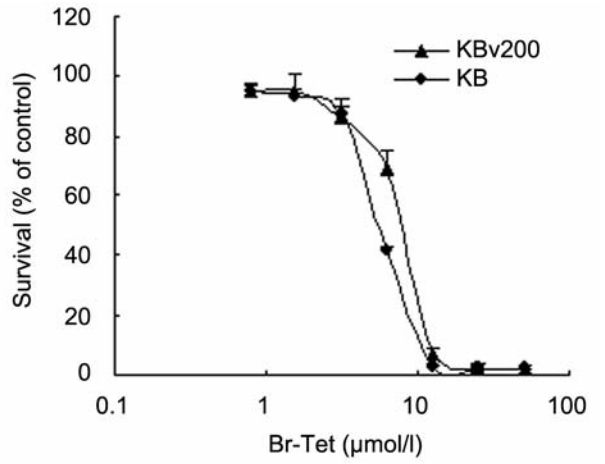

Figure 2. *Cytotoxicity of Br-Tet to KB and KBv200 cells. Cytotoxicity was determined by MTT assay as described in Materials and Methods.*

Flow cytometry. To measure the expression of P-gp, cells were incubated at room temperature for 0.5 h with monoclonal anti-P-gp antibody (UIC2; Immunotech, Marseille, France) at a concentration of 12.5 μg/ml or with an isotype-matched mIgG2α as control antibody (Immunotech) at a concentration of 10 μg/ml. After washing twice with PBS cells were analyzed by flow cytometry.

Western blot. Cells were treated with Br-Tet of 0.5, 1.0 or 1.5 μmol/l for the indicated times and protein extraction and Western blot was performed as described by Zhang *et al.* (8). Mouse monoclonal anti-P-glycoprotein antibody (Calbiochem) and HRP-linked secondary antibody were used to detect P-gp protein in this study.

Statistical analysis. All experiments were repeated at least three times and differences were determined using Student's *t*-test. **P*<0.05 was indicative of significant difference and ***p*<0.01 was indicative of very significant difference.

Results

Intrinsic cytotoxicity of Br-Tet. Br-Tet showed a steep curve between active and inactive concentrations with a sudden drop of cytotoxicity activity (Figure 2). At the concentration of 1.5 μmol/l, Br-Tet showed no growth-inhibiting effect on KB and KBv200 cells (survival rates of tumor cells were more than 90%). The IC_{50} of Br-Tet to KBv200 and KB cell lines were 6.167±0.630 and 5.137±0.266 μmol/l, respectively.

In vitro MDR reversal effect of Br-Tet. At the concentration used to modulate MDR (0.5, 1.0 and 1.5 μmol/l Br-Tet), Br-Tet had no growth-inhibiting effect on either cell line. No significant effect on the sensitivity to VCR, DOX, TAX,

Table III. *Modulation of the sensitivity to cytotoxic drugs by Br-Tet in MDR KBv200 cells and drug-sensitive KB cells.*

Cell line	Cytotoxic drug	IC_{50} (nmol/l)				Fold-resistance	Fold-reversal of MDR[a]		
		0*	0.5*	1.0*	1.5*		0.5*	1.0*	1.5*
KBv200	EADM	694.8±183.7	32.1±3.2**	27.3±7.4**	10.7±3.6**	22.1	21.3	25.1	64.0
	VCR	953.8±94.9	18.7±1.3**	8.7±1.1**	7.0±1.4**	244.6	51.0	109.4	135.4
	TAX	177.4±9.7	3.5±0.4**	2.3±0.2**	1.9±0.1**	55.4	50.8	78.4	93.4
	TXT	113.6±0.6	2.4±0.6**	2.0±0.6**	1.5±0.2**	75.7	47.1	57.8	74.3
	ADR	1387.3±222.9	124.8±15.9**	88.9±12.9**	59.2±21.5**	56.6	11.1	15.6	23.4
KB	EADM	31.5±2.5	23.3±1.4**	29.0±2.3	22.7±3.6		1.3	1.1	1.4
	VCR	3.9±1.6	3.7±1.2	3.9±1.1	3.5±1.1		1.1	1.0	1.1
	TAX	3.2±0.8	2.9±0.6	2.7±0.7	3.0±0.8		1.1	1.2	1.1
	TXT	1.5±0.2	1.5±0.1	1.4±0.2	1.4±0.1		1.0	1.1	1.1
	ADR	24.5±1.2	23.7±1.5	23.6±2.8	22.3±1.1		1.0	1.0	1.1

Each value represents the mean±standard deviation (S.D.) of at least three independent experiments. [a]Ratio of the IC_{50} for cytotoxic drug alone *versus* the IC_{50} in the presence of Br-Tet; *concentration of Br-Tet (μmol/l); **$p<0.01$ *versus* control group.

TXT and EADM was found after addition of Br-Tet to sensitive KB cells. However, Br-Tet restored the sensitivity of the resistant KBv200 cells to all five drugs to a significant degree. The IC_{50} of cytotoxic drugs with or without the presence of Br-Tet are listed in Table III. A concentration-dependent sensitization of KBv200 cells to anticancer drugs by Br-Tet is shown in Figure 3.

Br-Tet enhanced the intracellular accumulation of Dox in MDR KBv200 cells. The intracellular accumulation of Dox in KBv200 cells was only about one-fourth of that in KB cells. After the KBv200 and KB cells were treated with 0.5, 1.0 and 1.5 μmol/l Br-Tet, the intracellular accumulation of Dox was significantly increased in KBv200 cells. However, the Dox accumulation in KB cells was not affected by Br-Tet (Figure 4).

In vivo MDR reversal effect of Br-Tet. Br-Tet of 5 mg/kg, 7.5 mg/kg and 10 mg/kg inhibited the KBv200 tumor growth by –1.1%, 14.8%, 23.5%, respectively. EADM of 1 mg/kg and 2 mg/kg alone inhibited the growth of KBv200 xenografts by 16.9% and 29.0%. The combination of Br-Tet of 5 mg/kg, 7.5 mg/kg and 10 mg/kg with EADM of 1 mg/kg, 2 mg/kg respectively increased the IR of EADM. The curve of tumor growth is shown in Figure 5. The toxicity of EADM at the dose of 3 mg/kg was significantly increased (animal death and body weight loss) when co-administered with Br-Tet of 5 mg/kg, 7.5 mg/kg and 10 mg/kg, respectively (data not shown). These results suggested that 2 mg/kg of EADM was an optimal dose for use in nude mice. Furthermore, 10 mg/kg of Br-Tet enhanced the inhibition of KBv200 cell xenografts by EADM at doses of 1 and 2 mg/kg more potently than 5 and 7.5 mg/kg of Br-Tet did. These data demonstrated that the optimal dose of Br-Tet was 10 mg/kg (Table I).

Effect of Br-Tet on DOX accumulation in MDR human KBv200 cell xenograft tissue. Scan results of Dox samples showed that λ_{ex} was at 470 nm and λ_{em} was at 590 nm. There was no absorbance peak at 470/590 nm for Br-Tet, which indicated that Br-Tet would not interfere with the measurement of Dox. There were absorbance peaks at λ_{em}=590 nm for the tumor tissue sample from mice injected with Dox while no absorbance peak was seen for the tumor tissue samples of the control group.

Br-Tet increased the Dox accumulation of MDR cell xenograft tissue in nude mice (Figure 6). The results indicated that in the group receiving 8 mg/kg Dox alone, the Dox accumulation in KBv200 cell xenograft tissue remained at a low level and dropped after reaching a peak at 2 h, while that in KB cell xenograft tissue kept increasing in a time-dependent manner until 3 h. In the presence of Br-Tet, Dox accumulation in KBv200 and KB cell xenograft tissue continued to rise with increasing time within 3 h. Furthermore, compared with that of the group receiving 8 mg/kg Dox alone, the Dox accumulation in KBv200 cell xenograft tissue was significantly higher at the same time point in the group co-administered Dox and Br-Tet ($p<0.01$). However, there was no significant difference between the Dox accumulation of KB cell xenograft tissue in the absence and presence of Br-Tet.

Incubation with Br-Tet did not reduce the expressin of P-gp. To further understand the MDR reversal mechanism of Br-Tet, we assayed P-gp expression in KBv200 cells after they were exposed to 0.5, 1.0 and 1.5 μmol/l Br-Tet for 48 h by flow cytometry and Western blot. Results from flow cytometry showed an increased expression of P-gp in KBv200 cells, which indicated that Br-Tet did not down-regulate the expression of P-gp (Figure 7A). Consistent with

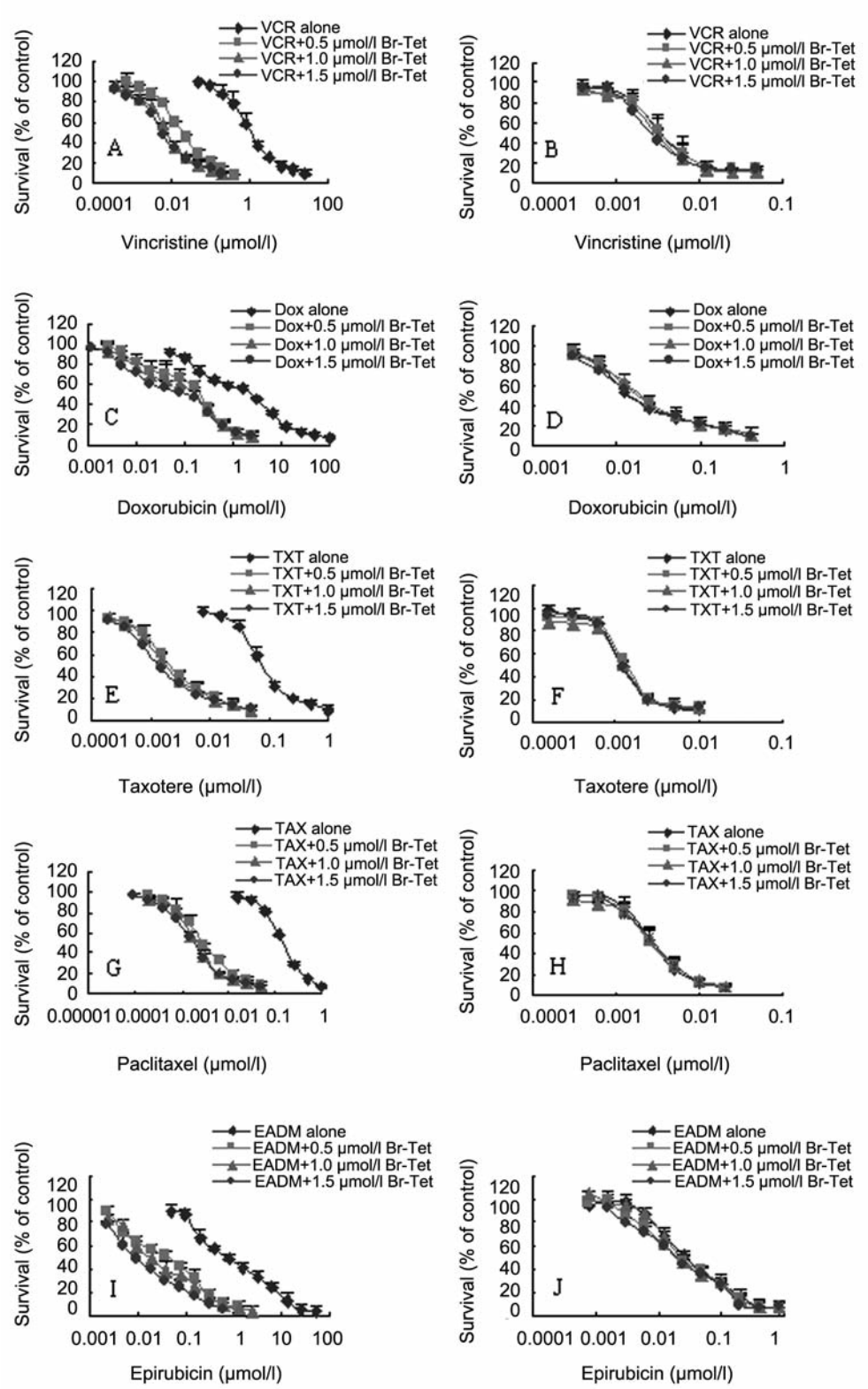

Figure 3. *The effect of Br-Tet on the sensitivity of KBv200 cells (A, C, E, G, I) and KB cells (B, D, F, H, J) to vincristine, doxorubicin, taxotere, paclitaxel and epirubicin in vitro, respectively. Cytotoxicity MTT assay was described in Materials and Methods. The cells were cultured with a full range of concentrations of anticancer drugs in the presence or absence of Br-Tet for 72 h. Data represent means and standard deviation of at least triplicate determinations.*

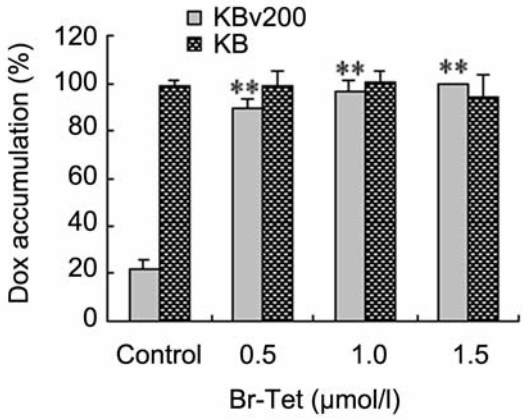

Figure 4. *Br-Tet increased Dox accumulation in KBv200 cell line in a concentration-dependent manner. Dox accumulation of Br-Tet-treated KBv200 cells reached 100% at 1.5 μmol/l. **p<0.01 versus control group.*

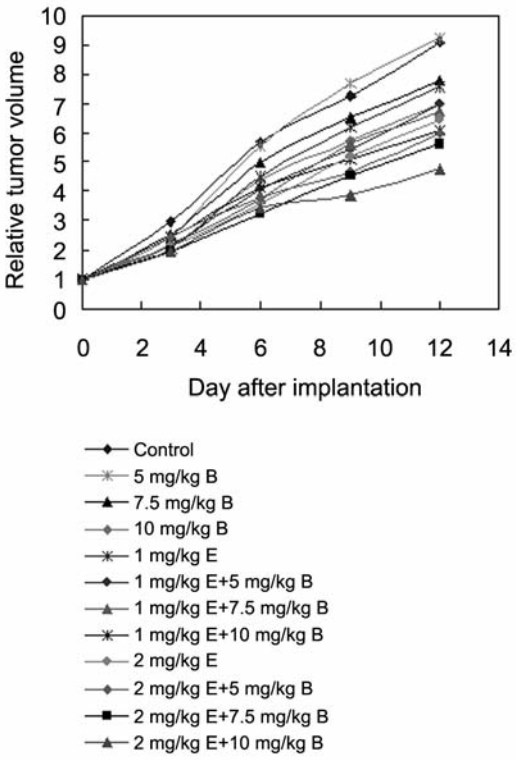

Figure 5. *Inhibition effect of Br-Tet and EADM on growth of KBv200 xenograft. The experiment was carried out using athymic mice implanted subcutaneously (s.c.) with KBv200 cells. The treatments were administered as indicated in the Materials and Methods. Each point represents mean relative tumor volume±standard deviation of each group. E is indicative of EADM; B is indicative of Br-Tet.*

the flow-cytometry results, Western blot analysis did not detect any decrease in P-gp expression after KBv200 cells were treated with Br-Tet (Figure 7B).

Discussion

Since the initial discovery that verapamil could reverse P-gp-mediated MDR was reported by Tsuruo and colleagues (9), there has been an intense search for compounds which could reverse the MDR phenotype of tumors. Many inhibitors of MDR transporters have been identified and some are undergoing clinical trials, but currently none are in clinical use.

Tet, a benzylisoquinoline alkaloid, has been used in China as an antifibrotic drug to treat the lesions of silicosis for over 30 years (1). Recent studies have shown that Tet has a reversal effect on P-gp-mediated MDR (2, 3). Br-Tet is a brominated derivative of Tet. In this study, we evaluated the MDR reversal potency of Br-Tet *in vivo* and *in vitro* and the mechanism involved in this reversal.

The *in vitro* reversal effects of Br-Tet were studied with conventional chemotherapeutical drugs that are all P-gp substrates in KBv200 cells and KB cells. Since Br-Tet was not cytotoxic for KBv200 cells and KB cells at concentrations up to 1.5 μmol/l, Br-Tet of 0.5, 1.0 and 1.5 μmol/l were chosen to modulate MDR. Br-Tet significantly restored the sensitivity of KBv200 to VCR, TAX, TXT, Dox and EADM in concentration-dependent manner. However, in KB cells, Br-Tet had no significant effect on drug cytotoxicity. These results demonstrated that Br-Tet was an efficacious reversal agent *in vitro*.

KBv200 cell xenografts were used to to evaluate the *in vivo* MDR reversal potency of Br-Tet. According to our preliminary experiments, Br-Tet of 7.5 mg/ml did not display reversal activity, while Br-Tet of 20 mg/kg demonstrated obvious toxicity (data not shown), therefore Br-Tet of 5, 7.5 and 10 mg/kg and EADM of 1 and 2 mg/kg were chosen for the *in vivo* study. The results showed that the growth of KBv200 cell xenograft in nude mice was affected little by Br-Tet of 5, 7.5 and 10 mg/kg alone or by EADM of 1 mg/kg alone. When given in combination, 10 mg/kg Br-Tet significantly increased the antitumor activity of 1 and 2 mg/kg EADM; 3 mg/kg EADM combined with Br-Tet caused a significant increase in animal death rate and obvious reduction in mouse body weight (data not shown). The most efficacious action was achieved when 10 mg/kg Br-Tet was combined with 2 mg/kg EADM, and there was no evidence of any increase of toxicity.

The mechanism of MDR reversal has been widely explored. MDR modulators are likely to reverse MDR through multiple pathways, such as by inhibiting P-gp function and/or expression. Most modulators can enhance the intracellular accumulation of anticancer drugs through inhibiting P-gp function. Recently some agents that reversed MDR through inhibition of P-gp gene expression were found (10, 11). In this study, Br-Tet enhanced intracellular Dox accumulation in the MDR KBv200 cell line in concentration-

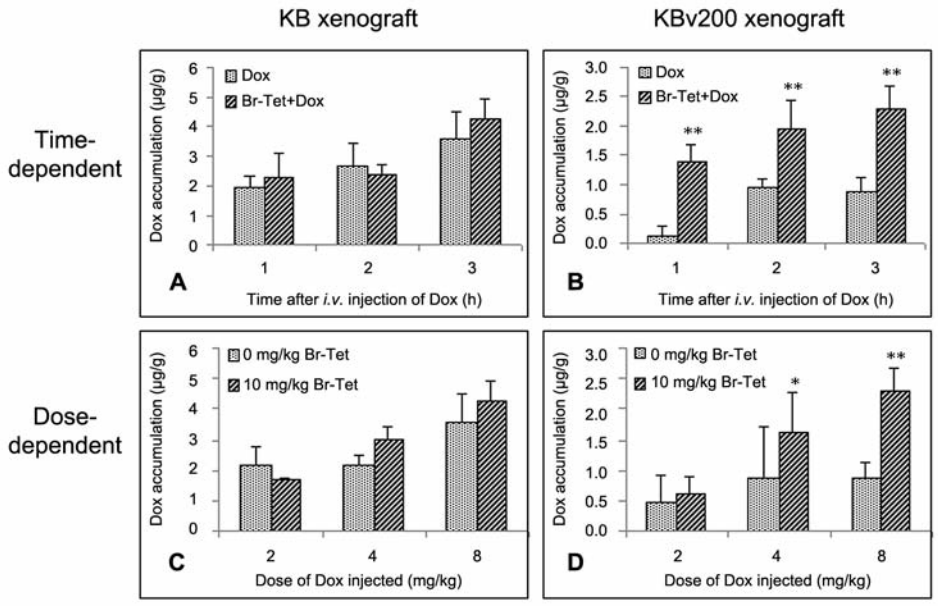

Figure 6. *Br-Tet increased Dox accumulation in KBv200 xenograft tissue in both time- and dose-dependent manners. The experiment was carried out as described in Materials and Methods. Dox accumulation (μg/g) in KB (A) and KBv200 (B) xenograft tissue in the absence and presence of 10 mg/kg Br-Tet at different time points after 8 mg/kg Dox was injected. Dox accumulation (μg/g) in KB (C) and KBv200 (D) xenograft tissue in the absence and presence of 10 mg/kg Br-Tet 3 h after Dox was injected. *p<0.05 and **p<0.01 versus corresponding group given Dox alone, respectively.*

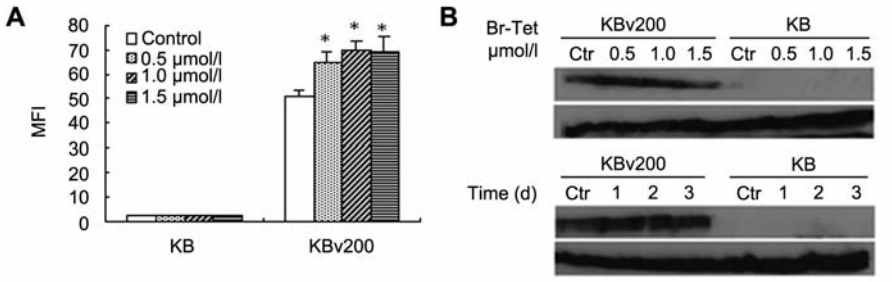

Figure 7. *The effect of Br-Tet on the expression of P-glycoprotein in KB and KBv200 cells. A, P-gp expression detected by flow cytometry. KB and KBv200 cells were treated with the indicated concentration of Br-Tet, then cells were harvested and incubated with monoclonal anti-P-gp antibody and the mean fluorescent intensity (MFI) of each sample was measured by flow cytometry. B, P-gp expression detected by Western blot. After KB and KBv200 cells were exposed to the indicated concentration of Br-Tet for 48 h or exposed to 1.5 μmol/l Br-Tet for the indicated time, the protein was extracted and analyzed by Western blot. The results showed Br-Tet did not decrease the expression of P-gp in KBv200 cells. *p<0.05 vs. control.*

dependent manner while it had no effect on Dox accumulation in the drug-sensitive KB cell line. The study carried out in mouse tumor tissue showed that Br-Tet also increased the Dox accumulation in KBv200 xenograft tissue in both a concentration-dependent and time-dependent manner. However, Br-Tet did not reduce P-gp expression in MDR cells, as shown by both flow cytometry and Western blot. These results indicated that Br-Tet reversed MDR through inhibiting P-gp function not by inhibiting P-gp expression. Notablely, P-gp expression in Br-Tet-treated KBv200 cells measured by flow cytometry showed a small but significant increase. This was probably a result of feedback to restore drug-transport function.

In conclusion, Br-Tet showed significant reversal of P-gp-mediated MDR *in vitro* and *in vivo* and this MDR reversal *in vivo* was associated with the enhancement of accumulation of chemotherapeutical drugs in tumor tissue.

Acknowledgements

This work was supported by a grant from the National Natural Sciences Foundation No. 30600769 and Key Project Foundation of Guangdong Province No. 2005B10401042.

References

1 Shen YC, Chou CJ, Chiou WF and Chen CF: Anti-inflammatory effects of the partially purified extract of radix *Stephaniae tetrandrae*: comparative studies of its active principles tetrandrine and fangchinoline on human polymorphonuclear leukocyte functions. Mol Pharmacol *60*: 1083-1090, 2001.

2 Fu L, Liang Y, Deng L, Ding Y, Chen L, Ye Y, Yang X and Pan Q: Characterization of tetrandrine, a potent inhibitor of P-glycoprotein-mediated multidrug resistance. Cancer Chemother Pharmacol *53*: 349-356, 2004.

3 Fu LW, Zhang YM, Liang YJ, Yang XP and Pan QC: Multidrug resistance of tumor cells was reversed by tetrandrine *in vitro* and in xenograft derived from human breast cancer MCF-7/adr cells. Eur J Cancer *38*: 418-426, 2002.

4 Dai CL, Tiwari AK, Wu CP, Su XD, Wang SR, Liu DG, Ashby CR Jr, Huang Y, Robey RW, Liang YJ, Chen LM, Shi CJ, Ambudkar SV, Chen ZS and Fu LW: Lapatinib (Tykerb, GW572016) reverses multidrug resistance in cancer cells by inhibiting the activity of ATP-binding cassette subfamily B member 1 and G member 2. Cancer Res *68*: 7905-7914, 2008.

5 Li-ming Chen, Yong-ju Liang, Ji-wu Ruan, Yan Ding, Xiu-wen Wang, Zhi Shi, Gu LQ, Yang XP and Fu LW: Reversal of P-gp mediated multidrug resistance *in vitro* and *in vivo* by FG020318. J Pharm Pharmacol *56*: 1061-1066, 2004.

6 Liang YJ, Fu LW and Pan QC: Establishment of a model of KBv200 nude mouse xenograft and the studies on its characterization of multidrug resistance. Chin Pharmacol Bull *16*: 705-707, 2000.

7 Jin J, Shahi S, Kang HK, van Veen HW and Fan TP: Metabolites of ginsenosides as novel BCRP inhibitors. Biochem Biophys Res Commun *345*: 1308-1314, 2006.

8 Zhang JY, Wu HY, Xia XK, Liang YJ, Yan YY, She ZG, Lin YC and Fu LW: Anthracenedione derivative 1403P-3 induces apoptosis in KB and KBv200 cells *via* reactive oxygen species-independent mitochondrial pathway and death receptor pathway. Cancer Biol Ther *6*: 1413-1421, 2007.

9 Tsuruo T, Iida H, Tsukagoshi S and Sakurai Y: Overcoming of vincristine resistance in P388 leukemia *in vivo* and *in vitro* through enhanced cytotoxicity of vincristine and vinblastine by verapamil. Cancer Res *41*: 1967-1972, 1981.

10 Miao ZH and Ding J: Transcription factor c-Jun activation represses *mdr-1* gene expression. Cancer Res *63*: 4527-4532, 2003.

11 Miao ZH, Tang T, Zhang YX, Zhang JS and Ding J: Cytotoxicity, apoptosis induction and down-regulation of MDR-1 expression by the anti-topoisomerase II agent, salvicine, in multidrug-resistant tumor cells. Int J Cancer *106*: 108-115, 2003.

Received April 8, 2009
Revised August 14, 2009
Accepted September 21, 2009