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ORIGINAL ARTICLE

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Characterization of tetrandrine, a potent inhibitor of P-glycoprotein-mediated multidrug resistance

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Abstract Multidrug resistance (MDR) is one of the main obstacles in tumor chemotherapy. A promising approach to solving this problem is to utilize a nontoxic and potent modulator able to reverse MDR, which in combination with anticancer drugs increases the anticancer effect. Experiments were carried out to examine the potential of tetrandrine (Tet) as a MDR-reversing agent. Survival of cells incubated with Tet at 2.5 μmol/l for 72 h was over 90%. Tet at 2.5 μmol/l almost completely reversed resistance to vincristine (VCR) in KBv200 cells. Tet at a concentration as low as 0.625 μmol/l produced a 7.6-fold reversal of MDR, but showed no effect on the sensitivity of drug-sensitive KB cells in vitro. In the KBv200 cell xenograft model in nude mice, neither Tet nor VCR inhibited tumor growth. However, VCR and Tet combined inhibited tumor growth by 45.7%, 61.2% and 55.7% in three independent experimental settings. In the KB cell xenograft model in nude mice, Tet did not inhibit tumor growth, but VCR and the combination of VCR and Tet inhibited tumor growth by 40.6% and 41.6%, respectively. Mechanism studies showed that Tet inhibited [^{3}H]azidopine photoaffinity labeling of P-gp and increased accumulation of VCR in MDR KBv200 cells in a concentration-dependent manner. The results suggest that Tet is a potent MDR-reversing agent in vitro and in vivo. Its mechanism of action is via directly binding to P-gp and increasing intracellular VCR accumulation.

Keywords Vincristine · Multidrug resistance · P-glycoprotein · Tetrandrine · Xenograft · Nude mice

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Abbreviations *DMSO* Dimethyl sulfoxide · *FBS* Fetal bovine serum · *MDR* Multidrug resistance · *MTT* 3-(4,5-Dimethylthiazol-yl)-2,5-diphenyltetrazolium bromide · *PBS* Phosphate-buffered saline · *P-gp* P-glycoprotein · *SDS* Sodium dodecyl sulfate · *Tet* Tetrandrine · *VCR* Vincristine

Introduction

Multidrug resistance (MDR) is characterized clinically as a broad cross-resistance to chemotherapeutic drugs and is the main reason for failure of chemotherapy. MDR is inherently expressed in some cancers such as colorectal, renal cell, hepatocellular, and adrenocortical cancers, and chronic leukemia. MDR develops in response to treatment in several additional tumor types such as breast carcinoma, acute myelogenous leukemia, lymphomas and ovarian carcinoma. Although the etiology of MDR is multifactorial, one of the main causes is the overexpression of the membrane-associated 170-kDa glycoprotein P-gp coded by the *mdr1* gene (Ling 1997; Szabo et al. 2000; Motoji et al. 2000). Recent studies have shown that tumor cells expressing MDR-associated protein (van den Heuvel-Eibrink et al. 2000) and lung-resistance protein (Scheper et al. 1993), and mutation of DNA topoisomerase II (Volm 1998), also show MDR.

The function of P-gp is as an ATP-dependent efflux pump of anticancer drugs, which decreases the intracellular drug accumulation. P-gp is a membrane transporter with broad specificity for many different hydrophobic drugs, such as vinca alkaloids (vinblastine, VCR, vinorelbine), anthracyclines (doxorubicin, daunorubicin, epirubicin, idarubicin), taxanes (paclitaxel and docetaxel), epipodophyllotoxins (etoposide, teniposide) and mitomycin C. Several noncytotoxic drugs can sensitize MDR cells to chemotherapeutic drugs in vitro and in vivo (Malayeri et al. 1996; Krishna and Mayer 2000; Sikic et al. 1997). They include calcium channel

blockers (e.g., verapamil, nifedipine), calmodulin antagonists (e.g., trifluoperazine, chlorpromazine), various steroids (e.g., progesterone, tamoxifen), quinolines (e.g., chloroquine, quinidine), immunosuppressive drugs (e.g., cyclosporine A, rapamycin), antibiotics (e.g., rifapicin, tetracyclines), surfactants (e.g., Tween 80, Cremophor EL), and yohimbine alkoids (e.g., reserpine, yohimbine), all of which have been shown to reverse MDR in vitro.

Combined therapy with MDR-related cytotoxins and modulators may inhibit tumor growth and prolong survival in animal models (Ling 1997). Unfortunately, data regarding clinical efficacy are not yet available from early clinical trials. Alternatively, the MDR-reversing agent may expose the patient to unacceptable side effects or toxicity at doses required for effectiveness and/or affect the pharmacokinetics of anticancer drugs (Sikic 1997; Krishna and Mayer 2000). These limitations have spurred efforts to search for new more effective compounds. In the development of new cancer chemotherapy drugs, specific inhibition of P-gp and modulation MDR are currently the most important strategies.

There have been a number of studies attempting to elucidate the structural requirements for MDR-reversing agents. These studies have focused on optimizing the activity of a particular class of compounds in reversing MDR. The results suggest that reversers are hydrophobic, contain two or more planar aromatic rings, a tertiary nitrogen, and a positive charge at physiological pH (Wiese and Pajeva 2001; Lawrence et al. 2001; Fu and Pan 1996). Theoretically, tetrandrine (Tet), a *bis*benzylisoquinoline alkaloid (Fig. 1), contains the ideal structural features associated with some modulators of MDR. In the study reported here, we investigated the characteristics of Tet, a P-gp inhibitor, in reversing MDR both in vitro and in vivo and the mechanisms involved.

Materials and methods

Reagents

Tet, $C_{38}H_{42}O_6N_2$, was obtained as a powder with a purity of >98% from Dr. X.P. Pan (Kuming Institute of Botany, Chinese Academy of Sciences). Tet was freshly made for use. VCR, sodium azide (NaN_3), deoxyglucose, DMSO and MTT were purchased from Sigma Chemical Company (St. Louis, Mo.). RPMI 1640 was purchased from Gibco BRL (Gaithersburg, Md.). [^{3}H]VCR and [^{3}H]azidopine were purchased from Amersham Pharmacia Biotech (Piscataway, N.J.).

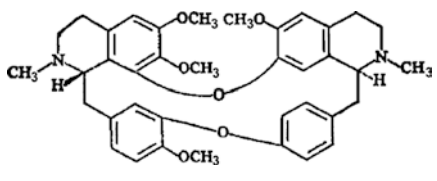

Fig. 1 The molecular structure of Tetrandrine

Cell lines and cell culture

KB and KBv200 cells are human epidermoid carcinoma cell lines. KBv200 cells, a classical MDR human epidermoid carcinoma cell line expressing high levels of P-gp (the main cause of MDR induction), was cloned from parental drug-sensitive KB cells by stepwise exposure to increasing doses of VCR and ethylmethane sulfonate mutagenesis. P-gp overexpression is a main cause of MDR induction in KBv200 cells. KBv200 cells and parental sensitive KB cells were obtained from the Chinese Academy of Medical Sciences, Beijing. KBv200 and KB cells were cultured with RPMI 1640 medium with 10% FBS, benzylpenicillin (50 kU/l), and streptomycin (50 mg/l) at 37°C in a humidified atmosphere comprising 5% CO_2 and 95% air (Zhang et al. 1994).

Cytotoxicity MTT assay

The cells were collected with trypsin and resuspended to a final concentration of 1×10^5 cells/ml, and 0.18-ml aliquots were seeded in 96-well multiplates. After 24 h of incubation, 10 µl modulator and 10 µl anticancer drug were added. After 68 h, 10 µl of 10 mg/ml MTT solution was added to each well, and the plate was further incubated for 4 h, allowing viable cells to reduce the yellow MTT to dark-blue formazan crystals, which were dissolved in 150 µl DMSO. Cell growth inhibition was evaluated by the MTT method in triplicate assays (Carmichael et al. 1987). IC_{50} values were calculated from cytotoxicity curves by the method of Bliss (Lella 2001). The degree of resistance was calculated by dividing the IC_{50} for MDR cells by that for parental sensitive cells. The fold reversal of MDR was calculated by dividing the IC_{50} of anticancer drug for cells in the absence of modulator by that in the presence of modulator.

In vivo studies

Balb/c nude mice were obtained from the Center of Experimental Animal, Sun Yat-Sen University, and maintained in the center. Animals were provided with sterilized food and water. Male mice 6–8 weeks old, weighing 20–25 g were used. Transplantable KBv200 and KB cells were collected and suspended at a concentration of 10^7/ml and implanted into the mice for the chemotherapeutic studies. In detail, mice received subcutaneous (s.c.) implantation of the KBv200 and KB cells using 0.5 ml per inoculation (about 10^7 cells/ml) under the shoulder. After 4 days, when the s.c. tumors were approximately 0.5×0.5 cm^2 (two perpendicular diameters) in size, mice were randomized into the following four treatment groups: control, VCR alone (0.2 mg/kg every 2 days), Tet alone (30 mg/kg every 2 days), and VCR plus Tet. Each animal was earmarked and followed individually throughout the experiments. Animals then received the desired drug and dosage. Tumor growth was monitored starting on the first day of treatment and then every 3 days by measuring the volume of tumor. Tumor volume (V) was determined from two perpendicular diameters (A and B) and was calculated according to the formula (Fu et al. 2003; Liang et al. 2000):

$$V = \frac{\pi}{6}\left(\frac{A+B}{2}\right)^3$$

The curve of tumor growth was drawn according to tumor volume and time of implantation. Then the mice were ethically killed and tumor tissues were removed and their weight measured. The rate of inhibition of tumor growth (IR) was calculated according to the formula:

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

Passive uptake, accumulation and efflux of drug

To determine the passive uptake of VCR, cells were preincubated at 37°C for 15 min in energy-free medium with 15 m*M* NaN_3 plus 10 m*M* deoxyglucose in PBS (pH 7.4) and 10% FBS. They were then incubated at 37°C for 2 h with 1 μ*M* of [^{3}H]VCR in the same buffer. The cells were then chilled on ice and washed with cold PBS three times. The cells were solubilized in 1% Triton X-100 and 0.2% SDS in 10 mmol/l phosphate buffer, pH 7.4, and the radioactivity determined (Sumizawa et al. 1997).

The accumulation and efflux of VCR were determined according to the method described by Chen et al. (1999). To measure drug accumulation, confluent monolayers of KB and KBv200 cells in 24-well plates were incubated in energy-supplied buffer (PBS plus 10% FBS added with 10 m*M* glucose) for 1 h at 37°C and then with 1 μmol/l [^{3}H]VCR in energy-supplied buffer with or without Tet for 2 h at 37°C. After washing with ice-cold PBS three times, the cells were solubilized in 1% Triton X-100 and 0.2% SDS in 10 mmol/l phosphate buffer, pH 7.4, and the radioactivity determined.

To measure drug efflux, cells were incubated overnight and then further incubated in the presence or absence of Tet for 1 h. The medium was then changed to energy-supplied buffer and incubated with 1 μmol/l [^{3}H]VCR for 2 h at 37°C in the presence or absence of Tet at 2.5 μmol/l. Each dish was washed once with PBS, and energy-supplied buffer without [^{3}H]VCR and with or without Tet was added. Then the cells were incubated for the indicated times at 37°C and harvested, and the radioactivity determined.

[^{3}H]Azidopine photoaffinity labeling of P-gp

KBv200 cell membranes were prepared as previously described (May et al. 1988), and the experiment was carried out as described by Hyafil et al. (1993). Membranes were incubated with Tet for 25 min in the dark, followed by a similar incubation with 0.6 μ*M* [^{3}H]azidopine. After UV irradiation for 2 min, the photolabeled membranes were subjected to SDS-PAGE on an 8% gel, followed by fluorography. P-gp was confirmed by Western blotting using monoclonal antibody to mdr1 at a dilution of 1:1000 (Santa Cruz Biotechnology, Santa Cruz, Calif.) and enhanced chemiluminescence detection.

Results

KBv200 cells are strongly resistant to VCR

To determine the degree of resistance of KBv200 cells to VCR, we compared the cytotoxicity of VCR in KBv200 cells and their parental sensitive KB cells. The IC_{50} values of VCR in KB and KBv200 cells were 38.9 and 3551.4 nmol/l, respectively (Fig. 2). So KBv200 cells were approximately 91-fold resistant to VCR compared to KB cells in our experimental system.

Tet itself inhibits growth of KB and KBv200 cells

The cytotoxic effect of Tet after a 72-h treatment is shown in Fig. 3. The IC_{50} values of Tet in KB and KBv200 cells were 10.3 and 13.0 μmol/l, respectively. Cell survival was approximately 90% or more in both KBv200 and KB cells at a Tet concentration of 2.5 μmol/l. So Tet concentrations of 2.5, 1.25 and 0.625 μmol/L were used to study MDR-reversing activity.

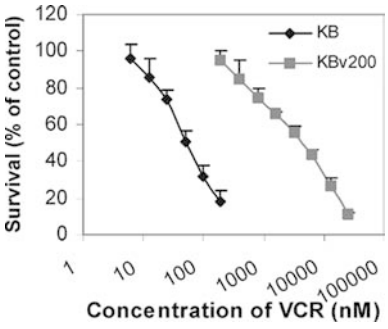

Fig. 2 Cytotoxicity of VCR in KB and KBv200 cells as determined by the MTT assay described in Materials and methods. The cells were cultured with a full range of concentrations of VCR for 72 h. The results are presented as the means and SD of at least triplicate determinations

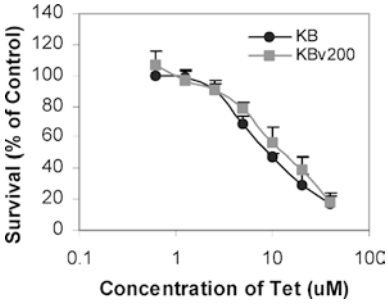

Fig. 3 Cytotoxicity of Tet in KB and KBv200 cells as determined by the MTT assay described in Materials and methods. The cells were cultured with a full range of concentrations of Tet for 72 h. The results are presented as the means and SD of at least triplicate determinations

Reversal of MDR in vitro by Tet

The cells were incubated with various concentrations of Tet and a full range of concentrations of the chemotherapeutic agent, VCR. The aim of the experiments was to see if Tet could modulate the sensitivity of MDR cells to VCR. The IC_{50} values and fold reversal are summarized in Table 1.

Almost complete reversal of MDR was typically seen with Tet at 2.5 μmol/l in KBv200 cells. Tet at 1.25 μmol/l lowered the IC_{50} of VCR from 3551.4 nmol/l to 181.0 nmol/l, i.e., about a 20-fold reversal of MDR. At 0.625 μmol/l Tet still reversed MDR 7.6-fold. In contrast, Tet had no effect on the sensitivity of KB cells to VCR (Fig. 4).

Reversal of MDR in vivo by Tet

Tet potently reversed MDR in KBv200 cells in vitro. To determine whether Tet in vivo could reverse MDR, tumor growth suppression experiments were conducted in nude mice bearing KBv200 and KB solid tumors. When the xenografts were established (4 days), the

Table 1 Modulation of sensitivity to VCR in KBv200 and KB cells by Tet. The IC_{50} of VCR was determined in the presence of various concentrations of Tet as described in Material and methods. Each value is the mean ± SD of at least three independent experiments. The fold-reversal of MDR is defined as the ratio of the IC_{50} for VCR alone to that in the presence of the modulating agent

Group	Concentration (μmol/l)	IC_{50} of VCR (mean ± SD, nmol/l)		Fold-reversal of MDR	
		KBv200	KB	KBv200	KB
Control		3551.4 ± 412.3	38.9 ± 7.4		
Tet	0.625	466.0 ± 65.4	51.0 ± 4.2	7.6	0.8
	1.25	181.0 ± 20.6	34.9 ± 8.1	19.6	1.1
	2.5	58.6 ± 7.9	40.9 ± 5.8	60.6	1.0

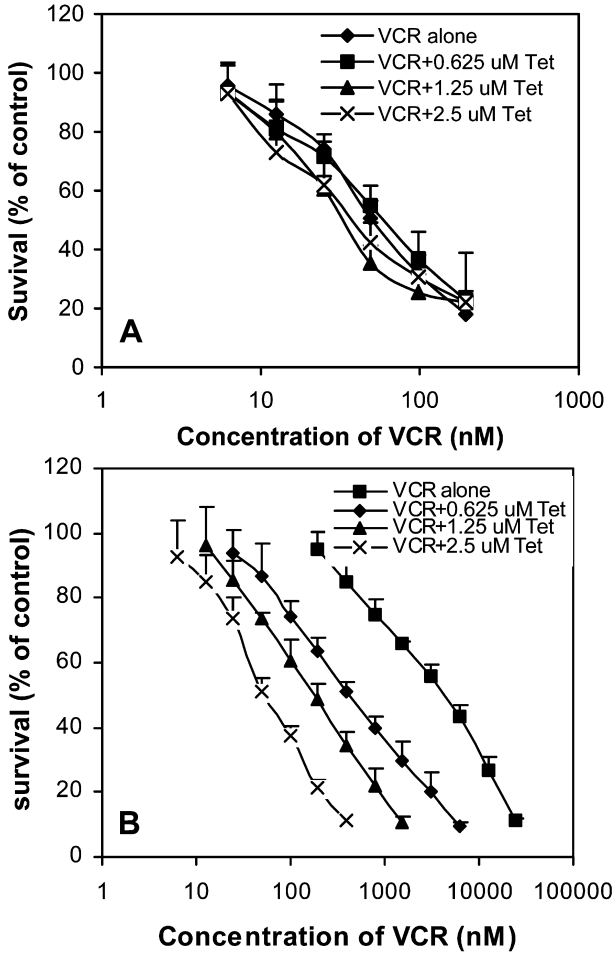

Fig. 4A, B Enhancement by Tet of the sensitivity of KB cells (**A**) and KBv200 cells (**B**) to VCR in vitro as determined by the MTT assay described in Materials and methods. The cells were cultured with a full range of concentrations of VCR in the presence or absence Tet for 72 h. The results are presented as the means and SD of at least triplicate determinations

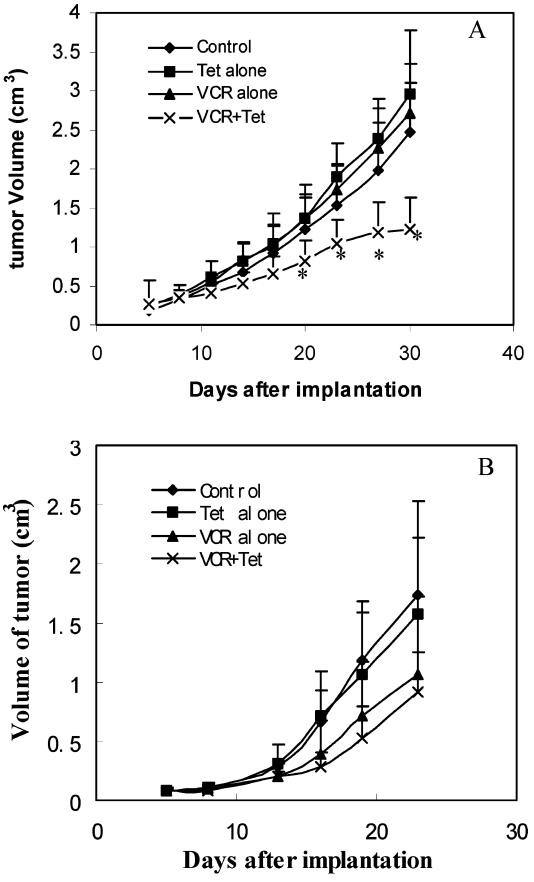

Fig. 5A, B Reversal of MDR in vivo by Tet. The experiment was carried out with athymic mice implanted s.c. with KBv200 cells (**A**) or KB cells (**B**). The treatments were administered as indicated in Materials and methods. The results are presented as the means ± SD of the tumor sizes in each group. $^*P < 0.05$ vs VCR alone. Similar results were obtained in three independent experiments

following treatments were initiated: VCR only (0.2 mg/kg, every 2 days, intraperitoneal), Tet only (30 mg/kg, every 2 days, intraperitoneal), VCR plus Tet (VCR 0.2 mg/kg, Tet 30 mg/kg, both every 2 days, intraperitoneal), control (equal volume saline).

Neither Tet alone nor VCR alone inhibited tumor growth. However, combined VCR and Tet significantly inhibited tumor growth. The tumors were much smaller in the mice receiving VCR plus Tet than in others and the rates of inhibition of tumor growth were 45.7%, 61.2% and 55.7% in three independent experimental settings (Fig. 5A, Table 2).

In the KB cell xenograft model in nude mice, Tet did not inhibit tumor growth, but VCR and the combination of VCR and Tet significantly inhibited tumor growth. The rates of inhibition of tumor growth were 40.6% and 41.6% in mice receiving VCR and in mice receiving VCR plus Tet, respectively (Fig. 5B, Table 3).

Table 2 Effect of combination treatment with Tet and VCR on the growth of KBv200 cell xenografts in nude mice. The experiments were conducted as described in Materials and methods. The values presented are the mean ± SD for each group. The results of three experiments are shown

Group	Before experiment		After experiment		Weight of tumor (g)	Inhibition (%)
	Animals	Weight (g)	Animals	Weight (g)		
Control	6	18.3 ± 1.1	6	21.1 ± 2.8	1.53 ± 0.77	
VCR	6	18.2 ± 1.2	6	19.8 ± 2.1	1.12 ± 0.75	27.2
Tet	6	18.6 ± 1.1	6	21.4 ± 1.0	1.27 ± 0.83	17.4
VCR + Tet	6	18.6 ± 1.3	6	20.2 ± 2.1	0.83 ± 0.25*,**	45.7
Control	6	20.6 ± 2.5	6	25.6 ± 2.9	3.02 ± 0.81	
VCR	7	19.9 ± 1.3	7	24.4 ± 2.5	2.41 ± 1.08	20
Tet	6	21.0 ± 2.2	6	25.0 ± 2.9	2.93 ± 1.33	2.8
VCR + Tet	7	20.8 ± 3.2	7	23.4 ± 4.1	1.17 ± 0.82*,**	61.2
Control	6	19.7 ± 1.7	6	23.2 ± 2.8	1.48 ± 0.64	
VCR	7	22.6 ± 2.7	7	23.6 ± 3.0	1.48 ± 0.64	0
Tet	6	21.0 ± 2.6	6	23.3 ± 1.6	1.52 ± 0.79	−2
VCR + Tet	7	21.8 ± 3.3	7	23.3 ± 2.6	0.66 ± 0.37*,**	55.7

*$P < 0.05$ vs control, **$P < 0.05$ vs VCR

Table 3 Effect of combination treatment with Tet and VCR on the growth of KB cell xenografts in nude mice. The experiments were conducted as described in Materials and methods. The values presented are the mean ± SD for each group

Group	Before experiment		After experiment		Weight of tumor (g)	Inhibition (%)
	Animals	Weight (g)	Animals	Weight (g)		
Control	10	25.2 ± 2.6	10	25.5 ± 3.5	1.01 ± 0.31	
VCR	9	25.9 ± 2.4	9	24.7 ± 2.2	0.60 ± 0.31*	40.6
Tet	9	24.1 ± 2.2	9	24.0 ± 2.7	0.92 ± 0.34	8.9
VCR + Tet	9	24.2 ± 2.2	9	22.1 ± 2.3	0.59 ± 0.20*	41.6

*$P < 0.05$ vs control

Mechanism of action studies

Tet inhibited the efflux of VCR and enhanced the accumulation of VCR in KBv200 cells

The decrease in cellular drug accumulation induced by P-gp is one of the main causes of MDR. To determine how Tet overcomes resistance to VCR in KBv200 cells, we examined its effects on VCR accumulation and passive uptake and efflux in KB cells and KBv200 cells (Fig. 6). VCR accumulation was much greater (3.1-fold) in sensitive KB cells than in MDR KBv200 cells. There was a high level of VCR accumulation in the drug-sensitive KB cells that was unaffected by Tet (0.9, 0.9, 1.1-fold at 2.5, 1.25, 0.625 μmol/l of Tet). There was a low level of accumulation in KBv200 cells, and Tet restored in a concentration-dependent manner the level of accumulation to levels approaching those in the drug-sensitive KB cells. Cellular VCR accumulation was up to 2.4-, 2.1- and 1.5-fold in KBv200 cells in the presence of 2.5, 1.25 and 0.625 μmol/l of Tet. However, intracellular VCR accumulation showed no significant difference in the presence or absence of Tet in energy-depleted KBv200 cells treated with NaN_3/deoxyglucose. We sought to determine whether the increased accumulation of VCR in KBv200 cells caused by Tet was due to inhibition of VCR efflux. The time-course of release of VCR after 2 h of accumulation is shown in Fig. 6C, which shows that KBv200 cells released a higher percentage of accumulated VCR than KB cells. At 30 min, 36% of accumulated VCR had been lost from KBv200 cells, whereas only 17% had been lost from KB cells. Tet at 2.5 μmol/l apparently inhibited the efflux of VCR from KBv200 cells, but not that from KB cells. These results suggest that Tet inhibits efflux of VCR induced by P-gp rather than passive uptake of VCR.

P-gp [^{3}H]azidopine photolabeling

Direct interaction of Tet with P-gp was assessed by examining the ability of Tet to prevent [^{3}H]azidopine photoaffinity labeling of P-gp in KBv200 cell membranes. Tet caused a significant inhibition of azidopine binding to P-gp in a dose-dependent manner (Fig. 7).

Discussion

A large number of compounds can reverse the P-gp-mediated MDR phenotype in vitro. Calcium channel blockers such as verapamil were the first drugs developed with the aim of reversing P-gp-mediated MDR in cell lines (Tsuruo et al. 1981). Similar observations were subsequently made with cyclosporin A (Wigler 1996). Although having some efficacy, these agents are relatively weak P-gp inhibitors, are often substrates for P-gp, and exhibit dose-limiting side effects that severely restrict their clinical utility. To address the problems described above, there has been considerable interest in second-generation P-gp inhibitors. VX-710 (Germann et al. 1997) and PSC833 (Atadja et al. 1998) are 3-fold to 100-fold more potent than verapamil in the reversal of MDR. However, an additional problem with most

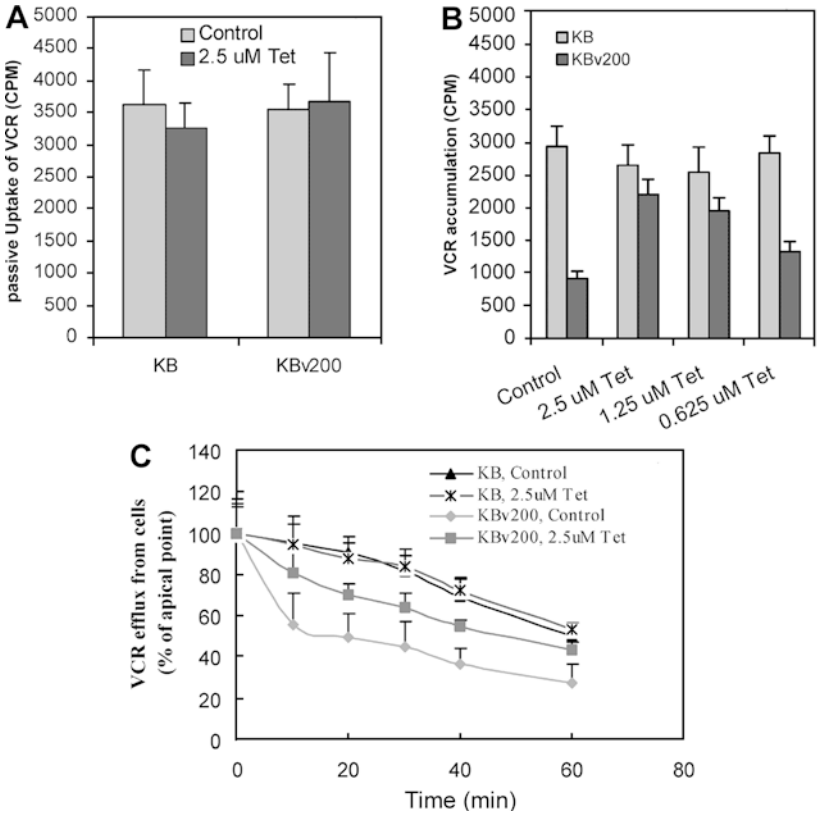

Fig. 6A–C Effect of Tet on the passive uptake, accumulation and efflux of VCR in KB and KBv200 cells. **A** VCR passive uptake in KB and KBv200 cells measured after incubation with 1 μM [^{3}H]VCR for 2 h in energy-free medium in the presence or absence of Tet; **B** VCR accumulation in KB and KBv200 cells measured after incubation with 1 μM [^{3}H]VCR for 2 h with or without Tet (*bars* means ± SD of triplicate determinations); $^*P < 0.05$ vs control. **C** Efflux of VCR: VCR retained after 2 h of accumulation in KB and KBv200 cells in the absence or presence of Tet. Values are percentages in relation to the start-point at time zero

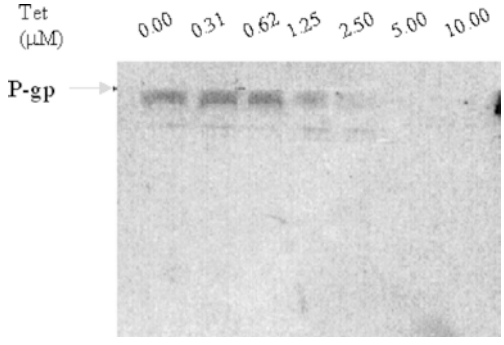

Fig. 7 Effect of Tet on [^{3}H]azidopine photoaffinity labeling of P-gp in KBv200 cell membranes. P-gp was identified by Western blotting using a monoclonal antibody (Mdr1) and ECL detection

second-generation P-gp inhibitors is that they significantly alter the plasma pharmacokinetics of coadministered antitumor agents, increasing blood levels by reducing clearance and/or metabolism. For example, PSC833 (Atadja et al. 1998) and VX-710 (Germann et al. 1997) have been reported to produce significant pharmacokinetic interactions with agents such as paclitaxel.

Alterations in anticancer drug excretion by the effects of MDR modulators on drug transport proteins often lead to increased anticancer drug exposure of healthy tissues and have necessitated dose reduction in many preclinical and clinical studies. Changes in the dose or schedule of anticancer drugs may be able to address toxicity or efficacy alterations brought about by MDR modulators. Due to this fact, an ability to avoid such alterations in anticancer drug clearance may be considered to be a significant advantage of MDR modulation strategies. Third-generation P-gp inhibitors that potently reverse MDR and lack significant pharmacokinetic interactions with anticancer drugs have been reported, such as S9788 (Tranchand et al. 1998), OC144-093 (Newman et al. 2000) and LY335979 (Dantzig et al. 1996; Starling et al. 1997).

Our experimental results showed that Tet had potent MDR-reversing activity in KBv200 cells in vitro at concentrations at which by itself it is not cytotoxic. In vivo, neither Tet nor the anticancer drug VCR alone showed significant ($P > 0.05$ versus the control group) tumor growth-inhibiting activity in the KBv200 xenograft nude mouse model. However, the combination of Tet and conventional anticancer drug potently inhibited ($P < 0.05$ versus anticancer drug alone) the growth of the xenografts. These results suggest that Tet is able to potently reverse MDR induced by P-gp in vitro and in vivo. Importantly, our experimental results showed that the toxicity of the anticancer drug VCR coadministered with Tet was endurable in the experimental animals. Therefore, no evidence of changes in the toxicity of VCR induced by Tet was observed. Furthermore, Tet has been used as an antifibrotic drug to treat the lesions of silicosis in China since the 1960s. It does not have any side effects even at a dose of 180 mg three times daily in long-term use (Fang and Fang 1996). The clinical data showed that it would be clinically safe. On the other

hand, the cytotoxicity of Tet was similar in drug-sensitive KB and drug-resistant KBv200 cells. This implies that Tet is not a substrate of P-gp. Additional studies are required to determine whether Tet has an affect on the pharmacokinetics of anticancer drugs. Taken together, these results suggest that Tet is a promising modulator of MDR in combination with conventional anticancer drugs.

The mechanism of action of P-gp has been investigated extensively from a biochemical perspective in the past few years. The use of purified P-gp and functionally reconstituting it into liposomes to investigate its properties show that P-gp alone is sufficient to transport different drugs. The decrease in cellular anticancer drug accumulation was due to n increase in the efflux of drug mediated by P-gp. So it was key to improve the accumulation of anticancer drug in MDR cells to reverse MDR. Our experiments showed that VCR accumulation in KBv200 cells was significantly increased in the presence of Tet and VCR efflux and accumulation was not affected in the parental cell lines that lack P-gp. Thus reversal of drug resistance by Tet is probably attributable to the inhibition of P-gp-mediated efflux. The inhibition of drug efflux as a result of direct interaction of Tet with P-gp was implied by the potent displacement of a photoaffinity label, [^{3}H]azidopine.

In summary, we observed an improvement in MDR reversal in vitro and in vivo when Tet was coadministered with a conventional anticancer drug. Also, the toxic effect of Tet was endurable. These results suggest that Tet is a promising modulator of MDR. The mechanism of MDR modulation by Tet is associated with an increase in intracellular drug accumulation induced by direct binding to P-gp. These results indicate promising clinical potential.

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References

Atadja P, Watanabe T, Xu H, Cohen D (1998) PSC-833, a frontier in modulation of P-glycoprotein mediated multidrug resistance. Cancer Metastasis Rev 17:163–168

Carmichael J, DeGraff WG, Gazadar AF, Minna JD, Mitchell JB (1987) Evaluation of a tetrazolium-based semiautomated colorimetric assay: assessment of chemosensitivity testing. Cancer Res 47:943–946

Chen ZS, Kawabe T, Ono M, Aoki S, Sumizawa T, Furukawa T, Uchiumi T, Wada M, Kuwano M, Akiyama SI (1999) Effect of multidrug resistance-reversing agents on transporting activity of human canalicular multispecific organic anion transporter. Mol Pharmacol 56:1219–1228

Dantzig AH, Shepard RL, Cao J, Law KL, Ehlhardt WJ, Baughman TM, Bumol TF, Starling JJ (1996) Reversal of P-glycoprotein-mediated multidrug resistance by a potent cyclopropyldibenzosuberane modulator, LY335979. Cancer Res 56:4171–4179

Fang JH, Fang YH (1996) Tetrandrine: pharmacology and clinical usefulness. Chin Pharm J 31:454–457

Fu LW, Pan QC (1996) The properties shared by compounds with reversing tumor multidrug resistance. Chin J Cancer 15:475–477

Fu LW, He LR, Liang YJ, Chen LM, Xiong HY, Yang XP, Pan QC (2003) Experimental chemotherapy against xenografts derived from multidrug resistant KBv200 cells and parental drug-sensitive KB cells in nude mice by annonaceous acetogenin 89-2. Yao Xue Xue Bao 38:565–570

Germann UA, Shlyakhter D, Mason VS, Zelle RE, Duffy JP, Galullo V, Armistead DM, Saunders JO, Boger J, Harding MW (1997) Cellular and biochemical characterization of VX-710 as a chemosensitizer: reversal of P-glycoprotein-mediated multidrug resistance in vitro. Anticancer Drugs 8:125–140

Hyafil F, Vergely C, Du Vignaud P, Grand-Perret T (1993) In vitro and in vivo reversal of multidrug resistance by GF120918, an acridonecarboxamide derivative. Cancer Res 53:4595–4602

Krishna R, Mayer LD (2000) Multidrug resistance (MDR) in cancer. Mechanisms, reversal using modulators of MDR and the role of MDR modulators in influencing the pharmacokinetics of anticancer drugs. Eur J Pharm Sci 11:265–283

Lawrence DS, Copper JE, Smith CD (2001) Structure-activity studies of substituted quinoxalinones as multiple-drug-resistance antagonists. J Med Chem 44:594–601

Lella JW (2001) Constructing history in biography: a symposium on William Osler: a life in medicine. Introduction. Bull Hist Med 75:740–744

Liang YJ, Fu Lw, Pan QC (2000) Establishment of the model of KBv200 nude mice xenograft and the studies on its characterization of multidrug resistance. Chin Pharmacol Bull 16:705–707

Ling V (1997) Multidrug resistance: molecular mechanisms and clinical relevance. Cancer Chemother Pharmacol [Suppl] 40:S3

Malayeri R, Filipits M, Suchomel RW, Zochbauer S, Lechner K, Pirker R (1996) Multidrug resistance in leukemias and its reversal. Leuk Lymphoma 23:451–458

May GL, Wright LC, Dyne M, Mackinnon WB, Fox RM, Mountford CE (1988) Plasma membrane lipid composition of vinblastine sensitive and resistant human leukaemic lymphoblasts. Int J Cancer 42:728–733

Motoji T, Motomura S, Wang YH (2000) Multidrug resistance of acute leukemia and a strategy to overcome it. Int J Hematol 72:418–424

Newman MJ, Rodarte JC, Benbatoul KD, Romano SJ, Zhang C, Krane S, Moran EJ, Uyeda RT, Dixon R, Guns ES, Mayer LD (2000) Discovery and characterization of OC144-093, a novel inhibitor of P-glycoprotein-mediated multidrug resistance. Cancer Res 60:2964–2972

Scheper RJ, Broxterman HJ, Scheffer GL, Kaaijk P, Dalton WS, van Heijningen TH, van Kalken CK, Slovak ML, de Vries EG, van der Valk P (1993) Overexpression of a M(r) 110,000 vesicular protein in non-P-glycoprotein-mediated multidrug resistance. Cancer Res 53:1475–1479

Sikic BI (1997) Pharmacologic approaches to reversing multidrug resistance. Semin Hematol 34 [4 Suppl 5]:40–47

Sikic BI, Fisher GA, Lum BL, Halsey J, Beketic-Oreskovic L, Chen G (1997) Modulation and prevention of multidrug resistance by inhibitors of P-glycoprotein. Cancer Chemother Pharmacol [Suppl] 40:S13–19

Starling JJ, Shepard RL, Cao J, Law KL, Norman BH, Kroin JS, Ehlhardt WJ, Baughman TM, Winter MA, Bell MG, Shih C, Gruber J, Elmquist WF, Dantzig AH (1997) Pharmacological characterization of LY335979: a potent cyclopropyldibenzosuberane modulator of P-glycoprotein. Adv Enzyme Regul 37:335–347

Sumizawa T, Chen ZS, Chuman Y, Seto K, Furukawa T, Haraguchi M, Tani A, Shudo N, Akiyama SI (1997) Reversal of multidrug resistance-associated protein-mediated drug resis-

tance by the pyridine analog PAK-104P. Mol Pharmacol 51:399–405
Szabo D, Keyzer H, Kaiser HE, Molnar J (2000) Reversal of multidrug resistance of tumor cells. Anticancer Res 20:4261–4274
Tranchand B, Catimel G, Lucas C, Sarkany M, Bastian G, Evene E, Guastalla JP, Negrier S, Rebattu P, Dumortier A, Foy M, Grossin F, Mazier B, Froudarakis M, Barbet N, Clavel M, Ardiet C (1998) Phase I clinical and pharmacokinetic study of S9788, a new multidrug-resistance reversal agent given alone and in combination with doxorubicin to patients with advanced solid tumors. Cancer Chemother Pharmacol 41:281–291
Tsuruo T, Iida H, Tsukagoshi S, Sakurai Y (1981) 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
van den Heuvel-Eibrink MM, Sonneveld P, Pieters R (2000) The prognostic significance of membrane transport-associated multidrug resistance (MDR) proteins in leukemia. Int J Clin Pharmacol Ther 38:94–110
Volm M (1998) Multidrug resistance and its reversal. Anticancer Res 18:2905–2917
Wiese M, Pajeva IK (2001) Structure-activity relationships of multidrug resistance reversers. Curr Med Chem 8:685–713
Wigler PW (1996) Cellular drug efflux and reversal therapy of cancer. J Bioenerg Biomembr 28:279–284
Zhang XH, Zhang FY, Ji XJ, Li ZY (1994) Vincristine-resistant human KB cell line and mechanism of multidrug resistance. Yao Xue Xue Bao 29:246–251