PERGAMON

European Journal of Cancer 38 (2002) 418–426

European Journal of Cancer

www.ejconline.com

The multidrug resistance of tumour cells was reversed by tetrandrine *in vitro* and in xenografts derived from human breast adenocarcinoma MCF-7/adr cells

L.W. Fu[a,*], Y.M. Zhang[b], Y.J. Liang[a], X.P. Yang[a], Q.C. Pan[a]

[a]*Cancer Center, Sun Yat-Sen University of Medical Sciences, Guangzhou 510060, China*
[b]*Department of Pharmaceutics, Third Affiliated Hospital, Sun Yat-Sen University of Medical Sciences, Guangzhou 510089, China*

Received 6 June 2001; received in revised form 29 August 2001; accepted 5 October 2001

Abstract

Multidrug resistance (MDR) is one of the main obstacles limiting the efficacy of chemotherapy treatment of tumours. One of the main causes of MDR is linked to the overexpression of P-glycoprotein (P-gp). This study aimed to characterise tetrandrine (Tet), a potent inhibitor of P-gp mediated MDR. Cytotoxicity was determined by the tetrazolium (MTT) assay. A MCF-7/adr cell xenograft model was established to investigate the effect of Tet on reversing MDR *in vivo*. Mechanistic experiments were conducted to examine the uptake, efflux and accumulation of doxorubicin (Dox) and Fura-2, and to assess lipid membrane fluidity. Tet potentiated the cytotoxicity of Dox; a 20.4-fold reversal of resistance was achieved in the presence of 2.5 μmol/l of Tet. Accumulation and efflux studies with the P-gp substrates, Dox and Fura-2, demonstrated that Tet inhibited the P-gp-mediated drug efflux. In addition, Tet lowered cell membrane fluidity in a concentration-dependent manner. In mice bearing the MDR MCF-7/adr cell xenografts, coadministration of Tet potentiated the antitumour activity of doxorubicin without a significant increase in toxicity. Tet was an extremely potent MDR modulator both *in vitro* and *in vivo*, without apparently enhancing the toxicity of the co-administered drugs. Hence, Tet holds great promise as a MDR modulator for the treatment of P-gp-mediated MDR cancers.

Keywords: Tetrandrine; Multidrug resistance; P-Glycoprotein; Xenograft; Doxorubicin; Breast cancer; Cellular drug accumulation; Cell membrane fluidity

1. Introduction

Multidrug resistance (MDR) is a major obstacle for successful chemotherapy treatment, MDR is often the result of overexpression of a 170 kD plasma membrane glycoprotein known as P-glycoprotein (P-gp). P-gp belongs to the superfamily of adenosine triphosphate (ATP)-binding cassette (ABC) transporters and actively pumps out a wide range of structurally and functionally diverse amphipathic anticancer drugs from the inside of tumour cells thereby decreasing their intracellular accumulation [1]. Recent studies showed that tumour cells expressing MDR-associated protein (MRP), lung resistant protein (LRP) and mutation of DNA topoisomerase II are likely to be MDR [2,3].

A plethora of agents are able to reverse the MDR phenotype *in vitro* [4–6]. Many natural and synthetic products of various structures, including calcium channel blockers (e.g. verapamil, nifedipine), calmodulin antagonists (e.g. trifluoperazine, chlorpromazine), quinolines (e.g. chloroquine, quinidine), immunosuppressive drugs (e.g. cyclosporin A, rapamycin) and yohimbine alkoids (e.g. reserpine, yohimbine) have been shown to partly or competitively inhibit the function of P-gp.

In vivo, combined therapy with MDR-related cytotoxins and modulators could shrink tumours and prolong the life-span in animal models. Unfortunately, the data regarding clinical efficacy are not yet available from the early clinical trials. Moreover, the MDR reversal agent may expose the patient to unacceptable side-effects

* Corresponding author. Tel.: +86-20-877-653-68x7333; fax: +86-20-877-545-06.
E-mail address: fulw@gzsums.edu.cn or fuliwu@hotmail.com (L.W. Fu).

0959-8049/02/$ - see front matter
PII: S0959-8049(01)00356-2

or toxicity at the doses required for effectiveness and/or affect the pharmacokinetics of the anticancer drug [1,7]. These limitations have spurred on efforts to search for new, more effective compounds. New drugs developed specifically to inhibit P-gp and modulate MDR are currently one of the most important strategies in the field of cancer chemotherapy.

Tetrandrine (Tet), one of the bisbenzylisoquinoline alkaloids, has been used as an antifibrotic drug to treat the lesions of silicosis in China since the 1960s. It is relatively non-toxic to humans, even at the administration of 180 mg, intramuscularly (i.m.) three times daily (t.i.d.) [8]. Our previous studies showed Tet was able to reverse MDR *in vitro* [9]. The aim of this study was to determine whether Tet can enhance the efficacy of doxorubicin (Dox), both *in vitro* and in nude mice bearing tumours, and to probe the mechanism of the reversal of MDR.

2. Materials and methods

2.1. Materials

Tet was generously provided by Dr Pan XP (Kuming Institute of Botany, Chinese Academy of Sciences). The powder of Tet, $C_{38}H_{42}O_6N_2$, with a purity of >98% was obtained by chromatography. Tet was freshly made before use. Fura 2-AM, Fura 2, 1,6-diphenyl-1,3,5-hexatriene (DPH) and Dox were purchased from Sigma Chemical Co. Dulbecco's modified Eagle's medium (DMEM) was purchased from Gibco BRL.

2.2. Cell lines and cell culture

The MDR cell lines MCF-7/adr and the parental sensitive cell line MCF-7 were generously provided by Prof. Liu XY (from the National Cancer Institute, USA). The MDR cell line MCF-7/adr and the parental cell line MCF-7 were grown as adherent monolayers in flasks in DMEM with 10% fetal bovine serum (FCS), benzylpenicillin (50 KU/L), and streptomycin (50 mg/1) at 37 °C in a humidified atmosphere of 5% CO_2 + 95% air. MCF-7/adr cells were approximately 48-fold resistant to Dox, and overexpression of P-gp was the main cause for the MDR.

2.3. MTT cytotoxicity assay

The cells were collected with trypsin and re-suspended in a final concentration of 4×10^5 cells/ml, 0.18 ml aliquots were seeded in 96-well multiplates. 10 μl of modulator and 10 μl of anticancer drug were added after a 24 h incubation. After 68 h, 10 μl of 10 mg/ml dimethyl thiazolyl-2,5-diphenyltetrazolium bromide (MTT) solution were added to each well, and the plate was incubated for 4 h, allowing the viable cells to reduce the yellow MTT into dark-blue formazan crystals, which were dissolved in 150 μl of dimethyl sulphoxide (DMSO). The cell growth inhibition was evaluated by the MTT method using triplicate assays [10]. The concentrations required to inhibit growth by 50% (IC_{50} values) were calculated from the cytotoxicity curves (Bliss's software). The degree of resistance was calculated by dividing the IC_{50} for the MDR cells by that for the parental sensitive cells. The fold-reversal of MDR was calculated by dividing the IC_{50} for cells to the anticancer drug in the absence of the modulator by that in the presence of the modulator.

2.4. Animals

Athymic nude mice were used for the MCF-7/adr xenografts. Mice were obtained from the Center of Experimental Animals, Sun Yat-Sen University of Medical Sciences, and maintained and brought up in the Center. Animals were provided with sterilised food and water. Male mice 5–7 weeks old, weighing 18–22 g, were used.

2.5. Reversal of MDR in the MCF-7/adr cell xenografts

Ullmann and colleagues established the model of xenografts of MCF-7/adr in nude mice and proved that like the MDR cells in culture, MCF-7/adr nude mice xenografts are extremely resistant to Dox and retain the characteristics of the MDR phenotype. In our experiments, the model of xenografts of MCF-7/adr was established as described by of Ullmann [11]. In detail, transplantable MCF-7/adr cells were collected and suspended at a concentration of 10^7/ml and transferred to the mice for the chemotherapeutic studies. Mice received a subcutaneous (s.c.) injection of the cells using 0.5 million cells/inoculation under the shoulder. After s.c. implantation of the cells, when the s.c. tumour was approximately 5×5 mm in size, mice were randomised into treatment groups of four including controls and groups receiving Dox alone (4 mg/kg), intraperitoneally (i.p.) every 2 days (q2d), Tet alone (30 mg/kg), i.p. q2d, or both Dox (4 mg/kg) + Tet(30 mg/kg), i.p. q2d. The animal's weight was measured every 4 days for modulation of the drug dosage. Each animal was tagged in the ear and followed individually throughout the experiments, with the animals receiving the desired drug and dosage. We monitored tumour growth starting on the first day of treatment and measured the volume of the xenograft every 4 days. Tumour volume was measured in two perpendicular diameters (A and B). Tumour volume (V) was estimated according to the formula:

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

The curve of tumour growth was drawn according to tumour volume and time of implantation. The mice were anaesthetised and killed when the mean tumour weights were over 1 g in the control group. Tumour tissue was excised from the mice and its weight was measured. The rate of inhibition (IR) was calculated according to the formula:

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

2.6. Fura 2-AM fluorescence measurements for detecting the function of P-gp

Fura 2-AM is a substrate of the MDR transporter (P-gp). Fura 2-AM shares several characteristics with substrates for the outward transport system associated with MDR. MDR cells showed impaired accumulation of Fura-2, the accumulation of this dye was promoted with modulators. Impaired accumulation of Fura-2 by the multidrug resistant cells has suggested the use of this highly fluorescent dye as a fluorescent probe for MDR [12,13]. Actually, Fura 2-AM is a non-fluorescent and highly lipid-soluble dye precursor that rapidly penetrates the plasma membrane of cells. Once inside the cell, ester bonds are cleaved by endogenous esterases, transforming Fura 2-AM into the hydrophilic and intensely fluorescent Fura-2. Cells expressing high levels of mdr1 rapidly extrude non-fluorescent Fura 2-AM from the plasma membrane, thereby reducing accumulation of fluorescent Fura-2 in the cytosol. The amount of P-gp activity is therefore inversely proportional to the accumulation of intracellular Fura-2 fluorescence. However, the extrusion of Fura 2-AM could be blocked in MDR cells when the function of P-gp was decreased or lost, therefore, increasing the cellular accumulation of Fura-2 in the MDR cells. The measurement of the accumulation of Fura-2 can therefore be used as a method for studying P-gp function and screening modulators of MDR induced by P-gp. In detail, the cells were collected and incubated in the medium containing Hepes-Na of 10 mmol/l (pH 7.4), NaCl of 120 mmol/l, KCl of 5 mmol/l, $MgCl_2$ of 0.4 mmol/l, $CaCl_2$ of 0.04 mmol/l, $NaHCO_3$ of 10 mmol/l, glucose of 10 mmol/l, Na_2HPO_4 of 5 mmol/l, with 2.5 μmol/l of Fura 2-AM in the absence or presence of Tet for 20 min at 25 °C. The cells were centrifuged and washed with phosphate-buffered solution (PBS) three times. The values of fluorescence were measured (λ_{ex}: 340 nm, λ_{em}: 500 nm) in the presence of $CaCl_2$ and 0.5% Triton X-100.

2.7. Dox passive uptake, accumulation and efflux

To examine the accumulation of Dox in cells, logarithmically growing MCF-7 cells and MCF-7/adr cells were exposed to Dox of 10 μmol/l in the absence or presence of Tet of 2.5, 1.25, 0.625 μmol/l at 37 °C for 3 h in the medium with PBS plus 10% FCS added with 10 mM glucose. The cells were then collected, centrifuged and washed 3 times with cold PBS. The cells were resuspended in HCl 0.3 mol/l in 60% ethanol. Following centrifugation, the supernatant was removed and assayed spectrofluorometrically at λ_{ex} 470 nm and λ_{em} 590 nm [14]. Tet did not affect the absorbance or emission spectra of Dox. The accumulation of Dox was calculated by the standard curve of Dox. The fold accumulation of Dox was calculated by dividing the value in the presence of Tet by that without Tet.

To determine the passive uptake of Dox, cells were preincubated at 37 °C for 15 min with 15 mM sodium azide (NaN_3) plus 10 mM deoxyglucose in PBS (pH 7.4) and 10% FCS. They were then incubated at 37 °C for 3 h with 10 μmol/l of Dox in the same buffer in the absence or presence of Tet of 2.5 μmol/l. The cells were washed and the intracellular Dox content was quantified as described above.

To measure drug efflux, cells were incubated in energy-supplied buffer and incubated with 10 μmol/l Dox for 3 h at 37 °C. Each dish was washed once with PBS, and energy-supplied buffer without Dox and with or without Tet was added. Then the cells were incubated for the indicated times at 37 °C and harvested, then quantified as described above.

2.8. Plasma membrane lipid fluidity

The fluidity of the cell membrane was determined by measuring the fluorescence polarisation of the hydrophobic probe, DPH, as previously described in Ref. [15]. In brief, an aliquot (25 μl) of a stock solution of DPH (2 mM in tetrahydrofuran) was diluted 1:2000 into the suspension buffer with vigorous mixing. This dispersion was mixed with the cell suspension and a given concentration of Tet in PBS, the final cell density being 1×10^6/ml. Probe uptake was for 1 h at room temperature in dark. The cells were washed by PBS, and resuspended by PBS. Fluorescence polarisation was measured in a fluorimeter (λ_{ex}: 362 nm, λ_{em}: 432 nm).

The intensity of the fluorescence that was parallel (I_{ll}) and perpendicular ($I_{\perp}$) to the direction of the vertically polarised excitation light was determined by measuring the emitted light through polarisers oriented vertically and horizontally. The polarisation (P) was obtained from intensity measurements using $P=(I_{ll}-GI_{\perp})/(I_{ll}+GI_{\perp})$ where G is a grating correction factor for the monochromator's transmission efficiency for vertically and horizontally polarised light. The value is given by

the ratio of the fluorescence intensities of the vertical to horizontal components when the exciting light is polarised in the horizontal direction. The polarisation was expressed as the anisotropy ($r=2P/(3-P)$).

2.9. Statistic analysis

All experiments were repeated at least twice and differences determined using the Student's *t*-test. Significance was determined at $P<0.05$.

3. Results

3.1. Tet itself inhibits cell growth in the MCF-7 and MCF-7/adr cells

The cytotoxic effect of Tet after a 72-h treatment is shown in Fig. 1. The IC_{50} of Tet to MCF-7 and MCF-7/adr cells was 9.3 ± 1.7 and 11.5 ± 2.4 μmol/l, respectively. Cell survival was more than 90% in both the MCF-7/adr cells and their parental sensitive MCF-7 cells using Tet concentrations of $\leqslant 2.5$ μmol/l. So Tet concentrations of 2.5, 1.25, 0.625 μmol/l were used to study the reversal of MDR.

3.2. 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. The aim of the experiments was to see if Tet modulated the sensitivity of the cells to the chemotherapeutic agent, Dox. It was clear that the sensitivity to Dox was very different in the MCF-7 cells from that in the MCF-7/adr cells. The IC_{50} of Dox for the MCF-7/adr cells and MCF-7 cells were 16.13 and 0.33 μmol/l, respectively. So the MCF-7/adr cells in these experiments were approximately 48-fold more resistant to Dox compared with the parental drug-sensitive MCF-7 cells (Fig. 2).

Tet concentrations of 2.5, 1.25, 0.625 μmol/l, that are barely cytotoxic (more than 90% cell survival) to the MCF-7 and MCF-7/adr cells, lowered the IC_{50} of Dox to 0.79, 1.37 and 2.98 μmol/l in the MCF-7adr cells.

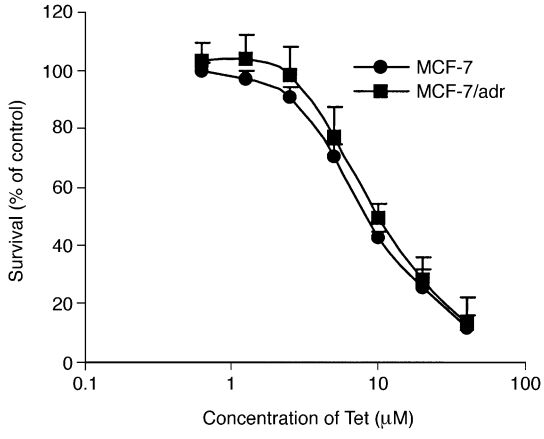

Fig. 1. Cytotoxicity effect of tetrandrine (Tet) on MCF-7 and MCF-7/adr cells. Cytotoxicity was measured using a dimethylthiazolyl-2,5-diphenyltetrazolium bromide (MTT) assay. The cells were cultured with a full range of concentrations of Tet for 72 h. Data represent means and standard errors of at least a triplicate determination.

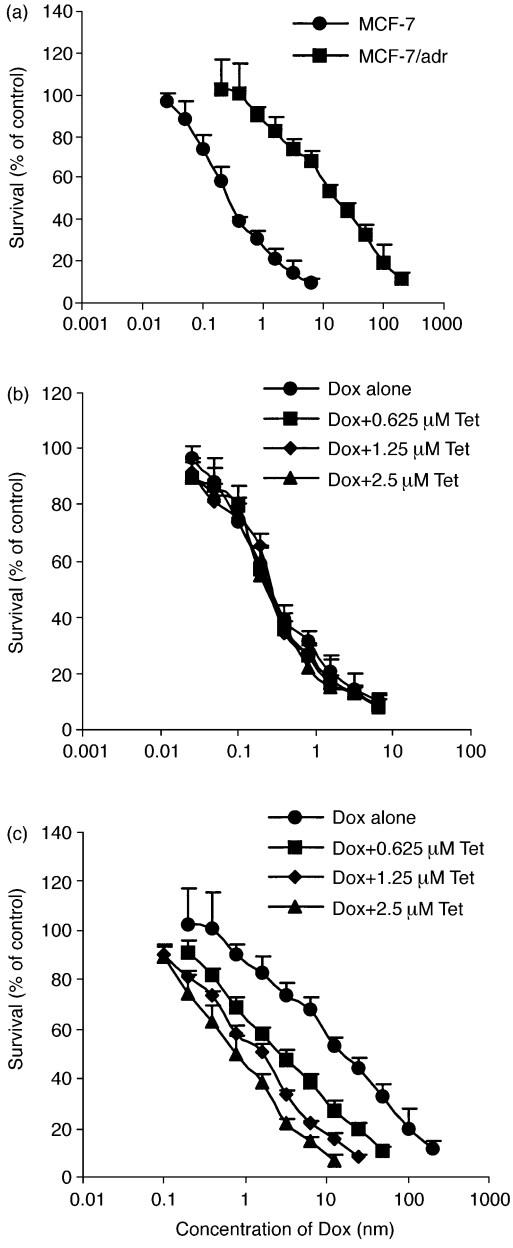

Fig. 2. Effect of tetrandrine (Tet) on enhancing the sensitivity of MCF-7 and MCF-7/adr cells to the chemotherapeutic agent *in vitro*. Cytotoxicity was measured using a MTT assay. The cells were cultured with a full range of concentrations of doxorubicin (Dox) in the presence or absence of Tet for 72 h. Data represent means and standard errors of at least triplicate determinations: (a) cytotoxicity of Dox alone in the MCF-7 and MCF-7/adr cells; (b) MCF-7 cells; (c) MCF-7/adr cells.

This gave a 20.4, 11.8 and 5.4-fold reversal respectively, in MDR. These results suggested Tet was very effective at reversing MDR *in vitro*. In the sensitive MCF-7 cells, the IC_{50} of Dox was 0.27, 0.29, 0.28 μmol/l in the presence of Tet concentrations of 2.5, 1.25, 0.625 μmol/l, respectively (Fig. 2, Table 1). These results suggested that Tet had no effect on the Dox sensitivity of these sensitive MCF-7 cells.

3.3. Reversal of MDR by Tet in xenografts

When MDR tumours were treated with the various regimens, tumour growth suppression was observed. Fig. 3 shows the curves of tumour growth in the nude xenografts of the MCF-7/adr cells. Tumour growth was much slower for the group given Dox and Tet compared with the other groups. Tumour weights were 0.94±0.33, 1.13±0.69, 0.98±0.32 and 0.40±0.18 g in the control group, Dox (4 mg/kg) alone, Tet (30 mg/kg) alone and co-administration groups at day 32 after implantation, respectively (Fig. 3, Table 2). Similar results are shown in Table 2 for another independent set of experiments. These show that neither Dox alone or Tet alone had any anticancer action in the nude mice xenografts of MCF-7/adr cells in two independent experiments. However, the combination of Dox and Tet significantly inhibited the growth of the xenograft and the inhibition (versus the control weight of tumour) was 52.8–57.5% ($P<0.05$ versus the control group, Dox alone or Tet alone). The results demonstrated that Tet could reverse MDR *in vivo*.

Importantly, the regimen of the combination of Dox and Tet did not cause any deaths in our experimental system. The weights of the animals were more in the post-experiments than in the pre-experiments in every group (with the exception of the Tet alone group). Furthermore, the weight of the animals was not significantly decreased in the combination of the group of Dox and Tet versus the control group. This suggests that this regimen did not result in increased toxic side-effects. Therefore, the toxicity co-administration of Dox and Tet was tolerable (Table 2).

3.4. Tet inhibited the extrusion of Dox and enhanced the accumulation of Dox and Fura-2 in the MCF-7/adr cells, not in the MCF-7 cells

The decrease of cellular drug accumulation induced by P-gp is thought to be one of the main causes of

Table 1
Modulation by Tetrandrine (Tet) of the sensitivity to Dox of MCF-7 and MCF-7/adr cells

Group	Concentration (μmol/l)	IC_{50} of Dox (μmol/l)±S.D.		Fold-reversal of MDR	
		MCF-7/adr	MCF-7	MCF-7/adr	MCF-7
Control	0	16.13±2.18	0.33±0.03	1.0	1.0
Tet	0.625	2.98±0.38*	0.28±0.03	5.4	1.2
	1.25	1.37±0.16**	0.29±0.03	11.8	1.2
	2.5	0.79±0.09**	0.27±0.03	20.4	1.2

Dox, doxorubicin; Tet, tetrandrine; MDR, multidrug resistance; IC_{50}, concentration required to inhibit growth by 50%.

The IC_{50} of Dox was determined in the presence of various concentrations of Tet. Each value represents the mean±standard deviation (S.D.) of at least three independent experiments. The fold-reversal of MDR is defined as the ratio of the IC_{50} for Dox alone versus the IC_{50} for Dox in the presence of the modulating agent.

** and * $P<0.01$ and $P<0.05$ respectively, for values versus those in the control group.

Table 2
Summary of two independent experiments on the effect of tetrandrine (Tet) on the reversal of multidrug resistance (MDR) in a xenograft model of MCF-7/adr cells in nude mice

Group	Pre-experiment		Post-experiment			Weight of tumour (g)	Inhibition (versus control weight of tumour) (%)
	Animals weight (g)		Animals weight (g)/change (%)				
Control	5	20.7±1.2	5	21.9±3.2	+5.8	0.94±0.33	
Dox	5	21.8±2.1	5	22.9±1.8	+5.0	1.13±0.69	−20.2
Tet	5	22.2±1.4	5	22.1±2.6	−0.5	0.98±0.32	−4.3
Dox+Tet	5	20.4±1.3	5	21.5±1.5	+5.4	0.40±0.18a,b	57.5
Control	7	19.7±2.8	7	21.0±2.6	+6.6	1.61±0.65	
Dox	7	22.6±3.2	7	25.7±3.2	+13.7	1.71±0.84	−6.2
Tet	7	21.0±2.6	7	23.3±1.6	+11.0	1.87±0.93	−16.1
Dox+Tet	7	21.8±3.3	7	23.9±2.8	+9.6	0.76±0.36a,b	52.8

Two experimental results are shown in Table 2. Data represents the mean for each group ±standard error. a $P<0.05$ for values versus those in the control group. b $P<0.05$ for values versus those in the group of Dox.

MDR. Fig. 4a illustrates the effect of Tet on the accumulation of Dox in the MCF-7/adr and MCF-7 cells. Dox accumulation was higher (5.2-fold) in the sensitive MCF-7 cells than in the MDR MCF-7/adr cells. There was a high level of Dox accumulation in the drug-sensitive MCF-7 cells that was unaffected by Tet (1.0, 1.1, 1.0-fold for 0.625, 1.25, 2.5 μmol/l of Tet). There was a low level of accumulation in the MCF-7/adr cells and Tet dose-dependently restored the level of Dox accumulation. Cellular Dox accumulation of 1.9-, 2.9-, 3.8-fold in the MCF-7/adr cells was observed in the presence of 0.625, 1.25, 2.5 μmol/l of Tet, respectively. However, intracellular Dox accumulation showed no significant difference in the presence or absence of Tet in energy-depleted MCF-7/adr cells that were treated with NaN_3/deoxyglucose (Fig. 4b). We examined whether the increased accumulation of Dox in the MCF-7/adr cells caused by Tet was due to inhibition of Dox efflux. The time course of release of Dox after 2 h of accumulation is shown in Fig. 4c. MCF-7/adr cells released a higher percentage of accumulated Dox than MCF-7 cells. At 40 min, 56% of the accumulated Dox was lost from the MCF-7/adr cells, whereas only 35% was lost from MCF-7 cells. Tet of 2.5 μmol/l apparently inhibited the efflux of Dox from the MCF-7/adr cells, but not so from the MCF-7 cells. This suggests that Tet inhibits the efflux of Dox induced by P-gp rather than the passive uptake of Dox.

Similarly, Fig. 4d shows the effect of Tet on Fura-2 accumulation in MCF-7 and MCF-7/adr cells. The cellular accumulation of Fura-2 was 4.2-fold in MCF-7 cells compared with that of MCF-7/adr cells. The cellular accumulation of Fura-2 was enhanced 2.1-, 2.9- and 3.5-fold in the MCF-7/adr cells in the presence of 0.625, 1.25, 2.5 μmol/l of Tet, respectively, but had no effect on the MCF-7 cells. This demonstrates that the function of P-gp for the extrusion of drug was decreased in the presence of Tet.

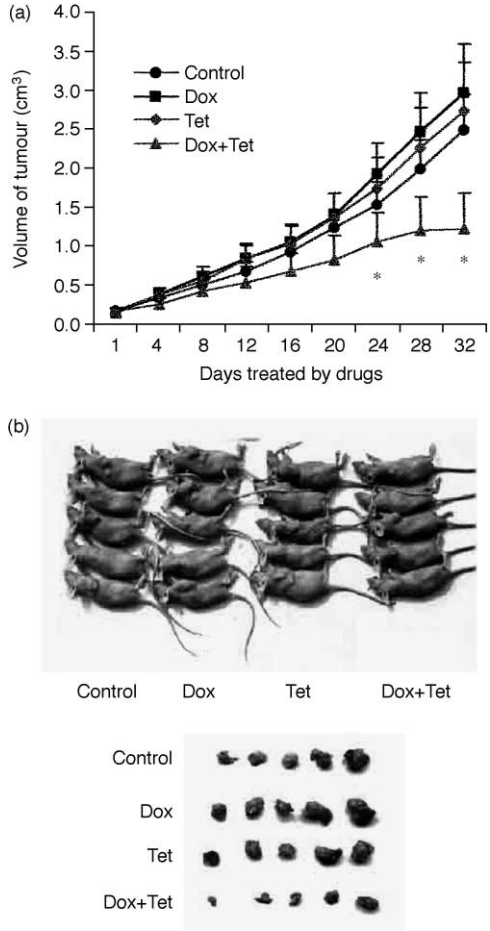

Fig. 3. Reversal of multidrug resistance (MDR) *in vivo* by tetrandrine (Tet). The experiment was carried out using athymic mice implanted subcutaneously (s.c.) with 10^7 MCF-7/adr cells. The treatments were administered as indicated in the Materials and methods. (a) Data represent the mean tumour size for each group ± standard error in 5–7 experimental animals. *, statistically significant difference in mean tumour size compared with the Dox alone. $P<0.05$. Similar results were obtained in two independent experiments. (b) The pictures were taken 32 days after implantation. The pictures show nude mice bearing tumours and the tumour size.

3.5. Cell membrane lipid fluidity is decreased in the presence of Tet

In an attempt to study the physical basis underlying the Tet-mediated effects, we postulated that Tet might affect membrane-fluidisation and that this might explain any disruption of function of integral membrane proteins such as P-gp. The measurement of the fluorescence polarisation reflected the microviscosity or fluidity of the lipid bilayers. So an increased fluorescence polarisation can be regarded as due to a decrease in the cell membrane fluidity. Table 3 shows that Tet dose-dependently increased the fluorescence polarisation and decreased the cell membrane fluidity in both the MCF-7/adr and MCF-7 cells. These results suggest the potentiation of cytotoxicity by Tet in MDR cells might involve a decrease in the lipid fluidity of the cell membrane.

Table 3
Effect of tetrandrine (Tet) on the anisotropy of cell membrane in MCF-7/adr cells and MCF-7 cells

	Concentration (μmol/l)	MCF-7 cells	MCF-7/adr cells
Control		0.128±0.005	0.101±0.005
Tet	0.625	0.128±0.003	0.128±0.003*
	1.25	0.137±0.008*	0.133±0.005**
	2.5	0.157±0.015**	0.139±0.011**

The experiments were conducted as described in Materials and methods. Data represents the mean for each group ± standard error for at least three independent experiments. *, **$P<0.05$, $P<0.01$ respectively, for values versus those in the control group.

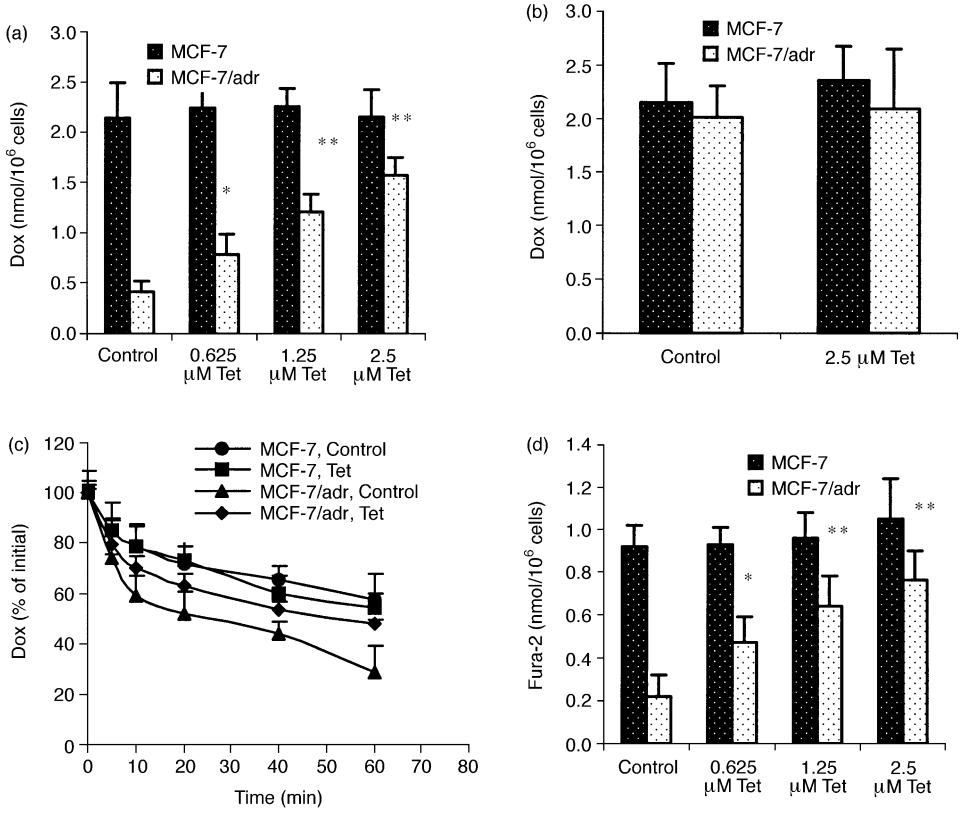

Fig. 4. Effect of tetrandrine (Tet) on doxorubicin (Dox) and Fura-2 accumulation, passive uptake, and efflux in MCF-7 and MCF-7/adr cells. Bars represent means±standard deviations (S.D.) of triplicate determinations; ** and * represent $P<0.01$ and 0.05, respectively, compared with the control group in the MCF-7/adr cells: (a) Dox accumulation, (b) Dox passive uptake, (c) Dox efflux, (d) Fura-2 accumulation. Values were expressed as percent compared with apical point.

4. Discussion

Simultaneous genetic knockout of *mdr1a* and *mdr1b* resulted in healthy mice, indicating that P-gp is not essential for basic physiological functions [16]. This suggested that the combination of anticancer drugs with a non-toxic and potent P-gp inhibitor may be a promising approach to solve the problem of resistance. A large number of compounds, such as verapamil and cyclosporin A, can reverse the MDR phenotype mediated by P-gp *in vitro* [17,18]. However, 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 [19] and PSC833 [20] are 3- to 100-fold more potent than verapamil in the reversal of MDR. However, an additional problem with most of the second-generation P-gp inhibitors is that they significantly alter the plasma pharmacokinetics when co-administered with antitumour agents, increasing blood levels by reducing clearance and/or metabolism. For example, PSC833 [20] and VX-710 [19] have been reported to produce significant plasma pharmacokinetics interactions with agents such as paclitaxel. This has necessitated significant reductions in the dose of the chemotherapeutic agent and, although feasible, interpatient variability in the metabolism and plasma pharmacokinetic interaction make this approach problematic. In addition, a requirement for dose reduction presents significant problems for the use of this approach in up-front therapy to kill cells expressing P-gp and/or to prevent the appearance of MDR after first diagnosis. The effects of a MDR modulator on drug transport proteins that causes alterations in the excretion of the anticancer drug often leads to increased anticancer drug exposure of healthy tissues and has necessitated a dose reduction in many preclinical and clinical studies. Changes in the dose or schedule of the anticancer drug may be able to address toxicity or efficacy alterations brought about by MDR modulators. Thus, an ability to avoid such alterations in anticancer drug clearance may be considered a significant advantage in MDR modulation strategies. Third generation P-gp inhibitors that exhibit potent action in the reversal of MDR and lack significant PK interaction with anticancer drugs have been reported, such as S9788 [21], GF120918 [22], OC144-093 [23] and LY335979 [24].

Our experimental results showed Tet itself had a similar cytotoxicity in MCF-7 cells and in MCF-7/adr cells.

This implies that Tet is not a substrate of P-gp. Tet enhanced the sensitivity of the MCF-7/adr cells to Dox *in vitro* using concentrations of Tet, which were not cytotoxic by themselves. *In vivo*, neither Tet alone nor Dox alone had any anticancer action ($P > 0.05$ versus the control group) in xenograft models of MCF-7/adr cells. In contrast, the combination of Tet and the conventional anticancer drug Dox had a potent inhibitory action. The % inhibition was 52.8–57.5% ($P < 0.05$ versus the groups given the anticancer drug alone or control or Tet alone) for the growth of the MCF-7/adr xenografts. This suggests that Tet is a potent MDR modulator not only *in vitro*, but also *in vivo*.

Furthermore, our experiments showed that the combination of Tet and Dox did not cause deaths amongst the animals and no decrease was seen in their body weight. Therefore, there was no evidence of an increase in Dox-associated toxicity induced by Tet.

Furthermore, Tet, a benzylisoquinoline alkaloid, has been used in China as an antifibrotic drug to treat the lesions of silicosis for over 30 years [8]. The dose of Tet recommended in the clinic was 180 mg t.i.d. for adults. Long-term treatment of Tet was safe for this recommended dose. This data is encouraging as regards its use in the clinic as a modulator of MDR.

Recently, the mechanism of action of P-gp has been extensively investigated. It is thought that the decreased cellular accumulation of anticancer drug is due to an increase in the extrusion of the drug mediated by P-gp. Thus, it is paramount that the accumulation of anticancer drugs in MDR cells is improved if MDR is to be reversed. In this study, the accumulation of Dox and Fura-2 was greatly increased in the MCF-7/adr cells in the presence of Tet. This demonstrates that the circumvention of MDR mediated by Tet is associated with an increased accumulation of the anticancer drug.

Biological membranes adjust their physical properties in order to function optimally [25]. The altered lipid fluidity of the membrane may have potentially important implications. Tet mediated membrane perturbations that alter the membrane fluidity may result in interference with either the ATPase activity of P-gp and/or with its drug-transport capability. This will consequently lead to an increased intracellular level of cytotoxic agents (e.g. Dox), thereby leading to a potentiation of the anticancer-drug cytotoxicity. Thus, our data suggests that one of the pathways of modulation of MDR by Tet resulted from the decrease of the cell membrane lipid fluidity, which then blocked the function of P-gp, caused a decreased extrusion of anticancer drug and an increased accumulation of anticancer drug in the cells.

In summary, MDR was partially reversed following the co-administration of Tet and the conventional anticancer drug Dox *in vitro* and *in vivo*. Tet may modulate MDR by increasing the intracellular drug accumulation by inducing a decrease in the fluidity of the cell membrane. These results therefore have potential future clinical significance.

Acknowledgements

This work was supported in part by 990B82 Key Science and Technology Project Research Grant of Guangdong Province, China and No. 9800182, natural science foundation of Guangdong province, China and No. 9905 Excellent Youth Grant of the Ministry of Health of China.

References

1. Ling V. Multidrug resistance: molecular mechanisms and clinical relevance. *Cancer Chemother Pharmacol* 1997, **40**(Suppl.), S3–S10.
2. Borst P, Kool M, Evers R. Do cMOAT (MRP2), other MRP homologues, and LRP play a role in MDR? *Semin Cancer Biol* 1997, **8**, 205–213.
3. Volm M. Multidrug resistance and its reversal. *Anticancer Res* 1998, **18**, 2905–2917.
4. Krishna R, Mayer LD. 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* 2000, **11**, 265–283.
5. Tan B, Piwnica-Worms D, Ratner L. Multidrug resistance transporters and modulation. *Curr Opin Oncol* 2000, **12**, 450–458.
6. Sikic BI, Fisher GA, Lum BL, Halsey J, Beketic-Oreskovic L, Chen G. Modulation and prevention of multidrug resistance by inhibitors of P-glycoprotein. *Cancer Chemother Pharmacol* 1997, **40**(Suppl.), S13–S19.
7. Kerb R, Hoffmeyer S, Brinkmann U. ABC drug transporters: hereditary polymorphisms and pharmacological impact in MDR1, MRP1 and MRP2. *Pharmacogenomics* 2001, **2**, 51–64.
8. Fang JH, Fang YH. Tetrandine: pharmacology and clinical usefulness. *Chin Pharmaceutic J* 1996, **31**, 454–457.
9. Pan QC, Tian H. Reversal multidrug resistance by various principles from Chinese herbal medicine. *Chin Sci Bull* 1996, **41**, 410–414.
10. Carmichael J, DeGraff WG, Gazadar AF, Minna JD, Mitchell JB. Evaluation of a tetrazolium-based semiautomated colormetric assay: assessment of chemosensitivity testing. *Cancer Res* 1987, **47**, 943–946.
11. Ullmann CA, Eliot HM, Sinha BK. Distribution, DNA damage and cytotoxic effects of etoposide in human tumor xenografts in vivo. *Anticancer Res* 1991, **11**, 1379–1382.
12. Fu LW, Pan QC, Lin GY. Compared study on Fura-2/AM assay and MTT assay for screening multidrug resistant modulators. *Acta Pharm Sin* 1997, **32**, 401–404.
13. Karaszi E, Jakab K, Homolya L, *et al*. Calcein assay for multidrug resistance reliably predicts therapy response and survival rate in acute myeloid leukaemia. *Br J Haematol* 2001, **112**, 308–314.
14. Dekker K, Kojima N, Yamamoto S, *et al*, Tatsumi K, Mino M, Yagi K. A simple fluorometric method to discriminate adriamycin-resistant subline from adriamycin-sensitive parental P388 murine leukemia cell line. *Biochem Int* 1987, **14**, 997–1001.
15. Kang JS, Choi CM, Yun I. Effects of ethanol on lateral and rotational mobility of plasma membrane vesicles isolated from cultured mouse myeloma cell line Sp2/0-Ag14. *Biochim Biophys Acta* 1996, **1281**, 157–163.

16. Schinkel AH. Pharmacological insights from P-glycoprotein knockout mice. *Int J Clin Pharmacol Ther* 1998, **36**, 9–13.
17. Tsuruo T, Iida H, Tsukagoshi S, 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* 1981, **41**, 1967–1972.
18. Wigler PW. Cellular drug efflux and reversal therapy of cancer. *J Bioenerg Biomembr* 1996, **28**, 279–284.
19. Germann UA, Shlyakhter D, Mason VS, *et al*. Cellular and biochemical characterization of VX-710 as a chemosensitizer: reversal of P-glycoprotein-mediated multidrug resistance in vitro. *Anticancer Drugs* 1997, **8**, 125–140.
20. Atadja P, Watanabe T, Xu H, Cohen D. PSC-833, a frontier in modulation of P-glycoprotein mediated multidrug resistance. *Cancer Metastasis Rev* 1998, **17**, 163–168.
21. Tranchand B, Catimel G, Lucas C, *et al*. 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* 1998, **41**, 281–291.
22. Hyafil F, Vergely C, Du Vignaud P, Grand-Perret T. In vitro and in vivo reversal of multidrug resistance by GF120918, an acridonecarboxamide derivative. *Cancer Res* 1993, **53**, 4595–4602.
23. Newman MJ, Rodarte JC, Benbatoul KD, *et al*. Discovery and characterization of OC144-093, a novel inhibitor of P-glycoprotein-mediated multidrug resistance. *Cancer Res* 2000, **60**, 2964–2972.
24. Starling JJ, Shepard RL, Cao J, *et al*. Pharmacological characterization of LY335979: a potent cyclopropyldibenzosuberane modulator of P-glycoprotein. *Adv Enzyme Regul* 1997, **37**, 335–347.
25. Hui SW, Sen A. Effects of lipid packing on polymorphic phase behavior and membrane properties. *Proc Natl Acad Sci USA* 1989, **86**, 5825–5829.