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Substituted tetrahydroisoquinoline compound B3 inhibited P-glycoprotein-mediated multidrug resistance in-vitro and in-vivo

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Abstract

P-glycoprotein (P-gp) mediated multidrug resistance (MDR) is one of the main obstacles in tumour chemotherapy. A promising approach to reverse MDR is the combined use of nontoxic and potent P-gp inhibitor with conventional anticancer drugs. We have examined the potential of a newly synthesized tetrahydroisoquinoline derivative B3 as a MDR-reversing agent. The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was used to examine the effect of B3 on the cytotoxicity in K562/A02 and MCF-7/ADM cells caused by doxorubicin (adriamycin). Accumulation and efflux of P-gp substrate rhodamine123 in K562/A02 and primary cultured rat brain microvessel endothelial cells (RBMECs) were measured to evaluate the inhibitory effect of B3 on P-gp. The K562/A02 xenograft model in nude mice was established to examine MDR-reversing efficacy of B3 in-vivo. The results indicated that co-administration of B3 resulted in an increase on chemosensitivity of K562/A02 and MCF-7/ADM cells to doxorubicin in a dose-dependent manner. Rhodamine123 accumulation in K562/A02 cells and RBMECs were significantly enhanced after the incubation with various concentrations of B3. Furthermore, B3 inhibited the efflux of rhodamine123 from RBMECs. Co-administration of B3 with doxorubicin significantly decreased weight and volume of tumour in nude mice. In conclusion, B3 is a novel and potent MDR reversal agent with the potential to be an adjunctive agent for tumour chemotherapy.

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Introduction

The achievement of successful chemotherapy in cancer patients is frequently impaired by either intrinsic or acquired resistance to anticancer drugs. In both cases, tumours can be refractory to a variety of drugs, a phenomenon termed multidrug resistance (MDR). MDR was first observed in experimental oncology by Biedler & Riehm (1970). MDR can be the result of a variety of mechanisms that are not fully understood, but the most widely implicated mechanism is concerned with altered membrane transport in tumour cells. At the moment, the best-known extrusion protein is P-glycoprotein (P-gp, 170–180 kDa, a highly conserved protein, a product of the MDR1 gene) that belongs to the ABC superfamily of transporters (Juliano & Ling 1976; Germann 1996). This protein has 12 transmembrane domains divided into two homologous halves, each of which includes an ATP-binding cassette domain that catalyses ATP hydrolysis.

P-gp is widely expressed in human tissues, including kidney, liver, the blood–brain barrier (BBB) and intestine (Thiebaut et al 1987). In rodents, two genes, mdr1a and mdr1b, have been reported to play a similar role in drug resistance to that of the MDR1 gene in man (Borst & Schinkel 1996). In the brain, P-gp expressed in the brain microvessel endothelial cells (BMECs) plays an important role in maintaining BBB integrity (Hegmann et al 1992; Regina et al 1998) by limiting the entrance of many endogenous and exogenous chemicals, which are P-gp substrates, into the brain (Wang et al 1995).

P-gp functions as an energy-dependent extrusion pump that efficiently effluxes various cationic and lipophilic chemotherapeutic agents used for cancer chemotherapy (Germann 1996) from tumour cells, resulting in decreased intracellular concentrations and decreased therapeutic efficacy.

Regardless of its action mechanism, the inhibition of P-gp functions has been rapidly recognized as a possible approach to circumvent MDR. Thus drugs possessing P-gp inhibitory properties have been highly sought (Teodori et al 2002). Calcium channel blockers (verapamil), calmodulin inhibitors (phenothiazines), and ciclosporins are known to effectively reverse the MDR phenotype in-vitro. However, the clinical use of these agents has been hampered by the unacceptable toxicity and side effects at the doses required to modulate P-gp. Therefore, searching for new compounds capable of modulating P-gp with high efficacy at nontoxic doses is the most active field in cancer chemotherapy research.

It has been shown that quinoline (Naito et al 2002) and isoquinoline alkaloid could effectively reverse MDR with low side effects. Based on these observations, a number of isoquinoline derivatives have been synthesized and their MDR reversing activity has been demonstrated in-vitro and in-vivo (Shiraishi et al 1987). Some tetrahydroisoquinoline-derived substances were characterized with a strong potency for inhibiting P-gp (50% inhibitory concentration (IC50) $< 0.4\ \mu$mol L^{-1}) with low cytotoxicity (Berger et al 1999; Mihalyi et al 2004). B3 is a novel tetrahydroisoquinoline derivative sharing a chemical structure similar to verapamil. However, compared with verapamil, B3 exhibits lower activity of Ca^{2+}-channel blocking with the evidence that B3 could not inhibit high KCl (60 mmol L^{-1})-induced contraction response of isolated thoracic aortic rings in rats at a concentration of 100 mmol L^{-1}. Therefore, it was expected that B3 may inhibit P-gp function at a concentration without producing cardiovascular side effects.

In this study, P-gp expressing cell lines including K562/A02 and MCF-7/ADM, and primary cultured rat brain microvessel endothelial cells (RBMECs) were adopted to examine MDR reversing effects of B3 in-vitro. In addition, in-vivo MDR reversing efficacy of B3 was evaluated in nude mice bearing K562/A02 xenografts.

Materials and Methods

Cell lines and cell culture

The drug-sensitive human leukaemia cell line K562, and its drug-resistant variant cell line K562/A02, were obtained from the Institute of Hematology of Chinese Academy of Medical Sciences. The human breast cancer cell line MCF-7, and its ADM-resistant subline MCF-7/ADM were provided by the National Center of New Drug Screening of China Pharmaceutical University. K562 and MCF-7 cell lines were cultured in RPMI-1640 medium supplemented with 10% bovine serum at 37°C in a humidified atmosphere of 5% CO_2 in air. K562/A02 and MCF-7/ADM cell lines were grown in the presence of 1 μmol L^{-1} doxorubicin (adriamycin), which was withdrawn for two generations before the experiments.

Rat brain microvessel endothelial cells (RBMECs) were isolated according to the method of Abbott et al (1992) and Ji et al (2006) with minor modifications. Firstly, cortex was obtained from the brains of Sprague–Dawley rats (d5-8; Jiangsu Province Animal Center) and placed in ice-cold phosphate-buffered saline (PBS). After the surface vessels and meninges were removed, the cortex grey matter was minced and incubated at 37°C for 25 min in D-Hanks containing 0.05% trypsin. After centrifugation at 800 *g* for 5 min, the samples were filtered using 150-μm and 75-μm nylon mesh. Microvessels were collected on the 75-μm nylon mesh and digested in D-Hanks containing 0.1% collagenase II at 37°C for 25 min. The microvessels were finally collected by centrifugation at 800 *g* for 5 min, and then the pellet was washed twice with PBS and cultured in DMEM/F12 (1:1) medium supplemented with 20% fetal bovine serum at 37°C in 5% CO_2. Approximately 10–14 days after primary culture, RBMECs could grow to monolayers.

Chemicals

B3 (Figure 1) was kindly provided by Dr Wenlong Huang (China Pharmaceutical University). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), rhodamine123, collagenase II, and Trition X-100 were purchased from Sigma. Trypsin was a product of AMRESCO. Verapamil and doxorubicin were purchased from Lianyungang Pharmaceutical Corporation and Pharmacia & Upjohn Corporation, respectively. RPMI-1640, DMEM/F12 (1:1), bovine serum, and fetal bovine serum were purchased from Hyclone. All other reagents were of analytical grade and commercially available.

MTT assay

The effects of B3 singly or co-administered with doxorubicin on the growth of K562, K562/A02, MCF-7 and MCF-7/ADM cells were evaluated by the MTT method after 48-h culture (Carmichael et al 1987; Lambert et al 1992; Chen et al 2004). Briefly, cells (1×10^5 mL^{-1}) were seeded in each well of a 96-well plate. After 24 h, B3 at the final concentrations of 1, 3.3, or 10 μmol L^{-1} with or without various concentrations of doxorubicin were added in a final volume of 200 μL per well. After 48-h incubation, the medium was replaced with an equal volume of fresh medium containing 0.5 mg mL^{-1} MTT, and incubated for a further 4 h at 37°C. The cytotoxicity was evaluated according to the optical density (OD) values determined by a microplate reader at an absorption wavelength of 570 nm. IC50 values were calculated from the cytotoxicity curves by the method of Bliss (Lella 2001). The reversal fold

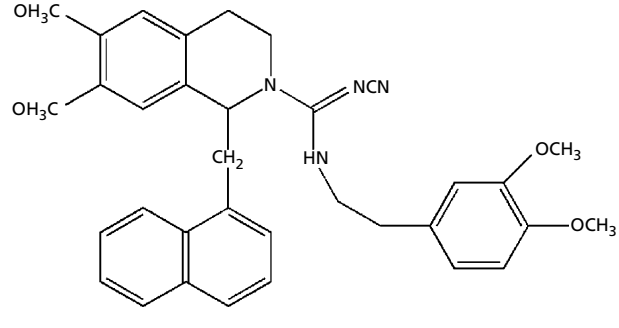

Figure 1 Chemical structure of B3.

of MDR was calculated by dividing the IC50 value of doxorubicin by that of co-administration of doxorubicin and P-gp inhibitors (Fu et al 2004).

Rhodamine123 accumulation assay (Ludescher et al 1992)

K562 and K562/A02 cell lines were incubated at 37°C with 5 μmol L^{-1} rhodamine123 in the absence or presence of 1, 3.3, or 10 μmol L^{-1} B3 or 10 μmol L^{-1} verapamil for 90 min. After incubation, the cells were washed twice with ice-cold PBS, resuspended in 200 μL PBS and then analysed by flow cytometry (FACS Callibur: BD) ($\lambda_{Ex}=488$ nm, $\lambda_{Em}=530$ nm).

The 14-day primary cultured RBMECs were seeded at a density of 5×10^4 mL^{-1} in 24-well plates. After reaching confluence, the cell monolayers were exposed to 5 μmol L^{-1} rhodamine123 in serum-free DMEM/F12 medium containing 1, 3.3, or 10 μmol L^{-1} B3 and 10 μmol L^{-1} verapamil at 37°C for 90 min. After incubation, the medium was removed, and all monolayers were washed three times with ice-cold PBS and then dissolved in 1% Triton X-100. Rhodamine123 fluorescence was determined using a Poly Immune Analysis System-1420 (Perkin Elmer) ($\lambda_{Ex}=488$ nm, $\lambda_{Em}=535$ nm), and the concentration of rhodamine123 was measured from the fluorescence value by using a rhodamine123 standard curve. The amount of rhodamine123 in the cell samples were normalized against the amount of protein in each sample as determined by the Coomassie brilliant blue G-250 dye binding method (Fontaine et al 1996).

Rhodamine123 efflux from RBMEC assay (Tsuji et al 1992)

When RBMECs were grown to monolayers, the culture medium was removed and the cells were washed three times with ice-cold incubation medium. Then cells were incubated with incubation medium containing 10 μmol L^{-1} rhodamine123 at 37°C for 90 min. Cells were washed with ice-cold incubation medium three times. After this, 10 μmol L^{-1} verapamil of 1, 3.3, or 10 μmol L^{-1} B3 were added to the micro-wells to initiate the efflux of rhodamine123 at 37°C. At various times termination of the efflux was performed by the same procedure as the uptake study mentioned above. Efflux was estimated from the amount of rhodamine123 remaining in the cells.

In-vivo antitumour activity assay (Gao et al 2006)

The antitumour activity of doxorubicin, either alone or in combination with B3, was examined in nude mice bearing K562/A02 xenografts. Approximately 1×10^7 cells were injected subcutaneously into the right flank of each nude mouse (Shanghai Institute of Materia Medica, Chinese Academy of Sciences). Mice bearing tumours were randomized into six mice per group. Groups of mice bearing K562/A02 xenografts were treated with: saline, doxorubicin (2 mg kg^{-1}), verapamil (8 mg kg^{-1}), B3 (8 mg kg^{-1}, 2 mg kg^{-1}), verapamil (8 mg kg^{-1}) + doxorubicin (2 mg kg^{-1}), B3 (8 mg kg^{-1}) + doxorubicin (2 mg kg^{-1}), B3 (4 mg kg^{-1}) + doxorubicin (2 mg kg^{-1}), or B3 (2 mg kg^{-1}) + doxorubicin (2 mg kg^{-1}). All the groups were injected intravenously every other day. Eventually the tumours in the mice were weighed and tumour volume (V, mm^3) was calculated as: $V=½\times l\times w^2$ (Nakajima et al 2003), where l and w are the longest and the shortest diameters of the tumour mass (in mm), respectively.

Statistical methods

Data are the means ± s.d. from at least three independent experiments. Individual differences between treatments were statistically identified using a non-parametric post-hoc Dunn's test after Kruskal–Wallis test. Results were considered to be statistically significant when $P<0.05$.

Results

B3 increased cytotoxic effect of doxorubicin in K562/A02 and MCF-7/ADM cells

Cell survival rate, which was calculated by dividing the OD values in the B3 group by those in the control group, was approximately 95% or more in K562, K562/A02, MCF/7 and MCF-7/ADM cells after the treatment of B3 at 1, 3.3, or 10 μmol L^{-1} (data not shown). At 30 μmol L^{-1}, B3 could not inhibit the growth of the cells mentioned above.

K562/A02 and MCF-7/ADM cells were incubated with various concentrations of B3 (1, 3.3, 10 μmol L^{-1}) and a full range of concentrations of the chemotherapeutic agent, doxorubicin. B3 reversed drug resistance of K562/A02 to doxorubicin in a dose-dependent manner. B3 at 10 μmol L^{-1} decreased the IC50 of doxorubicin from 71.30 to 1.73 μmol L^{-1}, i.e. approximately a 41-fold reversal of MDR, in K562/A02 cells, whereas the IC50 of doxorubicin was decreased from 72.51 to 5.42 μmol L^{-1}, i.e. approximately a 13-fold reversal of MDR, in MCF-7/ADM cells (Table 1).

Table 1 Effect of B3 on the cytotoxicity of doxorubicin in K562/A02 and MCF-7/ADM cell lines

Doxorubicin IC50 (μmol L^{-1})	K562	MCF-7
With no modulator	0.60 ± 0.02	1.86 ± 0.15
With verapamil (10 μmol L^{-1})	0.58 ± 0.04	1.79 ± 0.22
With B3 (1 μmol L^{-1})	0.66 ± 0.03	1.89 ± 0.24
With B3 (3.3 μmol L^{-1})	0.61 ± 0.03	2.01 ± 0.31
With B3 (10 μmol L^{-1})	0.54 ± 0.05	1.69 ± 0.29
Doxorubicin IC50 (μmol/L)	**K562/A02**	**MCF-7/ADM**
With no modulator	71.30 ± 8.56	72.51 ± 9.41
With verapamil (10 μmol L^{-1})	2.69 ± 0.11	12.13 ± 1.58
With B3 (1 μmol L^{-1})	14.78 ± 1.23	30.59 ± 3.34
With B3 (3.3 μmol L^{-1})	2.97 ± 0.25	16.48 ± 1.02
With B3 (10 μmol L^{-1})	1.73 ± 0.29	5.42 ± 0.57

The results shown in Table 1 indicated that the resistance to doxorubicin was significantly reversed by B3 at 1, 3.3, and 10 μmol L^{-1} with the reversal folds of 4.8, 24.0, and 41.2 in K562/A02 cells, and.2.4, 4.4, and 13.4 in MCF-7/ADM cells, respectively.

B3 increased rhodamine123 accumulation in K562/A02 cells and RBMECs

A significant difference was observed using the Kruskal–Wallis test, and then non-parametric post-hoc Dunn's test was used to analyse the difference between the control group and other groups. K562/A02 cells contained much less rhodamine123 than sensitive K562 cells due to the efflux of P-gp (group 1). B3 significantly increased the accumulation of rhodamine123 in K562/A02 cells (Figure 2) and RBMECs (Table 2), but not in P-gp-negative K562 cells.

B3 inhibited rhodamine123 efflux from RBMECs

It was assumed that B3 increased the uptake of rhodamine123 by inhibiting the efflux of rhodamine123 from the RBMECs. We therefore studied the effect of B3 on the efflux of rhodamine123 (Figure 3). In the presence of B3, the amount of rhodamine123 remaining in the cells at 60, 90 and 120 min significantly increased, suggesting that B3 inhibited the efflux of rhodamine123.

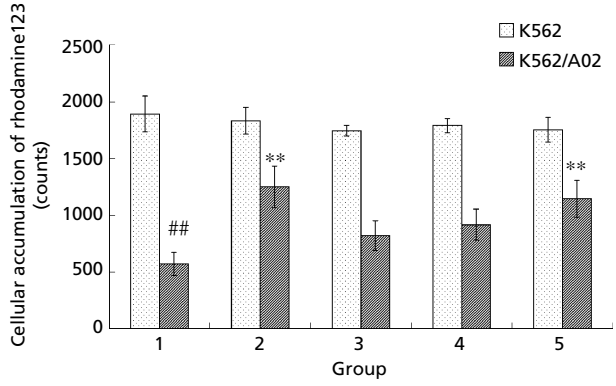

Figure 2 Rhodamine123 accumulation in K562 and K562/A02 cell lines in the presence of B3. Group 1, control (PBS); group 2, 10 μmol L^{-1} verapamil; group 3, 1 μmol L^{-1} B3; group 4, 3.3 μmol L^{-1} B3; group 5, 10 μmol L^{-1} B3. ##$P < 0.01$, control of K562/A02 cells compared with control of K562 cells; **$P < 0.01$, K562/A02 cells treated with B3 compared with control of K562/A02 cells.

Table 2 The effect of B3 on rhodamine123 accumulation in RBMECs

Group	Concn (μmol L^{-1})	Rhodamine123 concn (nmol (mg protein)$^{-1}$)
Control		0.565 ± 0.08
Verapamil	10	1.162 ± 0.01**
	1	0.893 ± 0.05**
B3	3.3	1.125 ± 0.11**
	10	1.217 ± 0.06**

**$P < 0.01$ compared with control.

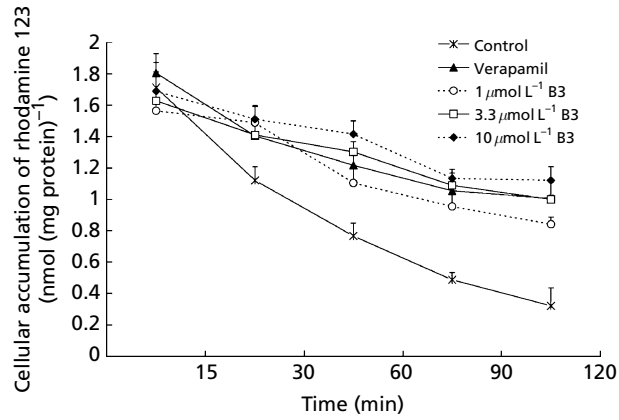

Figure 3 The effect of B3 on rhodamine123 efflux from RBMECs.

Effect of co-administration with B3 on the antitumour activity of doxorubicin against established tumour xenografts in mice

B3 (2, 4, or 8 mg kg^{-1}) and doxorubicin (2 mg kg^{-1}) were given intravenously every other day from day 1 to day 21. The results are shown in Figure 4, in which each point represents the mean ± s.d. of tumour volume from six mice in the

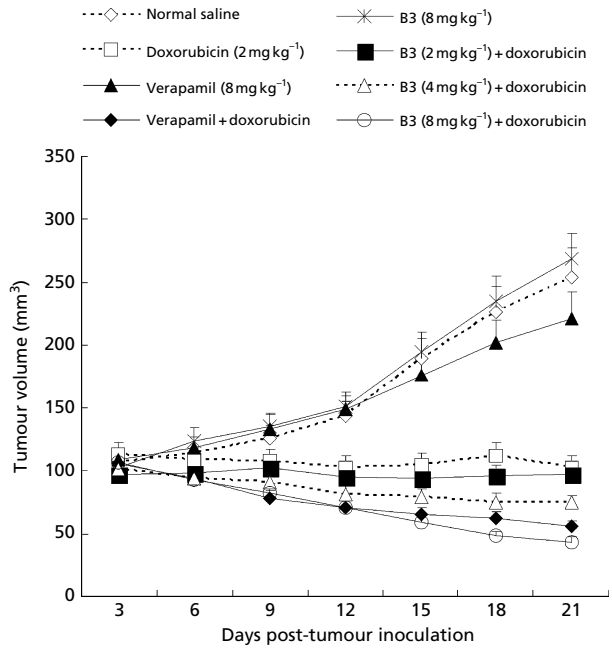

Figure 4 Effect of co-administration with B3 on the antitumour activity of doxorubicin in K562/A02 xenograft model in nude mice.

group. To monitor the toxicity of the treatment, the body weights of the mice were recorded every three days. When applied individually, B3 and verapamil were ineffective at inhibiting the growth of the tumour. However, when B3 was co-administrated with doxorubicin, the mean weight (data not shown) and volume of the tumours were significantly lower compared with control mice (Figure 4). These observations demonstrated that B3 was effectual in-vivo on reversing the resistance of tumour to doxorubicin.

Discussion

A major problem in the treatment of cancer is the development of resistance to chemotherapy and subsequent relapse. It is necessary to establish proper protocols for cancer chemotherapy in combination with appropriate MDR reversing agents that have low pharmacological potency and side effects.

B3 was designed and synthesized on the basis of tetrahydroisoquinoline, which has a structure similar to verapamil, a calcium-channel antagonist with potent MDR reversing activity by inhibiting P-gp function. However, the clinical application of verapamil in cancer therapy is hindered by its unacceptable clinical toxicity (such as heart block) at the dose required for P-gp inhibition in-vivo. Thus, a compound sharing a similar structure with verapamil might be an ideal candidate as a MDR reverser if its Ca^{2+}-blocking activity was considerably low. B3 did not inhibit high KCl (60 mmol L^{-1})-induced contraction response of isolated rat thoracic aorta rings even at the high concentration of 100 mmol L^{-1}, which indicated that B3 was not expected to produce any cardiovascular effects after a normal dose. In this study, the MDR reversing activity of B3 by inhibiting P-gp in-vitro and in-vivo has been investigated for the first time.

The in-vitro and in-vivo studies demonstrated that B3 was a potent reverser of P-gp-mediated MDR. The in-vitro potency was evaluated using human cell lines with high P-gp expression and primary cultured RBMECs (Takakura et al 1992). Cell culture has been proven to be an invaluable tool for the discovery and characterization of agents capable of altering the MDR phenotype (Dimmock et al 2005; Biscardi et al 2006). Due to P-gp efflux, K562/A02 and MCF-7/ADM cells are 118.8- and 39.0-times more resistant to doxorubicin compared with their parental cells, K562 and MCF-7, respectively.

The concentrations of doxorubicin required to achieve IC50 values for the K562/A02 and MCF-7ADM cells were higher than those of the corresponding parental cell lines when B3 was absent. Co-administration with B3 led to a significant decrease in the IC50 of doxorubicin in the aforementioned two cells, in a B3 dose-dependent manner. When 10 μmol L^{-1} B3 was present, the IC50 values of doxorubicin against the MDR cells became a little higher than those against the parental cells. The concentration of B3 in reversing MDR was comparable that of with verapamil and other isoquinoline derivatives in some reported articles. Suzuki et al (1997) reported that at low concentrations, some newly synthesized quinoline derivatives enhanced the accumulation of [^{3}H]vincristine in K562/ADM cells and reversed tumour cell MDR. Naito et al (2002) reported that MS-209 was one of the most potent quinoline derivatives that could reverse MDR in-vitro at a clinically achievable concentration of 3 μmol L^{-1}. It was undergoing clinical trials in Japan. Zhu et al (2005) reported E6, a derivative of berbamine, at 1, 3, 10, and 30 μmol L^{-1} significantly reversed the resistance of K562/DOX to doxorubicin. The IC50 of doxorubicin to K562/DOX in the presence of E6 (10 μmol L^{-1}) was approximately 6.31 μmol L^{-1}, which was higher than B3. Pyronaridine, a synthetic quinoline derivative, was capable of reversing MDR phenotype in P-gp-overexpressing tumour cells. Cytotoxicity of doxorubicin co-administration with pyronaridine was increased significantly in several drug-resistant cell lines. The IC50 of doxorubicin co-administration with 4 μmol L^{-1} pyronaridine to K562/A02 and MCF-7/ADM was approximately 5.6 and 11.0 μmol L^{-1} (Qi et al 2004). The study by Liu et al (2003) demonstrated that tetrandrine, a benzylisoquinoline alkaloid isolated from the Chinese herb 'Hanfangji', exhibited stronger activity to reverse drug resistance to doxorubicin and vinblastine multidrug-resistant human T lymphoblastoid leukaemia MOLT-4/DNR cells. Our results certified that B3 shared a similar, even higher, potency in reversing MDR with other effective P-gp inhibitors in-vitro. In contrast to the modulatory activity in the resistant cell lines, B3 had no effect on cytotoxic drug activity in non-P-gp-expressing parental cell lines.

The fluorescent dye, rhodamine123, a substrate of P-gp, was used to assess the functional activity of P-gp. B3 increased the intracellular accumulation of rhodamine123 in resistant K562/A02 cells and RBMECs, and inhibited rhodamine123 effluxes from RBMECs. The potential of B3 on rhodamine123 accumulation and efflux was similar to that of verapamil. Verapamil is a potent P-gp inhibitor capable of reversing MDR in man, but its cardiovascular side effects restrict its clinical use (Raderer et al 1993; Teodori et al 2002). B3 shares a similar potency to verapamil in MDR reversal but has a low Ca^{2+}-channel blocking activity and so would benefit from future research.

Although many pharmacological agents have been found to completely overcome drug resistance with in-vitro models, the number of reports showing such phenomena with in-vivo systems is limited (Mi et al 2001). Therefore, our in-vivo experiment was very important and necessary. The MDR reversing activity of B3 demonstrated in our in-vitro experiments was confirmed by in-vivo studies. B3 significantly increased the antitumour efficacy of doxorubicin in mice bearing human tumour xenografts without an apparent increase in toxicity. Moreover, maximum modulatory activity of B3 was achieved at 8 mg kg^{-1}, which was considerably lower than that of many other P-gp inhibitors reported previously. For example, doxorubicin co-administered with pyronaridine (20, 40, 60 mg kg^{-1}) orally significantly enhanced the antitumour activity of doxorubicin in the doxorubicin resistant K562/A02 model (Qi et al 2004). In an HCT-15 (colorectal adenocarcinoma)-bearing mouse model, oral administration of 200 mg kg^{-1} MS-209 30-min before injection of 15 mg kg^{-1} docetaxel produced significant inhibition of tumour growth (Naito et al 2002). The high validity with a low dose of B3 on reversing MDR in-vivo would, as far as possible, avoid side effects in future clinical use.

Conclusions

B3 effectively reversed MDR in-vitro and in-vivo. Given the strong MDR reversal effect and relatively weak calcium-channel blocking activity, B3 exhibited the potential to be developed as an adjunctive agent for cancer chemotherapy. However, further studies are warranted to elucidate its mechanism and pharmacokinetic properties.

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