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ORIGINAL ARTICLE

# Reversal of P-glycoprotein-mediated multidrug resistance of cancer cells by five schizandrins isolated from the Chinese herb *Fructus Schizandrae*

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**Abstract**
*Purpose* *Fructus Schizandrae* (FS) is commonly used as a tonic in traditional Chinese medicine. Recently, FS was found to significantly improve liver dysfunction in chronic hepatitis patients. The present study was to assess the reversal effect of five schizandrins and crude extract from FS (named LCC) on multidrug resistance (MDR) of cancer cells, both in vitro and in vivo. Chemically, the five schizandins are derivatives of dibenzo-(*a*, *c*)-cyclooctene lignan with distinct structures differing from any known MDR reversal agents.
*Methods* A panel of sensitive and resistant cancer cell lines were treated with various concentrations of LCC and schizandrins. Drug sensitivity, accumulation of Doxorubicin (Dox), expression of *P*-glycoprotein and protein kinase C (PKC), and apoptosis were determined in vitro. The in vivo effect was tested in nude mice grafted with sensitive and resistant human epidermal cancer cell line to vincristine (VCR) (KB, KBv200).
*Results* The tested five compounds at 25 μM showed various levels of MDR reversal activity, of which, schizandrin A (Sin A) was the most potent one. Sin A reversed VCR resistance in KBv200 cells, MCF-7/Dox cells and Bel7402 cells by 309-, 38-, and 84-folds, respectively. Also, Sin A reversed the resistance of Dox in the above cancer cell lines. LCC at 25 μg/ml reversed VCR resistance by 619-folds in KBv200, 181-folds in MCF-7/Dox cell line, and 1,563-folds in innate resistance of human hepatic cellular carcinoma Bel7402 cells to VCR. Furthermore, LCC and its active component Sin A potently reversed the cross-resistance to paclitaxel in those cell lines. Both Sin A and LCC markedly increased intracellular Dox accumulation and enhanced apoptosis, down-regulated Pgp protein and mRNA and total PKC expression in MDR cells. Coadministration of LCC (p.o.) significantly potentiated the inhibitory effect of VCR (i.p.) on tumor growth in nude mice bearing KBv200 xenograft.
*Conclusions* The LCC and its active component Sin A have remarkable reversal effect on MDR in cancer cells by inhibition of both the function and expression of Pgp and total PKC.

**Keywords** Multidrug resistance · Pgp · *Fructus Schizandrae* · Schizandrins

**Abbreviations**

| | |
|---|---|
| FS | *Fructus Schizandrae* |
| Sin A | Schizandrin A |
| Sin B | Schizandrin B |
| Sin C | Schizandrin C |
| Sol A | Schizandrol A |
| Sol B | Schizandrol B |
| VPL | Verapamil |
| Dox | Doxorubicin |

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| | |
|---|---|
| MDR | Multidrug-resistance |
| Pgp | P-glycoprotein |
| LCC | Crude extract of *Fructus Schizandrae* |
| PKC | Protein kinase C |
| VCR | Vincristine |
| MTT | 3-(4,5-Dimethylthiazole-2-yle)-2,5-diphenyltetrazolium bromide |
| Rho 123 | Rhodamine 123 |
| PI | Propidium iodide |
| FITC | Fluorescence isothiocyanate |
| LPS | Lipopolysaccharides |
| CAM | Camptothecin |
| ETO | Etoposide |
| NBT | Nitroblue tetrazolium chloride |
| BCIP | 5-Bromo-4-chloro-3-inDoxyl-phosphate |

## Introduction

Inherent or acquired resistance to anti-cancer drugs is a major cause of failure in cancer chemotherapy. Over-expression of Pgp confers resistance of tumor cells to a variety of structurally and functionally distinct classes of anti-cancer drugs, including the anthracyclines (Dox and daunomycin), Vinca alkaloids (VCR and vinblatine), podophyllotoxins, and actinomycin [1]. Pgp-induced MDR phenotype is induced by pumping these anti-cancer drugs out of cells through an energy-dependent mechanism [2–4]. A variety of compounds with different chemical structures are capable of suppressing Pgp expression, thereby increasing the sensitivity of MDR cancer cells to chemotherapy and are thus widely used as MDR reversal agents. The study of MDR reversal agent has gone through three generations. The first generation of MDR reversal agents includes verapamil (VPL), cyclosporine A, and progesterone [5]. Most of these MDR reversal agents were failures in clinical trials, mainly due to their insufficient MDR reversal potency at a tolerated dosage, solubility limitation, or unacceptable side effects [1, 6]. The second and third generations of MDR reversal agents are derivatives of the first-generation molecules. These agents include VX-710, PSC833, MS-209, GF120918, LY335979, R101933 XR9051, XR9576 and SB-T-110131 (IDN5109) [7, 8] The results from in vitro study and preliminary clinical trial suggest that these advanced generation of MDR modulators are more potent and less toxic than the first generation compounds. However, some are still prone to have adverse effects, poor solubility, and unfavorable changes on the pharmacokinetics of the anti-cancer drugs [9]. Continuing search for novel MDR reversal agent with lower side effects or unique reversal mechanisms may benefit chemotherapy in cancer patients.

Besides Pgp, Protein kinase C (PKC) over-expression in human MDR tumors may also affect the drug efflux activity of Pgp through its phosporylation [10–12]. Inhibition of PKC$\alpha$ and PKC$\beta$ message level by anti-sense oligomers was found capable of reversing paclitaxel resistance in a variety of different cancer cell lines [13]. Some therapeutic agents including camptothecin (CAM), etoposide (ETO) and VCR were found to modulate MDR phenotype through transient or sustained induction of typical or atypical PKC in a number of cancer cell lines and cancer patients [14, 15]. Therefore, PKC is also believed as a target for searching MDR reversal agent.

*Fructus Schizandrae* (FS) has been commonly used as a tonic in traditional Chinese medicine for thousands of years. Recently, FS was found to be effective in the improvement of abnormal liver function of chronic viral hepatitis patients in China [16]. The crude extract of FS (named LCC) and five schizandrins, derivatives of dibenzo-(*a*, *c*)-cyclooctene lignan isolated from LCC showed multi-bioactivities such as anti-liver injury [17], induction of hepatic cytochrome P450 [18], anti-oxidant activity [19], inhibition of xenobiotics metabolism and mutagenicity [20].

A number of accumulated evidence indicated that the development of human hepatic cellular carcinoma (HCC) is closely related to chronic hepatitis virus infection, and HCC responds to chemotherapy poorly. Our previous study found that human HCC Bel-7402 cell line is inherently resistant to multi-anti-cancer drugs [21]. These findings lead us to study whether FS and its active components can reverse MDR. In this report, we report the reversal activity of crude extract (LCC) and five components of FS to MDR in vitro and in vivo, and its effect on expression of Pgp protein, mdr1 gene and PKCa. The chemical structures of five schizandrins and VPL are shown below.

## Materials and methods

### Materials

5-Fluororacil (5-FU) was purchased from Shanghai Pudong Haipu Drug Limited Co. (Shanhai, China), VCR was from Minsheng Drug Manufacturing Factory, (Hangzhou, China), Dox was from Haimen Drug Manufacturing Factory (Zhejiang, China) and VPL was from Lianyun Gang Drug Manufacturing Factory (Jiangsu, China). 3-(4,5-Dimethylthiazole-2-yle)-2,5-diphenyltetrazolium bromide (MTT), trypsin, RNase A, agarose, rhodamine123 and propidium Iodide (PI) were purchased from Sigma Chemical Co. (St Lous, MO, USA). Pgp-specific MAbantibody C219 was from Centocor (Malvern, PA, USA). Goat-anti-mouse IgG conjugated to fluorescence, Isothiocyanate (FITC) was from

Jackson ImmunoResearch (West grove, PA, USA). All other chemicals used were of reagent grade. The five schizandrins are Sin A Sin B, Sin C, Sol A and Sol B, which were kindly provided by professors YR Chen and NL Li in our institute. The chemical structures of five lignans were characterized by MS and NMR spectrum [22]. LCC is an alcoholic extract of the kernels of FS [23].

## Cell culture

Human epidermal carcinoma KB cell, its MDR subline to VCR KBv200, and human hepatocellular carcinoma Bel7402 were all grown in RPMI1640 medium supplemented with 10% heat-inactivated new born calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. The KBv200 and MCF-7/Dox cell lines were obtained by multi-step drug selection in vitro [24, 25]. To maintain drug resistance, 200 nM VCR and 10 μM Dox were added to the culture of KBv200 and MCF-7/Dox cell, respectively. All experiments were performed after 7–10 days of incubation in drug-free medium.

## MTT assay

The cytotoxicity of compounds to KB, KBv200, MCF-7/Dox and Bel7402 cell lines was measured using the modified MTT assay [26]. Briefly, logarithmically growing cells were plated in 100 μl aliquots of growth medium into 96-well microtiter plates at a density of 800, 1,000, 1,500 cells per well, respectively. After acclimation overnight, 100 μl of various concentrations of test substance diluted in the medium was added. Four replicate wells were analyzed for each treatment. Control wells received 100 μl growth medium with the same volume of vehicle alone. After 96 h, the cytotoxicity was determined as previously described [26]. Reversal of the innate or acquired MDR by Sin A, LCC and VPL was studied by exposing the cells to a range of concentration of anti-tumor drug in the presence or absence of Sin A, LCC or VPL. The "fold reversal" for the test agent was calculated by the following formula: Fold reversal = $IC_{50}$ drug alone/$IC_{50}$ drug + test agent

## Analysis of apoptosis by flow cytometry

Apoptosis was analyzed quantitatively using flow cytometry as previously described [27, 28]. In each flask were plated $2 \times 10^5$ Bel7402 cells and cultured as described above. After incubation for 72 h, cells were exposed to various concentrations of Dox, or Dox combined with Sin A, LCC or VPL for 24 h before harvest. The cells were then rinsed twice with PBS. After 5-min incubation at room temperature in PBS with 0.02% EDTA, the cells were collected, washed twice with PBS, and fixed in cold 70% ethanol for at least 12 h at 4°C. The fixed cells were washed twice with PBS and incubated in 50 μg/ml RNAase A (diluted in PBS) for 1 h at 37°C. RNAase treated cells were washed with PBS, the cell pellets were resuspended in 1 ml of PBS containing 50 μg/ml PI for additional 1 h at 4°C. Cells were filtered through nylon net, and analyzed by flow cytometry (FACS 420). The percentage of apoptotic cells was calculated.

## Hoechst 33258 staining

Bel7402 cells were plated in 3.5 × 3.5 cm tissue culture disks at a concentration of $3.0 \times 10^5$ cells/ml. After overnight incubation, cells were rinsed to remove the detached cells, and then exposed to growth medium containing Dox either alone or combined with Sin A, LCC, and VPL for additional 24 h, respectively. The cells were then fixed in freshly prepared 4% paraformaldehyde in PBS for 20 min, washed with PBS, and stained in 5 μg/ml Hoechst 33258 in PBS for 15 min. Fixed cells were rinsed twice with PBS and examined under Confocal Laser Scanning Microscopy.

## Cellular accumulation of Dox

Bel7402 cells were seeded in triplicate at $2 \times 10^5$ cells/ml. Cells were grown to 70–80% of the confluence. Fresh medium containing Dox was added to obtain a final Dox concentration of 1,250 ng/ml in the absence or presence of 25/50 μM Sin A, 25 μg/ml LCC and 20 μM VPL. The cells were incubated at 37°C for additional 3 h. A total of $3.0 \times 10^5$ cells per sample were pelleted, washed three times with cold PBS, resuspended in 0.3 N HCl in 50% ethanol, and sonicated for 30 s. After centrifugation at 10,000 rpm for 15 min, the supernatants were removed and the Dox concentration was measured spectrofluorometrically with excitation and emission wavelengths of 470 nm and 585 nm, respectively [29]. The concentration of Dox (pmol/$10^6$ cells) was calculated from the standard curve prepared with known amounts of Dox.

## Rhodamine 123 (Rho123) accumulation assay

KB, KBv200, and Bel7402 cells were inoculated in 3.5 × 3.5 cm tissue culture disks at a concentration of $3.0 \times 10^5$ cells /ml, and incubated for 24 h. The cells were rinsed with PBS to remove detached cells, then loaded with 250 ng/ml Rh123 in growth medium for 30 min at room temperature in the presence or absence of Sin A, LCC, and

VPL. Background fluorescence of SinA and LCC was observed by exposure of cells to Sin A and LCC for 30 min at room temperature. Cells were washed twice with PBS, and images were captured and analyzed by Confocal Laser Scanning Microscopy.

### Indirect immunofluorescence of Pgp

Indirect immunofluorescent cell staining of Pgp was performed as previously described [30]. Briefly, KB or KBv200 cells were plated on glass cover slips placed in a sterile dish overnight and exposed to Sin A for 24 h. Cells were fixed either in freshly prepared 4% paraformaldehyde in phosphate-buffer saline (PBS) (for Pgp) or in ice-cold acetone/methanol (1:1) (for PKC) for 5 min, and washed three times with PBS, then blocked in 1% bovine serum albumin (BSA) or 10% goat serum in PBS for 30 min. Pgp-specific MAbC219 and rabbit-anti-human monoclonal antibody PKC (MC5) were used at 0.5 μg/ml and 1:200 in PBS containing 1.0% BSA, respectively. Secondary antibody (goat-anti-mouse or goat-anti-rabbit antibodies) conjugated to fluorescence isothiocyanate (FITC)) was diluted 1:300 in PBS containing 1% BSA. Stained slides were observed under confocal laser scanning microscope.

### RT-PCR analysis of mdr1 gene

Total RNAs were extracted from MCF-7 or MCF-7/Dox-cells with Trizol reagent (GIBCO) following the manufacturer's instruction. Two micrograms of total RNA was used for first-strand cDNA synthesis using oligo (DT) as primers and AcessQuick RT-PCR system (Promega), following the manufacturer's protocol. The primers used were 5′-TCG TAG GAG TAT CCG TGG AT-3′ (sense primer) and 5′-CAT TGG CGA GCC TGG TAG-3′ (anti-sense primer) for mdr1, 5′-ACG GAT TTG GTC GTA TTG GG-3′ (sense primer) and 5′-CGC TCC TGG AAG ATG GTG AT-3′ (anti-sense primer) for glyceraldehyde-3-phosphate dehydrogenase (GADPH) control. PCR conditions were as follows: 94°C for 3 min, followed by denaturation at 94°C for 30 s; annealing at 58°C (mdr1) or 55.5°C (GAPDH) for 1 min, extension at 72°C for 1 min (28 cycles), and a final extension step at 72°C for 7 min. PCR products were analyzed by 2% agarose gel and visualized by staining with ethidium bromide.

### Western blot analysis of Pgp and PKC proteins

Cells were treated with Sin A for 24 h and lysed in lysis buffer containing 25 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 5 mM EDTA, 5 mM EGTA, 10 mM NaF, 1 mM PMSF, 0.5% NP-40, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM pepstatin for 15 min. Cell lysates were frozen-thawed in liquid nitrogen for three times and centrifuged. Supernatants were harvested, and protein concentrations were assayed by the Lowry method [31]. The 150 μg of total protein was electrophoresesed on 10% SDS-polyacrylamide gels, and transferred onto nitrocellulose membrane. Non-specific binding sites were blocked by 5% milk powder dissolved in TBS at room temperature for 1 h. The primary antibody was rabbit monoclonal anti-human antibody PKC (MC5). The secondary antibody was alkaline phosphatase labeled goat-anti-rabbit IgG. Membranes were exposed in nitroblue tetrazolium chloride (NBT)/5-bromo-4-chloro-3-inDoxyl-phosphate (BCIP) as color detection reagents.

### Growth inhibition studies in vivo

KB and KBv200 xenograft were established in BALB/c nude mice (provided by Center of Experimental Animals, Chinese Academy of Medical College) as recently described [32]. All the animal experiments were approved by the Animal Ethics Committee at the college and performed following Animal Care and Use guidelines set by the NIH (National Institute of Health, USA). Each group contained seven mice with KB xenograft on the left flank and KBv200 xenograft on the right flank having a diameter of 5–16 mm. From day 1, a dose of 0.4 mg/Kg VCR was administered every other day for five doses. From day 1, 3% (v/v) Tween 80 in distilled water was given once daily for 14 days (q1d × 14). The 100, 200 and 300 mg/Kg of crude extract of FS (LCC) (dissolved in distilled water containing 2% Tween 80 as an auxiliary solvent) was given orally once daily. The LCC or 3% Tween 80 was given 3 h before the injection of VCR. A group of nude mice were orally administered 300 mg/Kg LCC only. A control group of nude mice received normal saline (0.2 ml/20 g body weight) every other day for five doses as the control. Tumor growth and body weight were monitored as previously described [32].

### Effect of LCC on Pharmacokinetics of Dox in mice bearing S180 sarcoma

ICR mice were s.c. injected with a S180 sarcoma cell suspension containing $4 \times 10^6$ cells in 0.2 ml on the right flank. Nine days later, the mice were divided randomly into two groups. The control group of mice was orally administered 0.5% CMC 10 ml/kg once daily for 3 days. The other group of mice received 300 mg/kg/10 ml of

LCC for 3 days. On day 3, 3 h after the last administration of LCC or CMC, all mice were i.p. injected a dose of 10 mg/kg Dox. Blood samples were taken from mice at 0, 2.5, 5, 10, 15, 20 and 30 min, and 1, 2, 3, 4, 6, 8 and 10 h after Dox injection, and put onto plastic tube containing heparin. The blood was centrifuged at 4°C for preparation of plasma. After the blood was taken, the mice were killed immediately, and the tumor was taken for homogenization. Then 0.4 ml of HCl (0.3N)-ethanol (50%) was added to 0.2 ml of the plasma sample or 0.2 ml of tumor homogenate. After 5–10 min the mixture was centrifuged at 10,000 rpm at 4°C for 15 min. The 100 µl of the supernatant was transferred to 96-well plates and read on a fluorescent microplate reader (Spectra MAX Gemini XS, Molecular Devices, Sunnyvale, CA, USA). Because this fluorometrical assay cannot discriminate Dox itself and its fluorescent metabolites, all data (three mice for each time-point) was expressed as Dox equivalents [33]. The concentration of Dox equivalents was calculated from standard curve prepared using appropriate biological fluids. The $AUC_{0-10\ h}$ of Dox in serum and tumor tissues was calculated for Dox equivalents using Drug and Statistics (DAS) 2.0 software.

## Results

### The MDR reversal efficacy of five components and crude extracts of FS

The cytotoxicity of the five components and crude extract (LCC) of FS were tested first. The five components Sin A, Sin B, Sin C, Sol A, and Sol B at concentrations up to 100 µM and LCC up to 100 µg/ml, were not toxic to MDR KBv200, MCF-7/Dox, Bel7402, KB and MCF-7 cancer cell lines. The cell survival rate for all the five cell lines was over 90% (data not shown). Both KBv200 and MCF-7/Dox are two acquired multi-drug resistant cell lines of man. Bel7402 is an innate multi-drug resistant human hepatocellular carcinoma cell line. KB and MCF-7 are sensitive cancer cell lines. The efficacy of above five compounds and LCC on MDR cancer cell lines was evaluated on the three cell lines. As shown in Table 1, KBv200 and MCF-7/Dox cell lines were 272-fold and 168-folds more resistant to VCR, respectively, compared to their parental cell lines. The addition of LCC at 25 µg/ml reversed VCR resistance by 619-folds in KBv200 cells and 181-folds in MCF-7 cells. Similarly, LCC at 25 µg/ml reversed the innate resistance of Bel7402 cells to VCR by 1,563-folds (Table 1).

To determine which components of FS are responsible for the MDR reversal activity, five dibenzo-(*a*, *c*)-cyclooctene lignans isolated from LCC, Sin A, Sin B, Sin C, Sol A and Sol B were screened. The reversing potency to VCR for five test lignans at concentration of 25 µM in KBv200 cells was ranked: Sin A (309-fold) > Sin B (23.5-fold) > Sol A (3.8-fold). Sin C and Sol B showed no detectable MDR reversal activity in KBv200 cells. Of five compounds, Sin A was the most efficient one for its MDR reversal activity. Therefore, the reversal activity to VCR resistance of Sin A (25 µM) was further studied in MCF-7/Dox cells and BeL7402 cells (Table 2). In both the cell lines, Sin A showed significant reversal activities. We then investigated the reversal activity of Sin A at cross-resistance to Dox. The reversal activity of Sin A was 32-fold in KBv200 cells, 23-fold in MCF-7/Dox cells, and 32.4-fold in Bel7402 cells, respectively. Furthermore, Sin A at 25 µm significantly reversed cross-resistance to paclitaxel resistance in KBv200 and MCF-7/Dox cell lines (Table 3). Similarly, the reversal activity of LCC at 25 ug/ml for Dox cross-resistance was 14-fold in KBv200 cells, 27-fold in MCF-7 cells, and 9-fold in Bel7402 cells. LCC at 25µg/ml also reversed cross-resistance to paclitaxel in both cells of KBv200 and MCF-7/Dox. MCF/Dox and Bel7402 cell

**Table 1** Reversal of resistance and cross-resistance of several tumor cell lines to VCR by crude extract of *Fructus Schizandrae* (LCC)

| Cell line | Drug concentration (µg/ml) | | IC50 (nM) | Fold reversal | *P* |
|---|---|---|---|---|---|
| KBv200 | VCR alone | | 2,479 ± 423 | | |
| | VCR + LCC | 25 | 4 ± 3 | 619 | |
| | | 12.5 | 36 ± 4 | 69 | |
| | | 6.25 | 229 ± 164 | 11 | <0.01 |
| | VPL | 10 µM | 65 ± 53 | 38 | |
| KB | VCR alone | | 9.1 ± 2.3 | | |
| | VCR + LCC | 12.5 | 0.4 ± 0.1 | 23 | |
| | | 6.25 | 1.3 ± 0.9 | 7 | <0.01 |
| | VPL | 10 µM | 6.4 ± 1.4 | 1.4 | >0.05 |
| MCF-7/Dox | VCR alone | | 2,905 ± 118 | | |
| | VCR + LCC | 25 | 16 ± 7 | 181 | |
| | | 12.5 | 71 ± 28 | 40.9 | |
| | | 6.25 | 1,069 ± 224 | 3 | <0.01 |
| | VPL | 10 µM | 22 ± 5 | 134 | |
| MCF-7 | VCR alone | | 17.3 ± 11.2 | | |
| | VCR + LCC | 25 | 2.4 ± 1.9 | 7.1 | |
| | | 12.5 | 12.8 ± 2.6 | 1.4 | >0.05 |
| | | 6.25 | 13.9 ± 8.4 | 1.2 | >0.05 |
| | VPL | 10 µM | 5.3 ± 4.3 | 3.3 | <0.01 |
| Bel 7402 | VCR alone | | 1,776.8 ± 695.4 | | |
| | VCR + LCC | 25 | 1.2 ± 1.1 | 1,480 | |
| | | 12.5 | 25.9 ± 16.1 | 69 | |
| | | 6.25 | 214.0 ± 86.7 | 8 | <0.01 |
| | VPL | 10 µM | 27.8 ± 31.3 | 64 | |

**Table 2** Reversal of resistance and cross-resistance of several tumor cell lines to vincristcin (VCR) and doxorubicin (Dox) by Sin A

| Cell line | Drug concentration (μM) | | IC50 (nM) | Fold reversal | *P* |
|---|---|---|---|---|---|
| KBv200 | VCR alone | | 1,949 ± 256 | | |
| | VCR + Sin A | 50 | 1.2 ± 0.3 | 1,624 | |
| | | 25 | 6.3 ± 1.3 | 309 | |
| | VCR + VPL | 20 | 1.9 ± 0.3 | 1,026 | |
| KB | VCR alone | | 10.4 ± 1.8 | | |
| Bel 7402 | VCR alone | | 2450 ± 654 | | |
| | VCR + Sin A | 25 | 29.0 ± 22 | 84 | |
| | | 12.5 | 78.0 ± 32 | 31 | <0.01 |
| MCF-7/Dox | Dox alone | | 26,022 ± 5,966 | | |
| | Dox + Sin A | 50 | 229 ± 31 | 113 | |
| | | 25 | 1,153 ± 216 | 23 | |
| | VPL | 10 | 3,164 ± 2021 | 8 | <0.01 |
| MCF-7 | Dox alone | | 105 ± 32 | | |
| | Dox + Sin A | 50 | 11 ± 8 | 10 | |
| | | 25 | 44 ± 19 | 2.4 | <0.05 |
| | VPL | 10 | 47 ± 21 | 2.3 | <0.05 |
| KBv200 | Dox alone | | 656 ± 209 | | |
| | Dox+Sin A | 50 | 20 ± 3 | 32 | |
| | | 25 | 83 ± 39 | 7.9 | <0.01 |
| | VPL | 10 | 73 ± 41 | 9 | <0.01 |
| | Dox alone | | 62 ± 5 | | |
| | Dox + LCC | 25 | 6.5 ± 1.2 | 9 | |
| | | 12.5 | 16.7 ± 2.7 | 2.7 | <0.01 |
| | Dox + Sin A | 50 | 18 ± 2 | 4 | <0.01 |
| | | 25 | 17 ± 5 | 4 | <0.01 |
| | VPL | 10 | 51 ± 25.4 | 1.2 | >0.05 |

**Table 3** Reversal of cross-resistance of several tumor cell lines to paclitaxel (Pacl) by Sin A

| Cell line | Drug Concentration (μM) | | IC50 (ng/ml) | Fold reversal | *P* |
|---|---|---|---|---|---|
| KBv200 | Pacl alone | | 1,243 ± 3.4 | | |
| | Pacl + Sin A | 50 | 51 ± 39 | 53.5 | |
| | | 25 | 61 ± 35 | 49.7 | |
| KB | Pacl alone | | 13.7 ± 6.6 | | |
| | Pacl + Sin A | 50 | 1.4 ± 1.1 | 10 | <0.01 |
| | | 25 | 3.3 ± 2.4 | 4.2 | <0.05 |
| MCF-7/Dox | Pacl alone | | 2,6022 ± 5966 | | |
| | Pacl + Sin A | 50 | 229 ± 31 | 113 | |
| | | 25 | 1,153 ± 216 | 23 | <0.01 |
| MCF-7 | Pacl alone | | 105 ± 32.0 | | |
| | Pacl + Sin A | 25 | 11 ± 8 | 10 | <0.01 |
| | | 12.5 | 44 ± 19 | 2.4 | <0.05 |

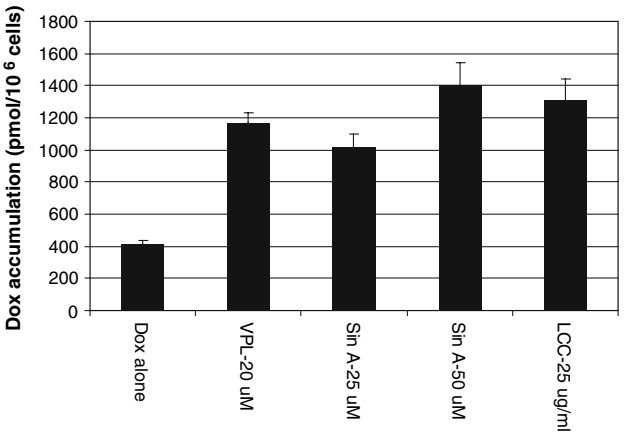


**Fig. 1** Enhancing effect of Sin A and LCC on cellular Dox accumulation in Bel7402 cells. $Bel_{7402}$ cells were treated with Dox alone or Dox plus Sin A, LCC, or VPL at the indicated concentrations for 3 h, respectively. Cells were washed, lysed and the intracellular concentrations of Dox were measured as described in "Materials and methods"

lines to paclitaxel were also potently reversed by both Sin A and LCC. In the above reversal assays, the reversal activity of Sin and LCC was stronger than that of verapamil (VPL) at non-toxic concentrations. LCC and Sin A also showed some reversal activity in sensitive KB and MCF-7 cancer cell lines but much weaker than their effect on resistance KBv200 and MCF-7/Dox cells.

## Enhanced intracellular accumulation of Dox by Sin A and LCC

It is well recognized that overcoming the Pgp-mediated efflux in drug-resistant cells will lead to enhance tumor cytotoxicity. In order to evaluate the inhibitory effects of Sin A and LCC on Pgp-mediated drug efflux, the intracellular accumulation of Dox was determined in Bel7402 cells. Co-treatment of Bel7402 cells with Dox and 25, 50 μM of Sin A or 25 μg/ml LCC resulted in an increase of intracellular Dox accumulation by 2–3-folds in comparison with Dox treatment alone group (Fig. 1).

## Increase of accumulation of Rhodamine 123 by Sin A and LCC

Rhodamine123 (Rho), a fluorescent molecule, is an excellent substrate for Pgp. Cells which over express Pgp fail to accumulate Rho123 because of enhanced drug-efflux activity by Pgp. Therefore, Rho123 accumulation was used as an imaging agent to screen and evaluate compound as inhibitor of Pgp activity at a single cell level [34]. To determine the effect of Sin A and LCC on the function of

Pgp, the intracellular accumulation of Rho123 was examined by confocal laser scanning microscope. Addition of Rho123 led to a significant increase of its accumulation in sensitive KB cells, but not in the innate resistant Bel7402 cells and resistant KBv200 cells. Preincubation of Pgp-over expressing Bel7402 and KBv200 cells with either 50 μM Sin A or 20 μM VPL at room temperature for 30 min, led to a marked increase of Rho123 accumulation in both the cell lines, but had little effect in the sensitive KB cells. The accumulation of Rho123 elicited by Sin A was significantly higher than that of VPL.

## Potentiation of Dox-induced apoptosis and chromatin condensation by Sin A and LCC

The appearance of a sub-G1 peak prior to the normal G1 peak is one of the characteristic features of apoptosis. To ascertain the percentage of cells possessing sub-G1 peaks, cellular DNA contents of Bel7402 cells stained with propidium iodide (PI) were analyzed by flow cytometry. There was only 1.8% the sub-G1 peak in Bel7402 cells, a cell line inherently resistant to Dox, treated with 1,250 ng/ml Dox alone. However, when the cells were treated with Sin A, LCC or VPL, and the same concentration of DOX (1250 ng/ml), the sub-G1 peak increased to 10–14% (Fig. 2). Exposure of Bel7402 cells to Dox at 1,250 ng/ml for 24 h had little, if any, effect on the morphology of chromatin. The degree of chromatin condensation, one of the few morphological characteristics of apoptosis, was increased in cells treated with a combination of Dox and Sin A or Dox and LCC as compared to cells treated with Dox alone.

## Effect of Sin A on the expression of Pgp and *mdr1*, which encodes Pgp

The results of immunofluorescent cell staining for Pgp and RT-PCR of *mdr1* mRNA showed that the expression of Pgp protein in KBv200 cells was markedly inhibited after treatment of Sin A at 25 μM for 24 h, and the *mdr1* mRNA level in MCF-7/Dox cells was also potently inhibited, indicating that transcriptional down-regulation of *mdr1* gene expression may be responsible for the reduced expression of Pgp protein after sustained exposure to Sin A or LCC.

## Induction of PKC translocation and down-regulation of PKC expression by Sin A

In addition to Pgp, PKC is another key component involved in induction and inhibition of MDR phenotype [10], the effects of Sin A and LCC on the expression and

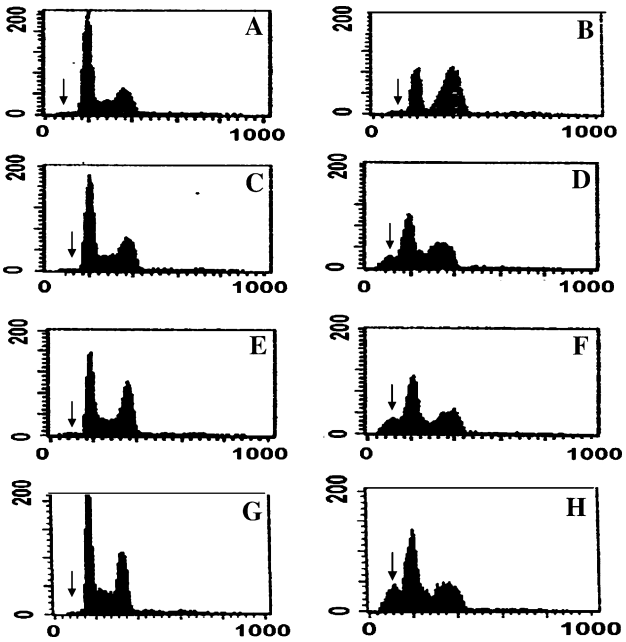


**Fig. 2** Flow cytometry detection of the enhancing effect of Sin A on apoptosis of Bel7402 cells. Histographs of propidium iodide stained small molecular DNA in $Bel_{7402}$ cells. Bel7402 cells were treated with Dox and Dox plus Sin A and VPL for 24 h, respectively. Cryptographs of propidium iodide stained cultured $Bel_{7402}$ cells treated with Dox alone or Dox combined with Sin A, LCC, or VPL for 24 h, respectively. The position of apoptotic cells was quantitied as a function of their reduced DNA content (sub-G1). DNA contents are indicated on the *x*-axis and the *y*-axis represents the intensity of forward light scatter (FSC), which indicates the size of the cells. **a** Control, **b** Dox 1250 ng/ml, **c** 20 μM VPL, **d** Dox 1250 ng/ml + VPL 20 μM, **e** 25 μM Sin A, **f** Dox 1,250 ng/ml + 25 μM Sin A, **g** LCC 25 μg/ml, **h** Dox 1,250 ng/ml + LCC 25 μg/ml

localization of PKC were detected by Western blot and immunostaining methods. As predicted, an increased expression of PKC was detected in KBv200 cells as compared to the parental KB cells. Sustained exposure of KBv200 cells to 12.5, 25 and 50 μM of Sin A for 24 h resulted in a dose-dependent reduction of total PKC expression to a level near to the parental KB cells (Fig. 3a). LCC showed similar inhibitory effects. Moreover immunostaining in the above cells revealed that over expression of PKC occurs in both cytosol and cell membranes of KBv200 cells (Fig. 3b). Addition of 50 μM Sin A almost completely inhibited PKC translocation. The results suggested that Sin A not only inhibited the total cellular PKC expression, but also inhibited PKC translocation in KBv200 cells.

## Inhibition of MDR tumor growth by LCC in vivo

Since LCC and Sin A were shown capable of reversing MDR in vitro, we next assessed their MDR reversal effect

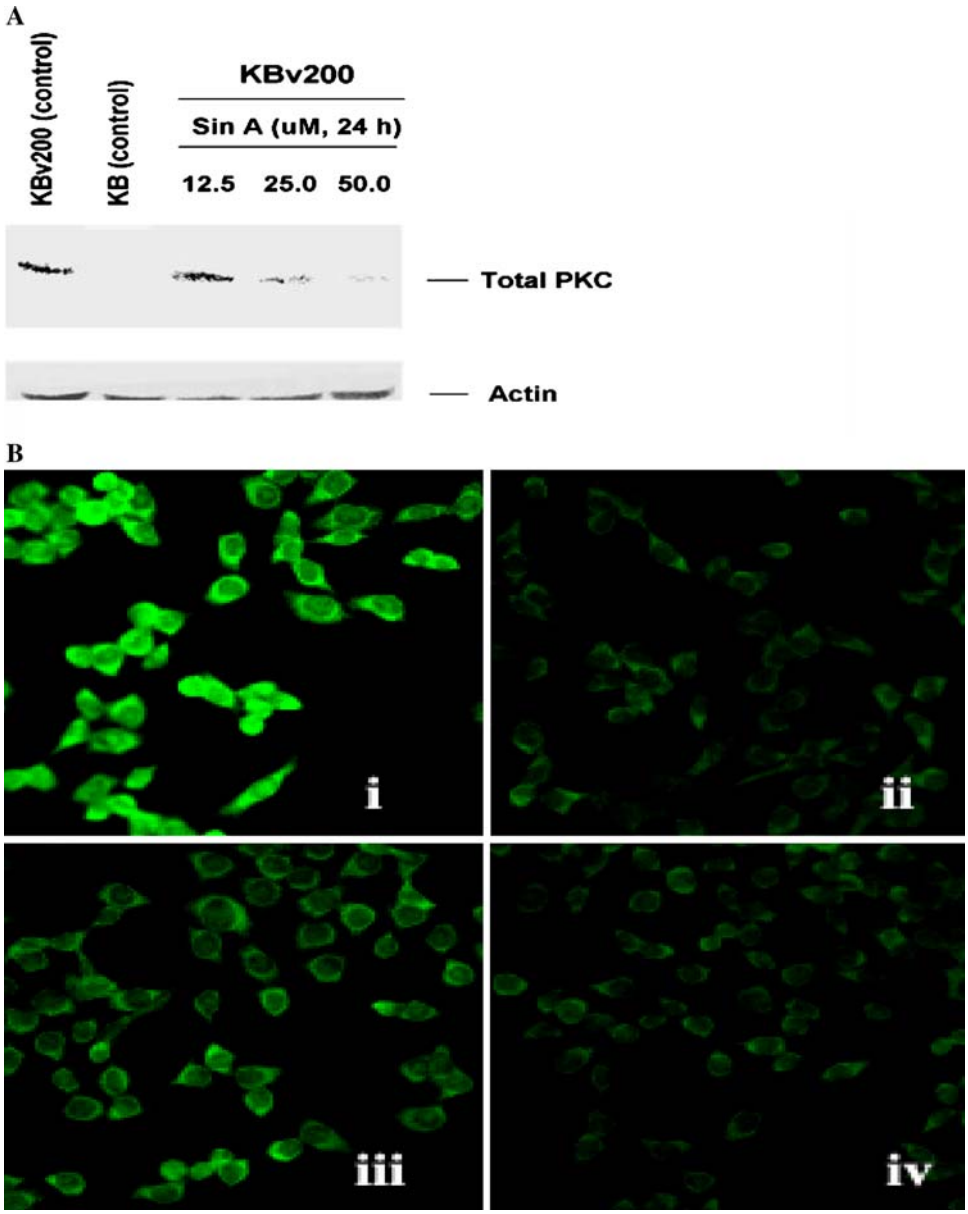


**Fig. 3** Suppressing effect of Sin A on PKC expression in KBv200 cells. **a** The expression of total PKC was detected by Western blot using anti-total PKC polyclonal antibody. *Lane 1* KBv200 control cells, *Lane 2* KB control cells, *Lane 3* KBv200 cells treated with 12.5 μM Sin A, *Lane 4* KBv200 cells treated with 25 μM Sin A, *Lane 5* KBv200 cells treated with 50 μM Sin A. **b** Visualization of PKC translocation by immunofluorescent cell staining. (*i*) KBv200 cells, (*ii*) KB cells, (*iii*) KBv200 cells treated with 25 μM Sin A, (*iv*) KBv200 cells treated with 50 μM Sin A

in vivo. Owing to limited available resource of Sin A, all in vivo experiments were conducted with LCC (crude extract of FS). Nude mice bearing KBv200 xenograft were i.p. injected VCR 0.04 mg/Kg every other day for 10 days (five doses). The tumor growth of KBv200 xenograft was inhibited by approximately 12% only as compared with control mice. Co-administration of LCC at 100, 200 and 300 mg/Kg body weight enhanced the anti-tumor activity of VCR in a dose-dependent manner (Fig. 4). LCC 300 mg/kg markedly potentiated the anti-cancer effect of co-administered VCR in KBv200 xenograft as indicated with 41.9% inhibition of tumor size on day 15 of treatment. The 41.9% inhibition of tumor growth by LCC plus VCR is significantly different from the 12% inhibition by VCR ($P < 0.05$). No significant body weight loss or signs of intoxication were observed during the combined treatment of LCC with VCR, suggesting that this regime was safe and effective in terms of inhibition of MDR tumor growth. The same dosages of LCC showed a little inhibitory effect on sensitive KB cells.

## Efficacy of LCC on Dox concentrations in plasma and tumor tissues for mice bearing S180 sarcoma

The efficacy of LCC on Dox concentration in plasma and tumor tissues in ICR mice bearing S180 sarcoma was studied. The area under curve (AUC) within 10 h was calculated as a marker of pharmacokinetics. The plasma $AUC_{0\text{-}10\ h}$ was 204.9 μg/ml for mice treated with Dox

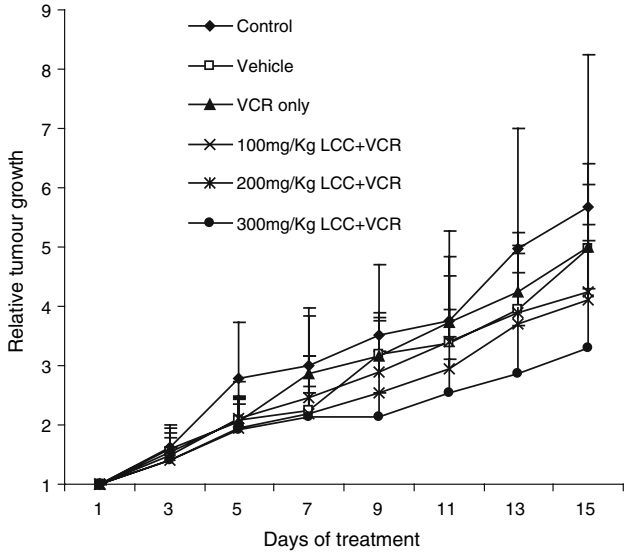


**Fig. 4** Inhibitory effect of LCC treatment on anti-tumor activity of VCR in KBv200-bearing nude mice. BALB-C nude mice bearing KBv200 xenograft were treated with VCR 0.04 mg/Kg (VCR only) injection every other day from day 1 for five doses, VCR plus LCC at the doses of 100, 200 and 300 mg/Kg, respectively (LCC 100 mg/kg + VCR, LCC 200 mg/kg + VCR and 300 mg/kg + VCR). LCC and the vehicle 3% Tween 80 were taken orally once every day from day 1 to 14 when the tumor size reached to 75–150 $mm^3$. The values represented mean ± SD of the relative tumor volume (RTV) calculated as described in "Materials and methods"

alone, and 186.0 μg/ml for mice treated with Dox plus LCC. Similarly, co-administration of LCC also did not affect Dox concentration in tumor tissues within 10 h. The $AUC_{0\text{-}10\ h}$ of Dox in tumor tissues was 90.5, and 87.5 μg/g for Dox alone and Dox plus LCC treatment groups, respectively. There were no significant differences in Dox concentration of blood and tumor tissues ($P > 0.05$). The results indicated that co-administration of LCC showed no significant effect on the pharmacokinetics of Dox in mice bearing S180 sarcoma.

## Discussion

In the past two decades, a number of natural and synthetic compounds have been tested for their ability to reverse MDR. Although several chemosensitizers have been shown to be very potent in reversal of MDR in both in vitro and in vivo experiments, the results of clinical trials with these compounds were disappointing because of the side effects and/or weak potency [4–6]. Therefore, development of new and more potent reversal agents with fewer side effects is a high priority of research on drug resistance. Using several inherently and acquired multi-drug resistant cell lines, this study has found that Sin A, one of the most active dibenzo-(*a*, *c*)-cyclooctene lignans isolated from herb *Fructus Schizandrae* (FS), exhibited a similar reversal activity to VCR, Dox and paclitaxel as verapamil, a calcium channel blocker and potent inhibitor of Pgp, even at a relatively non-toxic concentrations. Strikingly, LCC, the crude extract of FS, potently enhanced the susceptibility of these cell lines to VCR, Dox, and paclitaxel cytotoxicity, meanwhile significantly inhibited the MDR KBv200 tumor growth in nude mice. In general, the efficacy of LCC and Sin A was more potent than VPL in reversal VCR, Dox and paclitaxel resistance in the above assays.

Analysis of structure–activity relationship for five schizandrins isolated from FS, suggested that (1) Sin A, a component with two phenyl and three methoxyl (-$OCH_3$) groups (Fig. 5), is the most potent reversing compound of MDR in our study; (2) Sin A and Sol A share similar structural characteristics with only one exception, Sol A has a hydroxyl group (-OH) presented on the cyclooctene ring. The presence of this hydroxyl group in Sol A resulted in about a 100-fold reduction of the MDR reversal activity as compared with Sin A. Similarly, addition of a hydroxyl group in the same position of the cyclooctene ring in Sol B can completely abolish the MDR reversal activity. The sole structural difference between Sol A and Sol B is that two methoxyl groups on phenyl in Sol B is replaced by a methylenedioxy ring  ; (3) In contrast to high reversing effect of Sin A (309-fold) on MDR, Sin B and Sin C exhibited moderate (23.5-fold) and slight (3.9-fold) MDR reversal activity, respectively. A noticeable difference in the structure of Sin A and Sin B is the replacement of two methoxyl groups in one benzo-ring of Sin B by a methylenedioxy ring, and two dimethoxyl groups in both the benzo-ring of Sin C were replaced by methylenedioxy ring. It appears that the presence of dimethoxylphenyl groups in dibenzo-(*a*, *c*)-cyclooctene lignans could enhance the MDR reversal activity. The replacement of a dimethoxyl group by a methylenedioxy ring in Sin B and Sin C probably increases the bulky property or reduces the partially negative charges, and, therefore, leads to reduction of MDR reversal activity.

In comparison with the differences in chemical structure of verapamil and Sin A, the first reported reversal agent of MDR, the verapamil series of compounds typically have two dimethoxylphenyl [35], whereas Sin A, the most effective MDR reversal compound in our study, has two trimethoxylphenyl. Another striking difference between Sin A and verapamil is that a cyclooctene (an eight hydrocarbon ring structure) locates between the two phenyl with trimethoxyl groups in Sin A, whereas, nitrogen containing hydrocarbon side chain locates between two phenyl with dimethoxyl groups in verapamil. These differences in chemical structure might be the reason why Sin A has lower cytotoxicity and stronger reversing effect when

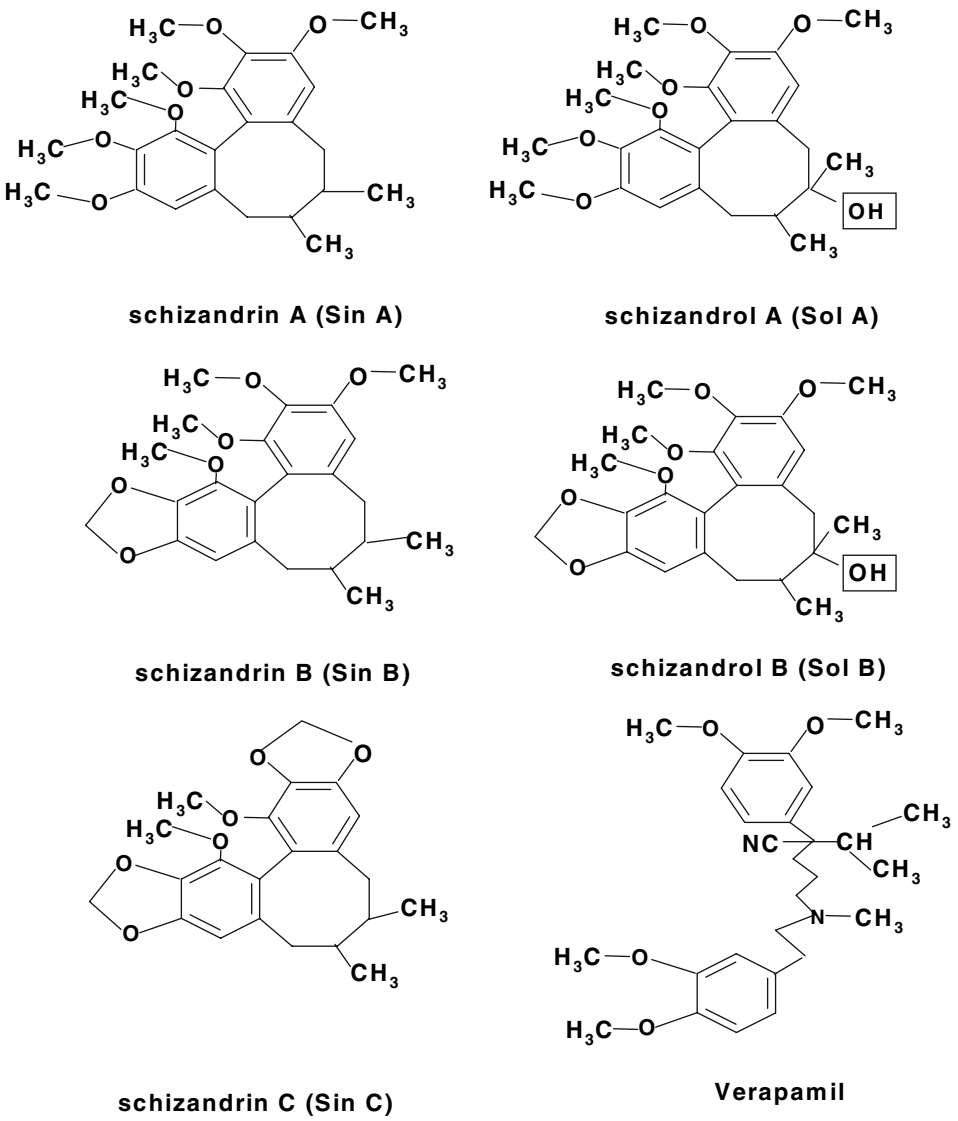


**Fig. 5** Chemical structure of five schizandrins isolated from *Fructus Schizandrae* as well as verapamil

compared to verapamil. A potential interaction between the partially positively charged hydrogen of the aromatic residues of Pgp and the partially negatively charged oxygen of the *O*-dimethoxylphenyl group on MDR reversal agents may be favorable for their binding to Pgp.

As a novel category of chemicals with distinct structural characteristics from the known MDR reversal agents so far identified [1], Sin A shows several distinct pharmacological properties that may favor their development as clinically effective MDR reversal agent: Firstly, like most other MDR reversal agents documented, Sin A could interact directly with membrane Pgp, and inhibit the efflux capability of Pgp. This prediction is supported by the fact that transient exposure of MDR cells to Sin A and LCC for 30 min induced a significant increase of intracellular accumulation of Dox and Rhodamine 123, Pgp substrates and in innate resistant human liver carcinoma cell line (Bel7402). Prolongation of the exposure of these cells to Sin A and LCC for 24 h markedly inhibited the expression of mdr1 mRNA and Pgp protein, indicating that Sin A and LCC is capable of inhibition the de novo production of Pgp. The inhibition of Pgp protein and mdr1 gene expression should be the main mechanism of both Sin A and LCC in reversal MDR in cancer cells. Secondly, a major challenge in confirming the clinical application of MDR reversal agents, particularly Pgp inhibitors, is their influence to the pharmacokinetics of the target anti-cancer drugs. Any change in pharmacokinetics of anti-cancer drugs by a MDR reversal agent will result in alteration of their efficacy and/or toxicity. It is interesting to note that LCC did not alter the Dox concentration in blood and tumor tissues in mice bearing S180 sarcoma. Thirdly, the present results indicated that Sin A and LCC were also able to inhibit the expression level of total PKC and its translocation from cytosol to membrane in MDR tumor cells The resultant reduction of membrane bound PKC may also

be partially involved in the mechanism of the reversing effect of Sin A and LCC on MDR in tumor cell lines and tumor animal models. It appears that the inhibition of Pgp and PKC by both Sin A and LCC contribute to their reversing activity to MDR. In addition, the increase of susceptibility of tumor cells to anti-cancer drugs-induced apoptosis by Sin A and LCC should be also responsible for their reversal MDR action. Fourthly, VPL is an anti-arrhythmic and the side effects are related to its primary pharmacodynamic activity (blocking effect on calcium channel), whereas Sin A and LCC showed no $Ca^{2+}$ antagonizing activity at a concentration tested that is capable of reversing MDR (data not shown). In order to further confirm the MDR reversal activity of LCC, a combination therapy of LCC with a lower and non-toxic dose of VCR in nude mice was performed. This combination regimen demonstrated a significantly reduced tumor size without causing significant body weight loss or signs of toxicity in the nude mice. The treatment of LCC 300 mg/kg in association with VCR 0.4 mg/kg reduced tumor growth of KBv200 in nude mice by 41%, while VCR 0.4 mg/kg given alone only reduced tumor growth by 12%. The difference between both the groups is significant statistically ($P < 0.05$). To fully evaluate the chemosensitizing effect of LCC or Sin A, further in vivo study may consider a combination regimen of LCC or Sin A with various concentrations of Dox and VCR.

Moreover, the presence of one or more nitrogen atoms in the hydrocarbon side chain of a compound, like VLP, is believed to be one of several indispensable constraints for enhancing its MDR reversal activity. There is no nitrogen atom in the structure of Sin A, but Sin A is still active in reversal of MDR cell lines. It is possible that derivatives of Sin A with nitrogen atom generated by chemical modification of Sin A side chains may further enhance Pgp binding and increase drug sensitivity.

Taken together, Sin A and LCC exert their reversal activity of multi-drug resistance through affecting multiple MDR targets, including direct interaction with the efflux pump of Pgp, inhibition of Pgp and PKC de novo production, inhibition of PKC activity and enhancing apoptosis of MDR tumor cells. Moreover, LCC showed no effect on pharmacokinetics of Dox in mice bearing tumor. All these make Sin A and LCC as promising candidates for further investigation. Regrettably, the currently available amount of natural Sin A was limited to permit us to perform MDR reversal test in animals. However, the promising MDR reversing activity of LCC in nude mice suggests the possibility that LCC or Sin A may become clinically effective chemosensitizer in MDR tumors. Alternatively, Sin A may serve as a novel leading compound for developing structural derivatives to overcome MDR. Clinical trial of the crude extract of FS will be applied soon in China. The discovery of Sin A and LCC acting on multiple targets involved in MDR may represent a new direction in the development of reversal agent of MDR from medicinal plants.

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