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ORIGINAL ARTICLE

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Dibenzocyclooctadiene lingnans: a class of novel inhibitors of P-glycoprotein

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Abstract *Purpose*: To determine if five dibenzocyclooctadiene lingnans, a class of naturally occurring compounds from Schisandra chinensis (Turcz.) Baill, have the activities to reverse P-glycoprotein (P-gp) mediated multidrug resistance (MDR). *Methods*: The IC_{50}s of four MDR cell lines (K562/Adr, MCF-7/Adr, KBv200, and Bcap37/Adr) toward daunorubicin, vincristine, and paclitaxel in the presence or absence of one of the dibenzocyclooctadiene lingnans were determined by a FACscan assay. The intracellular daunorubicin accumulation in the four MDR cell lines was determined by incubation of cells with daunorubicin (2 μg/ml) in the presence or absence of one of the dibenzocyclooctadiene lingnans by a FACscan assay. The interaction of the five dibenzocyclooctadiene lingnans with P-gp was assayed by their inhibition of ^{3}H-azidopine photoaffinity labeling of P-gp. *Results*: Among the five lingnans, while schisandrin A and B, and schisantherin A demonstrated strong and comparable activities to reverse the drug resistance and the intracellular drug accumulation in four MDR cell lines, schisandrol A and B showed very limited activities. The poor activities of schisandrol A and B are possibly caused by the hydroxyl groups on the cyclooctadiene ring, because the activities of the molecules resumed when the hydroxyl group was esterified to form a benzoate. Further studies demonstrated that these compounds physically interacted with P-gp. *Conclusion*: Schisandrin A and B, and schisantherin A are potent P-gp inhibitor and is of potential for future clinical application.

Keywords Schisandrin A · Schisandrin B · Schisantherin A · Schisandrol A · Schisandrol B · P-glycoprotein · Multidrug resistance · Cancer

Abbreviation P-gp: P-glycoprotein · MDR: Multidrug resistance · DNR: Daunorubicin · VCR: Vincristine

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Introduction

Cancer multidrug resistance (MDR) is one of the major obstacles hampering the success of cancer chemotherapy. P-glycoprotein (P-gp) is the most frequent cause for the clinically intrinsic and acquired MDR [1–3]. P-gp, functioning as an ATP-dependent drug pump, efficiently extrudes intracellular anticancer drugs out of cells, and keeps a sublethal drug concentration within cells. In addition, P-gp displayed broad substrate specificities toward many structurally and functionally unrelated compounds, such as vinca alkaloids, anthracyclines, epipodophyllotoxins, taxans, and therefore, rationalized the MDR caused by P-gp.

P-glycoprotein-mediated drug resistance could be overcome by an inhibitor antagonizing its drug pump function [4–6]. In the screening of naturally occurring compounds with potential efficacies to reverse cancer drug resistance, we found that schisandrin B, a major compound present in Schisandra chinensis (Turcz.) Baill, demonstrated a strong capacity to reverse cancer MDR (X. Hu and T. Wang. The application of schisandrin B in the preparation of medications against cancer, China patent application no. 200410059607). We then further investigated if the other compounds (Fig. 1) sharing the same core structure of dibenzocyclootadiene also displayed similar activities. Our results demonstrated that the five lingnans (schisandrin A and B, schisantherin A, schisandrol A and B) exhibited differential potencies to reverse P-gp mediated drug resistance.

Materials and methods

Chemicals

Schisandrin A and B, schisandrol A and B, and schisantherin A possessing a purity of over 99% were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China).

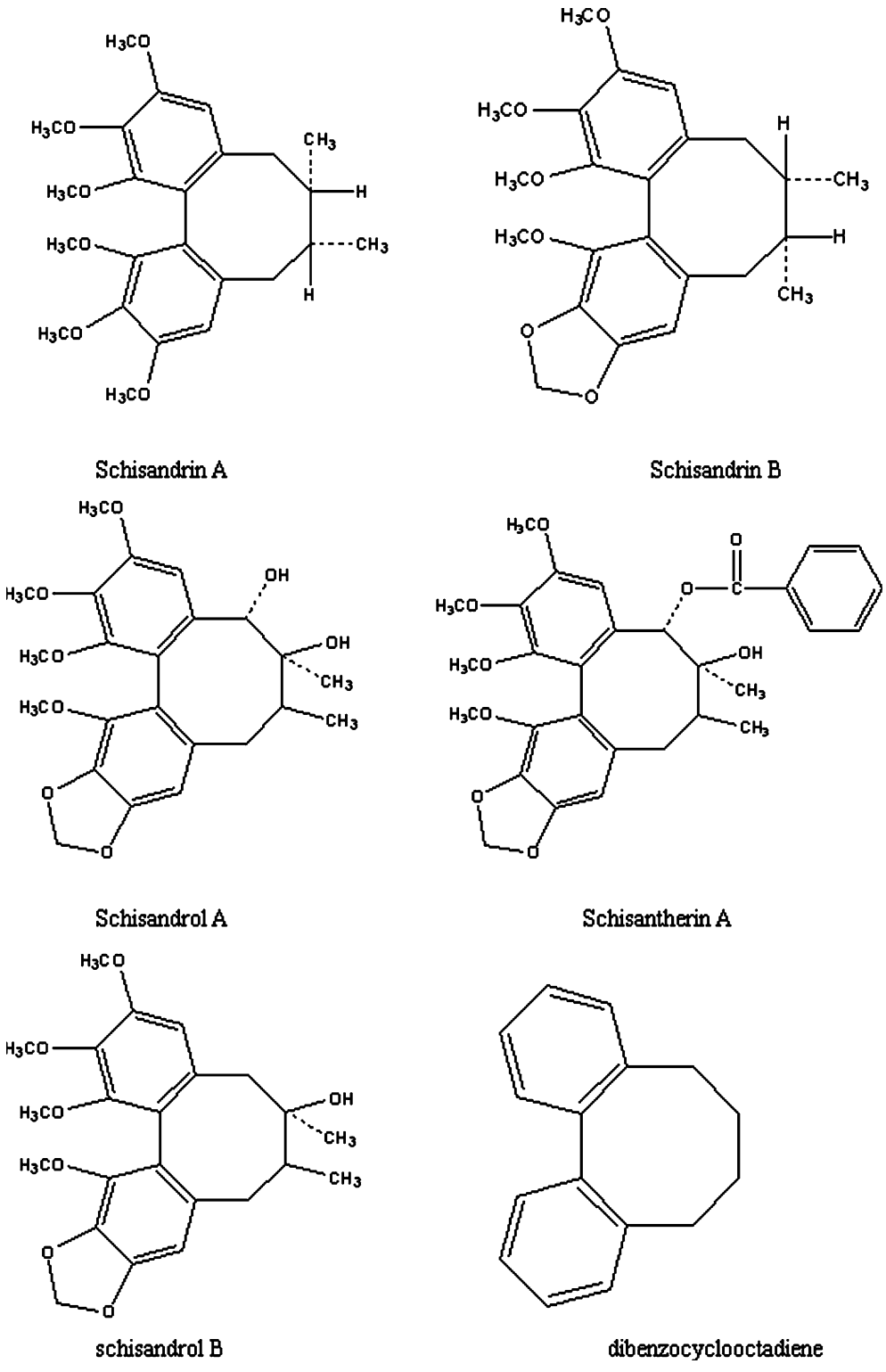

Fig. 1 The structures of five lingnans and dibenzocyclooctadiene

Cell lines

MDR cell lines

Human erythroleukemia cell line K562/Adr [7], human breast cancer cell line MCF-7/Adr [8], human epidermoid carcinoma cell line KBv200 [9], and Bcap37/Adr [10] were grown in an RPMI-1640 containing 10% FBS and 100 ng/ml doxorubicin, and their drug sensitive parental cell lines were maintained in an RPMI-1640 medium containing 10% FBS. The expression of P-gp in these MDR cell lines was confirmed by labeling an R-phycoerythrin (R-PE)-conjugated mouse antihuman P-gp monoclonal antibody (Becton-Dickinson, Holbrook, NY, USA), which was analyzed using a Becton Dickinson FACScan (BD Immunocytometry Systems, San Jose,

CA, USA). At least 50,000 cells were counted in each assay. The nonspecific labeling of cells was corrected by the monoclonal immunoglobulin isotype control (R-PE-conjugated mouse IgG2b, BD PharMingen).

Proliferation inhibiting assay

Inhibition of cell proliferation by anticancer drugs in the presence or absence of tested compounds was determined by a 3-day dose–response curve carried out in quadruplicates in 24-well plates. Cells were plated into 24-well plates in an RPMI-1640 medium containing 10% FBS at 4×10^4 cells/well. After a 2-h incubation for nonadherent cells (K562/adr) and an overnight incubation for adherent cells (Bcap37/adr, MCF-7/adr, KBv200) at 37°C in a humidified CO_2 incubator, schisandrin A, schisandrin B, schisantherin A, schisandrol A, or schisandrol B, or vehicles, and anticancer agents, were added to the plates upto the desired concentrations, and the incubation was continued for an additional 72 h. Final cell density in each well was counted with a Becton Dickinson FACScan, using which IC_{50}s were calculated.

Drug accumulation

Cells (1×10^6) were suspended in an RPMI-1640 medium containing 2 μg/ml daunorubicin (DNR) in the presence or absence of schisandrin A, schisandrin B, schisantherin A, schisandrol A, or schisandrol B, and incubated in a humidified CO_2 incubator at 37°C for 60 min. Cells were collected, washed twice with ice-cold PBS by centrifugation, and immediately subjected to intracellular DNR determination at an excitation wavelength of 488 nm and an emission wavelength of 530 nm using a Becton Dickinson FACScan. A minimum of 10,000 cells were counted for each assay, which was corrected by subtracting the auto-fluorescence of the cells.

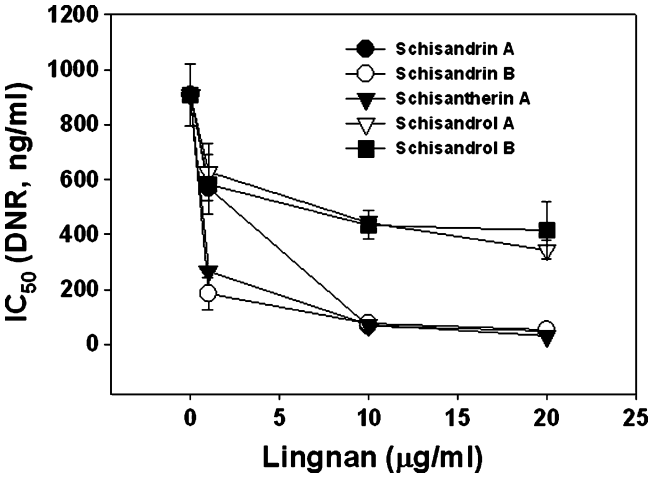

Fig. 2 The dose-dependent reversal of drug resistance of K562/Adr by schisandrin A, schisandrin B, schisantherin A, schisandrol A and schisandrol B. Cells were incubated in a complete RPMI-1640 medium containing a series of concentrations of DNR (0, 1, 10, 100, 1,000, 10,000 ng/ml) in the presence or absence of the indicated lingnans. After a 3-day incubation, cell densities were counted as described in Materials and methods and the IC_{50} s were calculated. The data are mean ± SD from three experiments

^{3}H-azidopine photoaffinity labeling of P-gp

The experiment was carried out as described by Hyafil et al. [11]. Briefly, 5×10^7 K562/adr cells were collected by centrifugation and the plasma membrane proteins were purified by a Plasma Membrane Protein Extraction Kit (BioVision, Mountain View, CA, USA) according to the

Table 1 The reversal of the drug resistance of the four MDR cell lines by five indicated dibenzocyclooctadiene lignans (10 μg/ml)

	IC_{50} (ng/ml)					
	Vehicle	Schisandrin A	Schisandrin B	Schisantherin A	Schisandrol A	Schisandrol B
Daunorubicin						
K562/ADR	908 ± 478	67 ± 3(14)[a]	63 ± 13(14)[a]	71 ± 23(13)[a]	444 ± 128(2)	434 ± 52(2)
MCF-7/ADR	875 ± 309	214 ± 32(4)	79 ± 3(11)	59 ± 16(15)		
KBV200	27 ± 3	2 ± 1(14)	6 ± 1(5)	3 ± 1(9)		
BCAP37/ADR	327 ± 127	53 ± 2(6)	49 ± 10(7)	70 ± 8(5)		
Vincristine						
K562/ADR	613 ± 71	7 ± 1(87)[a]	7 ± 1(88)[a]	8 ± 1(77)[a]	94 ± 1(7)	96 ± 10(6)
MCF-7/ADR	992 ± 341	8 ± 1(124)	22 ± 1(45)	25 ± 4(40)		
KBV200	22 ± 2	3 ± 1(7)	8 ± 1(3)	4 ± 1(6.0)		
BCAP37/ADR	911 ± 166	3 ± 1(304)	8 ± 1(114)	7 ± 2 (130)		
Paclitaxel						
K562/ADR	41 ± 14	6 ± 2(7)[a]	5 ± 2(8)[a]	< 1(> 41)[a,b]	10 ± 1(4)	16 ± 2(3)
MCF-7/ADR	53 ± 8	6 ± 1(9)	8 ± 4(7)	6 ± 1(9)		
KBV200	20 ± 2	2 ± 2(10)	3 ± 1(7)	2 ± 1(10)		
BCAP37/ADR	59 ± 9	4 ± 1(15)	8 ± 2(7)	7 ± 2(8)		

The numbers in the bracket are the reversal folds of drug resistance, the IC_{50} in the absence of the indicated lingnans divided by the IC_{50} in the presence of the indicated lingnans. Data are mean ± SD from three independent experiments

[a]Significantly different from schisandrol A and B, $P < 0.05$

[b]Significantly different from schisandrin A and B, and schisandrol A and B, $P < 0.05$

manufacturer's instruction. Plasma membrane protein of 10 µl (0.4 mg/ml in labeling buffer containing 50 mM Tris–HCl, pH 7.4, 0.1 mM 4-(2-aminoethyl)-benzenesulfong fluoride, 0.25 mM sucrose, 5 mM $MgCl_2$) was mixed with 5 µl labeling buffer with or without schisandrin A, schisandrin B, or schisantherin A for 40 min in the dark at 25°C in a humidified chamber, and followed by the addition of 5 µl containing 1.0 µmol (10 μCi/µmol) ^{3}H-azidopine (Amersham, Piscataway, NJ, USA). After UV irradiation at 254 nm for 2 min, the reaction was terminated by the addition of 20 µl electrophoretic loading buffer containing 4% SDS, and the photolabeled plasma membrane proteins were subjected to SDS-PAGE on a 7.5% gel; the band corresponding to 170 kD was cut and the radioactivity was measured using a liquid scintillation counter.

Induction of P-gp by dibenzocyclooctadiene lignans

K562 cells (2×10^5/6 ml) in six-well plates were incubated in a complete RPMI-1640 medium containing 10 µg/ml

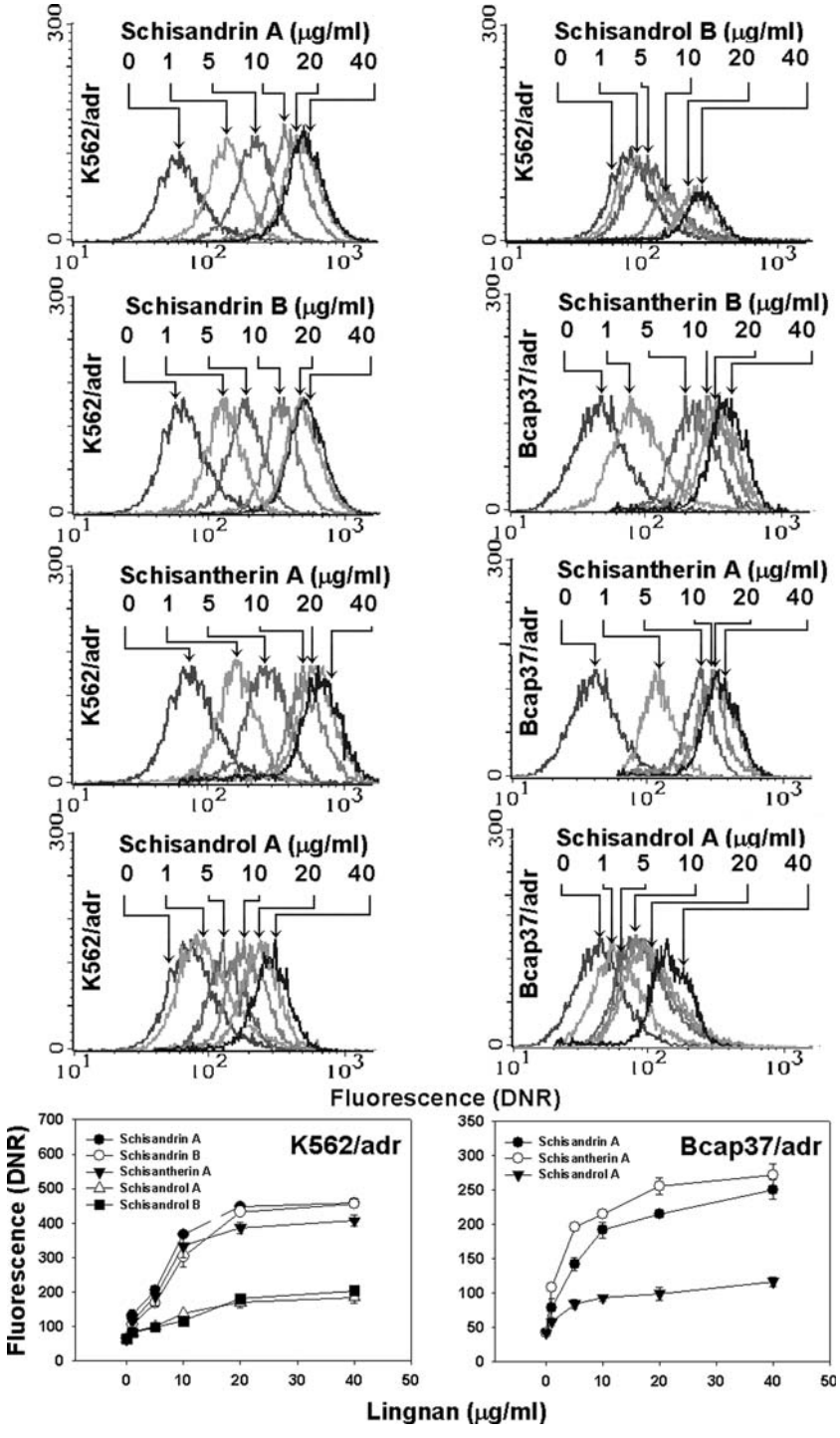

Fig. 3 Dose-dependent effects of the five lingnans on the intracellular DNR accumulation in MDR cell lines. Cells were incubated with DNR (2 µg/ml) in the presence or absence of the indicated lingnans at different concentrations for 60 min and collected for the determination of intracellular DNR as described in Materials and methods. Data are mean ± SD of three experiments

schisandrin A, schisandrin B, or schisantherin A in triplicates. After every 48 h, three-fourth of the cells and medium in the wells were removed, and the wells were supplemented with a fresh medium containing 10 μg/ml schisandrin A, schisandrin B, or schisantherin A. After pretreatment with the lingnans for 15 days, the cells were collected for P-gp determination by labeling an R-PE-conjugated mouse antihuman P-gp monoclonal antibody (Becton-Dickinson, Holbrook, NY, USA) according to the manufacturer's instructions, which was analyzed using a Becton Dickinson FACScan (BD Immunocytometry Systems, San Jose, CA, USA). At least 10,000 cells were counted in each assay. The nonspecific labeling of the cells was corrected by the monoclonal immunoglobulin isotype control (R-PE-conjugated mouse IgG2b, BD PharMingen).

Statistical analysis

Data was expressed as the mean ± SD, and analyzed by the Student's *t* test. *P*-values below 0.05 were regarded as statistically significant.

Results and discussion

Dibenzocyclooctadiene lingnans differentially reverse P-gp-mediated drug resistance

Firstly, we tested the direct cytotoxicity of the five dibenzocyclootadiene lingnans toward the MDR cell lines. The results indicated that concentrations of these compounds below 20 μg/ml had no significant effect on the growth of these cells (Data not shown).

Secondly, we did the concentration-dependent reversal of DNR resistance of K562/Adr by these lingnans. The results showed that with the increase in the concentration of the compounds, the IC_{50}s toward DNR reduced accordingly. At the concentration of 10 μg/ml, the activities of these compounds to reverse drug resistance reached the desired effectiveness (Fig. 2). Therefore, unless otherwise indicated, 10 μg/ml of these compounds was used for subsequent studies.

Thirdly, we tested the activities of the five lingnans to reverse the drug resistance of four MDR cell lines. As shown in Table 1, schisandrin A, schisandrin B, and schisantherin A exhibited strong and comparable activities to reverse the drug resistance of the four MDR cell lines, whereas schisandrol A and B demonstrated very limited activities.

Fourthly, we asked if these compounds could completely reverse the drug resistance of MDR cell lines. The IC_{50}s of DNR, vincristine (VCR), and paclitaxel toward Bcap37 were 44 ± 5, 7 ± 1, and 7 ± 4 (ng/ml), respectively. Schisandrin A and B and schisantherin A appear to be able to completely restore the drug sensitivities of Bcap37/Adr (Table 1). On the other hand, in the presence of schisandrin A or B or schisantherin A, the IC_{50}s of DNR toward MCF-7/Adr, although greatly reduced (Table 1), were yet significantly higher than that (22 ± 7 ng/ml) toward MCF-7. The differential reversal of drug resistance of Bcap37/Adr and MCF-7/Adr by these compounds is not surprising. Since Bcap37/Adr is an MDR cell line derived by transfection of Bcap37 with a

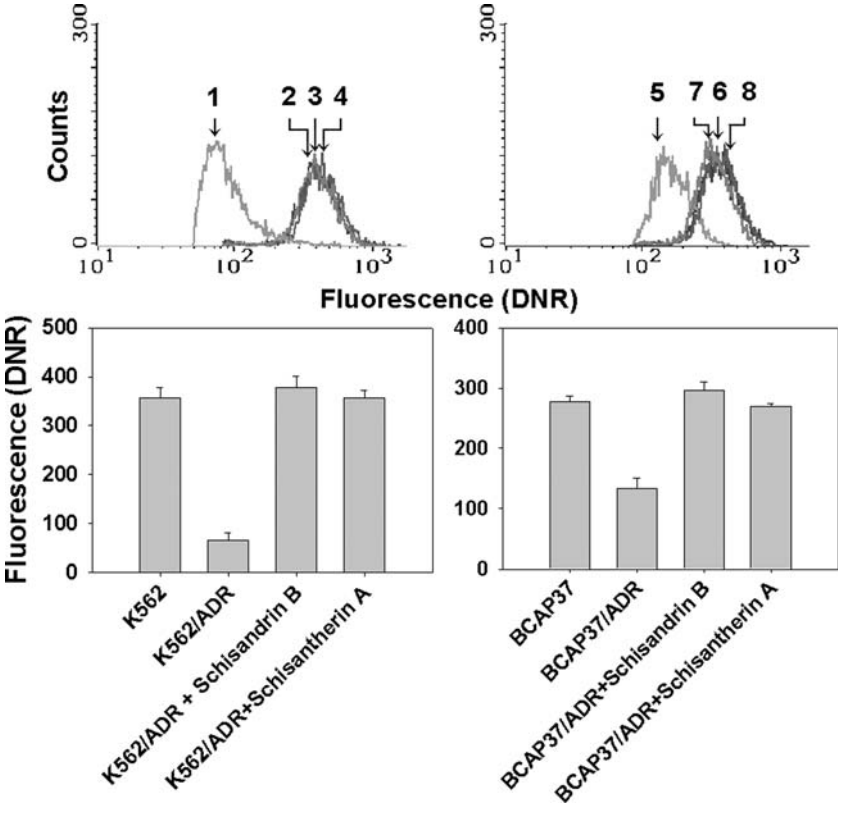

Fig. 4 The effects of schisandrin B and schisantherin A on DNR accumulation in K562/Adr and Bcap37/Adr and their parental cells. Cells were incubated with DNR (2 μg/ml) in the presence or absence of the indicated lingnans at 10 μg/ml for 60 min and collected for the determination of intracellular DNR as described in Materials and methods. *1* K562/Adr, *2* K562, *3* K562/adr + schisantherin A, *4* K562/adr + schisandrin B, *5* Bcap37/Adr, *6* Bcap37, *7* Bcap37/Adr + schisantherin A, *8* Bcap37/Adr + schisandrin B. Data are mean ± SD of three experiments

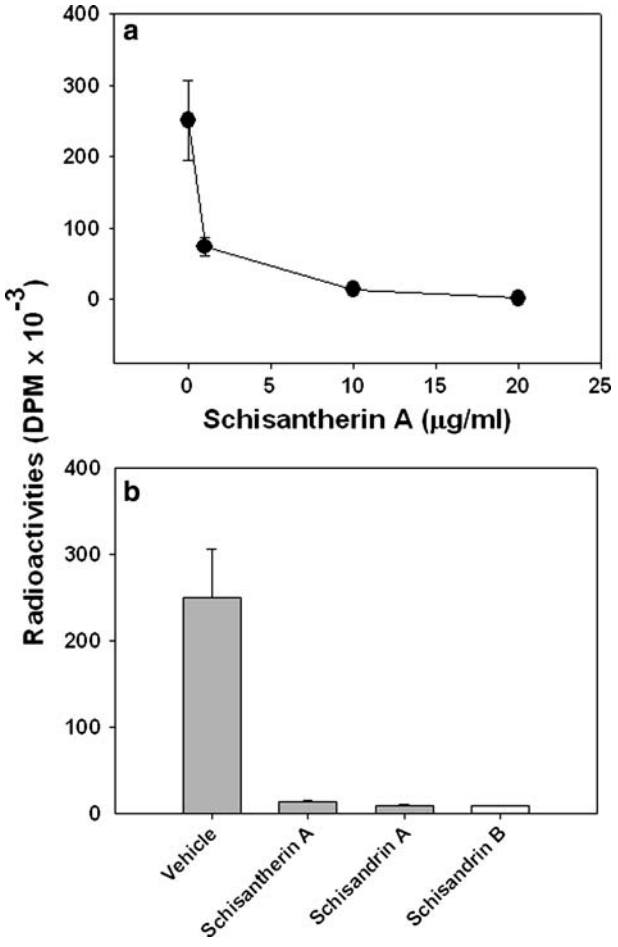

Fig. 5 Inhibition of photolabeling of P-gp with ^{3}H-azidopine by the indicated lingnans. **a** Dose dependent inhibition of photolabeling of P-gp with ^{3}H-azidopine by schisantherin A. **b** Inhibition of photolabeling of P-gp with ^{3}H-azidopine by the indicated lingnans at 10 μg/ml. Data are mean ± SD of three experiments

full-length MDR1 cDNA, its drug resistance, theoretically, is solely conferred by P-gp [10]. If these compounds function as P-gp inhibitors, they should completely reverse the drug resistance of this MDR cell line. In the case of MCF-7/Adr, which was derived by repeatedly exposing MCF-7 to an anticancer drug, more complex drug resistant mechanisms could be developed, although P-gp expression accounts for the major part. Therefore, the drug resistance of MCF-7/Adr could be overcome to the maximum by schisandrin A and B, and schisantherin A, but not fully.

Dibenzocyclootadiene lingnans differentially inhibit P-gp mediated drug efflux

K562/Adr and Bcap37/Adr were incubated with DNR in the presence or absence of dibenzocyclooctadiene compounds, to test their ability to affect the intracellular drug accumulation. The results showed that intracellular DNR accumulation in K562/Adr and Bcap37/Adr increased with an increase in the concentrations of these compounds, thereby representing a dose-dependent manner (Fig. 3). Schisandrin A and B, and schisantherin A displayed similar activities to increase the intracellular drug accumulation, which was much stronger than schisandrol A or B ($P < 0.05$). Therefore, the results obtained from assays of drug accumulation and drug resistance reversal are agreeable with each other, i.e., the compounds with stronger potency to increase the intracellular drug accumulation are also stronger to reverse drug resistance. To confirm if these compounds could fully restore DNR accumulation in MDR cells, we compared the DNR accumulation in MDR cells in the presence and absence of these compounds with that in their corresponding drug sensitive cells. As shown in Fig. 4, schisandrin B and schisantherin A at 10 μg/ml completely recover the DNR accumulation in K562/Adr and Bcap37/Adr cells. These results rationalize how these compounds reverse the drug resistance of MDR cancer cells.

Dibenzocyclooctadiene lingnans physically interact with P-gp

To test if these compounds physically interact with P-gp, we did the competitive inhibition of the photoaffinity labeling of P-gp with ^{3}H-azidopine by these compounds. The results showed a dose-dependent inhibition of ^{3}H-azidopin labeling of P-gp by schisantherin A (Fig. 5a). Schisandrin A and B and schisantherin A showed the comparable activities to inhibit ^{3}H-azidopin labeling of P-gp (Fig. 5b). The inhibitory efficiencies of the three lingnans at 10 μg/ml all exceeded 95% of the total labeling. These results proved the physical interaction of the tested compounds with P-gp.

Dibenzocyclooctadiene lingnans do not induce P-gp expression

In order to exclude the possibility that the dibenzocyclootadiene lingnans could induce the expression of P-gp, we examined the expression of P-gp in K562 cells pretreated with schisandrin A, schisandrin B, or schisantherin A. As shown in Fig. 6, the fluorescent peaks corresponding to P-gp in K562 cells pretreated with the dibenzocyclooctadienes were overlapped with the isotype control, and were the same as that of control K562 cells, indicating that these lingnans had no activities to induce P-gp. We did not test schisandrol A and B for their potential activities in the induction of P-gp because they lack the adequate efficacies for further development.

Dibenzocyclooctadiene lingnans represent a class of novel P-gp inhibitors

All the five compounds used in this study have a core structure of dibenzocyclooctadiene (Fig. 1). Although they have methoxyphenol group, which is also present in most of the P-gp inhibitors, the compounds do not contain any basic nitrogen atom which is the functional

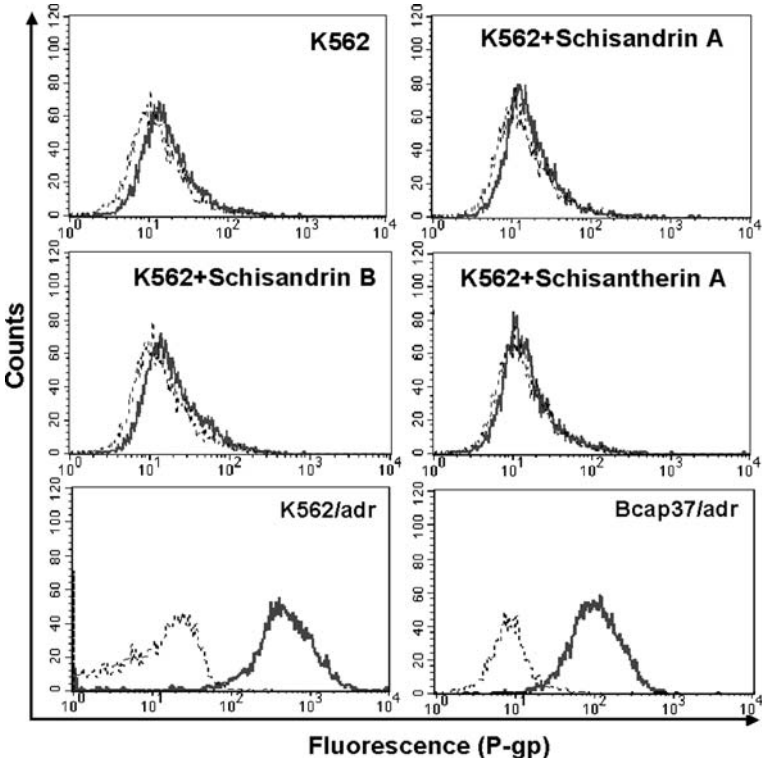

Fig. 6 Representative profiles of P-gp in K562 cells pretreated with schisandrin A, schisandrin B, or schisantherin A (10 μg/ml) for 15 days. *The solid line* P-gp; *the dotted line* isotype control

group associated with potency and present in most of the P-gp inhibitors [4, 5]. Because aromatic rings and nitrogen atoms are common features shared by the P-gp modulators, the five dibenzocyclooctadienes lacking the nitrogen atom, may represent a new class of P-gp inhibitor. The potency of the compound reduced significantly when a hydroxyl group was introduced into the cyclooctadiene ring (schisandrol B). Introducing the second hydroxyl group (schisandrol A) did not further weaken the activities of the molecule to inhibit P-gp. When the hydroxyl group is esterized to form a benzoate (schisantherin A), the potency is resumed, indicating that the hydroxyl and benzoic group were critically associated with the potency of the molecule.

Safety and potency are the two most important criteria to evaluate a drug. The five lingnans are the major components in Schisandra chinensis, which is listed in the Chinese Pharmacopoeia and indexed as a tonic and sedative. This herb has been widely used as a medication for the last several thousand years in China without reports of significant adverse-effects. Schisandrin B is a strong liver protective agent with very low toxicities. The dose of schisandrin B used to protect the liver against carbon tetrachloride was as high as 3 mmol/kg (1,200 mg/kg) in mice [12, 13]. The other data regarding the low toxicities related to these compounds are referred to in the review article [14].

As for the potency of schisandrin A, schisandrin B, and schisantherin A, our results proved that these compounds can fully restore the intracellular drug accumulation in four MDR cell lines, and overcome the P-gp mediated drug resistance. Taken together, the three lingnans are potent P-gp inhibitors with high safety and have the potential for future clinical application.

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