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Reversal of P-glycoprotein-mediated multidrug resistance by Alisol B 23-acetate

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Abstract

Herbal drugs were screened for their activity in reversing multidrug resistance (MDR) in P-glycoprotein (P-gp) over-expressing cancer cells. Through bio-assay guided fractionation an active compound was isolated from Rhizoma Alismatis, the underground part of *Alisma orientale* and the chemical structure of the isolate compound was confirmed by HPLC, LC-MS and NMR as Alisol B 23-acetate (ABA). ABA restored the sensitivity of MDR cell lines HepG2-DR and K562-DR to anti-tumor agents that have different modes of action but are all P-gp substrates. It restored the activity of vinblastine, a P-gp substrate, in causing G_2/M arrest in MDR cells. In a dose-dependent manner, ABA increased doxorubicin accumulation and slowed down the efflux of rhodamin-123 from MDR cells. ABA inhibited the photoaffinity labeling of P-gp by [^{125}I]iodoarylazidoprazosin and stimulated the ATPase activity of P-gp in a concentration-dependent manner, suggesting that it could be a transporter substrate for P-gp. In addition, ABA was also a partial non-competitive inhibitor of P-gp when verapamil was used as a substrate. Our results suggest that ABA may be a potential MDR reversal agent and could serve as a lead compound in the development of novel drugs.

Keywords: Alisol B 23-acetate; Multidrug resistance; P-glycoprotein; Chemosensitizer; Triterpene

1. Introduction

Much effort is being spent towards the identification of natural compounds that can reverse the multidrug resistance phenotype (MDR) in cancer cells and/or sensitize MDR cancer cells to chemotherapy without undesirable side effects. Medicinal herbs are excellent starting materials since they are not only rich sources of diverse chemicals but have been prescribed to humans and used for hundreds of years. We have started an extensive natural product drug discovery program involving initially the screening of herbal medicines for their ability to reverse MDR. Here we report the identification of Rhizoma Alismatis, the underground part of *A. orientale*, as one such herb. Bio-assay guided fractionation led to the isolation of Alisol B 23-acetate (ABA) and molecular and cellular studies led us to conclude that ABA is a novel P-gp modulator.

Intrinsic or acquired resistance of tumor cells to anticancer drugs is one of the major causes of failure of cancer chemotherapy. When acquired resistance occurs it often takes the form of MDR, i.e., the resistance would be against a spectrum of drugs with different structures and modes of action [1,2]. A number of cellular and molecular alterations may contribute to the development of the MDR phenotype and one that has been well studied is the over-expression of a 170-kDa P-glycoprotein (P-gp) which serves as a xenobiotics exporter [1]. Using ATP as the energy source, P-gp is known to facilitate the efflux of a broad range of cytotoxic drugs including anthracyclines, *Vinca* alkaloids, epipodophyllotoxins and tanxanes [2]. Agents that fully or partly block P-gp activity thus may prevent the undesirable loss of intracellular drugs and may have beneficial effects during chemotherapy.

A number of P-gp inhibitors or modulators have been described. Although many of these agents have been

Abbreviations: MDR, multidrug resistance; P-gp, P-glycoprotein; ABA, Alisol B 23-aceatate; Rh-123, rhodamine 123; CI, combination index; [^{125}I]IAAP, [^{125}I]iodoarylazidoprazosin

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found to overcome drug resistance in in vitro models, their in vivo uses have been disappointing [3,4]. The main reason for the failure is a low host tolerance to experimental MDR inhibitors that precluded the attainment of active intracellular levels [5,6]. For example full reversion of MDR by verapamil, a calcium channel blocker, requires a concentration of approximately 10 μM in vitro, whereas plasma levels above 1 μM result in atrioventricular blocks [7].

In this study cell growth inhibition assays were employed to identify plant extracts that could increase the cell-killing activity of doxorubicin in P-gp over-expressing MDR cells. We demonstrated for the first time that ABA inhibited P-glycoprotein activity and reversed P-gp-mediated MDR in vitro. It potentiated the cell killing effects of various anticancer drugs that are P-gp substrates, and reduced efflux of P-gp substrates in drug-resistant human HepG2-DR hepatoma and K562-DR leukemia cells. Furthermore, it was shown that ABA acted most likely through interacting with and inhibiting the P-gp.

2. Material and methods

2.1. Chemicals

Doxorubicin, vinblastine, puromycin, paclitaxel, verapamil, sulforhodamin B (SRB), MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetazolium bromide) and cell culture grade agarose were purchased from Sigma Chemicals. Actinomycin D was from Acros Organics. Propidium iodide (PI) and rhodamine 123 (Rh-123) were from Molecular Probes. [^{125}I]Iodoarylazidoprazosin (2200 Ci/mmol) was from Perkin-Elmer. RPMI-1640, fetal bovine serum and antibotic-antimycotic were from GibcoBRL. Nitrocellulose membranes and secondary antibody (horseradish-peroxidase-conjugated anti-rabbit IgG) were from Bio-Rad, and the anti-P-gp antibody was from Calbiochem.

2.2. Cells and cultures

Human HepG2 hepatoma and K562 leukemia cells and their multidrug-resistant, P-gp over-expressing, sublines HepG2-DR (maintained in 1.2 μM doxorubicin) and K562-DR (maintained in 0.1 μM doxorubicin), were kindly supplied by Judy Chan, Chinese University of Hong Kong [8]. Cells were maintained at 37 °C in 5% CO_2 in RPMI-1640 medium supplemented with 10% fetal bovine serum and 100 units/mL antibotic-antimycotic.

2.3. Bio-assay guided isolation of Alisol B 23-acetate

Plant extracts, fractions and purified compounds were monitored for their biological activity by an in vitro growth-inhibition assay. Powdered Rhizoma Alismatis(1 kg) was extracted with 95% ethanol twice at room temperature. The extracts were combined and ethanol was removed by a rotary evaporator under reduced pressure. Chloroform extract of the residue was fractionated on a silica gel column eluted with a discontinuous gradient of petroleum ether/ethyl acetate (10:2, 10:5, 2:10). The bioactive fractions were pooled and by thin layer chromatography (TLC) (silica gel-60, benzene/acetone, 3:1) was shown to contain a major spot with several minor components. This fraction was repeatedly purified on a silica gel column eluted with benzene/ethyl acetate (4:1) until one the major TLC spot remained. After removing the solvent, the residue was crystallized in *n*-hexane/benzene to give a white powder (130 mg) that was confirmed to be Alisol B 23-acetate by HPLC, LC-MS and NMR analyses.

2.4. In vitro growth inhibition assay

The growth inhibitory effects of drugs were estimated in adhered HepG2 and HepG2-DR cells by sulforhodamine B (SRB) cytotoxicity assay [9], and in suspended K562 and K562-DR cells by tetrazolium-based colorimetric MTT assay [10] in 96-well plates. Each experiment was independently performed at least three times.

2.5. Drug interactions

To detect synergism or antagonism a modified method of Chou and Talalay (1984) was used [10,11]. HepG2 and HepG2-DR cells were seeded into 96-well plates at 5×10^3 cells per well and cultured overnight. The medium was replaced with fresh medium containing ABA mixed with cancer drugs at the ratio of 1:1. Cells were further incubated for 72 h and the cytotoxic effect was analyzed by SRB assay. Combination index (CI) of combined drug effect was determined and the CI-isobologram was constructed [12]. In the presents study a CI greater than 4 was taken as antagonism and a CI smaller than 0.8 as synergism [13]. Effects of verapamil were also studied in parallel as a positive control. All experiments were repeated at least three times.

2.6. Cell cycle analysis

Approximately 1×10^6 cells in 5 mL of complete growth medium were treated with vinblastine (300 nM) alone or in combination with various concentrations of ABA for 24 h. Cells were harvested, washed twice with iced-cold phosphate buffered saline (PBS) and fixed in 70% ethanol at −20 °C overnight. Cells were washed with PBS and incubated with 200 μg/mL RNase at 37 °C for 30 min. PI solution was added at a final concentration of 40 μg/mL and analysis was performed immediately using a FACSCAN flow cytometer.

2.7. Estimation of cellular Rh-123 and doxorubicin by flow cytometry

Rh-123 retention assay was performed as described previously [14]. Rh-123 was added to 1×10^6 cells in 5 mL complete growth medium to a final concentration of 5 μg/mL and cells were incubated at 37 °C for 1 h to allow Rh-123 uptake. Rh-123 loaded cells were washed with ice-cold PBS, resuspended in fresh medium with or without ABA and further incubated for 1 h at 37 °C to allow Rh-123 efflux. Florescence of cellular Rh-123 was examined by a FACSCAN flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA) and data were analyzed with the Macintosh CellQuest software.

For doxorubicin accumulation assay, 1×10^6 cells in 5 mL of complete growth medium were incubated with 10 μM doxorubicin in combination with various concentrations of ABA at 37 °C for 1 h. After incubation, the amount of cellular doxorubicin was estimated by fluorescence flow cytometry [15]. Verapamil, a known P-gp inhibitor, was also studied as a positive control.

2.8. Western blot analysis of P-glycoprotein expression

HepG2 and HepG2-DR cells were treated with 5 μM ABA for up to 72 h and then incubated in ice-cold lysis buffer (50 mM Tris pH 7.4, 100 mM NaCl, 2 mM EDTA, 1% sodium deoxycholate, 0.1% SDS, 1% Triton X-100, 2 mM PMSF, 1% aprotinin) for 30 min. Protein concentration was determined by Bradford assay and samples containing 50 μg of protein were subjected to 6% SDS–PAGE and electro-transferred to nitrocellulose membranes. Membranes were blocked with 3% non-fat milk/0.05% Tween-20/TBS (10 mM Tris pH 7.5, 100 mM NaCl), incubated with anti-P-gp antibody for 1 h at room temperature followed by horseradish-peroxidase-conjugated secondary antibody for another 1 h at room temperature. Protein bands were detected by the ECL method.

2.9. Membrane vesicle preparation

Vesicles were prepared from cells by nitrogen cavitation as previously described [16]. Briefly, cells were grown to confluence in 24 cm × 24 cm dishes. Cell monolayers (10^9–10^{10} cells) were washed and scraped into DPBS (see above) containing 1% aprotinin (Sigma), washed and collected by centrifugation (4000 × *g*, 10 min) in DPBS and then in vesicle buffer (0.01 M Tris–HCl, pH 7.5, 0.25 M sucrose, 0.2 mM $CaCl_2$). Cells resuspended in vesicle buffer were equilibrated under 800 psi nitrogen at 4 °C for 15 min and cavitated twice. Under these conditions, greater than 95% of the cells were lysed. EDTA (1 mM) was added and the homogenate was diluted 1/4 with 0.01 M Tris–HCl, pH 7.5, 0.025 M sucrose and centrifuged at 1000 × *g* for 10 min to remove nuclei and unlysed cells. The supernatant was centrifuged for 30 min at 16,000 × *g* on a 35% sucrose cushion (0.01 M Tris–HCl, pH 7.5, 35% sucrose, 1 mM EDTA). The interface was collected and diluted 1/5 in 0.01 M Tris–HCl, pH 7.5, 0.25 M sucrose, then centrifuged for 45 min at 100,000 × *g*. The vesicle pellet was resuspended in 0.01 M Tris–HCl, pH 7.5, 0.25 M sucrose using a 25-gauge needle and stored at −80 °C.

2.10. Photoaffinity labeling of P-gp

Plasma membranes from HepG2 and HepG2-DR cells were photo-labeling with [^{125}I]IAAP (2200 Ci/mmol) as described previously [17] and effects of ABA and verapamil were studied. The labeled membrane samples were analyzed by 8% SDS–PAGE. Gels were dried and exposed to Kodak (Rochester, NY) X-AR films for 18–24 h at −80 °C. The quantitative radiographic imaging data was analyzed by Fluor-S™ MultiImager System (Bio-Rad, USA) using Multi-Analyst® software (Bio-Rad, USA).

2.11. ATP hydrolysis

P-gp ATPase activity was measured in a 96-well plate format and based on phosphate-release catalyzed by membrane microsome preparations with all other major membrane ATPases inhibited [18]. Microsomes were thawed on ice and diluted to 3.5 μg protein per well in ice-cold ATPase buffer [sodium ATP, 3 mM; KCl, 10 mM; $MgSO_4$, 10 mM; DTT, 3 mM; Tris–HCl, 50 mM (pH 7.0)] containing 0.5 mM EGTA (to inhibit Ca-ATPase), 0.5 mM ouabain (to inhibit the Na/K-ATPase), and 3 mM sodium azide (to inhibit the mitochondrial ATPase). The total incubation volume was 100 μl and the reaction was initiated by bringing plates to 37 °C and lasted for 30 min. The reaction was terminated by the addition of 50 μl of 12% SDS solution, followed by the addition of 50 μl of a 1:1 mixture of 18% fresh ascorbic acid in 1N HCl and 3% ammonium molybdate in 1N HCl. After 4 min, 100 μl of a fixing solution (2% sodium citrate and 2% sodium meta-arsenite in 2% acetic acid) was added and incubated for 30 min. OD at 750 nm was obtained by a microplate reader. Respective background values for the assay were subtracted and the amount of phosphate released, and hence ATP consumed, was obtained by comparing to a standard curve. Water-insoluble drugs were dissolved in ethanol and the maximum ethanol concentration in the reaction mixture (2% v/v) was shown not to affect the ATPase activity.

2.12. Soft-agar colony formation assay

Assays were performed in 35-mm dishes containing a 3-mL underlayer (0.6% agar in RPMI-1640 medium and 10% FBS). Samples of 1×10^4 cells suspended in 2 mL of drug-containing 0.3% agar-medium were then plated onto the underlayers. Plates were incubated at 37 °C in humidified 5% CO_2 atmosphere for 2 weeks.

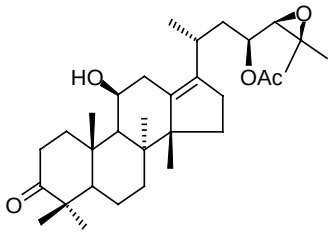


Fig. 1. Chemical structure of Alisol B 23-acetate.

3. Results

3.1. Identification of Alisol B 23-acetate

The NMR spectral data of our crystal was identical with those of Alisol B 23-acetate [19] (Fig. 1). The characteristic spectra data of our crystallized compound are as follow: Pos. FABMS *m/z*: 537 (M + Na), 515 (M + H). 1H NMR δ ($CDCl_3$, ppm): 4.61 (1H, td, $J = 8.8$, 2.4 Hz, 23-H), 3.81 (1H, td, $J = 10.8$, 5.6 Hz, 11-aH), 2.74 (1H, d, $J = 8.8$ Hz, 24-H), 2.55 (1H, dd, $J = 13.2$, 5.6 Hz, 12-eH), 2.16 (1H, ddd), 2.08 (3H, s, CH_3CO), 1.72 (1H, d, $J = 10.8$ Hz, 9-aH), 1.34, 1.32, 1.15, 1.08, 1.06, 1.05, 0.98 (3H each, s, CH_3), 1.03 (3H, d, $J = 6.8$ Hz, 21-CH_3). Table 1 listed all the ^1H and ^{13}C chemical shift data of our crystal in $CDCl_3$. The molecular weight of our crystallized compound as determined by LC/MS was 514 which was identical to Alisol B 23-acetate.

3.2. Modulation of drug resistance in MDR cell lines

The reversal of vinblastine resistance by ABA in MDR cells was shown in Fig. 2. The two MDR cell lines showed much higher tolerance to vinblastine compared to their parental, drug-sensitive cell lines (Fig. 2A). In MDR cells low concentrations (≈1 μM) of ABA significantly reversed resistance to vinblastine (100 and 300 nM) and a complete reversal could be observed at 10 μM ABA (Fig. 2B and C).

The effects of ABA on the actions of several clinically relevant drugs were also examined in the drug-resistant HepG2-DR cells and the effects were compared with that of verapamil. As summarized in Table 2, ABA significantly increased the sensitivity of HepG2-DR cells to actinomycin D, puromycin, paclitaxel and doxorubicin. For example the IC_{50} value of puromycin in MDR cells was lowered by ABA to a value comparable to that of puromycin alone in drug sensitive cells. The IC_{50} values of actinomycin D, paclitaxel and doxorubicin were lowered by 10- to about 40-folds. Compared to verapamil, ABA exhibited a greater reversal effect.

The effect of Alisol B 23-actate as a chemosensitizer was further assessed by the combination index (CI). In the drug-resistant cells strong synergism (CI < 0.8) was observed when ABA was added to actinomycin D, puromycin, paclitaxel or doxorubicin.

The above drug enhancement effects of ABA were not observed in the drug sensitive HepG2 cells (Table 2). In addition, the cytotoxicity of 5′-fluorouracil and cisplatin that are not P-gp substrates, in MDR cells was not affected by ABA. These results strongly suggested that ABA acts by interfering the P-gp activity.

At the concentrations tested, ABA showed a relatively low in vitro toxicity to both HepG2 and HepG2-DR cells ($IC_{50} \approx 19$ and 20 μM, respectively, Table 2). In the presence of verapamil, the cytotoxicity of ABA was enhanced ($IC_{50} \approx 4.7$ μM) and these two compounds showed a synergistic interaction.

3.3. Restoration of specific action of vinblastine in MDR cells

If ABA's main action is to maintain the intracellular concentration of cancer drugs, it should also restore the

Table 1
^1H and ^{13}C chemical shift data in $CDC1_3$ (all values in ppm and coupling constants in Hz)

N	C	H	R	*N*	C	H	R
1	30.9		CH_2	17	134.1		C
2	36.7		CH_2	18	23.1		CH_3
3	220.08		C	19	25.6		CH_3
4	46.9		C	20	27.8		CH
5	48.42		CH	21	20.0	1.06 (3H, d)	CH_3
6	19.98		CH_2	22	33.7		CH_2
7	34.13		CH_2	23	71.4	4.61 (1H, td)	CH
8	40.71		C	24	65.04	2.74 (1H, d)	CH
9	49.92	1.72 (1H, d)	CH	25	58.42		C
10	36.91		C	26	19.36		CH_3
11	70.17	3.81 (1H, td)	CH	27	24.66		CH_3
12	34.38		CH_2	28	29.52		CH_3
13	138.08		C	29	20.06		CH_3
14	57.00		C	30	23.79		CH_3
15	30.62		CH_2		169.93	2.08 (3H, s)	CH_3CO
16	29.13		CH_2		21.15		

s: singlet; *N*: C numbering in this system; d: doublet; C: ^{13}C chemical shift data; t: triplet; H: ^1H chemical shift data.

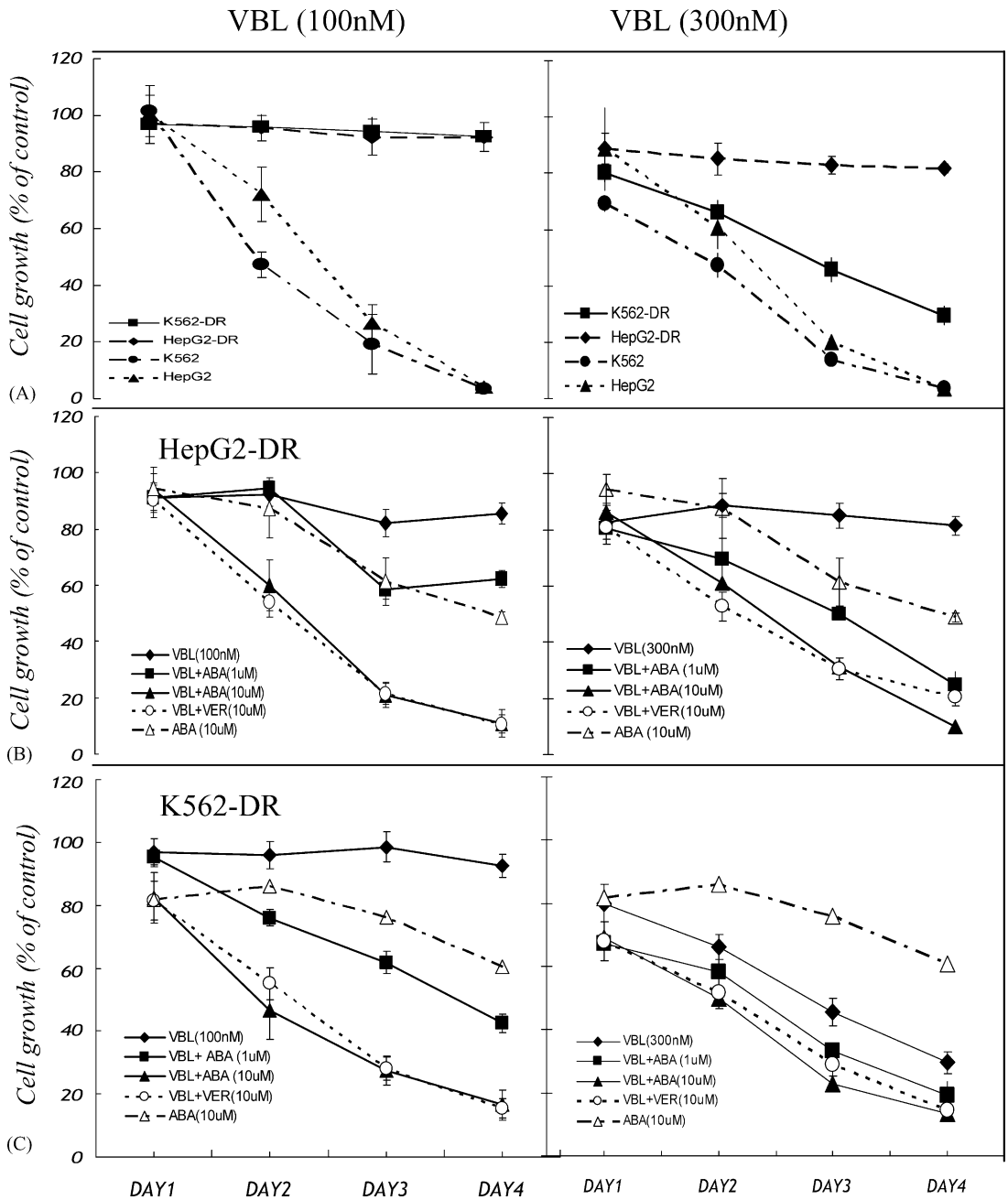


Fig. 2. Alisol B 23-acetate enhanced vinblastine action in drug-resistant cells. Cells were treated with vinblastine (VBL) for 96 h in the presence and absence of Alisol B 23-acetate (ABA) and verapamil (VER) and cell growth was estimated by MTT assay. Data were collected from three separate experiments and S.D. are indicated by vertical bars. (A) Effect of vinblastine alone. (B) Effects of ABA and VER on VBL-treated HepG2-DR cell. (C) Effects of ABA and VER on VBL-treated K562-DR cells.

original action of respective drugs. This was demonstrated by the effect on the specific action of vinblastine. Vinblastine is a microtubule inhibitor which impedes mitotic spindle formation, induce G_2/M arrest and apoptosis [20]. After a 24 h exposure to 300 nM vinblastine, over 86% of drug sensitive HepG2 cells were in the G_2/M phase but only 25% of the P-gp over-expressing HepG2-DR cells were in the G_2/M phase (Fig. 3). With the addition of 5 μM Alisol B 23-aceatate to the vinblastine treatment, MDR cells arrested in the G_2/M phase were increased to 68%. A comparable effect was achieved by adding 10 μM verapamil to vinblastine treatment. ABA alone, at concentrations as high as 25 μM, has no noticeable effect on cell cycle distribution. Similar results were observed in the K562/K562-DR cells (data not shown).

3.4. Doxorubicin accumulation and Rh-123 efflux in MDR cells

The drug (doxorubicin) accumulation assay generated information on the final and total result after drug exposure, while the (Rh-123) efflux assay offers quantitative

Table 2
Effects of MDR modulators on the cytotoxicity of anti-tumor agents in HepG2 and HepG2-DR cells

	IC_{50}[a] (μM)		CI[b]	
	HepG2	HepG2-DR	HepG2	HepG2-DR
Actinomycin D	0.002 ± 0.0004	4.06 ± 0.55		
With ABA (1:1)	0.002 ± 0.0003	0.31 ± 0.18	0.99 < CI < 1.01 (A)	0.03 < CI < 0.29 (S)
With verapamil (1:1)	0.002 ± 0.0008	0.51 ± 0.10	0.79 < CI < 1.06 (A)	0.03 < CI < 1.72 (S)
Puromycin	0.35 ± 0.03	281 ± 36		
With ABA (1:1)	0.24 ± 0.10	1.93 ± 0.74	0.87 < CI < 1.08 (A)	0.04 < CI < 0.39 (S)
With verapamil (1:1)	0.290 ± 0.061	5.26 ± 1.60	0.58 < CI < 1.44 (A)	0.05 < CI < 0.96 (S)
Paclitaxel	0.003 ± 0.001	4.80 ± 1.10		
With ABA (1:1)	0.003 ± 0.002	0.60 ± 0.09	0.90 < CI < 1.76 (A)	0.11 < CI < 0.60 (S)
With verapamil (1:1)	0.002 ± 0.001	0.86 ± 0.28	0.70 < CI < 1.20 (A)	0.14 < CI < 0.77 (S)
Doxorubicin	0.055 ± 0.016	46 ± 10		
With ABA (1:1)	0.053 ± 0.019	1.18 ± 0.25	0.78 < CI < 0.96 (A)	0.13 < CI < 0.43 (S)
With verapamil (1:1)	0.046 ± 0.018	1.58 ± 0.25	0.80 < CI < 1.02 (A)	0.06 < CI < 0.66 (S)
5′-Fluorouracil	6.95 ± 4.90	5.00 ± 3.20		
With ABA (1:1)	N.D.	5.18 ± 0.22	N.D.	0.19 < CI < 7.95 (A)
With verapamil (1:1)	N.D.	5.16 ± 1.76	N.D.	0.57 < CI < 5.09 (A)
Cisplatin	0.90 ± 0.21	0.22 ± 0.01		
With ABA (1:1)	N.D.	0.27 ± 0.02	N.D.	1.20 < CI < 1.65 (A)
With verapamil (1:1)	N.D.	0.23 ± 0.03	N.D.	0.97 < CI < 1.54 (A)
Alisol B 23-acetate (μM)	18.69 ± 3.56	19.44 ± 3.86		
With verapamil (1:1)	N.D.	4.27 ± 0.49	N.D.	0.14 < CI < 0.90 (S)
Verapamil (μM)	19.63 ± 6.39	25.91 ± 5.39		

HepG2 and HepG2-DR cells were exposed to drugs for 72 h. Values are means ± S.D. of three experiments. S: synergism; A: additive; N.D.: not determined.
[a] IC_{50} was drug concentration causing 50% growth inhibition and were determined by SRB assay.
[b] CI (combination index) shows the interaction relationship between modulators and chemotherapeutic agents. $CI = (C_c)_A/(C_a)_A + (C_c)_B/(C_a)_B$, where C_c is the concentration of agent A (or B) in the mixture to achieve a certain cell inhibition rate (fa); C_a is the concentration of agent A (or B) alone to require a same cell inhibition rate. When CI is greater than 4, it indicates antagonism; when it is smaller than 0.8, it indicates synergism and in the care of additive, the CI is between 0.8 and 4.

assessment on the outward transport activity. In drug accumulation assay cells were incubated with 10 μM doxorubicin alone or in combinations with various concentrations of P-gp modulator for 1 h at 37 °C and cellular doxorubicin was estimated by flow cytometry. As shown in Fig. 4A and B, drug-resistant K562-DR and HepG2-DR cells accumulated more doxorubicin in the presence of ABA or verapamil.

The effect of ABA on Rh-123 extrusion in drug-resistant HepG2-DR and K562-DR cells was illustrated in Fig. 5A and B. First there was no extensive decrease of Rh-123 in both parental, drug-sensitive cell lines. On the other hand, dramatic decreases in cellular fluorescence were observed in both MDR, P-gp over-expressing, cell lines, presumably due to an active P-gp-mediated outward transport of Rh-123. The outward movement of Rh-123 was noticeably decreased in the presence of ABA in a does-dependent manner, whereas no such effect was observed in both sensitive cell lines.

The inhibitory effect of ABA on the outward transport of Rh-123 in K562-DR cells was quantitatively assessed by applying simple Michaelis–Menten kinetics (Fig. 6):

$$i = \frac{I_{max} S}{K_m + S}$$

where I_{max}, S and K_m represent the maximal inhibition, concentration of inhibitor and Michaelis–Menten constant (equivalent to IC_{50} in this case), respectively. It was calculated from these data that $K_m \approx 6$ μM, i.e., the IC_{50} of ABA on Rh-123 transport in drug-resistant K562-DR cells was ≈6 μM. As a result of the inhibition on Rh-123 transport by ABA, after the first hour five times more Rh-123 remained associated with MDR cells.

3.5. Lack of an effect of Alisol B 23-acetate on MDR1 expression

HepG2-DR expressed a high level of P-gp compared to their parental cells [8]. MDR1 expression in HepG2 and HepG2-DR cells was studied by Western blot and no alteration on the expression level of MDR1 was detected in HepG2-DR cells treated with 5 μM ABA for 72 h (Fig. 7).

3.6. Interaction of Alisol B 23-acetate with P-gp: photoaffinity labeling with [^{125}I]IAAP and ATPase activity

Our data suggested that ABA inhibited the P-gp transport function, the following experiments were performed

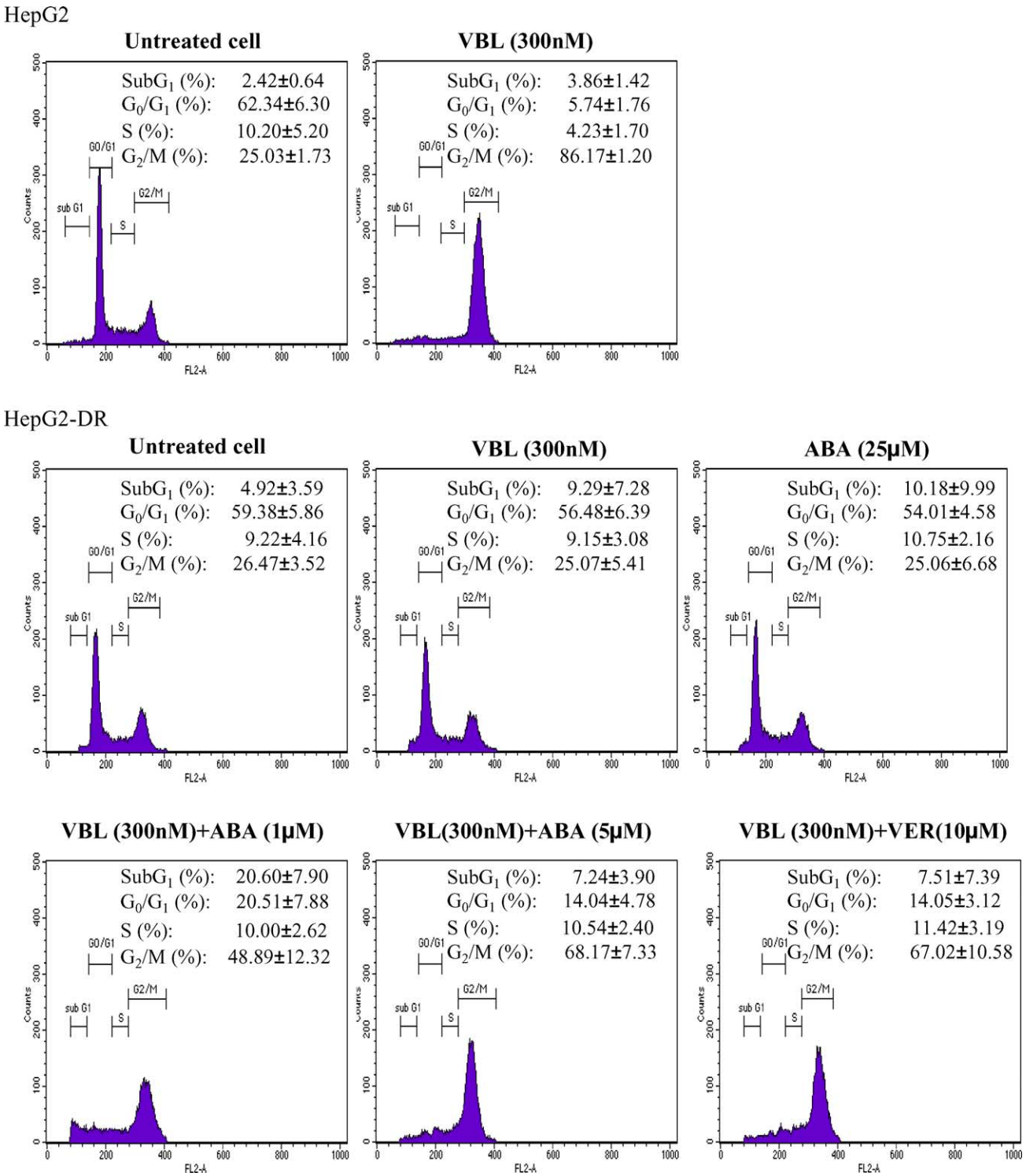


Fig. 3. Alisol B 23-acetate restored vinblastine-induced G_2/M arrest in drug-resistant HepG2-DR cells. Cells were incubated with vinblastine (VBL) with and without the addition of Alisol B 23-acetate (ABA) or verapamil (VER) for 24 h. Flow cytometry results of one typical experiment are shown. Numerical data are means ± S.D. of three independent experiments.

to verify whether there is any physical interaction between ABA and P-gp. Photoaffinity labeling of P-gp by [^{125}I]IAAP, an analog of prazosin, are reduced by P-gp substrates and substances that directly interact with P-gp [21]. As shown in Fig. 8, the binding of IAAP to P-gp was noticeably inhibited by ABA in a dose-dependent manner. At 100 μM, ABA completely inhibited IAAP labeling of P-gp.

As ATP was consumed during the transport event, the measurement of the ATP hydrolysis is a good indicator of transport activity [21]. We studied ATP hydrolysis rate catalyzed by membrane vesicle prepared from P-gp

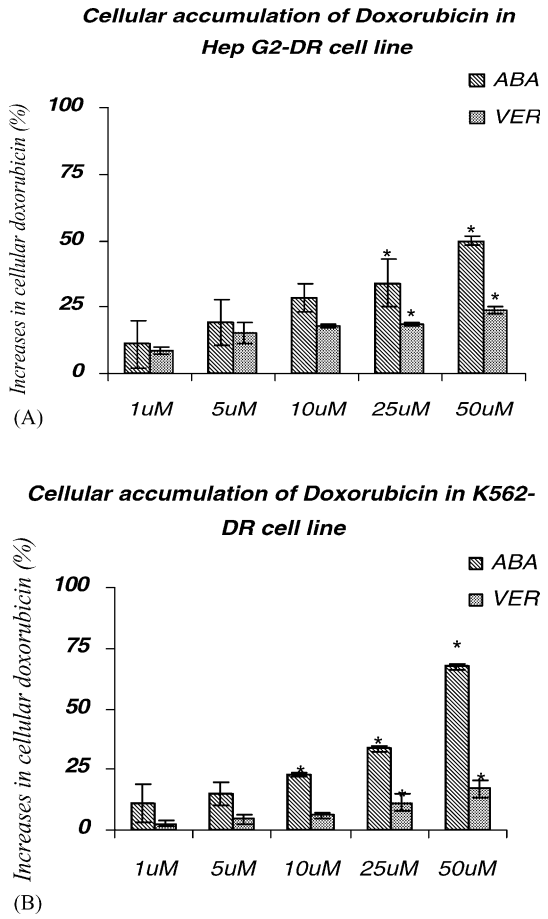


Fig. 4. Alisol B 23-acetate increased doxorubicin accumulation in drug-resistant cells. HepG2-DR (A) and K562-DR (B) cells were incubated with 10 μM doxorubicin with or without the addition of ABA or VER for 1 h and cellular fluorescence of doxorubicin was estimated by flow cytometry. Effects of ABA or VER are expressed as percent over doxorubicin-treated control and shown as means ± S.D. of three independent experiments. Significantly different at $^{*}P < 0.05$ compared with the doxorubicin control.

over-expressing HepG2-DR cells while suppressing the activities of other major membrane ATPases. As shown in Fig. 9A, ATPase activity was increased by the addition of ABA to the reaction mixture in a does-dependent manner and the reaction kinetics fitted well with the Michaelis–Menten pattern with a $K_m \approx 3.79$ μM. In addition, ABA can inhibit verapamil-stimulated ATPase activity in a concentration-dependent manner (Fig. 9B), where V_{max} was gradually decreased but the K_m was relatively constant with increasing ABA.

3.7. Effects of Alisol B 23-acetate on colony-forming ability of HepG2 and HepG2-DR cells

Soft agar colony formation assay measures cancer cell survival in a chronic cytotoxicity environment. As shown in Fig. 10, ABA at 5 μM had no noticeable effect on colony formation. At 100 nM, doxorubicin or vinblastine reduced drug sensitive HepG2 colonies to 13 and 0.28% of untreated control, respectively. Under the same conditions, drug-resistant HepG2-DR cells survived better and the numbers of colonies was only reduced to 60 and 38%, respectively. With the addition of 5 μM ABA or 10 μM verapamil, doxorubicin-treated resistant HepG2-DR colonies were reduced from 60% to below 30% and from 38 to 0.3% in vinblastine-treated cells.

4. Discussion

Through bio-assay guided screening and fractionation, we isolated ABA from Rhizoma Alismatis and demonstrated for the first time that this natural compound can inhibit the P-glycoprotein function and reverse the P-gp-mediated MDR in vitro. The MDR reversal activity of ABA was evaluated by several assays in two human MDR tumor cell lines over-expressing P-gp and their respective drug-sensitive maternal cell lines. The drug-resistant HepG2-DR, originally derived from HepG2, is resistant to a broad spectrum of anti-cancer drugs (Table 2) primarily due to the over-expression of P-gp on the cell membrane [8]. The over-expression of P-gp in HepG2-DR was confirmed in our laboratory (Fig. 7). The study on the human leukemia cells K562 (drug-sensitive) and K562-DR (P-gp over-expressing and MDR) was include to ascertain whether ABA's action is cell type specific. And verapamil, a well known reversal agent for P-glycoprotein-mediated MDR [6] was included in this study as a positive control.

As demonstrated by the cell growth inhibitory assay, 1–10 μM ABA can significantly reverse the resistance of HepG2-DR and K562-DR cells to vinblastine (Fig. 2). ABA could also potentiate the cytotoxicity of several other clinically relevant chemotherapeutic drugs, such as puromycin in HepG2-DR cells. Analysis of drug interaction between ABA and these chemotherapeutic agents using drug combination index (CI), showed a strong synergism in each pair of the combinations between Alisol B 23-acetate and tested anti-cancer drugs (Table 2). Moreover, co-treatment of ABA and vinblastine could almost completely restore the ability of vinblastine to induce G_2/M arrest in resistant cell lines (Fig. 3). In soft agar colony formation assay, 100 nM of vinblastine was sufficient to inhibit colony formation by MDR cells (Fig. 10) in the presence of 5 μM ABA. These data sufficiently demonstrate a potent restoration of drug activity by ABA in resistant cancer cell lines.

The action of Alisol B 23-acetate is highly specific against P-gp related functions. First there was no synergistic effect between ABA and non-P-glycoprotein substrate such as cisplatin and 5′-fluorouracil [22]. Second ABA did not affected actions of anti-cancer drugs in drug-sensitive parental cell lines (Table 2). In addition, ABA was only mildly toxic to HepG2 and HepG2-DR ($IC_{50} = 18.7$ and 19.4 μM, respectively) (Table 2), and had no significant effect on cell cycle distribution (Fig. 3), although it

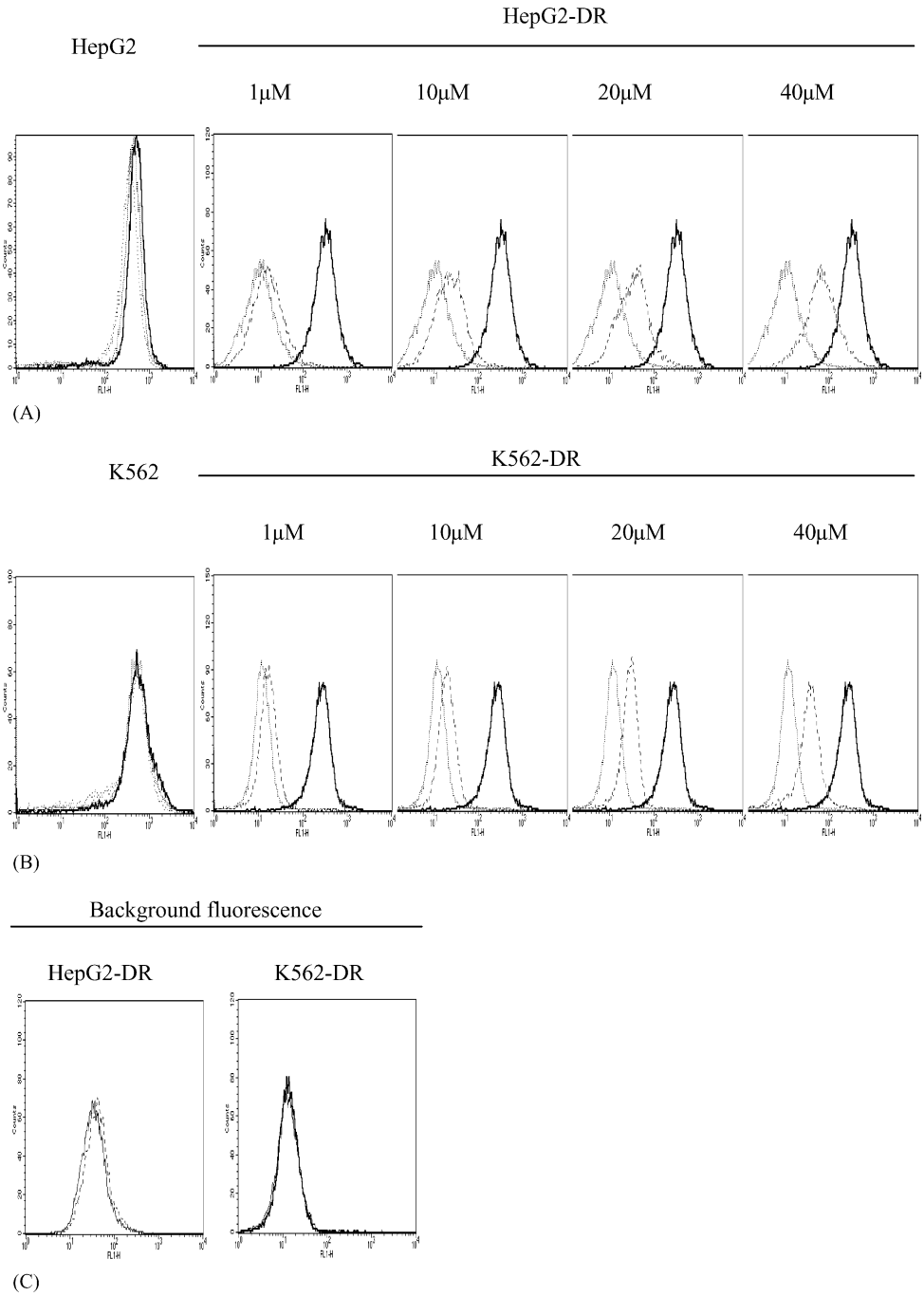


Fig. 5. Alisol B 23-acetate decreased the Rh-123 loss in drug-resistant cells. ''Loaded'' and ''residual'' Rh-123 levels were estimated in cells incubated with Rh-123 (5 μg/mL) for 1 h and in cells incubated for an additional hour in Rh-123 free medium, respectively. Cellular Rh-123 fluorescence was analyzed by flow cytometry. (A) HepG2 and HepG2-DR cells; (B) K562 and K562-DR cells. *Heavy solid line*: Rh-123 loaded cells. *Light solid line*: residual fluorescence. *Dashed line*: residual fluorescence in the presence of ABA at the concentrations indicated. (C) Background fluorescence of 20 μM ABA (dashed line) and unlabeled control cells (solid line).

was previously found to be able to induce apoptosis in vascular smooth muscle cells and lymphocytes [23] and showed some toxicity against SK-OV3, B16-F10, and HT1080 cells [24].

The activity of P-gp can be studied by measuring the transportation of P-gp substrates. In this study, we showed that in P-gp over-expressing MDR cells ABA significantly increased the accumulation of doxorubicin and blocked the efflux of Rh-123 ($IC_{50} \approx 6$ μM in K562-DR) (Figs. 4 and 5). However, these were not observed in drug sensitive parental cell lines. Thus it is conceivable that non-specific effects, such as changes in membrane fluidity, are not major contributing factors in the action of Alisol B 23-aceatate, for otherwise both drug-sensitive and MDR P-gp over-expressing cells should be affected the same.

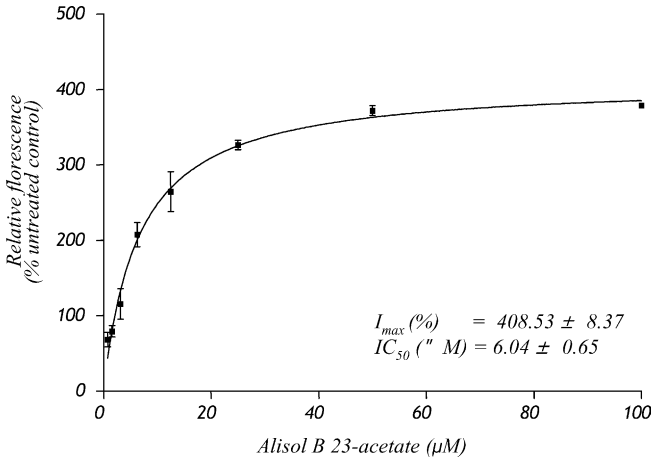


Fig. 6. Inhibitory effect of Alisol B 23-acetate on Rh-123 loss in K562-DR cells. The inhibitory effect of ABA is expressed as relative florescence in percent of untreated control. Kinetic parameters were calculated by the Michaelis–Menten equation: $i = I_{max}S/(K_m + S)$ as described in the text. Data points are mean ± S.D. of three experiments.

To test our hypothesis that ABA could exhibit its inhibitory activity on P-gp function by interfering the binding of P-gp with its substrate, we performed a photo-affinity label study with [^{125}I]IAAP in HepG2-DR cell membrane vesicles which contains enriched P-glycoprotein. Under the conditions as described in the material and methods, [^{125}I]IAAP will bind with P-gp, moving together with this protein when subjected to non-denature gel electrophoresis [17]. But when ABA was included into the reaction in series concentrations, [^{125}I]IAAP displacement from P-gp occurred in a concentration-dependent manner indicating that ABA had interfered with the binding of [^{125}I]IAAP to P-gp. This may be the result of direct interaction between ABA with P-glycoprotein. To confirm this, an ATP hydrolysis study showed that ABA served as a substrate of P-gp with a K_m at about 3.8 μM.

Previous studies have suggested a multi-site model for the binding of P-gp modulators and that the binding sites in the P-gp for cytotoxic drugs (e.g. vinblastine) are distinctly different from those for reversal agents (e.g. verapamil) [25]. We have shown that ABA inhibited the verapamil-stimulated ATPase activity in a dose-dependent manner, with a gradually decreasing V_{max} and a relatively constant K_m, a typical pattern for non-competitive enzyme inhibition [26]. Co-treatment of ABA and verapamil (a P-gp substrate and inhibitor) reduced the IC_{50} of the former from 20 to 5 μM (Table 2), this is in line with the suggestion that ABA is a P-gp substrate and may interact with P-gp. Furthermore, both ABA and verapamil inhibited the binding of IAAP to P-gp (Fig. 8). IAAP covalently binds to possibly three sites on P-gy: amino acids 248–312 (within TM4–TM5), 750–800 (beyond TM8) and 1160–1218

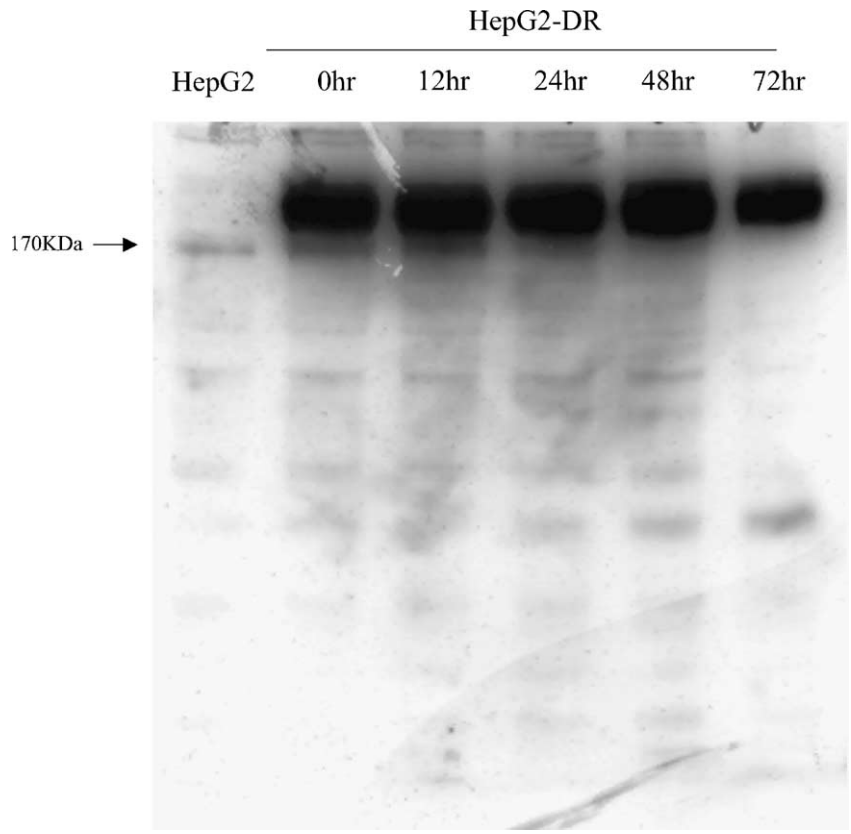


Fig. 7. Alisol B 23-acetate did not affect P-gp expression. Drug-resistant HepG2-DR cells were treated with 5 μM ABA for the times indicated and samples containing 50 μg of total cellular protein was analyzed by 6% SDS–PAGE. P-gp was detected with rabbit anti-P-gp antibody. The sample in lane 1 was from untreated HepG2 cells.

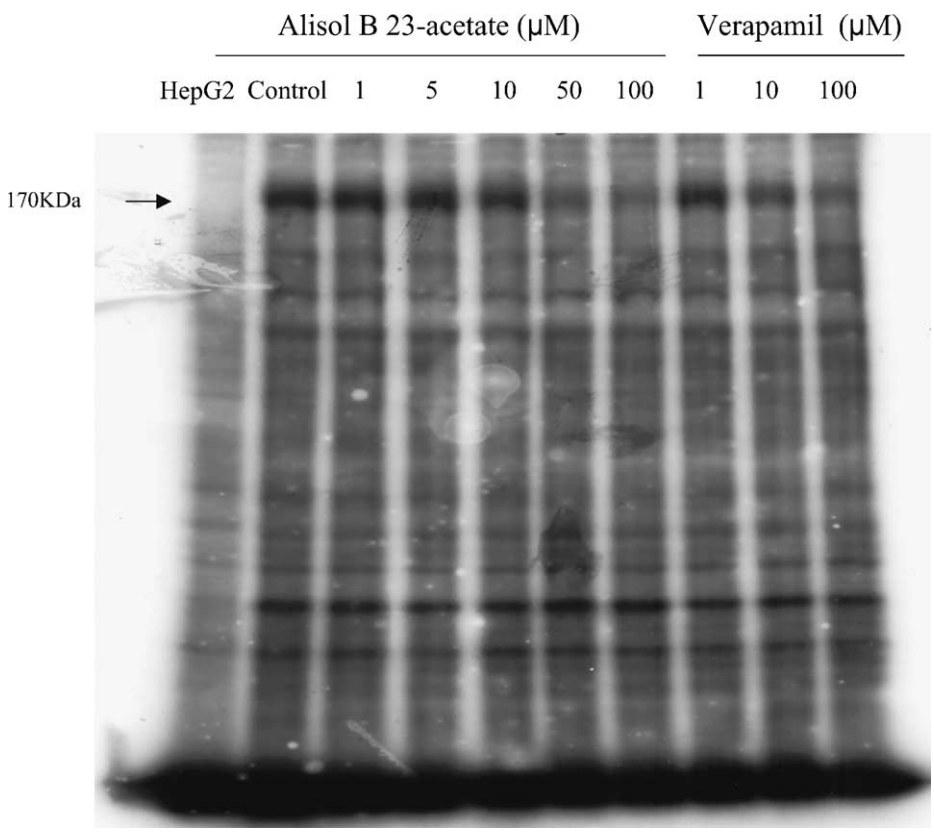
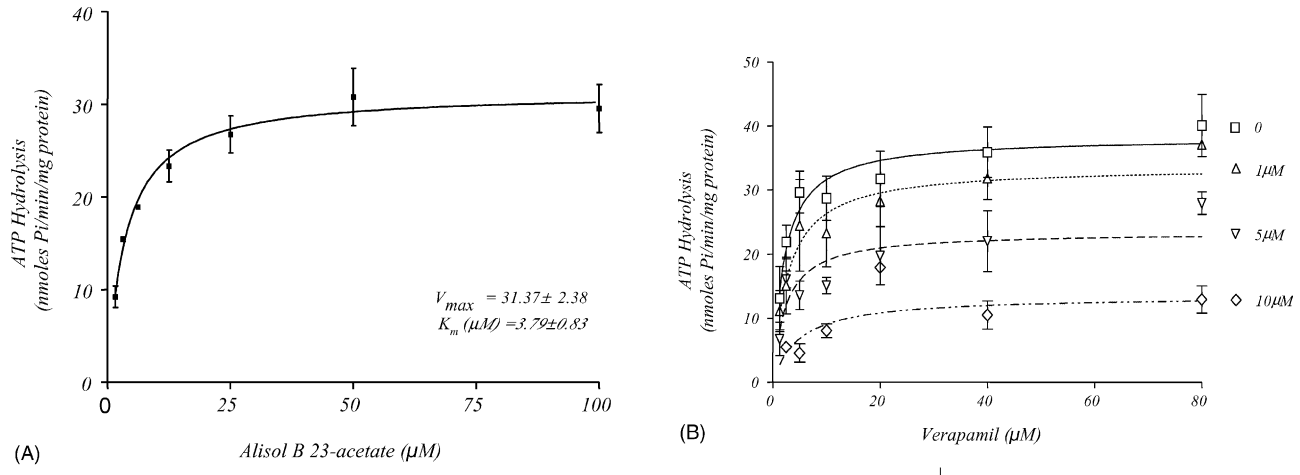


Fig. 8. Alisol B 23-acetate decreased [^{125}I]IAAP photoaffinity labeling of membrane preparations. Membrane preparations containing 50 μg proteins were pretreated with Alisol B 23-acetate or verapamil for 10 min at room temperature and were labeled with 30 nM IAAP. Lane 1: untreated drug sensitive HepG2 cells. Lane 2–10: drug-resistant, P-gp over-expressing, HepG2-DR cells treated by ABA or VER at concentration indicated.



ABA	I_{max}	K_m (μM)
0	38.2±1.49	2.08±0.38
1μM	33.67±1.79	2.80±0.62
5μM	23.42±1.51	2.35±0.77
10μM	13.45±1.58	3.00±2.14

Fig. 9. Effect of Alisol B 23-acetate on basal and verapamil-stimulated ATPase activity of P-gp. Membrane preparations were obtained from drug-resistant, P-gp over-expressing, HepG2-DR cells and ATPase activity was measured with the other major membrane ATPases inhibited. (A) Alisol B 23-acetate stimulated ATPase activity. (B) Effect of Alisol B 23-acetate on verapamil stimulated ATPase activity. The Michaelis–Menten parameters were means ± S.D. of three independent experiments.

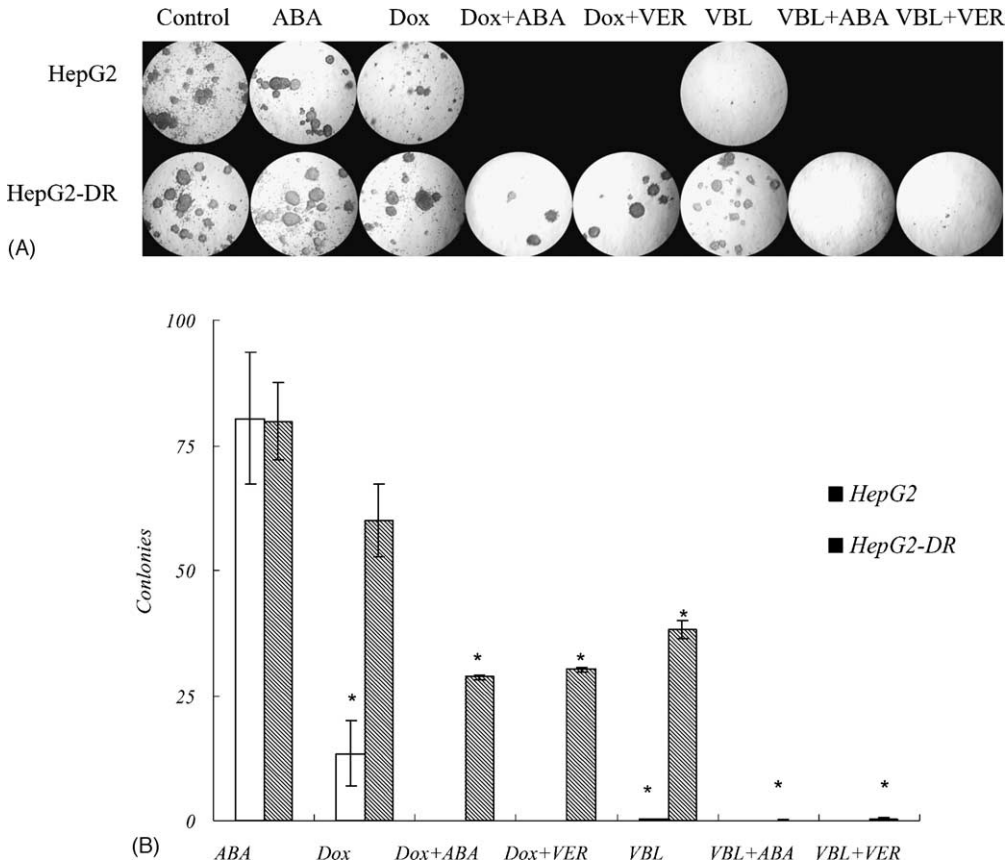


Fig. 10. Effects of Alisol B 23-acetate on colony-forming ability of HepG2 and HepG2-DR cells. (A) Pictures from a typical experiment showing colonies formed in untreated control plate, and plates treated by 5 μM Alisol B 23-acetate (ABA), 100 nM doxorubicin (Dox), 100 nM vinblastine (VBL) or their combinations as shown. (B) Relative colony numbers (percent of untreated control) from three independent experiments are shown as means ± S.D. Significantly different to the untreated control group at $^{*}P < 0.05$.

(within the second cytosolic NBD) [27]. Collectively our results suggest that verapamil and ABA are able to bind simultaneously probably to distinct but interacting sites on the P-gp.

It has been suggested that planar aromatic rings are common features shared by all P-gp modulators [28]. ABA is a member of triterpene compounds and has a common steroid-like structure with four planar aromatic rings. Additional studies to elucidate other structural factors contributed to the reversing effect of ABA on P-gp-mediated MDR are currently underway. The physicochemical properties, such as lipophilicity of ABA are also needed to be clarified [29].

Reduction of the expression of P-gp at either the transcriptional or protein level has been suggested to be one of the mechanisms for certain modulators or agents to reverse MDR phenotype [30]. Since incubation of HepG2-DR cells with 5 μM Alisol B 23-aceate up to 72 h did not significantly alter the protein level of P-gp, it is unlikely that ABA reverses MDR at either the transcriptional or the protein level.

In conclusion, we have provided evidence here showing that ABA is a modulator of P-gp-mediated MDR in vitro and could be considered as a potential lead compound for the design of more efficacious MDR chemosensitizers. Animal experiments to test its efficacy in vivo are now in progress.

Acknowledgments

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