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Gomisin A alters substrate interaction and reverses P-glycoprotein-mediated multidrug resistance in HepG2-DR cells

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Abbreviations:
[^{125}I]IAAP, [^{125}I]iodoarylazidoprazosin
MDR, multidrug resistance
Pgp, P-glycoprotein
PI, propidium iodide
Rh-123, rhodamine-123
SDS-PAGE, sodium doedecyl sulphate-polyacrylamide gel electrophoresis
SRB, sulforhodamine B

ABSTRACT

Through an extensive herbal drug screening program, we found that gomisin A, a dibenzocyclooctadiene compound isolated from *Schisandra chinensis*, reversed multidrug resistance (MDR) in in Pgp-overexpressing HepG2-DR cells. Gomisin A was relatively non-toxic but without altering Pgp expression, it restored the cytotoxic actions of anticancer drugs such as vinblastine and doxorubicin that are Pgp substrates but may act by different mechanisms. Several lines of evidence suggest that gomisin A alters Pgp-substrate interaction but itself is neither a Pgp substrate nor competitive inhibitor. (1) First unlike Pgp substrates gomisin A inhibited the basal Pgp-associated ATPase (Pgp-ATPase) activity. (2) The cytotoxicity of gomisin A was not affected by Pgp competitive inhibitors such as verapamil. (3) Gomisin A acted as an uncompetitive inhibitor for Pgp-ATPase activity stimulated by the transport substrates verapamil and progesterone. (4) On the inhibition of rhodamine-123 efflux the effects of gomisin A and the competitive inhibitor verapamil were additive, so were the effects of gomisin A and the ATPase inhibitor vanadate. (5) Binding of transport substrates with Pgp would result in a Pgp conformational change favoring UIC-2 antibody reactivity but gomisin A impeded UIC-2 binding. (6) Photocrosslinking of Pgp with its transport substrate [^{125}I]iodoarylazidoprazosin was inhibited by gomisin A in a concentration-dependent manner. Taken together our results suggest that gomisin A may bind to Pgp simultaneously with substrates and alters Pgp-substrate interaction.

1. Introduction

Multidrug resistance (MDR) is defined as the simultaneous resistance to various structurally and functionally unrelated drugs and is believed to be one of the major obstacles of successful cancer chemotherapy [1]. MDR can be resulted from molecular alterations in drug targets, apoptotic pathways, drug metabolizing enzymes and expression of drug transporters [2].

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One of the better-understood mechanisms is the over-expression of the membrane P-glycoprotein (Pgp), an ATP-dependent xenobiotic exporter that causes a reduced cellular retention of drugs. Pgp is a 170-kDa ATP binding cassette (ABC) family protein that acts on a wide range of compounds including various chemotherapeutic agents such as anthracyclines, *Vinca* alkaloids, epipodophyllotoxins and tanxanes [1–3]. Pgp up-regulation is often found in patients with cancer relapse after chemotherapy and in cultured cells that become drug resistant after stepwise selection by resistance to chemotherapeutic agents [4].

There is an intense interest in the identification of compounds that specifically block the function of Pgp. Verapamil and cyclosporine A are the typical examples of the first generation MDR modulators that unfortunately have limited clinical usefulness mainly due to their severe side-effects and toxicity. Subsequently, a number of second and third generation modulators such as R-verapamil [5], PSC833 (a cyclosporin analog) [6], GF120918 [7], XR9576 [8,9] and LY335979 [10,11] have been discovered and are being studied for their clinical efficacy.

At the present time the three-dimensional atomic structure of Pgp has not been resolved therefore the identification of new Pgp inhibitors relies mainly on screening. It is generally accepted that there are least three substrate binding sites and one allosteric site on Pgp, which are independent but interacting to each other [12]. A large portion of reported modulators mainly reversibly interact with one or more of the three presumed substrate binding sites and thus are competitive inhibitors. Recently, there has been a report that thioxanthene derivative *cis*-(*Z*)-flupentixol inhibits drug transport by an allosteric mechanism [13].

Medicinal herbs are rich sources of compounds with diverse chemical structures and are abundantly used in treating human diseases. In recent years, herbs are being used increasingly in conjunction with chemotherapeutic agents, particularly in terminally ill patients, with beneficial outcomes. We have been screening Chinese medicinal herbs traditionally used in treating cancers for their complementarity to chemotherapeutic agents. In particular, we are interested in the identification of compounds that may modulate Pgp-mediated multidrug resistance (MDR) and in agents that induce cell differentiation. Successful examples are the isolation of alisol B 23-acetate from *Alisma orientale* as a Pgp inhibitor [14] and honokial from *Magolia officinalis* as an inhibitor of NF-κB and inducer of cancer cell differentiation [15,16].

Gomisin A is a small molecular weight lignan present in the Chinese medicine Fructus Schisandrae, the dried seed of *Schisandra chinensis* that is widely used as a health food product. The LD_{50} of gomisin A in ICR mice by p.o. and s.c. administrations were 878 and 855 mg/kg, respectively [17], indicated that gomisin A is relatively non-toxic. Previous studies have shown that gomisin A protected CCl_4- and acetaminophen-induced hepatotoxicity and glutamate-induced oxidative neuronal damage [18,19]. Gomisin A has also been shown to inhibit TPA-induced tumor formation in animals [17]. In this report, we demonstrate that gomisin A reverses Pgp mediated MDR by altering Pgp-substrate interaction.

2. Materials and methods

2.1. Chemicals and antibodies

Doxorubicin, vinblastine, taxol, 5-fluoruracil, verapamil, sulforhodamine B (SRB), propidium iodide (PI), rhodamine-123 (Rh-123) and cell culture grade agarose were purchased from Sigma Chemicals, U.S. Materials for cell culture were purchased from Invitrogen. Anti-Pgp antibodies clone c-494 and UIC-2 were purchased from Calbiochem and Immunotech, respectively. Anti-β-tubulin, Anti-mouse IgG_{2a}-PE and normal IgG_{2a} were obtained from Santa Cruz Biotechnology.

2.2. Cell cultures

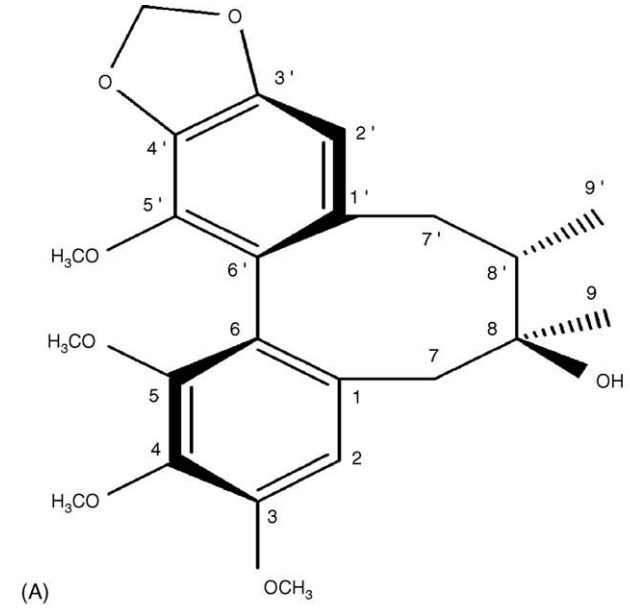
Human HepG2 hepatic carcinoma and its multidrug resistant, Pgp overexpressing subline HepG2-DR were kindly provided by Prof. K.P. Fung. The Chinese University of Hong Kong. Cells were maintained at 37 °C in 5% CO_2 in RPMI-1640 medium containing antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin), 10% fetal bovine serum and 1.2 μM doxorubicin.

2.3. Bio-assay guided isolation of gomisin A

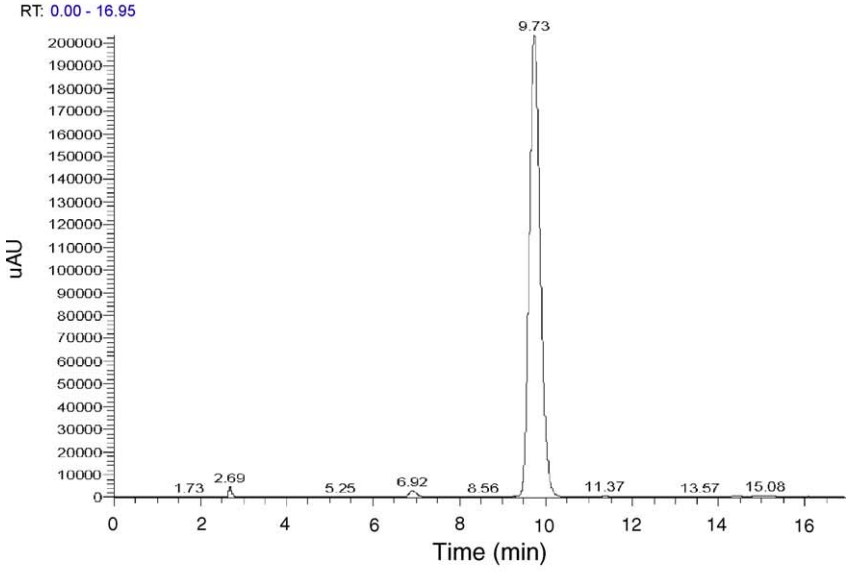
Biological activity of the plant extracts, fractions and purified compounds were monitored by in vitro growth inhibition assay and drug retention assay. Powdered Fructus Schisandrae (the dried fruit of *S. chinensis*) was extracted with 95% ethanol twice at room temperature. The extracts were combined and ethanol was removed by a rotary evaporator under reduced pressure. The residue was first separated by solvent extraction using petroleum ether and water and the petroleum ether portion was separated on a silica gel column eluted with a discontinuous gradient of petroleum ether (PE):ethyl acetate (EA) = 10:0, 10:1 and 2:1. The bioactivity was found in the PE:EA = 2:1 fraction. This fraction was further purified on a silica gel column eluted with a continuous gradient of PE/EA. Fractions were checked by HPLC and combined if they contained the same compounds. Gomisin A was isolated from one of three combined fractions by reverse phase HPLC. The chemical structure of gomisin A was verified by LC-MS and NMR analysis (Fig. 1A) and its purity was determined by HPLC (Fig. 1B). The solid form of gomisin A was dissolved in 50% DMSO and 50% ethanol to make a 10 mg/ml (24 mM) stock that was freshly diluted in culture medium in all experiments described below.

2.4. In vitro growth inhibition assay

Cell number was estimated by sulforhodamine B (SRB) protein assay and cell growth under chronic toxicity was assessed by soft-agar colony formation assay. In SRB assay, 5×10^5 cells were seeded in 96-well plates. After 18 h various concentrations of chemotherapeutic agents were added together with gomisin A. The plates were further incubated for 72 h and cell number estimated as described. In soft-agar colony formation assay, tests were performed in 35-mm dishes containing 2 ml underlayer (0.6% agar in RPMI-1640 medium and 10% FBS). Cell suspensions (1×10^4 cells per 1.5 ml) in drug-containing 0.3%



(A)



Apex RT	Start RT	End RT	Area	%Area	Height	%Height
6.92	6.64	7.29	41050.05	1.12	2988.82	1.45
9.73	9.11	10.48	3621940.60	98.88	203446.15	98.55

(B)

Fig. 1 – Identification of gomisin A. (A) Structure of gomisin A. m.w. = 416. (B) HPLC analysis showed that the isolated compound has a purity >98%.

agar-medium were then plated onto the underlayer. Plates were incubated at 37 °C in humidified 5% CO_2 atmosphere for 2 weeks. Numbers of colony formed were quantified by Gel Doc 2000 System (BioRad, CA).

2.5. *Cell cycle analysis*

Approximately 5×10^5 cells in 2 ml of complete growth medium were treated with 5 ng/ml (for drug sensitive HepG2 cells) or 300 ng/ml (fro drug resistant HepG2-DR cells) vinblastine either alone or in combination with gomisin A for 24 h. Cells were harvested, washed twice with ice-cold phosphate buffered saline (PBS) and fixed in 70% ethanol at −20 °C overnight. Cells were washed with PBS, incubated with 100 μg/ml RNase A at 37 °C for 30 min, stained with 25 μg/ml propidium iodide solutions and analyzed with a FACSCalibur flow cytometer (Becton Dickinson, CA).

2.6. *Estimation of cellular Rh-123 by flow cytometry*

Rhodamine-123 (Rh-123) is a fluorescent Pgp substrate that is frequently employed to evaluate Pgp activity [20,21]. Rh-123

was added to 8×10^5 cells in 1 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, re-suspended in fresh medium with or without gomisin A and further incubated for 1 h at 37 °C to allow Rh-123 efflux. Fluorescence of intracellular Rh-123 was examined by flow cytometry and data were analyzed with the Macintosh CellQuest software.

2.7. Western blot analysis

Cells were treated with various concentrations of gomisin A for 24 and 48 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, 1 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml pepstatin A, 2 mM sodium orthovanadate, Na_3VO_4). After 30 min of incubation on ice, lysates were centrifuged at 10,000 × *g* for 20 min at 4 °C and supernatants were collected and stored at −80 °C. Protein concentration was determined using Lowry assay and samples containing 50 μg of proteins were loaded and separated by sodium dodecyl sulphate-polyacrylaminde gel electrophoresis (SDS-PAGE). Protein bands were transferred to nitrocellulose membranes (BioRad, CA), blocked for 30 min at room temperature with 5% non-fat milk in PBS and incubated with specified primary antibodies at 4 °C overnight. After washing in PBS containing 0.05% Tween-20, membranes were incubated with HRP-conjugated secondary antibody (Zymed Laboratories, CA) and protein bands were visualized using a chemiluminescence system (Amersham, NJ). β-tubulin was used as loading control.

2.8. RT-PCR analysis

RT-PCR was performed to evaluate the mRNA expression level of Pgp after gomisin A treatment. Total RNA was isolated by SV Total RNA Isolation kit (Promega, WI) according to the manufacture's protocol, and the quality of RNA was checked by optical density measurement with $A_{260}/A_{280} > 1.8$. One microgram total RNA was subjected to a RT reaction using random oligonucleotide primers and M-MLV reverse transcriptase (Promega, WI). RT reaction product was then amplified by PCR using Taq DNA polymerase under the following conditions: 30 cycles of 94 °C for 2 min, 60 °C for 1 min, and 72 °C for 1 min. The PCR primers were: 5′-GCCTGGCAGCTGGAAGACAAATACACAAAATT-3′ and 5′-CAGACAGCAGCTGACAGTCCAAGAACAGGACT-3′ for Pgp (285 bp); 5′-GATGATATCGCCGCGCTCGTCGTCGAC-3′, and 5′-AGCCAGGTCCAGACGCAGGATGGCATG-3′ for β-actin (538 bp). PCR products were run on a 1% agarose gel and visualized by ethidium bromide staining.

2.9. Determination of Pgp-associated ATPase activity

Membrane vesicle of HepG2-DR cells were prepared by nitrogen cavitation as previously described [14]. ATPase activity was measured in a 96-well plate format based on inorganic phosphate-release catalyzed by membrane vesicle preparations with all other major membrane ATPases inhibited [22,23]. Briefly, drugs were diluted in ATPase buffer (50 mM Tris pH 7.4, 50 mM KCl, 2.5 mM $MgSO_4$, 3 mM DTT, with 0.5 mM EGTA to inhibit Ca-ATPase, 3 mM sodium azide to inhibit the mitochondrial ATPase, and 2 mM ouabain to inhibit the Na/K-ATPase) and added to each well. Membrane vesicles were thawed and diluted, 10 μg proteins were added per well. The plates were incubated at 37 °C for 5 min to activate Pgp. The reaction was initiated by adding 4 mM MgATP and the plates were incubated at 37 °C for 30 min. Reaction was terminated by adding 200 μl detecting reagent (35 mM ammonium molybdate: 10% ascorbic acid = 4:1, pH 5.0). The plates were further incubated at 37 °C for 20 min for color development. Absorbance at 750 nm was determined using ELISA reader. Vanadate sensitive ATPase activities were analyzed using non-linear regression analysis (GraphPad Prism Version 2.0).

2.10. UIC-2 reactivity shift assay

The UIC-2 shift assay was performed as described [13] with minor modifications. Monolayer cells were harvested, washed, and re-suspended in UIC-2 binding buffer (1 × PBS, 1% BSA). Approximately 1×10^6 cells were pre-warmed at 37 °C for 10 min, incubated with drugs at 37 °C for another 10 min, and 1 μg of the monoclonal antibody UIC-2 was added. After 10 min at 37 °C, 1 ml of ice-cold UIC-2 buffer was added to stop the reaction. Cell samples were washed twice, re-suspended in 500 μl ice-cold UIC-2 buffer and 2 ml of goat anti-mouse IgG_{2a}-PE was added. After 15 min at 4 °C in the dark, samples were washed, re-suspended in 1 ml ice-cold UIC-2 binding buffer and analyzed by flow cytometer. The fluorescence intensity associated with the cells was expressed on a log scale. Normal mouse IgG_{2a} served as a negative control.

2.11. Baculovirus mediated expression of human Pgp and photoaffinity labeling of Pgp with [^{125}I]IAAP

Recombinant baculoviruses harboring either the wild type BV-MDR1-(H6) [24] or the F983A mutant BV-MDR1-F983A-(H6)[25] of human MDR1 cDNA, with 6 × His-tag at the C-terminal end, were used to infect High Five insect cells grown in serum free Excell 400 medium as described [26]. Cells were grown to 80% confluency at 27 °C, transfected with the recombinant baculovirus with multiplicity of infection of 10 and harvested after 72 h. Crude membranes were prepared and photoaffinity labeled with the Pgp substrate [^{125}I]IAAP following an established protocol [27]. Briefly, membrane preparations (3 μg protein) expressing mutant or wild-type human Pgp were incubated with 5 nM [^{125}I]IAAP in 10 mM Tris–HCl (pH 7.5), 50 mM NaCl, 300 mM mannitol and 1% aprotinin (labeling buffer) at room temperature for 10 min under subdued light. After exposure to UV at 365 nm (General Electric F15T8-BLB) for 30 min at room temperature, 5× SDS-PAGE sample buffer was added to the reaction mixture and samples were held at room temperature (21–23 °C) for another 30 min and mixed well by vortexing before analyzed by SDS-PAGE. Whenever indicated, membranes were pre-incubated with gomisin A for 5 min prior to the addition of [^{125}I]IAAP and photocrosslinking.

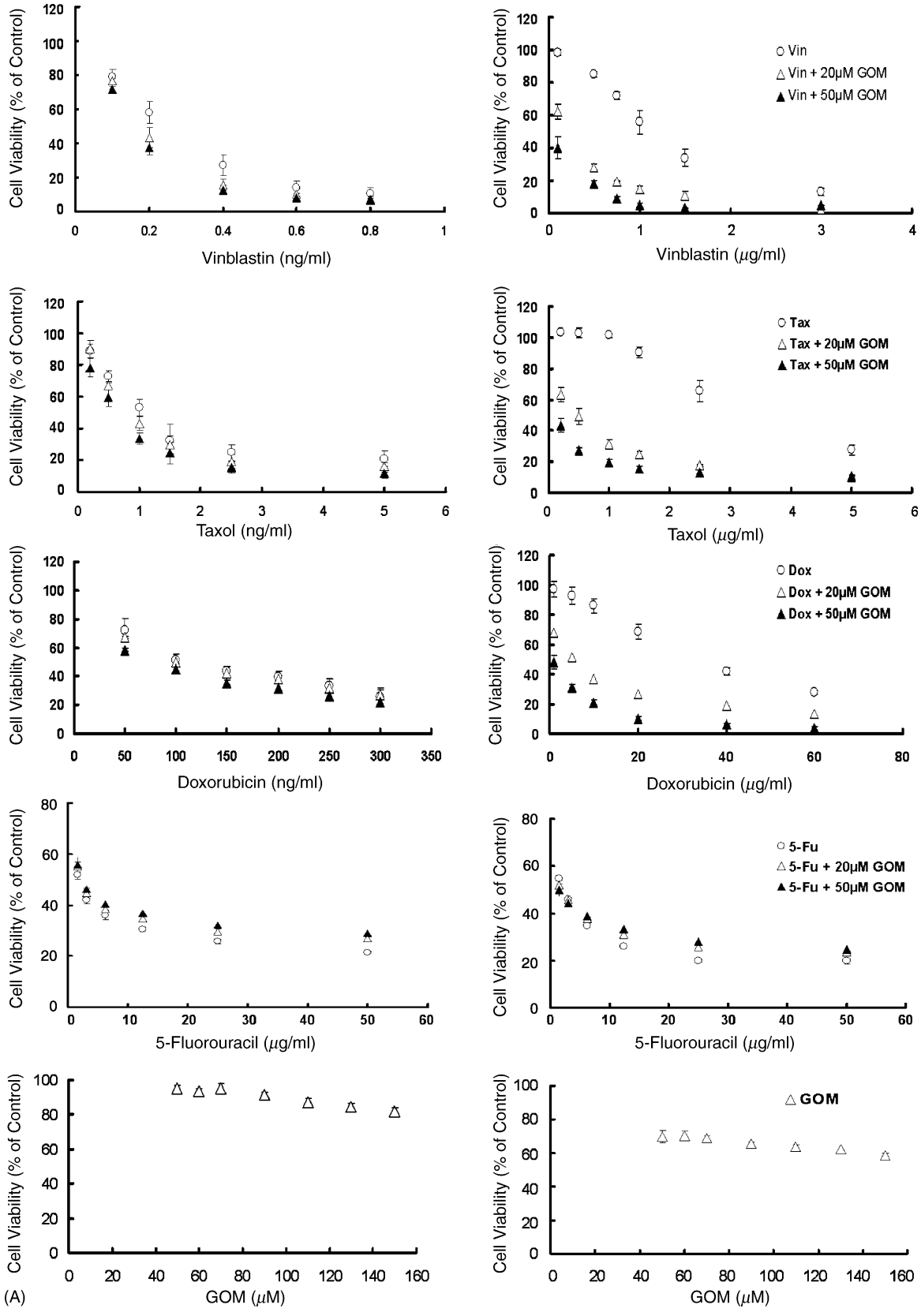


Fig. 2 – Gomisin A enhanced the cytotoxic effects of various Pgp substrates in HepG2-DR (Right) but not in HepG2 (Left) cells. (A) Cells were treated for 72 h with gomisin A (GOM) alone (bottom panels) or vinblastine (Vin), taxol (Tax), doxorubicin (Dox) and 5-fluorouracil (5-FU) in the presence and absence of 20 and 50 μM gomisin A (GOM). (B) The cytotoxic effect of gomisin A in HepG2-DR cells in the present of 10 and 20 μM verapamil. Cell growth was estimated by SRB assay and data are expressed as mean ± S.D. of three independent experiments.

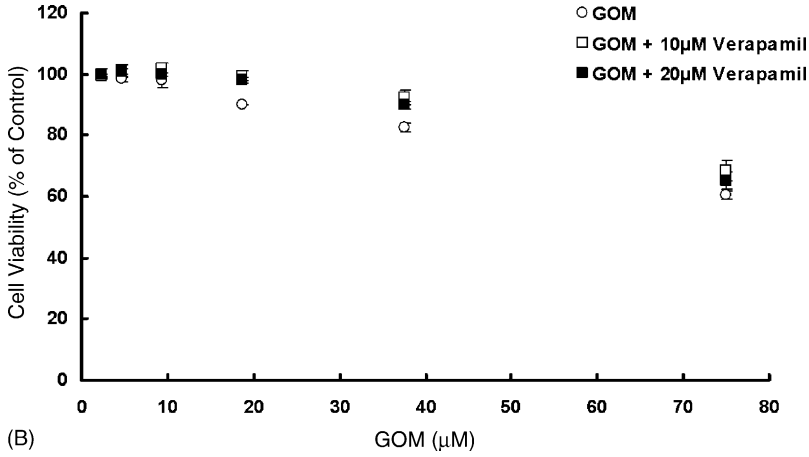


Fig. 2 (Continued).

3. Results

3.1. *Identification of gomisin A*

The NMR spectral data of our crystal was identical with those of gomisin A [28]. The characteristic spectra data of our crystallized compound are as follows: ESIMS (positive) *m/z* 417.4 $[M+H]^+$; ^{1}H NMR (400 MHz, CDCl3): δ 6.62 (1H, s, H-2), 6.48 (1H, s, H-2′), 5.97, 5.96 (2H, s,-OCH2O-), 3.91 (6H, s,-OCH3), 3.84 (3H, s,-OCH3), 3.52 (3H, s,-OCH3), 2.69 (1H, d, J = 13.5 Hz, H-7a), 2.59 (1H, d, J = 14.0 Hz, H-7′a), 2.35 (1H, d, J = 13.5 Hz, H-7b), 2.33 (1H, dd, J = 14.0, 6.0 Hz, H-7′b), 1.86 (1H, m, H-8′), 1.25 (3H, s, H-9), 0.82 (3H, d, J = 7.2 Hz, H-9′); ^{13}C NMR (100 MHz, CDCl3): δ 152.3 (C-3), 152.1 (C-5), 147.9 (C-3′), 141.2 (C-5′), 140.8 (C-4), 134.9 (C-4′), 132.5 (C-1′), 132.0 (C-1), 124.2 (C-6), 121.9 (C-6′), 110.3 (C-2), 105.9 (C-2′), 100.8 (C-OCH2O), 71.6 (C-8), 61.0 (C-OCH3), 60.6 (C-OCH3), 59.7 (C-OCH3), 55.9 (C-OCH3), 42.0 (C-8′), 40.5 (C-7), 33.7 (C-7′), 30.1 (C-9), 15.8 (C-9′). The molecular weight of our crystallized compound as determined by LC/MS was 416 which was identical to gomisin A [28].

3.2. *Modulation of drug resistance in HepG2-DR cells*

Through bioassay-guided fractionation, gomisin A was identified as a potential MDR modulator in Pgp-overexpressing cells. SRB assay showed that gomisin A was synergistic with well-known anticancer agents and Pgp substrates doxorubicin, vinblastine and taxol in Pgp over-expressing drug resistant HepG2-DR cells, but not in drug sensitive HepG2 cells. Meanwhile gomisin A had no effect when combined with 5-fluoruracil, a non-Pgp substrate, suggesting that the gomisin A effect was Pgp-dependent (Fig. 2A). It is worthy to note that although gomisin A was relatively non-toxic ($IC_{50} > 150$ μM for 72 h), the inhibitory effect of gomisin A alone in drug resistant HepG2-DR cells was markedly higher than in HepG2 cells (Fig. 2A). Furthermore, the cytotoxicity of gomisin A itself was not enhanced by a well-known Pgp inhibitor verapamil (Fig. 2B), implying that gomisin A is not a substrate of Pgp.

Colony formation assay is frequently used to evaluate cancer cell survival in a chronic cytotoxicity environment. As shown in Fig. 3, while 1 μg/ml doxorubicin and 200 ng/ml vinblastine alone had no noticeable effect, inhibitory effects were clearly observed when gomisin A was added in combination. In particular colony formation was reduced from 82 to 0.1% when vinblastine was combined with gomisin A (Fig. 3B).

3.3. *Gomisin A restored vinblastine's cell cycle action*

If gomisin A simply acts by increasing the cellular contents of anticancer drugs, it should restore their original cellular effects. In HepG2 cells, 5 ng/ml of vinblastine could increase G_2/M phase cells from 17 to 60% in 24 h. In drug resistant HepG2-DR cells, no effect was observed even if vinblastine concentration was increased to 300 ng/ml. By adding gomisin A, G_2/M arrested HepG2-DR cells were increased in a dose-dependent manner and 50 μM of gomisin A increased G_2/M phase cells from 28 to 77% (Fig. 4).

3.4. *Gomisin A did not affect the expression level of Pgp*

Modulation of Pgp-dependent MDR can be achieved by either decreasing Pgp expression or by inhibiting Pgp activity. As shown by Western blotting and RT-PCR, the protein and mRNA levels of Pgp were not altered after gomisin A treatment (Fig. 5).

3.5. *Gomisin A inhibited Pgp-ATPase activity*

The drug transport function of Pgp is coupled to ATP hydrolysis by the ATPase domain that is stimulated in the presence of Pgp transport substrates. We measured the rate of Pgp-mediated ATP hydrolysis in isolated membrane vesicles prepared from Pgp over-expressing HepG2-DR cells while suppressing the activities of other major membrane ATPase. Remarkably, gomisin A strongly inhibited the endogenous Pgp-ATPase activity (Fig. 6A), and unlike most of the well-known Pgp modulators such as verapamil, it did not appear to stimulate ATPase activity over a wide concentration range. These results indicate that gomisin A is not an effective transport substrate.

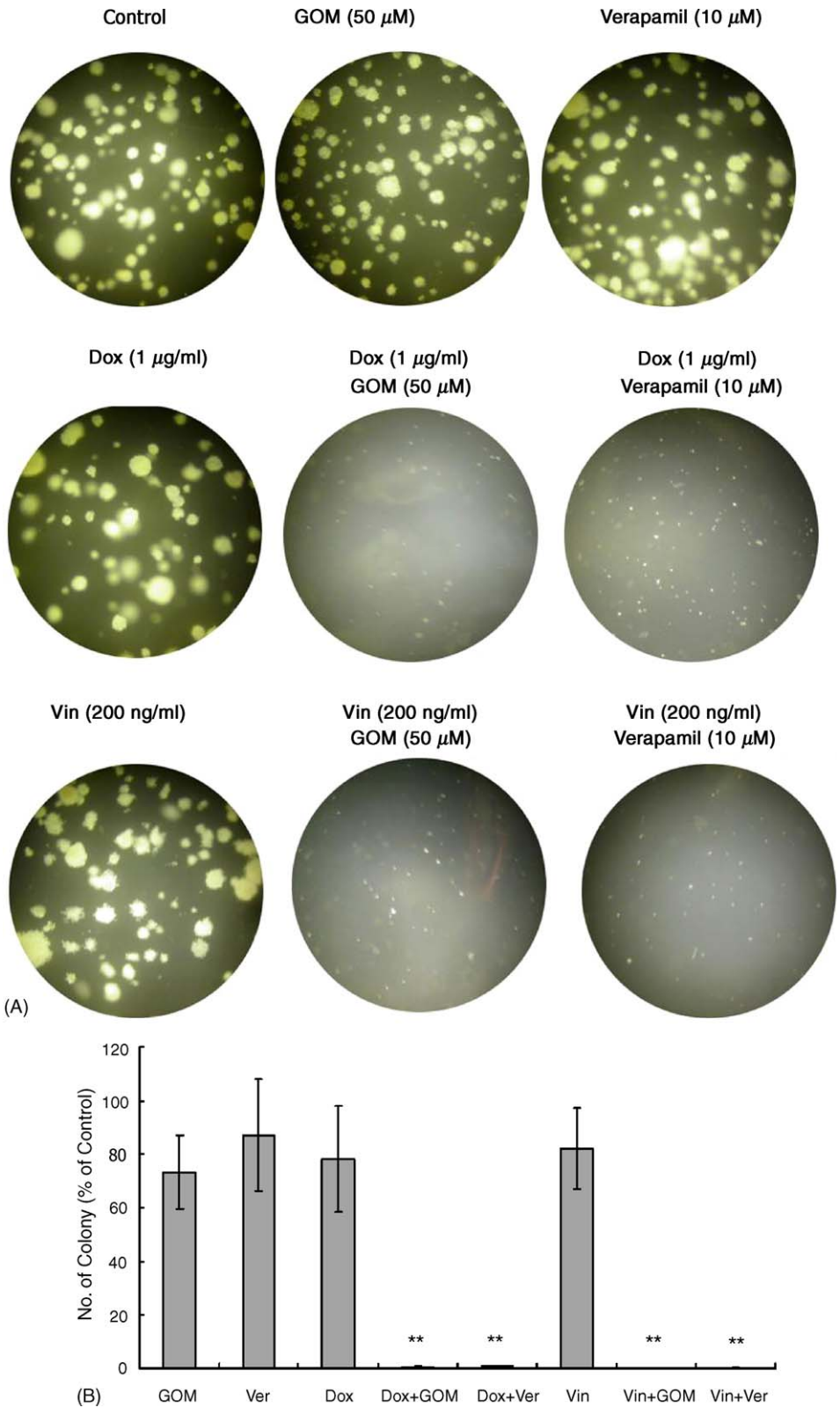


Fig. 3 – Effect of gomisin A on colony-formation ability of HepG2-DR cells. (A) Results of a representative experiment showing colony formation in untreated control plate and plates treated by gomisin A (GOM), doxorubicin (Dox), vinblastine (Vin) or their combinations as indicated. Verapamil (Ver) served as a positive control. (B) Relative numbers of colony (percentage of untreated control) from three independent experiments are shown as mean ± S.D. $^{**}p < 0.01$ significantly different at compared to Dox and Vin treatments alone.

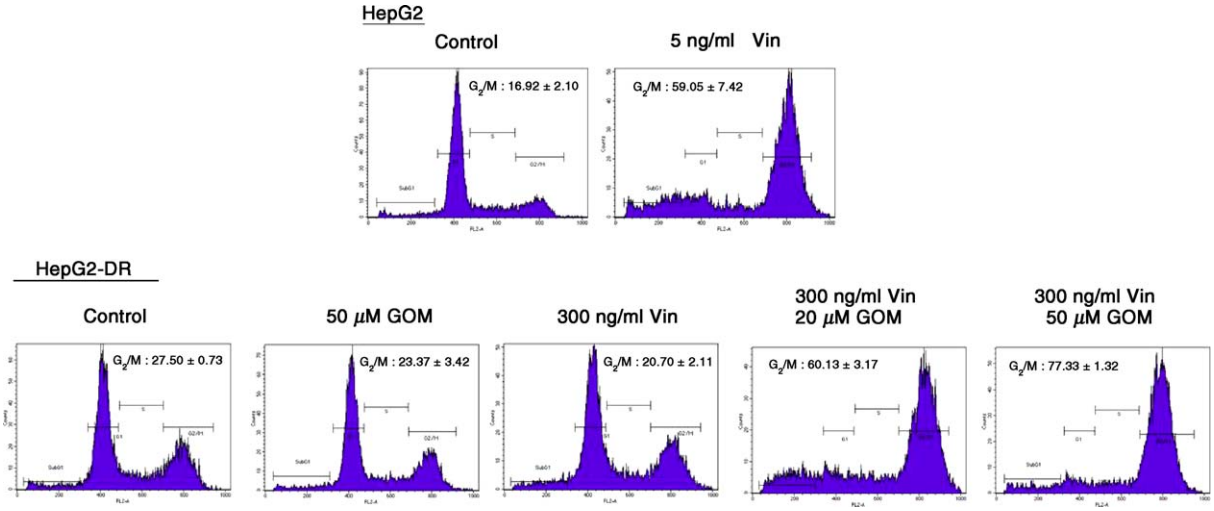


Fig. 4 – Gomisin A restored vinblastine-induced G_2/M arrest in drug-resistant HepG2-DR cells. Cells were incubated with vinblastine (Vin) with or without gomisin A (GOM) for 24 h. Flow cytometry results of a typical experiment are shown. Numerical data are mean ± S.D. of three independent experiments.

It can be speculated that gomisin A might bind either to one or more of the three substrate-binding sites without being transported, or to the presumed allosteric inhibitory site. To answer this, we studied the effects of gomisin A on progesterone- and verapamil-stimulated ATPase activity. Progesterone and verapamil are two Pgp substrates that bind to different transport sites of Pgp [22,29]. Interestingly, we found that gomisin A decreased both the V_{max} and the K_m of both progesterone- and verapamil-stimulated ATPase activities (Fig. 6B). Lineweaver–Burk plot of the above data showed a classical uncompetitive mode of inhibition of gomisin A on verapamil- or progesterone-dependent ATP hydrolysis (Fig. 6B, inset). The classical explanation of an uncompetitive inhibition is that the inhibitor binds to the enzyme–substrate complexes and thus affects the catalytic function of the enzyme without negatively affecting substrate affinity. The mechanism is common for multisubstrate enzymes.

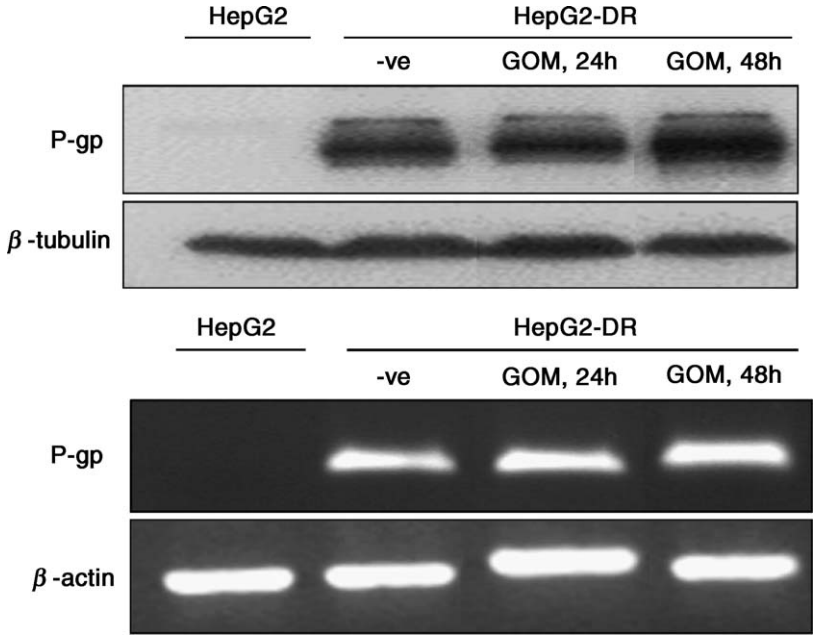


Fig. 5 – Gomisin A did not affect Pgp mRNA and protein expressions. Drug-resistant HepG2-DR cells were treated with 50 μM gomisin A (GOM) for 24 and 48 h. *Upper panel:* samples containing 50 μg of total cellular protein was analyzed by 8% SDS-PAGE. Expression level of Pgp was detected by mouse anti-Pgp antibody c-494. β-tubulin was included as an loading control. *Lower panel:* mRNA level of Pgp was determined by reverse transcription of 1 μg of total RNA and amplified by Pgp specific primers. β-actin served as an internal control.

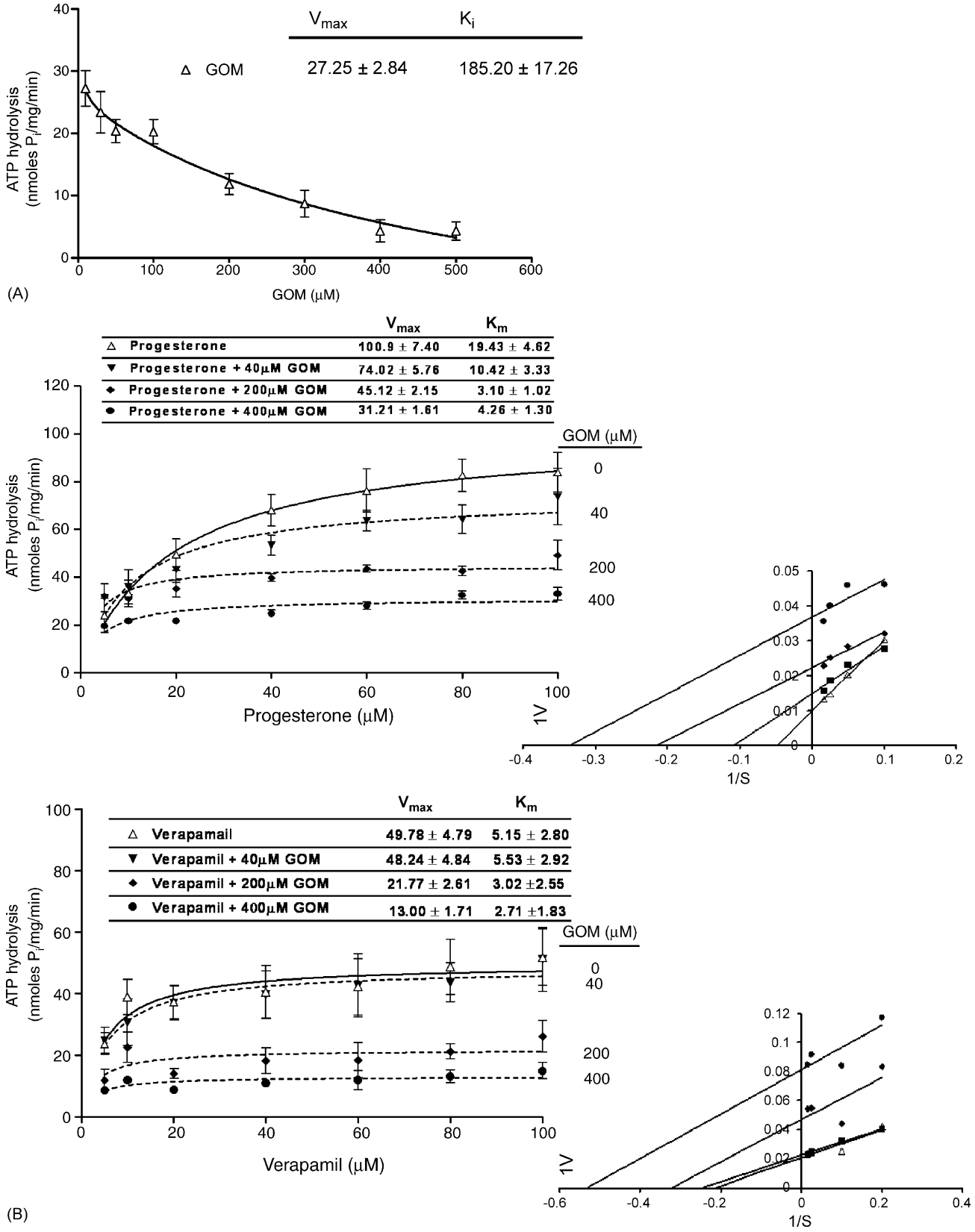


Fig. 6 – Effect of gomisin A on basal and substrate-stimulated Pgp-ATPase activity. Membrane preparations were obtained from Pgp-overexpressing HepG2-DR cells and the ATPase activity was measured by inhibiting other major membrane ATPase. (A) Gomisin A (GOM) alone showed inhibitory effect on ATPase activity. (B) Effect of gomisin A (GOM) on progesterone- and verapamil-stimulated ATPase activity. The Michaelis–Menten parameters are presented as mean ± S.D. of three independent experiments. *Inset:* same data presented by Lineweaver–Burk plot.

3.6. Additive effects of gomisin A and verapamil on rhodamine-123 (Rh-123) retention

Rh-123 efflux reflects the pumping activity of Pgp which was blocked by gomisin A or the competitive inhibitor verapamil in Pgp over-expressing HepG2-DR cells. Gomisin A alone increased Rh-123 retention by 600% (Fig. 7A). Fig. 7B showed that the maximal effect of verapamil and gomisin A on Rh-123 retention were basically additive, suggesting the two drugs were acting by different mechanisms.

3.7. Gomisin A caused a Pgp conformational change distinct from that caused by substrates

UIC-2 is a monoclonal antibody specific to a conformation-sensitive extracellular Pgp epitope with a higher level of reactivity to substrate-bound Pgp. Presumably, UIC-2 binding traps P-gp in an otherwise transient conformation and renders Pgp inactive [13,30,31]. It has been shown that the presence of Pgp substrates and/or competitive inhibitors, as well as ATP depletion or defects in ATP-binding sites, increase the reactivity of UIC-2 [30]. Allosteric modulators that indirectly interfere with substrate or competitive inhibitor binding also reduce UIC-2 reactivity [13]. Our results demonstrated that unlike Pgp substrates vinblastine and cyclosporine A that increased UIC-2 binding, gomisin A decreased UIC-2 binding (Fig. 8). Thus, gomisin A might cause a conformation change distinct from that caused by substrates or competitive inhibitors. UIC-2 binding was also decreased by vanadate, which inhibits Pgp function by trapping it in a Pgp–ADP–V_i complex (see Section 3.8 below). Total amount of Pgp, as evaluated by another Pgp antibody c494, was not altered (Fig. 8). UIC-2 binding activity was insignificant in HepG2 cells (data not shown).

3.8. Additive effects of gomisin A and vanadate on Rh-123 retention

Since both gomisin A and the ATPase inhibitor vanadate decreased UIC-2 binding, to differentiate the actions of the two inhibitors, we studied the combined effect of gomisin A and vanadate on the retention of intracellular Rh-123 in HepG2-DR cells. We have shown above that when verapamil-stimulated Pgp-ATPase activity was measured, gomisin A served as an uncompetitive inhibitor. Under the same experimental conditions vanadate served as a non-competitive inhibitor [22]. At 500 μM vanadate that maximally inhibited Rh-123 efflux (Fig. 9, inset), the addition of gomisin A produced an additive effect on the inhibition of Rh-123 efflux (increased retention) (Fig. 9). This observation supports the notion that their modes of action are different.

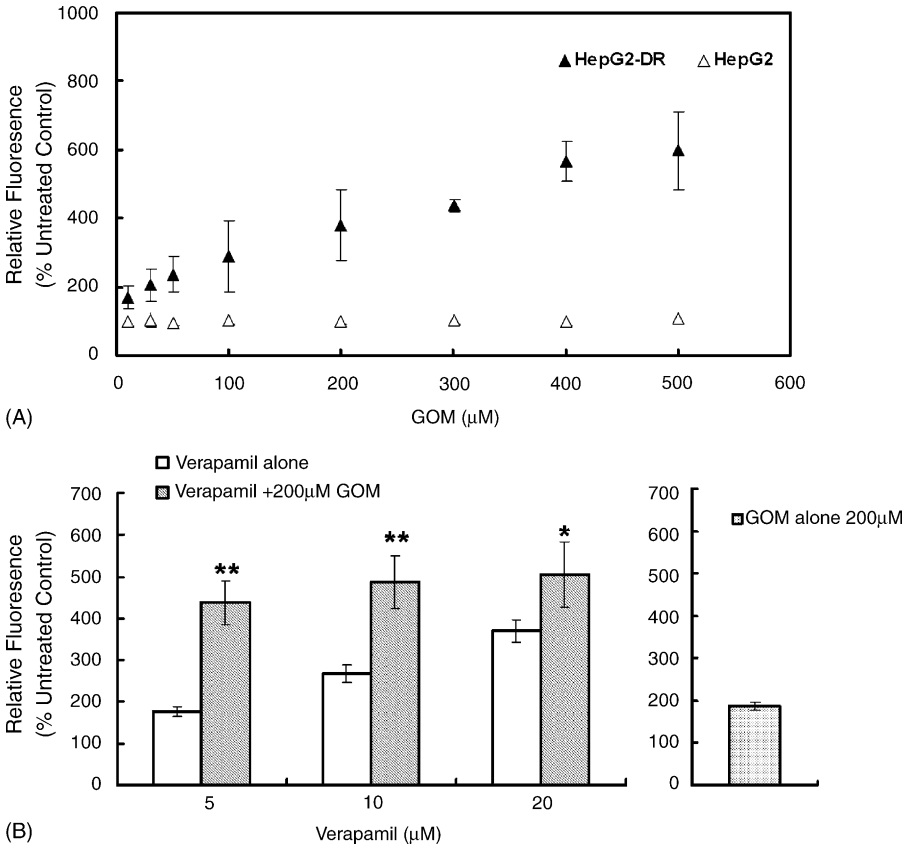


Fig. 7 – Effects of gomisin A on Rh-123 efflux. (A) HepG2 and HepG2-DR cells were incubated with 5 μg/ml of Rh-123 for 1 h, washed and incubated with individual drugs for another hour. Intracellular Rh-123 fluorescence was determined by flow cytometer. The inhibitory effect of gomisin A (GOM) on Rh-123 efflux was expressed as relative fluorescence in percent of untreated control. (B) Combined effect of gomisin A and verapamil on Rh-123 efflux. Various concentrations of verapamil were combined with 200 μM of gomisin A (GOM) and intracellular fluorescence was determined.

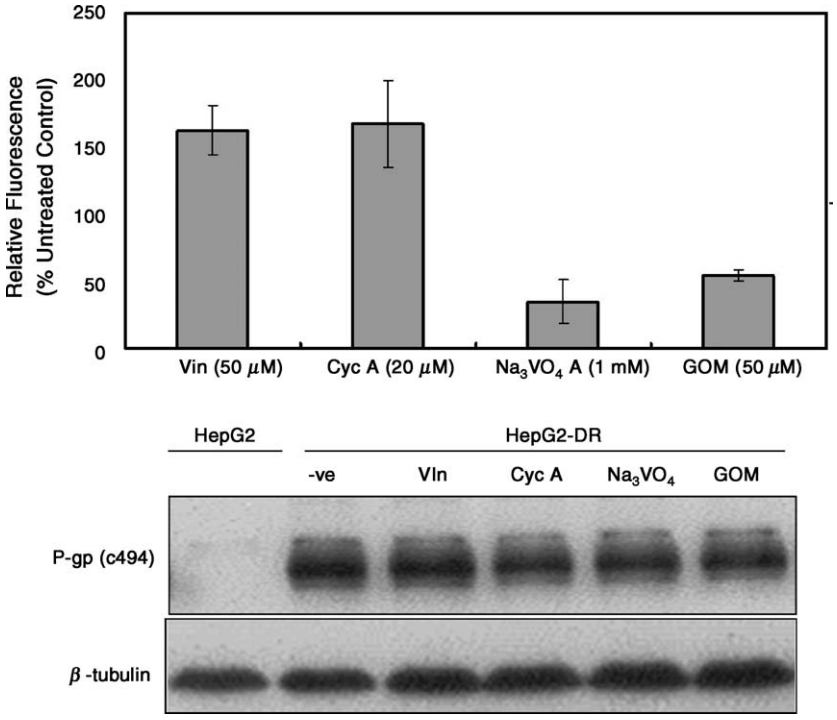


Fig. 8 – *Upper panel:* monoclonal antibody UIC-2 reactivity with Pgp in HepG2-DR cells. Pgp-overexpressing HepG2-DR cells were incubated with the monoclonal antibody UIC-2 in the presence of various drugs and analyzed by flow cytometry. Data are presented as mean ± S.D. of percent of untreated control in three independent experiments. *Lower panel:* drug-treated samples were analyzed by Western blotting. Total amount of Pgp was determined by c-494 anti-Pgp antibody.

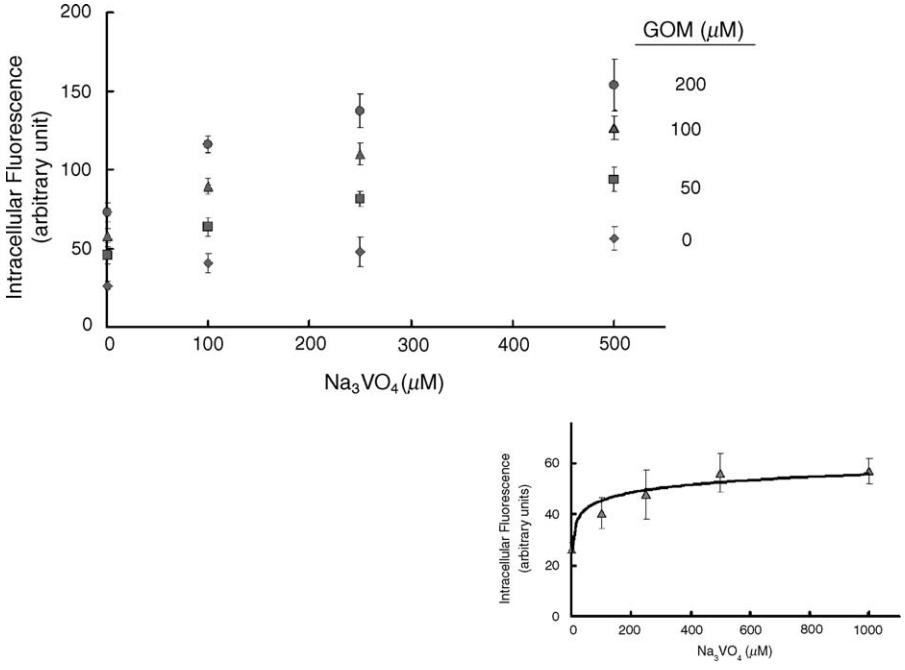


Fig. 9 – The combined effect of gomisin A and vanadate on Rh-123 efflux. HepG2-DR cells were incubated with 5 μg/ml of Rh-123 for 1 h, washed and incubated with various concentrations of gomisin A (GOM) and vanadate for another hour. Intracellular Rh-123 fluorescence was monitored by flow cytometer. *Inset:* effect of vanadate alone on intracellular Rh-123 retention.

3.9. Gomisin A inhibited [^{125}I]IAAP photo-crosslinking of Pgp

Pgp can be photocrosslinked to its transport substrate [^{125}I]IAAP. Pgp substrates and modulators compete with [^{125}I]IAAP for binding to Pgp. To understand the nature of interaction of gomisin A with the substrate interaction site of Pgp, we studied [^{125}I]IAAP labeling of Pgp in isolated membrane preparations in the presence of varying concentrations of the modulator. As indicated in Fig. 10, gomisin A effectively inhibited [^{125}I]IAAP labeling of Pgp in a concentration-dependent manner in the wild type Pgp (Fig. 10A, top) with near maximal inhibition occurring at a concentration of 100 μM and K_i of about 75 (±35.0) μM (Fig. 10B). This result suggested that gomisin A affects substrate binding to Pgp, which is consistent with the fact that the substrate-stimulated ATPase activity of Pgp is inhibited by gomisin A.

It has been demonstrated that alanine substitution of a single phenylalanine residue at position 983 in the transmembrane region 12 of Pgp caused reduced modulatory potential of thioxanthene based Pgp modulators thioxanthenes and phenothiazines [13]. To understand whether F983 has any role in the interaction of gomisin A with Pgp, we studied [^{125}I]IAAP labeling of Pgp F983A mutant in the presence of varying concentrations of gomisin A. Our results indicate no remarkable effect of gomisin A on [^{125}I]IAAP binding to the mutated Pgp ($K_i = 26 \pm 6.8$) (Fig. 10B). A maximum of 80% inhibition was observed for both the wild type and the F983 A mutated Pgp's (Fig. 10B). Thus, the F983 residue is probably not involved in gomisin A's action.

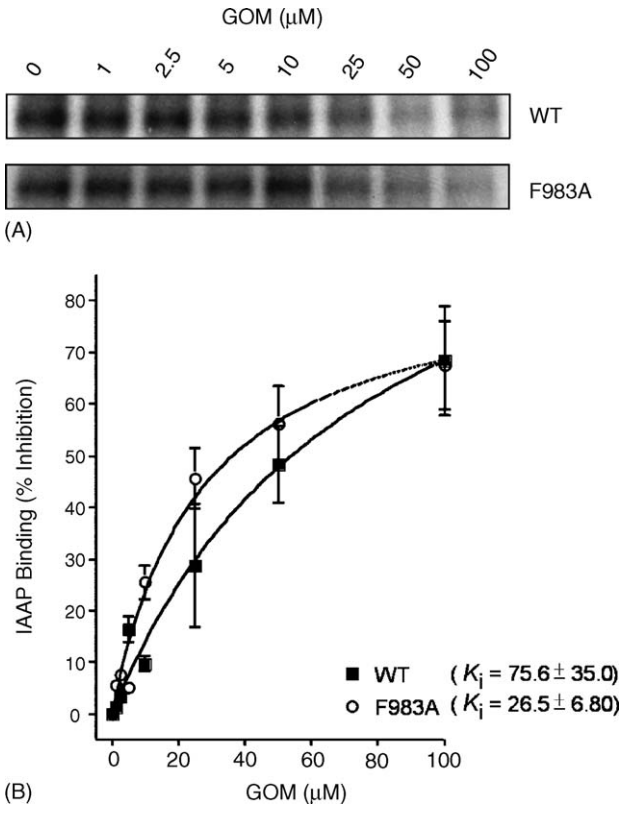


Fig. 10 – Effect of gomisin A on [^{125}I]IAAP photo-labeling of the wild type and F983A mutant Pgp. Wild type (Fig. 10A, top panel) or F983A mutant (Fig. 10B, bottom panel) Pgp in isolated membranes was photoaffinity labeled with 5 nM of [^{125}I]IAAP in the presence of varying concentrations (0–100 μM) of gomisin A (GOM). Labeled proteins (3 μg of protein per lane) were analyzed by SDS-PAGE in 8% Tris–glycine gel. Radio-labeled protein bands were visualized on a X-ray film (A) and quantified by phosphorimager (B). Data are averages of two independent experiments and plotted as percent inhibition against gomisin A (GOM) concentration.

4. Discussion

There have been few reports on the MDR modulating effects of natural lignans. During the preparation of this manuscript, there is a report showing that schisandrin B, another lignan found in *S. chinensis*, reverses Pgp-dependent MDR [32]. These reports suggest that compounds with the dibenzocyclooctadiene core are potential Pgp modulators.

The exact numbers and locations of transport and regulatory sites on Pgp are still uncertain. Since the three-dimensional atomic structure of Pgp has not been resolved, current information is largely based on biochemical and functional studies. An earlier model has suggested the existence of two separate sites [33] and it was demonstrated by competitive studies that, for example, verapamil and progesterone bind to two different sites, while cyclosporin A and verapamil bind onto a common site [22,29]. The more recent and widely accepted "multiple sites" model suggests that Pgp might contain at least three transport sites and in addition there might exist one allosteric site for the binding of allosteric modulators [12]. Transport substrates are being assigned to each of the three transport sites according to their competitiveness with the standard substrates verapamil, rhodamine 123 and H33342.

Based on results obtained from cysteine-scanning mutagenesis experiments an expanded substrate-induced fit mechanism was proposed. In this model, Pgp transport substrates with vastly different chemical structures may bind to a common pocket consisting of amino acid residues from different transmembrane domains [34,35]. This binding pocket could be large enough for simultaneous binding of two substrate molecules [36]. Supporting this model is a recent report showing that LDS-751 and rhodamine 123 are able to bind onto Pgp at the same time [37]. Indeed the "multiple sites" model and "substrate-induced fit" model are not mutually exclusive.

Verapamil and progesterone interact with different transport sites of Pgp and the interaction stimulates Pgp-ATPase activity [22,29]. Our kinetic data demonstrated that gomisin A acted as an uncompetitive inhibitor for Pgp-ATPase stimulated by verapamil or progesterone. Since gomisin A's inhibitory effect could not be overcome by increasing the concentrations of verapamil or progesterone (V_{max} decreased), and since gomisin A itself is apparently not an effective transport substrate (it inhibited rather than stimulated ATPase activity), it is conceivable that gomisin A could be binding to the enzyme alone or to the enzyme–substrate complexes.

Using a Pgp-specific antibody we also showed that gomisin A may alter Pgp conformation. UIC-2 monoclonal antibody recognizes an extracellular epitope of Pgp molecules actively engaged in ATP-dependent transport [13]. Pgp allosteric inhibitors such as Na_3VO_4 and *cis*-(Z)-flupentixol decrease UIC-2 reactivity [13]. We showed that gomisin A also decreased UIC-2 reactivity, suggesting that gomisin A affected the substrate-induced P-gp conformational change. However, gomisin A's effect on [^{125}I]IAAP labeling of Pgp was not altered in the Pgp F983A mutant that is impaired of modulation by *cis*-(Z)-flupentixol. This suggested that the specific Pgp-interaction contact sites of gomisin A and *cis*-(Z)-flupentixol are not identical.

The inhibitory effect of vanadate on Pgp is achieved by replacement of the hydrolyzed inorganic phosphate in the nucleotide binding domain and trap Pgp into a Pgp–ADP–V_i complex [38,39]. Both gomisin A and vanadate inhibit the Pgp-ATPase activity (this paper and [40]) and decrease UIC-2 binding (this paper and [13]). This prompted us to verify whether these two compounds modulate Pgp by the same mechanism. On the inhibition of Rh-123 efflux we found that the two compounds were basically additive without interfering each other, indicated that they likely modulated Pgp at different sites (Fig. 9).

The uncompetitive inhibition by gomisin A on substrate-stimulated Pgp-ATPase suggests the possibility of a simultaneous interaction of gomisin A and transport substrates (verapamil or progesterone) with Pgp. Three steps can be distinguished in Pgp catalyzed ATP-dependent transport as follows [13]: (1) substrate recognition, (2) substrate translocation that is coupled to ATP hydrolysis, and (3) substrate dissociation. The above observations, as well as the fact that gomisin A prevents Pgp-[^{125}I]IAAP association, suggest that gomisin A affects step one.

In conclusion, we showed that gomisin A isolated from *S. chinensis* reverses Pgp-mediated MDR by uncompetitive inhibition of substrate-Pgp association and inducing Pgp conformational changes that impede substrate transport. The non-toxic nature of this compound would suggest its potential use in vivo and this aspect is being studied in our laboratory.

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