# *Ganoderma lucidum* derived ganoderenic acid B reverses ABCB1-mediated multidrug resistance in HepG2/ADM cells

DAO-LU LIU[1*], YING-JIE LI[1*], DONG-HUA YANG[2], CHEN-RAN WANG[1], JUN XU[1], NAN YAO[1], XIAO-QI ZHANG[1], ZHE-SHENG CHEN[3], WEN-CAI YE[1] and DONG-MEI ZHANG[1]

[1]College of Pharmacy, Jinan University, Guangzhou 510632, P.R. China; [2]Biosample Repository, Core Facility, Fox Chase Cancer Center, Philadelphia, PA; [3]College of Pharmacy and Health Sciences, St. John's University, Queens, NY, USA

Received December 12, 2014; Accepted February 5, 2015

DOI: 10.3892/ijo.2015.2925

**Abstract.** Chemotherapy is one of the most common therapeutic option for metastatic tumors and hematological malignancies. ABCB1-mediated multidrug resistance is the major obstacle for chemotherapy. Natural products with diversified structures are ideal source of ABCB1 modulators. Ganoderenic acid B, a lanostane-type triterpene isolated from *Ganoderma lucidum*, exhibited potent reversal effect on ABCB1-mediated multidrug resistance of HepG2/ADM cells to doxorubicin, vincristine and paclitaxel. Similarly, ganoderenic acid B could also significantly reverse the resistance of ABCB1-overexpressing MCF-7/ADR cells to doxorubicin. Furthermore, ganoderenic acid B notably enhanced intracellular accumulation of rhodamine-123 in HepG2/ADM cells through inhibition of its efflux. ABCB1 siRNA interference assay indicated that the reversal activity of ganoderenic acid B was dependent on ABCB1. Further mechanistic investigations found that ganoderenic acid B did not alter the expression level of ABCB1 and the activity of ABCB1 ATPase. Molecular docking model displayed that the positions of ganoderenic acid B binding to ABCB1 were different from the region of verapamil interacted with ABCB1. Collectively, ganoderenic acid B can enhance the cytotoxicity of chemotherapeutics towards ABCB1-mediated MDR cancer cells via inhibition of the transport function of ABCB1. These findings provide evidence that ganoderenic acid B has the potential to be developed into an ABCB1-mediated multidrug resistance reversal agent.

*Correspondence to:* Dr Dong-Mei Zhang or Professor Wen-Cai Ye, College of Pharmacy, Jinan University, Guangzhou 510632, P.R. China
E-mail: dmzhang701@foxmail.com
E-mail: chywc@gmail.com

*Contributed equally

*Key words:* ganoderenic acid B, ABC transporter, multidrug resistance, HepG2/ADM, ABCB1

## Introduction

Chemotherapy is the ultimate therapeutic tool for metastatic and hematological malignancies. Also, postoperative chemotherapy is indispensable after surgical treatment. However, multidrug resistance (MDR) remains a major impediment for chemotherapy, accounting for >90% treatment failure in clinic (1). Genovariation, epigenetic changes and microenvironmental changes display the complexity of MDR mechanisms. These include: overexpression of the ATP-binding cassette (ABC) superfamily transporters; reduction of drug intake or increase drug metabolism; change of drug targets; activation of DNA repair mechanisms, and suppression of apoptosis pathways (2). Among them, the most dominant mechanism is the overexpression of ABC transporters. Three prominent ABC transporters (ABCB1, ABCC1 and ABCG2) are common in MDR tumors (3). Of these, ABCB1, a 170-kDa transmembrane glycoprotein encoded by the *MDR1* gene, has been most extensively identified in MDR cancer cells. ABCB1 has a broad spectrum of substrates that are chemotherapeutics, including doxorubicin (DOX), vinblastine (VCR), paclitaxel, etoposide, and bisantrene (4).

Currently, it is believed that inhibition of the ABC drug transporters is the most feasible strategy to overcome MDR. Taken a panoramic view of the three generations of ABCB1 modulators, the clinical limitations come from the poor specificity, less potency and unpredictable toxicity (5,6). Therefore, there is an urgent need to develop more specific, potent and relatively non-toxic modulators. Natural products such as plants, fungi and marine organisms possess a great diversity of compounds and are ideal source of ABCB1 modulators. Of those, the extraction-separation of traditional Chinese medicine (TCM) with traditional efficacy is one of the most important approaches to discover new biological active components. For instance, β-D-glucopyranoside has been developed as TCM monomer (7). Artemisinin is used as an effective antimalarial agent worldwide (8). A large number of TCM monomers and their derivatives which are ABCB1 modulators have been found. Clitocine, an alkaloid from *Clitocybe inversa*, reversed ABCB1-associated MDR in HepG2/ADM by downregulation of NF-κB and ABCB1 (9). Our group is devoted to find ABCB1 modulators from TCM, and has found

that 23-hydroxybetulinic acid derivatives BBA (10), DABB, DHBB (11) and B5H7 (12) could significantly reverse ABCB1-mediated MDR cells via inhibition of the function of ABCB1. In addition, we found that acerinol, isolated from *Cimicifuga acerina*, acts as a competitive inhibitor for ABCB1 to sensitize drug resistant cells HepG2/ADM and MCF-7/ADR to DOX, VCR and paclitaxel (13).

*Ganoderma lucidum* (Leyss. ex Fr.) Karst, a notable Chinese medicine, has been used as a folk remedy for health improvement. It has long been used for prevention and treatment of multiple diseases, such as cancer, neurasthenia, hepatopathy, aging, and diabetics (14). In the past two decades, over one hundred of triterpenoids in the fungus have been extracted and identified from the fruiting bodies, cultured mycelia, and spores of *Ganoderma lucidum*, which have been considered to be responsible for the biological activities of *Ganoderma lucidum* (15). Accumulated data show that these triterpenoids exhibit a broad spectrum of antitumor properties. For instance, *Ganoderma lucidum* extracts containing triterpenoids (GLCTs) exhibited anti-proliferative activity in several hematological cell lines such as HL-60, K562 and Nalm-6 via induction of cell cycle arrest and apoptosis (16). Moreover, GLCTs activated multiple signaling pathways to reduce secretion of vascular endothelial growth factor (VEGF) and suppress the activities or expression of matrix metalloproteinase (MMP) relative proteins and urokinase plaminogen activator (uPA), thereby inhibiting the invasion and angiogenesis of tumors *in vitro* and *in vivo* (17). Ganoderic acid DM, a lanostane-type triterpenoid, exhibited activity of inhibition of tumor proliferation and metastasis. Further mechanistic investigations indicated that Ganoderic acid DM had a hormonal-like function modulating estrogen receptor (ER) and androgen receptor (AR), subsequently inducing DNA damage, apoptosis and G1 phase cell cycle arrest in MCF-7 cells (18).

Ganoderenic acid B (GAB, Fig. 1A), a lanostane-type triterpene isolated from *Ganoderma lucidum* (19), exhibited potent antineoplastic activity in p388, BEL-7402, SGC-7901 and HeLa cells *in vitro*, with $IC_{50}$ values of 13.6, 18.6, 20.4 and 10 $\mu$M, respectively (14). However, the cellular and molecular mechanisms behind its anticancer action need to be further illuminated. So far, there is no report on its MDR reversal activity. In this report, we demonstrate that GAB is able to sensitize ABCB1-overexpressing MDR cells HepG2/ADM, and we reveal the mechanisms of action for its MDR reversal activity.

## Materials and methods

*Materials*. GAB was isolated from the fruit bodies of *Ganoderma lucidum* and the purity is >95% determined by HPLC. Doxorubicin (DOX), rhodamine-123 (Rhm-123), cisplatin, 3-(4,5-dimethylthiazol-2-yl)-2,5-dipheyltetrazolium bromide (MTT) and paclitaxel were obtained from Sigma-Aldrich (St. Louis, MO, USA). Recombinant human ABCB1 membranes, Pgp-Glo™ assay systems and CYP3A4 P450-Glo™ assay kit were acquired from Promega (Madison, WI, USA). Vincristine (VCR) was a product from Perivinkle Pharmaceutical (Haikou, Hainan, China). ABCB1 siRNA kit was obtained from GenePharma (Pudong, Shanghai, China). Verapamil (VRP) and mouse anti-ABCB1 antibody were purchased from Merck Calbiochem (Darmstadt, Hessen, Germany). Monoclonal anti-β-actin antibody was acquired from Multisciences (Hangzhou, Zhejiang, China). Other chemical reagents were purchased from Sigma-Aldrich.

*Cell lines and cell culture*. Human hepatocellular carcinoma cell line (HepG2) and drug-resistant cell line (HepG2/ADM) were kindly provided by Professor Kwok-Pui Fung (The Chinese University of Hong Kong, Hong Kong, China). ABCB1-overexpressing HepG2/ADM was established from HepG2 by progressive induction of DOX as described previously (20). Human breast cancer cell line MCF-7 and DOX-induced drug resistant cell line MCF-7/ADR was provided generously by Professor Li-Wu Fu (Sun Yat-Sen University, China) (21). All cell lines were maintained in RPMI-1640 containing 10% FBS and 1% penicillin streptomycin at 37˚C in a humidified incubator with 5% $CO_2$. In order to keep the drug resistant feature, HepG2/ADM and MCF-7/ADR cells were maintained in the culture medium containing 1.2 $\mu$M DOX. Cells in logarithmic phase were collected to use in cellular experiments.

*Cell viability assay*. The viability of cells treated with various concentrations of GAB was evaluated by MTT assay. Cells in logarithmic phase were plated into 96-well plates at a density of $5x10^3$ per well and maintained for 24 h. Cells were incubated in the medium containing various concentrations of GAB for 72 h. MTT solution (20 $\mu$l) (5 mg/ml in PBS) was added and cultured for another 4 h. The optical density of formazan at 595 nm was recorded by a microplate reader (DTX880, Beckman, USA). The viability of cells treated with dimethyl sulfoxide (DMSO) was defined as 100%, and the concentrations at which >90% cells were viable, were considered as non-toxic and used in the MDR reversal assay.

*MDR reversal assay*. Cells were treated with GAB (5 and 10 $\mu$M) or VRP (10 $\mu$M) combined with different concentrations of DOX, VCR, paclitaxel and cisplatin in 96-well plates. Cell viability was detected using MTT assay as described above. The half maximal inhibitory concentrations ($IC_{50}$) were calculated using GraphPad Prism 5.0 software according to their survival curves. The reversal folds of GAB and VRP were calculated by dividing the $IC_{50}$ value of DOX, VCR, paclitaxel and cisplatin in the absence or presence of GAB or VRP.

*Rhm-123 accumulation assay*. Intracellular Rhm-123 accumulation assay was evaluated as previously described (10). Briefly, cells ($1x10^4$ per well) were seeded into 96-well black clear-bottom plates for 24 h. Cells were pre-incubated with GAB (5 and 10 $\mu$M) or VRP (10 $\mu$M) for 4 h at 37˚C. After that Rhm-123 (5 $\mu$M) was added and cultured for another 2 h. After cells were washed three times with ice-cold PBS, cellular Rhm-123 fluorescence was detected by a microplate reader (DTX880, Beckman) or a fluorescence microscopy (Axio Imager A2, Zeiss, Germany).

*Rhm-123 efflux assay*. Cells ($1x10^4$ per well) were plated into 96-well black clear-bottom plates. After overnight attachment, cells were pre-treated with GAB (10 $\mu$M) or VRP (10 $\mu$M) for 2 h and cultured with Rhm-123 (5 $\mu$M) for another 2 h at

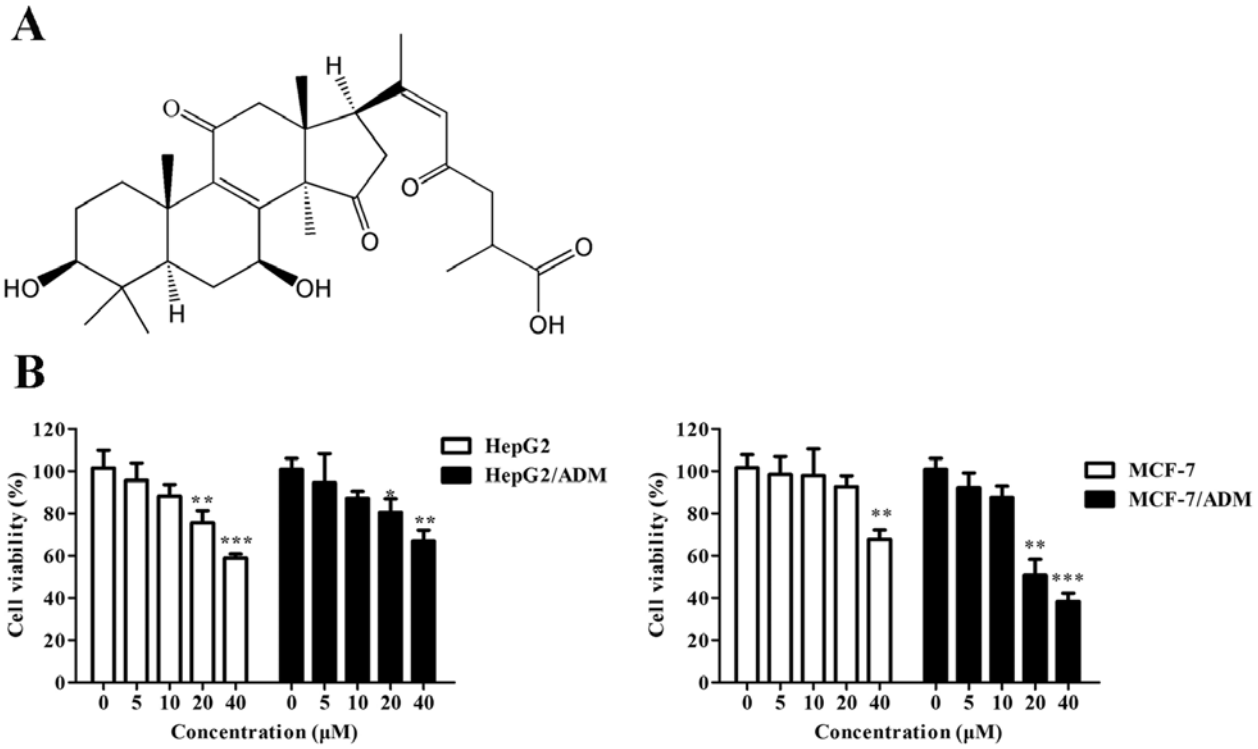


Figure 1. (A) Chemical structure of GAB. (B) Effect of GAB on HepG2/ADM, MCF-7/ADR and their parental cells HepG2 and MCF-7. Cells were treated alone with GAB for 72 h. Cytotoxicity of GAB was tested by MTT assay. Results are presented as mean ± SD of three independent experiments. $^{*}$P<0.05, $^{**}$P<0.01, $^{***}$P<0.001 compared with control.

37˚C, then washed 3 times with PBS at various time-points (0, 15, 30 and 60 min). Finally, the retention of intracellular Rhm-123 was evaluated by DTX 880 Multimode Detector. The fluorescence of Rhm-123 in cells at 0 min was considered to be 100%.

*ABCB1 siRNA interference*. HepG2/ADM cells ($3x10^5$ per well) were plated in 6-well plates. When they were 60-80% confluent, cells were incubated with the medium of Opti-MEM with Lipofectamine reagent, 100 nM ABCB1 siRNA or scrambled control siRNA for 6 h at 37˚C. Cells were then further incubated for 48 h, and cellular ABCB1 level was detected by western blot analysis as described below. To determine whether reversal effect of GAB were associated with ABCB1, the siRNA-transfected cells was exposed to VCR in the presence or absence of GAB and VRP for 48 h and cell viability was determined by MTT assay as described above.

*Western blot analysis*. Total protein was extracted from HepG2/ADM and MCF-7/ADR cells after treated with GAB (5 and 10 $\mu$M) or VRP (10 $\mu$M) for 72 h. The concentrations of total cellular protein were quantified using BCA protein assay kit. Proteins were separated by 10% SDS-PAGE and then transferred into PVDF membranes. After being blocked with 5% non-fat milk in TBS-T buffer for 1 h at room temperature, the membranes were immunoblotted with primary antibody (1:1,000) at 4˚C overnight and probed with secondary antibody (1:1,000) for 1 h at room temperature. The bands were enhanced using enhanced chemiluminescence solutions and imaged by X-ray film processor (Kodak X-102, Kodak, USA).

*ABCB1 ATPase activity assay*. To investigate the influence of GAB on ABCB1 ATPase activity, ABCB1-Glo™ ATPase assay kit was used following the instructions of the manufacturer. Total ATPase inhibitor $Na_3VO_4$ (0.25 mM), ABCB1 ATPase stimulator VRP (0.5 mM) and various concentrations of GAB (2.5, 5, 10 and 20 $\mu$M) in assay buffer were prepared and then added to opaque flat bottom 96-well plates, then recombinant human ABCB1 membranes were added and treated for 5 min at 37˚C. Ten $\mu$l of MgATP (25 mM) was added to each well. After incubation for 40 min at 37˚C, the reaction was stopped by adding 50 $\mu$l of ATP detection buffer and then incubated at room temperature for 20 min. The luminescence with positive proportion to ATP was tested by a microplate reader (DTX880, Beckman).

*CYP3A4 activity assay*. CYP3A4 P450-Glo assay kit was used to detect the influence of GAB on CYP3A4 according to the manufacturer's instructions. Briefly, GBA (20 and 40 $\mu$M) or ketoconazole (20 $\mu$M) diluted 4-fold by luciferin-free water were added into opaque flat bottom 96-well plates and co-treated with 4X CYP3A4 reaction mixture (0.5 M potassium phosphate, 12 $\mu$M luciferin-IPA and 0.008 pM recombinant human CYP3A4 membranes) for 10 min at 37˚C. 2X NADPH regeneration system (10% solution A and 2% solution B) was used to start reactions. The luminescent signal was detected after the reactions were stopped by luciferin detection reagent with esterase.

*Docking analysis*. Sybyl 8.0 in Surflex-dock module, a parented docking engine to explore binding modes and sites of ligands and proteins, was used to simulate the docking of GAB and

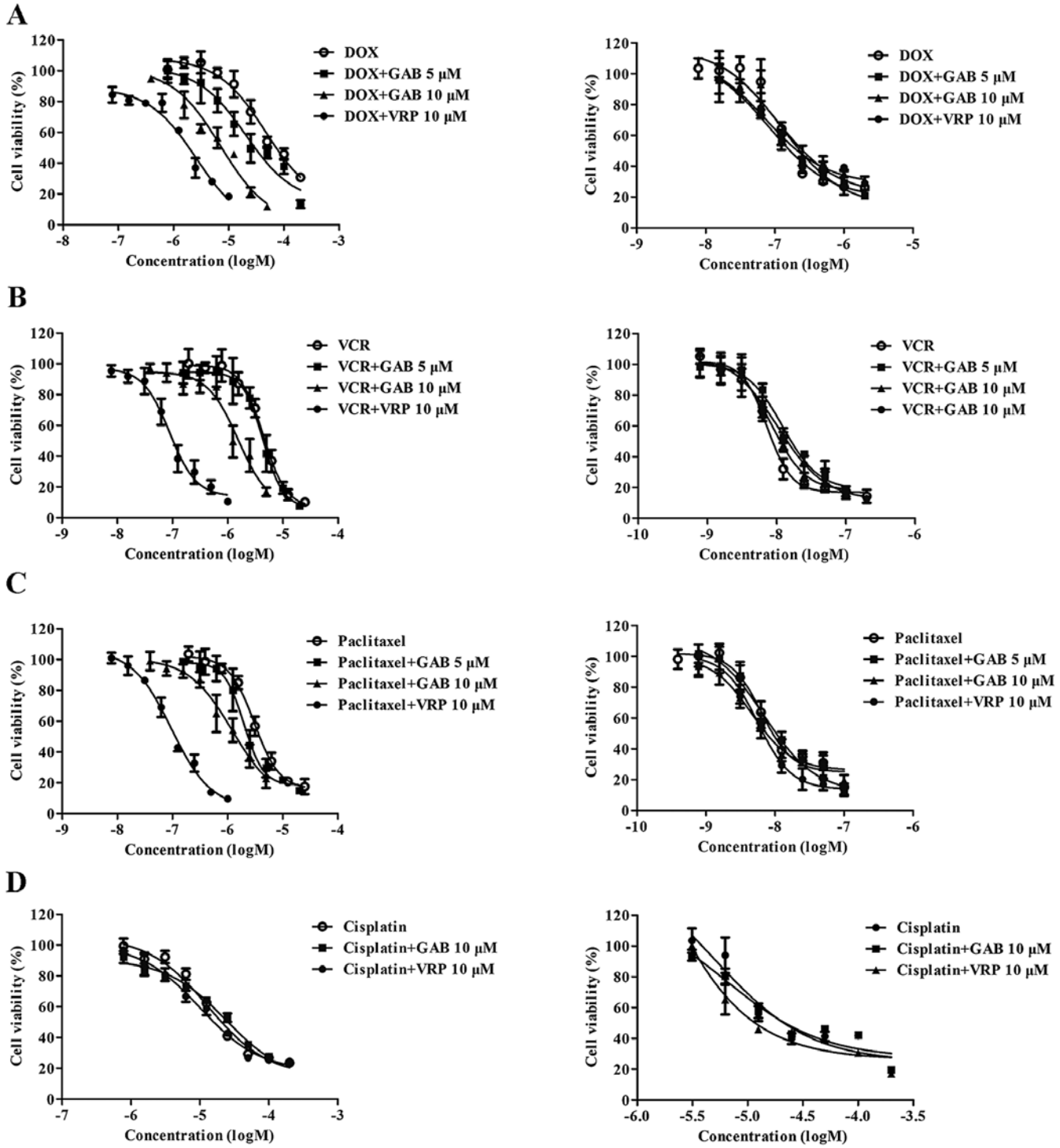


Figure 2. The MDR reversal effect of GAB on HepG2/ADM cells. (A) The influence of GAB on the sensitivity of HepG2/ADM (left) and HepG2 (right) towards DOX (A), VCR (B), paclitaxel (C) and cisplatin (D). VRP (10 $\mu$M) was used as a positive control. Results are presented as means ± SD of three independent experiments.

ABCB1. VRP was defined as reference standard. The charge of ABCB1 structure was calculated in MMFF94 protein structure-module without energy optimization. Three-dimensional structures of GAB and VRP with energy optimization were carried out using Tripos molecular mechanics force field. In the docking process, the preferable docking conformation and the best docking score using an empirical scoring function, were guidelines in the docking of GAB and VRP to ABCB1 binding site residues. GAB and VRP were docked into the idealized active sites of ABCB1 and further binding energy was calculated.

*Statistical analysis*. All experiments were performed at least three times, and results are shown as means ± SD. Data were analyzed using Graphpad Prism 4.0 with Student's t-test. $P<0.05$ was considered to be significant.

Table I. Reversal effect of GAB on HepG2/ADM cells and their parent cells.

| | $IC_{50}$ ± SD[a] ($\mu$M) (fold-reversal) | |
|---|---|---|
| | HepG2/ADM | HepG2 |
| DOX | 77.4565±4.2154 (1.00) | 0.1715±0.0116 (1.00) |
| + GAB 5 $\mu$M | 23.0050±1.7477 (3.37) | 0.1595±0.0106 (0.93) |
| + GAB 10 $\mu$M | 6.0446±0.1176 (12.81) | 0.2024±0.0215 (1.18) |
| +VRP[b] 10 $\mu$M | 1.4247±0.3601 (54.36) | 0.1727±0.0243 (1.01) |
| VCR | 5.2046±0.6377 (1.00) | 0.0098±0.0012 (1.00) |
| + GAB 5 $\mu$M | 4.4045±0.2575 (1.18) | 0.0111±0.0005 (0.88) |
| + GAB 10 $\mu$M | 1.1627±0.0265 (4.48) | 0.0106±0.009 (0.92) |
| +VRP[b] 10 $\mu$M | 0.1084±0.0066 (48.03) | 0.0103±0.0011 (0.94) |
| Paclitaxel | 4.0754±0.2169 (1.00) | 0.0081±0.0009 (1.00) |
| + GAB 5 $\mu$M | 2.2603±0.0831 (1.81) | 0.0097±0.0016 (0.83) |
| + GAB 10 $\mu$M | 1.3232±0.0594 (3.08) | 0.0071±0.0006 (1.13) |
| +VRP[b] 10 $\mu$M | 0.0942±0.0121 (42.23) | 0.0072±0.0007 (1.13) |
| Cisplatin | 18.5201±2.7032 (1.00) | 15.5270±2.3874 (1.00) |
| + GAB 10 $\mu$M | 18.9788±1.3646 (0.98) | 15.0938±1.7880 (1.03) |
| +VRP[b] 10 $\mu$M | 16.4769±2.8430 (1.12) | 18.1916±1.2672 (0.85) |

[a]Data are shown as means ± SD of at least three independent experiments performed in six replicates. [b]VRP was used as positive control.

## Results

*Effect of GAB on cell viability.* To examine the reversal capacity of GAB under non-toxic concentrations on ABCB1-mediated multidrug resistance of HepG2/ADM and MCF-7/ADR cancer cells, the cytotoxicity of GBA towards all cell lines was determined firstly. The relative cell viability of HepG2, HepG2/ADM, MCF-7 and MCF-7/ADR treated with or without GAB is shown in Fig. 1B. After treated with GBA less than the concentration of 10 $\mu$M for 72 h, the cell viability was >90% in all four cell lines, whereas GAB at the concentration >20 $\mu$M was toxic. Thus, the concentrations of 10 and 5 $\mu$M were considered to be the optimal concentrations for GAB in the following MDR reversal experiments.

*GAB sensitizes ABCB1-overexpressing MDR cells to DOX, VCR and paclitaxel.* To evaluate the reversal activity of GAB in HepG2/ADM, MCF-7/ADR and their parental sensitive cell lines, $IC_{50}$ values of DOX, VCR, paclitaxel and cisplatin in the presence or absence of GAB were determined by MTT assay. Reversal activity comparison experiments were conducted using the same concentration of VRP (10 $\mu$M) as a positive control, which is a known ABCB1 inhibitor (22). As shown in Fig. 2 and Table I, while HepG2 cells were sensitive to DOX, VCR and paclitaxel, HepG2/ADM cells exhibited significant resistance characteristics to those traditional antitumor drugs with resistance-fold of 451, 531 and 503, respectively. GAB at concentrations of 10 and 5 $\mu$M remarkably reversed the resistance of HepG2/ADM cells to DOX in a concentration-dependent manner, the $IC_{50}$ values were decreased to 6.0446±0.1176 and 23.0050±1.7477 $\mu$M. Similarly, the increased sensitivity of HepG2/ADM to VCR and paclitaxel was found when cells were co-treated with GAB at 10 $\mu$M. However, GAB at lower concentration (5 $\mu$M) could not significantly reverse HepG2/ADM to VCR and paclitaxel. GAB was still able to reverse resistance of MCF-7/ADR cells to DOX, producing 3.36- and 1.90-fold reversal activity, respectively, at concentrations of 10 and 5 $\mu$M. In comparison, the MDR reversal effect of GBA in HepG2/ADM and MCF-7/ADR at 10 $\mu$M was a little weaker than VRP. In their parental sensitive cell lines HepG2 and MCF-7, the cytotoxicity of DOX, VCR and paclitaxel showed no significant difference in the presence or absence of GAB, similarly to VRP. Cisplatin, a water-soluble chemotherapeutic drug, which can not be transported by ABCB1, was not sensitive to HepG2/ADM and HepG2 cells, which was not affected by GAB. The above data suggest that GAB significantly sensitizes ABCB1 substrates to ABCB1-overexpressing MDR cells, and its reversal effect is related to ABCB1.

*GAB stimulates the accumulation of Rhm-123 and attenuates Rhm-123 efflux velocity in HepG2/ADM cells.* MDR reversal assay suggests that GAB is able to sensitize ABCB1-overexpressing cells to ABCB1 substrates. To understand the underlining mechanism of this effect, we examine the accumulation of an ABCB1 specific fluorescent substrate, Rhm-123, in HepG2/ADM and HepG2 cells. It was found that GAB treatment increased the intracellular content of Rhm-123, similarly to VRP (Fig. 3A). Similarly to the trend shown in Fig. 3B, the fluorescence intensity was much higher in HepG2 cells than that in HepG2/ADM cells. HepG2/ADM cells could strongly pump out intracellular Rhm-123 to medium, displaying weak fluorescence in cells. GAB at a concentration of 10 $\mu$M obviously induced intracellular accumulation of Rhm-123 in

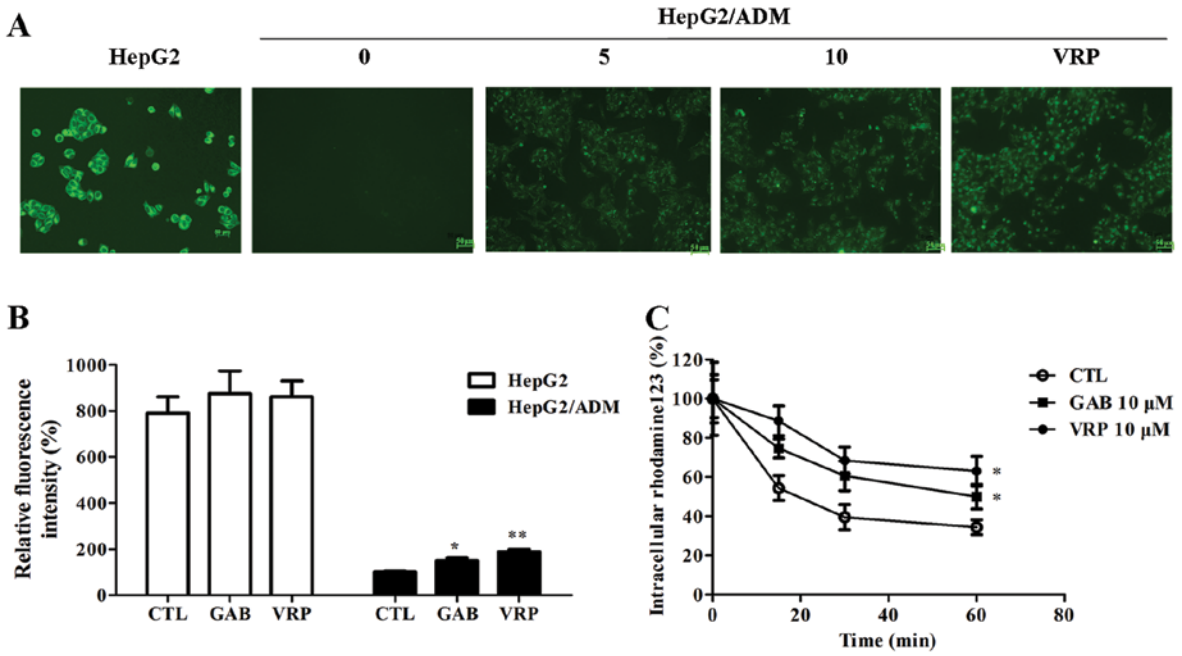


Figure 3. GAB enhances the accumulation of Rhm-123 and inhibits its efflux in HepG2/ADM cells. (A and B) Effect of GAB on the accumulation of Rhm-123 in HepG2/ADM cells. Cells were pre-incubated with or without GAB for 4 h at 37˚C, and then incubated with Rhm-123 for another 2 h at 37˚C. The cells were washed with cold PBS three times, the accumulation of Rhm-123 was measured by fluorescence microscope (A) and DTX 880 Multimode Detector (B). (C) Effect of GAB on the efflux of Rhm-123. Cells were incubated with or without GAB for 2 h, then Rhm-123 was added into each wells. After incubation for 2 h, cells were washed three times with PBS at different time-points (0, 15, 30 and 60 min). Intracellular Rhm-123 level was measured by DTX 880 Multimode Detector. VRP (10 $\mu$M) was used as a positive control. Results are presented as means ± SD of three independent experiments. $^{*}$P<0.05, $^{**}$P<0.01 compared with control.

HepG2/ADM, with a relative Rhm-123 level ≤148% compared with GAB untreated cells. However, GAB can not alter the accumulation of Rhm-123 in HepG2 cells. In addition, GAB inhibited Rhm-123 efflux velocity in HepG2/ADM, while Rhm-123 level in GAB-untreated cells was rapidly transported to extracellular medium (Fig. 3C). Consistent with the results in reversal activity assay, the effect of GAB on stimulation of accumulation of Rhm-123 and inhibition of its efflux was weaker than that of VRP. These data suggest that GAB may modulate the drug transport of ABCB1, thereby leading to the enhancement of the intracellular Rhm-123 accumulation and inhibition of Rhm-123 efflux.

*GAB reverses ABCB1-mediated MDR to VCR is dependent on ABCB1*. The results above indicated that the reversal effect of GAB may relate to influence of ABCB1 transporter. To further clarify whether the MDR reversal activity of GAB was dependent on ABCB1, ABCB1 gene was silenced by specific siRNA. As shown in Fig. 4A, ABCB1 protein level decreased significantly in HepG2/ADM cells after ABCB1 siRNA transfection. Scrambled control siRNA had no effect on the expression of ABCB1. As shown in Fig. 4B, ABCB1 siRNA-transfected HepG2/ADM cells showed attenuated reversal effect to VCR in the presence of GAB or VRP, indicating that GAB reverses ABCB1-mediated MDR depending on ABCB1.

*GAB can not alter ABCB1 expression*. Downregulation of expression and inhibition of function of ABCB1 are main reversal mechanisms of ABCB1 modulators. To further investigate the MDR reversal mechanisms of GAB, ABCB1 expression level in HepG2/ADM and MCF-7/ADR cells were measured by western blot analysis. As shown in Fig. 5A, the expression level of ABCB1 was not altered after GAB or VRP treatment. Our results indicate that GAB reverses ABCB1-

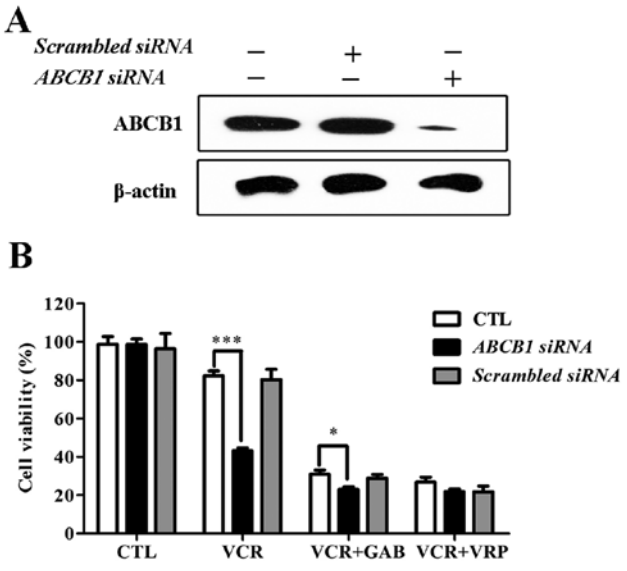


Figure 4. GAB reverses the resistance of HepG2/ADM cells to VCR partially dependent on ABCB1. (A) The expression of ABCB1 in ABCB1 siRNA-transfected HepG2/ADM cells was tested by western blot analysis. β-actin was used as an internal control. (B) Effect of GAB on the sensitivity of ABCB1 siRNA-transfected HepG2/ADM cells to VCR. ABCB1 siRNA- or scrambled siRNA-transfected cells were exposed to VCR in the presence or absence of GAB (10 $\mu$M) or VRP (10 $\mu$M), and cell viability was determined by MTT assay. Results are presented as means ± SD of three independent experiments. $^{*}$P<0.05, $^{***}$P<0.001 compared with control.

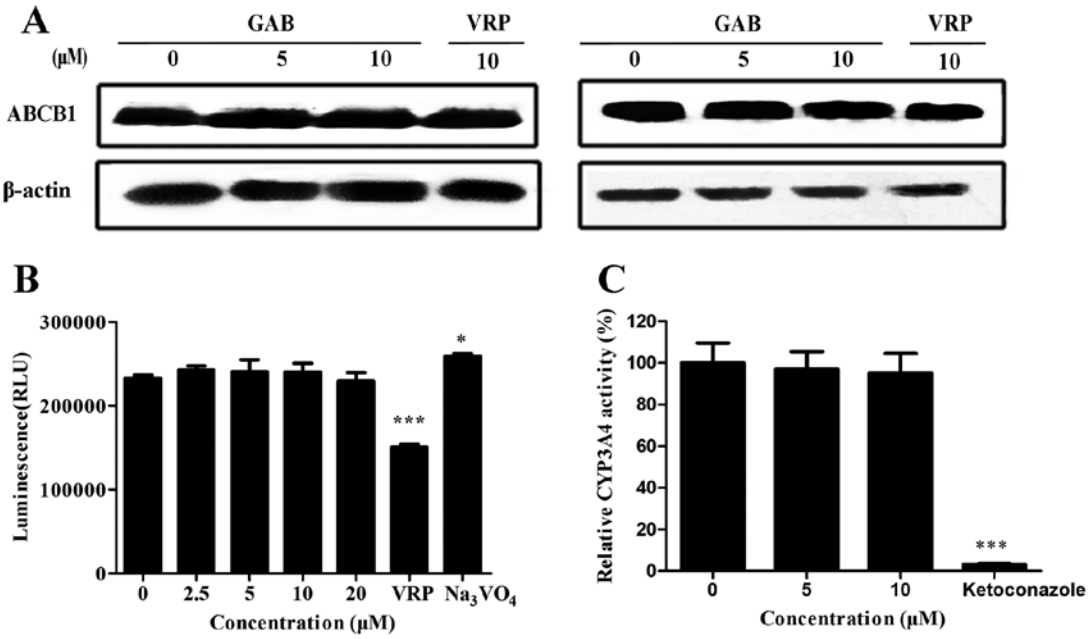


Figure 5. GAB does not alter ABCB1 expression or ABCB1 ATPase activity. (A) The protein level of ABCB1 was tested by western blot analysis with or without GAB in HepG2/ADM (left) and MCF-7/ADR (right) cells. β-actin was used as an internal control. (B) ABCB1 ATPase activity was detected using Pgp-Glo ATPase assay kit in the presence or absence of GAB. VRP (10 $\mu$M) was used as a positive control. (C) CYP3A4 activity was tested using CYP3A4 P450-Glo assay kit in the presence or absence of GAB. Ketoconazole was used as a positive control. Results are presented as means ± SD of three independent experiments. $^{*}$P<0.05, $^{***}$P<0.001 compared with control.

mediated MDR without downregulation of ABCB1, further suggesting that GAB may influence the function of ABCB1 to reverse MDR.

*GAB does not affect ABCB1 ATPase activity*. Because GAB does not alter the expression of ABCB1, GAB is likely to inhibit its transport function. Two ATP hydrolysis events are needed to change the conformation when ABCB1 transports the substrate out of the cells (23). ABCB1 substrates stimulate the ATPase activity in nucleotide-binding domains (NBD). Thus, the ABCB1 ATPase activity assay may contribute to reflect the action mode of GAB. As shown in Fig. 5B, ABCB1 substrate VRP was able to stimulate the activity of ABCB1, whereas ATP consumption was completely inhibited by $Na_3VO_4$. GAB at the reversal concentrations (10 and 5 $\mu$M) did not alter the ATPase activity of ABCB1. Moreover, the influence of ABCB1 ATPase was not observed even at higher or lower concentrations (20 and 2.5 $\mu$M). These data illustrate that GAB does not impact the activity of ABCB1 ATPase, and is not a competitive inhibitor for ABCB1.

*GAB does not alter CYP3A4 activity*. CYP3A4, one of the members of P450 family, is an important metabolic enzyme for anticancer drugs. Its broad spectrum substrate overlaps with ABCB1 modulators. A relative non-toxic ABCB1 modulator has the characteristic of no effect on CYP3A4 activity. We tested whether GAB inhibits the activity of CYP3A4. Ketoconazole was used as a positive control. GAB, even up to a concentration of 10 $\mu$M, did not inhibit CYP3A4 activity. Inversely, ketoconazole almost completely inhibited its activity (Fig. 5C). Taken together, these results indicate that GAB is not a CYP3A4 inhibitor.

*Molecular docking model of GAB binding to ABCB1*. To further explore the interaction mode and binding sites of GAB with ABCB1, GAB and VRP were docking toward ABCB1. As shown in Fig. 6A, GAB (blue) could bind to various conformation of ABCB1 transmembrane domain (TMD), far away from the VRP (green) binding position located to substrate binding region. ABCB1 residues and GAB binding sites are shown in Fig. 6B, GAB was surrounded by residues of LEU189, GLY190, SER192, ALA193, LYS198, PHE267, LEU268, ILE270, TYR271, TYR274, LEU303, ALA306, PHE307, GLY310, GLN311 and PRO314. The residual amino acids around VRP were LEU64, MET68, PHE724, ILE864, ALA865, ALA867, GLY868, THR941, GLN942, MET944, MET945, TYR949, PHE974, VAL978, ALA981 and VAL984, which were matched with previous reports (Fig. 6C) (23). Among these residues, none was around the GAB. This result further confirmed that GAB binding site to ABCB1 was different with the site of VRP.

## Discussion

Chemotherapy is thought to be the most effective treatment for disseminated cancer. Long-term continuous chemotherapy in clinic predisposes individuals to the development of acquired or intrinsic MDR. Overexpression of ABCB1 (P-glycoprotein, MDR1), which can pump out intracellular drugs to lead to reduce therapeutic dose in tumor cells, has been confirmed to be correlated with chemotherapeutic resistance and poor prognosis (24). In search for the third generation of ABCB1 modulators, steps have been taken to develop targeted, more specific modulators, with high selectivity and great potency. ABCB1 inhibitors or modulators obtained from natural

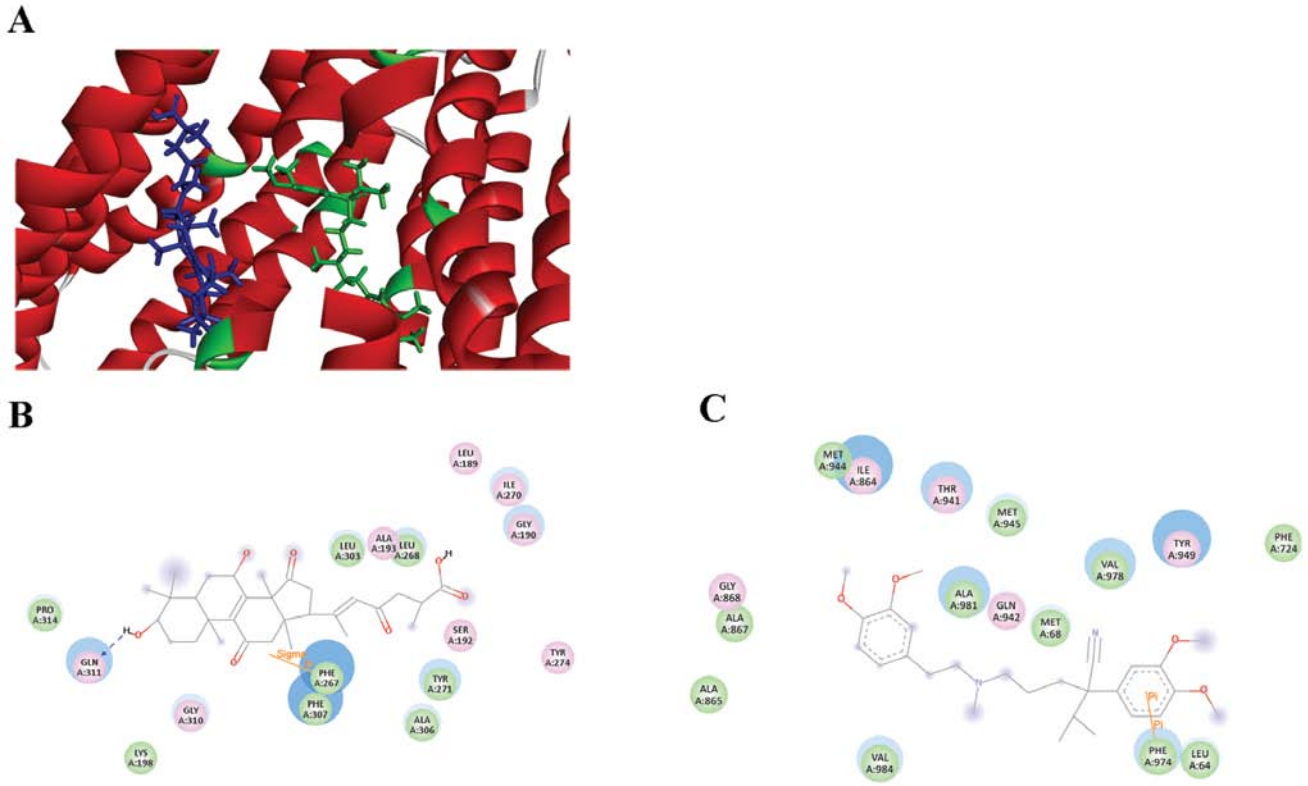


Figure 6. Docking analysis of GAB and VRP binding to ABCB1. (A) The binding mode and position of GAB (blue) and VRP (green) with ABCB1. The residual amino acids around GAB (B) and VRP (C). Key residues are labeled.

Table II. Reversal effect of GAB in MCF-7/ADR cells and their parent cells.

| | $IC_{50}$ ± SD[a] ($\mu$M) (fold-reversal) | |
|---|---|---|
| | MCF-7/ADR | MCF-7 |
| DOX | 40.0992±5.8447 (1.00) | 0.4472±0.0441 (1.00) |
| + GAB 5 $\mu$M | 21.0516±1.0390 (1.90) | 0.5301±0.0354 (0.85) |
| + GAB 10 $\mu$M | 11.9206±0.7707 (3.36) | 0.4854±0.0412 (0.92) |
| +VRP[b] 10 $\mu$M | 2.5183±0.3560 (15.92) | 0.4535±0.0378 (0.98) |

[a]Data are shown as means ± SD of at least three independent experiments performed in six replicates. [b]VRP was used as positive control.

sources usually have low toxicity and are well tolerated by humans. TCMs provide some natural products with novel structure, diverse skeleton and promising biological activities, that are suitable to be developed into potent, selective and low-toxic ABCB1 inhibitors (25). *Ganoderma lucidum* are precious fungi with good nutrition and medicinal properties, and have a long-historical utilization in China for promoting health and longevity. Some biologically active components of *Ganoderma lucidum* were found to have antitumor activity. Ethanol extracts from *Ganoderma lucidum*, inclusive of nucleosides, triterpenoids and sterols, showed significant antiproliferative activities on myriad tumor cell lines (15). Polysaccharides from *Ganoderma lucidum* have been reported to be able to attenuate DOX resistance to K562/ADM cells by inhibiting ABCB1 expression (26). The present study is the first to report that ganoderenic acid B (GAB), a lanostane type triterpene isolated from *Ganoderma lucidum*, markedly increase the cytotoxicity of DOX, VCR and paclitaxel to reverse ABCB1-mediated MDR cells by disrupting the function of ABCB1.

Previously, we reported that drug resistant cancer cells HepG2/ADM and MCF-7/ADR developed by long-term treatment of DOX, overexpress the ABCB1 transporter. These cells showed marked resistance to multiple chemotherapeutics, such as DOX, VCR and paclitaxel (10). In the present study, we found that GAB strongly enhanced the sensitivity of HepG2/ADM to several ABCB1 substrates such as DOX, VCR and paclitaxel (Fig. 2 and Table I). GAB was able to sensitize MCF-7/ADR cells to DOX (Table II). However, GAB had no such effect on their parental sensitive cells HepG2 and MCF-7 (Fig. 2 and Tables I and II). Moreover, GAB did not alter the sensitivity of HepG2/ADM and MCF-7/ADR cells to

cisplatin which is a non-ABCB1 substrate, indicating that the reversal efficacy of GAB is related to ABCB1-mediated drug resistance. Indeed, the speculation was strongly supported by the fact that GAB could make the retention of ABCB1 specific substrate Rhm-123 in HepG2/ADM cells, and this fluorescence accumulation study was consistent with the cytotoxic results. Simultaneously, inhibitive effect of GAB on the Rhm-123 efflux was also detected. RNA interference could decrease the expression level of ABCB1 in HepG2/ADM, following attenuation of the reversal effect of GAB in transfected HepG2/ADM. The results suggested ABCB1 is required in the reversal effect of GAB in MDR cells. Future studies on reversal effect of GAB will involve evaluation of MDR reversal effect in HepG2/ADM xenograft models.

Some active ingredients derived from the medical plants, such as icaritin, astragaloside II/IV and oroxylinA (27), could potentiate the cytotoxicity of anticancer drugs to MDR cells by downregulation of ABCB1 mRNA and protein levels, leading to the changes in MDR phenotype. Different from above active components, GAB did not alter the mRNA and protein expression levels of ABCB1 even after treatment for 72 h in HepG2/ADM and MCF-7/ADR cells (Fig. 5), suggesting that GAB may influence the drug transport function instead of expression of ABCB1. Structure of ABCB1 contains two ATP-binding sites (NBD) and two ATP hydrolysis events which are changed in the conformation when ABCB1 transports a substrate (23). ABCB1 substrates can stimulate the ATPase activity in NBD regions to pump them to the outer membrane. Some tyrosine kinase inhibitors such as erlotinib (28), apatinib (29) and lapatinib (30), inhibit ABCB1 transport function by stimulating ABCB1 ATPase activity. On the contrary, some of ABCB1 modulators, including 23-hydroxybetulinic acid derivatives DABB and DHBB, could inhibit the activity of ABCB1 ATPase thereby leading to dysfunction of ABCB1 (11). However, GAB neither stimulate nor inhibit the activity of ABCB1 ATPase (Fig. 5), ruling out the possibility that GAB binds to substrate-binding pocket to act as a competitive inhibitor of ABCB1 or that it binds to ATP-binding pocket to act as a ABCB1 ATPase inhibitor. The investigation on XR9576 binding to ABCB1 suggested the interaction between XR9576 and ABCB1 substrates vinblastine and paclitaxel in a non-competitive manner, indicating that vinblastine and paclitaxel could only fractionally displace [$^3$H]-XR9576 binding to ABCB1 (31). The longer term investigation of GAB showed that the reversal activity of GAB was retained after removal, suggesting its high binding affinity for ABCB1 and GAB was different from VRP reported as a typical substrate of ABCB1. The data showed that GAB does not appear to be a substrate for ABCB1, but may bind to ABCB1 in a non-competitive or uncompetitive manner. This hypothesis was probed further by the binding sites and style between GAB and ABCB1 in docking analysis. The most suitable docking conformation between GAB and ABCB1 has the best docking score far away from the binding sites of the substrate pocket (VRP binding region). Indeed, the characterization of the molecular interaction of GAB with ABCB1 needs to be further conducted by analysis of the inhibition kinetics of GAB on DOX transport by ABCB1. The properties of bilayer lipid membranes, including lipid composition, fluidity and cholesterol content, could influence ABCB1 function of ATP hydrolysis, drug interaction and transport (23). The relationship between GAB, lipid membrane and ABCB1 still remains an open question. Future efforts should focus on investigation of the interaction between GAB and ABCB1 to elucidate its underlying mechanism.

CYP3A4, a major anticancer drug metabolic enzyme *in vivo*, shares largely the same spectrum to substrates of ABCB1 modulators. In the case of the second generation reversal agent, PSC-338 inhibited CYP3A4 and induced the detention of 6-hydroxypaclitaxel in plasma during paclitaxel therapy, thus greatly increasing the risk of adverse effects (32). Fortunately, GAB did not markedly affect the CYP3A4 activity at reversal concentrations, indicating that it may interact with ABCB1 but not CYP3A4. Nevertheless, further clinical pharmacokinetics and toxicity assessment were expected to evaluate the side effects in human body.

Collectively, we for the first time report that GAB, a lanostane type triterpene from *Ganoderma lucidum*, can reverse ABCB1-mediated MDR by effectively inhibiting the transport function of ABCB1 and increasing the intracellular drug accumulation in MDR cells. GAB does not affect ABCB1 expression or ATPase in cells.

## Acknowledgements

This study was supported by Science and Technology Program of China (2012ZX09103101-053), Guangzhou City (2011Y1-00017-11 and 2011J2200045), National Science Foundation of China (30901847) and Guangdong Province (S2013050014183 and 2013CXZDA006), and Program for New Century Excellent Talents in University (D.M. Zhang).

## References

1. Gottesman MM, Fojo T and Bates SE: Multidrug resistance in cancer: role of ATP-dependent transporters. Nat Rev Cancer 2: 48-58, 2002.
2. Wu Q, Yang Z, Nie Y, Shi Y and Fan D: Multidrug resistance in cancer chemotherapeutics: mechanisms and lab approaches. Cancer Lett 347: 159-166, 2014.
3. Dean M, Hamon Y and Chimini G: The human ATP-binding cassette (ABC) transporter superfamily. J Lipid Res 42: 1007-1017, 2001.
4. Breier A, Gibalova L, Seres M, Barancik M and Sulova Z: New insight into p-glycoprotein as a drug target. Anticancer Agents Med Chem 13: 159-170, 2013.
5. Palmeira A, Sousa E, Vasconcelos MH and Pinto MM: Three decades of P-gp inhibitors: skimming through several generations and scaffolds. Curr Med Chem 19: 1946-2025, 2012.
6. Lee CH: Reversing agents for ATP-binding cassette drug transporters. Methods Mol Biol 596: 325-340, 2010.
7. Wang YS, Liao Z, Li YA, *et al*: New megastigmane diglycoside from Litsea glutinosa (Lour.) C. B. Rob. J Brazil Chem Soc 22: 2234-2238, 2011.
8. Ansari MT, Saify ZS and Sultana N: Malaria and artemisinin derivatives: an updated review. Mini-Rev Med Chem 13: 1879-1902, 2013
9. Sun J, Yeung CA, Co NN, *et al*: Clitocine reversal of P-glycoprotein associated multi-drug resistance through down-regulation of transcription factor NF-κB in R-HepG2 cell line. PLoS One 7: e40720, 2012.
10. Zhang DM, Shu C, Chen JJ, *et al*: BBA, a derivative of 23-hydroxybetulinic acid, potently reverses ABCB1-mediated drug resistance in vitro and in vivo. Mol Pharm 9: 3147-3159, 2012
11. Zhang DM, Li YJ, Shu C, *et al*: Bipiperidinyl derivatives of 23-hydroxybetulinic acid reverse resistance of HepG2/ADM and MCF-7/ADR cells. Anticancer Drugs 24: 441-454, 2013.

12. Yao N, Liu DL, Li YJ, *et al*: B5H7, a morpholine derivative of 23-hydroxybetulinic acid, reverses doxorubicin resistance in HepG2/ADM. J Cancer Res Updates 3: 59-66, 2014.
13. Liu DL, Li YJ, Yao N, *et al*: Acerinol, a cyclolanstane triterpenoid from *Cimicifuga acerina*, reverses ABCB1-mediated multidrug resistance in HepG2/ADM and MCF-7/ADR cells. Eur J Pharmacol 733: 34-44, 2014.
14. Wu GS, Guo JJ, Bao JL, *et al*: Anti-cancer properties of triterpenoids isolated from *Ganoderma lucidum* (Review). Expert Opin Inv Drug 22: 981-992, 2013.
15. Guan SH, Xia JM, Yang M, Wang XM, Liu X and Guo DA: Cytotoxic lanostanoid triterpenes from *Ganoderma lucidum*. J Asian Nat Prod Res 10: 695-700, 2008.
16. Wu G, Qian Z, Guo J, *et al*: *Ganoderma lucidum* extract induces G1 cell cycle arrest, and apoptosis in human breast cancer cells. Am J Chinese Med 40: 631-642, 2002.
17. Wang J, Chan JY, Fong CC, Tzang CH, Fung KP and Yang M: Transcriptional analysis of doxorubicin-induced cytotoxicity and resistance in human hepatocellular carcinoma cell lines. Liver Int 129: 1338-1347, 2009.
18. Wu GS, Lu JJ, Gu JJ, *et al*: Ganoderic acid DM, a natural triterpenoid, induces DNA damage, G1 cell cycle arrest and apoptosis in human breast cancer cells. Fitoterapia 83: 408-414, 2012.
19. Wang YL, Zhang XQ, Wang GC and Ye WC: Chemical constituents of the fruiting bodies of *Ganoderma lucidum*. Jiangsu Pharm Clin Res 14: 349-351, 2006.
20. Chan JY, Chu AC, Fung KP, *et al*: Inhibition of P-glycoprotein expression and reversal of drug resistance of human hepatoma HepG2 cells by multidrug resistance gene (mdr1) antisense RNA. Life Sci 67: 2117-2124, 2000.
21. Fu LW, Zhang YM, Liang YJ, Yang XP and Pan QC: The multidrug resistance of tumor cells was reversed by tetrandrine in vitro and in xenografts derived from human breast adenocarcinoma MCF-7/adr cells. Eur J Cancer 38: 418-426, 2002.
22. Massart C, Gibassier J, Lucas C, Pourquier P and Robert J: Expression of the MDR1 gene in five human cell lines of medullary thyroid cancer and reversal of the resistance to doxorubicine by ciclosporin A and verapamil. B Cancer 83: 39-45, 1996.
23. Aller SG, Yu J, Ward A, *et al*: Structure of P-glycoprotein reveals a molecular basis for poly-specific drug binding. Science 323: 1718-1722, 2009.
24. Sharom FJ: Complex interplay between the P-glycoprotein multidrug efflux pump and the membrane: its role in modulating protein function. Front Oncol 4: 41, 2014.
25. Wu CP, Ohnuma S and Ambudkar SV: Discovering natural product modulators to overcome multidrug resistance in cancer chemotherapy. Curr Pharm Biotechnol 12: 609-620, 2011.
26. Li WD, Zhang BD, Wei R, Liu JH and Lin ZB: Reversal effect of *Ganoderma lucidum* polysaccharide on multidrug resistance in K562/ADM cell line. Acta Pharmacol Sin 29: 620-627, 2008.
27. Wang PP, Xu DJ, Huang C, Wang WP and Xu WK: Astragaloside IV reduces the expression level of P-glycoprotein in multidrug-resistant human hepatic cancer cell lines. Mol Med Rep 9: 2131-2137, 2014.
28. Shi Z, Peng XX, Kim IW, *et al*: Erlotinib (Tarceva, OSI-774) antagonizes ATP-binding cassette subfamily B member 1 and ATP-binding cassette subfamily G member 2-mediated drug resistance. Cancer Res 67: 11012-11022, 2007.
29. Mi YJ, Liang YJ, Huang HB, *et al*: Apatinib (YN968D1) reverses multidrug resistance by inhibiting the efflux function of multiple ATP-bindingcassette transporters. Cancer Res 70: 7981-7991, 2010.
30. Dai CL, Tiwari AK, Wu CP, *et al*: Lapatinib (Tykerb, GW572016) reverses multidrug resistance in cancer cells by inhibiting the activity of ATP-binding cassette subfamily B member 1 and G member 2. Cancer Res 68: 7905-7914, 2008.
31. Martin C, Berridge G, Mistry P, Higgins C, Charlton P and Callaghan R: The molecular interaction of the high affinity reversal agent XR9576 with P-glycoprotein. Br J Pharmacol 128: 403-411, 1999.
32. Kang MH., Figg WD, Ando Y, *et al*: The P-glycoprotein antagonist PSC 833 increases the plasma concentrations of 6alpha-hydroxypaclitaxel, a major metabolite of paclitaxel. Clin Cancer Res 7: 1610-1617, 2001.