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Original Article

Wallichinine reverses ABCB1-mediated cancer multidrug resistance

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Abstract: Overexpression of ABCB1 in cancer cells is one of the main reasons of cancer multidrug resistance (MDR). Wallichinine is a compound isolated from *piper wallichii* and works as an antagonist of platelet activiating factor receptor to inhibit the gathering of blood platelet. In this study, we investigate the effect of wallichinine on cancer MDR mediated by ABCB1 transporter. Wallichinine significantly potentiates the effects of two ABCB1 substrates vincristine and doxorubicin on inhibition of growth, arrest of cell cycle and induction of apoptosis in ABCB1 overexpressing cancer cells. Furthermore, wallichinine do not alter the sensitivity of non-ABCB1 substrate cisplatin. Mechanistically, wallichinine blocks the drug-efflux activity of ABCB1 to increase the intracellular accumulation of rhodamine 123 and doxorubicin and stimulates the ATPase of ABCB1 without alteration of the expression of ABCB1. The predicted binding mode shows the hydrophobic interactions of wallichinine within the large drug binding cavity of ABCB1. At all, our study of the interaction of wallichinine with ABCB1 presented herein provides valuable clues for the development of novel MDR reversal reagents from natural products.

Keywords: Wallichinine, ABCB1, multidrug resistance, cancer

Introduction

Multidrug resistance (MDR) is a phenomenon wherein cancer patients do not respond to chemotherapy from multiple anticancer drugs with diverse structures and mechanisms of action, and it is the main reason of chemotherapy failure and high mortality rate of cancer [1]. One major mechanism contributing to MDR is the overexpression of ABCB1 (MDR1/P-glycoprotein) in cancer cells [2]. ABCB1 encodes a 170-KD transmembrane glycoproteid and belongs to the ATP-binding cassette (ABC) transporters family. It functions as an efflux pump that transports a wide variety of substrates across extra- and intracellular membranes, ranging from ions, vitamins, lipids, sugars, amino acids and drugs to larger molecules including oligopeptides, oligosaccharides and even higher molecular weight proteins [3]. Drugs transported by ABCB1 include vinca alkaloids, epipodophyllotoxins, anthracyclines, taxanes and so on, and this process is coupled to the energy from ATP hydrolysis via the ATPase domains of ABCB1 which is stimulated in the presence of substrates [4]. Theoretically, inhibition of ABCB1 activity may restore the sensitivity of MDR cancer cells to chemotherapeutic drugs and lead to an effective chemotherapy for patients with MDR tumor. Currently, a number of ABCB1 inhibitors are reported to enhance the effect of chemotherapeutic drugs on MDR cancer cells *in vitro* and *in vivo*, including verapamil, cyclosporine A, quinidine, PSC-833 (valspodar), R101933 (laniquidar), VX-710 (biricodar), oc-144-093 (ONT-093), GF-120918 (elacridar), LY335979 (zosuquidar) and XR9576 (tariquidar) [5, 6]. Unfortunately, most of these inhibitors were ineffective in clinical trials due to their unfavorable side effects, pharmacokinetic interference or non-significant clinical benifits. Consequently, it is necessary to develop more efficient and non-toxic agents to reverse cancer MDR.

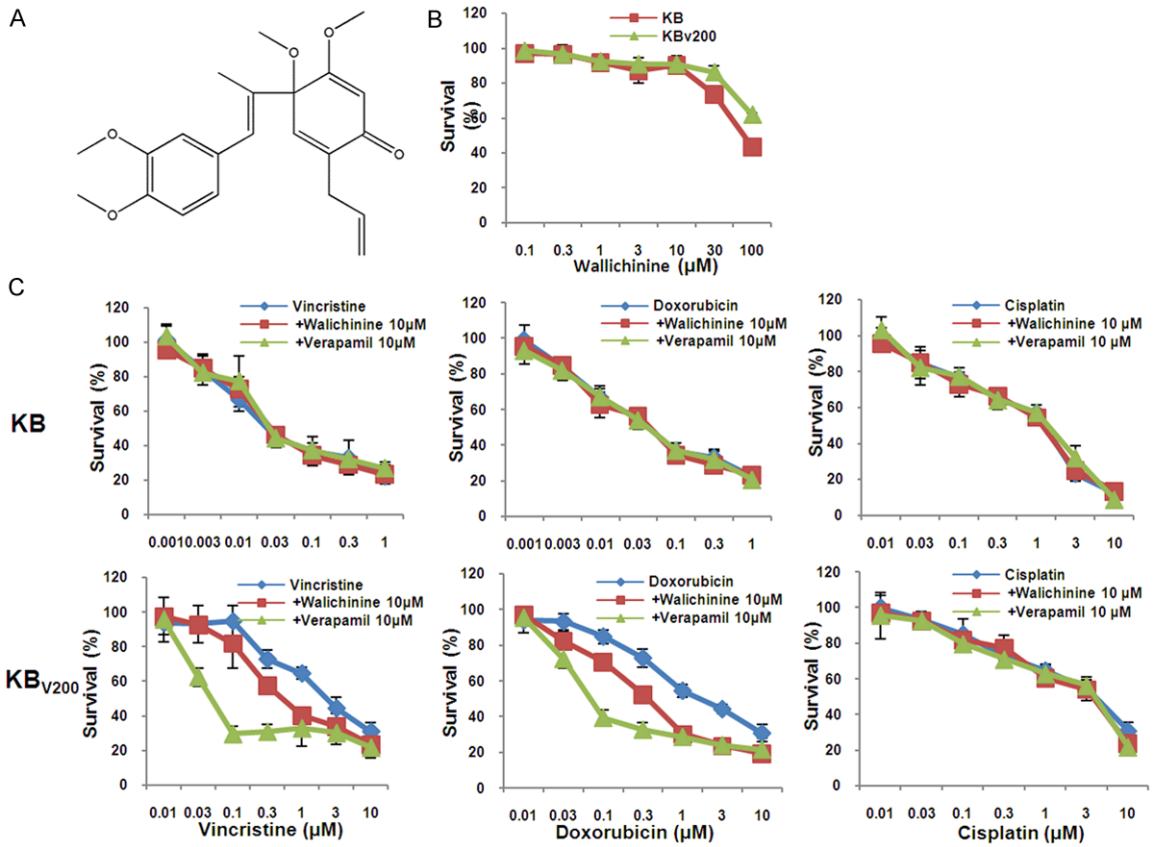

Figure 1. Wallichinine enhances the sensitivity of ABCB1-substrate chemotherapeutic agents in the ABCB1-overexpressing cells. Cells were treated with the indicated concentrations of wallichinine (A) or other agents for 72 hours, and cell survival was measured by MTT assay. The representative growth curve of KB and KB_{V200} cells treated with wallichinine alone (B) or in combination with vincristine, doxorubicin and cisplatin (C) are shown.

Wallichinine (**Figure 1A**) is a compound isolated from *piper wallichii* and works as an antagonist of platelet activiating factor receptor to inhibit the gathering of blood platelet [7-9]. In this study, we found that wallichinine is also an inhibitor of ABCB1 and demonstrated that wallichinine can reverses ABCB1-mediated multidrug resistance by in MDR cancer cells.

Materials and methods

Cell culture and reagents

The ABCB1-overexpressing MDR cancer cells KB_{V200} were generated from human cancer cells KB by stepwise exposure to increasing doses of vincristine respectively [10, 11]. All cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin (100 ng/ml) in a humidified incubator at 37°C with 5% CO_2. Vincristine, doxorubicin and cisplatin were ordered from LC Laboratories. Verapamil and rhodamine 123 were purchased from Sigma-Aldrich. Methylthiazolyldiphenyl-tetrazolium bromide (MTT) was from ApexBio Technology. Propidium iodide (PI) and other chemicals were purchased from Shanghai Sangon Biotech. Wallichinine (**Figure 1A**) was dissolved in DMSO as the stock concentration of 10 mM. P-gp-Glo™ Assay Systems (V3601) was acquired from Promega. Anti-PARP (9542), Anti-cleaved PARP (5625) antibodies were from Cell Signaling Technologies. Anti-ABCB1 (SC-13131) antibody was from Santa Cruz Biotechnology. Anti-GAPDH (KM9002) antibody was from Tianjin Sungene Biotech.

Cell viability assay

Cells were firstly seeded into a 96-well plate at a density of 5000 cells per well, and incubated with drugs in three parallel wells for 72 hours. Then MTT was added to each well at a final concentration of 0.5 mg/ml. After incubation for 4

Table 1. Summary of the effects of wallichinine on enhancing the sensitivity of vincristine, doxorubicin and cisplatin in KB and KB_{V200} cells

Compounds (μM)	IC_{50} ± SD (fold-reversal)	
	KB	KB_{V200}
Vincristine	0.029±0.009 (1.00)	1.705±0.066 (1.00)
+Wallichinine 10 μM	0.028±0.004 (1.04)	0.231±0.025 (7.38)*
+Verapamil 10 μM	0.027±0.001 (1.07)	0.032±0.001 (53.28)**
Doxorubicin	0.036±0.004 (1.00)	1.546±0.124 (1.00)
+Wallichinine 10 μM	0.032±0.002 (1.12)	0.260±0.015 (5.95)*
+Verapamil 10 μM	0.036±0.003 (1.00)	0.082±0.026 (18.85)**
Cisplatin	2.465±0.332 (1.00)	6.664±0.523 (1.00)
+Wallichinine 10 μM	2.478±0.147 (0.99)	6.482±0.376 (1.03)
+Verapamil 10 μM	2.369±0.224 (1.04)	6.612±0.451 (1.01)

*P<0.05 and **P<0.01 *vs.* corresponding control.

hours, formazan crystals were dissolved in 100 μl of DMSO, and absorbance at 570 nm was measured by plate reader. The concentrations required to inhibit growth by 50% (IC_{50}) were calculated from survival curves using the Bliss method [12, 13].

Cell cycle analysis

Cells were harvested and washed twice with cold phosphate-buffered saline (PBS), then fixed with ice-cold 70% ethanol for 30 minutes at 4°C. After centrifugation at 200 × g for 10 minutes, cells were washed twice with PBS and resuspended with 0.5 ml PBS containing PI (50 μg/ml), 0.1% Triton X-100, 0.1% sodiumcitrate, and DNase-free RNase (100 μg/ml), and detected by FCM after 15 minutes incubation at room temperature in the dark. Fluorescence was measuredat an excitation wavelength of 480 nm through a FL-2 filter (585 nm). Data were analyzed using ModFit LT 3.0 software (Becton Dickinson) [14, 15].

Apoptosis assay

Cell apoptosis was evaluated with flow cytometry (FCM) assay. Briefly, cells were harvested and washed twice with PBS, stained with Annexin V-FITC and propidium iodide (PI) in the binding buffer, and detected by FACS Calibur FCM (BD, CA, USA) after 15 minutes incubation at room temperature in the dark. Fluorescence was measured at an excitation wave length of 480 nm through FL-1 (530 nm) and FL-2 filters (585 nm). The early apoptotic cells (Annexin V positive only) and late apoptotic cells (Annexin V and PIpositive) were quantified [16, 17].

Western blot analysis

Cells were harvested and washed twice with cold PBS, then resuspended and lysed in RIPA buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 10 ng/ml PMSF, 0.03% aprotinin, 1 μM sodium orthovanadate) at 4°C for 30 minutes. Lysates were centrifuged for 10 minutes at 14,000 × g and supernatants were stored at -80°C as whole cell extracts. Total protein concentration was determined with Bradford assay. Proteins were separated on 12% SDS-PAGE gels and transferred to polyvinylidene difluoride membranes. Membranes were blocked with 5% BSA and incubated with the indicated primary antibodies. Corresponding horseradish peroxidase-conjugated secondary antibodies were used against each primary antibody. Proteins were detected using the chemiluminescent detection reagents and films [18, 19].

Rhodamine 123 and doxorubicin accumulation assay

Cells were seeded into a 6-well plate at a density of 2.5×10^5 cells/well, pre-incubated with or without inhibitors for 1 hour at 37°C, and incubated with 10 μM rhodamine 123 or doxorubicin for another 2 hours at 37°C. Verapamil was used as the positive inhibitor of ABCB1. After washing three times with PBS, cells were analyzed with FCM as previously described [20, 21].

ABCB1 ATPase assay

The Vi-sensitive ATPase activity of ABCB1 in the membrane vesicles of High Five insect cells was measured as describedin protocol. The membrane vesicles (100 μg of protein/ml) were incubated in ATPase assay buffer (50 mM MES, pH 6.8, 50 mM KCl, 5 mM sodium azide, 2 mM EGTA, 2 mM dithiothreitol, 1 mM ouabain, and 10 mM $MgCl_2$) with or without 0.3 mM vanadate at 37°C for 5 minutes, then incubated with different concentrations of drugs at 37°C for 3 minutes. The ATPase reaction was incubated by the addition of 5 mM Mg-ATP. After incubating at 37°C for 20 minutes, the reactions were stopped by adding 0.1 ml of 5% SDS solution. The liberated inorganic phosphate (Pi)

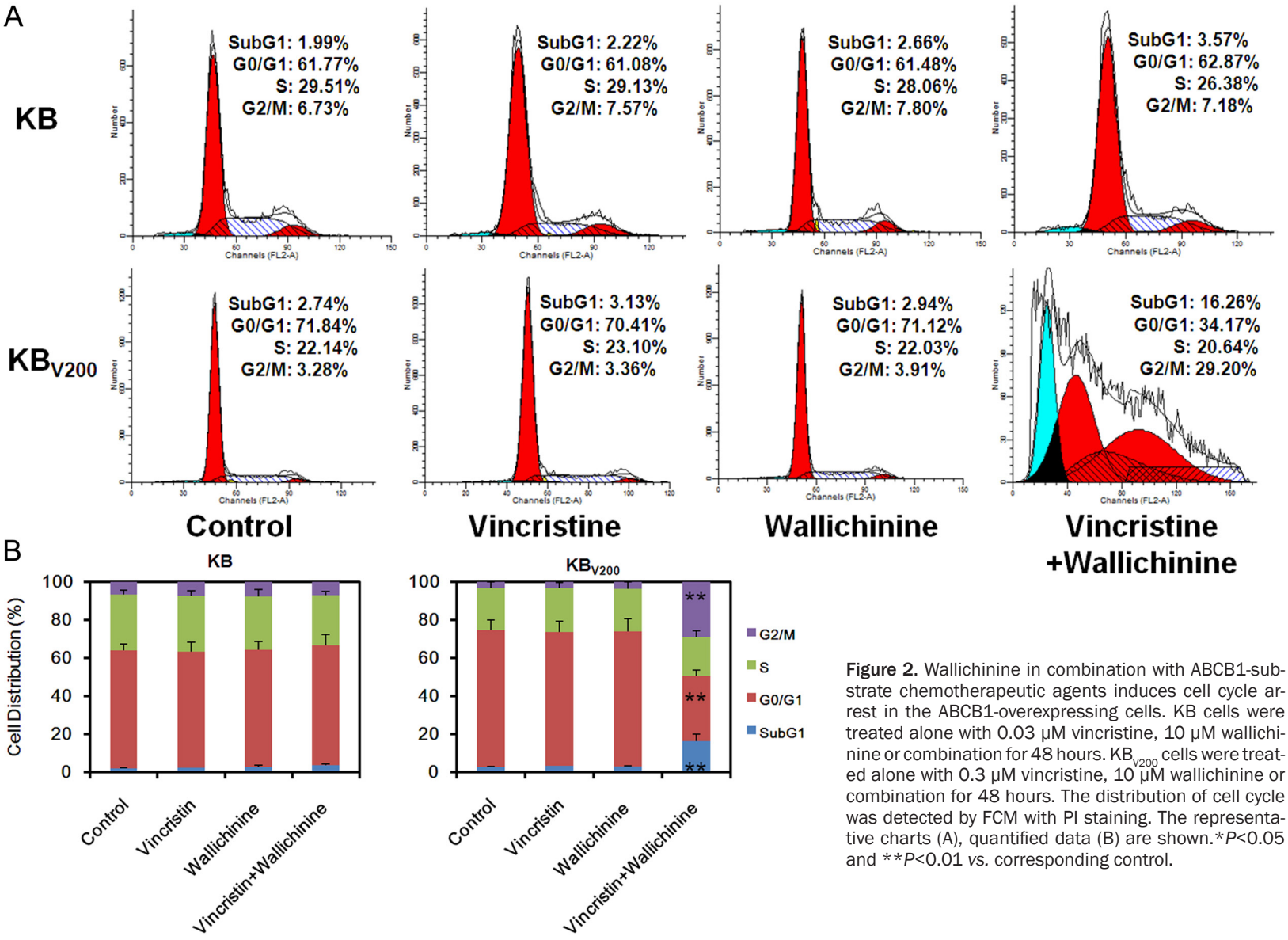

Figure 2. Wallichinine in combination with ABCB1-substrate chemotherapeutic agents induces cell cycle arrest in the ABCB1-overexpressing cells. KB cells were treated alone with 0.03 μM vincristine, 10 μM wallichinine or combination for 48 hours. KB_{V200} cells were treated alone with 0.3 μM vincristine, 10 μM wallichinine or combination for 48 hours. The distribution of cell cycle was detected by FCM with PI staining. The representative charts (A), quantified data (B) are shown.*P<0.05 and **P<0.01 *vs.* corresponding control.

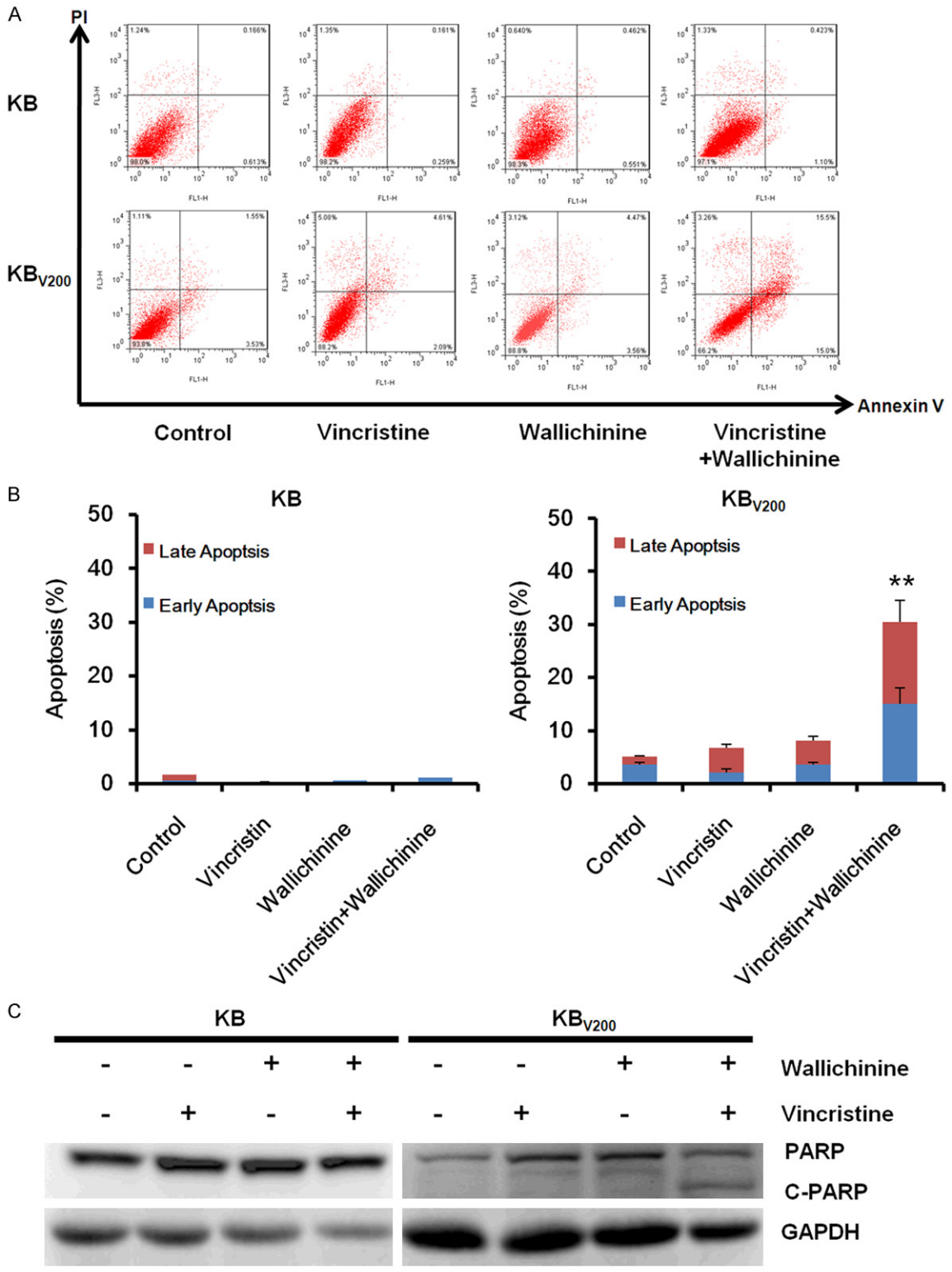

Figure 3. Wallichinine in combination with ABCB1-substrate chemotherapeutic agents induces apoptosis in the ABCB1-overexpressing cells. KB cells were treated alone with 0.03 μM vincristine, 10 μM wallichinine or combination for 48 hours. KB_{V200} cells were treated alone with 0.3 μM vincristine, 10 μM wallichinine or combination for 48 hours. The apoptosis was detected by FCM Annexin V/PI staining. The proportions of AnnexinV+/PI- and AnnexinV+/PI+ cells indicated the early and late stage of apoptosis. The protein expression was examined by Western blot after lysing cells, and GAPDH was used as loading control. The representative charts (A), quantified data (B) and Western blot results (C) are shown. *P<0.05 and **P<0.01 *vs.* corresponding control.

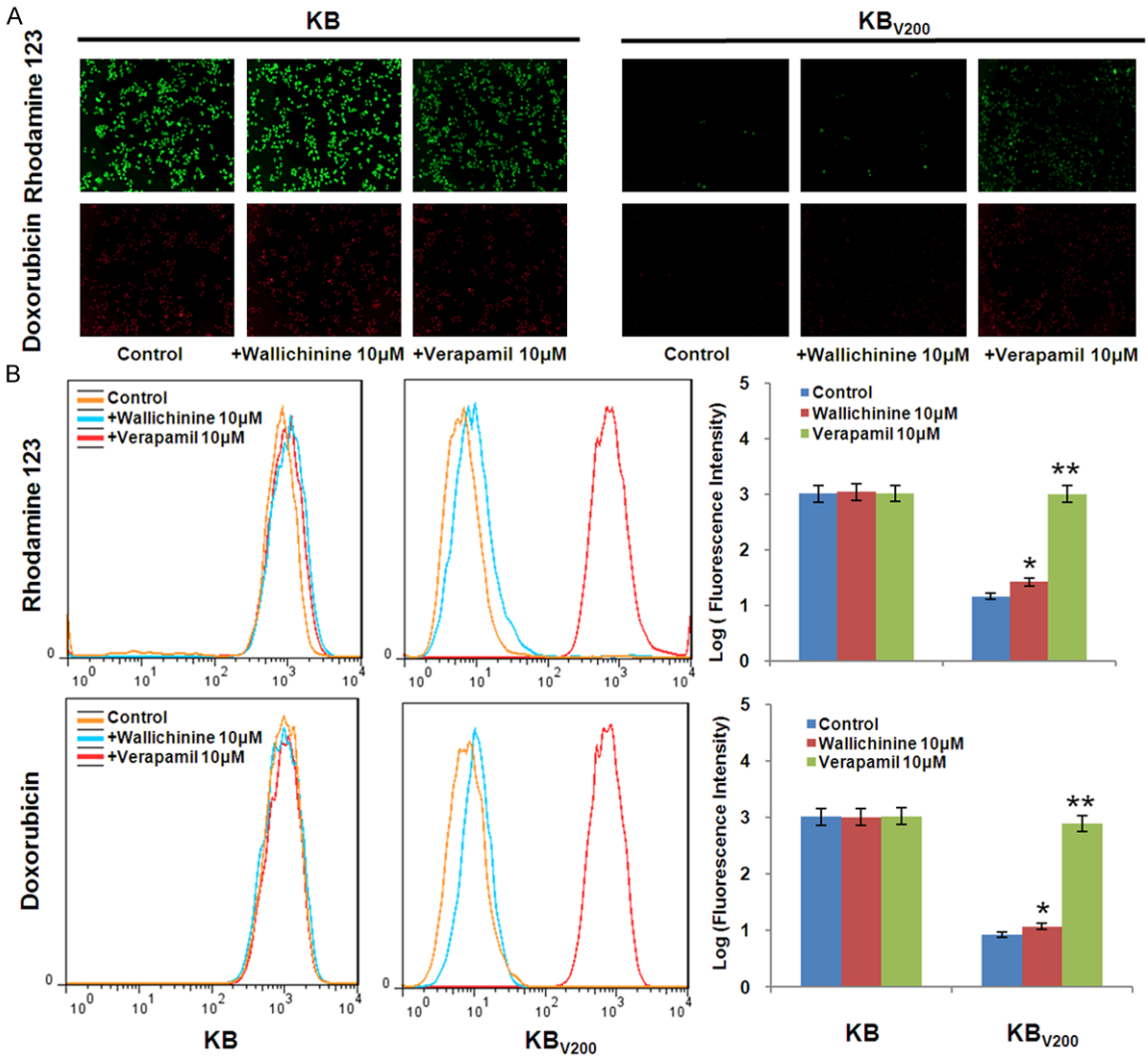

Figure 4. Wallichinine increases the intercellular accumulation of rhodamine 123 and doxorubicin in ABCB1-overexpressing cells. Cells were incubated with 10 μM rhodamine 123 or doxorubicin for another 2 hours at 37°C after pre-treated with 10 μM wallichinine or verapamil for 1 hour at 37°C, measured by FCM and photographed by fluorescent microscope. The representative graphs (A), charts and quantified data (B) are shown.*P<0.05 and **P<0.01 *vs.* corresponding control.

was measured as previously described [22, 23].

Docking protocol

The 3D structure of wallichinine was obtained from the software ChemDraw 7.0. The refined crystal structure of mouse ABCB1 in complex with QZ59-RRR (PDB ID: 4M2S) and QZ59-SSS (PDB ID: 4M2T) [24] was obtained from the RCSB Protein Data Bank. Docking experiments were performed with Discovery Studio 3.0. The top-scoring pose ABCB1 complex was then subjected to energy minimization and used for graphical analysis.

Statistical analysis

A student's t-test was used to compare individual data points among each group. A P-value of <0.05 was set as the criterion for statistical significance.

Results

Wallichinine enhances the sensitivity of ABCB1-substrate chemotherapeutic agents in the ABCB1-overexpressing cells

To investigate the effects of wallichinine on ABCB1-mediated MDR in cancer cells, we firstly examined the cytotoxicity of wallichinine in two

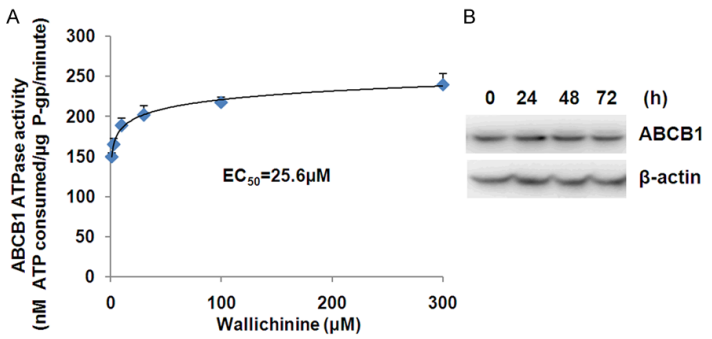

Figure 5. Wallichinine stimulates the ATPase activity of ABCB1 and does not alert the expression of ABCB1. The Vi-sensitive ATPase activity of ABCB1 (A) in membrane vesicles was determined with different concentration (3, 10, 30, 100 and 300 μM) of wallichinine. Cells were treated with wallichinine at 10 μM for the indicated time points, and the protein expression was examined by Western blot after lysing cells, and GAPDH was used as loading control. The representative Western blot results (B) were shown.

ABCB1-overexpressing cells KB_{V200} and its parental cells KB by MTT assay. As shown in **Figure 1B**, over 80% of two cells were viable after treated with wallichinine at 10 μM, indicating that this dose could be used as the highest concentration to explore the ability of wallichinine on enhancing the sensitivity of chemotherapeutic drugs in ABCB1-overexpressing MDR cancer cells. We then tested the cytotoxicity of combination of wallichinine with two ABCB1 substrates vincristine and doxorubicin and one non-ABCB1 substrate cisplatin at the various concentrations. The summary IC_{50} values and survival curves were shown in **Table 1** and **Figure 1C**. Compared with KB, KB_{V200} cells exhibited high resistance to vincristine and doxorubicin but not to cisplatin. Wallichinine at 10 μM significantly decreased the IC_{50} values of vincristine and doxorubicin in KB_{V200} cells but not in KB cells, although its effect is weaker than the known ABCB1 inhibitor verapamil. Furthermore, wallichinine did not significantly alter the cytotoxicity of cisplatin in either MDR or parental cells. Together, our results demonstrated that wallichinine significantly enhanced the sensitivity of ABCB1-substrate chemotherapeutic agents in the ABCB1-overexpressing cells, suggesting wallichinine is able to antagonize ABCB1-mediated cancer MDR.

Wallichinine in combination with ABCB1-substrate chemotherapeutic agents induces cell cycle arrest in the ABCB1-overexpressing cells

To evaluate the effects of wallichinine in combination with chemotherapeutic agents in the ABCB1-overexpressing cells, cell cycle distribution and the related proteins were detected by FCM, respectively. As shown in **Figure 2A** and **2B**, co-treatment with wallichinine and vincristine significantly increased the cell population of sub-G1 and G2/M phase in comparison with wallichinine or vincristine alone treatment in KB_{V200} cells but not in KB cells.

Wallichinine in combination with ABCB1-substrate chemotherapeutic agents induces apoptosis in the ABCB1-overexpressing cells

To further estimate the effects of wallichinine in combination with chemotherapeutic agents in the ABCB1-overexpressing cells, cell apoptosis and the related proteins were also detected by FCM and Western blot, respectively. As shown in **Figure 3A** and **3B**, co-treatment withwallichinineandvincristinedramaticallyenhanced the early apoptosis (Annexin V+/PI-) and late apoptosis (Annexin V+/PI+) and the protein levels of apoptotic marker cleaved PARP (C-PARP) in comparison with wallichinine or vincristine alone treatment in KB_{V200} cells but not in KB cells.

Wallichinine increases the intercellular accumulation of rhodamine 123 and doxorubicin in ABCB1-overexpressing cells

To examine whether wallichinine antagonizing ABCB1-mediated cancer MDR is owing to inhibition of the transporter activity of ABCB1, we measured the intracellular levels of two ABCB1 substrates rhodamine 123 and doxorubicin in the presence or absence of wallichinine. As shown in **Figure 4A** and **4B**, the intracellular levels of both rhodamine 123 and doxorubicin in KB_{V200} cells were significantly lower than those in KB cells, respectively. Wallichinine increased the intracellular levels of rhodamine 123 and doxorubicin in KB_{V200} cells but not in KB cells, although its effect is weaker than verapamil, suggesting that wallichinine is able to directly inhibiting the drug efflux function of ABCB1.

Wallichinine stimulates the ATPase activity of ABCB1 and does not alert the expression of ABCB1

The transporter function of ABCB1 is coupled to ATP hydrolysis, which is stimulated in the

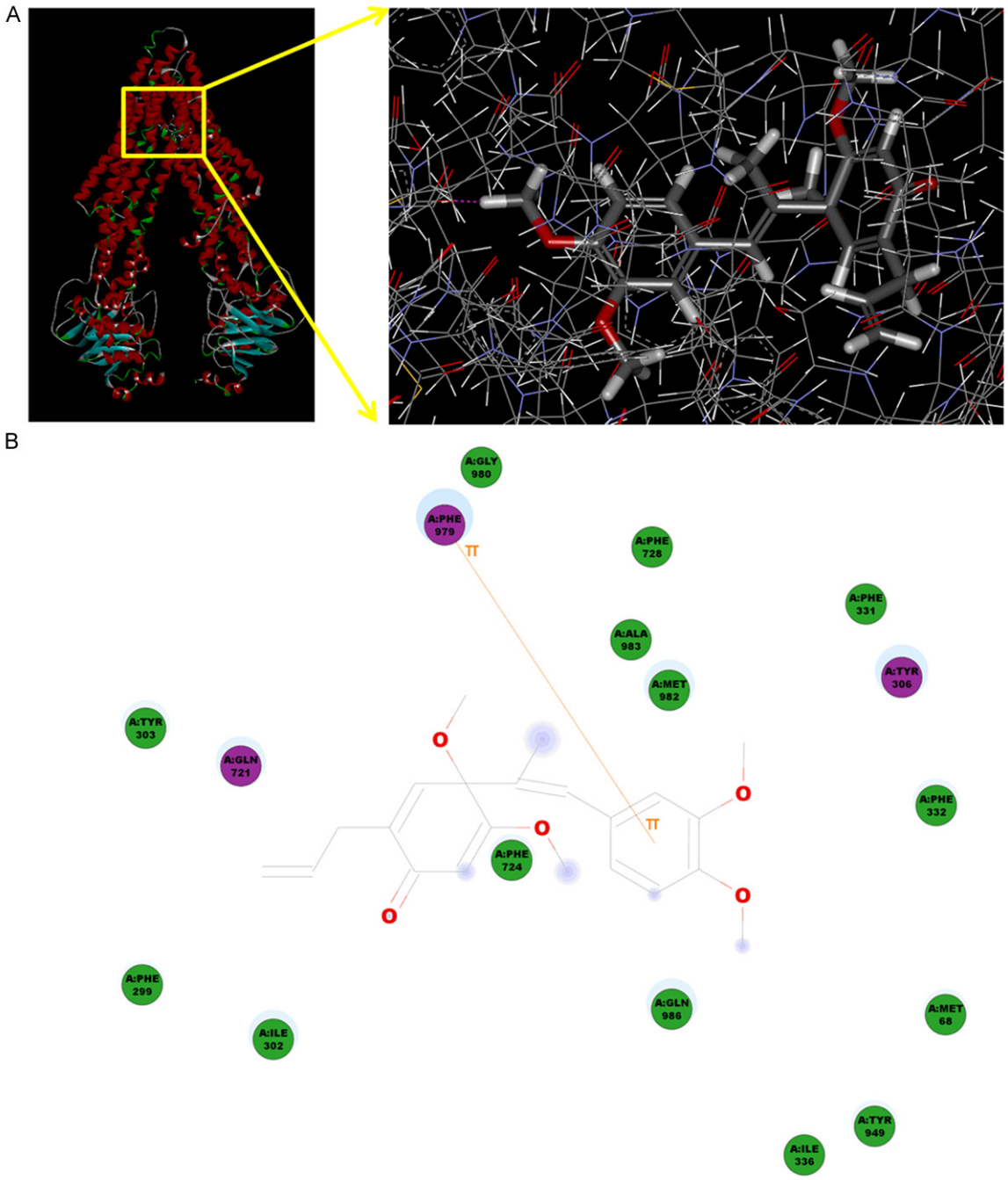

Figure 6. Model for binding of wallichinine to ABCB1. The ribbon diagram of 3D structure conformation (A) and the optimal predicted binding mode (B) of wallichinine within mouse ABCB1 binding site are shown. Important amino acids are depicted as lines with the atoms colored (carbon, gray; hydrogen, white; nitrogen, blue and oxygen, red), and wallichinine is shown as ball and stick model with the atoms colored (carbon, gray; hydrogen, white; nitrogen, blue; oxygen, red; iodine; violet and fluorine; sky-blue). Dotted green line indicates hydrogen bonding interaction. The 2D plane diagram of wallichinine-ABCB1 interaction is also presented. Dotted blue line represents the interaction site of wallichinine and ABCB1.

presence of ABCB1 substrates. To assess the effects of wallichinine on the ATPase activity of ABCB1, we detected the ABCB1-mediated ATP hydrolysis with various concentrations of wallichinine. As shown in **Figure 5A**, wallichinine enhanced the ATPase activity of ABCB1 in the dose-dependent manner with the EC_{50} value of 25.6 μM, suggesting that wallichinine is the

Table 2. Summary of the potential residues in transmembrane (TM) domains of ABCB1 interacting with wallichinine in comparison with QZ59-RRR, QZ59-SSS and verapamil

TM	Residue	QZ59-RRR	QZ59-SSS	Verapamil	Wallichinine
1	His 60			x	
	Ala 63			x	
	Leu 64	x	x	x	
	Met 67		x		
	Met 68	x	x		x
	Phe 71	x	x		
2	Tyr114		x		
	Val121			x	
4	Ser218			x	
5	Met295		x		
	Phe299		x		x
	Ile302		x	x	x
	Tyr303		x		x
	Tyr306	x	x		x
	Phe310				x
6	Phe331				x
	Phe 332	x	x		x
	Leu 335		x	x	x
	Ile 336	x	x		x
	Ala 338			x	
	Phe 339	x	x		
7	Asn 717		x		
	Gly718				
	Gln 721	x	x		x
	Phe 724	x	x	x	x
	Ala725			x	x
	Phe 728	x	x		x
8	Phe 766		x		
9	Gln834				
	Ala837			x	
	Asn838			x	
10	Ile 864			x	
	Ala 867			x	
	Gly 868			x	
11	Phe938			x	
	Thr941			x	
	Met945		x		
	Tyr949	x	x		x
	Phe953		x		
12	Leu 971		x	x	
	Phe 974	x	x		
	Ser 975	x			x
	Val 978			x	
	Phe979	x	x		x
	Gly 980			x	x
	Ala 981			x	
	Met 982	x	x		x
	Ala983	x	x		x
	Gln 986	x	x		x
	Val987		x		

Wallichinine facing the internal cavity of P-gp within the lipid bilayer for the P-gp structure. The interaction residues in TM helices are indicated. Residues that interact with QZ59-RRR and QSZ59-SSS models, as well as those protected by MTS-verapamil labeling by verapamil are marked. "x" stands for the residues of QZ59-RRR model interacting with wallichinine. "x" stands for the residues of QZ59-SSS model interacting with wallichinine. "x" stands for the residues of both QZ59-RRR and QZ59-SSS model interacting with wallichinine.

substrate of ABCB1. In addition, the reversal of ABCB1-mediated MDR can be achieved either by inhibiting its pump activity or by decreasing its expression. To study the effect of wallichinine on ABCB1 expression, the protein levels were detected by Western blot after treatment with wallichinine at 10 μM at the various time points. The results showed that the protein levels of ABCB1 were not altered in KB_{V200} cells after wallichinine treatment even up to 72 hours (**Figure 5B**). These data indicate that wallichinine is able to inhibit the activity of MEK but unable to alter the protein expression of ABCB1.

Model for binding of wallichinine to ABCB1

To understand the mechanism of binding of wallichinine to ABCB1 at a molecular level, docking studies were performed with the crystal structure of mouse Mdr3 as represented by ABCB1-QZ59-RRR, ABCB1-QZ59-SSS and ABCB1-verapamil. As shown in **Figure 6A** and **6B**, the predicted binding mode showed the hydrophobic interactions of wallichinine within the large drug binding cavity of ABCB1. Wallichinine is stabilized through specific interactions such as hydrogen bonding and nonspecific interactions such as hydrophobic interactions with residues in the hydrophobic cavity of ABCB1. The benzene ring of wallichinine showed ligand bumps interaction with the side chain of Phe979. The other groups of wallichinine are mainly stabilized through hydrophobic contacts within the large hydrophobic pocket formed by the side chains of Tyr303, Tyr306, Gln721, Ala725, Phe979, and Gln986 (**Figure 6C** and **Table 2**).

Discussion

Natural products play an important role in the prevention and treatment of cancer and other disease in the world. A large varieties of natural products not only provide useful tools for the study of major pharmacological properties, but also contribute to discovering a number of drugs [25-27]. Currently, natural products account for over half of clinical drugs, especially anticancer drugs, where camptothecin analogues, vinca alkaloidsand taxanes are widely used to treat multiple types of cancers. We and others have found a lot of natural products are substrates or modulators of ABCB1, including sipholenol A [28], tetrandrine [29], FG020326 [30], ONO-1078 [31], agosterol A (AG-A) [32], etc. In this study, we have showed that wallichinine potentiatesthe effects of vincristine and doxorubicin on inhibition of growth, arrest of cell cycle and induction of apoptosis in ABCB1 overexpressing cancer cells. Mechanistically, wallichinine blocks the drug-efflux activity of ABCB1 to increase the intracellular accumulation of rhodamine 123 and doxorubicin and stimulates the ATPase of ABCB1 without alteration of the expression of ABCB1. The transport process of ABCB1 is coupled to ATP hydrolysis, and ABCB1 has both basal and stimulated ATPase activity [33, 34]. The interaction of compounds with ABCB1 may affect its ATPase activity [35]. Different compounds may have different effects on the ATPase activity and affinity degrees of ABCB1 [36]. Our data showed that the continuous increase of ATPase activity of ABCB1 was accompanied with the increase of wallichinine concentration, suggesting that wallichinine strongly stimulated the ATPase activity of ABCB1 dose-dependently to enhance the efflux of ABCB1 substrates out of cells. Therefore, wallichinine may work as the substrate to inhibit ABCB1 function.

Quantitative structure-activity relationship analyses on some ABCB1 inhibitors have clearly revealed the major contribution of lipophilicity towards potent ABCB1 inhibitory activity [37, 38]. Indeed, these inhibitors mostly bind to the large hydrophobic cavity in the membrane bilayer portion of the ABCB1. Additionally, number of pharmacophore models for ABCB1 inhibitors have identified features such as hydrophobic, hydrogen bond acceptor, aromatic ring center and positive ionizable groups [39]. Importantly, although these properties are present in several ABCB1 inhibitors, they seem to be only partially overlapping in the spatial arrangement of the pharmacophoric groups, pointing towards the existence of multiple binding sites at ABCB1 [40]. In our binging model, wallichinine is stabilized through specific interactions such as hydrogen bonding and nonspecific interactions such as hydrophobic interactions with residues in the hydrophobic cavity of ABCB1. The benzene ring of wallichinine showed ligand bumps interaction with the side chain of Phe979, and other groups of wallichinine are mainly stabilized through hydrophobic contacts within the large hydrophobic pocket formed by the side chains of Tyr303, Tyr306, Gln721, Ala725, Phe979, and Gln986.

In conclusion, our results shows that wallichinine significantly reverses ABCB1-mediated MDR in cancer cells by directly inhibiting the drug efflux function of ABCB1, resulting in an increase of the intracellular concentration of anticancer drugs. Further study showed that wallichinine stimulated the ATPase activity of ABCB1, which is supported by the predicted binding mode that showed the hydrophobic interactions of wallichinine within the large drug binding cavity of ABCB1. Whether wallichinine contributes to reversal of cancer MDR mediated by ABCB1 *in vivo* remains to be determined. At all, our study of the interaction of wallichinine with ABCB1 presented herein provides valuable clues for the development of novel MDR reversal reagents from natural products.

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Disclosure of conflict of interest

None.

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