Isoreserpine Reverses Multidrug Resistance Mediated by ABCB1

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Abstract: One of the major obstacles to successful cancer chemotherapy is the development of multidrug resistance (MDR) that is associated with the overexpression of ATP-binding cassette (ABC) drug transporter ABCB1 (P-glycoprotein/ MDR1). Currently, the most efficient way to overcome ABCB1-mediated MDR in cancer is by direct inhibition of ABCB1 function. Many drugs with known biological activities have been discovered to inhibit the function of ABCB1 and reverse ABCB1-mediated MDR in cancers. However, clinical trial results suggested that many of these clinically active drugs should not be used as ABCB1 modulators due to direct toxicity or undesirable side effects. In this study, we demonstrated that isoreserpine, an indole alkaloid with relatively low toxicity, can significantly inhibit ABCB1-mediated efflux of calcein-AM, a known substrate of ABCB1, in a dose-dependent manner. Moreover, we showed that at non-toxic concentrations, isoreserpine potently reversed ABCB1-mediated resistance to doxorubicin and colchicine in ABCB1-overexpressing human KB-V-1 epidermal cancer cells. Collectively, our findings revealed that by inhibiting the transport function of ABCB1, isoreserpine can restore drug sensitivity of ABCB1-overexpressing cells to conventional chemotherapeutic drugs. In conclusion, isoreserpine should be further developed into a promising reversal agent for the treatment of MDR in ABCB1-overexpressing cancers.

Keywords: Multidrug resistance, Isoreserpine, ABCB1.

INTRODUCTION

One of the major obstacles to successful cancer chemotherapy is the development of multidrug resistance (MDR). A key feature of this phenomenon is that cancer cells spontaneously become insensitive to multiple structurally unrelated drugs [1]. Some members of the ATP-binding cassette (ABC) protein family can utilize energy derived from ATP hydrolysis to actively transport anticancer agents out of cancer cells, which lead to reduced intracellular drug concentration and cytotoxicity [2]. ABCB1 (P-glycoprotein/ MDR1), a classical ABC transporter composed of two transmembrane domains and two nucleotide-binding domains, is the first human ABC protein discovered to confer resistance to anticancer drugs [3, 4]. Subsequently, other transporters such as the multidrug resistant protein 1 (MRP1/ABCC1) and ABCG2 (BCRP/MXR) were discovered to also reduce the effect of chemotherapy [2]. Collectively, they are capable of transporting the majority of clinically active anticancer agents, including conventional anticancer drugs [5] and many protein kinase inhibitors [6]. Moreover, based on the localization of these transporters in human tissues, their physiological functions can significantly affect the adsorption, distribution, metabolism, elimination and toxicity of a large variety of drugs [7]. Overall, the overexpression of ABC drug transporters can significantly reduce the efficacy of anticancer drugs, which lead to the development of MDR and treatment failure [2]. Therefore, developing ways to modulate the function or expression of these drug transporters has great clinical significance.

Among the various strategies that have been explored, direct and transient inhibition of MDR-linked ABC drug transporters still provides the most efficient way to potentially overcome MDR in cancer patients [8]. The concept of "chemosensitization" here is to utilize low concentrations of a compound that interacts selectively with a particular drug transporter (such as ABCB1) and blocks transporter-mediated drug efflux, thus elevates drug accumulation in MDR cancer cells [2]. For decades, the task of finding a suitable modulator of ABCB1 has been hindered by the lack of specificity and potency, complex pharmacokinetics and the high intrinsic toxicity of candidate compounds [2, 9]. Therefore, researchers are currently investigating the potential of discovering new inhibitors of ABCB1 through various drug screening methods. In this study, isoreserpine was identified using a cell-based chemical screening of bioactive compounds. We demonstrated that isoreserpine selectively inhibits the transport function of ABCB1 and restores drug sensitivity of ABCB1-overexpressing MDR cancer cells to conventional chemotherapeutic drugs.

MATERIALS AND METHODS

Materials

Cell Counting Kit-8 (CCK-8), MTT dye, calcein-AM, pheophorbide A (PhA) isoreserpine, reserpine,

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doxorubicin, colchicine, Ko143 and other chemicals were purchased from Sigma (St. Louis, MO, USA), unless stated otherwise. Tariquidar was purchased from MedKoo Biosciences Inc (Chapel Hill, NC). Dulbecco's Modified Eagle Medium (DMEM), fetal calf serum (FCS), trypsin-EDTA, penicillin, streptomycin and phosphate buffered saline (PBS) were purchased from Gibco, Invitrogen (CA, USA).

Cell Lines

The pcDNA3.1-HEK293, MDR19-HEK293 (HEK293 cells transfected with human ABCB1) and R482-HEK293 (HEK293 cells transfected with human ABCG2) cells were cultured in DMEM, supplemented with 10% FCS, 2 mM L-glutamine, 100 units of penicillin/streptomycin/mL and 2 mg/mL G418 [10]. The ABCG2-overexpressing human breast MCF7-FLV1000 cells, human epidermal tumor line KB-3-1 and its drug-selected ABCB1-overexpressing variant KB-V-1 subline were cultured in DMEM, supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine and 100 units of penicillin/streptomycin/mL (Invitrogen, Carlsbad, CA). MCF7-FLV1000 cells were cultured in the presence of 1 μg/mL flavopiridol, whereas KB-V-1 cells were maintained in media containing 1 mg/mL vinblastine [11]. The ABCG2-overexpressing human colon carcinoma S1-M1-80 cells were cultured in RPMI 1640 medium (Gibco, Invitrogen), supplemented with 10% FCS and 100 units of penicillin/streptomycin/mL (Invitrogen, Carlsbad, CA) and 80 μM of mitoxantrone. Cell lines were generous gifts from Dr. Suresh V. Ambudkar (National Cancer Institute, NIH, Bethesda, MD, USA), all maintained at 37 °C in 5% CO_2 humidified air. Cells were placed in drug-free medium 7 days prior to assay.

Fluorescent Drug Accumulation Assay

ABCB1 and ABCG2-mediated efflux were measured using a FACSort flow cytometer equipped with Cell Quest software (Becton-Dickinson, Franklin Lakes, NJ) as described previously [12]. Fluorescent substrate calcein-AM was used to study ABCB1-mediated drug efflux, whereas PhA was used to study ABCG2-mediated drug efflux. Briefly, cells were harvested after trypsinization by centrifugation at 500 x g and then resuspended in Iscove's modified Dulbecco's medium (IMDM) (Gibco, Invitrogen) supplemented with 5% FCS. Calcein-AM or PhA or was added to 3 x 10^5 cells in 4 mL of IMDM in the presence or absence of tested compound or ABCB1 reference inhibitor tariquidar or ABCG2 reference inhibitor Ko143. The effect of tested compounds or reference inhibitors on fluorescent drug efflux mediated by ABCB1 or ABCG2 was measured and analyzed according to the method described by Gribar *et al.* [13].

Cytotoxicity Assay

CCK-8 and MTT assays were used to determine the sensitivity of cells to the tested compounds according to the method described by Ishiyama *et al.* [14]. Briefly, cells were plated at a density of 5,000 cells per well in 100 μL of culture medium into 96-well plates at 37 °C for 24 h before adding drugs to make a final volume of 200 μL. Cells were incubated for an additional 72 h with various concentrations of drugs before developed with CCK-8 reagent or MTT as described previously [15]. For the reversal of cytotoxicity assays, a nontoxic concentration of tested compound was added to the cytotoxicity assay, and the extent of reversal was then calculated based on the relative resistance values. IC_{50} values were calculated from fitted dose-response curves obtained from at least three independent experiments.

Immunoblotting

Cells were treated with increasing concentrations of isoreserpine for 72 h before harvesting. Crude membrane protein was prepared and subjected to electrophoresis on a 7.5% SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane. Each blot was then incubated in blocking buffer containing 5% (w/v) milk powder in 0.1% TBS-Tween (25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween-20) for an hour prior to the addition of the ABCB1-specific primary antibody C219 or anti-α-tubulin primary antibody for the detection of ABCB1 or tubulin as positive control. The secondary antibodies used were the Horseradish peroxidase-conjugated goat anti-mouse IgG and anti-rabbit IgG. Signals were detected as described previously [10, 16, 17].

Statistical Analysis

GraphPad Prism software (La Jolla, CA, USA) was used to plot the curves and statistical analysis. Data are presented as mean ± S.E.M, whereas IC_{50} values were calculated as mean ± SD from at least three independent experiments. Differences between any mean values were analyzed by two-sided Student's t-test and results were considered statistically significant at $P < 0.05$.

RESULTS

Isoreserpine Inhibits Fluorescent Drug Efflux Mediated by ABCB1

In an attempt to identify potential inhibitor of ABCB1, we performed a cell-based chemical screening of bioactive compounds according to the method described by Ansbro *et al.* [18]. Isoreserpine was a candidate identified from the screen that exhibited strong interaction with human ABCB1. Consequently, we sought to investigate the potential application of isoreserpine to re-sensitize ABCB1-overexpressing multidrug resistant carcinoma cells to chemotherapy. Moreover, since the overexpression of ABCG2 has

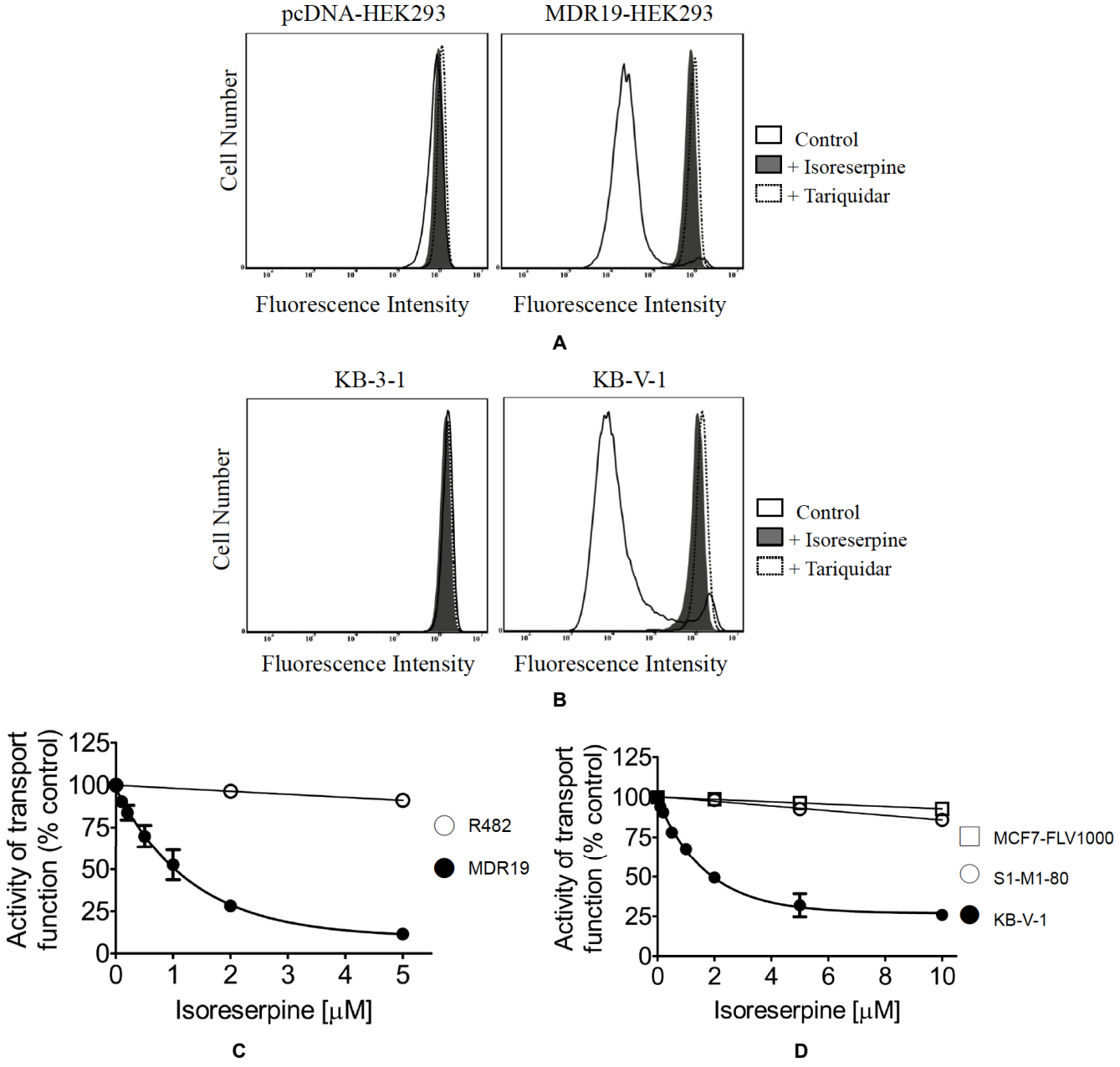

Figure 1: Effect of isoreserpine on the fluorescent substrate transport mediated by ABCB1. The accumulation of fluorescent calcein in drug sensitive parental HEK293 (**A**, *left panel*), ABCB1-transfected MDR19-HEK293 (**A**, *right panel*), parental human epidermal KB-3-1 cancer cells (**B**, *left panel*) and ABCB1-overexpressing MDR KB-V-1 (**B**, *right panel*) cells was measured in the absence (*solid lines*) or presence of 10 μM isoreserpine (*shaded*) or 3 μM of a known ABCB1 inhibitor tariquidar (*dotted lines*). Cells were analyzed immediately by flow cytometry as described in *Materials and Methods*. Representative histograms of three independent are shown. Concentration-dependent inhibition of ABCB1-mediated calcein-AM efflux or ABCG2-mediated PhA efflux by isoreserpine in (**C**) MDR19-HEK293 (*filled circles*) or ABCG2-transfected R482-HEK293 (*open circles*) and in (**D**) ABCB1-overexpressing KB-V-1 (*filled circles*) or ABCG2-overexpressing MCF7-FLV1000 (*open squares*) and S1-M1-80 (*open circles*) cancer cells. The values represent mean ± S.E.M. from at least three independent experiments.

also been shown to play a role in the development of MDR in cancer cells and that ABCB1 interacting compounds often interact with ABCG2 as well [19], we thus examined the selectivity of isoreserpine against both ABCB1 and ABCG2.

First, we determined the effect of isoreserpine on the accumulation of fluorescent dye calcein in HEK293 cells and HEK293 transfected with human ABCB1 (MDR19-HEK293), and in KB-3-1 human epidermal cancer cells and ABCB1-overexpressing MDR KB-V-1 cancer cells. As a positive control, 3 μM of ABCB1 reference inhibitor tariquidar was used for the complete inhibition of ABCB1 function. We discovered that the function of ABCB1 was completely blocked by 10 μM of isoreserpine, which significantly increased the level of calcein accumulation in MDR19-HEK293 cells (Figure **1A**, *right panel*) and KB-V-1 cells (Figure **1B**, *right panel*) without having significant effect on drug sensitive parental HEK293 and KB-3-1 cells (Figure **1A-B**, *left panels*). Moreover, we demonstrated that ABCB1-mediated calcein-AM efflux was inhibited by isoreserpine in a concentration dependent manner, with calculated IC_{50} values of approximately 0.9 μM and 2.3 μM in MDR19-HEK293 cells (Figure **1C**) and KB-V-1 (Figure **1D**), respectively. In contrast, isoreserpine had minor effect on ABCG2 fluorescent substrate pheophorbide A (PhA) accumulation in ABCG2-transfected R482-HEK293 cells (Figure **1C**) and in ABCG2-overexpressing MCF7-FLV1000 and S1-M1-80 cancer cells (Figure **1D**). Of note, Ko143 is known to completely inhibit the function of ABCG2 and was used here as a positive control [20].

Isoreserpine Reverses ABCB1-Mediated Multidrug Resistance in Cancer Cells

Knowing that isoreserpine can selectively inhibit the function of ABCB1, we next determined whether non-toxic concentrations of isoreserpine can selectively re-sensitize ABCB1-overexpressing KB-V-1 to substrate drugs of ABCB1 [2]. We discovered that isoreserpine significantly restored the sensitivity of KB-V-1 cells to doxorubicin (Figure **2A**) and colchicine (Figure **2B**) in a concentration-dependent manner. In Table **1**, the relative resistance factor (R.R) was calculated by dividing the IC_{50} value of drug resistant cells by the IC_{50} value of the respective parental cells in the presence of a particular drug. As a positive control, tariquidar was used here to demonstrate complete reversal of drug resistance mediated by ABCB1. We demonstrated that even at a concentration as low as 500 nM, isoreserpine was able to reverse ABCB1-mediated resistance to doxorubicin and colchicine in KB-V-1 cells, reducing the R.R values from 29 and 39 to values of 6 and 8, respectively (Table **1**).

Short-Term Exposure of Human KB Cancer Cells to Isoreserpine does not Affect ABCB1 Protein Expression

In addition to direct inhibition of ABCB1 function, reversal of ABCB1-mediated MDR can also be achieved by transient downregulation of ABCB1 expression [21, 22]. Therefore, we examined the effect of isoreserpine on ABCB1 protein expression by treating KB-V-1 cells with increasing concentrations of isoreserpine (0 - 1 μM) over 72 h as described in

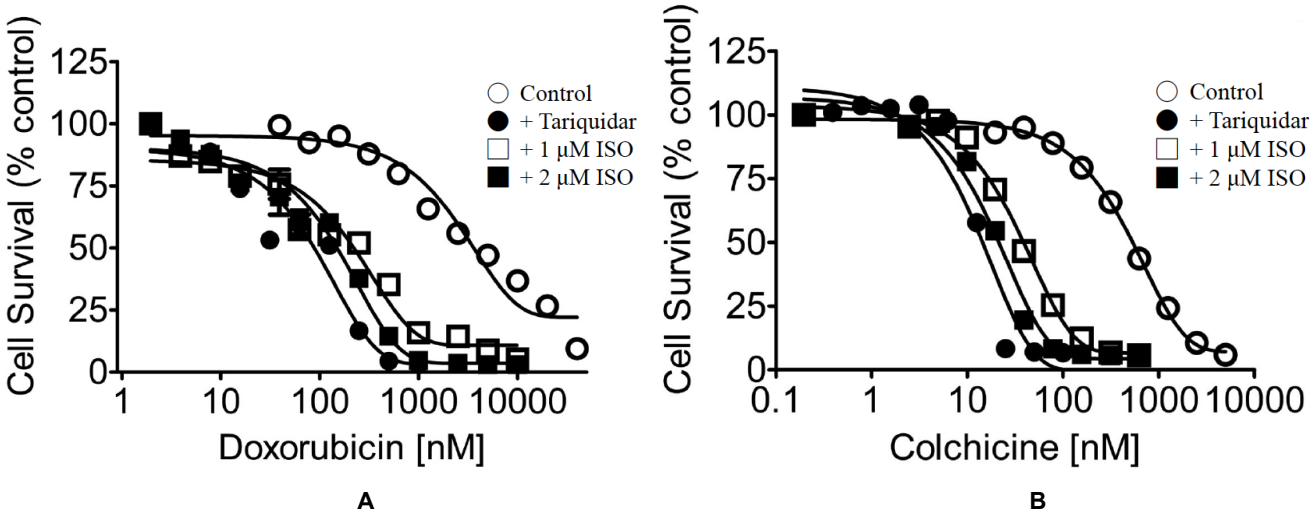

Figure 2: Effect of isoreserpine on ABCB1-mediated MDR in ABCB1-overexpressing KB-V-1 cancer cells. Cytotoxicity of (**A**) doxorubicin or (**B**) colchicine in KB-V-1 cells was measured in the absence (*open circles*) or presence of isoreserpine at 1 μM (*open squares*), 2 μM (*filled squares*) or 1 μM of tariquidar (*filled circles*). Points, means from at least three independent experiments; bars; SEM.

Table 1: The Effect of Isoreserpine on ABCB1-Overexpressing Human Epidermal KB-V-1 Cancer Cells

Treatment	Concentration (μM)	IC_{50} (nM)[†]		R.R[‡]
		KB-3-1 (parental)	KB-V-1 (resistant)	
Doxorubicin	-	154.8 ± 36.9	4552.5 ± 668.8	29
+ Isoreserpine	0.5	169.0 ± 67.9	934.5 ± 197.8***	6
+ Isoreserpine	1.0	170.7 ± 64.7	346.5 ± 54.8***	2
+ Isoreserpine	2.0	129.3 ± 47.6	173.3 ± 28.6***	1
+ Tariquidar	1.0	118.9 ± 36.3	73.1 ± 19.5***	1
Colchicine	-	12.2 ± 4.8	476.7 ± 46.6	39
+ Isoreserpine	0.5	10.9 ± 4.4	88.3 ± 6.4***	8
+ Isoreserpine	1.0	10.1 ± 4.2	32.5 ± 4.9***	3
+ Isoreserpine	2.0	9.1 ± 4.0	17.1 ± 4.2***	2
+ Tariquidar	1.0	11.6 ± 4.8	11.7 ± 4.4***	1

[†]IC_{50} values are mean ± SD calculated from dose-response curves obtained from at least three independent experiments. [‡]R.R,. relative resistance values were calculated by dividing IC_{50} values of ABCB1-overexpressing KB-V-1 cells by IC_{50} values of parental KB-3-1 cells. ***P < 0.001.

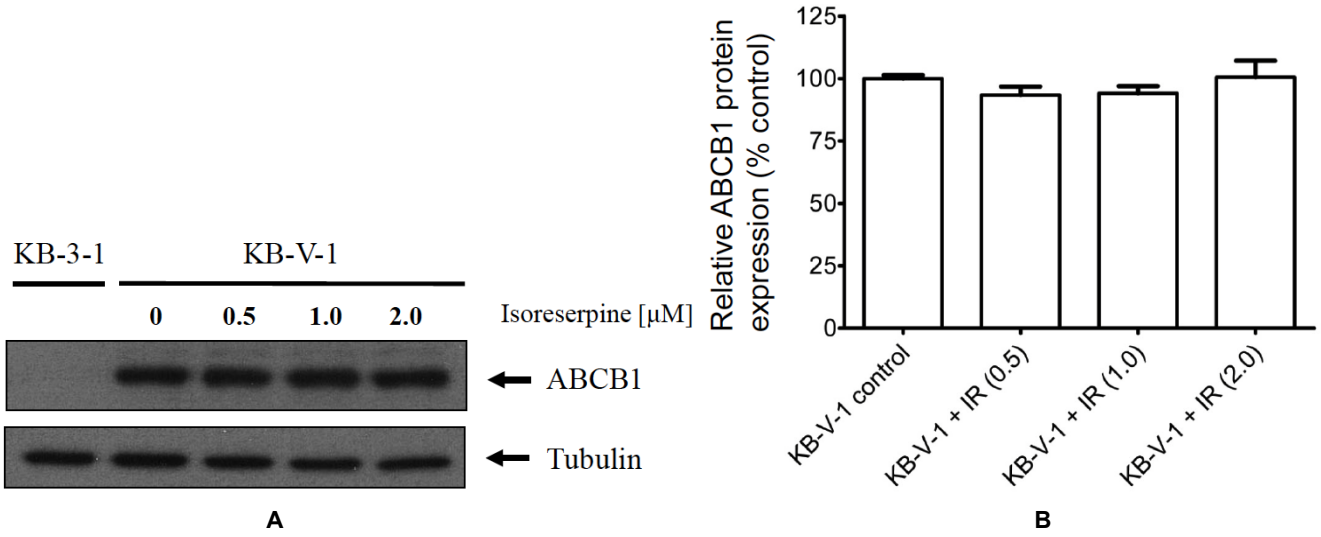

Figure 3: Effect of isoreserpine on protein expression of ABCB1 in human KB epidermal cancer cells. (**A**) Immunoblot detection of human ABCB1 and (**B**) quantification of total lysate protein (10 μg) from KB-3-1 and KB-V-1 cells treated with increasing concentrations of isoreserpine for 72 h as described previously [15]. α-tubulin was used as an internal control for equal loading. Values are presented as mean ± SEM calculated from three independent experiments.

Materials and Methods. As shown in Figure **3**, short-term exposure to isoreserpine had no significant effect on the protein expression level of ABCB1 in KB-V-1 cancer cells. Our data indicate that by inhibiting the function of ABCB1, isoreserpine was able to restore drug sensitivity of human MDR cancer cells to conventional chemotherapeutics without affecting the protein expression of ABCB1.

DISCUSSION

ABCB1 is an effective transporter of many important anticancer agents. The overexpression of ABCB1 has been linked to the development of clinical drug resistance. Moreover, the localization of ABCB1 in normal tissues suggests that ABCB1 may have a significant role in drug adsorption, distribution, metabolism and elimination [7]. Therefore, developing potential agents to modulate the function or expression of ABCB1 has great clinical significance.

Isoreserpine is an indole alkaloid discovered in a screen for potential inhibitors that selectively target the function of ABCB1. In this study, we demonstrated that isoreserpine can inhibit the transport function of ABCB1 to the same extent as the reference ABCB1 inhibitor tariquidar (Figure **1**). Structurally, isoreserpine is a

stereoisomer of reserpine. Previous studies have shown interaction between reserpine and ABCB1 [23], enhanced cytotoxicity of vinblastine in ABCB1-expressing MDR leukemic cells [24, 25] and inhibition of ABCG2 [26, 27] by reserpine. However, in comparison to reserpine, we discovered that isoreserpine was considerably more potent in inhibiting the activity of ABCB1 and had only minor effect on the function of ABCG2 (Figure **1C**, **1D**). The differences between reserpine and isoreserpine were not entirely surprising considering isoreserpine has been reported previously to lack the reserpine-like activity, such as sedative and anti-hypertensive effects [28]. Since the overexpression of ABCB1 has been linked to the development of MDR in cancer, we thus evaluated whether isoreserpine could reverse resistance to known substrate drugs of ABCB1. We found that inhibition of ABCB1-mediated drug efflux by isoreserpine was translated into full restoration of drug sensitivities in ABCB1-overexpressing MDR cells. At non-toxic concentrations, isoreserpine was able to significantly reverse ABCB1-mediated resistance to doxorubicin and colchicine in ABCB1-overexpressing human KB-V-1 cancer cells (Figure **2**). Moreover, we confirmed that expression level of ABCB1 played no role in the re-sensitization process given that ABCB1-overexpressing KB-V-1cells exposed to isoreserpine for 72 hrs did not show a loss of MDR phenotype or protein expression of ABCB1 (Figure **3**).

In conclusion, we identified isoreserpine as a potent modulator of ABCB1 that can be developed into a promising reversal agent for the treatment of MDR in ABCB1-overexpressing cancers.

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