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A novel potent ABCB1 modulator, phenethylisoquinoline alkaloid, reverses multidrug resistance in cancer cell

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Abstract

ATP-binding cassette (ABC) transporters, which are concerned with the efflux of anticancer drugs from cancer cells, have a pivotal role in multidrug resistance (MDR). In particular, ABCB1 is a well-known ABC transporter that develops MDR in many cancer cells. Some ABCB1 modulators can reverse ABCB1-mediated MDR, however no modulators with clinical efficacy have been approved. The aim of this study was to identify novel ABCB1 modulators by using high-throughput screening. Of 5861 compounds stored at Tohoku University, 13 compounds were selected after the primary screening via a fluorescent plate reader-based calcein acetoxymethylester (AM) efflux assay. These 13 compounds were validated in a flow cytometry-based calcein AM efflux assay. Two isoquinoline derivatives were identified as novel ABCB1 inhibitors, one of which was a phenethylisoquinoline alkaloid, ($\pm$)-7-benzyloxy-1-(3-benzyloxy-4-methoxyphenethyl)-1,2,3,4-tetrahydro-6-methoxy-2-methylisoquinoline oxalate. The compound, a phenethylisoquinoline alkaloid, was subsequently evaluated in the cytotoxicity assay and shown to significantly enhance the reversal of ABCB1-mediated MDR. In addition, the compound activated the ABCB1-mediated ATP hydrolysis and inhibited the photolabeling of ABCB1 with [¹²⁵I]iodoarylazidoprazosin. Furthermore, the compound also reversed the resistance to paclitaxel without increasing the toxicity in the ABCB1-overexpressing KB-V1 cell xenograft model. Overall, we concluded that the newly identified phenethylisoquinoline alkaloid reversed ABCB1-mediated MDR through direct interaction with the substrate-binding site of ABCB1. These findings may contribute to the development of more potent and less toxic ABCB1 modulators, which could overcome ABCB1-mediated MDR.

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SUPPORTING INFORMATION

Synthetic procedure for isoquinoline **2** and the spectrum data of synthetic metofoline and Ro-04-2359. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Keywords

Multidrug resistance (MDR); ABCB1 modulator; High-throughput screening; Isoquinoline derivatives; phenethylisoquinoline alkaloid

INTRODUCTION

Chemotherapy has a highly important role in treating patients with advanced cancer. However, although some patients are cured, most respond transiently or incompletely. After acquiring resistance to a certain chemotherapeutic drug, cancer cells have cross-tolerance to functionally and structurally unrelated drugs. Due to this phenomenon, so-called multidrug resistance (MDR),^{1,2} chemotherapy is an insufficiently curative treatment. Several different mechanisms involved in the resistance to anticancer drugs have been characterized, and the up-regulation of drug efflux transporters is a representative one.³

Drug efflux transporter, an important MDR mechanism, is represented by ATP-binding cassette (ABC) transporters, which export chemotherapeutic agents from cancer cells.^{4,5} Forty-eight human ABC transporters have been specified and distinctly grouped into 7 subfamilies (ABCA–ABCG) based on their structural features.⁶ Currently, at least 15 ABC transporters are known to export anticancer drugs from cancer cells.⁷ Moreover, ABCB1 (P-glycoprotein, MDR1) is a pivotal ABC transporter that mediates MDR in cancer cells.^{8,9} Conversely, ABCB1 is also expressed in many normal cells, as previously described.¹⁰

Many different types of compounds that inhibit the efflux of anticancer drugs through ABCB1 are known to redress ABCB1-mediated MDR. The first-generation modulators of ABCB1 are represented by verapamil, a calcium channel blocker, and cyclosporin A, an immunosuppressant.^{11,12} The main problem with these drugs was toxicity, as high concentrations are needed to inhibit the transport function of ABCB1. Subsequently, second-generation modulators were developed by structure-activity relationship (SAR)-based studies.^{13,14} Some clinical trials actually demonstrated advantages over the previous compounds, while adverse events related to the chemotherapy were still common.^{15,16} To improve the selectivity and inhibitory effect on ABCB1, third-generation modulators, including elacridar (GF120918),¹⁷ zosuquidar (LY335979),¹⁸ tariquidar (XR9576),¹⁹ and dofequidar (MS-209)²⁰, were developed. However, owing to chemotherapy-related toxicity, no ABCB1 modulators with clinical efficacy have been approved.²¹ Therefore, the development of novel potent ABCB1 modulators is urgently needed to overcome ABCB1-mediated MDR.

To identify novel ABCB1 modulators from a large library, the development of a high-throughput assay to perform primary screening is necessary. Fluorescent substrate efflux assays based on flow cytometry, microscopy cell imaging, or fluorescent microplate readers were previously performed to evaluate the inhibitory effect on ABCB1. Calcein acetoxymethylester (AM) is a known ABCB1 substrate is employed for fluorescent substrate efflux assays because of its non-fluorescence and cell-permeability. The hydrolysis of calcein AM to calcein by intracellular esterase produces green fluorescence, because fluorescent calcein is entrapped in the cells owing to its hydrophilicity.²² Flow cytometry-

based calcein AM efflux assays for high-throughput screening have the capability to screen numerous compounds at once, however the required systems are not widely available,^{23,24} and microscopy-based imaging systems need dedicated equipment for the measurements.²⁵ In contrast, fluorescent plate reader-based calcein AM efflux assays for high-throughput screening, which are less sensitive, can provide a simple screening method for ABCB1 modulators.²⁶

In the present study, a fluorescent plate reader-based calcein AM efflux assay was used for the initial screening, and a flow cytometry-based calcein AM efflux assay was carried out to validate the candidates identified from the screening. Furthermore, each identified ABCB1 modulator was analyzed for its potency to restore ABCB1-mediated MDR and to interact with ABCB1 *in vitro*. Finally, the reversal activity of this ABCB1 modulator to the anticancer drug resistance of an ABCB1 substrate was examined *in vivo*.

EXPERIMENTAL SECTION

Screening chemical compounds.

Tohoku University Graduate School of Pharmaceutical Science own the original chemical library that consists of original chemical compounds, such as alkaloids, macrolides, cyclic peptides, flavones, polyphenols, and heterocyclic compounds, including biologically active natural products.²⁷ The compounds were dissolved in DMSO to a final concentration of 2 $\mu\text{mol/L}$ and 5 μL were aliquoted into 384-well plates. A total of 5861 compounds were provided.

Chemicals.

Calcein AM and cyclosporin A were obtained from Sigma-Aldrich (St. Louis, MO). Rhodamine 123, paclitaxel, vinblastine, doxorubicin, and cisplatin were purchased from Wako (Tokyo, Japan). Tariquidar was purchased from AdooQ BioScience (Irvine, CA). CellTiter 96 AQueous One Solution Reagent was purchased from Promega (Madison, WI). [¹²⁵I]-Iodoarylazidoprazosin (IAAP) was obtained from Perkin-Elmer Life Science (Wellesley, MA).

Cell lines.

KB-3-1, a parental human epidermal carcinoma cell line, and the ABCB1-overexpressing KB-V1 cell line, previously selected from KB-3-1, were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich) supplemented with 10% FBS and 1% penicillin-streptomycin (PS) at 37 °C in 5% CO₂.^{28,29} These cell lines were gifts from Dr. Michael M. Gottesman of the National Cancer Institute, Bethesda, MD. KB-V1 cells were ordinarily cultured in medium with vinblastine (1 $\mu\text{g/mL}$), then the medium was changed to a vinblastine-free one 2 days before each experiment. HCT-15, a human colon adenocarcinoma cell line expressing endogenous ABCB1 without exposure to any chemotherapy drugs, was cultured in RPMI 1640 (Sigma-Aldrich) supplemented with 10% FBS and 1% PS. This cell line was obtained from Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer Tohoku University (Sendai, Japan).

Reverse transcription and quantitative PCR.

The mRNA expression of ABC transporters in each cell line was evaluated by reverse transcription (RT) and quantitative PCR (qPCR), as previously described.³⁰ Total RNA isolation from each cell line was made by the RNeasy Mini Kit (Qiagen, Hilden, Germany). Then, reverse transcription was performed by the PrimeScript RT reagent kit (Takara Bio, Shiga, Japan). Subsequently, the samples were mixed with SYBR Premix Ex Taq II (Takara Bio) and real-time PCR analysis was performed by the StepOnePlus Real Time PCR System (Life Technologies, Foster City, CA). The primers for ABCB1 were 5'-CAGAGGGGATGGTCAGTGTT-3' (forward) and 5'-CCTGACTCACCACACCAATG-3' (reverse), the primers for ABCG2 were 5'-GACTTATGTTCCACGGGCCT-3' (forward) and 5'-TCTCTGTTTAATGCCACAGCA-3' (reverse), the primers for ABCC1 were 5'-GGTCAGCCCAACTCTCTTGG-3' (forward) and 5'-CACACACTAGGGCTACCAGC-3' (reverse), and the primers for GAPDH were 5'-GCACCGTCAAGGCTGAGAAC-3' (forward) and 5'-TGGTGAAGACGCCAGTGGA-3' (reverse).

Plate reader-based calcein AM efflux assay for high-throughput screening.

KB-V1 cells were seeded at 1×10^4 cells/well (20 μ L) in 384-well black plates. After incubation for 30 min, the screening compounds and calcein AM were added to each well. Instead of the screening compounds, cyclosporin A and phosphate buffered saline (PBS, Thermo Fisher Scientific, Waltham, MA) as a control were also used. In this assay, the Biomek NXP (Beckman Coulter, Indianapolis, IN) was used to dispense the cells and chemicals into 384-well plates. The final reaction volume of each well was 40 μ L and the final concentration of screening compounds, cyclosporin A, and calcein AM were 10 μ mol/L, 10 μ mol/L, and 0.25 μ mol/L, respectively. After incubation for 2 h, the fluorescence intensity of the plate (λ excitation = 490 nm, λ emission = 515 nm) was measured by the SpectraMax M2e (Molecular Devices, Sunnyvale, CA). To select the candidate compounds that interacted with ABCB1, the fold-changes in calcein fluorescence of KB-V1 cells incubated with the screening compounds relative to the control were calculated.

Flow cytometry-based assay.

KB-3-1, KB-V1, or HCT-15 cells (5×10^5 cells/mL) were incubated with an ABCB1 inhibitor (a candidate compound or cyclosporin A) and a fluorescent substrate (0.25 μ mol/L calcein AM or 1.5 μ mol/L rhodamine 123) for 45 min at 37 °C in Iscove's Modified Dulbecco's Medium (IMDM, Thermo Fisher Scientific) supplemented with 5% FBS. Subsequently, the cells were washed and resuspended in PBS. Then, the fluorescence intensity was immediately measured by the BD FACS Verse™ flow cytometer (BD Biosciences, San Jose, CA). To validate the candidate compounds that interacted with ABCB1, the fold-changes in the calcein fluorescence of KB-V1 cells incubated with the screening compounds relative to the control were calculated.

Cytotoxicity assay.

The reversal effects of ABCB1 modulators on the cytotoxicity of anticancer drugs in KB-3-1, KB-V1, or HCT-15 cells were evaluated by CellTiter 96 AQueous One Solution Cell Proliferation Assay (MTS assay). Briefly, 4×10^3 KB-3-1 cells, 6×10^3 KB-V1 cells, or $3 \times$

10³ HCT-15 cells, were dispensed in 96-well plates and then incubated for 24 h. Subsequently, both the ABCB1 modulators and varying concentrations of anticancer drugs (paclitaxel, vinblastine, doxorubicin, and cisplatin) were added in each well. After an additional 72 h incubation, each well was resuspended in 100 μ L of culture medium with 20 μ L CellTiter 96 AQueous One Solution Reagent and incubated for 1 h. The absorbance at 492 nm was measured by the Multiskan™ FC (Thermo Fisher Scientific). The cell viability (%) was calculated from the following formula: ((absorbance in ABCB1 modulators with anticancer drugs) – (absorbance in blank)) ÷ ((absorbance in ABCB1 modulators only) – (absorbance in blank)) × 100. GraphPad Prism 7 (GraphPad Software Inc., La Jolla, CA) was used to calculate the IC₅₀ values.

ATPase assay of ABCB1.

To evaluate the ABCB1-mediated ATP hydrolysis activity, ATPase assay was performed as previously described.^{31,32} After the addition of varying concentrations of the novel ABCB1 modulator, ABCB1-expressing membrane vesicles (6–10 μ g protein) were incubated in ATPase buffer with or without sodium orthovanadate (Vi). Subsequently, 5 mmol/L ATP was added to initiate the reaction and incubated for 20 min at 37°C. Further, the reaction was finished by the addition of sodium dodecyl sulfate solution. Then, the amount of inorganic phosphate released was determined by colorimetric method as previously described.³¹

Photolabeling of ABCB1 with [¹²⁵I]IAAP.

To assess the novel ABCB1 modulator that interacts with the substrate-binding site of ABCB1, [¹²⁵I]IAAP photolabeling assay was carried out as previously described.^{33,34} Crude membranes from Hi-five cells overexpressing human ABCB1 were incubated with varying concentrations of the novel ABCB1 modulator for 10 min at 37°C. The samples were treated with 3 nmol/L [¹²⁵I]IAAP (2,200 Ci/mmol) and irradiated with a UV lamp (365 nm) on ice cold water for 10 min. Then, photolabeling of ABCB1 with [¹²⁵I]IAAP was evaluated as previously described.³³

Reversal of ABCB1-mediated MDR in xenograft model.

KB-V1 cells (2×10⁶ cells, 0.1 mL) in FBS-free DMEM and 0.1 mL Matrigel Matrix (Corning, NY) were mixed and subcutaneously injected into the shoulder of 6-week-old BALB/c nu/nu mice (CLEA Japan, Inc., Tokyo, Japan). When the tumor diameter reached to 0.5–1.0 cm, the mice were allocated to four groups: a) control (saline), b) isoquinoline **2** (15 mg/kg), c) paclitaxel (18 mg/kg), and d) the simultaneous administration of isoquinoline **2** (15 mg/kg) and paclitaxel (18 mg/kg). The treatments were performed by intraperitoneal injection every 3 days and repeated six times (day 0, 3, 6, 9, 12, 15). The body weight of the mice and tumor volume were measured every 3 days. TV was calculated from the following formula: tumor volume = length × width² × 0.5. This animal experiment was approved by the Animal Care and Use Committee of Tohoku University Graduate School of Medicine (2017MdA-174). All mice were handled according to the Guidelines for the Care and Use of Laboratory Animals of Tohoku University

Statistical analysis.

The screening chemical compounds were treated in two independent experiments. At least three independent experiments were conducted with the novel ABCB1 modulator identified from the screening and validation. The data were presented as the mean $\pm$ SE and two-tailed Student's *t* test was performed to assess the differences between the means. The results were considered to be statistically significant at $P < 0.05$. Statistical analyses were conducted with R Statistics version 3.2.2 or GraphPad Prism 7.

RESULTS

Confirmation of the mRNA expression of ABC transporters on each cancer cell line.

The mRNA expression of ABCB1 in KB-V1 cells and HCT-15 cells was 16906-fold and 1292-fold higher than that in KB-3-1 cells, respectively, as determined by RT-qPCR (Figure 1A). In contrast, the mRNA expression of ABCG2 and ABCC1 in KB-V1 cells was similar to that of parental KB-3-1 cells (1.24-fold and 0.80-fold, respectively). Both the KB-V1 and HCT-15 cell lines, which express a high level of ABCB1, are well-characterized, as previously described.^{25,35}

Fluorescent plate reader-based calcein AM efflux assay for primary high-throughput screening.

To exclude the compounds that exhibited green fluorescence similar to calcein before screening, the fluorescence intensity of 5861 compounds was measured in each plate. Only one compound was highly fluorescent. In this assay, the fold-change in fluorescence intensity of each of the 5860 compounds relative to the control in ABCB1-overexpressing KB-V1 cells was measured to identify novel ABCB1 inhibitors. The results of the primary high-throughput screening are presented in a dot plot (Figure 1B). Fifty-three compounds showed a 2-fold or greater increase in fluorescence intensity. Furthermore, 13 compounds showed a 3-fold change fluorescence intensity, which was the same as that induced by cyclosporin A (3.31-fold $\pm$ 0.33).

Candidate compound validation in flow cytometry-based calcein AM efflux assay.

To validate the candidate compounds, a flow cytometry-based calcein AM efflux assay was performed. A fluorescent plate reader measures one signal from the whole contents of each well, while flow cytometry can measure thousands of signals from individual cells at once. In this assay, 10 μ mol/L of two candidate compounds increased the calcein fluorescence of KB-V1 cells by 61-fold (isoquinoline **1**) and 69-fold (isoquinoline **2**) respectively, which was similar to the 66-fold increase observed in 10 μ mol/L cyclosporin A (Figure 1C). As a result, two compounds were selected for the candidate ABCB1 inhibitors (Figure 1D). The chemical structures of isoquinoline **1** and isoquinoline **2**, shown in Figure 1E, contain the same core isoquinoline structure. Both isoquinoline **1** and isoquinoline **2** induced the increased calcein fluorescence of KB-V1 cells concentration-dependently at 1, 5, and 10 μ mol/L (Figure 2A and B). In contrast, the high calcein fluorescence of parental KB-3-1 cells was not increased in the presence of either 10 μ mol/L of isoquinoline **1** or isoquinoline

2 (Figure 2C and D). Therefore, these two isoquinoline derivatives were identified from the screening of 5861 compounds as novel ABCB1 inhibitors.

The effect of isoquinoline **2** as an ABCB1 inhibitor was demonstrated in another ABCB1-overexpressing cell line, HCT-15 cells. Isoquinoline **2** enhanced the fluorescence intensity of calcein in HCT-15 cells concentration-dependently at 0.1, 1, and 10 $\mu\text{mol/L}$ (Figure 2E).

Effect of isoquinoline 2 in flow cytometry-based rhodamine 123 accumulation assay.

Rhodamine 123, a cell-permeant and green-fluorescent compound, is a known ABCB1 substrate. ABCB1 contains multiple binding sites for many different drugs, and rhodamine 123 interacts with a different binding site from calcein AM. However, data about the number of binding sites for each drug are scarce.³⁶ As shown in Figure 2F, isoquinoline **2** increased rhodamine 123 fluorescence (accumulation) in KB-V1 cells concentration-dependently similar to calcein fluorescence (a fluorescent product of calcein AM). However, 1 $\mu\text{mol/L}$ of isoquinoline **2** was insufficient to increase the fluorescence intensity of rhodamine 123.

Isoquinoline 2, a potent ABCB1 modulator that increases the chemo-sensitivity.

The cytotoxic and modulatory effects of the two isoquinoline derivatives (isoquinoline **1** and **2**) in ABCB1-overexpressing cells were determined by MTS assay. Five $\mu\text{mol/L}$ isoquinoline **2** (IC_{50} : $0.036 \pm 0.0019 \mu\text{mol/L}$) enhanced the paclitaxel-induced cytotoxicity to a greater extent than isoquinoline **1** (IC_{50} : $1.08 \pm 0.060 \mu\text{mol/L}$) in KB-V1. In addition, 10 $\mu\text{mol/L}$ isoquinoline **2** strongly enhanced the paclitaxel-induced cytotoxicity (IC_{50} : $0.0091 \pm 0.00016 \mu\text{mol/L}$), whereas no cells survived, regardless of the paclitaxel concentration, in 10 $\mu\text{mol/L}$ isoquinoline **1**. Furthermore, for the cytotoxicity of isoquinoline **1**, the mean IC_{50} value was $6.47 \pm 0.22 \mu\text{mol/L}$ in KB-V1. These results show that isoquinoline **2** strongly and safely modulated the effect of paclitaxel.

Isoquinoline **2**, ($\pm$)-7-Benzyloxy-1-(3-benzyloxy-4-methoxyphenethyl)-1,2,3,4-tetrahydro-6-methoxy-2-methylisoquinoline oxalate, was synthesized from 2-(4-(benzyloxy)-3-methoxyphenyl)ethanamine and 3-(3-(benzyloxy)-4-methoxyphenyl)propanoic acid via condensation, following Bischler-Napieralski reaction, and reductive methylation. The detailed experimental procedures and chemical data of the synthetic compounds are in the supporting information.

To examine the cytotoxic effect of isoquinoline **2** in other cell lines, MTS assay was also performed. The mean IC_{50} values of isoquinoline **2** for cytotoxicity were $11.5 \pm 0.54 \mu\text{mol/L}$ in KB-3-1, $15.1 \pm 0.07 \mu\text{mol/L}$ in KB-V1, and $17.9 \pm 0.19 \mu\text{mol/L}$ in HCT-15 (Table 1). Furthermore, the concentration of isoquinoline **2**, in which 90% or more of the cells were alive, was up to 5 $\mu\text{mol/L}$ in KB-3-1 cells and 10 $\mu\text{mol/L}$ in KB-V1 and HCT-15 cells. In parental KB-3-1 cells, isoquinoline **2** did not enhance the cytotoxicity of paclitaxel, vinblastine, or doxorubicin (Table 2). In KB-V1 cells, isoquinoline **2** significantly induced a dose-dependent enhancement of the cytotoxic effect of paclitaxel, vinblastine, and doxorubicin; however, isoquinoline **2** did not enhance the cytotoxicity of cisplatin that shows no interaction as a substrate of ABCB1 (Table 2). The MDR reversal activity of known ABCB1 modulators, cyclosporin A and tariquidar, was estimated in comparison with

isoquinoline **2**. As shown in Table 2, 0.1 $\mu\text{mol/L}$ tariquidar increased the chemo-sensitivity of cells to paclitaxel, vinblastine, and doxorubicin more strongly than 10 $\mu\text{mol/L}$ cyclosporin A. Moreover, 10 $\mu\text{mol/L}$ isoquinoline **2** reversed the resistance to paclitaxel by 1555-fold, which was similar to the level of the MDR reversal activity exhibited by 0.1 $\mu\text{mol/L}$ tariquidar (1790-fold). Similarly, isoquinoline **2** reversed the resistance to vinblastine and doxorubicin concentration-dependently. When the experiments were repeated in HCT-15 cells, the level of the MDR reversal activity was found to be lower (Table 2); this was attributed to the lower ABCB1 expression in HCT-15 than in KB-V1 (Figure 1A).

Isoquinoline 2 activates ABCB1-mediated ATP hydrolysis.

An ABCB1 substrate stimulates ATP hydrolysis when it interacts with ABCB1. The ATPase activity was measured with varying concentrations of isoquinoline **2** to investigate the influence of isoquinoline **2** on the ATP hydrolysis on ABCB1. As shown in Figure 3A, isoquinoline **2** stimulated the ATP hydrolysis of ABCB1 concentration-dependently. In addition, the concentration of isoquinoline **2** required for 50% stimulation (EC_{50}) was $4.09 \pm 0.89 \mu\text{mol/L}$.

Isoquinoline 2 affects photolabeling of ABCB1 with [^{125}I]IAAP.

[^{125}I]IAAP, a photoaffinity analogue of prazosin, interacts with the substrate binding site of ABCB1. Then, substrates and modulators that interact with ABCB1 inhibit the photolabeling of ABCB1 with [^{125}I]IAAP. As shown in Figure 3B, isoquinoline **2** suppressed the photoaffinity labeling of ABCB1 with [^{125}I]IAAP concentration-dependently. In addition, the concentration of isoquinoline **2** required for 50% inhibition (IC_{50}) was $0.063 \pm 0.012 \mu\text{mol/L}$.

Influence of isoquinoline 2 on the mRNA expression of ABCB1.

Isoquinoline **2** may reverse ABCB1-mediated MDR caused by either a decrease of the ABCB1 expression or inhibition of the ABCB1 function. Therefore, the mRNA expression of ABCB1 after the treatment of isoquinoline **2** was evaluated by RT-qPCR. KB-V1 and HCT-15 cells were cultured with 1 $\mu\text{mol/L}$ isoquinoline **2** for 10 days. In this experiment, KB-V1 cells were cultured without vinblastine to evaluate the influence of isoquinoline **2**, although the KB-V1 cells were usually maintained with vinblastine to maintain the ABCB1 expression. As shown in Figure 3C, no significant difference in the mRNA expression of ABCB1 was detected in the cells manipulated with isoquinoline **2** for 10 days when compared to untreated cells. Isoquinoline **2** did not inhibit ABCB1 expression in ABCB1-overexpressing cells.

Isoquinoline 2 reverses MDR in a KB-V1 cell xenograft model.

The efficacy of isoquinoline **2** in the reversal of resistance to paclitaxel was examined by using an ABCB1-overexpressing KB-V1 cell xenograft model. The intraperitoneal injection of the four regimens was performed and the amount of the tumor volume was measured every 3 days. After treatment for 18 days, the mean tumor volumes in the four treatment groups were $2804 \pm 553 \text{ mm}^3$ for saline treatment, $2709 \pm 194 \text{ mm}^3$ for isoquinoline **2** treatment, $2398 \pm 280 \text{ mm}^3$ for paclitaxel treatment, and $1244 \pm 446 \text{ mm}^3$ for the

combination (isoquinoline **2** and paclitaxel) treatment (Figure 4A). No significant difference was observed in the tumor volume between the saline group and the isoquinoline **2** group. The tumor volume in the paclitaxel-treated group was slightly smaller than that in the saline group. However, the combination treatment remarkably suppressed the tumor growth compared to saline. In addition, the mean tumor weight in the combination treatment group (0.896 ± 0.358 g) was remarkably lower than that in the saline group (2.068 ± 0.359 g) (Figure 4B). Furthermore, no significant body weight loss (Figure 4C) and no mortality were observed at the administered dose in the combination treatment group, which indicated that the combination treatment did not enhance the toxicity.

DISCUSSION

We newly identified a phenethylisoquinoline alkaloid (isoquinoline **2**), ($\pm$)-7-benzyloxy-1-(3-benzyloxy-4-methoxyphenethyl)-1,2,3,4-tetrahydro-6-methoxy-2-methylisoquinoline oxalate, as a novel potent ABCB1 modulator. The primary high-throughput screening of 5861 compounds was performed using a fluorescent plate reader-based calcein AM efflux assay. Subsequently, the ABCB1 inhibitors selected from the screening were validated in a flow cytometry-based calcein AM efflux assay. Isoquinoline **2**, a phenethylisoquinoline alkaloid, has not previously been reported to inhibit ABCB1. As shown in the cytotoxicity assay, isoquinoline **2** also enhanced the reversal of ABCB1-mediated MDR. Furthermore, isoquinoline **2** activated the ABCB1-mediated ATP hydrolysis and affected the photolabeling of ABCB1 with [125 I]IAAP. However, isoquinoline **2** did not inhibit the ABCB1 expression in ABCB1-overexpressing cells. These findings suggested that isoquinoline **2** may have direct interaction with the substrate-binding site of ABCB1. In the next step, the animal experiment was organized to determine if the observed *in vitro* inhibitory effect of isoquinoline **2** on ABCB1 could be extended to the *in vivo* environment. Isoquinoline **2** also improved the resistance to paclitaxel in the ABCB1-overexpressing KB-V1 cell xenograft model.

Two isoquinoline derivatives, isoquinoline **1** and **2**, were identified as novel ABCB1 inhibitors from screening a chemical library of 5861 compounds. The possibility of other isoquinoline derivatives in the chemical library exerting an inhibitory effect on ABCB1 was also considered, because fluorescent plate reader-based calcein AM efflux assays for high-throughput screening have low sensitivity, as previously reported.²⁶ To supplement the results of the high-throughput screening, additional experiments were performed on 20 of the isoquinoline derivatives (except isoquinoline **1** and **2**) in the chemical library. To evaluate the interaction of these isoquinoline derivatives with ABCB1, they were assessed in the flow cytometry-based calcein AM efflux assay. Of these 20 isoquinoline derivatives, nine compounds increased calcein fluorescence intensity from 3-fold to 20-fold (data not shown), which was a smaller increase than that caused by isoquinoline **1** and **2** (> 60-fold). In this study, candidate ABCB1 inhibitors were screened by a fluorescent plate reader-based calcein AM efflux assay for primary high-throughput screening, followed by a flow cytometry-based calcein AM efflux assay for validation. This screening method was simple and useful for the detection of the most potent ABCB1 inhibitors.

Isoquinoline derivatives have been previously reported to inhibit the ABCB1 function.³⁷ Chong et al. reported that metofoline, which is an isoquinoline derivative, inhibited the ABCB1 activity.³⁸ Ramu et al. reported the structural features that interfered with the reversal activity of MDR, which included isoquinoline derivatives. In their article, Ro-04–2359, an isoquinoline derivative, reversed the MDR activity.³⁹ Therefore, we synthesized metofoline and Ro-04–2359 (Supporting information), and evaluated their interactions with ABCB1. In the flow cytometry-based calcein AM efflux assay, these compounds slightly increased the fluorescence intensity (data not shown). In the cytotoxicity assay, 10 $\mu\text{mol/L}$ metofoline or Ro-04–2359 reversed the resistance to paclitaxel by 26.4-fold and 3.9-fold respectively, which was a weaker effect than that by 10 $\mu\text{mol/L}$ isoquinoline **2** (1555-fold). Furthermore, elacridar and tariquidar, which are third-generation modulators, are also isoquinoline derivatives. In the present study, we showed that isoquinoline **2** showed a similar activity to tariquidar in the reversal of ABCB1-mediated MDR (Table 2). SAR studies have been attempted to discover more potent and less toxic modulators of ABCB1. However, SAR studies are extremely hard to perform and, to date, the exact portion contributing to the amelioration of ABCB1-mediated MDR has not been clarified.⁴⁰ Indeed, SAR studies on isoquinoline derivatives have been performed.^{41,42} Although the inhibitory effect of some isoquinoline derivatives for ABCB1 has been reported,^{43–45} no novel compounds have yet been tested in clinical trials. So far, two phase III clinical trials for chemotherapy with tariquidar, a potent ABCB1 inhibitor, were initiated, and both trials were terminated because of chemotherapy-linked toxicity.²¹ In our study, isoquinoline **2** did not show a stronger inhibitory effect on ABCB1 than tariquidar. However, isoquinoline **2**, a newly identified phenethylisoquinoline alkaloid, seemed to have less toxicity in our animal experiments and may be a lead compound for the exploitation of more convincing and less toxic ABCB1 modulators in future studies.

Isoquinoline **2** activated the ABCB1-mediated ATP hydrolysis and abrogated the photolabeling of ABCB1 with [¹²⁵I]IAAP. Therefore, isoquinoline **2** may have direct interaction with the substrate-binding site of ABCB1 as a competitive ABCB1 inhibitor. Because the substrate-stimulated ATPase activity is tightly related to the transport of the drug,³¹ isoquinoline **2** might be a substrate of ABCB1. However, it is not certain that isoquinoline **2** is a substrate of ABCB1, because it was not possible to demonstrate the transport of isoquinoline **2**. To prove that isoquinoline **2** is a substrate of ABCB1, other transport assays are needed, for instance, by using radiolabeled or fluorescent probe conjugated isoquinoline **2**.

To show the effect of isoquinoline **2** in animal experiments, a xenograft model of ABCB1-overexpressing KB-V1 cells in nude mice was established and treated by the injection of paclitaxel and/or isoquinoline **2** into the intraperitoneal cavity. To the best of our knowledge, we are the first to examine the administration of isoquinoline **2**, a phenethylisoquinoline alkaloid, in living mice.^{46,47} Therefore, this was important for evaluating both the reversal of ABCB1-mediated MDR and the safety in living organisms. The combination treatment of isoquinoline **2** and paclitaxel produced a significant inhibitory effect compared to the saline treatment on tumor volume and weight, although the paclitaxel monotherapy at the same concentration did not inhibit the tumor growth. Moreover, no significant body weight loss

and no mortality were observed at the administered dose in the combination treatment. Therefore, the administration of isoquinoline **2** at the dose required to reverse ABCB1-mediated MDR in mice was determined to be safe.

In conclusion, isoquinoline **2**, a phenethylisoquinoline alkaloid identified as a novel ABCB1 modulator, directly interacted with ABCB1, resulting in the restoration of ABCB1-mediated MDR *in vitro*. This reversal also inhibited the tumor growth in the animal experiment. Importantly, these findings may contribute the development of more effective and less toxic ABCB1 modulators, which could overcome ABCB1-mediated MDR.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

High-throughput screening and validation identified two isoquinoline derivatives. **A**, mRNA expression levels were measured by RT-qPCR relative to KB-3-1. Data are mean $\pm$ SE ($n = 3$). **B**, Dot plot representation of fluorescent plate reader-based calcein AM efflux assays for primary high-throughput screening. All screening compounds and cyclosporin A were used in 10 $\mu\text{mol/L}$. Fold fluorescence intensity was calculated relative to control (calcein AM only). Points, mean ($n = 2$ for screening compounds, $n = 36$ for cyclosporin A); bars, SE for cyclosporin A. **C**, In flow cytometry-based calcein AM efflux assay, KB-V1 cells were treated with 10 $\mu\text{mol/L}$ of the candidate compounds or cyclosporin A. Fold fluorescence intensity was calculated relative to control (calcein AM only). Data are from a representative experiment of two independent experiments. **D**, Flow diagram of screening. **E**, Chemical structures of two isoquinoline derivatives.

Figure 2.

Effect of two isoquinoline derivatives on flow cytometry-based efflux assay. Calcein fluorescence of KB-V1 cells treated with 1, 5, 10 μmol/L of isoquinoline 1 (A) or isoquinoline 2 (B). The fluorescence intensities (mean ± SD) at 1, 5, 10 μmol/L of isoquinoline 1 were 433 ± 988 , 2318 ± 1832 , and 4200 ± 2588 , respectively. Similarly, the fluorescence intensities at 1, 5, 10 μmol/L of isoquinoline 2 were 452 ± 867 , 2498 ± 1773 , and 4206 ± 2158 , respectively. As a reference, the calcein fluorescence with 10 μmol/L cyclosporin A (4867 ± 2942) and control (83 ± 871) is included (A and B). Calcein fluorescence of parental KB-3-1 cells in the presence of 10 μmol/L of isoquinoline 1 (7527 ± 4431) (C) or isoquinoline 2 (7454 ± 3664) (D), and control (6261 ± 3518). Calcein fluorescence of HCT-15 cells treated with 0.1, 1, 10 μmol/L of isoquinoline 2 (E). The fluorescence intensities (mean ± SD) at 0.1, 1, 10 μmol/L of isoquinoline 2 were 1518 ± 1435 , 3750 ± 2381 , and 4669 ± 2886 , respectively, and that of control was 786 ± 921 .

Rhodamine 123 fluorescence of KB-V1 cells treated with 1, 5, 10 $\mu\text{mol/L}$ of isoquinoline **2**; the fluorescence intensities (mean $\pm$ SD) at 1, 5, 10 $\mu\text{mol/L}$ of isoquinoline **2** were 65 ± 245 , 658 ± 504 , and 1470 ± 739 , respectively, and the control was 79 ± 251 (**F**). The representative data of three independent experiments are shown. *P* values were determined with the two-tailed Student's *t* test (*, $P < 0.05$ compared with control).

Figure 3.

Effect of isoquinoline 2 on ATPase activity of ABCB1 and [^{125}I]IAAP photolabeling of ABCB1. **A**, ABCB1 expressing membrane vesicles was incubated with varying concentrations of isoquinoline 2 in the presence or absence of 0.25 mmol/L vanadate. The ABCB1-specific activity was recorded as the vanadate-sensitive ATPase activity. The result is a typical experiment of three independent experiments. Data are mean $\pm$ SE of three independent experiments performed in duplicate. **B**, Membrane vesicles from Hi-five cells overexpressing human ABCB1 were incubated with varying concentrations of isoquinoline 2 and treated with 3 nmol/L [^{125}I]IAAP (2,200 Ci/mmol). The autoradiogram and quantification of incorporation of IAAP into the ABCB1 band from three independent experiments. Points, mean ($n = 3$); bars, SE. **C**, KB-V1/ Isoquinoline 2 and HCT-15/ Isoquinoline 2 cells were cultured with 1 μmol/L isoquinoline 2 for 10 days. KB-V1/ Isoquinoline 2 cells were compared with KB-V1 cells cultured without vinblastine. mRNA expression levels of ABCB1 were measured by RT-qPCR relative to the cells in the absence of isoquinoline 2. Data are mean $\pm$ SE ($n = 3$).

Figure 4.

Efficacy of isoquinoline **2** to reverse the resistance to paclitaxel was examined using the KB-V1 cell xenograft model in nude mice. Tumor volume (**A**) and body weight (**C**) were measured every 3 days. **B**, Tumor weight on day 18 post treatment is shown. Columns, mean ($n = 5$); bars, SE. The intraperitoneal injection every 3 days were performed as follows: a) control (saline), b) isoquinoline **2** (15 mg/kg), c) paclitaxel (18 mg/kg), and d) paclitaxel (18 mg/kg) + isoquinoline **2** (15 mg/kg). The treatments were repeated six times (day0, 3, 6, 9, 12, 15). Points, mean ($n = 5$); bars, SE. P values were determined with the two-tailed Student's t test (*, $P < 0.05$ compared with control).

Table 1.

Cytotoxicity of ABCB1 modulators

	$IC_{50} \pm SE$ ($\mu\text{mol/L}$)		
	KB-3-1	KB-V1	HCT-15
Isoquinoline 2	11.5 ± 0.54	15.1 ± 0.07	17.9 ± 0.19
Cyclosporin A	7.95 ± 0.23	23.9 ± 0.60	15.0 ± 0.80
Tariquidar	0.36 ± 0.007	0.21 ± 0.005	0.59 ± 0.012

The values are mean $\pm$ SE of three independent experiments performed in triplicate.

Table 2.

Effect of ABCB1 modulators on reversing ABCB1-mediated drug resistance

	IC ₅₀ ± SE (μmol/L)	Fold reversal
KB-3-1		
Paclitaxel	0.0075 ± 0.00018	1.0
+ 5 μmol/L isoquinoline 2	0.0065 ± 0.00001	1.1
+ 5 μmol/L cyclosporin A	0.0047 ± 0.00001	1.6
+ 0.1 μmol/L tariquidar	0.0050 ± 0.00001	1.5
Vinblastine	0.099 ± 0.0047	1.0
+ 5 μmol/L isoquinoline 2	0.035 ± 0.0036	2.8
+ 5 μmol/L cyclosporin A	0.084 ± 0.0020	1.2
+ 0.1 μmol/L tariquidar	0.071 ± 0.0097	1.4
Doxorubicin	0.17 ± 0.020	1.0
+ 5 μmol/L isoquinoline 2	0.24 ± 0.014	0.7
+ 5 μmol/L cyclosporin A	0.11 ± 0.026	1.5
+ 0.1 μmol/L tariquidar	0.15 ± 0.023	1.1
KB-V1		
Paclitaxel	14.2 ± 1.331	1.0
+ 1 μmol/L isoquinoline 2	0.72 ± 0.031	19.7
+ 5 μmol/L isoquinoline 2	0.036 ± 0.0019	394
+ 10 μmol/L isoquinoline 2	0.0091 ± 0.00016	1555
+ 10 μmol/L cyclosporin A	0.14 ± 0.033	102
+ 0.1 μmol/L tariquidar	0.0079 ± 0.00019	1790
Vinblastine	1.89 ± 0.143	1.0
+ 1 μmol/L isoquinoline 2	0.30 ± 0.018	6.3
+ 5 μmol/L isoquinoline 2	0.0017 ± 0.00017	1115
+ 10 μmol/L isoquinoline 2	0.0017 ± 0.00001	1138
+ 10 μmol/L cyclosporin A	0.024 ± 0.0082	77.3
+ 0.1 μmol/L tariquidar	0.0021 ± 0.00010	908
Doxorubicin	169 ± 71.50	1.0
+ 1 μmol/L isoquinoline 2	12.2 ± 1.800	13.8
+ 5 μmol/L isoquinoline 2	1.34 ± 0.119	126
+ 10 μmol/L isoquinoline 2	0.87 ± 0.034	194
+ 10 μmol/L cyclosporin A	0.36 ± 0.061	468
+ 0.1 μmol/L tariquidar	0.16 ± 0.010	1069
Cisplatin	150 ± 18.60	1.0
+ 1 μmol/L isoquinoline 2	90.7 ± 2.980	1.7
+ 1 μmol/L cyclosporin A	105 ± 3.100	1.4

	IC₅₀ ± SE (μmol/L)	Fold reversal
KB-3-1		
+ 0.1 μmol/L tariquidar	73.1 ± 3.370	2.1
HCT-15		
Paclitaxel	0.24 ± 0.014	1.0
+ 1 μmol/L isoquinoline 2	0.012 ± 0.0008	20.0
+ 5 μmol/L isoquinoline 2	0.0097 ± 0.00173	25.2
+ 10 μmol/L isoquinoline 2	0.0063 ± 0.00219	38.8
Vinblastine	0.085 ± 0.0056	1.0
+ 1 μmol/L isoquinoline 2	0.011 ± 0.0014	7.9
+ 5 μmol/L isoquinoline 2	0.0027 ± 0.00062	31.3
+ 10 μmol/L isoquinoline 2	0.0011 ± 0.00036	80.7
Doxorubicin	2.05 ± 0.127	1.0
+ 1 μmol/L isoquinoline 2	0.28 ± 0.020	7.3
+ 5 μmol/L isoquinoline 2	0.30 ± 0.017	6.7
+ 10 μmol/L isoquinoline 2	0.26 ± 0.033	7.8

The fold reversal of MDR was calculated by dividing the IC₅₀ for cells with the anticancer drug in the absence of ABCB1 modulator by that obtained in the presence of this modulator.