CEP-33779 antagonizes ATP-binding cassette subfamily B member 1 mediated multidrug resistance by inhibiting its transport function

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ABSTRACT

The overexpression of ATP-binding cassette (ABC) transporters often leads to the development of multidrug resistance (MDR), which is the major factor contributing to the failure of chemotherapy. The objective of this study was to investigate the enhancement of CEP-33779, a small-molecule inhibitor of Janus kinase 2 (JAK2), on the efficacy of conventional chemotherapeutic agents in MDR cells with overexpression of P-glycoprotein (ABCB1), multidrug resistance-associated protein 1 (ABCC1) and breast cancer resistance protein (ABCG2). Our results showed that CEP-33779, at nontoxic concentrations, significantly sensitized ABCB1 overexpressing MDR cells to its anticancer substrates. CEP-33779 significantly increased intracellular accumulation and decreased the efflux of doxorubicin by inhibiting the ABCB1 transport function. Furthermore, CEP-33779 did not alter the expression of ABCB1 both at protein and mRNA levels but did stimulate the activity of ABCB1 ATPase. CEP-33779 was predicted to bind within the large hydrophobic cavity of homology modeled ABCB1. In addition, the down-regulation of JAK2 by shRNA altered neither the expression of ABCB1 nor the cytotoxic effect of chemotherapeutic agents in ABCB1-overexpressing cells. Significantly, CEP-33779 enhanced the efficacy of vincristine against the ABCB1-overexpressing and drug resistant KBv200 cell xenograft in nude mice. In conclusion, we conclude that CEP-33779 enhances the efficacy of substrate drugs in ABCB1-overexpressing cells by directly inhibiting ABCB1 transport function. The findings encouraged to further study on the combination therapy of CEP-33779 with conventional chemotherapeutic agents in ABCB1 mediated-MDR cancer patients.

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Abbreviations: ABC, ATP-binding cassette; ABC1, multidrug resistance-associated protein 1; ABC2, breast cancer resistance protein; CCS, Cancer stem cell; DOX, doxorubicin; FTC, fumitremorgin C; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Rho123, Rhodamine 123; MDR, multidrug resistance; JAK2, Janus kinase 2; MTT, 3-(4,5-dimethylthiazol-2-yi)-2,5-diphenyltetrazolium bromide; P-gp/ABCB1, P-glycoprotein; STAT3, Signal transducer and activator of transcription 3; TKI, tyrosine kinase inhibitor; Vi, vanadate; VFP, verapamil; VCR, vincristine.

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1. Introduction

Chemotherapy is an important therapeutic strategy for cancer. However, multidrug resistance (MDR), the ability of cancer cells to acquire resistance to a broad spectrum of structurally and functionally unrelated anticancer drugs, remains one of the primary obstacles to successful cancer chemotherapy [1]. A number of cellular and molecular alterations may contribute to the development of the MDR phenotype. One of the most common causes of MDR results from the overexpression of ATP-binding cassette (ABC) transporters, which actively extrude a variety of chemotherapeutic drugs out of the cancer cells, thereby decreasing the intracellular drug accumulation and resulting in drug resistance [2,3]. Currently, in the human genome, 49 different members of ABC transporter family have been identified and classified into seven subfamilies (A–G) based on the sequence similarities as well as structural organization [4]. Among which, the ATP-binding cassette subfamily B member 1 (ABCB1/P-gp), subfamily C member 1 (ABCC1/MDR1) and subfamily G member 2 (ABCG2/BCRP) play a major role in producing MDR in cancer cells [5,6].

Using the energy provided by ATP hydrolysis, these ABC transporters actively pump out a wide range of anticancer drugs from the inside of cancer cells, thereby attenuating their cytotoxic actions [7]. ABCB1, a 170 kD membrane glycoprotein, is overexpressed in a broad range of solid human tumors and hematologic malignancies, such as liver, colon, kidney and pancreas cancers [8]. It can pump out a wide spectrum of compounds including vinca alkaloids, epipodophyllotoxins, taxanes and some tyrosine kinase inhibitors (TKIs) [9,10]. ABC1, a 190 kD transmembrane protein, confers resistance to anthracyclines, vinca alkaloids, epipodophyllotoxins, camptothecins and methotrexate [11]. In contrast to ABCB1, ABCG2, a 72 kD transmembrane protein, is a half transporter that consists of only one transmembrane domain with six helices and one ATP-binding site, conferring resistance to mitoxantrone, indolocarbazole, topoisomerase I inhibitors and anthracyclines, as well as fluorescent dyes such as Hoechst 33342 [12].

A logical strategy to overcome ABC transporters mediated MDR is to develop inhibitors to prevent the efflux of anticancer drugs from the cancer cells [13]. TKIs are a novel class of anticancer agents, functioning by competing with ATP for binding to the ATP sites of the catalytic domain of tyrosine kinase. Interestingly, it has been reported that several TKIs, such as gefitinib [14], lapatinib [15], apatinib [16] and sunitinib [17] could interact with specific ABC transporters, thereby inhibiting those drug transport activity and enhancing the anticancer efficacy of conventional chemotherapeutic agents. Thus, it is possible that TKIs could be used as promising MDR inhibitors in cancer cells.

CEP-33779 is a novel, highly selective and orally bioavailable, ATP-competitive and small-molecule Janus kinase 2 (JAK2) inhibitor with a favorable preclinical profile [18]. Fueled by previous encouraging sensitized effect of TKIs to MDR cancer cells, we hypothesized that CEP-33779 may inhibit the function of specific ABC transporters and help circumventing anticancer drugs resistance. Therefore, in this study, we conducted experiments to determine whether CEP-33779 could potentiate the efficacy of specific conventional antineoplastic drugs through interaction with ABCB1, ABCC1 and ABCG2 in vitro and in vivo.

2. Materials and methods

2.1. Chemicals and reagents

CEP-33779, whose molecular structure was shown in Fig. 1B, was purchased from Selleck Chemicals (Houston, TX, USA) and prepared as a 50 mM stock solution in DMSO (Sigma-Aldrich, St. Louis, MO, USA) for in vitro studies. Dulbecco’s modified Eagle’s medium (DMEM) and RPMI-1640 were purchased from Gibco BRL (Gaithersburg, MD, USA). Monoclonal antibodies against ABCB1 (sc-55510) was purchased from Santa Cruz Biotechnology, Inc. (California, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody was purchased from Kangchen Co. (Shanghai, China). Phosphorylated-STAT3 (#9145), STAT3 (#4904) and JAK2 (#3230) antibody were purchased from Cell Signaling Technology, Inc. (Danvers, MA). SYBER Green was product of Invitrogen (Minneapolis, USA). Four individual GV112 lenti viral shRNAs targeting JAK2 were obtained from Genechem (Shanghai, China). The shRNA sequences were as follows: JAK2 shRNA I, 5’-CAGTTTGAGAGAAGACATT-3’; JAK2 shRNA II, 5’-AGATCTTCAAAACCTTTA-3’; JAK2 shRNA III, 5’-TGGTTCTTTCTGTCTATTAC-3’; JAK2 shRNA IV, 5’-CTGACCCCTAATAATCAC-3’ and control shRNA, 5’-TCTCGGCTTGGCGGAGAAGTC-3’. Fueled by previous encouraging sensitized effect of TKIs to MDR cancer cells, we hypothesized that CEP-33779 may inhibit the function of specific ABC transporters and help circumventing anticancer drugs resistance. Therefore, in this study, we conducted experiments to determine whether CEP-33779 could potentiate the efficacy of specific conventional antineoplastic drugs through interaction with ABCB1, ABCC1 and ABCG2 in vitro and in vivo.

2.2. Cell lines and cell culture

The human oral epidermoid carcinoma cell line KB and its vincristine-selected ABCB1-overexpressing derivative KBv200 were gifts from Dr. Xu-Yi Liu (Cancer Hospital of Beijing, Beijing, China) [19]. The following cell lines were gifts from Dr. S.E. Bates (National Cancer Institute, NIH, Bethesda, MD, USA): human breast carcinoma cell lines MCF-7 and its doxorubicin-selected ABCB1-overexpressing derivative MCF-7/adr [20], human colon carcinoma cell lines S1 and its mitoxantrone (MX)-selected ABCB2-overexpressing derivative S1-Mi-80 [21], human leukemia cell lines HL60 and its doxorubicin-selected ABCCI-overexpressing derivative HL60/adr [22], human primary embryonic kidney cell line HEK293 and its pcDNA3.1, ABCB1 stable gene-transfected cell line HEK293/ABCB1 (cultured in medium with 2 μg/ml G418) [23]. Cell lines used in this study were thawed from early passage stocks and were passaged for less than 6 months. Cell lines were periodically monitored for mycoplasma by Hoechst staining. All cell lines were cultured in DMEM or RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and with 1% antibiotic solution (penicillin-streptomycin). All cells were grown in drug free culture medium for more than 2 weeks before assay.

2.3. Experimental animals

Athymic nude mice (BALB/c-nu/nu), 5–6 weeks of age and weighing 18–20 g, were obtained from the Center of Experimental Animals, Sun Yat-Sen University (Guangzhou, China). All animals received sterilized food and water. All animal care and experimental procedures have been approved by the ethics committee for animal experimentation and were carried out in accordance with the guidelines on animal care and experiments of laboratory animals (Center of Experimental Animals, Sun Yat-Sen University, China), and conformed to the National Institutes of Health Guide for Care and Use of Laboratory Animals.

2.4. Cell cytotoxicity assay

The MTT assay was done as previously described to assess the sensitivity of cells to drugs [24]. The concentration required to inhibit cell growth by 50% (IC50) was calculated from survival curves using the Bliss method [25]. The degree of resistance was estimated by dividing the IC50 for the MDR cells by that of the parental sensitive cells. 10 μM VPA, 50 μM MK571 and 2.5 μM FTC
Fig. 1. Cytotoxicity of CEP-33779 in pairs of sensitive and drug-resistant cells. The protein level of ABCB1 was measured by Western blot analysis. (A) The protein expression of ABCB1 in KB, KBv200, MCF-7, MCF-7/adr, HEK293/pcDNA 3.1 and HEK293/ABCB1 cells, the expression of GAPDH was used as a loading control; (B) the chemical structure of CEP-33779; the cytotoxicity of CEP-33779 alone was tested by MTT assay as described in Section 2. (C) ABCB1-negative KB and ABCB1-overexpressing KBv200 cells; (D) ABCB1-negative MCF-7 and ABCB1-overexpressing MCF-7/adr cells; (E) ABCG2-negative S1 and ABCG2-overexpressing S1-Mi-80 cells; (F) ABCC1-negative HL60 and ABCC1-overexpressing HL60/adr cells and (C) HEK293/pcDNA 3.1 and HEK293/ABCB1 cells which were exposed to the indicated concentrations of CEP-33779 for 72 h. Data shown are the means ± SD for three independent determinations. Each experiment was performed in four replicate wells.
(inhibitor for ABCB1, ABCC1 and ABCG2, respectively) were used in place of CEP-33779 as positive control to confirm the mechanism of drug resistance in the MDR cell line models [26].

2.5. Nude mouse tumor xenograft model

The KBv200 cells inoculated nude mouse tumor xenograft model was established as previously described [27]. The xenograft model was found to maintain the MDR phenotype in vivo and was extremely resistant to vincristine treatment. Briefly, KBv200 cells (2 × 10^6) were implanted subcutaneously (sc) under the shoulder in the nude mice. The mice were randomized into four groups if the tumors reached a mean diameter of 0.5 cm, and then received various regimens: (a) Control (vehicle alone); (b) Vincristine (q2d × 6, ip, 0.2 mg/kg); (c) CEP-33779 (q2d × 6, p.o, 30 mg/kg) and (d) Vincristine (q2d × 6, ip, 0.2 mg/kg) plus CEP-33779 (q2d × 6, p.o, 30 mg/kg, given an hour before Vincristine administration). The body weights of the animals and the two perpendicular diameters (A and B) were recorded every 2 days, and tumor volume (V) was estimated according to the following formula [27]:

\[ V = \frac{1}{2} \left( A + B \right)^2 \]

The curve of tumor growth was drawn according to tumor volume and time of implantation. On the last day of the experiment, the mice were sacrificed and tumor tissue was excised from the mice and weighted. The ratio of growth inhibition (IR) was calculated according to the following formula [27]:

\[ IR = \left[ 1 - \frac{\text{Mean tumor weight of experimental group}}{\text{Mean tumor weight of control group}} \right] \times 100\% \]

2.6. DOX and Rho 123 accumulation

The effect of CEP-33779 on the intracellular accumulation of DOX and Rho 123 in ABCB1-overexpressing KBv200 cells, MCF-7/adr cells and their parental sensitive KB cells, MCF-7 cells was evaluated by flow cytometry as previously described [28]. First, cells were treated with CEP-33779 at various concentrations at 37 °C for 3 h. Then, 10 μM DOX or 5 μM rhodamine 123 was added to the medium and another 3 h or 0.5 h incubation was continued, respectively. After that, the cells were collected, washed twice with ice-cold PBS, and analyzed with flow cytometric analysis (Beckman Coulter, Cytomics FC500, USA). VRP was used as a positive control inhibitor of ABCB1 [29].

2.7. DOX efflux studies

DOX efflux was determined following a modification of methods described previously [26]. Briefly, KB and KBv200 cells were treated with 10 μM DOX for 3 h at 37 °C. The cells were then washed twice with ice-cold PBS and subsequently maintained in DOX-free medium at 37 °C, in absence or presence of 1.5 μM CEP-33779. Thereafter, at 0, 15, 30, 60, 90 and 120 min, cells were collected and washed again twice with ice-cold PBS, and analyzed with flow cytometric analysis.

2.8. Preparation of cell lysates and Western blot analysis

Western blot analysis was performed following a modification of methods described previously [15]. All samples used in the Western blot assays were whole cell lysates from various treated groups. The cell extracts were collected in cell lysis buffer (PBS containing 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 100 mg/ml phenylmethylsulfonyl fluoride, 10 mg/ml aprotinin, 10 mg/ml leupeptin) for 20 min on ice, with occasional rocking, followed by centrifugation at 12,000 rpm at 4 °C for 15 min [30]. The supernatant containing the total cell lysates was stored at −80 °C. The protein concentration was determined using a BCA assay (Beyotime Biotechnology, Haimen, China) and equalised before loading. Equal amounts (50 μg of protein) were resolved by SDS-PAGE and transferred onto PVDF membranes (Pall, USA) through electrophoresis. After blocked in 5% non-fat milk in TBST buffer (10 mM Tris-HCL, 150 mM NaCl, and 0.1% Tween20 pH 8.0) for 2 h at room temperature, the membranes were incubated overnight with the primary monoclonal antibody against ABCB1 (at a 1:1000 dilution), STAT3 (at a 1:1000 dilution), p-STAT3 (at a 1:1000 dilution) JAK2 (at a 1:1000 dilution) or GAPDH (at a 1:1000 dilution) at 4 °C. The membranes were then washed thrice with TBST buffer and incubated with HRP-conjugated secondary antibody at a 1:1000 dilution for 2 h at room temperature. After washed thrice with TBST buffer, the protein antibody complex were visualized by the enhanced Phototope TM-HRP Detection Kit (Cell Signaling, USA) and exposed to Kodak medical x-ray processor (Carestream Health, USA). The expression of GAPDH was used as a loading control. The protein expression level was quantified using the gray value analysis Software.

2.9. Real-time quantitative PCR

ABCB1 expression in mRNA level was assayed as previously described [31]. After CEP-33779 treatment, total cellular RNA was isolated by Trizol Reagent RNA extraction kit following the manufacturer’s instruction (Molecular Research Center, Cincinnati, USA). The first strand cDNA was synthesized by Oligo dT primers with reverse transcriptase (Promega Corp.). Real-time quantitative PCR primers were 5'-CAGGCTTGGTGAATACCCA-3' (forward) and 5'-TCAAAGAAAACAGGTTCCG-3' (reverse) for ABCB1; 5'-GACTCAAGATTGTGCTGT-3' (forward) and 5'-GATCGCTGCTGGAA-GATG-3' (reverse) for GAPDH, respectively.

Real-time quantitative PCR was performed with Real-time PCR Master Mix containing SYBR GREEN I and hotstart Taq DNA polymerase. Real-time detection of the emission intensity of SYBR GREEN bound to double-stranded DNAs was performed using the iCycler Instrument (Bio-Rad, Hercules, CA, USA). The geometric mean of GAPDH was used as an internal control to normalize the variability in expression levels. The PCR reactions were performed at 50 °C for 2 min, at 95 °C for 5 min, and then at 95 °C for 15s and 60 °C for 30s for 40 cycles. All procedures were carried out according to the instructions. Relative quantification of ABCB1 was performed using the 2^(-ΔΔCt) method [32]. The results were obtained from three reactions in each sample and analysed by the SPSS software (Version 16.0) (SPSS Inc., Chicago, IL, USA).

2.10. ABCB1 ATPase activity assay

The vanadate (Vi)-sensitive ATPase activity of ABCB1 in the membrane vesicles of High Five insect cells was measured by endpoint inorganic phosphate (Pi) assay as previously described [33]. Briefly, the membrane vesicles (10 μg protein) were incubated in ATPase assay buffer with or without 0.3 mM vanadate at 37 °C for 5 min and then incubated with different concentrations of CEP-33779 at 37 °C for 3 min. The ATPase reaction was induced by the addition of 5 mM Mg-ATP, and the total volume was 0.1 mL. After incubation at 37 °C for 20 min, the reactions were stopped by loading 0.1 mL of 5% SDS solution. The liberated inorganic phosphate (Pi) was measured as described previously [34].
2.11. RNA interference

KBv200 cells or MCF-7/adr cells were seeded in 6-well plates and allowed to attach overnight. Upon discarding the medium, each well was washed thrice with PBS. Afterward, transfected with shRNA using Lipofectamine TM2000 complexes diluted in OptiMEM® I Medium. Followed by incubation at 37 °C for 6 h, the cells were switched to culture in the normal medium. After 48 h of incubation cells were harvested for Western blot and MTT analyses.

2.12. Molecular modeling of ABCB1

CEP-33779 structure was built using the fragment dictionary of Maestro v9.9 and subjected to energy minimization by Macro-model program v10.1 (Schrödinger, LLC, New York, NY, 2013). The low-energy 3D structures of CEP-33779 were generated by LigPrep v2.7 and the parameters were defined based on different protonation states at physiological pH ± 2, and all possible tautomers and ring conformations. Ligand structures obtained were further used for generating 100 ligand conformations for each protonated structure using the default parameters of torsional/low-mode sampling function. The conformations were filtered with a maximum relative energy difference of 5 kcal/mol to excluding redundant conformers. The conformational library of CEP-33779 was used as input for docking simulations at human homology ABCB1.

The homology model based on mouse ABCB1 was generated and kindly provided by S. Aller. Receptor docking site was generated by selecting all amino acid residues identified previously as interacting with drugs or with cyclic peptides [35] using Glide v6.0 program. The receptor docking grid was confined to the enclosing box with length of 20 Å. Docking simulation of CEP-33779 was carried using the “Extra Precision” (XP) mode of Glide docking program v6.0 (Schrödinger, LLC, New York, NY, 2013) and the default parameters. The top-scoring pose-ABCB1 complex structures were then used for graphical analysis. All computations were carried out a Dell Precision 490n dual processor with Linux OS (Ubuntu 12.04 LTS).

2.13. Statistics

Microsoft Office Excel 2010 and SPSS 16.0 statistical software were used in data processing and analyzing. Results were shown as means ± SD. All experiments were repeated at least three times and the differences were determined by using the Student's t-test. The statistical significance was determined to be * P < 0.05, ** P < 0.01.

3. Results

3.1. Demonstration of multidrug resistance in cell line models

ABCB1 is overexpressed in KBv200, MCF-7/adr and HEK293/ABCB1 cell lines, whereas ABCC1 and ABCG2 are overexpressed in HL60/adr and S1-Mi-80 cell lines, respectively. MTT analysis confirmed that these five MDR cell lines exerted much higher tolerance to multiple chemotherapeutic agents than their parental sensitive cell lines. The mean IC_{50} values of chemotherapeutic agents were shown in Table 1 and Table 2. Western blot analysis confirmed that ABCB1 was overexpressed in KBv200, MCF-7/adr and HEK293/ABCB1 cells, while the expressions of ABCB1 in their parental sensitive cells were undetectable (Fig. 1A).

3.2. CEP-33779 sensitizes ABCB1-overexpressing cells to chemotherapeutic agents in vitro

We investigated the cytotoxicity of CEP-33779 in different cell lines by MTT assay. The IC_{50} values were 8.45 ± 0.21, 10.89 ± 0.69, 9.53 ± 0.66, 12.93 ± 1.00, 4.22 ± 0.73, 5.51 ± 0.59, 5.27 ± 0.10, 6.38 ± 0.56, 18.25 ± 1.29 and 24.09 ± 1.56 μM for KB, KBv200, MCF-7, MCF-7/adr, S1, S1-Mi-80, HL60, HL60/adr, HEK293/pDNA3.1 and HEK293/ABCB1 cells, respectively (Fig. 1C–G). More than 85% of cells survived at the concentration of 1.5 μM CEP-33779 in KB, KBv200, MCF-7, MCF-7/adr, HEK293/pDNA3.1 and HEK293/ABCB1 cells and 1.0 μM in S1, S1-Mi-80, HL60 and HL60/adr cells (Fig. 1C–G). Therefore, CEP-33779 was tested in drug combination assays at a maximum concentration of 1.5 μM in KB, KBv200, MCF-7, MCF-7/adr, HEK293/pDNA3.1 and HEK293/ABCB1 cells and 1.0 μM in S1, S1-Mi-80, HL60 and HL60/adr cells, respectively. The IC_{50} values of the antineoplastic drugs in the resistant and parental cells when treated alone or combined with different concentrations of CEP-33779 were shown in Table 1 and Table 2. CEP-33779 at 1.5 μM could significantly sensitize KBv200, MCF-7/adr and stably transfected HEK293/ABCB1 cells to their substrates but did not alter the cytotoxicity of cisplatin (a non-ABCB1 substrate). However, no sensitized effect of CEP-33779 was observed in the parental cells (Table 1). Furthermore, CEP-33779 also had no significant sensitized effect on ABCB1-mediated drug resistance in HL60/adr cells or ABCG2-mediated drug resistance in S1-Mi-80 cells (Table 2). These results indicate that CEP-33779 is a selective modulator of ABCB1.

3.3. CEP-33779 sensitizes KBv200 cell xenograft model to vincristine in vivo

The ABCB1-overexpressing KBv200 cell xenograft model in nude mouse was employed to examine whether CEP-33779 could enhance the efficacy of vincristine in vivo. As shown in Fig. 2, there was no significant difference in tumor sizes and weights among animals treated with saline, vincristine alone, and CEP-33779 alone. However, the combination of CEP-33779 and vincristine group produced a significant inhibition of tumor growth compared with other groups (P < 0.01, Fig. 2A and B). The inhibition rate in the combination group was 63.53% (Table 3). Importantly, no mortality or apparent body weight loss was observed in the combination treatment group at all dose levels tested (Fig. 2C), suggesting that the combination regimen does not give rise to toxic side effects.

3.4. CEP-33779 significantly increases the accumulation of DOX and Rho 123 in ABCB1-overexpressing cells

These results above indicated that CEP-33779 could sensitize the MDR cells to antineoplastic drugs in vitro and in vivo. To understand the underlying mechanisms, the intracellular accumulation of DOX and Rho 123 in presence or absence of CEP-33779 was examined by flow cytometric analysis. In absence of CEP-33779, the fluorescence of DOX and Rho 123 was significantly higher in the parental sensitive cells than in the MDR cells (Fig. 3A and B). However, CEP-33779 significantly increased the accumulation of DOX and Rho 123 in KBv200 and MCF-7/adr cells in a concentration-dependent manner without affecting their parental sensitive cells (Fig. 3A and B). At the concentration of 0.375, 0.75 and 1.5 μM, CEP-33779 increased the fluorescence index of DOX by 1.27, 1.98 and 2.96 folds in KBv200 cells and by 1.38, 2.0 and 2.5 folds in MCF-7/adr cells, respectively (Fig. 3A). The same doses of CEP-33779 increased the fluorescence index of Rho 123 by 2.00, 3.30 and 8.31 folds in KBv200 cells and by 2.29, 3.32 and 7.62 folds in MCF-7/adr cells, respectively (Fig. 3B). These results suggest that CEP-33779 increases the accumulation of these anticancer drugs in ABCB1-overexpressing cells, which may be related to a modulating effect on ABCB1-mediated transport.
Table 1
Effect of CEP-33779 on reversing ABCB1-mediated drug resistance.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>IC_{50} ± SD (μM)(^a) (fold reversal)(^b)</th>
<th>KB</th>
<th>KBv200 (ABC1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Doxorubicin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ 0.375 μM CEP-33779</td>
<td>0.1082 ± 0.0072</td>
<td>(1.00)</td>
<td>5.0054 ± 0.0196</td>
</tr>
<tr>
<td>+ 0.75 μM CEP-33779</td>
<td>0.1119 ± 0.0077</td>
<td>(0.97)</td>
<td>2.3122 ± 0.0331</td>
</tr>
<tr>
<td>+ 1.5 μM CEP-33779</td>
<td>0.0916 ± 0.0058</td>
<td>(1.18)</td>
<td>1.0856 ± 0.0261</td>
</tr>
<tr>
<td>+ 10 μM Verapamil</td>
<td>0.0746 ± 0.0065</td>
<td>(1.45)</td>
<td>0.4463 ± 0.0198</td>
</tr>
<tr>
<td>+ 0.75 μM CEP-33779</td>
<td>0.0823 ± 0.0105</td>
<td>(1.30)</td>
<td>0.3112 ± 0.0162</td>
</tr>
<tr>
<td>+ 1.5 μM CEP-33779</td>
<td>0.0084 ± 0.0001</td>
<td>(1.00)</td>
<td>0.3462 ± 0.0066</td>
</tr>
<tr>
<td>+ 0.75 μM CEP-33779</td>
<td>0.0050 ± 0.0006</td>
<td>(0.98)</td>
<td>0.1385 ± 0.0136</td>
</tr>
<tr>
<td>+ 1.5 μM CEP-33779</td>
<td>0.0048 ± 0.0005</td>
<td>(1.02)</td>
<td>0.0433 ± 0.0035</td>
</tr>
<tr>
<td>+ 10 μM Verapamil</td>
<td>0.0047 ± 0.0001</td>
<td>(1.32)</td>
<td>0.0142 ± 0.0003</td>
</tr>
<tr>
<td>+ 0.75 μM CEP-33779</td>
<td>0.0023 ± 0.0004</td>
<td>(2.13)</td>
<td>0.0061 ± 0.0005</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>0.0021 ± 0.0001</td>
<td>(1.00)</td>
<td>0.1994 ± 0.0015</td>
</tr>
<tr>
<td>+ 0.75 μM CEP-33779</td>
<td>0.0022 ± 0.0001</td>
<td>(0.95)</td>
<td>0.1351 ± 0.0034</td>
</tr>
<tr>
<td>+ 0.75 μM CEP-33779</td>
<td>0.0016 ± 0.0001</td>
<td>(1.31)</td>
<td>0.1111 ± 0.0273</td>
</tr>
<tr>
<td>+ 1.5 μM CEP-33779</td>
<td>0.0016 ± 0.0001</td>
<td>(1.31)</td>
<td>0.0281 ± 0.0045</td>
</tr>
<tr>
<td>+ 10 μM Verapamil</td>
<td>0.0014 ± 0.0001</td>
<td>(1.50)</td>
<td>0.0095 ± 0.0004</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>1.194 ± 0.2198</td>
<td>(1.00)</td>
<td>2.3382 ± 0.3182</td>
</tr>
<tr>
<td>+ 1.5 μM CEP-33779</td>
<td>1.199 ± 0.2520</td>
<td>(0.99)</td>
<td>2.1510 ± 0.2641</td>
</tr>
<tr>
<td>+ 10 μM Verapamil</td>
<td>1.150 ± 0.2234</td>
<td>(1.04)</td>
<td>2.3182 ± 0.2982</td>
</tr>
</tbody>
</table>

\(^a\) IC_{50} values are represented as mean ± SD of three independent experiments performed in triplicate.

\(^b\) The fold-reversal of MDR (values given in parenthesis) was calculated by dividing the IC_{50} for cells with the anticancer drugs in the absence of inhibitor by that obtained in the presence of inhibitor. Cell survival was performed by MTT assay as described in “Materials and Methods”. Verapamil was used as a positive control of ABCB1 inhibitor.

\(^1\) P < 0.05, significantly different from those obtained in the absence of inhibitor.

\(^2\) P < 0.01, significantly different from those obtained in the absence of inhibitor.

Table 2
Effect of CEP-33779 on reversing ABCG2- and ABCG2-mediated drug resistance.

| Compounds      | IC_{50} ± SD (μM)\(^a\) (fold reversal)\(^b\) | HL60/60 | HL60/60|ABC1 |
|----------------|---------------------------------------------|---------|--------|
| Doxorubicin    |                                             |         |        |
| + 0.25 μM CEP-33779 | 0.0294 ± 0.0017 | (0.91) | 1.9081 ± 0.1062 | (0.95) |
| + 0.5 μM CEP-33779  | 0.0215 ± 0.0021 | (1.24) | 1.5143 ± 0.2081 | (1.19) |
| + 1.0 μM CEP-33779   | 0.0258 ± 0.0044 | (1.03) | 1.3473 ± 0.0361 | (1.30) |
| + 5.0 μM MK571      | 0.0233 ± 0.0021 | (1.15) | 1.1744 ± 0.0087 | (10.42) |
| Topotecan         |                                             |         |        |
| + 0.25 μM CEP-33779  | 0.2501 ± 0.0037 | (1.00) | 13.1670 ± 0.4260 | (1.00) |
| + 0.5 μM CEP-33779  | 0.3126 ± 0.0130 | (0.94) | 12.5442 ± 0.3219 | (1.04) |
| + 1.0 μM CEP-33779   | 0.2713 ± 0.0023 | (1.08) | 7.3826 ± 0.3694 | (1.78) |
| + 2.5 μM FTC        | 0.2817 ± 0.0019 | (1.03) | 0.6273 ± 0.0843 | (21.00) |

\(^a\) IC_{50} values are represented as mean ± SD of three independent experiments performed in triplicate.

\(^b\) The fold-reversal of MDR (values given in parenthesis) was calculated by dividing the IC_{50} for cells with the anticancer drugs in the absence of inhibitor by that obtained in the presence of inhibitor. Cell survival was performed by MTT assay as described in “Materials and Methods”. MK571 and FTC were used as a positive control of ABCG1 and ABCG2 inhibitor, respectively.

\(^1\) P < 0.01 versus the control group.

3.5. CEP-33779 significantly inhibits the efflux of intracellular DOX in ABCB1-overexpressing cells

To ascertain whether the increase in the intracellular DOX accumulation caused by CEP-33779 was due to an inhibition of DOX efflux, we conducted a time course study to determine DOX efflux in the presence of CEP-33779 in KBv200 and its parental sensitive KB cells. Our results indicated that KBv200 cells extruded a significantly higher percentage of intracellular accumulated DOX than parental KB cells (Fig. 3C, P < 0.05). When we incubated cells with CEP-33779 at 1.5 μM, it significantly blocked the intracellular DOX efflux at different time periods (0, 15, 30, 60, 90, 120 min) from KBv200 cells, but not in the parental KB cells (Fig. 3C). The accumulation of DOX at 0 min was set as 100%, at 15, 30, 60, 90 and 120 min, the percentages were 74.60%, 67.15%, 58.64%, 48.49% and 44.09%, respectively, of the accumulated DOX that remained in KBv200 cells in the absence of CEP-33779. When KBv200 cells were incubated with 1.5 μM CEP-3379, the percentages of DOX remained at 15, 30, 60, 90, and 120 min were increased by 89.88%, 82.87%, 76.7%, 70.49% and 63.27% respectively (Fig. 3C, P < 0.05 for the same time point comparison). These results indicate that CEP-33779 inhibits the efflux function of ABCB1.

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Fig. 2. Potentiation of the antitumor effects of vincristine by CEP-33779 in a KBv200 cell xenograft model in nude mice. (A) The changes in tumor volume with time after tumor cell implantation. (B) The photograph of tumor size was taken on the 21st day after implantation. (C) Average % body weight change of body weight after tumor cell implantation. Data shown are means ± SD for each group of eight mice after implantation. The various treatments were as follows: (a) Control (vehicle alone); (b) Vincristine (q2d × 6, ip, 0.2 mg/kg); (c) CEP-33779 (q2d × 6, p.o, 30 mg/kg); and (d) Vincristine (q2d × 6, ip, 0.2 mg/kg) plus CEP-33779 (q2d × 6, p.o, 30 mg/kg, given an hour before Vincristine administration).

Table 3

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean tumor volume (mm³)</th>
<th>Mean xenograft weight (g)</th>
<th>IR (%) a</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Start day 9</td>
<td>End day 21</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>111.3 ± 53.5</td>
<td>2502.3 ± 1029.9</td>
<td>1.81 ± 0.73</td>
</tr>
<tr>
<td>Vincristine</td>
<td>121.5 ± 26.5</td>
<td>2467.1 ± 1017.7</td>
<td>1.72 ± 0.70</td>
</tr>
<tr>
<td>CEP-33779</td>
<td>109.0 ± 61.0</td>
<td>2388.0 ± 903.6</td>
<td>1.61 ± 0.68</td>
</tr>
<tr>
<td>Combination</td>
<td>118.7 ± 44.1</td>
<td>992.7 ± 413.2</td>
<td>0.66 ± 0.35</td>
</tr>
</tbody>
</table>

a IR (%): Inhibition rate (IR), IR = [1 – (mean tumor weight for experimental group/mean tumor weight for control group)] × 100.

3.6. CEP-33779 stimulates the ATPase activity of ABCB1

It is well known that the drug-efflux function of ABCB1 is linked to ATP hydrolysis, which is stimulated in the presence of ABCB1 substrates. For this reason, we evaluated the effect of CEP-33779 on ABCB1 ATPase activity. We found that CEP-33779 was an activator of ABCB1 ATPase. As shown in Fig. 4, CEP-33779 produced up to ~6-fold stimulation of ABCB1 ATPase activity in a concentration-dependent manner.

3.7. CEP-33779 does not significantly alter the expression level of ABCB1

Drug-induced up-regulation or down-regulation of ABCB1 drug transporter can potentially affect the drug sensitivity of cancer cells to chemotherapeutics. Hence, we determined the effect of CEP-33779 on the expression of ABCB1 at both mRNA and protein levels. Results from Western blot and real-time quantitative PCR analyses demonstrated that CEP-33779 did not affect the expression of ABCB1 in KBv200 and MCF-7/adr cells (Fig. 5). This data also confirmed that CEP-33779 sensitized ABCB1-overexpressing MDR cells to chemotherapeutic agents by inhibiting the function of ABCB1, and not by downregulating the protein level of this transporter.

3.8. The downregulation of JAK2 does not alter the expression of ABCB1 and the cytotoxic effect of chemotherapeutic agents in ABCB1-overexpressing cells

Signal transducer and activator of transcription 3 (STAT3), the major downstream markers of JAK2, is used to assess targeted activity of JAK2 TKIs [36]. JAK2 inhibition was monitored by determining levels of STAT3 phosphorylation. Previous studies have shown that the inhibition of the JAK2/STAT3 pathway could enhance the efficacy of chemotherapeutic agents in cancer cells [37,38]. Therefore, we examined the effect of CEP-33779 on the expression of total and phosphorylated STAT3 in KB, KBv200, MCF-7 and MCF-7/adr cells, respectively. As shown in Fig. 6A–C, after treated with 0.375, 0.75, 1.5 and 3.0 μM CEP-33779 for 48 h, there were different degrees inhibitory effect on phosphorylated STAT3 in tested cells. So it is conceivable that the blockade of JAK2/STAT3 signaling pathway may contribute to the sensitization of KBv200 and MCF-7/adr cells to chemotherapeutic agents.

To explore this issue, we designed shRNA to silence JAK2 target gene and observed the cytotoxicity alteration of conventional chemotherapeutic agents in absence or presence of CEP-33779. Western blot analysis revealed that targeted shRNA could significantly down-regulate JAK2 after transfection for 48 h (Fig. 6D and E). Nevertheless, there was no significant change in ABCB1 expression (Fig. 6D and F) or anticancer activity of chemotherapeutic agents in the JAK2-silenced ABCB1-overexpressing cells (Fig. 6G and H). Thus, we concluded that CEP-33779 could block JAK2/STAT3 signaling pathway at the MDR sensitized concentrations, but this effect was not related to the enhancing efficacy of chemotherapeutic agents by CEP-33779 in ABCB1-overexpressing cells.

3.9. Molecular docking simulation of CEP-33779 into human homology ABCB1

The docked pose showed that CEP-33779 could fit into large drug-binding cavity of human homology ABCB1. The top-scoring pose (XP Glide score: −9.68) was shown in Fig. 7. The 4-methylpiperazine group was stabilized into a hydrophobic pocket formed by residues Ile306, Tyr307, Tyr310 and Phe728. Moreover, the protonated tertiary amine of the piperazine ring formed
hydrogen bond interaction with the hydroxyl group of Tyr307 (-NH--OH-Tyr307, 2.71 Å). The aniline group along with the [1,2,4]triazolo[1,5-a]pyridine group seem to take part in hydrophobic interactions with residues Ile340, Gln725, Phe983, Met986, Ala987 and Gln990. The 4-methysulfonyl phenyl group was found fitted into another hydrophobic pocket formed by Met68, Met69, Phe72, Phe336, Tyr953, Phe957 and Phe978.

4. Discussion

There is growing evidence demonstrating that JAK2/STAT3 signaling pathway plays a critical role in the growth and progression of many solid and hematopoietic cancers and often correlate with a poor prognosis and resistance to multiple therapies [39]. Interestingly, recent advances have highlighted the role of JAK2/STAT3 signaling in the maintenance of Cancer stem cell (CSCs), which reinforces the importance of this pathway in tumor recurrence and chemoresistance. For example, blockade of JAK2 activation in breast cancer resulted in a reduction of the CD44⁺/CD24⁻ CSC population and a loss of tumorigenicity in vivo [40]. Therefore, targeted inhibition of JAK2 or/and STAT3 may be an effective therapeutic strategy in cancers.

CEP-33779 is a novel ATP-competitive, highly selective and small-molecule JAK2 inhibitor with IC50 of 1.8 nM. In preclinical study, Seavey et al. [18] showed for the first time that CEP33779 orally administered twice daily at dose of 55 mg/kg induces regression of established colorectal tumors, reduces angiogenesis, and reduces proliferation of tumor cells in a mouse model of colitis-induced colorectal cancer. In addition, CEP-33779 exerted potent anticancer effect in multiple JAK2 driven tumor xenografts.
and efficacy in inflammation models in mouse and had potential as a preclinical development candidate [41].

A large number of TKIs targeting different oncogenic signaling pathways have been developed, and are currently used in the clinic or being tested in clinical trials. Interestingly, previous studies have shown that several TKIs could interact with specific ABC transporters, thereby inhibiting those drug transport activity and enhancing the anticancer efficacy of conventional chemotherapeutic agents. This modulatory property may make TKIs promising compounds for use in combination with other anticancer drugs, allowing for an effective enhancement of various cytotoxic agents. The objectives of this study were to determine the effect of CEP-33779 on enhancement of conventional chemotherapeutic agents in ABC transporter-mediated MDR cells and to investigate the underlying mechanisms.

In the present study, we examined the effect of CEP-33779 on ABCB1-, ABCC1- and ABCG2-overexpressing cancer cells. Our data shows that CEP-33779 has different effects on these three ABC transporters. CEP-33779 potently sensitized ABCB1-overexpressing cells to its respective substrate anticancer drugs. However,

**Fig. 4.** Effect of CEP-33779 on the ATPase activity of ABCB1. The ATP hydrolysis in membrane vesicles was determined with different concentrations of CEP-33779, as described in Section 2. Data shown are means ± SD for independent determinations in triplicate.

**Fig. 5.** Effect of CEP-33779 on the expression of ABCB1 in MDR cancer cells. KBv200 cells (ABCB1) and MCF-7/adr cells (ABCB1) were treated with CEP-33779 at various concentrations for 48 h and at 1.5 μM for various times. (A) Equal amounts of total cell lysates were loaded and detected with Western blotting. Representative blots from three independent experiments were shown. (B) The gray value analysis of ABCB1 in the absence and presence of increasing concentrations of CEP-33779 in KBv200 and MCF-7/adr cells, respectively. Values are presented as mean ± SD calculated from three independent experiments. (C) The averaged relative mRNA levels for ABCB1 in KBv200 and MCF-7/adr cells in the absence and presence of increasing concentrations of CEP-33779. Values are presented as mean ± SD calculated from three independent experiments. GAPDH was used as an internal control for equal loading.
Fig. 6. Effect of modulating ABCB1 with silencing JAK2 by shRNA. Equal amounts of whole cell lysates were loaded and detected with Western blotting. The effects of CEP-33779 on JAK2/STAT3 signaling were measured by monitoring the levels of p-STAT3. The inhibitory effect of p-STAT3 in KB, KBv200, MCF-7 and MCF-7/adr cells treated with CEP-33779 at various concentrations after 48 h (A). Representative immunoblots of total STAT3, p-STAT3 and GAPDH, are showed here. The averaged relative expression levels of total STAT3 (B), p-STAT3 (C) treated with increasing concentrations of CEP-33779 in KB, KBv200, MCF-7 and MCF-7/adr cells. Values are presented as mean ± SD calculated from three independent experiments. **, P < 0.01, versus control group. The expression of ABCB1 and JAK2 in KBv200 and MCF-7/adr transfected with control shRNA, JAK2 shRNA I, JAK2 shRNA II, JAK2 shRNA III and JAK2 shRNA IV after 48 h (D). Representative immunoblots of ABCB1, JAK2 and loading control tubulin, are showed here. The
CEP-33779 had no significant effect on sensitivity of ABCG2- and ABCC1-overexpressing cells to their substrate drugs. To determine whether the in vitro effects of CEP-33779 can be extended to an in vivo paradigm, we have examined the effect of CEP-33779 on the antitumor activity of vincristine in KBv200 cells xenograft model in nude mice. Consistent with the in vitro findings, our results indicated that CEP-33779 significantly increased the antitumor activity of vincristine (Fig. 2A and B), without a loss of body weight in the combination group (Fig. 2C). These results indicated that CEP-33779 could enhance the efficacy of conventional chemotherapeutic agents in ABCB1-overexpressing cells in vitro and in vivo.

To investigate the mechanism of CEP-33779 on sensitizing ABCB1-overexpressing MDR cells to chemotherapeutic agents, ABCB1 transport activity and its expression were examined. As demonstrated by the flow cytometric analyses, CEP-33779 significantly increased the intracellular accumulation of DOX and Rho123 in ABCB1-overexpressing KBv200 and MCF-7/adr cells, without any observable effect on the corresponding parental KB and MCF-7 cells (Fig. 3A and B). Furthermore, our results indicated that CEP-33779 also significantly blocked the DOX efflux at different time periods (0, 15, 30, 60, 90, 120 min) from KBv200 cells, but not in the parental KB cells (Fig. 3C). However, the incubation of ABCB1-overexpressing cells with CEP-33779 did not alter the expression of ABCB1 at both mRNA and protein levels (Fig. 5). Thus, we concluded that CEP-33779 potentiated the sensitivity of anticancer agents in ABCB1-overexpressing cells may result from the direct interference of the efflux function of ABCB1 as opposed to alteration in protein expression levels.

ABCBC1 move substrates out of cells using ATP hydrolysis as the energy source. Therefore, the rate of ATP hydrolysis is directly proportional to the transport activity of the transporters [7]. A variety of transporter modulators can be categorized into three distinct classes, based on their effect on ATPase activity of ABC transporters. The first class of compounds stimulates ATPase activity at low concentrations but inhibits the activity at high concentrations, the second class of compounds enhances ATPase activity in a concentration-dependent manner without any inhibition, whereas the third class of compounds inhibits both basal and stimulated ATPase activity [7,34]. Previous reports have demonstrated that several TKIs can modulate the ABCB1 mediated efflux by inhibiting [42,43] or stimulating [16,26,44] ABCB1 ATPase activity. In this study, we found that CEP-33779 stimulated ATPase activity of ABCB1 transporter in a concentration-dependent manner. These data suggest that CEP-33779 belongs to the second class of compounds to interact with ABC transporters and is likely to be a substrate. Collectively, we conclude that CEP-33779 competitively bound to the substrate-binding site of ABCB1, leaving little place for other substrate agents to bind to the transporter, which resulted in decreased activity of ABCB1 transporter, thus enhancing the intracellular drug concentration.

Previous research demonstrated that hyperactivation of JAK2/STAT3 signaling pathway was correlated with poor prognosis and resistance to conventional anticancer drugs [45,46]. Therefore, it is averaged relative expression levels of JAK2 (G) and ABCB1 (F) transected with control shRNA, JAK2 shRNA I, JAK2 shRNA II, JAK2 shRNA III and JAK2 shRNA IV after 48 h in KBv200 and MCF-7/adr cells. Values are presented as mean ± SD calculated from three independent experiments. *, P < 0.05; **, P < 0.01, versus control group. MTT assay was used to draw the survival rate curve (G, H) after silencing JAK2 by shRNA III and JAK2 shRNA IV. Each point represents the means ± SD for three determinations. GAPDH was used as an internal control for equal loading. VCR: vincristine, DOX: doxorubicin.
important to clarify whether this pathway is related to the reversal effect of CEP-33779 on ABCB1 mediated MDR. Our Western blot analysis revealed that CEP-33779 inhibited the phosphorylation of STAT3 in different degrees in KB, KBv200, MCF-7 and MCF-7/adr cells, respectively (Fig. 6A–C). It is possible that blockage of JAK2/STAT3 pathway by CEP-33779 confers to the sensitization of KBv200 and MCF-7/adr cells to chemotherapeutic agents. To explore this issue, we knocked down JAK2 by shRNA, and MT assay were done in the presence of JAK2 RNAi duplex. Nevertheless, JAK2 down-regulation altered neither the expression of ABCB1 (Fig. 6D and F) nor the sensitivity of chemotherapeutic agents (Fig. 6G and H). Therefore, we concluded that JAK2/STAT3 pathway blocked by CEP-33779 did not confer to the sensitization of ABCB1-overexpressing cells to chemotherapeutic agents.

Since the crystal structure of human ABCB1 is currently unavailable, our docking simulations mainly relied on homology model of newly refined mouse ABCB1 [35]. CEP-33779 is a hydrophobic molecule with calculated logP of 3.6. It also exhibits several aromatic ring centers, which have been described as critical for binding at ABCB1 transporter [47]. Since ATPase assay showed that CEP-33779 is a competitive inhibitor of ABCB1, the hydrophobic property of CEP-33779 may also contribute to distribute itself within the membrane from which ABCB1 normally exerts its substrates in order to fulfill its inhibitory effect (Fig. 7).

Overall, docking simulation is helpful to provide clues to understand and optimize further derivatives.

Until now, multiple non-cytotoxic agents can sensitize MDR tumor cells to chemotherapeutic drug, with promising preclinical and early clinical data. Unfortunately, several problems, including unacceptable cytotoxic effects, altered pharmacokinetics of anticancer drugs, and adversely modified drug distribution have prevented their successful translation to the clinic [48,49]. To overcome these shortcomings, new generation of ABCB1 inhibitors with more efficacious, non-toxic and high-affinity for ABCB1 that could antagonize ABCB1 mediated MDR in the clinic are ongoing. More recently, attention has been paid to the development of other strategies for inhibiting MDR and benefiting patients, such as use of TKIs, drug delivery systems, nanomedicines, RNAi, or pharmacogenomics [49,50].

In conclusion, this study provides the first in vitro and in vivo evidence that CEP-33779 significantly enhances the efficacy of chemotherapeutic agents in ABCB1-overexpressing MDR cells by directly inhibiting ABCB1 drug efflux function resulting from stimulating its ATPase activity. In addition, the MDR circumvention seems to be independent of inhibiting ABCB1 expression or JAK2 phosphorylation. Collectively, our results advocate the combination use of CEP-33779 with ABCB1 substrate anticancer drugs in the clinic to circumvent MDR and enhance the chemotherapeutic response.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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