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Cancer Lett. Author manuscript; available in PMC 2019 October 01.

Published in final edited form as:

Cancer Lett. 2018 October 01; 433: 259–272. doi:10.1016/j.canlet.2018.07.004.

SIS3, a specific inhibitor of Smad3 reverses ABCB1- and ABCG2-mediated multidrug resistance in cancer cell lines

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CONFLICT OF INTEREST

None.

AUTHOR DECLARATION

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us. We confirm that we have given due consideration to the protection of intellectual property associated with this work and that there are no impediments to publication, including the timing of publication, with respect to intellectual property. In so doing we confirm that we have followed the regulations of our institutions concerning intellectual property.

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Abstract

One of the major challenges in cancer chemotherapy is the development of multidrug resistance phenomenon attributed to the overexpression of ATP-binding cassette (ABC) transporter ABCB1 or ABCG2 in cancer cells. Therefore, re-sensitizing MDR cancer cells to chemotherapy by directly inhibiting the activity of ABC transporters has clinical relevance. Unfortunately, previous attempts of developing clinically applicable synthetic inhibitors have failed, mostly due to problems associated with toxicity and unforeseen drug-drug interactions. An alternative approach is by repositioning drugs with known pharmacological properties as modulators of ABCB1 and ABCG2. In this study, we discovered that the transport function of ABCB1 and ABCG2 is strongly inhibited by SIS3, a specific inhibitor of Smad3. More importantly, SIS3 enhances drug-induced apoptosis and resensitizes ABCB1- and ABCG2-overexpressing cancer cells to chemotherapeutic drugs at non-toxic concentrations. These findings are further supported by ATPase assays and by a docking analysis of SIS3 in the drug-binding pockets of ABCB1 and ABCG2. In summary, we revealed an additional action of SIS3 that re-sensitizes MDR cancer cells and a combination therapy with this drug and other chemotherapeutic agents may be beneficial for patients with MDR tumors.

Keywords

Multidrug resistance; ABCB1; ABCG2; SMAD3; SIS3

1. Introduction

One of the major challenges in cancer chemotherapy is the development of multidrug resistance (MDR) attribute to the overexpression of one of the ATP-Binding Cassette (ABC) transporters such as ABCB1 (P-glycoprotein/ MDR1) and ABCG2 (BCRP; MXR) [15, 16, 54]. These transporters can generate energy from ATP hydrolysis to actively transport anticancer drugs away from their targets within a cancer cell, resulting in MDR phenotype [15, 45, 68]. Together, ABCB1 and ABCG2 are capable of transporting a majority of conventional anticancer drugs that are functionally and structurally unrelated, including but not limited to Vinca alkaloids, anthracyclines, methotrexate, topotecan, etoposide, SN-38, as well as many protein kinase inhibitors [17, 19, 37, 63]. The overexpression of ABCB1 and/or ABCG2 has been linked to the MDR phenotype in a variety of blood and solid tumors, including acute lymphocytic leukemia (ALL), acute myelogenous leukemia (AML) [44, 52, 60], chronic lymphocytic leukemia (CLL) [33], multiple myeloma (MM) [20, 35, 39, 40, 46, 58, 59], advanced non-small cell lung cancer [71] and metastatic breast cancer [27]. Moreover, both ABCB1 and ABCG2 are highly expressed in cells forming the blood-tissue barrier sites such as gastrointestinal tract, liver, kidney and the blood-brain barrier (BBB), affecting the oral bioavailability, distribution, metabolism and elimination of most drugs in patients [8, 17, 54].

One of the most effective approaches to overcome MDR mediated by ABCB1 or ABCG2 is to directly inhibit the drug efflux function of the transporter in cancer cells [64]. Although substantial efforts have been invested in recent years to develop novel compounds that can inhibit the function of ABCB1 and ABCG2, there is still no modulator available to treat

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patients with MDR tumors. The problems with developing clinically applicable synthetic inhibitors are often associated with the lack of potency or selectivity, high intrinsic toxicity or adverse drug-drug interactions [50, 54]. As a result, we and others have applied the drug repositioning (or drug repurposing) approach to discover drugs with known pharmacological and toxicological properties in the treatment of MDR cancer [34, 38, 50, 64]. In recent years, many inhibitors of protein kinases and signaling molecules, especially inhibitors of the epidermal growth factor receptor (EGFR), have been shown to reverse MDR mediated by ABCB1 or ABCG2 in cancer cells [23, 26, 48, 49, 51, 65]. However, many of these inhibitors are both transport substrates and/or inhibitors of an ABC drug transporter [19]. For instance, gefitinib (Iressa) inhibits the function of ABCB1 [26], whereas ABCG2 effluxes and reduces the efficacy of gefitinib in cancer cells [13].

In the present study, we investigated the interaction of SIS3 with ABCB1 and ABCG2 in cancer cell lines. SIS3 is a specific inhibitor of Smad3, known for its potent inhibitory activity against transforming growth factor (TGF)-β1-induced phosphorylation of Smad3 [25]. Therefore, SIS3 has been used frequently as a benchmark inhibitor in studies evaluating TGF-β-regulated cellular mechanisms [9, 28, 30, 32, 41, 61, 62, 72]. Our data show that by inhibiting the drug transport function of ABCB1 and ABCG2, SIS3 enhances drug-induced apoptosis and reverses MDR in cancer cells overexpressing ABCB1 or ABCG2. In summary, our results suggest that combination therapy of SIS3 and anticancer agents may be beneficial for patients with MDR tumors, and should be further investigated.

2. Materials and methods

2.1. Chemicals

RPMI medium, Iscove's modified Dulbecco's medium (IMDM), Dulbecco's Modified Eagle's medium (DMEM), Phosphate-buffered saline (PBS), fetal calf serum (FCS), trypsin-EDTA, penicillin, and streptomycin were purchased from Gibco, Invitrogen (CA, USA). Annexin V : FITC Apoptosis Detection Kit was purchased from BD Pharmingen (San Diego, CA, USA). Tools Cell Counting (CCk-8) Kit was purchased from Biotools Co., Ltd (Taipei, Taiwan). SIS3, verapamil, Ko143 and all other chemicals were purchased from Sigma (St. Louis, MO, USA), unless stated otherwise.

2.2. Cell culture conditions

The human OVCAR-8 ovarian cancer cell line and the ABCB1-overexpressing variant NCI-ADR-RES, human KB-3–1 epidermal cancer cell line and the ABCB1-overexpressing variant KB-V-1, human MCF-7 breast cancer cell line and the ABCG2-overexpressing variants MCF7-FLV1000 and MCF7-AdVp3000, human H460 non-small cell lung cancer cell line and the ABCG2-overexpressing variant H460-MX20, as well as pcDNA3.1-HEK293, ABCB1-transfected MDR19-HEK293 and ABCG2-transfected R482-HEK293, were cultured in DMEM. The human S1 colon cancer cell line and the ABCG2-overexpressing variant S1-M1–80 were cultured in RPMI-1640. HEK293 and HEK293 transfected cells were maintained in media containing 2 mg/mL G418 [69], KB-V-1 cells were maintained in 1 mg/mL vinblastine [47], MCF7-FLV1000 cells were maintained in media containing 1 μg/mL flavopiridol [22, 42], MCF7-AdVp3000 cells were maintained in

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media containing 3 μg/mL doxorubicin and 5 μg/mL verapamil [22], and S1-M1–80 cells were maintained in 80 μM of mitoxantrone [69]. All cell lines were cultured in medium supplemented with 10% FCS, 2 mM L-glutamine and 100 units of penicillin/streptomycin/mL, maintained at 37 °C in 5% CO_2 humidified air and placed in drug-free medium 7 days prior to assay.

2.3. Fluorescent drug accumulation assay

The intracellular accumulation of fluorescent substrates was recorded using a FACScan flow cytometer (BD Biosciences) and analyzed using CellQuest software (Becton-Dickinson) according to the method described by Gribar *et al* [18]. Briefly, after harvesting cells by trypsinization and centrifugation, 3×10^5 cells were resuspended in 4 mL of IMDM supplemented with 5% FCS before ABCB1 substrate calcein-AM (0.5 μM) or ABCG2 substrate pheophorbide A (1 μM) was added to the cell suspension in the presence or absence of SIS3, verapamil (an inhibitor of ABCB1), or Ko143 (an inhibitor of ABCG2), as described previously [43]. Calcein fluorescence was detected with excitation and emission wavelengths of 485 and 535 nm, whereas pheophorbide A fluorescence was detected with excitation and emission wavelengths of 395 and 670 nm.

2.4. Immunoblotting

Primary antibodies C219 (1: 3000), BXP-21 (1:15000) and α-tubulin (1:100000) were used in Western blot immunoassay to detect ABCB1, ABCG2 and positive control tubulin, respectively. The horseradish peroxidase-conjugated goat anti-mouse IgG (1:100000) was used as the secondary antibody. Signals were detected as described previously [69].

2.5. Cytotoxicity assay

Cytotoxicity assays were carried out to determine the sensitivities of cells to tested drugs according to the method described by Ishiyama *et al* [24]. Briefly, cells were plated in each well of 96-well plates at a density of 5000 cells per well in 100 μL of culture medium and maintained at 37 °C. After 24 h, an additional 100 μL of tested drug at various concentrations was added to each well and incubated for an additional 72 h before developing with either Cell Counting Kit-8 (CCK) or MTT reagent. CCK assay was used to determine the cytotoxicity of drugs in HEK293 and HEK293 transfected cells, whereas MTT assay was used to determine the cytotoxicity of drugs in human cancer cell lines. For the MDR reversal assays, nontoxic concentrations of SIS3 or a known inhibitor of ABCB1 or ABCG2, were added to the cytotoxicity assays. The extent of reversal was determined based on the calculated fold-reversal (FR) values, as described previously [12].

2.6. Apoptosis assay

The percentage of apoptotic cells in the total cell population induced by the indicated regimens was determined using the conventional Annexin V-FITC and propidium iodide (PI) staining method, as described previously [23]. Briefly, cells were first treated with colchicine, topotecan, SIS3 or in combinations as indicated for 48 h before harvested, centrifuged and resuspended in FACS buffer containing 1.25 μg/mL Annexin V-FITC (PharMingen) and 0.1 mg/mL PI and incubated for 15 min at room temperature. The labeled

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cells (10000 per sample) were analyzed by FACScan using CellQuest software (BD Biosciences). Phosphatidylserine PS-positive and PI-negative cells were counted as early apoptotic cells with intact plasma membranes, whereas PS-positive and PI-positive cells are considered as either necrotic or late apoptotic with leaky membranes [4].

2.7. ATPase assay

The effect of SIS3 on vanadate (Vi)-sensitive ATPase activity of ABCB1 or ABCG2 was determined using membrane vesicles of High-Five cells expressing ABCB1 or ABCG2 based on the endpoint P_i assay as described previously [1].

2.8. Docking analysis of SIS3 with ABCG2 and modeled structure of ABCB1

The three dimensional structure of human ABCB1 was predicted using an automated protein homology-modeling server SWISS-MODEL. The amino acid sequence of the protein was submitted to SWISS-MODEL server and templates were searched with BLAST and HHBlits against SWISS-MODEL template library. For each identified template, its quality was predicted from features of the target-template alignment. The templates with the highest quality were then selected and built based on the target-template alignment using ProMod3 [5–7]. The energy was minimized for ABCB1 homology modeled structure based on the structure of mouse Abcb1a and ABCG2 protein structure (PDB:5NJG) [57] using Acclerys Discovery Studio 4.0. Ligand preparation and docking was performed by the CDOCKER module of the same software.

2.9. Quantification and statistical analysis

Experimental values including IC_{50} are presented as mean ± standard deviation (SD) calculated from at least three independent experiments. In some cases where indicated, the values are given as mean ± standard error of the mean (SEM). Curve plotting and statistical analysis were performed with KaleidaGraph (Reading, PA, USA) and GraphPad Prism (La Jolla, CA, USA) software. The improvement in fit was analyzed by two-sided Student's t-test and labeled "statistically significant" if the probability, *p*, was less than 0.05.

3. Results

3.1. SIS3 inhibits ABCB1- and ABCG2-mediated drug transport

We first examined the effect of SIS3 on the drug transport function and the protein expression of ABCB1 or ABCG2. As shown in Fig. 1, the accumulation of calcein, a fluorescent product of calcein-AM, which is a substrate drug of ABCB1[21], was measured in human KB-3–1 epidermal cancer cells and the ABCB1-overexpressing variant KB-V-1cells, as well as in HEK293 cells and HEK293 cells transfected with human ABCB1 (MDR19-HEK293) treated with DMSO (*solid lines*), 10 μM of SIS3 (*shaded, solid lines*) or 10 μM of ABCB1 reference inhibitor verapamil (*dotted lines*). On the other hand, the accumulation of pheophorbide A (PhA), a fluorescent substrate drug of ABCG2[43], was measured in human H460 non-small cell lung cancer cells and the ABCG2-overexpressing variant H460-MX20 cells, as well as in HEK293 cells and HEK293 cells transfected with human ABCG2 (R482-HEK293) treated with DMSO (*solid lines*), 10 μM of SIS3 (*shaded, solid lines*) or 10 μM of ABCG2 reference inhibitor Ko143 (*dotted lines*), and analyzed as

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described in *Materials and methods.* SIS3 significantly inhibited ABCB1-medaited transport of calcein from KB-V-1 cells (Fig. 1A, right panel) and MDR19-HEK293 (Fig. 1B, right panel), as well as ABCG2-mediated transport of PhA fom H460-MX20 cells (Fig. 1C, right panel) and R482-HEK293 cells (Fig. 1D, right panel). Of note, SIS3 had no significant effect on the accumulation of fluorescent substrates in any of the drug-sensitive parental cells (Fig. 1B–1D, left panels).

3.2. SIS3 reverses multidrug resistance mediated by ABCB1 and ABCG2

Providing that SIS3 is able to block the drug efflux function of both ABCB1 and ABCG2, we next examined the chemosensitization effect of SISs in cells overexpressing ABCB1 or ABCG2. We found that at non-toxic concentrations and without affecting the proliferation of parental cells (Fig. 2, *left panels*), SIS3 re-sensitized KB-V-1 cells and MDR19-HEK293 cells to paclitaxel (Fig. 2A, right panel and Table 1) and colchicine (Fig. 2B and 2C) in a concentration-dependent manner. Similarly, SIS3 reversed ABCG2-mediated drug resistance to mitoxantrone and SN-38 in S1-M1–80 cells (Fig. 2D and 2E, right panels) and R482-HEK293 cells (Table 1 and Fig. 2C). The MDR reversal effect of SIS3 was further tested in ABCB1-overexpressing NCI-ADR-RES and ABCG2-overexpressing H460-MX20 cancer cell lines. The IC_{50} values and the extent of reversal of multidrug-resistant cells to a particular drug by SIS3, which was calculated as the fold-reversal (FR) values [12], are summarized in Table 1. Of note, verapamil and Ko143 were used as positive controls for the reversal of drug resistance mediated by ABCB1 and ABCG2 [66]. Our results revealed that SIS3 is capable of reversing multidrug resistance mediated by ABCB1 and ABCG2 in cancer cells at concentrations below 1 μM.

Knowing that transient down-regulation of ABCB1 or ABCG2 can also lead to the re-sensitization of MDR cancer cells to conventional anticancer agents [11, 36], we examined effect of SIS3 on the protein expression of ABCB1 and ABCG2 in KB-V-1 and H460-MX20 cancer cells, respectively. We found that treating cancer cells with increasing concentrations of SIS3 (0 – 1 μM) for 72 h has no significant effect on ABCB1 protein expression in KB-V-1 cells (Fig. 3A) or ABCG2 protein expression in H460-MX20 cells (Fig. 3B).

3.3. SIS3 enhances drug-induced apoptosis in ABCB1- and ABCG2-overexpressing cancer cells

Next, we evaluated the effect of SIS3 on drug-induced apoptosis in drug-sensitive cancer cells and MDR cancer cells overexpressing ABCB1 or ABCG2. KB-3–1 and KB-V-1 cells were treated with colchicine in the presence or absence of SIS3 for 48 h, whereas S1 and S1-M1–80 cells were treated with topotecan in the presence or absence of SIS3 for 48 h, processed and analyzed as described previously [23, 65]. As shown in Fig. 4A, treatment with colchicine substantially increased the level of apoptosis from approximately 6% to 33% in KB-3–1 cells. In contrast, treatment with colchicine, a known substrate of ABCB1, only increased the level of apoptosis from approximately 8% to 10% in KB-V-1 cells. Interestingly, we found that co-treatment of SIS3 and colchicine significantly enhanced the level of apoptosis from approximately 6% to 49% in KB-V-1 cells. Similarly, treatment with topotecan increases the level of apoptosis in S1 cells, but not in S1-M1–80 cells (from approximately 6% to 7% total apoptosis), which can be significantly enhanced by SIS3,

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from approximately 6% to 28% total apoptosis (Fig. 4B). Of note, SIS3 alone had no significant effect on the level of apoptosis in all cell lines tested. Our results show that SIS3 enhances the chemosensitivity of MDR cancer cells overexpressing ABCB1 or ABCG2.

3.4. SIS3 stimulates the ATPase activity of ABCB1 and ABCG2

In order to further understand the interaction of SIS3 with the substrate-binding sites of ABCB1 and ABCG2, we examined the effect of SIS3 on vanadate (Vi)-sensitive ATPase activity of ABCB1 and ABCG2, as well as docking analysis of SIS3 with molecular modeling of ABCB1 and ABCG2. SIS3 stimulated Vi-sensitive ABCB1 ATP hydrolysis in a concentration-dependent manner, producing approximately 250% maximum stimulation (basal, 34.6 ± 4.0 nmole P_i/min/mg protein) and the concentration required to produce 50% of the maximum stimulation (EC_{50}) of approximately 160 nM (Fig. 5A). Similarly, SIS3 also stimulated ABCG2 ATP hydrolysis in a concentration-dependent manner, producing approximately 230% maximum stimulation (basal, 73.3 ± 3.9 nmole P_i/min/mg protein) and the EC_{50} of approximately 60 nM (Fig. 5B). Knowing that substrate transport mediated by ABCB1 or ABCG2 is coupled to ATP hydrolysis [2, 3], our results indicate that SIS3 is a substrate of both ABCB1 and ABCG2.

3.5. Docking analysis of SIS3 with ABCG2 and modeled structure of ABCB1

To better understand the binding of SIS3 with the transporter proteins, docking study was performed using crystal structure of ABCG2 and a homology model generated from high resolution mouse (*Mus musculus*) ABCB1 protein crystal structure (pdb.4Q9L) as template. Docking of SIS3 with modeled ABCB1 protein structure showed three main interactions in the probable substrate binding site [14, 55]: the aromatic rings interacted with hydrophobic residues Ala^{302} and Ala^{987} whilst the amino group on Asn^{842} may form hydrogen bond with the oxygen from methoxy moiety (Fig. 6A). In the docking study with ABCG2 protein, SIS3 was predicted to bind in the substrate binding cavity between two ABCG2 monomers. It is possibly stabilized by hydrophobic interactions between its aromatic rings and residues such as Phe^{439}, Ser^{440} on transmembrane domain 2 (TM2) and Val^{546} on TM5a (Fig. 6B). Results of our ATPase activity experiments and docking analysis indicate strong interaction of SIS3 with both ABCB1 and ABCG2.

3.6. Overexpression of ABCB1 or ABCG2 does not reduce the chemosensitivity of cancer cells to SIS3

Knowing that SIS3 behaves as a substrate for ABCB1 and ABCG2, we examined whether cancer cells overexpressing ABCB1 or ABCG2 are resistant to SIS3. As shown in Table 2, the resistance factor (RF) value represents the extent of acquired SIS3 resistance in cancer cells caused by the overexpression of ABCB1 or ABCG2, calculated by dividing the IC_{50} value of SIS3 in MDR subline by the IC_{50} value of SIS3 in the respective parental line. The calculated RF values revealed no significant differences in the IC_{50} values of SIS3 in drug-sensitive cancer cells and cancer cells overexpressing ABCB1 or ABCG2. Our results show that drug-sensitive cancer cells and MDR cancer cells are equally sensitive to SIS3 treatment, suggesting that SIS3 is not actively effluxed by ABCB1 or ABCG2 in cancer cells and behaves as a high-affinity substrate for ABCB1 and ABCG2.

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4. Discussion

One of the significant challenges to the effective treatment of cancer is the development of multidrug resistance to chemotherapy, which is in part contributed by the overexpression of ABCB1 or ABCG2 in cancer cells [15, 37, 68]. In recent years, many attempts of developing novel inhibitors to re-sensitize MDR cancer cells to anticancer agents have failed, mostly due to problems associated with toxicity and adverse drug-drug interactions [50, 54]. This prompted us to search for drugs with known pharmacological and toxicological properties that could be re-positioned to treat MDR cancer cells that overexpress ABCB1 or ABCG2. The specific inhibitor of Smad3, SIS3, has been used on a regular basis in studies evaluating various TGF-β-regulated cellular mechanisms [9, 28, 30, 32, 41, 61, 62, 72]. More recently, SIS3 has emerged as a potential therapeutic agent in cancer treatment, capable of suppressing cancer progression and attenuating resistance to anticancer drugs in cancer cells. For instance, a recent study demonstrated that systemic treatment with SIS3 in the tumor microenvironment suppresses tumor growth, invasion and metastasis [56]. Similarly, SIS3 was able to suppress TGF-β1-induced epithelial-mesenchymal transition (EMT) in lung carcinomas [31], whereas another study demonstrated that by interrupting the TGF-β-SMAD3 pathway, SIS3 prevents the development of anti-HER2 drugs resistance and restores trastuzumab sensitivity in trastuzumab-resistant cells [10].

In the present study, we demonstrate that SIS3 inhibits the drug efflux function of ABCB1 and ABCG2. More importantly, SIS3 enhances the drug-induced apoptosis and reverses MDR-mediated by ABCB1 and ABCG2 in MDR cancer cell lines at low, nontoxic concentrations that are considerably lower than the concentrations of SIS3 used in studies evaluating TGF-β-regulated cellular mechanisms [9, 28, 30, 32, 41, 61, 62, 72]. At the same concentrations, SIS3 did not alter the protein expression of ABCB1 or ABCG2 in MDR cancer cells over a period of 72 h. In addition, the ATPase activity of ABCB1 and ABCG2 was stimulated by SIS3 in a concentration-dependent manner, which is in the same manner as other known substrate drugs for ABCB1 and ABCG2 [48, 49, 51]. The apparent EC_{50} value for SIS3-stimulated ABCB1 ATPase activity is approximately 160 nM, and approximately 60 nM for SIS3-stimulated ABCG2 ATPase activity, indicating a high-affinity interaction of SIS3 with ABCB1 and ABCG2. In addition, results of our docking analysis and the fact that ABCB1 and ABCG2 do not confer resistant to SIS3 in cancer cells further support the notion that SIS3 binds to the substrate-binding site of ABCB1 and ABCG2 with high enough affinity to compete with the binding of another drug substrate at the same site.

In summary, our results revealed that SIS3 is an effective modulator of ABCB1 and ABCG2, and that concomitant administration of SIS3 could potentially improve the therapeutic efficacy of other chemotherapeutic agents. Nevertheless, knowing that favorable experimental results do not necessarily translate into clinical outcomes [50], and that combination therapy may sometimes lead to unfavourable clinical responses [29, 53], SIS3 should be further evaluated in preclinical animal model studies and in clinical studies.

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ACKNOWLEDGMENTS

This work was supported by funds from the Ministry of Science and Technology of Taiwan (MOST-105–2320-B-182–018 and MOST-106–2320-B-182–017 to CPW; MOST 103–2314-B-182A-099-MY1 and 103–2314-B-182A-099-MY2 to THH; MOST-102–2113-M-029–005 to YSW), Chang Gung Medical Research Program (BMRPC17, CMRPD1D0153 and CMRPD1G0112 to CPW), Taichung Veterans General Hospital (TCVGH-T1057805 to YSW) and the Intramural Research Program of the National Institutes of Health, National Cancer Institute, Center for Cancer Research to SVA and MM.

Abbreviations:

MDR	multidrug resistance
ABC	ATP-binding cassette
SMAD3	Mothers against decapentaplegic homolog 3
FCS	fetal calf serum
CCK-8	Cell Counting Kit-8
MTT	3-(4,5-dimethylthiazol-yl)-2,5-diphenyllapatinibrazolium bromide
Vi	sodium orthovanadate
IMDM	Iscove’s Modified Dulbecco’s Medium
FR	Fold reversal

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Highlights

- SIS3, a specific inhibitor of Smad3, inhibits the function of ABCB1 and ABCG2.
- SIS3 enhances drug-induced apoptosis in multidrug-resistant cancer cells.
- SIS3 resensitizes ABCB1 and ABCG2 overexpressing cancer cells to chemotherapeutics.
- ABCB1 and ABCG2 do not confer resistance to SIS3 in cancer cells.
- Inclusion of SIS3 in the drug treatment may benefit patients with multidrug-resistant tumors in the future.

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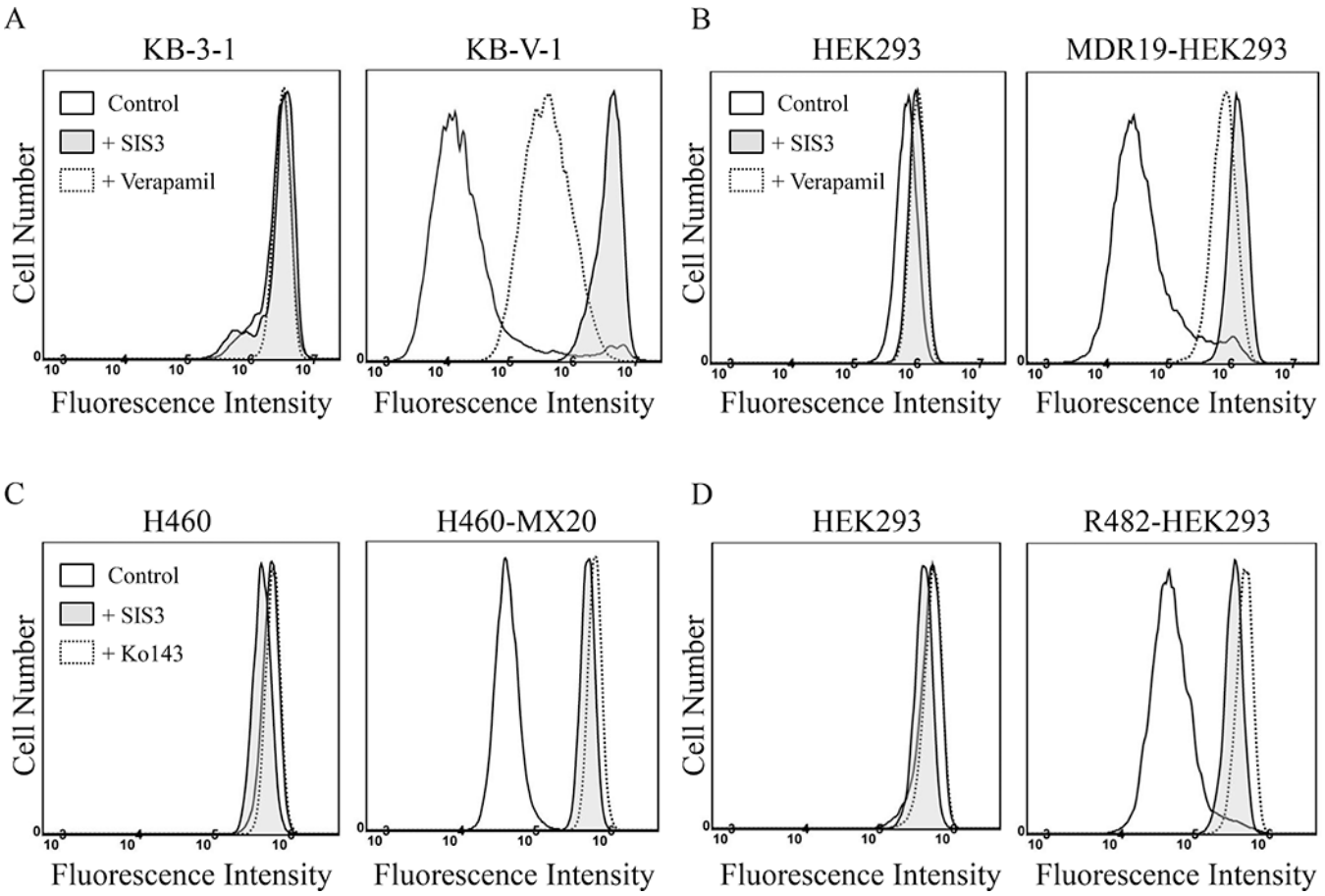

Fig. 1. SIS3 inhibits drug efflux mediated by ABCB1 and ABCG2.

The accumulation of fluorescent calcein (0.5 μM calcein-AM) in human KB-3–1 epidermal cancer cells (A, left panel) and the ABCB1-overexpressing variant KB-V-1 cancer cells (A, right panel), as well as in human HEK293 cells (B, left panel) and MDR19-HEK293, HEK293 cells transfected with human ABCB1 (B, right panel), or 1 μM of fluorescent pheophorbide A (PhA) in human H460 lung cancer cells (C, left panel) and the ABCG2-overexpressing variant H460-MX20 cancer cells (C, right panel), as well as in human HEK293 cells (D, left panel) and R482-HEK293, HEK293 cells transfected with human ABCG2 (D, right panel), was measured and analyzed immediately by flow cytometry as described previously [70]. Experiments were carried out either in the absence (solid lines) or presence of SIS3 at10 μM (shaded, solid lines) or verapamil, a reference inhibitor for ABCB1at 20 μM (A and B, dotted lines), or Ko143, a reference inhibitor for ABCG2 at 3 μM (C and D, dotted lines). Representative histograms of at least three independent experiments are shown.

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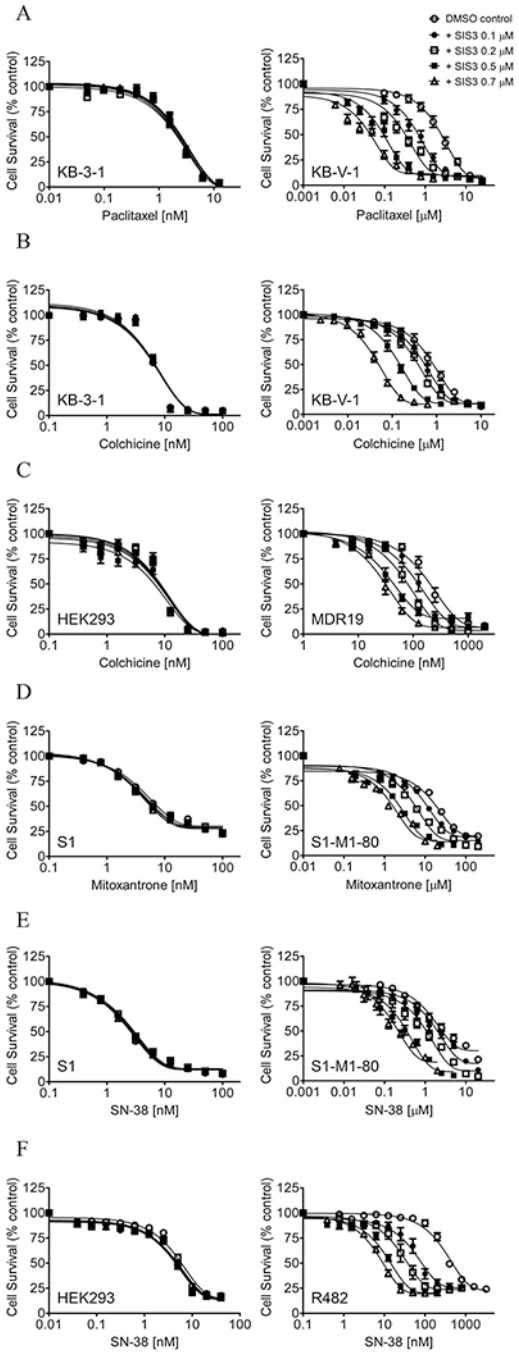

Fig. 2. SIS3 reverses multidrug resistance mediated by ABCB1and ABCG2 in cancer cell lines.

Drug-sensitive KB-3–1 cells (A and B, left panel) and MDR variant KB-V-1 cancer cells (A and B, right panel), HEK293 cells (C, left panel) and MDR19-HEK293 cells (C, right panel), drug-sensitive S1 colon cancer cells (D and E, left panel) and MDR variant S1-M1–80 cancer cells (D and E, right panel), as well as HEK293 cells (F, left panel) and R482-HEK293 cells (F, right panel), were treated with increasing concentrations of paclitaxel (A), colchicine (B and C), mitoxantrone (D), SN-38 (E and F) in the presence of DMSO (empty circles), or SIS3 at 0.1 μM (filled circles), 0.2 μM (empty squares), 0.5 μM (filled squares)

or 0.7 μM (empty triangles). Points, mean values from at least three independent experiments; bars; SEM.

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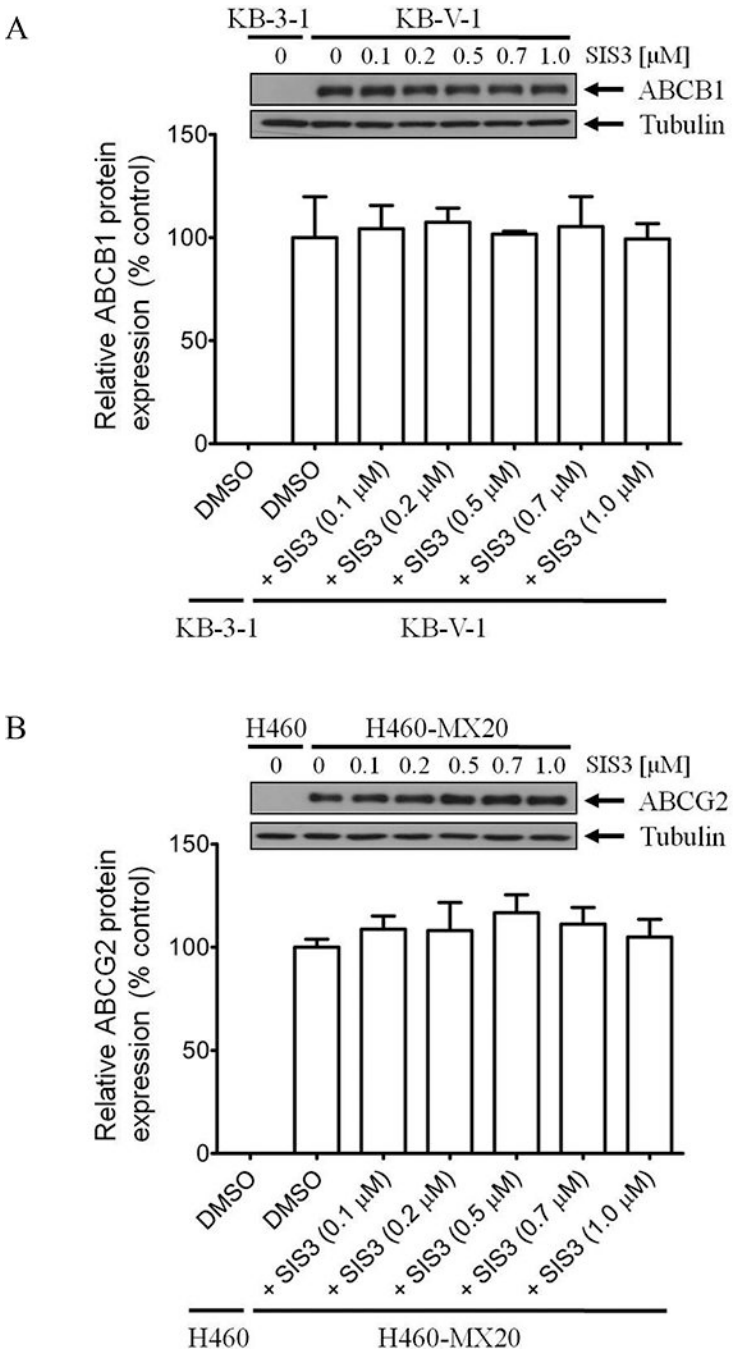

Fig. 3. Effect of SIS on ABCB1 protein expression in human KB-V-1 epidermal cancer cells and ABCG2 in human H460-MX20 lung cancer cells.

Immunoblot detection and quantification of (A) human ABCB1 in drug-sensitive human KB-3–1 cells and ABCB1-overexpressing KB-V-1 cells, or (B) human ABCG2 in drug-sensitive H460 cells and ABCG2-overexpressing H460-MX20 cells treated with DMSO (vehicle control) or increasing concentrations (0.1 – 1.0 μM) of SIS3 for 72 h according to the method described previously [65]. α-Tubulin was used as an internal loading control. Values are presented as mean ± S.D. calculated from three independent experiments.

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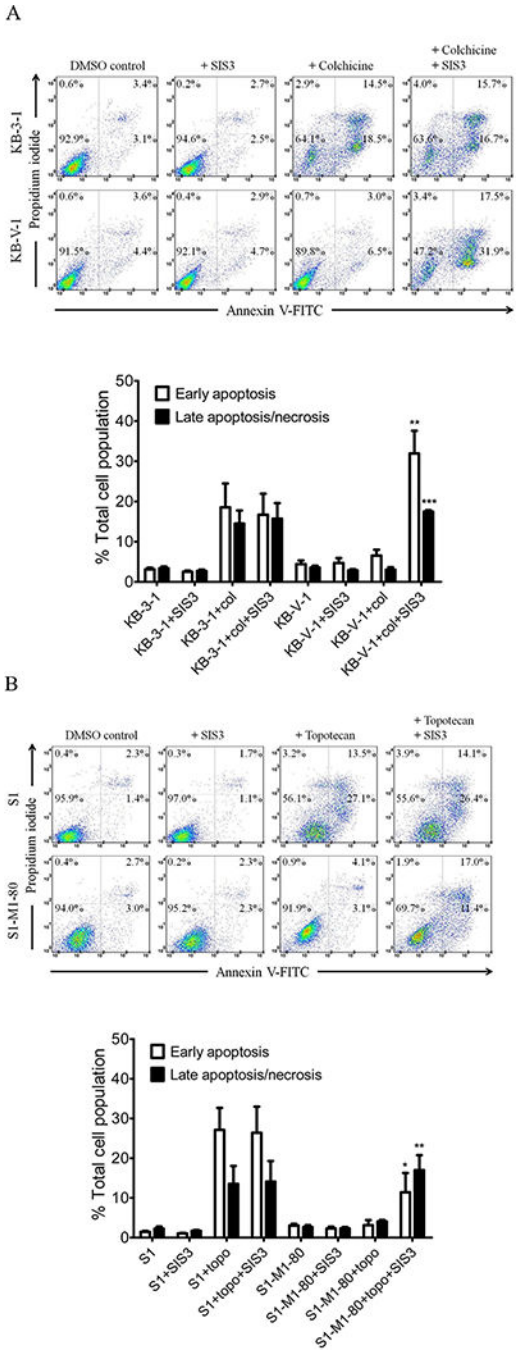

Fig. 4. SIS3 enhances drug-induced apoptosis in ABCB1- and ABCG2-overexpressing cancer cells.

(A) Human KB-3–1 epidermal cancer cells (top dot plots panels) and MDR variant KB-V-1 cancer cells (lower dot plots panels) were treated with either DMSO (control), 1.0 μM SIS3 (+ SIS3), 0.5 μM colchicine (+ colchicine) or a combination of 0.5 μM colchicine and 1.0 μM SIS3 (+ colchicine + SIS3) for 48 h. (B) Human S1 colon cancer cells (top dot plots panel) and MDR variant S1-M1–80 cancer cells (lower dot plots panels) were treated with either DMSO (control), 1.0 μM SIS3 (+ SIS3), 5.0 μM topotecan (+ topotecan) or a combination of 5.0 μM topotecan and 1.0 μM SIS3 (+ topotecan + SIS3) for 48 h. Cells were

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isolated and analyzed by flow cytometry according to the method described previously [66]. Representative dot plots and the mean values of three independent experiments are shown. Quantifications of drug-induced apoptosis in cancer cell lines are presented as mean ± S.D. calculated from three independent experiments. $^{**}P < 0.01$; $^{***}P < 0.001$, versus the same treatment in the absence of SIS3.

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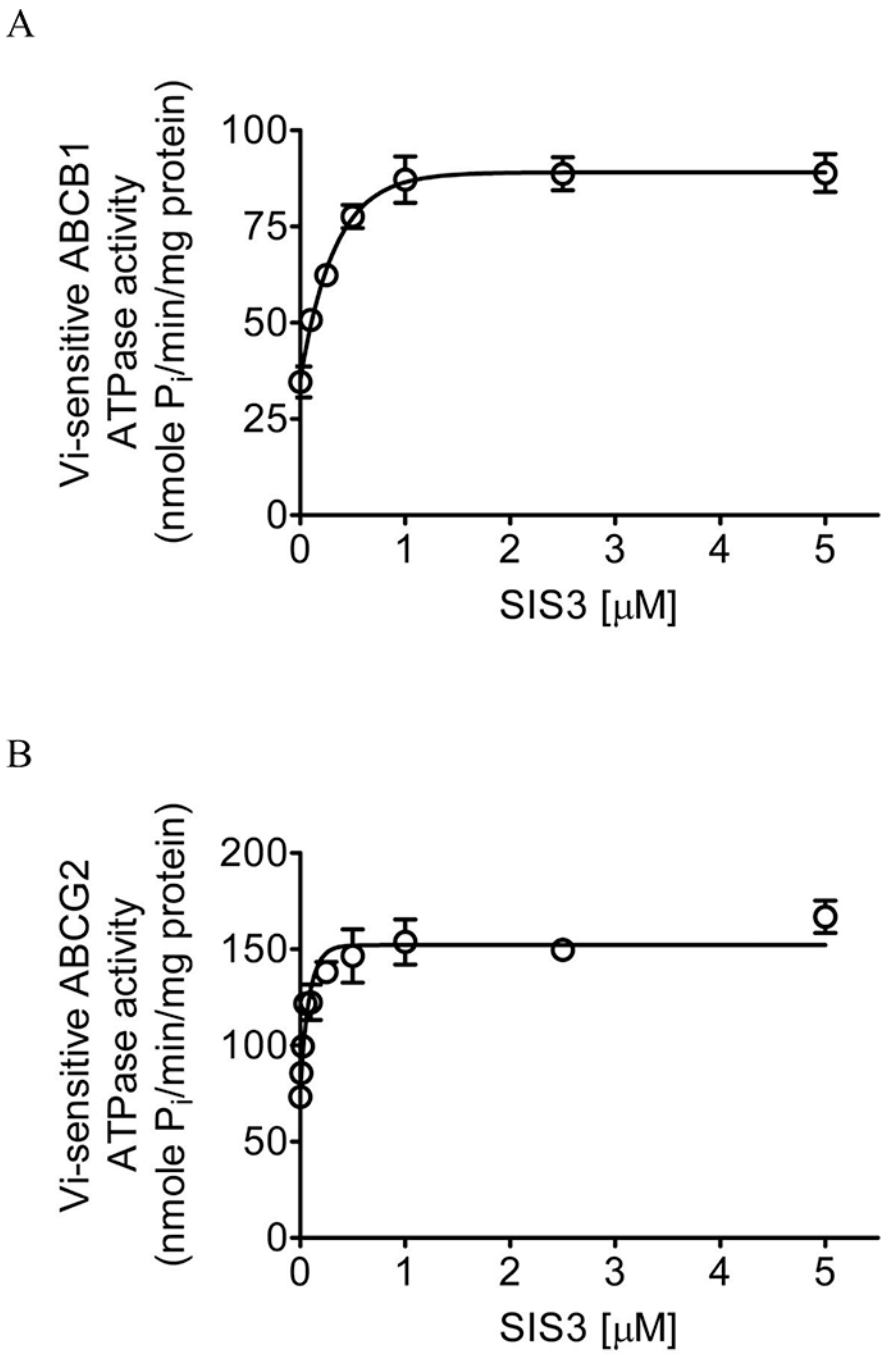

Fig. 5. SIS3 stimulates vanadate-sensitive ATPase activity of ABCB1 and ABCG2.

The effect of SIS3 (0 – 5 μM) on vanadate-sensitive (A) ABCB1 and (B) ABCG2 ATP hydrolysis was determined by endpoint P_i assay, as described previously [1, 67]. Values are presented as mean ± S.D. calculated from three independent experiments.

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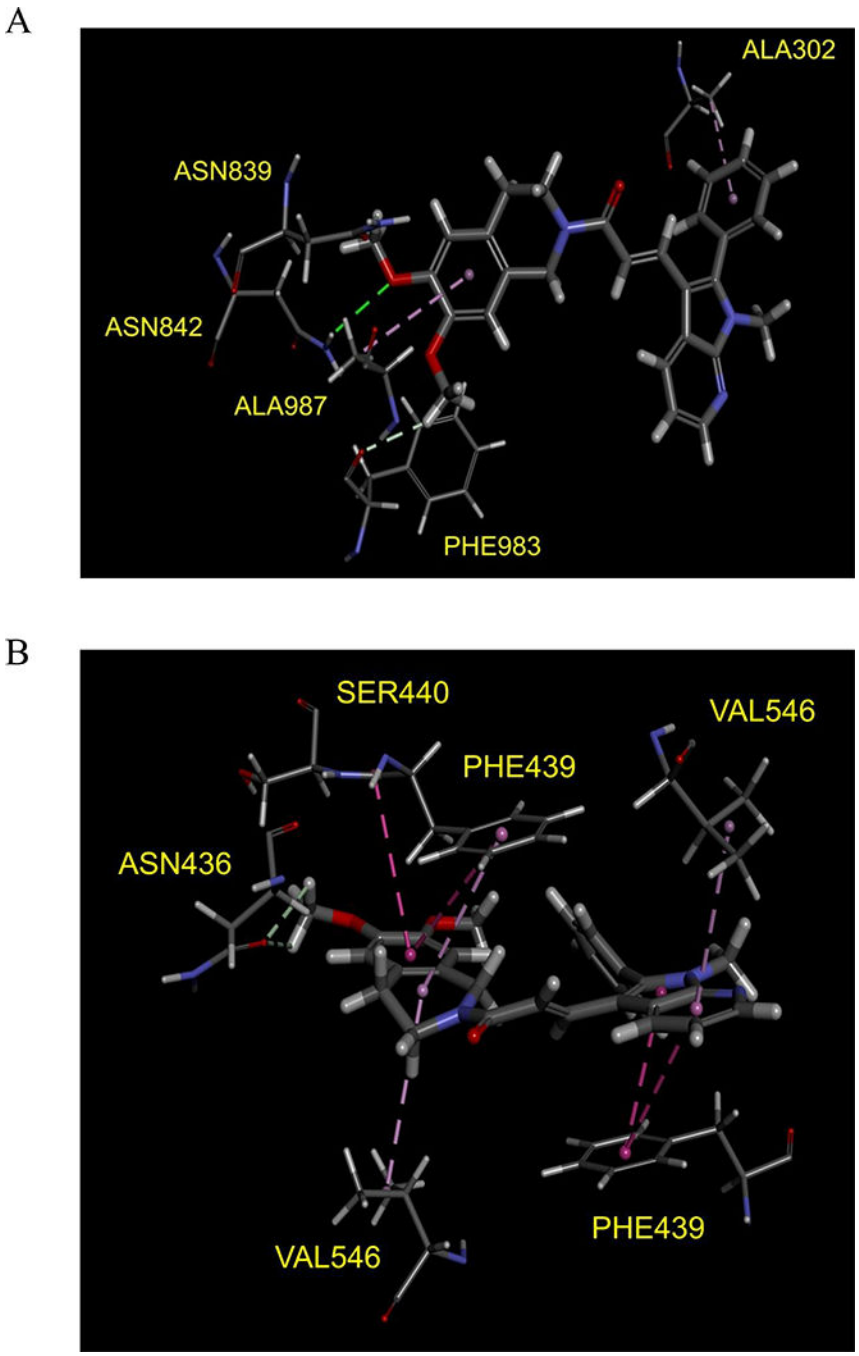

Fig. 6. Docking of SIS3 in the drug-binding pocket of ABCB1 and ABCG2.

Binding modes of SIS3 with (A) homology modeled ABCB1 and (B) ABCG2 protein structure (PDB:5NJG) were predicted by Acclerys Discovery Studio 4.0 software as described in *Materials and methods.* SIS3 is shown as a molecular model with the atoms colored as carbon- gray, hydrogen-light gray, nitrogen-blue and oxygen-red. The same color scheme is used for interacting amino acid residues. Dotted lines indicate proposed interactions.

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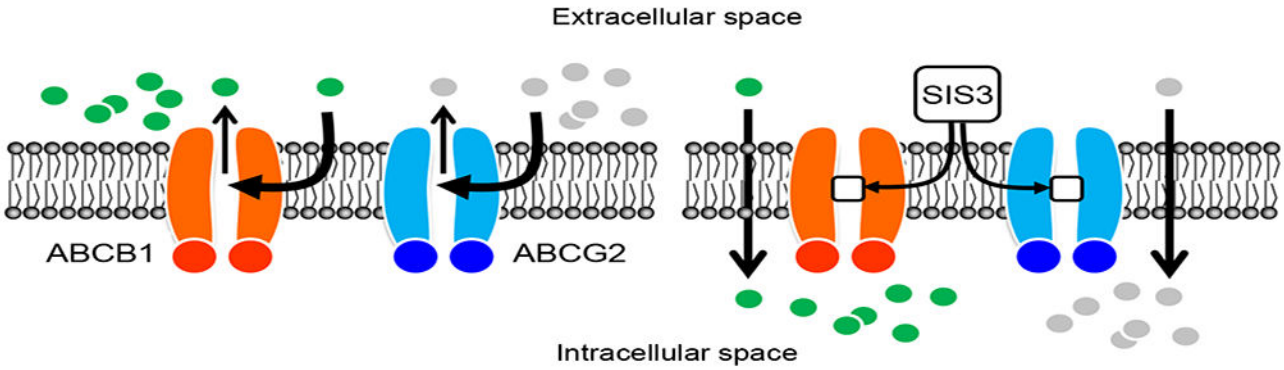

Fig. 7. A schematic diagram illustrating how SIS3 reverses drug resistance mediated by ABCB1 and ABCG2.

In the absence of SIS3 (left panel), ABCB1 substrate drugs (green circles) and ABCG2 substrate drugs (gray circles) are actively pumped out by ABCB1 (orange) and ABCG2 (blue), respectively, resulting in reduced intracellular drug accumulation and drug resistance. In contrast, in the presence of SIS3 (right panel), ABCB1 substrate drugs and ABCG2 substrate drugs are no longer able to access the drug binding pockets of ABCB1 and ABCG2 since SIS3 (white squares) has already occupied the sites, consequently restoring intracellular drug concentration and chemosensitivity.

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Table 1

Reversal effect of SIS3 on drug resistance mediated by ABCB1 and ABCG2.

Treatment	Concentration (μM)	Mean IC_{50} † ± SD and (FR‡)	
		KB-3–1 [nM]	KB-V-1 [nM]
Colchicine	-	5.09 ± 1.88 (1.0)	808.13 ± 41.30 (1.0)
+ SIS3	0.1	5.11 ± 1.85 (1.0)	523.77 ± 40.35 ** (1.5)
+ SIS3	0.2	5.16 ± 1.92 (1.0)	367.13 ± 43.11 *** (2.2)
+ SIS3	0.5	5.31 ± 2.07 (1.0)	136.60 ± 15.69 *** (5.9)
+ SIS3	0.7	5.10 ± 2.02 (1.0)	45.51 ± 6.19 *** (17.8)
+ Verapamil	5.0	2.86 ± 1.17 (1.8)	71.44 ± 7.96 *** (11.3)
Paclitaxel	-	1.99 ± 0.53 (1.0)	2273.60 ± 218.05 (1.0)
+ SIS3	0.1	1.90 ± 0.46 (1.0)	635.44 ± 37.69 *** (3.6)
+ SIS3	0.2	1.89 ± 0.48 (1.1)	323.36 ± 42.78 *** (7.0)
+ SIS3	0.5	2.15 ± 0.50 (1.0)	92.37 ± 12.79 *** (24.6)
+ SIS3	0.7	2.08 ± 0.48 (1.0)	48.19 ± 9.47 *** (47.2)
+ Verapamil	5.0	2.56 ± 0.73 (0.8)	89.06 ± 12.26 *** (25.5)
		OVCAR-8 [nM]	NCI-ADR-RES [nM]
Colchicine	-	18.62 ± 3.45 (1.0)	1730.54 ± 317.81 (1.0)
+ SIS3	0.1	19.29 ± 3.28 (1.0)	1019.80 ± 216.52 * (1.7)
+ SIS3	0.2	19.74 ± 3.14 (1.0)	681.85 ± 148.18 ** (2.5)
+ SIS3	0.5	17.65 ± 3.09 (1.1)	277.86 ± 56.22 ** (6.2)
+ SIS3	0.7	16.56 ± 3.09 (1.1)	142.47 ± 16.16 ** (12.1)
+ Verapamil	5.0	11.92 ± 2.56 (1.6)	518.26 ± 96.19 ** (3.3)
Paclitaxel	-	79.60 ± 23.93 (1.0)	6385.00 ± 959.34 (1.0)
+ SIS3	0.1	73.43 ± 25.60 (1.1)	3147.20 ± 267.22 ** (2.0)
+ SIS3	0.2	62.07 ± 20.33 (1.3)	2102.74 ± 267.40 ** (3.0)
+ SIS3	0.5	58.53 ± 16.68 (1.4)	466.38 ± 46.62 *** (13.7)
+ SIS3	0.7	53.56 ± 12.95 (1.5)	328.34 ± 51.72 *** (19.4)
+ Verapamil	5.0	46.90 ± 15.15 (1.7)	184.35 ± 22.73 *** (34.6)
		pcDNA-HEK293 [nM]	MDR19-HEKS293 [nM]
Colchicine	-	8.20 ± 2.42 (1.0)	183.66 ± 35.56 (1.0)
+ SIS3	0.1	8.14 ± 2.26 (1.0)	111.59 ± 23.66 * (1.6)
+ SIS3	0.2	8.09 ± 2.33 (1.0)	66.03 ± 15.18 ** (2.8)
+ SIS3	0.5	7.18 ± 1.80 (1.1)	45.05 ± 8.65 ** (4.1)
+ SIS3	0.7	7.98 ± 2.38 (1.0)	26.76 ± 5.17 ** (6.9)

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Treatment	Concentration (μM)	Mean IC_{50} † ± SD and (FR‡)	
		KB-3–1 [nM]	KB-V-1 [nM]
+ Verapamil	5.0	8.14 ± 2.44 (1.0)	40.40 ± 9.30 ** (4.5)
Paclitaxel	-	1.53 ± 0.26 (1.0)	835.30 ± 128.85 (1.0)
+ SIS3	0.1	1.24 ± 0.18 (1.2)	479.04 ± 36.49 ** (1.7)
+ SIS3	0.2	1.25 ± 0.19 (1.2)	297.83 ± 48.57 ** (2.8)
+ SIS3	0.5	1.27 ± 0.20 (1.2)	73.94 ± 9.18 *** (11.3)
+ SIS3	0.7	1.20 ± 0.17 (1.3)	60.43 ± 5.05 *** (13.8)
+ Verapamil	5.0	0.90 ± 0.16 * (1.7)	6.14 ± 1.23 *** (136.0)
		H460 [nM]	H460-MX20 [nM]
SN-38	-	4.49 ± 1.02 (1.0)	138.71 ± 32.27 (1.0)
+ SIS3	0.1	4.15 ± 1.07 (1.1)	36.67 ± 8.01 ** (3.8)
+ SIS3	0.2	5.14 ± 0.90 (0.9)	20.52 ± 5.35 ** (6.8)
+ SIS3	0.5	3.76 ± 0.67 (1.2)	11.65 ± 3.48 ** (11.9)
+ SIS3	0.7	3.34 ± 0.58 (1.3)	4.84 ± 1.28 ** (28.7)
+ Ko143	1	4.10 ± 0.75 (1.1)	4.45 ± 1.06 ** (31.2)
Mitoxantrone	-	13.73 ± 1.19 (1.0)	488.15 ± 81.40 (1.0)
+ SIS3	0.1	20.54 ± 5.79 (0.7)	342.70 ± 85.71 (1.4)
+ SIS3	0.2	15.61 ± 4.68 (0.9)	227.99 ± 58.92 * (2.1)
+ SIS3	0.5	16.70 ± 4.84 (0.8)	77.22 ± 18.04 ** (6.3)
+ SIS3	0.7	17.00 ± 5.41 (0.8)	55.71 ± 21.69 ** (8.8)
+ Ko143	1	15.93 ± 5.27 (0.9)	70.14 ± 19.37 ** (7.0)
		S1 [nM]	S1-M1–80 [μM]
SN-38	-	2.32 ± 0.28 (1.0)	3.83 ± 0.65 (1.0)
+ SIS3	0.1	2.58 ± 0.30 (0.9)	2.33 ± 0.30 * (1.6)
+ SIS3	0.2	2.76 ± 0.32 (0.8)	1.05 ± 0.12 ** (3.6)
+ SIS3	0.5	2.36 ± 0.26 (1.0)	0.32 ± 0.03 *** (12.0)
+ SIS3	0.7	2.31 ± 0.29 (1.0)	0.23 ± 0.02 *** (16.7)
+ Ko143	1	2.22 ± 0.28 (1.0)	0.05 ± 0.01 *** (76.6)
Mitoxantrone	-	11.48 ± 2.70 (1.0)	24.09 ± 5.80 (1.0)
+ SIS3	0.1	9.60 ± 2.21 (1.2)	15.17 ± 2.82 (1.6)
+ SIS3	0.2	9.61 ± 2.49 (1.2)	6.95 ± 1.22 ** (3.5)
+ SIS3	0.5	8.20 ± 2.01 (1.4)	2.79 ± 0.48 ** (8.6)
+ SIS3	0.7	8.03 ± 2.09 (1.4)	1.88 ± 0.33 ** (12.8)
+ Ko143	1	10.42 ± 2.56 (1.1)	0.87 ± 0.13 ** (27.7)

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Treatment	Concentration (μM)	Mean IC_{50} † ± SD and (FR‡)	
		KB-3–1 [nM]	KB-V-1 [nM]
		pcDNA-HEK293 [nM]	R482-HEKS293 [nM]
SN-38	-	6.53 ± 0.98 (1.0)	535.00 ± 63.14 (1.0)
+ SIS3	0.1	5.88 ± 0.95 (1.1)	114.69 ± 21.08*** (4.7)
+ SIS3	0.2	5.51 ± 0.84 (1.2)	50.09 ± 11.35*** (10.7)
+ SIS3	0.5	5.38 ± 0.83 (1.2)	17.61 ± 3.69*** (30.4)
+ SIS3	0.7	5.17 ± 0.83 (1.3)	10.96 ± 2.16*** (48.8)
+ Ko143	1	5.50 ± 0.87 (1.2)	7.18 ± 1.73*** (74.5)
Mitoxantrone	-	0.74 ± 0.11 (1.0)	122.15 ± 16.21 (1.0)
+ SIS3	0.1	1.00 ± 0.13 (0.7)	29.48 ± 2.72*** (4.1)
+ SIS3	0.2	0.90 ± 0.13 (0.8)	15.94 ± 2.14*** (7.7)
+ SIS3	0.5	0.62 ± 0.09 (1.2)	10.95 ± 1.65*** (11.2)
+ SIS3	0.7	0.65 ± 0.07 (1.1)	8.74 ± 1.42*** (14.0)
+ Ko143	1	0.81 ± 0.12 (0.9)	7.14 ± 1.10*** (17.1)

Abbreviation: FR, fold-reversal.

† IC_{50} values are mean ± SD calculated from dose-response curves obtained from three independent experiments using cytotoxicity assay as described in *Materials and methods.*

‡ FR values were calculated by dividing IC_{50} values of cells treated with a particular anticancer drug in the absence of SIS3 or a reference inhibitor by IC_{50} values of cells treated with the same anticancer drug in the presence of SIS3 or a reference inhibitor.

* $P < 0.05$;

** $P < 0.01$;

*** $P < 0.001$

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Table 2

Cytotoxicity of SIS3 in human cancer cell lines overexpressing ABCB1 or ABCG2.

Cell line	Type	Transporter expressed	IC_{50} (nM)†	R.F‡
KB-3–1	epidermal	-	10.73 ± 4.14	1.0
KB-V-1	epidermal	ABCB1	9.56 ± 3.70	0.9
OVCAR-8	ovarian	-	7.58 ± 1.07	1.0
NCI-ADR-RES	ovarian	ABCB1	8.79 ± 1.77	1.2
H460	lung	-	9.97 ± 3.53	1.0
H460-MX20	lung	ABCG2	8.30 ± 2.12	0.8
S1	colon	-	4.06 ± 0.93	1.0
S1-M1–80	colon	ABCG2	3.54 ± 0.48	0.9
MCF7	breast	-	8.90 ± 2.22	1.0
MCF7-FLV1000	breast	ABCG2	11.21 ± 1.72	1.3
MCF7-AdVp3000	breast	ABCG2	10.11 ± 2.79	1.1

Abbreviation: RF, resistance factor.

† IC50 values are mean ± SD calculated from dose-response curves obtained from three independent experiments using cytotoxicity assay as described in *Materials and methods.*

‡ RF values were obtained by dividing IC_{50} values of ABCB1- or ABCG2- overexpressing cells by IC_{50} values of respective parental cells.

$^{*}P < 0.05$;

$^{**}P < 0.01$;

$^{***}P < 0.001$