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KIT and PDGFRA inhibitor avapritinib (BLU-285) overcomes ABCB1- and ABCBG2-mediated MDR in cancer cells

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The development of multidrug resistance (MDR) due to the overexpression of ATP-binding cassette (ABC) transporters remains one of the major obstacles to the success of chemotherapy in clinics. It is of paramount importance to identify novel drug combinations that could inhibit the multidrug efflux of ABC transporters and enhance the chemo-sensitivity of substrate anticancer drugs. In this study, we evaluated avapritinib, a KIT and PDGFRA blocker, for its reversal effects on the drug sensitivity of ABCB1 and ABCG2 overexpressing cells. Our results show that avapritinib significantly enhanced the cytotoxicity of the substrates of both ABCB1 and ABCG2. Mechanistic studies revealed that avapritinib enhances the intracellular accumulation of the substrates of ABCB1 or ABCG2 by directly decreasing their efflux from the cells overexpressing ABCB1 or ABCG2. Moreover, avapritinib did not change the expressional levels or translocation of ABCB1 or ABCG2 protein from the cell membrane to the cytoplasm and stimulates the ATP cleaving activity of both ABCB1 and ABCG2. Taken together, our results open new avenues for the use of avapritinib as cancer chemotherapy, when used in combination with the substrates of ABCB1 or ABCG2.

Keywords

Avapritinib; multidrug resistance; ATP-binding cassette transporters; ABCB1; ABCG2

1. Introduction

It has long been known that the cancer cells adapt and become insensitive to long exposures of chemotherapeutic drugs [1, 2, 3, 4]. This phenomenon of cancer cells becoming resistant to a wide variety of structurally and mechanistically different classes of chemotherapeutic drugs has been reported in a number of studies and it is termed multidrug resistance (MDR) [5, 6, 7, 8].

Studies conducted in the past have reported a number of biological reasons that lead to cancer cells becoming resistant and researchers have been exploiting these factors as potential drug targets. Yet, the existence and overexpression of ATP-binding cassette (ABC) transporters remains by far one of the leading factors contributing to MDR [9, 10, 11, 12, 13]. The ABC transporters are efflux transporters which are located on the plasma membrane and uses the energy derived from the hydrolysis of ATP into ADP and Pi. [14, 15, 16]. As a result, the intracellular concentration of the chemotherapeutic drug decreases, which then necessitates a higher dose to produce the same anti-cancer effect, eventually causing MDR. Studies done in the past have identified ABC transporters to be present in a number of organs such as, the kidney, liver, small intestine, brain, and placenta. Interestingly, in these organs, the ABC transporters exert cytoprotective effects by preventing the accumulation of exogenous and endogenous cytotoxins. [7, 12, 16]. Contrastingly, in tumors, the efflux function of ABC transporters renders low concentration of chemotherapeutic drugs to be present inside the cells which results in the development of MDR.

The ABC transporter superfamily consists of a total of 49 members subdivided into seven families (ABCA-G). The ABCB1, ABCG2, and members of ABCC family are the most extensively studied ABC transporters for their role in inducing MDR in cancer cells [17, 18, 19].

ABCB1 (also known as P-glycoprotein), was the first identified ABC transporter. ABCB1, a 170-kDa protein, mediates resistance to a variety of antitumor drugs including anthracyclines (e.g. doxorubicin and daunorubicin), taxanes (e.g. paclitaxel and docetaxel), epipodophyllotoxins (e.g. etoposide and teniposide), *Vinca* alkaloids (e.g. vinblastine and vincristine), and antibiotics (e.g. actinomycin D) [1, 2]. ABCG2, which is also known as BCRP (breast cancer resistance protein), is a 72-kDa protein. Studies have shown that ABCG2 is present in the placenta, liver, and small intestine [9, 20, 21]. The substrates of ABCG2 include cytotoxic drugs like anthracyclines, camptothecin-derived topoisomerase I inhibitors, and methotrexate as well as organic anion conjugates, organic dyes, and tyrosine kinase inhibitors (TKIs), [22, 23]. Owing to their distribution in a number of major organs and their role in multiple cancer types, it is pertinent to develop and investigate novel modulators of ABCB1 and ABCG2.

Over the past thirty years, innovative drug combinations have been developed with the hope of combating MDR caused by ATP transporters. We and others have identified a number of sensi-

tizing agents that antagonizes the efflux function of ABC transporters and thus overcome MDR in cancer cells. [5, 15, 16, 20, 24]. Avapritinib, a KIT and PDGFRA inhibitor, is under phase 3 investigation in patients with locally advanced, unresectable or metastatic GIST and aggressive systemic mastocytosis [25, 26]. Here, we report the chemosensitizing effects of avapritinib in cancer cells overexpressing ABCB1 or ABCG2. The findings of the current study will reposition avapritinib to be used in combination with conventional chemotherapeutic drugs to overcome ABC transporter-mediated MDR.

2. Material and methods

2.1 Chemicals

Avapritinib was obtained from ChemieTek (Indianapolis, IN). Fetal bovine serum (FBS), penicillin/streptomycin, trypsin 0.25%, and Dulbecco's modified Eagle's Medium (DMEM), were bought from Hyclone (Pittsburgh, PA). Antibodies against ABCB1, ABCG2, and GAPDH were bought from Thermo Fisher Scientific Inc. (Rockford, IL). Paclitaxel, doxorubicin, mitoxantrone, SN-38, cisplatin, and verapamil were purchased from Sigma-Aldrich (St. Louis, MO). [^{3}H]-paclitaxel (15 Ci/mmol) and [^{3}H]-mitoxantrone (2.5 Ci/mmol) were purchased from Moravek Biochemicals, Inc. (Brea, CA). Alexa Fluor 488 anti-mouse IgG antibody and 4′,6-diamidino-2-phenylindole (DAPI) were purchased from Thermo Fisher Scientific Inc. (Rockford, IL). Formaldehyde was purchased from J.T. Baker Chemical Co. (Phillipsburg, NJ). Bovine Serum Albumin (BSA) and 10X phosphate buffered solution (PBS) were obtained from VWR chemicals, LLC (Solon, OH). The chemicals utilized for the ATPase assay were as described in our previous study [27].

2.2 Cells and their culture conditions

The SW620 cell line which is obtained from a human colorectal adenocarcinoma source, its doxorubicin-resistant SW620/AD300 cell line, the HEK293/pcDNA3.1 cell line which is obtained from a human embryonic kidney source, and HEK293/ABCB1 (transfected with human ABCB1 cDNA [28]) were used in this study to test for ABCB1 reversal activity. The NCI-H460 cell line which is obtained from a non-small cell lung cancer source, its mitoxantrone-resistant NCI-H460/MX20, HEK293/pcDNA3.1, and HEK293/ABCG2, transfected with wild-type (WT) *ABCG2* (HEK/ABCG2-R482) or mutants of *ABCG2* (HEK/ABCG2-R482G and HEK/ABCG2-R482T) were used for ABCG2 reversal study. The transfected cells were cultured in a growth medium containing 2 mg/mL G418. All cell lines were cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin, at 37 °C and 5% CO_2.

2.3 MTT assay

In order to assess drug sensitivity, modified MTT colorimetric assay was used as previously described [4, 15]. The IC_{50} values, that is, the drug concentration required to limit the the growth of cells by 50% were calculated from the cytotoxicity curves using a modified Bliss method [29]. Resistance fold (Rf) was estimated using the following formula:

$$\frac{IC_{50}\text{of the in}-\text{sensitive (resistant) cells in the presence or absence of a drug (inhibitor)}}{IC_{50}\text{ of sensitive (parental) cells without a drug (inhibitor)}}$$

Avapritinib was tested at concentrations of 1 μM and 5 μM. Verapamil (5 μM) and fumitremorgin C (FTC, 5 μM) were employed as as a positive control inhibitors of ABCB1 and ABCG2, respectively.

2.4 [^{3}H]-paclitaxel or [^{3}H]-mitoxantrone accumulation assay

Cells were trypsinized and treated with avapritinib, or verapamil or FTC at the indicated concentrations for 2 h. After 2 h, the cells were further treated with either 0.1 μM [^{3}H]-paclitaxel or 0.1 μM [^{3}H]-mitoxantrone for additional 2 h. Following this, the cells were washed twice in ice-cold PBS and lysed with 10 mM lysis buffer (pH 7.4, containing 1% Triton X-100 and 0.2% SDS). Cell lysates were aliquoted in vials containing 5 mL scintillation fluid and radiation was estimated using a Packard TRI-CARB 1900CA liquid scintillation analyzer from Packard Instrument Company, Inc. (Downers Grove, IL).

2.5 [^{3}H]-paclitaxel or [^{3}H]-mitoxantrone efflux assay

To assess the efflux of [^{3}H]-paclitaxel or [^{3}H]-mitoxantrone from parental and MDR cells, cells were treated with 0.1 μM [^{3}H]-paclitaxel or [^{3}H]-mitoxantrone as mentioned in the accumulation experiment above. The cells were washed twice and cultured in fresh media with or without avapritinib or positive control inhibitors. 1 ml of cell suspension was aliquoted at 0, 30, 60, and 120 min. The aliquots were washed twice with ice-cold PBS and analyzed by the liquid scintillation analyzer as previously described [30].

2.6 Preparation of total cell lysate

The cells were harvested and washed with PBS. Cells were lysed using lysis buffer as previously described [31, 32]. The lysates were centrifuged at 12000 rpm for 20 min and the supernatant was collected and stored at 80 °C for Western blot analysis. Protein concentration was determined using the bicinchoninic acid (BCA™) protein assay (Thermo Scientific, Rockford, IL).

2.7 Western blotting

Equal amounts of total cell lysates (40 μg protein) were resolved by sodium dodecyl sulfate polyacrylamide gel (4-12% gradient gel) electrophoresis (SDS-PAGE). The gel was electroblotted onto polyvinylidene fluoride (PVDF) membranes at 90V for 2 h. The membranes were blocked with 5% skim milk for 2 h followed by incubation with an ABCB1 specific (dilution of 1 : 500), an ABCG2 specific (dilution of 1 : 500), or a GAPDH specific (dilution 1 : 1000) primary antibody, overnight at 4 °C. The following day, membranes were treated with secondary antibody which was HRP (horseradish peroxidase)-conjugated. The protein-antibody complex was detected using the enhanced chemiluminescence detection system.

2.8 Immunofluorescence

The two pairs of cell lines were seeded as 10,000 cells per well in 24-well plates and cultured at 37 °C for 24 h. The cells were then incubation with 5 μM avapritinib at the indicated time points. The cells were then washed twice with cold PBS and fixed in 4% formaldehyde for 15 min. Subsequently, the cells were permeabilized with 0.25% Triton X-100 followed by incubation with BSA (6% with PBS) for 1 h. Then the cells were treated with antibodies specific for ABCB1 (dilution 1 : 1000) or ABCG2 (dilution

1 : 1000) overnight at 4 °C. The next day, cells were treated with secondary antibody conjugated with Alexa Fluor 488 for 2 h in the dark. DAPI was used to stain the nuclei for 15 min. Cell images were taken by a Nikon TE-2000S fluorescence microscope (Nikon Instruments Inc., Melville, NY, USA).

2.9 ATPase assay

The ATP cleaving ability of ABCB1 and ABCG2 in the presence avapritinib was assessed using PREDEASY ATPase Kits. In brief, the crude membranes of High-five insect cells were incubated with avapritinib (0 to 40 μM) and the ATPase activity was measured as previously described [6, 33].

2.10 Molecular Modeling

Molecular simulations were performed using Maestro v11.1 (Schrödinger, LLC, Cambridge, MA). Ligands and proteins were prepared using default protocols as previous described [34]. The human ABCB1 homology model was established on the basis of the refined mouse ABCB1 protein (PDB ID: 4M1M) which was kindly provided by S. Aller [35]. The docking grid (length: 25 Å) for human ABCB1 was refined by setting the centroid with the amino acid residues that are suggested to have interaction with substrates [35]. The cryo-EM structure of human ABCG2 (PDB ID: 6ETI) is available, and the docking grid was generated at the drug binding cavity by selecting residues of importance, including Ala397, Val401, Leu405, Leu539, Ile543 and Phe547 (A397, V401, L405, L539, I543 and F547) [36]. Glide v 7.4 XP (extra precision) docking default protocol (Schrödinger, LLC, New York, NY) was adopted, then induced-fit docking (IFD) was conducted using the best scored binding pose of ligand and protein from the Glide XP docking to get an optimal binding simulation. The top scoring docked pose of ligand-protein complex was subjected to graphical analysis. The docking scores were calculated and expressed as kcal/mol.

2.11 Statistical analysis

All the studies were performed minimum three independent times. Student's *t*-test was used to determine the statistical significance between two groups using Microsoft Excel 2017. The *a priori P* value was $P < 0.05$.

3. Results

3.1 Cytotoxic effect of avapritinib

In order to assess the cytotoxic effects of avapritinib, the MTT assay was performed. Our results showed that about 80-85% of both sensitive and resistant cells survived treatment with 5 μM of avapritinib (Fig. 1), indicating that avapritinib is relatively non-toxic up to a concentration of 5 μM. Thus, avapritinib was used at concentrations of 1 and 5 μM to test its chemosensitizing and reversal effects in combination with chemotherapeutic drugs.

3.2 Effect of avapritinib on the sensitization of ABCB1-overexpressing cells

Cell survival assay was conducted to determine the effect of avapritinib on drug sensitivity of ABCB1 substrates. As shown in Table 1, the substrate anti-cancer drugs of ABCB1 exerted a significantly higher IC_{50} in SW620/AD300 (ABCB1-overexpressing) cells than SW620 (parental) cells. Avapritinib (1 and 5 μM) restored the sensitivity of ABCB1 substrates as evident from a decrease in the IC_{50} values of paclitaxel and doxorubicin, in SW620/AD300 cells . Similarly, reduction in the IC_{50} values were observed in HEK293/ABCB1 cells upon treatment with 1 and 5 μM avapritinib (Table 1). For ABCB1 substrates, paclitaxel and doxorubicin were used and for non-ABCB1 substrate, cisplatin was used. Verapamil (5 μM) was used as a positive control inhibitor of ABCB1.

3.3 Effect of avapritinib on the drug sensitivity of ABCG2-overexpressing cells

Studies have reported that ABCG2 exists in wild type and mutant forms which differ in their substrate and transport inhibitor specificity [8, 37]. To this end, we tested if avapritinib could overcome both wild-type and mutant ABCG2. Our results indicated that avapritinib at 1 and 5 μM decreased the IC_{50} values of mitoxantrone and SN38 in HEK/ABCG2-R482, HEK/ABCG2-R482G and HEK/ABCG2-R482T cells (Table 2). Additionally, avapritinib sensitized the resistant NCI-H460/MX20 cells (Table 3). However, avapritinib did not exerted any change in the IC_{50} values of mitoxantrone and SN-38 in the parental cells that do no overexpress ABCG2. FTC, a known ABCG2 blocker, was used as a positive control inhibitor at 5 μM.

3.4 Effect of avapritinib on the intracellular accumulation of [^{3}H]-paclitaxel or [^{3}H]-mitoxantrone

To ascertain that avapritinib sensitizes ABCB1 or ABCG2-overexpressing cells by increasing the accumulation of their respective substrates, we conducted the intracellular accumulation assay. The incubation of SW620/AD300 cells with avapritinib (1 or 5 μM) enhances the intracellular accumulation of [^{3}H]-paclitaxel as compared to parental cells (Fig. 2A). Verapamil (5 μM) exerted similar effects. Likewise, avapritinib (1 or 5 μM) displayed a significantly higher accumulation of [^{3}H]-mitoxantrone NCI-H460/MX20 cells than in parental cells (Fig. 2B). FTC (5 μM) showed similar effect.

3.5 Effect of avapritinib on the efflux of [^{3}H]-paclitaxel or [^{3}H]-mitoxantrone

In order to determine whether the increase in accumulation of ABCB1 and ABCG2 substrates is due to the blockade of efflux function, we conducted the efflux assay. Our results showed that in the absence of avapritinib, a significantly lower intracellular concentration of [^{3}H]-paclitaxel accumulated in ABCB1 overexpressing cells than in parental cells, due to the efflux of [^{3}H]-paclitaxel by ABCB1 (Figs. 3A and 3B). Interestingly, avapritinib (1 or 5 μM) significantly decreased the efflux of [^{3}H]-paclitaxel from ABCB1 overexpressing cells. Moreover, avapritinib, at 1 or 5 μM, exhibited a similar reduction in efflux of [^{3}H]-mitoxantrone from ABCG2 overexpressing cells (Figs. 3C and 3D). The positive control inhibitors, verapamil or FTC, showed similar effect.

3.6 Effect of avapritinib on the expression and translocation of ABCB1 or ABCG2 protein

It is important to understand if the reversal effect of avapritinib is by the inhibition of the efflux function or a downregulation of ABCB1 or ABCG2 expression. To this end, we assessed the effect of avapritinib on the expression levels of ABCB1 or ABCG2 protein. Our results showed that avapritinib did not change the expression levels of ABCB1 or ABCG2 (Figs. 4A and 4B) upon avapritinib (5 μM) treatment for 72h.

Although our Western blot results confirmed that avapritinib

Table 1. The effect of Avapritinib on the reversal of ABCB1-mediated MDR

Treatment	$IC_{50} \pm SD^a$			
	SW620 (μM, FR[b])	SW620/AD300 (μM, FR[b])	HEK293/pcDNA3.1 (nM, FR[b])	HEK293/ABCB1 (nM, FR[b])
Paclitaxel	0.07 + 0.01 (1)	22.86 + 2.03 (326.57)	81.10 + 7.17 (1)	3012.1 + 129.62 (37.13)
+ 1 μM Avapritinib	0.07 + 0.02 (1)	4.22 + 0.31 (60.28)[c]	87.63 + 8.19 (1.08)	1403.14 + 102.3 (17.3)[c]
+ 5 μM Avapritinib	0.08 + 0.01 (1.1)	0.68 + 0.09 (9.71)[c]	89.91 + 9.02 (1.1)	382.86 + 21.12 (4.71)[c]
+ 5 μM Verapamil	0.07 + 0.01 (1)	1.11 + 0.12 (15.85)[c]	83.81 + 7.99 (1.03)	491.56 + 33.23 (6.06)[c]
Doxorubicin	0.09 + 0.02 (1)	18.01 + 1.89 (200.12)	124.98 + 9.87 (1)	5918 + 209.84 (47.34)
+ 1 μM Avapritinib	0.08 + 0.02 (0.8)	4.18 + 0.56 (46.45)[c]	138.2 + 11.89 (1.1)	2654.31 + 186.56 (21.2)[c]
+ 5 μM Avapritinib	0.09 + 0.01 (1)	0.23 + 0.04 (2.56)[c]	117.65 + 10.18 (0.9)	409.65 + 37.86 (3.27)[c]
+ 5 μM Verapamil	0.08 + 0.01 (0.8)	0.84 + 0.05 (9.34)[c]	123.48 + 12.87 (0.9)	510.18 + 43.62 (4.11)[c]
Cisplatin	1.92 + 0.93 (1)	2.09+ 0.98 (1.08)	1578.14 + 149.45 (1)	2014.71 + 199.14 (1.2)
+ 1 μM Avapritinib	1.87 + 0.12 (0.9)	1.98 + 0.22 (1.03)	1994.70 + 109.70 (1.2)	2004.06 + 213.08 (1.2)
+ 5 μM Avapritinib	1.89 + 0.19 (0.9)	1.91 + 0.13 (0.9)	1693.67 + 106.31(0.9)	1876.05 + 174.92 (1.18)
+ 5 μM Verapamil	1.95 + 0.21 (1.01)	2.06 + 0.19 (1.07)	1708.18 + 123.56 (0.9)	1906.60 + 143.71 (1.2)

[a] IC_{50} values are presented as mean ± SD of at least three independent experiments (n = 3) performed in triplicates.

[b] FR: Resistance fold was calculated by dividing the IC_{50} values of substrate drugs in the presence or absence of transport inhibitor by the IC_{50} of parental cells without inhibitor.

[c] $P < 0.05$ versus the control group without reversal agent.

Table 2. The effect of Avapritinib on reversal of ABCG2-mediated MDR

Treatment	$IC_{50} \pm SD^a$ (nM) (FR[b])			
	HEK293/pcDNA3.1	HEK/ABCG2-R482	HEK/ABCG2-R482G	HEK/ABCG2-R482T
Mitoxantrone	34.23 ± 3.12 (1)	498.38 ± 29.62(14.55)	983.51 ± 43.87 (28.73)	671.11 ± 53.02 (19.6)
+ 1 μM Avapritinib	38.92 ± 2.97 (1.1)	197.64 ± 21.32(5.78)[c]	391.71 ± 30.87 (11.45)	304.40 ± 28.56 (8.8)[c]
+ 5 μM Avapritinib	32.95 ± 3.05 (0.9)	43.95 ± 3.86 (1.2)[c]	53.25 ± 3.04 (1.56)	29.82 ± 3.01 (0.87)[c]
+ 5 μM FTC	29.98 ± 2.17 (0.8)	51.18 ± 4.92 (1.49)[c]	60.12 ± 5.04 (1.75)	33.47 ± 2.99 (0.97)[c]
SN-38	12.04 ± 1.01 (1)	98.94 ± 8.61 (8.21)	131.34 ± 9.87 (10.9)	183.45 ± 14.65 (15.23)
+ 1 μM Avapritinib	13.34 ± 1.08 (1.1)	59.31 ± 4.87 (4.9)[c]	52.34 ± 4.18 (4.35)	96.68 ± 7.62 (8.02)[c]
+ 5 μM Avapritinib	11.97 ± 0.97 (0.9)	16.49 ± 1.38 (1.36)[c]	14.56 ± 1.42 (1.2)	23.99 ± 2.12 (1.99)[c]
+ 5 μM FTC	13.06 ± 1.02 (1.08)	18.82 ± 1.03 (1.56)[c]	21.23 ± 1.80 (1.7)	28.8 ± 1.83 (2.39)[c]
Cisplatin	813.39 ± 73.8 (1)	882.3 ± 80.64 (1.08)	1305.18 ± 108.5 (1.6)	1037.83 ± 98.4 (1.2)
+ 1 μM Avapritinib	834.01 ± 81.6 (1.02)	853.04 ± 72.5 (1.04)	1314.6 ± 187.49 (1.6)	965.18 ± 87.63 (1.18)
+ 5 μM Avapritinib	808.62 ± 79.93 (0.9)	831.9 ± 80.8 (1.02)	1145.78 ± 102.41 (1.4)	1123.31 ± 102.5 (1.38)
+ 5 μM FTC	819.1 ± 74.45 (1)	844.5 ± 82.8 (1.03)	1229.1 ± 108.92 (1.5)	901.75 ± 86.45 (1.1)

[a] IC_{50} values are presented as mean ± SD of at least three independent experiments (n = 3) performed in triplicates.

[b] FR: Resistance fold was calculated by dividing the IC_{50} values of substrates in the presence or absence of inhibitor by the IC_{50} of parental cells without transport inhibitor.

[c] $P < 0.05$ versus the control group without reversal agent.

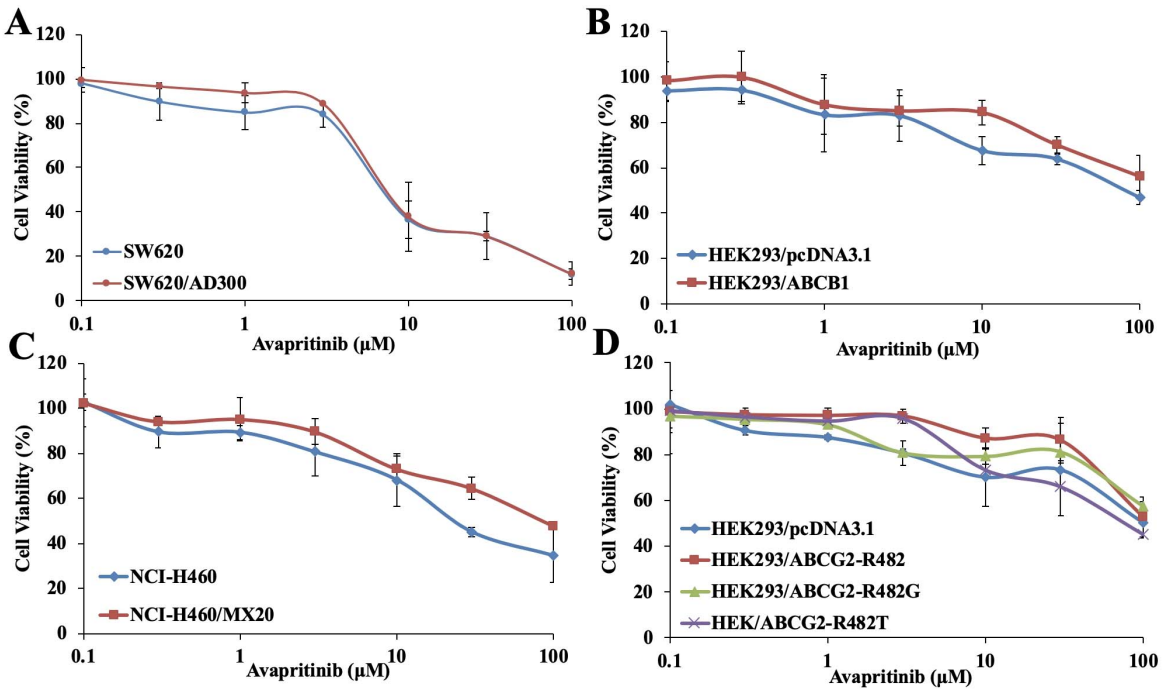

Figure 1. Cytotoxicity of avapritinib in parental and drug-resistant cells. Percent cell survival was measured after treatment with avapritinib for 72 h in parental and MDR cells: SW620 and SW620/AD300 cells (A), HEK293/pcDNA3.1 and HEK293/ABCB1 (B), NCI-H460 and NCI-H460/MX20 cells (C), and HEK293/pcDNA3.1 and HEK/ABCG2-R482, HEK/ABCG2-R482G, HEK/ABCG2-R482T (D). Points with error bars represent the mean ± SD for independent determinations in triplicate. The above figures are representative of three independent experiments.

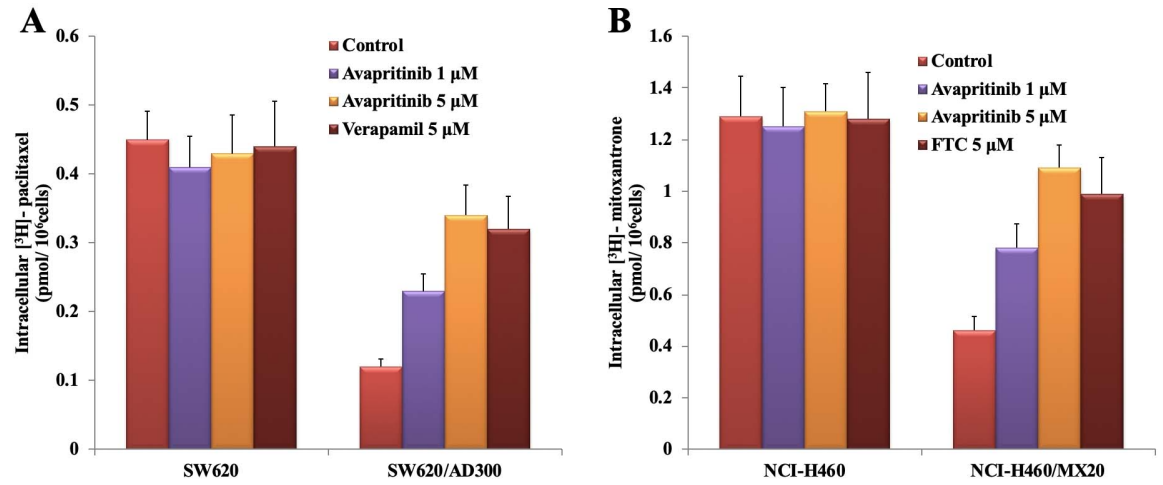

Figure 2. Effect of avapritinib on the accumulation of drug transport substrates of ABCB1 and ABCG2. The effect of avapritinib on the accumulation of [^{3}H]-paclitaxel in SW620 and SW620/AD300 cells (A) and effect of avapritinib on accumulation of [^{3}H]-mitoxantrone in NCI-H460 and NCI-H460/MX20 cells (B). Columns are the means of triplicate determinations (n = 3), error bars represent the SD, and $*P <$ 0.05 versus the control group. Verapamil (5 μM) or FTC (5 μM) were used as positive controls for ABCB1- or ABCG2-overexpressing cells, respectively.

does not affect the expression of ABCB1 or ABCG2, it could be argued that avapritinib translocases the ABC transporter protein from the cell membrane to the cytoplasm of cells. We performed staining of the resistant cells and confirmed that avapritinib (5μM) did not alter the native subcellular localization of ABCB1 or ABCG2 (Figs. 4C and 4D).

3.7 Effect of avapritinib on the ATPase activity of ABCB1 or ABCG2

In order to study the effect of avapritinib on the ATP cleaving activity of ABCB1 or ABCG2, we measured the ability to hydrolyze ATP in the presence of avapritinib (0-40 μM). Avapritinib stimulated the vanadate-sensitive ATPase activity of ABCB1 or

Table 3. The effect of Avapritinib on reversal of ABCG2-mediated MDR

Treatment	$IC_{50} \pm SD^a(\mu M)(FR^b)$	
	NCI-H460	NCI-H460/MX20
Mitoxantrone	0.38 ± 0.04 (1)	49.8 ± 2.48 (131.05)
+ 1 μM Avapritinib	0.36 ± 0.02 (0.9)	17.92 ± 1.52 (47.15)^c
+ 5 μM Avapritinib	0.37 ± 0.01 (0.9)	1.92 ± 0.94 (5.05)^c
+ 5 μM FTC	0.35 ± 0.01 (0.9)	2.02 ± 1.72 (5.31)^c
SN-38	0.49 ± 0.02 (1)	89.91 ± 5.8 (183.48)
+ 1 μM Avapritinib	0.45 ± 0.01 (0.9)	20.12 ± 3.91 (41.06)^c
+ 5 μM Avapritinib	0.48 ± 0.02 (0.9)	1.44 ± 0.95 (2.93)^c
+ 5 μM FTC	0.40 ± 0.01 (0.8)	1.92 ± 0.85 (3.91)^c
Cisplatin	18.48 ± 1.01 (1)	22.73 ± 1.12 (1.2)
+ 1 μM Avapritinib	17.04 ± 0.96 (0.9)	20.04 ± 1.72 (1.08)
+ 5 μM Avapritinib	16.58 ± 0.91 (0.9)	19.88 ± 1.86 (1.07)
+ 5 μM FTC	16.01 ± 0.82 (0.8)	22.4 ± 2.05 (1.2)

[a] IC_{50} values are presented as mean ± SD of at least three independent experiments (n=3) performed in triplicates.

[b] FR: Resistance fold was calculated by dividing the IC_{50} values of substrates in the presence or absence of inhibitor by the IC_{50} of parental cells without transport inhibitor.

[c] $P < 0.05$ versus the control group without reversal agent.

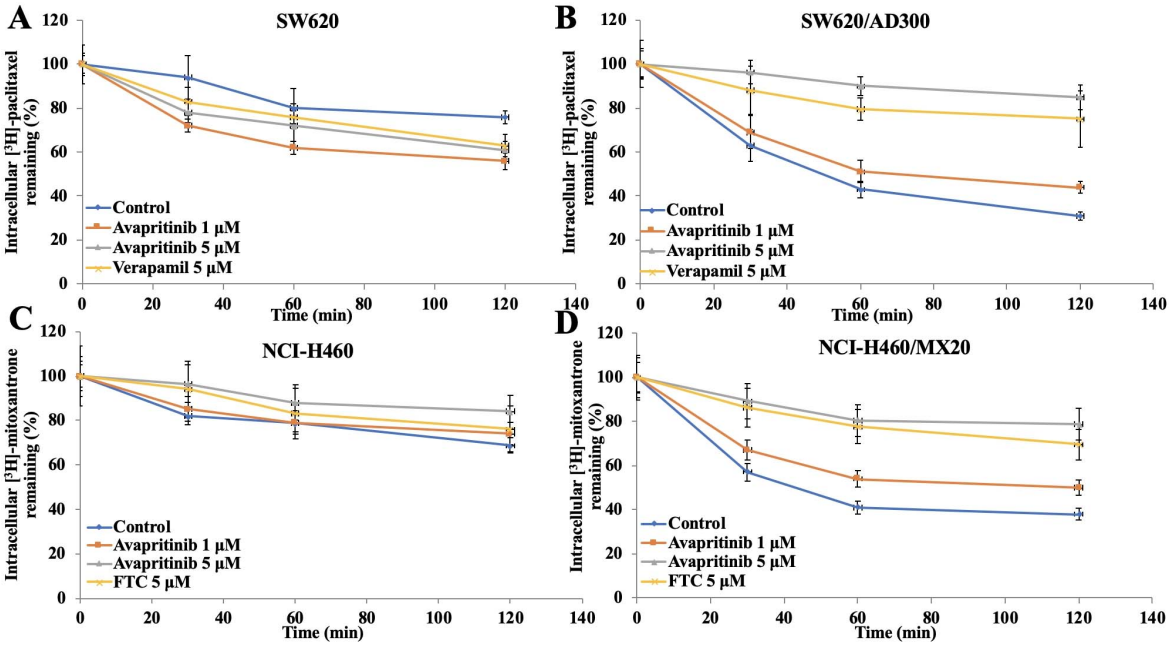

Figure 3. Effect of avapritinib on the efflux of the drug transport substrates of ABCB1 and ABCG2. A time course (0, 30, 60, 120 min) *versus* percentage of intracellular [^{3}H]-paclitaxel remaining (%) was plotted to show the effect of avapritinib in SW620 (A) and SW620/AD300 cells (B). A time course (0, 30, 60, 120 min) *versus* percentage of intracellular [^{3}H]-mitoxantrone remaining (%) was plotted to show the effect of avapritinib in NCI-H460 (C) and NCI-H460/MX20 cells (D). Lines are the means of triplicate determinations (n = 3). Error bars represent the SD and *$P < 0.05$ versus the control group. Verapamil (5 μM) or FTC 5 (μM) were used as positive controls for ABCB1- or ABCG2-overexpressing cells, respectively.

ABCG2 with a fold-stimulation of 2.3 or 1.7-fold, respectively, as compared to the basal activity (Fig. 5). Avapritinib at 2.7 μM or 1.5 μM exhibited a 50% of maximal stimulation or ABCB1 or ABCG2, respectively.

3.8 Docking

The top-scored avapritinib docking pose within homology-modeled human ABCB1 (Fig. 6A) as predicted by IFD simulation had a Glide gscore of -9.564 kcal/mol. The core structure of avapritinib was mainly stabilized into a hydrophobic cavity of the

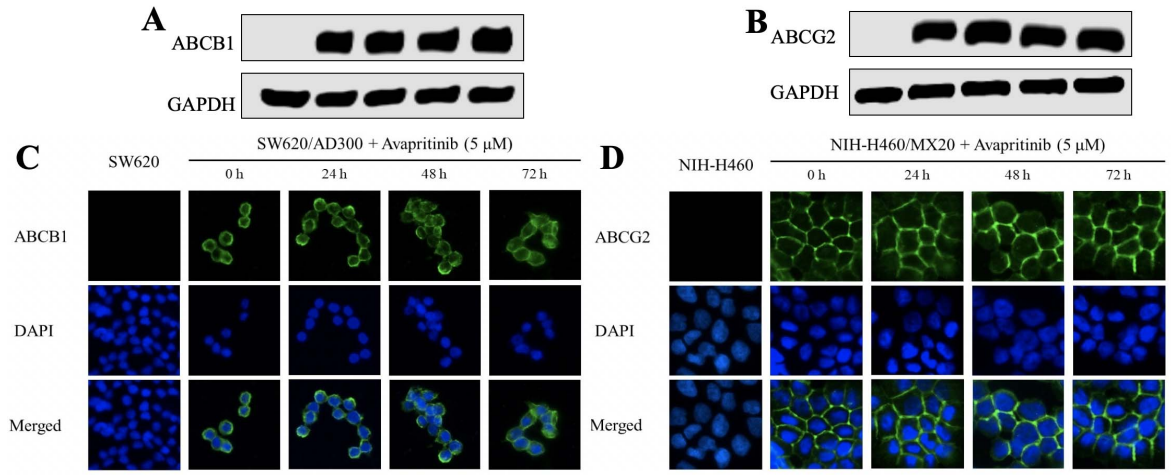

Figure 4. Effect of avapritinib on the expression and subcellular localization of ABCB1 and ABCG2. The effect of avapritinib on the expression of ABCB1 and ABCG2 was tested after the cells were treated with 5 μM avapritinib for 0, 24, 48, 72, and 96 h (A, B). Equal amounts of total cell lysates were used for each sample and Western blot analysis was performed. The differences were not statistically significant (P > 0.05). The effect of avapritinib at 5 μM on the subcellular localization of ABCB1 or ABCG2 in ABCB1-overexpressing SW620/AD300 (C) or ABCG2-overexpressing NCI-H460/MX20 cells (D). Scale bar, 10 μm. DAPI (blue) was used to counterstain the nuclei.

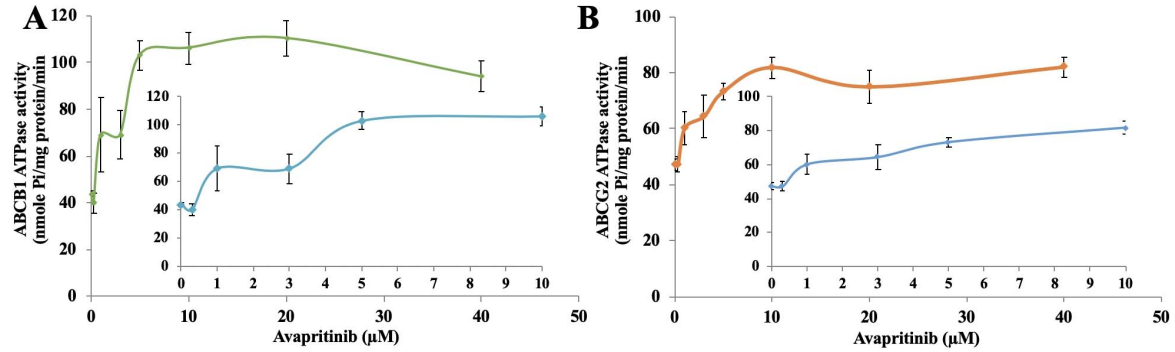

Figure 5. Effect of avapritinib on orthovanadate (Vi)-sensitive ABCB1 and ABCG2 ATPase activity. Crude membranes (10 μg protein/reaction) from High-five cells expressing ABCB1 or ABCG2 were incubated with increasing concentrations of avapritinib (0-40 μM). The concentration of avapritinib was plotted for ABCB1 (A) or ABCG2 (B). The inset shows stimulation of ATP hydrolysis at concentrations of 0-10 μM avapritinib. The mean values are plotted, and error bars depict SD obtained from the average of at list three independent experiments.

transmembrane domain (TMD) formed by residues Met68, Met69, Phe72, Tyr118, Phe303, Ile306, Tyr307, Tyr310, Phe336, Phe343, Phe728, Phe732, Tyr953, Phe957, Leu975, Phe978, and Phe983 (Fig. 6B). The ethanamine group of avapritinib was involved in hydrogen-bonding interaction with Gln725, Tyr310 and Phe728, as well as in π-cation interactions with Tyr310 and Phe728, respectively. Another hydrogen bond was observed between the 4'-nitrogen atom on the triazine ring of avapritinib and the hydroxyl group at the side chain of Ser929. Moreover, π-π stacking interaction was observed between the phenyl ring of avapritinib and the side chain of Phe303. Similarly, the predicted ABCG2-avapritinib complex showed that avapritinib was predominantly settled at a hydrophobic drug-binding pocket at the TMD of ABCG2 (Fig. 6C), with a Glide gscore of -12.089 kcal/mol. Like in its interaction with ABCB1, the ethanamine group, the triazine ring, and the phenyl ring structures of avapritinib all played critical roles in forming interactions with the nearby residues in ABCG2 (Fig. 6D). The ethanamine group of avapritinib formed hydrogen-bonding interactions with Phe432 (chain A) and Thr435 (chain A). The other predicted hydrogen bond was between the 2'-nitrogen atom on the triazine ring of avapritinib and Asn436 (chain B). The Phe432 residue at chain A of ABCG2 was also predicted to be involved in π-π stacking interaction with the phenyl ring of avapritinib.

4. Discussion

KIT and PDGFRA are complex multi-target kinases that have been studied for their key cellular effects, such as altering cell cycle events and modulating DNA damage repair mechanisms [38, 39, 40]. KIT and PDGFRA inhibitors, including imatinib, have also been employed as modulators of ABC transporters-mediated MDR in cancer cells [41, 42]. Here, we report for the first time, the chemosensitizing effects of avapritinib, a phase 3 KIT and PDGFRA inhibitor, in cells overexpressing the MDR efflux transporters ABCB1 or ABCG2.

Our findings revealed that avapritinib (1 and 5 μM) sensitized ABCB1- or ABCG2-overexpressing cells to their respective sub-

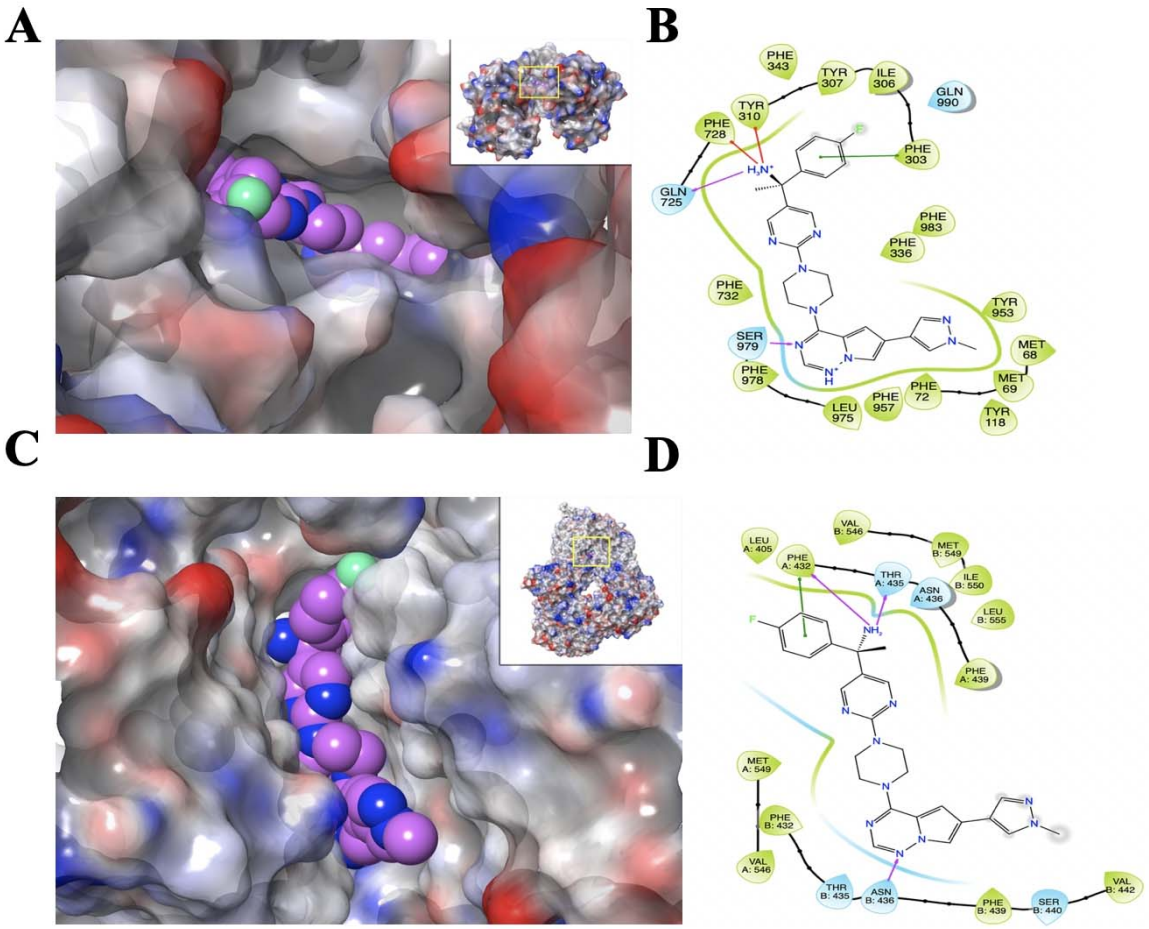

Figure 6. Best-scored binding poses of avapritinib to human ABCB1 homology model (A) and human ABCG2 model (C), predicted by IFD simulation. Homology modeled human ABCB1 and human ABCG2 are represented by surface colored by electrostatic potential. The avapritinib molecule is shown by CPK representation with the atoms colored as carbon–violet, hydrogen–white, oxygen–red, and nitrogen–blue. Only polar hydrogens of the ligand are shown. (B) The two-dimensional ligand−transporter interaction diagram of avapritinib and human ABCB1. The amino acids within 3Å are shown as colored bubbles, blue indicates polar residues, and green indicates hydrophobic residues. Grey circles indicate solvent exposure. Hydrogen bonds are shown by the purple arrow, and π-π stacking aromatic interactions are shown by green lines, and π-cation interactions are shown by red lines. (D) Two-dimensional ligand−transporter interaction diagram of avapritinib and human ABCG2, with the same color scheme as in panel (C).

strate anti-cancer drugs. The results of the drug sensitivity MTT assay showed that avapritinib exhibited a lower IC_{50} values of paclitaxel and doxorubicin (bona fide ABCB1 transport substrates) or mitoxantrone and SN-38 (established ABCG2 transport substrates) in ABCB1 or ABCG2 overexpressing cells. Researchers have shown that a mutation at the 482 position of ABCG2 produces an inhibitory effect [21, 37, 43]. Convincingly, our results indicated that avapritinib enhances chemosensitivity by inhibiting the activity of both wild-type and mutant forms of ABCG2.

We next investigated the mechanism by which avapritinib exerts its chemosensitization effects. To this end, we tested the effect of avapritinib on the accumulation and efflux of [^{3}H]-paclitaxel and [^{3}H]-mitoxantrone in ABCB1- or ABCG2-overexpressing cells. In accord with studies done in the past, avapritinib produced a concentration-dependent increase in the intracellular concentration of transport substrate drugs in the resistant cells but not in their parental cells [8, 11, 18]. Next, we sought to understand whether the chemosensitizing effects of avapritinib were due to the blockade of ABCB1 or ABCG2 functions or due to a decrease in the protein expression of ABCB1 or ABCG2. Our results indicated that avapritinib (at 5 μM and for 72 h) did not alter the expression of ABCB1 or ABCG2. The results of the immunofluorescence assay further confirmed that avapritinib had no effect on the translocation of ABCB1 or ABCG2 transporters away from the plasma membrane in MDR cells at a concentration of 5 μM. These results collectively revealed that avapritinib, without altering the protein expression and subcellular localization, could reverse the chemotherapy resistance induced by ABCB1 or ABCG2 overexpression in MDR cancer cells.

It is well known that the ABC transporters function through utilization of energy from the hydrolysis of ATP via their ATPase activity [44, 45, 46, 47, 48]. Thus, we determined if avapritinib could block the ATPase activity of ABCB1 or ABCG2. The findings of this study showed that avapritinib enhances the ATPase activity

of both ABCB1 and ABCG. These results suggested that avapritinib could competitively inhibit ABCB1 and ABCG2 activity by interacting with the drug-substrate-binding site. Previous studies have reported a number of ABC transporter modulators to competitively inhibit ABCB1 or ABCG2 by stimulating the ATPase activity of ABCB1 or ABCG2 [9, 16, 18, 19].

5. Conclusion

In summary, the results of our current study indicate that avapritinib potently enhances the chemosensitivity of ABCB1 or ABCG2 cytotoxic substrates. Mechanistically, avapritinib inhibits the efflux function of ABCB1 or ABCG2 without altering their expression or their native plasma membrane localization. It would be interesting to further explore the combination of avapritinib and chemotherapeutic agents *in vivo* as well as in patient derived xenograft models of ABC transporter mediated MDR.

Authors' contributions

P.G. and Z.-S.C. designed the experiments, P.G., Y.A., Q.T. Z.L., and B.C. performed the experiments, P.G., Y.A., Q.T., Z.L, S.R., J.W., and Z.-S.C. analyzed the data, P.G., Y.A., Q.T., Z.L., and Z.-S.C. wrote the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript.

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Conflicts of interest

The authors declare no potential conflicts of interest.

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