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The third-generation EGFR inhibitor almonertinib (HS-10296) resensitizes ABCB1-overexpressing multidrug-resistant cancer cells to chemotherapeutic drugs

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ABSTRACT

The overexpression of the human ATP-binding cassette (ABC) drug transporter ABCB1 (P-glycoprotein, P-gp) or ABCG2 (breast cancer resistance protein, BCRP) in cancer cells often contributes significantly to the development of multidrug resistance (MDR) in cancer patients. Previous reports have demonstrated that some epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs) could modulate the activity of ABCB1 and/or ABCG2 in human cancer cells, whereas some EGFR TKIs are transport substrates of these transporters. Almonertinib (HS-10296) is a promising, orally available third-generation EGFR TKI for the treatment of EGFR T790M mutation-positive non-small cell lung cancer (NSCLC) in patients who have progressed on or after other EGFR TKI therapies. Additional clinical trials are currently in progress to study almonertinib as monotherapy and in combination with other agents in patients with NSCLC. In the present work, we found that neither ABCB1 nor ABCG2 confers significant resistance to almonertinib. More importantly, we discovered that almonertinib was able to reverse MDR mediated by ABCB1, but not ABCG2, in multidrug-resistant cancer cells at submicromolar concentrations by inhibiting the drug transport activity of ABCB1 without affecting its expression level. These findings are further supported by in silico docking of almonertinib in the drug-binding pocket of ABCB1. In summary, our study revealed an additional activity of almonertinib to re-sensitize ABCB1-overexpressing multidrug-resistant cancer cells to conventional chemotherapeutic drugs, which may be beneficial for cancer patients and warrant further investigation.

1. Introduction

Human ABCB1 (MDR1/P-glycoprotein) and ABCG2 (BCRP/MXR/ABCP) are ATP-binding cassette (ABC) transporters that are able to derive energy from ATP hydrolysis to transport a large variety of therapeutic agents across biological membranes [1–3]. Together, these two drug transporters are capable of effluxing key conventional cytotoxic anticancer drugs such as *Vinca alkaloids*, anthracyclines and taxanes, as well as molecularly targeted therapeutic agents including nilotinib, gefitinib and ricolinostat, out of cancer cells [3–9]. Consequently, the overexpression of ABCB1 and/or ABCG2 is associated with multidrug resistance (MDR) [3,4] and poor clinical outcome in patients with multiple myeloma (MM) [10–12], chronic lymphocytic leukemia (CLL), acute lymphocytic leukemia (ALL), acute myelogenous leukemia (AML) [13–16] or metastatic breast cancer (MBC) [17].

Considering that cancer MDR remains one of the most serious obstacles in clinical cancer chemotherapy, developing therapeutic agents against the activity of ABCB1 and/or ABCG2 is therefore of great clinical significance [3,4]. Previous attempts to develop novel synthetic inhibitors against ABCB1 and/or ABCG2 have met with less success, often due to adverse drug-drug interactions or the lack of selectivity [3,18–21]. Consequently, there are currently no U.S. Food and Drug

Abbreviations: ABC, ATP-binding cassette; MDR, multidrug resistance; EGFR, epidermal growth factor receptor; Vi, sodium orthovanadate; FR, fold-reversal.

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Administration (FDA)-approved drugs to treat MDR in cancer patients. Interestingly, studies have reported previously that several human epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs) interact strongly with ABCB1 and/or ABCG2, and were able to reverse MDR mediated by ABCB1 and ABCG2 [22–29]. Therefore, rather than synthesizing novel inhibitors, we explore the prospects of repurposing approved EGFR TKIs to resensitize ABCB1- and ABCG2-overexpressing multidrug-resistant cancer cells to conventional cytotoxic anticancer agents.

Almonertinib (HS-10296) is an orally available, third-generation EGFR TKI [30–32]. In 2020, the Chinese National Medical Products Administration (NMPA) approved the use of almonertinib for the treatment of EGFR T790M mutation-positive non-small cell lung cancer (NSCLC) in patients who have progressed on or after other EGFR TKI therapies, based on results from a phase II APOLLO study [33]. Moreover, almonertinib is currently being evaluated as monotherapy in clinical trials for patients with epidermal growth factor receptor mutation-positive, locally advanced or metastatic NSCLC (ClinicalTrials.gov Identifier: NCT03849768 and NCT02981108), EGFR mutation-positive locally advanced or metastatic pulmonary adenosquamous carcinoma (ARISE) (NCT04354961), and as a combination therapy in patients with EGFR-mutant NSCLC (NCT03904823).

In the present work, we examined the interaction between almonertinib and ABCB1 and ABCG2, as well as evaluating whether almonertinib could resensitize ABCB1- and ABCG2-overexpressing multidrug-resistant cancer cells to cytotoxic therapeutic drugs. We found that the overexpression of ABCB1 or ABCG2 had no significant effect on the cytotoxicity of almonertinib in human cancer cell lines. More importantly, we discovered that by selectively inhibiting the transport function of ABCB1, almonertinib was able to resensitize ABCB1-overexpressing cancer cells to drug-induced apoptosis and reverse ABCB1-mediated MDR in human cancer cell lines at submicromolar concentrations. In summary, our data indicate that modulation of ABCB1-mediated transport is an additional mode of action for almonertinib in combination therapies for patients with multidrug-resistant cancers and warrant further studies.

2. Materials and methods

2.1. Chemicals

Rosewell Park Memorial Institute (RPMI) 1640 medium, Dulbecco's Modified Eagle's Medium (DMEM), Iscove's Modified Dulbecco's Medium (IMDM), fetal calf serum (FCS), phosphate-buffered saline (PBS), trypsin-EDTA, penicillin, and streptomycin were obtained from Gibco, Invitrogen (Carlsbad, CA, USA). Fluorescein isothiocyanate (FITC) Annexin V Apoptosis Detection Kit was purchased from BD Pharmingen (San Diego, CA, USA). Almonertinib (HS-10296) was purchased from Selleckchem (Houston, TX, USA). Tools Cell Counting (CCK-8) kit was obtained from Biotools Co., Ltd. (Taipei, Taiwan). All other chemicals were purchased from Sigma (St. Louis, MO, USA) unless stated otherwise.

2.2. Cell culture conditions

The human embryonic kidney HEK293 cell lines stably transfected with either empty pcDNA 3.1 vector (pcDNA3.1-HEK293), human ABCB1 (MDR19) [34] or human ABCG2 (482R- 5) [35] were maintained in DMEM supplemented with 10% FCS, 2 mM L-glutamine, 2 mg/mL G418, and 100 units of penicillin/streptomycin/mL. The human ovarian cancer cell line OVCAR-8 and NCI-ADR-RES subline [36]; the human epidermal cancer cell line KB-3–1 and KB-V-1 subline [37], were maintained in DMEM supplemented with 10% FCS, 2 mM L-glutamine, and 100 units of penicillin/streptomycin/mL. NCI-ADR-RES cells and KB-V-1 cells were maintained in media containing 0.85 μM doxorubicin [36] and 1 μg/mL vinblastine [38], respectively. The human NSCLC cell line H460 and H460-MX20 subline [39]; the human NSCLC cell line A549 and A549-Bec150 subline [40], were maintained in RPMI-1640 supplemented with 10% FCS, 2 mM L-glutamine, and 100 units of penicillin/streptomycin/mL. H460-MX20 cells and A549-Bec150 cells were maintained in media containing 20 nM of mitoxantrone [41] or 150 nM of becatecarin [40], respectively. Cell cultures were screened periodically for mycoplasma contamination using TOOLS Mycoplasma Detection Kit. The KB-3–1, KB-V-1, OVCAR-8 and NCI-ADR-RES cell lines were generous gifts from Dr. Michael Gottesman (NCI, NIH, Bethesda, MD, USA). The H460, H460-MX20, A549, A549-Bec150, HEK293 and HEK293 transfected lines were generous gifts from Dr. Susan Bates (NCI, NIH, Bethesda, MD, USA). All cells were cultured at 37 °C in 5% CO_2 humidified air and maintained in drug-free medium for 7 days before assay.

2.3. Cell viability assay

The cytotoxicity of almonertinib alone or in combination with other therapeutic drugs was determined by cytotoxic MTT assay [42] or Cell Counting Kit-8 (CCK-8) assay. In brief, cells were seeded in 96-well flat-bottom plates and allowed to attach for 24 h at 37 °C in 5% CO_2 humidified air. Different concentrations of almonertinib or drug combinations were added into each plate with 0.5% (v/v) final concentration of DMSO in all wells and incubated for an additional 72 h before processed as described previously [43]. At least three independent experiments were performed to obtain the IC_{50} values, calculated using the fitted concentration–response curve of each drug regimen. The extent of chemosensitization by a modulator was presented as a fold-reversal (FR) value, determined by adding almonertinib or a reference inhibitor to the cytotoxicity assays as described previously [23].

2.4. Apoptosis assay

The effect of almonertinib on colchicine-induced apoptosis in cancer cells was determined by concurrent staining of annexin V–FITC and propidium iodide (PI) according to the manufacturer's instructions (BD Pharmingen) and as previously described [44]. In short, KB-3–1 and KB-V-1 cells were treated with DMSO, 5 μM of almonertinib alone, 500 nM of colchicine alone, or the combination of 500 nM of colchicine and 5 μM of almonertinib as indicated for 48 h before stained with annexin V–FITC (1.25 μg/mL) and PI (0.1 mg/mL) for 15 min at room temperature. Labeled cells were analyzed by FACScan equipped with the CellQuest software (Becton-Dickinson Biosciences, San Jose, CA, USA) as described previously [25].

2.5. Fluorescent substrate accumulation assay

The effect of almonertinib on the intracellular accumulation of fluorescent calcein (485 nm excitation and 535 nm emission), a known substrate of ABCB1 [45], was determined according to the method described by Gribar *et al.* [46]. In brief, cells were first trypsinized and resuspended in IMDM containing 5% FCS before calcein was added to 3×10^5 cells in 4 mL of IMDM in the presence of DMSO (control), 20 μM of almonertinib or tariquidar. The relative fluorescence intensity was detected and analyzed using a FACSort flow cytometer equipped with the CellQuest software (Becton-Dickinson) and the FlowJo software (Tree Star, Inc., Ashland, OR, USA), as described previously [47,48].

2.6. Immunoblotting

KB-V-1 and NCI-ADR-RES cancer cells were treated with DMSO (control) or almonertinib at 50 nM, 100 nM, 200 nM, or 500 nM for 72 h, harvested and subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting. Membranes were incubated with anti-ABCB1 C219 (1:3000 dilution, #517310, Merck Millipore, Burlington, MA, USA) primary antibody to detect ABCB1 or anti-α-tubulin

Table 1
Parameters used for docking of almonertinib to ABCB1.

PDBID	6QEX
Flexible residues	L65, M68, M69, F72, Q195, W232, F303, I306, Y307, Y310, F314, F336, L339, I340, F343, Q347, N721, Q725, F728, F732, F759, F770, F938, F942, Q946, M949, Y953, F957, L975, F978, V982, F983, M986, Q990, F993 and F994.
Center of the receptor grid	x = 19, y = 53, and z = 3
Inner box dimensions	44 Å × 44 Å × 44 Å
Exhaustiveness	100

(1:100000 dilution, #T6199, Sigma-Aldrich, St. Louis, MO, USA) primary antibody to detect the positive loading control tubulin. Membranes were subsequently incubated with horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (IgG) secondary antibody (1:100000 dilution). Signals were detected using the enhanced chemiluminescence (ECL) kit (Merck Millipore, Billerica, MA, USA).

2.7. Docking analysis

AutoDock Vina [49] was used to dock almonertinib to the recently published cryo-EM structure of ABCB1 bound to taxol (pdb id: 6QEX) [50]. Proteins and ligands were prepared using the MGLtools software package (Scripps Research Institute) [51]. Table 1 shows the parameters used for docking. Analysis of the docked poses was performed using the Pymol molecular graphics system, Version 1.7 (Shrödinger, LLC, NY, USA).

2.8. Quantification and statistical analysis

Experimental data are presented as mean ± standard deviation (SD) or as mean ± standard error of the mean (SEM) as indicated from at least three independent experiments. GraphPad Prism software (GraphPad Software, La Jolla, CA, USA) was used for curve plotting. KaleidaGraph software (Synergy Software, Reading, PA, USA) was used for statistical analysis. The difference between mean values of experimental and control or improvement in fit was analyzed by two-tailed Student's *t*-test and labeled with asterisks as "statistically significant" if the probability, *p*, was <0.05.

3. Results

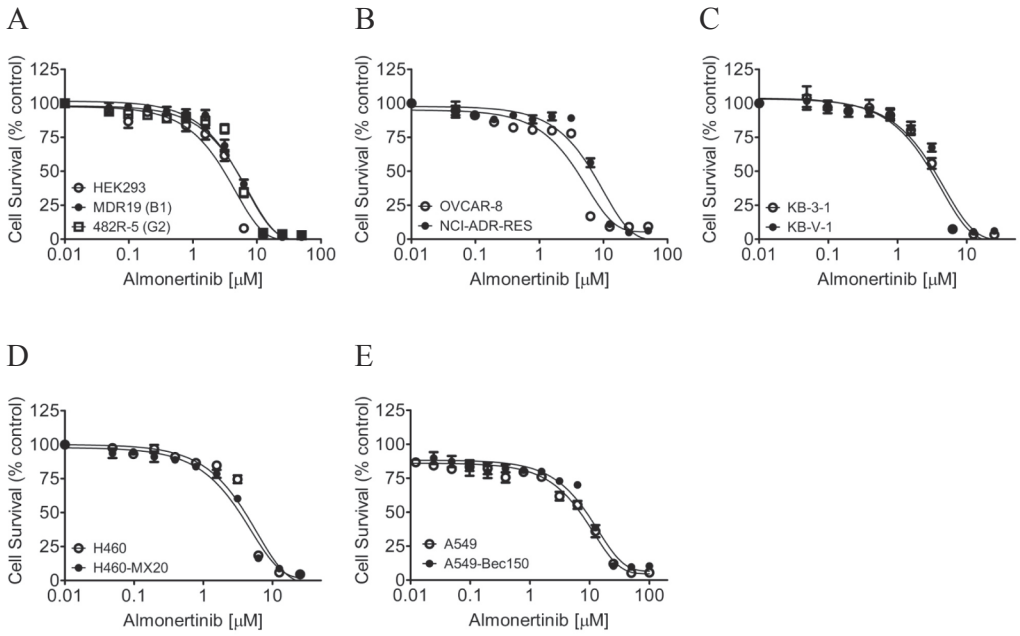
3.1. Multidrug-resistant cells overexpressing ABCB1 or ABCG2 are equally sensitive to almonertinib as drug-sensitive cells.

Previous studies have reported that selected EGFR inhibitors are substrates for ABCB1 and/or ABCG2 [52–58]. Therefore, we examined whether cells overexpressing ABCB1 or ABCG2 are less susceptible to almonertinib. The cytotoxicity of almonertinib was determined in HEK293 stably transfected with either empty pcDNA 3.1 vector (pcDNA3.1-HEK293), human ABCB1 (MDR19), or human ABCG2

Table 2
Sensitivity of drug-sensitive and multidrug-resistant cells overexpressing ABCB1 or ABCG2 to the epidermal growth factor receptor (EGFR) inhibitor almonertinib.

Cell line	Type	Transporter expressed	IC_{50} (μM) [†]
pcDNA3.1-HEK293	–	–	2.76 ± 0.82
MDR19	–	ABCB1	4.19 ± 1.03
482R-5	–	ABCG2	4.50 ± 1.36
KB-3–1	epidermal	–	2.54 ± 0.73
KB-V-1	epidermal	ABCB1	2.94 ± 0.92
OVCAR-8	ovarian	–	3.89 ± 1.20
NCI-ADR-RES	ovarian	ABCB1	6.37 ± 1.97
H460	lung	–	3.57 ± 1.09
H460-MX20	lung	ABCG2	3.06 ± 0.71
A549	lung	–	7.75 ± 1.09
A549-Bec150	lung	ABCG2	9.73 ± 1.73

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



Points, mean values from at least three independent experiments; bars; S.E.M.

Fig. 1. The multidrug-resistant cells overexpressing ABCB1 or ABCG2 are equally sensitive to almonertinib as their respective drug-sensitive parental cells. The cytotoxicity of almonertinib was determined in (A) parental pcDNA-HEK293 cells (open circles) and HEK293 cells transfected with human ABCB1 (MDR19, closed circles) or human ABCG2 (482R-5, open squares), (B) ABCB1-overexpressing multidrug-resistant NCI-ADR-RES human ovarian cancer cell line (closed circles) and its drug-sensitive parental OVCAR-8 cancer cell line (open circles), (C) ABCB1-overexpressing multidrug-resistant KB-V-1 human epidermal cancer cell line (closed circles) and its drug-sensitive parental KB-3–1 cancer cell line (open circles), (D) ABCG2-overexpressing multidrug-resistant H460-MX20 human NSCLC cell line (closed circles) and its drug-sensitive H460 cancer cell line (open circles), as well as (E) ABCG2-overexpressing multidrug-resistant A549-Bec150 human NSCLC cell line (closed circles) and its drug-sensitive parental A549 cancer cell line (open circles).

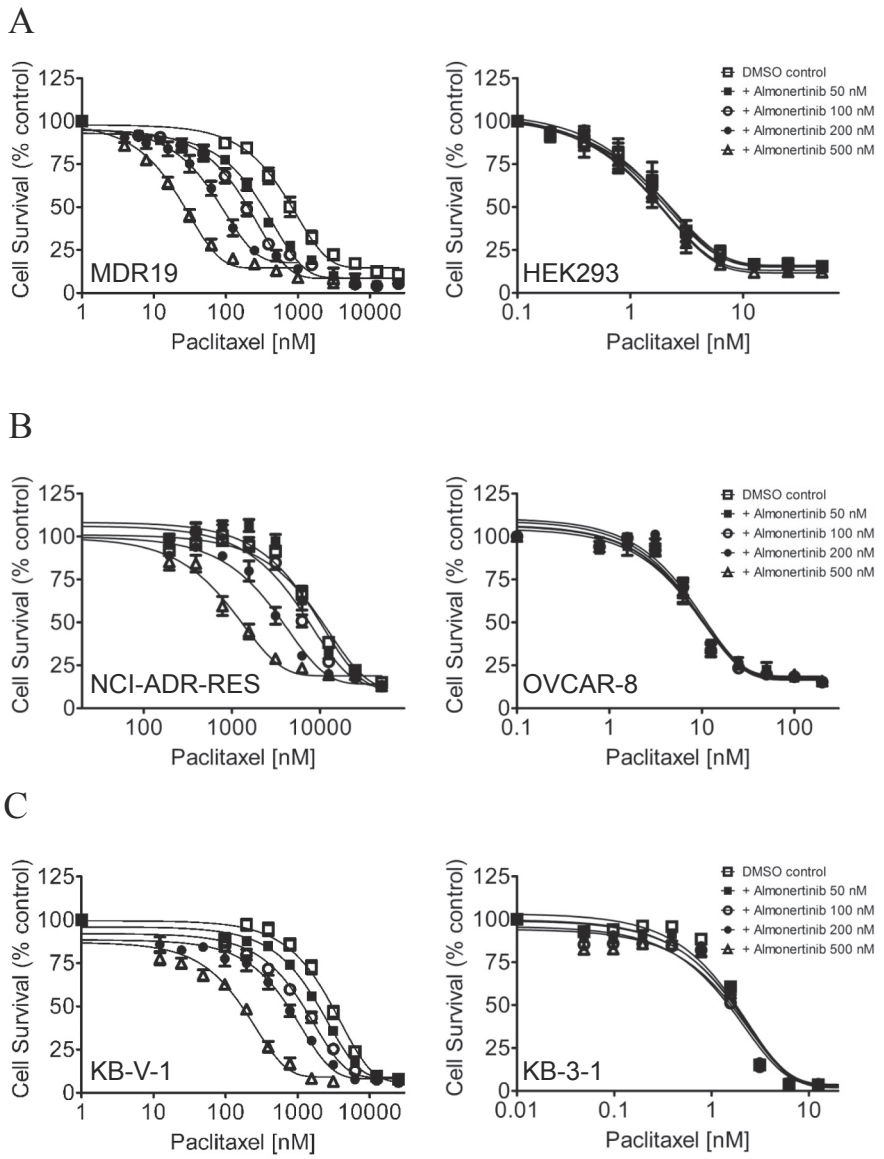


Fig. 2. Almonertinib resensitizes ABCB1-overexpressing multidrug-resistant cells to paclitaxel in a concentration-dependent manner. The chemosensitizing effect of almonertinib on paclitaxel resistance mediated by ABCB1 was examined in (A) HEK293 cells transfected with human ABCB1 (MDR1, left panel) and parental HEK293 cells (right panel), (B) ABCB1-overexpressing NCI-ADR-RES cancer cell line (left panel), and its drug-sensitive parental OVCAR-8 cancer cell line (right panel), as well as (C) ABCB1-overexpressing KB-V-1 cancer cell line (left panel) and its drug-sensitive parental KB-3–1 cancer cell line (right panel). Cells were treated with various concentrations of paclitaxel in the presence of DMSO (open squares) or almonertinib at 50 nM (closed squares), 100 nM (open circles), 200 nM (closed circles), or 500 nM (open triangles). Points, mean values from at least three independent experiments; bars; S.E.M.

(482R- 5), as well as in drug-sensitive KB-3–1 human epidermal cancer cell line and its ABCB1-overexpressing multidrug-resistant variant KB-V-1, drug-sensitive OVCAR-8 human ovarian cancer cell line and its ABCB1-overexpressing multidrug-resistant NCI-ADR-RES, drug-sensitive H460 human NSCLC cell line and its ABCG2-overexpressing multidrug-resistant H460-MX20 subline, and drug-sensitive A549 human NSCLC cell line and its ABCG2-overexpressing multidrug-resistant A549-Bec150 subline (Fig. 1). We found that cells overexpressing ABCB1 or ABCG2 are equally sensitive to almonertinib as their corresponding drug-sensitive parental cells (Table 2).

3.2. *Almonertinib resensitizes ABCB1-overexpressing cancer cells to therapeutic drugs*

Previous reports have demonstrated that some EGFR inhibitors are capable of reversing ABCB1- and/or ABCG2-overexpressing multidrug-resistant cancer cells to conventional cytotoxic anticancer agents [22–29]. Therefore, we examined the potential chemosensitizing of almonertinib in multidrug-resistant cells overexpressing ABCB1 or ABCG2. We found that almonertinib significantly reversed ABCB1-mediated resistance to its substrates paclitaxel (Fig. 2), vincristine and colchicine [59] in ABCB1-overexpressing NCI-ADR-RES, KB-V-1, and MDR19 cell lines at submicromolar concentrations (Table 3). In contrast, we found that almonertinib had no significant effect on ABCG2-mediated resistance to ABCG2 substrates topotecan, SN-38, or mitoxantrone [60,61] in ABCG2-overexpressing H460-MX20, A549-Bec150 and 482R-5 cell lines (Table 4). Of note, the fold-reversal (FR) value in Table 3 and Table 4 [23] represents the degree of resensitization of a particular cell line to a particular therapeutic drug by almonertinib or by the ABCB1 reference inhibitor verapamil or the ABCG2 reference inhibitor Ko143. Moreover, almonertinib did not significantly affect the proliferation of drug-sensitive parental cells at submicromolar concentrations. It is worth noting that consistent with previous reports illustrating an enhanced cytotoxic effect of vincristine by verapamil at nontoxic concentrations [62,63], we also observed that verapamil amplified the toxicity of vincristine in drug-sensitive cancer cell lines (Table 3). Our results revealed that almonertinib selectively reversed MDR in ABCB1-overexpressing cancer cells. However, this drug had no effect on ABCG2-mediated drug resistance suggesting that almonertinib is not interacting with ABCG2 in the tested concentration range.

Table 3
Chemosensitizing effect of almonertinib on multidrug resistance mediated by ABCB1 in ABCB1-overexpressing human cell lines.

Treatment	Concentration (nM)	Mean $IC_{50}^{\dagger} \pm$ SD and ($FR^{\ddagger}$)	
		OVCAR-8 (parental) [nM]	NCI-ADR-RES (resistant) [μM]
Paclitaxel	–	10.60 ± 2.46 (1.0)	10.20 ± 1.91 (1.0)
+ almonertinib	50	10.47 ± 2.52 (1.0)	8.78 ± 2.21 (1.2)
+ almonertinib	100	10.04 ± 2.41 (1.1)	6.76 ± 1.26 (1.5)
+ almonertinib	200	10.50 ± 3.01 (1.0)	3.82 ± 0.59** (2.7)
+ almonertinib	500	10.31 ± 2.74 (1.0)	1.54 ± 0.35** (6.6)
+ verapamil	5000	3.85 ± 1.12* (2.8) [nM]	0.39 ± 0.06*** (26.2) [μM]
Vincristine	–	14.19 ± 1.89 (1.0)	7.03 ± 1.31 (1.0)
+ almonertinib	50	12.33 ± 1.47 (1.2)	6.14 ± 1.20 (1.1)
+ almonertinib	100	12.24 ± 1.42 (1.2)	4.66 ± 0.92 (1.5)
+ almonertinib	200	11.36 ± 1.36 (1.3)	3.33 ± 0.72* (2.1)
+ almonertinib	500	12.24 ± 1.75 (1.2)	1.73 ± 0.43** (4.1)
+ verapamil	5000	2.91 ± 0.86*** (4.9) [nM]	0.29 ± 0.06*** (24.2) [μM]
Colchicine	–	20.86 ± 5.87 (1.0)	3.34 ± 0.82 (1.0)
+ almonertinib	50	22.33 ± 6.76 (0.9)	2.88 ± 0.73 (1.2)
+ almonertinib	100	24.23 ± 8.15 (0.9)	2.68 ± 0.59 (1.2)
+ almonertinib	200	23.64 ± 7.04 (0.9)	1.87 ± 0.55 (1.8)
+ almonertinib	500	21.91 ± 7.29 (1.0)	1.12 ± 0.31* (3.0)
+ verapamil	5000	15.20 ± 3.33 (1.4)	0.80 ± 0.25** (4.2)
Treatment	Concentration (nM)	KB-3-1 (parental) [nM]	KB-V-1 (resistant) [μM]
Paclitaxel	–	1.55 ± 0.46 (1.0)	2.73 ± 0.49 (1.0)
+ almonertinib	50	1.54 ± 0.42 (1.0)	1.85 ± 0.18* (1.5)
+ almonertinib	100	1.42 ± 0.40 (1.1)	1.18 ± 0.09** (2.3)
+ almonertinib	200	1.57 ± 0.44 (1.0)	811.07 ± 93.83** [nM] (3.4)
+ almonertinib	500	1.55 ± 0.45 (1.0)	182.60 ± 29.33*** [nM] (15.0)
+ verapamil	5000	1.31 ± 0.33 (1.2) [nM]	44.21 ± 6.47*** [nM] (61.8) [nM]
Vincristine	–	0.73 ± 0.21 (1.0)	871.55 ± 140.69 (1.0)
+ almonertinib	50	0.79 ± 0.22 (0.9)	806.07 ± 107.72 (1.1)
+ almonertinib	100	0.77 ± 0.20 (0.9)	550.93 ± 80.46* (1.6)
+ almonertinib	200	0.79 ± 0.26 (0.8)	182.56 ± 18.08** (4.8)
+ almonertinib	500	0.76 ± 0.16 (1.0)	41.39 ± 5.09*** (21.1)
+ verapamil	5000	0.12 ± 0.03** (6.1) [nM]	12.00 ± 2.11*** (72.6) [nM]
Colchicine	–	9.97 ± 3.74 (1.0)	988.30 ± 78.56 (1.0)
+ almonertinib	50	10.21 ± 3.67 (1.0)	701.27 ± 98.35* (1.4)
+ almonertinib	100	10.14 ± 3.75 (1.0)	598.14 ± 83.54** (1.7)
+ almonertinib	200	9.92 ± 3.66 (1.0)	459.00 ± 44.63*** (2.2)
+ almonertinib	500	9.43 ± 3.41 (1.1)	274.91 ± 46.86*** (3.6)
+ verapamil	5000	6.35 ± 2.38 (1.6)	225.56 ± 41.34*** (4.4)
Treatment	Concentration (nM)	pcDNA3.1-HEK293 (parental) [nM]	MDR19 (resistant)[nM]
Paclitaxel	–	2.24 ± 0.39 (1.0)	879.51 ± 107.25 (1.0)
+ almonertinib	50	2.01 ± 0.36 (1.1)	345.19 ± 28.18** (2.5)
+ almonertinib	100	2.06 ± 0.38 (1.1)	207.16 ± 17.37*** (4.2)
+ almonertinib	200	1.69 ± 0.32 (1.3)	98.61 ± 10.58*** (8.9)
+ almonertinib	500	1.61 ± 0.26 (1.4)	28.89 ± 3.78*** (30.4)
+ verapamil	5000	1.57 ± 0.29 (1.4) [nM]	8.68 ± 1.95*** (101.3) [nM]
Vincristine	–	2.61 ± 0.35 (1.0)	497.19 ± 79.78 (1.0)
+ almonertinib	50	2.67 ± 0.48 (1.0)	228.25 ± 35.92** (2.2)
+ almonertinib	100	2.14 ± 0.39 (1.2)	145.99 ± 30.22** (3.4)
+ almonertinib	200	1.99 ± 0.37 (1.3)	76.93 ± 12.31*** (6.5)
+ almonertinib	500	1.61 ± 0.31* (1.6)	25.81 ± 4.14*** (19.3)
+ verapamil	5000	0.61 ± 0.14*** (4.3) [nM]	4.30 ± 0.93*** (115.63) [nM]
Colchicine	–	16.14 ± 3.08 (1.0)	238.81 ± 31.59 (1.0)
+ almonertinib	50	14.77 ± 3.28 (1.1)	171.94 ± 35.83 (1.4)
+ almonertinib	100	13.66 ± 3.31 (1.2)	116.51 ± 25.09** (2.0)
+ almonertinib	200	14.37 ± 3.04 (1.1)	105.39 ± 21.28** (2.3)
+ almonertinib	500	13.81 ± 3.36 (1.2)	65.42 ± 13.46*** (3.7)
+ verapamil	5000	15.71 ± 3.15 (1.0)	69.84 ± 13.68** (3.4)

Abbreviation: FR, fold-reversal. $^{\dagger}IC_{50}$ values are mean ± SD calculated from dose-response curves obtained from at least 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 chemotherapeutic drug by IC_{50} values of cells treated with the same chemotherapeutic drug in the presence of almonertinib or verapamil. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

3.3. Almonertinib preserves the effect of drug-induced apoptosis in ABCB1-overexpressing multidrug-resistant cancer cells

Growth retardation initiated by almonertinib could also lead to the seemingly resensitization of ABCB1-overexpressing cancer cells. Therefore, in order to confirm that almonertinib reverses ABCB1-mediated MDR was by restoring the cytotoxicity of therapeutic drugs in ABCB1-overexpressing cancer cells, we examined the effect of almonertinib on

Table 4
Chemosensitizing effect of almonertinib on multidrug resistance mediated by ABCG2 in ABCG2-overexpressing human cell lines.

Treatment	Concentration (nM)	Mean $IC_{50}^{\dagger}$ ± SD and ($FR^{\ddagger}$)	
		H460 (parental) [nM]	H460-MX20 (resistant) [nM]
Topotecan	–	58.55 ± 7.77 (1.0)	1648.90 ± 465.88 (1.0)
+ almonertinib	50	54.50 ± 6.71 (1.1)	1551.00 ± 433.33 (1.1)
+ almonertinib	100	54.10 ± 6.30 (1.1)	1800.21 ± 503.43 (0.9)
+ almonertinib	200	50.22 ± 6.38 (1.2)	1613.53 ± 451.17 (1.0)
+ almonertinib	500	46.73 ± 6.29 (1.3)	1219.52 ± 331.87 (1.4)
+ Ko143	1000	27.85 ± 5.03** (2.1) [nM]	121.54 ± 40.90** (13.6) [nM]
SN-38	–	16.30 ± 2.03 (1.0)	524.35 ± 137.66 (1.0)
+ almonertinib	50	16.91 ± 2.14 (1.0)	454.85 ± 105.21 (1.2)
+ almonertinib	100	17.23 ± 2.39 (0.9)	454.36 ± 108.72 (1.2)
+ almonertinib	200	16.47 ± 2.07 (1.0)	372.35 ± 85.17 (1.4)
+ almonertinib	500	15.17 ± 2.14 (1.1)	319.94 ± 84.68 (1.6)
+ Ko143	1000	4.85 ± 1.08*** (3.4) [nM]	12.34 ± 4.68** (42.5) [nM]
Mitoxantrone	–	38.83 ± 8.37 (1.0)	851.68 ± 113.26 (1.0)
+ almonertinib	50	41.02 ± 7.87 (0.9)	937.54 ± 90.37 (0.9)
+ almonertinib	100	37.80 ± 7.53 (1.0)	890.69 ± 116.69 (1.0)
+ almonertinib	200	34.67 ± 6.04 (1.1)	726.15 ± 127.91 (1.2)
+ almonertinib	500	28.28 ± 5.21 (1.4)	815.60 ± 168.21 (1.0)
+ Ko143	1000	27.69 ± 7.59 (1.4)	109.17 ± 43.52*** (7.8)
Treatment	Concentration (nM)	A549 (parental) [μM]	A549-Bec150 (resistant) [μM]
Topotecan	–	0.53 ± 0.12 (1.0)	1.79 ± 0.26 (1.0)
+ almonertinib	50	0.69 ± 0.18 (0.8)	1.80 ± 0.30 (1.0)
+ almonertinib	100	0.61 ± 0.16 (0.9)	1.52 ± 0.25 (1.2)
+ almonertinib	200	0.58 ± 0.15 (0.9)	1.34 ± 0.23 (1.3)
+ almonertinib	500	0.71 ± 0.21 (0.7)	1.42 ± 0.23 (1.3)
+ Ko143	1000	0.28 ± 0.08* (1.9) [nM]	0.12 ± 0.02*** (14.9) [nM]
SN-38	–	22.51 ± 6.36 (1.0)	200.14 ± 44.67 (1.0)
+ almonertinib	50	22.92 ± 6.03 (1.0)	220.71 ± 48.70 (0.9)
+ almonertinib	100	22.26 ± 6.19 (1.0)	213.95 ± 48.30 (0.9)
+ almonertinib	200	24.24 ± 7.08 (0.9)	200.75 ± 46.44 (1.0)
+ almonertinib	500	19.11 ± 5.22 (1.2)	145.86 ± 33.70 (1.4)
+ Ko143	1000	14.73 ± 4.23 (1.5) [nM]	6.44 ± 1.80** (31.1) [nM]
Mitoxantrone	–	7.07 ± 1.93 (1.0)	471.92 ± 111.19 (1.0)
+ almonertinib	50	6.74 ± 1.93 (1.0)	528.13 ± 116.32 (0.9)
+ almonertinib	100	6.81 ± 2.09 (1.0)	430.80 ± 87.59 (1.1)
+ almonertinib	200	7.14 ± 2.17 (1.0)	324.75 ± 68.12 (1.5)
+ almonertinib	500	6.61 ± 2.11 (1.1)	336.82 ± 66.32 (1.4)
+ Ko143	1000	5.01 ± 1.51 (1.4)	46.15 ± 10.30** (10.2)
Treatment	Concentration (nM)	pcDNA3.1-HEK293 (parental) [nM]	482R-5 (resistant) [nM]
Topotecan	–	30.06 ± 5.27 (1.0)	672.58 ± 182.46 (1.0)
+ almonertinib	50	32.66 ± 5.61 (0.9)	1010.80 ± 314.06 (0.7)
+ almonertinib	100	33.64 ± 6.86 (0.9)	829.73 ± 321.37 (0.8)
+ almonertinib	200	30.81 ± 6.04 (1.0)	537.47 ± 170.83 (1.3)
+ almonertinib	500	31.98 ± 6.07 (0.9)	402.33 ± 174.25 (1.7)
+ Ko143	1000	30.42 ± 5.81 (1.0) [nM]	70.43 ± 9.31** (9.5) [nM]
SN-38	–	2.30 ± 0.51 (1.0)	66.52 ± 10.34 (1.0)
+ almonertinib	50	2.79 ± 0.58 (0.8)	65.17 ± 10.10 (1.0)
+ almonertinib	100	2.92 ± 0.76 (0.8)	70.73 ± 8.39 (0.9)
+ almonertinib	200	3.41 ± 0.88 (0.7)	56.77 ± 4.70 (1.2)
+ almonertinib	500	3.42 ± 1.08 (0.7)	57.59 ± 5.95 (1.2)
+ Ko143	1000	2.52 ± 0.60 (0.9) [nM]	3.48 ± 0.83*** (19.1) [nM]
Mitoxantrone	–	1.77 ± 0.21 (1.0)	36.12 ± 6.15 (1.0)
+ almonertinib	50	1.37 ± 0.22 (1.3)	37.04 ± 6.85 (1.0)
+ almonertinib	100	1.36 ± 0.18 (1.3)	31.91 ± 6.33 (1.1)
+ almonertinib	200	1.41 ± 0.23 (1.3)	32.42 ± 6.57 (1.1)
+ almonertinib	500	2.15 ± 0.27 (0.8)	25.83 ± 7.07 (1.4)
+ Ko143	1000	2.08 ± 0.27 (0.9)	3.86 ± 1.34*** (9.4)

Abbreviation: FR, fold-reversal. $^{\dagger}IC_{50}$ values are mean ± SD calculated from dose-response curves obtained from at least 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 chemotherapeutic drug by IC_{50} values of cells treated with the same chemotherapeutic drug in the presence of almonertinib or Ko143. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

drug-induced apoptosis. Parental KB-3–1 and ABCB1-expressing KB-V-1 cancer cells were treated with 0.5 μM of colchicine, a known inducer of apoptosis [64] and a substrate of ABCB1 [59], 1 μM of tariquidar, a known inhibitor of ABCB1 [65], or 5 μM of almonertinib alone or in combinations for 48 h before processed as detailed in Materials and methods. As shown in Fig. 3, the extent of total apoptosis induced by colchicine increased substantially from 6% basal to approximately 51% in KB-3–1 cells, in contrast to an increase of merely 2% in ABCB1-

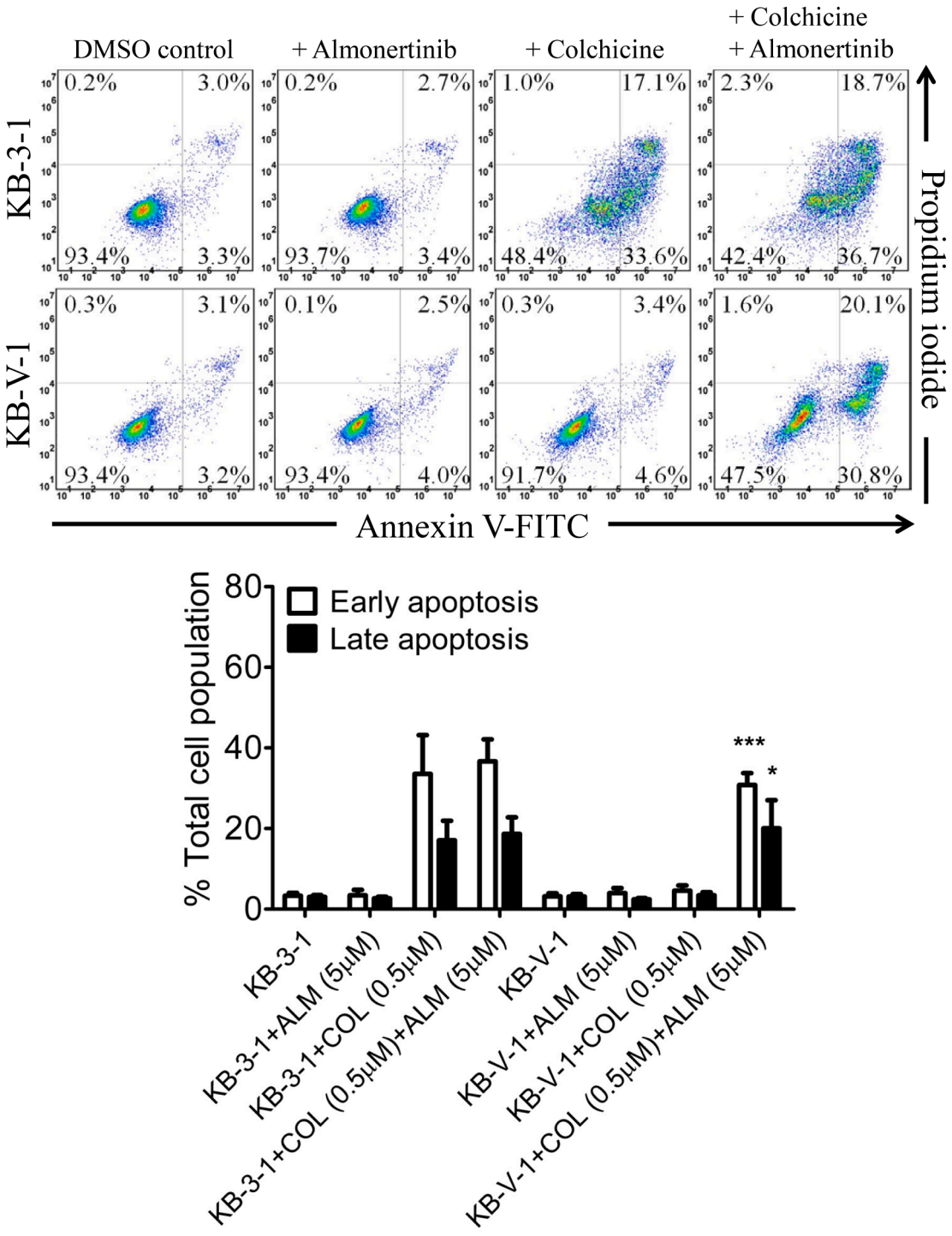


Fig. 3. Almonertinib resensitizes ABCB1-overexpressing multidrug-resistant KB-V-1 cancer cells to drug-induced apoptosis. Drug-sensitive KB-3–1 and multidrug-resistant KB-V-1 cancer cells were treated with either DMSO (control), 5 μM of almonertinib (+ALM), 500 nM of colchicine (+COL), or a combination of 500 nM of colchicine with 5 μM of almonertinib (+COL + ALM) and analyzed by flow cytometry as described in Materials and methods. The quantification data are presented as mean ± SD calculated from at least three independent experiments. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$, versus the same treatment in the absence of tariquidar or almonertinib.

overexpressing KB-V-1 cells. More importantly, we found that without inducing apoptosis itself, the colchicine-induced apoptosis was significantly restored in KB-V-1 cancer cells by tariquidar (from 8% basal to approximately 56% total apoptosis, data not shown) and almonertinib (from 8% basal to approximately 51% total apoptosis).

3.4. Almonertinib inhibits the drug efflux function of ABCB1 without affecting its expression in cancer cells

Two of the most common mechanisms for reversing ABCB1-mediated MDR in cancer cells are transiently inhibiting the transport function of ABCB1 and down-regulating the protein expression of ABCB1 in cancer cells [66–68]. Therefore, we examined the effect of

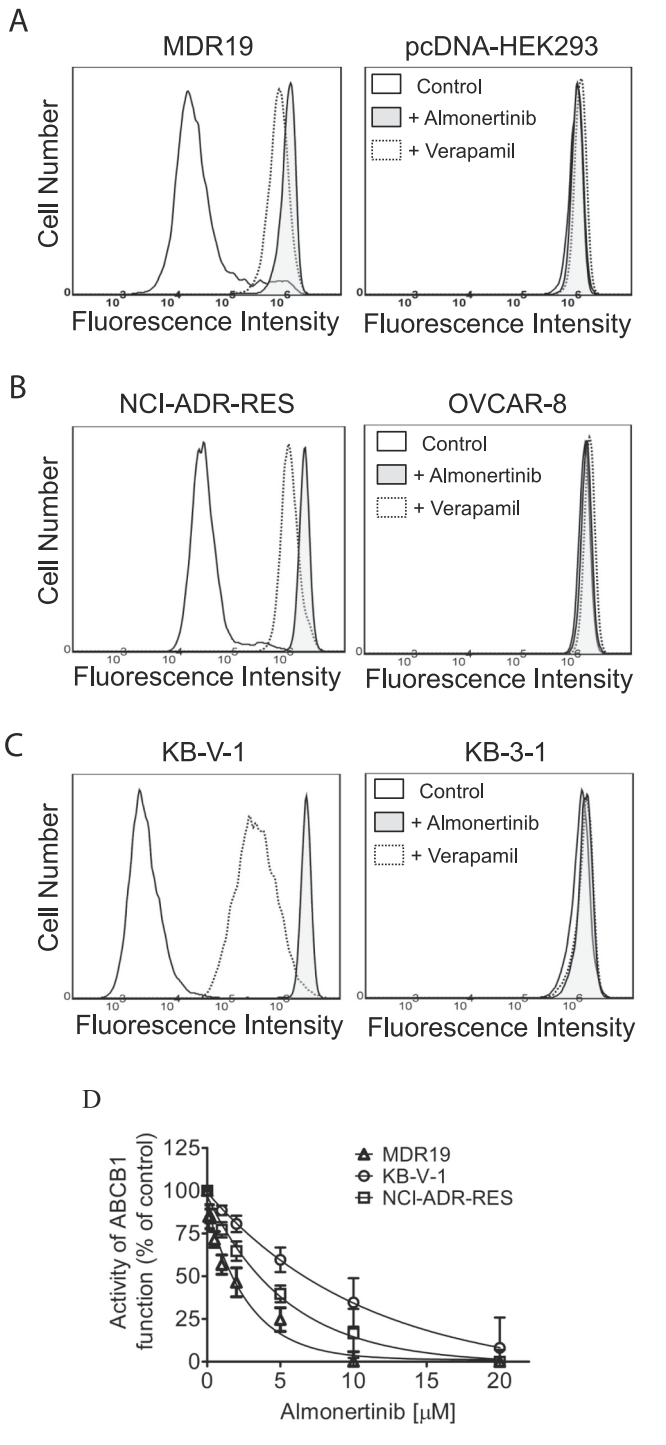


Fig. 4. Almonertinib inhibits ABCB1-mediated transport of calcein-AM. (A) HEK293 cells transfected with human ABCB1 (MDR19, left panel), (B) ABCB1-overexpressing NCI-ADR-RES cancer cells (left panel), and (C) ABCB1-overexpressing KB-V-1 cancer cells (left panel), as well as in respective parental cells (A-C, right panels). Cells were treated with 0.25 μM calcein-AM and DMSO (A-C, solid line), 20 μM of almonertinib (A-C, filled solid line), or 20 μM of verapamil (A-C, dotted line) as a positive control for ABCB1. Representative histograms of at least three independent experiments are shown. (D) Effect of increasing concentrations (0–20 μM) of almonertinib on ABCB1-mediated calcein-AM efflux in MDR19 (open triangles), KB-V-1 (open circles), and NCI-ADR-RES (open squares) cells. Points, mean values from at least three independent experiments; bars; S.D.

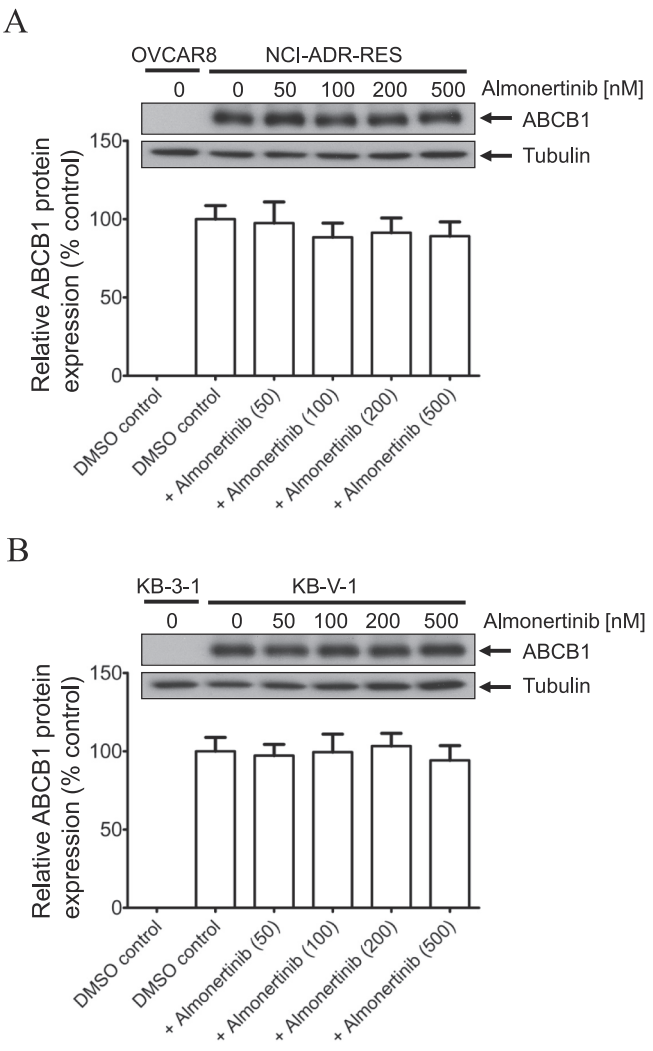


Fig. 5. Almonertinib does not significantly affect the protein expression of ABCB1 in human (A) NCI-ADR-RES or (B) KB-V-1 cancer cells. Cells were treated with DMSO (vehicle control) or almonertinib at 50 nM, 100 nM, 200 nM, or 500 nM for 72 h and processed for Western blotting as described in Materials and methods. The representative immunoblots (upper panel) and the corresponding quantification (lower panel) human ABCB1 protein are shown. α-Tubulin was used as an internal loading control. Values are presented as mean ± S.D. calculated from at least three independent experiments.

almonertinib on ABCB1-mediated drug efflux and the protein expression of this transporter in KB-V-1 cancer cells. ABCB1-overexpressing KB-V-1 and NCI-ADR-RES cancer cells, as well as ABCB1-transfected MDR19 cells, were treated with calcein-AM in the absence or presence of almonertinib or verapamil and processed as described in Materials and methods. We found that 20 μM of almonertinib completely inhibited ABCB1-mediated efflux of calcein-AM and increased the intracellular accumulation of calcein, a fluorescent product of an ABCB1 substrate calcein-AM [45], in ABCB1-overexpressing MDR19 (Fig. 4A), NCI-ADR-RES (Fig. 4B) and KB-V-1 (Fig. 4C) cells, and in a concentration-dependent manner (Fig. 4D), with the IC_{50} values of approximately 2, 4 and 8 μM, respectively. Of note, 20 μM of almonertinib and 20 μM of verapamil alone had no significant effect on the intracellular accumulation of calcein in parental cell lines (Fig. 4A - C, right panels). Next, we examined the effect of almonertinib on ABCB1 protein expression by treating NCI-ADR-RES and KB-V-1 cancer cells with increasing concentrations of almonertinib (0–500 nM) for 72 h followed by Western blot analysis as described in Materials and methods. We found that the

Pose	Docking score (kcal/mol)
1	-10.4
2	-10.3
3	-10.3
4	-10.3
5	-10.3
6	-10.2
7	-10.1
8	-10.1
9	-10.0

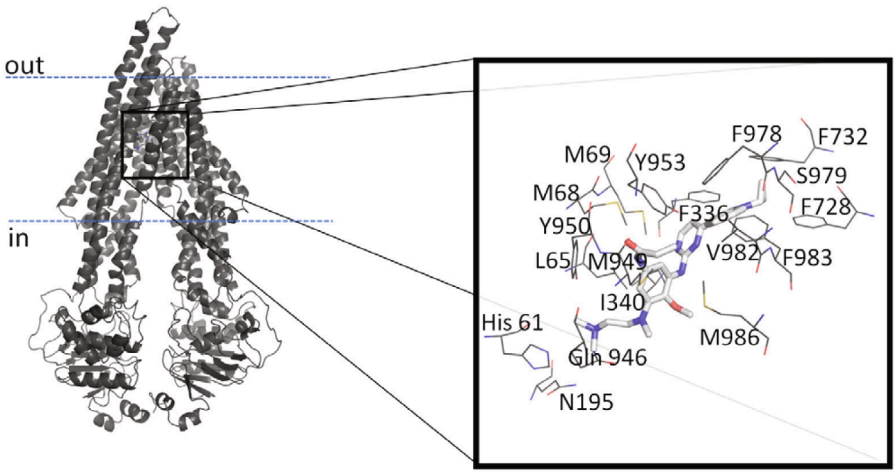


Fig. 6. Docking of almonertinib in the drug-binding pocket of ABCB1. The AutoDock Vina software was used to dock almonertinib in the substrate-binding site of ABCB1 as described in the methods section. Cryo-EM structure of ligand-bound ABCB1 in the inward-open conformation (pdb id.6QEX) was used for the docking. The lowest energy pose of almonertinib bound to ABCB1 is presented in stick representation. Residues within 4.5 Å of the ligand are shown in line representation. Colors as follow carbon-gray, nitrogen-blue, oxygen-red, hydrogen-white, sulfur-yellow. The energy values of the lowest nine dockings are presented on the left. The figure was prepared using Pymol molecular graphics system. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

protein expression of ABCB1 was not significantly affected by almonertinib over a period of 72 h in NCI-ADR-RES (Fig. 5A) and KB-V-1 (Fig. 5B) cancer cells.

3.5. *Docking of almonertinib in the drug-binding pocket of ABCB1*

Knowing that almonertinib blocks ABCB1-mediated drug efflux (Fig. 4), we performed docking studies of almonertinib to ABCB1 to identify the potential site(s) where almonertinib interacts with the drug-binding pocket of ABCB1. The most energy favorable docking pose of almonertinib with human ABCB1 protein structure (pdb.6QEX) is shown in Fig. 6. The nine lowest energy poses had scores ranging from −10.0 to −10.4 kcal/mol. This inward-open structure of ABCB1 is the conformation with the binding of substrate Taxol (paclitaxel) in the drug-binding pocket. These in silico results indicated several hydrophobic and aromatic interactions between almonertinib and the residues within the transmembrane domain of ABCB1.

4. Discussion

The development of MDR against cytotoxic anticancer drugs in cancer cells is often mediated by the overexpression of ABCB1, a well-known factor that is associated with poor therapeutic response in patients receiving conventional anticancer drugs [3,69]. Despite tremendous efforts in developing novel synthetic inhibitors of ABCB1, MDR mediated by ABCB1 remains a substantial obstacle in cancer chemotherapy due to the lack of clinically safe and effective modulators [3,21,69–71]. Alternatively, we and others have discovered that numerous TKIs, including EGFR inhibitors, could reverse ABCB1-mediated MDR in cancer cells through inhibition of its drug efflux function [25,28,72–76]. Interestingly, studies have suggested that the co-administration of FDA-approved TKIs could be beneficial for cancer patients undergoing conventional chemotherapy. For instance, in trials comparing the outcome of monotherapy to combination-therapy, the combination-therapy of gemcitabine with erlotinib was superior to monotherapy with gemcitabine in patients with advanced pancreatic cancer [77,78], while the combination-therapy of capecitabine with lapatinib was superior to monotherapy with capecitabine in patients with human epidermal growth factor receptor 2 (HER2)-positive advanced breast cancer [79,80]. Moreover, promising results from a more recent phase I trial of using doxorubicin with nilotinib as a co-adjuvant treatment to inhibit the drug efflux function of ABCB1 in patients with sarcomas [81] signify the importance of using combination therapies against multidrug-resistant cancers. Collectively, these findings prompted us to investigate the interaction between almonertinib and the MDR-linked ABC drug transporters ABCB1 and ABCG2.

Knowing that some of the EGFR inhibitors, such as gefitinib [52,53], afatinib [55] and osimertinib [56], are substrates of ABCB1 and/or ABCG2 [57], we compared the chemosensitivity of multidrug-resistant cells overexpressing ABCB1 or ABCG2 and respective drug-sensitive parental cells to almonertinib. We discovered that cells overexpressing ABCB1 or ABCG2 are equally sensitive to almonertinib (Fig. 1). Although the effect of prolonged treatment with almonertinib on ABCB1 and ABCG2 in patients remains to be determined in clinical studies, our results here suggest that almonertinib is not rapidly effluxed by ABCB1 or ABCG2, and both transporters are unlikely to contribute significantly to the development of almonertinib resistance in cancer patients. Moreover, studies have also reported that some EGFR inhibitors, such as dacomitinib, olmutinib, and rociletinib, could inhibit the drug efflux function of ABCB1 and ABCG2, thus re-sensitize ABCB1- and ABCG2-overexpressing cancer cells to various cytotoxic drugs [27–29]. Similar to those findings, we discovered that at subtoxic concentrations (<1 μM), almonertinib was able to reverse MDR mediated by ABCB1 in multidrug-resistant cancer cells overexpressing this transporter and cells transfected with human ABCB1 (MDR19-HEK293) in a concentration-dependent manner (Table 3). However, almonertinib did not resensitize ABCG2-overexpressing multidrug-resistant cancer cells or cells transfected with human ABCG2 (482R- 5-HEK293) to known drug substrates of ABCG2 (Table 4), indicating that almonertinib is selective to ABCB1 relative to ABCG2. Cmax (peak serum concentration) values of 522 ng/mL and 492 ng/mL were reported in a clinical trial for 220 mg and 260 mg single dose of almonertinib, respectively [32]. Thus, the inhibitory concentration of almonertinib (50 – 500 nM) used in this study to reverse ABCB1-mediated drug resistance is likely to be physiologically relevant. However, further studies with *in vivo* model are required. Furthermore, our data showed that almonertinib inhibits the drug efflux function of ABCB1 (Fig. 4), and consequently restores the susceptibility of ABCB1-overexpressing cancer cells to drug-induced apoptosis (Fig. 3) and resensitizes ABCB1-overexpressing cancer cells to cytotoxic drugs (Table 3). These findings are further supported by the *in silico* docking analysis of almonertinib binding to the drug-binding pocket of ABCB1 in the inward-open conformation (Fig. 6), indicating

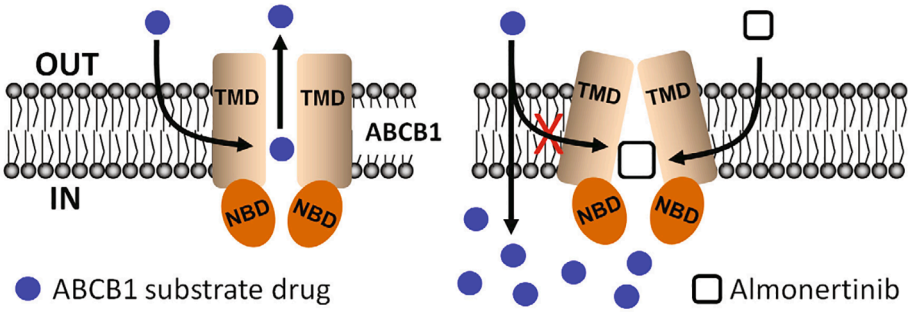


Fig. 7. Schematic diagram showing almonertinib resensitizing ABCB1-overexpressing multidrug-resistant cancer cells to conventional anticancer drugs. The efficacy of ABCB1 substrate anticancer drugs (blue circles) is reduced by ABCB1-mediated drug transport in ABCB1-expressing cancer cells (left). In the presence of almonertinib (white square), almonertinib reduces ABCB1-mediated drug efflux by outcompeting the binding of ABCB1 substrate anticancer drugs to the drug-binding pocket of ABCB1, thus restoring the efficacy of these drugs in ABCB1-expressing cancer cells. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

that almonertinib could outcompete another drug substrate for binding to the transmembrane domain of ABCB1 (Fig. 7).

In summary, despite the potential adverse drug reactions that may occur in combination therapies and the presence of other mechanisms that may also contribute to MDR [3,70,71,82], our data indicate that the third-generation EGFR TKI almonertinib is capable of modulating the drug efflux function of ABCB1 and reversing ABCB1-mediated MDR, and can potentially be used to battle against multidrug-resistant cancers associated with the overexpression of ABCB1. Concomitant administration of almonertinib with ABCB1 substrate cytotoxic anticancer drugs warrants further investigation.

CRediT authorship contribution statement

Chung-Pu Wu: Conceptualization, Methodology, Software, Writing - original draft, Supervision. **Tai-Ho Hung:** Conceptualization, Methodology, Supervision. **Sabrina Lusvarghi:** Data curation, Visualization, Investigation, Writing - review & editing. **Yi-Hsuan Chu:** Data curation, Visualization, Investigation. **Sung-Han Hsiao:** Data curation, Visualization, Investigation. **Yang-Hui Huang:** . **Yu-Tzu Chang:** Data curation, Visualization, Investigation. **Suresh V. Ambudkar:** Conceptualization, Methodology, Supervision, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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