The Overexpression of ABCG2 Reduces the Efficacy of Volasertib (BI 6727) and GSK641364 in Human S1-M1-80 Colon Carcinoma Cells

Sung-Han Hsiao[a], Shi-Yu Luo[a], Ching-Ya Su[a], Wei-Cherng Tuo[a], Cheng-Ting Chiang[a], Yan-Qing Li[b], Yang-Hui Huang[c] and Chung-Pu Wu[a,b,c,*]

[a]*Graduate Institute of Biomedical Sciences,* [b]*Department of Physiology and Pharmacology, and* [c]*Molecular Medicine Research Center, College of Medicine, Chang Gung University, Tao-Yuan 333, Taiwan*

Abstract: The polo-like kinase 1 (Plk1) is one of the key regulators in cell cycle progression. Plk1 is overexpressed in many types of cancer and promotes the proliferation of cancer cells. Inhibition of Plk1 activity induces G2/M cell cycle arrest and reduces cancer cell viability. Volasertib and GSK461364 are selective inhibitors of Plk1, active against a wide range of tumor cells at nanomolar concentrations. In this study, while examining the effectiveness of Plk1 inhibitors against multiple human colon cancer cell lines, we discovered that the overexpression of ATP-binding cassette (ABC) drug transporter ABCG2 in human S1-M1-80 colon cancer cells confers resistance to volasertib and GSK461364. Moreover, we found that ABCG2-transfected HEK293 cells were also resistant to both Plk1 inhibitors. We revealed that volasertib and GSK461364 inhibited the function of ABCG2 in a concentration dependent manner, and had no significant effect on the protein expression of ABCG2. More importantly, we showed that the G2/M cell cycle arrest induced by volasertib or GSK461364 was significantly reduced in S1-M1-80 cells, and that ABCG2-mediated drug resistance to Plk1 inhibitors can be restored by inhibition of ABCG2 function. Therefore, the development of ABCG2-mediated drug resistance to volasertib and GSK461364 in cancer clearly present a significant therapeutic challenge, and a better treatment strategy should be further investigated.

Keywords: ABCG2, multidrug resistance, Polo-like kinase 1, volasertib, GSK641364.

INTRODUCTION

The Polo-like kinase 1 (Plk1) is one of the member of the serine/threonine kinase Plk family. Plk1 plays a crucial role in the regulation of mitosis [1-3], including mitotic entry [4], bipolar spindle formation [5], centrosome maturation [6] and mitotic exit [7]. Plk1 has been found overexpressed in many human tumors, including colorectal cancers [8], and has been shown to promote the proliferation of tumors, thus is recognized as a good candidate for targeted cancer therapy [3, 9-17]. Inhibition of Plk1 activity has been shown to cause cell cycle arrest, subsequent induction of apoptosis and cell death [6, 18, 19]. Therefore, small-molecule inhibitors of Plk1 were developed to specifically inhibit the activity of Plk1, which leads to induced G2/M cell cycle arrest, apoptotic and cancer cell death cell death. Volasertib (BI 6727) and GSK461364 are selective Plk1 inhibitors that induce G2/M mitotic arrest and inhibit the proliferation of various types of human cancer cells [12, 20-22], including bladder cancer [21] and glioblastoma [23, 24]. Moreover, both inhibitors showed promising results in preclinical studies and clinical trials, as well as favorable pharmacological properties [9, 20, 25, 26]. However, the potential risk of cancer cells developing drug resistance to these Plk1 inhibitors has yet to be addressed.

The overexpression of major ATP-binding cassette (ABC) drug transporters ABCB1, ABCC1 and ABCG2, is one of the most common mechanisms for the development of multidrug resistance (MDR) in cancers [27, 28]. These ABC drug transporters utilize ATP hydrolysis to actively efflux a wide range of therapeutic agents out of cancer cells, causing MDR, cancer relapse and death [28]. Human ABCG2 (BCRP, MXR) is the last of the three major ABC drug transporters identified [29, 30], and is overexpressed in many cancers. It is known for transporting a large variety of conventional anticancer agents and newly developed targeted therapy drugs, including many tyrosine kinase inhibitors [31-33], and is linked to the development of MDR in patients with advanced non-small cell lung cancer or acute myeloid leukemia (AML) [28, 34]. Moreover, ABCG2 is localized at barrier sites such as the luminal membrane of brain capillaries and the blood-brain barrier (BBB) signify that it has significant physiological and pharmacological impact on drug bioavailability, drug distribution and protecting cells or tissues, such as the brain, from xenobiotics and chemotherapeutics [35, 36].

Here, we discovered that volasertib and GSK461364 interacted with human ABCG2 without affecting its protein expression, and the overexpression

*Address correspondence to this author at the 259 Wen-Hwa 1st Road, Kwei-Shan, Tao-Yuan 333, Taiwan; Tel: 886-3-2118800, ext. 3754; Fax: 886-3-2118700; E-mail: wuchung@mail.cgu.edu.tw

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of ABCG2 led to reduced G2/M cell cycle arrest induced by volasertib or GSK461364 and decreased the chemosensitivity to both Plk1 inhibitors in human colon cancer S1-M1-80 cells. Lastly, the reduced sensitivity of S1-M1-80 cancer cells to volasertib and GSK461364 can be significantly restored by inhibiting the function of ABCG2.

MATERIALS AND METHODS

Materials

DMEM, RPMI medium, fetal calf serum (FCS), trypsin-EDTA, penicillin, streptomycin and PBS were purchased from Gibco, Invitrogen. Pheophorbide A, mitoxantrone, MTT dye, Cell Counting Kit-8 (CCK-8) and all other chemicals were purchased from Sigma (St. Louis, MO, USA), unless stated otherwise. Volasertib and GSK461364 (99% purity by HPLC, Chiral HPLC) were purchased from ChemieTek (Indianapolis, IN, USA). FTC was a generous gift from Dr Susan Bates (National Cancer Institute, NIH, Bethesda, MD, USA).

Cell Lines and Culture Conditions

pcDNA3.1-HEK293 and R482-HEK293 cells were cultured in DMEM, supplemented with 10 % FCS, 2 mM L-glutamine and 100 units of penicillin/streptomycin/mL. S1 and S1-M1-80 cells were cultured in RPMI-1640, supplemented with 10 % FCS, 2 mM L-glutamine and 100 units of penicillin/ streptomycin/mL. S1-M1-80 cells were cultured in 80 μM of mitoxantrone, whereas R482-HEK293 cell were maintained in the presence of 2 mg/mL G418, as described previously [37]. All cell lines were maintained at 37 °C in 5 % CO_2 humidified air.

Cytotoxicity Assay

CCK-8 and MTT assays were used to determine the general sensitivities of cells to the tested chemicals as described previously [37]. For the reversal of cytotoxicity assays, a nontoxic concentration of ABCG2 inhibitor FTC was added into the cytotoxicity assay, and the extent of reversal was then calculated based on the relative resistance values.

Fluorescent Drug Accumulation Assay

The effect of volasertib, GSK461364 and FTC on ABCG2-mediated efflux of mitoxantrone was carried out using a FACSort flow cytometer equipped with CellQuest software (Becton-Dickinson) and analyzed as described previously [38]. The mean fluorescence intensity was calculated with the histogram stat program in CellQuest software.

Immunoblotting

Antibodies BXP-21 (1:500) and anti-α-tubulin (1:2000) were used to detect ABCG2 and tubulin. Tubulin was as positive control for Western blotting. The secondary antibody used was the Horseradish peroxidase-conjugated goat anti-mouse IgG (1:10,000). Signals were detected as described previously [39].

Cell Cycle Analysis

For the determination of drug-induced cell cycle arrest, a standard propidium iodide (PI) staining method and analyzed using a FACSort flow cytometer equipped with CellQuest software. Briefly, cells were treated with indicated regimens for 24 hours before harvested in PBS and fixed in ethanol overnight. Cells were washed once with PBS, then treated with 0.5 % TritonX-100 and 0.05 % RNase in PBS at 37 °C for 1 hr. Cells were washed, propidium iodide (50 μg/mL) added, then incubated at 4 °C for at least 20 min before analysis.

Statistical Analysis

Data are presented as mean ± S.E.M. The IC_{50} values were calculated as mean ± SD from at least three independent experiments. Differences between any mean values were analyzed by two-sided Student's t-test and results were considered statistically significant at $P < 0.05$.

RESULTS

Human ABCG2 Reduced the Effectiveness of Volasertib and GSK461364

Volasertib and GSK461364 have been shown highly effective against the proliferation of multiple human cancer cell lines [20, 25, 26]. While examining their activity against a panel of human colon cancer cell lines, we discovered that the ABCG2-overexpressing human colon S1-M1-80 cells were less sensitive to volasertib and GSK461364 than the parental S1 cells (Figure **1A**, **1B**). The resistance factor (RF) values for volasertib and GSK461364 in S1-M1-80 cells were 3 and 4, respectively. The RF value was calculated by dividing the IC_{50} value of ABCG2-overexpressing subline by the IC_{50} value of the respective parental line, which represents the degree of resistance to volasertib

or GSK461364 caused by the presence of ABCG2. Our data suggested that human ABCG2 confers resistance to both Plk1 inhibitors in cancer cells. In order to confirm our findings, the cytotoxicities of volasertib and GSK461364 in ABCG2-transfected R482-HEK293 cells and parental HEK293 cells were also determined. R482-HEK293 cells were more resistant to both Plk1 inhibitors than parental HEK293 cells, with calculated RF values of 3 and 4, respectively (Figure **1C**, **1D**). The calculated IC_{50} values for volasertib and GSK461364 for each cell lines are summarized in Table **1**.

Volasertib and GSK461364 Inhibit ABCG2-Mediated Transport

Next, we evaluated the biochemical interactions between volasertib, GSK461364 and ABCG2. We measured the effect of both Plk1 inhibitors on ABCG2-mediated efflux of Pheophorbide A (PhA), a known fluorescent substrate of human ABCG2 [37], in ABCG2-overexpressing cancer cells and HEK293 cells transfected with human ABCG2, as described in *Materials and methods*. The presence or absence of volasertib, GSK461364 or FTC had no significant effect on the accumulation of PhA in drug sensitive S1 and pcDNA-HEK293 cells (Figure **2A-D**, *left panels*). In contrast, the PhA accumulation levels in ABCG2-expressing S1-M1-80 (Figure **2A**, **2B**) and R482-HEK293 (Figure **2C**, **2D**) in the presence of 10 μM volasertib or GSK461364 (shaded, solid lines) or 5 μM of ABCG2 inhibitor FTC (*dotted lines*) were significantly higher than cells in the absence of Plk1 inhibitors or FTC (*solid lines*). In addition, we revealed that

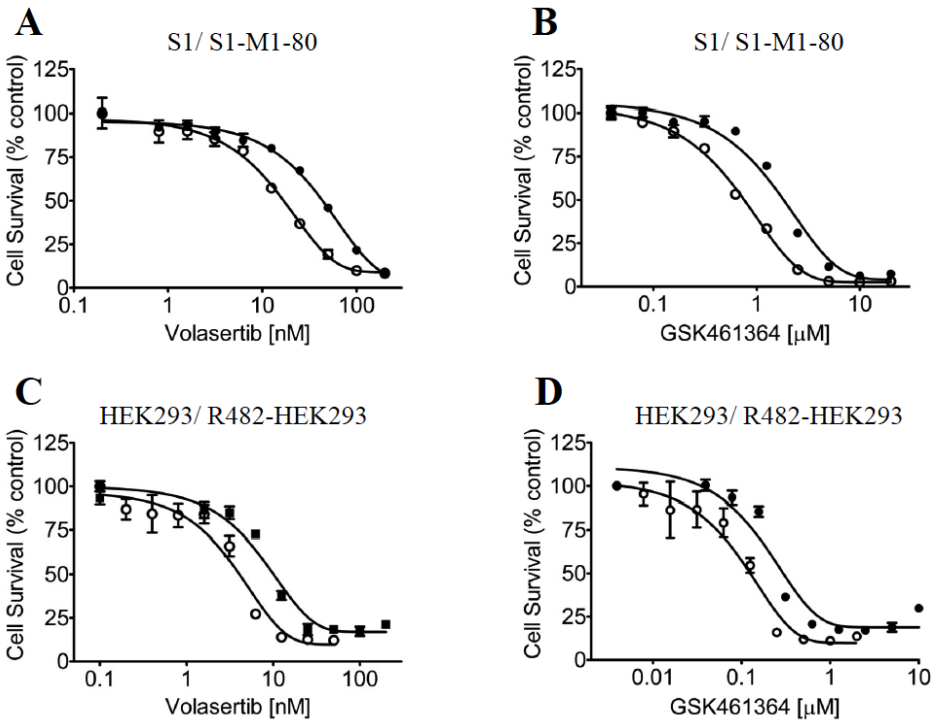

Figure 1: Overexpression of human ABCG2 confers resistance to volasertib and GSK461364. The cytotoxicity of (**A**) volasertib and (**B**) GSK461364 in sensitive human colon S1 (○) and ABCG2-overexpressing resistant S1-M1-80 (●) cells; as well as (**C**) volasertib and (**D**) GSK461364 in parental HEK293 (○) and ABCG2-tranfected R482-HEK293 (●) cells, was determined as described previously [37]. *Points*, mean from at least three independent experiments; *bars*, SEM.

Table 1: Sensitivity of Cell Lines to Polo-Like Kinase 1 Inhibitors Volasertib and GSK461364

Cell line	Cancer origin	Transporter expressed	Volasertib IC_{50} (nM)[†]	GSK461364 IC_{50} (μM)[†]
S1	colon	-	15.76 ± 1.71	0.55 ± 0.06
S1-M1-80	colon	ABCG2	40.99 ± 5.56**	2.18 ± 0.56**
pcDNA-HEK293	-	-	6.26 ± 1.75	0.11 ± 0.03
R482-HEK293	-	ABCG2	16.56 ± 4.94*	0.44 ± 0.10**

[†]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*. *P < 0.05; **P < 0.01.

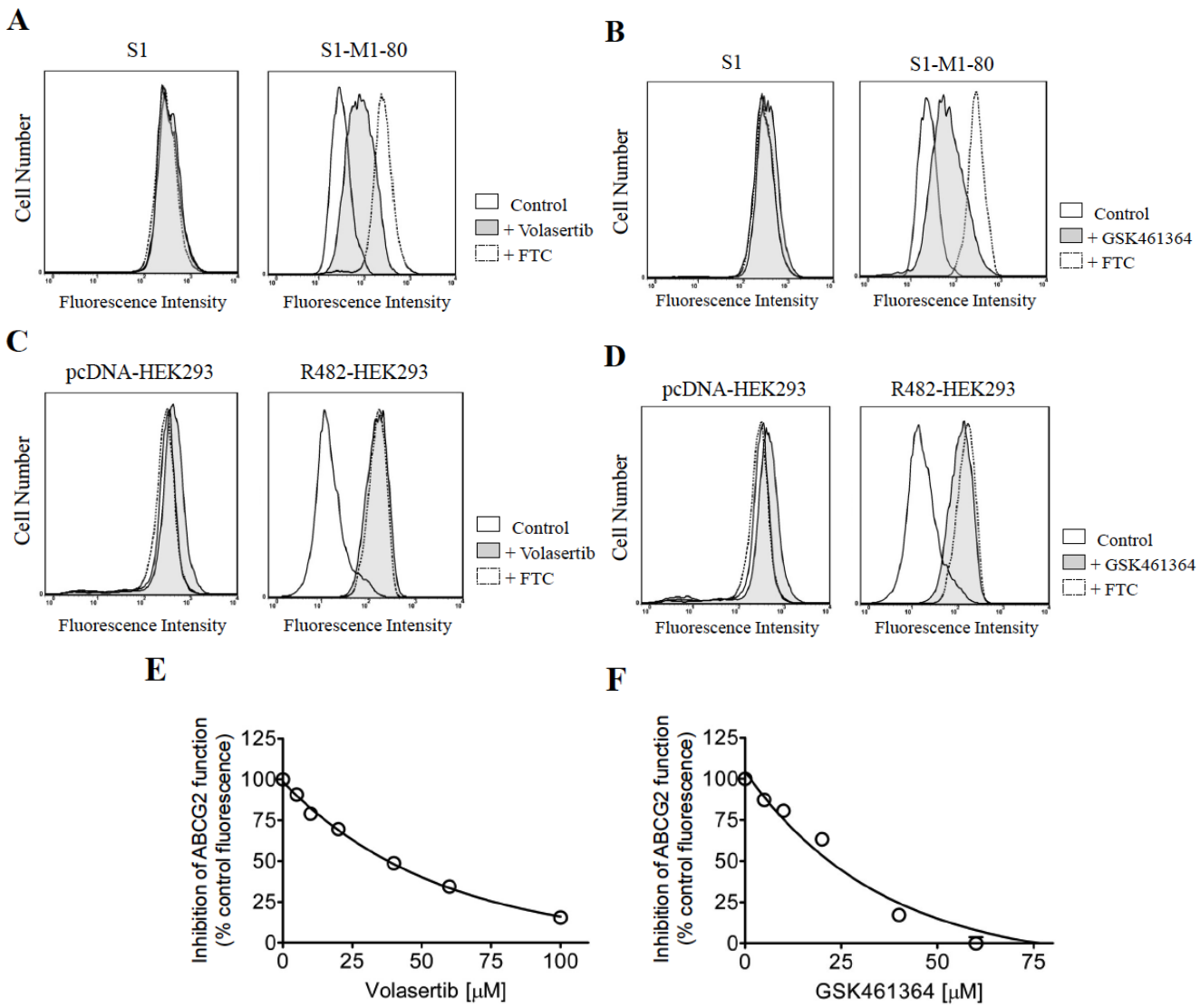

Figure 2: Volasertib and GSK461364 inhibit ABCG2-mediated transport of PhA. The level of fluorescent PhA accumulation in parental S1 (**A** and **B**, *left panel*) and ABCG2-overexpressing S1-M1-80 (**A** and **B**, *right panel*); parental HEK293 (**C** and **D**, *left panel*) and ABCG2-transfected R482-HEK293 (**C** and **D**, *right panel*) cells was measured in the presence or absence of volasertib (**A** and **C**) or GSK461364 (**B** and **D**) or a known ABCG2 inhibitor FTC, and analyzed immediately by flow cytometry as described in *Materials and methods*. Representative histograms of three independent experiments are shown; solid lines represent cells in the absence of tested compound, shaded solid lines represent cells in the presence of 10 μM volasertib or GSK461364, whereas dotted lines represent cells in the presence of 5 μM FTC. Concentration-dependent inhibition of ABCG2-mediated PhA efflux by (**E**) volasertib or (**F**) GSK461364 in S1-M1-80 cells was also determined. Data points represent the mean ± SEM from at least three independent experiments. The IC_{50} values were calculated as the concentration that inhibited the efflux to 50 % of the control values.

volasertib and GSK461364 inhibited the function of ABCG2 in a concentration dependent manner, with calculated IC_{50} values of approximately 39 and 24 μM, respectively (Figure **2E**, **2F**).

Volasertib and GSK461364 had no Significant Effect on the Protein Expression of ABCG2 in Human Colon Cancer Cells

Given that several ABC protein-interacting compounds have been reported to affect the protein expression of ABC proteins [40-43], we investigated whether volasertib and GSK461364 have any regulatory effect on the protein expression of ABCG2 in human colon S1 and S1-M1-80 cancer cells (Figure **3A**, **3B**). Cells were treated with increasing concentrations of either volasertib or GSK461364 for 72 hours, then harvested and processed for immunodetection of ABCG2 as detailed in *Materials and methods*. Our results showed that the protein expression of ABCG2 in S1 and S1-M1-80 cancer cells were not affected by either volasertib or GSK461364 over a period of 72 hours.

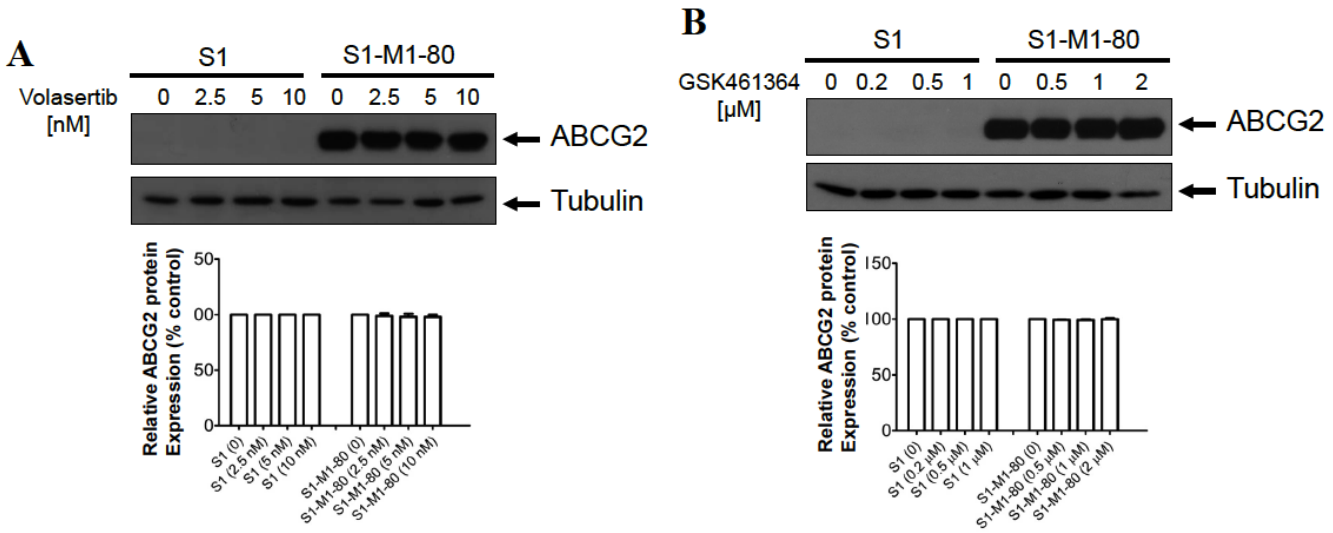

Figure 3: Effect of volasertib and GSK461364 on the protein expression ABCG2 in human colon S1 and S1-M1-80 cancer cells. The representative immunoblots (*top panels*) and averaged relative protein expression levels (*lower panels*) obtained from immunoblot detection of ABCG2 in total cell lysate protein (10 μg) in S1 and S1-M1-80 cells, treated with (**A**) increasing concentrations (0 - 10 nM) of volasertib and (**B**) concentrations (0 - 1 μM) or (0 - 2 μM) of GSK461364, respectively for 72 hours as described previously [39]. α-Tubulin was used as an internal control for equal loading. Values are presented as mean ± SD calculated from three independent experiments.

The Reduced Volasertib or GSK461364-Induced G2/M Cell Cycle Arrest and Cytotoxicity in ABCG2-Overexpressing Cancer Cells can be Restored by Inhibiting the Function of ABCG2

One of the key features of volasertib and GSK461364 is that they induce G2/M cell cycle arrest in many human cancer cells [2, 20, 44]. Therefore, we investigated the effect of ABCG2 overexpression on the ability of volasertib and GSK461364 to induce G2/M arrest in human colon S1 and S1-M1-80 cells. In the presence of volasertib (10 nM) and GSK461364 (500 nM), G2/M arrest was significantly induced in drug sensitive S1 cells (Figure **4A**, *upper panels*), from 7 % basal level to 73 % and 55 %, respectively. In contrast, the levels of G2/M arrest induced by volasertib and GSK461364 in drug resistant S1-M1-80 cells were considerably less than in S1 cells (Figure **4A**, *lower panels*), from 22 % to 44 % and 26 %, respectively. Moreover, we found that in the presence of FTC (3 μM), the levels of G2/M arrest induced by volasertib and GSK461364 can be recovered significantly (Figure **4B**), from 44 % and 26 % to 90 % and 81 %, respectively. The overall effect of volasertib, GSK461364 or FTC on the phases of cell cycle in S1 and S1-M1-80 cells is summarized in Table **2**. To ensure ABCG2-mediated resistance to Plk1 inhibitors can be reversed by inhibiting the function of ABCG2, we examined the effect of FTC on the cytotoxicity of volasertib and GSK461364 in ABCG2-overexpressing S1-M1-80 cells. In the presence of 3 μM FTC, the sensitivity of S1-M1-80 cells to volasertib and GSK461364 was reversed from RF value of 3 to 1, and from RF value of 4 to 1, respectively (Table **3**).

DISCUSSION

The search for a new therapeutic target for cancer treatment has been ongoing for decades. The fact that Plk1 is a key regulator in cell cycle progression and is overexpressed in different tumor types, making Plk1 a good target for cancer chemotherapy. Studies have shown that the inhibition of Plk1 activity by inhibitors such as volasertib and GSK461364 can induce significant G2/M cell cycle arrest, which led to onset of apoptosis and eventual cell death [13, 20, 44, 45]. Volsertib and GSK461364 are highly effective against multiple human cancer cell lines and inhibit Plk1 activity in the nanomolar range [12, 20].

While evaluating the effectiveness of volasertib and GSK461364 against multiple human colon cancer cell lines, we discovered that both Plk1 inhibitors were less effective in ABCG2-overexpressing human S1-M1-80 colon cancer cells when compared to the drug sensitive parental human S1 colon cancer cells (Table **1**). Since the overexpression of ABCG2 is one of the most common mechanisms of developing drug resistance in cancer cells [31-33], we suspect the reduced efficacy of Plk1 inhibitors in S1-M1-80 is a direct result of ABCG2 function. First, we confirmed our findings by determining the cytotoxicity of volasertib and

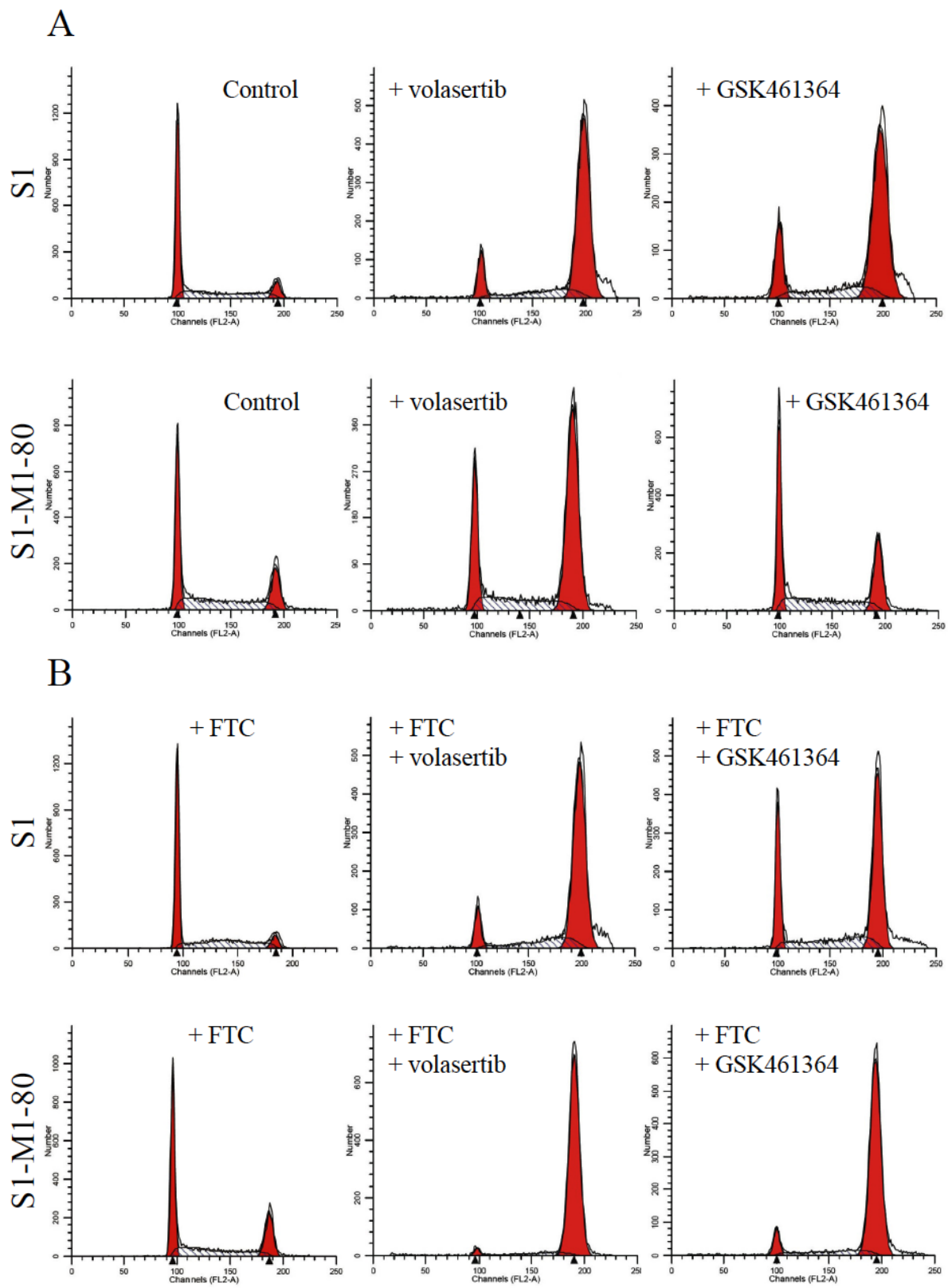

Figure 4: ABCG2 inhibitor FTC restores volasertib- or GSK461364-induced G2/M cell cycle arrest in ABCG2-overexpressing drug resistant human colon cancer cells. Human S1 (*top panels*) and ABCG2-overexpressing S1-M1-80 (*lower panels*) cells were plated and maintained in the absence or presence of Plk1 inhibitors alone, or Plk1 inhibitors in combination with FTC for 24 h before harvest for cell cycle analysis. S1 and S1-M1-80 cells were treated with either DMSO (**A**, *left panels*), 10 nM of volasertib (**A**, *middle panels*), 500 nM of GSK461364 (**A**, *right panels*), 3 μM of FTC (**B**, *left panels*), combination of 3 μM FTC and 10 nM of volasertib (**B**, *middle panels*) and 3 μM FTC and 500 nM of GSK461364 (**B**, *right panels*). Representative histograms of three independent experiments are shown.

Table 2: The Percentage Distribution of Cells in Cycle Phases

	Cell cycle phase		
	G1 (%)	**S (%)**	**G2/M (%)**
S1			
Control	49.70 ± 4.76	43.04 ± 4.38	7.27 ± 0.48
+ FTC	44.73 ± 2.96	47.96 ± 2.39	7.31 ± 1.37
+ Volasertib	11.46 ± 2.01	15.56 ± 2.73	72.98 ± 4.52
+ Volasertib + FTC	12.73 ± 4.01	17.64 ± 0.82	69.63 ± 4.79
+ GSK461364	22.18 ± 6.02	23.05 ± 4.28	54.77 ± 10.02
+ GSK461364 + FTC	24.84 ± 1.49	24.22 ± 1.62	50.94 ± 3.06
S1-M1-80			
Control	44.33 ± 1.22	33.31 ± 3.11	22.36 ± 2.28
+ FTC	46.90 ± 6.60	31.51 ± 2.20	21.59 ± 6.03
+ Volasertib	28.10 ± 8.08	27.54 ± 5.10	44.37 ± 12.00
+ Volasertib + FTC	2.33 ± 0.84	7.56 ± 1.77	90.12 ± 2.61
+ GSK461364	39.99 ± 3.58	34.51 ± 4.91	25.51 ± 4.35
+ GSK461364 + FTC	7.11 ± 1.24	11.56 ± 2.53	81.33 ± 3.56

The percentage values were calculated from three independent experiments.

Table 3: Effect of ABCG2 Inhibitor FTC on the Chemosensitivity of Volasertib and GSK461364 in Human S1 and ABCG2-Overexpressing S1-M1-80 Colon Cancer Cell Lines

Cell line	Transporter overexpressed	IC_{50} (nM)†		IC_{50} (μM)†	
		Volasertib	**Volasertib + FTC (3 μM)**	**GSK461364**	**GSK461364 + FTC (3 μM)**
S1	-	15.76 ± 1.71	23.95 ± 4.17*	0.55 ± 0.06	0.69 ± 0.08
S1-M1-80	ABCG2	40.99 ± 5.56	21.45 ± 3.15**	2.18 ± 0.56	0.63 ± 0.08**

ND, Not determined.
†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. *$P < 0.05$; **$P < 0.01$.

GSK461364 in HEK293 cells and ABCG2-transfected HEK293 (R482-HEK293) cells (Figure **1C**, **1D**). We found that ABCG2 conferred significant resistance to both Plk1 inhibitors in ABCG2-transfected R482-HEK293 cells (Table **1**). In order to study the biochemical interactions between Plk1 inhibitors and ABCG2, we examined the effect of volasertib and GSK461364 on ABCG2-mediated transport of pheophorbide A, a known substrate of ABCG2. Our results showed that both Plk1 inhibitors inhibited ABCG2-mediated drug efflux from S1-M1-80 cell (Figure **2A**, **2B**) and R482-HEK293 (Figure **2C**, **2D**), in a concentration-dependent manner (Figure **2E**, **2F**). Moreover, since some compounds are known to modulate the protein expression of ABC transporters [40-42], we studied the effect of Plk1 inhibitors on the expression level of ABCG2. We discovered that 72 hour treatment with volasertib and GSK461364 had no significant effect on the expression of ABCG2 in human S1 cancer cells (Figure **3**). Lastly, we studies the effect of ABCG2 on G2/M cell cycle arrest induced by Plk1 inhibitors and whether inhibition of ABCG2 function can restore sensitivity of ABCG2-overexpressing S1-M1-80 cancer cells to both volasertib and GSK461364. We found that the chemosensitivity and G2/M arrest induced by volasertib or GSK461364 were significantly reduced in S1-M1-80 when compared to the drug sensitive parental S1 cells (Figure **4**), and that we can restore the chemosensitivity (Table **3**) and drug-induced G2/M arrest (Table **2**) in S1-M1-80 by inhibiting the function of ABCG2 with FTC, an established inhibitor of human ABCG2.

In summary, our study showed that ABCG2-mediated drug resistance to volasertib and GSK461364 in cancer presents a potential therapeutic challenge, and a better treatment strategy should be further investigated.

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ABBREVIATIONS

MDR = multidrug resistance

ABC = ATP-binding cassette

Plk-1 = Polo-like kinase 1

PhA = Pheophorbide A

FTC = Fumitremorgin C

MTT = 3-(4,5-dimethylthiazol-yl)-2,5-diphenyllapatinibrazolium bromide

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