Original Paper

PCI29732, a Bruton's Tyrosine Kinase Inhibitor, Enhanced the Efficacy of Conventional Chemotherapeutic Agents in ABCG2-Overexpressing Cancer Cells

Chunlei Ge^{a,b} Fang Wang^b Chaochu Cui^b Xiaodong Su^b
Kenneth Kin Wah To^c Xiaokun Wang^b Hui Zhang^b Xin Song^a
Liwu Fu^b

^aCancer Biotherapy Center, the Third Affiliated Hospital of Kunming Medical University (Tumor Hospital of Yunnan Province), Kunming, ^bState Key Laboratory of Oncology in South China, Sun Yat-Sen University Cancer Center, Guangzhou, ^cSchool of Pharmacy, The Chinese University of Hong Kong, Hong Kong, China

Key Words

Pci29732 • MDR • ABCG2 • ABC transporter • Cancer chemotherapy

Abstract

Background/Aims: Multidrug resistance (MDR) induced by the ABC transporter subfamily B member 1 (ABCB1) and subfamilyG member 2 (ABCG2) limits successful cancer chemotherapy and no commercially available MDR modulator is used in the clinic. In the current study, we aimed to investigate the effects of PCI29732 on the enhancement of chemotherapeutic agents.

Methods: Cell cytotoxicity and reversal effect were measured with MTT assay. Additionally, flow cytometry was employed to detect the accumulation and efflux of the drugs. We investigated the interaction of PCI29732 and the substrate binding sites of ABCG2 was investigated via the photo-labeling of ABCG2 with the [¹²⁵I] iodoarylazidoprazosin. The vanadate-sensitive ATPase activity of ABCG2 was measured to identify whether the drug affected the ATPase activity. RT-PCR and Western blot were utilized to analyze mRNA and protein expression respectively.

Results: Here, we found that PCI29732 significantly enhanced the efficacy of substrate chemotherapeutic agents in ABCG2-overexpressing cells and also in xenografts harboring the H460/MX20 cell that overexpress ABCG2, but not in their parental sensitive cells and ABCB1-overexpressing cells. Mechanistically, the intracellular accumulations of doxorubicin and Rhodamine 123 were increased in ABCG2-overexpressing S1-MI-80 cells with the presence of PCI29732. PCI29732 stimulated the ATPase activity of ABCG2 at low concentrations. However at the high concentrations, PCI29732 inhibited the ATPase activity, and competed with [¹²⁵I]-iodoarylazidoprazosin for photo-affinity labeling of ABCG2. PCI29732 did neither alter the

Professor Liwu Fu
and Professor Xin Song

Cancer Center, Sun Yat-Sen University
Guangzhou, 510060, Guangdong Province, (China)
Tel. +86-20-873-431-63, E-Mail Fulw@mail.sysu.edu.cn; songxin68@126.com

mRNA or protein expression levels of ABCG2 nor the phosphorylation levels of AKT and ERK1/2. **Conclusion:** Our study demonstrates that PCI29732 inhibits the function of ABCG2 by competitively binding to the ATP-binding site of ABCG2 and enhances the anti-tumor efficacy of substrate chemotherapeutic agents. This findings encourages the development of combinational chemotherapy for the treatment of ABCG2- overexpressing cancer patients.

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Introduction

Chemotherapy remains an important treatment for human cancers. Among the factors affecting the efficacy of chemotherapy, multidrug resistance (MDR) profoundly limits the effectiveness of numerous chemotherapeutic drugs. Although some compounds have been confirmed to be very effective at reversing MDR in the *in vitro* and *in vivo* experiments, results of clinical trials have been unsatisfactory, which is mostly due to side effects and/or weak efficacy [1]. Hence, in attempts to overcome drug resistance in cancer therapy, exploring for new and more potent MDR reversal agents and uncovering the underlying mechanisms of MDR reversal have been given a high priority.

Overexpression of ATP-binding cassette (ABC) transporters is one of the most importantly and well-studied mechanisms of MDR [2]. ABC transporters, can pump various chemotherapeutic drugs out of cells, reducing their intracellular accumulation and diminishing their anticancer activity. Among the 49 ABC transporters discovered so far, the ABC transporters, including subfamily B member 1 (ABCB1, MDR1, P-glycoprotein, P-gp), subfamily C member 1 (ABCC1, multidrug resistance associated protein 1, MRP1), and subfamily G member 2 (ABCG2, breast cancer resistance protein, BCRP), are considered the most important in conferring MDR in cancer cells [3, 4].

ABCB1, a 170-kDa glycoprotein, is constitutively expressed in many normal cells, including hematopoietic stem cells, natural killer cells, and cells in the intestinal mucosa as well as other tissues (particularly cells of the brain, testes, kidneys, liver, and muscle). The main function of ABCB1 is associated with detoxification and secretion [5]. ABCB1 can transport a wide range of anti-neoplastic drugs (e.g., doxorubicin, vincristine, paclitaxel, and epipodophyllotoxins) out of cancer cells [6]. ABCG2/BCRP, also known as mitoxantrone resistance-associated protein (MXR), was initially cloned from the human breast cancer cell line MCF-7, with the function to be found to confer resistance to several chemotherapeutic agents, including doxorubicin, mitoxantrone, and topotecan [7]. ABCG2 is widely expressed in normal human tissues, including the intestines, liver, mammary glands, placenta, and brain [8, 9], where it contributes to the absorption, distribution, and elimination of drugs and endogenous compounds as well as protection of the tissues from xenobiotic exposure [10]. Additionally, ABCG2 is one of the key drug transporters for cancer chemotherapy [11-13]. It is also responsible for the "side population" (SP) phenotype [14], which is often used for the identification and isolation of cancer stem cells (CSCs). The CSCs are spared by their high expression of multidrug transporters (especially ABCG2), mediating their chemo-resistance and eventually leading to cancer relapse and metastasis [15]. Eliminating CSCs that enhance the resistance to chemotherapy through ABCG2 would be a new therapeutic strategy for human cancers. Tyrosine kinase inhibitors (TKIs) represent a group of therapeutic agents that are aimed at novel targets for the treatment of various human cancers. TKIs such as Ibrutinib, can bind to the ATP binding site to the domain of kinase, so it is conceivable that TKIs may also be capable of binding to the ATP binding site of ABC transporter. Recently several studies have reported that various TKIs can block the drug efflux activity of ABCB1 [16]. For instance, ceritinib remarkably enhanced the efficacy of chemotherapeutic drugs in cancer cells with ABCB1 or ABCG2 overexpression *in vitro* and *in vivo* [17]. Osimertinib (AZD9291), a third-generation irreversible TKI, reversed MDR through inhibition of ABCB1 and ABCG2, thus limiting the pumping out of chemotherapeutic agents [18]. Afatinib could reverse ABCG2-mediated drug resistance via competitive blockage of substrate transportation and downregulation of ABCG2 expression [19].

Bruton's tyrosine kinase (BTK), a member of the Tec family of kinases, is specifically required for the B-cell antigen receptor (BCR) signaling pathway [20]. It has been identified as an essential signaling kinase for cell survival of a subtype of diffuse large B-cell lymphoma [21]. Recently, PCI32765 (Ibrutinib), a novel BTK inhibitor, was approved by the United States Food and Drug Administration (FDA) for treating patients with mantle cell lymphoma (MCL) [22], and it has recently been shown by many *in vitro*, *in vivo*, and *ex vivo* cancer models that it can increase the effectiveness of several chemotherapeutic agents that are ABCC1/MRP1 substrates in MRP1-overexpressing cells [23].

Another BTK inhibitor, PCI29732, can irreversibly inhibits BTK by interacting with the BTK gatekeeper residue T474 [24]. It has a potent inhibitor of interleukin-2 inducible T cell kinase (Itk) and other kinases from the Tec and Src families [25]. In addition, PCI29732 can block the transcriptional up-regulation of a panel of B-cell activation genes, thus inhibiting B-cell signaling and activation [25, 26].

The present study was designed to investigate the effects of PCI29732, both *in vitro* and *in vivo*, on the enhancing the tumor suppression activity of chemotherapeutic agents in cancer cells that overexpress ABCG2 *in vitro* and *in vivo*.

Materials and Methods

Ethics approval and consent to participate

The ethics approval statements for animal work were provided by the Institutional Animal Care and Use Committee of Sun Yat-Sen University Cancer Center. The ethics approval numbers of animal work was L102012015090V.

Availability of data and materials

The dataset(s) supporting the findings of this study are included within the article.

Chemicals and reagents

Dulbecco's modified Eagle medium (DMEM) and Gibco® RPMI-1640 were purchased from Thermo Fisher Scientific Inc. (MA, USA). Chemicals 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), mitoxantrone, topotecan, doxorubicin, paclitaxel, verapamil, fumitremorgin C (FTC), and Rhodamine 123 were purchased from Sigma-Aldrich (MO, USA); PCI29732 was purchased from Med Chem Express (NJ, USA). Antibodies against ABCG2 (sc-377176), AKT (sc-8312), p-AKT (sc-7985-R), ERK (sc-514302), and p-ERK (sc-7383) were purchased from Santa Cruz Biotechnology, Inc. (CA, USA). Antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody was purchased from Kangcheng Co. (Shanghai, China). SYBR Green qPCR Master Mix was purchased from ExCell Bio (Shanghai, China).

Cell lines and cell culture

The human colon carcinoma cell-line S1 and its mitoxantrone-selected ABCG2-overexpressing derivative S1-MI-80 [27], human non-small cell lung cancer cell line NCI-H460 (H460) and its mitoxantrone-selected ABCG2-overexpressing subline NCI-H460/MX20 (H460/MX20) [28], stably transfected HEK293/pcDNA3.1, HEK293/ABCG2-482-R2 (wild-type), and HEK293/ABCG2-482-T7 cells carrying either an empty pcDNA3.1 vector or a pcDNA3.1 vector containing full-length ABCG2 coding either arginine (R) or threonine (T) at the amino acid 482 position, respectively [29], were kindly provided by Dr. Susan Bates (Division of Hematology and Oncology, Columbia University Medical Centre, NY, USA) and cultured in DMEM.

The human oral epidermoid carcinoma cell line KB and its vincristine-selected ABCB1-overexpressing derivative KBv 200 were generous gifts from Dr. Xu-Yi Liu (Cancer Hospital of Beijing, Beijing, China) and cultured in RPMI 1640 [30]. Cell culture media was supplemented with 10% fetal bovine serum (FBS), 100 U/mL of penicillin, and 100 U/mL of streptomycin. All the cells were cultured at 37°C in a humidified atmosphere of 5% CO₂.

Cytotoxicity assay

The sensitivity of the parental cancer cells and their derivatives to drugs was determined by using the MTT assay as described previously [31]. Cells were seeded onto 96-well plates and cultured for 24 hours. Cells were incubated with various concentrations of PCI29732 for 1 hour and then, without removal, exposed to various concentrations of chemotherapeutic agents for an additional 68 hours. MTT (5 mg/mL, 20 μ L) was then added to each well and the cells were cultured for 4 hours. The cell culturing media was discarded and 120 μ L of DMSO was added to each well. Finally, the absorbance at 540 nm was determined using a Model 550 Microplate Reader (Bio-Rad, CA, USA) with background subtraction.

The IC₅₀ value, which means the drug concentration under which cell growth was inhibited by 50% was calculated from the survival curves using the Bliss method [32]. FTC (2.5 μ M) and verapamil (10 μ M) were used as positive controls for ABCG2 and ABCB1, respectively. As described previously [32], the fold change in the MDR was calculated by dividing the IC₅₀ values of the anticancer agents obtained in the absence of PCI29732 by those obtained in the presence of PCI29732. All the experiments were repeated at least three times.

Cancer xenograft model in nude mice

In the present study, H460/MX20 cells were used to establish a xenograft mouse model in mice. Athymic nude mice (5–6 weeks old) were purchased from Nanjing Medical University, Nanjing, China. All the animal experiments were performed in accordance with the guidelines for the use of laboratory animals of the Institutional Animal Care and Use Committee of the Sun Yat-Sen University, Guangzhou, China, who reviewed and approved the animal protocol.

Briefly, H460/MX20 cells (3.0×10^6) in 200 μ L of phosphate buffer saline (PBS) were implanted subcutaneously into the right flank of each mouse. Tumor sizes and body weights were measured every 3 days. On day 7 post cell inoculation, the tumor volume of each animal was measured and the mice were randomly assigned to four groups and treated as follows: (1) saline (every 3 d $\times$ 5 times, intraperitoneally, i.p.); (2) topotecan (every 3 d $\times$ 5 times, i.p., 3 mg/kg); (3) PCI29732 (every 3 d $\times$ 5 times, by mouth p.o., 20 mg/kg); and (4) PCI29732 (every 3 d $\times$ 5 times, p.o., 20 mg/kg) plus topotecan (every 3 d $\times$ 5 times, i.p., 3 mg/kg; topotecan was given 1 h after PCI29732 administration). Two perpendicular diameters (A and B) of each tumor were recorded every 3 days, and the tumor volume (V) was estimated according to the following formula [33]:

$$V = \frac{\pi}{6} \left(\frac{A+B}{2} \right)^3$$

The tumor growth curves were plotted according to mean tumor volumes measured in each group. The mice were anesthetized and euthanized on day 25. Tumors were excised and weighed. The ratio of growth inhibition (IR) was calculated according to the following formula:

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

Assays for doxorubicin and Rhodamine 123 accumulation

The effects of PCI29732 on the intracellular accumulation of doxorubicin and Rhodamine 123 in S1 and S1-M1-80 cells were measured by flow cytometry as described previously [34]. The cells were incubated in six-well plates overnight and then treated with 3 different concentrations of PCI29732 (0.75, 1.5, and 3 μ M) for 3 hours. Doxorubicin (final concentration of 10 μ M) or Rhodamine 123 (5 μ M) was added and the cells were cultured for an additional 3 hours or 0.5 hour, respectively. Cells were then collected, washed with ice-cold PBS three times, and then analyzed by flow cytometric analysis (Cytomics FC500; Beckman Coulter Inc., CA, USA). FTC was used as a positive control inhibitor of ABCG2.

Rhodamine 123 efflux assay

To measure drug efflux, a previously described method [35] using Rhodamine 123 was slightly modified. S1 and S1-MI-80 cells were incubated with 5 μ M of Rhodamine 123 for 0.5 hour, washed with ice-cold PBS, and then cultured in medium with or without 3 μ M of PCI29732 for 0, 15, 30, 60, and 120 min. The cells were collected, washed with ice-cold PBS, and then immediately analyzed with flow cytometry immediately.

ABCG2 ATPase assay

A colorimetric ATPase assay was performed as previously described [36], with a minor modification. Briefly, the crude membranes isolated from High Five™ insect cells expressing ABCG2 (100 μ g protein/mL; a kind gift from Dr. Suresh Ambudkar, National Cancer Institute, NIH, Bethesda, MD) were incubated at 37°C with various concentrations of PCI29732 in the presence or absence of 1.2 mM of sodium orthovanadate in ATPase assay buffer (50 mM KCl, 5 mM sodium azide, 2 mM EDTA, 10 mM MgCl₂, 1 mM DTT, pH 6.8) for 5 min.

The ATP hydrolysis reaction was triggered off with the addition of 5 mM Mg-ATP (concentration in a final volume of 60 μ L). The reaction was continued for 10 min, and terminated by adding 30 μ L of a 10% sodium dodecyl sulfate (SDS). The absorbance at 750 nm was measured after the addition of a detection reagent (35 mM ammonium molybdate, 15 mM zinc acetate, 10% ascorbic acid) followed by incubation at 37°C for 20 min. The amount of released inorganic phosphate was quantified by reading from a standard curve.

The specific PCI29732-stimulated ABCG2 ATPase activity (*i.e.*, vanadate-sensitive) was determined as the difference between the amounts of inorganic phosphate released from ATP in the absence and presence of sodium orthovanadate.

Photo-affinity labeling of ABCG2 with [¹²⁵I]-iodoarylazidoprazosin (IAAP)

The crude membrane from High Five™ insect cells expressing ABCG2 (50 μ g protein) was incubated with 0-10 μ M of PCI29732 in 50 mM Tris-HCl (pH 7.5) at room temperature for 5 min. After [¹²⁵I]-IAAP (2200 Ci/nmole, 3 nM) was added, the mixture was incubated for an additional 5 min under subdued light. The samples were then cross-linked by UV illumination (365 nm) on ice. The labeled ABCG2 was immunoprecipitated using the BXP21 antibody (ab3380, Abcam, Cambridge, UK). The samples were then subjected to separation on SDS-polyacrylamide gel electrophoresis (PAGE) using a 7% Tris-acetate NuPAGE gel, followed by drying the gel, and exposure of the gel to Bio-Max MR film (Eastman Kodak Co., Rochester, NY) at -80°C for 4 hours. The radioactivity incorporated into the ABCG2 band was quantified using the Storm 860 PhosphorImager system (Molecular Dynamics, Sunnyvale, CA).

Western blot analysis

To determine whether PCI29732 affected ABCG2 protein expression, the S1-MI-80 cells were incubated with various concentrations of PCI29732 (0.75, 1.5, 3, and 6 μ M) for 48 hours or incubated with 3 μ M of PCI29732 for different times (0, 24, 48, and 72 hours). The whole cell lysate was then harvested and protein concentration was determined using the BCA assay reagent (#23227, Thermo Fisher Scientific Inc., MA, USA). The lysates were determined by 50 μ g of total protein were resolved in 10% SDS-polyacrylamide gel and transferred onto polyvinylidene fluoride (PVDF) membranes (#ISEQ00010, Millipore, MA, USA). After blocking with 5% non-fat milk for 2 hours at room temperature, the membranes were incubated with primary antibodies against ABCG2 (1:1000), ERK1/2 (1:1000), p-ERK (1:1000), AKT (1:1000), and p-AKT (1:1000) overnight at 4°C. The membranes were washed with TBST buffer three times and then incubated with a specific secondary anti-mouse IgG (1:5000) (#7076, Cell Signaling Technology, Inc, MA, USA) for 2 hours at room temperature. After washing the membrane for three times with TBST, the bands for protein of interest were visualized by using the Phototope-HRP Western Blot Detection System (Cell Signaling Technology, MA, USA) and Kodak medical X-ray processor (Carestream Health Inc., NY, USA). GAPDH (1:5000) was used as an internal loading control.

RT-PCR and real-time PCR

Total cellular RNA was isolated using the Trizol Reagent RNA extraction kit (#15596026, Thermo Fisher Scientific Inc, MA, USA). The RNA samples were then reversely transcribed into cDNA using a thermoscript RT-PCR system (#18080-051, Invitrogen, CA, USA). The PCR primers used in the present study were as follows: 5'-TGGCTGTCATGGCTTCAGTA-3' (forward) and 5'-GCCACGTGATTCTTCCACAA-3' (reverse) for ABCG2; 5'-GAGTCAAGGATTTGGTCGT-3' (forward) and 5'-GATCTCGCTCCTGGAAGATG-3' (reverse) for GAPDH. The reactions were carried out using the GeneAmp PCR system 9700 (PE Applied Biosystems, CA, USA) at 94°C for 2 min for initial denaturation, and then 94°C for 30 s, 58°C for 30 s, and 72°C for 1 min. After 35 cycles of amplification, the PCR products were separated by 1.5% agarose gel electrophoresis.

Real-time PCR was performed by Bio-Rad CFX96™ Real-Time (Bio-Rad, CA, USA) with Real-time PCR Master Mix containing SYBR® Green I and Hot Start *Taq* DNA Polymerase. PCR was performed at 50°C for 2 min, 95°C for 5 min and then recycled at 95°C for 15 s, and 60°C for 30 s for 40 cycles. The gene encoding GAPDH was used as an internal control. Relative quantification of ABCG2 was performed using the $2^{-\Delta\Delta Ct}$ method. The results were obtained from three reactions for each sample.

Statistical analysis

The data are shown as means $\pm$ standard deviation (SD). Statistical analyses were performed using SPSS v16.0 statistical software (SPSS, Inc., IL, USA). All of the experiments were repeated at least three times. Significant differences were analyzed using Student's *t*-test, with $P < 0.05$ considered statistically significant. The authenticity of this article has been validated by uploading the key raw data onto the Research Data Deposit public platform (www.researchdata.org.cn), with the approval RDD number as RDDB2018000348.

Results

Cytotoxicity of PCI29732 in S1-MI-80, H460/MX20, KBv 200, HEK293/ABCG2, and their parental sensitive cells

The cytotoxicity of PCI29732 in different cells was determined by the MTT assay. The IC_{50} values were $7.94 \pm 0.62 \mu M$ for S1, $7.79 \pm 0.58 \mu M$ for S1-MI-80, $6.55 \pm 0.19 \mu M$ for H460, $6.34 \pm 0.17 \mu M$ for H460/MX20, $6.14 \pm 0.25 \mu M$ for KB, $6.02 \pm 0.53 \mu M$

Fig. 1. The structure of PCI29732 and the cytotoxicity of PCI29732 in the drug-resistant and parental sensitive cancer cells. Structure of PCI29732 (A); MTT assays were used to assess the cell survival in pairs of parental and transporter-overexpressing cells, H460 and ABCG2-overexpressing H460/MX20 cells (B), S1 and ABCG2-overexpressing S1-MI-80 cells (C), KB and ABCB1-overexpressing KBv 200 cells (D), and HEK293/pcDNA3.1 and HEK293/ABCG2-482-R2 and HEK293/ABCG2-482-T7 cells stably overexpressing ABCG2 (E). All the cells were exposed to various concentrations of PCI29732 for 72 h. The data are shown as means $\pm$ SD of at least three independent experiments performed in quadruple.

for KBv200, 12.45 ± 0.98 μ M for HEK293/pCDNA3, 14.58 ± 1.03 μ M for HEK293-ABCG2-482-R2, and 13.24 ± 0.83 μ M for HEK293-ABCG2-482-T7 cells. The IC_{50} values of PCI29732 were significantly different among the tested cells. More than 90% of S1 and S1-MI-80, H460 and H460/MX20, HEK293/pCDNA3.1, HEK293/ABCG2-482-R2, and HEK293/ABCG2-482-T7 cells survived upon exposure to 3 μ M of PCI29732 and more than 90% of KB and KBv200 cells survived upon treatment with 1.5 μ M of PCI29732 (Fig. 1B-E).

Based on the results, 3 μ M was chosen as the maximum concentration of PCI29732 for combination treatment with anticancer drugs in S1 and S1-MI-80, H460 and H460/MX20, HEK293/pCDNA3.1, HEK293/ABCG2-482-R2, and HEK293/ABCG2-482-T7 cells, and 1.5 μ M was used in the assays with KB and KBv200 cells.

Effects of PCI29732 on enhancement of chemotherapeutic agents activity in ABCG2-overexpressing cells

After being combined with various concentrations of PCI29732, the IC_{50} values of the chemotherapeutic agents in the resistant and the parental sensitive cells in combination with various concentrations of PCI29732 are shown in Tables 1 and 2. In a concentration-dependent manner, PCI29732 at 0.75, 1.5, and 3 μ M decreased the IC_{50} values of ABCG2 substrate chemotherapeutic agents (mitoxantrone and topotecan) in the S1-MI-80 and H460/MX20 cells, but not in their parental sensitive S1 and H460 cells, respectively. PCI29732 did not change the cytotoxicity of cisplatin, a non-ABCG2 substrate agent. However, PCI29732 did not enhance the efficacy of chemotherapeutic agents in ABCB1-overexpressing KBv200 cells (Table 1). These results indicated that PCI29732 could selectively sensitize

Table 1. Effects of PCI29732 on chemotherapeutic agents in ABC transporter-overexpressing cells. Note: The cell viability was assessed by the MTT assay. The data are shown as the mean $\pm$ standard deviation (SD) of at least three independent experiments. The fold-reversal of MDR was calculated by dividing the IC_{50} value for cells treated with the anticancer agents in the absence of PCI29732 by that obtained in the presence of PCI29732. * $P < 0.05$, ** $P < 0.01$ vs. control group

Compounds	$IC_{50} \pm SD, \mu M$ (Fold reversal)			
	S1		S1-MI-80 (ABCG2)	
Topotecan	0.3275 $\pm$ 0.0384	(1.00)	27.0030 $\pm$ 1.9167	(1.00)
+0.75 μ M PCI29732	0.3215 $\pm$ 0.0266	(1.02)	12.4971 $\pm$ 0.7806**	(2.16)
+1.5 μ M PCI29732	0.3082 $\pm$ 0.0240	(1.06)	8.6102 $\pm$ 0.1957**	(3.14)
+3 μ M PCI29732	0.2846 $\pm$ 0.0195	(1.15)	5.5443 $\pm$ 0.9135**	(4.87)
+2.5 μ M FTC	0.2873 $\pm$ 0.0193	(1.14)	3.6226 $\pm$ 0.3105**	(7.45)
Mitoxantrone	0.1483 $\pm$ 0.0088	(1.00)	4.0626 $\pm$ 0.4058	(1.00)
+0.75 μ M PCI29732	0.1491 $\pm$ 0.0178	(0.99)	2.1233 $\pm$ 0.1149**	(1.91)
+1.5 μ M PCI29732	0.1458 $\pm$ 0.0271	(1.01)	1.5289 $\pm$ 0.0191**	(2.66)
+3 μ M PCI29732	0.1441 $\pm$ 0.0228	(1.03)	0.8561 $\pm$ 0.1554**	(4.75)
+2.5 μ M FTC	0.1342 $\pm$ 0.0141	(1.11)	0.6233 $\pm$ 0.0274**	(6.51)
Cisplatin	7.0195 $\pm$ 0.6654	(1.00)	10.9883 $\pm$ 0.1617	(1.00)
+3 μ M PCI29732	5.9225 $\pm$ 0.4347	(1.18)	10.3702 $\pm$ 0.3350	(1.06)
H460				
H460/MX20 (ABCG2)				
Mitoxantrone	0.0477 $\pm$ 0.0065	(1.00)	1.2818 $\pm$ 0.0079	(1.00)
+0.75 μ M PCI29732	0.0460 $\pm$ 0.0041	(1.02)	0.7173 $\pm$ 0.0031**	(1.78)
+1.5 μ M PCI29732	0.0312 $\pm$ 0.0063*	(1.51)	0.4687 $\pm$ 0.0086**	(2.73)
+3 μ M PCI29732	0.0300 $\pm$ 0.0074*	(1.57)	0.2521 $\pm$ 0.0291**	(5.07)
+2.5 μ M FTC	0.0283 $\pm$ 0.0038*	(1.66)	0.2252 $\pm$ 0.0166**	(5.68)
KB				
KBv200 (ABCB1)				
Doxorubicin	0.0195 $\pm$ 0.0015	(1.00)	0.8953 $\pm$ 0.1305	(1.00)
+0.375 μ M PCI29732	0.0183 $\pm$ 0.0015	(1.06)	0.7978 $\pm$ 0.0597	(1.12)
+0.75 μ M PCI29732	0.0181 $\pm$ 0.0011	(1.07)	0.7138 $\pm$ 0.0982	(1.25)
+1.5 μ M PCI29732	0.0182 $\pm$ 0.0014	(1.07)	0.6998 $\pm$ 0.0941	(1.28)
+10 μ M Verapamil	0.0185 $\pm$ 0.0011	(1.05)	0.0756 $\pm$ 0.0067**	(11.84)
Paclitaxel	0.0013 $\pm$ 0.0003	(1.00)	0.4600 $\pm$ 0.0553	(1.00)
+0.375 μ M PCI29732	0.0014 $\pm$ 0.0003	(0.92)	0.4332 $\pm$ 0.0204	(1.06)
+0.75 μ M PCI29732	0.0011 $\pm$ 0.0001	(1.14)	0.4129 $\pm$ 0.0153	(1.11)
+1.5 μ M PCI29732	0.0013 $\pm$ 0.0002	(0.97)	0.3784 $\pm$ 0.0378	(1.22)
+10 μ M Verapamil	0.0013 $\pm$ 0.0002	(1.00)	0.0363 $\pm$ 0.0036**	(12.68)
Cisplatin	0.4258 $\pm$ 0.1000	(1.00)	1.8861 $\pm$ 0.1557	(1.00)
+1.5 μ M PCI29732	0.3814 $\pm$ 0.0391	(1.12)	1.6653 $\pm$ 0.1324	(1.13)

Table 2. Effects of PCI29732 on reversing ABCG2-mediated multidrug resistance in transfected cells. Note: The cell viability was assessed by the MTT assay. The data are shown as the mean $\pm$ standard deviation (SD) of at least three independent experiments. The fold-reversal of MDR was calculated by dividing the IC_{50} value of the cells treated with the anticancer agents in the absence of PCI29732 by that obtained in the presence of PCI29732. * $P < 0.05$, ** $P < 0.01$ vs. control group in the absence of PCI 29732

Compounds	$IC_{50} \pm SD, \mu M$ (Fold reversal)			
	HEK293/pCDNA3.1		ABCG2-482-R2	
Topotecan	0.0389 $\pm$ 0.0032	(1.00)	0.3727 $\pm$ 0.0430	(1.00)
+0.75 μ M PCI29732	0.0400 $\pm$ 0.0057	(0.97)	0.2171 $\pm$ 0.0360*	(1.72)
+1.5 μ M PCI29732	0.0339 $\pm$ 0.0030	(1.15)	0.0915 $\pm$ 0.0111**	(4.07)
+3 μ M PCI29732	0.0307 $\pm$ 0.0031*	(1.27)	0.0531 $\pm$ 0.0105**	(7.02)
+2.5 μ M FTC	0.0355 $\pm$ 0.0029	(1.10)	0.0323 $\pm$ 0.0055**	(11.53)
Mitoxantrone	0.0176 $\pm$ 0.0042	(1.00)	0.3883 $\pm$ 0.0341	(1.00)
+0.75 μ M PCI29732	0.0173 $\pm$ 0.0023	(1.02)	0.1693 $\pm$ 0.0080**	(2.29)
+1.5 μ M PCI29732	0.0136 $\pm$ 0.0024	(1.29)	0.0812 $\pm$ 0.0024**	(4.78)
+3 μ M PCI29732	0.0130 $\pm$ 0.0019	(1.35)	0.0620 $\pm$ 0.0028**	(6.27)
+2.5 μ M FTC	0.0061 $\pm$ 0.0008**	(2.89)	0.0332 $\pm$ 0.0043**	(11.68)
Cisplatin	3.8256 $\pm$ 0.1479	(1.00)	1.4099 $\pm$ 0.1168	(1.00)
+3 μ M PCI29732	3.3171 $\pm$ 0.2826	(1.15)	1.2558 $\pm$ 0.0379	(1.12)

ABCG2-overexpressing cells to substrate anticancer drugs.

To further confirm the effects of PCI29732 in ABCG2-overexpressing cells, cells stably transfected with ABCG2 were generated. Previous studies have demonstrated that amino-acid 482 was decisive in determining the effectiveness of ABCG2 antagonists as well as in the transport of selected anticancer drugs [29]. Therefore, HEK293 cells were stably transfected with pcDNA3.1 vector carrying wild-type (482R2) and mutant (482T7) ABCG2. Cells transfected with the empty pcDNA3.1 vector was also generated as a control.

As shown in Table 2, HEK293/ABCG2-482-R2 and HEK293/ABCG2-482-T7 cells exhibited moderate levels of resistance to ABCG2 substrates (topotecan or mitoxantrone) compared with cells transfected with an empty pcDNA3.1 vector. PCI29732 increased the cytotoxicity of mitoxantrone and topotecan in both HEK293/ABCG2-482-R2 and HEK293/ABCG2-482-T7 cells, while the IC_{50} values of topotecan or mitoxantrone were not altered in HEK293/pcDNA3.1 cells in the presence or absence of PCI29732. These results further confirmed that PCI29732 selectively sensitized the cancer cells to ABCG2 substrate chemotherapeutic drugs.

Effects of PCI29732 on the efficacy of topotecan in H460/MX20 xenograft model

To examine whether PCI29732 can enhance the efficacy of topotecan *in vivo*, a xenograft mouse model was established using ABCG2-overexpressing H460/MX20 cell xenograft model was established in nude mice and used to examine whether PCI29732 could enhance the efficacy of topotecan *in vivo*. As shown in Fig. 2A and 2B, there were no significant changes in tumor sizes in the groups treated with topotecan or PCI29732 alone, compared with the control group. However, significant reductions in tumor weight and volume were observed in the group treated with PCI29732 in combination with topotecan ($P < 0.01$). The mean tumor weights were 1.07 ± 0.44 , 0.77 ± 0.32 , 0.80 ± 0.35 , and 0.51 ± 0.21 g for the saline control, topotecan, PCI29732, and topotecan and PCI29732 combination groups, respectively (Fig. 2C). The IR of the combination group was 52.53%. We did not observe any body weight loss in the combination group or drug-related deaths in this experiment (Fig. 2D). These results suggest that PCI29732 can enhance the efficacy of topotecan in cancer cells overexpressing ABCG2 overexpression *in vivo*.

Effects of PCI29732 on intracellular accumulation of doxorubicin and Rhodamine 123 in S1-MI-80 cells

Overcoming the ABC transporter-mediated efflux in drug-resistant cells is known to enhance tumor cytotoxicity [2]. To evaluate the potential mechanisms of PCI29732-mediated MDR reversal, we examined the accumulation of doxorubicin and Rhodamine 123 in S1 and S1-MI-80 cells using flow cytometry. The intracellular concentrations of doxorubicin

Fig. 2. Potentiation of the anticancer efficacy of topotecan by PCI29732 in the H460/MX20 cell xenograft nude mice model. Tumor volumes after the xenograft size reached a mean diameter of 0.5 cm on day 7. The data are shown as means $\pm$ SD ($n=6$) (A). Tumors were excised from the nude mice on the 25th day after cell implantation (B). The average tumor weight of each group was calculated after the tumors were excised from the nude mice on the 25th day after cell implantation (C). The data are shown as means $\pm$ SD ($n=6$). Body weight of the xenograft nude mice model were measured every 2 days, and the average percentage changes were calculated (D).

Fig. 3. Effects of PCI29732 on intracellular accumulation of doxorubicin and Rhodamine 123 in S1-MI-80 cells and its parental sensitive S1 cells. The intracellular accumulation of doxorubicin (A, C) and Rhodamine 123 (B, D) in S1 and S1-MI-80 cells as measured by flow cytometric analysis, respectively. The results are presented as fold-change in fluorescence intensity relative to control MDR cells (means $\pm$ SD of triplicate determinations). * $P < 0.05$, ** $P < 0.01$, vs. the control group.

and Rhodamine 123 in S1 cells were significantly higher than those in the S1-MI-80 cells untreated with PCI29732. The intracellular accumulation of doxorubicin increased by 1.78-, 3.79-, and 4.33-fold, and that of Rhodamine 123 increased by 2.27-, 5.43-, and 7.50-fold in a concentration-dependent manner, respectively (Fig. 3). Furthermore, PCI29732 did not increase the intracellular accumulation of doxorubicin and Rhodamine 123 in the parental sensitive S1 cells. These results indicate that PCI29732 significantly inhibits the efflux of chemotherapeutic drugs and increases the efficacy of these chemotherapeutic agents in ABCG2-overexpressing MDR cells.

Effects of PCI29732 on the efflux of Rhodamine 123

We next examined whether the increased accumulation of anticancer agents with PCI29732 was due to an inhibition of efflux of the drugs. The time course for Rhodamine 123 efflux during the 120 min after accumulation is shown in Fig. 4A. After 120 min, the level of Rhodamine 123 dropped to 45.47% of the initial level in the S1-MI-80 cells. However, the decrease in the level of Rhodamine 123 was significantly less in the parental S1 cells (about 68.09%). More importantly, the level of Rhodamine 123 dropped to 73.69% in S1-MI-80 cells in the presence of 3 μ M PCI29732. These results indicate that PCI29732 inhibits the efflux of Rhodamine 123 in the ABCG2-overexpressing S1-MI-80 cells.

Effects of PCI29732 on the ATPase activity of ABCG2

The substrates of ABC transporters can either stimulate ATPase activity or inhibit ATP hydrolysis [37]. ATP consumption has been generally used to reflect the ATPase activity of the transporters [38]. To assess whether PCI29732 influenced the ATPase activity of ABCG2, we measured the vanadate-sensitive ATPase activity of ABCG2 with various concentrations of PCI29732. We found that PCI29732 stimulated the ATPase activity of ABCG2 at low concentrations but inhibited the ABCG2 ATPase activity at higher concentrations (Fig. 4B), suggesting that PCI29732 may be a substrate of ABCG2.

Fig. 4. Effects of PCI29732 on efflux of Rhodamine 123, ABCG2 ATPase activity, and [^{125} I]-IAAP photoaffinity labeling of ABCG2. Time course of Rhodamine 123 efflux as measured in the S1 and S1-MI-80 cells treated with or without 3 μ M PCI29732 (A). Effects of PCI29732 on the ATPase activity of ABCG2 (B). The vanadate-sensitive ABCG2 ATPase activity was evaluated in the presence of the indicated concentrations of PCI29732. The data are shown as means and standard error values from three independent experiments. PCI29732 competed for photolabeling of ABCG2 by [^{125} I]-IAAP (C). The crude membranes from High Five^{TM} insect cells expressing ABCG2 were incubated with [^{125} I]-IAAP and various concentrations (0 – 10 μ M) of PCI29732. Samples were then cross-linked by UV illumination, subjected to electrophoresis, and analyzed. A representative autoradiogram from three independent experiments is shown. The relative amount of [^{125} I]-IAAP incorporated is plotted against the concentration of PCI29732. 100% incorporation refers to results obtained in the absence of PCI29732.

Fig. 5. Effects of PCI29732 on the expression of ABCG2 in S1-MI-80 cells. The S1-MI-80 cells were treated with various concentrations (0–3 μM) of PCI29732 for 48 h or with 1.5 μM of PCI29732 for 24, 48, or 72 h. mRNA expression was measured by RT-PCR, with GAPDH as a control (A); q-PCR was further applied to confirm the mRNA levels in the S1-MI-80 cells (B). The level of ABCG2 mRNA was normalized to GAPDH in the same sample. The protein level of ABCG2 was measured by western blot analysis, with GAPDH as a loading control (C).

Fig. 6. Effects of PCI29732 on total and phosphorylated levels of AKT and ERK1/2. S1-MI-80 cells were treated with various concentrations (0–3 μM) of PCI29732 for 48 h, or with 1.5 μM of PCI29732 for 24, 48, or 72 h. Equal amounts of protein were used for western blot analysis. Independent experiments were performed at least three times, and results from a representative experiment are shown.

Effects of PCI29732 on the photo-affinity labeling of ABCG2 with [¹²⁵I] IAAP

It has been reported that [¹²⁵I]-IAAP photolabeling of ABCG2 and its binding can be competitively inhibited by substrates or inhibitors of the respective transporters [39]. To determine whether PCI29732 interacts with the drug-binding pocket of ABCG2, the membrane vesicles of these transporters were incubated with [¹²⁵I]-IAAP in the absence or presence of various concentrations of PCI29732. As shown in Fig. 4C, PCI29732 inhibited the photo-affinity labeling of ABCB1 with [¹²⁵I]-IAAP in a concentration-dependent manner, demonstrating that PCI29732 interacts directly with the drug-binding pocket of ABCG2.

Examination of the effects of PCI29732 on ABCG2 protein and mRNA expression

The reversal of ABCG2-mediated MDR can usually be achieved either by decreasing transporter expression or by inhibiting transporter function [38]. Therefore, we determined the possible effects of PCI29732 on the expression of ABCG2 at the mRNA and protein level via RT-PCR, real-time PCR, and Western blot analyses. As shown in Fig. 5A and 5B, PCI29732 did not alter the ABCG2 mRNA level in either concentration-dependent or time-dependent assays. In parallel, at the protein level, we also found that ABCG2 expression was not affected with increasing concentrations of PCI29732 or incubation times (Fig. 5C). Therefore, these results suggest that PCI29732 reversed MDR by inhibiting the transport function of ABCG2, but not its expression.

Evaluation of the possible effects of PCI29732 on the blockage of AKT and ERK1/2 phosphorylation at the concentration of MDR reversal

Main pathways downstream of BTK involves in AKT and ERK phosphorylations which activated survival and proliferation pathways. Previous studies have shown that inhibition of the AKT and ERK1/2 pathways may increase the efficacy of chemotherapeutic agents in cancer cells [40, 41]. We therefore tested total and phosphorylated of AKT or ERK1/2 levels after treatment of S1-MI-80 cells with various concentrations of PCI29732. As shown in Fig. 6, PCI29732 did not significantly alter the levels of the total or phosphorylated forms of AKT or ERK1/2 in either concentration-dependent or time-dependent assays, suggesting that the PCI29732-induced MDR reversal in ABCG2-overexpressing S1-MI-80 cells occurs independently of the changes in AKT and ERK1/2 phosphorylation.

Discussion

In the current study, we provided *in vitro* and *in vivo* evidence supporting that PCI29732 can induce MDR reversal in ABCG2-overexpressing cells by inhibiting ABCG2 function to increase the intracellular accumulation of chemotherapeutic agents (Fig. 7), which is not associated with changes in the mRNA or protein expression of ABCG2 or blockage of the AKT and ERK1/2 pathways.

Overexpression of the ABC family membrane proteins is one of the most common reasons for MDR [42]. Indeed, ABC transporters can extrude a wide range of structurally and functionally diverse amphipathic anticancer drugs from cells. ABCB1, ABCG2, and ABCC1 are known as the most important ABC transporters associated with MDR in cancer cells [43]. ABCB1 preferentially extrudes hydrophobic molecules that are either uncharged or slightly positively charged, such as anthracyclines, vinca alkaloids, anthracyclines, epipodophyllotoxins, and taxanes [5]. Drugs transported by ABCG2 include anthracyclines, mitoxantrone, antifolates, and flavopiridol [10]. ABCC1 can transport a broad spectrum of substrate anticancer drugs mainly conjugated to glutathione, glucuronate and sulphate, including vincristine and doxorubicin [44]. Therefore, compounds that can fully or partly block ABC transporter activities may prevent the undesirable loss of intracellular substrate anticancer drugs and could be beneficial when used in combination chemotherapy.

Several recent studies have reported that TKIs have additional benefits in combination with cytotoxic anticancer drugs [45] and that TKIs such as lapatinib [46], gefitinib [47], and erlotinib [48] are also inhibitors of ABCB1 and ABCG2. BTK is an essential cytoplasmic component of the B-cell receptor signaling

Fig. 7. Schematic of PCI29732 function in drug-resistant cancer cells. PCI29732 could enhance the efficacy of chemotherapeutic drugs in drug ABCG2-overexpressing cancer cells, which is likely due to an increase in the intracellular accumulation and decrease in the efflux of ABCG2 substrate drugs.

pathway, which is an attractive target for cancer chemotherapy [20]. Ibrutinib is often used in combination with rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone in patients newly diagnosed with the non-germinal center B-cell subtype of diffuse large B-cell lymphoma [49]. Furthermore, Ibrutinib significantly increases the efficacy of the chemotherapeutic agents that are MRP1 substrates, in MRP1-overexpressing cells [23].

As a BKT inhibitor, PCI29732 has also been shown to reverse MRP1, although its effect is modest [23]. In the present study, we investigated the interactions between PCI29732 and ABCB1 or ABCG2 proteins by using various cell lines with normal or overexpression of the transporters, including S1, S1-MI-80, H460, H460/MX20, HEK293, HEK293/ABCG2-482-R2, HEK293/ABCG2-482-T7, KB, and KBv 200 cells. Our results indicated that PCI29732 increased the sensitivity of ABCG2-overexpressing cells to substrate cancer chemotherapeutic agents in a concentration-dependent manner. However, PCI29732 did not enhance the cytotoxicity of doxorubicin and paclitaxel in ABCB1-overexpressing cells, suggesting that PCI29732 did not have any reversal effects on ABCB1-mediated MDR. We therefore concluded that PCI29732 is a more specific inhibitor of ABCG2. Furthermore, we found that PCI29732 significantly increased the accumulation of doxorubicin and Rhodamine 123 in S1-MI-80 cells, but not in the sensitive parental S1 cells, which was consistent with the cytotoxicity results seen in these cells.

To further understand the underlying mechanism of ABCG2-mediated MDR reversal by PCI29732, we checked on its effects on the ATPase activity. PCI29732 stimulated the ATPase activity of ABCG2 at low concentrations but inhibited the ABCG2 ATPase activity at higher concentrations. These results were further confirmed by observations that PCI29732 competed with [¹²⁵I]-IAAP for the photo-affinity labeling of ABCG2.

The reversal of ABCG2-mediated MDR can usually be achieved either by decreasing transporter expression or by inhibiting its function. In the present study, we found that PCI29732 did not reduce ABCG2 expression at the mRNA or protein levels, in either concentration-dependent or time-dependent assays. Of note, previous studies reported that the blockage of phosphorylation in the PI3K/AKT and/or ERK pathways could enhance the efficacy of chemotherapeutic agents [50-52]. In the present study, we found that PCI29732 did not alter the expression or phosphorylation levels of AKT and ERK1/2, demonstrating that the reversal of MDR by PCI29732 is most likely not associated with inhibition of the AKT and ERK pathways.

Conclusion

The present study demonstrates that PCI29732 enhances the efficacy of chemotherapeutic drugs in ABCG2-overexpressing MDR cells through the inhibition of ABCG2 function, which suggests that combination therapy using PCI29732 and conventional chemotherapeutic drugs may benefit to the clinical management of drug resistance that are mediated by the ABCG2 transporter. These results may significantly enhance the efficacy of several clinically used chemotherapeutic agents.

Abbreviations

MDR (multidrug resistance); ABCB1 (the ABC transporter subfamily B member 1); ABCC1 (the ABC transporter subfamily C member 1); ABCG2 (the ABC transporter subfamily G member 2); MXR (mitoxantrone resistance-associated protein); CSCs (cancer stem cells); TKIs (tyrosine kinase inhibitors); BTK (bruton's tyrosine kinase); BCR (B-cell antigen receptor); FDA (the United States Food and Drug Administration); MCL (mantle cell lymphoma); Itk (interleukin-2 inducible T cell kinase); MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide).

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Disclosure Statement

The authors declare that they have no competing interests.

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