Paliperidone, a relatively novel atypical antipsychotic drug, is a substrate for breast cancer resistance protein

YANGANG ZHOU[1,2], HUANDE LI[1,2], PING XU[1,2], LI SUN[1-3], QING WANG[1,2], QIONG LU[1,2], HAIYAN YUAN[1,2] and YIPING LIU[1,2]

[1]Department of Pharmacy, The Second Xiangya Hospital, Central South University; [2]Institute of Clinical Pharmacy, Central South University, Changsha, Hunan 410011; [3]Department of Pharmacy, The Maternal and Child Health Hospital of Hunan Province, Changsha, Hunan 410008, P.R. China

Received December 22, 2017; Accepted June 22, 2018

DOI: 10.3892/etm.2018.6847

Abstract. Paliperidone (PAL) is a relatively novel atypical antipsychotic drug for schizophrenia that induces markedly varying responses. Breast cancer resistance protein (BCRP) is a recently discovered member of the ATP-binding cassette superfamily that has been used to control drug absorption, distribution and elimination, and especially to impede drug entry into the brain. To the best of our knowledge, the present study is the first to investigate the possibility of using PAL as a BCRP substrate. The intracellular accumulation and bidirectional transport were investigated using transfected 293 cell/BCRP and porcine renal endothelial cell (LLC-PK1)/BCRP cell monolayers and BCRP overexpression was confirmed by reverse transcription-quantitative polymerase chain reaction and western blot analysis. The *in vitro* affinity to BCRP was assessed in human BCRP (Arg482) membranes. The intracellular accumulation and bidirectional transport investigations demonstrated that BCRP can efflux PAL from cells and significantly decrease its cellular concentration over a concentration range of 0.1-50 μM. The *in vitro* affinity experiments indicated that PAL has a moderate affinity to BCRP at 0.1-100 μM. These results together suggest that PAL is a substrate for BCRP and that it can affect the blood-brain barrier penetration of PAL at therapeutic dosages.

Introduction

Schizophrenia is a serious psychotic disease which leads to both physical and psychotic symptoms (1). Treatments for schizophrenia cost US$ 94 million-102 billion worldwide annually (2), with atypical antipsychotic drugs, including olanzapine, risperidone and aripiprazole, representing the most commonly administered medicines due to their abilities to control positive syndromes, including hallucination and delusion, and negative syndromes, including emotional blunting, emotional withdrawal and emotional exchange disorder, of schizophrenia (3). In addition, these drugs reduce side effects, including extrapyramidal reactions caused by typical antipsychotic drugs. (3). However, the responses to these drugs vary markedly between patients, which makes it difficult for doctors to predict drug effects (4,5).

Paliperidone (PAL) is the metabolite of risperidone and is a relatively novel atypical antipsychotic drug that is effective in improving the positive and negative syndrome of schizophrenia, and can also improve the associated cognitive impairment (6,7). Similarly to other atypical antipsychotics, the responses to PAL vary widely; the effective PAL daily dose typically varies from 3-15 mg, and 13-26% patients reportedly exhibit side effects, including dyskinesia, in the extrapyramidal system (8,9). There is therefore a need to identify the mechanisms underlying the differences in individual responses to PAL treatment.

A number of factors can affect the outcome of antipsychotic medication, with one of the most important being the drug penetration into the brain. Atypical antipsychotic drugs have to cross the blood-brain barrier (BBB) prior to exerting their effects, with their concentration within the brain being affected by the drug molecular weight and lipophilicity, and the presence of transport proteins in the BBB (10). There are a number of efflux proteins in the BBB, such as P-glycoprotein (P-gp), multidrug resistance-associated protein (MRP), breast cancer resistance protein (BCRP), organic anion-transporting polypeptides (OATs), and organic anion transporting polypeptides (OATPs) (11,12). As PAL exists as a positive ion, MRP, OATs and OATPs, which mainly efflux negative ions, might not influence its penetration into the brain (13). Both *in vitro* and *in vivo* studies have demonstrated that P-gp may impede the penetration of PAL into the brain, with the ATP binding cassette (ABC) subfamily B member 1 genetic polymorphism possibly influencing the plasma concentration of PAL (14,15). BCRP is a member of the ABC superfamily that has been associated with the phenomenon of multidrug resistance (16). Wang *et al* (17) identified that PAL can inhibit the function of

Correspondence to: Dr Ping Xu, Department of Pharmacy, The Second Xiangya Hospital, Central South University, 139 Middle Renmin Road, Changsha, Hunan 410011, P.R. China
E-mail: xuping1109@163.com

Key words: paliperidone, breast cancer resistance protein, LLC-PK1/BCRP, substrate, affinity

BCRP *in vitro*, but the efflux effect of BCRP on PAL has not yet been reported.

In the present study the affinity of PAL with BCRP was investigated *in vitro*, and the uptake and transport of PAL in 293 and 293/BCRP cells, porcine renal endothelial cell (LLC-PK1) and LLC-PK1/BCRP cell monolayers was also investigated.

Materials and methods

Materials. Human BCRP (Arg482) membranes (5 mg/ml) and a control membrane preparation for ABC transporters were purchased from Gentest; Corning Incorporated (Corning, NY, USA). Anti-BCRP mouse monoclonal antibody (clone BXP21; cat. no. MC-236) and horseradish peroxidase-labeled goat anti-mouse secondary antibody (cat. no. 5220-0341) were purchased from Kamiya Biomedical Company (Tukwila, WA, USA) and SeraCare Life Sciences (Milford, MA, USA), respectively. β-Actin mouse monoclonal antibody (cat. no. AF0003) was purchased from Beyotime Institute of Biotechnology (Shanghai, China). PAL, Ko143, sulfasalazine, and dimethyl sulfoxide were purchased from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany). Fetal bovine serum (FBS), trypsin, Dulbecco's modified Eagle's medium (DMEM), Ham's F12 nutrient (F12) medium, PBS and TRIzol reagent were obtained from Gibco; Thermo Fisher Scientific, Inc. (Waltham, MA, USA). Microplates (with 6 and 96 wells) and cell culture flasks were obtained from Corning Costar; Merck KGaA. Methanol of high-performance liquid chromatography (HPLC) grade was obtained from Merck KGaA and the other solvents used were of analytical grade. The lentiviral vector encoding the BCRP gene was purchased from Shanghai GeneChem Co., Ltd. (Shanghai, China).

Cell culture and transfection. The porcine renal endothelial cell line LLC-PK1 and LLC-PK1/BCRP cells were provided by Professor Zeng Su from the College of Pharmaceutical Sciences, Zhejiang University (Hangzhou, China). LLC-PK1/BCRP cells are transgenic cells that were established by using liposome as vehicles to import human BCRP genes into LLC-PK1 cells (18). Both types of cell were cultured in F12 medium supplemented with 20% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37˚C in an atmosphere of 5% CO_2 and 95% relative humidity. Cells were supplemented with fresh media every 2-3 days. All of the cultured cells used in subsequent experiments were at passages 5-25.

In addition, 293 cells were seeded in 6-well plates at a density of $1x10^4$ cells/well and were cultured in DMEM supplemented with 20% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37˚C in an atmosphere of 5% CO_2 and 95% relative humidity. Cells were exposed to lentiviral vector encoding the BCRP gene with a green fluorescence protein (GFP)-tag at a multiplicity of infection of 50 in the presence of 8 μg/ml polybrene (Sigma-Aldrich; Merck KGaA) added to enhance the transduction efficiency. The transfection medium was replaced with DMEM after 24 h. Cells were observed under a fluorescence microscope at 4 days following transfection, at which time >90% of the cells were found to be positive for green fluorescence protein-tag fluorescence. Both 293 cells and BCRP-transfected 293 (293/BCRP) cells were then cultured in DMEM supplemented with 20% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37˚C in an atmosphere of 5% CO_2 and 95% relative humidity. Cells were supplemented with fresh media every 2-3 days. All of the cultured cells used in the subsequent experiments were at passages 2-5.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analyses. RT-qPCR was used to detect gene expression levels in 293 cells and lentivirus-BCRP-transduced 293 cells at 10 and 20 days following transduction. Total RNA was isolated from cells using TRIzol reagent (Life Technologies; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. RNA samples (0.5 μg) were used for RT using the Prime Script™ 174 RT reagent kit (Takara, Biotechnology, Dalian, China) according to the manufacturer's instructions. SYBR Green Realtime PCR Master mix (Qiagen, Inc., Valencia, CA, USA) was used to detect the expression level of ABC subfamily G member 2 (*ABCG2)*, with a 20 μl reaction mixture containing 2 μl synthesized cDNA, 10 μl SYBR Premix Ex Taq, 0.2 μl each primer and 7.6 μl ddH_2O. The cycling conditions were as follows: Initial denaturation at 95˚C for 30 sec followed by 36 cycles of 95˚C for 5 sec and 60˚C for 30 sec. The primers used for human *ABCG2* were forward, 5'-GTTGTGATGGGCACTCTGAC-3' and reverse), 5'-CCCTGTTAATCCGTTCGTTT-3'; and for β-actin forward, 5'-CTCTTCCAGCCTTCCTTCCT-3' and reverse), 5'-AGCACTGTGTTGGCGTACAG-3' were used for human β-actin (GenBank accession no. NM 01101). Each sample was analyzed in triplicate. Endogenous β-actin mRNA was used to determine the relative quantitative expression using the $2^{-\Delta\Delta Cq}$ method (19).

Western blotting. 293 and lentivirus-BCRP-transduced 293 cells were cultured in 6-well plates for harvesting the cells and the cell monolayer was lysed at 4˚C for 30 min in radioimmunoprecipitation assay buffer with protease inhibitors (Sigma-Aldrich; Merck KGaA) and the amount of protein was determined by bicinchoninic acid assay. Proteins (60 μg) were separated on 12% SDS-PAGE gels and transferred onto a polyvinylidene difluoride membrane (EMD Millipore, Billerica, MA, USA). The membrane was blocked in Dulbecco's PBS containing 0.1% Tween-20 and 5% non-fat dry milk (Bio-Rad Laboratories, Inc., Hercules, CA, USA) for 2 h at room temperature. The PVDF membranes were then washed three times for 10 min with tris-buffered saline with Tween-20 (TBST) buffer and incubated overnight at room temperature with a mouse monoclonal antibody (1:1,000) against human BCRP and β-actin. Following extensive washing, the membranes were further incubated with secondary antibody (1:5,000) for 1 h at room temperature. The membranes were then washed three times with TBST and immunoreactivity was visualized using an enhanced chemiluminescence kit and the membranes were exposed to photographic film (Kodak, Rochester, NY, USA). Protein levels were expressed as the ratio of the band intensities of BCRP to the endogenous control β-actin. Each sample was analyzed in triplicate.

Cellular accumulation. Intracellular accumulation experiments were conducted with 293 and 293/BCRP cells. Cells

were cultured in 6-well plates at a density of $2x10^5$ cells/well for 2 days and on the day of the experiment the culture medium was replaced with DMEM with, containing PAL at varying concentrations (0.1, 1, 10, 25 and 50 μM) with or without BCRP inhibitor Ko143 (5 μM). Following incubation for 2 h in a humidified atmosphere of 5% CO_2 at 37˚C, the cells were washed three times with PBS and then were lysed by repeated freezing and thawing. The lysed cells were centrifuged at 16,770 x g at 4˚C for 5 min and 150 μl liquid supernatant was collected for PAL detection by high performance liquid chromatography/mass spectrometry (HPLC/MS), whereas 20-μl samples were taken for bicinchoninic acid assays to determine the protein concentration. Data are presented as the mean ± standard deviation (n=3) following normalization to the total protein concentration in each well.

Bidirectional transport experiments. For the bidirectional transport experiments, LLC-PK1 and LLC-PK1/BCRP cells were seeded into Transwell inserts in 6-well plates at a density of $2x10^5$ cells/well. The cells were then cultured in F12 medium supplemented with 20% FBS, at 37˚C for 7 days, at which time the integrity of the monolayer was evaluated by measuring the transepithelial electrical resistance (TEER) as described previously (20) across the monolayer (EVOM2; World Precision Instruments, Inc., Sarasota, FL, USA). Monolayers with TEER values exceeding 200 Ω/cm^2 were chosen for use in the transport experiments.

On the day of transport experiments, the culture medium was replaced with F12 medium containing PAL at varying concentrations (0.1, 1, 10, 25 and 50 μM) on the upper or lower side and with blank Hank's balanced salt solution (HBSS; Thermo Fisher Scientific, Inc.) on the other side. Cells were incubated at 37˚C. Six sequential samples (0.2 ml) were collected at different times (0, 0.5, 1, 2, 3 and 4 h) from both sides of the cell monolayer. The same volume of testing solution or blank HBSS was immediately added to replace the samples obtained. Transport experiments were also performed in the presence of the BCRP inhibitor (5 μM Ko143) loaded on the same side of the monolayers with PAL.

The permeability of PAL was estimated by calculating the apparent permeability coefficient (Papp) as follows: $Papp=dQ/dtxAxC_0$, where C_0 is the concentration of PAL added to the upper or lower side, A is the surface area of the monolayer (4.67 cm^2 in the present study) and dQ/dt is the rate at which the drug(s) appeared on the opposite side.

The efflux ratio was calculated as: $Efflux\ ratio=Papp_{(BL\rightarrow AP)}/Papp_{(AP\rightarrow BL)}$, where apical (AP) and basal (BL) are the two sides of the compartment, with the indicated transport direction. Data are presented as the mean ± standard deviation (n=3).

HPLC/MS analysis. PAL concentrations following cellular accumulation and bidirectional transport experiments were determined using HPLC/MS. An 80-μl aliquot obtained from the transport investigation and 20 μl internal standard moclobemide (100 ng/ml; Sigma-Aldrich; Merck KGaA) in the mobile phase were mixed on a high-speed vortex for 30 sec. Methyl tert-butyl ether (400 μl) was added to the mixture and vortexed for 5 min. Following centrifugation at 22,160 x g for 10 min at 4˚C, the supernatant (300 μl) was transferred to an 1.5-ml Eppendorf tube and solvent was evaporated to dryness for 40 min at 37˚C using a vacuum drying system (RVT4104-230; Thermo Fisher Scientific, Inc.). The dry residue was reconstituted in 80 μl mobile phase and mixed on a vortex for 5 min. The mixture was then centrifuged at 22,160 x g for 5 min at 4˚C, and a 5-μl aliquot of the supernatant was injected into an HPLC-MS system for analysis. The calibration standards ranged from 2-5,000 ng/ml.

PAL concentration was measured using an HPLC system (2690; Waters Corporation, Milford, MA, USA) and a mass spectrometer (Micromass; Waters Corporation) equipped with an electrospray ionization (ESI) ion source. Separation was performed on an Ultimate XB-C18 column (3 μm; 2.1x 100 mm; Welch Materials, Inc., Shanghai, China). The mobile phase was water (25 mM ammonium acetate, 0.06% ammonium) and acetonitrile (68:32) at a flow rate of 0.25 ml/min. The total run time was 3 min and the column and autosampler were maintained at 40 and 25˚C, respectively.

The compounds were ionized in the positive ESI ion source mode of the mass spectrometer. The detection conditions were a capillary voltage of 3.00 kV, a cone voltage of 22.00 V, an extractor voltage of 2.00 V, a source temperature of 120˚C, a desolvation temperature of 350˚C and cone and desolvation gas flows of 50 and 500 l/h, respectively. The selected ion recording mode was used for quantification, with m/z=427.5 for PAL and m/z=269.5 for moclobemide.

In vitro BCRP affinity for PAL. The *in vitro* BCRP affinity for PAL was investigated as described by Boulton *et al* (21) with residue R482/BCRP membranes analyzed for both basal and drug-stimulated ATP hydrolysis by colorimetric detection of inorganic phosphate release. The reaction was conducted in 96-well plates, and initiated by adding 20 μl Mg-ATP (12 mM) solution (heated to 37˚C) and 20 μl (heated to 37˚C for 5 min) BCRP membrane (2 mg/ml) to the reaction system (20 μl), which contained 100 μM sodium orthovanadate and PAL or sulfasalazine (a substrate for ABCG2) at varying concentrations (0.1, 1, 10, 25, 50 and 100 μM). All of the reagents were made up in Tris and 2-(N-morpholino) ethanesulfonic acid buffer (pH 6.8). Following 60 min of incubation at 37˚C, the reaction was terminated by the addition of 30 ml ice-cold 10% sodium dodecylsulfate plus 0.1% antifoam A. The phosphate concentration (Pi) was assayed by adding 200 μl 35 mM ammonium molybdate in 15 mM zinc acetate: 10% ascorbic acid (pH 5.0, freshly prepared) at a ratio of 1:4 to each well and the mixture was incubated for 20 min at 37˚C. The liberation of Pi was detected by the ultraviolet absorption of the Pi-molybdate complex at 620 nm by a spectrophotometer and the Pi concentration was calculated from the standard curve. Each sample was analyzed in triplicate. Data were plotted as drug concentration vs. Pi release and analyzed by least-squares non-linear regression fits using the Michaelis-Menten equation. The maximal reaction velocity (V_{max}) and the substrate concentration at 50% of V_{max} (K_m) were determined using GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA, USA).

Statistical analyses. Statistical analyses were performed with SPSS software (version 17.0; SPSS, Inc., Chicago, IL, USA). The effect of BCRP on PAL accumulation in 293 and 293/BCRP cells was analyzed with one-way analysis of variance, using Fisher's least significant difference test for

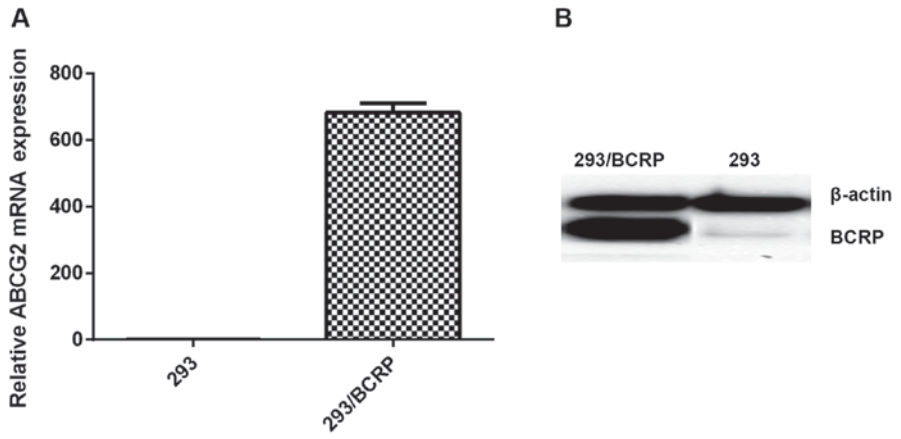

Figure 1. ABCG2 gene expression in 293 and 293/BCRP cells as analyzed by (A) reverse transcription-quantitative polymerase chain reaction and (B) western blotting. ABCG2, ATP-binding cassette subfamily G member 2; BCRP, breast cancer resistance protein. Data are presented as the mean ± standard deviation (n=3).

post hoc comparisons. P<0.05 was considered to indicate a statistically significant difference.

Results

Expression of BCRP in 293/BCRP cells. The results from the RT-qPCR and western blotting assays indicated high expression levels of BCRP mRNA and protein in 293 cells transfected with the BCRP gene, whereas no notable BCRP was detected in the 293 cells (Fig. 1).

Cellular accumulation. The cellular accumulation of PAL in both 293 and 293/BCRP cells increased markedly with the drug concentration. However, the rate of increase was markedly lower in 293/BCRP cells than in 293 cells (Fig. 2), indicating that the cellular bioavailability of PAL was attenuated by BCRP. Conversely, combination treatment with 5 μM Ko143 significantly increased the PAL accumulation in 293/BCRP cells but had no influence on its accumulation in 293 cells. These results suggest that PAL can be effluxed by BCRP.

Bidirectional transport. Table I lists the permeability parameters of the transcellular transport of PAL. The transport of PAL was asymmetric, with the transport rates being distinctly lower in the AP→BL direction than in the BL→AP direction. Transfection of the BCRP gene further reduced the transport rates in the AP→BL direction, while it increased the rates in the BL→AP direction. The net efflux ratios of PAL in were >1.7 for concentrations from 0.1 to 25 μM, which indicated that PAL may be a substrate of BCRP. The net efflux ratio of PAL decreased as the concentration increased to 50 μM, for which the net efflux ratio was 1.22, suggesting that the BCRP in PAL transport had saturated.

The addition of the BCRP inhibitor Ko143 (5 μM) increased transport rates in the AP→BL direction but decreased those in the BL→AP direction were observed in LLC-PK1/BCRP cells and the net ratios of PAL were markedly decreased. These results further support that PAL is a substrate of BCRP.

In vitro BCRP affinity for PAL. It is known that ABC drug transporters utilize the energy of ATP hydrolysis to transport drugs outside cells (11,21). As presented in Fig. 3, both

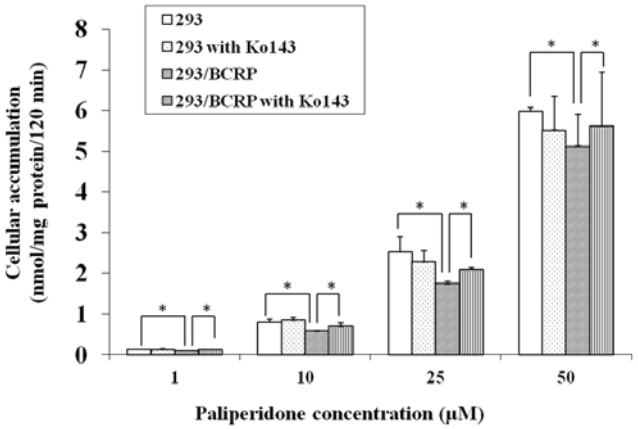

Figure 2. Cellular accumulation of paliperidone in 293 and 293/BCRP cells. Data are presented as the mean + standard deviation (n=3). *P<0.05. BCRP, breast cancer resistance protein.

sulfasalazine and PAL stimulated vanadate-sensitive BCRP ATPase activity in a concentration-dependent manner, with the degree of stimulation being less for PAL than for sulfasalazine at all concentrations. The concentrations of sulfasalazine and PAL required for half-maximal stimulation (i.e., EC_{50} values) of the vanadate-sensitive ATPase activity were 25.09±4.96 and 31.68±9.60 μM, respectively, and the V_{max} values were 108.5 and 98.01 nM/min; the V_{max}/K_m values of the two compounds were therefore 4.32×10^{-3} and 3.09×10^{-3} min^{-1}.

Discussion

PAL is a relatively novel atypical antipsychotic drug that has been demonstrated to be effective in reducing the symptoms of schizophrenia and in improving personal and social functioning in both short- and long-term studies (6). However, like other atypical antipsychotic drugs, there are significant individual differences in the treatment efficacy and adverse effects of PAL (8). A number of studies have demonstrated that ABC transporters are associated with the efflux of an antipsychotic, which may influence the drug absorption and transport *in vivo*, and hence may be associated with the individual differences in clinical treatment responses (22,23). The present

Table I. Permeability of PAL across LLC-PK1 and LLC-PK1/BCRP cell monolayers.

A, Without Ko143

	LLC-PK1/BCRP			LLC-PK1			
PAL (μM)	Pab ($x10^{-6}$ cm/sec)	Pbax (10^{-6} cm/sec)	ER	Pab ($x10^{-6}$ cm/sec)	Pba ($x10^{-6}$ cm/sec)	ER	Net ER
0.1	3.38±0.27	17.61±1.24	5.21	6.75±0.60	9.97±0.71	1.47	3.53
1	3.68±0.10	17.72±0.72	4.80	7.07±0.58	13.02±0.85	1.84	2.61
10	8.24±0.60	33.38±0.54	4.05	11.34±0.44	21.68±0.75	1.91	2.12
25	8.69±0.77	35.29±3.51	4.06	10.14±0.72	24.13±1.32	2.38	1.71
50	11.66±1.51	29.81±1.40	2.56	10.80±0.54	22.55±0.97	2.09	1.22

B, With Ko143 (5 μM)

	LLC-PK1/BCRP			LLC-PK1			
PAL (μM)	$Papp_{(BL\rightarrow AP)}$ ($x10^{-6}$ cm/sec)	$Papp_{(BL\rightarrow AP)}$ ($x10^{-6}$ cm/sec)	ER	$Papp_{(BL\rightarrow AP)}$ ($x10^{-6}$ cm/sec)	$Papp_{(BL\rightarrow AP)}$ ($x10^{-6}$ cm/sec)	ER	Net ER
0.1	5.74±0.37	9.30±0.92	1.62	7.15±0.72	12.21±0.70	1.71	0.95
1	7.32±0.57	17.19±0.68	2.35	7.12±0.73	14.07±0.52	1.98	1.19
10	11.84±0.80	21.21±0.68	1.79	11.09±0.89	23.11±0.54	2.08	0.85
25	13.89±0.87	25.98±0.81	1.87	11.46±1.13	25.31±1.26	2.21	0.85
50	15.14±0.39	23.99±1.33	1.58	13.16±0.64	20.75±1.35	1.57	1.01

Data are presented as the mean ± standard deviation (n=3). PAL, paliperidone; BCRP, breast cancer resistance protein; AP, apical; BL, basal; $Papp_{(AP\rightarrow BL)}$, PAL permeability in the AP to BL direction; $Papp_{(BL\rightarrow AP)}$, PAL permeability in the BL to AP direction; ER, efflux ratio for bidirectional transportation.

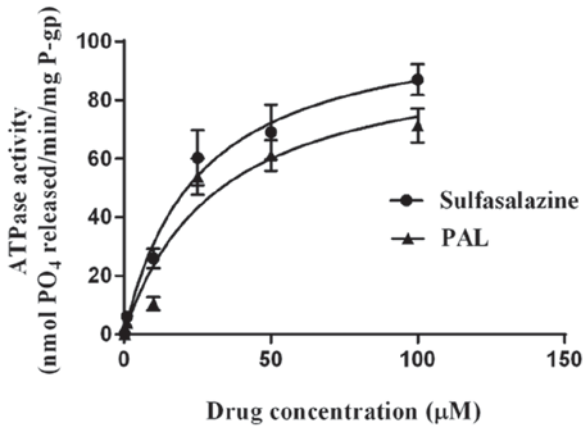

Figure 3. Concentration dependency of orthovanadate-sensitive substrate-induced breast cancer resistance protein ATPase activity as measured by inorganic phosphate release by PAL and sulfasalazine. Data are presented as the mean ± standard deviation (n=3). Pal, paliperidone; P-gp, P-glycoprotein.

study is, to the best of our knowledge, the first to use three independent methods to investigate the potency of PAL transport by BCRP.

The cellular accumulation experiments performed in the present study were based on BCRP being able to pump its substrates out of cells so as to decrease their intracellular concentrations (11). The results demonstrated that the concentration of PAL was significantly lower in 293/BCRP cells than in 293 cells, and that this difference could be ameliorated by the addition of 5 μM Ko143. These observations indicate that BCRP can export PAL and reduce its intracellular concentration. It was also demonstrated that the difference in the intracellular accumulation between wild-type (293) and 293/BCRP cells decreased for 50 μM PAL concentrations compared with 25 μM PAL, which may be due to saturation of the transporter.

LLC-PK1/BCRP transgenic cells were used to explore the association of BCRP with the transport of PAL, as this cell line exhibits high BCRP protein expression and strongly maintains the characteristics of the parent LLC-PK1 cell (11). The results demonstrated that for concentrations ranging from 0.1-50 μM, the bidirectional transport of PAL was highly polarized in LLC-PK1/BCRP cells, and could be eliminated by the BCRP inhibitor Ko143, with this not being observed in the LLC-PK1 cells. These results were highly consistent with those from the PAL accumulation experiments, with both sets of results indicating that PAL can be effluxed by BCRP.

The LLC-PK1 cells were obtained from pig kidney cells to form a polarity monolayer spontaneously in the cultivation condition, which differentiates the characteristics of microvilli and tight junctions so as to simulate a membrane barrier *in vivo* (18,24). When transfected with BCRP, the protein was

located on the apical side of the cells, which is similar to the findings for BCRP under physiological conditions (25). This indicates that the results from *in vitro* studies may predict the efflux effect of BCRP on PAL in the BBB.

ATPase assays were used to investigate the direct interaction between PAL and BCRP, and they confirmed that PAL may have a moderate affinity for BCRP (with $V_m/K_m=3.09x10^3$ min^{-1}). Three classes of drug-stimulated ATPase activity in ABC transporters have been proposed previously (26,27): Class I compounds, which stimulate ATPase activity at low concentrations but inhibit the activity at high concentrations predominate; class II compounds, which exhibit concentration-dependent ATPase activity with no inhibition at high concentrations; and class III compounds, which inhibit both basal and class I or II ATPase activity. Wang *et al* (15) previously reported that PAL could inhibit the function of BCRP with a concentration where the response is reduced by half of 51 μM; combined with the present results, this indicates that PAL acts as class I compounds, being substrates at lower concentrations and competitive inhibitors at higher concentrations.

It has been reported that residue 482 of BCRP may be the site of drug interaction in human cell lines, and that mutation of the ABCG2 gene in exon 5 leads to the substitution of Arg482 for a threonine (R482T) or a glycine (R482G) (28). The affinity of BCRP to several substrates has also been demonstrated to be altered in the wild-type and mutant cell lines (29,30). Cells having a threonine or glycine at position 482 were able to efflux rhodamine 123, whereas cells having an arginine at that position were not (29). The findings of cross-resistance studies suggest that cells carrying an R482T mutation exhibit higher anthracycline resistance, whereas an R482G mutation seems to confer lower resistances to SN-38 and topotecan along with a higher affinity to etoposide (30).

The Arg482 BCRP membrane was used in the ATPase affinity assay, and the ABCG2 gene was transfected to both 293 and LLC-PK1. Therefore, the present results only demonstrated the substrate affinity of wild-type BCRP to PAL, and so the affinity of PAL to the mutant $ABCG_2$ gene may require further studies that may elucidate the possible genetic reason for the individual variations in the responses to PAL.

Pharmacokinetics studies have demonstrated that a PAL dosage of 6 mg/day produces a plasma concentration of ~0.1 μM (13,31). A previous *in vitro* study demonstrated that BCRP may significantly efflux PAL at that concentration, and then may block it through the BBB. It has also been demonstrated that PAL is the substrate of P-gp, and that the 3435C>T SNP impacts the pharmacokinetics of PAL (14). These two proteins may therefore act together in effluxing PAL out of the brain, and this may be the reason for the significantly higher concentration of risperidone, which is a substrate of P-gp but not of BCRP, compared with PAL, considering the similarity of their structures (31).

Multiple psychotropic drugs are reportedly combined in approximately 50% of patients, and especially in treatment-resistance cases (32). Furthermore, a number of antipsychotics have been demonstrated to interact with BCRP (16,17), and so when used together with other drugs they may inhibit the function of BCRP, thereby increasing the plasma and brain concentrations of PAL and eventually leading to better efficacy or serious adverse reactions.

In conclusion, the novel atypical antipsychotic drug PAL is a substrate of BCRP that may have moderate affinity for BCRP at clinical dosages, and its penetration through the BBB may be influenced by BCRP. These mechanisms may provide a clue for explaining the significant individual differences observed clinically in the treatment efficacy and adverse effects of PAL.

Acknowledgements

The present authors would like to thank Professor Zeng Su for providing the LLC-PK1 and LLC-PK1/BCRP cells used in the present experiments.

Funding

The present study was supported by the National Natural Science Foundation of China (grant no. 81101001).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YZ performed cellular accumulation experiments and prepared the manuscript. HL was involved in the study design and data analysis. PX and LS performed cell culturing and transfection experiments. QW and QL performed polymerase chain reaction and western blot analysis. HY and YL performed the bidirectional transports experiment and *in vitro* BCRP affinity assays. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Conflict of interest

The authors declare that they have no conflicts of interest.

References

1. Tandon R, Belmaker RH, Gattaz WF, Lopez-Ibor JJ Jr, Okasha A, Singh B, Stein DJ, Olie JP,Fleischhacker WW and Moeller HJ; Section of Pharmacopsychiatry, World Psychiatric Association: World Psychiatric Association Pharmacopsychiatry Section statement on comparative effectiveness of antipsychotics in the treatment of schizophrenia. Schizophr Res 100: 20-38, 2008.
2. Chong HY, Teoh SL, Wu DB, Kotirum S, Chiou CF and Chaiyakunapruk N: Global economic burden of schizophrenia: A systematic review. Neuropsychiatr Dis Treat 12: 357-373, 2016.
3. Samara MT, Dold M, Gianatsi M, Nikolakopoulou A, Helfer B, Salanti G and Leucht S: Efficacy, acceptability, and tolerability of antipsychotics in treatment-resistant schizophrenia: A network meta-analysis. JAMA Psychiatry 73: 199-210, 2016.
4. Owen MJ, Sawa A and Mortensen PB: Schizophrenia. Lancet 388: 86-97, 2016.

5. Suzuki T, Remington G, Mulsant BH, Rajji TK, Uchida H, Graff-Guerrero A and Mamo DC: Treatment resistant schizophrenia and response to antipsychotics: A review. Schizophr Res 133: 54-62, 2011.
6. Chwieduk CM and Keating GM: Paliperidone extended release: A review of its use in the management of schizophrenia. Drugs 70: 1295-1317, 2010.
7. Wang SM, Han C, Lee SJ, Patkar AA, Pae CU and Fleischhacker WW: Paliperidone: A review of clinical trial data and clinical implications. Clin Drug Investig 32: 497-512, 2012.
8. Nussbaum AM and Stroup TS: Paliperidone for treatment of schizophrenia. Schizophr Bull 34: 419-422, 2008.
9. Spina E and Cavallaro R: The pharmacology and safety of paliperidone extended-release in the treatment of schizophrenia. Expert Opin Drug Saf 6: 651-662, 2007.
10. Breedvel P, Benjnen JH and Schellens JH: Use of P-glycoprotein and BCRP inhibitors to improve oral bioavailability and CNS penetration of anticancer drugs. Trends Pharmacol Sci 27: 17-24, 2006.
11. Ueno M, Nakagawa T, Wu B, Onodera M, Huang CL, Kusaka T, Araki N and Sakamoto H: Transporters in the brain endothelial barrier. Curr Med Chem 17: 1125-1138, 2010.
12. Begley DJ: ABC transporters and the blood-brain barrier. Curr Pharm Des 10: 1295-1312, 2004.
13. Vermeir M, Naessens I, Remmerie B, Mannens G, Hendrickx J, Sterkens P, Talluri K, Boom S, Eerdekens M, van Osselaer N and Cleton A: Absorption, metabolism, and excretion of paliperidone, a new monoaminergic antagonist, in humans. Drug Metab Dispos 36: 769-779, 2008.
14. Suzuki Y, Tsuneyama N, Fukui N, Sugai T, Watanabe J, Ono S, Saito M and Someya T: Impact of the ABCB1 gene polymorphism on plasma 9-hydroxyrisperidone and active moiety levels in Japanese patients with schizophrenia. J Clin Psychopharmacol 33: 411-414, 2013.
15. Wang JS, Ruan Y, Taylor RM, Donovan JL, Markowitz JS and DeVane CL: The brain entry of risperidone and 9-hydroxyrisperidone is greatly limited by P-glycoprotein. Int J Neuropsychopharmacol 7: 415-419, 2004.
16. Jani M, Ámbrus C, Magnan R, Jakab KT, Beéry E, Zolnerciks JK and Krajcsi P: Structure and function of BCRP, a broad specificity transporter of xenobiotics and endobiotics. Arch Toxicol 88: 1205-1248, 2014.
17. Wang JS, Zhu HJ, Markowitz JS, Donovan JL, Yuan HJ and Devane CL: Antipsychotic drugs inhibit the function of breast cancer resistance protein. Basic Clin Pharmacol Toxicol 103: 336-341, 2008.
18. Tian Y, Qu BX, Yao Y and Zeng S: Establishment of BCRP expressed pig kidney cell line LLC-PK1/BCRP and its biological profile. Yao Xue Xue Bao 47: 1599-1604, 2012 (In Chinese).
19. Yan H, Zhang DY, Li X, Yuan XQ, Yang YL, Zhu KW, Zeng H, Li XL, Cao S, Zhou HH, *et al*: Long non-coding RNA GAS5 polymorphism predicts a poor prognosis of acute myeloid leukemia in Chinese patients via affecting hematopoietic reconstitution. Leuk Lymphoma 58: 1948-1957, 2017.
20. Feng B, Mills JB, Davidson RE, Mireles RJ, Janiszewski JS, Troutman MD and de Morais SM: In vitro P-glycoprotein assays to predict the in vivo interactions of P-glycoprotein with drugs in the central nervous system. Drug Metab Dispos 36: 268-275, 2008.
21. Boulton DW, DeVane CL, Liston HL and Markowitz JS: In vitro P-glycoprotein affinity for atypical and conventional antipsychotics. Life Sci 71: 163-169, 2002.
22. Kim KA, Joo HJ, Lee HM and Park JY: Influence of ABCB1 and CYP3A5 genetic polymorphisms on the pharmacokinetics of quetiapine in healthy volunteers. Pharmacogenet Genomics 24: 35-42, 2014.
23. Hoosain FG, Choonara YE, Tomar LK, Kumar P, Tyagi C, du Toit LC and Pillay V: Bypassing P-glycoprotein drug efflux mechanisms: Possible applications in pharmacoresistant schizophrenia therapy. Biomed Res Int 2015: 484963, 2015.
24. Tian Y, Qian S, Jiang Y, Shen Q, Zheng J, Zhou H and Zeng S: The interaction between human breast cancer resistance protein (BCRP) and five bisbenzylisoquinoline alkaloids. Int J Pharm 453: 371-379, 2013.
25. Takada T, Suzuki H and Sugiyama Y: Characterization of polarized expression of point- or deletion-mutated human BCRP/ABCG2 in LLC-PK1 cells. Pharm Res 22: 458-464, 2005. Ambudkar SV, Dey S, Hrycyna CA, Ramachandra M, Pastan I and Gottesman MM: Biochemical, cellular, and pharmacological aspects of the multidrug transporter. Annu Rev Pharmacol Toxicol 39: 361-398, 1999.
26. Ambudkar SV, Dey S, Hrycyna CA, Ramachandra M, Pastan I and Gottesman MM: Biochemical, cellular, and pharmacological aspects of the multidrug transporter. Annu Rev Pharmacol Toxicol 39: 361-98, 1999.
27. Hrycyna CA, Ramachandra M, Ambudkar SV, Ko YH, Pedersen PL, Pastan I and Gottesman MM: Mechanism of action of human P-glycoprotein ATPase activity. Photochemical cleavage during a catalytic transition state using orthovanadate reveals cross-talk between the two ATP sites. J Biol Chem 273: 16631-16634, 1998.
28. Ozvegy-Laczka C, Köblös G, Sarkadi B and Váradi A: Single amino acid (482) variants of the ABCG2 multidrug transporter: Major differences in transport capacity and substrate recognition. Biochim Biophys Acta 1668: 53-63, 2005.
29. Shukla S, Robey RW, Bates SE and Ambudkar SV: The calcium channel blockers, 1,4-dihydropyridines, are substrates of the multidrug resistance-linked ABC drug transporter, ABCG2. Biochemistry 45: 8940-8951, 2006.
30. Eddabra L, Wenner T, El Btaouri H, Baranek T, Madoulet C, Cornillet-Lefebvre P and Morjani H: Arginine 482 to glycine mutation in ABCG2/BCRP increases etoposide transport and resistance to the drug in HEK-293 cells. Oncol Rep 27: 232-237, 2012.
31. de Leon J, Wynn G and Sandson NB: The pharmacokinetics of paliperidone versus risperidone. Psychosomatics 51: 80-88, 2010.
32. Lindenmayer JP and Kaur A: Antipsychotic management of schizoaffective disorder: A review. Drugs 76: 589-604, 2016.