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

YANGANG ZHOU¹², HUANDE LI¹², PING XU¹², LI SUN¹⁻³, QING WANG¹², QIONG LU¹², HAIYAN YUAN¹² and YIPING LIU¹²

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

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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

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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 Sigma-Aldrich; 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% CO₂ 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.

In addition, 293 cells were seeded in 6-well plates at a density of 1x10⁴ 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% CO₂ 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% CO₂ 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 ddH₂O. 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'-GTTGTTGATGGGACACTGTGAC-3' and reverse, 5'-CCCTGTAACTCGGTGTTT-3'; and for β-actin forward, 5'-CTCTTCCAGCCTTCCTCTC-3' and reverse, 5'-AGACTGTGGTGGGTACAG-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⁻ΔΔCq 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% CO₂ 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 2x10⁵ 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/cm² 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 for PAL detection by high performance liquid chromatography/mass spectrometry (HPLC/MS) system (2690; Waters Corporation, Milford, MA, USA). The dry residue was reconstituted in 80 µl mobile phase and 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.

**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.).

**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
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 \( \mu \)M 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 \( \mu \)M, which indicated that PAL may be a substrate of BCRP. The net efflux ratio of PAL decreased as the concentration increased to 50 \( \mu \)M, 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 \( \mu \)M) 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 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 \( \mu \)M, respectively, and the \( V_{\text{max}} \) values were 108.5 and 98.01 nM/min; the \( V_{\text{max}}/K_{m} \) values of the two compounds were therefore 4.32x10\(^{-3}\) and 3.09x10\(^{-3}\) 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
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 \( V_{\text{m}}/K_m = 3.09 \times 10^4 \) 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 \( \mu \)M; 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 ABCG2 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 \( \sim 0.1 \) \( \mu \)M (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.

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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. HY and YL performed Western blot analysis. HY and YL performed polymerase chain reaction and western blot analysis. The authors declare that they have no conflicts of interest.

Conflict of interest

The authors declare that they have no conflicts of interest.

References


