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The potential role of human multidrug resistance protein 1 (MDR1) and multidrug resistance-associated protein 2 (MRP2) in the transport of Huperzine A *in vitro*.

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Running title: MDR1 and MRP2 transport Huperzine A.

Abstract

1. More than 30% of epilepsy patients suffer pharmacoresistance. Transport of antileptic drugs by P-glycoprotein (P-gp) and MRP2 plays an important role in drug-resistant epilepsy. Huperzine A (Hup-A) is a natural compound, which might have potential in treating neurological disorders including epilepsy and Alzheimer's disease. In this study, we investigated whether human P-gp and MRP2 transport Hup-A.

2. LLC-PK1 and MDCKII cells transfected with human P-gp or MRP2 were used to establish concentration equilibrium transport assays (CETAs) and determine the transport profile of Hup-A. The expression of P-gp and MRP2 was detected by qPCR and western blotting. The transport function of P-gp and MRP2 was measured by Rho123 and CDFDA cell uptake assay.

3. In CETAs, Hup-A at concentrations of 10 ng/ml or 2 μg/ml was transported by MDR1 and MRP2 from basolateral to apical sides of the cell monolayers. P-gp and MRP2 inhibitors completely blocked the efflux of Hup-A. There was no efflux of Hup-A in LLC-PK1 or MDCKII wild-type cells.

4. We demonstrate that Hup-A is a substrate of P-gp and MRP2. These results imply the efflux of Hup-A across the BBB *in vivo*, suggesting potential drug resistance of

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Hup-A.

Keywords: P-glycoprotein, Hup-A, drug-resistant epilepsy, drug transporter, cell monolayer

Introduction

Epilepsy, affecting approximately 68 million people worldwide, is one of the most common neurological disorders (Kwan and Brodie, 2000). More than 30% of epilepsy patients are drug-resistant, leading to increased risks of premature death, injuries, psychosocial dysfunction, and reduced quality of life (Kwan et al. , 2011, Loscher et al. , 2013). The transporter hypothesis of drug-resistant epilepsy proposes that overexpression of multidrug transporters in BBB may lower the concentration of antiepileptic drugs (AEDs) at epileptic foci (Kwan et al., 2011, Loscher and Potschka, 2005, Zhang et al. , 2012).

P-glycoprotein (P-gp, MDR1, or ABCB1) and multidrug resistance-associated protein 2 (MRP2), which are members of the ABC transporter superfamily, exhibit associations with drug-resistant epilepsy (Loscher and Potschka, 2005, Zhang et al., 2012). P-gp is a critical efflux transporter at the BBB that can pump most exogenous substances from the CNS (Miller et al. , 2008). It was demonstrated that P-gp was overexpressed in epileptic foci (Kwan and Brodie, 2005, Kwan et al. , 2010, Zhang et al., 2012). Overexpression of P-gp was associated with decreased concentration of AEDs in the brain and CSF (Loscher and Potschka, 2005, Zhang et al., 2012). Many AEDs, such as phenytoin, phenobarbital, topiramate, lamotrigine, and oxcarbazepine,

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are substrates of P-gp *in vivo* and *in vitro* (Luna-Tortos et al. , 2009, Zhang et al., 2012). MRP2 is another ABC transporter in the luminal surface of brain capillary endothelium. It was demonstrated that MRP2 was overexpressed in the capillary endothelial cells in patients with drug-resistant epilepsy and the epileptic foci of epileptic rats (Hoffmann et al. , 2006, Liu et al. , 2015, Loscher and Potschka, 2005, Potschka et al. , 2003b, van Vliet et al. , 2005, Yao et al. , 2012). Several AEDs, such as phenytoin and carbamazepine, may be substrates of MRP2 *in vitro* or *in vivo* (Kim et al. , 2010, Potschka et al. , 2003a, Potschka et al., 2003b, van Vliet et al., 2005). These observations indicate that P-gp and MRP2 may contribute to drug-resistant epilepsy (Chan et al. , 2014, Kwan and Brodie, 2005, Loscher and Potschka, 2005, Zhang et al. , 2013, Zhang et al. , 2010, Zhang et al., 2012, Zhang et al. , 2011).

Huperzine A (Hup-A) is a sesquiterpene alkaloid isolated from *Huperzia serrata*. It is an N-methyl-d-aspartate (NMDA) receptor antagonist and reversible acetylcholinesterase inhibitor (AChE) (Coleman et al. , 2008, Li et al. , 2007). Hup-A has therapeutic potential to treat multiple neurological conditions, such as Alzheimer's disease (AD), poisoning with organophosphate neurotoxins, and neuropathic pain (Lallement et al. , 2002, Ma et al. , 2007, Wang et al. , 2006, Yang et al. , 2013, Yu et al. , 2013). In recent years, several studies have demonstrated that Hup-A is a potential drug for epilepsy (Coleman et al., 2008, Damar et al. , 2016, Gersner et al. , 2015). Hup-A inhibited pentylenetetrazol (PTZ)-induced seizure in rats (Gersner et al., 2015). Hup-A also provided sustained protection against

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SCN1A-related seizures in mice and zebrafish with *Scn1a* mutations (Dinday and Baraban, 2015, Wong et al. , 2016). Hup-A had a clinical effect in treating putative complex partial seizures in dogs (Schneider et al. , 2009). As a potential drug, Hup-A has been studied in clinical trials to treat epilepsy, which has modified the formulation of Hup-A and obtained significant seizure protection (Bialer et al. , 2018, Bialer et al. , 2015, Damar et al., 2016, Golyala and Kwan, 2017).

It is important to determine whether P-gp and MRP2 transport Hup-A, which might affect its clinical efficacy. In the current study, we used the concentration equilibrium transport assay (CETA) to investigate whether Hup-A is transported by P-gp and MRP2 *in vitro*.

Materials and methods

Chemicals

Huperzine A was purchased from Solarbio Life Sciences (Beijing, China). MTT (3-[4,5dimethyl thiazolyl-2]-2,5-diphenyltetrazolium bromide) and Rho123 were supplied by Sigma Aldrich (St. Louis, MO, USA). CDFDA (2',7'-Dichlorodihydrofluorescein diacetate), MK571, and Tariquidar were purchased from MCE company (Shanghai, China). Verapamil was provided by Alexis Biochemicals (San Diego, CA, USA). CDFDA, Tariquidar, and MTT were dissolved in water, and other drugs were dissolved in dimethyl sulfoxide (DMSO) (<0.1% DMSO in final solution). Acetonitrile, triethylamine, and methanol were HPLC grade.

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All other reagents were at least analytical grade. Huperzine A was tested at concentrations covering the ranges of therapeutic plasma concentrations.

Cell lines and cell culture

LLC-PK1 (LLC-WT), MDCKII (MDCK-WT), and their human MDR1 or MRP2 gene transfected cell lines (LLC-MDR1, MDCK-MDR1, and MDCK-MRP2) were kindly provided by Professor P. Borst (The Netherlands Cancer Institute, Amsterdam, The Netherlands). Cell culture was performed as described previously (Zhang et al., 2011). Six-well Transwell (Transwell®, 0.4 μm, polycarbonate membrane, 24 mm insert, Corning, NY, USA) plates were used for the transport studies. Cells (2×10^6 MDCK cells or 1.5×10^6 LLC cells) were seeded on the Transwell plates and were grown in the relevant medium at 37℃ with 5% CO_2 for five days as described previously. The cell culture medium (M199 for LLC cells, DMEM for MDCK cells) was changed every day.

Cytotoxicity test

The cytotoxicity of Hup-A and other drugs was tested by MTT assay in all cell lines used in the current study (Chan et al., 2014, Zhang et al., 2011). Briefly, $1.5\text{x}10^4$ cells/well were seeded in 96-well plates and cultured for 48 h. After withdrawing the culture medium, 200 μl drug solution was added (various concentrations diluted in PBS) and incubated for four hr. The buffer was then replaced with 200 μl of 0.5 mg/ml MTT in PBS and incubated for another three hr. The solution was replaced

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with 200 μl DMSO, and the absorbance was determined at a wavelength of 590 nm on a Thermo microplate reader (MA, USA).

Cell uptake assay and flow cytometry

To verify the efflux function of P-gp and MRP2, Rho123 or CDFDA cell uptake assays were performed to detect the efflux activity of P-gp and MRP2 (Schexnayder and Stratford, 2015). For Rho123 cell uptake assay, 5×10^5 cells (MDCK-MDR1 and LLC-MDR1 cells) were collected and washed with warm PBS. Cells were resuspended in 1 ml DMEM medium containing Rho123 (1 μg/ml), then incubated at 37°C with 5% CO_2 for 20 min. For CDFDA cell uptake assay, 1.5×10^6 cells (MDCK-MRP2 cells) were collected by trypsin and washed once with warm PBS. Then, cells were resuspended in 1 ml DMEM medium with CDFDA (5 μg/ml), then incubated at 37°C with 5% CO_2 for 30 min. The cells were centrifuged and resuspended in cold PBS. Flow cytometry analysis of Rho123 fluorescence and CDFDA fluorescence were performed with the BECKMAN COULTER Cytomics FC 500 MCL (CA, USA) at 510 nm.

Real time-PCR

To quantify mRNA levels of MDR1 and MRP2, total RNA was isolated from cells by Trizol (Invitrogen, CA, USA). Reverse transcription was performed by using a Takara Reverse Transcription Kit (Takara, Japan) according to the manufacturer's protocol. Real-time PCR was performed with QIAGEN QuantiNova SYBR Green PCR Kit

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(QIAGEN, Germany) by using the following primers (Zhang et al., 2011): TCTCTCGATACTCTGTGGCAC and CTGGAATCCGTAGGAGATGAAGA for MRP2; CCCATCATTGCAATAGCAGG and TGTTCAAACTTCTGCTCCTGA for MDR1; CCTCTATGCCAACACAGTGC and ACATCTGCTGGAAGGTGGAC for β-Actin.

Western blotting

To detect the protein level of P-gp and MRP2 in different cell lines, western blotting was performed. Briefly, the cells were collected, and whole protein was extracted by ice-cold lysis RIPA buffer (150 mM NaCl, 50 mM Tris, 2 mM EDTA, 0.1% SDS, 1% Triton X-100). The protein concentration was measured by BCA Kit as described in the handbook. First antibodies for P-gp (Invitrogen, C219, MA1-26528), MRP2 (CST, R260) and β-actin (ZSGB-BIO, OTI1) and their corresponding secondary antibody were used (ZSGB-BIO, ZB05301, ZB-2305). Bands were visualized by Immobilon Western Chemoluminescent HRP substrate (Millipore, USA) and visualized with an imaging system ChemiScope 6000 Exp (CLINX, China).

Immunostaining

The location and level of P-gp in cells were detected by using immunostaining as described previously (Zhang et al., 2011). In brief, cells were fixed with 70% ethanol for 10 min at -20 ^{o}C and wished with cold PBS twice. The fixed cells were blocked with 10% BSA for 30 min at room temperature, then incubated with anti-P-gp

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antibody (C219, 1:100) in PBS with 0.5% BSA for 6 hr at 4 ^{o}C. Cells were washed twice with cold PBS and incubated with fluorescent-conjugated secondary antibody (Invitrogen, Alex 488-conjugated IgG, 1:400) for 30 min at room temperature. Cells were visualized by fluorescent microscopy (Nikon).

Cell transport assays

The concentration equilibrium transport assay (CETA) was performed as previously described (Zhang et al., 2010). Briefly, cells at densities of 1.8×10^6 (MDCK cells) or 1.4×10^6 (LLC cells) were seeded into 6-well Transwells and cultured in the relevant medium (DMEM with 10% FBS for MDCK cells; Medium 199 with 10% FBS for LLC cells) for five days. The integrity of monolayers was tested by measuring the transepithelial electrical resistance (TEER) with the EVOM2 (World Precision Instruments Inc, FL, USA). Monolayers with TEER > 150 $\Omega \cdot cm^2$ (subtracting the background value of a Transwell) were used for assays. After each experiment, TEER was tested, and only monolayers with TEER no less than 85% of the initial value were used. The drug was added to both sides of monolayers at equal concentrations. Volumes on apical and basolateral sides were 2 ml and 2.7 ml, respectively. The concentrations of Huperzine A were 2 μg/ml and 10 ng/ ml for LLC-MDR1 cells and MDCK-MDR1 cells. The concentrations of Huperzine A were 10 μg/ml, 2 μg/ml and 10 ng/ ml for MDCK-MRP2 cells; aliquots of 100 μl and 130 μl from apical and basolateral side were collected at various time points of drug exposure (30, 60, 90, 120, and 180 min). Aliquots of samples did not affect the hydrostatic pressure on the

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cell monolayers. The above transport assays were repeated in the presence of the P-gp inhibitors tariquidar (2 μM) and MRP2 inhibitors MK571 (50 μM), respectively. All transport assays were performed in triplicate. The samples were stored at -20°C until analysis.

Drug analysis

Huperzine A was quantified by high performance liquid chromatography (HPLC) with UV Detection (HPLC/UV) and an Agilent 1260 HPLC system (Agilent, CA, USA) with a Dalian Elite Hypersil BDS C18 column (Dalian, China) (5 μM pores, 150 mm long, 4.6 mm inner diameter). The mobile phase consisted of methanol and 0.1% triethylamine in water (60:40). The detection wavelength of Huperzine A is 313 nm. The limits of quantification (LOQs) were 10 ng/ml for Huperzine A; the relative standard deviation (RSD) of both intraday and interday precision for all the drugs was below 5%. For the LC-MS/MS system, Agilent 1200 series LC pumps (Agilent, CA, USA) and an ABI 2000 Q-Trap triple quadrupole mass spectrometer (AB Sciex Instruments, CA, USA) were used. A Hypersil BDS C18 column (5 μM pores, 150 mm long, 4.6 mm inner diameter, Dalian, China) was used. The MS/MS system was positive mode. The mobile phases were acetonitrile and 0.1% formic acid in water. The concentration of Hup-A was calculated by using the area ratio compared to the internal standard.

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Data analysis

For the cytotoxicity test, when the survival rate of cells exposed to an antiepileptic drug is higher than 80%, the drug can be considered safe (Chan et al., 2014). The concentration of drug was much higher than the therapeutic doses in the blood of patients according to quantitative in vitro to in vivo extrapolation (Wambaugh et al. , 2018). For real time-PCR, relative expression levels of MDR1 or MRP2 mRNA in MDCKII cells and LLC cells were scaled to the mean relative expression level of housekeeping gene β-actin, relative levels of MDR1 or MRP2 in wild-type cells were regarded as 1 respectively. For western blot, the protein level of P-gp was quantified and scaled to β-actin, and relative levels of P-gp in MDCK-WT and LLC-WT cells were regarded as 1 respectively.

For Rhodamine-123 efflux, fluorescence values for MDCK-MDR1 cells, LLC-MDR1 cells, and LLC-WT cells were scaled to the median fluorescence value of MDCK-WT cells, which was defined as 100. For CDFDA uptake, fluorescence values for MDCK-MRP2 cells were scaled to the median fluorescence value of MDCK-WT cells, which was defined as 100.

In the CETA, the data were presented as the percentage of the drug loading concentration in either apical or basolateral chamber *vs.* time, as described previously (Zhang et al., 2012, Zhang et al., 2011). At various time intervals, differences of drug concentration between the two chambers of each well were compared between

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wild-type (WT) cells and either MDR1-transfected cells or MRP2-transfected cells.

Values are shown as means ± SEM with an independent biological repeat. Significant differences between two groups or more than two groups were calculated by Student's t-test or one-way ANOVA, respectively, with $P < 0.05$ considered as significant.

Results

The cytotoxicity of Hup-A was tested by MTT. Hup-A at 10 μg/ml for 4 hours was not toxic to any of the five cell lines (data not shown). In order to verify the expression of P-gp and MRP2 in different cell lines, western blotting, immunostaining, and real-time PCR were performed (Fig.1). The protein levels of P-gp in MDR1-transfected cell lines (MDCK-MDR1 and LLC-MDR1 cells) were significantly higher than in wild-type cell lines (MDCKII and LLC cells) (Fig.1A,B). P-gp was mainly located in the cell membrane (Fig. 1G). MRP2 protein level in MRP2-transfected cells (MDCK-MRP2 cells) was significantly higher than in wild-type cell lines (MDCKII cells) (Fig.1C,D). P-gp mRNA levels in MDR1-transfected cell lines were also significantly higher than in their respective wild-type cell lines (Fig.1E). MRP2 mRNA levels in MDCK-MR2 cell lines was significantly higher than in MDCKII cell lines (Fig.1F).

The efflux activity of P-gp and MRP2 in different cell lines was verified by detecting the cellular level of fluorescent substrates. Rho-123 and CDFDA are the fluorescent

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substrates of P-gp and MRP2, respectively. The density of fluorescent substrate in the transfected cells (MDCK-MDR1, LLC-MDR1, and MDCK-MRP2) was significantly lower than that of the corresponding wild-type cells, demonstrating the significant efflux of fluorescent substrates by P-gp and MRP2 (Fig.2). These results demonstrated that the expression and efflux function of P-gp and MRP2 in their transfected cell lines were normal (Fig.1&2).

In order to verify the integrity of the monolayer system of all cell lines (MDCKII, MDCK-MDR1, MDCK-MRP2, LLC, and LLC-MDR1) used in this study, the apparent permeability values of propranolol and atenolol were tested. The P_{app} values were in the range of 0.5×10^{-6} - 1.2×10^{-6} cm/s (atenolol) and 2×10^{-5} - 3×10^{-5} cm/s (propranolol) respectively, which were similar to previous results(Zhang et al., 2010, Zhang et al., 2011).

In the concentration equilibrium transport assay (CETA), the apical concentration of Hup-A was significantly increased at 60, 90, 120, and 180 min after initial adding of Hup-A at concentrations of 10 ng/ml and 2 μg/ml to MDCK-MDR1 and LLC-MDR1 cells, respectively (Fig.3). There was no significant difference of the Hup-A concentrations between apical and basolateral sides of wild-type MDCKII or LLC cells (Fig.3). Also, the transport of Hup-A (10 ng/ml and 2 μg/ml) was inhibited by tariquidar (2 μM) in MDCK-MDR1 and LLC-MDR1 cells, indicating that Hup-A is a substrate of P-gp *in vitro* (Fig.3).

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Hup-A was transported by MRP2 at concentrations of 10 μg/ml, 2 μg/ml and 10 ng/ml in MDCK-MRP2 cells (Fig.4). There was no significant transportation of Hup-A by MDCKII cells. Moreover, transportation of Hup-A was inhibited by MRP2 inhibitor MK571 (50 μM), indicating that Hup-A is a substrate of MRP2 *in vitro* (Fig.4).

Discussion

Hup-A, a natural compound isolated from an herb used in Traditional Chinese Medicine, has potential to treat epilepsy, AD, and other neurological diseases. In this study, we demonstrated that Hup-A was transported by P-gp and MRP2 in cell monolayer models *in vitro*, suggesting the potential efflux of Hup-A across the BBB *in vivo*.

Drug resistance is one of the biggest challenges in the current medical treatment of epilepsy (Kwan et al., 2011). High expression of ABC transporters such as MRP2 and P-gp at the BBB may contribute to pharmacoresistance of AEDs (Grewal et al. , 2017, Liu et al., 2015, Miller, 2015, van Hoppe et al. , 2017). In this study, we firstly demonstrated that Hup-A was transported by MRP2 (Fig.4). At concentrations of 10 ng/ml, 2 μg/ml and 10 μg/ml, Hup-A was significantly transported from the basolateral to the apical side of MDCK-MRP2 cell monolayers in the CETA assay (Fig.4). Similarly, Hup-A was transported by P-gp at concentrations of 10 ng/ml and 2 μg/ml (Fig.3). These results indicated that Hup-A is a substrate of MRP2 and P-gp.

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Consistent with our results, it was reported that the brain-to-plasma concentration ratio of Hup-A was higher in *Abcb1a*$^{-/-}$ mice than in wild-type mice (Li et al. , 2017). Hup-A was also transported by P-gp in a bi-directional transport assay in Caco-2 and MDCKII cell lines at a concentration of 2.42 μg/ml (Li et al., 2017).

We previously demonstrated that the concentrations of AEDs might affect their P-gp transport profile (Zhang et al., 2010, 2012). The brain concentration of Hup-A was low (less than 50 ng/ml) in patients and animals (Jiang et al. , 2003, Li et al., 2017, Ye et al. , 2008). In the current study, we determined whether a Hup-A in this concentration range was transported by P-gp in a sensitive cell monolayer transport assay (CETA). We found that Hup-A was transported by P-gp at a low concentration, 10 ng/ml (Fig.3C&D), which is in the range of brain concentrations found in animal models and at a high concentration, 2 μg/ml (Fig.3A&B). As with P-gp, MRP2 transported Hup-A at a range of concentrations (10 ng/ml, 2 μg/ml and 10 μg/ml) in the CETA (Fig.4), indicating that Hup-A is a substrate of MRP2 *in vitro*.

Recent studies have shown that Hup-A can inhibit N-methyl-D-aspartate (NMDA) receptors and AChE (Coleman et al., 2008), suggesting that Hup-A has promise for treating epilepsy. Hup-A has antiepileptic effects in patients and multiple animal models, including zebrafish, mice, rats, and dogs (Coleman et al., 2008, Dinday and Baraban, 2015, Gersner et al., 2015, Schneider et al., 2009, Wong et al., 2016). A clinical trial, with the extended-release formulation of Hup-A, improved the

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pharmacokinetics and increased the seizure protection of Hup-A (Bialer et al., 2018). Hup-A can also treat AD and improve cognitive deficits (Yang et al., 2013, Zhang et al. , 2002). Phase IV clinical trials conducted in China have demonstrated that Hup-A significantly improved the memory of older adults and patients with AD (Xu et al. , 1995, Zhang et al., 2002), although the methodological quality of clinical trials is not high and some of them had a risk of bias (Li et al. , 2008, Yang et al., 2013). In some brain diseases, up-regulation of P-gp in the BBB has been associated with decreased concentration of P-gp substrates and with drug-resistance (Loscher and Potschka, 2005). The expression of MRP2 was significantly increased in the hippocampus of patients with hippocampal sclerosis (Aronica et al. , 2005). MRP2 was also significantly upregulated in rat brains after electrical stimulation and pilocarpine-induced seizures (van Vliet et al., 2005, Zhang et al. , 2004). In the current study, we demonstrated that P-gp and MRP2 transported Hup-A *in vitro*, implying that P-gp and MRP2 might affect the therapeutic efficacy of Hup-A and the druggability in drug development. However, there have been no studies on the substrate status of Hup-A for MRP2 *in vivo*. Moreover, the substrate status of Hup-A for P-gp has only been studied in *Abcb1a*$^{-/-}$ mice. More studies are needed to investigate efflux of Hup-A by MRP2 and P-gp *in vivo*. In addition, the influence of MRP2 and P-gp on drug resistance of Hup-A needs further study.

Conclusions

This study provided evidence that Hup-A is a substrate of human P-gp and MRP2 *in*

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vitro. This suggests the potential efflux transportation of Hup-A by P-gp and MRP2 at the BBB, and implies the potential for resistance to Hup-A in treating neurological diseases in animals and patients.

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Declaration of interest

The authors declare that there are no conflict interests.

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Figures Legends

Figure 1. P-gp and MRP2 expression in different cell lines. (A) Protein expression of P-gp was detected by western blot. (B) Quantification of P-gp by scanning of the protein bands in A. (C) Protein expression of MRP2 was detected by western blot. (D) Quantification of MRP2 by scanning of the protein bands in C. (E) mRNA expression of P-gp in MDCK, MDCK-MDR1, LLC, and LLC-MDR1 cells. (F) mRNA expression of MRP2 in MDCK-WT and MDCK-MRP2 cells. Relative mRNA and protein levels of P-gp or MRP2 were scaled to the mean relative expression level of β-actin. The relative levels of P-gp or MRP2 in wild-type cells were regarded as 1. (G) Immunostaining of P-gp in wild type and *MDR1*-transfected cells. Experiments were performed in triplicate, and values are shown as mean ± SEM ($n = 3$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

Figure 2. Rho123 and CDFDA efflux assay in different cell lines. (A) Rho123 efflux from MDCK cell lines (MDCK-WT and MDCK-MDR1 cells) and LLC cell lines (LLC-WT and LLC-MDR1) was detected by flow cytometry. (B) The density of Rho123 (A.U.) in different cell lines was quantified. Fluorescence values for MDCK-MDR1 cells, LLC-MDR1 cells and LLC-WT cells were scaled to the median fluorescence value of MDCK-WT cells, which was defined as 100. (C) CDFDA

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efflux from MDCK-WT and MDCK-MRP2 cells. The density of CDFDA (A.U.) in the two cell lines was detected by flow cytometry. (D) Fluorescence values for MDCK-MRP2 cells were scaled to the median fluorescence value of MDCK-WT cells, which was defined as 100. Experiments were performed in triplicate, and values are shown as mean ± SEM (n = 3, $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

Figure 3. Concentration equilibrium transport assays of Huperzine A in cells transfected with the human MDR1 gene and their wild-type cells. (A) Huperzine A was significantly transported by MDCK-MDR1 at the concentration of 2 μg/ml. (B) Huperzine A was significantly transported by LLC-MDR1 at 2 μg/ml. (C) Huperzine A was significantly transported by MDCK-MDR1 cells when the initial concentration was 10 ng/ml. (D) Huperzine A was significantly transported by LLC-MDR1 cells when the initial concentration was 10 ng/ml. P-gp inhibitor tariquidar (2 μM) blocked the efflux of Hup-A in MDR1 transfected cells. Data are given as the percentage of the initial drug concentration in either apical or basolateral chamber versus time. Experiments were performed in triplicate, and values are shown as mean ± SEM (n = 3, $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

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Figure 4. Concentration equilibrium transport assays of Huperzine A for MDCK cells transfected with the human MRP2 gene. Huperzine A was significantly transported by MDCK-MRP2 cells when the initial Huperzine A concentrations were 10 ng/ml (A), 2 μg/ml (B), and 10 μg/ml (C). MRP2 inhibitor MK571 (50 μM)

blocked the efflux of Hup-A in MRP2 transfected cells. Data are given as the percentage of the initial drug concentration in either apical or basolateral chamber versus time. Experiments were performed in triplicate, and values are shown as mean ± SEM ($n = 3$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

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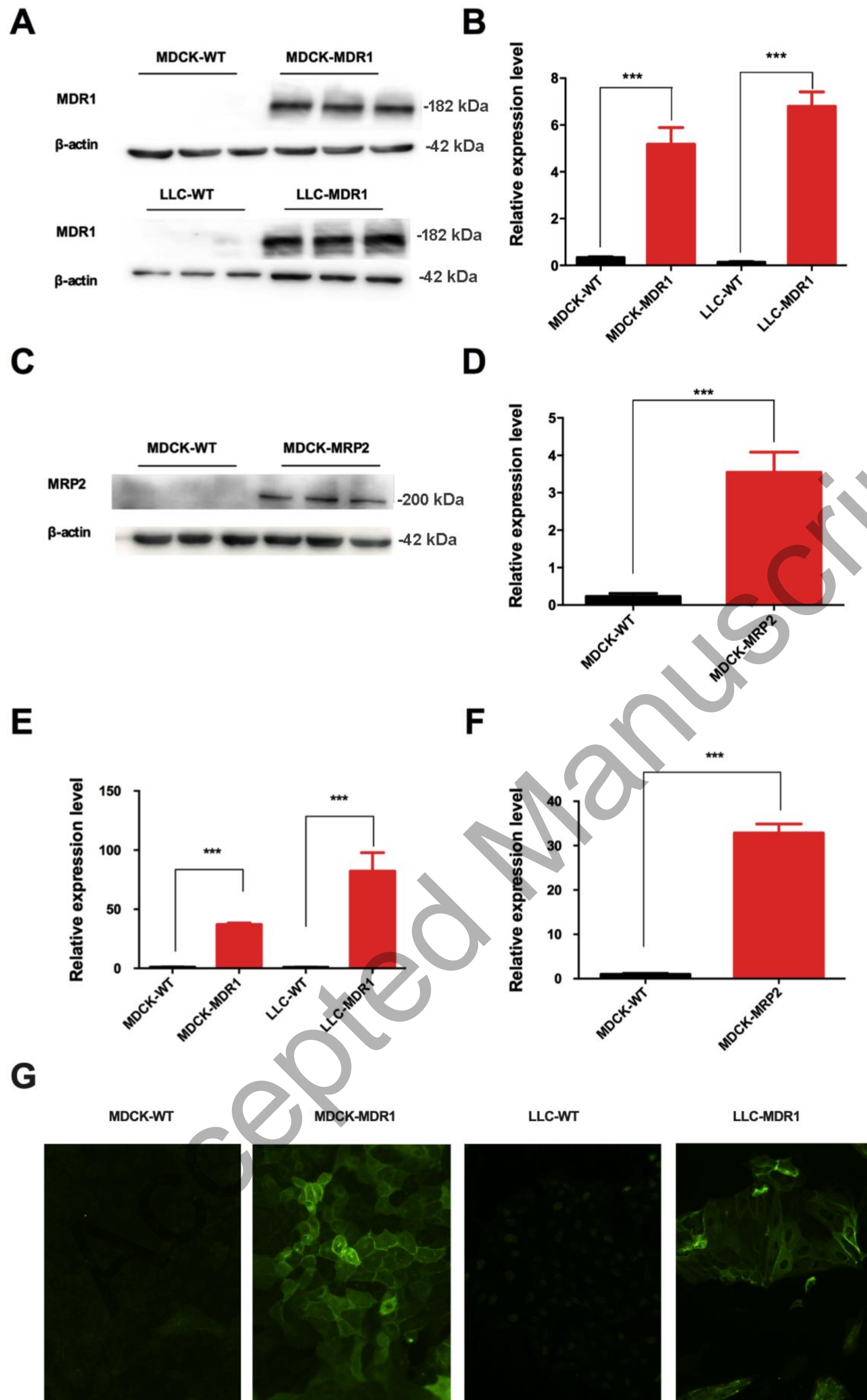

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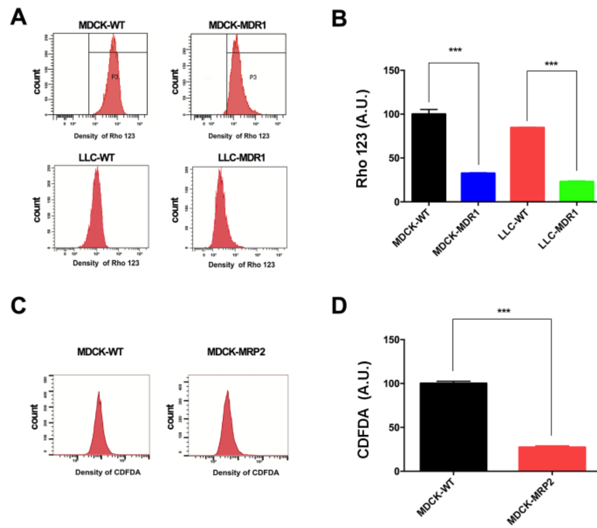

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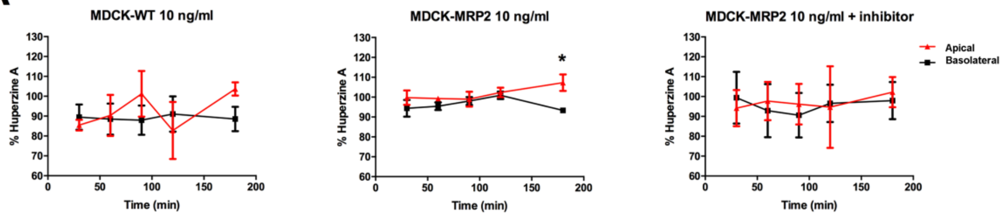

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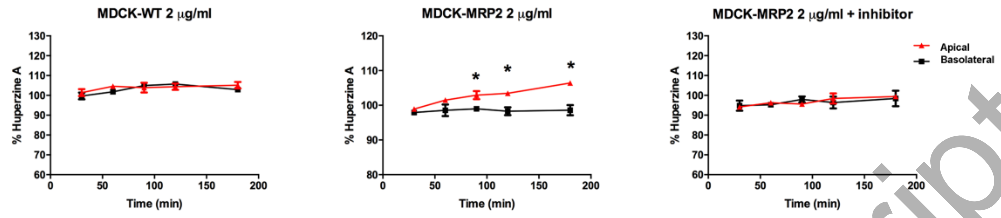

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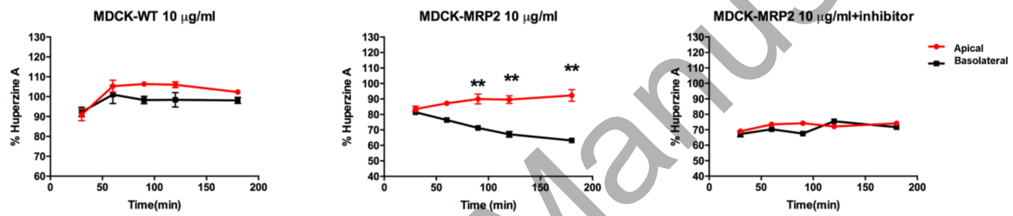

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