College of Pharmaceutical Sciences[1], Zhejiang University, Zhejiang[2]; School of Pharmacy, Tianjin TCM University, Tianjin, China

Construction and application of double-transfected cells expressing the human transporter P-glycoprotein and cytochrome P450 3A4

H.H. Hu[1], C. Su[1], Y. Jiang[1], L.S. Yu[1], Y. Liu[1], Y. Tian[1], S.Y. Xu[1], H. Zhou[1], X. He[2], H.D. Jiang[1], S. Zeng[1]

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Prof. Lushan Yu; Prof. Su Zeng, Department of Drug Metabolism and Pharmaceutical Analysis, Zhejiang University, Hangzhou, Zhejiang, 310058, China
yuls@zju.edu.cn

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Intestinal P-glycoprotein (P-gp) and cytochrome P450 (CYP) enzymes are known to influence oral bioavailabilities of drugs. Recombinant plasmids pcDNA3.1/ Hypgro/CYP3A4 were transfected into MDCK and MDCK-MDR1 cells to construct the single-transfected cell line MDCK-CYP3A4 and double-transfected cell line MDCK-MDR1/CYP3A4. The expression of CYP3A4 in the double-transfected cell line was determined by Western blot and its activity was detected by the metabolism assays of three substrates of CYP3A4, which were 7-benzyloxy-4-trifluoro-methylcoumarin (BFC), testosterone and midazolam. In addition, the selection of monoclones with high CYP3A4 activities in the single-tranfected cell line was performed by the P450 Glo CYP3A4 assay. Through MTT assay, the interaction between P-gp and CYP3A4 was preliminarily determined based on the changes of IC_{50} values. The results showed that paclitaxel detoxified in the single-transfected MDCK-MDR1 cell because of P-gp efflux. And it was also less toxic in the single-transfected CYP3A4 cell line due to the metabolism by CYP3A4. In the double-transfected MDCK-MDR1/CYP3A4 cell line, the toxicity decreased dramatically because of the interplay between P-gp and CYP3A4. Therefore, the cell model could be applied to study the toxicity and detoxification of chemicals due to the metabolism by CYP3A4 and the efflux through P-gp.

1. Introduction

Recently it has become evident that in the small intestine, drug efflux pumps and cytochrome P450 (CYP) enzymes, particularly P-glycoprotein (P-gp) and CYP3A, are forming a cooperative barrier against the absorption of xenobiotics (Cummins et al. 2002; Benet et al. 2003; Benet and Cummins 2001). P-gp is localized to the apical membrane of the intestinal epithelial cells (Thiebaut et al. 1987), consistent with the role in effluxing compounds back into the intestinal lumen. CYP3A4 is the most prominent oxidative cytochrome P450 enzyme (Watkins et al. 1987; Zhang et al. 1999), localized to the columnar epithelial cells lining the intestinal lumen (Kolars et al. 1994). CYP3A4 and P-gp have common substrates, demonstrating the mutually broad selectivity of these proteins (Wacher et al. 1995). Co-induction of CYP3A and P-gp by rifampin was shown in human LS180 colon carcinoma cells (Schuetz et al. 1996) and human intestine (Kolars et al. 1992; Greiner et al. 1992), but not in rat liver (Salphati and Benet 1998). Resolution of these findings is now possible with the knowledge that the nuclear receptor PXR /SXR, which can activate both CYP3A4 and P-gp, has species specific patterns of induction (Lehmann et al. 1998; LeCluyse EL 2001; Yu et al. 2009; Yu et al. 2011). These characters imply that there may be an interplay between P-gp and CYP3A4 (Kivistö KT et al. 2004 Pang et al. 2009). However, this has not been clarified. In our study, developing and characterizing the double-transfected MDR1 and CYP3A4 MDCK cells could resolve this problem. Experimental studies on the metabolism and transport were performed in vitamin D3-induced Caco-2 cells (Hochman et al. 2000) or CYP3A4 transfected Caco-2 cells (Cummins et al. 2004) with a high expression of CYP3A4 and P-gp. However, there was the drawback that the drugs were transported by other transporters simultaneously. Therefore, the model illustrated in this article was more suitable to study the interplay between P-gp and CYP3A4, which contributes to interference on the disposition and metabolism in the intestine.

2. Investigations and results

2.1. Expression of CYP3A4 in double-transfected monoclone cells

Recombinated plasmids pcDNA3.1/ Hypgro/CYP3A4 were successfully constructed and confirmed by being digested with *Kpn* I and *Xho* I. The fragment lengths of vector and CYP3A4 were consistent with those before ligation, which were 5400 bp and 1512 bp respectively (Fig. 1). Through DNA sequencing, the sequence of CYP3A4 was aligned with NCBI database.

The protein expression of human CYP3A4 in the transfected MDCK-MDR1 monoclone cells was analyzed by immunoblotting (Fig. 2).The control cells (MDCK-MDR1) had no expression of CYP3A4, while the expression of CYP3A4 was high in the selected monoclones, especially in number 2, 7 and 39 monoclones. Human liver microsomes were used as positive control.

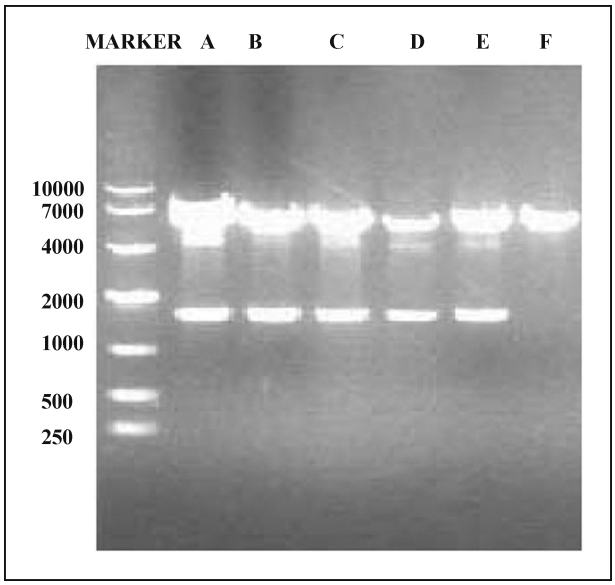

Fig. 1: Electrophoresis identification of plasmid pcDNA3.1 (+)/Hygro/CYP3A4. The left lane was DNA marker. Five recombinant plasmids (A-E) were identified correctly by digestion of *Kpn* I and *Xho* I. And the right lane (F) was the vector pcDNA3.1 (+) fragment from *Kpn* I and *Xho* I digestion (negative control).

2.2. Determination of cytochrome P450 3A4 activity

Fluorescence measurement was used as a rapid and convenient method to determine the CYP3A4 activities of MDCK-MDR1/CYP3A4 cells. In this experiment, 7-benzyloxy-4-trifluoromethylcoumarin (7-BFC) was incubated with MDCK-MDR1/CYP3A4 cell lysates as a substrate of CYP3A4. The concentration of its metabolite (7-HFC) was determined to screen the CYP3A4 catalytic activities of the monoclones, as shown in Fig. 3A. Monoclones such as p3.1–1, 2, 7, 11, 14 and 33, which were more active than control (MDCK-MDR1cells), were then co-incubated with CYP3A4 inhibitor ketoconazole in the incubation system, as shown in Fig. 3B. The results showed that the enzyme activities of these monoclones were all inhibited to varying degrees. Moreover, CYP3A4 classic substrates (testosterone and midazolam) were applied to further estimate the activities. The results showed that the monoclone p3.1–33 had a highest activity toward the two classic substrates (Fig. 3C, D).

The functional activities of the MDCK-CYP3A4 monoclones were measured under similar treatment conditions by P450 Glo CYP3A4-IPA assay kit containing luciferin-IPA substrate. Fluorescent metabolite formation in the positive control MDCK-MDR1/ CYP3A4 cell line was almost 4 times higher than that in the host cells (MDCK cells) suggesting enhanced expression and activity of CYP3A4 (Fig. 4). The results showed that the MDCK-CYP3A4 monoclones had CYP3A4 activities of different levels, especially number 5 monoclone, which had the highest activity.

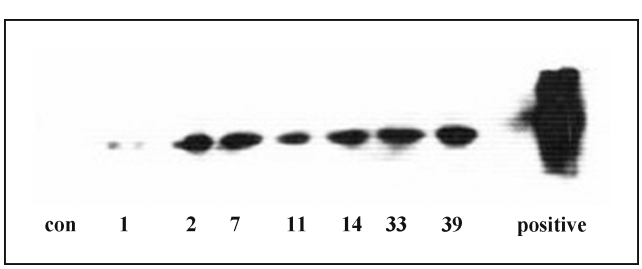

Fig. 2: CYP3A4 expression in total cell lysates from parent MDCK-MDR1 and CYP3A4 transfected MDCK-MDR1 cells, analyzed by Western blot. MDCK-MDR1 cells and human liver microsomes were the negative and positive control, respectively. Seven MDCK-MDR1/CYP3A4 monoclone cells were obtained by tranfecting expression vector pcDNA3.1 (+)/Hygro/CYP3A4 into the MDCK-MDR1 cells.

2.3. MTT assay

To estimate and compare the functions of MDCK, MDCK-CYP3A4, MDCK-MDR1, and MDCK-MDR1/CYP3A4 cells, MTT assay was performed with paclitaxel. The results showed that IC_{50} values toward paclitaxel were 0.04 μM, 0.08 μM, 0.11 μM, 0.18 μM, respectively, in the cell lines of MDCK, MDCK-CYP3A4, MDCK-MDR1, and MDCK-MDR1/CYP3A4. Due to paclitaxel effluxed by P-gp in the MDCK-MDR1 cells, the IC_{50} value increased significantly compared with the cells MDCK. Moreover, paclitaxel was metabolized by CYP3A4 to less toxic metabolites, which resulted in increase of the IC_{50} values compared with control cells. Therefore, the IC_{50} value increased dramatically in the double-transfected MDCK-MDR1/CYP3A4 cells compared with the cells MDCK-MDR1 and MDCK-CYP3A4. The results indicated that a double-transfected MDCK cell line stably expressing CYP3A4 and P-gp was established.

3. Discussion

Transporters and metabolic enzymes play an important role in the disposition of drugs in various organs of the body. The interplay between P-gp and CYP3A4 can influence the bioavailability of the drug. Since the double-transfected cells can over-express P-gp and CYP3A4 with no significant expression of other efflux transporters, the cells can be identified as an *in vitro* model to study their synergism.

Figure 1 shows that the *CYP3A4* gene was successfully cloned into the vector pcDNA3.1(+)/Hypgro, and the lengths of bands were correct after digestion. The plasmids pcDNA3.1(+)/Hypgro/CYP3A4 were transfected into the MDCK and MDCK-MDR1 cells. Figure 2 clearly indicates that transfection resulted in a higher expression of CYP3A4 at protein level. The fluorescent substrate 7-BFC was applied to select the monoclones roughly for its rapid and convenient advantage. The inhibitor ketoconazole was chosen to further confirm the presence of active CYP3A4 protein in the *in vitro* system. Several monoclones with relatively high activities were further characterized with two classic substrates, testosterone and midazolam. Through the selection, the final monoclone was identified, which were shown in Fig. 3 (A-D).

Figure 4 shows the expression of CYP3A4 at protein levels in the single-transfected cell lines MDCK-CYP3A4. The determination was performed with the P450 Glo CYP3A4-IPA assay kit, which had a very high sensitivity. The inhibitor ketoconazole, whose concentration was 1 μM, was chosen to further confirm the activity of protein in an *in vitro* system. MDCK and MDCK-MDR1/CYP3A4 were the negative and positive control, respectively. Through the selection, several monoclones had a relatively high expression of CYP3A4.

The toxicity of CYP3A4/P-gp substrates, such as paclitaxel, decreased for two pathways, which were metabolized by CYP3A4 (Vignati et al. 2005) and effluxed by P-gp. The toxicity clearance pathway plays an important role in the intestine. P-gp interacts with drugs and effluxes them to the intestinal lumen, so it could decrease the toxicity of this kind of drugs. The metabolic enzyme CYP3A4 detoxifies drugs through metabolizing them to less toxic metabolites. In the intestine, there might be interplay between P-gp and CYP3A4 in the course of biotransformation. The double-transfected cells were used to elaborate the mechanism of drug detoxification. As shown

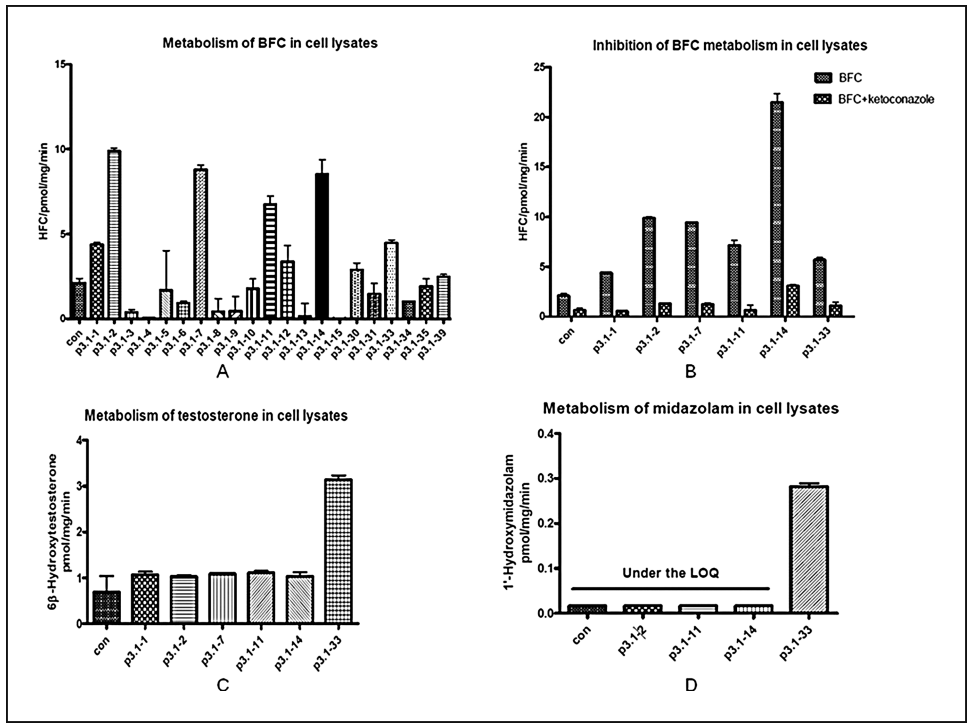

Fig. 3: Selection and determination of high-activity CYP3A4 monoclones .A. Selection of MDCK-MDR1/CYP3A4 clones by BFC metabolism activities in cell lysates. The control was metabolism activities in MDCK-MDR1 cells (n = 3). B. Inhibition of BFC metabolism by ketoconazle. The control was metabolism activities in MDCK-MDR1 cells (n = 3). C. Selection of MDCK-MDR1/CYP3A4 clones by testosterone 6β-hydroxylase activity in cell lysates. The control was metabolism activities in MDCK-MDR1 cells (n = 3).D. Selection of MDCK-MDR1/CYP3A4 clones by midazolam 1-hydroxylase activity in cell lysates. The control was metabolism activities in MDCK-MDR1 cells (n = 5).

in Fig. 5, due to paclitaxel interaction with P-gp or CYP3A4 in the cells, the IC_{50} value increased significantly compared with the MDCK cells. The IC_{50} value increased dramatically in the double-transfected MDCK-MDR1/CYP3A4 cells compared with the single-transfected cells. Therefore, an interplay between P-gp and CYP3A4 was suggested.

4. Experimental

4.1. Materials

MDCK cells were purchased from the Peking Union Medical College. The pcDNA^{TM}3.1(+) /Hygro and Lipofectamine^{TM} LTX were purchased from Invitrogen. The pMD19-T was obtained from Takara. T4 DNA ligase and two restriction enzymes *Kpn* I and *Xho* I were purchased from MBI Fermantas Company. Monoclonal antibody against His tag, horseradish peroxidase conjugated goat anti-mouse IgG antibody were separately received from Merck and Biosciences Santa Cruz Biotechnology. Hypgro B solution was obtained from Roche Molecular Biochemicals, and its concentration was 50 mg/mL. 6β-Hydroxylation testosterone, 1-hydroxylation midazolam, citrate dehydrogenase, citric acid trisodium salt, NADPH and NADP were all purchased from Sigma-Aldrich. 7-Hydroxy-4-(trifluoromethy) coumarin (HFC) was obtained from ACROS. P450-Glo^{TM} CYP3A4 Assay (Luciferin-IPA) was purchased from Promega Company. All solvents used were HPLC grade and all chemicals were analytical grade.

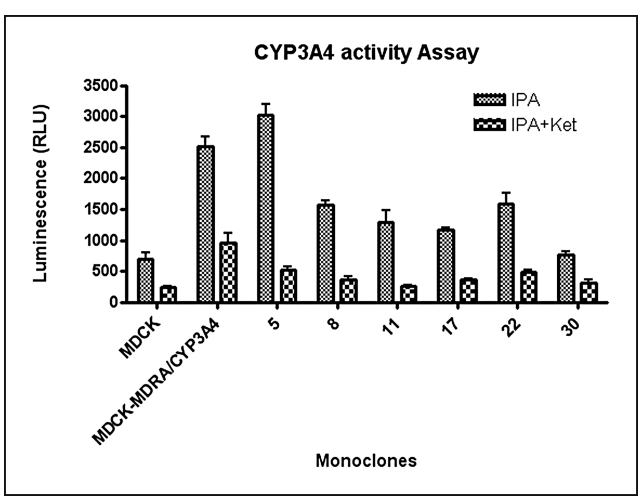

Fig. 4: Determination of CYP3A4 in the single-transfected cell lines MDCK-CYP3A4. MDCK and MDCK-MDR1/CYP3A4 were the negative and positive control respectively. 5,8,11,17,22,30 represented the monoclones number. Compared with the negative control cells MDCK, monoclone number 5 had the highest activities, which especially exceeded the positive control. Ketoconazole was chosen as the inhibitor to inhibit the CYP3A4's activity. In conclusion, monoclone number 5 was identified as the right cells, named as MDCK-CYP3A4.

4.2. Methods

4.2.1. Construction of plasmid vector

Human CYP3A4 cDNA was firstly cloned into pMD19-T vector by T-A clone. After being correctly sequenced, the pMD19-T /CYP3A4 plasmids

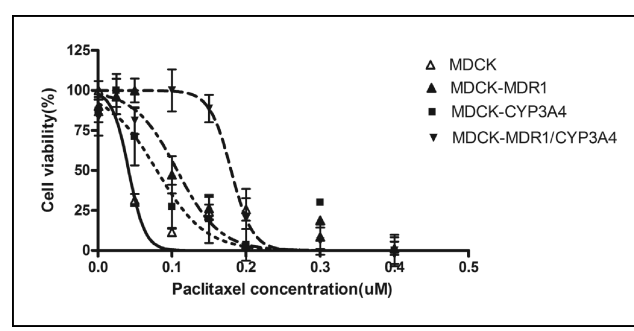

Fig. 5: Cell viability assay of paclitaxel in four cell lines. The results showed that IC_{50} values toward paclitaxel were 0.04 μM, 0.08 μM, 0.11 μM, 0.18 μM in the cell lines of MDCK, MDCK-CYP3A4, MDCK-MDR1, and MDCK-MDR1/CYP3A4 respectively. Due to paclitaxel effluxed by P-gp in the MDCK-MDR1 cells, the IC_{50} value increased significantly compared with the cells MDCK. Moreover, paclitaxel was metabolized by CYP3A4 to be less toxic metabolites, which resulted in increase of the IC_{50} values compared with control cells. The IC_{50} value increased dramatically in the double-transfected MDCK-MDR1/CYP3A4 cells compared with the single-transfected cells.

and pcDNA3.1 (+)/ Hypgro vectors were digested by *Kpn* I and *Xho* I restriction enzymes at the same time. The two fragments were extracted and ligated overnight at 16 °C. The colonies were picked; the plasmids were extracted and identified by double digestion. Recombinant plasmid was constructed, named pcDNA3.1/ Hypgro/CYP3A4.

4.2.2. Cell culture and transfection

MDCK-MDR1 cells stably expressing P-gp have been constructed in our laboratory previously (Liu et al. 2009). MDCK, MDCK-MDR1 cells were both cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, California, USA) supplemented with 10% fetal bovine serum (Invitrogen, California, USA), 100 U/ml penicillin, and 100 U/ml streptomycin at 37 °C, 95% humidity, and 5% CO_2. The transfection of plasmids into MDCK and MDCK-MDR1 cells was performed using Lipofectamine™ LTX (Invitrogen,California,USA). At 50%∼70% confluency, cells in 6-well plate were exposed to serum-free DMEM containing plasmid and lipofectamine. After 6 h transfection, the former plasmid-lipofectamine solution was removed, and the complete medium, consisting of DMEM supplemented with 10% fetal bovine serum, was added. After 24 h of Hygro B selection (400 mg/L), monocolonies were screened for the expression of CYP3A4.

4.2.3. Immunoblot analysis

Cells were harvested from the cell culture plates and collected in ice-cold PBS (Ca^{2+} and Mg^{2+} free). Then, the samples were washed twice with PBS, resuspended in lysis buffer (10 mM Tris-HCl, 10 mM KCl, and 1.5 mM $MgCl_2$, pH 7.4, with protease inhibitors: 2 mg/ml leupeptin, 2 mg/ml aprotinin, and 1 mM PMSF), sonicated on ice, and stored at − 80 °C until analysis. Protein concentrations were measured using the BCA protein assay with BSA as a standard. The samples were electrophoresed on 8%–12% gradient gels and transferred to nitrocellulose using standard techniques. The blot was blocked in 5% non-fat milk and incubated with primary antibody anti-his tag (1:1500 dilution) (Merck), washed with TBST (Tris-buffered saline containing 0.05% Tween-20), and incubated with secondary HRP conjugated antibody, goat anti-mouse for CYP3A4 (1:10,000 dilution). The signal was observed using ECL detection.

4.2.4. Analysis of CYP3A4 enzymatic activity

Three substrates were applied to determine cytochrome P450 3A4 activity, which were BFC, testosterone and midazolam.

The NADPH-dependent metabolism of BFC was studied in incubation mixtures containing 50 μM BFC, 0.5 mg/mL of microsomal protein, 50 mM phosphate buffer (pH 7.4) and an isocitrate/isocitric dehydrogenase regenerating system in a final volume of 100 μL. Stock solutions of BFC were prepared in methanol and diluted in 1:1000 with 50 mM phosphate buffer, and accordingly, the final methanol concentration in the incubations was 0.1% (v/v). After pre-incubation at 37 °C for 5 min, 2 mM NADPH was added to initiate the reaction. After incubation at 37 °C for 120 min, 0.2 mL ice-cold methanol (Merck. Germany) was added into the reaction mixture to terminate the reaction. The samples were centrifuged at 13,000 g for 10 min at 4 °C and with 200 μL aliquot removed into a black 96-well plate. All fluorescence measurements were performed with a luminescence spectrophotometer (Molecular Devices, California, U.S.A) equipped with a Spectramax M2 Microplate reader. HFC concentration of the samples was detected by fluorescence measurement at an excitation wavelength of 410 nm and an emission wavelength of 510 nm.

The concentrations of testosterone and midazolam were 20 μM and 50 μM, respectively. The other incubation conditions were the same as for 7-BFC. The detection of testosterone and its metabolite (6β-hydroxylation testosterone) was performed on an Agilent HPLC system. Chromatography was performed on an Agilent C_{18} column (4.6 × 250 mm, 5 μm). The mobile phase consisted of methanol–water with a gradient elution starting at 5% methanol and progressing linearly to 70% methanol over 15 min and then returning to 5% methanol. The flow rate was 1.0 mL/min. The sample volume was 100 μL and the column effluent was monitored by ultraviolet absorbance at 220 nm. The detection of midazolam and its metabolite were performed on a triple-quadruple tandem mass spectrometer (Waters,Acquity TQD) using positive ion mode electrospray ionization (ESI) in the multiple reaction monitoring (MRM) mode. The MS/MS ion transitions monitored were *m/z* 341.79 > 202.98 for 1-hydroxylation midazolam. Chromatographic separation was achieved by an Xbridge™ C18 column (2.1 mm × 50 mm, 3.5 μm; Waters). The mobile phase consisted of acetonitrile and 10 mM ammonium acetate solution containing 0.1% formic acid with a gradient elution starting at 25% acetonitrile and progressing linearly to 90% acetonitrile over 2.5 min and then returning to 25% acetonitrile. The total runtime was 3.5 min. The mobile phase was delivered at a flow rate of 0.2 mL/min and introduced into ESI source with no split, and the effluent from the chromatographic column was switched to the ESI^+ MS/MS. Operating parameters used in this study were as follows: nitrogen was used as the desolvation and cone gas with a flow rate of 500 and 0 L/h, respectively; the source and desolvation gas temperature were set at 120 °C and 350 °C, respectively; capillary voltage was set at 4 kV, cone voltage was 23 V, and collision energy was 25 eV (1-hydroxylation midazolam). Data acquisition and processing were performed using Masslynx 4.1 software and Quanlynx V4.1 (Micromass, Manchester, UK).

4.2.5. P450 Glo Assay

The activity of CYP3A4 in the single-transfected cell line MDCK-CYP3A4 was assayed using the luciferin-IPA substrate (Promega, cat V9001), which was prepared as a 3 mM stock solution in dimethyl sulfoxide (DMSO). Cells were plated in the 96-well plates and the culture medium was replaced with fresh medium containing a luminogenic CYP substrate IPA (1:1000 dilution). After 1 h incubation at 37 °C, 25 μL of culture medium was transfered to the 1.5 mL centrifuge tube and 25 μL of luciferin detection reagent was added to initiate a luminescent reaction. The plate was incubated at room temperature for 20 min, and then the luminescence was read using a luminometer. One second per well was chosen as an integration time. To determine background luminescence, luminogenic substrate in medium was added to a set of empty wells (no cells). Net signals were calculated by subtracting background luminescence values (no-cell control) from test compound-treated and untreated (vehicle control) values.

4.2.6. MTT Assay

Four cells (MDCK, MDCK-CYP3A4, MDCK-MDR1 and MDCK-MDR1/CYP3A4) were plated in 96-well plates (Costar, Ann Arbor, MI) and 100 μL medium with different concentrations of drugs or chemicals was added into each well. A serial of paclitaxel concentrations were set at 0.025–0.4 μM. The incubation time was 72 h. MTT was dissolved in PBS, usually at a concentration of 5 mg/ml, sterilized by filtration. It was diluted with culture medium by 10 times and added to each well. After a further period of incubation, the medium was aspirated from the wells, and 150 μL DMSO was added to each well. The plates were vigorously shaken for 10 min to solubilize the MTT-formazan product. The absorbance was read using SpectraMax M2 reader (Molecular Devices, California, U.S.A) at a wavelength of 490 nm. Values IC_{50} were calculated using Prism 4.0 soft. Each experiment was conducted in triplicate; the data were analyzed separately from each experiment.

4.2.7. Statistical analysis

All values were expressed as mean ± S.D. Statistical analyses were conducted using one-way ANOVA with Dunnett's posttest, and multiple variances were analyzed by two-way ANOVA (GraphPad Prism). P values less than 0.05 was considered as statistically significant.

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