Inhibition of P-glycoprotein, multidrug resistance-associated protein 2 and cytochrome P450 3A4 improves the oral absorption of octreotide in rats with portal hypertension

XIAO-YU SUN[1], ZHI-JUN DUAN[1], ZHEN LIU[1], SHUN-XIONG TANG[2], YANG LI[3,4], SHOU-CHENG HE[1], QIU-MING WANG[1] and QING-YONG CHANG[5]

[1]Department of Gastroenterology, First Affiliated Hospital of Dalian Medical University, Dalian, Liaoning 116011; [2]Department of Invasive Technology, Affiliated Zhongshan Hospital of Dalian University, Dalian, Liaoning 116001; [3]Department of Clinical Pharmacology, College of Pharmacy, Dalian Medical University, Dalian, Liaoning 116023; [4]Department of Breast Surgery, Liaoning Cancer Hospital & Institute, Shenyang, Liaoning 110042; [5]Department of Neurosurgery, Affiliated Zhongshan Hospital of Dalian University, Dalian, Liaoning 116001, P.R. China

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Abstract. The aim of the present study was to increase the intestinal transport of octreotide (OCT) by targeting the first-pass impact to identify a potential method for decreasing portal vein pressure (PVP) using oral OCT. Thus, the bioavailability of intestinally absorbed OCT was evaluated in normal rats and rats with portal hypertension (PH) that had been administered P-glycoprotein/multidrug resistance-associated protein 2/cytochrome P450 3A4 (P-gp/MRP2/CYP3A4) inhibitors. The mRNA and protein expression levels of P-gp, MRP2 and CYP3A4 were evaluated in normal and PH rats with or without OCT and the inhibitors using RT-PCR, western blot and immunohistochemical analyses. The potential effects of the inhibitor administration on PVP were also examined. The results suggest that P-gp, MRP2 and CYP3A4 play important roles in prohibiting the enteral absorption of OCT, particularly under a PH environment. Moreover, inhibitors of P-gp, MRP2 and CYP3A4 decrease the first-pass effects of OCT and effectively reduce PVP under PH conditions. Therefore, the present results suggest P-gp, MRP2 and CYP3A4 are key factors in the intestinal absorption of OCT. The inhibition of P-gp, MRP2 and CYP3A4 can markedly decrease the first-pass effects of OCT, and their use may facilitate the use of orally administered OCT.

Correspondence to: Professor Zhi-Jun Duan, Department of Gastroenterology, First Affiliated Hospital of Dalian Medical University, 222 Zhongshan Road, Dalian, Liaoning 116011, P.R. China
E-mail: cathydoctor@sina.com

Professor Qing-Yong Chang, Department of Neurosurgery, Affiliated Zhongshan Hospital of Dalian University, 222 Zhongshan Road, Dalian, Liaoning 116001, P.R. China
E-mail: qychang0409@163.com

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Introduction

Portal hypertension (PH) is the primary factor resulting in serious complications and mortality in patients with liver cirrhosis (1-3). Early and consistent intervention of PH has been recommended in the clinic to reduce morbidity and mortality of patients with cirrhosis; however, currently, there are no ideal oral medicines for limiting PH due to side effects and poor efficacy (4).

Octreotide (OCT; Fig. 1), an octapeptide that pharmacologically mimics natural somatostatin, can alleviate PH through intravenous (i.v.) or intramuscular infusion, with limited side effects (1). As OCT is stable against enzymatic degradation, it may partially overcome the problems associated with therapeutically active peptides, which are often limited by their short biological half-lives (5). OCT may be a potential oral medicine for long-time use that can persistently decrease portal vein pressure (PVP). A research study by Tuvia *et al* suggested that orally administered OCT may be an alternative to parenteral OCT treatment for patients with acromegaly (6). However, the extensive intestinal and hepatic first-pass elimination of OCT limits its clinical application via oral administration (2,7).

Previous studies have attempted to improve the intestinal absorption of OCT by changing its chemical structure or adding an absorption enhancer to increase paracellular absorption, but with little effect (2,8,9). The elucidation of the factors that affect the oral absorption of OCT would facilitate the development of strategies to improve its absorption. In addition, it is unclear whether orally administrated OCT can decrease PVP effectively. In a previous study, we have indicated that the effect of hepatic first-pass is minor on the PH state (unpublished data). Furthermore, the intestinal transporters of drugs, the ATP-driven drug efflux pumps, P-glycoprotein (P-gp; MDR1-gene product, mdr1a and mdr1b subtypes) (10,11), multidrug resistance-associated protein 2 (MRP2; mrp2-gene

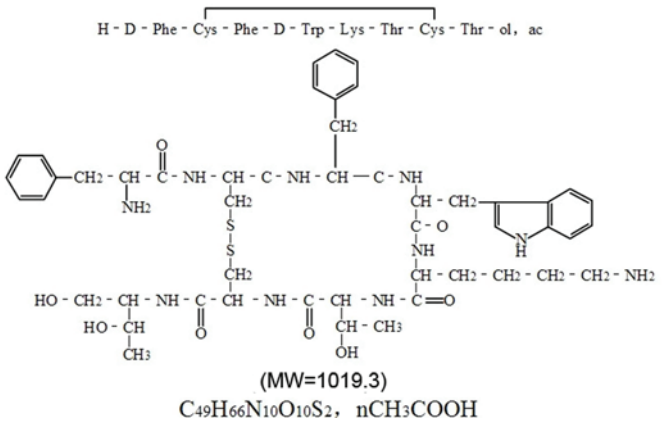

Figure 1. Chemical structure of octreotide.

product) and cytochrome P450 3A4 (CYP3A4; cyp3A1-gene product in rats) are among the most important CYP enzymes in the small intestine, functioning as a barrier against enteral absorption of OCT under the conditions of PH. P-gp, MRP2 and CYP3A4 are crucial factors in the intestinal first-pass effects of OCT, thus affecting its oral absorption (data to be published elsewhere). However, the inhibition of the enteral absorption of OCT by P-gp, MRP2 and CYP3A4 has not been evaluated, particularly with respect to the stage of cirrhosis with PH. The aim of the current study was to determine whether inhibitors of intestinal first-pass elimination can be used to effectively increase OCT absorption, thus decreasing PVP.

Materials and methods

Animal care. Male Sprague Dawley rats (n=52; age, 4-6 months; weight, 200-230 g) were obtained from the Experimental Animal Centre at Dalian Medical University (Liaoning, China). This study was performed in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the Canadian Council on Animal Care. The protocol was approved by the Committee on the Ethics of Animal Experiments of Dalian Medical University (permit no. L2008102001) in accordance with the Code of Ethics of the World Medical Association. Ether anaesthesia was persistently used for all surgeries, and all efforts were made to mitigate animal suffering. Under ether anaesthesia, the animals were euthanised via cervical dislocation.

Animal model. The rats were fed in a specific pathogen-free centre at 24-26˚C with a relative humidity of 60-65%. The rats were allowed free access to water and fed a chow diet for 3 days prior to any experimental protocols. Prior to the pharmacokinetic experiments, the animals were fasted for 12 h with water available *ad libitum*. Biliary cirrhosis with PH was induced via bile duct ligation (BDL) as previously described (12). The surgical procedures were approved by the Animal Care and Use Committee of Dalian Medical University. The laparotomy was performed under anaesthesia with ether. The bile duct was isolated and double-ligated using a 3-0 silk suture. The abdominal wall and skin were closed with a 4-0 silk suture, and the antibiotic gentamicin (0.3 ml; Sigma-Aldrich) was injected intramuscularly. The rats were continuously fed and housed for a four-week period after surgery. The administration methods and measurements for each group will be further specified when mentioned. The jejunum was selected in the present study as it is the main site of OCT absorption in the intestine (9). For the experiment of intrajejunal (i.j.) injection, a mid-line laparotomy was made and the caecum was removed to create a more open operating field. A 15-cm loop of mid-jejunum was located ~30 cm distally from the pylorus. Then the portex tubing was cannulated through stab incisions in the left side of the abdominal wall.

In vivo absorption evaluation. Normal rats were randomly assigned to two groups: i) I.j. injection of 2 ml OCT (0.1 mg/kg; purity, 99%; Chengdu Xinlinbang Bio-pharmaceutical Co., Ltd., Chengdu, China); and ii) i.v. injection (bolus method) of 0.2 ml OCT (0.01 mg/kg) through the internal jugular vein (n=4 per group). The PH rats were randomly assigned to three groups (n=4 per group): i) I.j. injection of 2 ml OCT (0.1 mg/kg); ii) i.v. infusion of 0.2 ml OCT (0.01 mg/kg); and iii) i.j. injection of 4 ml OCT (0.1 mg/kg) and P-gp/MRP2/CYP3A4 mixed inhibitors (4.9 mg/kg verapamil hydrochloride, 300 mg/kg probenecid and 5.3 mg/kg ketoconazole; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). The beginning of the jejunum was localised 5 cm distal to the Treitz ligament. A 0.3-ml blood sample was collected from the external jugular vein at 1, 5, 10, 15, 30, 45, 60, 120, 240, 360, 480 and 720 min for OCT determination via liquid chromatography-tandem mass spectrometry (LC-MS/MS). The main pharmacokinetic parameters were calculated according to the method described by Thanou *et al* (13), using the Bioavailability Program Package. Absolute bioavailability values (*F*) and absorption enhancement ratios (*ER*) were obtained according to the following formulae:

$$F\ (\%)=AUC_{i.j.} \times D_{i.v.}/AUC_{i.v.} \times D_{i.j.} \times 100.$$
$$ER=F_{(OCT+inhibitors)}/F_{(OCT\ alone).}$$

in which D is the administered dose and AUC (area under the curve) is the individual concentration-time curve.

Effects of OCT and P-gp/MRP2/CYP3A4 mixed inhibitors on the expression of P-gp/MRP2/CYP3A4 in the intestinal mucosa of rats with or without PH. Rats were randomly assigned to the following groups (n=4 per group): i) Normal rats (N group); ii) normal rats with oral administration of OCT (N + OCT group); iii) PH rats (PH Group); iv) PH rats with oral administration of OCT (PH + OCT Group); and v) PH rats with oral administration of OCT and P-gp/MRP2/CYP3A4 mixed inhibitors (verapamil hydrochloride, probenecid, and ketoconazole) (PH + OCT + I group). The administered drug doses were identical to those used in the *in vivo* absorption experiment and were administered for 3 days; untreated rats were fed *ad libitum* for 3 days. The tissue samples collected from the upper jejunum of each group were used for reverse transcription-polymerase chain reaction (RT-PCR), western blot and immunohistochemistry analyses.

RT-PCR. RT-PCR was performed as previously described (14). The jejunum tissue samples collected from the upper jejunum

Table I. Nucleotide sequences and cycling conditions of rat reverse transcription-polymerase chain reaction primers.

Gene	Primer sequence (5'-3')	Denaturation	Annealing	Elongation	Product size (bp)
mdr1a					
F	GATGGAATTGATAATGTGGACA	94˚C (30 sec)	48˚C (30 sec)	72˚C (45 sec)	352
R	AAGGATCAGGAACAATAAA				
mdr1b					
F	GAAATAATGCTTATGAATCCCAAAG	94˚C (30 sec)	54˚C (30 sec)	72˚C (45 sec)	327
R	GGTTTCATGGTCGTCGTCTCTTGA				
mrp2					
F	ACCTTCCACGTAGTGATCCT	94˚C (30 sec)	56˚C (30 sec)	72˚C (45 sec)	449
R	ACCTGCTAAGATGGACGGTC				
cyp3A1					
F	GAGGAGTAATTTGCTGACAGAACCTGC	95˚C (15 sec)	57˚C (30 sec)	72˚C (30 sec)	149
R	CCAGGAATCCCCTGTTTCTTGAA				

F, forward; R, reverse; mrp2, multidrug-resistance associated protein 2; cyp3A1, cytochrome P450 3A1.

of each group (normal rats as a control group) were stored in an RNA stabiliser (Dalian Pauley Shield Bio-Engineering Co., Ltd., Dalian, China) and were rapidly frozen to prevent possible RNA degradation. Total RNA was extracted from each perfused sample using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Carlsbad, CA, USA) according to the manufacturer's recommendation and analysed by ultraviolet spectrophotometry. RT-PCR was performed using the PrimeScript™ RT-PCR kit (cat. no. RR014A) as recommended by the manufacturer (Takara Biotechnology Co., Ltd., Dalian, China). After treatment with DNAse (D7076; Beyotime Institute of Biotechnology, Shanghai, China), cDNA was subsequently amplified using a TC512 thermal cycler (Bibby Scientific Ltd., Stone, UK). RNA samples (500 ng) were reverse transcribed and immediately amplified by PCR. Reverse transcription was performed for 10 min at 30˚C, followed by 30 min at 42˚C, and the samples were subsequently heated for 5 min at 95˚C to terminate the reverse transcription reaction. The primers for β-actin were 5'-GGGACCTGACAGACTACCTC-3' (forward) and 5'-GACAGCACTGTGTTGGCATAG-3' (reverse), which yielded a PCR product of 351 bp. The primers of mdr1, mdr2, mrp2 and cyp3A1 (Takara Biotechnology Co., Ltd.) and PCR cycling conditions are summarised in Table I. Quantity One software (version 4.40; Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used to analyse the densities of the bands on the gel. β-actin served as a housekeeping gene constitutively expressed at a constant amount, and the level of each mRNA in each group was normalised against the corresponding β-actin mRNA level. All samples were amplified in triplicate.

Western blot analysis. Tissue samples of the upper jejunums were frozen and stored in rapid immunoprecipitation assay buffer (Beyotime Institute of Biotechnology). Protein concentration was determined according to the Lowry method using bovine serum albumin as a standard. The western blot assay was performed as previously described (12). Total protein (20 μg/ml) was separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (EMD Millipore, Bedford, MA, USA). The membrane was blocked with 5% skimmed milk powder with Tris-buffered saline-Tween 20 (TBST) for 2 h at 37˚C and then incubated with primary antibodies for for 2 h at 37˚C. The primary antibodies were mouse P-gp monoclonal antibody (1:75; MA1-26528; Pierce Biotechnology; Thermo Fisher Scientific, Inc., Waltham, MA, USA), rabbit-anti-mouse MRP2 monoclonal antibody (1:100; ab203397), rabbit-anti-mouse CYP3A4 polyclonal antibody (1:1,000; ab3572; Abcam, Cambridge, MA, USA) and monoclonal antibody for β-actin (1:1,000; TA-09; Beijing Zhongshan Golden Bridge Biological Technology Co., Ltd., Beijing, China). After incubation, the membrane was washed three times with TBST and then incubated with horseradish peroxidase (HRP)-linked secondary antibody, anti-rabbit (1:800; A0208) or anti-mouse (1:1,000; A0208) IgG (Beyotime Institute of Biotechnology) at room temperature for 2 h. The membrane was immersed for 1 min in enhanced chemiluminescence detection reagent (Amersham; GE Healthcare Life Sciences, Chalfont, UK). Protein bands were visualised and photographed under transmitted ultraviolet light for semiquantitative measurement based on band densitometry. Quantity One software was used to analyse the densities of the bands.

Immunohistochemistry. Tissue samples of the upper jejunums were prepared for immunohistochemical analysis as previously described (12). The primary antibodies for P-gp, MRP2 and CYP3A4 were the same as those used in the western blot analysis, and were used at dilutions of 1:58, 1:100 and 1:70, respectively, with incubation at room temperature for 1-2 h. The primary antibody was replaced with phosphate-buffered saline (Beyotime Institute of Biotechnology) to serve as a negative control. Goat anti-mouse HRP (IgG, H&L) (1:1,000; ab6789; Abcam, Cambridge, MA, USA) was used as the secondary antibody, with incubation at room temperature for 15 min. DAB was used as the chromogen. Yellow staining in the membranes and cytoplasm of cells indicated P-gp and MRP2 positivity, while yellow material in the membrane only

indicated CYP3A4-positivity. A total of five high-power microscopic fields were randomly chosen per slide. Cell staining was assigned via four potential scores, and cell staining intensity was scored based on its colour. The final score was defined as the staining intensity x percentage of positive cells (15). The mean score of five fields was used to compare the five groups.

Effects of P-gp/MRP2/CYP3A4 mixed inhibitors on PVP. The PH rats were randomly assigned to three groups (n=4 per group): i) Without OCT (PH group); ii) with oral administration of OCT (PH + OCT group); and iii) with oral administration of OCT and P-gp/MRP2/CYP3A4 mixed inhibitors (PH + OCT + I group). The oral doses were the same as those used in the previously described *in vivo* absorption experiment and were administered for 3 days. The rats were anaesthetised, and a catheter connected to a pressure transducer (BL-420F; Chengdu Technology and Market Co., Ltd., Chengdu, China) was placed in the portal vein to measure the PVP.

Determination of OCT by LC-MS/MS. An Agilent LC system (HP1200; Agilent Technology, Inc., Palo Alto, CA, USA) was used to analyse the samples. Isocratic chromatographic separation was performed using a Hypersil BDS-C18 column (150 x 4.6; i.d., 5 μm; Dalian Elite Analytical Instruments Co. Ltd., Dalian, China), which was maintained at room temperature. A mixture of methanol and water (60:40, v/v) with 0.1% formic acid was used as the mobile phase at a flow rate of 0.5 ml/min. An API 3200 triple-quadrupole mass spectrometer (Applied Biosystems; Thermo Fisher Scientific, Inc., Foster City, CA, USA) was operated using a turbo ion spray interface in positive ion mode. The instrument was operated using an ion spray voltage at +4 psi, heater gas temperature of 500˚C, nebuliser gas (Gas 1) at 40 psi, heater gas (Gas 2) at 40 psi, curtain gas at 10 psi and collision gas at 16 psi.

The declustering potential was set at 30 V for the analyses and internal standard (1 μg/ml Gly-Sar, IS). Multiple reaction monitoring (MRM) was employed for data acquisition. The optimised MRM fragmentation transition was 510.2-120.1 m/z with a collision energy of 50 eV for OCT. The dwell time for each transition was 200 msec. Data processing was performed using the Analyst 1.4.1 software package (Applied Biosystems).

Sample preparation for LC-MS/MS analysis. Frozen samples were thawed at room temperature prior to preparation. Cell lysate (200-μl aliquot) contained 1 ml Tris (Beyotime Institute of Biotechnology), 99 ml D-Hanks and 200 μl methyl cyanides (both Sigma-Aldrich). The mixture was vortexed for 1 min and centrifuged at 12,000 x g for 10 min, and the supernatants were transferred to another glass tube. The supernatants were then evaporated to dryness at 40˚C under nitrogen. A 10-μl aliquot was injected for LC-MS/MS analysis. A total of 200 μl methanol was added to 50-μl samples of blood plasma, which were vortexed and centrifuged as described above to remove the protein precipitate. A total of 200 μl of the supernatants was transferred and evaporated. The residues were then reconstituted with 100 μl mobile phase. An aliquot of 10 μl was used for LC-MS/MS analysis.

Statistical analysis. SPSS version 11.5 (SPSS, Inc., Chicago, IL, USA) was used for data analysis. All measurements are expressed as the mean ± standard deviation. A one-way analysis of variance was performed to test for significant differences between multiple treatments for a given parameter. The non-paired *t*-test was used to assess significant differences between mean values. Values of P<0.05 or P<0.01 were considered to indicate a statistically significant difference.

Table II. Intravenous administration of octreotide in rats.

Parameter	Normal rats	PH rats
$t_{1/2}$ (min)	81.47±6.51	82.34±4.91
V_d (ml/kg)	2.95±0.18	1.53±0.03[a]
AUC (ng/ml x min)	88,283.17±7,062.72	628,615±3,756.92[b]

Data are presented as the mean ± standard deviation. [a]P<0.05, PH rats vs. normal rats. [b]P<0.01, PH rats vs. normal rats. PH, portal hypertension; $t_{1/2}$, half life; V_d, apparent volume of distribution; AUC, area under curve.

Results

Influence of PH and P-gp/MRP2/CYP3A4 mixed inhibitors on OCT absorption in rats. *In vivo* experiments were performed to evaluate the effects of P-gp/MRP2/CYP3A4 mixed inhibitors on the rate and efficiency of OCT absorption in rats and to compare the absorption differences between normal and PH rats. As shown in Tables II and III, OCT was rapidly dispelled from plasma following i.v. infusion, and OCT underwent slower elimination or more rapid absorption in normal rats compared with PH rats. The rapid elimination in the PH state may be due to increased expression or activities of transporters and metabolic enzymes in the liver. Longer T_{max} and lower C_{max} and AUC values were observed in PH rats compared with normal rats upon i.j. administration of OCT, indicating that increased expression levels or activities of transporters and metabolic enzymes may occur in the intestines of PH rats, thereby inhibiting OCT absorption. Shorter T_{max} and higher C_{max} and AUC values were observed in the group administered OCT with P-gp/MRP2/CYP3A4 mixed inhibitors compared with normal or PH rats without inhibitors. In addition, *F* and *ER* increased ~4-fold in PH rats when administered mixed inhibitors. These results indicate that mixed inhibitors may markedly improve OCT absorption and that P-gp, MRP2 and CYP3A4 may function as key factors in the transportation and metabolism of OCT in the intestine.

Effects of OCT and P-gp/MRP2/CYP3A4 mixed inhibitors on mRNA and protein expression levels of P-gp/MRP2/CYP3A4 in the intestinal mucosa of rats with or without PH. RT-PCR analysis revealed that the mRNA expression levels of P-gp/MRP2/CYP3A4 was significantly higher in group PH than in group N (P<0.05). The mRNA expression of these genes was also increased by the use of OCT (group N + OCT > group N, group PH + OCT > group PH; P<0.05) and was the lowest in group PH + OCT + I due to inhibitor usage (P<0.01). No significant difference in mRNA expression was observed between group N + OCT and group PH (P>0.05; Fig. 2A). The results of the western blot analysis of

Table III. Intestinal absorption of octreotide in rats by intra-jejunal injection.

Group	T_{max} (min)	C_{max} (ng/ml)	AUC_{0-12h} (ng/ml x min)	*F* (%)	*ER*
Normal rats	30	414±20.71	74,442.75±8,188.7	1.18±0.06	
PH rats	45	264.37±31.75	34,893.75±2,442.6[a]	3.95±0.24	3.35
PH rats with inhibitors	15	726.33±58.14	127,777.9±11,500.4[b,c]	14.47±1.01[b]	12.26

Data are presented as the mean ±standard deviation. [a]P<0.05 for PH rats vs. normal rats. [b]P<0.01 for PH rats with octreotide (OCT) and mixed inhibitors (verapamil hydrochloride, probenecid and ketoconazole) vs. PH rats with OCT. [c]P<0.01 for PH rats with OCT and mixed inhibitors (verapamil hydrochloride, probenecid and ketoconazole) vs. normal rats with OCT. T_{max}, time to reach plasma peak concentration; C_{max}, plasma peak concentration; AUC, area under curve; *F*, absolute bioavailability; *ER*, absorption enhancement ratio; PH, portal hypertension.

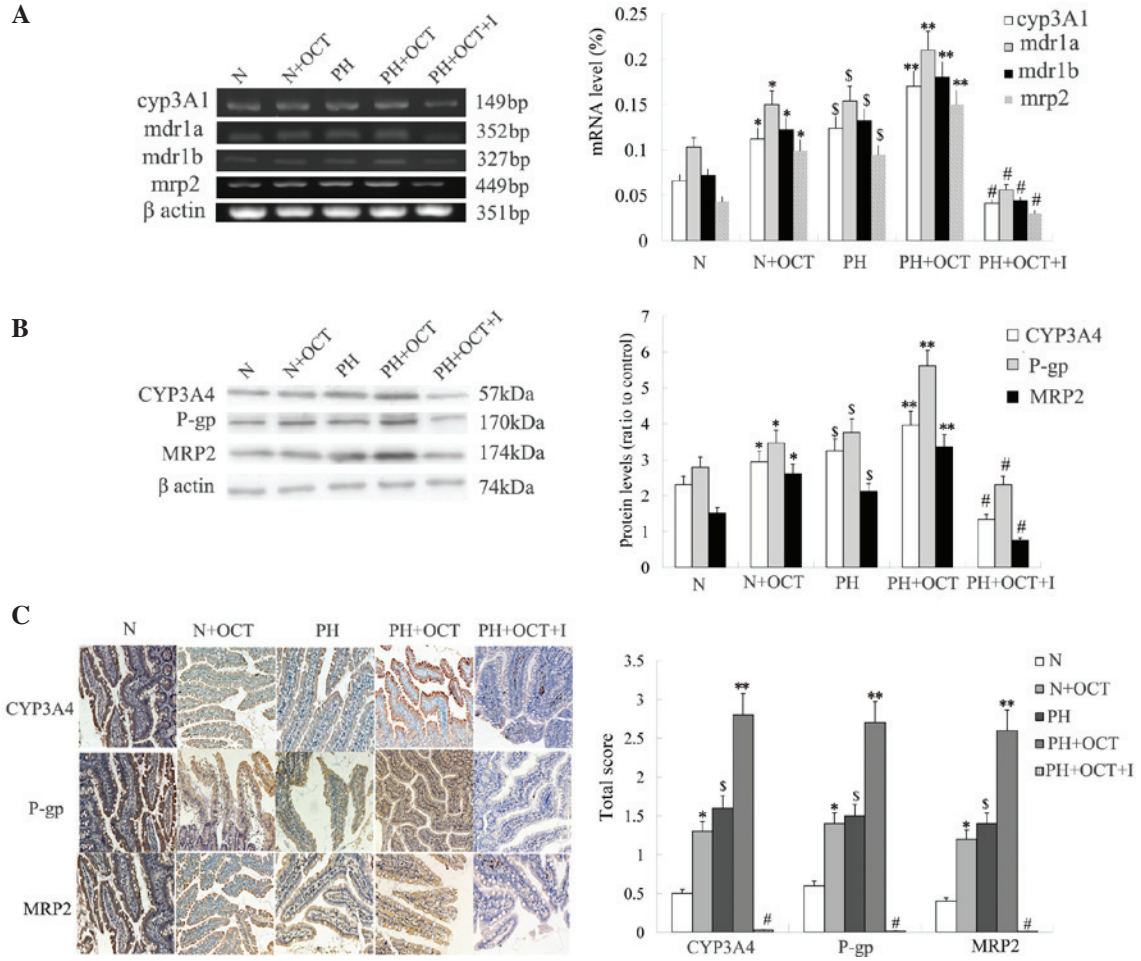

Figure 2. mRNA and protein expression levels of P-gp/MRP2/CYP3A4 in the intestinal mucosa of rats with or without PH. Data are expressed as the means ± standard deviation (n=4). A two-tailed unpaired *t*-test was used to assess significant differences. (A) Reverse transcription-polymerase chain reaction data showing the CYP3A1, MDR1 (mdr1a, mdr1b) and MRP2 expression levels in the rat intestine in each group. (B) Western blot data showing the CYP3A4, P-gp and MRP2 protein expression levels in the rat intestine in each group. (C) Immunohistochemistry showing the location and extent of CYP3A4, P-gp and MRP2 protein expression in the rat intestine in each group (magnification, x100). *P<0.05, Group N + OCT vs. Group N. **P<0.05, Group PH + OCT vs. Group PH; $P<0.05, Group PH vs. Group N; #P<0.01, Group PH + OCT + I vs. each other group. N, normal control; OCT, octreotide; PH, portal hypertension; I, inhibitors; cyp3a1, cytochrome P450 3A1; MRP2, multidrug-resistence associated protein 2; P-gp, P-glycoprotein.

protein expression were consistent with the RT-PCR results (Fig. 2B). Immunohistochemical analysis showed that the P-gp/MRP2/CYP3A4 scores for group PH + OCT were significantly higher than for the other groups (P<0.01; Fig. 2C) and were significantly higher in group PH compared with group N (P<0.05). Among all subgroups, the P-gp/MRP2/CYP3A4 scores in group PH + OCT + I were the lowest (P<0.01), and there was no significant difference between group N + OCT and group PH (P>0.05). These results indicate that the extended use of OCT could increase the mRNA and protein expression levels of P-gp/MRP2/CYP3A4 and that P-gp/MRP2/CYP3A4 are upregulated in PH rats.

Table IV. PVP in each group (n=4).

Group	PVP (mmHg)
N	9.24±0.76
PH	15.56±2.36[a]
PH + OCT	12.51±1.50[b]
PH + OCT + I	6.95±1.12[c,d]

Data are presented as the mean ± standard deviation. [a]P<0.01, PH vs. normal; [b]P<0.05, PH vs. PH + OCT; [c]P<0.01, PH + OCT + I vs. PH; [d]P<0.01, PH + OCT + I vs. PH + OCT. PVP, portal vein pressure; N, normal; PH, portal hypertension; OCT, octreotide; I, inhibitors (verapamil hydrochloride, probenecid and ketoconazole).

Effect on PVP of co-administration of OCT and mixed P-gp/MRP2/CYP3A4 inhibitors. The mean PVP level in group PH (15.56±2.36 mmHg) was higher than that in normal (9.24±0.76 mmHg; P<0.01) and group PH + OCT rats (12.51±1.50 mmHg; P<0.05). The PVP level in group PH + OCT + I (6.95±1.12 mmHg) was significantly lower than that in group PH or group PH + OCT (P<0.01) and was within normal levels. Oral administration of OCT with P-gp/MRP2/CYP3A4 mixed inhibitors thus effectively decreased PVP.

Discussion

In the present study, a rat model of biliary cirrhosis with PH was induced via BDL. This method was selected to reduce cholestasis in the intestine, easily induce pathological conditions of PH and diminish rat suffering.

To decrease the influence of gastric acid and enzymes, the i.j. injection method was used in the *in vivo* experiment. Verapamil hydrochloride and probenecid are specific inhibitors of P-gp and MRP2, respectively. Ketoconazole inhibits a wide range of CYP enzymes. However, the results of our previous study, involving experiments of intestinal microsome and recombinant human P450 CYP3A4, indicate that OCT is a substrate of CYP3A4 and that ketoconazole can decrease CYP3A4 content effectively (unpublished data).

In the present study it was observed that the gastrointestinal absorption of OCT markedly decreased under PH but was significantly increased upon administration of P-gp/MRP2/CYP3A4 mixed inhibitors. In addition, an approximately four-fold increase in absorption was observed when mixed inhibitors were administered to PH rats. Previous studies have indicated that OCT was a substrate for P-gp, MRP2 and CYP3A4 (4,16-18). Increased expression or activities of these were observed under a PH state to decrease the intestinal absorption of OCT. To investigate the mechanism underlying the effect of the mixed inhibitors on the first pass effects, we also evaluated the expression levels of P-gp/MRP2/CYP3A4 using RT-PCR, western blot and immunohistochemistry analyses. The results found that the mRNA and protein expression levels of P-gp/MRP2/CYP3A4 increased in PH model rats, and that the extended use of OCT further increased this expression under some conditions of liver damage due to physiological environment changes (such as PH, PH enteropathy, PH gastropathy and rich collateral circulation), as well as alterations in the intestinal flora. Moreover, mixed inhibitors were able to markedly decrease these expression levels. As OCT is clinically used to reduce PVP, the co-administration of OCT with mixed inhibitors to PH rats was evaluated in this study to examine its capacity to increase oral OCT bioavailability in a PH state. The study found that the oral administration of OCT with P-gp/MRP2/CYP3A4 mixed inhibitors effectively decreased PVP. Previous research has demonstrated that the decreased oral bioavailability of OCT was potentially associated with the transporters P-gp and MRP2 and the metabolic enzyme CYP3A4 (16-18). The upregulated expression or activities of P-gp/MRP2/CYP3A4 further decreased OCT bioavailability in PH model rats; the present study further indicated the extended use of OCT may affect this process. In addition, mixed inhibitors of P-gp, MRP2 and CYP3A4 markedly decreased the PVP.

Certain changes of efflux transporters and metabolic enzymes have been identified in the intestine and liver in liver diseases by previous studies (18-20). Wang *et al* found that P-gp expression and CYP isoenzymatic activities of the small intestine were enhanced in liver fibrosis, thus inducing decreased bioavailability and increased elimination of orally administered ofloxacin (21). A study has also indicated that the canalicular export pumps MRP2 are preserved and that MDR P-gp (MDR1, MDR3) is enhanced in patients with advanced-stage primary biliary cholangitis (22). Various adaptive responses, including the induction of intestinal, hepatic and renal bile acid transport proteins, could be triggered under conditions of cholestatic liver disease and increased concentrations of serum bile acid (20). The results of previous studies were consistent with those of the present study, indicating that the increased expression or activities of export pumps (P-gp and MRP2) and CYP enzymes (CYP3A4) may occur in cirrhosis complicated by PH in the intestine (21-23). P-gp, CYP3A4 and MRP2 may have been involved in intestinal first-pass effects. In addition, P-gp, MRP2 and CYP3A4 may affect the intestinal absorption of OCT.

Previous studies have primarily focused on investigating the desirable effects of P-gp and CYP3A4 on mediating drug transport through their inhibition (18,24,25). The results of a prior study suggested that the intestinal absorption of OCT may be increased and its bioavailability increased 23-fold by means of OCT microspheres with polyoxyethylene-24-cholesterol ether (9). Moreover, cationic polymer-chitosan and its derivatives, such as N-trimethyl chitosan chloride, could increase OCT bioavailability to five-fold (5) and chenodeoxycholic acid could increase it to 1.26%, with the absorption rate reaching 20.2% (26). Unexpectedly, the present study found that the inhibition of P-gp, MRP2 and CYP3A4 is a viable method for improving the efficacy of orally administered OCT to effectively decrease PH. Inhibiting P-gp, MRP2 and CYP3A4 may represent a general method for improving polypeptide absorption by oral administration.

In the present study, we did not clarify the optimal proportion of OCT and mixed inhibitors, and no preliminary toxicity studies were performed for pharmaceutical safety testing to consider the side effects. In addition, the specific molecular mechanisms by which P-gp/MRP2/CYP3A4 inhibitors are regulated in the intestines of PH rats remain unclear. It may be associated with the pregnane X receptor, retinoid X receptor alpha, protein kinase C, radixin protein, nuclear

factor kB, human cAMP response element-binding protein or CAATT box enhancer binding protein, which are reported to be the key factors involved in regulating CYP3A4, P-gp or MRP2 (19,27-31). Further studies are required to address these questions to ultimately achieve the oral absorption of OCT.

In conclusion, the present results suggest that P-gp, MRP2 and CYP3A4 may be involved in prohibiting the intestinal absorption of OCT. The effective inhibition of P-gp, MRP2 and CYP3A4 could be regarded as a targeted therapy to improve the oral absorption of OCT. The current study is also partially elucidates the oral absorption of polypeptides under pathological conditions of PH, thus supporting a basic theory to increase oral bioavailability by overcoming intestinal first-pass effects. Further studies are required to investigate the transportation and enzymolysis mechanisms that occur in the intestines of patients with cirrhosis complicated by PH. The appropriate clinical combination of OCT with the presently investigated inhibitors may yield an improved treatment approach for PH.

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