Decreased Oral Absorption of Cyclosporine A after Liver Ischemia-Reperfusion Injury in Rats: The Contribution of CYP3A and P-Glycoprotein to the First-Pass Metabolism in Intestinal Epithelial Cells

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ABSTRACT

The bioavailability of orally administrated cyclosporine A (CsA) is poor and variable in liver transplantation recipients. Little information is available about the effect of liver ischemia-reperfusion (I/R) injury, which is associated with liver transplantation, on the intestinal first-pass metabolism of CsA. In the present study, we investigated the pharmacokinetics of CsA after liver I/R and assessed the effect of liver I/R via CYP3A and P-glycoprotein (P-gp) on its intestinal first-pass metabolism. When CsA alone was administrated orally, the area under the concentration-time curve (AUC) in the I/R rats was significantly decreased compared with that in the sham rats. On the other hand, there were no significant differences in the AUC between I/R and sham rats when CsA was administrated intravenously or orally with ketoconazole. After intraloop administration of CsA to the small intestine (upper, middle, and lower portions) of the I/R and sham rats, the AUC0–15 min in the upper intestine was significantly lower in the I/R rats than in the sham rats. CYP3A activity and the expression levels of P-gp in the upper intestine of the I/R rats were significantly higher than those of the sham rats. Our study clearly demonstrates for the first time that liver I/R decreases the oral bioavailability of CsA and that this is attributable principally to increased first-pass metabolism mediated by CYP3A and P-gp in the upper small intestine. The present findings provide useful information for the etiology of liver I/R injury and appropriate use of CsA after liver transplantation.

Cyclosporine A (CsA) is a calcineurin inhibitor that is widely used to prevent acute rejection after liver transplantation (Kelly et al., 2004). CsA has a narrow therapeutic range and shows large inter- and intraindividual pharmacokinetic variability (Ptachcinski et al., 1986). Thus, therapeutic drug monitoring is clinically essential to achieving effective immunosuppressive therapy while avoiding toxicity and maintaining tolerability.

CsA is metabolized in the liver and small intestine through cytochrome P450 3A subfamilies, including CYP3A4 and CYP3A5 (Hebert, 1997). The activity of CYP3A accounts for approximately 30 and 70% of total cytochrome P450 activity in the liver and small intestine, respectively (Watkins et al., 1987; de Waziers et al., 1990; Paine et al., 1997), and intestinal first-pass metabolism mediated by CYP3A has been shown to be clinically important for CsA (Hebert et al., 1992; Wu et al., 1995). Moreover, it was reported that the intestinal absorption of CsA is also limited by intestinal P-glycoprotein (P-gp), which is an efflux pump encoded by the multidrug resistance 1 gene (Kaplan et al., 1999). We previously reported that the absorption of tacrolimus, which is a calcineurin inhibitor, is regulated by both P-gp and CYP3A in recipients of living donor liver transplantation and that it is important to know the expression level of P-gp and CYP3A to establish an appropriate dosage regimen (Goto et al., 2004; Masuda et al., 2005). Therefore, the pharmacokinetic variability of CsA could be explained in part by marked inter- and intraindividual heterogeneity in intestinal and hepatic CYP3A and P-gp expression and/or their function. Masuda et
reported that increased intestinal P-gp expression leads to a reduction in the bioavailability of orally administered CsA in recipients of living donor liver transplantations. However, the contribution of intestinal CYP3A activity to the changes of CsA pharmacokinetics in these patients is not fully understood.

Liver ischemia-reperfusion (I/R) injury associated with liver transplantation is an unsolved problem. Liver I/R injury is mediated by several processes that lead to hepatocellular damage, which is triggered when the liver is transiently deprived of oxygen during the organ procurement for transplantation and is later reoxygenated during reperfusion. Numerous factors have been reported to be involved in this pathophysiology, such as reactive oxygen species (Galaris et al., 2006), inflammatory cytokines (Frangogiannis, 2007), and activated leukocytes (Pacher and Gao, 2008).

In our previous report, we used the ileum I/R injury model in rats to evaluate the permeability of tacrolimus and to examine the molecular and functional variations of ileum P-gp. The results indicated that the absorption of tacrolimus is decreased by transient up-regulation of P-gp after ileum I/R (Omae et al., 2005). On the other hand, it was reported that the expression levels of CYP3A in the liver tissue decreased (Izushi et al., 2000), whereas multidrug resistance 1 mRNA increased (Tanaka et al., 2006) after liver I/R injury. However, no information is available to date on the relationship between the changes in the intestinal CYP3A or P-gp activity and the pharmacokinetics of CsA after liver I/R injury. In the present study, we used rats after liver I/R injury as a model for the conditions within a graft liver immediately after liver transplantation. Using this animal model, we examined the pharmacokinetics of CsA after liver I/R and assessed the effect of liver I/R on the first-pass metabolism of CsA by intestinal CYP3A and P-gp.

Materials and Methods

Materials. Cyclosporine A (Sandimmun injection, 50 mg/ml) was obtained from Novartis (Basel, Switzerland). Testosterone and ketocconazole (KCU) were purchased from Nacalai Tesque (Kyoto, Japan). 6β-Hydroxytestosterone was purchased from Sigma-Aldrich (St. Louis, MO). All of the other chemicals used were of the highest purity available.

Animals. Nine-week-old male Wistar rats (SLC Japan Co., Shizuoka, Japan) were used for these experiments. The rats were fasted for 12 h before the experiments but allowed free access to water. The experiments were approved by the Mie University Review Board for animal experiments of the National Institutes of Health (Institute of Laboratory Animal Resources, 1996).

After the rats were anesthetized with an intraperitoneal injection of 50 mg/kg pentobarbital, a silicon catheter was implanted into the right external jugular vein to facilitate frequent blood collection and to administer the drug, and then the abdomen was opened through a midline incision. Partial hepatic ischemia was achieved by clamping the blood vessels of the left portal vein and hepatic artery using a microvessel clip. Ischemia was confirmed by the pale branching of the ischemic lobes, and then the abdomen was closed. The vascular clip was released at 60 min of hepatic ischemia. The sham-operated rats were treated in the same way as the liver I/R rats without occlusion of the left portal vein and hepatic artery. The rats were fasted until the pharmacokinetic studies at 12 h after reperfusion.

The Effects of Liver I/R on Bile Flow Rate. At 12 h after reperfusion, the I/R and sham rats were anesthetized with an i.p. injection of 50 mg/kg pentobarbital. A catheter was inserted into the common bile duct to collect bile samples. All bile samples were collected into preweighted sample tubes twice every 30 min. Bile volumes were determined gravimetrically by assuming a density of 1.0 g/ml. After the collection of bile samples, liver tissues were obtained, and the weight of the entire liver was measured.

Cyclosporine A Administration and Sample Collection. The CsA dosing solutions used in the pharmacokinetic studies were made by diluting Sandimmun 10-fold with saline containing 0.2% bovine serum albumin for injection. At 12 h after reperfusion, the rats received CsA at doses of 5 mg/kg for the bolus injection (intravenous) and 10 mg/kg for the oral and in situ closed loop experiments. All animals were provided food at 2 h after CsA administration.

The intravenous of CsA was injected rapidly into the jugular vein cannula, before being flushed with 0.5 ml of saline containing 0.2% bovine serum albumin. In the intravenously administered rats, the first 0.25 ml of blood was discarded during blood withdrawal. By using this flush and discard technique, the residual drug content in the cannula was negligible.

For whole-blood pharmacokinetic analysis of CsA, 0.3-ml blood samples were withdrawn from the cannula at 5, 20, and 40 min and 1, 2, 4, 6, 8, 12, and 24 h after intravenous administration. In the cases of oral administration of CsA with or without concomitant oral KCZ (30 mg/kg), blood samples were obtained at 0.5, 1, 1.5, 2, 3, 4, 6, 8, 12, and 24 h after administration. In addition, 0.3-ml blood samples were withdrawn from the cannula before and 6, 12, and 24 h after administration of CsA to measure alanine aminotransferase (ALT) activity in plasma. Plasma ALT activity was measured with a Transaminase Test Wako kit (Wako Pure Chemicals, Osaka, Japan).

In Situ Closed Loop Experiments. The I/R and sham rats were fasted for 12 h before the experiments and were anesthetized with an i.p. injection of 50 mg/kg pentobarbital. A catheter was inserted into the portal vein to collect blood samples. An in situ closed loop (15-cm segments of the upper, middle, and lower intestine) was prepared in bile duct-ligated rats, and CsA was administered at 10 mg/kg into the loop. Blood samples (0.5 ml) were drawn 1, 3, 5, 10, and 15 min after drug administration.

Measurement of Testosterone Hydroxylation Activity. Microsomes from the small intestine and liver were prepared according to the method of Mohri and Uesawa (2001). The protein concentration was measured by the method of Bradford (1976), using a Coomassie Brilliant Blue protein assay kit with bovine γ-globulin as a standard. The activity of CYP3A was assessed by formation of 6β-hydroxytestosterone from testosterone by a previously reported method (Purdon and Lehman-McKeeman, 1997). In brief, a reaction mixture (final volume of 500 μl) containing 0.4 mg of intestinal microsome or 0.01 mg of hepatic microsome protein prepared from rats, 100 mM potassium phosphate buffer, pH 7.4, NADPH-generating system (3.3 mM MgCl2, 1.3 mM β-NADPH, 3.3 mM glucose 6-phosphate, and 0.4 U/ml glucose-6-phosphate dehydrogenase), and 0.2 mM testosterone was incubated at 37°C for 30 min, before 1 ml of ice-cold ethyl acetate was added. The organic phase was evaporated to dryness, and the residue was dissolved in 50% methanol and subjected to HPLC analysis.

Determination of 6β-Hydroxytestosterone by HPLC. HPLC analyses were performed using an LC-9A pump, SPD-6A UV detector (Shimadzu, Kyoto, Japan), and C-R6A integrator (Shimadzu) equipped with a C18 5-μm octadecylsilane column (ZORBAX, 150 × 4.6-mm i.d.; Agilent Technologies, Santa Clara, CA). The column temperature was set at 40°C in a CTO-6A column oven (Shimadzu). The elution of 6β-hydroxytestosterone was conducted with 50% (v/v) methanol at 1 ml/min. The detection wavelength was 240 nm.

Western Blotting for P-gp. Crude plasma membrane fractions from the small intestine and liver were isolated according to the method of Nishio et al. (2005). The protein (12.5 μg) isolated from the intestine and liver was separated by 7.5% SDS-polyacrylamide gel electrophoresis (PAGE). Western blotting using the C219 monoclonal antibody (Calbiochem, San Diego, CA) for P-gp was performed as described.
described previously (Shimomura et al., 2002), and a polyclonal antibody for villin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was used according to the manufacturer’s instructions. The relative densities of the bands in each lane were determined using Image J 1.38 (National Institutes of Health, Bethesda, MD), and the densitometric ratios of P-gp to those of villin were calculated (Omae et al., 2005).

**Determination of Whole-Blood CsA.** The amounts of CsA in whole blood were measured by an affinity column-mediated immunoassay method using Dimension Xp and-HM with a Cyclosporine Flex reagent cartridge (lot nos. DB7296, GC7318, DB7324, CK8241, and CB8008; Dade Behring, Inc., Deerfield, IL). The cross-reactivities with metabolites of CsA ranged from 0 to 5.7% according to the affinity column-mediated immunoassay method (Terrell et al., 2002).

**Pharmacokinetic Analysis.** The AUC was calculated using the trapezoidal rule. The total body clearance (CLtot) and the oral clearance (CL/F) were calculated by D/AUC, where D represents the intravenous or oral dose. Oral bioavailability was estimated from the ratio of the dose-corrected AUC after a single oral or intravenous administration of CsA.

**Statistical Analyses.** All data were expressed as the mean ± S.D. Statistical analyses were carried out using the unpaired Student’s t test with GraphPad Prism 4.03 (GraphPad Software Inc., San Diego, CA). The level of significance was p < 0.05.

**Results**

**Assessment of Liver Injury by Liver I/R.** Liver injury was assessed by measuring plasma ALT activity. Plasma ALT activity was significantly higher in the I/R rats than that in the sham rats at all time points. Specifically, the plasma ALT activity of the I/R rats was approximately 16-fold higher than that of the sham rats at 12 h after reperfusion, suggesting accomplishment of liver injury by liver I/R (Table 1).

**The Effect of Liver I/R on Bile Flow Rate.** The bile flow rates from the common duct in the I/R and sham rats are shown in Table 1. The bile flow rate in the I/R rats was significantly lower than that in the sham rats (1.87 ± 0.17 and 1.21 ± 0.31 μl/min/g liver for the sham and I/R rats, respectively).

**In Vivo Pharmacokinetics of CsA after Oral or Intravenous Administration.** Figure 1 shows the whole-blood concentrations of CsA after oral administration of CsA (10 mg/kg) with or without oral KCZ (30 mg/kg) in the I/R and sham rats, and Fig. 2 shows the whole-blood concentrations of CsA after intravenous administration at a dose of 5 mg/kg in the I/R and sham rats. The pharmacokinetic parameters from the I/R and sham rats are summarized in Table 2. The whole-blood concentration of CsA was significantly lower in the I/R rats than in the sham rats at 1.5, 2, 3, 4, 6, 8, and 24 h after oral administration of CsA alone. When CsA alone was administrated orally, the AUC_0→∞ of CsA in the I/R rats was markedly decreased compared with that of the sham rats, whereas the CL/F of CsA in the I/R rats was significantly higher than that in the sham rats. The bioavailability of CsA in the I/R and sham rats after a single oral administration was 61.7 and 74.4%, respectively. When CsA and KCZ were coadministered orally, the AUC_0→∞ of CsA was markedly increased in both the I/R and sham rats compared with that in the rats without KCZ, and CL/F was significantly decreased in both rats (Table 2). However, there were no significant differences between the I/R and sham rats with respect to the AUC_0→∞ or CL/F of CsA in the presence of KCZ. Moreover, there were no significant differences in the AUC_0→∞ or CLtot of CsA between the I/R and sham rats after intravenous administration.

**TABLE 1**

<table>
<thead>
<tr>
<th>Time after Reperfusion</th>
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<td>ALT activity (IU/l)</td>
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<td>34 ± 11</td>
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<td></td>
<td>36</td>
<td>26 ± 4</td>
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<tr>
<td>Bile flow rate (μl/min/g liver)</td>
<td>12</td>
<td>1.87 ± 0.17</td>
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* p < 0.05 compared with sham rats.

*** p < 0.001 compared with sham rats.

**Fig. 1.** Time course of whole-blood CsA concentrations after oral administration at a dose of 10 mg/kg with or without oral KCZ (30 mg/kg). Each point represents mean ± S.D. of five (without KCZ) and four (with KCZ) rats. Solid line (circles), without KCZ; dashed line (squares), with KCZ. I/R (closed circles and squares) and sham (open circles and squares) rats. *, p < 0.05; **, p < 0.01; ***, p < 0.001 compared with sham rats without KCZ. Lower mean ± S.D. values were included in the symbols.

**Fig. 2.** Time course of whole-blood CsA concentrations after intravenous administration at a dose of 5 mg/kg. Each point represents mean ± S.D. of three rats. I/R (closed circles) and sham (open circles) rats. *, p < 0.05 compared with sham rats. Lower mean ± S.D. values were included in the symbols.
The Effects of Liver I/R on CYP3A Activity and the Expression Level of P-gp in the Liver. The testosterone 6β-hydroxylation (CYP3A) activity of the hepatic microsomes is shown in Fig. 3A. The CYP3A activity in hepatic microsomes was not significantly different between the I/R and sham rats. The expression levels of P-gp and villin proteins were confirmed by Western blot analysis using crude plasma membranes isolated from the livers of I/R and sham rats (Fig. 3B). Figure 3C shows the expression levels of P-gp, normalized by the corresponding villin signals in the livers of I/R and sham rats. The protein expression level of P-gp in the liver was slightly but significantly higher in the I/R rats than the sham rats.

In Situ Absorption Profiles of CsA after Intraloop Administration. Sites for intestinal absorption of CsA in the I/R and sham rats were investigated by an in situ closed loop method. The whole-blood concentrations of CsA in the portal vein after administration of CsA (10 mg/kg) into a loop containing the upper, middle, and lower small intestine (from duodenum to ileum) and the AUC0–15 min of CsA are shown in Fig. 4, A to C, and Table 3, respectively. The absorption of CsA in the upper intestine was higher than that in the middle or lower intestine in the I/R and sham rats. The rank order of the absorption site according to the observed portal vein concentrations and AUC0–15 min of CsA in the I/R and sham rats was upper > middle > lower intestine. The profiles of the whole-blood concentrations and AUC0–15 min of CsA in the portal vein after administration of CsA into the loop via the upper intestine were markedly lower in the I/R rats than in the sham rats (Fig. 4A; Table 3), whereas there were no significant differences in the absorption of CsA from the middle and lower intestine between I/R and sham rats (Fig. 4, B and C; Table 3).

The Effect of Liver I/R on CYP3A Activity in the Intestine. The testosterone 6β-hydroxylation (CYP3A) activity of intestinal microsomes is shown in Fig. 5. The CYP3A activity in microsomes isolated from the upper part of the intestine in the I/R rats was significantly higher than that in the sham rats, whereas that from the middle part of the small intestine was significantly lower in the I/R rats than the sham rats. Moreover, the CYP3A activity in the lower intestine was not significantly different between I/R and sham rats.

The Effect of Liver I/R on the Expression Level of P-gp. The expression levels of P-gp and villin proteins were confirmed by Western blot analysis using crude plasma membranes isolated from the small intestines of I/R and sham rats (Figs. 6, A–C). Figure 6, D to F, show the expression levels of P-gp normalized by the corresponding villin signals in the upper, middle, and lower parts of small intestine in sham and I/R rats. The protein expression levels of P-gp in all parts of the intestine were significantly higher in the I/R rats than the sham rats.

Discussion

The bioavailability of orally administrated CsA is poor and variable in liver transplantation recipients (Masuda and Inui, 2006). Liver I/R injury is a considerable factor involved...
in its poor and variable absorption and first-pass metabolism of CsA in the intestine and liver. However, the effect of liver I/R injury, which is associated with liver transplantations, on the first-pass metabolism of CsA and the roles of intestinal CYP3A and P-gp have not been clarified. In the present study, we investigated the effect of liver I/R on the pharmacokinetics of CsA and the first-pass metabolism of CsA mediated by intestinal CYP3A and P-gp.

The present study demonstrated that the AUC of CsA in I/R rats was decreased compared with that in sham rats when CsA alone was administrated orally (Table 2). To clarify the contribution of the first-pass metabolism mediated by CYP3A on the decreased oral bioavailability of CsA after liver I/R, we evaluated the effect of orally coadministered KCZ, a potent inhibitor of CYP3A, on the oral absorption of CsA in I/R and sham rats. KCZ markedly increased the AUC of CsA both in the sham and I/R rats, and no significant difference in the AUC of CsA was observed between the I/R and sham rats (Table 2). These results suggest that the decreased oral absorption after liver I/R may be because of increased first-pass metabolism mediated by CYP3A. On the other hand, there were no significant differences in the pharmacokinetic parameters of CsA or CYP3A activity in hepatic microsomes between the I/R and sham groups, when CsA was administered intravenously (Figs. 2 and 3A; Table 2). These results suggest that the decreased oral absorption of unchanged CsA after liver I/R may be because of decreased absorption or increased first-pass metabolism of CsA mediated by intestinal CYP3A and P-gp.

Moreover, KCZ is also reported to be a modest inhibitor of P-gp (Takano et al., 1998; Zhang et al., 1998). Therefore, KCZ may partly inhibit P-gp-mediated CsA efflux, and this may also contribute to the increased CsA levels observed in our study. However, oral administration of KCZ caused no significant change in T_{max} (the time of peak blood concentration) of CsA in the sham rats (Table 2). Furthermore, it was reported that KCZ is a 10 times more potent inhibitor of
CYP3A-mediated metabolism (IC_{50} = 0.15 \mu M) than it is of P-gp-mediated transport (IC_{50} = 1.4 \mu M) (Wandel et al., 2000). These findings suggest that KCZ is the main contributor to the inhibition of CYP3A-mediated metabolism of CsA, although the effect of KCZ on the contribution of P-gp to the oral bioavailability of CsA in vivo may not be excluded.

On the other hand, concomitant administration of KCZ led to prolonged T_{max} in the I/R rats compared with the sham rats, although there was no difference in the T_{max} between the I/R rats and sham rats in the absence of KCZ (Table 2). It is known that bile acid is an important factor affecting the absorption of CsA from the intestinal lumen (Mehta et al., 1988; Lindholm et al., 1990). In our present study, the bile flow rate was decreased to approximately 64% by liver I/R similar to the results reported by Accatino et al. (2003). Thus, it may be possible that the decreased bile flow associated with liver I/R injury delays the absorption of CsA and, therefore, prolongs the T_{max} of oral CsA in I/R rats in the presence of KCZ. Moreover, the CL/F values of CsA in the absence of KCZ were 1.9- and 2.6-fold higher than those in the presence of KCZ in the sham and I/R rats, respectively, suggesting that the effect of decreased bile flow on the T_{max} of CsA in the I/R rats was limited in the absence of KCZ because of the high first-pass metabolism in the intestine. Therefore, in the presence of metabolism mediated by intestinal CYP3A, the decreased bile flow that accompanies liver I/R injury should not be significant in the decreased oral absorption of CsA induced by liver I/R.

To clarify the relative contributions of each portion of the small intestine, i.e., upper, middle, and lower intestine, on the decreased absorption of CsA after liver I/R, we evaluated the intestinal absorption of CsA in the I/R and sham rats. Our results clearly indicate that the absorption of CsA in the upper intestine of I/R and sham rats was markedly higher than that in other portions (Table 3), which is comparable with the report by Jin et al. (2006). In addition, the absorption of CsA from the upper small intestine was decreased in the I/R rats compared with the sham rats (Fig. 4A). In contrast to the decreased absorption of CsA in the upper small intestine, there were no differences between I/R and sham rats in the absorption of CsA from the middle and lower intestine (Table 3). Considering that orally ingested CsA is absorbed mostly from the upper small intestine, decreased oral absorption of CsA after liver I/R should be attributable to the decreased absorption of CsA in the upper small intestine.

In addition, we examined the effect of liver I/R on CYP3A activity and the expression level of P-gp in each portion of the small intestine. As shown in Fig. 5, CYP3A activity in the upper intestine of the I/R rats was approximately 1.5-fold higher than that of the sham rats. Moreover, as shown in Fig. 6, the expression levels of P-gp were significantly increased in all portions of the intestine in the I/R rats compared with those in the sham rats. It is well documented that CYP3A activity is more abundant in the upper portion of the intestine than the lower portion of intestine (Takemoto et al., 2003), whereas P-gp is expressed at higher levels in the lower
intestine than the upper intestine (Makhey et al., 1998). Our results also indicate that CYP3A activity in the upper and middle small intestine was higher than that in the lower intestine, and the expression levels of P-gp were highest in the lower intestine (data not shown), which matches the findings of previous reports. Cummins et al. (2003) reported that P-gp increased intestinal metabolism of CYP3A/P-gp substrates by CYP3A and that intestinal drug metabolism is dependent not only on the activity of CYP3A in the intestine but also on the activity of P-gp. Therefore, the present results suggest that the decreased absorption of CsA in the upper intestine after liver I/R is because of both increased CYP3A activity and increased expression levels of P-gp, and it is likely that no differences between the sham and I/R on the absorption of CsA from the middle and lower intestine should be because of the counterbalance of increased P-gp and decreased CYP3A activities.

Moreover, in our present study, the CYP3A activity in liver microsomes slightly but not significantly decreased, and the expression levels of P-gp slightly and significantly increased in liver crude membranes after liver I/R, which is comparable with reports by Izuishi et al. (2000) and Tanaka et al. (2006). It is likely that the increased P-gp expression levels induced by liver I/R have little effect on the pharmacokinetics of orally administrated CsA because there was no significant difference in the AUC0–∞ of CsA between the I/R and sham rats after it was administered intravenously (Fig. 2; Table 2).

In conclusion, our study clearly demonstrates for the first time that liver I/R decreases the oral bioavailability of CsA, mainly because of the increased first-pass metabolism of CsA mediated by CYP3A and P-gp in the upper small intestine. The present findings provide useful information for the etiology of liver I/R injury and appropriate use of immunosuppressants after liver transplantation.

References


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