Pharmacokinetics of Tacrolimus and Mycophenolic Acid Are Altered, but Recover at Different Times during Hepatic Regeneration in Rats

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Running title page

Running title: Pharmacokinetics of tacrolimus and mycophenolic acid

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Abbreviations: LDLT, living donor liver transplantation; PHx, partial hepatectomy; CYP, cytochrome P450; UGT, uridine diphosphate glucuronosyltransferase; M1, 13-demethyleled tacrolimus; MPA, mycophenolic acid; MPAG, mycophenolic acid glucuronide; Mrp2, multidrug resistance-associated protein 2; Mrp3, multidrug resistance-associated protein 3; PCR, polymerase chain reaction; beta-2-m, beta-2-microglobulin.
Abstract

Hepatic regeneration is very critical to the success of living donor liver transplantation, which allows a reduced size liver to grow in size to accommodate the requirements of both the donor and the recipient. The objectives of this study were to evaluate 1) the hepatic metabolism of the two immunosuppressive drugs, tacrolimus and mycophenolic acid (MPA), and 2) the pharmacokinetics of tacrolimus and mycophenolic acid at various time points after initiation of hepatic regeneration by partial hepatectomy in rats. The hepatic intrinsic clearance of tacrolimus was decreased to 70% and 51% of control levels at the 24th hour and the 6th day, respectively but returned to normal level by day 14. The total body clearance of tacrolimus was reduced transiently but recovered completely by day 18. The hepatic intrinsic clearance of MPA was decreased to 52% and 51% of that in control rats at the 24th hour and the 6th day, respectively but recovered to normal level by day 14. The total body clearance of MPA was reduced at the 24th hour but recovered by day 6. The magnitude of reduction in the clearance of tacrolimus and MPA was much lower than what was predicted from in vitro data. The elimination clearance of MPA glucuronide was also impaired during hepatic regeneration but recovered to normal with time. In conclusion, the pharmacokinetics of tacrolimus and mycophenolic acid was altered during hepatic regeneration but recovered completely at different rates over time. Caution must be exercised in extrapolating in vitro data to in vivo conditions during hepatic regeneration.
Introduction

The increased number of patients requiring transplantation and the stagnant number of cadaveric organs available for transplantation has increased the waiting time and has led to high mortality for patients on the waiting list (Shiffman et al, 2002). Since the first adult living donor liver transplantation (LDLT) performed in the United States (US) in 1998, LDLT has emerged as an effective alternative strategy to overcome the organ shortage (Wachs et al, 1998; Bak et al, 2001). The number of LDLT performed in the US has increased from less than 100 in 1998 to more than 500 in 2001 (Wiesner et al, 2003).

Successful LDLT takes advantage of the ability of the resected liver to regenerate with time. The liver regeneration process involves proliferation of all the existing mature cellular populations within the liver, including hepatocytes, biliary epithelial cells, fenestrated endothelial cells, kupffer cells, and cells of Ito (Michalopoulos and DeFrances, 1997). Hepatic regeneration is believed to be triggered or activated by hepatocyte growth factor (HGF) (Lindroos et al, 1991), transforming growth factor-α (TGF-α) (Mead and Fausto et al 1989), tumor necrosis factor-α (TNF-α) (Diehl et al, 1994), and interleukin-6 (IL-6) (Matsunami et al, 1992). Many genes such as c-fos, c-jun, c-myc, bclx, p53, p21, mdm2, cyclin D1 and cyclins E, C, B are also activated during hepatic regeneration (Fausto, 2000).

Liver plays an important role in the elimination of drugs and xenobiotics. During hepatic regeneration, the reduction in total liver mass due to partial hepatectomy (PHx) is expected to reduce the ability of the liver to metabolize drugs. In addition, cytokines such
as TNF-α and IL-6 that are released during the regeneration process are also known to regulate the drug metabolizing enzymes in the liver (Abdel-Razzak et al., 1993; Monshouwer et al., 1996; Morgan et al., 2002). Previous studies in rats have shown suppression of several cytochrome P450s (CYPs) and uridine diphosphate glucuronosyltransferases (UGTs) during the initial phase of liver regeneration (Tamasi et al., 2001; Starkel et al., 2000; Ishizuka et al., 1997; Favre et al., 1998; unpublished observations). The impaired activities of CYPs and UGTs in the remaining liver will further reduce the ability of the regenerating liver to clear drugs from the body.

Cytochrome P450 3A enzyme plays a significant role in the metabolism of approximately 50% of the drugs in use and is also involved in the metabolism of several immunosuppressive drugs such as cyclosporine A, tacrolimus and sirolimus that are used in liver transplant patients. In addition, UGT mediated drug metabolism is very critical in the detoxification of drugs and UGT is involved in the metabolism of the immunosuppressive drug, mycophenolic acid (MPA). So it is important to understand whether the magnitude of change in the pharmacokinetics of immunosuppressive drugs metabolized by CYP3A or UGT during the hepatic regeneration process matches the predicted magnitude of changes due to a decrease of liver mass and a decrease in the intrinsic activity of CYP3A and UGT. Nothing is known about the pharmacokinetics of immunosuppressive drugs during hepatic regeneration. Such knowledge will help in optimizing not only the immunosuppressive drug therapy but also therapy with other drugs that are metabolized by CYP3A or UGT.
In this study, we have utilized partial hepatectomized rats to study the effect of hepatic regeneration on the hepatic intrinsic clearance and the pharmacokinetics of tacrolimus and MPA.

Materials and Methods

Chemicals

Testosterone and 6β-hydroxytestosterone were purchased from Steraloids Inc. (Newport, RI). Mycophenolic acid was purchased from Sigma Chemical Co. (St. Louis, MO). Tacrolimus, (10 mg/ml tacrolimus injection - Lot No. 711337K) and 13-demethylated tacrolimus (M1) were generous gifts from Fujisawa Pharmaceutical Company (Osaka, Japan). Mycophenolic acid glucuronide was a generous gift from Roche Bioscience (Palo Alto, CA). Heparin injection (Lot No. 322024) was obtained from American Pharmaceutical Partners, Inc. (Los Angeles, CA). IMx system and related reagents for measurement of tacrolimus blood concentration were obtained from Abbott Laboratories (Abbott Park, IL). Mouse anti-human M2III-6 monoclonal antibody was obtained from ID Labs Inc. (London, ON, Canada). Horseradish peroxidase-conjugated donkey anti-rabbit IgG was purchased from Amersham Biosciences, (Piscataway, NJ). Western Chemiluminescence reagent was obtained from Perkin Elmer Life Sciences, Inc. (Boston, MA). All solvents used were of HPLC grade and were obtained from Fisher Scientific Inc. (Pittsburgh, PA).

Animals
Partial hepatectomy was performed according to the method of Higgins and Anderson (Higgins and Anderson, 1931). Male Sprague-Dawley rats weighing 225-250 g were anesthetized with methoxyflurane inhalation and the ventral surface was shaved along the mid line and swabbed with betadine. A midline incision of 3-4 cm was made. Blood vessels supplying the medial and left lateral lobes of the liver were sutured and these lobes were excised. This accounts for the removal of 65-75% of the total liver, leaving the right lateral lobe and the small caudate lobe. The removed liver lobes, used as the control were perfused with ice-cold 0.15 M KCl, frozen in liquid nitrogen immediately and stored at -80ºC. After surgery, the rats had free access to food and water and were maintained on a 12-hour light and 12-hour night cycle. Rats were sacrificed by CO₂ inhalation at 24 hours, 6 days and 14 days after sham operation or PHx. Livers and kidneys were harvested as mentioned earlier and small intestines were collected and processed immediately as per the method described by Emoto et al (Emoto et al., 2000).

**Liver Mass**

The liver mass recovery was calculated as wet weight of the remaining liver lobes after PHx / estimated total liver mass (calculated by multiplying body weight at the time of sacrifice X ratio of liver mass to body weight in normal rats). Here the ratio of liver mass to body weight for normal rats was considered to be 0.04 (Davies and Morris, 1993).

**Preparation of Microsomes**

The livers and kidneys were homogenized in an ice-cold buffer of 50 mM Tris-HCl (pH 7.4) containing 0.15 M KCl and 1 mM EDTA. The homogenate was centrifuged at
10,000g for 30 min and the supernatant fraction was further centrifuged at 100,000g for 65 min. The microsomal pellet was resuspended in a buffer of 0.15 M KCl/50 mM Tris-HCl (pH 7.4) containing 1 mM EDTA and 20% glycerol. Intestinal microsomes were prepared as per the method described previously (Emoto et al., 2000). Microsomes were stored at -80°C before use. Microsomal protein concentrations were determined with bovine serum albumin as the standard. The protein content was determined by Lowry’s method using bovine serum albumin as a standard (Lowry et al., 1951). Microsomes were stored at -80°C until used in incubation studies.

**Measurement of $V_{\text{max}}$, $K_m$, and CL$_{\text{int}}$ for the Formation of 13-demethylated Metabolite of Tacrolimus in Hepatic Microsomes**

For the calculation of $V_{\text{max}}$ (maximal velocity of the formation of 13-demethylated metabolite (M1) of tacrolimus) and $K_m$ values (the affinity of tacrolimus for the enzyme), tacrolimus was incubated at various concentrations (0-25 µM) with liver microsomes at a protein concentration of 2.4 mg/ml, in the presence of MgCl$_2$ (10 mM) for 10 min at 37°C in 0.1 mM phosphate buffer (pH 7.4). The metabolism of tacrolimus was initiated with the addition of NADPH (1 mM). After 20 min, the reaction was terminated by placing the tubes into ice and equal volume of methanol was added to precipitate the proteins. After centrifugation at 13,000 rpm for 5 min, the supernatant was directly injected on to a HPLC column and the metabolite (M1) formed was measured using the reported method (Perotti et al., 1994a). The retention time for metabolite M1 was 9.3 minutes and the total run time was 43 minutes. The standard curve was linear over a range of 2.5-50 µg/ml.
The recovery (%) at 5 µg/ml and 50 µg/ml was within range of 87%-96% (n = 5). The intra-day and inter-day CV% at 5 µg/ml and 50 µg/ml was less than 10% (n = 5).

**Pharmacokinetic Study of Tacrolimus**

Pharmacokinetic studies were carried out in different sets of rats for tacrolimus and MPA and included a control group, 24 hours after PHx, 14 days after PHx and 18 days after PHx for tacrolimus studies. Since there was no significant difference between the control group and the sham group in the *in vitro* intrinsic clearance of tacrolimus at any time, pharmacokinetic studies were not conducted in the sham group (Table 1). A silastic tubing was inserted into the jugular vein 24 hours before the study. Tacrolimus (0.6 mg/kg) was administered intravenously as a bolus through the jugular vein catheter. Multiple blood samples (150 µl) were collected in heparinized tubes at 0, 0.5, 1, 2, 4, 6, 8, 12 and 24 hours after intravenous administration of tacrolimus. Tacrolimus concentration in the blood was measured by a microparticulate enzyme immunoassay (MEIA) using Abbott’s IMx analyzer. The antibody used in the assay does not cross-react with the major metabolite, (representing more than 75% of total metabolites formed) M1. Cross reactivity has been observed only with a few minor metabolites. Based on this, the antibody used primarily measures only tacrolimus. The calibration curve, ranging from 2 ng/ml to 30 ng/ml, was generated using reagents supplied in the kit. The recovery (%) at 3 ng/ml, 12 ng/ml and 25 ng/ml was within range of 82-97% (n = 5). The intra-day and inter-day CV (%) at 3 ng/ml, 12 ng/ml and 25 ng/ml was less than 14% (n =5). For each run of samples, three controls with concentrations of 5 ng/ml, 11 ng/ml and 22 ng/ml were also run.
Measurement of V\textsubscript{max}, K\textsubscript{m}, and CL\textsubscript{int} for the Formation of Mycophenolic Acid Glucuronide in Hepatic Microsomes

The incubation was carried out in a glass culture tube using varying concentrations of mycophenolic acid (MPA) (0-7.5 mM), 0.4 mg/ml microsomal protein, 10 mM MgCl\textsubscript{2} and Brij58 (0.1 mg/mg microsomal protein) with the final volume adjusted to 0.2 ml by the addition of 0.05 M phosphate buffer (pH 7.4). The tubes were pre-incubated for 5 minutes at 37°C and 4 mM UDPGA was added to initiate the reaction. After incubation for 30 minutes, the reaction was stopped by the addition of equal volume (0.2 ml) of ice-cold methanol. The tubes were centrifuged at 13000 rpm at 4°C for 5 minutes and the supernatants were frozen immediately at -80°C. The formation of MPA glucuronide (MPAG) was measured using a HPLC method developed in our laboratory. (Column: LiChrospher column, C18, 5 µ, 250 mm x 4.6 mm; column temperature: 38°C; mobile phase: 25% acetonitrile:75% water containing 0.05% phosphoric acid, 1.00 ml/min; UV detector at 254 nm; injection volume: 30 µl.) The standard curve was linear over the range of 1-100 µg/ml. The recovery (%) at 2.5 µg/ml, 25 µg/ml and 100 µg/ml was within range of 91-99% (n = 4). The intra-day and inter-day CV% at 2.5 µg/ml, 25 µg/ml and 100 µg/ml was less than 4% (n = 4).

Measurement of the Formation of Mycophenolic Acid Glucuronide in Small Intestine and kidney Microsomes

The incubation was conducted using the method described above with saturable MPA concentration of 5 mM (Km is less than 1 mM for both small intestine and kidney
microsomes). The formation of MPAG was measured using the HPLC method as described above.

**Pharmacokinetics Study of MPA**

Pharmacokinetic studies were carried out in rats in the control group, 24 hours after PHx, 6 days after PHx and 13 days after PHx. Since there was no significant difference between the control group and the sham group in the *in vitro* intrinsic clearance of MPA at any time during hepatic regeneration, pharmacokinetic studies were not conducted in the sham group. A silastic tubing was inserted into the jugular vein 24 hours before the study. MPA (20 mg/kg) was administered intravenously as a bolus through the jugular vein catheter. Multiple blood samples (150 µl) were collected in heparinized tubes at 0, 1, 5, 10, 15, 20, 30, 60, 90, 120, 240 and 480 minutes after intravenous administration of MPA. The concentration of MPA and MPAG in plasma was determined using the method described by Shipkova et al with minor modifications (Shipkova et al, 1998). The linearity of the method was from 2 µg/ml to 100 µg/ml for both MPA and MPAG in plasma. The recovery (%) at 5 µg/ml, 25 µg/ml and 100 µg/ml for both MPA and MPAG was within range of 94-105% (n = 4). The intra-day and inter-day CV% at 5 µg/ml, 25 µg/ml and 100 µg/ml was less than 7% (n = 4).

**Western Blot for Multidrug Resistance-Associated Protein (Mrp) 2 Protein Expression**

Liver membrane preparations were made as previously described (Ogawa et al, 2000). Protein concentration was measured using Lowry’s method (Lowry et al, 1951). Western blot was performed using the published method (Slitter et al, 2003) with minor
modifications: 45 µg of membrane protein (without boiling) loaded onto 7.5% SDS-PAGE.

**Real-Time PCR for Mrp3 mRNA Expression**

Total RNA was extracted from 50-100 mg livers using 1 ml Trizol (Invitrogen, Carlsbad, CA) according to the instructions of the manufacturer. Extracted RNA was quantified spectrophotometrically at 260/280 nm and the integrity was checked using agarose gel. After removal of DNA using RNase-Free DNase, 2 µg of RNA was reversely transcribed using 0.5 µg of random hexamer (Promega, Madison, WI) heated to 70ºC for 5 minutes, and then cooled to 4ºC. A reaction mixture containing 200 U Moloney murine leukemia virus reverse transcriptase, 1 mM dNTPs and 25 U RNasin (Promega, Madison, WI) was added to the previous mixture and incubated at 37ºC for 60 minutes. The resulting cDNA was diluted 10 fold and stored at -20ºC. Polymerase chain reaction (PCR) was performed on Applied Biosystems 7700 cycler using 5 µl of cDNA, 7.25 pmol of forward and reverse primers and 12.5 µl of SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) for a total volume of 25 µl. Forward and reverse primers, designed using Primer Express 2.0 (Applied Biosystems), are 5’-TCCCACTTCTCGGAGACAGTAAC-3’ (Forward), 5’-CTTAGCATCACTGAGGACCTTGAA-3’ (Reverse) for Mrp3 and 5’-CGTGCTTGCCATTCAAGAAA-3’ (Forward), 5’-GAAGTTGGGCTTCCCATTCTC-3’ for beta-2-microglobulin (beta-2-m). Cycling conditions were 1 cycle in 50ºC for 2 min, 1 cycle at 95ºC for 10 min, 50 cycles at 95ºC with 1 min annealing at 60ºC. The relative
cDNA content was determined in duplicate using standard curves created from cDNA and normalized to beta-2-m for each sample.

**Data Analysis**

Enzyme kinetics analysis was carried out by nonlinear regression analysis using Prism 3.0 (GraphPad Software Inc., San Diego, CA). The intrinsic formation clearance (CL\text{int}) was calculated by dividing the $V_\text{max}$ by the $K_m$. Pharmacokinetics of tacrolimus was analyzed by fitting the data to a two compartment model ($C = (Ae^{-\lambda_1 t} + Be^{-\lambda_2 t})$) and pharmacokinetics of MPA was analyzed by a non-compartmental model using WinNonlin 3.1 (Pharsight Co., Mountain View, CA). The selection of the kinetic model was made using AKAIKE information criterion (AIC) and the precision of the estimated parameters. Area under curve (AUC) was calculated using trapezoidal method. Clearance (CL) was calculated by Dose/AUC. Parameters such as $K_{10}$, $K_{12}$, $K_{21}$, $V$, MRT and half life were estimated as per standard pharmacokinetic principles. All data are reported by mean ± SD. Comparisons between two groups were made by student t-test and comparison among multiple groups were made by one way analysis of variance with Tukey post hoc analysis ($P < 0.05$).

**Results**

**Liver Mass Recovery**

Liver mass was 31.3 ± 2.0% at 24-hours, 50.5 ± 4.0% on day 6 and 72.3 ± 5.1% on day 14 (N = 6). The recovery of liver mass approached a plateau by day 18.
**Measurement of V\text{max}, K_m, and CL_{int} for the Formation of 13-demethylated Metabolite of Tacrolimus in Hepatic Microsomes**

Both $V_{\text{max}}$ and $K_m$ values were not different between the control group and the sham group at all time points studied (Table 1). The $V_{\text{max}}$ for the formation of M1 in hepatic microsomal fraction obtained at the 24th hour after PHx was significantly decreased compared to control value (Table 1). On day 6, the $V_{\text{max}}$ still remained at the lower level (50% of the control level) but recovered back completely by day 14 during hepatic regeneration (90% of the control level). However, the $K_m$ values were similar among all the groups. The intrinsic clearance (CL_{int}) for the formation of M1 in hepatic microsomal fraction was significantly decreased during hepatic regeneration at the 24th hour and on day 6.

**Pharmacokinetics of Tacrolimus**

The blood concentration vs. time curve of tacrolimus followed a biexponential process after intravenous administration of tacrolimus (Figure 1). The pharmacokinetic parameters of tacrolimus at different time points after initiation of the regeneration are summarized in Table 2. The area under the blood concentration vs time curve (AUC), the total body clearance (CL), the terminal disposition rate constant ($\beta$) and the terminal disposition half life ($T_{1/2}(\beta)$) were significantly different between control and PHx rats. The total body clearance of tacrolimus at the 24th hour was much lower than that in the control group. The clearance of tacrolimus increased significantly on day 14 from values observed at the 24th hour but was still lower than the clearance in the control group. There was no significant difference in total body clearance on day 18 and the control
group. The volume of distribution and other rate constants such as $K_{12}$ and $K_{21}$ were not altered at any time point.

**Measurement of $V_{\text{max}}$, $K_m$, and $CL_{\text{int}}$ for the Formation of MPAG in Hepatic Microsomes**

Both $V_{\text{max}}$ and $K_m$ values were not different between the control group and the sham group at all time points studied. The $V_{\text{max}}$ for the formation of MPAG in hepatic microsomal fraction obtained at 24 hours after PHx was significantly decreased compared to the control value (Table 3). On day 6, the $V_{\text{max}}$ still remained at the lower level (51% of the control level), but recovered back completely by day 14 with hepatic regeneration (96% of the control level). However, the $K_m$ values were similar among all the groups. The intrinsic clearance ($CL_{\text{int}}$) for the formation of MPAG in hepatic microsomal fraction was significantly decreased during hepatic regeneration at the 24th hour and on day 6.

**Pharmacokinetics of MPA**

The plasma concentration vs. time curves of MPA and MPAG after intravenous administration of MPA are shown in Figure 2. The pharmacokinetic parameters of MPA at different time points after initiation of hepatic regeneration are summarized in Table 4. The area under the plasma concentration vs time curve (AUC) for MPA, the total body clearance (CL) for MPA, the mean residence time (MRT) for MPA, the area under the plasma concentration vs time curve (AUC) for MPAG and the total body clearance (CL) for MPAG were significantly different between control and PHx rats. The total body
clearance of MPA and MPAG at the 24th hour was significantly lower than that in the control group. The clearance of MPA and MPAG recovered completely by day 6. The volume of distribution at steady state (Vss) was not altered.

**Measurement of Expression of Mrp2 and Mrp3**

The expression of Mrp2 was comparable among all the groups at the 24th hour after initiation of hepatic regeneration (Figure 3, panel A). The mRNA expression of Mrp3 also remained similar among groups at the 24th hour after PHx (Figure 3, panel B).

**Measurement of the Formation of Mycophenolic Acid Glucuronide in Small Intestine and Kidney Microsomes**

The formation of MPAG by small intestine microsomes at the 24th hour after initiation of hepatic regeneration was not different from that in the sham group (N = 5 rats); (PHx 24-hour vs. sham: 1.15 ± 0.50 vs. 1.04 ± 0.48 nmol/mg protein/min, P > .05, t-test). The formation of MPAG by kidney microsomes from PHx 24-hour group was similar to that in the sham group (N = 5 rats). (PHx 24-hour vs. sham: 0.29 ± 0.07 vs. 0.23 ± 0.06 nmol/mg protein/min, P > .05, t-test)

**Discussion**

In this study we have used partially hepatectomized rats to evaluate the effect of hepatic regeneration on the pharmacokinetics and metabolism of tacrolimus and MPA. This study simulates what is likely to happen to the drug metabolizing capacity in the donor or the recipient in a living donor liver transplant program. In addition to hepatic regeneration,
additional factors such as cold ischemia, warm reperfusion injury and immunosuppressive drug therapy can also modify drug metabolizing capacity in the recipient. In this study, tacrolimus and MPA were used as representative marker drugs for CYP3A and UGT enzyme.

Tacrolimus is primarily eliminated by hepatic metabolism (only 0.5% unchanged tacrolimus was recovered in human after IV dosing of tacrolimus, Moller et al, 1999) through the formation of M1 (represents 75% of the total metabolites, Perotti et al, 1994b) and CYP3A enzyme appear to be responsible for the formation of M1 (Shiraga T et al, 1994). Alterations in the hepatic function due to drug or disease state have been associated with altered ability of the liver to clear tacrolimus (Venkataramanan et al, 1995). Since the magnitude of change in the total body clearance of tacrolimus at the 24th hour was smaller (52%) than the magnitude in reduction in the liver mass (70%), we evaluated the ability of the liver to metabolize tacrolimus in vitro. The metabolism of tacrolimus was also reduced in the regenerating liver at 24 hours and on day 6, but recovered to normal by day 14. Taking into consideration the decrease in the hepatic intrinsic clearance of tracrolimus normalized to protein amount (assuming that hepatic M1 formation clearance approximates the hepatic intrinsic clearance of tacrolimus because majority of tacrolimus is metabolized to M1) and the reduction in liver mass, the whole liver intrinsic clearance for tacrolimus must decrease to 21% of normal liver at the 24th hour after initiation of hepatic regeneration. Based on the total body clearance of tacrolimus in control rats (9.22 ml/min/kg) and reported blood flow 55.2 ml/min/kg (Davies and Morris, 1993), assuming lack of any change in the unbound fraction of
tacrolimus as red blood cells are primarily responsible for binding tacrolimus in blood (Venkataramanan et al., 1995) and hematocrit does not change during hepatic regeneration (Okano et al., 2001; Kurata et al., 2000; Eguchi et al., 1998), the total body clearance of tacrolimus at the 24th hour should have been at most 2.21 ml/min/kg. However, while the total body clearance of tacrolimus was significantly decreased twenty four hours after PHx, the magnitude was much less than what was predicted based on in vitro data. Taking account of the low activity (at least 10 fold less than that in liver, data not shown) and small organ mass, the contribution of small intestine and kidney to the metabolism of tacrolimus is expected to be negligible in rats after intravenous administration. When normalized to the predicted liver weight at the 24th hour, the clearance per unit liver weight was increased significantly during hepatic regeneration (24-PHx vs. control: 0.33 ml/min/g vs. 0.23 ml/min/g). This observation points to the significant reserve capacity of the liver to clear drugs from the body. Since tacrolimus is a low hepatic extraction ratio drug (0.167, total body clearance 9.22 ml/min/kg divided by reported blood flow 55.2 ml/min/kg (Davies and Morris, 1993)), increased blood flow per unit weight of the liver per se had little effect on the clearance of tacrolimus. It is interesting to note that while higher blood level of tacrolimus normalized to unit dose have been reported in LDLT recipients compared to those receiving cadaveric livers, this increase was also much smaller (26%) (Trotter et al., 2002) than what is expected based on the smaller liver volume and the expected impairment in the metabolic activity of the liver. It is possible that increased blood flow per unit weight of the liver due to PHx might in some manner increase the functional capacity of the remaining liver mass. The precise mechanism for this observation needs to be evaluated in future studies.
The magnitude of change in the total body clearance of MPA (37%) was also much less than that in the liver mass (70%) at the 24th hour. Taking into consideration the decrease in the hepatic intrinsic clearance of MPA normalized to protein amount (assuming that hepatic M1 formation clearance approximates the hepatic intrinsic clearance of MPA because only less than 1% of MPA was recovered in 48-hour urine after both PO and IV dosing of MPA in human, Bullingham et al, 1996) and the reduction in liver mass, the whole liver intrinsic clearance for MPA must decrease to 16% of normal liver at the 24th hour after initiation of hepatic regeneration. Based on the total body clearance of MPA in control rats (9.29 ml/min/kg) and the hepatic plasma flow 25.4 ml/min/kg in rats (estimated based on the reported hepatic blood flow of 55.2 ml/min/kg and hematocrit of approximately 46% in rats (Davies and Morris, 1993)), assuming lack of significant change (< 20%) in the unbound fraction of MPA as albumin, the major MPA binding protein is decreased by only 20% at the 24th hour after PHx (Bullingham et al, 1996; Fouad et al, 1992), the total body clearance of MPA at the 24th hour should have been at most 2.12 ml/min/kg (If accounting for the three times increase of blood flow per unit liver weight (only 30% liver remaining), the total body clearance should have been at most 2.25 ml/min/kg). However, while the total body clearance of MPA was significantly decreased twenty four hours after PHx, the magnitude of reduction was much less than what was predicted based on in vitro data. Based on in vitro studies it was clear that extrahepatic pathways did not change and did not compensate for reduction in hepatic metabolic capacity. When normalized to the predicted liver weight at the 24th hour, the clearance per unit liver weight was increased significantly during hepatic regeneration.
(24-PHx vs. control: 0.49 ml/min/g vs. 0.23 ml/min/g). This further supported the hypothesis that the presence of significant reserve capacity of the liver to clear drugs.

Even though the pharmacokinetics of tacrolimus and MPA were altered in a similar manner at the 24th hour during hepatic regeneration, the recovery profile for the pharmacokinetic parameters of tacrolimus and MPA was different at later stages of hepatic regeneration: the total body clearance of MPA recovered much earlier than that of tacrolimus (6 days for MPA vs. 18 days for tacrolimus). The reason for the differentially recovery of the pharmacokinetic profile of tacrolimus and MPA may be due to the different reserve capacity of the regenerating livers for different metabolism pathways in which different metabolizing enzymes with different abundance are involved.

The specific reason why the intrinsic clearance for tacrolimus reaches a nadir on day 6 and not at the 24th hour is not clear at this point. The possible reason may be the fact that a regenerating liver is a dynamic system and that there is a time delay between changes in the mRNA level and changes in the activity of an enzyme. In fact, cytokines that are likely to decrease mRNA expressions are elevated up to 48 hours after PHx (Fulop et al, 2001; Iwai et al, 2001). Our result is also consistent with the reports in the literature. In rats, CYP3A activity measured using nifedipine and CYP3A protein expression level were much lower on day 4 and day 5 compared to the activity and the expression of CYP3A at the 24th hour after PHx (Favre et al, 1998; Ishizuka et al, 1997).
In addition, we also observed a lower total body clearance of MPAG at the 24th hour during hepatic regeneration. In order to determine the reason(s) for this decrease, we evaluated the expression of Mrp2 and Mrp3, two transporters involved in the biliary excretion of MPAG (Kobayashi et al, 2004). However, no change was detected in the expression of Mrp2 or Mrp3 at the 24th hour, which ruled out any possible roles of Mrp2 and Mrp3 in the reduced clearance of MPAG. It is likely that the decrease in total body clearance of MPAG is due to the dramatic reduction in the number of hepatocytes leading to decreased bile formation in the regenerating liver.

To the best of our knowledge this is the first study to evaluate the pharmacokinetics of tacrolimus and MPA and to analyze the recovery profile of the pharmacokinetics of tacrolimus and MPA over time after initiation of hepatic regeneration in an animal model. Our study provides several implications for use of drugs in LDLT patients. First, the reduction in clearance of the drug in vivo is not proportional to the reduction in liver mass. Second, caution must be excised in using in vitro data to predict in vivo clearance of drugs by the regenerating liver. Finally, drug dosing in LDLT patients must be routinely monitored and a reduction in dose for different drugs that is less than the reduction in liver mass may be necessary.
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regeneration stimulated by partial hepatectomy and carbon tetrachloride administration.

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Footnote

Part of this work was presented as the poster at the 2003 American College of Clinical Pharmacology Meeting in Tampa, Florida, September 22, 2003, and it also appeared as an abstract in *J Clin Pharmacol* 2003 43:1024.
Legends for figures

Figure 1. Blood concentration of tacrolimus vs time profile at different time points after initiation of hepatic regeneration. Data were represented by mean + SD (N = 6, 6, 5 and 4 for control, PHx 24-hour, PHx 14-day and PHx 18-day respectively).

Figure 2. Plasma concentration of MPA (panel A) and MPAG (panel B) vs time profile at different time points after initiation of hepatic regeneration. Data were represented by mean + SD (N = 5, 5, 5, and 4 for control, PHx 24-hour, PHx 6-day and PHx 13-day respectively).

Figure 3. Immunochemical analysis of Mrp2 protein expression (panel A) and the mRNA expression of Mrp3 (panel B) from control livers, livers of the sham group, and livers of 24-hour PHx group. Equal amounts of microsomal protein were loaded in each lane. Microsomal proteins from 6 rats were pooled together in each group. The relative mRNA level was determined by real time PCR as described in Methods using pooled cDNAs generated from total RNAs from 6 normal livers with different dilutions as the standards. The arbitrary mRNA values were normalized with their respective beta-2-m values. N = 4-5.
TABLE 1. Mean (± SD) Vmax, Km and CLint for the formation of 13-demethylated metabolite (M1) of tacrolimus in hepatic microsomes.

<table>
<thead>
<tr>
<th>Time after PHx</th>
<th>Groups</th>
<th>Vm (nmol/mg protein/min)</th>
<th>Km (µM)</th>
<th>CLint (ml/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>24-hour</td>
<td>Control</td>
<td>0.40 ± 0.06</td>
<td>4.87 ± 0.69</td>
<td>0.082 ± 0.007</td>
</tr>
<tr>
<td></td>
<td>(N = 4)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sham</td>
<td>0.41 ± 0.03</td>
<td>5.28 ± 0.55</td>
<td>0.079 ± 0.011</td>
</tr>
<tr>
<td></td>
<td>(N = 4)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>PHx</td>
<td>0.30 ± 0.03</td>
<td>5.10 ± 0.85</td>
<td>0.059 ± 0.005&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>(N = 4)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6-day</td>
<td>Control</td>
<td>0.36 ± 0.07</td>
<td>5.29 ± 0.80</td>
<td>0.068 ± 0.008</td>
</tr>
<tr>
<td></td>
<td>(N = 5)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sham</td>
<td>0.38 ± 0.05</td>
<td>5.16 ± 0.73</td>
<td>0.075 ± 0.018</td>
</tr>
<tr>
<td></td>
<td>(N = 5)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>PHx</td>
<td>0.18 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.18 ± 0.74</td>
<td>0.035 ± 0.003&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>(N = 5)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>14-day</td>
<td>Control</td>
<td>0.42 ± 0.03</td>
<td>5.04 ± 0.88</td>
<td>0.085 ± 0.018</td>
</tr>
<tr>
<td></td>
<td>(N = 6)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sham</td>
<td>0.40 ± 0.03</td>
<td>5.00 ± 0.68</td>
<td>0.081 ± 0.013</td>
</tr>
<tr>
<td></td>
<td>(N = 6)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>PHx</td>
<td>0.38 ± 0.04</td>
<td>4.90 ± 0.75</td>
<td>0.079 ± 0.011</td>
</tr>
<tr>
<td></td>
<td>(N = 6)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> P < .01; <sup>b</sup> P < .05 (vs. control, Tukey post hoc analysis).
**TABLE 2. Pharmacokinetic parameters of tacrolimus (0.6 mg/kg, i.v. bolus) 24 hours, 14 days and 18 days after PHx.**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control (N = 6)</th>
<th>PHx (24-hour) (N = 6)</th>
<th>PHx (14-day) (N = 5)</th>
<th>PHx (18-day) (N = 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC (hr•ng/ml)**</td>
<td>1089.29 ± 83.05</td>
<td>2297.60 ± 260.51a</td>
<td>1575.97 ± 144.99a</td>
<td>1160.75 ± 74.62</td>
</tr>
<tr>
<td>CL (ml/min/kg)**</td>
<td>9.22 ± 0.72</td>
<td>4.40 ± 0.50a</td>
<td>6.39 ± 0.61a</td>
<td>8.64 ± 0.55</td>
</tr>
<tr>
<td>β ((hr⁻¹))</td>
<td>0.31 ± 0.12</td>
<td>0.10 ± 0.05a</td>
<td>0.24 ± 0.04</td>
<td>0.24 ± 0.10</td>
</tr>
<tr>
<td>t₁/₂β (hr)*</td>
<td>2.53 ± 0.97</td>
<td>8.67 ± 4.48b</td>
<td>2.98 ± 0.52</td>
<td>3.14 ± 1.10</td>
</tr>
<tr>
<td>K₁₀ (hr⁻¹)**</td>
<td>2.31 ± 0.74</td>
<td>0.83 ± 0.25b</td>
<td>2.88 ± 0.51</td>
<td>2.67 ± 0.28</td>
</tr>
<tr>
<td>K₁₂ (hr⁻¹)</td>
<td>1.12 ± 0.40</td>
<td>1.25 ± 0.17</td>
<td>1.03 ± 0.50</td>
<td>1.43 ± 0.54</td>
</tr>
<tr>
<td>K₂₁ (hr⁻¹)</td>
<td>0.40 ± 0.25</td>
<td>0.49 ± 0.20</td>
<td>0.35 ± 0.04</td>
<td>0.29 ± 0.07</td>
</tr>
<tr>
<td>Vss (ml/kg)</td>
<td>718.53 ± 258.20</td>
<td>895.74 ± 272.98</td>
<td>653.37 ± 192.74</td>
<td>658.31 ± 274.50</td>
</tr>
</tbody>
</table>

**P < .01, * P < .05 for ANOVA; " P < .01, " P < .05 (vs. control) was obtained from Turkey post hoc analysis.**

Abbreviations: AUC, area under the blood concentration vs time curve; CL, total body clearance; t₁/₂ = half life; Vss = volume of distribution at steady state.
TABLE 3. Mean (± SD) Vmax, Km and CLint for the formation of MPAG in hepatic microsomes.

<table>
<thead>
<tr>
<th>Time after PHx</th>
<th>Groups</th>
<th>Vm (nmol/mg protein/min)</th>
<th>Km (mM)</th>
<th>CLint (µl/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (N = 4)</td>
<td>1.75 ± 0.40</td>
<td>1.07 ± 0.22</td>
<td>1.62 ± 0.06</td>
<td></td>
</tr>
<tr>
<td>Sham (N = 4)</td>
<td>1.88 ± 0.24</td>
<td>1.07 ± 0.18</td>
<td>1.79 ± 0.33</td>
<td></td>
</tr>
<tr>
<td>PHx (N = 4)</td>
<td>0.89 ± 0.17&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.06 ± 0.21</td>
<td>0.85 ± 0.20&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Control (N = 5)</td>
<td>1.84 ± 0.23</td>
<td>1.00 ± 0.19</td>
<td>1.87 ± 0.35</td>
<td></td>
</tr>
<tr>
<td>Sham (N = 5)</td>
<td>1.90 ± 0.29</td>
<td>0.99 ± 0.18</td>
<td>1.98 ± 0.57</td>
<td></td>
</tr>
<tr>
<td>PHx (N = 5)</td>
<td>0.94 ± 0.10&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.00 ± 0.23</td>
<td>0.96 ± 0.17&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>Control (N = 6)</td>
<td>1.84 ± 0.23</td>
<td>0.90 ± 0.13</td>
<td>2.08 ± 0.36</td>
<td></td>
</tr>
<tr>
<td>Sham (N = 6)</td>
<td>1.77 ± 0.30</td>
<td>0.94 ± 0.16</td>
<td>1.96 ± 0.60</td>
<td></td>
</tr>
<tr>
<td>PHx (N = 6)</td>
<td>1.76 ± 0.19</td>
<td>1.01 ± 0.24</td>
<td>1.80 ± 0.38</td>
<td></td>
</tr>
</tbody>
</table>
\(^a\) \(P < .01\) (vs. control, Tukey post hoc analysis).
TABLE 4. Pharmacokinetic parameters of MPA (20 mg/kg, i.v. bolus) 24 hours, 6 days and 13 days after PHx.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control (N = 5)</th>
<th>PHx (24-hour) (N = 5)</th>
<th>PHx (6-day) (N = 5)</th>
<th>PHx (13-day) (N = 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC (min•µg/ml)**</td>
<td>2174.00 ± 229.02</td>
<td>3411.10 ± 250.01a</td>
<td>1821.50 ± 124.67</td>
<td>1987.03 ± 70.71</td>
</tr>
<tr>
<td>CL (ml/min/kg)**</td>
<td>9.29 ± 1.07</td>
<td>5.89 ± 0.42a</td>
<td>10.67 ± 0.43</td>
<td>10.07 ± 0.24</td>
</tr>
<tr>
<td>MRT (min)**</td>
<td>37.84 ± 2.07</td>
<td>53.04 ± 8.41a</td>
<td>33.87 ± 9.40</td>
<td>36.37 ± 3.93</td>
</tr>
<tr>
<td>Vss (ml/kg)</td>
<td>348.43 ± 44.18</td>
<td>293.01 ± 60.39</td>
<td>369.97 ± 88.76</td>
<td>429.03 ± 107.59</td>
</tr>
<tr>
<td>(AUC&lt;sub&gt;MPAG&lt;/sub&gt;)&lt;sub&gt;MPA&lt;/sub&gt; (min•µg/ml)**</td>
<td>4417.91 ± 850.02</td>
<td>7667.38 ± 1088.84a</td>
<td>4283.66 ± 569.63</td>
<td>5153.61 ± 455.53</td>
</tr>
<tr>
<td>CL&lt;sub&gt;MPAG&lt;/sub&gt; (ml/min/kg)**</td>
<td>4.66 ± 0.90</td>
<td>2.72 ± 0.41a</td>
<td>4.73 ± 0.57</td>
<td>3.90 ± 0.34</td>
</tr>
</tbody>
</table>

**P < .01 for ANOVA; aP < .01, bP < .05 (vs. control) was obtained from Turkey post hoc analysis. CL<sub>MPAG</sub> was calculated using CL<sub>MPAG</sub> = fm*AUC*CL/(AUC<sub>MPAG</sub>)<sub>MPA</sub>

(assuming fm = 1 because more than 99% MPA is metabolized, Bullingham et al, 1996).

Abbreviations: AUC, area under the plasma concentration of MPA vs time curve; CL, total body clearance of MPA; MRT = mean residence time of MPA; Vss = volume of distribution at steady state; (AUC<sub>MPAG</sub>)<sub>MPA</sub>, area under the plasma concentration of MPA.
MPAG vs time curve after intravenous administration of MPA; $\text{CL}_{\text{MPAG}}$, total body clearance of MPAG after intravenous administration of MPA.
Blood concentration of tacrolimus (ng/ml) on a log scale as a function of time (hours) for different groups: Control, PHx (24-hour), PHx (14-day), and PHx (18-day).
Figure 2

A

Plasma concentration of MPA (µg/ml)

0 50 100 150 200 250 300

Time (minutes)

B

Plasma concentration of MPAG (µg/ml)

0 10 20 30 40 50 60 70 80

Time (minutes)
Figure 3

A

Mrp2

Control  Sham  PHx

B

Mrp 3 mRNA expression (normalized to beta-2-m)

0.000005  0.00001  0.000015  0.00002  0.000025  0.00003

Control  Sham  PHx