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Change in Pharmacokinetics of Mycophenolic Acid as a Function of Age in Rats and Effect of Coadministered Amoxicillin/Clavulanate

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Changes of mycophenolic acid (MPA) pharmacokinetics with aging were investigated in rats. We also compared the effect of concomitant amoxicillin/clavulanate combination (CVA/AMPC) on the pharmacokinetics of MPA in 4-week-old and 12-week-old rats (the package insert of CVA/AMPC warns of possible interaction with MPA). Four-week-old rats showed a 1.4-fold higher total body clearance of MPA and a lower volume of distribution of MPA (65%), compared to the values in 12-week-old rats. However, the difference in MPA pharmacokinetics disappeared when enterohepatic circulation was eliminated by bile duct cannulation (BDC). Concomitant CVA/AMPC significantly reduced plasma MPA concentration in intact rats of both age groups, and the age-dependent difference of MPA pharmacokinetics was no longer apparent. The effect of CVA/AMPC was not seen in rats that had undergone BDC, suggesting that the drug–drug interaction can be attributed to inhibition of enterohepatic circulation by CVA/AMPC. These results indicate that the aging-related alteration of MPA pharmacokinetics is a consequence of immature enterohepatic circulation in 4-week-old rats. Higher doses of MPA may be necessary in juveniles.

Key words mycophenolic acid; enterohepatic circulation; amoxicillin; clavulanate; pharmacokinetics

Mycophenolic acid (MPA) is widely used in clinical practice, in the form of the prodrug mycophenolate mofetil (MMF, brand name Cellcept), for suppression of organ rejection after transplantation in both adult and pediatric patients. MMF is better absorbed than MPA from the intestine (or gastrointestinal tract), and is quickly transformed into MPA in the gastrointestinal mucosa, liver, blood and plasma.[1] One of the issues in the clinical use of MPA is the large inter- and intra-individual variation in blood MPA pharmacokinetics, which makes it difficult to achieve a proper dosing regimen, because the efficacy and safety of MPA are related to area under the concentration–time curve (*AUC*) and trough level. Further, the inter- and intra-individual variations in pediatric patients are as large as in adults, and these patients require a higher dose as compared to adults.[2,3]

The possibility of drug–drug interactions must also be taken into account in post-transplantation patients, because these patients are generally on multiple medications, including other immunosuppressants and prophylactic drugs against infection. In August 2009, the MMF package insert in Japan was amended to advise caution regarding concomitant use of amoxicillin/clavulanate combination (CVA/AMPC, combination of penicillin antibiotic and β-lactamase inhibitor), which may interact with MMF through inhibition of enterohepatic circulation. Although CVA/AMPC is widely used in patients of all ages, its interaction with other drugs has not been compared between children and adults. Thus, our aim here was to conduct a fundamental study to examine the age dependence of MPA pharmacokinetics and the effect of coadministered CVA/AMPC in rats. The results are expected to be useful in planning a future clinical trial.

MATERIALS AND METHODS

Materials MPA, mycophenolic acid glucuronide (MPA-G) and indomethacin were purchased from Sigma-Aldrich Inc. (St. Louis, MO, U.S.A.). β-Glucuronidase/arylsulfatase was purchased from Roche Diagnostics GmbH (Mannheim, Germany). All other chemicals were of reagent grade.

Animal Experiments Four- and twelve-week old male Wistar rats were purchased from Sankyo Laboratory Animal Co., Ltd. (Hamamatsu, Japan). All animal experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of Kanazawa University.

A dose of 5 mg/kg of MPA in polyethylene glycol 400 was injected *via* the femoral vein. Blood and urine samples were collected at designated time intervals from the jugular vein under light ether anesthesia. Bile samples were collected *via* bile duct cannulation (BDC), at the same time as the blood samples for time course analysis. Plasma was separated by centrifugation at 2700×*g* for 20 min. Samples were stored at −30°C until assay.

In experiments with concomitant administration, CVA/AMPC (CLAVAMOX, GlaxoSmithKline, Co., Ltd., Tokyo, Japan) was orally administered at a dose of 48.2 mg/kg (45 mg/kg as AMPC) 5 times with intervals of 12 h, followed by a single injection of MPA at 5 mg/kg *via* the jugular vein 10 min after the last CVA/AMPC dose. Rats in the control group were orally administered purified water at an amount of 1.5 mL/kg 5 times, followed by a single dose of MPA in the same manner as above.

Assay for MPA Concentrations of MPA in plasma, bile and urine were determined with an HPLC system (LC-10AD, Shimadzu Co., Ltd., Kyoto, Japan). The assay for MPA was carried out according to Seebacher *et al.*[4] Aliquots of 90 μL of

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sample were mixed with 10 μL methanol, 10 μL of 50 μg/mL indomethacin in methanol as an internal standard and 1 mL of acetonitrile. They were vortexed, put on ice for 10 min and centrifuged at 23145×g for 5 min at 4°C. Eight hundred microliters of the upper layer was separated and dried under nitrogen. The residue was reconstituted with 150 μL of the mobile phase and a 50 μL aliquot was injected into the HPLC system.

All samples were analyzed on an HPLC system equipped with a Shim-pack CLC-ODS(M) column (150×4.6 mm i.d., Shimadzu). The absorbance was detected at a wavelength of 214 nm. The mobile phase was a mixture of 50 mmol/L *O*-phosphoric acid and acetonitrile (50:50) at flow rate of 1 mL/min.

For the estimation of glucuronides, plasma, urine and bile samples were incubated with β-glucuronidase/arylsulfatase at 37°C for 70 min, and MPA was extracted and analysed as described above. The concentration of MPA-G was obtained by subtracting the concentration of the unconjugated form from that of the hydrolysate.

The limit of quantification was 0.09 μg/mL and the linear regression coefficients were 0.998–1.000. The coefficients of variance for the within-run and between-run precisions were below 5%.

Data Analysis Pharmacokinetic parameters were estimated according to model-independent moment analysis as described by Yamaoka *et al.*[5] The data were analysed by the use of Student's *t*-test to compare the unpaired mean values of two sets of data. The number of determinations is noted in each table and figure. A value of $p<0.05$ was taken to indicate a significant difference.

RESULTS

Comparison of Plasma Concentration–Time Courses in Rats of Different Ages and Effect of Bile Duct Cannulation (BDC) Figure 1 shows the time courses of plasma MPA concentration following intravenous MPA at 5 mg/kg in 4-week-old and 12-week-old rats with BDC and without BDC (no treatment, NT). Table 1 presents pharmacokinetic parameters estimated from the plasma concentration time course by moment analysis. In rats without BDC, the plasma MPA concentration was significantly lower in 4-week-old rats than in 12-week-old rats at 8 h after administration and thereafter: the total body clearance (CL_{tot}) was increased by 40% and the volume of distribution (V_{dss}) was reduced to 70%, compared with the values in the older animals. In 4-week-old rats, the plasma concentrations were significantly reduced by BDC at 2 h, 6 h and thereafter. Significant changes were also observed in the pharmacokinetic parameters: mean residence time (*MRT*) was reduced to 30%, CL_{tot} was increased approximately 1.3-fold, and V_{dss} was lowered to 50% (Fig. 1, Table 1). In 12-week-old rats, the plasma concentrations were significantly reduced by BDC at 4 h and thereafter. Significant changes were also seen in pharmacokinetic parameters: $AUC_{0\to12h}$ was reduced to 70%, *MRT* was shortened to 20%, CL_{tot} was increased approximately 2-fold, and V_{dss} was reduced to 35% (Fig. 1, Table 1). In rats with BDC, there was no significant difference between age groups with regard to plasma concentration and pharmacokinetic parameters.

Comparison of Urinary and Biliary Excretions of MPA

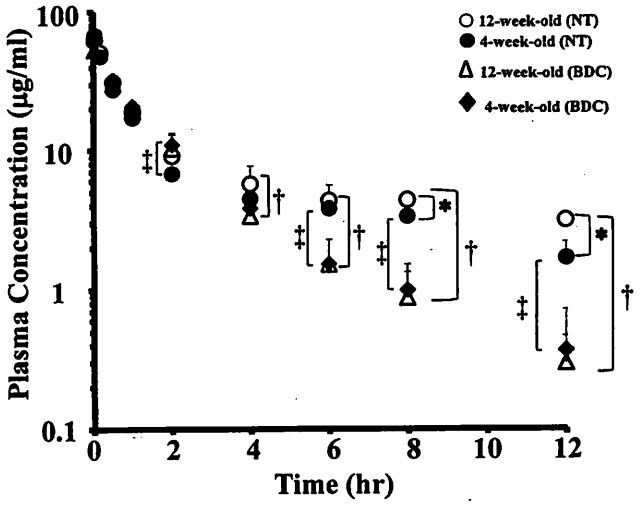

Fig. 1. Plasma Concentration–Time Courses of Mycophenolic Acid (MPA) after Single Intravenous Administration at a Dose of 5 mg/kg in 4-Week-Old (Closed Symbols) and 12-Week-Old Rats (Open Symbols) without Bile Duct Cannulation (NT; ○, ●) or with Bile Duct Cannulation (BDC; △, ◆,)

Symbols and bars represent means and SDs (n=4). *: Difference between 4- and 12-week-old rats within the NT or BDC group is statistically significant ($p<0.05$). †: Difference between NT and BDC rats at 12 weeks of age is statistically significant ($p<0.05$). ‡: Difference between NT and BDC rats at 4 weeks of age is statistically significant ($p<0.05$).

Table 1. Pharmacokinetic Parameters of MPA

Parameter	4-Week-old rats				12-Week-old rats			
	Control		CVA/AMPC		Control		CVA/AMPC	
	NT	BDC	NT	BDC	NT	BDC	NT	BDC
AUC_{0-12h} (μg min/mL)	81.7±4.9	77.7±11.5	66.7±6.4[c]	77.9±26.1	99.3±13.9	71.9±15.9[b]	74.9±16.7[c]	72.2±13.2
MRT (h)	5.99±1.94	1.72±0.33[b]	1.41±0.16[c]	1.66±0.52	9.36±2.44	1.72±0.33[b]	1.94±0.63[c]	1.48±0.15
CL_{tot} (mL/h/kg)	50.7±4.7	65.5±10.0[b]	70.1±7.5[c]	71.8±31.1	36.8±8.3[a]	73.1±18.2[b]	66.5±16.7[c]	70.9±13.7
V_{dss} (mL/kg)	221±38	110±5[b]	98.4±6.1[c]	109±15	334±55[a]	121±14[b]	123±20[c]	104±10

Pharmacokinetic parameters of MPA in 4- and 12-week-old rats after single intravenous administration of MPA 5 mg/kg alone (Control) or with concomitant CVA/AMPC (CVA/AMPC). The same experiment was performed using rats that had undergone bile duct cannulation to eliminate the effect of enterohepatic circulation (BDC) and the data are presented along with those obtained in rats without surgical treatment (NT). Pharmacokinetic parameters were estimated according to model-independent moment analysis. Each value represents mean±S.D. (n=3–9). *a*) Difference between 4-week-old and 12-week-old rats (4W *vs.* 12W) is statistically significant ($p<0.05$) within the NT-Control group. *b*) Difference between NT and BDC rats (NT *vs.* BDC) is statistically significant ($p<0.05$) in the Control or CVA/AMPC group within each age group. *c*) Difference between Control and CVA/AMPC rats (Control *vs.* CVA/AMPC) is statistically significant ($p<0.05$) in the NT group within each age group.

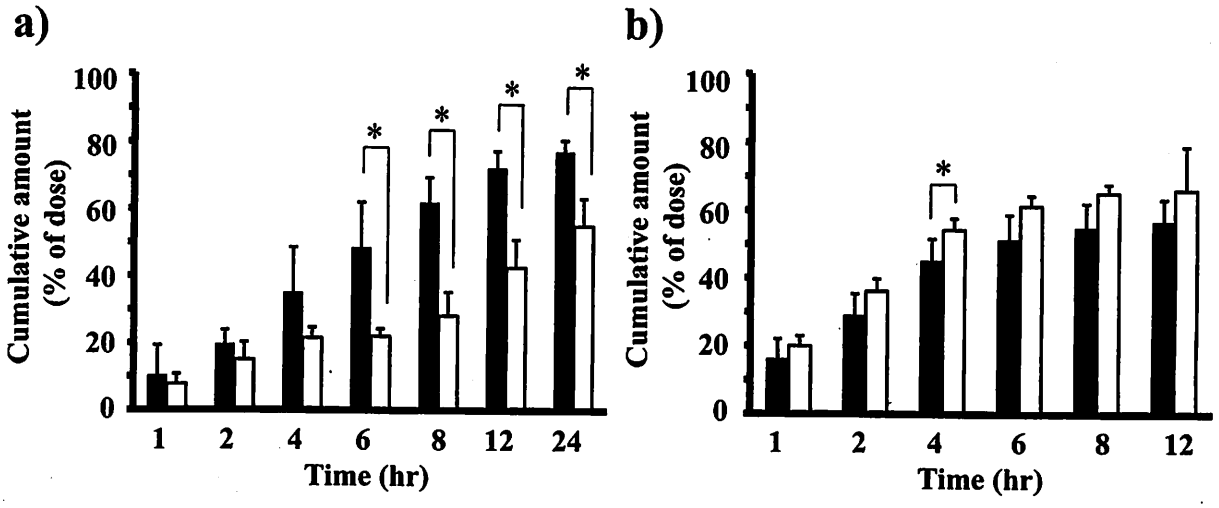

Fig. 2. Cumulative Urinary (2a) and Biliary (2b) Excretions of Mycophenolic Acid Glucuronide (MPAG) after Single Intravenous Administration of MPA 5 mg/kg in 4-Week-Old (Closed Bars) and 12-Week-Old Rats (Open Bars) as Percent of Dose

Urinary samples were taken from NT rats, and bile samples were collected from BDC rats. Bars represent means±S.D. (n=4). *: Difference between the 4- and 12-week-old rats is statistically significant (p<0.05).

and MPAG Urinary samples were taken from rats without BDC (NT) (Fig. 2a), and bile samples were collected from rats with BDC (Fig. 2b). Figure 2a illustrates the cumulative urinary excretion of MPAG up to 24 h after intravenous administration of MPA 5 mg/kg in 4- and 12-week-old rats without BDC. Cumulative urinary excretion in 4-week-old rats was higher than that in 12-week-old rats from 1 h after administration, and the difference between the two groups was significant at 6 h and thereafter. Cumulative urinary MPAG excretion at 24 h after administration reached 77% and 55% of the dose in 4- and 12-week-old rats, respectively. Cumulative urinary excretion of unchanged MPA at the same time point was 0.9% and 1.5% of the dose in 4- and 12-week-old rats, respectively.

Figure 2b shows cumulative biliary MPAG excretion up to 24 h following intravenous administration of MPA 5 mg/kg in 4- and 12-week-old rats with BDC. Cumulative biliary excretion in 4-week-old rats was lower than that in 12-week-old rats from 1 h after administration and the difference between the two groups was significant at 4 h after administration. Cumulative biliary MPAG excretion at 12 h after administration reached 57% and 66% of the dose in 4- and 12-week-old rats, respectively. Cumulative biliary excretion of unchanged MPA at the same time point was 1.6% and 1.2% of the dose in 4- and 12-week-old rats, respectively.

Effect of CVA/AMPC on MPA Pharmacokinetics The pharmacokinetic parameters of MPA with concomitant CVA/AMPC are summarized by age group in rats with and without BDC in Table 1. In rats without BDC, concomitant CVA/AMPC significantly changed MPA's pharmacokinetic parameters: $AUC_{0\to12h}$ was reduced to 75–80%, MRT was shortened to 20–25%, CL_{tot} was increased approximately 1.4–1.8-fold, and V_{dss} was lowered to 40–45% (Table 1). In rats with BDC, however, the pharmacokinetic parameters were not significantly altered by concomitant CVA/AMPC (Table 1).

DISCUSSION

In our study, MPA was administered to 4- and 12-week-old rats and the pharmacokinetic characteristics were compared. Rats are generally considered to be weaned at around 3 weeks and to become reproductively mature at around 10 weeks after birth.[6] In this study, 4-week-old rats were considered to be representative of the immature stage after weaning and before puberty, and 12-week-old rats were considered to be representative of the mature stage.

MPA is metabolized mainly in the liver by uridine diphosphate glucuronosyltransferase (UGT), of which UGT1A9 is dominant in human, whereas 1A1 and 1A7 are the principal forms in rat.[7] The resulting MPA-G is excreted in urine and bile.[8] It has been reported that a higher dose of MPA is required for pediatric patients than for adults,[2,3] but significantly lower expression of UGT1A9 was identified in pediatric liver,[9] so a difference in metabolic activity can likely be ruled out as a cause of the observed age dependence of MPA pharmacokinetics in humans. In rat liver, UGT activity measured with p-nitrophenol as the acceptor peaked during the first week after birth, and declined to the adult level at 3–4 weeks after birth.[10,11] On the other hand, activity of UGT1A1 gradually increases after birth, and the activity of 4-week-old rats is 70% of that of adult rats.[12] In accordance with the clinical reports, we found a marked difference in the disposition kinetics of MPA between 4- and 12-week-old rats. However, the difference in disposition kinetics between age groups in our study also did not appear to be due to a difference of UGT activity.

To investigate the influence of enterohepatic circulation, the pharmacokinetics of MPA was compared in rats with and without BDC. We found that BDC altered plasma MPA concentration and pharmacokinetic parameters in both 12- and 4-week-old rats, and also abrogated the difference according to age (Fig. 1, Table 1). Also, CL was significantly increased by BDC, by 1.3-fold and approximately 2-fold in 4- and 12-week-old rats, respectively. These results indicate that the difference in MPA pharmacokinetics observed between 4- and 12-week-old rats without BDC is likely to be attributable to a difference in enterohepatic circulation, which seems to have greater impact with increasing age.

The enterohepatic circulation consists of biliary excretion of glucuronate conjugates, hydrolysis of the conjugates by

β-glucuronidase produced by intestinal bacterial flora, and reabsorption of the regenerated MPA from the intestinal tract. A study of gastrointestinal absorption in 5–30-week-old rats indicated that drug permeability *via* passive diffusion through the small intestine did not vary with age.[13]

In human liver, β-glucuronidase activity to hydrolyze MPAG into MPA is the highest in newborns and falls to the adult level by the age of 4 months.[14] In rat liver, β-glucuronidase activity shows a sharp increase 2–3 d after birth, peaks at 20–30 d and remains at a plateau during 50–90 d.[15] If the difference in disposition kinetics between 4- and 12-week-old rats were caused by β-glucuronidase activity in liver, the plasma concentration of 4-week-old rats should be higher than that of 12-week-old rats, whereas the opposite was the case. On the other hand, the level of β-glucuronidase activity in rat intestine appeared to be almost constant.[15] Thus, the difference in enterohepatic circulation between age groups in our study is not considered to be due to a difference in β-glucuronidase activity.

MPA is excreted as the glucuronate conjugate MPAG in urine and bile. The proportion of unchanged form in the urine was reported to be less than 2%,[8] which is approximately the same as the result in this study. Also, the biliary excretion rate at 1 h after administration in 12-week-old rats was 26%, which is consistent with a previous report.[16] On the other hand, the plasma MPAG/MPA ratio during 12 h after administration ranged from 0.1 to 0.5, indicating no accumulation of MPAG, irrespective of age (data not shown). From these results, the smaller effect of enterohepatic circulation and faster elimination in 4-week-old rats compared to 12-week-old rats were considered to be attributed to quicker excretion of plasma MPAG into urine, even though the plasma MPAG level is relatively high due to low biliary MPAG excretion in 4-week-old rats.

MPAG is excreted into the bile *via* multidrug resistance-associated protein 2 (Mrp2).[16,17] We previously reported that a rat model of liver disorder had a lower level of hepatic Mrp2 and a higher level of renal Mrp2, as well as increased urinary excretion and decreased plasma concentration of the antiepileptic drug valproate, which undergoes glucuronate conjugation and urinary excretion similarly to MPA. The results demonstrated that drug pharmacokinetics are affected by alteration of Mrp2 expression.[18] On the other hand, a study in Sprague-Dawley (SD) rats found that Mrp2 expression at both the mRNA and protein levels was lower in liver from newborn and 1-week-old rats as compared to adult rats.[19] These reports suggests that changes of Mrp2 expression over the course of development affect the disposition kinetics of MPA. The influence of Mrp2 was not examined here, but should be investigated in the future, as the effect of enterohepatic circulation may vary among drugs depending on whether or not they are substrates for efflux transporter(s).

While the interaction between CVA/MAPC and MPA has not been investigated in detail, the Cellcept package insert states that concomitant CVA/AMPC apparently inhibits the enterohepatic circulation of MPA. On the other hand, concomitant use of warfarin and CVA/AMPC increased international normalized ratio in humans, which was considered to be due to a reduction in vitamin K producing bacteria in the intestinal flora.[20] Therefore, we examined whether the drug interaction of MPA with CVA/AMPC varies with age, and whether the interaction is still observed when the effect of enterohepatic circulation is eliminated by BDC. In humans, there are reports that trough MPA concentration was lowered by concomitant use of an oral antimicrobial agent,[8] and that trough level was reduced by 46% after concomitant administration of CVA/AMPC for 3 d.[21] Based on information about CVA/AMPC pharmacokinetics in rats[22] and humans,[23,24] the dosing regimen in this study was determined so that plasma concentrations similar to those reported in humans would be achieved in rats: CVA/AMPC was orally administered at a dose of 45 mg/kg as AMPC 5 times with intervals of 12 h, followed by a single intravenous administration of MPA at 5 mg/kg 10 min after the last CVA/AMPC dose.

Concomitant CVA/AMPC showed no significant interaction with MPA in rats with BDC, and there was no significant difference between NT and BDC in the CVA/AMPC group. The level of β-glucuronidase activity in rat intestine is markedly different in 4- and 12-week-old rats.[15] Thus, we consider that CVA/AMPC inhibited β-glucuronidase activity, thereby blocking enterohepatic circulation of MPA under the experimental conditions used in this study, suggesting that similar drug–drug interaction occurs in both rats and humans. Since the effect of CVA/AMPC becomes stronger as the contribution of the enterohepatic circulation increases, it may be necessary to monitor the plasma concentration or effect of drugs that undergo extensive enterohepatic circulation, *e.g.* MPA and valproate, if they are coadministered with CVA/AMPC.

In conclusion, our results indicate that the pharmacokinetics of MPA in rats differs with age, and this difference is due to a difference in enterohepatic circulation, *i.e.*, the capacity of the enterohepatic circulation seems to increase with age. Further, drug–drug interaction of MPA with CVA/AMPC is due to inhibition of the enterohepatic circulation by CVA/AMPC.

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