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molecular pharmaceutics

Characterization of Gastrointestinal Drug Absorption in Cynomolgus Monkeys

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Received July 19, 2007; Revised Manuscript Received November 28, 2007; Accepted November 28, 2007

Abstract: Possible factors of species differences in gastrointestinal drug absorption between cynomolgus monkeys and humans were examined using several commercial drugs. Oral bioavailability (BA) of acetaminophen, furosemide, and propranolol in cynomolgus monkeys was significantly lower than that in humans. From the pharmacokinetic analysis, these drugs were found to show the low fraction absorbed into portal vein (F_aF_g), suggesting that the low BA in cynomolgus monkeys was attributed mainly to the gastrointestinal absorption processes. The gastric emptying rate (GER) calculated from plasma concentration profiles after oral administration of acetaminophen in cynomolgus monkeys was similar in humans. The gastrointestinal transit time (GITT) in cynomolgus monkeys was only slightly shorter than that in humans. On the other hand, it was demonstrated that the apparent intestinal permeability (P_{app}) of five drugs to cynomolgus monkey intestine was lower than that to rat intestine; especially propranolol and furosemide showed the remarkably low P_{app}. The expression levels of mRNAs of efflux transporters analyzed by real-time RT-PCR indicated that mRNA expression levels of MDR1, MRP2, and BCRP in monkey intestine were significantly higher than those in human intestine. This result suggested that low oral absorption of furosemide in cynomolgus monkeys was attributed to the high activities of efflux transporters in its intestinal membrane. Results of *in vivo* PK analysis clearly showed that F_aF_g values of propranolol and acetaminophen in cynomolgus monkeys were markedly lower than those in humans. Since propranolol and acetaminophen were the drug with high membrane permeability, it was considered that the high first-pass metabolism in the enterocytes was a main factor of their low F_aF_g in cynomolgus monkeys. In conclusion, it was demonstrated that the high activities of efflux transporters and/or metabolizing enzymes in the intestinal membrane are possible factors to cause poor oral absorption of drugs in cynomolgus monkeys.

Keywords: Cynomolgus monkey; species difference; oral bioavailability; intestinal permeability; gastrointestinal physiology; Ussing-type chamber

Introduction

Rats, dogs and monkeys are often used for pharmacokinetics (PK) studies during the drug discovery and development process in the pharmaceutical industries. Since oral bioavailability (BA) is a key factor for development of oral new chemical entities, the oral BA in human should be predicted accurately to enhance the success probability in the clinical study. However, these

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10.1021/mp700095p CCC: $40.75
Published on Web 02/02/2008

experimental animals sometimes show significant species differences in the oral BA of drugs that might lead to the erroneous prediction of the BA in humans. Especially in monkeys, remarkably low oral BA has often been shown even if the high BA was shown in other species for the same drugs. In general, oral BA of drugs can be expressed as

$$\mathrm{BA} = F_a F_g F_h$$

where F_a is a fraction dose absorbed from the gastrointestinal tract, F_g is intestinal availability (a fraction not metabolized in the gut wall), and F_h is a hepatic availability (a fraction not metabolized in the liver). Species differences in oral BA are attributed to any of these processes in oral drug absorption. In order to improve the predictability of drug BA in humans from the preclinical animal studies, it is important to determine the factors involved in the species differences in oral drug absorption.

Chiou et al. have carefully surveyed the literature and calculated F_a of various drug in rats,[1] dogs,[2] and cynomolgus monkeys[3] and compared them with that in humans. Since F_a in animals correlated well with that in humans, they have concluded that species differences were not significant in F_a. In contrast, insufficient correlations in drug absorption between experimental animals and humans were reported due to the differences in gastric pH[4–6] and/or the gastric emptying rate and gastrointestinal motility.[7–11] Also, the differences in the activity of influx such as PepT1 or efflux transporters such as MDR1 might cause the species differences in F_a.

Differences in the activity of metabolic enzymes in GI tract and liver cause the species differences in F_g or F_h. Chiou et al. have also shown the higher nonrenal clearance in monkeys than that in humans and thus concluded that low oral BA in cynomolgus monkeys is mainly attributed to the low F_h of drugs due to the high first-pass metabolism in the liver.[3] Contrary to this report, Ward and Smith have identified the clearance of 103 drugs in rats, dogs, monkeys, and humans after intravenous administration and evaluated the predictability of each animal for human drug clearance.[12] In all instances, the monkey tended to provide the most qualitatively and quantitatively accurate predictions of human clearance and also afforded the least biased prediction compared with other species. According to this report, F_h in monkeys is not necessarily higher than that in humans.

In contrast, several reports have demonstrated the high metabolic activity of monkey intestine for CYP3A4 substrate drugs. In the case of midazolam, a typical substrate of CYP3A, $F_a F_g$ value in cynomolgus monkeys was calculated as 0.03–0.1[13,14] and markedly lower than that in humans (approximately 0.5). Although the exact value of F_g was not calculated, the low oral absorption of midazolam in cynomolgus monkeys was considered to be attributed to the high first-pass metabolism in the intestine.[13] Also, it was reported that the oxidation activities and conjugation activities in the cynomolgus monkey intestine were higher than those in human intestine.[15,16] These findings have implied the possibility of high intestinal first-pass metabolism in monkeys for various drugs. Therefore, as the test drugs in this study, we have included the substrates of CYP (CYP2D6) and UGT-glucuronosyltransferase (UGT), such as propranolol and acetaminophen, respectively, in addition to the drugs with high and low permeability to the intestinal membrane

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(naproxen and atenolol).[17,18] Furosemide was also included as a substrate of efflux transporters.

In the present study, in order to consider the first-pass metabolism in cynomolgus monkeys, PK parameters of several drugs were calculated from the time-course of plasma concentration after intravenous and oral administration. Since F_a and F_g are obtained as a hybrid (F_aF_g) and cannot be isolated only from the *in vivo* data, we have measured the intestinal permeability of drugs in cynomolgus monkeys by the *in vitro* Ussing-type chamber method. Lennernäs et al. have demonstrated a good correlation between the effective permeability of the human jejunum obtained with *in vivo* single pass perfusion and that of rat jejunum obtained with the Ussing-type chamber method.[19] Therefore, it seems that the species differences in F_a can be evaluated by measuring the membrane permeability *in vitro*. In addition, GI transit time and expression levels of mRNAs of the efflux transporters in cynomolgus monkeys were evaluated and compared with those in humans to elucidate its effects on F_a.

Materials and Methods

Materials. Acetaminophen, salitylazosulfapyridine, sulfapyridine, propranolol, atenolol, furosemide, naproxen, *p*-anisamide, and warfarin were purchased from Sigma Chemical Co., Ltd. (St. Louis, MO). D82-7319, an internal standard for the LC/MS/MS analysis, was synthesized by the Medicinal Chemistry Research Laboratory, Daiichi Pharmaceutical Co., Ltd. (Tokyo, Japan). All other chemicals were obtained from commercial sources and were of analytical or the highest available grade.

***In Vivo* Pharmacokinetic Studies in Monkeys.** Female cynomolgus monkeys weighing 2.5–3.5 kg were used. Cynomolgus monkeys ($n = 3$–5) were fasted overnight prior to drug administration, whereas access to water was provided ad libitum. Each compound was suspended in 0.5% methylcellulose solution and administered orally with a gastric tube. The intravenous dose was injected in the forearm vein as saline solution. The cynomolgus monkeys were restricted to a chair only at the time of drug administration and blood collection. Approximately 1 mL blood samples were collected using heparinized syringes at the designated time intervals after drug administration via the femoral vein. Plasma was separated by centrifugation of the blood sample at 3000 rpm (1800*g*) for 10 min at 4 °C and stored at −20 °C until analysis.

Evaluation of the Gastrointestinal Transit Time (GITT) in Cynomolgus Monkeys. Female cynomolgus monkeys weighing 2.5–3.5 kg ($n = 5$) were fasted overnight, and 100 mg of salitylazosulfapyridine was administered with a gastric tube. Approximately 1 mL blood samples were collected using heparinized syringes every hour after administration for 8 h. The time of appearance of sulfapyridine in plasma after oral administration of salitylazosulfapyridine was evaluated. Plasma sulfapyridine concentrations were analyzed by HPLC according to the method of Mizuta et al.[7] The average time of appearance of sulfapyridine in plasma of five cynomolgus monkeys was used as GITT.

Evaluation of Blood to Plasma Concentration Ratio (R_b). Each drug solution (100 μg/mL in 20% methanol solution) was diluted with 20% methanol solution, and finally a 2 μg/mL solution of each compound was prepared. Forty microliters of each drug solution was added to 360 μL of blank blood, and it was incubated for 10 min at 37 °C. The mixture was centrifuged at 3000 rpm (1800*g*) for 10 min at 4 °C. To 100 μL aliquots of supernatant, 50 μL of IS (warfarin, 200 ng/mL) acetonitrile solution and 50 μL of acetonitrile were added. The supernatant was transferred to MultiScreen (GV, 0.22 μm, Millipore, Billerica, MA) and filtered by centrifugation at 3000 rpm (1800*g*) for 10 min at 4 °C. Each filtrate was transferred to autosampler vials, and 10 μL portions were injected into the LC/MS/MS system.

The R_b value was calculated by the following equation

$$R_b = C_b/C_p \quad (1)$$

where C_b is the plasma concentration when the compound is added to plasma and C_p is the plasma concentration when the compound is added to blood.

***In Vitro* Intestinal Permeability.** Membrane permeability was evaluated by the Ussing-type chamber method according to the previous reports.[19,20] Experiments were performed in modified Ringer solution (pH 7.4) of the following composition: NaCl, 125 mM; KCl, 5 mM; $CaCl_2$, 1.4 mM; NaH_2PO_4, 1.2 mM; $NaHCO_3$, 10.0 mM; and D-glucose 11.1 mM in an atmosphere of a 95% O_2/5% CO_2 gas. The small intestinal tract of cynomolgus monkey was removed under light anesthesia with ketamine. A portion of the jejunum segment from a cynomolgus monkey was removed the muscle layer and was mounted to the Ussing-type chamber. The viability of tissue was checked by the permeability of sulfasalazine, the paracellular maker, and membrane conductance (data not shown). The initial drug concentration on the mucosal side was set at 0.1 mM for all compounds. Samples (100 μL) were withdrawn from both sides every 20 min up to 120 min. The apparent permeability coefficient (P_{app}) across the excised jejunum segment was calculated using the following equation:

$$P_{app} = dX_R/dT \times 1/A \times C_0 \quad (2)$$

where dX_R/dT is the steady-state appearance rate on the

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serosal side, A is the exposed membrane surface area, and C_0 is the initial concentration on the mucosal side.

All animal experiments were conducted with the approval of the Animal Experiment Ethics Committee of Daiichi Pharmaceutical Co., Ltd.

Pharmacokinetic Analysis. The area under the concentration−time curve (AUC), total body plasma clearance (CLtot.plasma), and the mean residence time (MRT) after intravenous and oral administration were calculated using the noncompartmental method without extrapolation using a validated program developed in-house.

Oral BA was calculated as the ratio of AUC after oral and intravenous administration. Hepatic clearance (CL_h), F_h, and F_aF_g were calculated using eqs 3−6 according to the theory of Rowland.[21] The R_b of each drug was experimentally measured as described above. The hepatic blood flow (Q_h) in cynomolgus monkeys (43.6 mL/min/kg) used the reported value.[22] In the calculation, the urinary excretion ratios (fe) of each drug in cynomolgus monkeys was assumed to be the same as those in humans,[23] 0% for acetaminophen, naproxen and propranolol, 65% for furosemide, and 94% for atenolol, respectively.

$$CL_{tot.blood} = CL_{tot.plasma}CL/R_b \quad (3)$$

$$CL_h = CL_{tot.blood}(1 - fe) \quad (4)$$

$$F_h = 1 - CL_h/Q_h \quad (5)$$

$$F_aF_g = BA/F_h \quad (6)$$

The gastric emptying rate (GER) of cynomolgus monkeys was estimated by means of mean absorption time (MAT) calculated as follows

$$MAT = MRT_{po} - MRT_{iv} \quad (7)$$

where MRT is the mean residence time after oral or intravenous administration of acetaminophen in cynomolgus monkey.

The function that indicates a relationship between the P_{app} of drugs and F_aF_g in humans and cynomolgus monkeys is described by eq 8.

$$F_aF_g = 1 - \exp(-P_{app}f) \quad (8)$$

The relationship between P_{app} and F_aF_g, which is expressed as the correction factor f, was determined by nonlinear least-squares regression analysis (Levenbeg-Marquardt least-squares algorithm; Delta Graph 4.0 by SPSS, Inc., Chicago, IL).

HPLC Analysis. Acetaminophen and sulfapyridine concentrations in plasma were measured by HPLC according to the methods of Mizuta et al.[7] A liquid chromatograph (Model 600E, Waters, Milford, MA) equipped with UV detector (Model 484, Waters, Milford, MA), autosampler (Model 717, Waters), and autointegrator (Millennium, Waters) was used. For the stationary phase, a reversed-phased column (L-column ODS, 4.6 mm i.d. × 150 mm length, Chemicals Inspection and Testing Institute, Tokyo, Japan) warmed to 40 °C was used. The mobile phase was a mixture of 50 mM phosphate buffer adjusted to pH 6.8 and acetonitrile (100:14) containing 5 mM octylamine. The flow rate was 1.0 mL/min.

Sample Preparation for HPLC Analysis. For the analysis of plasma concentrations of acetaminophen and sulfapyridine, 1 mL of 50 mM phosphate buffer, 0.1 mL of IS solution (*p*-anisamide, 20 μg/mL), and 2 mL of ethyl acetate were added to 0.2 mL of plasma. The mixture was shaken for 10 min and centrifuged at 3000 rpm (1800*g*) for 10 min at 4 °C. The organic layer was evaporated to dryness under reduced pressure. The residue was dissolved with mobile phase and transferred to autosampler vials, and a 10 μL portion was injected onto the HPLC system. Standard working solutions (10 μL) were added to blank plasma to give standard calibration samples at concentrations of 200–5000 ng/mL.

LC/MS/MS Analysis. Concentrations of propranolol, atenolol, furosemide, naproxen in plasma and Ussing-type chamber medium, and acetaminophen in Ussing-type chamber medium were measured by the LC/MS/MS method consisting of Quattro Ultima (Waters) with Alliance 2695 separation module (Waters). Multiple reaction monitoring (MRM) mode was used as follows to monitor ion (precursor ion → product ion): atenolol (267.0 → 144.8), acetaminophen (152.2 → 109.8), propranolol (260.0 → 182.8), furosemide (329.07 → 285.04), naproxen (230.88 → 184.93), and D82–7319 (IS, 488.2 → 350.2). Samples were injected into a Symmetry Shield RP8 column (3.5 μm φ, 2.1 mm × 30 mm i.d., Waters) with Symmetry Shield RP8 guard column (3.5 μm φ, 2.1 mm × 10 mm i.d., Waters) warmed to 40 °C. Elution was conducted at a flow rate of 0.5 mL/min by a linear gradient with the mobile phase, which consisted of a mixture of A (10 mM ammonium formate solution) and B (methanol). The gradient conditions for elution were as follows: gradient [min, B%] = [0, 30]−[0.5, 30]−[1.5, 90]−[3.5, 90]−[4, 30]−[5, 30].

Sample Preparation for LS/MS/MS Analysis. Methanol (150 μL) containing IS (D82-7319, 62.5 ng/mL) was added to the plasma and medium samples (50 μL) of Ussing-type chamber experiments and centrifuged at 3000 rpm (1800*g*) for 10 min at 4 °C. The supernatant was transferred to MultiScreen and filtered by centrifugation at 3000 rpm (1800*g*) for 10 min at 4 °C. The filtrate was transferred to autosampler vials, and a 10 μL portion was injected onto the LC/MS/MS system. Standard working solutions (10 μL) were added to blank plasma or medium to give standard

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Table 1. Gene-Specific Primers of Monkey for SYBR Green PCR

gene	forward primer	reverse primer	product size (bp)
GAPDH	5′-ATTCCACCCATGGCAAGTTC-3′	5′-ACGTACTCAGCGCCAGCAT-3′	136
MDR1	5′-AGCGGCTCCGATACATGGT-3′	5′-GGCGAGCCTGGTAGTCAAT-3′	101
BCRP	5′-AGCGGGATAAGCCACTCGTA-3′	5′-CTCACCCCCGGAAAGTTGAT-3′	101
MRP2	5′-ATGGCAGTGAAGAAGAAGACGAT-3′	5′-TGCTGCTGGACCTAGAACTG-3′	138

calibration samples at concentrations of 1–5000 ng/mL for LC/MS/MS.

Data Analysis of HPLC and LC/MS/MS Data. The peak area of the compound was divided by the peak area of the IS to obtain the peak-area ratio. The calibration curve for the compound was constructed from the least-squares linear regression of the peak−area ratios of standards versus the compound concentrations.

RNA Extraction and RT Reaction. Segments of jejunum, colon, and liver tissues were isolated from cynomolgus monkeys ($n = 7$) and were quickly stripped of connective tissue, snap-frozen, and stored at −80 °C until processing. Total RNA was extracted from tissue samples using TRIzol reagent (Invitrogen Japan K.K., Tokyo, Japan) and chloroform. The RT reaction was conducted in 10 μL of two-step RT reaction mix containing 2 μL of the extracted total RNA (200 μg/mL), 1 × TaqMan RT buffer, 5.5 mM $MgCl_2$, 500 μM dATP, 500 μM dGTP, 500 μM dCTP, 500 μM dUTP, 2.5 μM oligo $(dT)_{16}$ primer, 0.4 U/μL of Rnase inhibitor, and 1.25 U/μL MultiScribe reverse transcriptase (Applied Biosystems, Foster City, CA). The mixture was incubated at 25 °C for 10 min and subsequently at 48 °C for 30 min. The RT reaction was terminated by heating at 95 °C for 5 min followed by cooling at 4 °C for 5 min, giving the RT product.

Real-Time PCR with SYBR Green. Primer pairs for MDR1, BCRP, MRP2, and GAPDH were designed using the Primer Express (Ver. 1.0, Applied Biosystems) as shown in Table 1 and were synthesized by Invitrogen (Tokyo, Japan). A commercial reagent, QuantiTect SYBR Green PCR kit (QIAGEN, Hilden, Germany), was used for PCR.

Each reaction mixture contained 300 nM each primer, 6.5 μL of RT product, and SYBR Green PCR Master Mix (QIAGEN) in a total volume of 25 μL. PCR conditions were 10 min at 95 °C followed 40 cycles of 15 s at 95 °C and 1 min at 60 °C. The relative increase of the reporter fluorescent dye emission was monitored in real time using an ABI prism 7000 sequence detector (Applied Biosystems). The fluorescent dye emission was a function of cycle number and was determined using the sequence detector software (Applied Biosystems), giving the threshold cycle number (C_T) at which PCR amplification reached a significant threshold. The mRNA levels of MDR1, BCRP, and MRP2 are expressed as values relative to GAPDH mRNA.

Results

The oral BA of five drugs in cynomolgus monkeys are summarized in Table 2 with that in humans (cited from the literature). Almost the same BA was obtained in both cynomolgus monkeys and humans for atenolol and naproxen, whereas the BA of propranolol, furosemide, and acetaminophen was much lower in cynomolgus monkeys. Although the oral BA of acetaminophen was reported as 80% in humans[25] and 100% in beagle dogs,[5] it was only 16% in cynomolgus monkeys in this study. In the case of propranolol, plasma concentrations were not detected after oral administration to cynomolgus monkeys even at a dose of 1 mg/kg. The correlation of the oral BA of these drugs between humans and cynomolgus monkeys was therefore poor and not significant, as shown in Figure 1.

Table 2. Oral BA of Five Drugs in Cynomolgus Monkeys and Humans[a]

	cynomolgus monkeys		humans[b]	
compound	dose	BA (%)	dose	BA (%)
acetaminophen	7.7 mg/kg	16	20 mg/kg	88
atenolol	1 mg/kg	57	100 mg/man	56
furosemide	1 mg/kg	32	40 mg/man	71
propranolol	1 mg/kg	<1	80 mg/man	26
naproxen	1 mg/kg	100	250 mg/man	99

[a] Each value in cynomolgus monkeys represents the means of three to five experiments. [b] Human data were cited from the literature.[23]

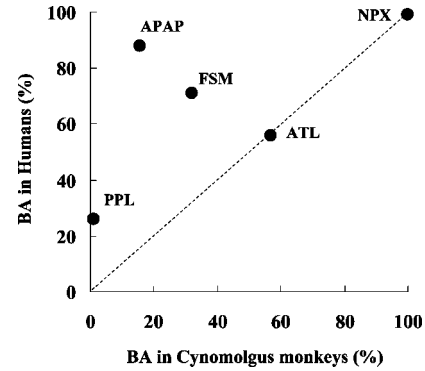

Figure 1. Correlation of the oral bioavailability of acetaminophen (APAP), atenolol (ATL), furosemide (FSM), propranolol (PPL), and naproxen (NPX) between humans and cynomolgus monkeys. BA values in cynomolgus monkeys represent the mean of the data from three to five experiments. Human data were cited from the literature.[23]

In order to determine the factors involved in the low BA of these drugs in cynomolgus monkeys, at first, PK analysis

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Table 3. Oral BA, F_h, and F_aF_g Values of Each Drug Calculated with Pharmacokinetic Parameters after Oral and Intravenous Administration to Cynomolgus Monkeys[a]

	cynomolgus monkeys					humans[b]		
	BA (%)	Rb	CL_{tot} (mL/min/kg)	F_h	F_aF_g	BA (%)	F_h	F_aF_g
acetaminophen	16	1.34	7.3	0.83	0.19	88	0.77	1
atenolol	57	1.08	7.5	0.98	0.58	56	0.99	0.56
furosemide	32	0.66	9.1	0.83	0.38	71	0.98	0.73
propranolol	<1	1.17	37.6	0.14	0	26	0.24	1
naproxen	100	0.60	0.83	0.97	1	99	0.99	0.99

[a] Each value in cynomolgus monkeys represents the means of three to five experiments. [b] Human data were cited from the literature.[23]

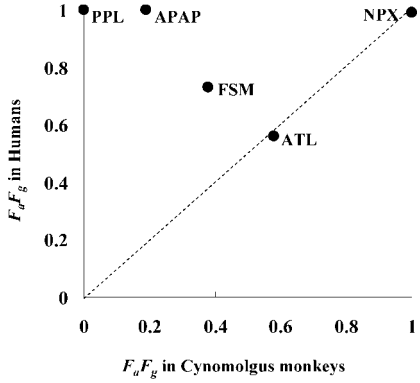

Figure 2. Correlation of the F_aF_g value of acetaminophen (APAP), atenolol (ATL), furosemide (FSM), propranolol (PPL), and naproxen (NPX) between humans and cynomolgus monkeys. F_aF_g values in cynomolgus monkeys represent the mean of the data from three to five experiments. Human data were cited from the literature.[23]

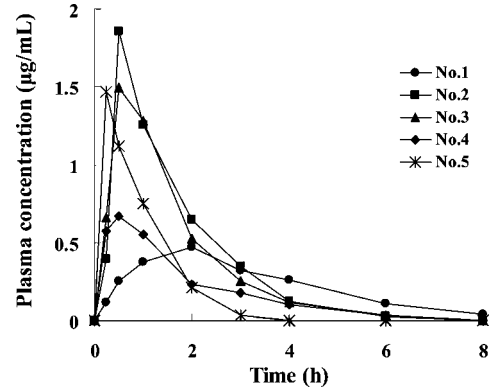

Figure 3. Plasma concentration−time profiles of acetaminophen after oral administration at a dose of 7.7 mg/kg in fasted cynomolgus monkeys.

was performed to estimate the F_aF_g and F_h. The oral BA, F_h, and F_aF_g values of each drug are shown in Table 3. F_h, which is an index of hepatic first-pass metabolism, was close to unit except for propranolol and was almost consistent with that in humans. Only propranolol showed a significantly smaller F_h value in cynomolgus monkeys than in humans. On the other hand, F_aF_g of acetaminophen, furosemide, and propranolol were markedly smaller in cynomolgus monkeys than those in humans as shown in Figure 2. This result indicating that the low oral BA of these drugs in cynomolgus monkeys was mainly attributed to the drug absorption processes from the GI tract, but not to hepatic first-pass metabolism.

GER and GITT were evaluated from the rate of absorption of acetaminophen and from the appearance time of sulfapyridine in plasma after oral administration of salitylazosulfapyridine, respectively. Plasma concentration−time profiles of acetaminophen after oral administration as the solution at a dose of 7.7 mg/kg in cynomolgus monkeys are shown in Figure 3. Although large interindividual deviations in the plasma concentration of acetaminophen were observed, the mean absorption time (MAT) of 1.02 h in cynomolgus monkeys was only slightly longer than that in humans (Table 4). GITT in cynomolgus monkeys, calculated from the average appearance time of sulfapyridine in plasma after oral administration of salitylazosulfapyridine, was 2.7 ± 0.4 h (mean ± S.E., $n = 6$). GITT was considered to be only slightly shorter in cynomolgus monkeys than in humans since GITT in humans was reported to be 3–4 h.[20]

Then, P_{app} of these drugs to the intestinal membrane of cynomolgus monkeys was measured by the *in vitro* Ussing-type chamber method and was compared with that of rat intestine (jejunum) measured by the same method in our previous report.[20] The P_{app} of cynomolgus monkey intestine was low compared with that to rat intestine in all five drugs; in particular, propranolol and furosemide showed the remarkably low P_{app} value (Table 5). The P_{app} of propranolol and furosemide in cynomolgus monkey intestine was markedly low and deviated from the correlation to human F_aF_g obtained for other three drugs ($F_a = 1 - \exp(-P_{app}(4.98 \times 10^5))$, $r^2 = 0.923$) (Figure 4). This correlation curve was similar with that obtained between P_{app} in rat intestine and human F_a in the previous report.[20] When the correlation was calculated for all five drugs, it became very poor ($F_aF_g = 1 - \exp(-P_{app}(42.6 \times 10^5))$, $r^2 = 0.056$). Furthermore, in Figure 5, the correlation between F_aF_g calculated from the PK data in cynomolgus monkeys and P_{app} in cynomolgus monkey intestine was shown. Both acetaminophen and propranolol were plotted significantly down below the correlation curve for other drugs ($F_aF_g = 1 - \exp(-P_{app}(5.80 \times 10^5))$, $r^2 = 0.871$), suggesting the small F_g values of acetaminophen and propranolol in cynomolgus monkeys.

Additionally, in order to investigate the cause of low F_aF_g and P_{app} of furosemide in cynomolgus monkeys, expression levels of mRNAs of efflux transporters in jejunum, colon, and liver of cynomolgus monkeys were quantified by real-

Table 4. Pharmacokinetic Parameters after Oral Administration of Acetaminophen in Cynomolgus Monkeys and Humans

	dose (mg/kg)	AUC (μg*h/mL)	C_{max} (μg/mL)	MRT (h)	MAT (h)	BA (%)
cynomolgus monkeys	7.7	2.1	1.19	1.75	1.02	16
humans[a]	5	13.3	4.18	2.52	0.7–0.8	100

[a] Human PK parameters were cited from the reference literature.[25]

Table 5. Apparent Permeability Coefficients (P_{app}) of Five Drugs Obtained through the in Vitro Ussing-Type Chamber Method Using Cynomolgus Monkeys and Rats Intestinal Tissues[a]

	P_{app} ($\times 10^{-6}$ cm/s)	
	cynomolgus monkeys	rats
acetaminophen	4.38 ± 0.93	8.67 ± 0.01
atenolol	1.92 ± 1.01	6.95 ± 3.07
furosemide	0.33 ± 0.30	6.06 ± 0.74
propranolol	0.66 ± 0.32	11.80 ± 3.51
naproxen	7.36 ± 1.05	30.13 ± 3.15

[a] Each value represents the mean ± SD of three experiments.

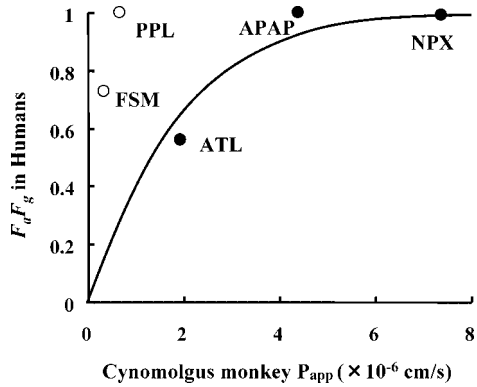

Figure 4. Plot of the fraction dose absorbed from the GI tract (F_aF_g) in humans versus apparent permeability coefficients (P_{app}) to cynomolgus monkey intestine of acetaminophen (APAP), atenolol (ATL), furosemide (FSM), propranolol (PPL), and naproxen (NPX). F_aF_g values were calculated from the clinical data by PK analysis. P_{app} values were measured by theUssing-type chamber method in vitro. The theoretical line was calculated by nonlinear least-squares regression analysis using the data of three drugs excluding those of propranolol and furosemide.

time RT-PCR using GAPDH as an endogenous gene. Expression levels of mRNA of MDR1, MRP2, and BCRP were significantly higher in cynomolgus monkey intestine than those in human intestine (Table 6).

Discussion

Cynomolgus monkeys or beagle dogs are often used for PK studies in the drug discovery and development processes in pharmaceutical companies. Amidon et al. have demonstrated a good correlation of apparent drug permeability obtained by *in situ* perfusion of rat intestine to F_a in humans.[26] Recently, an *in vitro* method using Caco-2 cell monolayer has been used widely as a high-throughput screening for intestinal drug absorption since drug permeability to Caco-2 monolayers shows the good correlation to F_a in humans.[27,28] Chiou et al. have carefully investigated the F_a of 43 drugs in cynomolgus monkeys correlated well with that in humans.[3] These reports indicate the possibility to predict F_a in humans from *in vivo* animal studies or *in vitro* membrane permeation studies. However, in our company, remarkably low oral BA has often been shown in cynomolgus monkeys compared with that in other animals at the preclinical studies. In some cases, since the compounds were fairly stable in monkey liver microsomes, the reason of low oral BA in cynomolgus monkeys was not fully explained by the hepatic first-pass metabolism. If the factors that cause species differences in oral BA were not determined, it is difficult to judge the validity to advance to the

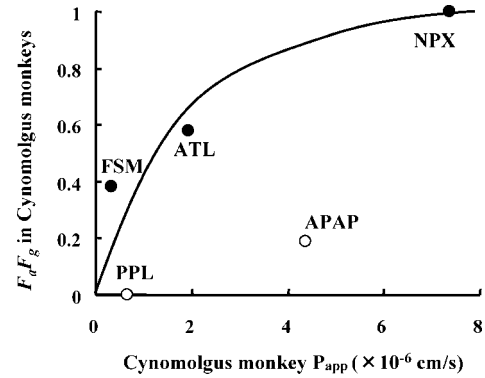

Figure 5. Plot of the fraction dose absorbed from the GI tract (F_aF_g) in cynomolgus monkeys versus apparent permeability coefficients (P_{app}) to cynomolgus monkey intestine of acetaminophen (APAP), atenolol (ATL), furosemide (FSM), propranolol (PPL), and naproxen (NPX). F_aF_g values were calculated from the data of in vivo experiments by PK analysis. P_{app} values were measured by the Ussing-type chamber method in vitro. The theoretical line was calculated by nonlinear least-squares regression analysis using the data of three drugs, excluding those of acetaminophen and propranolol.

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Table 6. Relative Expression of mRNA for MDR1, MRP2, and BCRP in Intestine and Liver of Cynomolgus Monkeys and Humans[a]

	MDR1		MRP2		BCRP	
tissue	monkey	human[b]	monkey	human[b]	monkey	human[b]
small intestine	0.479 ± 0.212	0.017	0.302 ± 0.117	0.014	1.450 ± 0.462	0.024
colon	0.846 ± 0.430	0.007	0.0029 ± 0.0020	0.0003	0.278 ± 0.246	0.004
liver	0.039 ± 0.022	0.013	0.216 ± 0.129	0.121	0.161 ± 0.100	0.008

[a] Relative expression of mRNA were determined by real-time PCR. GAPDH was selected as an endogeneous RNA to normalize for differences in the amount of total RNA. Each result of cynomolgus monkeys represents the mean ($n = 7$) ± SD. [b] Human data were cited from the reference literature.[24]

clinical trials from the data of preclinical animal studies for such candidate compounds.

The gastric pH and the gastrointestinal motility affect the oral drug absorption. It was reported that the relation of oral drug absorption between dogs and humans could be improved by adjusting the gastric pH or by controlling the gastrointestinal transit of dogs.[9,25] These reports suggest that the influence of the physiological factors on gastrointestinal absorption of drugs is not negligible. In this study, at first, GER and GITT of cynomolgus monkeys were evaluated. Although GITT in cynomolgus monkeys was slightly shorter than that in humans, it would be difficult to explain the low oral BA of furosemide, acetaminophen, and propranolol in cynomolgus monkeys only by the difference in GITT. Since the oral BA of atenolol, a low permeability drug, and that of naproxen, a high permeability drug, were similar between humans and cynomolgus monkeys, both GER and GITT were considered not to be the main factors for low oral BA.

Acetaminophen is a drug with a high permeability to the intestinal membrane and is absorbed quickly from the upper small intestine in humans; thus, this drug is used to evaluate GER.[10,11,25] As shown in Table 1, although the oral BA of acetaminophen was only 16% in cynomolgus monkeys, the mean absorption time (MAT) value was almost the same with that in humans. This result suggested that, in cynomolgus monkeys, the rate of acetaminophen absorption was still high and acetaminophen was absorbed quickly from the upper part of the small intestine. From the plasma concentrations after intravenous and oral administration, F_h of acetaminophen in cynomolgus monkeys was calculated as 0.83. Together with the above results, the main cause of low oral BA of acetaminophen might be attributed to low F_g.

In order to investigate the cause of species differences of the intestinal absorption of cynomolgus monkeys in detail, we have measured the permeability (P_{app}) of test drugs to the intestinal membrane of cynomolgus monkey and evaluated their relation to F_aF_g. Amidon et al. have demonstrated a good correlation of drug P_{app} obtained by *in situ* perfusion of rat intestine to F_a in humans.[26] In the previous report, we have observed the same type of correlation using the P_{app} to rat jejunum measured by Ussing-type chamber method *in vitro*.[20] In this study, the P_{app} values of tested drugs to cynomolgus monkey intestine were plotted against F_aF_g in human (Figure 4) and in cynomolgus monkeys (Figure 5). Although monkey intestine showed the P_{app} lower for all drugs than rat intestine (Table 5), a similar correlation was obtained for acetaminophen, atenolol, and naproxen to human F_aF_g as shown in Figure 4. However, propranolol and furosemide were deviated from the correlation curve and were plotted on the left side of the curve. Furthermore, in Figure 5, a similar correlation was observed for furosemide, atenolol, and naproxen between monkey P_{app} and monkey F_aF_g, but propranolol and acetaminophen were plotted on the lower side due to the low F_aF_g in cynomolgus monkeys for both drugs.

The reason for the low P_{app} of furosemide to cynomolgus monkey intestine (Figure 4) was uspected to be contribution of efflux transporters since furosemide was reported to be actively secreted in Caco-2 cells and rat jejunal tissue.[29–31] This consideration was strongly supported in this study by the observation of expression levels of mRNA of MDR1, MRP2, and BCRP in cynomolgus monkey intestine. As shown in Table 6, the mRNA levels of these efflux transporters in the intestine were much higher in cynomolgus monkeys than those in humans. Although the corresponding transporter for furosemide has not been molecularly identified yet, it is reasonable to consider that those drugs are the substrates of efflux transporters such as P-gp and MRP2, show low permeability to the intestinal membrane of cynomolgus monkeys, and may result in the low BA after oral administration.

Results of *in vivo* PK analysis clearly showed that F_aF_g values of propranolol and acetaminophen in cynomolgus monkeys were markedly lower than those in humans (Figure 2). Propranolol was reported to be a substrate of CYP1A2, 2D6, and UGT,[32–35] and acetaminophen[36] and naproxen[37] were also the substrate of UGT in humans. Several reports

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have demonstrated that the intestinal first-pass metabolism of midazolam, a typical substrate of CYP3A, in cynomolgus monkeys is markedly higher than that in humans and is a main factor of the low BA in cynomolgus monkeys.[13,14] It was also reported that the conjugative activities in the intestine were higher in cynomolgus monkeys than in humans.[15,16] Since the membrane permeability of propranolol and acetaminophen were considered to be high enough, it is obvious that the high first-pass metabolism in the enterocytes is a main factor of their low F_aF_g in cynomolgus monkeys.

In Figure 5, since both acetaminophen and propranolol were plotted down below the correlation curve for other drugs, high metabolism of these drugs in the enterocytes of cynomolgus monkeys were not fully reflected *in vitro* P_{app} values.

Bergren et al. have reported that ropivacaine, a substrate of CYP3A4, was metabolized in the human small intestine *in vitro*.[38] In addition, Johnson et al. have demonstrated that metabolites of verapamil were accumulated in the apical side of rat small intestine in the permeation study of verapamil in Ussing-type chambers.[39] However, in our preliminary study in which intestinal microsomes were used to examined the *in vitro* metabolic stability of several drugs, any significant degradation were not detected for acetaminophen, propranolol, and naproxen in the intestinal microsomes of cynomolgus monkeys and human. Significant degradation was detected only for CYP3A4 substrates, such as midazolam and verapamil, in the intestinal microsomes of cynomolgus monkeys (data not shown). In the *in vitro* study, the intestinal metabolism of drugs has often been evaluated by detecting the metabolites. However, some reports have suggested that the amount of metabolites detected was too little to explain the high first-pass intestinal metabolism of drugs *in vivo*.[15,16] Especially, in the case of glucuronidation, CL_{int} values obtained by *in vitro* microsome studies were significantly lower (10- to 30-fold) than those observed *in vivo*.[41,42]

These facts indicated the difficulty to detect the intestinal metabolism of drugs by *in vitro* experiments. In this study, it is possible to consider that P_{app} of propranolol to the cynomolgus monkey intestine was lowered by the influence of metabolism because the P_{app} value was much lower in the intestine of cynomolgus monkey than that of rat. Also in Figure 4, the plot of propranolol was significantly deviated from the correlation curve for other drugs. Further studies on *in vitro* activity of CYP and conjugative enzymes in the intestinal membrane should be necessary to clarify the reason of *in vitro*−*in vivo* incompatibility in intestinal metabolism.

From these findings, the following considerations are possible about the low oral BA of furosemide, acetaminophen, and propranolol in cynomolgus monkeys,

(1) Low oral BA of furosemide in cynomolgus monkeys was attributed to its low membrane permeability to monkey small intestine. The difference in the function of efflux transporters such as MDR1, MRP2, or BCRP in the small intestine might be the factor of low P_{app} and thus low oral BA of furosemide in cynomolgus monkeys.

(2) Acetaminophen seems to be metabolized in the monkey small intestine during the absorption process *in vivo* that leads to the low oral BA in cynomolgus monkeys. In human, intestinal metabolism was not significant for acetaminophen.

(3) Although it was impossible to calculate the exact value of F_aF_g in cynomolgus monkeys, propranolol was considered to be significantly metabolized in the intestine during the absorption process *in vivo*.

In conclusion, it was demonstrated that the oral drug absorption in cynomolgus monkeys was strongly affected by the intestinal metabolism and/or the efflux transport. High activities of metabolic enzymes and transporters are the possible factors to cause poor oral absorption of drugs in cynomolgus monkeys.

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