Studies on the contribution of drug transporters and metabolic enzymes in the pharmacokinetics of oral pharmaceutical drugs

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LIST OF PUBLICATIONS

1. Studies on the intestinal absorption characteristics of sulfasalazine, a breast cancer resistance protein (BCRP) substrate : Atsuko Tomaru, Nozomi Morimoto, Mariko Morishita, Kozo Takayama, Takuya Fujita, Kazuya Maeda, Hiroyuki Kusuhara, Yuichi Sugiyama. *Drug Metab. Pharmacokinet.*(accepted) <presented in Chapter 1 of this dissertation>

2. Analysis of the pharmacokinetic boosting effects of ritonavir on oral bioavailability of drugs in mice : Atsuko Tomaru, Mariko Takeda-Morishita, Hirokazu Banba, Kozo Takayama. *Drug Metab. Pharmacokinet.*(submitted) <presented in Chapter 2 of this dissertation>

ABBREVIATIONS

ASBT	apical sodium-dependent bile acid transporter
ANOVA	analysis of variance
AUC	area under the plasma concentration-time curve
BCRP (Bcrp)	breast cancer resistance protein
C_{max}	the maximum plasma concentration
CYP3A (cyp3a)	Cytochrome P450, family 3, subfamily A
DMSO	dimethyl sulfoxide
HPLC-UV	high performance liquid chromatography with ultraviolet detection
LC-MS/MS	liquid chromatography tandem mass spectrometry
PEPT	peptide transporter
P-gp	P-glycoprotein
MRP2 (Mrp2)	multidrug resistance-associated protein
T_{max}	the time to reach the maximum plasma concentration
HIV	human immunodeficiency virus
IC_{50}	half maximal (50%) inhibitory concentration
Ki	inhibition constant
Km	michaelis constant
OATP (Oatp)	organic anion transporter

GENERAL INTRODUCTION

Pharmacokinetics and pharmacodynamic effects of drugs are influenced by multiple process including absorption, distribution, metabolism and excretion. Recent significant advances in the research and technology have demonstrated the expression of various metabolic enzymes and transporters in the small intestine and the liver, and its important role in determining drug disposition and pharmacological effects. Furthermore, recent studies have demonstrated that drug transporters are also important determinations of drug disposition and response by affecting cellar exposure and distribution. Therefore, the interplay between uptake/efflux transporters and metabolic enzymes needs to be carefully investigated in order to obtain a better understanding of molecular mechanisms underlying variable drug response.

The intestinal absorption is determined by the physicochemical properties of a drug, such as its solubility, the permeability of epithelial cell membranes, and its first-pass metabolism in the liver and small intestine. The most important factor involved in the first-pass effect in the small intestine has been considered to be CYP3A and P-gp. In addition, the importance of other efflux transporters, such as BCRP and MRP2, and influx transporters, such as PEPT and ASBT, expressed on the apical membrane has been recognized. CYP3A is responsible for the majority of phase I drug metabolism and is predominantly expressed in the human small intestine and liver. In the liver, metabolic enzymes such as CYP3A has been considered to play a significant role for the drug metabolism. However, recent study demonstrates influx transporters expressed on sinusoidal membrane is also important for the metabolism in the liver because the inhibition of these influx transporters increases the plasma concentration of drugs in the liver, causing adverse events.

BCRP is expressed at the apical membrane in the small intestine and has been found recently. The mRNA level of BCRP in human small intestine is the same or higher compared to that of other efflux transporters[1)]. Urquhart *et al.* and Yamasaki *et al.*

demonstrated a prominent role of BCRP polymorphisms in the intestinal absorption of SASP in humans[2,3]. Therefore, the probe substrate of BCRP is required to examine the relation between pharmacokinetics and drug efficacy.

Sulfasalazine (SASP) has long been used in the treatment of inflammatory bowel diseases such as ulcerative colitis and Crohn's disease, and rheumatoid disease[4,5]. A recent study demonstrated that long-term treatment of human T-cells with SASP causes the development of cellular drug resistance that is mediated by the induction of BCRP, suggesting that SASP is a substrate of human BCRP[6]. Furthermore, animal studies using $Bcrp^{-/-}$ mice have shown the area under the plasma concentration (AUC) of SASP after oral administration was 111-fold higher in $Bcrp^{-/-}$ mice compared with wild-type mice, whereas the AUC of SASP after intravenous administration was only 13-fold higher in $Bcrp^{-/-}$ mice[7]. These results suggest that Bcrp limits oral absorption of SASP in the intestine. However, the recent report demonstrated the involvement of MRP2[8,9] and an influx transporter in the intestinal absorption of SASP[10]. Therefore, to elucidate the mechanism of intestinal absorption of SASP is important to use it as the probe substrate of BCRP. In chapter 1, the intestinal absorption characteristic of sulfasalazine (SASP) was investigated.

In chapter 2, the mechanism of ritonavir (RTV) boosting using each probe substrate was investigated. RTV boosting is a drug therapy used in the treatment of HIV that utilizes the principal of drug-drug interaction. RTV was first developed as an HIV protease inhibitor (PI) and used for a single PI treatment. However, since its potent inhibitory effect on the CYP3A was clarified, RTV has been used in multidrug treatment regimens for HIV to obtain an adequate plasma concentration for effective anti-HIV activity[11,12]. In addition, the inhibitory effects of RTV to various efflux transporters such as P-gp, BCRP, MRP2, and OATP were also reported[13,14,15,16]. Therefore, RTV boosting involves multiple mechanisms. The contribution of RTV to the first-pass effect in the small intestines and livers of mice was investigated by using several probe substrates.

Chapter 1

Studies on the intestinal absorption characteristics of sulfasalazine, a breast cancer resistance protein (BCRP) substrate

1. Introduction

In this chapter, I investigated the characteristic of SASP, which is used as a probe substrate of BCRP, in the small intestinal absorption.

After oral administration, SASP is broken down into sulfapyridine and 5-aminosalicylic acid by bacterial azo reductases in the colon and cecum [17, 18]. 5-aminosalicylic acid is effective for inflammatory bowel diseases, while SASP and sulfapyridine are effective for rheumatoid disease [19, 20, 21]. The targeting of SASP to the colon is critical for demonstrating its pharmacological action.

In humans, the bioavailability of orally administered SASP is less than 15% [22]. The cause of low bioavailability of SASP has been investigated and was attributed to low solubility and poor permeability [23]. However, the recent study demonstrated that SASP is a substrate of the efflux transporter, BCRP [6]. More recently, Kusuhara *et al.* reported the non-linearly in AUC of plasma SASP concentration between microdose and therapeutic dose in the clinical study. As one of possible mechanisms underlying the nonlinear pharmacokinetics of SASP, the saturation of the influx transporter, was considered[10]. However, the contribution of influx transporters on the intestinal SASP absorption has not been elucidated in detail, and only the limited information is available on the characterization of SASP intestinal absorption. Therefore, to elucidate the mechanism of SASP in the small intestine is important to use a probe substrate of BCRP. Thus, this study aimed to examine the intestinal absorption of SASP by using everted sacs from wild-type and Bcrp$^{-/-}$ mice.

2. Materials and Methods

2.1 Materials

SASP was purchased from Sigma-Aldrich (St Louis, MO, USA). Ko134 was kindly provided by SOLVO biotechnology (Budaörs, Hungary). All other chemicals were of analytical grade and are commercially available.

2.2 Animals

Male ddY mice were purchased from Sankyo Labo Service Corp. (Tokyo, Japan). Female Bcrp$^{-/-}$ mice were purchased from Taconic Farms (Germantown, NY, USA) and were bred by Shimizu Laboratory Supplies Co., Ltd. (Kyoto, Japan). Age-matched wild-type mice (FVB strain) were purchased from CLEA Japan (Tokyo, Japan). All mice (10-40 weeks) were housed in rooms maintained at 23 °C and 55±5 % relative humidity, and were allowed free access to food and water during the acclimatization period. The animal work was performed at Hoshi University and complied with the regulations of the Committee on Ethics in the Care and Use of Laboratory Animals.

2.3 Preparation of everted mouse ileum sacs

Everted sacs were prepared by a modification of the procedure described previously[24]. Mice were anesthetized with ether and sacrificed by exsanguination of the abdominal aorta. The ileum was removed immediately and rinsed in ice-cold Krebs-Ringer-Henseleit bicarbonate buffer (KRB; 118 mM NaCl, 4.75 mM KCl, 2.50 mM $CaCl_2$, 1.19 mM KH_2PO_4, 1.19 mM $MgSO_4$, 25 mM $NaHCO_3$, 11 mM D-glucose, pH 6.5). Approximately 5-cm segments of the ileum was isolated and everted using a stainless steel rod. Polyethylene tubes were inserted into both ends of the everted segments and ligated.

2.4 *In vitro* absorptive transport (mucosal-to-serosal) study using everted mouse ileum sacs

The everted sac was placed in 30 mL of KRB and gassed with O_2/CO_2 (95:5) at 37°C.

The everted ileum was filled initially with 1.5 mL of KRB and perfused with the buffer at 0.1 mL/min using an infusion pump (KD Scientific Inc., Holliston, MA, USA) throughout the transport study. After preincubation in the buffer containing 2 μM Ko134/ DMSO or DMSO for 30 min, SASP was added to the mucosal side to give a final concentration of 0.1, 0.3, 1, 3 or 50 μM. The outflow perfusate was then collected for 5 min at 15 minute intervals for up to 90 min.

The absorptive clearance of SASP in the everted sac was calculated according to the following equation:

$$\text{Absorption rate} = (C_{out} \times Q) / L$$

$$\text{Absorptive clearance} = \text{Absorption rate} / C_m$$

where C_{out} represents the SASP concentration in the outflow solutions, Q is the perfusion rate (0.1 mL/min), L is the length of the intestinal segment, and C_m is the concentration of SASP in the mucosal medium.

2.5 Quantification of SASP concentration in samples

The SASP concentration was analyzed by LC-MS/MS in turbo ion spray negative ion mode equipped with the Prominence LC system (Shimadzu Co., Kyoto, Japan) coupled to an API4000 mass spectrometer (AB SCIEX, San Jose, CA, USA). The mass transition was from m/z 397 to 197. A CAPCELL PAK MGII C_{18} (2.1×50 mm, 5 μm; Shiseido Co., Ltd., Tokyo, Japan) maintained at 40°C was used for the chromatographic separation. The LC separation was achieved using a mobile phase comprising of 10 mM ammonium acetate (pH 8) and acetonitrile (2:8, v/v) at a flow rate of 0.2 mL/min. The data acquisition was performed using Analyst ver. 1.4.2 (AB SCIEX, San Jose, CA, USA).

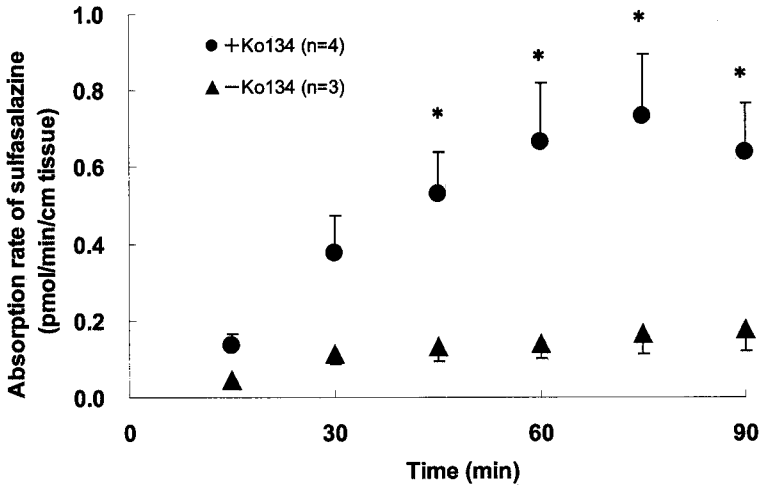
2.6 Statistical analysis

Each value is expressed as the mean ± standard error (S.E.) of 4-7 determinations.

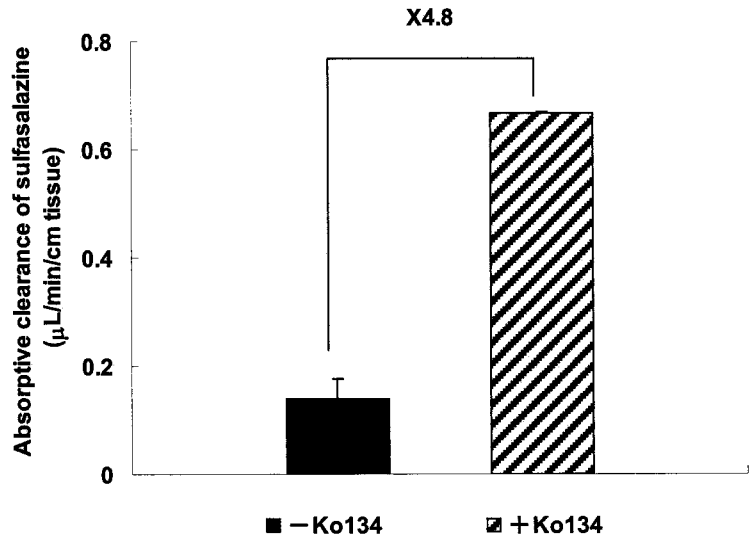
For group comparisons, ANOVA with a one-way layout was applied. Significant differences in the mean values were evaluated by Student's unpaired *t*-test or Dunnett's test for multiple comparisons. A p value of less than 0.05 was considered significant.

3. Results

In wild-type mice, the intestinal absorption rate of 10 μM SASP reached a plateau at 60 min in absence and presence of 2 μM Ko134, a known inhibitor of Bcrp (Figure 1A). The absorptive clearance at 60 min was 0.14 μL/min/cm in the absence of Ko134. The reported IC_{50} value for the inhibition of BCRP by Ko134 is 0.07 μM [25]. Therefore, we used 2 μM Ko134 to inhibit completely the efflux transport by Bcrp. The absorptive clearance was increased by 4.8-fold in the presence of Ko134 (Figure 1B). These results indicate that Bcrp limits the intestinal absorption of SASP as reported previously.



(A)



(B)

Figure 1

(A)Intestinal absorption rate of SASP (10 μM) in the ileum of wild-type mice with or without Ko134

The data are expressed as mean ± S.E. (n=3-4). *p<0.05

(B)Steady-state absorptive clearance of SASP (at 60 min) in the ileum of wild-type mice with or without Ko134

The data are expressed as mean ± S.E. (n=4).

The intestinal SASP absorption was also examined at various SASP concentrations. In wild-type mice, the absorptive clearance of SASP was similar in the range of 0.1 to 50 μM (Figuer 2). By contrast, the absorptive clearance of SASP decreased significantly along with the SASP concentration in the mucosal side in the presence of Ko134 (Figure 3). Such concentration dependence in the absorptive clearance of SASP was also observed in Bcrp$^{-/-}$ mice (Figure 4). Thus, the saturation of the absorptive rate at a high concentration of SASP in the presence of the specific Bcrp inhibitor and in Bcrp$^{-/-}$ mice suggests the involvement of some influx transporters in the absorption of SASP.

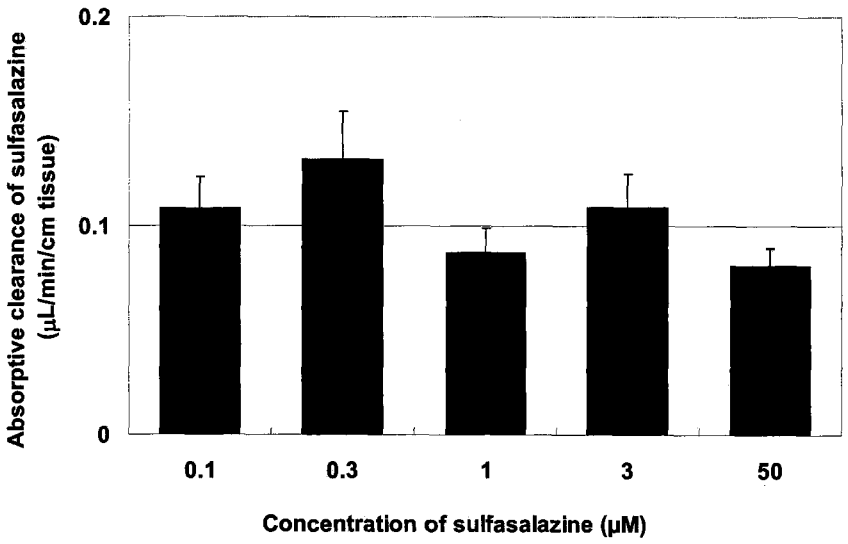


Figure 2

Steady-state absorptive clearance of SASP at various concentrations in the everted ileum sac in wild-type mice

The data are expressed as mean ± S.E. (n=4).

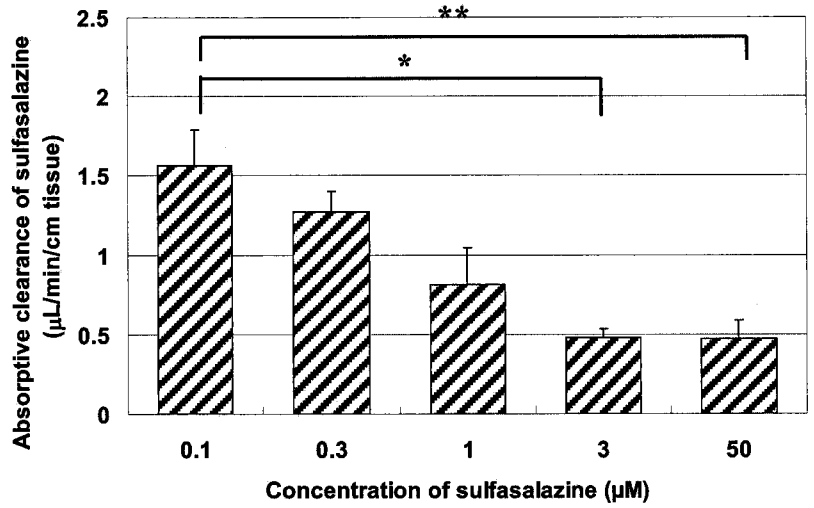


Figure 3

Steady-state absorptive clearance of SASP at various concentrations in the everted ileum sac in the presence of Ko134 in wild-type mice

The data are expressed as mean ± S.E. (n=4). *p<0.05, **p<0.01

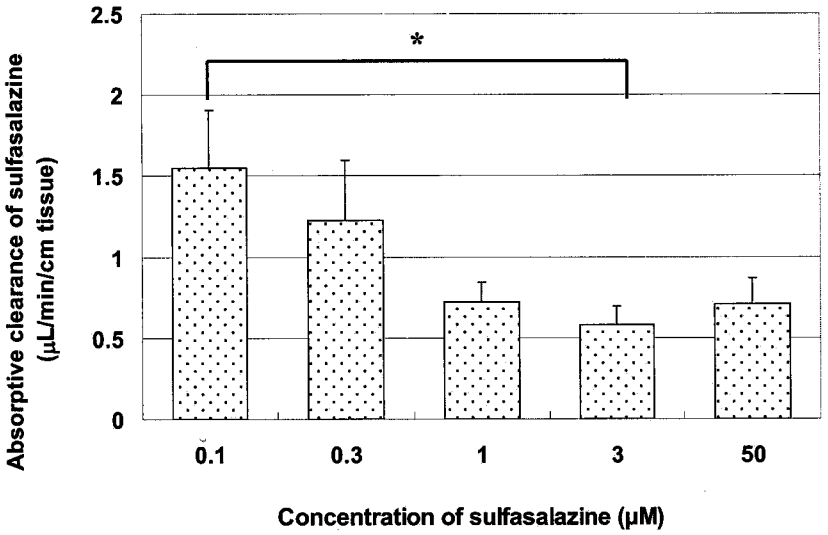


Figure 4

Steady-state absorptive clearance of SASP at various concentrations in the everted ileum sac in Bcrp$^{-/-}$ mice

The data are expressed as mean ± S.E. (n=4-7). *p<0.05

4. Discussion

Our data suggest that both influx and efflux transporters are involved in the absorption of SASP. As a result, each process of intestinal absorption of SASP mediated by influx and efflux transporters was not observed clearly in wild-type mice. Recently, involvement of MRP2 in the intestinal absorption of SASP has been reported[8,9]. However, considering the affinity and the expression of these transporters[26], the contribution of Mrp2 to SASP intestinal absorption is considered to be low in our study because we used the ileum for the experiments.

Kusuhara *et al.* reported that SASP was a substrate of OATP2B1 and its Km was 1.7 μM[10]. In our study, the Km of influx transporter mediated SASP absorption was estimated to be approximately 1μM (Figure 3) and this value was almost the same value calculated in their study. Jani *et al.* investigated the detailed kinetic characterization of SASP using membrane vesicles prepared from mammalian cells selectively overexpressing ABCG2, and calculated Km of SASP to ABCG2 was about 0.7 μM[27]. Therefore, the influx and efflux transporters involved in SASP absorption may have similar affinity for SASP.

In clinical use, the intestinal concentration of SASP was calculated to be 2.5 mM when the intestinal volume was assumed to be as 1.92 L[28], which was far greater than its Km to OATP2B1 and saturated. Under this estimation, SASP may inhibit the absorption of OATP2B1 substrate drugs. In a clinical study of talinolol, which is substrates of OATP1A2 and OATP2B1 in human and Oatp1a5 in rat[29, 30], concomitant use of SASP decreased the AUC for talinolol[31]. Although this mechanism is unclear, the drug-drug interaction must be noted when SASP is coadministered with a substrate of OATP2B1. Furthermore, in the clinical study of Kusuhara *et al.*, the dose-normalized AUC of SASP was much smaller in therapeutic dose than that in microdose. As they described in their reports, BCRP is considered not to be saturated even in the therapeutic dose and this nonlinearity is caused by the saturation of the influx transporter OATP2B1 at a therapeutic dose. Therefore, the absorption of SASP is mainly regulated by BCRP in clinical use.

5. Conclusion

The intestinal absorption of SASP involved not only BCRP but also the influx transporter. In the clinical use, the contribution of this influx transport on the intestinal absorption of SASP may be low. However, the drug-drug interaction must be noted when SASP is coadministered with the substrate of this influx transporter.

Chapter 2

Analysis of the pharmacokinetic boosting effects of ritonavir on oral bioavailability of drugs in mice

1. Introduction

In the previous chapter, the elucidation of the characteristics of SASP could explain the findings in the clinical study. In this chapter, the inhibition mechanisms involved in the RTV boosting was investigated. RTV dramatically increases the bioavailability of a variety of concurrently administered drugs by inhibition of metabolic enzymes and drug transporters. In this study the extent to which ritonavir's inhibition of drug transporters and/or CYP3A contributes to the increased oral bioavailability in mice was investigated.

In antiretroviral therapies, adequate plasma concentrations of PIs are required for their effective anti-HIV activity. The potent inhibition of CYP3A in the small intestine and liver by RTV improves the bioavailability of drugs and prolongs their elimination half-lives. As a result, RTV boosting allows both dose and dosing frequency of the concurrently administered PI to be reduced.

Saquinavir (SQV) is also a highly selective HIV PI and is administered as one of the RTV boosted PIs. The bioavailability of orally administered SQV was only 4 % in healthy volunteers after a single dose and this poor bioavailability of SQV is attributed to the extensive first-pass metabolism in the small intestine and liver, because SQV is a substrate of CYP3A[32]. The concurrent administration of SQV with RTV enhances the plasma concentration of SQV, and so SQV is used as a combination therapy with RTV. The boosting effect of RTV is considered to be mediated through its inhibitory effect on CYP3A. However, RTV also inhibits many transporters[13,14,15,16] and SQV is also a substrate of P-gp[33]. Therefore, the inhibition of P-gp by RTV in the small intestine might enhance the bioavailability of SQV. Recently, it was reported that SQV is a substrate of OATP1B1 and OATP1B3[34]. OATP1B1 and OATP1B3 are influx transporters and localized on the basolateral membrane of hepatocytes. As observed in the drug-drug interaction between cerivastatin and gemfibrozil[35], the inhibition of the influx transporters expressed on sinusoidal membrane increases the plasma concentration, causing adverse events.

In this chapter, the contribution of the RTV effect on the first-pass effect in the small

intestines and livers of mice was assessed. The RTV boosting effects on several probe substrates were also investigated. In addition, the *in vitro* study using mouse intestinal and liver microsomes were performed to confirm the results obtained from the *in vivo* study.

2. Materials and Methods

2.1 Materials

RTV purchased as Norvir® (80 mg/mL) from Abbot Laboratories (Abbott Park, IL, USA) and midazolam (MDZ) as Dormicum Injection® (5 mg/mL) purchased from Astellas Pharma Inc. (Tokyo, Japan) were used in the *in vivo* study. In the *in vitro study*, RTV purchased from BIOTREND Chemikalien GmbH (Cologne, Germany) and MDZ purchased from Wako Pure Chemical Industries, Inc. (Tokyo, Japan) were used. SQV was purchased from Sequoia Research Products Ltd. (Pangbourne, UK) and pravastatin (PRV) sodium was purchased from Wako Pure Chemical Industries, Inc. Itraconazole (ITZ) was purchased from Sigma-Aldrich Co. (St Louis, MO, USA). Fexofenadine hydrochloride was purchased from Tronto Research Chemicals Inc. (Ontario, Canada). All other chemicals were of analytical grade and are commercially available.

2.2 Animals

Female ddY mice (11-13 weeks old) were purchased from Sankyo Labo Service Corp (Tokyo, Japan). The mice were housed in rooms maintained at 23 °C and 55 ± 5 % relative humidity, and allowed free access to food and water during the acclimatization period. In the *in vivo* study, the mice were fasted overnight for at least 12h, with free access to water. The animal study was performed at Hoshi University and complied with the regulations of the Committee on Ethics in the Care and use of Laboratory Animals.

2.3 *In vivo* studies

2.3.1 Preparation of dosing solutions

The SQV dosing solution was prepared as described previously[36]. Briefly, SQV was dissolved in an 8 % ethanol/4.2 % glucose solution for intravenous administration and in a 16.4 % ethanol/3 % glucose/15.6 % Cremophor EL solution for oral administration. Norvir® was diluted with ethanol and water to prepare a 8 mg/mL solution. As the control vehicle for

Norvir®, a 43 %(v/v) ethanol solution containing Cremophor EL (105 mg/mL), propylene glycol (0.25 mg/mL), peppermint oil (3.5 mg/mL), and water-free citric acid (2.8 mg/mL) were used. This control vehicle was 10-fold diluted by ethanol and water to contain the same volume of vehicle constituents as RTV dosing solution. For the dose-dependency of RTV inhibition effect study, Norvir® was diluted with control vehicle to prepare 2.4, 8 and 40 mg/mL solutions. Then, these solutions were diluted with ethanol and water to make each RTV dosing solution (0.24, 0.8 and 4 mg/mL). The dosing solution for each probe substrate was prepared as follows. Fexofenadine hydrochloride and pravastatin sodium were dissolved in water to concentrations of 1.5 and 20 mg/mL, respectively. Dormicum Injection® was diluted with water to concentration of 3 mg/mL and used as the MDZ dosing solution. The ITZ dosing solution was prepared as described previously[37]. Briefly, 20 mg of ITZ was dissolved with 0.1 mL of 12 N HCl, and 1.75 mL of polyethylene grycol 400 and 0.15 mL of 8 N NaOH were added to prepare a 10 mg/mL ITZ solution. As the control vehicle for ITZ, the solution which contains the same contents of vehicle constituents was used.

2.3.2 Effect of RTV on the pharmacokinetic of SQV

The dose dependent effects of RTV on the pharmacokinetics of SQV were investigated. The administered doses of RTV were set at 1.5, 5, 25 and 50 mg/kg. The dose of 1.5 mg/kg is the clinically relevant dose (RTV boosting dose : 100 or 200 mg/dose, i.e.,1.5-3 mg/kg for a 70 kg man). SQV (20 mg/kg) was administered orally 30 min after the oral administration of RTV. In addition, to investigate the contribution of RTV on the first-pass effect in the liver and small intestine, SQV was administered orally (20 mg/kg) or intravenously (2 mg/kg) 30 min after the oral administration of 50 mg/kg RTV. For oral administration, mice were given each solution by sonde needle into the stomach. For intravenous administration, each solution was injected into the tail vein of mice. Thirty μL of blood was taken from the tail vein by using micro-haematocrit capillary tubes and centrifuged at 15,400×g for 10 min to obtain the plasma samples. The plasma samples were stored at -20 °C

until analysis.

2.3.3 Effect of RTV on the pharmacokinetic of probe substrates

FEX (10 mg/kg) was used as the probe substrate for P-gp, MDZ (10 mg/kg) for CYP3A, and PRV (100 mg/kg) for OATP1B1. Each probe substrate was administered orally 30 min after the oral administration of 1.5 or 50 mg/kg RTV. The effect of ITZ, an inhibitor of CYP3A in humans, on the pharmacokinetics of SQV was also investigated. SQV (20 mg/kg) was administered orally 30 min after the oral administration of 50 mg/kg ITZ. Thirty μL of blood was taken from the tail vein by using micro-haematocrit capillary tubes and centrifuged at 15,400×g for 10 min to obtain the plasma samples. The plasma samples were stored at -20 °C until analysis.

2.3.4 Quantification of the plasma concentration of SQV, FEX, MDZ and PRV

For the determination of each substrate concentration in plasma, 100 μL of acetonitrile was added to the 10 μL of plasma sample. After the protein precipitation by vortex mixing and centrifugation, 80μL of supernatant was evaporated at 40 °C under nitrogen. The residue was reconstituted with the 100 μL of mobile phase and subjected to LC–MS/MS analysis on an API4000 system (AB SCIEX, Foster City, CA, USA) equipped with the Prominence LC system (Shimadzu Co., Kyoto, Japan). The LC-MS/MS conditions are summarized in Table 1. The data were acquired with Analyst ver. 1.4.2 (AB SCIEX). The limit of quantification for all compounds was 1ng/mL.

Table1 LC-MS/MS conditions for each substrate

Compounds	Column	Mobile phase	Ionization	m/z
SQV	CAPCELL PAK MGII C18	10 mM acetic acid in 40% acetonitrile	Positive	671 to 570
FEX	(2.1×50 mm, 5 μm)	10 mM acetic acid in 35% acetonitrile	Positive	502 to 466
MDZ	(Shiseido Co., Ltd., Tokyo, Japan)	10 mM acetic acid in 30% acetonitrile	Positive	326 to 291
PRV	Hypersil Gold (2.1×50 mm, 5 μm) (Thermo fisher Co., Ltd., Tokyo, Japan)	10 mM acetic acid in 40% acetonitrile	Negative	423 to 321

2.4 *In vitro* studies

2.4.1 Preparation of mouse microsomes

Pooled mouse liver and intestinal microsomes were prepared according to a previously reported method[38]. Briefly, livers and intestinals were homogenized separately in three volumes of ice-cold buffer (50 mM Tris-HCl, pH7.4) containing 150 mM KCl, 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, 1 mg/mL trypsin inhibitor, 10 μM leupeptin, 0.04 unit/mL aprotinin, 1 μM bestatin, and 20 %(v/v) glycerol using Teflon-tipped pestle. The homogenates were centrifuged at 9,000×g for 20 min at 4°C, and the supernatants were then centrifuged at 105,000×g for 60 min at 4°C. The microsomal pellets were resuspended in the same buffer and stored at -80 °C. The protein concentrations were determined using a BCA Protein Assay Kit (Pierce Chemical, Rockford, IL, USA).

2.4.2 Inhibition study

In preliminary experiments, we confirmed that substrate depletion increased proportionally with time (to 20 min) and with protein concentration (in the range of 0.1 to 1 mg/mL protein) for both the liver and intestinal microsomes.

The mouse liver and intestinal microsomes were incubated with SQV or MDZ with or without an inhibitor in an NADPH-generating system containing 2.5 mM NADP, 25 mM glucose 6-phosphate, 2 units of glucose-6-phosphate dehydrogenase, and 10 mM $MgCl_2$ in a total volume of 0.2 mL of 100 mM phosphate buffer (pH7.4). After preincubation for 5 min at 37°C, the reaction was initiated by the addition of the

NADH-generating system. After incubation, the reaction was terminated by the addition of 0.4 mL of ice-cold acetonitrile and briefly vortex mixed. The samples were centrifuged at 15,400×g for 15 min to precipitate the protein. The supernatant was evaporated at 40 °C under nitrogen. The residue was reconstituted with the mobile phase and the depletion of SQV or MDZ was analyzed by HPLC-UV.

2.4.3 Quantification of SQV and MDZ concentration in the in vitro samples

HPLC was performed with a Prominence LC system (Shimadzu Co.), equipped with a TSKgel® ODS-100V column (2.1×50 mm, 5 μm; Tosoh Co., Tokyo, Japan) for SQV and a CAPCELL PAK MGII C18 column (2.1×50 mm, 5 μm; Shiseido Co., Ltd., Tokyo, Japan) for MDZ. 10 mM Na_2HPO_4 in 35 % acetonitrile was used as the mobile phase for SQV and 10 mM phosphate buffer (pH6) in 40 % acetonitrile were used as the mobile phase for MDZ. The flow rate was 0.2 mL/min at 40 °C and the elution was monitored at wavelengths of 254 nm for SQV and 230 nm for MDZ.

2.5 Pharmacokinetic calculations

2.5.1 *In vivo* study

The C_{max} was obtained directly from the raw data. The area under the plasma concentration-time curve from time 0 to the last time point (AUC_{0-t}) was calculated using the linear trapezoidal rule. The AUC_{inf}, with extrapolation to infinity was calculated by dividing the last measured concentration by the elimination rate constant, which was determined as the slope of regression for the terminal log-linear portion of the concentration versus time curve. The bioavailability (F), hepatic availability (F_h), fraction absorbed (F_a), and intestinal availability (F_g) were calculated using equations 1-4. The blood-to-plasma ratio (R_b) and the hepatic blood flow (Q_h) values used were 0.6 [39] and 5.4 L/h/kg [40], respectively. Hepatic clearance was regarded as the total clearance because the urinary excretion was negligible[32].

$F=(AUC_{po} \cdot Dose_{iv})/(AUC_{iv} \cdot Dose_{po})$ (eq.1)

$CL_{tot}=CL_h=Dose_{iv}/(AUC_{iv} \cdot R_b)$ (eq.2)

$F_h=1-CL_h/Q_h$ (eq.3)

$F_a \cdot F_g = F/F_h$ (eq.4)

2.5.2 *In vitro* study

The initial reaction rate was determined under linear conditions. The inhibition constant (ki) was determined by plotting the slope of the Dixon plot versus inhibitor concentration.

The increase in the AUC (R) caused by the drug-drug interactions was calculated with eq.5 for the intravenous administration and with eq.6 for the oral administration[41].

$R=1+(I_{in,u} \cdot F_h) / ki$ (eq.5)

$R=1+I_{in,u}/ ki$ (eq.6)

Where, $I_{in,u}=(I_{max}+K_a \cdot F_a \cdot F_g \cdot D/Q_h) \cdot f_b$

I_{max} represents the maximum concentration of the inhibitor in the systemic blood and used C_{max} of inhibitor corrected R_b. The first-order rate constant (K_a), the fraction absorbed from the gastrointestinal tract into the portal vein (F_a), and the unbound fraction in the blood (f_b) used for calculations are summarized in Table 2.

Table2 Parameters used in the prediction of the increased in the AUC from ki values

Parameters	Unit	RTV	ITZ
I_{max}	μM	24	2 [42]
K_a	min^{-1}	0.007	0.004 [4]
$F_a \cdot F_g$	-	0.8	1
Q_h	L/h/kg	5.4 [40]	5.4 [40]
R_b	-	0.7 [43]	0.6 [44]
f_b	-	0.026 [43]	0.003 [37]

I_{max} represents the maximum concentration of the inhibitor in the systemic blood and used C_{max} of inhibitor corrected R_b (The C_{max} for RTV was obtained from our study). The K_a and $F_a \cdot F_g$ for RTV was calculated using the AUC_{inf} in this study and bioavailbility [45]. The maximum value was used as $F_a \cdot F_g$ for ITZ.

2.6 Statistical analysis

Each value is expressed as a mean ± standard deviation (SD). Statistical significance was examined by one-way ANOVA followed by Dunnett's *post hoc* multiple-comparison test. A p value of less than 0.05 was considered significant.

3. Results

3.1 Effect of RTV on the pharmacokinetics of SQV

RTV increased the C_{max} and AUC_{inf} for SQV in a dose-dependent manner. The AUC_{inf} for SQV was increased three-, 26-, 241-, and 325-fold by the coadministration of 1.5, 5, 25, and 50 mg/kg RTV, respectively. The plasma concentration-time profiles and pharmacokinetic parameters are shown in Figure 1 and Table 3, respectively. High dose of RTV was administered and significant interaction was observed in this study, however, the animals were well tolerated.

To assess the effect of RTV on the first-pass effect in the liver and small intestine,

$F_a \cdot F_g$ and F_h were calculated from the pharmacokinetic parameters after the intravenous and oral administration of SQV with and without 50 mg/kg RTV. The plasma-concentration time profiles and pharmacokinetic parameters for SQV are presented in Figure 2 and Table 4, respectively. After the intravenous administration of SQV, the coadministration of RTV caused only fivefold increase in the AUC_{inf} for SQV. In contrast, after the oral administration of SQV, the AUC_{inf} increased dramatically (325-fold) with the coadministration of 50 mg/kg RTV. As a result, the bioavailability of orally administered SQV increased from 0.0093 to 0.675. F_h and $F_a \cdot F_g$ for SQV were increased 1.7- and 38-fold, respectively, with the coadministration of RTV.

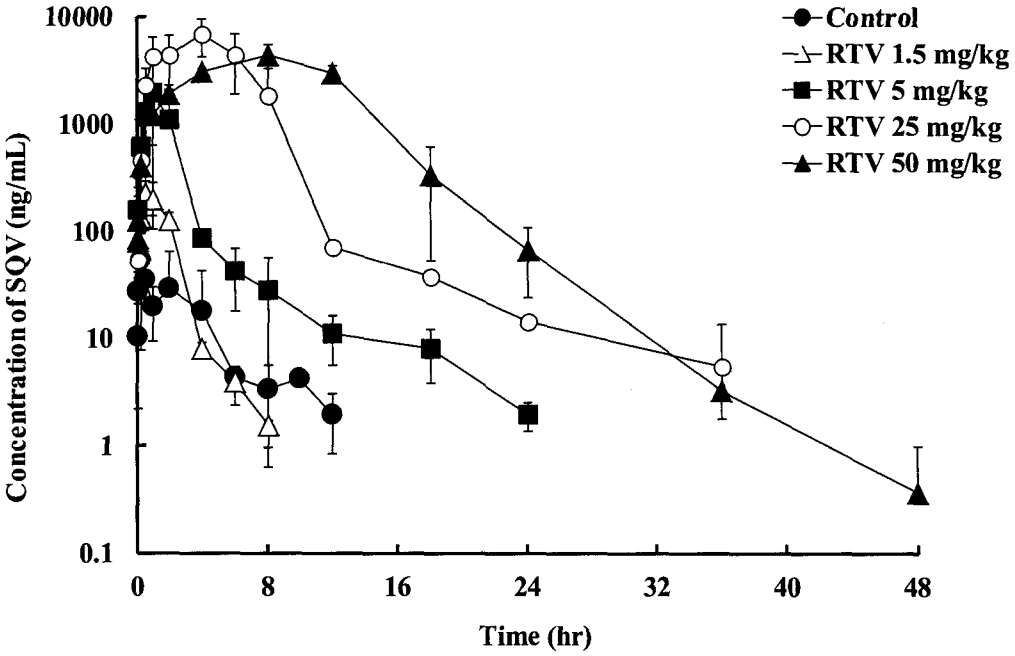


Figure 1

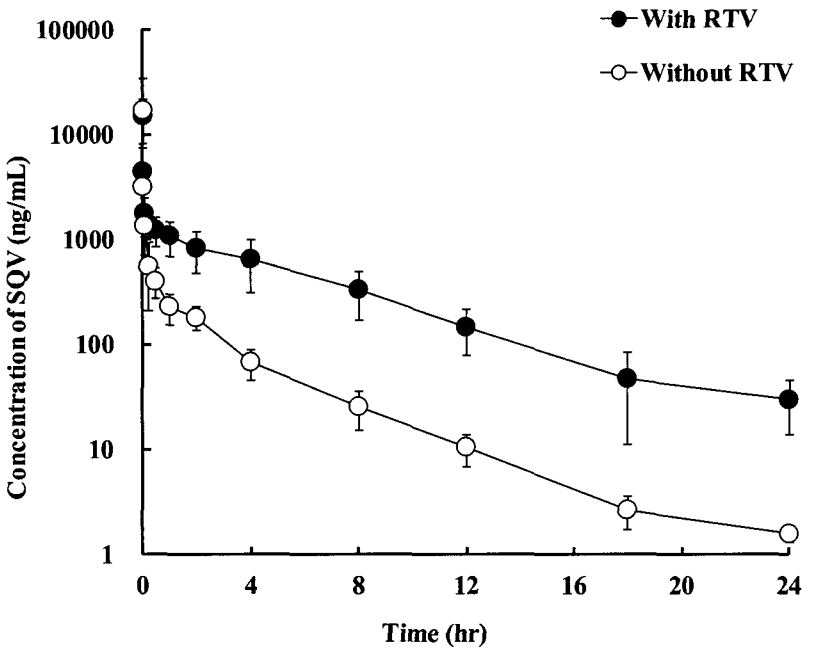
Plasma SQV concentration - time profiles after oral administration of SQV with various dose of RTV

SQV (20 mg/kg) was given orally after oral administration of RTV at a dose of 1.5, 5, 25 and 50 mg/kg. Each point represents the mean ± SD (n=3).

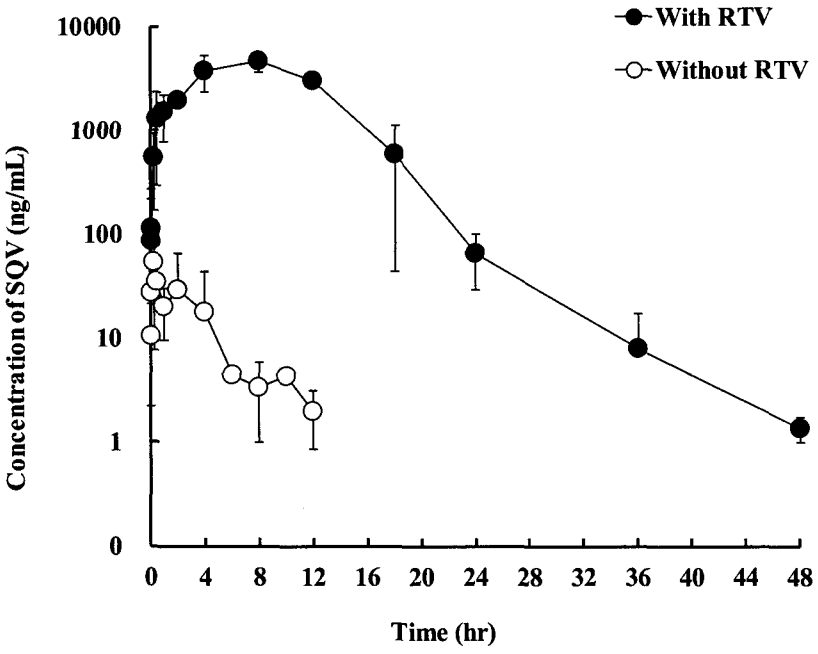
Table3 Pharmacokinetic parameters for SQV after oral administration of SQV with or without oral administration of RTV in mice

SQV		
RTV	C_{max}	AUC_{inf}
Control	65.6 ± 43.9	165.6 ± 128.7
1.5	266.6 ± 43.5	500.7 ± 78.0
Fold increase	4	3
5	1969.9 ± 1829.5	4335.0 ± 3580.6
Fold increase	30	26
25	6921.5 ± 2691.7*	39966.6 ± 21426.0*
Fold increase	106	241
50	4779.7 ± 1151.2*	53850.1 ± 12758.9*
Fold increase	73	325

Values are mean ± SD (n=3-4). *: $p<0.01$



(A)



(B)

Figure 2

Plasma SQV concentration - time profiles after intravenous (A) or oral (B) administration of SQV with or without RTV

SQV was given intravenously (2 mg/kg) or orally (20 mg/kg) after oral administration of RTV at a dose of 50 mg/kg. Each point represents the mean ± SD (n=4-6).

Table4 Pharmacokinetic parameters of SQV after intravenous and oral administration of SQV with or without oral administration of RTV in mice

Oral	C_{max} (ng/mL)	T_{max} (h)	AUC_{0-t} (ng·h/mL)	AUC_{inf} (ng·h/mL)
Control	65.6 ± 43.9	0.6 ± 0.7	153.4 ± 133.1	165.6 ± 128.7
with RTV	4779.7 ± 1151.2**	7.0 ± 2.0**	53839.0 ± 12757.3**	53850.1 ± 12758.9**

Intravenous	AUC_{0-24} (ng·h/mL)	AUC_{inf} (ng·h/mL)	CL_{tot} (L/hr/kg)
Control	1516.7 ± 608.0	1525.4 ± 606.7	2.46 ± 0.93
with RTV	7685.0 ± 3127.7*	7882.0 ± 3180.2*	0.47 ± 0.16*

Values are mean ± SD (n=4-6). * : $p<0.05$ ** : $p<0.01$

3.2 Effect of RTV on the pharmacokinetics of probe substrates

The effects of RTV on the pharmacokinetics of each probe substrate were investigated. The plasma concentration-time profiles and pharmacokinetic parameters for each probe substrate are shown in Figures 3 - 5 and Tables 5 - 7, respectively. At the clinically relevant dose (1.5 mg/kg), the AUC_{inf} for FEX was not affected with the coadministration of RTV. However, the AUC_{inf} for MDZ and PRV increased twofold when they were coadministered with 1.5 mg/kg RTV. The AUC_{inf} for MDZ, FEX and PRV increased five-, 13- and sevenfold, respectively, when they were coadministered with 50 mg/kg RTV.

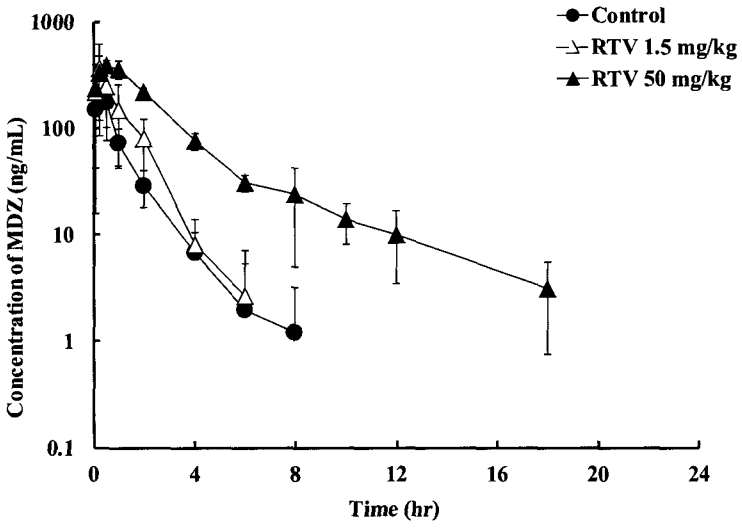


Figure 3

Plasma MDZ concentration - time profiles after oral administration of MDZ with or without RTV

MDZ (10 mg/kg) was given orally after oral administration of RTV at a dose of 1.5 and 50 mg/kg. Each point represents the mean ± SD (n=3).

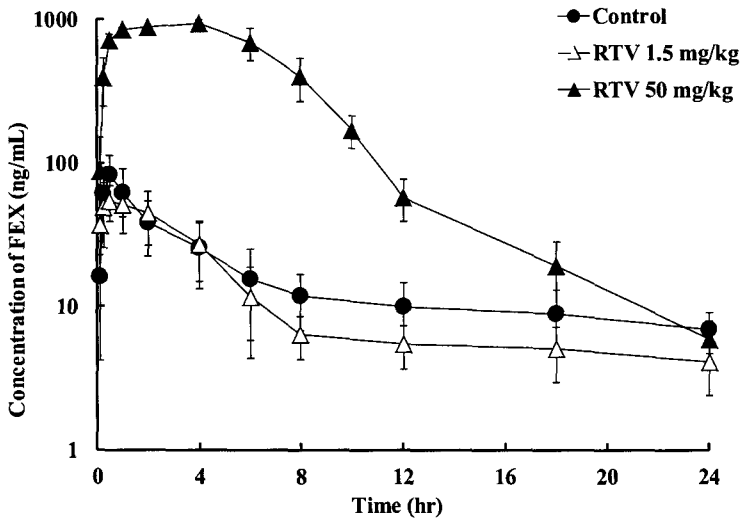


Figure 4

Plasma FEX concentration - time profiles after oral administration of FEX with or without RTV

FEX (20 mg/kg) was given orally after oral administration of RTV at a dose of 1.5 and 50 mg/kg. Each point represents the mean ± SD (n= 3).

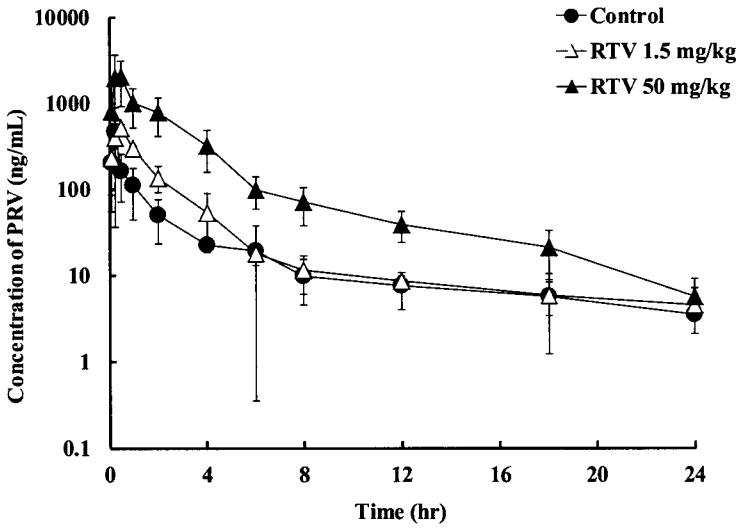


Figure 5

Plasma PRV concentration - time profiles after oral administration of PRV with or without RTV

PRV (100 mg/kg) was given orally after oral administration of RTV at a dose of 1.5 and 50 mg/kg. Each point represents the mean ± SD (n=3).

Table5 Pharmacokinetic parameters of MDZ after oral administration of MDZ with or without oral administration of RTV in mice

	MDZ	
RTV	C_{max}	AUC_{inf}
Control	226.7 ± 100.6	245.2 ± 88.3
1.5	371.3 ± 252.1	463.4 ± 296.3
Fold increase	2	2
50	427.6 ± 61.8	1220.3 ± 117.9*
Fold increase	2	5

Values are mean ± SD (n=3). *p<0.01

Table6 Pharmacokinetic parameters of FEX after oral administration of FEX with or without oral administration of RTV in mice

FEX		
RTV	C_{max}	AUC_{inf}
Control	85.6 ± 28.9	542.3 ± 211.7
1.5	63.5 ± 14.6	398.4 ± 84.6
Fold increase	1	1
50	939.8 ± 42.9*	7169.2 ± 586.9*
Fold increase	11	13

Values are mean ± SD (n=3). * $p<0.01$

Table7 Pharmacokinetic parameters of PRV after oral administration of PRV with or without oral administration of RTV in mice

PRV		
RTV	C_{max}	AUC_{inf}
Control	737.3 ± 557.1	636.3 ± 97.1
1.5	534.9 ± 58.1	1045.7 ± 164.2
Fold increase	1	2
50	2433.7 ± 1532.3	4604.1 ± 1736.3*
Fold increase	3	7

Values are mean ± SD (n=3). *$p<0.01$

3.3 Effect of ITZ on the pharmacokinetics of SQV

The inhibitory effect of 50 mg/kg ITZ on the pharmacokinetics of SQV after its oral administration was investigated. The plasma concentration-time profile and pharmacokinetic parameters are shown in Figure 6 and Table 8, respectively. There was no significant difference in the C_{max} and AUC_{inf} after treatment with and without 50 mg/kg ITZ and this result indicates that cyp isoforms other than cyp3a are involved in the metabolism of SQV in mice.

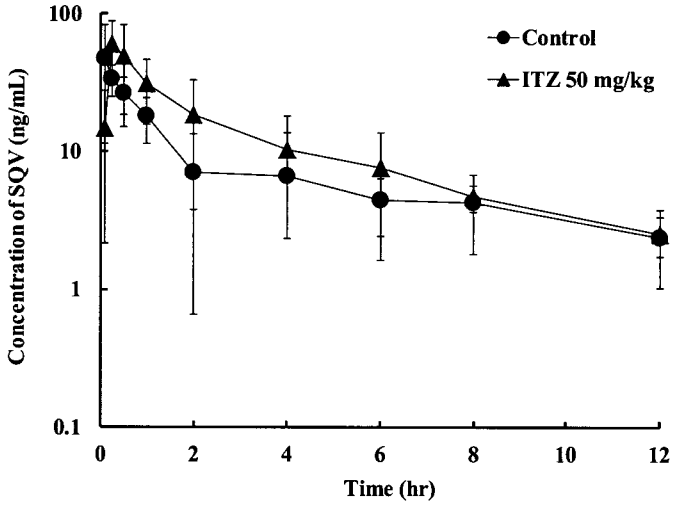


Figure 6

Plasma SQV concentration - time profiles after oral administration of SQV with or without ITZ

SQV (20 mg/kg) was given orally after oral administration of ITZ at a dose of 50 mg/kg. Each point represents the mean ± SD (n=3).

Table8 Pharmacokinetic parameters of SQV after oral administration of SQV with or without oral administration of ITZ in mice

	SQV	
ITZ (mg/kg)	C_{max} (ng/mL)	AUC_{inf} (ng·h/mL)
Control	50.6 ± 33.2	86.7 ± 65.8
50	70.0 ± 19.9	138.6 ± 55.0
Fold increase	1.4	1.6

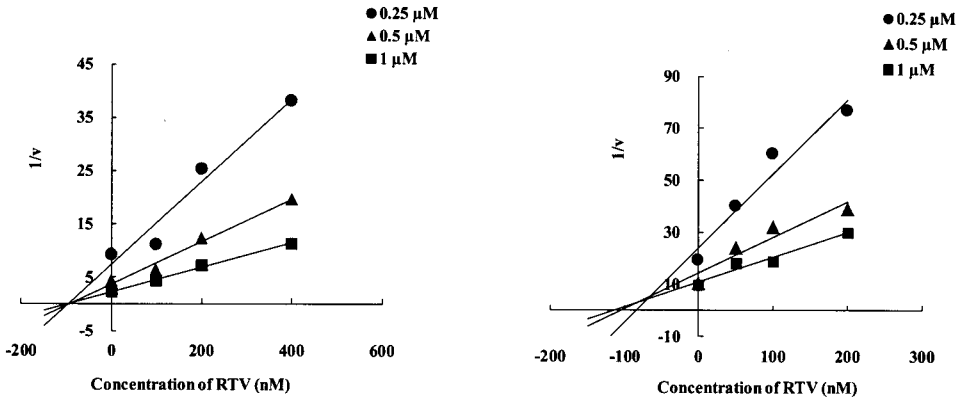
Values are mean ± SD (n=3).

3.4 *In vitro* study

Before performing the inhibition study, the apparent Km of SQV in the mouse liver was calculated and the concentration of substrate in the inhibition study was set below

the Km value (data not shown). The same concentration was used in the inhibition study with mouse intestinal microsomes. Figures 7 and 9 show the Dixon plots for the inhibition by RTV of SQV or MDZ metabolism, respectively, in mouse liver and intestinal microsomes. The SQV and MDZ concentrations used were 0.25, 0.5 and 1 μM. The inhibition by RTV or ITZ was considered to be competitive or non-competitive. The Ki values for the inhibition of SQV metabolism by RTV were 95 and 75 nM in the liver and intestinal microsomes, respectively (Table 8). The Ki value for the inhibition of MDZ metabolism was 65 nM in both the liver and intestinal microsomes (Table 9). The inhibitory effect of ITZ on the metabolism of SQV was also investigated (Figure 9). The Ki values were 3.6 and 2.7 μM in the liver and intestinal microsomes, respectively.

The fold increases in the AUC predicted from the *in vitro* study and their comparison with the values observed in the *in vivo* study are shown in Table 10. A fourfold increase in the AUC for SQV was predicted from the Ki value using eq.5, which is close to the value observed after the intravenous administration of SQV. In contrast, a ninefold increase in the AUC for SQV was predicted from the Ki value using eq.6. The remaining increase in the AUC for SQV derived from the inhibition of metabolic enzymes in the small intestine was estimated about 36-fold. This result indicates that the effect of RTV was greater in the small intestine than in the liver, which consistent with the results obtained in the *in vivo* study.

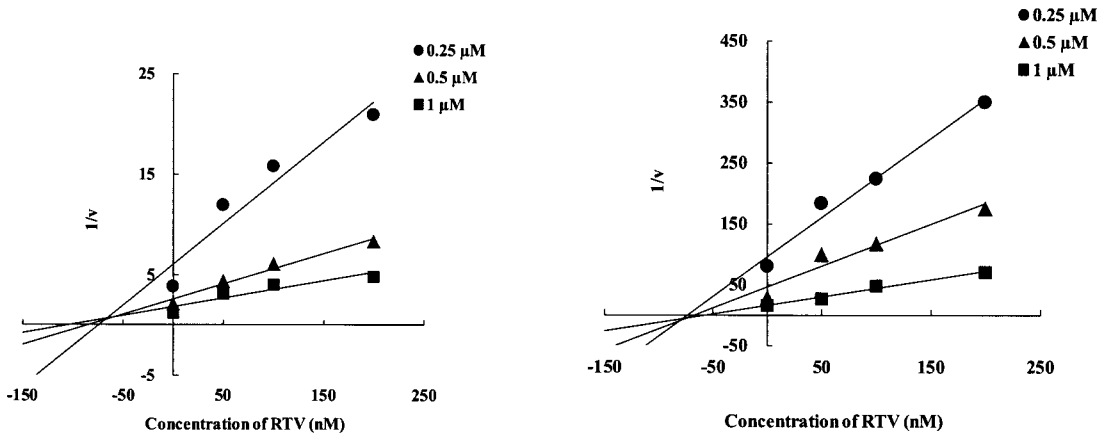


(A) (B)

Figure 7

Dixon plots for the inhibition of SQV metabolism by RTV in mouse liver (A) and intestinal microsomes (B)

SQV (0.25, 0.5, 1 μM) was incubated at 37°C for 10 min with liver microsomes (0.2 mg /mL protein) (A) and 20 min with intestinal microsomes (0.2 mg/mL protein) (B) The data represents the mean of n=3.

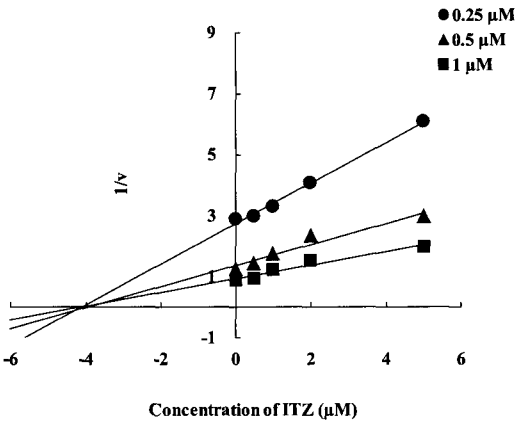


(A) (B)

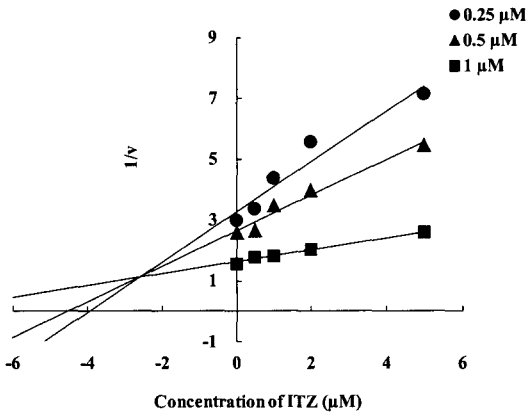
Figure 8

Dixon plots for the inhibition of MDZ metabolism by RTV in mouse liver (A) and intestinal (B) microsomes

MDZ (0.25, 0.5, 1 μM) was incubated at 37°C for 5 min with liver microsomes (0.1 mg /mL protein) (A) and 15 min with intestinal microsomes (0.2 mg/mL protein) (B) The data represents the mean of n=3.



(A)



(B)

Figure 9

Dixon plots for the inhibition of SQV metabolism by ITZ in mouse liver (A) and intestinal (B) microsomes

SQV (0.25, 0.5, 1 μM) was incubated at 37°C for 10 min with liver microsomes (0.2 mg /mL protein) (A) and 20 min with intestinal microsomes (0.2 mg/mL protein) (B) The data represents the mean of n=3.

Table9 The ki values of SQV and MDZ inhibition by RTV and ITZ in mouse liver and intestinal microsomes

Substrates	SQV	SQV	MDZ
Inhibitors	RTV	ITZ	RTV
Liver microsomes	95 nM	3.7 μM	65 nM
Intestinal microsomes	75 nM	2.6 μM	65 nM

Values are mean of n=3.

Table10 Comparison of the fold increase in the AUC between observed value and predicted value

Substrates		SQV	SQV	MDZ
Inhibitors		RTV	ITZ	RTV
Oral	Fold increase in AUC (Observed)	325	2	5
	Fold increase in AUC (Predicted)	9	1	12
Intravenous	Fold increase in AUC (Observed)	5	-	-
	Fold increase in AUC (Predicted)	4	-	-

The dosage of each substrate and inhibitor are follows.

SQV: 2 mg/kg for intravenous administration and 20 mg/kg for oral administration,

MDZ: 10 mg/kg, RTV and ITZ : 50 mg/kg

4. Discussion

In this study, *in vivo* and *in vitro* experiments were performed in mice to clarify the underlying mechanisms of RTV boosting. Escalating doses of RTV had dose-dependent effects on the pharmacokinetics of SQV. The increasing in the C_{max} for SQV reached a plateau at 25-50 mg/kg RTV and the AUC_{inf} showed a tendency to increase even at 50 mg/kg RTV. There was a threefold increase in the AUC_{inf} for SQV when administered with 1.5 mg/kg RTV, the clinically relevant dose. In the clinical dose, it is reported that the increase in the AUC for SQV at SQV/RTV doses of 1200/100 mg is six- to sevenfold[46, 47]. Therefore, our results indicate the effect of RTV boosting is similar in humans and in mice.

The contribution of 50 mg/kg RTV boosting to the first-pass effect on SQV in the small intestine and liver was accessed. As a result, $F_a \cdot F_g$ increased 38-fold, whereas F_h increased only twofold. These results suggest that RTV mainly influences the first-pass effect in the small intestine, resulting in an increase in the bioavailability of orally administered SQV.

Factors affecting the first-pass effect in the liver are metabolic enzymes and drug transporters. Among the metabolic enzymes in the liver, CYP3A is particularly important because it is responsible for the majority of phase I drug metabolism reaction, including those of PIs. CYP3A is also an important factor in the first-pass effect in the small intestine. In addition to the metabolic enzymes, the efflux transporters localized in the apical membrane are also important factors in the small intestine. Therefore, the inhibition of P-gp/CYP3A in the small intestine and the inhibition of CYP3A in the liver are considered to increase the oral bioavailability of SQV. However, regarding to the probe substrate of CYP3A, the increase in the AUC_{inf} for MDZ after its oral administration with 50 mg/kg RTV was only fivefold. In addition, the increase in the AUC for SQV after its oral administration with ITZ was only twofold, and did not differ significantly from the control. Yamano *et al.* evaluated the extent of drug-drug interactions involving metabolic inhibition in the rat liver[37]. In their report, the increase in the AUC for MDZ in the presence of ITZ was

about twofold, which is consistent with our result of the inhibition study by ITZ.

MDZ is predominantly biotransformed to the 1'-hydroxy MDZ rather than to 4-hydroxy MDZ in both humans and mice [48]. In humans, CYP3A mediates its biotransformation to both 1'-hydroxy and 4-hydroxy MDZ. On the other hands, there is a report that cyp3a mediates only its biotransformation to 4-hydroxy MDZ in mice, and cyp isoforms other than cyp3a mediate its biotransformation to 1'-hydroxy MDZ [49]. Furthermore, Waterschoot *et al.* even reported that MDZ metabolism was observed in liver microsomes prepared from *cyp3a*$^{-/-}$ mice [50]. These data indicate a species difference in the MDZ metabolism in mice and humans and suggest that the cyp isoforms involved in the metabolism of SQV in mice differ from those involved in humans.

Perloff *et al.* reported that the Ki values for the inhibition by ketoconazole of 1'-hidroxy and 4-hydroxy MDZ biotransformation were 1.7 and 0.066 μM, respectively, in mouse liver microsomes. In humans, these Ki values were 0.0054 and 0.039 μM, respectively[49]. These results show the Ki value for the inhibition by ketoconazole of 1'-hydroxy MDZ biotransformation in mice differed greatly from that in humans. Moreover, the Ki value for the inhibition by ketoconazole of 1'-hidroxy MDZ biotransformation was the same as our result for the inhibition by ITZ of SQV metabolism. In addition, Yamano *et al.* determined the Ki value for the inhibition of 1'-hidroxy biotransformation by ketoconazole and ITZ, and they found those were the same[37]. In our study, the inhibitory effect of RTV on the metabolism of SQV and MDZ was the same. Therefore, RTV and ITZ inhibit the cyp isoforms involved in the metabolism of SQV and MDZ, and these cyp isoforms may be the same or have a same affinity to RTV or ITZ. Komura and Iwaki reported that the cyp mRNA expression levels differ in the livers and small intestines of mice, and that cyp3a13 is predominantly expressed in the small intestine and is responsible for the first-pass metabolism of CYP3A substrates in the mouse small intestine[37]. They also evaluated the relationships of the Km values for the CYP3A4 mediated biotransformations of 13 drugs in intestinal and liver microsomes, and a good relationship was observed,

although the mRNA expression levels differed. This observation supports our finding that the Ki values were the same for the liver and intestinal microsomes in the *in vitro* study.

When we considered a substrate of P-gp, there was no significant difference in the C_{max} or AUC_{inf} for FEX when it was coadministered with 1.5 mg/kg of RTV. In humans, twofold increase in the AUC for FEX was observed when given with 100 mg of RTV [51], so there are also species differences in the effects of RTV on the pharmacokinetics of FEX. The oral bioavailability, $F_a \cdot F_g$ and F_h of FEX in humans has been reported as 0.28, 0.31 and 0.9 [52], respectively. Therefore, it is estimated that the maximum increase in the AUC for FEX is about threefold when the inhibition by RTV is completed. In our study, the increase in the AUC_{inf} for FEX in mice coadministered with 50 mg/kg RTV was 13-fold, which was higher than that estimated for humans. In addition, it has been reported the oral bioavailability , $F_a \cdot F_g$ and F_h in mice were 0.014, 0.022 and 0.641 [53], respectively. The maximum increase in the AUC for FEX is estimated to be about 50-fold, with complete inhibition by RTV. Therefore, even 50 mg/kg RTV may not inhibit the first-pass effect in the small intestine and liver completely in mice. Tahara *et al.* reported that the increase in the AUC for FEX in Mdr1a/1b P-gp knockout mice was about sixfold [54]. Considering our result and the very small $F_a \cdot F_g$ values of FEX in mice, efflux transporters other than P-gp may be involved in the intestinal absorption of FEX in mice. Bcrp and Mrp2 are the efflux transporters expressed at the apical membrane in the mouse small intestine. It has been reported that FEX is not a substrate of BCRP in human [55]. Furthermore, Mrp2/3 are involved in the hepatic disposition of FEX in mice [55, 56] and MRP2 mediates the absorption of FEX in humans [57, 58]. These reports suggest that efflux transporters other than P-gp, such as Mrp2, might be involved in the absorption of FEX in mice and that the differences in the RTV boosting effects on these transporters may result in the species differences observed in the pharmacokinetics of FEX.

An additional experiment was performed using mouse liver and intestinal

microsomes to confirm the result obtained in the *in vivo* study. The fourfold increase in the AUC for SQV was predicted from the *in vitro* Ki value for intravenously administered SQV, which is consistent with the results of the *in vivo* study. This result indicates that the inhibitory effect of RTV in the liver is mainly attributable to the inhibition of metabolic enzymes with a minor contribution on the inhibition of influx transporters in the liver. On the other hand, a significant increase in the AUC_{inf} for PRV was observed after the oral administration of 50 mg/kg RTV in the *in vivo* study. According to the package information of Karetra®, which is a PI formed by the combination of lopinavir and RTV, there are no reports that Karetra® enhances the plasma concentration of PRV. However, it has been reported that the AUC for rosuvastatin, a substrate of OATP1B1, is increased twofold by its coadministration with Karetra®[59]. In this report, they concluded this interaction might be mediated by the inhibition by lopinavir and/or ritonavir of rosuvastatin uptake at the level of absorption by BCRP or at the level of uptake into the hepatocytes by OATP1B1, by both, or by neither.

After the oral administration of SQV together with RTV, a ninefold increase in the AUC for SQV was predicted from the *in vitro* Ki value, and this is derived from the inhibition of metabolic enzymes in the liver. From this result, the remaining 36-fold increase in the AUC was considered to be derived from the inhibition of metabolic enzymes in the small intestine. This result corresponds to the result obtained in the *in vivo* study, and the inhibitory effect of RTV boosting is shown to be higher in the small intestine than in the liver.

5. Conclusion

RTV mainly affects the first-pass effect in the small intestine, increasing the bioavailability of orally administered SQV. The effects of RTV boosting are same between in humans and in mice. However, the cyp isoforms involved in the metabolism of SQV in mice differ from those in human.

SUMMARY

Currently, most drugs are developed as oral formulations and the intestinal absorption has become important for understanding the pharmacokinetics of orally administered drugs. Recent significant advances in the research and technology have revealed the expression of various metabolic enzymes and transporters in the small intestine, and these findings make it complex the understanding of intestinal absorption. However, to obtain the appropriate efficacy and avoid an unexpected adverse event, underlying mechanism involved in the pharmacokinetic of xenobiotics is significantly important.

In chapter 1, the characteristics of SASP transport in the mouse intestine were investigated. SASP is used as a probe substrate of BCRP and the recent clinical study indicated the nonlinearity of SASP pharmacokinetic. The absorptive clearance of SASP did not change in a concentration-dependent manner in wild-type mice. By contrast, the absorptive clearance of SASP decreased significantly in a concentration-dependent manner in the presence of Ko134 which is an inhibitor of BCRP. Similar results were obtained in $Bcrp^{-/-}$ mice. These results suggest the possible involvement of some influx transporters in the intestinal absorption of SASP and both the influx and efflux transporters are involved in the intestinal absorption of SASP, which would explain why the absorptive clearance did not appear to change at various SASP concentrations in wild-type mice.

In chapter 2, the extent to which RTV's inhibition of drug transporters and/or CYP3A contributes to the increased oral bioavailability in mice was investigated by using each probe substrate. RTV dramatically increases the bioavailability of a variety of concurrently administered drugs by inhibition of metabolic enzymes and drug transporters and used as RTV boosting for HIV treatment. Escalating doses of RTV had dose-dependent effects on the pharmacokinetics of orally administered SQV and the increase in the AUC for SQV was 325-fold after its coadministration with RTV. As a result, $F_a \cdot F_g$ increased 38-fold, whereas F_h increased only twofold. These results

indicate ritonavir mainly affects the first-pass effect of SQV in the small intestine, increasing the bioavailability of orally administered SQV. In addition, similar result was demonstrated by the prediction using the the *in vitro* Ki value. Furthermore, the AUC for the probe substrates MDZ, FEX, and PRV increased after the oral administration of RTV by only five-, 13-, and sevenfold, respectively. Moreover, the AUC for SQV was affected negligibly by ITZ which was an inhibitor of CYP3A in human.

In conclusion, the intestinal absorption of SASP involved both the influx and efflux transporters. Moreover, RTV mainly affects the first-pass effect of SQV in the small intestine, increasing the bioavailability of orally administered SQV. Furthermore, cyp isoforms other than CYP3A, which contribute to the metabolism of saquinavir in human, are involved in the metabolism of saquinavir in mice. In this thesis, the pharmacokinetics of the oral drugs which metabolic enzymes and drug transporters are involved was analyzed using the probe substrates, and the useful basic knowledge utilized for future pharmacokinetic analysis was acquired.

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