

## **DISPOSITION OF ORAL AND INTRAVENOUS PRAVASTATIN IN Mrp2-DEFICIENT TR- RATS**

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Abbreviations:

Ae, amount excreted unchanged into urine; AUC, area under the plasma concentration-time curve; CL, total clearance; CL<sub>R</sub>, renal clearance; EHBR, Eisai hyperbilirubinemic Sprague-Dawley; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; Mrp, multidrug resistance-associated protein; Oatp, organic anion-transporting polypeptide; TR<sup>-</sup>, Mrp2 transport-deficient; V, apparent volume of distribution

**ABSTRACT:**

The aim of this study was to characterize the role of the efflux transporter Mrp2 (Abcc2) in the pharmacokinetics of orally and intravenously administered pravastatin in rats. Eight Mrp2-deficient TR- rats and eight wild-type rats were given an oral dose of 20 mg/kg pravastatin. Four TR- animals and four wild-type animals were studied after intravenous administration of pravastatin (5 mg/kg). The TR- rats showed a 6.1-fold higher mean AUC of pravastatin ( $p < 0.001$ ) after oral administration and a 4.7-fold higher AUC ( $p < 0.01$ ) after intravenous administration of pravastatin as compared with the wild-type animals. The mean CL of pravastatin was 4.6-fold higher (39.2 vs 8.50 l/h/kg,  $p < 0.001$ ) and the mean V 4.3-fold higher (14.1 vs 3.29 l/kg,  $p < 0.01$ ) in the wild-type rats. The mean CL<sub>R</sub> of pravastatin in the TR- rats was 16.5-fold increased as compared with the wild-type animals (0.695 vs 0.042 l/h/kg,  $p < 0.05$ ). The increased systemic exposure to oral pravastatin in the TR- rats was associated with a greater inhibitory effect on HMG-CoA reductase, as shown by smaller lathosterol to cholesterol concentration ratios. These results suggest that the reduced biliary pravastatin excretion in the Mrp2-deficient TR- rats is partly compensated for by increased urinary excretion of pravastatin. Further, intestinal Mrp2 does not appear to play a major role in the oral absorption of pravastatin in normal rats.

Pravastatin, a semisynthetic inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, is well established as an agent that reduces serum cholesterol concentration and thereby decreases cardiovascular morbidity and mortality. A considerable interindividual variability is evident in the pharmacokinetics and cholesterol-lowering effects of pravastatin in man (Pazzucconi et al., 1995; Niemi et al., 2004). The origins of this variation are not completely understood. The pharmacokinetics of pravastatin in rats are characterized by a low bioavailability, selective oatp-mediated uptake by the liver, substantial biliary excretion, and enterohepatic circulation (Komai et al., 1992; Yamazaki et al., 1996a,b, 1997; Hatanaka et al., 1998; Hsiang et al., 1999; Tokui et al., 1999). Only a small amount of pravastatin is excreted unchanged in the urine and contribution of urinary excretion to total clearance is small (Komai et al., 1992; Hatanaka et al., 1998).

Transport of pravastatin across the canalicular membrane of hepatocytes into bile is mediated to a considerable extent by the multidrug resistance-associated protein 2 (rodents, Mrp2/Abcc2; humans, MRP2/ABCC2) (Yamazaki et al., 1997). Mrp2 is an ATP-binding cassette (ABC) transporter that mediates the biliary excretion of numerous organic anions (conjugated and unconjugated), including many drugs and their metabolites. It is expressed in the apical (canalicular) membrane of proximal renal tubular cells, hepatocytes, and enterocytes of the proximal small intestine, and in many other tissues (König et al., 1999; Gerk and Vore, 2002). Mrp2 is absent in TR<sup>-</sup> rats and Eisai hyperbilirubinemic Sprague-Dawley (EHBR) rats due to distinct mutations which create premature termination codons in the *Abcc2* gene (Büchler et al., 1996; Paulusma et al., 1996; Ito et al., 1997).

Yamazaki et al. (1997) showed that the biliary excretion clearance and total clearance of pravastatin at steady-state (during an intravenous infusion) were higher and the steady-state plasma concentration was about 2-fold lower in normal rats as compared with EHBR rats.

Likewise, Fukumura et al. (1998) found a considerably reduced biliary excretion of intravenously administered pravastatin in EHBR rats. However, the pharmacokinetics of oral pravastatin in Mrp2-deficient rats have not been well characterised. In the rat small intestine, Mrp2 expression is concentrated at the tips of the villi, with the highest concentrations seen in the proximal jejunum (Gotoh et al., 2000; Mottino et al., 2000). We postulated that absence of this efflux transporter in the intestinal enterocytes might result in increased absorption and oral bioavailability of pravastatin in Mrp2-deficient rats. We have therefore investigated the pharmacokinetics of pravastatin in Mrp2-deficient TR<sup>-</sup> rats and wild-type rats after oral and intravenous administration of a single dose of pravastatin.

## Materials and Methods

**Materials.** Pravastatin sodium salt was obtained from Merck KGaA (Darmstadt, Germany), pentobarbital sodium from Synopharm (Barsbüttel, Germany), and pancuronium bromide from Curamed Pharma (Karlsruhe, Germany). Other chemicals were purchased from Sigma-Aldrich Corporation (St Louis, MO). All solvents used for experimental and analytical purposes were of the highest commercially available quality.

**Animals.** Male wild-type Lewis rats (280-407 g) and genetically matched male Mrp2-deficient ( $TR^-$ ) Lewis rats (360-423 g; in-house breeding colony) were obtained from Dr. Ingrid Klöting (Department of Laboratory Animal Science, Ernst-Moritz-Arndt University Greifswald, Karlsburg, Germany). Genotyping confirmed presence of the premature termination codon in the  $TR^-$  rats only and Western blotting indicated absence of Mrp2 protein in the  $TR^-$  animals. The total plasma bilirubin concentration averaged  $4.7 \pm 0.21$  (SD) mg/dl and  $0.16 \pm 0.14$  mg/dl ( $p < 0.0001$ ) in the  $TR^-$  and control animals, respectively. The animal research protocol was in accordance with the current version of the German Law on the Protection of Animals and was approved by the appropriate local authority (Landesveterinär- und Lebensmitteluntersuchungsamt, Rostock, Germany).

**Determination of Pharmacokinetics of Oral and Intravenous Pravastatin.** Oral pravastatin (20 mg/kg) was investigated in eight wild-type rats and in eight  $TR^-$  rats and intravenous pravastatin in four wild-type rats and in four  $TR^-$  rats. The rats were allowed free access to water and laboratory rat chow and were housed in individual cages in a room with a 12 h light-dark cycle.

In the oral study, each rat was given after an overnight fast 20 mg/kg pravastatin sodium dissolved in sterile water, administered by direct injection into the stomach using a blunt-ended needle inserted via the esophagus. A 0.3-ml blood sample was obtained by orbital bleeding 0.5, 1, 1.5, 2, 4, and 10 h after administration of pravastatin. The animals were anesthetized using diethyl ether before blood was drawn. They were killed at the end of the experiment by cervical dislocation under anesthesia.

In the intravenous study, anesthesia was induced after an overnight fast by intraperitoneal injection of 50 mg/kg pentobarbital sodium. When stable anesthesia was achieved, animals were placed on a servo-controlled heating pad (Ugo Basile, Comerio, Italy) to maintain body temperature at 37°C. The neck was opened via a 2-cm midline incision and a PE50 catheter filled with isotonic saline containing 100 IU heparin/ml was inserted into the right carotid artery for arterial pressure recording and blood sampling. Another PE50 catheter was placed into the right jugular vein for continuous infusion (100 µl/kg/min) of isotonic saline containing 2% bovine serum albumin to replace fluid losses occurring during the experiment. A cannula was inserted into the trachea. Two catheters (PE10 fused to PE50) were inserted into the left femoral vein for administration of supplemental pentobarbital and pravastatin. The lower abdomen was opened via a 3-cm midline incision and a PE10 catheter was inserted into each ureter for urine sampling. After completion of the surgery, the tracheal cannula was connected to a small animal respirator (Ugo Basile) and the rats were paralyzed with pancuronium bromide (1 mg/kg/h) and allowed to stabilize for 30 min.

Pravastatin sodium dissolved in isotonic saline (5 mg/ml) was administered as an intravenous bolus injection at a dose of 5 mg/kg. To ensure complete administration of the dose, the catheter was flushed with 100 µl of isotonic saline immediately after drug injection. Blood samples (0.5 ml) were taken before pravastatin administration and 15 min, 30 min, 45 min, 60

min, 90 min, and 120 min after pravastatin into tubes containing 750 µg EDTA. Urine was collected in fractions of 0-30 min, 30-60 min, 60-90 min, and 90-120 min in pre-weighted test tubes. The hemodynamics of all animals remained stable throughout the experiment, with a mean arterial pressure between 87 and 113 mmHg. No animal experienced a drop in blood pressure. At the end of the experiment, the animals were killed with an overdose of pentobarbital.

**Determination of Plasma Pravastatin Concentrations.** Pravastatin concentrations were determined by gas chromatography-tandem mass spectrometry (GC-MS-MS), using lovastatin as the internal standard. Plasma samples (100 µl) were spiked with 5 ng of lovastatin and 0.5 ml of acetate buffer (0.1 M, pH 5.0). After extraction with 5 ml of diethyl ether/2-propanol (9:1 v/v), the organic layer was evaporated to dryness in a stream of nitrogen. Pentafluorobenzyl (PFB) derivatives were prepared by treating the residue with 30 µl of PFB bromide (30% in acetonitrile) and 10 µl of diisopropyl ethyl amine for 20 min at room temperature. After evaporation to dryness, 20 µl of *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) was added and the samples were kept at room temperature for 10 min before GC-MS-MS analysis.

For GC-MS-MS, a TSQ 700 mass spectrometer (Finnigan MAT, Bremen, Germany) coupled to a 5890 II gas chromatograph (Hewlett Packard, Waldbronn, Germany) was used. Gas chromatography was performed on a Rtx-5MS column (30 m, 0.25 mm i.d., dimethylpolysiloxane with 5% phenyl groups, 0.25 µm film thickness; Restek, Bad Homburg, Germany) in the splitless mode (280°C), with helium as carrier gas at an inlet pressure of 100 kPa. The initial oven temperature of 120°C was held for 1 min, increased by 15°C/min to 240°C and then by 30°C/min to 300°C. The final temperature was held for 7 min. Mass spectrometry was performed in the negative ion chemical ionisation (NICI) mode. Precursor

and product ions were  $m/z$  639 and  $m/z$  459 for pravastatin and  $m/z$  565 and  $m/z$  385 for lovastatin. The method was linear over the studied concentration range and intra- and inter-day coefficients of variation were below 10% at relevant concentrations. The limit of quantification was 2 ng/ml.

**Pharmacokinetic Analysis.** The pharmacokinetics of pravastatin were characterized, as appropriate, by peak concentration in plasma ( $C_{max}$ ), time to  $C_{max}$  ( $T_{max}$ ), area under the plasma pravastatin concentration-time curve (AUC), terminal half-life ( $T_{1/2}$ ), total amount of pravastatin excreted unchanged into urine ( $A_e$ ), systemic (total) clearance (CL), renal clearance ( $CL_R$ ), and apparent volume of distribution (V). The elimination rate constant ( $k_{el}$ ) was determined by a linear regression analysis of the terminal log-linear part of the concentration-time curve. The  $T_{1/2}$  was calculated by the equation  $T_{1/2} = \ln 2/k_{el}$ . AUC values were calculated by the linear trapezoidal method. CL was calculated by the equation  $CL = Dose_{IV}/AUC_{IV}$ ,  $CL_R$  by  $CL_R = A_e/AUC_{IV}$ , and V by the equation  $V = CL/k_{el}$ .

**Pharmacodynamic Analysis.** Plasma concentrations of cholesterol and the cholesterol precursor lathosterol were determined in the oral study in samples obtained 10 h after pravastatin administration and in the intravenous study in samples obtained before pravastatin administration and 15 min, 30 min, 45 min, 60 min, 90 min, and 120 min after pravastatin. Cholesterol and lathosterol were measured by gas-liquid chromatography-mass spectrometry (GLC-MS), as described in detail previously (Lütjohann et al., 2004). The effects of pravastatin on cholesterol synthesis were characterized by the ratio of lathosterol to cholesterol in plasma. This ratio is an established indicator of the activity of hepatic HMG-CoA reductase and the rate of total cholesterol synthesis *in vivo* (Björkhem et al., 1987; Kempen et al., 1988).

**Statistical Analysis.** Data are presented as mean  $\pm$  SD. The unpaired *t* test (two-tailed) or the Mann-Whitney test was employed for statistical analysis of the results as appropriate. *p* values  $< 0.05$  were considered statistically significant.

## Results

**Pharmacokinetics of Oral and Intravenous Pravastatin.** The TR<sup>-</sup> rats showed a 6.1-fold higher mean pravastatin AUC ( $p < 0.001$ ) after oral pravastatin (20 mg/kg) and a 4.7-fold higher AUC ( $p < 0.01$ ) after intravenous pravastatin (5 mg/kg) as compared with the wild-type rats (Table 1, Figs. 1 and 2). Accordingly, the mean CL of pravastatin was 4.6-fold higher in the wild-type animals (39.2 vs 8.50 l/h/kg,  $p < 0.001$ ). The terminal  $T_{1/2}$  of pravastatin after intravenous administration averaged 16 min in the TR<sup>-</sup> rats and 15 min in the controls. After oral administration of pravastatin, the terminal  $T_{1/2}$  could not be calculated in the wild-type rats due to fluctuating plasma drug concentrations. Among the TR<sup>-</sup> rats, the pravastatin concentration-time curve displayed a log-linear terminal phase in only five out of the eight animals, resulting in an average terminal  $T_{1/2}$  of 3.6 h. The mean V of pravastatin was 4.3-fold higher in the wild-type animals than in the TR<sup>-</sup> animals (14.1 vs 3.29 l/kg,  $p < 0.01$ ). The mean CL<sub>R</sub> of pravastatin in the TR<sup>-</sup> rats was 16.5-fold increased as compared with the controls (0.695 vs 0.042 l/h/kg,  $p < 0.05$ ).

**Pharmacodynamics.** The lathosterol to cholesterol concentration ratio determined 10 h after oral administration of pravastatin was significantly lower in the TR<sup>-</sup> rats as compared with the wild-type rats ( $0.67 \pm 0.12$  vs  $1.27 \pm 0.22$   $\mu\text{g}/\text{mg}$ ,  $p < 0.001$ ). In the intravenous study, there were no significant differences in this ratio between the two groups at any time point (e.g. baseline,  $0.34 \pm 0.015$  vs  $0.33 \pm 0.026$   $\mu\text{g}/\text{mg}$ ; 1 h,  $0.35 \pm 0.024$  vs  $0.36 \pm 0.024$   $\mu\text{g}/\text{mg}$ ; 2 h,  $0.35 \pm 0.020$  vs  $0.38 \pm 0.017$   $\mu\text{g}/\text{mg}$ ).

## Discussion

Limited information is available regarding the impact of intestinal Mrp2 expression on the oral bioavailability of pravastatin. We explored the possibility that intestinal Mrp2 might serve as an absorption barrier to pravastatin in rats, by investigating the pharmacokinetics of pravastatin after oral and intravenous administration in normal rats and Mrp2-deficient TR<sup>-</sup> rats. This is the first study to investigate disposition of both orally and intravenously administered pravastatin in Mrp2-deficient rats. The AUC of oral pravastatin was about 6-fold higher and the CL of pravastatin was about 5-fold lower in the TR<sup>-</sup> rats as compared with the normal rats. On the other hand, the CL<sub>R</sub> of pravastatin in the TR<sup>-</sup> animals was >10-fold increased as compared with the wild-type animals.

The increased systemic exposure to pravastatin in the Mrp2-deficient TR<sup>-</sup> rats was associated with a greater inhibitory effect on HMG-CoA reductase after oral administration of pravastatin, as demonstrated by smaller lathosterol to cholesterol concentration ratios as compared with the wild-type animals. These results are in line with previous studies showing potent inhibition of hepatic cholesterol synthesis by pravastatin in the rat (Tsujita et al., 1986; Hatanaka et al., 1998), and suggest that hepatic pravastatin concentrations were considerably higher in the Mrp2-deficient animals sometime between the drug administration and end of the experiment. This would also be expected considering the marked differences in plasma pravastatin concentrations between the groups together with the fact that absorbed pravastatin is selectively distributed to the liver (Komai et al., 1992; Yamazaki et al., 1996b; Hatanaka et al., 1998). That intravenous pravastatin had no effect on the lathosterol to cholesterol ratio might be due to the rapid elimination of pravastatin observed after intravenous administration. In addition, the intravenous dose was 4-fold lower than the oral one.

The terminal  $T_{1/2}$  could not be determined in the wild-type rats in the oral study due to fluctuating plasma drug concentrations, probably resulting from enterohepatic recirculation and reabsorption of pravastatin from the intestine. However, data from five Mrp2-deficient animals for which the elimination rate constant could be determined suggested a terminal  $T_{1/2}$  on the order of 3–4 h. A recent study reported a terminal  $T_{1/2}$  of about 7 h for pravastatin in three normal rats and a  $T_{1/2}$  of about 15 h in three EHBR rats after a very large oral dose of pravastatin (200 mg/kg) (Naba et al., 2004). The terminal  $T_{1/2}$  of pravastatin after intravenous administration was short, about 15 min, in both rat groups. However, the fact that >95% of the total amount of intravenously administered pravastatin found in the urine was excreted within 30 min of pravastatin administration in each animal concurs with the half-lives determined from plasma pravastatin concentrations. Further, Hatanaka et al. (1998) found a terminal  $T_{1/2}$  of about 10 min in normal rats after intravenous doses ranging from 10 to 200 mg/kg, although the CL of pravastatin decreased with increasing dose. Saturation of pravastatin uptake into the liver might partly explain the lack of a relationship between pravastatin CL and  $T_{1/2}$ , as Yamazaki et al. (1996c) showed that hepatic uptake was the rate-limiting step in the overall hepatic elimination of intravenous pravastatin at steady-state in rats. As for oral pravastatin, the terminal half-lives found in this and other studies for oral pravastatin may not represent the true elimination  $T_{1/2}$ , possibly due to a saturable absorption from the intestine (Hatanaka et al., 1998).

The  $T_{1/2}$  after intravenous pravastatin administration did not differ between the TR<sup>−</sup> and wild-type rats, which was unexpected considering the considerably lower CL of pravastatin in the Mrp2-deficient animals. However, the CL and V were decreased to a similar degree, 4- to 5-fold, in the Mrp2-deficient rats as compared with the wild-type rats, which would leave the  $T_{1/2}$  largely unchanged as these primary pharmacokinetic parameters determine together the  $T_{1/2}$ . Moreover, it is possible that adaptive changes in expression of other transporters

contributed to the pharmacokinetic results obtained in the TR<sup>-</sup> rats. For example, it is known that the hepatic expression of Mrp3, which mediates transport of conjugated organic anions across the hepatocyte basolateral membrane into sinusoidal blood (König et al., 1999), is upregulated in Mrp2-deficient rats (Hirohashi et al., 1998; Ogawa et al., 2000). In a recent study, hepatic and renal expression of Mrp3 protein was 2- to 3-fold higher in EHBR rats than in Sprague-Dawley rats (Kuroda et al., 2004). In contrast, hepatic expression of both oatp1 and oatp2 protein, which are responsible for the hepatic uptake of pravastatin in rats (Hsiang et al., 1999; Tokui et al., 1999), was about 50% lower in EHBR rats (Kuroda et al., 2004). These changes may represent a physiologically important compensatory mechanism to restrict accumulation of potentially toxic organic anions in hepatocytes in Mrp2 deficiency. However, it is not known whether pravastatin is a Mrp3 substrate.

That the systemic exposure to pravastatin (as determined by AUC) was increased in the Mrp2-deficient rats to nearly the same extent after intravenous and oral pravastatin administration suggests that intestinal Mrp2 does not appear to play a major role in the oral absorption of pravastatin in normal rats. This was unexpected, as Mrp2 serves as an efflux pump for xenobiotics and is expressed on the apical membrane of the epithelial cells lining the intestinal lumen (Gotoh et al., 2000; Mottino et al., 2000). Our findings are supported by a recent study (Chen et al., 2003), suggesting that Mrp2 does not play a significant role in hindering oral absorption of some of its substrate drugs. Importantly, Wu and Benet (2005) have suggested that the contribution of efflux transporters to drug disposition depends on other characteristics of the drug such as permeability and solubility in gastrointestinal fluids. Therefore, it should not be generalized that Mrp2 does not influence drug absorption.

The CL<sub>R</sub> of pravastatin was markedly increased in the TR<sup>-</sup> rats as compared with the wild-type animals, suggesting that the reduced biliary pravastatin excretion in Mrp2-deficient rats

is to some extent compensated for by increased urinary excretion. In any event, the contribution of  $CL_R$  to CL was small even in the TR<sup>-</sup> animals (about 8%), indicating that other transporters are capable of mediating the biliary excretion of pravastatin in the absence of Mrp2.

In conclusion, the considerably higher plasma pravastatin concentrations observed in the Mrp2-deficient TR<sup>-</sup> rats resulted mainly from reduced CL due to the absence of Mrp2 from the canalicular membrane of hepatocytes. Intestinal Mrp2 expression does not appear to play a major role in the oral absorption of pravastatin in normal rats.

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## **Footnotes**

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(No numbered footnotes)

### Legends for figures

Fig. 1. Pravastatin concentration-time profiles in plasma after oral administration of pravastatin (20 mg/kg) in eight Mrp2-deficient TR<sup>-</sup> rats and eight wild-type rats (mean ± SD).

Fig. 2. Pravastatin concentration-time profiles in plasma after intravenous administration of pravastatin (5 mg/kg) in four Mrp2-deficient TR<sup>-</sup> rats and four wild-type rats (mean ± SD).

TABLE 1

*Pharmacokinetics of pravastatin after oral (20 mg/kg) and intravenous (5 mg/kg) administration to Mrp2-deficient TR<sup>-</sup> rats and wild-type rats*

	Oral Study		Intravenous Study	
Variable	TR <sup>-</sup> rats (n = 8)	Wild-type rats (n = 8)	TR <sup>-</sup> rats (n = 4)	Wild-type rats (n = 4)
C <sub>max</sub> (ng/ml)	240 ± 93.2***	19.2 ± 8.03	-	-
T <sub>max</sub> (h)	0.5 (0.5-0.5)	0.5 (0.5-0.5)	-	-
AUC (ng · h/ml)	387.0 ± 97.7***	63.0 ± 34.3	609.3 ± 137.6**	128.6 ± 13.1
CL (l/kg/h)	-	-	8.50 ± 1.72***	39.2 ± 4.33
V (l/kg)	-	-	3.29 ± 0.72**	14.1 ± 2.62
T <sub>1/2</sub> (min)	-	-	16.1 ± 0.97	14.9 ± 1.5
Ae (μg)	-	-	166 ± 89.5*	1.87 ± 1.58
CL <sub>R</sub> (l/kg/h)	-	-	0.695 ± 0.325*	0.042 ± 0.034

Results are shown as mean ± SD. T<sub>max</sub> is given as median with range.

\*p < 0.05 versus wild-type rats (control)

\*\*p < 0.01 versus wild-type rats (control)

\*\*\*p < 0.001 versus wild-type rats (control)

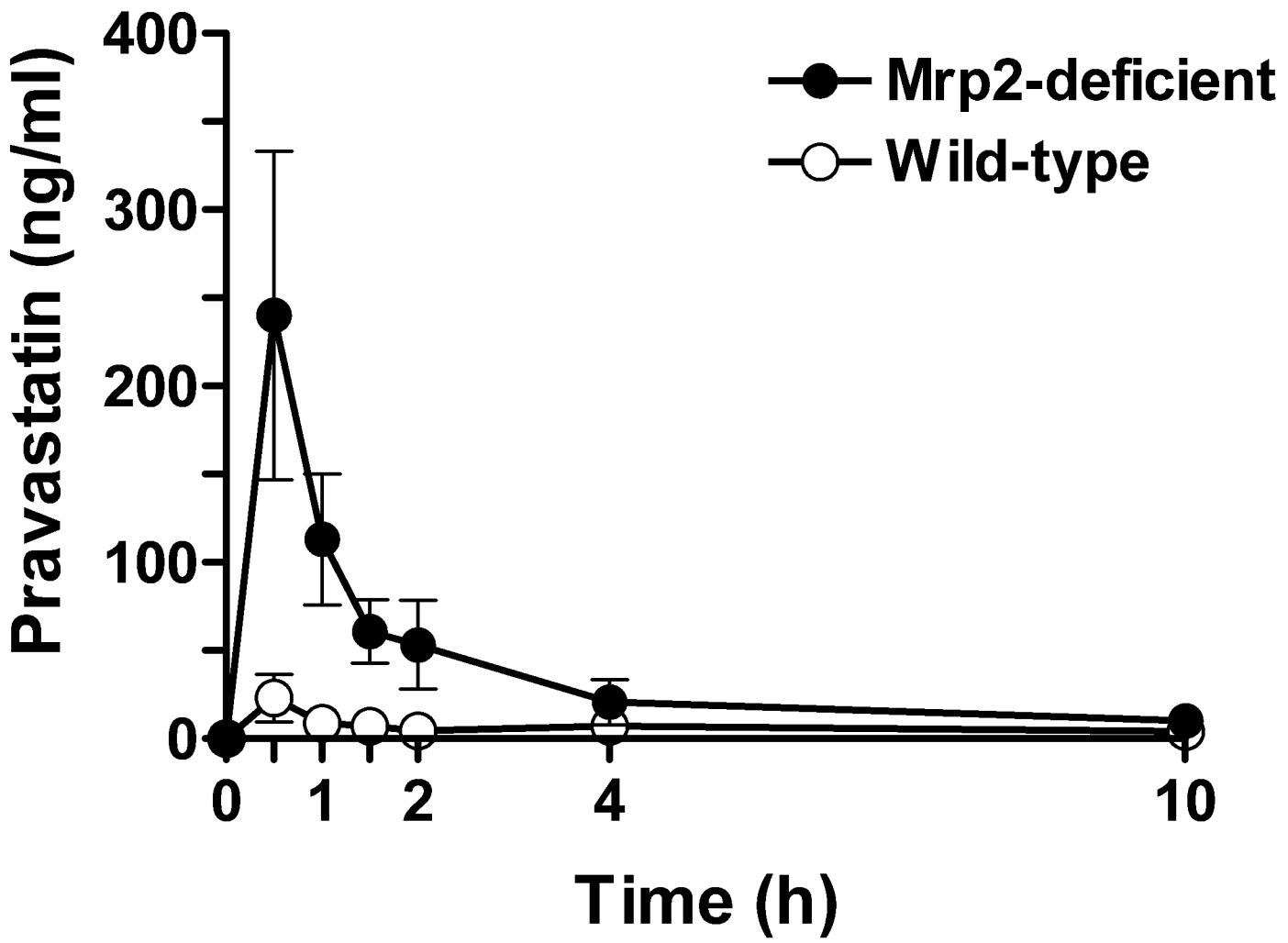


Fig. 1.

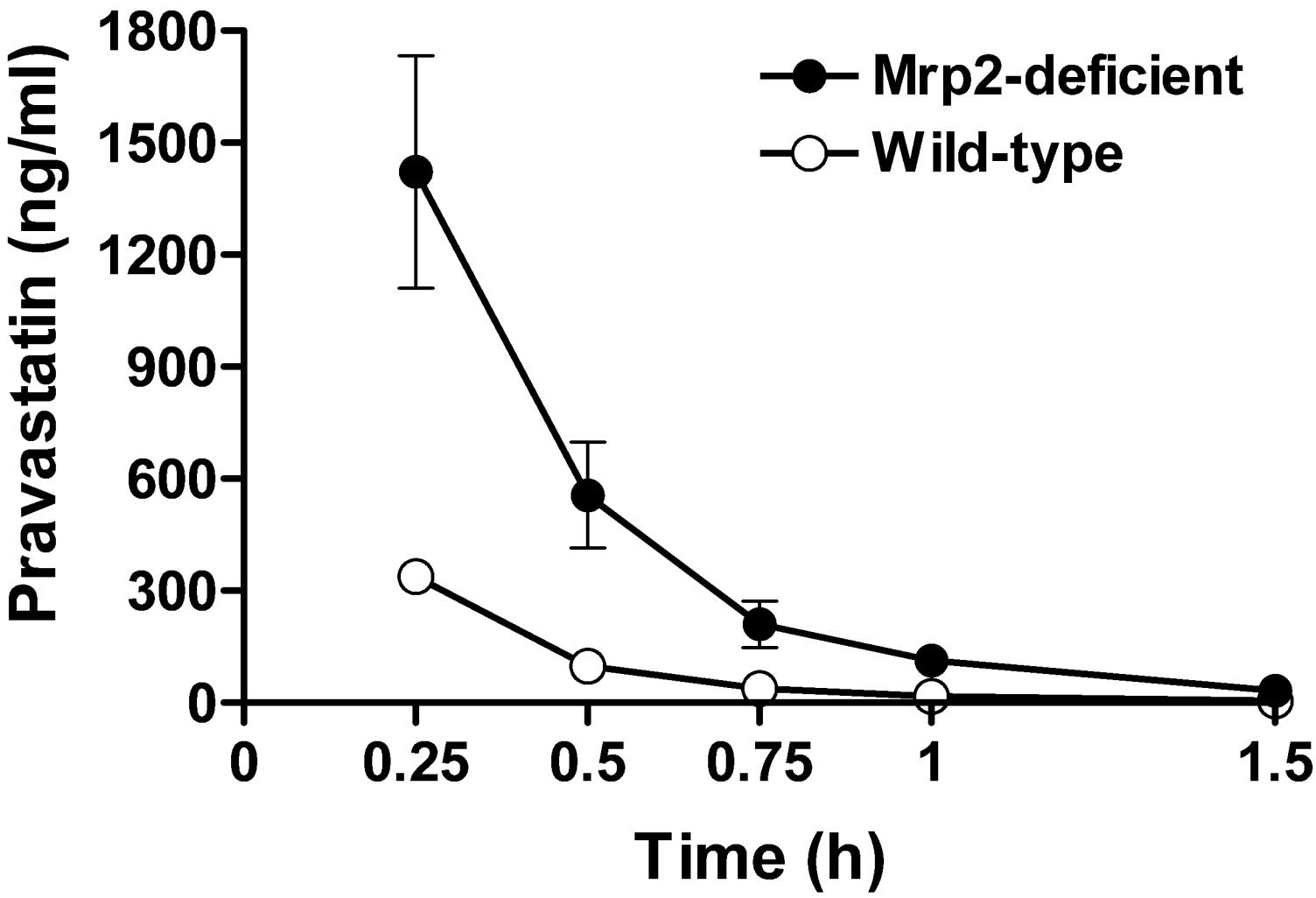


Fig. 2