Disposition of the Cholesterol Absorption Inhibitor Ezetimibe in *mdr1a/b* (−/−) Mice

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ABSTRACT: The lipid lowering agent ezetimibe (EZ) and its intestinally formed glucuronide (GLUC) were shown to be substrates of the efflux transporters P-glycoprotein (P-gp) and the multidrug resistance associated protein 2 (MRP2) which markedly influences the disposition and efficacy of EZ in man. This study aims to elucidate the unique meaning of P-gp in the pharmacokinetics of EZ in mice. In brief, serum concentrations, organ distribution and elimination of EZ were determined in 10 male wild-type and *mdr1a/b* (−/−) mice after oral treatment with EZ (10 mg/kg, 10 days). EZ and GLUC were quantified in serum, urine, feces and various tissues using a validated LC-MS/MS method. Compared to wild-type mice, *mdr1a/b* knockout was associated with significantly increased serum concentrations of GLUC (5.58 ± 2.07 versus 2.09 ± 0.83 ng/ml, $p < 0.001$) but not of EZ (0.92 ± 0.73 versus 0.55 ± 0.40 ng/ml, n.s.). Consequently, urinary excretion of GLUC was about three-fold increased (9.96 ± 0.27 versus 3.10 ± 1.37 μg/day, $p = 0.049$) whereas renal clearance and the amount excreted via feces remained unchanged. Both EZ and GLUC were not over-proportionally distributed into investigated organs. P-glycoprotein primary influences the oral absorption of ezetimibe in mice. Distribution, renal and fecal excretion of the drug seems not to be markedly affected by P-glycoprotein. © 2007 Wiley-Liss, Inc. and the American Pharmacists Association J Pharm Sci 96:3478–3484, 2007

Keywords: ezetimibe; mdr1; P-glycoprotein; absorption; drug transport; disposition

INTRODUCTION

Bioavailability and pharmacodynamic effects of drugs with intensive presystemic clearance are influenced by many factors that are poorly predictable. These mechanisms include the physiological conditions for uptake at the intestinal site of drug liberation, the substrate concentration at the sites of intestinal and hepatic uptake, the presystemic biotransformation and the efflux drug transport from enterocytes and/or hepatocytes. An example for this complex interplay is ezetimibe, that inhibits cholesterol absorption by interaction with the Niemann-Pick C1 like 1 protein (NPC1L1) in enterocytes along the small intestine.[1,2] Disposition and cholesterol-lowering effect of ezetimibe are markedly influenced by the activity of intestinal UDP-glucuronosyltransferases (mainly UGT1A1) and the efflux transport proteins P-glycoprotein (P-gp) and the multidrug resistance associated protein 2 (MRP2) (Fig. 1). Cytochrome P450 enzymes are not involved.[3,4] Intestinal conjugation followed by secretion of the glucuronide into gut lumen initiates intensive entero-systemic circulation thus maintaining a long-lasting cholesterol-lowering effect. Consequently, pregnane X receptor (PXR) type upregulation of P-gp and MRP2 by rifampicin leads to lower serum levels and reduced sterol-lowering effect of ezetimibe in man whereas modulation of

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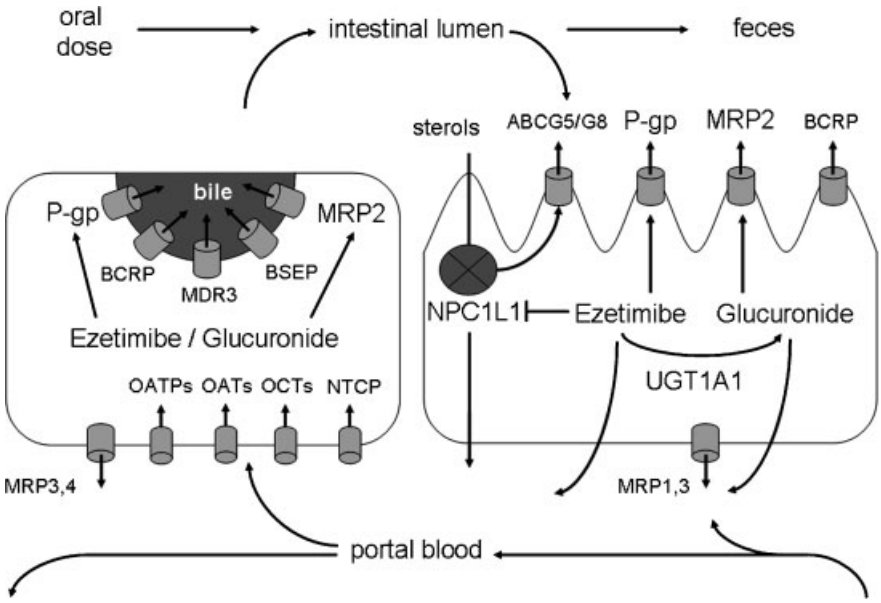

Figure 1. Schematic overview of the local pharmacokinetics of ezetimibe in enterocytes (right) and hepatocytes (left). The molecular target of ezetimibe is the Niemann-Pick C1 like 1 (NPC1L1) protein mediating the sterol uptake into the enterocytes, which is limited by the sterol-efflux pumps ABCG5/G8. Within the enterocytes, ezetimibe is metabolized predominately by the isoenzyme UGT1A1 and subjected to active efflux transport via P-gp and MRP2. From the portal blood flow, ezetimibe is either taken up into hepatocytes to undergo subsequent biliary secretion or reaches the systemic circulation. Furthermore, several other important intestinal and hepatic transporters are shown. Abbreviations: ABC, ATP-binding cassette; BCRP, breast cancer resistance protein; BSEP, bile salt exporting pump; MRP, multidrug resistance associated protein; MDR, multidrug resistance; OAT, organic anion transporter; OATP, organic anion transporting polypeptide; OCT, organic cation transporter; NTCP, Na^+-taurocholate cotransporting polypeptide; P-gp, P-glycoprotein; UGT, UDP-glucuronosyltransferase.

these carriers by ciclosporin, fibrates and single dose rifampicin increase systemic exposure and accelerate its effect.[4–8] However, the individual, quantitative role of P-gp and MRP2 in overall disposition and pharmacological effect of ezetimibe remains unclear because both induction and inhibition by mentioned interaction partners concomitantly influence P-gp and MRP2. Recent *in vitro* experiments in P-gp overexpressing MDCKII cells, P-gp- or MRP2-containing vesicles confirmed that the glucuronide is a high affinity substrate of MRP2 whereas the parent compound binds with low affinity to MPR2 and P-gp.[4] Appropriate experimental models to assess the distinct *in vivo* meaning of P-gp and MRP2 in the disposition of drugs are transporter deficient animals. We have recently shown that Mrp2 deficiency in rats leads to manifold increased serum levels of the glucuronide which in turn caused higher amounts excreted into urine.[9] The serum levels of ezetimibe, however, were significantly decreased and, consequently, the sterol-lowering effect was minimized. Thus, it was shown that Mrp2 appears to be the major determinant for ezetimibe glucuronide elimination and of outstanding importance for the intended drug effects. Referring to P-gp, *mdr1a/b* knockout mice are a suitable rodent model which is characterized by artificial gene disruption and which was extensively used for the assessment of P-gp substrates as elegantly shown for several anticancer drugs, HIV-protease inhibitors, digoxin and ciclosporin.[10–13] In contrast to the absence of Mrp2, P-gp deficiency is expected to cause higher serum concentrations of ezetimibe due to the lack of intestinal drug efflux and the consequently enhanced drug absorption. Furthermore, a wider distribution of parent ezetimibe into organs with P-gp-mediated blood-organ barriers (e.g. brain, testes) is anticipated. The impact of *mdr1* knockout on the disposition of ezetimibe glucuronide so far is not predictable because there is no infor-

mation about the rate-limiting step of the presystemic metabolism within murine enterocytes (i.e. glucuronidation and transport).

MATERIALS AND METHODS

Chemicals and Reagents

Ezetimibe (Ezetrol® tablets) was obtained from MSD Sharp & Dohme (Haar, Germany). Acetonitrile and diethyl ether were purchased from Merck (Darmstadt, Germany) and methyl cellulose from Sigma–Aldrich (Taufkirchen, Germany).

Animals

The study was performed in 10 male wild-type (26–36 g) and 10 male *mdr1a/b* (−/−) FVB/NTac@Bom mice (24–29 g), which were purchased from the Department of Pathology of the University of Greifswald. The animals were housed under standard laboratory conditions in the life island box A 110 (Flufrance, Wissous, France) with mass-air displacement (temperature 25°C, 12 h light–dark cycle with light on at 8 a.m.). The mice were hold in polycarbonate cage (three to four animals per cage; bedding, ssniff, Soest, Germany) with free access to acidified water and to rodent chow (ssniff, Soest, Germany). The study was permitted by the Federal Authorities.

Animal Study

After adaptation for 14 days, the mice were treated orally with 10 mg/kg ezetimibe for 10 days. In order to realize reproducible drug administration, mortared Ezetrol® tablets were suspended in 0.5% methyl cellulose solution to avoid drug sedimentation. For adequate dispersion of the drug, suspensions were incubated into an ultrasonic bath for 1 min. This drug suspension was prepared immediately before each daily drug administration via gavage (administration volume 20 ml/kg). To assess the intestinal and renal excretion of ezetimibe and its glucuronide at steady state, feces and urine was collected at the 10th study day while housing the animals in metabolic cages (three to four mice per cage). In the morning of the 11th study day (24 h after last drug administration), animals were anesthetized with diethyl ether and sacrificed by cervical dislocation and decapitation for blood sampling and dissection of liver, testes, kidneys and brain. All samples were stored until the quantitative analysis at least at −20°C. All animals included in our experiment finished the study.

Quantitative Assay for Ezetimibe

Ezetimibe and its glucuronide in serum, urine and feces were quantified using liquid chromatography-tandem mass spectrometry as described elsewhere.[14] For the determination of tissue concentrations, the thawed organs were mixed 1 + 5 (mass related) with distilled water and subsequently homogenized using the Ultra Turrax T25 (IKA, Staufen, Germany). Briefly, 200 mL of each organ homogenate was used for the quantitative assay as described for the other matrices. The glucuronide was quantified indirectly after hydrolysis using ß-glucuronidase/sulfatase (Sigma–Aldrich). The validation ranges for ezetimibe in serum was 0.05–10 ng/mL, for urine 0.01–5.0 μg/mL, for feces 0.01–10 μg/mL, and for tissues homogenates 0.05–500 ng/mL. In the present study, within-day and between-day accuracy for serum were within −5.1 and 12.4% and −4.8 and 2.7%, respectively, of the nominal concentrations of parent and total ezetimibe. Between-day precision for unchanged and total ezetimibe in serum was 3.6–15.3% and 2.1–8.0% of the mean values. For urine, feces and tissues, accuracy was −8.5 to 7.5%, −9.1 to 3.0% and −3.2 to 8.6% of nominal values and precision 3.1–10.1%, 4.4–11.1% and 10.8–5.3% of means.

Pharmacokinetic and Statistical Evaluation

Serum trough concentrations of ezetimibe and its glucuronide at steady-state (C_{ss}) were taken from the study data. Renal clearance (CL_R) and apparent fecal clearance ($CL_{F(app)}$) was derived from the amount excreted (A_e) into the urine and feces over $C_{ss} \times 24$ h which represents approximately $AUC_{0-24\,h}$ of ezetimibe and the glucuronide, respectively. It is important to note that according to the urinary and fecal A_e no individual but pooled samples were collected due to the usage of metabolic cages with three to four animals. Therefore, for the urinary and fecal A_e only three values for each group were available for analysis. Data are presented as arithmetic means ± S.D. Samples were compared using the non-parametric Mann–Whitney-test, which was calculated by the SPSS software, version 12 (SPSS Inc., Chicago).

Table 1. Pharmacokinetic Data of Ezetimibe and its Glucuronide in Mice after Administration of 10 mg/kg for 10 days

			Ezetimibe		Ezetimibe Glucuronide	
		N	Wild-type	*mdr1a/b* (−/−)	Wild-type	*mdr1a/b* (−/−)
C_{SS}	(ng/mL)	10	0.55 ± 0.40	0.92 ± 0.73	2.09 ± 0.83	$5.58 \pm 2.07^{**}$
Ae_{feces}	(μg/day)	3[a]	276 ± 17.6	271 ± 5.90	77.2 ± 1.72	85.3 ± 23.4
Ae_{urine}	(μg/day)	3[a]	0.81 ± 0.24	1.46 ± 0.64	3.10 ± 1.37	$9.96 \pm 0.27^{*}$
CL_R	(mL/min)	10	1.41 ± 1.23	1.39 ± 1.16	1.03 ± 0.40	1.24 ± 0.05
$CL_{F(app)}$	(mL/min)	10	430 ± 253	231 ± 108	26.2 ± 4.58	$10.6 \pm 3.04^{*}$

Means ± standard deviations and *p*-values (Mann–Whitney-test) for comparisons with wild-type are given ($^{*}p < 0.05$, $^{**}p < 0.01$). *N* indicates the number of analyzed samples.
[a]Values were generated from pooled urine and feces of three to four animals, respectively.

RESULTS

Mdr1a/b knockout was associated with a 2.7-fold, significant increase of the serum concentrations of ezetimibe glucuronide whereas the serum levels of the parent compound were not significantly different (Table 1). This was associated with a significant increase of the urinary excretion of both ezetimibe and its glucuronide. The renal clearances remained unchanged. Furthermore, fecal excretion was also not different. Contrary to this, the apparent fecal clearance was nearly halved for ezetimibe and its glucuronide in *mdr1* knockout mice, which was statistically significant only for the glucuronide. As a result of the increased serum concentrations, the distribution of the parent ezetimibe into the liver and kidney and of the glucuronide into all organs studied was significantly increased (Table 2). However, the ratios obtained with organ concentrations over the respective serum concentrations of ezetimibe and its glucuronide showed no over-proportional organ distribution of ezetimibe and its glucuronide in *mdr1a/b* knockout mice.

DISCUSSION

To our understanding, ezetimibe after oral administration is taken up into enterocytes after liberation from the dosage form in the upper small intestine in dependence on the regional P-gp and MRP2 expression and activity. However, its bioavailability is so far unknown due to the fact that there is no parenteral application form for this very lipophilic compound available. Once in the enterocytes, the parent compound undergoes nearly complete phase II metabolism to a phenolic glucuronide, which is predominately subjected to intestinal secretion by MRP2. Efflux of the glucuronide into gut lumen seems to be the rate-determining step in ezetimibe disposition that initiates the long-lasting entero-hepatic and entero-systemic recirculation of the active

Table 2. Tissue Concentrations and Organ to Serum Ratios of Ezetimibe and the Glucuronide (ng/g wet weight) After Oral Administration of 10 mg/kg for 10 days

		Ezetimibe		Ezetimibe glucuronide	
	N	Wild-type	*mdr1a/b* (−/−)	Wild-type	*mdr1a/b* (−/−)
Liver	10	82.8 ± 49.6	$173 \pm 76.9^{**}$	142 ± 72.1	$357 \pm 98.9^{**}$
Kidney	10	6.69 ± 3.27	$9.80 \pm 2.57^{*}$	55.6 ± 19.5	$95.7 \pm 21.1^{**}$
Testes	10	4.64 ± 4.50	4.18 ± 4.81	8.01 ± 17.2	$11.5 \pm 10.9^{**}$
Brain	10	0.45 ± 1.04	0.51 ± 0.94	5.12 ± 1.79	$6.11 \pm 1.04^{*}$
Liver/serum	10	151 ± 90.2	188 ± 83.6	67.8 ± 34.5	63.9 ± 17.7
Kidney/serum	10	12.2 ± 5.94	10.7 ± 2.79	26.6 ± 9.35	$17.2 \pm 3.78^{**}$
Testes/serum	10	8.44 ± 8.17	4.54 ± 5.23	3.83 ± 8.21	2.06 ± 1.96
Brain/serum	10	0.82 ± 1.89	0.55 ± 1.02	2.45 ± 0.86	$1.10 \pm 0.19^{**}$

Means ± standard deviations and *p*-values (Mann–Whitney-test) for comparisons with wild-type are given ($^{*}p < 0.05$, $^{**}p < 0.01$). *N* indicates the number of analyzed samples.

ezetimibe back to the cholesterol absorption compartment along the entire small intestine.[4,8] Therefore, Mrp2 deficiency is associated with cessation of the recycling processes and, consequently, with decreased serum levels and sterol-lowering effects of ezetimibe as shown in our recent study using the spontaneous mutant TR^- rat model. The non-secreted portion of the glucuronide appears via the portal vein in the blood and leads to manifold increased serum levels and a compensatory increase of its urinary excretion.[9]

In contrast to the absence of Mrp2, ezetimibe is in the lack of intestinal P-gp obviously better absorbed from the small intestine. Hence, a higher amount of unchanged ezetimibe undergoes immediate glucuronidation within the enterocytes leading to significantly three-fold increased glucuronide levels in serum and concomitantly elevated urinary excretion. Consequently, the serum levels of ezetimibe were not different in *mdr1a/b* knockout mice. Also the fecal excretion of ezetimibe was unchanged. However, the apparent fecal clearance of both compounds were markedly reduced but in a significant manner only according to the glucuronide. This is most likely caused by the fact that ezetimibe is only poorly absorbed from the alimentary tract as a result of its insufficient water solubility (0.007 g/L). Thereby, actively secreted compound increase the drug concentration within the gut lumen only negligibly (ezetimibe gut concentration: ~200 μg/g versus serum concentration: 0.6 ng/mL). We concluded from this pharmacokinetic pattern that the extent of oral absorption of ezetimibe is controlled by intestinal P-gp and that this efflux transport protein is involved at least in part in long-lasting recirculation of the drug to the pharmacodynamic site of action. P-gp in the tubular cells of the kidneys seems not to be involved in renal elimination of ezetimibe and the glucuronide, because renal clearance of both were not significantly decreased in *mdr1a/b* (−/−) mice. A further conclusion from our data in *mdr1a/b* (−/−) mice is that P-gp seems not to be involved in tissue distribution of ezetimibe and its major metabolite. In the organs of P-gp deficient animals, the organ/serum concentration ratios as an indicator for over-proportional drug accumulation were not significantly increased. On the contrary, this was revealed for other P-gp substrates such as HIV-protease inhibitors, digoxin, cerivastatin, grepafloxacin and dexamethasone.[10,11,15–17] However, these drugs are not subjected to such tremendous and immediate intestinal metabolism as it is the case for ezetimibe.

In discussing our pharmacokinetic data with ezetimibe it must be considered that potential adaptation processes in expression of drug metabolizing enzymes and transport proteins in transporter-deficient animals may appear as shown in our recent study with Mrp2-deficient rats. Mrp2-deficiency was associated with up-regulation of hepatic *Mdr1*, *Mrp3* and *Ugt1a1*.[9] *Mdr1a/b* (−/−) mice are known to express markedly increased levels of CYP450 enzymes although this is not relevant for ezetimibe which does not undergo oxidative biotransformation.[3,18] Moreover, Lam et al. showed that *Bsep*-knockout in mice leads to increased hepatic expression of P-gp, Mdr2, Mrp2, and Mrp3.[19] Therefore, it can not be excluded that adaptive processes in the expression of Ugts and/or other transport proteins may have influenced the disposition of ezetimibe in our study with *mdr1a/b* (−/−) mice. A further limitation of our study is that there is so far no information about dose-dependent inhibitory effects of ezetimibe on P-gp and MRP2. Thus, the high dose of applied ezetimibe in this study (10 mg/kg) may be capable to inhibit P-gp or other transporters and in turn may lead to over- or under-estimation of its *in vivo* relevance. Accordingly, modulation of P-gp in the wild type mice could mask the impact of this efflux pump, whereas blocking of other transporters may result in pharmacokinetic effects that are not caused by P-gp. Furthermore, the administered dose used in our investigation is far in excess of the applied dose in humans (10 mg/day in human → 70 kg, 0.14 mg/kg vs. 10 mg/kg in mice), which makes it difficult to draw conclusions from our observations for the situation in humans. It is gradually being recognized that it is challenging to predict the bioavailability in human using rodent models.[20] However, despite all limitations, these experimental models may provide deeper *in vivo* insights into the functional relevance of transporters on drug disposition which is the case for our study.

CONCLUSION

The activity of intestinal P-glycoprotein in mice influences the extent of oral absorption of ezetimibe and controls at least in part the long-lasting drug recirculation and bioavailability of the drug in the cholesterol-absorbing compartment

along the small intestine. Distribution and renal excretion of the drug are not dependent on P-glycoprotein in the investigated rodent model.

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