Synergistic Influence of Abcb1 and Abcc2 on Disposition and Sterol Lowering Effects of Ezetimibe in Rats

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ABSTRACT: Pharmacokinetics of the sterol-lowering drug ezetimibe (EZ) is influenced by intestinal ABCB1 and ABCC2. This study in Lew.1W rats with "chemical" and genetic Abcb1 and Abcc2 deficiency was initiated to evaluate the individual contribution of both efflux carriers to the overall disposition and sterol-lowering effects of EZ. Disposition and sterol-lowering effects of EZ (5 mg/kg, 14 days) were measured in wild-type (WT) and Abcc2-deficient (Abcc2-) rats (N = 8 per group) and in animals treated with PSC833 (20 mg/kg) to generate "chemical" Abcb1-deficiency (Abcb1-, Abcb1-/Abcc2-). EZ serum levels decreased in the order WT (3.11 ± 1.09 ng/mL), Abcb1- (1.94 ± 1.10 ng/mL), Abcc2- (1.42 ± 0.42 ng/mL, $p = 0.003$ vs. WT), Abcb1-/Abcc2- (1.17 ± 0.53 ng/mL, $p = 0.002$ vs. WT) whereas the serum EZ glucuronide levels increased as follows: WT (23.2 ± 24.6 ng/mL), Abcb1- (119 ± 74.5 ng/mL, $p = 0.002$ vs. WT), Abcc2- (195 ± 76.5 ng/mL, $p < 0.001$ vs. WT), Abcb1-/Abcc2- (676 ± 207 ng/mL, $p < 0.001$ vs. WT, Abcb1- and Abcc2-). Abcb1 and Abcc2 protein deficiency resulted synergistically in lower fecal but increased renal excretion of total EZ although to a much lower extent. The sterol-lowering effects of EZ were significantly correlated to serum levels of EZ. In conclusion, Abcb1 and Abcc2 deficiency leads to lower levels of the active EZ and in turn to decreased sterol-lowering effects. © 2009 Wiley-Liss, Inc. and the American Pharmacists Association J Pharm Sci 99:422–429, 2010

Keywords: ezetimibe; disposition; ABCB1; ABCC2; MRP2; P-gp; transporter; PSC833

INTRODUCTION

Disposition and pharmacological effects of many drugs are markedly affected by the complex interplay between intestinal and hepatic metabolism and transport.[1–3] One characteristic example is the frequently prescribed cholesterol-lowering drug ezetimibe which modulates the uptake of nutritional and biliary cholesterol via the intestinal Niemann Pick C 1 like 1 (NPC1L1) transporter.[4] Major variables in disposition of ezetimibe in man are intestinal glucuronidation by enzymes of the UDP-glucuronosyltransferase (UGT) 1A family, intestinal efflux transport via ABCB1 (former name: P-glycoprotein) and ABCC2 (former name: MRP2) and hepatic uptake via organic anion transporting polypeptides (OATPs).[5–7] Coordinate interplay of intestinal and hepatic glucuronidation and efflux by ABCB1 and ABCC2 as well as hepatic OATP-mediated uptake are the rationale behind intensive entero-intestinal and entero-hepatic circulations and the long lasting cholesterol-lowering effects of ezetimibe. Accordingly, upregulation of ABCB1, ABCC2 and UGT1A1 results in markedly decreased serum concentrations of the active ezetimibe and nearly

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abolished its sterol-lowering effects.[6] On the contrary, modulation of intestinal efflux and hepatic uptake of ezetimibe and/or its glucuronide leads to significantly increased maximum serum levels of ezetimibe and accelerated the onset of the sterol-lowering effects.[8] Finally, deficiency of the hepatic glucuronide uptake as caused by polymorphisms of OATP1B1 is associated with lower systemic ezetimibe exposure.[7]

However, it is nearly impossible to distinguish the individual contribution of the pharmacokinetic variables to overall disposition and pharmacological effect of ezetimibe because selective inhibitors are not available for studies in man. Therefore, we recently evaluated the impact of ABCB1 and ABCC2 in experimental animal studies using congenital Abcc2-deficient rats and *Abcb1a/b(−/−)* knock-out mice.[9,10]

To overcome the species-related limitations of these studies and to distinguish the role of ABCB1 and ABCC2 in disposition of ezetimibe in one species, we amended our former study in Abcc2-deficient rats [9] by two study arms and treated both wild-type rats (WT) and Abcc2-deficient rats (Abcc2-) with the ABCB1 inhibitor PSC833 (valspodar), to generate animals with "chemically" induced Abcb1-deficiency (Abcb1-) and rats with "chemical" Abcb1-deficiency in addition to congenital Abcb2-deficiency (Abcb1-/Abcc2-). Using this experimental model, we will show in this paper, how ABCB1 and ABCC2 influence synergistically serum levels and sterol-lowering effects of ezetimibe.

MATERIALS AND METHODS

Animals

Male wild-type and Abcc2-deficient Lew.1W rats (250–350 g) were purchased from the Department of Pathophysiology (University of Greifswald, Germany) and hold 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 08.00 h a.m.). The animals had free access to acidified water and to a sterol enriched diet containing 16% fat, 1% cholesterol, 21% proteins and 0.5% sodium cholate (ssniff, Soest, Germany). The body weight at the time of sacrifice was 375–406 g (wild-type) and 328–411 g (Abcc2-deficient), respectively. The study was permitted by the Local Authorities of Mecklenburg/Pommerania, Germany.

Experimental Protocol

The animal experiment was performed in eight wild-type and eight Abcc2-deficient rats. After adaptation for 14 days, the animals were orally treated with 5 mg/kg ezetimibe and 20 mg/kg PSC833 for 14 days (administration volume 5 mL/kg). This dose of PSC833 was shown to be highly potent in terms of Abcb1 inhibition and was well tolerated in rats during chronic oral administration.[11,12] Ezetimibe was obtained from MSD Sharp & Dohme (Haar, Germany). PSC833 was kindly provided by Novartis (Basel, Switzerland). Feces were collected between the 9th and 14th treatment day and urine on the 14th treatment day and stored at least at −20°C until quantitative analysis. Twenty-four hours after last drug administration, the animals were anesthetized with diethyl ether. Then, blood was sampled from the retrobulbar plexus (about 3 mL) before the animals were sacrificed by cervical dislocation. The study was performed 1 year after our recently published study, which was performed under identical experimental conditions and which results were used for comparison.[9]

Quantitative Assays for Ezetimibe and Serum Sterols

Ezetimibe and its glucuronide in serum, urine and feces were determined by liquid chromatography-tandem mass spectrometry as described elsewhere by using a XTerra® MS column (C_{18}, 2.1 mm × 100 mm, particle size 3.5 μm, Waters, Milford, MA) and the MDS Sciex API 4000 mass spectrometer (Applied Biosystems, Darmstadt, Germany).[13] The validation ranges of ezetimibe were 0.25–500 ng/mL for serum, 0.001–2.5 μg/mL for urine and 0.005–10 μg/mL for feces. The coefficients of variations for within-day and between-day accuracy and precision of the ezetimibe assay in serum, urine and feces were lower than 8% of the respective nominal values and mean values, respectively. Serum concentrations of total cholesterol were determined by gas chromatography with flame ionization detection. Lathosterol and the plant sterols campesterol and sitosterol were analyzed by gas chromatography–mass spectrometry (GC–MS) as described.[14] The limit of quantification for cholesterol was 10 μg/mL and 0.05 μg/mL for campesterol, sitosterol and lathosterol. The within-day and between-day coefficients of variation for all sterols were below 10% of the respective means (precision).

Pharmacokinetic and Statistical Evaluation

Study data are presented as arithmetic means ± SD. The Mann–Whitney *U*-test was used for evaluation of sample differences and Spearman's rank test for correlations between samples (SPSS, version 15, Chicago, IL).

RESULTS

In rats with "chemical" Abcb1-deficiency, ezetimibe trough serum concentrations 24 h after last administration on the 14th treatment day were numerically somewhat lower (1.94 ± 1.10 ng/mL vs. 3.11 ± 1.09 ng/mL, $p = 0.093$), whereas the glucuronide levels were about fivefold increased (119 ± 74.5 ng/mL vs. 23.2 ± 24.6 ng/mL, $p < 0.001$) compared to wild-type animals. Congenital Abcc2-deficiency led to significantly lower serum levels of ezetimibe by about 50% (1.42 ± 0.42 ng/mL, $p = 0.003$) and to a more than an eightfold increase of the glucuronide levels (195 ± 76.5 ng/mL, $p < 0.001$). In animals with "chemical" Abcb1-deficiency in addition to congenital Abcc2-deficiency, the serum levels of ezetimibe were not significantly different from the levels in animals with congenital Abcc2-deficiency alone but significantly lower than in wild-type rats (1.17 ± 0.53 ng/mL, $p = 0.002$). The serum levels of the glucuronide (676 ± 207 ng/mL) in these animals were significantly increased ($p < 0.001$) compared to wild-type rats (about 30-fold) and to animals which were deficient for either Abcb1 (about 6-fold) and Abcc2 alone (about 3.5-fold).

Abcc2-deficiency (with and without Abcb1-deficiency) resulted in a marked decrease of the fecal excretion of total ezetimibe (ezetimibe plus glucuronide) by about 70%. In chemical Abcb1-deficiency, the fecal excretion was decreased by about 30% both in wild-type and Abcc2-deficient rats (Fig. 1). The percentage of total ezetimibe excreted via urine increased only from 0.04% in wild-type and Abcb1-, 0.58% in Abcc2- to 3.33% of the given oral dose in Abcb1-/Abcc2- rats. The cumulative amount of total ezetimibe, calculated from ezetimibe and ezetimibe glucuronide, excreted into urine and feces has been $110 \pm 34\%$ in wild-type rats, $56 \pm 9.4\%$ in Abcb1-, $29 \pm 19\%$ in Abcc2- and $23 \pm 2.6\%$ in Abcb1-/Abcc2- rats.

Treatment with ezetimibe for 14 days caused significantly lower serum concentrations of cholesterol (46%) and the plant sterols campesterol (49%) and sitosterol (49%) in wild-type rats (each $p = 0.018$) compared to the baseline values before treatment. "Chemical" Abcb1-deficiency was associated with a significantly reduced sitosterol-lowering effect (-71%, $p = 0.025$) of ezetimibe, whereas its effects on serum cholesterol and campesterol remained unchanged (Fig. 2). Abcc2-deficiency was associated with markedly reduced lowering effects on cholesterol (-56%, $p = 0.006$) and campesterol (-66%, $p = 0.021$). Deficiency of Abcb1 and Abcc2 resulted in a significant reversal of the sterol-lowering effects of ezetimibe for all measured sterols (cholesterol: -36%, $p = 0.021$; campesterol: -44%, $p = 0.037$; sitosterol: -80%, $p = 0.004$). Furthermore, we observed significant correlations between the serum concentrations of ezetimibe and the lowering effect on campesterol ($r = 0.606$, $p < 0.001$) and sitosterol ($r = 0.515$, $p = 0.004$) but not between the serum levels of the glucuronide and sterol-lowering (Fig. 3).

DISCUSSION

In this study was clearly shown that "chemical" deficiency of Abcb1 as induced by chronic treatment with PSC833 did not cause, as expected, increased serum concentrations of the parent ezetimibe. There was even a tendency for lower serum levels, also in Abcc2-deficient rats which were treated with PSC833. Congenital Abcc2-deficiency leads to significantly lower systemic ezetimibe exposure, whereas combined deficiency of Abcb1 and Abcc2 results in the lowest ezetimibe serum levels among all animal groups. In contrast to this, serum levels of ezetimibe glucuronide increased in the order WT < Abcb1- < Abcc2- < Abcb1-/Abcb2-. Ezetimibe was nearly completely eliminated via feces in wild-type rats. In animals with deficient function of the efflux carriers, the percentage of the dose excreted by the feces was markedly decreased, which was only negligibly compensated by increased urinary excretion. Thus, up to nearly 75% of the dose could not be balanced in double deficient animals.

For understanding this phenomenon, our conception on ezetimibe pharmacokinetics is presented briefly: *In vitro* studies in ABCB1 and ABCC2-containing inside-out vesicles pointed out that ezetimibe binds to ABCB1 and ABCC2 with a low affinity whereas the glucuronide is a high-affinity substrate of ABCC2 and has a moderate affinity to ABCB1.[6] Therefore, intestinal ABCB1 and ABCC2 that are both localized at the apical (luminal) membrane of enterocytes may influence oral absorption of ezetimibe and secretion of the

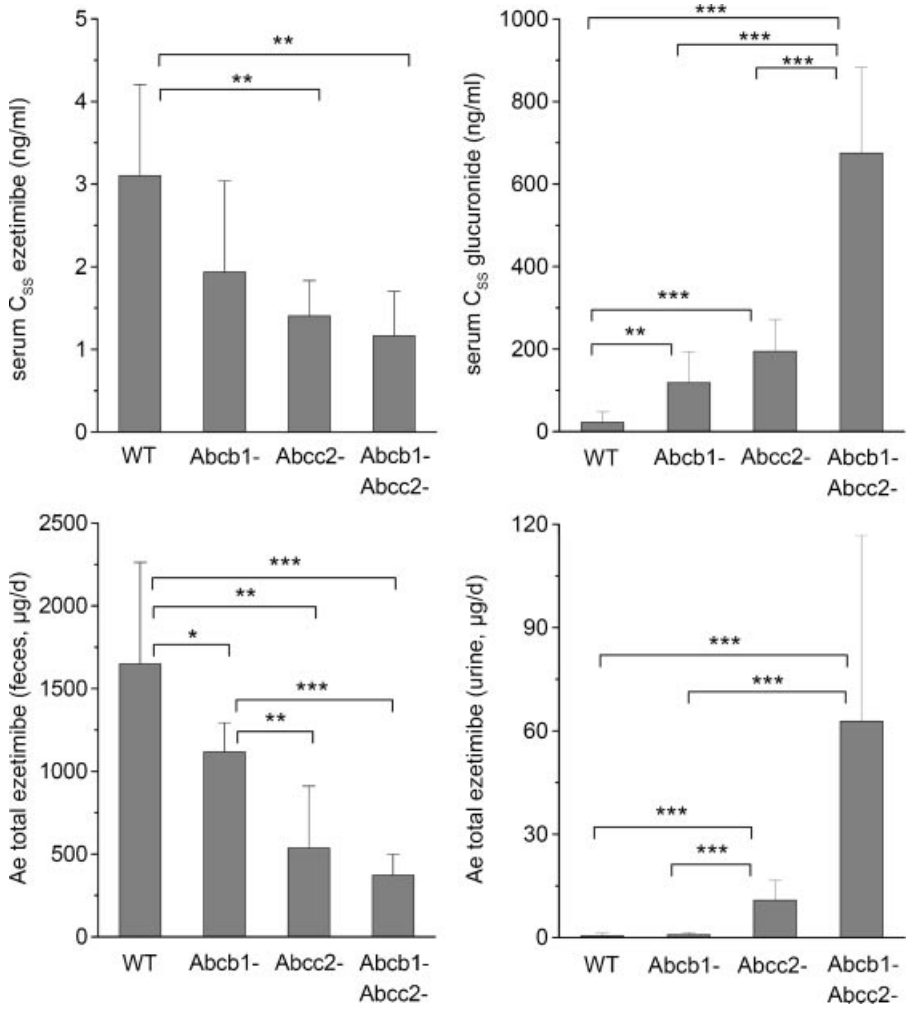

Figure 1. Serum trough concentrations (Css) of ezetimibe and its glucuronide and cumulative fecal and renal excretion of total ezetimibe (parent ezetimibe and ezetimibe glucuronide) after chronic treatment with ezetimibe (5 mg/kg, 14 days) in wild-type (WT) and congenital Abcc2-deficient Lew.1W rats (Abcc2-) and in wild-type and Abcc2-deficient rats which were co-medicated with PSC833 (20 mg/kg) to produce "chemical" Abcb1-deficiency (Abcb1-) and Abcb1-/Abcc2-, respectively. Columns and error bars indicate arithmetic means ± SD (each group, N = 8) and asterisks the significance levels $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ for differences (Mann-Whitney test).

glucuronide back to gut lumen. The highly lipid-soluble ezetimibe ($\log P$ 3.49, BCS class II compound) is rapidly absorbed in the proximal small intestine in dependence of the activity of ABCB1 and ABCC2. Within the enterocytes, ezetimibe undergoes nearly complete phenolic glucuronidation by intestinal UGT1A enzymes.[15] The drug appears in portal plasma nearly completely in its conjugated form.[16] From portal blood, the glucuronide is partially extracted into the liver by organ-specific uptake OATP carriers.[7] From enterocytes and hepatocytes, the glucuronide is secreted back to gut lumen predominantly by ABCC2 and to a lower extent by ABCB1. After bacterial hydrolysis of the glucuronide in proximal colon, the parent ezetimibe is reabsorbed and redistributed, amongst others to the receptor site of the NPC1L1 transporter in the cholesterol absorbing compartment along the small intestine. These entero-systemic and entero-hepatic circulations are considered to be the mechanism behind the long-lasting sterol-lowering effects of ezetimibe.[5,6] We assume that ezetimibe appears in the blood predominantly via these circulations of the glucuronide, because glucuronidation seems to be the rate-limiting process in the enterocytes.[6]

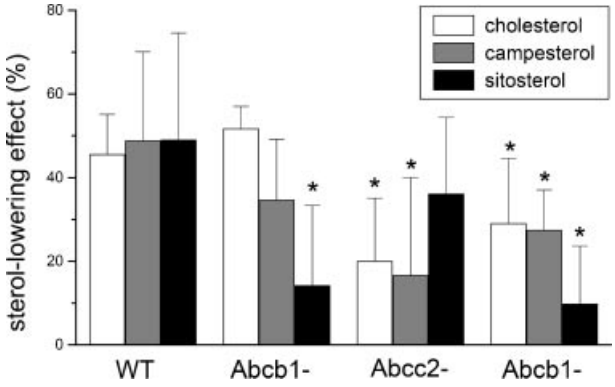

Figure 2. Sterol-lowering effects of ezetimibe after chronic treatment with ezetimibe (5 mg/kg, 14 days) in percentage of the pretreatment (baseline) serum concentrations of wild-type (WT) and congenital Abcc2-deficient Lew.1W rats (Abcc2-) and in wild-type and Abcc2-deficient rats which were co-medicated with PSC833 (20 mg/kg) to produce "chemical" Abcb1-deficiency (Abcb1-) and Abcb1-/Abcc2-, respectively. Columns and error bars indicate arithmetic means ± SD (each group, N = 8) and asterisks the significance level $^{*}p < 0.05$ for differences to wild-type animals (Mann-Whitney).

Deficiency of Abcc2 and/or of Abcb1 is consistently associated with lower intestinal secretion, lower recycling of the active ezetimibe and decreased sterol-lowering effect. The intestinal ABC efflux carriers exert obviously additive effects as shown in our study (Abcb1-/Abcc2- > Abcc2- > Abcb1-). The nonsecreted glucuronide fraction leads to higher serum concentrations and, in compensation, to an increased amount excreted via the kidneys at unchanged renal clearance (data not shown). This process drains the active ezetimibe from the recirculation system thus decreasing its serum concentrations and in turn its sterol-lowering effects.[8,9] These conclusions from the animal experiment agree well with data from drug interaction studies in man. If substrates and/or modulators of ABCB1 or ABCC2 are given concomitantly with ezetimibe, the glucuronide concentrations were also significantly elevated in human beings as shown for gemfibrozil, fenofibrate, cyclosporine, and single-dose rifampicin.[8,17–19]

On the other site, there exists probably an additional metabolism that competes with glucuronidation in the enterocytes. There is evidence, that significant amounts of a ketone (SCH 57871) and a lactone (SCH 59566) of ezetimibe are formed by oxidation and nonenzymatic rearrangement, respectively, at least in animals.[20] Additional metabolic transformation could be the reason why fecal excretion of total ezetimibe drops markedly down in rats with deficient efflux carriers without adequate increase of the urinary recovery rate (Abcb1- < Abcc2- < Abcb1/Abcc2). In these rats, an increasing dose fraction is obviously absorbed into enterocytes and saturates the UDP-glucuronosyltransferases. The residual ezetimibe may undergo an increased oxidative and nonenzymatic metabolism. In our model, genetic deficiency of Abcc2 had an about two times stronger influence on absorption and entero-systemic circulation of ezetimibe than chemical deficiency of Abcb1 as caused by PSC833.

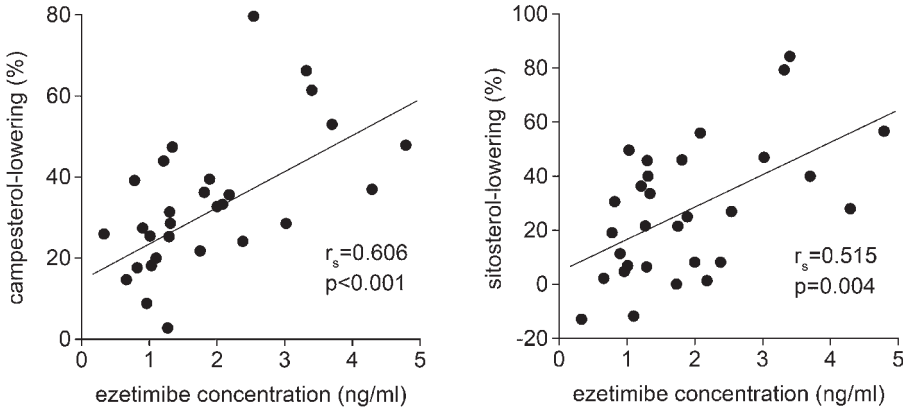

Figure 3. Correlations between the serum concentrations of ezetimibe and its plant sterol-lowering effects after chronic treatment with ezetimibe (5 mg/kg, 14 days) in wild-type and congenital Abcc2-deficient Lew.1W rats (Abcc2-) and in wild-type and Abcc2-deficient rats which were co-medicated with PSC833 (20 mg/kg) to produce "chemical" Abcb1-deficiency (Abcb1-) and Abcb1-/Abcc2-, respectively. *p*-values and correlation coefficients are given (Spearman-rank correlation).

Associated to this apparent metabolic shift in transporter deficient animals, one has to consider that several cytochrome P450 enzymes were shown to be markedly higher expressed in Abcc2-deficient rats than wild-type animals, which may lead to several-fold increased formation of oxidative metabolites as shown for a tobacco-specific carcinogen by Leslie et al.[21–23]

In well agreement to our conception, we found reduced sterol-lowering effects by inhibition of Abcb1, Abcc2 deficiency or the combination of both in parallel to decreased serum levels of ezetimibe. As expected, only the lowering effects of the plant sterols campesterol and sitosterol were positively correlated to the serum concentrations of ezetimibe because, in contrast to cholesterol, these sterols are exclusively of nutritional origin and represent suitable markers for intestinal sterol absorption.[24] The different influence of Abcb1 and Abcc2 on campesterol and sitosterol lowering by ezetimibe is not plausibly explained. It seems that PSC833 may have an own effect on sitosterol disposition in rats. However, final conclusions are not appropriate because of the high variability of all data and the descriptive intention of our study.

In this context, it appears confusing that induction of ABCB1 and ABCC2 in man by chronic pre-treatment with rifampicin leads to very similar effects as in our genetic or chemical knockout rats, that is, decreased serum levels of ezetimibe and reduced sterol-lowering effects. However, the mechanisms behind this phenomenon are quite different. Up-regulation of efflux transporter proteins caused markedly increased and accelerated fecal elimination of ezetimibe and its glucuronide by intestinal and hepatic ABCB1 and ABCC2. Consequently, serum levels of ezetimibe and its major metabolite were reduced at about 60%, which resulted in lower ezetimibe levels at its pharmacological target, namely the NPC1L1 sterol uptake protein, and in turn to markedly reduced sterol-lowering effects.[6]

We are aware of some limitations of our study: First, the influence of PSC833-induced "chemical" Abcb1-deficiency in Abcc2-deficient rats might be overestimated because Abcb1a, Abcb1b and Ugt1a1 are significantly higher expressed in Abcc2-deficient rats compared to wild-type rats.[9,25] Second, there are species differences in protein structure of human and rat ABCB1 (homology 77%) and ABCC2 (homology 79%) which must be considered for prediction of the situation in man. Third, PSC833 is a second generation inhibitor of ABCB1 with limited potency and some nonspecificity which might have influenced our results by weak inhibition of ABCC2 or OATPs.[26–28] We cannot fully exclude effects of PSC833 on Abcc2 and hepatic uptake transporters. Therefore, the role of Abcb1 in ezetimibe disposition might have been overestimated. However, the nonspecificity seems to be low because we observed in our recent study in *Abcb1a/b(−/−)* mice nearly the same pharmacokinetic changes than in PSC833 treated rats.[10]

Finally, it has to be considered that there are markedly differences in the expression and function of metabolizing enzymes and drug transporters between man and rats which may counteract final conclusions about the impact of ABCB1 and ABCC2 in the pharmacokinetics of ezetimibe in humans.[3,29] Nevertheless, due to the fact that there are no specific inhibitors of ABCB1 and ABCC2 available for use in humans, we believe that our experimental approach is appropriate to understand the complex interplay of drug metabolism and transport *in vivo*.

CONCLUSIONS

Deficiency of Abcb1 and Abcc2 in rats leads to higher serum concentrations of ezetimibe glucuronide, lower levels of the active ezetimibe and in turn to a decreased sterol-lowering effect.

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