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Title page

Disposition and sterol-lowering effect of ezetimibe in multidrug resistance associated protein (Mrp) 2-deficient rats

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Running title page

Running title: Ezetimibe disposition in TR-negative rats

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Abbreviations : Mrp, multidrug resistance associated protein; UGT, UDP-glucuronosyl transferase; Bsep, bile salt exporting pump; Mdr, multidrug resistance; Ntcp, sodium taurocholate transporting polypeptoide; Oatp, organic anion transporting polypeptide; RT-PCR, real time-polymerase chain reaction, NPC1L1, Niemann-Pick C 1 like 1; MDCK, Madine Darby canine kidney; FXR, farnesoid X receptor; PXR, pregnane X receptor; CAR, constitutive androstane receptor

Abstract

Disposition of the lipid lowering agent ezetimibe (EZ) and its glucuronide (GLUC) which is mainly formed by UDP-glucuronosyltransferase (UGT) 1A1 is influenced by the intestinal efflux transporters P-glycoprotein (P-gp) and multidrug resistance associated protein (MRP) 2. To evaluate the role of Mrp2 in overall disposition and pharmacodynamic effects of EZ, wild-type and Mrp2-deficient TR-negative Lew.1W rats (each 8 males) fed with a cholesterol-enriched diet were orally treated with 5 mg/kg EZ for 14 days. EZ and GLUC in serum, urine and feces and cholesterol, campesterol and sitosterol in serum were assayed using LC-MS/MS and LC-MS methods, respectively. Gene expression of *Bsep, Mdr1a, Mdr1b, Mrp2, Mrp3, Ntcp, Oatp1, 2, 4* and *Ugt1a1* was quantified in several tissues using real-time RT-PCR (*Taq*Man®). Mrp2-deficiency resulted in lower serum levels and fecal excretion of EZ (1.4±0.4 vs. 3.1±1.1 ng/ml; 115±48 vs. 361±102 µg/d, both $p<0.01$) whereas serum concentrations of GLUC were manifold increased compared to wild-type (196±76 vs. 23±25 ng/ml; $p<0.01$) associated with elevated renal excretion and decreased intestinal clearance (7.8±3.1 vs. 0.4±0.4 µg/d, $p<0.01$; 0.3±0.3 vs. 15±17 ml/min; $p<0.05$). The sterol-lowering effect of EZ was reduced in correlation to EZ serum levels (cholesterol $r=0.449$, $p=0.093$; campesterol $r=0.717$, $p=0.003$; sitosterol $r=0.507$, $p=0.054$) whereas GLUC was inversely correlated ($r=-0.743$, $p=0.002$; $r=-0.768$, $p=0.001$; $r=-0.634$, $p=0.011$). Disposition of EZ may have been additionally influenced by hepatic P-gp, Mrp3 and Ugt1a1 which were significantly higher expressed in Mrp2-deficient rats. Mrp2-deficiency in rats is associated with decreased sterol-lowering effect of ezetimibe obviously caused by lower intestinal clearance of the glucuronide and decreased entero-systemic and entero-hepatic recycling of the parent ezetimibe to the intestinal NPC1L1 sterol-uptake compartment.

Introduction

The efficacy of drug therapy depends on pharmacokinetic processes that provide pharmacological active concentrations in the vicinity of the respective pharmacological receptor(s) and on expression and function of the effectuation pathways. An example is cholesterol-lowering therapy with ezetimibe that selectively reduces intestinal sterol uptake by inhibition of the recently discovered sterol uptake transporter Niemann-Pick C 1 like 1 protein (NPC1L1) in the brush border of the jejunum and, to a lower extent, in the duodenum and the ileum (Altmann et al., 2004;Garcia-Calvo et al., 2005). Ezetimibe is widely used in patients with hypercholesterolemia and sitosterolemia. Additional benefit is expected from combinations with low dose statins (Gagne et al., 2002;Knopp et al., 2003;Salen et al., 2004). After oral administration, ezetimibe is rapidly absorbed from gut lumen and nearly completely conjugated by UDP-glucunonosyltransferases (UGT). It was shown that the parent drug is conjugated to a major phenolic glucuronide and only in traces to a benzylic and ketone glucuronide involving UGT1A1, 1A3 and 2B15 of which UGT1A1 was shown to be the most active isoform and whose activity is highest in jejunal sterol absorbing enterocytes (Patrick et al., 2002;Ghosal et al., 2004;Shelby et al., 2003;Tukey and Strassburg, 2001). The glucuronide undergoes intestinal and hepatic secretion thus initiating via colonic cleavage intensive enteral recirculation of ezetimibe back to the side of NPC1L1 action. Accordingly, fluctuation of the serum concentration-time curve is observed and the sterol-lowering effect is long lasting (Ezzet et al., 2001a;Patrick et al., 2002). Recent data from an in-vitro study with transfected MDCKII (Madine Darby canine kidney), LLC (Lewis lung carcinoma) and 2008 (human ovarian carcinoma) cells confirmed that ezetimibe glucuronide is a high-affinity substrate of the multidrug resistance associated protein (MRP2) 2 and has low affinity to P-glycoprotein (P-gp) whereas ezetimibe interacts moderately with

both P-gp and MRP2 (Oswald et al., 2006a). Therefore, we assume that pre-systemic elimination of ezetimibe and the glucuronide by intestinal and/or hepatic UGT1A1, P-gp and MRP2 is the major variable for the bioavailability of ezetimibe. Inhibition of P-gp and MRP2 by gemfibrozil, fenofibrate and cyclosporine leads consistently to elevation of ezetimibe serum concentrations (Bergman et al., 2005;Kosoglou et al., 2004;Reyderman et al., 2004). In contrary, up-regulation of intestinal UGT1A1, P-gp and MRP2 in healthy subjects by the PXR-ligand rifampicin is associated with markedly decreased serum concentrations and sterol-lowering effect of ezetimibe (Oswald et al., 2006a). However, the rate-limiting elimination pathway in overall disposition and lipid-lowering effect of ezetimibe could not be derived from the results of the so far published drug interaction studies because P-gp, MRP2 and UGT1A1 are obviously co-regulated in man by the nuclear receptors pregnane X receptor (PXR) and constitutive androstane receptor (CAR) (Tirona and Kim, 2005;Xu et al., 2005).

A suitable approach to differentiate between the role of P-gp, MRP2 und UGT1A1 in disposition and pharmacological effect of ezetimibe is the use of hereditary variant animals such as Mrp2-deficient rats. Such rats do not synthesize Mrp2 because of mutations in the *Abcc2* gene that creates premature termination codons (Keppler and Konig, 1997;Paulusma et al., 1996). However, in studies with Mrp2-deficient rats, complex changes in the expression of other multidrug transporters and drug metabolizing enzymes must be considered which may have additional influence on the disposition and pharmacological effect of ezetimibe (Hirohashi et al., 1998;Johnson et al., 2006;Kuroda et al., 2004;Newton et al., 2005;Ogawa et al., 2000). Overall, we hypothesized that Mrp2-deficiency should result (1) in a substantially reduced intestinal and hepatic secretion of ezetimibe and its glucuronide and, in turn (2) to a decreased enteral recirculation of the active ezetimibe to the

intestinal NPC1L1 receptor compartment and, (3) to a reduction of the sterol-lowering effect. The Mrp2-related differences might be (4) augmented by increased organ expression of *Mdr1, Mrp3* and *Ugt1a1*.

Materials and Methods

Chemicals and Reagents

Ezetimibe (Ezetrol®) was obtained from MSD, Haar, Germany. Acetonitrile and diethyl ether were purchased from Merck (Darmstadt, Germany) and methyl cellulose, cholesterol, campesterol and sitosterol from Sigma-Aldrich (Taufkirchen, Germany). All other reagents were obtained from commercial sources.

Animals

Disposition and sterol-lowering effect of ezetimibe was evaluated in eight male wild-type (236-328 g) and eight male congenic Mrp2-deficient, TR-negative Lew.1W rats (358-390 g) which were purchased from the Department of Pathophysiology of the University of Greifswald. The presence of the premature termination codon in the *Mrp2* gene and the absence of Mrp2 were confirmed by genotyping and Western blot analysis, respectively. 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 hours light-dark cycle with light on at 8 a.m., with one rat per polycarbonate cage, bedding (ssniff, Soest, Germany) and with free access to acidified water and to a sterol enriched diet containing 16.1 % fat, 1 % cholesterol and 20.7 % proteins (ssniff). The study was permitted by the Federal Authorities.

Experimental protocol

The study was performed in eight wild-type and eight Mrp2-deficient rats. After adaptation for 14 days, approximately 0.5 ml blood was obtained by puncture of the retrobulbar venous plexus in ether narcosis. After that, the animals were orally treated with 5 mg/kg ezetimibe for 14 days. The drug was suspended in 0.5 % methyl cellulose suspension and administered orally via a gavage (administration volume 5 ml/kg). To measure intestinal and renal excretion of ezetimibe and its glucuronide

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at steady-state, feces were collected between the 9th and 14th treatment day and urine on the 14th treatment day and stored at least at -80 °C until analysis. In the morning of the 15th study day (24 hours after last drug administration), animals were anaesthetized with diethyl ether for blood sampling from the retrobulbar plexus (about 0.5 ml) and for sacrifice by cervical dislocation and dissection. Aliquots of liver, kidney, duodenum, jejunum, ileum and colon were immediately frozen with liquid nitrogen to measure mRNA expression. The further storage of the samples until analysis was at least at -80 °C.

Quantification of gene expression

Approximately 20-30 mg of the respective frozen tissue were homogenized (Mikro-Dismembrator S, B. Braun, Melsungen, Germany) in the presence of a guanidine isothiocyanate containing buffer and total mRNA was isolated using the RNeasy® Mini Kit (Qiagen, Hilden, Germany) and assayed for content and purity using the spectrometry (Biophotometer, Eppendorf, Hamburg, Germany). Reverse transcription of 200 ng mRNA was performed with random hexamers and *Taq*Man Reverse Transcription Reagents (Applied Biosystems). *Abcb1a* mRNA and *Abcc2* mRNA expression was quantified by real-time reverse transcriptase-polymerase chain reaction according to the *Taq*Man® technology using the ABI Prism 7700 cycler, the *Taq*Man® Universal PCR MasterMix (Applied Biosystems). For *Abcb1a*, 5`-CCCATGGCCGGAACAGT was used as forward primer, 5`-ATGGGCTCCTGGGACACA-3' as reverse primer and 5`-FAM-TGGCTCCGCGCCCACCTGT TAMRA-3` as probe and for *Abcc2* 5`-TGTGGGCTTTGTT-CTGTCCA-3` as forward primer, 5`-CAGCCACAATGTTGGTCTCG-3` as reverse primer and 5`-FAM-CTCAATATCACACAAACCCTGAACTGGCTGT TAMRA-3` as probe (Molbiol, Berlin, Germany). To quantify mRNA expression of *Bsep, Mdr1b, Mrp3, Ntcp, Oatp1, Oatp2,*

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Oatp4, and *Ugt1a1*, *Taq*Man® Gene Expression Assays were used according to the recommendations of the manufacturer (Applied Biosystems). The reference gene for all quantifications was *18S* rRNA by use of the ΔΔCT (cycles of threshold) method (Livak and Schmittgen, 2001).

Western blot analysis

For protein preparation liver tissue (20 g) of wild-type and TR-negative rats was homogenized by 20 strokes (1.000 rpm) using the Potter S homogenizer (B. Braun, Melsungen, Germany). Thereafter, the homogenate was centrifuged at 100.000 x g for 30 min and the resulting membrane pellets were resuspended in Tris/HCL buffer (5mM, pH 7.4). Protein concentration of these crude membrane fractions were analysed by the BCA method.

For Western blot analysis 50 µg of each sample were loaded onto a 7.5 % sodium dodecylsulfate-polyacrylamid gel after incubation in Laemmli buffer at 95 °C for 10 min. After electrophoretic separation for 2 h at 180 V the immunoblotting on a nitrocellulose membrane (Schleicher & Schüll, Dassel, Germany) was performed using a tank blotting system (BioRad, Hercules, CA, USA). After a protein control staining with PonceauS, unspecific binding sites were blocked over-night at 4°C using a blocking solution containing 5 % milk powder and 5 % fetal calf serum in TBST (Tris buffered saline containing 0.05 % Tween 20). For primary antibody incubation membranes were washed with TBST and incubated with the respective antibody diluted in TBST containing 5 % bovine serum albumin. The detection of Mrp2 was performed using the EAG15 anti Mrp2 antibody (kindly provided by Dr. D. Keppler, Deutsches Krebsforschungszentrum Heidelberg, Germany; dilution: 1:5000), for P-gp with the C219 monoclonal mouse antibody (Alexis, Grünberg, Germany; dilution: 1:500), for Mrp3 with FDS anti-Mrp3 rabbit antiserum (kindly provided by Dr. D. Keppler, Deutsches Krebsforschungszentrum Heidelberg,

Germany; dilution: 1:1000) and for UGT1a a cross-reactive human UGT1A family antibody from BD Gentest (Heidelberg, Germany; dilution: 1:500) was used as recently described (Johnson et al., 2006). The secondary horse-radish peroxidase-conjugated goat anti-rabbit and anti-mouse antibodies (BioRad, Hercules, CA, USA) were used at a 1:2000 dilution. Finally, the immobilized antibodies were stained using an enhanced chemiluminescence (ECL) system (Amersham Biosciences, Freiburg, Germany) and exposed to X-ray films. For quantification the blots were scanned and the respective band-density was calculated using the Sigmagel software (Jandel Scientific, San Rafael, USA).

Quantitative assay for ezetimibe

Ezetimibe and its glucuronide in serum, urine and feces were determined using liquid chromatography-tandem mass spectrometry as recently described (Oswald et al., 2006b). Thereby ezetimibe glucuronide was quantified indirectly after hydrolysis using ß-glucuronidase. The validation ranges of ezetimibe were 0.5 – 500 ng/ml for serum, 5 – 1000 ng/ml for urine and 0.1-15 µg/ml for feces. Within-day and between-day accuracy and precision of calibration values and quality control were for serum lower than 10.7 % of the nominal values and means, respectively, and for urine and feces lower than 15 %.

Quantitative assay for cholesterol, campesterol and sitosterol

Serum concentrations of total cholesterol and the plant sterols campesterol and sitosterol were quantified by means of a validated LC-MS method with 4-hydroxychalcone as internal standard. In brief, 0.1 ml serum was diluted with 0.9 ml water. Of this, 0.2 ml was mixed with 25 µl internal standard solution (50 µg/ml) and incubated with 2 ml ethanol (96 %, v/v) and 0.4 ml potassium hydroxide (10 M) for 3 hours at room temperature (22-25 °C) to hydrolyze any sterol esters. After neutralization with formic acid, the samples were extracted with chloroform that was

evaporated to dryness under a gentle nitrogen stream at 60 °C. The residue was dissolved in acetonitrile/0.1% formic acid (90/10, v/v) for quantification using a liquid chromatography-mass spectrometry system consisting of the pump series 1100 (Hewlett Packard, Waldbronn, Germany), the autosampler series 200 (Perkin Elmer, Überlingen, Germany), the column thermostat L-5025 (Merck-Hitachi, Darmstadt, Germany) and the PE Sciex API 2000 mass spectrometer equipped with the Analyst 1.2 software (Applied Biosystems, Darmstadt, Germany). The chromatography was performed isocratically using acetonitrile/0.1% formic acid (90/10; v/v) as mobile phase (flow rate 200 µl/min) and the column XTerra® MS (C_8, 2.1 x 50 mm, particle size 3.5 µm, Waters, Milford, U.S.A.). The mass spectrometer was used with the Heated Nebulizer (APCI) interface in the positive ion mode. The following *m/z* values were monitored for quantification: 396.3 for cholesterol, 384.3 for campesterol, 397.3 for sitosterol and 225.1 for 4-hydroxychalcone. The method was validated for 10 - 500 µg/ml cholesterol and for 0.1-7.5 µg/ml campesterol and sitosterol. Within-day and between-day accuracy and precision for all sterols were within -7.8 % and 10 % of the nominal values and lower 14.3 % of the mean values, respectively.

Pharmacokinetic and statistical evaluation

Trough serum concentrations of ezetimibe and its glucuronide at steady-state (C_{ss}) were taken from the study data. Renal clearance (CL_R), metabolic clearance (CL_M) and intestinal clearance ($CL_{intestinal}$) were derived from the amounts (A_e) excreted into the urine and feces (average values from 9^{th} to 14^{th} study day) over the respective product of $C_{ss} \times 24$ hours (represents approximately $AUC_{0\text{-}24h}$) of ezetimibe and the glucuronide, respectively. Samples are presented as arithmetic means ± SD or medians and 75 % percentiles. Mann-Whitney, Wilcoxon, Jonckheere-Terpstra and Spearman´s rank test were used for statistical analysis as appropriate.

Results

Gene and protein expression in Mrp2-deficient rats

Mrp2 mRNA as well as protein was not expressed in organs of Mrp2-deficient rats (Figure 1, Figure 2). In wild-type animals, *Mrp2* mRNA levels were highest in the liver. Compared to liver, intestinal expression was markedly lower with a minimum in the colon. Regional differences in intestinal mRNA expression were also observed for *Mdr1a* and *Ugt1a1* in wild-type as well as TR-negative animals. *Mdr1a* mRNA was expressed with significantly increasing levels along the small intestine. Maximum levels were found in the ileum. In contrast, *Ugt1a1* mRNA content was highest in duodenum and decreased significantly towards distal parts of the gut. In the colon, *Mdr1a* and *Ugt1a1* were expressed in significantly lower extent. *Mrp3* mRNA was well expressed in all parts of the intestine with highest levels in the colon but failed to reach level of significance caused by the huge standard deviations. Wild-type and Mrp2-deficient rats were not different in intestinal mRNA content of *Mdr1a*, *Mrp3* and *Ugt1a1* with exception of *Mdr1a* in the ileum. In the liver of Mrp2-deficient rats, however, *Mdr1a, Mdr1b*, *Mrp3* and *Ugt1a1* were significantly higher expressed that in wild-type animals (Figure 1). Expression of the hepatic uptake transporters *Oatp1, Oatp2, Oatp4*, *Ntcp* and the major bile salt elimination pump *Bsep* was not changed in the Mrp2-deficient rats (Table 1).

Furthermore Western blot analysis revealed significant higher protein levels of Mrp3 (5.1-fold, p=0.02) and Ugt1a (4.2-fold, p=0.02) while P-gp content was only numeric increased (1.3-fold, p=0.24) in Mrp2-deficient animals (Figure 2).

Disposition of ezetimibe

Mrp2 deficiency resulted in an about 8.5-fold increase of the glucuronide serum concentrations but in markedly lower levels of unchanged ezetimibe (Table 2). This was associated with a manifold higher renal but lower fecal excretion of the

glucuronide and a significantly elevated metabolic clearance of ezetimibe into the urine. The amount of glucuronide in feces was significantly lower in *Mrp2*-deficient rats whereas the amount excreted into urine was manifold higher. Renal clearance and urinary excretion of ezetimibe were also markedly increased in TR^- animals (Table 2).

Sterol lowering effect

Mrp2-deficiency was associated with significantly lower baseline serum concentrations of cholesterol (119 mg/dl vs. 177 mg/dl, $p<0.05$) and the plant sterols campesterol and sitosterol (39.8 mg/dl vs. 51.2 mg/dl and 10.4 vs. 12.7 mg/dl, both $p<0.05$). Treatment of wild-type rats with ezetimibe resulted in significant lowering of the mean cholesterol and plant sterol serum concentrations by about 40 – 50 %. In Mrp2-deficient rats, the sterol-lowering effect of ezetimibe was markedly less expressed and reached for cholesterol even not the level of statistical significance (Figure 3). Furthermore, the effect of ezetimibe on sterol absorption was correlated to serum concentrations of ezetimibe (significant only according campesterol) but significant inversely correlated to the serum concentration of the glucuronide (Figure 4).

Discussion

This study in Mrp2-deficient rats clearly demonstrates that the activity of the multidrug transporter Mrp2 is a major determinant for the disposition and sterol-lowering effect of ezetimibe. Mrp2 is co-localized with P-gp to the luminal membrane of the villous enterocytes, the canalicular membrane of hepatocytes, the proximal tubular cells in the kidney and the luminal surface of blood capillaries (Chan et al., 2004;Takano et al., 2006). Mrp2 serves as an export pump for a wide range of conjugated and unconjugated anionic compounds and shares some degree of overlapping substrate specificity with P-gp (Keppler and Konig, 1997;Keppler and Konig, 2000). Ezetimibe has low affinity to MRP2 whereas the glucuronide is a high-affinity substrate (Oswald et al., 2006a). After oral administration ezetimibe is intensively conjugated by intestinal and/or hepatic UGTs resulting to a major phenolic glucuronide which undergoes intestinal and/or hepatic secretion followed by bacterial hydrolysis in the colon and reuptake of the parent ezetimibe into systemic blood (van Heek et al., 2000;Ghosal et al., 2004;Patrick et al., 2002). The mechanism behind intestinal secretion of the glucuronide seems to be MRP2-mediated efflux as up-regulation of intestinal MRP2 by chronic treatment with the PXR-ligand rifampicin leads to a markedly increased intestinal clearance and a reduction of glucuronide serum concentrations for about 70% (Oswald et al., 2006a). In this study, the absence of intestinal and hepatic Mrp2 in our TR-negative rats resulted in manifold increased serum glucuronide concentrations, a significantly increase of urinary excretion at unchanged renal clearance and a nearly abolished intestinal clearance. From this pattern of pharmacokinetic changes, we conclude that Mrp2 is the major pathway for intestinal and hepatic secretion of the glucuronide. Furthermore, it seems very likely that that the glucuronide is the source for the long-lasting circulation of ezetimibe via hepatic and intestinal secretion of the glucuronide, bacterial hydrolysis

in the colon and re-absorption of the parent drug into the systemic circulation. This recycling scheme yields in man characteristic multiple serum concentration peaks that are to our interpretation amplified by gastro-ileo-cecal reflex that results in bolus propulsion of chyme from the terminal ileum to the hydrolytic environment of the colon after meals (Schiller et al., 2005). Furthermore food-triggered emptying of the gall bladder most likely enforces this effect by delivering glucuronized drug to the upper intestine.

With respect to P-gp and Mrp2 the physiological preconditions for colonic absorption of the parent drug ezetimibe are well comparable for wild-type and Mrp2-deficients rats. Ezetimibe is a substrate of P-gp and less of Mrp2 but P-gp is low expressed in the colon of wild-type and TR^- rats; Mrp2 is low expressed in wild-type rats and missing in TR^- rats. We assume that the ezetimibe permeability across the mucosa of the colon is much better than in distal parts of the small intestine in which P-gp and Mrp2 are highest expressed. Furthermore, pre-systemic glucuronidation in the colon is low because Ugt1a1, for which ezetimibe is a high affine substrate, is lowest expressed in colonic enterocytes (Tukey and Strassburg, 2001;Ghosal et al., 2004). Therefore, the reduced serum concentrations of ezetimibe assessed in Mrp2-deficient animals may the consequence of diminished glucuronide secretion in the intestine and increased biliary ezetimibe secretion by P-gp which was found to be slightly up-regulated in our congenic rats. Interestingly, the renal clearance of parent ezetimibe was markedly increased in TR-negative rats. This can not be addressed to glucuronide cleavage during the 24 hours lasting urine collection period because corresponding stability tests revealed that only a negligible amount (0.9 %) of the glucuronide was degraded to free ezetimibe. Due to this, the Mrp2-deficient rats seem to exhibit an enhancement of other renal elimination pathways because *Mdr1* was not found to be induced (data not shown) in accordance to findings of Johnson

et al. (Johnson et al., 2006). Alternatively, differences in expression of other renal drug transporters like organic anion transporters (OAT), organic cation transporters (OCT) or Mrp4 which was shown to be markedly higher expressed in the kidney of Mrp2-deficient rats may contribute to this phenomenon (Chen et al., 2005).

The regioselective expression of *Mdr1, Mrp2, Mrp3* and *Ugt1a1* along the rat intestine observed in our study is in well accordance with published data (Brady et al., 2002;Dietrich et al., 2003;Ho et al., 2003;Rost et al., 2002;Tukey and Strassburg, 2001). One might speculate, that a high colonic expression of the basolateral efflux transporter Mrp3 may contribute to the re-absorption of ezetimibe because it shares a considerable overlap in substrate specificity with P-gp and Mrp2 (Chan et al., 2004). However, the systemic concentration of ezetimibe in blood significantly predicts the extent of the sterol-lowering effect which results from inhibition of NPC1L1 in the small intestinal epithelium (Ezzet et al., 2001b). Thereby, glucuronide exposure is inversely correlated to the pharmacological effect. That means according to our hypothesis that the decrease or the absence of intestinal (and hepatic) “first-pass” secretion of the glucuronide caused by Mrp2-deficiency reduces or even interrupts recycling of the active compound ezetimibe to the NPC1L1 receptor compartment. In the Mrp2-defcient rats the non-secreted portion of the glucuronide is now available in the systemic circulation yielding very high serum concentrations that cause a significantly increased amount to be excreted into urine by renal filtration. Renal clearance is consequently not influenced as shown by our data.

The magnitude of changes in ezetimibe disposition caused by the absence of Mrp2 may have been augmented by particularities in the expression of hepatic drug transport and metabolism in Mrp2-deficient rats. In our TR-negative rats, the expression of hepatic mRNA of *Mdr1a, Mdr1b, Mrp3* and *Ugt1a1* as well as protein content of Mrp3 and Ugt1a was significantly increased, probably by adaptation of

liver cells to deficient Mrp2-mediated canalicular export. Similar up-regulation of Mrp3 and Ugt1a was recently shown in GY/TR$^-$ Wistar rats (Johnson et al., 2006). A compensatory up-regulation of Mrp3 was also observed in patients with the Dubin-Johnson syndrome (Konig et al., 1999). Mrp3 serves in hepatocytes as a basolateral efflux transporter which activity is enhanced during pathophysiological situations of hepatic elimination like cholestasis leading to a boosted urinary detoxification pathway (Chan et al., 2004;Keppler and Konig, 2000). Increased activity of hepatic Ugt1a1 may have additionally lowered ezetimibe availability in blood but may have increased exposure with the glucuronide synergistically to the effects of Mrp2-deficiency and Mrp3 over-expression. Since there was no specific Ugt1a1 rat antibody available we used for our Western blot analysis a cross-reactive human UGT1A family antibody and could show a markedly higher expression of the according isoenzyme family.

The molecular mechanism behind P-gp, Mrp3 and Ugt1a1 up-regulation in Mrp2-deficient animals may result from induction of the nuclear farnesoid X receptor (FXR), pregnane X receptor (PXR) and constitutive androstane receptor (CAR) by bile acids which are elevated in Mrp2 deficient animals and known inducers of the mentioned nuclear receptors (Guo et al., 2003;Takikawa et al., 1991;Xie et al., 2001).

In conclusion Mrp2-deficiency in rats is associated with a decreased sterol-lowering effect of ezetimibe that is most probably caused by a reduced intestinal clearance of the glucuronide and a decreased entero-systemic recycling of the parent drug ezetimibe to the intestinal NPC1L1 sterol-uptake transporter compartment.

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Footnotes

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Legends for Figures

Figure 1: Expression of *Mdr1a, Mdr1b, Mrp2, Mrp3* and *Ugt1a1* mRNA relative to *18S* rRNA in liver and intestine of 8 wild-type (white columns) and 8 Mrp2-deficient TR-negative Lew.1W rats (grey columns). Columns and bars indicate medians ± 75%-quartile (*p<0.05, **p<0.01 relative to wild-type, Mann-Whitney test; †p<0.05 Jonckheere-Terpstra trend test for wild-type and ‡p<0.05 for Mrp2-deficient animals).

Figure 2: Western blots of Mrp2, Mrp3, P-gp and Ugt1a of liver homogenates from wild-type (WT) and Mrp2-deficient (TR-) animals. Blots were prepared as described under *Materials and Methods* in each case from four separate animals. Approximate molecular weights (MW) are shown.

Figure 3: Sterol-lowering effects of ezetimibe after oral administration of 5 mg/kg for 14 days in percent of the pre-treatment (baseline) serum concentrations of wild-type (white columns) and Mrp2-deficient TR-negative Lew.1W rats (grey columns). Columns and bars indicate arithmetic means ± SD (*p<0.05 compared to baseline, Wilcoxon test; ††p<0.01, compared to wild-type, Mann-Whitney test).

Figure 4: Correlations between trough serum concentrations of ezetimibe (left figures) and ezetimibe glucuronide (right figures) and sterol-lowering effects after oral administration of 5 mg/kg ezetimibe for 14 days in percent of the pre-treatment (baseline) levels. Open circles indicate

data from wild-type and solid circles from Mrp2-deficient TR-negative Lew.1W rats. Spearman rank correlations (r) and significance levels (p) are given.

Tables

Table 1: Hepatic mRNA expression of *organic anion transporting peptide* (O*atp*) *1*, O*atp2*, *Oatp4* and of *bile salt exporting pump* (*Bsep*) and *sodium taurocholate co-transporting polypeptide* (*Ntcp*) relative to *18S* rRNA of 8 wild-type and 8 Mrp2-deficient TR-negative Lew.1W rats. Arithmetic means ± SD are given.

	Oatp1	*Oatp2*	*Oatp4*	*Bsep*	*Ntcp*
wild type	1.05 ± 0.37	1.10 ± 0.45	1.04 ± 0.32	1.04 ± 0.31	1.04 ± 0.30
TR^--rats	0.87 ± 0.22	1.47 ± 0.42	0.80 ± 0.25	0.75 ± 0.26	0.93 ± 0.39

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Table 2: Pharmacokinetic characteristics of ezetimibe in wild-type and Mrp2-deficient TR-negative Lew.1W rats (each n=8) after oral administration of 5 mg/kg ezetimibe for 14 days. Arithmetic means ± SD are given (*p<0.05, **p<0.01, relative to wild-type, Mann-Whitney test).

		ezetimibe		ezetimibe glucuronide	
		wild type	TR^--rats	wild type	TR^--rats
AUC_{0-24}	(ng*h/ml)	74.6 ± 26.3	34.1 ± 10.1**	556 ± 591	4691 ± 1835**
CL_F	(ml/min)	97.4 ± 64.4	61.8 ± 37.0	15.0 ± 17.5	0.26 ± 0.34**
CL_R	(ml/min)	0.06 ± 0.04	1.41 ± 1.63**	0.02 ± 0.01	0.03 ± 0.03
$CL_{M(F)}$	(ml/min)	52.7 ± 52.9	29.7 ± 39.0	-	-
$CL_{M(R)}$	(ml/min)	0.08 ± 0.07	4.24 ± 2.77**	-	-
Ae_{feces}	(µg/d)	361 ± 102	115 ± 48.4**	189 ± 141	64.6 ± 82.6*
Ae_{urine}	(µg/d)	0.26 ± 0.22	3.10 ± 4.06*	0.38 ± 0.41	7.84 ± 3.05**

Figure 1

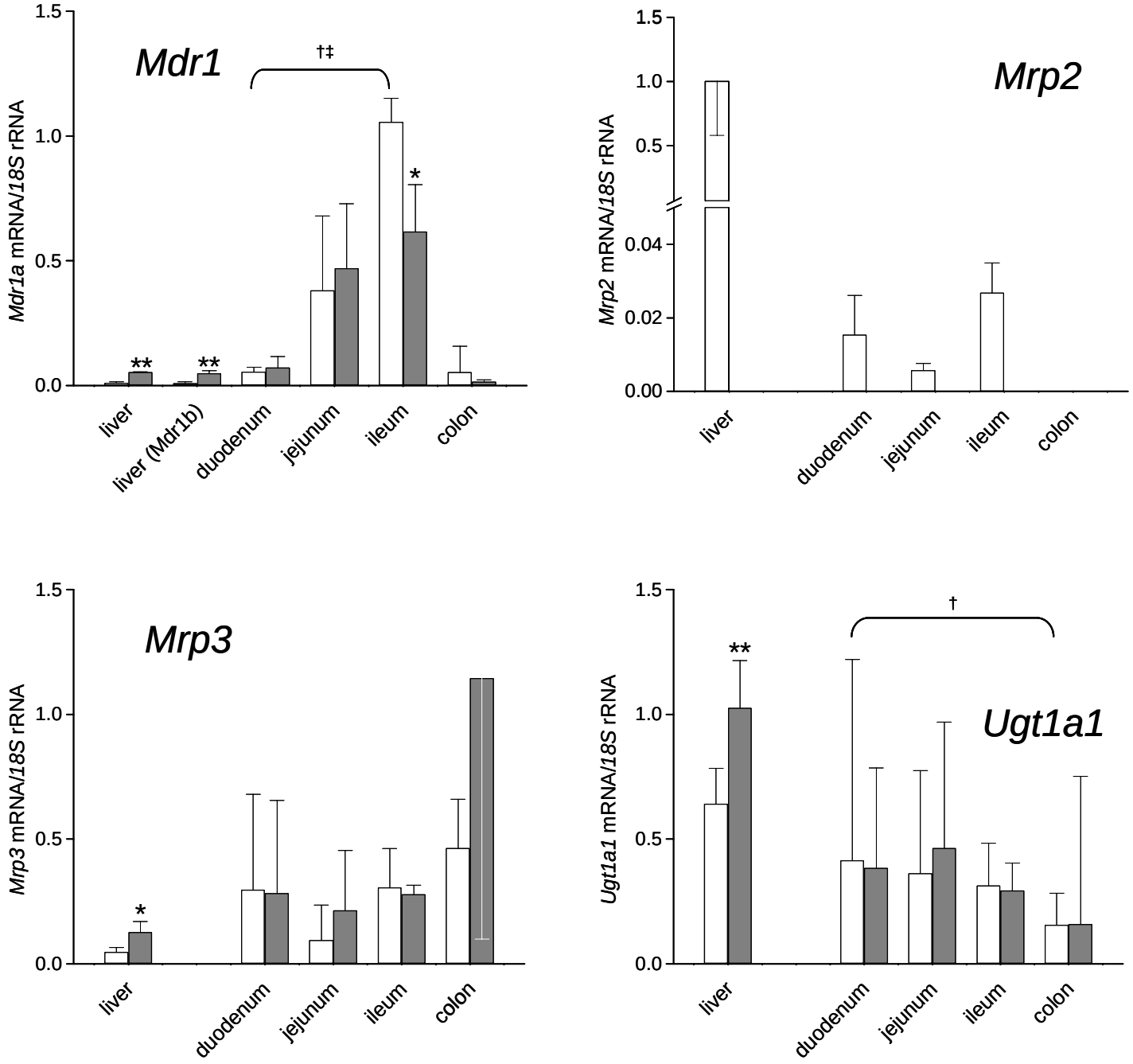

Figure 2

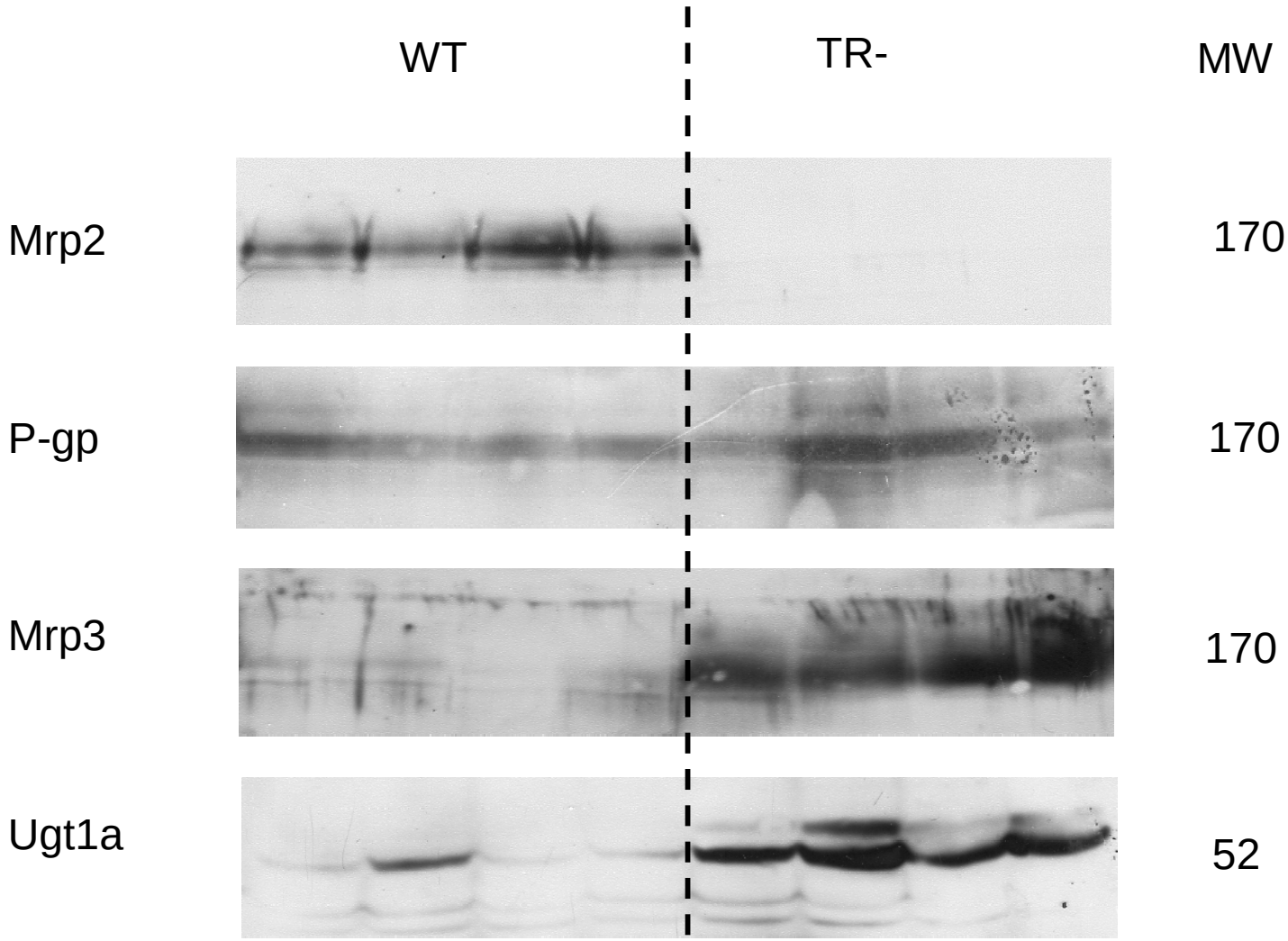

Figure 3

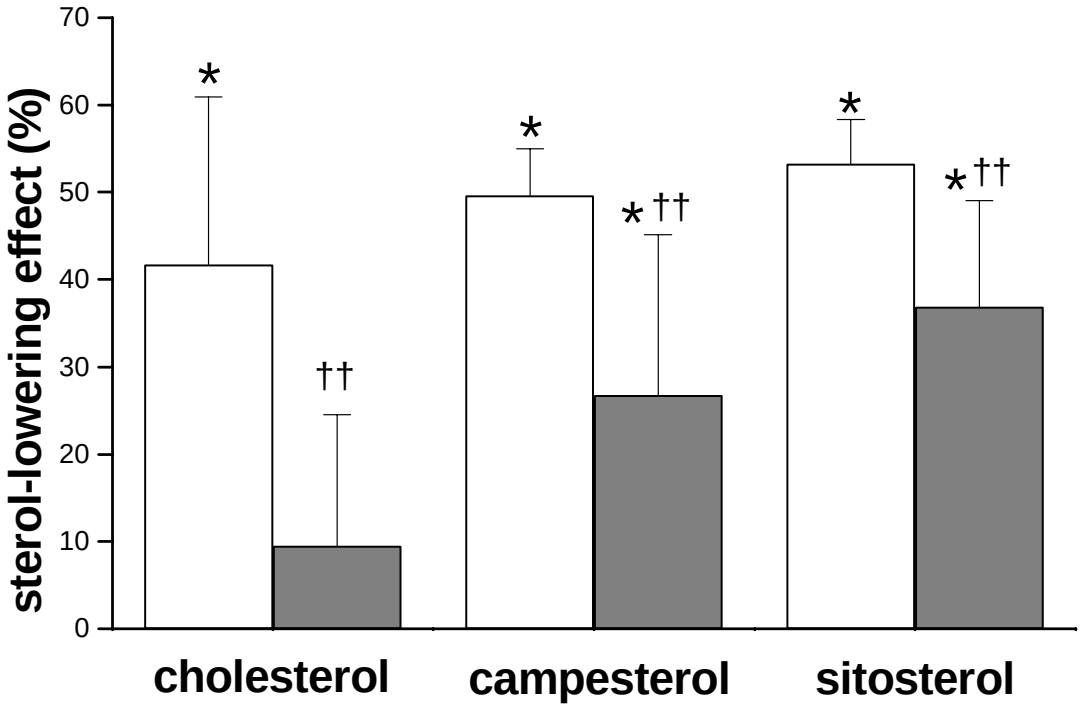

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Figure 4