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SHORT COMMUNICATION

Methotrexate pharmacokinetics in transgenic mice with liver-specific expression of human OATP1B1 (SLCO1B1)

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Running title:

Methotrexate transport by human OATP1B1 *in vivo*

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Abbreviations used within manuscript: OATP1B1, organic anion transporting polypeptide 1B1; MTX, methotrexate; ApoE, apolipoprotein E; kb, kilobase(s); HCR-1, hepatic control region-1; PCR, polymerase chain reaction; RT-PCR, real-time PCR; AUC, area under the plasma versus time curve; MRP2, multidrug resistance protein 2

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ABSTRACT

Human OATP1B1 is an important hepatic uptake transporter that can transport a wide variety of drugs. In the present study, we have generated and characterized a transgenic mouse model with specific and functional expression of human OATP1B1 (SLCO1B1) in the liver. Immunohistochemical staining revealed basolateral localization of transgenic OATP1B1 in the liver, whereas no expression of OATP1B1 was found in the kidney and small intestine. Using this transgenic model, the *in vivo* role of human OATP1B1 in the disposition of the anticancer drug methotrexate (MTX) was studied. In mice on a semi-synthetic diet, the area under the plasma concentration-time curve (AUC) for intravenous methotrexate in SLCO1B1 transgenic mice was 1.5-fold decreased compared to wild-type mice. Furthermore, the amount of MTX in the liver was markedly higher (~2-fold) in the SLCO1B1 transgenic mice compared to wild-type mice, resulting in 2- to 4-fold higher liver-plasma ratios of MTX. Some murine liver Slco genes were markedly downregulated on the semisynthetic diet compared to a standard diet, which likely reduced murine Oatp-mediated MTX uptake in the liver and therefore facilitated detection of the function of the transgenic OATP1B1. Collectively, these data demonstrate a marked and possibly rate-limiting role for human OATP1B1 in MTX elimination *in vivo*. Variation in OATP1B1 activity due to genetic polymorphisms, drug-drug interactions and possibly dietary conditions may therefore play a role in the severity of MTX-related toxicity. SLCO1B1 transgenic mice will provide a useful tool to study the *in vivo* role of human OATP1B1 in drug pharmacokinetics.

INTRODUCTION

Uptake transporters belonging to the superfamily of organic anion transporting polypeptides (rodents: Oatp/Slco, human: OATP/SLCO) are nowadays recognized as important transmembrane proteins that can have a profound impact on the systemic pharmacokinetics, tissue distribution and elimination of a wide range of drugs (König et al., 2006). Since the discovery of the first Oatp (rat Oatp1a1) in 1994 (Jacquemin et al., 1994), considerable effort has been put into the discovery of new substrates for Oatps/OATPs. However, most of these studies are performed *in vitro* and models to investigate the role of Oatps/OATPs *in vivo* are limited. The aim of this study was therefore to obtain a useful model to study human OATP1B1 *in vivo*, by generating SLCO1B1 transgenic mice.

Human OATP1B1 (previously called OATP-C, LST-1 or OATP2; gene name: SLCO1B1) is highly expressed at the basolateral (sinusoidal) plasma membrane of hepatocytes and could play a key role in the uptake of compounds into the human liver (Abe et al., 1999; König et al., 2000; Tamai et al., 2000). Recently, however, SLCO1B1 mRNA was also detected in human enterocytes (Glaeser et al., 2007). OATP1B1 has a broad substrate specificity and appears to be involved in the transport of bile salts, bromosulphthalein, steroid conjugates, the thyroid hormones T3 and T4 and drugs like benzylpenicillin, rifampicin, pravastatin, pitavastatin, rosuvastatin, fexofenadine and methotrexate (König et al., 2006; Matsushima et al., 2008). The importance of OATP1B1 in the therapeutic efficacy and toxic side effects of substrate drugs has been confirmed by several studies focusing on genetic polymorphisms in SLCO1B1. For example, a commonly occurring haplotype, SLCO1B1*15, containing SNPs A388G and T521C, has been associated with a strongly reduced transport functionality, markedly increased plasma levels (2-fold), and drastically reduced non-renal clearance of the drugs pravastatin and pitavastatin in Japanese, Korean and Caucasian populations (Chung et al., 2005; Niemi et al., 2004; Nishizato et al., 2003; Nozawa et al., 2002; Ho et al., 2007).

Methotrexate (MTX), a folate antimetabolite and a bicarboxylic organic anion, is widely used for the treatment of various types of cancer (i.e. breast cancer, head and neck cancer, lung cancer and non-Hodgkin's lymphoma). It is also used to treat non-malignant diseases, including psoriasis and rheumatoid

arthritis (van Outryve et al., 2002; Wessels et al., 2008). Two independent studies show that OATP1B1 is able to transport MTX *in vitro*, suggesting the importance of a basolateral uptake transporter in MTX pharmacokinetics (Abe et al., 2001; Sasaki et al., 2004).

In this study, we have generated and characterized a transgenic mouse model which shows substantial and functional expression of human OATP1B1 specifically in the liver. Using this transgenic model, the role of human OATP1B1 in MTX disposition *in vivo* was studied. Our results indicate that, *in vivo*, OATP1B1 can be a rate-limiting factor in the clearance of MTX, illustrating the potential use of this model in assessing drug pharmacokinetics.

MATERIALS AND METHODS

Animals: Mice were housed and handled according to institutional guidelines complying with Dutch legislation. The animals used in this study were male SLCO1B1 transgenic and wild-type mice of identical genetic background (FVB) between 9 and 14 weeks of age. Animals were kept in a temperature controlled environment with a 12-h light/12-hour dark cycle. Mice received a standard diet (AM-II; Hope Farms, Woerden, The Netherlands) and acidified water ad libitum. Three weeks prior to specified experiments, mice were fed with a semi-synthetic diet (Reference diet 20% casein, 4068.02; Hope Farms, Woerden, The Netherlands).

Chemicals and reagents: Methotrexate (100 mg/ml, Emthexate PF) was obtained from Pharmachemie (Haarlem, The Netherlands). Methoxyflurane (Metofane) was from Medical Develeopments Australia (Springvale, Victoria, Australia) and heparin (5,000 IE/ml) was from Leo Pharma BV (Breda, The Netherlands). Bovine serum albumin, Fraction V, was from Roche (Mannheim, Germany). Drug-free human plasma was obtained from healthy volunteers. The polyclonal antibody against human OATP1B1 (ESL clone) was a kind gift of Prof. Dr. D. Keppler (Deutsches Krebsforschungszentrum, Heidelberg, Germany) (König et al., 2000).

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Transgene construction: To achieve liver specific expression of human OATP1B1 we generated a transgene construct as follows (figure 1A). pLIV-LE6 (kindly provided by Dr. J. Taylor, Gladstone Institute, University of California, San Francisco, CA) (Simonet et al., 1993) was completely digested with ClaI followed by a partial digestion with Asp718. The 7.6 kb fragment of the vector was isolated and used for ligation with human OATP1B1 cDNA later on. PCR was used to generate the SLCO1B1 construct with ClaI and Asp718 recognition sites at the 5' and 3' ends respectively. The template, human SLCO1B1 cDNA in pBluescript SK-, was a kind gift of Dr. T. Abe (University Graduate School of Medical Science, Sendai, Japan). This cDNA represents the SLCO1B1*1a polymorphic variant, which is considered to be the wild-type variant (Iwai et al., 2004). The PCR product was digested with ClaI and Asp718 and ligated into the dephosphorylated 7.6 kb pLIV-LE6 fragment, yielding pLIV-LE6-SLCO1B1. Plasmid DNA was linearized and irrelevant plasmid DNA was removed by restriction digestion with SalI and NgOM IV, followed by pronuclear injection into fertilized oocytes of FVB mice. Two-cell stage embryos were implanted into oviducts of pseudopregnant F1 fosters and carried to term.

PCR and Southern blot analysis: Transgenic founder lines were initially detected by PCR screen with forward 5'-GAAGGCTAACCTGGGGTGAG-3' and reverse 5'-GGCAAAGCAGTCAAAACACC-3' primers located within the ApoE intron 1 and SLCO1B1 cDNA, respectively, to yield a 450-bp fragment (figure 1A). Southern blot analysis was used for the definitive identification of transgenic founders, as well as for the differentiation between heterozygous and homozygous individuals. DNA was extracted from the tail tips of mice (Laird et al., 1991) and a ~1.8-kb EcoRV pLIV-LE6-SLCO1B1 fragment was used as a probe (figure 1A).

Western Blot analysis: Crude membrane fractions from liver, kidney and small intestine were prepared as described (Ogihara et al., 1996). The microsomal protein was quantified by the Bio-Rad protein assay based on the method of Bradford (Bio-Rad, Veenendaal, The Netherlands) and 20 μg of the crude membrane protein was loaded on a 8% polyacrylamide Tris-HCL gel. After transfer, blots were probed

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with rabbit polyclonal antibody ESL (1:10000), followed by horseradish peroxidase-labeled goat-anti-rabbit secondary antibody (Dako, Glostrup, Denmark). Equal protein loading across the lanes was confirmed by Ponceau S staining of the membranes after transfer.

RT-PCR analysis: RNA isolation from mouse livers, subsequent cDNA synthesis and real-time PCR (RT-PCR) using specific primers (QIAGEN) for mouse Slco1a1, -1a4, 1b2 and -2b1 was performed as described (van Waterschoot et al., 2008b).

Clinical chemical and hematological analysis: Standard clinical chemical analyses on EDTA plasma were performed on a Roche Hitachi 917 analyzer to determine levels of total and conjugated bilirubin, alkaline phosphatase, aspartate aminotransaminase, alanine aminotransaminase, γ-glutamyl transferase, lactate dehydrogenase, creatinine, ureum, Na^+, K^+, Ca^{2+}, total protein, albumin, uric acid, cholesterol and triglyceride. Hemoglobin, hematocrit, mean corpuscular volume, red and white blood cells, and platelets were analyzed in peripheral blood on a Beckman Coulter Ac·T Diff analyzer (Beckman Coulter, Fullerton, CA).

Immunohistochemical analysis: Wild-type and SLCO1B1 transgenic livers were fixed in 4% phosphate-buffered formalin, embedded in paraffin, sectioned at 4 μm, and incubated with rabbit polyclonal ESL antibody (1:250 in PBS) followed by horseradish peroxidase-labeled secondary antibody (Dako, Glostrup, Denmark). Nuclei were stained with H&E according to standard procedures.

MTX pharmacokinetics: MTX (100 mg/ml saline) was diluted to 2 mg/ml in saline and was injected as a single bolus into the tail vein of male mice (n = 3-4 for each group) at a dose of 10 mg/kg (5 μl/g body weight). Animals were killed at indicated time points by terminal bleeding through cardiac puncture under methoxyflurane anesthesia and livers were isolated. Plasma fractions were isolated by centrifugation and organs were homogenized in 4% bovine serum albumin using a Polytron homogenizer.

Levels of MTX and 7-OH-MTX in plasma and homogenized organs were determined by HPLC analysis as described (van Tellingen et al., 1989).

Pharmacokinetic calculations and statistics: The two-sided unpaired Student's t test was used to assess the statistical significance of differences between two sets of data. Results are presented as the means ± SD. Differences were considered to be statistically significant when $P < 0.05$. Averaged concentrations for each time point were used to calculate the area under the plasma concentration versus time curve (AUC) from t = 0 to the last sampling time point by the linear trapezoidal rule. The SE of the AUC was calculated by the law of propagation of errors (Bardelmeijer et al., 2000). Results of the AUC measurements are presented as means ± SE.

RESULTS AND DISCUSSION

Transgenic mice show liver-specific and stable basolateral expression of human OATP1B1.

Stable and specific expression of human OATP1B1 in the liver of transgenic mice was achieved by using an ApoE promoter-HCR1-driven expression cassette containing human SLCO1B1 cDNA (figure 1A). A similar expression cassette was used before to generate liver-specific CYP3A4 transgenic mice (van Herwaarden et al., 2005). Integration of the transgenic construct into the mouse genome was confirmed by PCR and Southern Blot analysis (not shown). Transgene transmission occurred at the expected Mendelian ratios, and two independent homozygous SLCO1B1 transgenic founder lines were generated. Homozygous SLCO1B1 transgenic mice were fertile and did not differ from wild-type mice in life span or body weights. Clinical chemical, hematological and pathological analysis did not reveal any abnormalities. Crude membrane fractions of liver, small intestine and kidney of SLCO1B1 transgenic mice and wild-type mice were analyzed for the expression of OATP1B1 by Western blotting (figure 1B). Both founder lines showed abundant expression of human OATP1B1 in their livers, which was roughly comparable to expression of OATP1B1 in a pooled human crude liver fraction. Detection of OATP1B1 in

human crude liver fraction showed a main band around 80 kD, representing (N-glycosylated) OATP1B1 as was shown before (Ho et al., 2006; König et al., 2000). OATP1B1 detection in the transgenic liver also revealed a main band at ~80 kD (appearance of multiple bands in both human and transgenic samples probably reflects differences in N-glycosylation levels). We found no expression of the transgene in the small intestine and kidney of these mice (figure 1B). Immunohistochemical staining confirmed basolateral (sinusoidal) localization of human OATP1B1 throughout the liver lobules of transgenic mice (figure 2), as was shown for OATP1B1 expression in human liver (Ho et al., 2006; König et al., 2000). This supports the physiological relevance of this model. However, for SLCO1B1 transgenic livers, immunohistochemical staining was strongest around the portal vein (periportal; figure 2b) whereas weaker staining was found towards the central vein (centrolobular; figure 2c). Expression of transgenic OATP1B1 did not influence hepatic expression levels of murine Slco1a1, -1a4, -1b2 and -2b1 as measured by RT-PCR analysis (not shown). Transgenic OATP1B1 expression was monitored over approximately 5 generations and was found to be stable (not shown).

Expression levels of endogenous Slco genes in human SLCO1B1 transgenic mice on semi-synthetic diet.

Pilot studies with high-dose MTX (50 mg/kg) in SLCO1B1 transgenic mice fed with a standard diet resulted in only minor differences between transgenic and wild-type mice (17% decrease in plasma AUC (6194 ± 220 versus 7506 ± 517 nmol · h l^{-1}; P = 0.07) and maximally 2.0-fold increase in liver accumulation ($P < 0.01$) after intravenous administration; Supplemental data 1). These modest effects suggest that under standard conditions the transgenic OATP1B1 activity does not go much beyond the endogenous murine Oatp activity. We therefore switched the mice from the standard diet to a semi-synthetic diet, as we expected that this would result in downregulation of some Oatps, since the semi-synthetic diet contains less phytochemicals than the standard diet (composition of both diets are shown in Supplemental data 2). Phytochemicals are well-known inducers of detoxifying systems by activating PXR, CAR and possibly other xenobiotic nuclear receptors (*e.g.*, van Waterschoot et al., 2008a). RT-PCR analysis for a set of endogenous hepatic Oatp genes was performed to determine diet-dependent

alterations in mRNA levels in the liver of wild-type and SLCO1B1 transgenic mice. We found that mouse Slco1a1, -1a4, -1b2 and -2b1 were indeed (markedly) downregulated in livers of both wild-type and SLCO1B1 transgenic mice on the semi-synthetic diet. These observed decreases were of the same order of magnitude in the two strains, with 1.7- and 2.6-fold (Slco1a1), 3.9- and 6.3-fold (Slco1a4), 2.5- and 1.7-fold (Slco1b2) and 1.5- and 1.5-fold (Slco2b1) decreases in wild-type and SLCO1B1 transgenic mice on the semi-synthetic diet, respectively (figure 3). Since mouse Oatp1a4 is also a MTX transporter (Sasaki, et al., 2004), the marked downregulation of Oatp1a4 in mice fed with the semi-synthetic diet might reduce background murine Oatp-mediated MTX uptake in the liver. Importantly, protein expression of the transgenic human OATP1B1 (which is controlled by the ApoE promoter) was not affected by the semi-synthetic diet, as analyzed by Western blotting (not shown).

Incidentally, our results show that expression of some Slco1 and -2 genes can be markedly affected by dietary conditions. Given the impact of OATP on drug disposition (see also below) it will be interesting to investigate whether this also applies in humans.

SLCO1B1 transgenic animals show increased hepatic uptake of MTX and lower plasma concentrations.

To test the *in vivo* functionality of the transgene, we evaluated MTX disposition in SLCO1B1 transgenic versus wild-type mice fed with the semi-synthetic diet. At various time points after intravenous administration of 10 mg/kg MTX, blood samples were taken and livers were isolated. The amounts of MTX, and its main metabolite 7-OH-MTX, were determined by HPLC analysis. Plasma AUC for MTX in SLCO1B1 transgenic mice was 1.5-fold decreased compared to wild-type mice (1261 ± 30.3 versus 1857 ± 112 nmol · h l^{-1}; $P < 0.05$; figure 4A). The inset in figure 4 illustrates that the terminal elimination of plasma MTX is somewhat faster in SLCO1B1 transgenic mice compared to wild-type mice (semi-log scale). This supports a role of transgenic OATP1B1 not just in short-term liver accumulation, but also in longer-term plasma clearance. Furthermore, the amount of MTX in the liver was markedly increased (~2-fold) at all time points in the SLCO1B1 transgenic mice compared to wild-type mice (figure 4B). Liver to plasma ratios of MTX showed 2.2-, 2.6- and 4.2-fold increases in mice expressing human OATP1B1

compared to wild-type mice at 15, 30 and 60 minutes after injection, respectively ($P < 0.001$; figure 4C). Plasma concentrations of 7-OH-MTX, which is primarily formed by aldehyde oxidase in the liver, were low and only significantly decreased in SLCO1B1 transgenic mice compared to wild-types 30 minutes after MTX administration (93.3 ± 24.3 versus 192.7 ± 67.8 nmol/l; $P < 0.05$). 7-OH-MTX amount in the liver did not differ between transgenic and wild-type mice (not shown).

MTX was earlier identified as a substrate for human OATP1B1 *in vitro* (Abe et al., 2001; Sasaki et al., 2004). To the best of our knowledge, this is the first study that shows that OATP1B1 is an important hepatic uptake transporter for MTX *in vivo*, with a rate-limiting role in MTX plasma elimination. Interindividual variation in MTX efficacy and toxicity correlating with plasma levels (Gorlick and Bertino, 1999) is a well recognized obstacle in the treatment of patients with cancer and rheumatoid arthritis. As many genetic and functional variants in SLCO1B1 have been identified (Chung et al., 2005; Niemi et al., 2004; Nishizato et al., 2003; Nozawa et al., 2002; Tirona et al., 2001), the results of this study imply that variations in OATP1B1 activity, due to genetic polymorphism, dietary conditions, and perhaps drug-drug interactions, can have profound effects on plasma pharmacokinetics of MTX in patients and therefore partly explain interindividual variation. Thus OATP1B1, besides other hepatic transporters like MRP2 (Vlaming et al., 2006), might play an important role in MTX-related hepatic and/or plasma–exposure dependent toxicity. For example, MTX treatment is associated with acute and chronic liver damage (Hirvikoski et al., 1997; van Outryve et al., 2002). It would be interesting to see whether the observed toxicity could be correlated with genetic polymorphisms in SLCO1B1. At this moment, we can only speculate about this and further research needs to investigate the clinical implications of MTX as an OATP1B1 substrate.

In conclusion, this study describes a novel liver-specific SLCO1B1 transgenic mouse model that provides an appropriate tool to study the role of human OATP1B1 in drug pharmacokinetics *in vivo*. Recently, Slco1b2 knockout mice have been described (Lu et al., 2008; Zaher et al., 2008). Since mouse Slco1b2 is orthologous to human SLCO1B1 and SLCO1B3 (Hagenbuch and Meier, 2004), Slco1b2 knockout mice

might be a useful model to combine with our SLCO1B1 transgenic mice in order to generate a humanized model for analysis of human OATP1B1 function *in vivo*.

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Footnotes

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Figure 1.

A. Structure of the ApoE promoter-HCR1-driven expression cassette, containing human SLCO1B1 cDNA. Functional elements are represented approximately to scale. Translational start and stop codon and reading frame direction (bold arrow) for SLCO1B1 are indicated. Small arrows indicate the primers used for PCR detection. The probe used for Southern blot analysis (1.8 kb) is indicated below the cDNA. **B.** Expression of human OATP1B1 in the liver, but not in kidney and small intestine, of SLCO1B1 transgenic versus wild-type mice fed with the standard diet (male) as detected by Western blotting (upper panel). Two independently generated founder lines were analyzed (#1 and #2). HCLM, human crude liver membrane; Wt, wild-type; Tg, SLCO1B1 transgenic. Crude membrane protein (20 μg) was analyzed for all fractions. A molecular weight marker of 85 kD is indicated. Total protein staining (Ponceau S) confirmed equal loading across the lanes (lower panel).

Figure 2.

Immunolocalization of human OATP1B1 in the liver of SLCO1B1 transgenic mice. Paraffin embedded liver of a SLCO1B1 transgenic mouse fed with the standard diet (male, founder line 1) was sectioned (4 μm) and stained with a rabbit polyclonal antibody against human OATP1B1 (brown). Nuclei were stained with hematoxylin/eosin (blue). The picture shows a basolateral staining pattern throughout the liver lobule of SLCO1B1 transgenic mice (A), which was strongest around the portal vein (B) and weaker (but positive) at the centrolobular region (C). Wild-type liver (male) did not show staining of OATP1B1 (D). Scale bars are indicated.

Figure 3.

Slco1a1, -1a4, -1b2 and -2b1 mRNA expression measured by RT-PCR. Results are expressed as the fold change in expression of murine Slco genes in livers of wild-type and SLCO1B1 transgenic mice fed with the standard diet (male, n = 4) compared to mice fed with the semi-synthetic diet (male, n = 4). Data were

normalized against the expression of the endogenous control beta-actin. Each sample was assayed in duplicate. $^{**}P < 0.01$; $^{***}P < 0.001$.

Figure 4.

Plasma and liver pharmacokinetics of methotrexate (MTX) after intravenous injection (10 mg/kg) into wild-type and SLCO1B1 transgenic mice on the semi-synthetic diet (male, n = 3-4). Plasma concentration versus time curves of methotrexate (A), methotrexate concentration in the liver (presented as percentage of dose) (B) and liver to plasma ratios of methotrexate (C) are shown. Each point/bar represents mean ± SD. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

Supplemetal data 1.

Plasma and liver pharmacokinetics of methotrexate (MTX) after intravenous injection (50 mg/kg) into wild-type and SLCO1B1 transgenic mice fed with the standard diet (male, n = 3-4). Plasma concentration versus time curves of methotrexate (A), methotrexate concentration in the liver (presented as percentage of dose) (B) and liver to plasma ratios of methotrexate (C) are shown. Each point/bar represents mean ± SD. $^{*}P < 0.05$; $^{**}P < 0.01$.

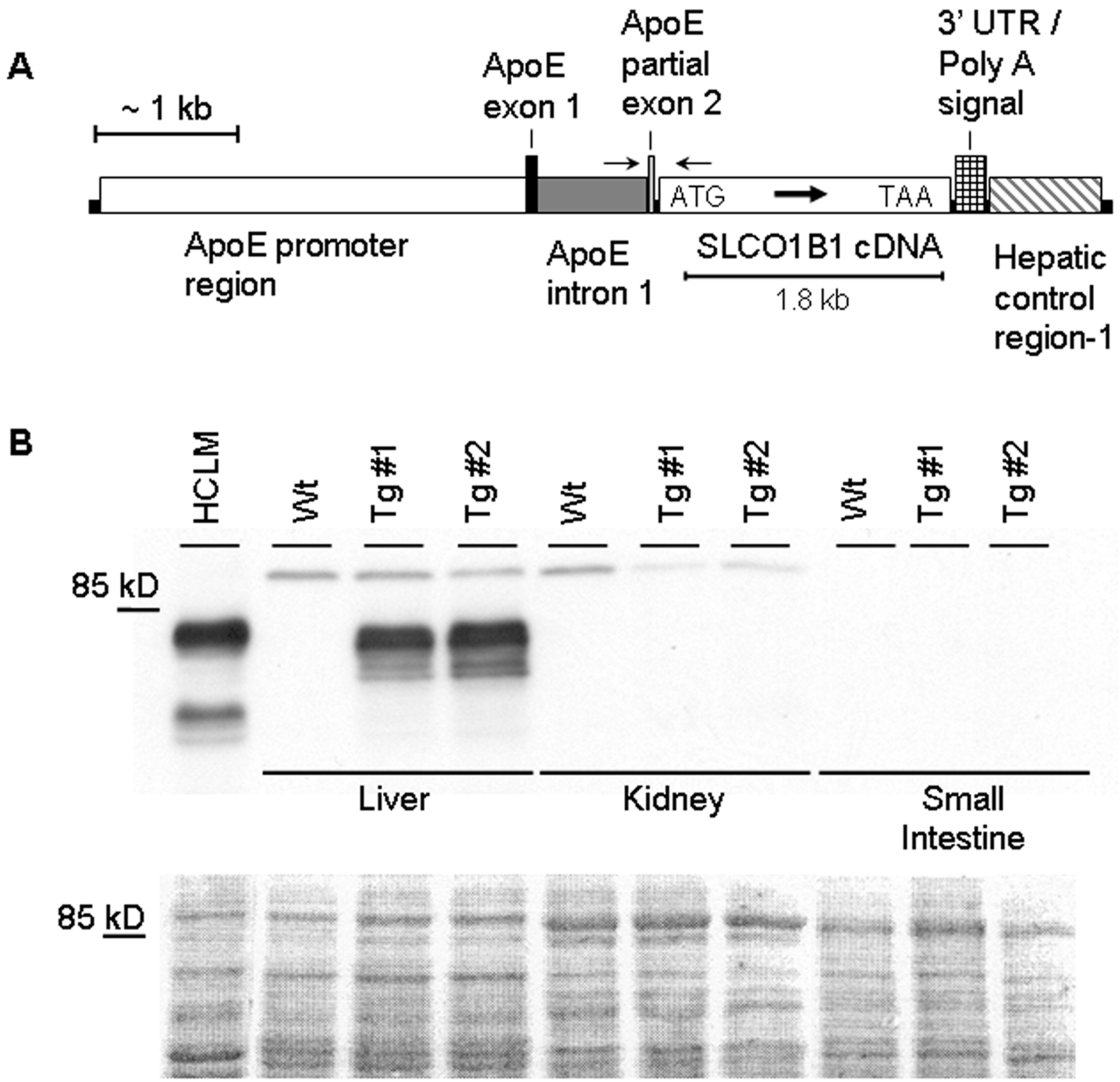
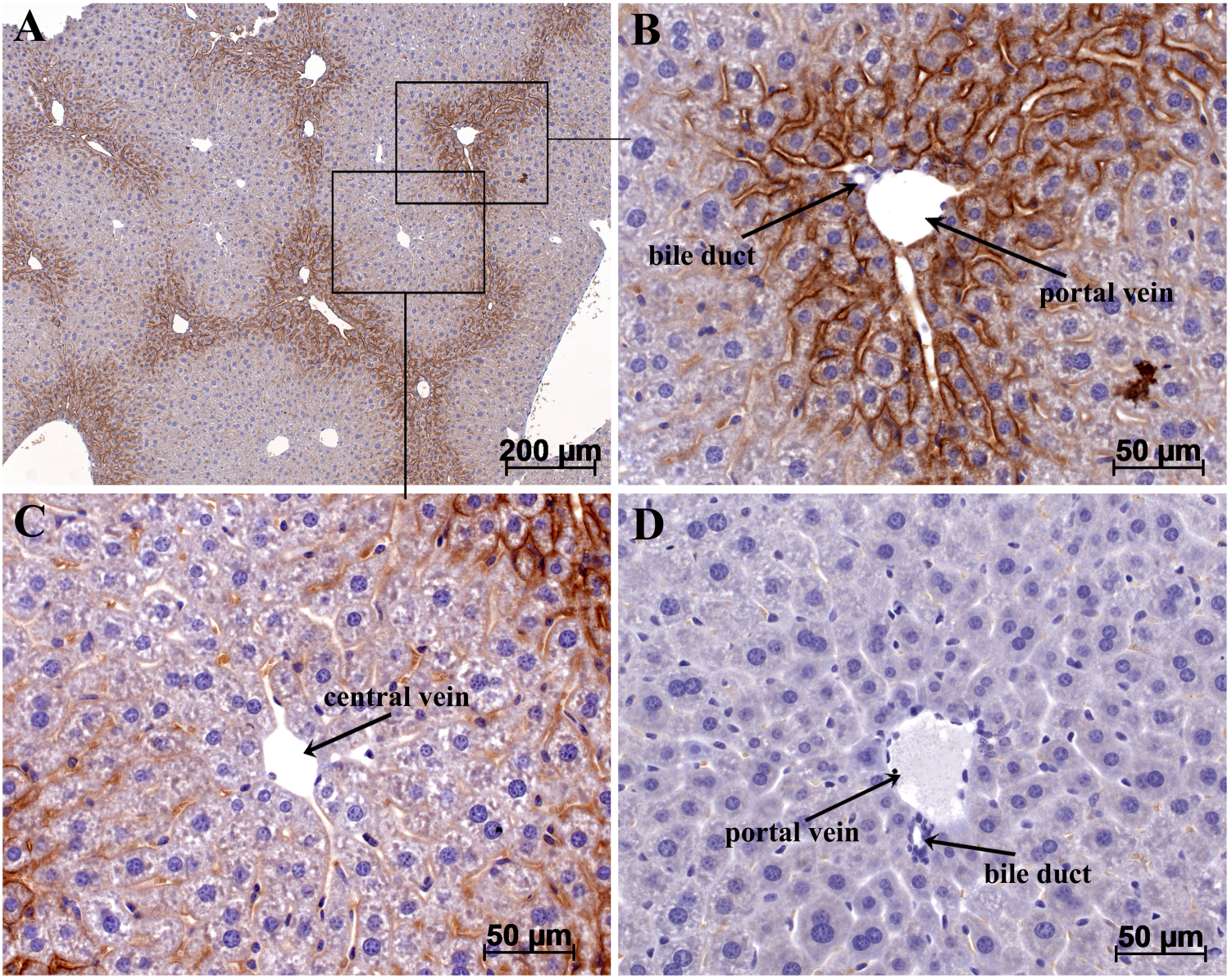


Fig. 1


A
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B
bile duct
portal vein
50 µm
C
central vein
50 µm
D
portal vein
bile duct
50 µm

Fig. 2

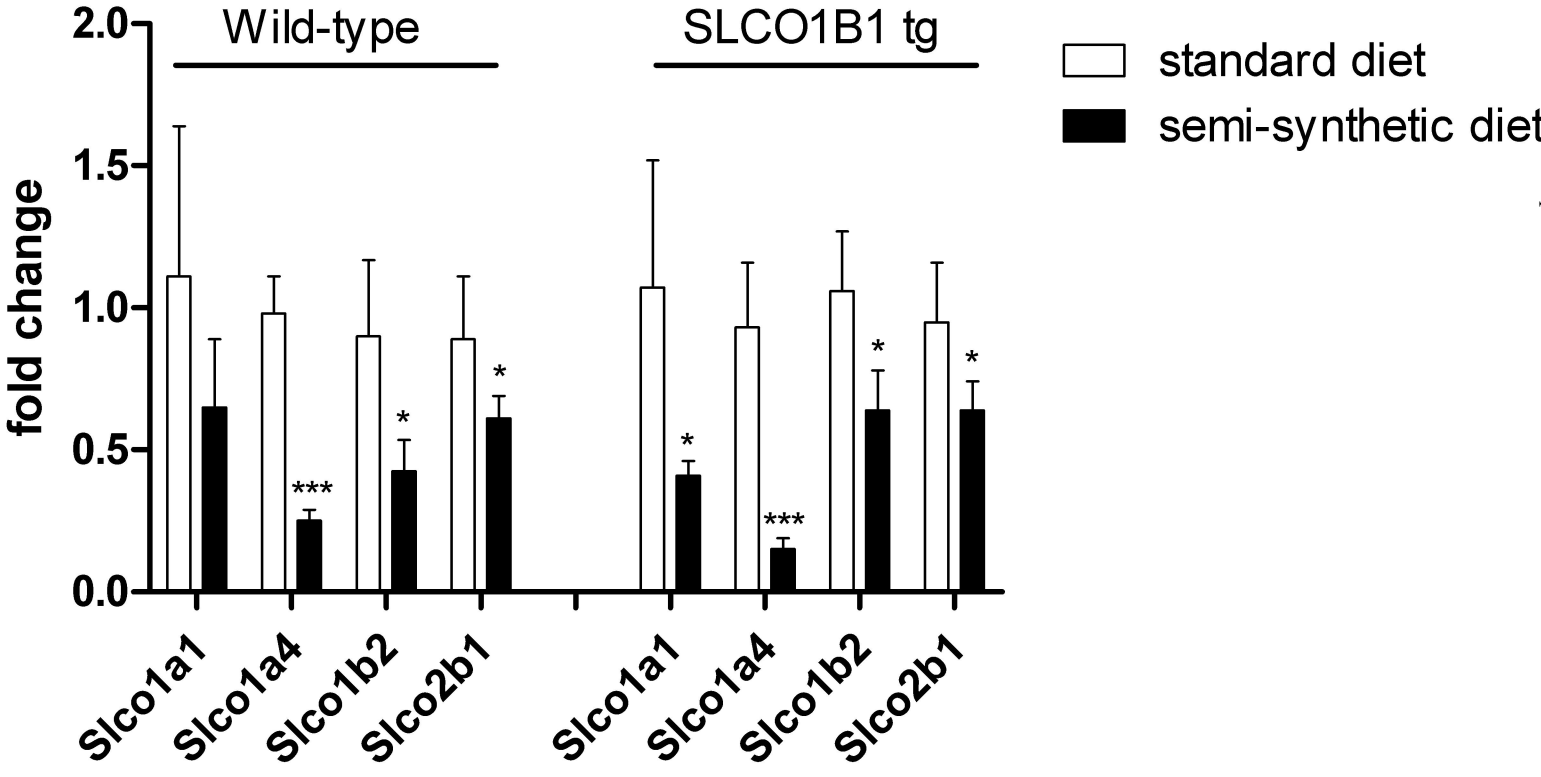
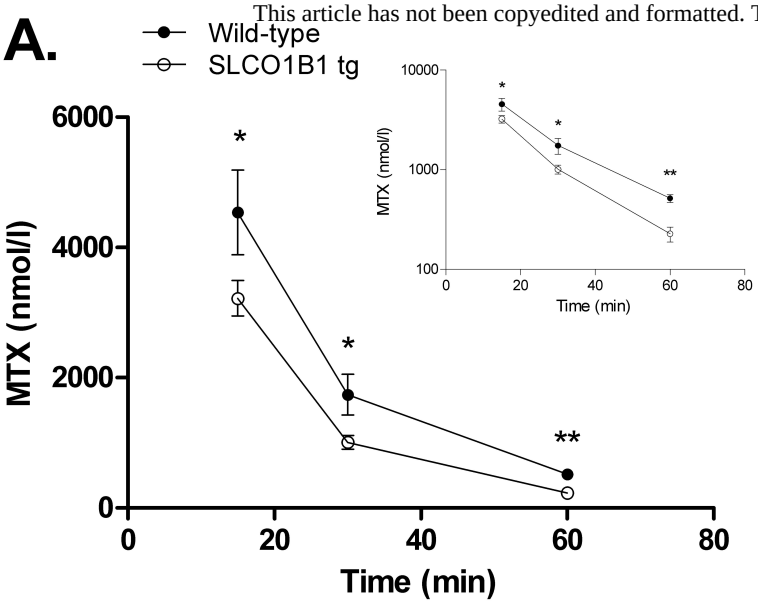
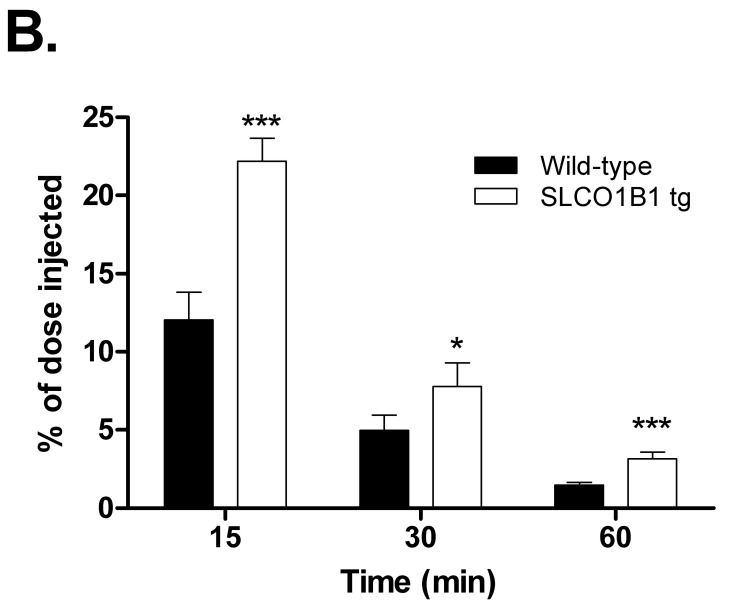


Fig. 3





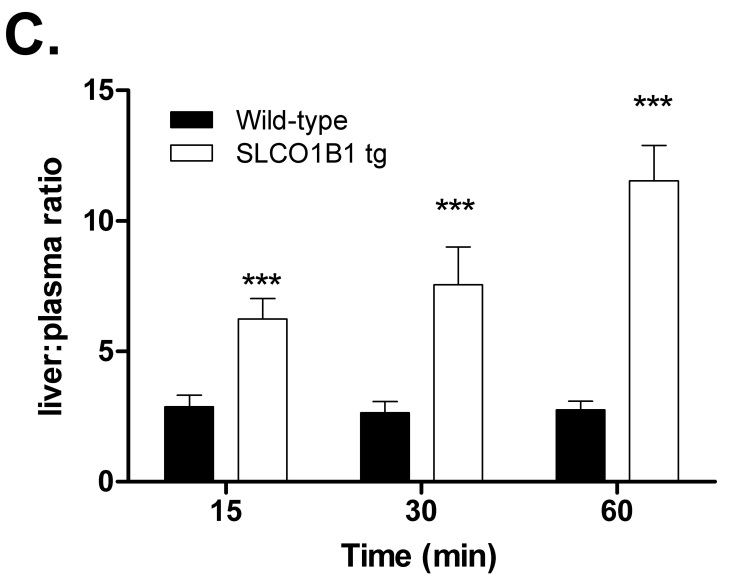


Fig. 4