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ABCB1 restricts brain accumulation of the novel RORγ agonist cintirorgon, while OATP1A/1B and CYP3A limit its oral availability

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ABSTRACT

Cintirorgon (LYC-55716), a first-in-class, small-molecule, oral selective RORγ agonist, has been developed as a new immuno-oncology drug for solid tumors. We here studied the functions of the ABCB1 and ABCG2 multidrug efflux transporters, the OATP1A/1B uptake transporters, and the drug-metabolizing CYP3A enzyme complex in cintirorgon pharmacokinetics using genetically modified mouse models. Cintirorgon was modestly transported by human ABCB1 and mouse Abcg2 *in vitro*. Upon oral administration at 40 mg/kg, net cintirorgon brain penetration was enhanced in *Abcb1a/1b*$^{-/-}$ (2.1-fold) and *Abcb1a/1b;Abcg2*$^{-/-}$ (2.7-fold) relative to wild-type mice. Deficiency of Oatp1a/1b led to a substantial (2.4-fold) increase in cintirorgon systemic exposure, with a corresponding (2.3-fold) decrease in hepatic distribution. However, these changes were not rescued in mice overexpressing human OATP1B1 or human OATP1B3 in liver, although this did partially reverse the altered cintirorgon glucuronide pharmacokinetics in *Oatp1a/1b*$^{-/-}$ mice. In *Cyp3a*$^{-/-}$ mice, the cintirorgon plasma $AUC_{0\text{-}8h}$ was 1.4-fold increased, and then decreased by 1.5-fold upon overexpression of transgenic human CYP3A4 in intestine and liver. Cintirorgon brain accumulation was thus markedly restricted by ABCB1. Mouse Oatp1a/1b mediated cintirorgon uptake into the liver, thus limiting its plasma exposure. Moreover, oral availability of cintirorgon was limited by CYP3A. These insights could help optimizing cintirorgon's clinical application.

1. Introduction

Retinoic acid receptor-related orphan receptors (RORs) are members of a subfamily of the nuclear receptor superfamily. They can affect, amongst others, neuronal cell development, circadian rhythm, and immune cell differentiation [1]. The master transcription factor RORγ is involved in type 17 effector cell differentiation into $CD4^+$ helper T (Th17) and $CD8^+$ cytotoxic T (Tc17) cells [2,3], which are associated with an improved outcome of many preclinical tumor models and human cancers [4–7]. Thus, RORγ represents a novel anticancer target. In preclinical studies, RORγ agonists, by enhancing Th17 and Tc17 cells, could stimulate a potent antitumor response, characterized by reduced immune suppression and increased immune activity [8–10].

Cintirorgon (LYC-55716, Supplemental Fig. 1A), a first-in-class, small-molecule, oral selective RORγ agonist, is currently under clinical development as an immuno-oncology agent for solid tumors. Based on results of preclinical studies and bioinformatics analyses, 6 types of tumor (NSCLC, ovarian, renal, bladder, head-and-neck, and gastro-esophageal cancers) showing greater sensitivity to RORγ agonists were selected for inclusion in ongoing clinical trials [11,12]. Orally administered cintirorgon or other RORγ agonists have shown promise in several syngeneic tumor models in both mono- and combination therapy [9]. In a first-in-human Phase 1/2a study (ARGON) of cintirorgon in patients with relapsed or refractory metastatic cancer not responding to current standard therapies, cintirorgon showed a good clinical activity, with 2 patients (8%) achieving partial response and 11 patients (44%)

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achieving stable disease for between 2 and 12 months [13]. Moreover, cintirorgon was well tolerated, with mainly Grade 1–2 gastrointestinal symptoms as treatment-related adverse events. No dose-limiting toxicities were seen for doses up to 450 mg BID, which was defined as a dosing regimen for future clinical trials [13].

Drug pharmacokinetics and efficacy can be influenced by transmembrane transporters including the ATP-binding cassette (ABC) and the organic anion transporting polypeptide (OATP) transporters [14]. P-glycoprotein (P-gp; ABCB1) and breast cancer resistance protein (BCRP; ABCG2), two main ABC drug efflux transporters, are highly expressed in the pharmacokinetically important organs (liver, small intestine, kidneys) and critical barriers (blood–brain barrier (BBB) and blood-testis barrier (BTB)) as well as in a number of tumors [15–17]. They can limit systemic, brain and intracellular tumor cell exposure of many clinically used anticancer drugs. This may well reduce the therapeutic efficacy of these drugs, especially against brain metastases, and may confer multidrug resistance to tumor cells [15–17].

OATP transporters belong to a superfamily of transporters which primarily take up various endogenous and exogenous compounds into cells. OATP1A/1B transporter proteins are of interest because of their broad substrate specificity and variation in activity due to drug-drug interactions and genetic polymorphisms [18].

Drug-metabolizing enzymes often work in concert with the drug transporters to modulate drug absorption, distribution, metabolism and elimination [19]. Cytochrome P450 3A4 (CYP3A4) is an important member of the CYP enzymes family as it represents approximately 80% of CYPs expressed predominantly in small intestinal mucosa. CYP3A4, together with its close relative CYP3A5, catalyzes the majority of phase I drug metabolism reactions and has a very broad substrate specificity [20,21]. Metabolism of drugs by CYP3A4 can variously lead to activation, inactivation, formation of toxic metabolites, as well as the risk of potential drug-drug interactions. There is currently no publicly available information regarding the interactions of cintirorgon with these transporters and CYP3A, or clinical-pharmacokinetic data concerning cintirorgon drug-drug interactions and pharmacogenetics.

Therefore, using genetically engineered cell lines and mouse models, we aimed to investigate whether and to what extent the plasma exposure and tissue accumulation of oral cintirorgon are influenced by ABCB1, ABCG2, OATP1A/1B, and CYP3A activity.

2. Materials and methods

2.1. Chemicals

Cintirorgon (LYC-55716; >99.5%) was purchased from ChemieTek (Indianapolis, IN, USA). Zosuquidar was obtained from Sequoia Research Products (Pangbourne, United Kingdom). Ko143 was purchased from Tocris Bioscience (Bristol, United Kingdom). Bovine Serum Albumin (BSA) Fraction V was obtained from Roche Diagnostics (Mannheim, Germany). Heparin (5000 IU·ml^{-1}) was purchased from Leo Pharma (Breda, The Netherlands) and isoflurane from Pharmachemie (Haarlem, The Netherlands). All other reagents and chemicals were purchased from Sigma-Aldrich (Steinheim, Germany).

2.2. Cell lines and transepithelial transport assay

Polarized Madin-Darby Canine Kidney (MDCK-II) cells (ECACC 00062107) stably transduced with either human ABCB1, human ABCG2 or mouse Abcg2 cDNA were previously generated in our institute. These polarized epithelial cells are continually used in our lab and demonstrate highly characteristic growth and drug transport properties, confirming their proper identity and functionality. Cells were routinely tested negative for mycoplasma. The passage number of cells used in these transport experiments was 10–15.

The transepithelial transport assay was conducted using a 12 mm transwell with 3.0 μm pore microporous polycarbonate membrane insert (Transwell 3402, Corning Incorporated, Kennebunk, ME) as previously described [22]. 2.5 × 10^5 cells from the parental and variant subclones were seeded on the membrane per well and cultured for 72 h to form an intact monolayer. Prior to and after the transport phase, transepithelial electrical resistance (TEER) was measured to check for the integrity and permeability of the membrane.

Cintirorgon, zosuquidar (ABCB1 inhibitor), or Ko143 (ABCG2 inhibitor) were first dissolved in dimethyl sulfoxide (DMSO) at 5 mM, which was diluted 1000-fold with DMEM medium containing 10% (v/v) fetal bovine serum (FBS) to obtain 5 μM. Where appropriate, zosuquidar and Ko143 were added during the transport experiments after pre-incubation with these inhibitors for 1 h in both compartments. The transepithelial transport phase was initiated (t = 0) by replacing the donor compartment medium with fresh DMEM that contained 10% FBS, 5 μM cintirorgon and inhibitors if applicable. The cells were kept in an environment of 5% CO2 (v/v), at 37 °C, pH ~ 7.4 during the experiments, and 50 μl aliquots were collected from the acceptor compartment at 1, 2, 4, and 8 h, and stored at the −30 °C until LC-MS/MS analysis. The transport ratio (*r*) was adopted to assess the active transport of cintirorgon and equals apically directed drug translocation divided by basolaterally directed drug translocation after 8 h.

2.3. Animals

Wild-type, *Abcb1a/1b*$^{-/-}$, *Abcg2*$^{-/-}$, *Abcb1a/1b;Abcg2*$^{-/-}$, *Slco1a/1b*$^{-/-}$, Slco1B1, Slco1B3, *Cyp3a*$^{-/-}$, and Cyp3aXAV mice, all of a > 99% FVB genetic background, were used between 9 and 15 weeks of age. Mouse housing and handling were in accordance with the institutional guidelines complying with Dutch and EU legislation. The animals were kept in a temperature-controlled and specific pathogen-free environment with a 12/12 hr light/dark cycle and they received standard diet (Transbreed, SDS Diets, Technilab – BMI, Someren, The Netherlands) and acidified water *ad libitum*. All animal experiment protocols designed under the nationally approved DEC/CCD project AVD301002016595 were reviewed and approved by the Institutional Animal Care and Use Committee. Welfare-related assessment was carried out prior to and during the experiments, with mice showing discomfort levels higher than mild being humanely sacrificed.

2.4. Drug stock and dosing solution

For *in vivo* studies, cintirorgon was dissolved in DMSO at a concentration of 50 mg/ml as the stock solution, and stored at −30 °C. The dosing solution was prepared by diluting the cintirorgon stock solution with a mixture of ethanol/ polysorbate 80 (1:1, v/v), and then 5% (w/v) glucose water to reach a concentration of 2 or 4 mg/ml. Final concentrations for DMSO, ethanol, polysorbate 80, and glucose were 4% (8%), 2% (4%), 2% (4%), and 4.6% (4.2%) (v/v/v/w), respectively, in the 2 (or 4) mg/ml dosing solution. All the dosing solutions were prepared freshly on the day of experiment.

2.5. Plasma pharmacokinetics and organ accumulation of cintirorgon in mice

Oral administration of cintirorgon to mice using oral gavage with a blunt-ended needle directly into the stomach was adopted in this study as cintirorgon is recommended to be taken orally in patients. To minimize the variation in absorption, mice were fasted for around 2–3 h prior to receiving cintirorgon (20 mg/kg or 40 mg/kg) at 10 μl/g body weight. Four or two hours after oral administration, tail vein serial blood samplings were performed at 0.125, 0.25, 0.5, 1 and 2 h or 3, 7.5, 15, 30, and 60 min, respectively, using microvette tubes containing heparin (Sarstedt, Nümbrecht, Germany). For the eight-hour experiment, tail vein serial blood samples were collected at 0.25, 0.5, 1, 2, 4 and 6 h in the same way. Two, four or eight hours after oral administration, 5 ml pipette tips were used to cover the snout of the mice and they were

deeply anesthetized by isoflurane evaporator using 2–3% isoflurane together with 0.2 L/min air and 0.1 L/min oxygen forced flow. Cardiac puncture was then performed to collect blood in Eppendorf tubes (Hamburg, Germany) containing heparin as an anticoagulant. After sacrificing the anesthetized mice by cervical dislocation, several tissues were rapidly collected. The small intestinal contents (SIC) were collected separately from small intestinal tissue (SI), which was rinsed by cold saline to remove any residual feces. Blood samples were centrifuged at 9000*g* for 6 min at 4 °C, and the plasma fraction was collected and stored at −30 °C. Brain, liver, spleen, kidney, small

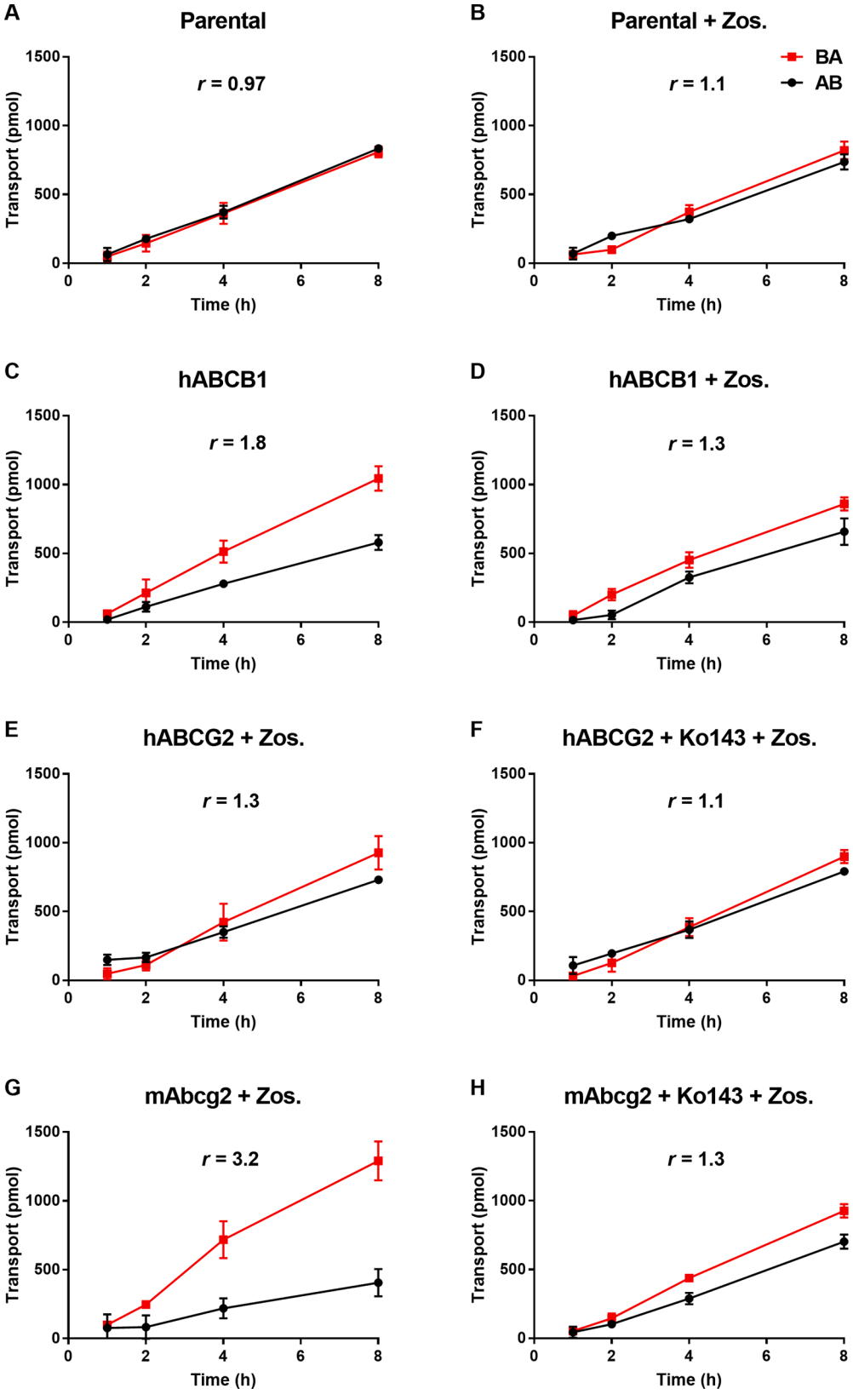

Fig. 1. Transepithelial transport of cintirorgon (5 μM) evaluated in MDCK-II cells either non-transduced (A, B), transduced with hABCB1 (C, D), hABCG2 (E, F) or mAbcg2 (G, H) cDNA. At t = 0 h, cintirorgon was applied in the donor compartment and the concentrations in the acceptor compartment at t = 1, 2, 4, and 8 h were determined and plotted as the cumulative amount of cintirorgon transport (pmol) per well (n = 3). B, D–H: Zos. (zosuquidar, 5 μM) was used to inhibit human and/or endogenous canine ABCB1. F and H: the ABCG2 inhibitor Ko143 (5 μM) was applied to inhibit ABCG2/Abcg2–mediated transport. *r*, relative transport ratio. BA (■), translocation from the basolateral to the apical compartment; AB (●), translocation from the apical to the basolateral compartment. Points, mean; bars, S.D.

intestinal tissue, small intestinal content (SIC), and testis were homogenized with 1, 3, 1, 2, 3, 2, and 1 ml of 2% (w/v) bovine serum albumin (BSA), respectively. All samples were kept at −30 °C until LC-MS/MS analysis.

2.6. LC-MS/MS analysis

The concentrations of cintirorgon in DMEM medium, mouse plasma and tissue homogenates were quantified using a recently developed and validated liquid chromatography-tandem mass spectrometry method [22].

2.7. Pharmacokinetic and statistical analysis

Non-compartmental analysis was adopted using the PKSolver add-in program for Microsoft Excel to calculate the pharmacokinetic parameters of cintirorgon [24]. The area under the curve (AUC) was determined using cintirorgon plasma concentration–time curves by the trapezoidal rule without extrapolating to infinity. The plasma peak concentration (C_{max}) and time to reach peak concentration (T_{max}) were assessed from the original data. The two-sided unpaired student's *t* test was applied to compare two groups using Graphpad Prism7 (Graphpad Software, La Jolla, CA). One-way analysis of variance (ANOVA) was adopted when differences between multiple groups were assessed, and the Bonferroni *post hoc* correction was used to accommodate multiple testing. Heteroscedastic data were first log-transformed before applying statistical analysis. Differences were considered statistically significant when $P < 0.05$. All data are presented as geometric mean ± SD.

3. Result

3.1. Human ABCB1 and mouse Abcg2 modestly transport cintirorgon

Transepithelial transport of cintirorgon was assessed in polarized monolayers of MDCK-II parental cells and engineered subclones overexpressing human (h)ABCB1, hABCG2, or mouse (m)Abcg2 transporters as described previously. These cell lines were chosen based on their availability and expression levels of transduced cDNA. These assays were performed primarily to evaluate whether it was justifiable to initiate *in vivo* mouse experiments, rather than aiming for a systematic species comparison.

There was little net transport of cintirorgon in parental cells ($r = 0.97$), which was not altered by the ABCB1 inhibitor zosuquidar ($r = 1.1$, Fig. 1A and B). Modest apically directed transport of cintirorgon was found in the cells overexpressing hABCB1 ($r = 1.8$, Fig. 1C), and this was partially reduced by zosuquidar ($r = 1.3$, Fig. 1D).

In transport experiments with MDCK-II cells overexpressing hABCG2 and mAbcg2, zosuquidar was used to inhibit any endogenous canine ABCB1 contribution. In cells overexpressing mAbcg2, there was clear apically directed transport of cintirorgon ($r = 3.2$, Fig. 1G), which was markedly suppressed by Ko143, a specific ABCG2 inhibitor ($r = 1.3$, Fig. 1H). Cintirorgon was not substantially transported in hABCG2-overexpressing cells with or without Ko143, although the qualitative shift upon Ko143 treatment does not exclude a minor transport contribution (Fig. 1E and F).

These data therefore indicate that cintirorgon is transported modestly by hABCB1 and mAbcg2, but not substantially by hABCG2. As cintirorgon has an estimated pK_a of 4.6, it will be mostly negatively charged at physiological pH. This is of interest, as only few negatively charged compounds are known to be ABCB1 substrates, whereas for ABCG2 this is more common. Also, the MDCK-II monolayer permeation of cintirorgon appears to be relatively good for a charged compound, suggesting the involvement of uptake systems.

3.2. ABCB1 restricts accumulation of cintirorgon in the brain

To investigate the combined impact of mAbcb1a/1b and mAbcg2 on cintirorgon pharmacokinetics, a 4-h pilot experiment was conducted in female wild-type and *Abcb1a/1b;Abcg2*$^{-/-}$ mice, with 20 mg/kg cintirorgon administered orally. Plasma exposure of cintirorgon over 4 h ($AUC_{0\text{-}4h}$) at this dose was significantly elevated by 1.7-fold ($P < 0.05$) in *Abcb1a/1b;Abcg2*$^{-/-}$ compared to wild-type mice (Fig. 2 and Supplemental Table 1).

Brain, liver, spleen, kidney, small intestine (SI), and small intestinal content (SIC) distribution of cintirorgon were also analyzed at 4 h. Cintirorgon brain concentration and brain-to-plasma ratios in *Abcb1a/1b;Abcg2*$^{-/-}$ mice were substantially elevated by 6.0-fold and 2.1-fold, respectively, in comparison with wild-type mice (Fig. 2C and D). However, we did not observe significant exposure differences in other tissues of cintirorgon between these two mouse strains based on the tissue-to-plasma ratios (Supplemental Figs. 2, 3). Intriguingly, the brain-to-plasma ratio was approximately 0.9 in wild-type mice, suggesting relatively good intrinsic brain penetration of cintirorgon, in spite of this compound being an organic anion. In fact, relative brain distribution was similar to that in spleen (1.0) and kidney (2.2), although liver showed far more extensive accumulation (20).

Of note, shortly after oral administration, the mice showed some mild toxicity. They constantly shook their head touching the bottom of cage, which looked as if they were trying to remove something from the mouth (Supplemental video). As there was no obvious difference in this respect between the strains, this may have been due to an unpleasant taste of the administered drug solution. Since the same formulation composition did not cause this problem with many other drugs, it must be the cintirorgon itself that caused the unpleasant stimulus.

To further study the separate and combined roles of mAbcb1a/1b and mAbcg2 in the oral availability and tissue distribution of cintirorgon, we performed an experiment in male wild-type, *Abcb1a/1b*$^{-/-}$, *Abcg2*$^{-/-}$, and *Abcb1a/1b;Abcg2*$^{-/-}$ mice. We also increased the dosage of cintirorgon to 40 mg/kg to obtain plasma concentrations in mice similar to the reported therapeutic plasma levels in patients. The experiment was terminated at 2 h after oral administration, when the plasma concentrations were still relatively high. Cintirorgon was rapidly absorbed, with the T_{max} falling around 30 min after oral administration. Cintirorgon plasma exposure was elevated in all the knockout strains, but a significant increase (1.7-fold) was only observed in single *Abcg2*$^{-/-}$ mice compared to wild-type mice (Fig. 3 and Table 1). These data suggest that mAbcb1a/1b and mAbcg2, at least at this cintirorgon dose, play at best a limited role in restricting the oral availability of cintirorgon.

Also at 2 h, significantly (~3-fold) higher brain concentrations of cintirorgon were found in *Abcb1a/1b*$^{-/-}$ and *Abcb1a/1b;Abcg2*$^{-/-}$ mice compared to wild-type mice (Fig. 3C; Table 1). Correcting for the plasma exposure, the brain-to-plasma ratio of cintirorgon in wild-type mice was around 0.7, which was markedly increased in *Abcb1a/1b*$^{-/-}$ and *Abcb1a/1b;Abcg2*$^{-/-}$ mice by 2.1-fold and 2.7-fold, respectively, but not in single *Abcg2*$^{-/-}$ mice (Fig. 3D; Table 1). This demonstrates that brain distribution of cintirorgon is primarily limited by mAbcb1a/1b, but not substantially by mAbcg2.

Cintirorgon testis distribution was also assessed in this experiment as testis resembles the brain regarding the abundant expression of ABCB and ABCG2 in the BTB. The testis-to-plasma ratios of cintirorgon were increased, but not significantly, in knockout mice compared to wild-type mice, indicating that ABCB1 and ABCG2 play only a limited role in restricting cintirorgon distribution into testis (Fig. 3F and Table 1). Moreover, no significant or meaningful differences in other tested organ distributions of cintirorgon were observed in Abcb1a/1b- and Abcg2-deficient mice (Supplemental Figs. 4, 5). We found similar but not more severe toxicity signs in mice in this experiment, in spite of the higher dosage of cintirorgon used.

In summary, it appears that mAbcb1 can markedly limit brain

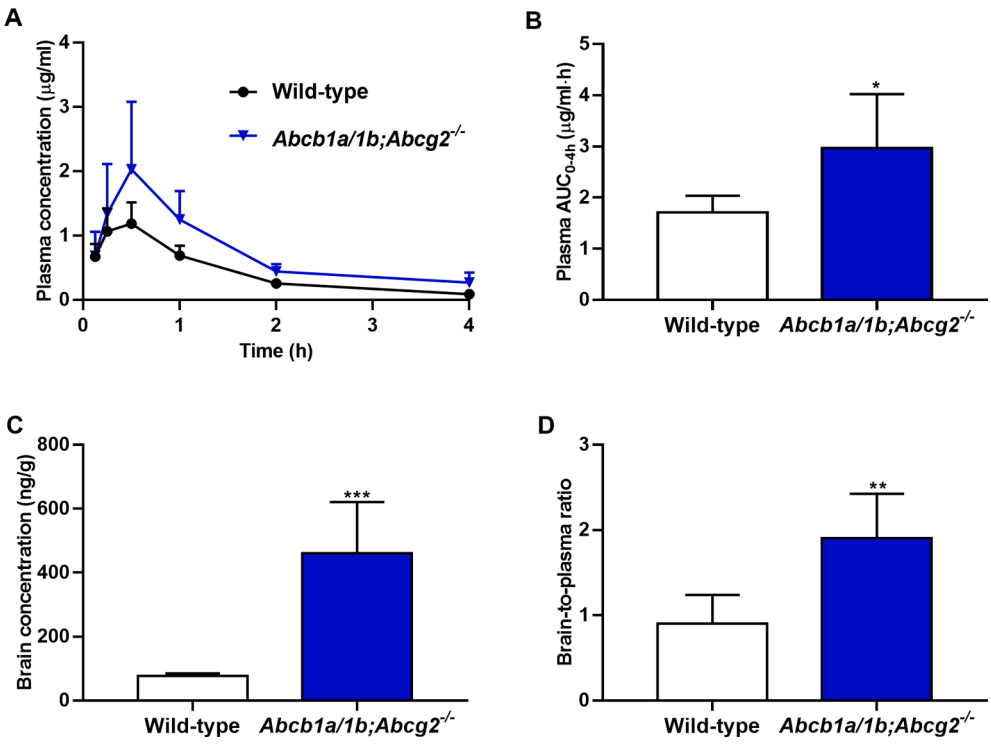

Fig. 2. Plasma concentration–time curves (A), plasma $AUC_{0\text{-}4h}$ (B), brain concentration (C), and brain-to-plasma ratio (D) of cintirorgon in female wild-type and *Abcb1a/1b;Abcg2*$^{-/-}$ mice over 4 h after oral administration of 20 mg/kg cintirorgon. Data are presented as mean ± SD (n = 6). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ compared to wild-type mice.

distribution of cintirorgon, but not its other relative tissue distributions. mAbcb1 and/or mAbcg2 may modestly limited the oral availability of cintirorgon at a dose of 20 mg/kg, and showed an even lower impact at a higher dose (40 mg/kg).

3.3. *OATP1A/1B mediates hepatic uptake of cintirorgon and its glucuronide and reduces their oral availability*

OATP-mediated hepatic uptake can influence (oral) availability and elimination of many substrate drugs. Since cintirorgon contains a carboxyl group and is negatively charged (anionic) under normal physiological conditions, it is a potential substrate of OATP transporters. About possible interactions of cintirorgon with OATP/SLCO uptake transporters is currently little known. We thus conducted a pilot experiment with oral cintirorgon (at 20 mg/kg) in wild-type and Slco1a/1b-deficient mice, and analyzed plasma levels up till 4 h and liver distribution at 4 h. Significantly higher plasma exposure and reduced liver-to-plasma ratios of cintirorgon were observed in Slco1a/1b-deficient compared to wild-type mice (data not shown). This suggests that the mouse Oatp1a/1b transporters may mediate hepatic uptake of cintirorgon, resulting in reduced plasma levels.

To further investigate the effects of mouse Oatp1a/1b, and human OATP1B1 and OATP1B3 transporters, we performed a more extensive experiment up to 2 h with oral cintirorgon (40 mg/kg). Male wild-type, *Slco1a/1b*$^{-/-}$, and *Slco1a/1b*$^{-/-}$ mice with specific transgenic overexpression of human OATP1B1 or human OATP1B3 in liver (Slco1B1 or Slco1B3 mice) were included. The peak plasma concentrations and plasma $AUC_{0\text{-}2h}$ of cintirorgon were significantly increased in *Slco1a/1b*$^{-/-}$, Slco1B1, and Slco1B3 mice relative to wild-type mice (Fig. 4A, B and Table 2). Accordingly, markedly lower liver-to-plasma ratios and SI-to-plasma ratios of cintirorgon were observed in *Slco1a/1b*$^{-/-}$, Slco1B1, and Slco1B3 mice than in wild-type mice. However, there were no significant differences in the humanized transgenic mice compared to their control *Slco1a/1b*$^{-/-}$ mice, either in systemic exposure or liver and SI distribution of cintirorgon. Furthermore, the relative tissue distribution (tissue-to-plasma ratios) of cintirorgon in other tested organs showed no meaningful differences between the wild-type and genetically modified strains (Supplemental Fig. 6).

Intriguingly, cintirorgon glucuronide was detected in the liver, kidney and small intestine in all four mouse strains. It was further found in plasma of *Slco1a/1b*$^{-/-}$, Slco1B1, and Slco1B3 mice, but not of wild-type mice. In view of the lack of a reference standard, we semi-quantified the levels of cintirorgon glucuronide using cintirorgon concentrations in these samples as a reference [22]. As shown in Fig. 5A and B, the plasma exposure of cintirorgon glucuronide was increased relative to wild-type, but markedly reduced in Slco1B1 and Slco1B3 transgenic mice compared to *Slco1a/1b*$^{-/-}$ mice. Although the concentrations of cintirorgon glucuronide were similar between the mouse strains in liver, kidney, and small intestine, we found significantly increased tissue-to-plasma ratios of cintirorgon glucuronide in Slco1B1 transgenic mice compared to *Slco1a/1b*$^{-/-}$ mice, but not in Slco1B3 mice, likely because of the relatively high experimental variation (Fig. 5). In theory, liver-to-plasma ratios of the glucuronide in wild-type mice would have been at least a factor 60-fold (Fig. 5) higher than in *Slco1a/1b*$^{-/-}$ mice, but these could not be calculated in the absence of measurable plasma levels. Of note, as both plasma and tissue concentrations of cintirorgon glucuronide were semi-quantified in the same way, tissue-to-plasma ratios of cintirorgon glucuronide could be quantitatively established. The absence of detectable cintirorgon glucuronide in plasma of wild-type but not of *Slco1a/1b*$^{-/-}$ mice suggested that mouse Oatp1a/1bs efficiently limit plasma exposure of cintirorgon glucuronide through hepatic uptake. Moreover, the reduced plasma exposure and increased tissue distribution of cintirorgon glucuronide in humanized transgenic mice compared to their control *Slco1a/1b*$^{-/-}$ mice suggests that human OATP1B1, and possibly OATP1B3, could take up the cintirorgon glucuronide into the liver, increasing its intestinal distribution through elevated biliary excretion, and also reducing its systemic exposure. Furthermore, since human OATP1B1 and OATP1B3 are also expressed in the kidney of transgenic mice due to the ApoE promoter we used [25], the increased kidney-to-plasma ratios of cintirorgon glucuronide further

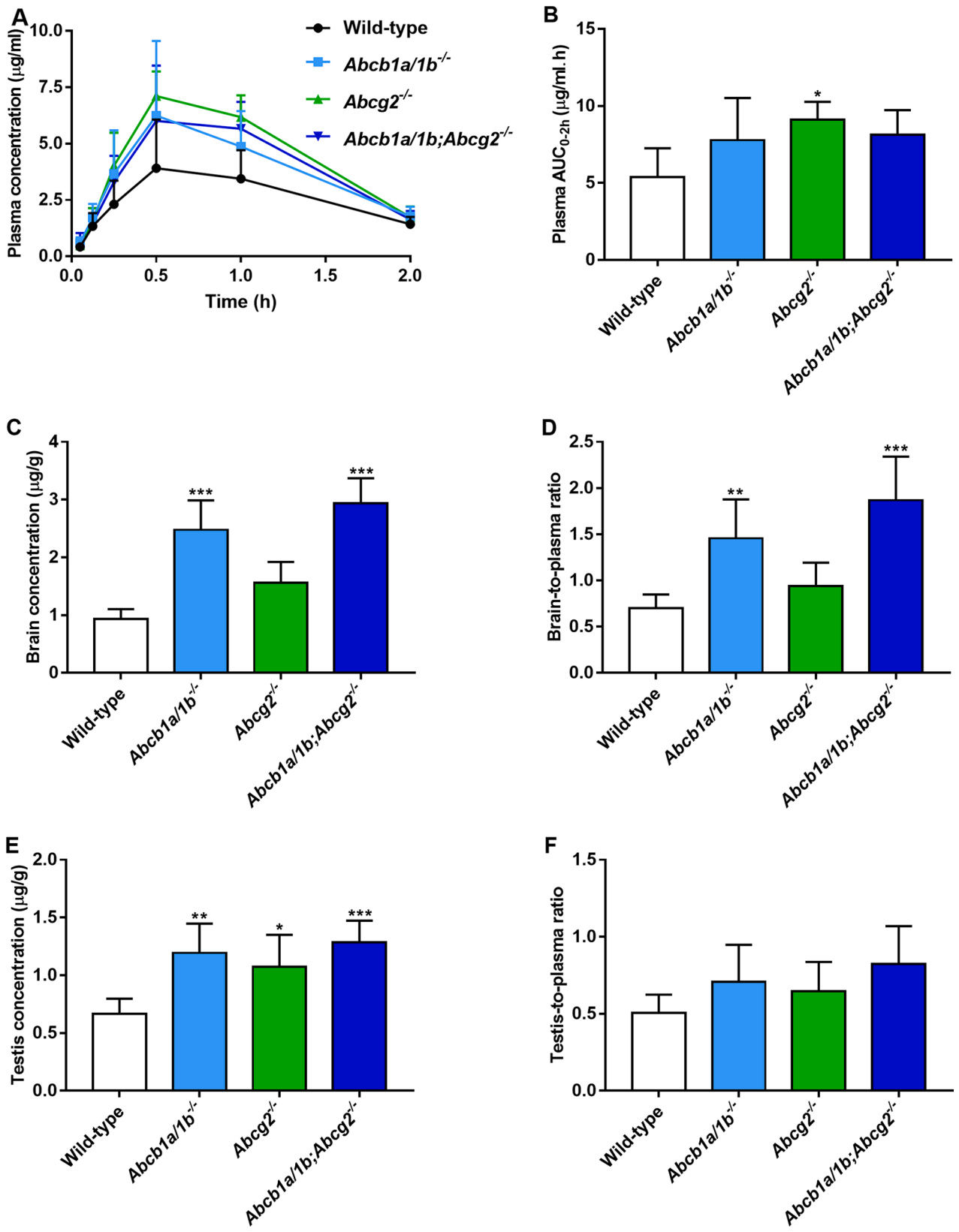

Fig. 3. Plasma concentration–time curves (A), plasma $AUC_{0\text{-}2h}$ (B), brain and testis concentrations (C, E), and brain- and testis-to-plasma ratios (D, F) of cintirorgon in male wild-type, *Abcb1a/1b*$^{-/-}$, *Abcg2*$^{-/-}$, and *Abcb1a/1b;Abcg2*$^{-/-}$ mice 2 h after oral administration of 40 mg/kg cintirorgon. Data are presented as mean ± SD (n = 6–7). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ compared to wild-type mice. $^{\#}$, $P < 0.05$; $^{\#\#}$, $P < 0.01$; $^{\#\#\#}$, $P < 0.001$ comparing *Abcb1a/1b;Abcg2*$^{-/-}$ mice to *Abcb1a/1b*$^{-/-}$ mice.

Table 1
Plasma, brain, liver, and testis pharmacokinetic parameters of cintirorgon 2 h after oral administration of 40 mg/kg cintirorgon to male wild-type, *Abcb1a/1b*$^{-/-}$, *Abcg2*$^{-/-}$, and *Abcb1a/1b;Abcg2*$^{-/-}$ mice.

Parameter	Genotype			
	Wild-type	*Abcb1a/1b*$^{-/-}$	*Abcg2*$^{-/-}$	*Abcb1a/1b;Abcg2*$^{-/-}$
$AUC_{0\text{-}2h}$, μg/ml·h	5.36 ± 1.90	7.78 ± 2.75	9.11 ± 1.16*	8.12 ± 1.61
Fold increase $AUC_{0\text{-}2h}$	1.00	1.45	1.70	1.50
C_{max}, μg/ml	4.11 ± 2.03	6.48 ± 2.95	7.34 ± 1.03	6.66 ± 2.11
T_{max}, h	0.5–1	0.5–1	0.5–1	0.5–1
C_{brain}, μg/g	0.93 ± 0.17	2.48 ± 0.51***	1.56 ± 0.37	2.941 ± 431***
Fold increase C_{brain}	1.00	2.67	1.67	3.16
Brain-to-plasma ratio	0.70 ± 0.15	1.46 ± 0.42**	0.94 ± 0.26	1.87 ± 0.47***
Fold change ratio	1.00	2.09	1.34	2.67
C_{liver}, μg/g	24.4 ± 4.9	23.1 ± 8.4	28.3 ± 7.3	23.5 ± 5.5
Fold change C_{liver}	1.00	0.95	1.16	0.96
Liver-to-plasma ratio	18.3 ± 2.8	13.2 ± 3.5	16.5 ± 1.7	14.5 ± 2.5
Fold change ratio	1.00	0.72	0.90	0.79
C_{testis}, μg/g	0.67 ± 0.13	1.19 ± 0.25**	1.07 ± 0.28*	1.28 ± 0.19***
Fold increase C_{testis}	1.00	1.78	1.60	1.91
Testis-to-plasma ratio	0.51 ± 0.12	0.71 ± 0.24	0.65 ± 0.19	0.82 ± 0.25
Fold change ratio	1.00	1.39	1.27	1.61

Data are presented as mean ± SD (n = 6–7). $AUC_{0\text{-}2h}$, area under the plasma concentration–time curve; C_{max}, maximum concentration in plasma; T_{max}, time point of maximum plasma concentration (range for individual mice); $C_{brain/liver/testis}$, brain/liver/testis concentration. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ compared to wild-type mice.

support that human OATP1B1 and perhaps OATP1B3 could transport cintirorgon glucuronide.

Taken together, these results indicate that the mouse Oatp1a/1b transporters can mediate hepatic uptake of cintirorgon and its glucuronide, and indirectly control their intestinal disposition by biliary excretion, leading to reduced oral availability of cintirorgon. A role for Oatp1a/1b transporters in the direct small-intestinal uptake of cintirorgon is unlikely, as we recently found negligible RNA levels in the small intestine for all these transporters in an RNA deep sequencing analysis of wild-type mice as kept in our facility (manuscript in preparation). In contrast, the human OATP1B1 and possibly OATP1B3 transporters can noticeably transport cintirorgon glucuronide into the liver, but not cintirorgon itself.

3.4. Roles of CYP3A in cintirorgon plasma pharmacokinetics and tissue distribution

CYP3A enzymes participate in metabolism of many drugs, often limiting their oral availability. To study the interaction between cintirorgon and CYP3A, a 4-h pilot experiment was performed with oral cintirorgon at 20 mg/kg in female wild-type, Cyp3a knockout (*Cyp3a*$^{-/-}$) mice, and *Cyp3a*$^{-/-}$ mice overexpressing transgenic human CYP3A4 in liver and intestine (Cyp3aXAV) mice. As shown in Fig. 6A–C and Supplemental Table 2, cintirorgon absorption was rapid, with peak plasma concentrations occurring at around 30 min after oral administration. The plasma $AUC_{0\text{-}4h}$ of cintirorgon was substantially increased by 1.7-fold in *Cyp3a*$^{-/-}$ mice compared to wild-type (Fig. 6C). Moreover, plasma exposure was then markedly reduced in Cyp3aXAV relative to *Cyp3a*$^{-/-}$ mice, reaching similar levels as seen in wild-type mice. This suggests that cintirorgon is likely a substrate of both mouse Cyp3a and human CYP3A4. Interestingly, in *Cyp3a*$^{-/-}$ mice the plasma concentration after 4 h was higher than after 2 h (Fig. 6A and B). This pattern was shared among all *Cyp3a*$^{-/-}$ mice, but was not seen in any of the wild-type or Cyp3aXAV mice (n = 6). Possibly, substantial second-phase absorption (enterohepatic cycling) of cintirorgon may take place in *Cyp3a*$^{-/-}$ mice, without being interrupted by Cyp3a/CYP3A4-mediated metabolism in liver and small intestine. In contrast, only minor effects of the CYP3A modifications were observed for the relative distribution of cintirorgon to brain, liver, spleen, kidney, small intestine and small intestine content, taking into consideration the marked deviation of plasma pharmacokinetics between *Cyp3a*$^{-/-}$ mice and the other two strains over the last two hours of the experiment (Supplemental Figure 7).

To further verify these findings and study what happens at a somewhat later stage after oral administration, we performed a follow-up experiment in female mice from these three mouse strains, administering 40 mg/kg cintirorgon. At this higher dosage, the peak concentrations of cintirorgon no longer showed significant differences among the three mouse strains, but the relative order remained the same as in the 20 mg/kg experiment, perhaps suggesting initial saturation of CYP3A-mediated metabolism of cintirorgon (Fig. 6D and Supplemental Table 3). The oral $AUC_{0\text{-}8h}$ in *Cyp3a*$^{-/-}$ was 1.4-fold higher ($P < 0.05$) than in wild-type mice. Moreover, cintirorgon plasma exposure in Cyp3aXAV mice was reduced by 1.5-fold ($P < 0.01$) compared to *Cyp3a*$^{-/-}$ mice (Fig. 6F and Supplemental Table 3). Intriguingly, the elimination was markedly decreased in all three strains from 2 h on after oral administration. This may further suggest that substantial enterohepatic circulation of cintirorgon occurred, leading to reduced net elimination. Interestingly, the plasma concentrations of cintirorgon in mice were quite similar at the 2 h and 8 h time points. This might reflect that the rate of reabsorption of cintirorgon from the small intestine lumen could nearly compensate the elimination rate of cintirorgon from the systemic circulation. Among the three mouse strains, no meaningful and significant differences were found in the relative accumulation of cintirorgon in a number of tested tissues (Supplemental Figure 8). This suggests that mouse Cyp3a and human CYP3A4 can mediate significant metabolism of cintirorgon, thus limiting its oral availability.

4. Discussion

We found that, *in vitro*, cintirorgon is modestly transported by mouse Abcg2 and human ABCB1, but not noticeably by human ABCG2. Oral cintirorgon was rapidly absorbed in all tested strains. Depending on the dose, the cintirorgon plasma AUC was significantly, albeit modestly, increased in *Abcg2*$^{-/-}$ and *Abcb1a/1b;Abcg2*$^{-/-}$ mice, but not in *Abcb1a/1b*$^{-/-}$ mice. Moreover, brain-to-plasma ratios of cintirorgon were markedly enhanced in *Abcb1a/1b*$^{-/-}$ and *Abcb1a/1b;Abcg2*$^{-/-}$ mice, but not in single *Abcg2*$^{-/-}$ mice. Therefore, ABCG2 may perhaps limit cintirorgon oral availability, whereas ABCB1 in the BBB can restrict the brain penetration of cintirorgon. We further observed an increased plasma exposure and reduced liver distribution of cintirorgon in *Slco1a/1b*$^{-/-}$ compared to wild-type mice, but humanized transgenic Slco1B1 and Slco1B3 failed to rescue these changes in *Slco1a/1b*$^{-/-}$ mice. This indicates that mouse Oatp1a/1b, but not human OATP1B1 or OATP1B3, can substantially mediate the hepatic uptake of cintirorgon, thus limiting its oral availability. Cintirorgon oral availability was further restricted by mouse Cyp3a and human CYP3A4, but its relative tissue distribution was not meaningfully altered. This suggests that CYP3A contributes to the metabolic clearance of cintirorgon in both species. Moreover, substantial enterohepatic circulation of cintirorgon was observed from 2 h on after oral administration, resulting in a low elimination rate.

The transcription factor RORγ regulates, amongst others, the expression of the pro-inflammatory cytokine IL-17 in human $CD4^+$ T helper 17 cells. The activation of RORγ can be useful for anticancer therapy but may induce multiple IL-17-mediated autoimmune diseases

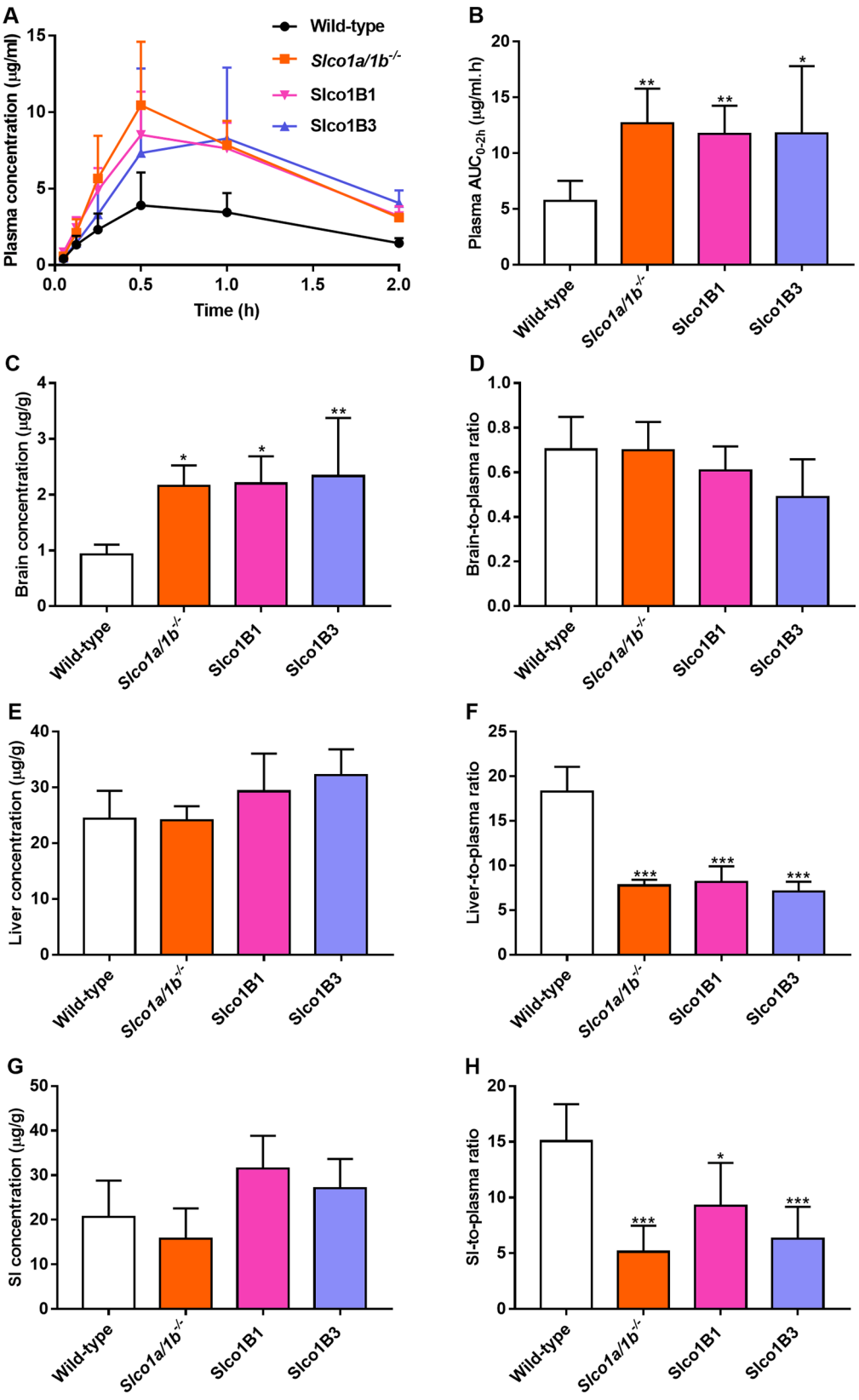

Fig. 4. Plasma concentration–time curves (A), plasma $AUC_{0\text{-}2h}$ (B), brain, liver, and small intestine (SI) concentrations (C, E, G), and brain-, liver-, and SI-to-plasma ratios (D, F, H) of cintirorgon in male wild-type, *Slco1a/1b*$^{-/-}$, Slco1B1, and Slco1B3 mice 2 h after oral administration of 40 mg/kg cintirorgon. Data are presented as mean ± SD (n = 6–7). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ compared to wild-type mice.

Table 2
Plasma, brain, liver, and small intestine pharmacokinetic parameters of cintirorgon 2 h after oral administration of 40 mg/kg cintirorgon to male wild-type, *Slco1a/1b*$^{-/-}$, Slco1B1, and Slco1B3 mice.

Parameter	Genotype			
	Wild-type	*Slco1a/1b*$^{-/-}$	Slco1B1	Slco1B3
$AUC_{0\text{-}2h}$, μg/ml·h	5.36 ± 1.90	12.67 ± 3.13**	11.73 ± 2.52**	11.79 ± 6.02*
Fold increase $AUC_{0\text{-}2h}$	1.00	2.36	2.19	2.20
C_{max}, μg/ml	4.11 ± 2.03	10.77 ± 3.64**	10.25 ± 3.22*	10.94 ± 7.46*
T_{max}, h	0.5–1	0.5–1	0.5–1	0.5–1
C_{brain}, μg/g	0.93 ± 0.17	2.16 ± 367*	2.20 ± 0.49*	2.33 ± 1.04**
Fold increase C_{brain}	1.00	2.32	2.37	2.51
Brain-to-plasma ratio	0.70 ± 0.15	0.70 ± 0.13	0.61 ± 0.11	0.49 ± 0.17
Fold change ratio	1.00	1.00	0.87	0.70
C_{liver}, μg/g	24.4 ± 4.9	24.1 ± 2.5	29.3 ± 6.7	32.2 ± 4.6
Fold change C_{liver}	1.00	0.99	1.20	1.32
Liver-to-plasma ratio	18.3 ± 2.8	7.8 ± 0.6***	8.1 ± 1.8***	7.1 ± 1.1***
Fold change ratio	1.00	0.43	0.44	0.39
C_{SI}, μg/g	20.6 ± 8.2	15.8 ± 6.7	31.5 ± 7.4	27.1 ± 6.5
Fold change C_{SI}	1.00	0.77	1.53	1.32
SI-to-plasma ratio	15.1 ± 3.3	5.1 ± 2.3***	9.3 ± 3.8*	6.3 ± 2.9***
Fold change ratio	1.00	0.34	0.62	0.42

Data are presented as mean ± SD (n = 6–7). $AUC_{0\text{-}2h}$, area under the plasma concentration–time curve; C_{max}, maximum concentration in plasma; T_{max}, time point of maximum plasma concentration (range for individual mice); SI, small intestine; $C_{brain/liver/SI}$, brain/liver/SI concentration. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ compared to wild-type mice.

[26]. However, in a recent phase I clinical trial, the synthetic RORγ agonist cintirorgon was considered safe to use in several types of cancer; most treatment-related adverse effects were observed at grade 1–2 severity levels, i.e., fairly mild [13]. No signs of severe toxicity of cintirorgon were found in our study, but we did find that some mice tended to continue rubbing their snouts to the bottom of the cage, suggesting they were trying to remove the dosing solution, perhaps due to an unpleasant taste or feel of the compound. This toxicity sign happened consistently immediately after oral administration both at 20 mg/kg and 40 mg/kg cintirorgon, and it disappeared within a few minutes. Even though there was a large amount of 5% glucose water (w/v) in our dosing solution, it appears that the flavor of cintirorgon was still not very pleasant to the mice. This might also be a factor to be considered for designing the formulation of cintirorgon for patients.

Upon oral administration of cintirorgon at 20 mg/kg, the peak concentrations and plasma exposure of cintirorgon were each markedly increased by 1.7-fold ($P < 0.05$) in *Abcb1a/1b;Abcg2*$^{-/-}$ mice relative to wild-type mice. The plasma levels of cintirorgon in wild-type mice, however, were well below those observed in patients. Since linear plasma pharmacokinetics of cintirorgon was observed in the clinic, we increased the dosage of cintirorgon from 20 mg/kg to 40 mg/kg to achieve plasma concentrations in mice more similar to plasma levels in patients [13]. With 40 mg/kg cintirorgon, a substantially more than linear increase in plasma $AUC_{0\text{-}2h}$ was found in wild-type mice compared with the 20 mg/kg dose, suggesting possible saturation of cintirorgon clearance mechanisms (Supplemental Table 4 and 5). At 40 mg/kg an increase in plasma $AUC_{0\text{-}2h}$ relative to wild-type mice was observed in all the ABC transporter knockout strains, but only in *Abcg2*$^{-/-}$ mice this was statistically significant. These results may therefore represent that Abcb1a/1b and Abcg2 could modestly limit cintirorgon oral availability. However, their relative impact appeared to be lower at a higher dose. This effect might be the result of partial saturation of the Abcb1 and Abcg2 systems normally limiting the oral availability of cintirorgon at the higher cintirorgon dose, thus contributing to a more than linear increase in cintirorgon plasma exposure. Nonetheless, the finding that the more-than-linear increase in plasma AUC was just as prominent in *Abcb1a/1b;Abcg2*$^{-/-}$ as in wild-type mice (3.6- vs 3.9-fold, respectively, Supplemental Table 4) suggests that other clearance mechanisms were more relevant for this saturation phenomenon.

Unexpectedly, even with a negatively charged carboxyl group, cintirorgon showed a good brain penetration in wild-type mice, with a brain-to-plasma ratio of 0.7. This ratio is comparable and even higher than that of lorlatinib (0.46), a quite hydrophobic ALK/ROS1 inhibitor with a compact macrocyclic structure and lower molecular weight (Supplemental Fig. 1B), and far higher than that of most other TKIs tested to date [23,27,28]. In general, hydrophobic molecules and small polar molecules can more easily diffuse through lipid layers than ions and large polar molecules. The relatively high intrinsic BBB permeability of cintirorgon might therefore be due to the existence of one or more uptake system(s), which could actively take up cintirorgon across the BBB from plasma. Although we do not have direct evidence for involvement of uptake systems from the *in vitro* uptake experiments, it seems very unlikely that a quite large, negatively charged compound like cintirorgon would readily pass the barrier without involvement of a facilitating transport system. It is, however, unlikely that OATP1A/1B transporters play a meaningful role in this process, as we did not see any significant change in brain-to-plasma ratios between wild-type and *Slco1a/1b*$^{-/-}$ mice. There are some indications that the OATP2B1 uptake transporter is expressed in the brain [29], which might perhaps contribute to the good brain penetration of cintirorgon. It is further worth noting that a relatively high transmembrane permeability of a compound, for whatever reason (extensive passive diffusion or net uptake transport), will tend to reduce the impact of the ABC efflux transporters, as they will need to work against a greater influx rate. It may thus be that the intrinsically high brain uptake of cintirorgon is in part responsible for the comparatively modest impact of ABCB1 on its brain penetration, as discussed below. These findings illustrate that, based only on physicochemical properties and molecular structure, it is still very difficult to reliably predict the intrinsic BBB permeability of drugs.

While cintirorgon showed a relatively good brain penetration in wild-type mice, this could still be increased by more than 2-fold by removing ABCB1 from the BBB. This finding might be relevant for improving the therapeutic efficacy of cintirorgon against brain malignancies, assuming that ABCB1 has a similar effect in the human brain as in the mouse brain. Moreover, due to longer patient survival resulting from newer, more effective treatment modalities, the incidence of brain metastases is on the increase, especially for lung cancer patients [30]. Considering a broader clinical use of cintirorgon, we could apply these insights to boost the brain penetration of cintirorgon using pharmacological inhibitors of ABCB1, such as elacridar. On the other hand, the comparatively modest interaction between cintirorgon and ABCB1 we observed also reduces the risk of dramatic changes in potential (CNS) toxicity of cintirorgon.

Of note, many ABCB1/P-glycoprotein substrates contain aromatic rings and are hydrophobic or possess a partially positively charged hydrophilic moiety. It is not common for ABCB1 substrate to be negatively charged, although some have been described. Similar to cintirorgon, at physiological pH, methotrexate (pKa = 3.04–4.99) and atorvastatin (pKa = 4.46) are negatively charged, and can also be transported by ABCB1 [31,32]. In addition, some zwitterionic compounds (e.g., phosphatidylcholine analogues) are also substrates of ABCB1 [33]. The diversity of ABCB1 substrates shares a common structural denominator in that these compounds nearly always contain spatially separated hydrophilic and hydrophobic moieties, as also applies to cintirorgon. These physicochemical characteristics may be related to the mechanism of drug transport by ABCB1, which possibly depends on the ability of the drug to insert into one hemileaflet of the plasma membrane. ABCB1 may

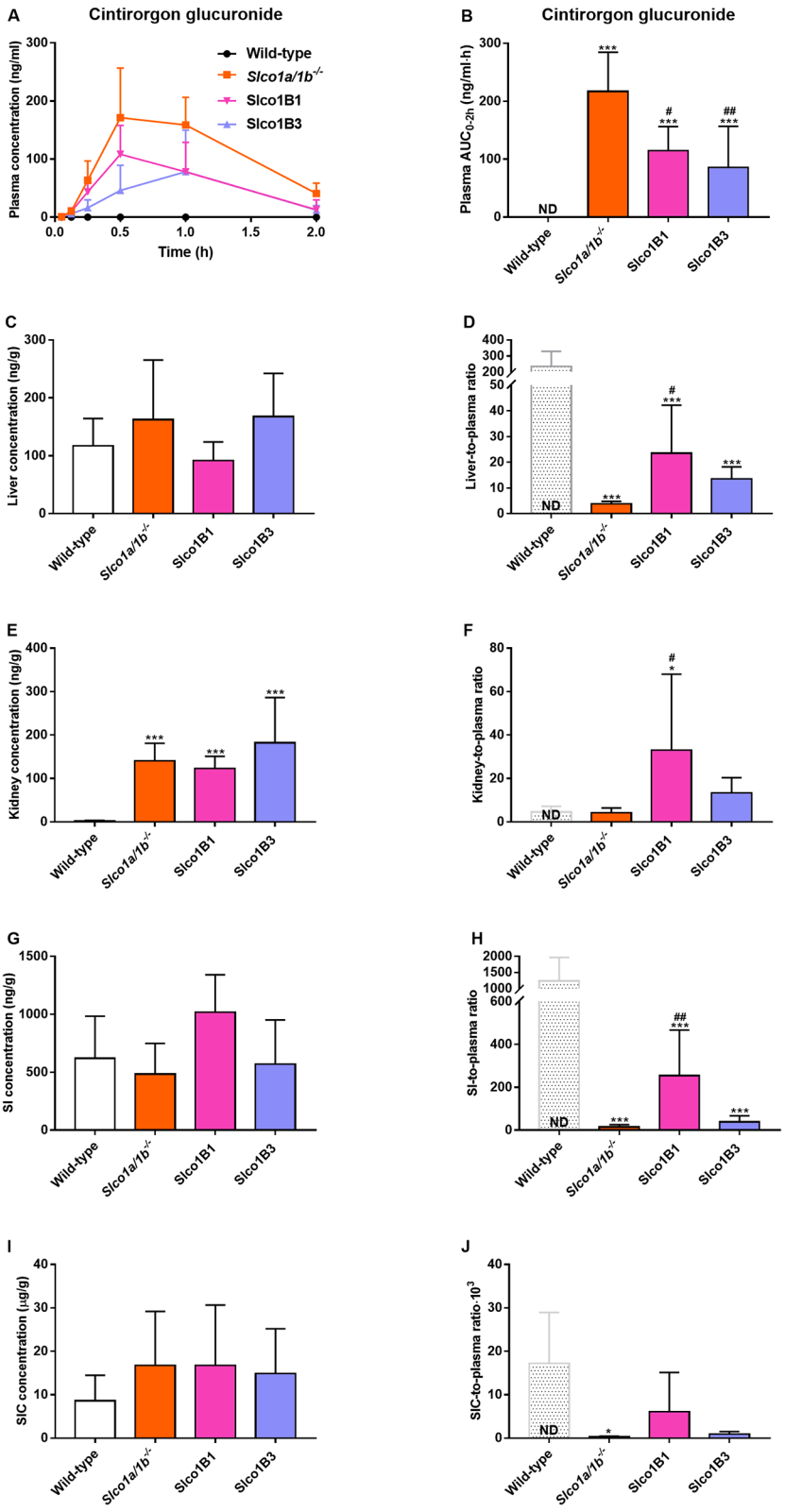

Fig. 5. Semi-quantified plasma concentration–time curves (A), plasma $AUC_{0\text{-}2h}$ (B), and tissue concentrations of cintirorgon glucuronide (C, E, G, I) based on the cintirorgon concentrations, and quantified tissue-to-plasma ratios of cintirorgon glucuronide (D, F, H, J) in male *Slco1a/1b*$^{-/-}$, Slco1B1, and Slco1B3 mice 2 h after oral administration of 40 mg/kg cintirorgon. No cintirorgon glucuronide was found in plasma of wild-type mice. The semi-quantified lower limit of quantification (LLoQ) of cintirorgon glucuronide was 0.5 ng/ml. The light-grey stippled bar value was a minimum estimate of the tissue-to-plasma ratio based on LLoQ values in wild-type plasma. ND, not detectable. ND with a stippled bar, not quantitatively determined. Data are presented as mean ± SD (n = 6). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ compared to wild-type mice; $^{\#}$, $P < 0.05$; $^{\#\#}$, $P < 0.01$ compared to *Slco1a/1b*$^{-/-}$ mice.

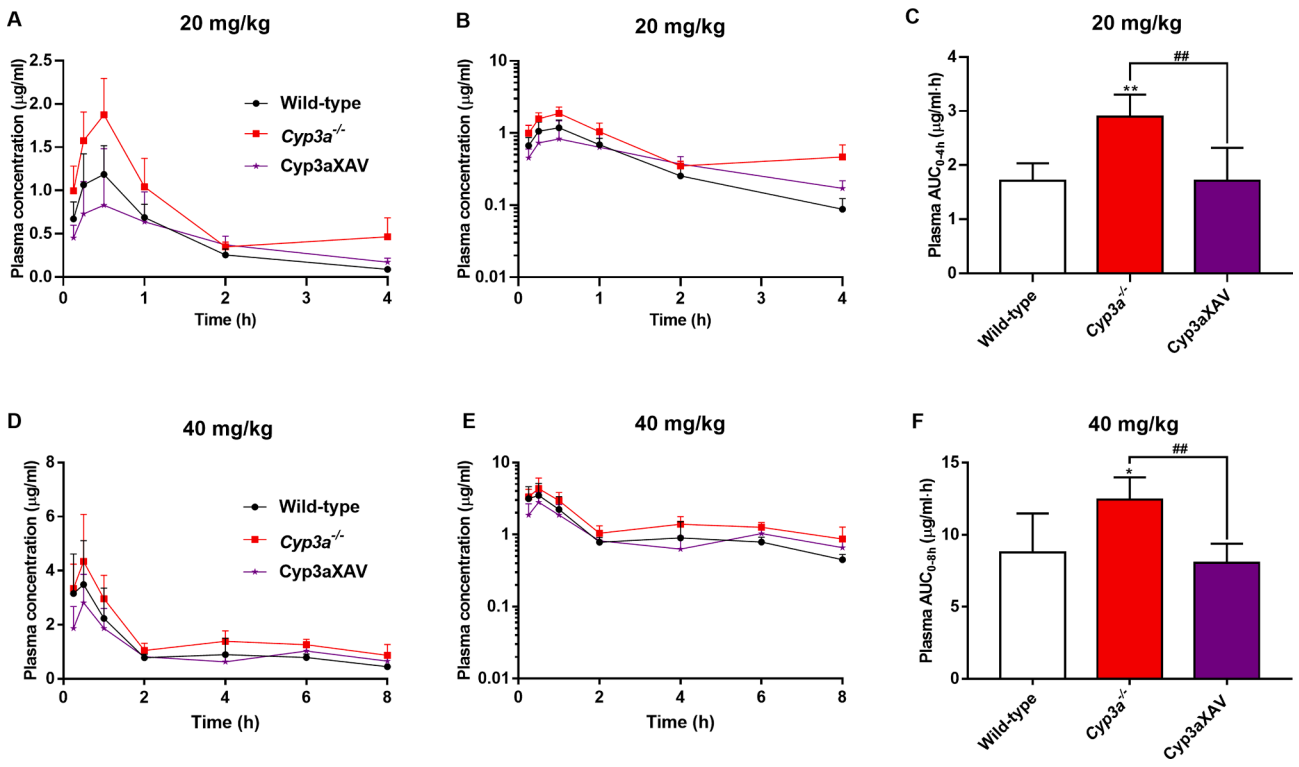

Fig. 6. Plasma concentration–time curves (A, D), semi-log plot of plasma concentration time curves (B, E) and plasma AUC (C, F) of cintirorgon in female wild-type, *Cyp3a*$^{-/-}$ and Cyp3aXAV mice after oral administration of 20 mg/kg (over 4 h, n = 6) and 40 mg/kg (over 8 h, n = 6–7) cintirorgon, respectively. Data are given as mean ± SD. *, $P < 0.05$; **, $P < 0.01$ compared to wild-type mice. $^{\#}$, $P < 0.05$; $^{\#\#}$, $P < 0.01$ comparing Cyp3aXAV to *Cyp3a*$^{-/-}$ mice.

translocate many of its substrates mainly by 'flipping' them actively from the inner to the outer leaflet of the lipid membrane bilayer, yielding a net efflux of drug [34].

Most OATP substrates are large hydrophobic anions with molecular weight >350 Da, which makes cintirorgon a potential OATP substrate based on its chemical structure [18]. Indeed, our current work demonstrates that mouse Oatp1a/1b transporters can markedly affect the plasma levels, liver distribution, and intestinal disposition of cintirorgon. However, human OATP1B1 and OATP1B3 showed limited impact on the plasma levels and tissue distribution of cintirorgon. This suggests that transgenic OATP1B1 or OATP1B3 expression may not be enough to noticeably compensate for the loss of mouse Oatp1a/1b function with respect to cintirorgon, although they do rescue the hyperbilirubinemia seen in Oatp1a/1b knockout mice [35]. Such a situation has been shown before for validated *in vitro* transport substrates such as rosuvastatin and pitavastatin [36]. Moreover, we performed an *in vitro* uptake experiment, in which we did not observe noticeable uptake transport of cintirorgon in HEK293 cells overexpressing human OATP1B1 or OATP1B3 compared to parental HEK293 cells (data not shown). However, as the background cintirorgon uptake in the parental HEK293 cells was already high, the sensitivity of this assay was probably low. In any case, it is reasonable to conclude that cintirorgon is not a strong substrate of human OATP1B1 and OATP1B3. In contrast, we found that hepatic overexpression of human OATP1B1, and perhaps OATP1B3, could partially rescue the altered pharmacokinetics of cintirorgon glucuronide in *Slco1a/1b*$^{-/-}$ mice. These data support that mouse and human OATP1A/1B transporters have a substantial, but certainly not complete overlap in substrate spectrum.

In addition, even though based on semi-quantification, one could hypothesize that the large amount of cintirorgon glucuronide detected in the small intestinal content in all mouse strains may partially explain the slow elimination of cintirorgon from the plasma, especially from 2 h on (Fig. 6). The cintirorgon glucuronide may behave as a deposit station releasing the cintirorgon upon hydrolysis by certain enzymes in the small intestinal lumen. The newly re-generated cintirorgon can then be reabsorbed into the system circulation, reducing the apparent overall elimination rate of cintirorgon.

To date, the possible interaction of cintirorgon and CYP3A is largely unknown based on publicly available information. In our study, we observed that mouse Cyp3a and human CYP3A4 can both modestly but significantly limit the plasma concentrations and thus the systemic exposure of cintirorgon, whereas the relative tissue distribution of cintirorgon was not meaningfully altered. Moreover, the much more than linear increases in plasma $AUC_{0\text{-}2h}$ upon dose increase in wild-type and Cyp3aXAV mice, but not in *Cyp3a*$^{-/-}$ mice (Supplemental Table 5) suggests that Cyp3a and CYP3A4 may be important factors in this saturation process. Based on the moderate impact of CYP3A on oral availability of cintirorgon we observed, coadministration of CYP3A inhibitors or inducers is likely to affect the pharmacokinetics of cintirorgon in patients, but probably only modestly. Therefore, possible effects on the therapeutic efficacy or toxicity of cintirorgon may also be modest. However, this still needs to be carefully assessed in humans first, especially as, based on our findings, the relative impact may be dose- or exposure-dependent.

In this study, we demonstrated various potentially therapeutically relevant effects of ABCB1 and ABCG2, OATP1A/1B, and Cyp3a and CYP3A4 on oral cintirorgon pharmacokinetics. Obviously, these findings will first need to be tested in their own right for their applicability in patients, before using them in efforts to further improve the therapeutic efficacy and safety of cintirorgon.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejpb.2022.06.008.

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