SUPPLEMENTARY MATERIALS AND METHODS

Chemicals and reagents
Everolimus and 7-ethyl-10-hydroxycamptothecin (SN-38) were purchased from Sequoia Research Products (Pangbourne, UK). Irinotecan HCl-trihydraat was from Hospira Benelux BVBA, Brussels, Belgium. Bis(4-nitrophenyl) phosphate (BNPP) was from Sigma-Aldrich (Steinheim, Germany). EDTA disodium salt pH 8.0 was from Cambrex BioScience Inc. (Rockland, ME). Bovine serum albumin (BSA), fraction V, was purchased from Roche (Mannheim, Germany). Tetra-n-butylammonium bromide (TBABr) was purchased from Merck Schuchardt (Hohenbrunn, Germany). Acetonitrile (HPLC grade) was from Biosolve (Valkenswaard, the Netherlands). Heparin (5000 IE/ml) was purchased from Leo Pharma BV (Breda, the Netherlands). Isoflurane (Forane) was from Abbott Laboratories (Queenborough, Kent, UK). All other chemicals and reagents were obtained from Sigma-Aldrich (Steinheim, Germany).

Animals
Mice were housed and handled according to institutional guidelines complying with Dutch legislation. Male wild-type, Abcb1a/1b(-/-) (1), Abcg2(-/-) (2), Abcb1a/1b;Abcg2(-/-) (3), Cyp3a(-/-) and Abcb1a/1b;Cyp3a(-/-) (4) mice, all of a >99% FVB genetic background, were used between 8 and 14 weeks of age. Animals were kept in a temperature-controlled environment with a 12-hr light/12-hr dark cycle and received a standard diet (AM-II, Hope Farms B.V., Woerden, The Netherlands) and acidified water ad libitum. Mice that were fed with a semi-synthetic diet (20% casein, 4068.02; Hope Farms B.V., Woerden, The Netherlands) received this for 8 weeks prior to oral administration of everolimus.

Everolimus and metabolite analysis
Everolimus concentrations in whole blood, plasma, blood cells, brain homogenates and liver homogenates were determined using liquid chromatography coupled to tandem mass
spectrometry (LC-MS/MS) in the selected reaction monitoring mode. Metabolite A was quantified semi-quantitatively.

The equipment consisted of an Accela pump and autosampler and a TSQ Quantum Ultra quadrupole mass spectrometer with heated electrospray ionization (Thermo Fisher Scientific, San Jose, CA, USA). Chromatographic separations of the analytes were carried out on an Aquity UPLC® BEH C18 300 column (50x2.1mm, dp = 1.7 µm, Waters, Milford, USA) with an Aquity UPLC® BEH C18 VanGuard pre-column (Waters, 5x2.1mm, dp = 1.7 µm). The column temperature was maintained at 60°C and the autosampler was maintained at 4°C. A mobile phase consisting of eluent A (1% (v/v) formic acid in water) and eluent B (methanol) was pumped through the column with a flow rate of 0.75 ml/min. Linear gradient elution was used from 50% to 80 % eluent B during 4 min, followed by a linear increase to 100% B during 0.5 min. Between 4.5 and 5 min, the composition was maintained at 100% eluent B. The percentage of eluent B was reduced to the initial composition (50% B) and from 5.01 to 6 min the column was reconditioned until the start of the next injection. The eluate was totally led into the electrospray probe from 2 to 4.96 min after injection. Electrospray settings were a 3900 V spray voltage, a 400°C capillary temperature, a 250°C vaporizer temperature and a 2.0 mTorr argon collision pressure. Dwell times were 0.05 s, skimmer off set was -8 V and the mass resolution was set at 0.7 full with at half height (unit resolution) for both separating quadrupoles. Mass transitions were m/z 980.6>389.2 for everolimus and metabolites and m/z 984.6>393.2 for the internal standard (IS; everolimus-d₄). The tube lens off set was 180 V and the collision energy -52 V for all compounds. The retention times were 3.7 min for everolimus and IS and 3.8 min for metabolite A.

Mouse blood, plasma or tissue homogenate samples (50 µl) were pipetted into a well of a 96-well plate (0.5 ml), 50 µl of 500 ng/ml IS in 50% (v/v) methanol was added. After vortex mixing shortly, adding 150 µl of acetonitrile and vortex mixing again, the precipitate was separated by centrifuging at 2,643 g for 5 min. The clear supernatant was transferred into a second plate (1 ml wells) and 300 µl of 25% methanol (v/v) was added. After closing the wells and shaking manually 2 µl of the samples was injected.
Stability of everolimus in mouse plasma preincubated with BNPP

Fresh plasma was obtained from wild-type, \textit{Abcb1a/1b}(-/-), \textit{Abcg2}(-/-) and \textit{Abcb1a/1b;Abcg2}(-/-) mice. Experiments were carried out in 1.5 ml Eppendorf tubes at a total volume of 200 µl. For the inhibition experiment, 10 µl of BNPP stock (20 mM) was added to 180 µl plasma and incubated for 15 min at 37°C with gentle shaking. The reaction was then initiated by mixing 10 µl of everolimus with 190 µl of mouse plasma with or without BNPP and allowed to proceed at 37°C for 8 hr with gentle shaking. Final concentrations were 4000 ng/ml (everolimus) and 1 mM (BNPP). Samples (30 µl) were collected at different time points until 8 hr, and stored frozen at -20°C until analysis.

Inhibitory effect of everolimus on irinotecan hydrolase activity by mouse plasma

\textit{Ces1} activity in individual mouse plasma samples was measured using irinotecan hydrolysis. Fresh plasma was obtained from wild-type, \textit{Abcb1a/1b}(-/-), \textit{Abcg2}(-/-) and \textit{Abcb1a/1b;Abcg2}(-/-) mice. Irinotecan hydrolysis was tested in 1.5 ml Eppendorf tubes in a total volume of 150 µl (containing 120 µl plasma). Irinotecan at 20 mg/ml was diluted in 20 mM Tris-HCl (pH 7.8) to 50 µM and pre-incubated at 37°C for 30 min to achieve equilibrium between the carboxylate and lactone form. 15 µl of everolimus vehicle, everolimus or BNPP solution was added to the reaction mixture. The final concentrations of ethanol and tween-80 in the reaction mixture were 0.25% and 0.25%, respectively. After a 15-min preincubation at 37°C, the reaction was initiated by mixing 15 µl of irinotecan with 135 µl of mouse plasma with or without inhibitors. The final irinotecan concentration was 5 µM and the mixture was incubated at 37°C for 30 min. Irinotecan and SN-38 concentrations were quantified by high performance liquid chromatography as described (5).

\textbf{siRNA screening and validation}

To select the most efficient siRNA against mouse \textit{Ces1c}, we first screened a pool of three siRNAs targeting mouse \textit{Ces1c} in primary mouse hepatocytes of FVB background. Pre-designed mouse \textit{Ces1c}-specific siRNA (si\textit{Ces1c}) were purchased from Ambion Applied Biosystems, Carlsbad, California, USA (Ambion Silencer® Pre-designed for Ces1c; catalogue numbers
s201305, s65663 and s65665). Ambion’s negative control siRNA (siNEG) (catalogue number 4404020) was used as negative control siRNA. Hepatocytes isolation and siRNA transfection were performed as previously described (6). Sequences from siRNA yielding maximal reduction of Ces1c transcript level at an siRNA concentration of 0.25 nM (catalogue number s65665) was selected for use in in vivo studies.

**Ces1c knockdown in mouse liver**

siNEG and siCes1c (Ambion In-Vivo-Ready, catalogue numbers 4404020 and s65665, respectively) with sense sequence CAG GGA AAC UGG UAC AGU ATT were complexed with Invivofectamine®2.0 Reagent (Invitrogen, Life Technologies Corporation, USA) according to the manufacturer’s protocol. To validate the finding that Ces1c is responsible for the tight binding and stabilization of everolimus, resulting in higher retention of this drug in plasma of knockout and “high” wild-type mice, male Abcb1a/1b(-/-) mice (weighing 27–30 grams) were intravenously injected via the tail with complexed siRNA at a dose of 7 mg siRNA per kg body weight (injection volume 12 ml per kg body weight); in total 12 mice, 5 mice for the siNEG-treated group and 7 mice for the siCes1c-treated group were injected. At three days post siRNA injection, mice were fasted for 3 hr before everolimus was administered (6.7 ml/kg) by gavage into the stomach, using a blunt-ended needle. Tail vein sampling was performed at 5, 15, 30, 60 and 120 min after oral administration, using microvettes containing dipotassium-EDTA. Three hours after oral administration, mice were anaesthetized with isoflurane and blood was collected by cardiac puncture. Blood samples were collected in eppendorf tubes containing 0.5 M disodium-EDTA as anticoagulant. Immediately thereafter, mice were sacrificed by cervical dislocation, and liver and brains were rapidly removed and weighed. A small piece of liver tissue was immediately submerged in RNAlater™ for RNA isolation and real time RT-PCR. Brains and the remaining part of livers were homogenized with 1 ml and 5 ml of 4% BSA, respectively, and stored at -20 °C until analysis.
Inhibitory effects of everolimus on \( p \)-nitrophenyl hydrolase activities by recombinant human CES1 and CES2

Everolimus concentrations that caused 50% inhibition (IC\(_{50}\)) of the \( p \)-nitrophenyl acetate hydrolase activities by recombinant human CES1 and CES2 were determined as described previously (7).

RNA isolation, cDNA synthesis and real-time RT-PCR

RNA isolation from mouse liver and subsequent cDNA synthesis and RT-PCR were performed as described (8). Specific primers (Qiagen, Hilden, Germany) were used to quantify the expression levels of the following mouse carboxylesterase genes: \( Ces1b \), \( Ces1c \), \( Ces1d \), \( Ces1e \), \( Ces1f \), \( Ces1g \), and \( Ces2a \).
Reference List


