P-glycoprotein, CYP3A and plasma carboxylesterase determine brain and blood disposition of the mTOR inhibitor everolimus (Afinitor) in mice

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ABSTRACT

Purpose: To clarify the role of ABCB1, ABCG2, and CYP3A in blood and brain exposure of everolimus using knockout mouse models.

Experimental Design: We used wild-type, Abcb1a/1b(-/-), Abcg2(-/-), Abcb1a/1b;Abcg2(-/-) and Cyp3a(-/-) mice to study everolimus oral bioavailability and brain accumulation.

Results: Following everolimus administration, brain concentrations and brain-to-liver ratios were substantially increased in Abcb1a/1b(-/-) and Abcb1a/1b;Abcg2(-/-), but not Abcg2(-/-) mice. The fraction of everolimus located in the plasma compartment was highly increased in all knockout strains. In vitro, everolimus was rapidly degraded in wild-type but not knockout plasma. Carboxylesterase 1c (Ces1c), a plasma carboxylesterase gene, was highly upregulated (~80-fold) in the liver of knockout mice relative to wild-type mice, and plasma Ces1c likely protected everolimus from degradation by binding and stabilizing it. This binding was prevented by preincubation with the carboxylesterase inhibitor BNPP. In vivo knockdown experiments confirmed the involvement of Ces1c in everolimus stabilization. Everolimus also markedly inhibited the hydrolysis of irinotecan and p-nitrophenyl acetate by mouse plasma carboxylesterase and recombinant human CES2, respectively. After correcting for carboxylesterase binding, Cyp3a(-/-), but not Abcb1a/1b(-/-), Abcg2(-/-), or Abcb1a/1b;Abcg2(-/-) mice, displayed highly (>5-fold) increased oral availability of everolimus.

Conclusions: Brain accumulation of everolimus was restricted by Abcb1, but not Abcg2, suggesting the use of coadministered ABCB1 inhibitors to improve brain tumor treatment. Cyp3a, but not Abcb1a/1b, restricted everolimus oral availability, underscoring drug-drug interaction risks via CYP3A. Upregulated Ces1c likely mediated the tight binding and stabilization of everolimus, causing higher plasma retention in knockout strains. This Ces upregulation might confound other pharmacological studies.
STATEMENT OF TRANSLATIONAL RELEVANCE

Everolimus is currently used to treat breast cancer patients, who have a high risk of developing brain metastases. We show here that brain accumulation of everolimus is markedly restricted by ABCB1 in mice, providing a rationale for combining everolimus with ABCB1 inhibitors to improve its therapeutic efficacy against primary and metastatic brain tumors. CYP3A also strongly restricted the oral availability of everolimus, underscoring drug-drug interaction risks via CYP3A. Unexpectedly, several carboxylesterase (Ces) enzymes were upregulated in Abcb1a/1b and Abcg2 knockout mice, causing a strong increase in everolimus blood levels, apparently by tight binding of everolimus to plasma Ces1c. Numerous pharmacological and pharmacokinetic studies of various drugs using these knockout strains in academia and pharmaceutical companies alike could be confounded by this Ces upregulation. Importantly, our results indicate that everolimus is a human CES1 and CES2 inhibitor, which might be relevant in modulating the efficacy of (pro-)drugs hydrolyzed by especially CES2.
INTRODUCTION

The mammalian target of rapamycin (mTOR) is a serine-threonine protein kinase and downstream effector of the phosphatidylinositol 3-kinase (PI3K)-protein kinase B signaling pathway (1, 2), which controls cell growth, proliferation, survival and metabolism (3, 4). Deregulation of the PI3K-AKT-mTOR signaling pathway occurs in many types of cancers (5-7). The macrocyclic lactone everolimus (Afinitor, Zortress/Certican, SDZ RAD or RAD001; Supplementary Figure 1A), a derivative of rapamycin (sirolimus), is an orally active inhibitor of mTOR used in cancer therapy and as an immunosuppressant to prevent transplanted organ rejection.

Everolimus is used either alone or in combination for treating multiple cancers including advanced renal cell carcinoma (8), subependymal giant cell astrocytoma (9), advanced pancreatic neuroendocrine tumors (10) and advanced hormone receptor-positive, HER-2-negative breast cancer (11). Clinical trials to assess its efficacy in gastric cancer, hepatocellular carcinoma and lymphoma are ongoing, and it appears beneficial in refractory graft-versus-host disease after bone marrow transplantation. Given the sensitivity of human glioma cell lines to everolimus (12, 13), and the alterations in the PI3K-AKT-mTOR pathway in >80% of glioblastoma (14); Cancer Genome Atlas Research Network (2008), it might also benefit the treatment of these primary brain tumors.

The ATP-binding cassette (ABC) drug efflux transporters P-glycoprotein (P-gp; ABCB1) and breast cancer resistance protein (BCRP; ABCG2) are highly expressed in the intestinal epithelium and in the blood-brain barrier (BBB), as well as in many tumors. They can thus confer multidrug resistance, and limit the oral absorption and brain penetration of many clinically used anticancer drugs (15-19), which may well limit their therapeutic efficacy, especially against brain metastases. It is therefore important to know whether everolimus interacts with these transporters. \textit{In vitro}, everolimus is transported by ABCB1 (20) and it inhibits ABCB1 and ABCG2 (21). However, the plasma AUC$_{0-24\text{hr}}$ of everolimus in Abcb1a/1b(-/-) mice was only 1.3-fold higher than in wild-type mice upon oral administration of 0.25 mg/kg everolimus (22), suggesting little influence of Abcb1 on oral availability. Nonetheless, everolimus coadministration could increase the brain accumulation of vandetanib, presumably by inhibiting Abcb1 and Abcg2 activity in wild-type mice (21). Although these studies suggest an interaction of everolimus with ABCB1 and ABCG2 \textit{in vitro} and \textit{in vivo}, the roles of Abcb1 and possibly Abcg2 in brain accumulation of everolimus remain unknown.
In vitro studies supported by clinical data established that everolimus is metabolized by cytochrome P450 3A (CYP3A) (23). This is a concern for drug-drug interactions, as coadministered drugs or food components may drastically alter CYP3A activity, and therefore the systemic levels of orally administered everolimus, resulting in either undertreatment, or serious (life-threatening) side effects of this potentially highly toxic drug. We aimed to clarify the in vivo roles of ABCB1, ABCG2 and CYP3A in oral availability and brain accumulation of everolimus using knockout mouse models, in order to investigate a potential improvement of the therapeutic efficacy of everolimus, especially for brain tumors positioned behind an intact BBB.
MATERIALS AND METHODS

Part of the materials and methods is presented in the supplementary materials and methods section.

Blood pharmacokinetics and tissue disposition of everolimus in mice

Everolimus was dissolved in ethanol:tween-80 (1:1, v/v) to 2 mg/ml and further diluted with saline to yield solutions of 0.3 mg/ml and 0.4 mg/ml for oral and intravenous administration, respectively. To minimize variation in absorption on oral administration, mice were fasted for 3 hr before everolimus was administered (6.7 ml/kg) by gavage into the stomach, using a blunt-ended needle. Three hours later, mice were anesthetized with isoflurane and blood was collected by cardiac puncture. Blood samples were collected in tubes containing Na$_2$EDTA as an anticoagulant. Immediately thereafter mice were sacrificed by cervical dislocation, and livers and brains were rapidly removed. Brains and livers were homogenized with 1 ml and 5 ml of 4% BSA, respectively and stored at -20°C until analysis. For intravenous administration, mice were injected with a single bolus of everolimus (5 ml/kg) via the tail vein. One hour later, mice were anesthetized with isoflurane and blood was collected by cardiac puncture. Immediately thereafter mice were sacrificed by cervical dislocation, and livers and brains were processed as described above.

Blood cell distribution and tissue disposition of intravenous everolimus in mice

To obtain complete plasma pharmacokinetics and tissue concentration curves of everolimus, the experiment was initially terminated at 5, 30 and 60 min, and later extended to 2, 4 and 8 hr. Everolimus was administered intravenously to mice, and at the aforementioned time-points, mice were anesthetized and blood was collected by cardiac puncture. Immediately thereafter mice were sacrificed by cervical dislocation, and livers and brains were processed as described above. 50 µl of blood samples was transferred to new eppendorf tubes and stored at -20°C for further analysis. The remaining blood samples were immediately centrifuged at 2,100 x g for 6 min at 4°C, and plasma and blood cell fractions were then collected and stored at -20°C for further analysis. The measured everolimus concentrations in plasma and blood cell fractions were adjusted to correspond to the volume ratio between plasma and red blood cells in total blood composition, which is 0.63 and 0.37, respectively.
Stability of everolimus in mouse plasma in vitro

Blood was freshly collected by cardiac puncture in anesthetized wild-type, Abcb1a/1b(-/-), Abcg2(-/-) and Abcb1a/1b;Abcg2(-/-) mice, followed by centrifugation at 2,100 x g for 6 min at 4°C for the separation of plasma from blood cells. The test was initiated by mixing 20 µl of everolimus solution with 980 µl of plasma pooled from mice of the same genotype to achieve final concentrations of 250, 1000 or 4000 ng/ml. The mixture was incubated at 37°C for 8 hr with gentle shaking. Samples (50 µl) were collected at different time points until 8 hr, and were stored frozen at -20°C until analysis.

Stability of everolimus in knockout plasma diluted with increasing amounts of wild-type plasma

Blood was freshly collected as described above. Pooled Abcb1a/1b(-/-), Abcg2(-/-) and Abcb1a/1b;Abcg2(-/-) plasma was diluted with increasing amounts of wild-type plasma, at dilution factors between 2 to 125-fold. The reaction was initiated by mixing 15 µl of everolimus with 735 µl of knockout plasma with or without increasing amounts of wild-type plasma. The final everolimus concentration was 4000 ng/ml and the mixture was incubated at 37°C for 8 hr with gentle shaking. Samples (50 µl) were collected at different time points until 8 hr, and stored frozen at -20°C until analysis.

Statistical analysis

Data are presented as means ± SD. One-way analysis of variance (ANOVA) was used to determine the significance between groups, after which post-hoc tests with Bonferroni correction were performed for comparison between individual groups. Differences were considered statistically significant when $P < 0.05$. 
RESULTS

Everolimus pharmacokinetics and tissue disposition in vivo

To assess the impact of Abcb1 and Abcg2 on oral bioavailability and tissue disposition of everolimus, we administered everolimus (2 mg/kg) orally or intravenously to wild-type, Abcb1a/1b(−/−), Abcg2(−/−) and Abcb1a/1b;Abcg2(−/−) mice, and measured blood and tissue concentrations by LC-MS/MS. 3 hr after oral administration, 7 out of 10 wild-type mice had low blood levels of everolimus, while 3 had ~50-fold higher levels (Figure 1A). No wild-type mice had intermediate blood everolimus levels, implying the existence of two clearly distinct groups. Also in later experiments a variable, but usually minor fraction of wild-type mice displayed much higher everolimus blood levels. We therefore separately present data for the “low” and “high” everolimus wild-type mice. No “high” everolimus wild-type mice were present in the parallel intravenous experiment, assessed 1 hr after administration (Figure 1B). Everolimus blood levels in all the knockout strains were ~80-fold higher upon oral administration, and ~16-fold higher upon i.v. administration than those obtained in the “low” wild-type mice.

In spite of the large differences in blood everolimus levels, the liver concentrations in wild-type (low and high) and knockout strains were quite similar, regardless of the route of administration (Figure 1C and D). This suggested that some factor(s) affecting the blood-tissue distribution behavior of everolimus had drastically changed in the knockout strains, and likely also in the “high” wild-type mice, relative to the “low” wild-type mice.

To correct for possibly altered blood-tissue distribution behavior of everolimus, we plotted both the direct brain concentrations (Figure 1E and F), and the brain-to-liver concentration ratios in the different strains (Figure 1G and H), rather than the brain-to-blood concentration ratios. We assumed that altered free everolimus concentrations in plasma of knockout strains would similarly affect drug distribution to liver and brain. We thus used liver as a probe for the level of free everolimus in plasma. The brain-to-liver concentration ratios suggested that Abcb1a/1b(−/−) and Abcb1a/1b;Abcg2(−/−) mice had 10-14-fold increased brain accumulation of everolimus relative to wild-type mice (both “low” and “high”, \( P < 0.001 \)), whereas Abcg2(−/−) mice had ~3-fold increased brain accumulation (\( P < 0.05 \)) upon oral administration (Figure 1G). Despite the 50-fold higher blood concentration of everolimus in the “high” versus “low” wild-type mice, brain concentrations and brain-to-liver ratios between these groups
were not significantly different (Figure 1E and G). Upon i.v. administration, brain-to-liver ratios were ~8-fold increased in both Abcb1-deficient strains \((P < 0.001)\), and not altered in the \(Abcg2(-/-)\) strain (Figure 1H). Collectively, the data suggest that Abcb1 strongly restricts the brain accumulation of everolimus, whereas Abcg2 has little, if any, impact on brain accumulation of everolimus.

**Blood cell distribution and tissue disposition of everolimus in vivo**

The discrepancy between blood concentration and liver (plus brain) accumulation data of everolimus between the strains suggested the existence of strong everolimus retention factors in the blood of the knockout strains, and presumably also the “high” wild-type mice. Since everolimus in blood can sometimes distribute very extensively to red blood cells (e.g., ~80% in humans) (24), we assessed the *in vivo* plasma to blood-cells distribution of everolimus in the different strains, as well as the liver and brain accumulation, at 5, 30, and 60 min after i.v. administration at 2 mg/kg. We again observed “low” and “high” wild-type mice in the 5 min and 60 min (but not the 30 min) groups (Figure 2A, B), and while there was obvious (and significant) everolimus clearance in blood and plasma of the “low” wild-type mice between 5 and 60 min (3- to 4-fold decrease), this was not seen in the other strains. Blood levels of everolimus were greatly and similarly increased in all the knockout strains. Importantly, there was only little distribution of everolimus to the blood cells relative to whole blood and plasma, ranging from about 6% in the “low” wild-type mice to well below 2% in all the knockout strains (Figure 2C, D). Altered retention in blood cells could therefore not explain the marked alterations in total blood levels of everolimus. To better understand the tissue concentration during the plasma clearance phase, especially in the “high” wild-type and knockout strains, the experiment was extended to 2, 4 and 8 hr. Liver and brain accumulation in this experiment (Figure 2E-H) reflected the patterns observed in Figure 1, with low plasma clearance in all strains except for the “low” wild-type mice, similar levels of everolimus in liver, and highly increased brain concentrations and brain-to-liver ratios in \(Abcb1a/1b(-/-)\) and \(Abcb1a/1b;Abcg2(-/-)\) mice. Note that in all strains a substantial fraction (~30% of the dose) of everolimus had accumulated in the liver within 5 min, which was then gradually cleared at similar rates (Figure 2F).

The higher blood cells-to-blood ratios in the “low” wild-type mice versus all the knockout strains and the “high” wild-type mice (Figure 2D) suggested higher retention of everolimus in the plasma of the latter strains. This might also explain why there was little clearance of everolimus from
plasma in the knockout strains after i.v. administration (Figure 2A, B and E). Note that, at 4000 ng/ml, a substantial fraction (~10%) of the administered everolimus dose was retained in the plasma.

**Stability of everolimus in plasma of wild-type and knockout mice in vitro**

Attempts to assess *in vitro* whether knockout and wild-type plasma had different levels of free and (protein-)bound amounts of everolimus failed because of the rapid disappearance of everolimus from wild-type plasma (data not shown). Indeed, a possible cause of the very different plasma levels of everolimus might be greater stability of everolimus in knockout plasma relative to the (“low”) wild-type plasma. We therefore incubated various concentrations of everolimus (roughly covering the concentration range seen in Figure 2B) in plasma of the different strains *in vitro* at 37°C, and measured presence of everolimus over time. Everolimus itself was quite stable in saline at all concentrations (data not shown). Interestingly, whereas there was very limited loss of everolimus at 250 ng/ml in plasma of all strains, at 4000 ng/ml there was marked loss in the wild-type plasma but not in the knockout plasmas. At 1000 ng/ml an intermediate pattern was observed (Figure 3A-C).

The stability of everolimus in wild-type plasma at low concentrations and its relative instability at high concentrations suggested that a stabilizing plasma protein fully protected low amounts of everolimus. Upon saturation of this protein, more free everolimus existed, that was degraded in wild-type plasma by an as yet unidentified plasma enzyme. The results of figure 3A-C imply a much higher level of the stabilizing protein in the knockout plasmas than in the wild-type plasma, whereas the level of the everolimus-degrading enzyme might be similar between the strains. Alternatively, wild-type plasma might have much higher levels of an everolimus-degrading plasma enzyme, whereas all the strains had similar (low) levels of everolimus-stabilizing protein. To distinguish between these two hypotheses, we repeated the everolimus stability experiment at 4000 ng/ml with mixtures at various ratios of wild-type and knockout plasmas. In case of much higher concentrations of an everolimus-degrading enzyme in wild-type plasma, a 1 to 1 (i.e., 2-fold) dilution of wild-type plasma with knockout plasma should result in a 2-fold lower degradation (loss) rate of everolimus. However, the results of Figure 3D-F show that the everolimus loss rate was much more than 2-fold decreased in the 2-fold dilution mixtures compared to undiluted wild-type plasma. For example, interpolation of the data in Figure 3D indicated an initial everolimus loss rate of 2500 ng/ml/hr in wild-type plasma, and 100 ng/ml/hr in *Abcb1a/1b(-/-)* plasma. The predicted everolimus degradation rate for the 1:1 dilution in
case of a higher concentration of everolimus-degrading enzyme in the wild-type plasma would have been: \( \frac{1}{2} \times (100 + 2500) = 1300 \) ng/ml/hr. This is clearly far higher than the interpolated measured rate of 350 ng/ml/hr in the 1:1 dilution samples (Figure 3D). We can thus reject the hypothesis of higher everolimus-degrading enzyme in wild-type plasma. As for the alternative hypothesis, depending on the amount of excess of the everolimus-protecting protein (over everolimus) in knockout plasma, one can easily envisage that a 5- to 10-fold reduction in the rate of everolimus degradation ensues upon 1:1 dilution of wild-type plasma with knockout plasma. We indeed observed a 7-fold reduced degradation rate, from 2500 ng/ml/hr to 350 ng/ml/hr (Figure 3D). Qualitatively similar data were obtained with the two other knockout strains (Figure 3E and F). Only when knockout plasmas were between 5- and 25-fold diluted with wild-type plasma did the everolimus loss rates approach those seen in undiluted wild-type plasma. These results are therefore more compatible with upregulation of an everolimus-stabilizing protein in the knockout plasmas, than with downregulation of an everolimus-degrading protein in the knockout plasmas relative to wild-type plasma.

Indirect information on the presumed everolimus-degrading activity in plasma came from the detection in the in vitro plasma incubations of Figure 3D-F of a prominent everolimus metabolite, metabolite A (Figure 3G-I). Based on LC-MS/MS detection properties, metabolite A had the same mass over charge ratio as everolimus, but an apparently opened ring structure. Because its product spectrum lacked the m/z 686.4 and m/z 518.3 peaks (25, 26), metabolite A was very likely the dehydrated ring-opened derivative of everolimus (Supplementary Figure 1B). The absolute amount of metabolite A could not be determined without reference material, but assuming an LC-MS/MS signal strength similar to that of everolimus, a substantial fraction (~50%) of everolimus was converted to metabolite A in undiluted wild-type plasma (Figure 3D-F and G-I). As with the disappearance rate of everolimus, the formation rate of metabolite A was much more than 2-fold decreased in the 2-fold diluted wild-type plasmas (Figure 3G-I). In fact, the metabolite A formation rate was already about 2-fold reduced in a mixture of 80% wild-type and 20% Abcb1a/1b(-/-) plasma (5-fold dilution, Figure 3G). This again suggests upregulation of an everolimus-stabilizing protein in the knockout plasmas. Still, metabolite A may be further metabolized, thus complicating interpretation of its appearance profile.

Increased levels of an everolimus-stabilizing (and presumably everolimus-binding) protein in the knockout plasmas would likely also cause increased plasma retention, and reduced levels of free everolimus relative to total blood concentrations of everolimus. This would be compatible with the
greatly reduced liver-to-blood ratios (as can be derived from Figure 1), and reduced blood cells-to-blood ratios (Figure 2A-D) in the knockout strains compared to (“low”) wild-type mice. Collectively, our data suggest that everolimus in plasma of knockout strains is protected from degradation by an everolimus-binding protein.

Liver expression of Ces1 genes is highly upregulated in Abcb1a/1b(-/-), Abcg2(-/-) and Abcb1a/1b;Abcg2(-/-) mice

While trying to identify the nature of the everolimus-stabilizing plasma protein, we discovered in an independent study that a range of carboxylesterase enzymes was highly upregulated in, amongst others, Abcb1a/1b(-/-) mice (27). As some carboxylesterases synthesized in the liver can be abundant in mouse plasma, perhaps one or more of these plasma carboxylesterases could tightly bind everolimus. One could even speculate that there could be recognition (but not hydrolysis) of the lactone ring-internal carboxylester bond of everolimus (see Supplementary Figure 1A), leading to a tight but non-processive protein-substrate complex. We therefore tested RNA levels of the main liver-expressed mouse Ces genes, i.e. Ces1b-Ces1g and Ces2a, in wild-type, Abcb1a/1b(-/-), Abcg2(-/-) and Abcb1a/1b;Abcg2(-/-) mice using RT-PCR. Interestingly, Ces1b was about 8- to 10-fold upregulated, and Ces1c was about 70-fold upregulated in all the knockout strains (Supplementary Figure 2). The basal expression of Ces1d was virtually undetectable in male wild-type liver, leading to nominally ~40,000-fold upregulation in all the knockout strains (Supplementary Figure 2), but the observed deltaCt values of these strains were in the same order as for the other upregulated Ces1 genes, suggesting that the final expression levels were not extremely high (Supplementary Table 1). Ces1e was 3- to 6-fold upregulated, whereas Ces1f, Ces1g and Ces2a were not upregulated, and perhaps sometimes even downregulated (Supplementary Figure 2). Strikingly, reminiscent of the everolimus pharmacokinetic data, there was one “high” Ces1 wild-type mouse, which consistently displayed clearly increased expression levels of Ces1b, Ces1c, Ces1d and Ces1e relative to the 2 “low” Ces1 wild-type mice, albeit not completely up to the level of the knockout strains (Supplementary Figure 2).

Of the upregulated Ces1 proteins, only Ces1b and Ces1c lack the ER retention signal that prevents protein secretion from liver into plasma, and they are thus likely to occur in plasma. As Ces1b is hardly expressed in mouse liver (28, 29), the substantially expressed carboxylesterase Ces1c is the
most likely candidate everolimus-binding protein in plasma. A range of other esterases, including Ces2e, Ces3a, Aadac, and Pon1, 2, and 3, were not upregulated in Abcb1a/1b(-/-) mice (27), and thus unlikely to be involved in everolimus protection in the knockout strains studied here.

**The carboxylesterase inhibitor BNPP reverses stabilization of everolimus in mouse plasma**

To provide more direct evidence that plasma carboxylesterase was responsible for protecting everolimus from degradation in knockout plasma, we tested whether the stabilization of everolimus could be reversed using the carboxylesterase inhibitor BNPP. This organophosphate irreversibly inhibits carboxylesterases through the generation of a stable phosphate ester covalently attached to the catalytic serine residue in the enzyme active site (30). If everolimus normally binds to the substrate binding site of carboxylesterase, one would expect it to bind no longer if the carboxylesterase has bound BNPP. We therefore preincubated BNPP (1 mM) for 15 min in vitro with freshly collected wild-type, Abcb1a/1b(-/-), Abcg2(-/-) and Abcb1a/1b;Abcg2(-/-) plasma, and measured disappearance of subsequently spiked everolimus (4000 ng/ml) over time. In the absence of BNPP, everolimus was rapidly decreased in 3 of 5 (“low”) wild-type plasmas, whereas everolimus concentrations remained similar over time in 2 of 5 (“high”) wild-type plasmas and all knockout plasmas (Figure 4A). After preincubation with BNPP, however, all knockout plasmas and the “high” wild-type plasmas displayed similar rapid degradation profiles as seen in the “low” wild-type plasma without or with BNPP preincubation (Figure 4B). These results indicate that upregulated carboxylesterases in the knockout and 2 “high” wild-type plasmas are responsible for stabilizing everolimus. Moreover, the similarity in everolimus degradation rates between all the strains in the presence of BNPP indicates that there were no pronounced differences in the potential plasma everolimus-degrading activity between the strains. Ces expression analysis of livers of the individual mice tested confirmed that the “high” wild-type mice had marked upregulation of Ces1b-e relative to the “low” wild-type mice, and this upregulation approached the levels seen in the knockout strains (Figure 4C-F and Supplementary Table 2).

**Everolimus inhibits the conversion of irinotecan to SN-38 by carboxylesterase 1c in knockout plasma**
If everolimus binds tightly to the active site of plasma carboxylesterase, it might also inhibit the hydrolytic activity towards carboxylesterase substrates. The anticancer prodrug irinotecan is hydrolyzed to its active derivative SN-38 primarily by plasma Ces1c in mice (31). We therefore tested the conversion of spiked irinotecan (5 µM) to SN-38 in individual wild-type and knockout plasmas in a 30 min *in vitro* incubation, and the effect of preincubation of these plasmas with everolimus (100 µM). Without inhibitors, we observed very little conversion of irinotecan to SN-38 in all wild-type plasmas, versus almost complete conversion in all knockout plasmas (Supplementary Figure 3). The hydrolase activity towards irinotecan in knockout plasmas was at most weakly inhibited by the everolimus vehicle (0.25% ethanol and 0.25% polysorbate 80), whereas it was strongly inhibited by both everolimus and the positive control inhibitor BNPP (Supplementary Figure 3). These results indicate that there is highly increased hydrolysis of irinotecan in knockout plasmas, most likely due to the highly upregulated Ces1c, and that preincubation with everolimus could effectively inhibit this hydrolase activity. This strongly supports that everolimus binds to plasma carboxylesterase 1c, most likely to its active site.

**In vivo knockdown confirms Ces1c involvement in everolimus pharmacokinetics**

To specifically test whether Ces1c was responsible for the altered pharmacokinetic behavior of everolimus, we performed an *in vivo* Ces1c knockdown experiment in the *Abcb1a/1b(-/-)* mice, which had the most consistent upregulation of Ces1c and altered everolimus pharmacokinetics. We compared everolimus pharmacokinetics in *Abcb1a/1b(-/-)* mice treated with either a specific Ces1c siRNA, or a negative control siRNA. Pilot experiments showed that the selected siRNA was efficient in knocking down Ces1c in cultured primary hepatocytes of FVB mice, whereas the negative control siRNA had no effect (data not shown). Subsequent *in vivo* siRNA experiments demonstrated a very extensive knockdown of hepatic Ces1c RNA 3 days after intravenous administration of the Ces1c siRNA, relative to the negative control siRNA, as judged by qRT-PCR (ΔCt 0.77 ± 0.33 versus -4.54 ± 0.21, a 40.2-fold linear decrease (*P* < 0.001) (Supplementary Table 3). Pharmacokinetic analysis performed at this day 3 of orally administered everolimus showed that in the Ces1c siRNA-treated *Abcb1a/1b(-/-)* mice plasma levels were extensively, albeit not completely, reversed to those seen in (low) wild-type mice, whereas in the negative control siRNA-treated mice everolimus plasma levels were roughly the same as seen previously in untreated *Abcb1a/1b(-/-)* mice (Figure 5A,
Supplementary Table 4). Liver and brain concentrations were only modestly affected by these changes in the Ces1c siRNA-treated mice: the slightly lower liver concentration may reflect faster overall everolimus elimination, and the somewhat higher brain concentration may reflect higher peak free plasma concentrations of everolimus (Figure 5B and C). Nonetheless, these data fully confirm that Ces1c was the main factor responsible for the anomalous everolimus plasma pharmacokinetics seen in the knockout strain.

**Everolimus inhibits hydrolysis by recombinant human CES1 and CES2**

Although there are no straightforward orthologues between the mouse Ces1 and Ces2 family members and the human CES1 and CES2 enzymes, and substrate and inhibitor specificity can differ between these species, we tested the inhibitory effect of everolimus on the p-nitrophenyl acetate hydrolase activity of recombinant human CES1 and CES2. A substrate concentration of 100 µM was used, similar to the $K_m$ values of recombinant CES1 and CES2 (32). Everolimus inhibited both enzymes, albeit with relatively high IC$_{50}$ values of 157.2 µM and 19.4 µM for CES1 and CES2, respectively (Figure 6). These results indicate that everolimus is a better inhibitor of human CES2 than CES1 in vitro.

**Cyp3a, but not Abcb1, limits the oral availability of everolimus in mice**

Notwithstanding the carboxylesterase upregulation and everolimus binding in the knockout mouse strains, we aimed to assess the impact of Abcb1 and CYP3A on the oral availability of everolimus in mice. Everolimus was orally administered at 2 mg/kg to wild-type and Abcb1a/1b, Cyp3a and combination Abcb1a/1b/Cyp3a knockout strains, and whole blood everolimus concentrations were assessed (Supplementary Figure 4). We again observed a “low” (n = 5) and “high” (n = 3) wild-type group. Importantly, the Abcb1a/1b knockout did not result in a significant increase in oral AUC relative to the “high” wild-type group (Supplementary Figure 4 and Supplementary Table 5). Considering the high upregulation of plasma Ces in both mouse groups, this indicates that Abcb1 had little impact on the oral availability of everolimus at this dosage. Cyp3a(-/-) mice, however, which have a similar level of hepatic Ces1 upregulation as Abcb1a/1b(-/-) mice, had a 7.8-fold higher everolimus blood AUC than the “high” wild-type group, indicating that Cyp3a markedly restricts the oral availability of everolimus (Supplementary Figure 4, Supplementary Table 5). Additional knockout of Abcb1a/1b in
Abcb1a/1b;Cyp3a(-/-) mice did not result in a further increase in blood AUC, consistent with the absence of a marked effect of Abcb1a/1b on everolimus oral availability.
DISCUSSION

We demonstrated that Abcb1a/1b markedly reduces the brain accumulation, but not the oral availability of everolimus, whereas Abcg2 does not affect either. Cyp3a, however, strongly reduced the oral availability of everolimus. Most remarkably, upregulation of plasma Ces1c in knockout and “high” wild-type mice had a pronounced effect on the plasma pharmacokinetics of everolimus, which could be reversed by in vivo siRNA-mediated Ces1c knockdown. Apparently, everolimus binds tightly to plasma Ces1c, which largely prevents degradation of everolimus by another plasma protein, and strongly reduces overall blood clearance. This is schematically illustrated in Supplementary Figure 5. Everolimus also inhibited human CES1 and especially CES2.

Although we knew that plasma Ces enzymes are upregulated in some knockout strains, affecting drugs hydrolyzed by these enzymes (27), we had not anticipated that strong Ces binding of otherwise unhydrolyzed drugs might profoundly affect their blood pharmacokinetics. Since many other drugs may be bound but not hydrolyzed by these multispecific enzymes, this confounder should be considered in studies with these knockout strains. Note that not all knockout strains for detoxifying proteins display hepatic Ces1 upregulation: whereas similar levels of Ces1b-e upregulation were observed in Abcb1a/1b, Abcg2, and Cyp3a knockout strains and combinations thereof (27 and the present study), Abcc2 and Abcc3 knockout strains did not show altered everolimus blood pharmacokinetics (data not shown), and are thus unlikely to have upregulated Ces1c.

The mechanism behind the upregulation of the Ces1b-e genes is currently unknown. The similarity in upregulation profiles between the different mouse strains suggests a shared induction mechanism between these genes. Since a semi-synthetic diet does not affect Ces1 upregulation (as judged by everolimus blood pharmacokinetics) in the various transporter knockout strains (Supplementary Figure 6) it is unlikely that altered exposure to some dietary xenobiotic is directly responsible. Perhaps some endogenous inducers are responsible, or possibly signaling pathways activated by xenotoxins derived from the intestinal microflora, but their nature remains speculative.

That some wild-type mice display very similar, albeit slightly lower, upregulation of the same group of Ces1 genes as many knockout strains do is also intriguing (genotypes of “high” wild-type mice were double-checked). There were no obvious external clues to which wild-type mice displayed a “high” or “low” everolimus or Ces1 phenotype, and it varied also among siblings from one litter. We
currently do not understand the mechanistic cause of incidental Ces1 upregulation in wild-type mice. We observed no intermediate Ces1c expression levels in wild-type mice, suggesting either that Ces1 upregulation is a fixed situation in individual wild-type mice, or that a switch from “low” to “high” Ces1 expression (or vice versa) occurs abruptly.

Regardless, the endogenous variation in Ces1 expression in wild-type mice will complicate pharmacokinetic analyses for any drug that is hydrolyzed or bound by the upregulated Ces enzymes. For instance, oral everolimus pharmacokinetics in nude female BALB/c mice (24) show everolimus blood levels that are compatible with the levels in our wild-type FVB “high” mice, but not the “low” mice. The tested strain apparently had constitutively “high” plasma Ces1 levels. This could also explain the extremely high plasma protein binding (99.9%) of everolimus reported for this mouse strain, as opposed to 92% in rats and 75% in humans. To test whether the “nude” mutation might be responsible for this presumed Ces1 upregulation, we tested FVB nude “wild-type” and Abcb1a/1b(-/-) and Abcb1a/1b;Abcg2(-/-) mice, but found similar low wild-type and high knockout Ces1 expression in liver as in the normal FVB background (data not shown). Also the level of irinotecan hydrolysis observed in plasma of wild-type B6D2 mice by Morton et al. (31) suggests a “high” wild-type plasma Ces1c level. As carboxylesterases are normally not substantially present in plasma of humans, and in view of the poorly predictable variation in plasma carboxylesterases in various mouse strains, it may be preferable to perform studies with drugs that may be hydrolyzed or bound by plasma carboxylesterases in Ces1c knockout strains, or in other species that lack plasma carboxylesterases (33), thus avoiding this potential confounder.

The prolonged retention of everolimus in blood and plasma of mice with high Ces1 plasma levels suggests a much reduced blood-to-tissue distribution of everolimus. However, our data indicate that, apart from the fraction of everolimus that is tightly bound to plasma Ces (~5% of the dose after oral, and ~10% of the dose after i.v. administration in Ces upregulated mice; values derived from Figure 1A and 1B), there is also a “free” fraction of the drug in blood. The concentration of this free fraction does not seem to differ much between all the mouse strains, judging from the similar levels of liver accumulation between the knockout strains and the “low” and “high” wild-type mice (Figure 1). Thus, although a significant fraction of everolimus is rapidly and tightly bound to plasma Ces1, the remainder (90-95% of the dose) seems to be normally available for distribution. The overall impact of
plasma binding of everolimus on tissue distribution of the drug is therefore limited, at least during the first few hours, and at the dosage tested.

The identity of the plasma enzyme that converts everolimus to metabolite A is unknown. Human liver microsomes in both the absence and presence of NADPH convert everolimus to a lactone ring-opened product that is subsequently dehydrated to its seco acid (34), which resembles metabolite A. The responsible enzyme is thus not a Cytochrome P450. A plasma-localized mouse analogue of this enzyme might be responsible for the metabolism of free everolimus in mouse plasma. Its activity towards everolimus when Ces1c was blocked by BNPP did not differ between all the wild-type and knockout mouse strains (Figure 4B). Of note, also in human plasma ring-opened everolimus metabolites predominate (FDA application 21-560s000).

Everolimus showed higher inhibitory effect towards human CES2 than towards human CES1. This difference could be due to size-limited access of the bulky everolimus, as the active site of CES1 is smaller than that of CES2 (35). The inhibitory effects of everolimus were also different between human CES1 and mouse Ces1c, possibly reflecting a similar size-access difference, although little is known about Ces1c structure. Species differences in inhibitor sensitivity between human and rat liver Ces have been demonstrated previously (36).

The profound plasma carboxylesterase binding of everolimus observed in mice is unlikely to play a role in humans as, unlike mouse Ces1c, human CES1 or CES2 are not normally substantially present in plasma (37). Also the inhibitory activity of everolimus towards human CES1 and CES2 is not very high, suggesting that it may bind less tightly to these proteins than to mouse Ces1c. However, inhibition of the hepatic CES1 and especially CES2, which is primarily found in the intestine, by everolimus might play a role in drug-drug interactions with coadministered drugs. CES1 and CES2 hydrolyze many drugs and prodrugs (30). Upon oral everolimus administration local concentrations of everolimus might be high especially in the intestine, and possibly surpass the Ki of ~20 µM towards CES2. CES2 is for instance thought to be a main enzyme responsible for the conversion of irinotecan to SN-38 in humans (38), and for hydrolysis of a prodrug of gemcitabine (39). Coadministration of everolimus with ester (pro-)drugs affected by carboxylesterases, including the 5-FU anticancer prodrug capecitabine (40), should thus be assessed very carefully.

The limited brain accumulation of everolimus due to the activity of ABCB1 may restrict the therapeutic efficacy of everolimus towards brain tumor parts or (micro-)metastases that are effectively
situated behind a functional blood-brain barrier. Very likely this limited accumulation could be improved by coadministration of an effective ABCB1 inhibitor such as elacridar (16). ABCB1 itself did not affect the oral availability of everolimus, when taking plasma Ces upregulation into account. Given the complications of plasma carboxylesterase upregulation, we think that the small (1.3-fold) reported effect of ABCB1 on low-dose everolimus oral availability assessed with Abcb1a/1b(-/-) mice (22) should be interpreted with caution.

Notwithstanding the plasma Ces1 upregulation, mouse Cyp3a can considerably reduce the oral availability of everolimus (Supplementary Figure 4 and Supplementary Table 5). This is consistent with the demonstrated metabolism of everolimus by recombinant human CYP3A4, CYP3A5 and CYP2C8 in vitro, with CYP3A4 being the major enzyme involved (41). Moreover, drug-drug interaction studies with various CYP3A inhibiting drugs further support that CYP3A is a major factor in the in vivo clearance of everolimus (FDA application 21-560s000). Accordingly, great caution is indicated in the clinical co-application of everolimus with drugs that affect CYP3A activity.

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REFERENCE LIST


FIGURE LEGENDS

Figure 1. Blood levels and tissue disposition of everolimus. Blood concentration (ng/ml; A and B), liver accumulation (ng/g; C and D), brain concentration (ng/g; E and F) and brain-to-liver concentration ratio (G and H) of everolimus in male wild-type, Abcb1a/1b(-/-), Abcg2(-/-) and Abcb1a/1b;Abcg2(-/-) mice 3 hr after oral (left panels) or 1 hr after intravenous (right panels) administration of 2 mg/kg everolimus. All data are presented as mean ± SD (n = 3-7; *, P < 0.05; ***, P < 0.001 when compared with wild-type mice with low everolimus blood levels; †, P < 0.05; ††, P < 0.01; †††, P < 0.001 when compared with wild-type mice with high everolimus blood levels). 1% of doses for liver are 452 ng/g and 562 ng/g for oral and intravenous administration, respectively.

Figure 2. Blood, blood cell, and plasma distribution and tissue disposition of everolimus in vivo. Blood concentrations (ng/ml; A), plasma concentrations (ng/ml; B), blood cells concentrations (ng/ml; C), blood cells-to-blood ratio (D), plasma concentration-time curve (ng/ml; E), liver concentration-time curve (ng/g; F), brain concentration-time curve (ng/g; G) and brain-to-liver ratio (H) of everolimus in male wild-type, Abcb1a/1b(-/-), Abcg2(-/-) and Abcb1a/1b;Abcg2(-/-) mice at 5, 30 or 60 min (panels A-D) as well as 2, 4, and 8 hr (panels E-H) after intravenous administration of 2 mg/kg everolimus. All data are presented as mean ± SD (n = 1-5; *, P < 0.05; **, P < 0.01; ***, P < 0.001 when compared with wild-type mice with low everolimus blood levels; ††, P < 0.01; †††, P < 0.001 when compared with wild-type mice with high everolimus blood levels). #, by chance no “high” wild-type mice were present in the 30 min group.

Figure 3. Stability of everolimus in plasma of wild-type and knockout mice in vitro. A-C, concentration-time curves of everolimus in male wild-type, Abcb1a/1b(-/-), Abcg2(-/-) and Abcb1a/1b;Abcg2(-/-) plasma after incubation of 250 ng/ml (A), 1000 ng/ml (B) or 4000 ng/ml (C) spiked everolimus. D-I, stability of everolimus in knockout mouse plasmas diluted with increasing amounts of wild-type plasma in vitro. Concentration-time curves of everolimus (ng/ml, D-F) and metabolite A (response relative to internal standard, G-I) after incubation of 4000 ng/ml everolimus in Abcb1a/1b(-/-) (D and G), Abcg2(-/-) (E and H) and Abcb1a/1b;Abcg2(-/-) (F and I) pooled plasma diluted with increasing amounts of pooled wild-type plasma. Values below lower limit of quantifications were replaced with 100 ng/ml and...
0.1 response relative to internal standard for everolimus and metabolite A, respectively. Each data point represents a single determination.

**Figure 4.** Stability of everolimus in mouse plasma after preincubation with the irreversible CES inhibitor BNPP *in vitro*. Concentration of everolimus (% of control) after incubation of 4000 ng/ml everolimus spiked into wild-type, Abcb1a/1b(-/-), Abcg2(-/-) and Abcb1a/1b;Abcg2(-/-) plasma either without (A), or with 1 mM BNPP pretreatment (B). All data are presented as mean ± SD (n = 2-3). Expression levels of Ces1b (C), Ces1c (D), Ces1d (E) or Ces1e (F) mRNA in livers of male wild-type, Abcb1a/1b(-/-), Abcg2(-/-) and Abcb1a/1b;Abcg2(-/-) mice used in the stability experiment, as determined by real-time RT-PCR. Data are normalized to GAPDH expression. Values represent mean fold change ± SD, compared to wild-type mice with low Ces expression (n = 2-5; *, P < 0.05; **, P < 0.01; ***, P < 0.001 when compared with wild-type mice with low plasma everolimus levels).

**Figure 5:** Effect of Ces1c knockdown on plasma pharmacokinetics and tissue disposition of everolimus *in vivo*. Plasma concentration-time curve (ng/ml; A), liver concentration (ng/g; B), brain concentration (ng/g; C) and brain-to-liver ratio (D) of everolimus in negative control siRNA (siNEG)- or siCes1c-treated male Abcb1a/1b(-/-) mice at 3 hr after oral administration of 2 mg/kg everolimus. All data are presented as mean ± SD (n = 5-7; *, ** and *** indicate P < 0.05, P < 0.01 and P < 0.001 when compared with siNEG-injected male Abcb1a/1b(-/-) mice, respectively).

**Figure 6.** Inhibitory effect of everolimus on hydrolase activities by recombinant human CES1 and CES2 *in vitro*. The activities were determined at 100 µM p-nitrophenyl acetate substrate concentrations. Each data point represents the mean of triplicate determinations. The control activities by recombinant CES1 and CES2 were 542 and 300 nmol/min/mg, respectively.
Figure 1

A. Oral (3 hr)  
B. Intravenous (1 hr)

C. Liver (mg/g)  
D. Liver (mg/g)

E. Brain concentration (mg/g)  
F. Brain concentration (mg/g)

G. Brain-to-liver ratio  
H. Brain-to-liver ratio
Figure 4

(A) No inhibition

![Graph showing Everolimus concentration (% of control) over time (hr) for different genotypes.]

(B) BNPP (1 mM)

![Graph showing Everolimus concentration (% of control) over time (hr) with BNPP treatment.]

(C) Ces1b

![Bar graph showing fold change in expression for Ces1b.]

(D) Ces1c

![Bar graph showing fold change in expression for Ces1c.]

(E) Ces1d

![Bar graph showing fold change in expression for Ces1d.]

(F) Ces1e

![Bar graph showing fold change in expression for Ces1e.]

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Figure 5

A

Plasma concentration (ng/ml)

Time (hr)

- siNEG
- siCes1c

B

Liver (ng/g)

siNEG

siCes1c

***

C

Brain (ng/g)

siNEG

siCes1c

*

D

Brain-to-liver ratio

siNEG

siCes1c

**
Figure 6

The graph shows the residual activity (% of control) of CES1 and CES2 enzymes in response to varying concentrations of Everolimus (μM). The x-axis represents the concentration of Everolimus ranging from 0.01 to 1000 μM, while the y-axis shows the residual activity ranging from 0 to 120%. The data points indicate a decrease in residual activity as the concentration of Everolimus increases.
Clinical Cancer Research

P-glycoprotein, CYP3A and plasma carboxylesterase determine brain and blood disposition of the mTOR inhibitor everolimus (Afinitor) in mice

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