BJP BRITISH JOURNAL OF PHARMACOLOGY

BPS BRITISH PHARMACOLOGICAL SOCIETY

British Journal of Pharmacology (2010), 160, 1224–1233

0007-1188/10
www.brjpharmacol.org

RESEARCH PAPER

Effects of cytochrome P450 3A (CYP3A) and the drug transporters P-glycoprotein (MDR1/ABCB1) and MRP2 (ABCC2) on the pharmacokinetics of lopinavir

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Background and purpose: Lopinavir is extensively metabolized by cytochrome P450 3A (CYP3A) and is considered to be a substrate for the drug transporters ABCB1 (P-glycoprotein) and ABCC2 (MRP2). Here, we have assessed the individual and combined effects of CYP3A, ABCB1 and ABCC2 on the pharmacokinetics of lopinavir and the relative importance of intestinal and hepatic metabolism. We also evaluated whether ritonavir increases lopinavir oral bioavailability by inhibition of CYP3A, ABCB1 and/or ABCC2.
Experimental approach: Lopinavir transport was measured in Madin-Darby canine kidney cells expressing ABCB1 or ABCC2. Oral lopinavir kinetics (+/– ritonavir) was studied in mice with genetic deletions of Cyp3a, Abcb1a/b and/or Abcc2, or in transgenic mice expressing human CYP3A4 exclusively in the liver and/or intestine.
Key results: Lopinavir was transported by ABCB1 but not by ABCC2 *in vitro*. Lopinavir area under the plasma concentration – time curve $(AUC)_{oral}$ was increased in Abcb1a/b$^{-/-}$ mice (approximately ninefold vs. wild-type) but not in Abcc2$^{-/-}$ mice. Increased lopinavir AUC_{oral} (>2000-fold) was observed in cytochrome P450 3A knockout (Cyp3a$^{-/-}$) mice compared with wild-type mice. No difference in AUC_{oral} between Cyp3a$^{-/-}$ and Cyp3a/Abcb1a/b/Abcc2$^{-/-}$ mice was observed. CYP3A4 activity in intestine or liver, separately, reduced lopinavir AUC_{oral} (>100-fold), compared with Cyp3a$^{-/-}$ mice. Ritonavir markedly increased lopinavir AUC_{oral} in all CYP3A-containing mouse strains.
Conclusions and implications: CYP3A was the major determinant of lopinavir pharmacokinetics, far more than Abcb1a/b. Both intestinal and hepatic CYP3A activity contributed importantly to low oral bioavailability of lopinavir. Ritonavir increased lopinavir bioavailability primarily by inhibiting CYP3A. Effects of Abcb1a/b were only detectable in the presence of CYP3A, suggesting saturation of Abcb1a/b in the absence of CYP3A activity.
British Journal of Pharmacology (2010) **160,** 1224–1233; doi:10.1111/j.1476-5381.2010.00759.x

Keywords: lopinavir; CYP3A; P-glycoprotein (ABCB1; MDR1); MRP2 (ABCC2); intestinal metabolism

Abbreviations: AUC, area under the plasma concentration – time curve; C_{max}, maximum drug concentration in plasma; CYP3A, cytochrome P450 3A; Cyp3a$^{-/-}$, cytochrome P450 3A knockout mice; Cyp3a$^{-/-}$Tg-3A4$_{Hep}$, Cyp3a knockout mice with liver-specific transgenic expression of human CYP3A4; Cyp3a$^{-/-}$Tg-3A4$_{Int}$, Cyp3a knockout mice with intestine-specific transgenic expression of human CYP3A4; HIV, human immunodeficiency virus; MDCK, Madin-Darby canine kidney; MDR1, multidrug resistance protein 1; MRP2, multidrug resistance protein 2; PI, HIV protease inhibitor; T_{max}, time to maximum drug concentration in plasma

Introduction

Lopinavir is currently one of the most widely used human immunodeficiency virus (HIV) protease inhibitors (PIs). The drug itself is rapidly metabolized and has very low oral bioavailability. The metabolism of lopinavir is primarily mediated by cytochrome P450 3A (CYP3A) enzymes

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Received 3 November 2009; revised 16 February 2010; accepted 19 February 2010

(nomenclature follows Alexander *et al.*, 2009) and yields metabolites that are less potent as PIs (Kumar *et al.*, 1999). CYP3A enzymes are not only involved in the metabolism of lopinavir but also in metabolism of about 50% of the currently marketed drugs (Guengerich, 1999). While the liver has long been considered to be the most important organ where CYP3A-mediated metabolism takes place, evidence is accumulating that intestinal CYP3A metabolism can also have a profound impact on the oral bioavailability of drugs (van Herwaarden *et al.*, 2007; Thummel, 2007).

Inhibition or induction of CYP3A can have profound clinical consequences, especially with drugs that have narrow therapeutic windows, such as PIs (Dresser *et al.*, 2000). Importantly, there is a high inter- but also intra-individual variation in CYP3A activity levels (Lamba *et al.*, 2002; Lin *et al.*, 2002). These factors, together with the wide substrate specificity of the enzyme, make CYP3A an important determinant of drug–drug interactions (Thummel and Wilkinson, 1998).

In addition to CYP3A, active drug transporters such as P-glycoprotein (ABCB1; MDR1, multidrug resistance protein 1) and the multidrug resistance protein 2 (MRP2; ABCC2) can have a strong effect on the oral bioavailability and elimination of administered drugs (Schinkel and Jonker, 2003). Recently, lopinavir was reported to be a substrate for ABCB1 and ABCC2 *in vitro* (Woodahl *et al.*, 2005; Agarwal *et al.*, 2007). Although this could have clinical implications, the impact of these drug transporters on the pharmacokinetics of lopinavir *in vivo* has not been elucidated.

It is noteworthy that many ABCB1 and/or ABCC2 substrates are also substrates for CYP3A, like lopinavir, and it has been hypothesized that the combined activity of drug transporters and CYP3A results in efficient first-pass metabolism of orally administered drugs (Watkins, 1997; Ito *et al.*, 1999a; Benet and Cummins, 2001; Kivisto *et al.*, 2004; Garmire and Hunt, 2008). Especially in the intestine, the apically located drug transporters such as ABCB1 or ABCC2 may reduce the probability of CYP3A4 saturation. However, little *in vivo* evidence for this possibility is currently available. Yet, we have recently demonstrated that the combined activity of CYP3A and Abcb1a/b is an important determinant of the low bioavailability of the anti-cancer drug docetaxel and that the absence of both systems leads to disproportionate increases in systemic docetaxel exposure with a concomitant higher toxicity risk (van Waterschoot *et al.*, 2009).

In humans, lopinavir is co-formulated with ritonavir, which improves the oral bioavailability of lopinavir dramatically. Although ritonavir is a PI as well, the ritonavir concentrations achieved in the combination therapy are not considered therapeutic. While ritonavir primarily inhibits CYP3A-mediated metabolism of lopinavir, it is noteworthy that ritonavir can also inhibit ABCB1 and ABCC2 (Drewe *et al.*, 1999; Gutmann *et al.*, 1999). Inhibition of ABCB1 and/or ABCC2 by ritonavir might therefore be in part responsible for the increased oral bioavailability of lopinavir.

In addition to the Cyp3a/Abcb1a/b combination knockout mouse model (van Waterschoot *et al.*, 2009), we recently generated Cyp3a/Abcb1a/b/Abcc2 combination knockout mice. In this study we have utilized these novel mouse models to investigate the individual and combined effects of CYP3A and the drug transporters Abcb1a/b and Abcc2 on lopinavir pharmacokinetics *in vivo*. Moreover, by using a set of 'humanized' CYP3A4-transgenic mouse strains, we assessed the relative importance of intestinal and hepatic CYP3A4-mediated metabolism of lopinavir. Finally, we aimed to obtain more insight into how ritonavir increases the oral bioavailability of lopinavir.

Methods

Cell lines and tissue culture

The polarized canine kidney cell line Madin-Darby canine kidney (MDCK)-II was used in transport assays. Human ABCB1- and ABCC2-transduced MDCK-II subclones were described previously (Evers *et al.*, 1997; 1998). The MDCK-II cells and transduced subclones were cultured in Dulbecco's modified Eagle's medium supplied with Glutamax (Invitrogen, Carlsbad, CA, USA) and supplemented with 50 units·mL^{-1} penicillin, 50 μg·mL^{-1} streptomycin and 10% (v/v) fetal calf serum (Invitrogen) at 37°C in the presence of 5% CO_2. The cells were trypsinized every 3 to 4 days for subculturing.

Transport assay

Transport assays were performed as reported previously with minor modifications (Huisman *et al.*, 2002). Briefly, cells were seeded on microporous polycarbonate membrane filters (Transwell 3414; Costar, Corning, NY, USA) at a density of 1.0 × 10^6 cells per well in 2 mL of complete medium and were subsequently grown for 3 days. Two hours before the start of the experiment, complete medium in the apical and basolateral compartments was replaced with Optimem medium (Life Technologies, Breda, the Netherlands), without serum, and with or without ritonavir (50 μM). In addition, 5 μM of elacridar was present in experiments with ABCC2 cells, to inhibit the endogenous P-glycoprotein activity. At t = 0 h the experiment was started by replacing the medium with fresh Optimem medium with 5 μM lopinavir in the appropriate compartment. Depending on the cell line and experiment, this was done in the presence of 50 μM ritonavir and/or 5 μM elacridar. Cells were incubated at 37°C in 5% CO_2, and 100 μL aliquots of medium were taken at t = 2 and 4 h. Samples were stored at –20°C until analysis by LC-MS as described below. Membrane tightness was assessed using [^{3}H]-inulin (Amersham, Little Chalfont, UK). Leakage was not allowed to be >1% of the total added radioactivity per hour.

Animals

Mice were housed and handled according to institutional guidelines complying with Dutch legislation. All mice used in this study had a >99% FVB genetic background and were between 8 and 14 weeks of age. All experiments were done using male mice. Animals were kept in a temperature-controlled environment with a 12 h light/12 h dark cycle and received a standard diet (AM-II, Hope Farms, Woerden, the Netherlands) and acidified water *ad libitum*.

Abcb1a/b$^{-/-}$ (Schinkel *et al.*, 1997), Abcc2$^{-/-}$ (Vlaming *et al.*, 2006) and cytochrome P450 3A knockout (Cyp3a$^{-/-}$) mice

(van Herwaarden *et al*., 2007) were crossed to obtain Cyp3a/Abcb1a/b/Abcc2$^{-/-}$ triple knockout mice. Genotypes of mice were evaluated by PCR as described in the original publications. In addition, homozygous Cyp3a$^{-/-}$Tg-3A4$_{Hep}$ [Cyp3a$^{-/-}$ mice expressing CYP3A4 exclusively in the liver (previously Cyp3a$^{-/-}$A)] and Cyp3a$^{-/-}$Tg-3A4$_{Int}$ [Cyp3a$^{-/-}$ mice expressing CYP3A4 exclusively in the intestine (previously Cyp3a$^{-/-}$V)] mice were crossed to obtain homozygous Cyp3a$^{-/-}$Tg-3A4$_{Hep/Int}$ (Cyp3a$^{-/-}$ mice expressing CYP3A4 in both liver and intestine) mice, and genotypes were verified as described (van Herwaarden *et al*., 2007). Intrinsic docetaxel clearance values of hepatic and intestinal microsomes derived from the CYP3A4-transgenic tissues were similar to those of wild-type mouse and human microsomes, suggesting physiologically relevant activity levels of the transgenic CYP3A4 (van Herwaarden *et al*., 2007).

Lopinavir plasma pharmacokinetics

Lopinavir with or without ritonavir was formulated in ethanol/propylene glycol [19:81 (v/v)] and administered by oral gavage into the stomach of male mice. To minimize variation in absorption, mice were fasted for 2 h before lopinavir was administered. Multiple blood samples (~40 μL) were collected from the tail vein at several time points up to 24 h using heparinized capillary tubes (Oxford Labware, St. Louis, MO, USA). Blood samples were centrifuged at 2100× *g* for 10 min at 4°C, and the plasma fraction was collected, made up to 50 μL with human plasma, and stored at –20°C until analysis.

Drug analysis

Lopinavir and ritonavir concentrations in plasma samples were determined using a previously described sensitive assay using liquid chromatography coupled to tandem mass spectrometry (ter Heine *et al*., 2007). Possible interference or matrix issues for analysing mouse plasma with this assay were not observed.

Statistical procedures

Pharmacokinetic calculations and statistical analysis were performed as described (van Waterschoot *et al*., 2009). The two-sided unpaired Student's *t*-test was used for statistical analysis. Data obtained with single and combination knockout mice were compared with data obtained with wild-type mice. Differences were considered statistically significant when $P < 0.05$. Data are presented as mean ± SD.

Materials

Lopinavir and ritonavir were purchased from Sequoia Research Products Ltd. (Pangbourne, UK). Elacridar (GF120918) was a kind gift of GlaxoSmithKline (Research Triangle Park, Durham, NC, USA). All other chemicals were of analytical grade and obtained from commercial sources.

Results

In vitro *transport of lopinavir by ABCB1 and ABCC2*

To determine whether lopinavir is transported by ABCB1 and ABCC2, we analysed transepithelial transport using monolayers of MDCK-II cells transfected with human ABCB1 or ABCC2. In MDCK-II parental cells, the translocation of lopinavir (5 μM) in the apical direction was higher than in the basolateral direction (Figure 1A). In the ABCB1-transfected MDCK-II cells, this apically directed translocation was significantly increased whereas basolaterally directed translocation was sharply decreased, resulting in an increase in apical over basolateral transport ratio (*r*) from 2 to 32 (Figure 1C). In the presence of ritonavir (50 μM), the lopinavir transport was completely inhibited in the parental cells, but only modestly in the ABCB1-transfected cells (Figure 1B and D). The apically directed transport in the parental cell line can most likely be attributed to low-level endogenous canine MDR1 activity (Ito *et al*., 1999b; Guo *et al*., 2002). Accordingly, the MDR1 inhibitor elacridar was added to parent and ABCC2-transfected cells to allow a fair assessment of ABCC2 transport activity (Figure 1E–H) (Huisman *et al*., 2002). The vectorial translocation of lopinavir, however, was not different in ABCC2-transfected cells compared with that in parental cells (Figure 1G and H). Taken together, these *in vitro* results demonstrated that lopinavir was efficiently transported by ABCB1 but not by ABCC2, and that ritonavir was a modest inhibitor of ABCB1-mediated lopinavir transport.

Individual and combined impact of CYP3A, ABCB1 and ABCC2 on lopinavir pharmacokinetics

We further evaluated the *in vivo* role of ABCB1 and ABCC2 in lopinavir oral pharmacokinetics by utilizing Abcb1a/b and Abcc2 knockout mice. Lopinavir was administered orally at 25 mg·kg^{-1} to wild-type and knockout mice and at various time points blood samples were taken. In line with our *in vitro* results, we found a marked increase (8.6-fold) in systemic exposure (area under the plasma concentration – time curve, AUC) in Abcb1a/b$^{-/-}$ mice when compared with wild-type mice (Table 1). In contrast, no significant increase in systemic exposure could be determined for Abcc2$^{-/-}$ mice (Table 1). Because Abcb1a/b and Abcc2 can sometimes take over each other's function (Lagas *et al*., 2006), we also administered lopinavir to Abcb1a/b/Abcc2 combination knockout mice. The AUC in Abcb1a/b/Abcc2$^{-/-}$ mice (Table 1), however, was not significantly different from that in Abcb1a/b$^{-/-}$ mice, further supporting the observation that Abcc2 is not an important determinant of lopinavir plasma pharmacokinetics.

In addition to the drug transporters, we also studied the dependence of lopinavir pharmacokinetics on CYP3A using Cyp3a$^{-/-}$ mice (van Herwaarden *et al*., 2007). We note that lopinavir metabolism appears highly comparable between mouse and human in terms of rate and metabolic profile (Kumar *et al*., 1999). Interestingly, a more than 2000-fold increase in systemic exposure was observed in Cyp3a$^{-/-}$ mice when compared with wild-type mice (Figure 2A; Table 1). These data illustrate that CYP3A is by far the most important determinant of lopinavir pharmacokinetics, greatly surpassing any effect of drug transporters.

We have recently demonstrated that the simultaneous absence of both CYP3A and P-glycoprotein leads to a disproportionate increase in systemic exposure for the anti-cancer drug docetaxel (van Waterschoot *et al*., 2009). By utilizing

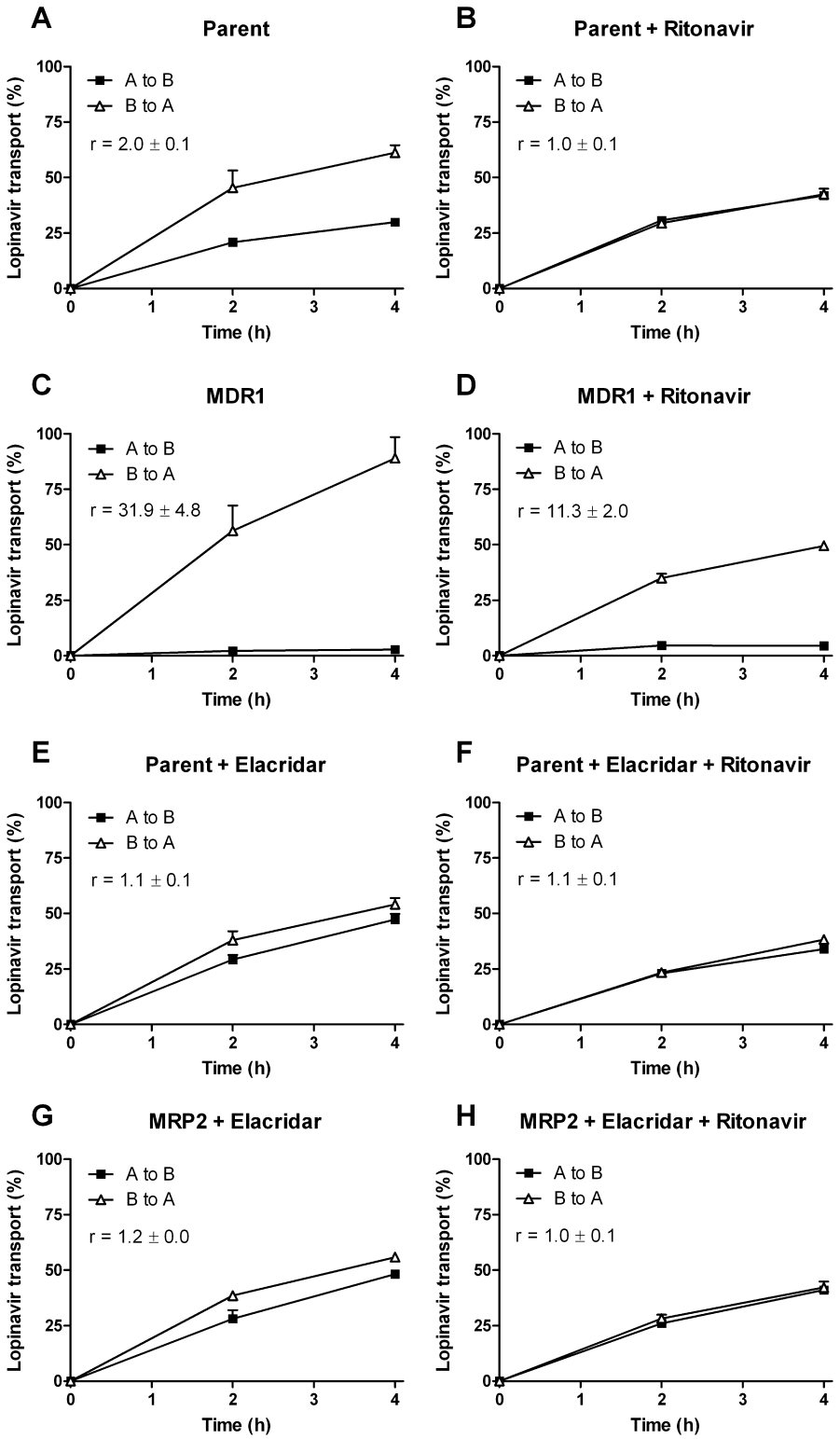

Figure 1 Transepithelial transport of lopinavir (5 μM) in MDCK-II cells either non-transfected (A, B, E and F), transfected with human ABCB1 (C and D) or human ABCC2 (G and H) cDNA in the absence (A, C, E and G) or presence (B, D, F and H) of ritonavir (50 μM). Parent and ABCC2 cells were incubated in the presence of 5 μM elacridar (C, D, G and H). At $t = 0$ h, lopinavir was applied in one compartment (apical or basolateral) and the percentage of lopinavir transported to the opposite compartment at $t = 2$ and 4 h was measured by LC-MS/MS ($n = 3$). Data represent means ± SD. *r* represents the relative transport ratio (i.e. the apically directed translocation divided by the basolaterally directed translocation) at $t = 4$ h. MDCK, Madin-Darby canine kidney; MDR1, multidrug resistance protein 1; MRP2, multidrug resistance protein 2.

Table 1 Plasma pharmacokinetic parameters after oral administration of 25 mg·kg^{-1} lopinavir with or without 25 mg·kg^{-1} ritonavir

	$AUC_{(0\text{-}24h)}$ (μg·h·mL^{-1})	*Fold vs. wild-type*	C_{max} (μg·mL^{-1})	T_{max} (h)	$AUC_{(0\text{-}24h)}$ (μg·h·mL^{-1})	*Fold vs. without ritonavir*	C_{max} (μg·mL^{-1})	T_{max} (h)
	+Vehicle				*+Ritonavir*			
Wild-type	0.054 ± 0.034	1	0.11 ± 0.095	0.17 ± 0.0	57.4 ± 26.2††	1063	5.42 ± 1.44†††	5.78 ± 3.85†
Abcb1a/b$^{-/-}$	0.465 ± 0.316*	8.6	0.34 ± 0.16*	0.17 ± 0.0	–	–	–	–
Abcc2$^{-/-}$	0.248 ± 0.249	4.6	0.31 ± 0.30	0.21 ± 0.08	–	–	–	–
Abcb1a/b/Abcc2$^{-/-}$	0.573 ± 0.281*	10.6	0.41 ± 0.17*	0.25 ± 0.10	120 ± 32†††	209	8.59 ± 2.21†††	6.22 ± 1.54 †††
Cyp3a$^{-/-}$	118 ± 52**	2185	8.14 ± 3.31**	4.33 ± 2.96*	148 ± 28	1.3	8.78 ± 2.67	8.0 ± 0.0
Cyp3a/Abcb1a/b$^{-/-}$	120 ± 16***	2222	7.52 ± 1.38***	6.67 ± 1.54***	–	–	–	–
Cyp3a/Abcb1a/b/Abcc2$^{-/-}$	145 ± 31***	2685	9.81 ± 2.56***	8.0 ± 0.0***	232 ± 32†	1.6	11.4 ± 1.88	11.1 ± 11.6

Area under the plasma concentration versus time curve (AUC), maximal concentration obtained after oral administration (C_{max}) and the corresponding time of maximum concentration (T_{max}) are indicated. Values represent the means ± SD (n = 3–4).
*P < 0.05, **P < 0.01, ***P < 0.001, compared with wild-type mice and $^{\dagger}P$ < 0.05, $^{\dagger\dagger}P$ < 0.01, $^{\dagger\dagger\dagger}P$ < 0.001, compared with the same strain without ritonavir.

Cyp3a/Abcb1a/b$^{-/-}$ and Cyp3a/Abcb1a/b/Abcc2$^{-/-}$ combination knockout mice, we evaluated whether an analogous effect could also be observed for oral lopinavir pharmacokinetics. It appeared, however, that plasma levels in both combination knockout strains were in the same range as observed in Cyp3a$^{-/-}$ mice with no significant differences in AUC between Cyp3a$^{-/-}$, Cyp3a/Abcb1a/b$^{-/-}$ and Cyp3a/Abcb1a/b/Abcc2$^{-/-}$ (Figure 2: Table 1). Thus, whereas we observed a marked (8.6-fold) effect of Abcb1a/b absence in a CYP3A-proficient situation, this was no longer seen in the CYP3A-deficient background (Table 1).

Effects of ritonavir co-administration on the oral bioavailability of lopinavir

Ritonavir is considered to be an inhibitor of not only CYP3A but also Abcb1a/b and Abcc2 (Drewe *et al*., 1999; Gutmann *et al*., 1999; Huisman *et al*., 2001). Although ritonavir is known to increase the oral bioavailability of lopinavir, it is not clear whether this can be solely attributed to CYP3A inhibition or that inhibition of ABCB1 and ABCC2 contributes to the overall result. To evaluate this, we co-administered ritonavir (25 mg·kg^{-1}) with lopinavir (25 mg·kg^{-1}) to wild-type, Abcb1a/b/Abcc2$^{-/-}$, Cyp3a$^{-/-}$ and Cyp3a/Abcb1a/b/Abcc2$^{-/-}$ mice and subsequently determined lopinavir plasma levels. Interestingly, the AUC in wild-type mice was more than 1000-fold increased when ritonavir was co-administered (Figure 2; Table 1), illustrating a major effect of ritonavir on the oral bioavailability of lopinavir. In contrast, no significant difference in systemic exposure was seen between Cyp3a$^{-/-}$ mice that received lopinavir in combination with ritonavir and Cyp3a$^{-/-}$ mice that only received lopinavir. In addition, in Abcb1a/b/Abcc2$^{-/-}$ mice plasma levels were highly increased (209-fold) when ritonavir was co-administered and the AUC reached a similar level as in Cyp3a$^{-/-}$ mice (Table 1). Collectively, these data indicate that ritonavir increases the oral bioavailability of lopinavir primarily by inhibition of CYP3A.

Relative importance of intestinal and hepatic lopinavir metabolism

Evidence is accumulating that intestinal CYP3A metabolism can often strongly affect the pharmacokinetics of orally administered drugs (Kolars *et al*., 1991; Wu *et al*., 1995; Paine *et al*., 1996; Lown *et al*., 1997; van Herwaarden *et al*., 2007). As our data above indicate that CYP3A is a major determinant of oral lopinavir pharmacokinetics, we further investigated how relevant intestinal versus hepatic metabolism was for the disposition of lopinavir. For this purpose, we used transgenic mice that have either hepatic or intestinal specific CYP3A4 expression in a murine Cyp3a knockout background, denoted as Cyp3a$^{-/-}$Tg-3A4$_{Hep}$ and Cyp3a$^{-/-}$Tg-3A4$_{Int}$ mice respectively (van Herwaarden *et al*., 2007). In addition, an analogous mouse strain with transgenic CYP3A4 expression in both intestine and liver (Cyp3a$^{-/-}$Tg-3A4$_{Hep/Int}$) was studied. The transgenic CYP3A4 activity in liver or intestine of these mouse strains is comparable with the endogenous CYP3A activity in the respective organs of wild-type mice (van Herwaarden *et al*., 2007), indicating physiologically relevant levels of CYP3A4. Consistent with our observations in

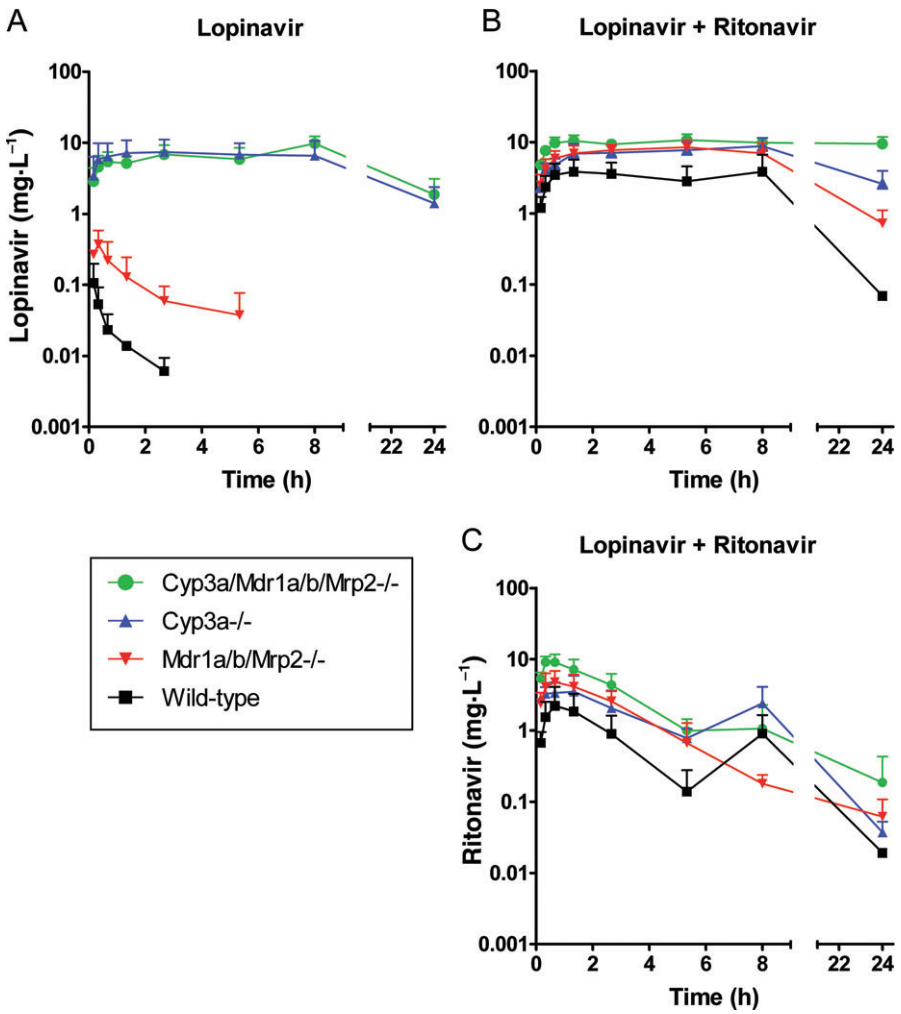

Figure 2 Plasma concentration–time curves of lopinavir in male wild-type, Abcb1a/b/Abcc2$^{-/-}$, Cyp3a$^{-/-}$ and Cyp3a/Abcb1a/b/Abcc2$^{-/-}$ mice after oral administration of lopinavir (25 mg·kg^{-1}) in the absence (A) and presence (B) of ritonavir (25 mg·kg^{-1}). The corresponding plasma concentration–time curves of ritonavir are given in (C). Data are shown as the mean concentration, and error bars represent the SD (n = 3–4 per time point). Cyp3a$^{-/-}$, cytochrome P450 3A knockout mice.

wild-type mice, we found that the systemic lopinavir exposure in Cyp3a$^{-/-}$Tg-3A4$_{Hep/Int}$ mice was dramatically reduced (>4400-fold) when compared with Cyp3a$^{-/-}$ mice (Table 2) and was in the same order as we observed for wild-type mice (Table 1). Note, however, that in this experiment we used a fourfold higher lopinavir dose (i.e. 100 mg·kg^{-1}) to allow quantification of the lopinavir plasma levels in all strains up to at least 4 h after administration. Interestingly, exclusive expression of CYP3A4 in either the intestine (Cyp3a$^{-/-}$Tg-3A4$_{Int}$) or in the liver (Cyp3a$^{-/-}$Tg-3A4$_{Hep}$) could each by itself already reduce the systemic exposure of lopinavir substantially (135- and 103-fold, respectively) when compared with Cyp3a$^{-/-}$ mice (Figure 3; Table 2).

Ritonavir increases lopinavir bioavailability by inhibiting intestinal and hepatic CYP3A

To assess whether ritonavir primarily inhibits intestinal or hepatic CYP3A4 we co-administered ritonavir (25 mg·kg^{-1}) with lopinavir (100 mg·kg^{-1}) to the CYP3A4-transgenic mouse strains. Ritonavir co-administration increased the lopinavir AUC roughly 20–30-fold in all strains that have transgenic CYP3A4 expression but not in Cyp3a$^{-/-}$ mice (Figure 3A and B; Table 2). When compared with the Cyp3a$^{-/-}$ mice, lopinavir plasma levels remained rather low in mice that have both intestinal and hepatic CYP3A4 expression (Cyp3a$^{-/-}$Tg-3A4$_{Hep/Int}$) (Figure 3A and B). Furthermore, the increase in lopinavir exposure in this strain was also much less pronounced than we observed when ritonavir was co-administered to wild-type mice (Figure 2; Table 1), which also had (mouse) CYP3A expression in both intestine and liver. A possible reason for this might be that ritonavir was rapidly degraded in the CYP3A4-expressing mouse strains, especially the Cyp3a$^{-/-}$Tg-3A4$_{Hep/Int}$ line. Indeed, ritonavir levels were highly affected by CYP3A4 and were much more reduced in Cyp3a$^{-/-}$Tg-3A4$_{Hep/Int}$ mice than in wild-type mice (Figures 2C and 3C). For example, whereas we could detect ritonavir levels up to 24 h after administration in wild-type mice, in Cyp3a$^{-/-}$Tg-3A4$_{Hep/Int}$ mice levels were already below the limit of quantification at 4 h. Most likely, this discrepancy between Cyp3a$^{-/-}$Tg-3A4$_{Hep/Int}$ and wild-type can be attributed to differences in the kinetic properties (K_m, V_{max}) of ritonavir metabolism by human

Table 2 Plasma pharmacokinetic parameters after oral administration of 100 mg·kg^{-1} lopinavir with or without 25 mg·kg^{-1} ritonavir

	$AUC_{(0-24h)}$ (μg·h·mL^{-1})	*Fold decrease vs. Cyp3a*$^{-/-}$	C_{max} (μg·mL^{-1})	T_{max} (h)	$AUC_{(0-24h)}$ (μ·h·mL^{-1})	*Fold vs. without ritonavir*	C_{max} (μg·mL^{-1})	T_{max} (h)
	+Vehicle				*+Ritonavir*			
Cyp3a$^{-/-}$	372 ± 20	1	23.3 ± 2.09	5.0 ± 2.0	317 ± 58	0.9	17.4 ± 4.3†	5.4 ± 3.2
Cyp3a$^{-/-}$Tg-3A4$_{Hep}$	3.60 ± 0.82***	103	0.27 ± 0.00***	0.44 ± 0.13**	97.6 ± 30.9††	27	8.3 ± 3.6††	5.3 ± 2.3††
Cyp3a$^{-/-}$Tg-3A4$_{Int}$	2.75 ± 1.93***	135	0.54 ± 0.44***	1.44 ± 1.74***	52.0 ± 11.8†††	19	6.74 ± 1.78†††	1.31 ± 0.38
Cyp3a$^{-/-}$Tg-3A4$_{Hep/Int}$	0.084 ± 0.068***	4429	0.10 ± 0.08***	0.25 ± 0.00**	1.78 ± 0.39†††	21	0.58 ± 0.18††	0.42 ± 0.29

Area under the plasma concentration versus time curve (AUC), maximal concentration obtained after oral administration (C_{max}) and the corresponding time of maximum concentration (T_{max}) are indicated. Values represent the means ± SD (n = 3–4).
*P < 0.05, **P < 0.01, ***P < 0.001, compared with Cyp3a$^{-/-}$Tg-3A4$_{Hep/Int}$ mice and $^{\dagger}P$ < 0.05, $^{\dagger\dagger}P$ < 0.01, $^{\dagger\dagger\dagger}P$ < 0.001, compared with the same strain without ritonavir.
Cyp3a$^{-/-}$, cytochrome P450 3A knockout mice; Cyp3a$^{-/-}$Tg-3A4$_{Hep}$, Cyp3a knockout mice with liver-specific transgenic expression of human CYP3A4; Cyp3a$^{-/-}$Tg-3A4$_{Int}$, Cyp3a knockout mice with intestine-specific transgenic expression of human CYP3A4.

CYP3A4 and the mouse CYP3A enzymes. Regardless of this difference, our data indicated that ritonavir could inhibit both intestinal and hepatic CYP3A4 and that CYP3A4 in both locations contributed equally to the lopinavir exposure.

Discussion and conclusions

Our results demonstrated that CYP3A was a major determinant of oral lopinavir pharmacokinetics, greatly surpassing the effects of Abcb1. In addition, Abcc2 had no noticeable effect on lopinavir exposure. Interestingly, no further increase in lopinavir systemic exposure could be observed when Abcb1a/b/Abcc2 was absent in addition to CYP3A. More detailed examination of the role of CYP3A indicated that both hepatic and intestinal CYP3A activity had a major impact on the oral bioavailability of lopinavir. Furthermore, our study demonstrated that ritonavir affected lopinavir pharmacokinetics by inhibition of both intestinal and hepatic CYP3A.

The systemic exposure of lopinavir in Cyp3a$^{-/-}$ mice was more than 2000-fold increased when compared with wild-type, unambiguously demonstrating the importance of CYP3A in lopinavir pharmacokinetics. Furthermore, our results show that both intestinal as well as hepatic CYP3A4 could reduce lopinavir exposure considerably. These results differ from our previous study in which we investigated the relative importance of intestinal versus hepatic CYP3A4 metabolism for docetaxel (van Herwaarden *et al*., 2007). Whereas for oral lopinavir we found a roughly comparable effect of intestinal and hepatic CYP3A4, this balance was much more in favour of intestinal CYP3A4 metabolism in case of orally administered docetaxel (van Herwaarden *et al*., 2007). So, perhaps not surprisingly, the relative importance of intestinal and hepatic CYP3A-mediated metabolism is substrate-dependent. Nonetheless, although our results show that both intestinal and hepatic CYP3A4 can limit the lopinavir exposure to a similar extent, one has to consider that after oral administration, lopinavir will first be subjected to intestinal metabolism and so only a minor fraction of the initial dose will reach the liver. As such, in terms of absolute amount of drug metabolized, intestinal CYP3A4 is still likely to account for the majority of the metabolism of lopinavir.

The majority of PIs are substrates for ABCB1 and it has been shown that ABCB1 can limit the oral bioavailability of PIs (see Kim *et al*., 1998). In addition to ABCB1, the drug transporter ABCC2 can also have an important effect on the oral bioavailability of drugs (see Lagas *et al*., 2006). Recently, Agarwal *et al*. (2007) reported that lopinavir, at low concentrations, was a substrate for ABCC2 *in vitro*. We, however, could not detect substantial active transport of lopinavir by ABCC2 at 5 μM. In addition, we also did not find a significant difference in systemic lopinavir exposure between Abcc2$^{-/-}$ and wild-type mice. Furthermore, no difference between Abcb1a/b$^{-/-}$ and Abcb1a/b/Abcc2$^{-/-}$ mice was observed. We note that many other PIs have been identified as ABCC2 substrates (Huisman *et al*., 2002). In the case of lopinavir, which is to our knowledge the first PI evaluated in Abcc2$^{-/-}$ mice, we thus found no obvious role for this drug transporter. Whether ABCC2 could affect the pharmacokinetics of other PIs remains to be determined.

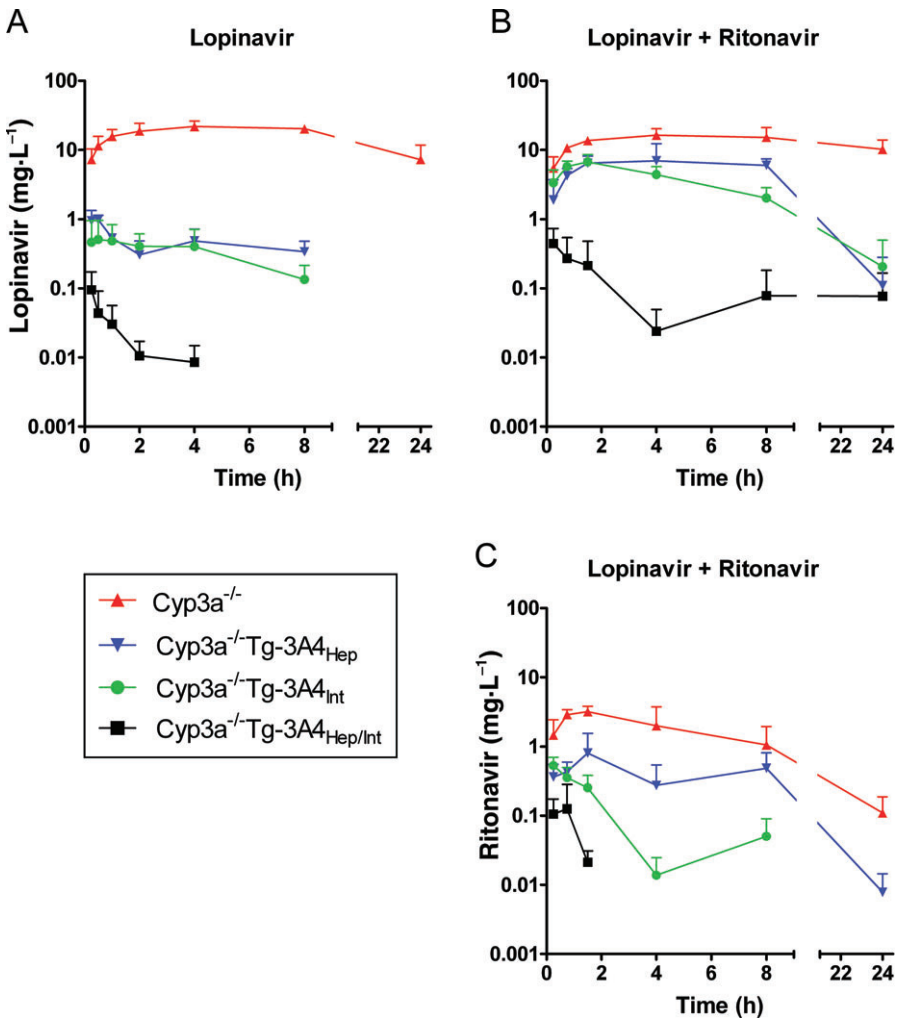

Figure 3 Plasma concentration–time curves of lopinavir in male Cyp3a$^{-/-}$Tg-3A4$_{Hep/Int}$, Cyp3a$^{-/-}$Tg-3A4$_{Hep}$, Cyp3a$^{-/-}$Tg-3A4$_{Int}$ and Cyp3a$^{-/-}$ mice after oral administration of lopinavir (100 mg·kg^{-1}) in the absence (A) and presence (B) of ritonavir (25 mg·kg^{-1}). The corresponding plasma concentration–time curves of ritonavir are given in (C). Data are shown as the mean concentration, and error bars represent the SD (n = 3–4 per time point). Cyp3a$^{-/-}$, cytochrome P450 3A knockout mice; Cyp3a$^{-/-}$Tg-3A4$_{Hep}$, Cyp3a knockout mice with liver-specific transgenic expression of human CYP3A4; Cyp3a$^{-/-}$Tg-3A4$_{Int}$, Cyp3a knockout mice with intestine-specific transgenic expression of human CYP3A4.

Given the prominent role of CYP3A in the pharmacokinetics of lopinavir, the known inter- and intra-individual variation in CYP3A activity can have profound effects on the therapeutic effectiveness and toxicity of lopinavir. There is indeed large variation in lopinavir plasma levels between patients (Guiard-Schmid *et al*., 2003). Ritonavir might reduce the inter-individual variation in lopinavir exposure to some extent but, as also observed in this study (Figure 3C), ritonavir levels itself can also be highly affected by CYP3A. Indeed, it has been reported that there is not only considerable inter-individual variation in lopinavir but also in ritonavir plasma levels in patients (Guiard-Schmid *et al*., 2003). Accordingly, lopinavir plasma levels have been found to positively correlate with ritonavir levels in patients (Guiard-Schmid *et al*., 2003; Crommentuyn *et al*., 2005).

Several drug–drug interactions affecting lopinavir have been documented (Crommentuyn *et al*., 2004; van der Lee *et al*., 2007; Corona *et al*., 2008; Falcon and Kakuda, 2008; Fulco *et al*., 2008; Kiser *et al*., 2008), which can probably all be attributed to interactions at the CYP3A level. For example, when lopinavir is given in combination with efavirenz or nevirapine, two known inducers of CYP3A4, the lopinavir exposure is significantly reduced (Bertz *et al*., 2002; Bergshoeff *et al*., 2005). Not only other drugs but also food constituents can affect CYP3A activity through either inhibition or induction of the enzyme (Mandlekar *et al*., 2006). For example, grapefruit juice can potently inhibit intestinal CYP3A (Lown *et al*., 1997; Paine and Oberlies, 2007). This interaction might be of particular relevance as we demonstrated that intestinal CYP3A4 significantly contributes to lopinavir metabolism. In addition, also some herbal products, which are widely used among HIV patients, are known to interfere with CYP3A activity (van den Bout-van den Beukel *et al*., 2006). An interesting case study of an HIV patient taking lopinavir in combination with herbal products (evening primrose, colayur and rheum frangula) was recently reported (van den Bout-van den Beukel *et al*., 2008). This patient attained toxic lopinavir levels and suffered from severe diarrhea. It was concluded that inhi-

bition of CYP3A4 by the herbals was the most plausible explanation for the observed toxic lopinavir levels (van den Bout-van den Beukel *et al*., 2008). Indeed, efamol, an ingredient of evening primrose, has been found to inhibit CYP3A4 (Zou *et al*., 2002), although little is known about the inhibition potential of the other herbs. These examples, together with our present data, underscore the importance of CYP3A in lopinavir treatment. Variable CYP3A activity as well as possible lopinavir–drug, food or herbal interactions could result in ineffective treatment or toxicity. Clearly, these interactions are not always easy to predict.

It has been suggested that the combined activity of CYP3A and drug transporters is important in limiting the systemic exposure of many orally administered drugs (Watkins, 1997; Ito *et al*., 1999a; Benet and Cummins, 2001; Kivisto *et al*., 2004; Garmire and Hunt, 2008). Recently, we demonstrated that when Cyp3a/Abcb1a/b$^{-/-}$ mice were challenged with docetaxel, a more than 70-fold increase in systemic exposure after oral administration was observed when compared with wild-type. Importantly, this effect was more than additive when compared with the single Cyp3a$^{-/-}$ (12-fold) and Abcb1a/b$^{-/-}$ (threefold) mice, illustrating a disproportionate increase in oral bioavailability when both systems are absent or inhibited. Interestingly, in the present study we also found a significant increase in lopinavir exposure in both Cyp3a$^{-/-}$ and Abcb1a/b/(Abcc2)$^{-/-}$ mice when compared with wild-type mice, but we did not observe a further increase in lopinavir exposure in Cyp3a/Abcb1a/b/(Abcc2)$^{-/-}$ mice compared with Cyp3a$^{-/-}$ mice. This indicates that the function of Abcb1a/b/(Abcc2)$^{-/-}$ is only noticeable at lower tissue and plasma levels but not at relatively higher levels reached when CYP3A is absent. The most likely explanation for this is that CYP3A activity, which drastically lowers lopinavir tissue and plasma concentrations, prevents saturation of Abcb1a/b and Abcc2 in intestine and liver. Apparently, both Abcb1 and Abcc2 have a relatively low maximal clearance rate for lopinavir and so these transporters become relatively easily saturated. Note that this is consistent with the *in vitro* study of Agarwal *et al*. (2007), who could readily detect lopinavir transport by ABCB1 and ABCC2 at concentrations of 0.5 μM, but scarcely any at higher concentrations (5–25 μM). An important theoretical argument of the proposed interplay between CYP3A and drug transporters is that the function of the drug transporter would prevent saturation of CYP3A (e.g. Benet and Cummins, 2001; Kivisto *et al*., 2004). Yet, in the case of lopinavir it seems that *in vivo* it is not so much the drug transporters that prevent saturation of CYP3A but rather that CYP3A prevents saturation of the drug transporters. This is an interesting finding of which, to our knowledge, no examples have been previously described. It also clearly illustrates how entangled and unpredictable the interplay between CYP3A and drug transporters can be. It is therefore important to be aware that the interplay between CYP3A and drug transporters cannot be simply generalized and will be highly substrate- and dose-dependent.

Acknowledgements

We thank Canan Karakaya for excellent technical assistance. This work was supported by the Technical Sciences Foundation of the Netherlands Organization for Scientific Research (NWO/STW).

Conflicts of interest

The authors declare no conflicts of interest.

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