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A Critical Analysis of the Interplay between Cytochrome P450 3A and P-Glycoprotein: Recent Insights from Knockout and Transgenic Mice

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***Abstract*——CYP3A is one of the most important drug-metabolizing enzymes, determining the first-pass metabolism, oral bioavailability, and elimination of many drugs. It is also an important determinant of variable drug exposure and is involved in many drug-drug interactions. Recent studies with CYP3A knockout and transgenic mice have yielded a number of key insights that are important to consider during drug discovery and development. For instance, studies with tissue-specific CYP3A-transgenic mice have highlighted the importance of intestinal CYP3A-dependent metabolism. They also revealed that intestinal CYP3A plays an important role in the regulation of various drug-handling systems in the liver. Intestinal CYP3A activity can thus have far-reaching pharmacological effects. Besides CYP3A, the active drug efflux transporter P-glycoprotein also has a strong effect on the pharmacokinetics of numerous drugs. CYP3A and P-glycoprotein have an extensive overlap in their substrate spectrum. It has been hypothesized that for many drugs, the combined activity of**

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CYP3A and P-glycoprotein makes for efficient intestinal first-pass metabolism of orally administered drugs as a result of a potentially synergistic collaboration. However, there is only limited in vitro and in vivo evidence for this hypothesis. There has also been some confusion in the field about what synergy actually means in this case. Our recent studies with Cyp3a/P-glycoprotein combination knockout mice have provided further insights into the CYP3A-P-glycoprotein interplay. We here present our view of the status of the synergy hypothesis and an attempt to clarify the existing confusion about synergy. We hope that this will facilitate further critical testing of the hypothesis and improve communication among researchers. Above all, the recent findings and insights into the interplay between CYP3A and P-glycoprotein may have implications for improving oral drug bioavailability and reducing adverse side effects.

I. Introduction

The cytochrome P450 (P450[1]) enzymes play a pivotal role in the metabolism of drugs and other xenobiotics. Members of the CYP3A subfamily are of particular interest because of their broad substrate specificity and high inter- and intraindividual variation in expression levels. Because CYP3A enzymes are strategically located in the liver and intestinal wall, they have a strong effect on the first-pass metabolism, oral bioavailability, and elimination of administered drugs. Furthermore, inhibition and induction of CYP3A enzymes are considered to be important determinants in the therapeutic efficacy and toxicity of numerous drugs (Dresser et al., 2000; Lamba et al., 2002). Accordingly, interactions at the CYP3A level are often the cause of pronounced drug-drug interactions (Thummel and Wilkinson, 1998).

In humans, four CYP3A genes and corresponding enzymes have been identified, but in adults, CYP3A4 and CYP3A5 are primarily relevant for drug metabolism. In general, CYP3A4 and CYP3A5 have similar substrate specificities (Williams et al., 2002). However, there are some notable exceptions, such as quinine and erythromycin, which are good substrates for CYP3A4 but not for CYP3A5 (Wrighton et al., 1990). It is noteworthy that CYP3A enzymes are involved in the metabolism of roughly 50% of currently marketed drugs (Guengerich, 1999). In addition to drugs and other xenobiotics, CYP3A enzymes are also involved in the synthesis and metabolism of a broad range of endogenous substrates, including cholesterol, steroid hormones (e.g., testosterone), bile acids, and vitamin D (Bodin et al., 2001; Xie et al., 2001; Gupta et al., 2005; Xu et al., 2006).

The extremely wide structural (and size) diversity of substrates metabolized by CYP3A4 has raised the question of how one protein can recognize such a diversity of molecules. Surprisingly, the first two CYP3A4 crystal structures suggested that the enzyme's active site was relatively small, considering the large substrates CYP3A4 is known to metabolize, and did not change much in shape upon ligand binding (Williams et al., 2004; Yano et al., 2004). However, more recent CYP3A4 crystal structures, including crystals with one or more substrate compounds bound, revealed a high degree of structural flexibility at the protein's active site (Ekroos and Sjögren, 2006). For example, it was found that, upon ligand binding, the active site volume can increase by more than 90%. This high flexibility of the CYP3A4 active site helps to explain the promiscuous behavior of the enzyme and its ability to accommodate substrates of widely different sizes.

In addition to CYP3A-mediated metabolism, many drugs are subject to active efflux from cells by drug-transporting proteins, of which P-glycoprotein (P-gp; MDR1; ABCB1) is one of the most important (Schinkel and Jonker, 2003). P-gp belongs to the family of ATP-binding cassette transporters and has very broad substrate specificity. It is noteworthy that there is a very large (albeit incomplete) overlap between CYP3A and P-gp substrate drugs. A recent crystal structure of P-gp revealed that it also has a very large drug binding site, allowing simultaneous binding of various drug ligands at different positions within the site (Aller et al., 2009), suggesting an organizational similarity to the CYP3A4 drug-binding site. In polarized (epithelial) cells, P-gp localizes to the apical (luminal) membrane. Activity of P-gp in the intestinal epithelium (enterocytes) can therefore strongly restrict the uptake of substrates from the gut, whereas in liver and kidney, it facilitates the excretion of substrates into bile and urine, respectively. In addition, P-gp is known to be expressed at blood-tissue barriers of various pharmacological sanctuary sites, such as the blood-brain, blood-placenta, and blood-testis barriers, where its activity prevents accumulation of potentially harmful compounds in these organs or compartments. Furthermore, expression of P-gp in tumor cells is known to confer drug resistance by extrusion of anticancer drugs out of the cell (Borst and Elferink, 2002; Szakács et al., 2006).

CYP3A and P-gp are both known to be major determinants of low and variable oral drug bioavailability for a range of drugs. Oral drug administration is generally more patient-friendly, cost-effective, and safer than intravenous administration. In addition, oral drug administration could also result in more favorable pharmacokinetics and may allow more long-term administration regimens. Given these advantages, enhancing oral bioavailability by inhibiting CYP3A or P-gp has received considerable interest in recent years (Malingré et al.,

[1]Abbreviations: AUC, area under the curve; BCRP, breast cancer resistance protein; CAR, constitutive androstane receptor; K77, *N*-methylpiperazine-urea-phenylalanine-homophenylalanine-vinylsulfone-benzene; M17, cyclosporine A metabolite; MRT, mean residence time; P450, cytochrome P450; P-gp, P-glycoprotein; PXR, pregnane X receptor.

2001; Kuppens et al., 2005; Oostendorp et al., 2009). For example, the widely used anticancer drug docetaxel has a very low oral bioavailability and is therefore administered only intravenously in the clinic. This low oral bioavailability is believed to be largely attributable to the fact that the drug is a very good substrate for both CYP3A and P-gp (Bardelmeijer et al., 2002). Accordingly, in a recent clinical proof-of-concept study, it was demonstrated that simultaneous oral coadministration of docetaxel with the CYP3A (and, to some extent, P-gp) inhibitor ritonavir resulted in a docetaxel exposure that was in the same range as achieved after intravenous administration (without ritonavir) (Oostendorp et al., 2009).

The strategic colocalization of CYP3A and P-gp in enterocytes and hepatocytes has given rise to models of the possible interaction between these proteins when dealing with shared substrates. Especially in enterocytes, the apically located P-gp might reduce the exposure of the microsomal (intracellular) CYP3A to drugs that are given orally. In contrast, in hepatocytes, orally administered drugs would first encounter (upon uptake into the liver) intracellular CYP3A, before being potentially transported into the bile by P-gp. These models have raised the question of whether CYP3A-P-gp interactions might have a substantial impact on pharmacokinetics, especially oral bioavailability. Although the individual contributions of CYP3A4 and P-gp to pharmacokinetics can be assessed reasonably well in model systems, it has been difficult to examine the effect of both systems functioning together in vivo. In this review, we discuss recent insights obtained from studies with *Cyp3a* knockout and humanized CYP3A4 transgenic mice. Furthermore, a critical survey of the currently available literature on the interplay between P-gp and CYP3A is presented. Special attention is given to recent studies that have used combination Cyp3a/P-gp(−/−) knockout mice, which were generated to obtain a better understanding of the functional interplay between CYP3A and P-gp. In addition to the fundamental insights, clinical implications for strategies to improve oral drug bioavailability and reduce adverse side effects are discussed.

II. Importance of CYP3A in Drug-Drug and Drug-Food Interactions

A. Inhibition

Given the plethora of drugs that are substrates and/or inhibitors for CYP3A, it is not surprising that the enzyme is an important determinant of many drug-drug and drug-food interactions (Thummel and Wilkinson, 1998; Dresser et al., 2000). The most common type of CYP3A-mediated drug-drug interaction is that one drug inhibits CYP3A-mediated metabolism of another drug, resulting in higher exposure levels of this second drug that are potentially toxic. The majority of inhibitors are competitive inhibitors that bind reversibly to CYP3A. In addition, mechanism-based (suicide) inhibitors require CYP3A-mediated metabolism toward a reactive intermediate that then binds irreversibly (covalently) to CYP3A (Zhou et al., 2005). This type of inhibition has a long-lasting effect, and enzyme activity can be restored only by de novo CYP3A synthesis.

Many clinically relevant drug-drug and drug-food interactions involving CYP3A inhibition have been documented (e.g., Dresser et al., 2000). For example, the widely used antihistamine drug terfenadine was withdrawn from the market because of CYP3A-mediated drug-drug interactions that resulted in several cases of lethal cardiotoxicity (Honig et al., 1993). Although most drug-drug interactions are undesirable, in particular cases, CYP3A can be purposely inhibited to improve drug therapy. For instance, the CYP3A inhibitor ritonavir, originally developed as an HIV protease inhibitor, is given in combination with another such inhibitor, lopinavir, which markedly improves the oral bioavailability of lopinavir (Kumar et al., 1999).

It is important to be aware that not only drugs but also food constituents can be potent inhibitors of CYP3A. For example, components of grapefruit juice potently inhibit (intestinal) CYP3A, and several clinically relevant drug-grapefruit juice interactions have been described previously (Dresser and Bailey, 2003; Paine and Oberlies, 2007). Likewise, some herbal remedies can interfere with CYP3A activity (e.g., Pal and Mitra, 2006), which is of extra concern in view of the generally unregulated use of these preparations.

B. Induction

A completely different type of drug-drug interaction is that a drug induces the expression of CYP3A. As a result, drugs that are CYP3A substrates will be more rapidly metabolized, which could lead to therapeutic inefficacy. Several nuclear receptors, including the pregnane X receptor (PXR) and constitutive androstane receptor (CAR) are known to play a key role in the regulation of CYP3A enzymes (Willson and Kliewer, 2002). In addition, activation of the glucocorticoid and vitamin D nuclear receptors has been indicated to result in an up-regulation of CYP3A enzymes (Schuetz et al., 1996; Makishima et al., 2002). PXR can be activated by a very broad range of xenobiotics, including many drugs, such as dexamethasone, rifampicin, and paclitaxel (Urquhart et al., 2007). In addition, hyperforin, the active ingredient in the herbal remedy St. John's wort, is a potent activator of PXR (Moore et al., 2000a). Furthermore, endogenous compounds such as bile acids (e.g., lithocholic acid) activate PXR and thereby increase CYP3A expression (Staudinger et al., 2001; Xie et al., 2001). In addition, various drugs (e.g., phenobarbital) can activate CAR, although its ligand specificity is generally considered to be less broad than that of PXR. It is important to note that these nuclear receptors regulate not only ex-

pression of CYP3A but also that of a range of other important detoxifying systems, including various drug-metabolizing enzymes and drug transporters such as P-glycoprotein (e.g., Urquhart et al., 2007).

Studies with transgenic and knockout mouse models for PXR and CAR have verified the importance of these receptors in CYP3A regulation (Xie et al., 2000; Wei et al., 2002). Furthermore, these animal studies have revealed important species differences in the ligand specificities for PXR and CAR. For instance, the synthetic steroid pregnelone-16α-carbonitrile is a potent inducer of mouse but not human PXR, whereas for the antitubercular drug rifampicin, this is the other way around (Jones et al., 2000; Xie et al., 2000). Likewise, the synthetic compound 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP) is an activator of mouse but not human CAR (Moore et al., 2000b). Several clinically relevant drug-drug interactions involving CYP3A induction have been described previously (Harmsen et al., 2007). For example, ineffectiveness of oral contraceptives, resulting in unwanted pregnancies, has been attributed to CYP3A induction by St. John's wort (Schwarz et al., 2003).

C. Stimulation

Another mechanism of drug-drug interaction is that a drug, by direct physical interaction with CYP3A, increases the rate of CYP3A-mediated metabolism of another drug. This direct stimulation of metabolism is also known as heterotropic positive cooperativity (Tang and Stearns, 2001; Hutzler and Tracy, 2002). This direct stimulation is believed to be caused by an allosteric effect of the stimulating drug that could even occur by simultaneous binding of the stimulator and the substrate drug to the large and flexible drug binding site of CYP3A (Harlow and Halpert, 1998). Stimulation of metabolism could be of clinical relevance, because the accelerated metabolism might result in subtherapeutic drug levels. A classic example of a compound that is known to stimulate several CYP3A-mediated reactions is 7,8-benzoflavone, which, for instance, increases the metabolism of diazepam and aflatoxin B1 in vitro (Andersson et al., 1994; Ueng et al., 1997). It has to be noted that, although many in vitro examples of CYP3A stimulation have been documented, there is only very limited in vivo, let alone clinical, evidence for this particular type of drug-drug interaction occurring (Hutzler and Tracy, 2002; Wienkers and Heath, 2005).

III. Insights from Cyp3a Knockout Mice

Given the prominent role of CYP3A in drug metabolism, the in vitro screening of novel drug candidates as potential substrates and/or inhibitors of CYP3A has become routine in the preclinical drug development stage. However, these in vitro studies are not always indicative for the in vivo situation. To get more insight into the pharmacokinetic but also the physiological role of CYP3A, mice lacking all *Cyp3a* genes [Cyp3a(−/−)] have recently been generated (van Herwaarden et al., 2007). Given the functional overlap of the CYP3A enzymes and the fact that there are no clear orthologs between the four human and the at least six (possibly seven or eight) mouse CYP3A enzymes, it was necessary to delete or inactivate all eight mouse *Cyp3a* (pseudo-)genes to obtain a useful knockout mouse model. Surprisingly, despite the fact that many endogenous physiological functions for CYP3A have been proposed, Cyp3a(−/−) mice seemed to be viable and fertile and did not show obvious physiological abnormalities. These observations suggest that the CYP3A enzymes do not have a vital endogenous physiological function besides their importance in the detoxification of xenobiotics.

A. Pharmacokinetic Studies in Cyp3a Knockout Mice

The widely used anticancer drug docetaxel is a well established CYP3A substrate and was the first drug studied in Cyp3a(−/−) mice. Docetaxel is metabolized by CYP3A to its primary metabolite hydroxydocetaxel, which can then be further metabolized by CYP3A to several other metabolites. Whereas hydroxydocetaxel formation could be readily determined in liver microsomes from wild-type mice, this formation was undetectable in microsomes from Cyp3a(−/−) mice (van Herwaarden et al., 2007). Accordingly, when docetaxel was given orally to Cyp3a(−/−) mice, an 18-fold increase in systemic exposure compared with wild-type mice was observed (van Herwaarden et al., 2007). In addition, a 7-fold higher systemic docetaxel exposure was found after intravenous administration. These results clearly illustrate the importance of CYP3A in the oral availability and clearance of docetaxel.

An even more striking increase in drug exposure was observed when the oral pharmacokinetics of the HIV protease inhibitor lopinavir was investigated in Cyp3a(−/−) mice. The oral AUC was more than 2000-fold higher in Cyp3a(−/−) mice than in wild-type mice (van Waterschoot et al., 2010). In addition, the C_{max} was increased by 74-fold, and lopinavir clearance was almost completely abolished. Because lopinavir is coformulated with the potent CYP3A inhibitor ritonavir, the extent to which ritonavir could increase the lopinavir exposure in wild-type mice was also evaluated. Ritonavir indeed substantially increased the lopinavir AUC in wild-type mice (~1000-fold), although this did not completely reach the levels seen in Cyp3a(−/−) mice. In contrast, ritonavir coadministration did not alter lopinavir exposure in Cyp3a(−/−) mice, suggesting that its effect was completely mediated through CYP3A. Overall, the docetaxel and lopinavir results clearly illustrate how Cyp3a(−/−) mice can be used to investigate the in vivo impact of CYP3A-dependent drug metabolism.

B. Compensatory Metabolism in Cyp3a Knockout Mice by CYP2C Enzymes

Despite the relatively modest changes in expression of other detoxifying systems observed in a microarray analysis of liver and intestine of Cyp3a(−/−) mice (van Herwaarden et al., 2007), puzzling results were found when the metabolism of midazolam in Cyp3a(−/−) mice was investigated. Midazolam is one of the most widely used probes to assess CYP3A activity in humans. The drug is considered highly specific because no other human P450s contribute significantly to its metabolism. In addition, the fact that midazolam is not a substrate for the drug transporter P-glycoprotein makes this drug very suitable as CYP3A probe (Kirby et al., 2006). The biotransformation of midazolam by CYP3A enzymes yields 1′-OH and 4-OH midazolam as the principal metabolites (Kronbach et al., 1989). Although it was therefore expected that the midazolam metabolism and clearance would be severely reduced in the absence of CYP3A enzymes, the metabolism seemed to be only marginally altered in Cyp3a(−/−) mice compared with wild type, both in vitro and in vivo (van Waterschoot et al., 2008).

Further study revealed that several of the murine CYP2C enzymes are primarily responsible for this compensatory metabolism. A number of individual murine CYP2C enzymes could be identified that catalyze the midazolam 1′- and/or 4-hydroxylation reactions. Furthermore, it was demonstrated that the *Cyp3a* knockout results in a significant up-regulation of some of these CYP2C enzymes (van Waterschoot et al., 2008). Overall, this study demonstrated that in the absence of an important detoxifying system such as CYP3A, organisms can still deal with some xenobiotics as a result of the overlapping substrate specificity of P450s and the potential up-regulation of these enzymes. We assert that such flexible compensatory interplay between functionally related detoxifying systems is probably essential to their biological role in xenobiotic protection. However, this interplay will often be strongly drug- and species-dependent. For instance, human CYP2C enzymes are not able to efficiently metabolize midazolam (van Waterschoot et al., 2008).

In contrast to midazolam, the closely related drug triazolam is more specific for CYP3A compared with other mouse P450 isoforms (Perloff et al., 2000) and might therefore be a better probe drug to characterize the Cyp3a(−/−) mouse strain. Indeed, compared with midazolam, the compensatory metabolism of triazolam by mouse CYP2C seemed to be much lower (van Waterschoot et al., 2009b). For example, the intrinsic clearance for the 1′-OH triazolam formation in Cyp3a(−/−) mouse liver microsomes was reduced by 40-fold. In vivo, the difference was less pronounced; nevertheless, a 1.6-fold increase in triazolam systemic exposure was found in Cyp3a(−/−) mice after oral administration compared with wild-type mice. Nonetheless, we demonstrated that in the case of triazolam, some residual triazolam metabolism remains, both in vitro and in vivo, even though the contribution of CYP2C enzymes in the wild-type situation is negligible (van Waterschoot et al., 2009b).

The above-mentioned studies indicate that Cyp3a(−/−) mice are potentially valuable tools to investigate the impact of CYP3A. However, it is also clear that other up-regulated P450 enzymes can obscure, for some drugs, the results obtained. Therefore, proper in vitro evaluation of the background metabolism of drugs of interest in Cyp3a(−/−) mice would be recommended to optimize application of the Cyp3a(−/−) mouse model. We also note that not only can the metabolism of particular xenobiotics possibly be taken over by other P450 enzymes (e.g., CYP2C) but also that of endogenous compounds. Indeed, not only CYP3A but also CYP2C enzymes are known to be involved in the metabolism of steroids (e.g., testosterone) and bile acids (e.g., lithocholic acid) (Schenkman, 1992; Deo and Bandiera, 2008). Although this requires further research, we cannot exclude the possibility that compensatory metabolism of endogenous compounds by CYP2C enzymes might also be accountable in part for the fact that we did not observe an overt, spontaneous physiological phenotype in Cyp3a(−/−) mice.

IV. Insights from Tissue-Specific CYP3A4-Transgenic Mouse Models

A. Relative Importance of Intestinal and Hepatic Metabolism

In addition to Cyp3a(−/−) mice, transgenic mice with expression of human CYP3A4 in either the intestine or the liver and on a mouse *Cyp3a* knockout background have been generated (van Herwaarden et al., 2007). The aim for making these mice was 2-fold. First, we wanted to obtain a "humanized" mouse model in which the human CYP3A4 was expressed in liver and/or intestine, so the function of this primary human CYP3A could be studied in an in vivo situation. This should yield optimal predictive power for pharmacological and toxicological functions and behavior of CYP3A4 in humans. Second, we wanted to obtain basic mechanistic insight into the separate and combined roles of intestinal and hepatic CYP3A in a defined in vivo situation. In case of the liver-specific transgenic strain, expression of CYP3A4 cDNA was under the control of an apolipoprotein E promoter, which results in constitutive expression of human CYP3A4 in the liver hepatocytes (Cyp3a(−/−) Tg-3A4$_{Hep}$ [previously Cyp3a(−/−)A]). In addition, transgenic mice expressing CYP3A4 cDNA under control of the villin promoter resulted in constitutive expression of human CYP3A4 in the villous epithelial cells of the intestine (Cyp3a(−/−)Tg-3A4$_{Int}$ [previously Cyp3a(−/−)V]) (van Herwaarden et al., 2007).

The CYP3A4 transgenic mice have subsequently been used to evaluate the relative impact of intestinal versus hepatic metabolism. Pharmacokinetic studies with do-

cetaxel revealed that intestinal CYP3A4 alone was sufficient to virtually abrogate net docetaxel entry from the gut after oral administration (causing a 17-fold reduction in oral AUC), whereas hepatic CYP3A4 was more important in systemic docetaxel clearance after intravenous dosing, causing a 5-fold drop in intravenous AUC (van Herwaarden et al., 2007). In contrast, hepatic CYP3A4 had only a modest impact on oral docetaxel (2-fold reduced oral AUC) whereas intestinal CYP3A4 had only a minor impact on intravenous docetaxel (1.4-fold decrease in intravenous AUC). The dramatic impact of intestinal CYP3A4 on the docetaxel oral AUC illustrates the profound effect that intestinal metabolism can have on overall oral bioavailability of a drug (reduction from 21 to 1.8%).

It is noteworthy that subsequent studies with lopinavir, which is also a shared CYP3A and P-gp substrate, showed a more balanced role of intestinal and hepatic metabolism after oral administration of this drug than for docetaxel. Either intestinal or hepatic CYP3A4 activity alone could reduce the systemic lopinavir exposure by approximately 100-fold compared with Cyp3a(−/−) mice (van Waterschoot et al., 2010). An additional reduction of lopinavir exposure was observed in transgenic mice expressing CYP3A4 in both intestine and liver [>4000-fold reduction compared with Cyp3a(−/−) mice]. Together, the docetaxel and lopinavir studies underscored the importance of intestinal metabolism in lowering the bioavailability of orally administered drugs. However, they also showed that the relative importance of intestinal and hepatic CYP3A metabolism is highly substrate-dependent.

A question that remained was whether intestinal metabolism can also be as dominant for drugs that, unlike docetaxel and lopinavir, are not P-gp substrates. To investigate this, we extended our studies with triazolam (van Waterschoot et al., 2009b). Transgenic expression of CYP3A4 in the intestine [Cyp3a(−/−)Tg-3A4$_{Int}$] significantly reduced the triazolam systemic exposure after oral administration compared with Cyp3a(−/−) mice (2.3-fold). However, no difference in exposure was observed in Cyp3a(−/−)Tg-3A4$_{Hep}$ and Cyp3a(−/−) mice. These results indicate that, after oral administration of the non-P-gp substrate triazolam, intestinal metabolism by CYP3A4 is still more significant than hepatic metabolism. Thus, a substantial impact of intestinal CYP3A4 metabolism is not necessarily dependent on whether a drug is simultaneously subject to P-gp-mediated efflux.

B. Evaluation of Drug-Drug Interactions in CYP3A4 Humanized Mice

To evaluate whether the CYP3A4 humanized mouse models could be used to study in vivo drug-drug interactions, the prototypical CYP3A4 inhibitor ketoconazole was coadministered with triazolam in pharmacokinetic studies (van Waterschoot et al., 2009b). Ketoconazole increased the triazolam exposure in all mouse strains with transgenic CYP3A4 expression but, as expected, not in Cyp3a(−/−) mice. Coadministration of the CYP3A inhibitor ritonavir with lopinavir also resulted in ~25-fold higher lopinavir exposures in all the different Cyp3a(−/−)Tg-3A4 strains but again had no effect in Cyp3a(−/−) mice. These findings confirmed that, at least for the tested substrate drugs, ketoconazole and ritonavir are relatively specific in vivo CYP3A inhibitors.

Interesting findings were obtained with the anticancer drug gefitinib (van Waterschoot et al., 2009b), which turned out to be a potent stimulator of the in vitro 1′-OH triazolam formation. Subsequent in vivo studies revealed that coadministration of gefitinib resulted in a significantly lower triazolam exposure in Cyp3a(−/−) Tg-3A4$_{Hep}$ and Cyp3a(−/−)Tg-3A4$_{Hep/Int}$ mice, but not in Cyp3a(−/−) and Cyp3a(−/−)Tg-3A4$_{Int}$ mice (van Waterschoot et al., 2009b). These results indicate that gefitinib-mediated stimulation of CYP3A4 activity primarily had an impact in the liver, and that direct CYP3A4 stimulation can have, under some circumstances, a substantial in vivo impact on the pharmacokinetics of a metabolized drug. So far, there are no recognized examples of clinically relevant drug-drug interactions that can be attributed to the direct stimulation of CYP3A-mediated metabolism. Because this phenomenon typically concerns only a specific combination of drugs, we expect that stimulation of CYP3A-mediated drug metabolism will be more rarely encountered than inhibition. These studies indicate that CYP3A4 transgenic mice are good models to investigate the potential in vivo impact of drug-drug interactions in a tissue-specific fashion and can be used for either analyzing inhibition or stimulation of CYP3A4.

C. Intestinal CYP3A Regulates Detoxifying Systems in the Liver by Modulating Exposure to Inducing Compounds

As described above, several CYP2C enzymes were up-regulated in Cyp3a(−/−) mice. Among these, CYP2C55 in particular seemed to be highly up-regulated. We hypothesized that transgenic expression of CYP3A4 could compensate for the loss of murine CYP3A activity and that, consequently, expression levels of CYP2C55 might be normalized. This was indeed the case (van Waterschoot et al., 2009c). We could thus use changes in CYP2C55 expression as a relatively sensitive probe for hepatic gene expression changes due to variable CYP3A activity. It is noteworthy that the pronounced induction of CYP2C55 observed in livers of Cyp3a(−/−) mice was reversed not only in transgenic mice with liver-specific CYP3A4 expression (Cyp3a(−/−)Tg-3A4$_{Hep}$) but also in mice that have only intestinal CYP3A4 expression (Cyp3a(−/−)Tg-3A4$_{Int}$) (Fig. 1). This suggests that CYP3A functionality in the intestine is important for limiting hepatic exposure to orally ingested inducing agents. In contrast, expression of CYP3A4 in the liver

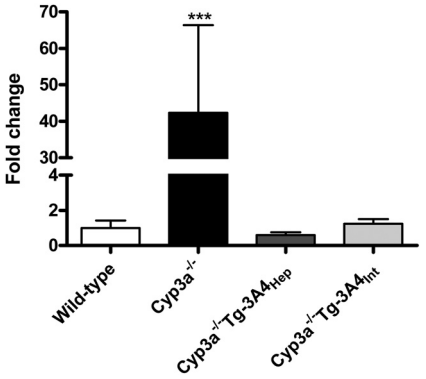

FIG. 1. Expression levels of CYP2C55 in liver of wild-type, Cyp3a(−/−), Cyp3a(−/−)Tg-3A4$_{Hep}$, and Cyp3a(−/−)Tg-3A4$_{Int}$ mice as determined by reverse transcription-polymerase chain reaction. Values represent means ± S.D. (n = 4 for all strains) expressed as fold change in comparison with wild type. Data are normalized to GAPDH expression. Asterisks indicate statistically significant differences in ΔC_t values compared with wild type: ***, $P < 0.001$ (van Waterschoot et al., 2009c).

did not influence the expression of intestinal CYP2C55 [which was 4.4-fold up-regulated in Cyp3a(−/−) mice], whereas intestinal CYP3A4 expression did result in reduced intestinal CYP2C55 levels. This suggests that local intestinal exposure to CYP2C55-inducing compounds is determined by intestinal rather than hepatic CYP3A activity. It is noteworthy that analysis of expression of a number of other genes demonstrated that intestinal CYP3A activity is not only relevant for the regulation of hepatic CYP2C55 but also for regulation of a broad range of other hepatic detoxifying systems, including drug metabolism, efflux, and uptake systems (van Waterschoot et al., 2009c).

Further studies indicated a prominent role for the nuclear receptors PXR and CAR in the in vivo regulation of CYP2C55 (van Waterschoot et al., 2009c). We subsequently hypothesized that CYP3A normally metabolizes one or more food-derived activators of PXR, CAR, and/or other nuclear xenobiotic receptors and that, consequently, levels of these activators are much higher in Cyp3a(−/−) mice. Many dietary phytochemicals are known to activate PXR and/or CAR (Mandlekar et al., 2006). The CYP3A-mediated breakdown of such phytochemicals clearly would be absent in Cyp3a(−/−) mice. It is therefore likely that plasma and tissue levels of a number of inducing phytochemicals that are normally degraded by CYP3A will be higher in Cyp3a(−/−) mice and that, consequently, PXR and CAR are more activated in these mice. It should be noted that the standard chow of the tested mice (as in most animal facilities) consists of a mixture containing several relatively crude plant-derived ingredients, including alfalfa hay, linseed, whole wheat, dent corn, rolled oats, and soy oil, undoubtedly resulting in a high dietary phytochemical load. Indeed, when mice were given instead a semisynthetic diet containing much lower levels of phytochemicals, the induction of CYP2C55 (and several other detoxifying systems) in Cyp3a(−/−) mice was markedly reduced (van Waterschoot et al., 2009c). Although the further identification of the dietary inducing compounds involved will be of interest, this could be quite difficult, especially because it is likely that several compounds work in concert, each possibly activating different nuclear receptors and corresponding target genes, making the assessment of individual contributions complicated.

Nonetheless, it seems likely that increased levels of food-derived compounds are primarily responsible for the induction of CYP2C55, as well as of several other detoxifying systems, in liver and intestine of Cyp3a(−/−) mice. Apparently, intestinal as well as hepatic CYP3A4 activity can efficiently limit the hepatic exposure to food-derived activators of PXR, CAR, and possibly other xenobiotic nuclear receptors. Extensive intestinal metabolism of orally ingested xenobiotics is, of course, in line with the previously demonstrated important role of intestinal CYP3A4 in first-pass metabolism of docetaxel (van Herwaarden et al., 2007). Intestinal CYP3A activity can thus not only directly affect xenobiotic availability but also indirectly affect expression levels of a broad range of other detoxifying systems in the liver. Furthermore, the induction level of detoxifying systems in the intestine is significantly reduced by intestinal CYP3A4 activity. Given the diversity and potential impact of the detoxifying systems affected, these findings indicate that intestinal CYP3A activity can have far-reaching biological effects.

V. Interplay between CYP3A and P-Glycoprotein

A. Theoretical Considerations

Both CYP3A and P-gp can have a strong effect in reducing the oral bioavailability and in mediating the elimination of numerous drugs, as documented in numerous preclinical and clinical studies. It is noteworthy that there is a very extensive overlap between their drug substrates, and between the tissue-specific expression of CYP3A and P-gp, especially in liver (hepatocytes) and intestine (enterocytes), as first pointed out by Wacher et al. (1995). Furthermore, both detoxifying systems are subject to (up-)regulation by prototypical nuclear xenobiotic receptors such as PXR and CAR (e.g., Urquhart et al., 2007), supporting a strong functional relationship. In view of these findings, it has been hypothesized that for many shared P-gp and CYP3A substrate drugs, the combination of back-transport by P-gp in the intestinal epithelial cell and the presence of CYP3A4-mediated metabolism within the same cell makes for efficient intestinal first-pass metabolism of orally administered drugs (Wacher et al., 1996; Watkins, 1997; Fisher et al., 1999; Ito et al., 1999; Benet and Cummins, 2001; Kivistö et al., 2004; Knight et al., 2006; Garmire and Hunt, 2008; Benet, 2009; Pang et al., 2009; Fan et al., 2010). Because the combined activity of both systems working together was initially expected to be more efficient than the sum of the separate activities, this would even result in a synergistic collaboration between P-gp and CYP3A.

Two mechanistic arguments have been brought forward to support a possible synergistic action of intestinal P-gp and CYP3A: first, the function of P-gp would lower the intracellular enterocyte concentration of a substrate drug and might thus prevent saturation of the CYP3A enzyme by keeping the concentration more within the linear range of the CYP3A metabolizing capacity. Consequently, a larger fraction of the intracellular drug can be metabolized, and eventually overall intestinal metabolism would be more extensive, because a smaller fraction of the drug can reach the circulation unchanged. Note, however, that the speed of metabolism would not be increased (rather even decreased), but P-gp activity would simply afford CYP3A more time to metabolize its substrates extensively. Thus, P-gp would allow CYP3A to efficiently metabolize drugs over a far higher dose range. Second, it has been suggested that the extrusion function of P-gp combined with subsequent drug re-uptake may allow drug substrates to have repeated and therefore prolonged access to enterocyte CYP3A, thereby increasing the probability that a drug will be metabolized. This repeated cycling of drugs per se would increase the total metabolism, even without considering saturating or nonsaturating conditions for CYP3A (e.g., Benet, 2009).

Intestinal P-gp activity might, of course, also reduce the rate at which oral drug reaches the liver and thus reduce the chance of saturating drug-metabolizing activity in the liver, but this is in essence a two-organ variant of the first mechanistic argument. In a further elaboration of P-gp-CYP3A interplay, it has also been hypothesized that CYP3A-generated metabolites are often better substrates for P-gp than the parent compound (Watkins, 1997; Christians et al., 2005), preventing product inhibition of CYP3A. An obvious additional possibility is that CYP3A-dependent metabolism yields metabolites that are efficiently transported by other (apical) efflux transporters such as MRP2 (ABCC2) or BCRP (ABCG2), thus also facilitating CYP3A functioning. Overall, these transport processes would lead to highly efficient intestinal metabolism by CYP3A.

1. Intermezzo: Definition of Synergy in CYP3A-P-gp Interplay. By now, many in vitro, some in vivo, and some in silico studies have tried to establish whether synergistic collaboration between enterocyte P-gp and CYP3A is something that occurs readily, with variable degrees of success. Our impression is that there can be substantial confusion among researchers as to what synergy actually means in this case, and this confusion can hinder communication between researchers.

Although the Greek roots of the equivalent words synergy and synergism simply mean "working together" (collaboration), the usual meaning in pharmacology is to describe that the effect of combining two (or more) drugs or processes exceeds the effect expected from the sum of their individual effects. We use this definition of synergy herein. In contrast, collaboration is a more neutral term that may also be used when the effect of combining two drugs or processes is simply additive or even subadditive.

We would like to point out that there is currently a substantial risk of confusion among researchers as to what synergism means in the interplay between P-glycoprotein and CYP3A. In some cases, this can mean that two researchers can interpret the same set of results as indicating synergism or lack of synergism, or even antagonism (e.g., Ito et al., 1999; Cummins et al., 2002; Garmire and Hunt, 2008). To avoid such confusion, it is crucial that a precise definition of synergy is provided in each case. We feel that synergism is a core component of the CYP3A-P-gp "interplay" concept. Without synergism, the CYP3A-P-gp interplay would amount only to a trivial addition of detoxifying effects and would thus have little added scientific value. Avoiding this term could even lead to obfuscation of the central questions to be addressed. In the interest of clarity, we therefore apply the term where appropriate according to the explanation below.

In this article, we use the term synergy as applied to the mechanistic collaboration between P-glycoprotein and CYP3A, because we are primarily interested in obtaining a basic understanding of the interaction of these proteins in the cell. Because collaboration in the enterocyte is thought to be critically dependent on the relative position of these proteins in the cell (P-gp in the apical membrane, CYP3A in cytoplasmic membranes) and relative to the direction of flux of the drug, we will call it positional synergy. Note that in our approach, we (and most other researchers in this field) do not postulate that P-gp or CYP3A directly affects the enzymatic capacity of the other protein (K_m, V_{max}) but just that they alter the drug exposure conditions of the other protein and therefore its relative contribution to the overall drug detoxification. We thus assume that P-gp and CYP3A function as autonomous enzymes.

To illustrate the potential confusion in the use of the term synergism, we present a simplified, theoretical set of outcomes of oral plasma AUCs of a dual CYP3A/P-gp substrate drug in wild-type and P-gp(−/−), Cyp3a(−/−), and Cyp3a/P-gp(−/−) mice (i.e., mice lacking P-gp, CYP3A, or both) in Table 1. The assumption of the enterocyte positional synergism model is that P-gp activity can enhance the contribution of CYP3A to reducing the plasma levels of drug, for instance by preventing saturation of the enzyme. The simplest case is that P-gp and CYP3A work in a purely additive manner (row "ad-

TABLE 1
Theoretical AUC values for an oral drug in various mouse strains assuming additive, synergistic, or competitive (= antagonistic) functioning of CYP3A and P-gp

	Cyp3a/P-gp(−/−) (−)	P-gp(−/−) (+CYP3A)	Cyp3a(−/−) (+P-gp)	WT (+CYP3A+P-gp)
Additive	100	5	20	1
Synergistic	100	25	20	1
Competitive	100	4	5	1

ditive" in Table 1). We assumed that CYP3A by itself can reduce the AUC by 20-fold, and P-gp by itself by 5-fold. Thus the AUC of Cyp3a/P-gp(−/−) animals that do not have any of these systems (taken here as 100) is reduced by 5-fold in the Cyp3a(−/−) mice due to P-gp addition and by 20-fold in the P-gp(−/−) mice due to CYP3A addition. In the wild-type mice (presence of both CYP3A and P-gp), the AUC is reduced by 20 × 5 = 100-fold. We emphasize that, if one wants to understand the mechanistic positional synergy between P-gp and CYP3A, it is essential that one reasons from the fully deficient situation and then sees what happens when adding one system, or the other, or both. After all, the exposure (AUC) in the fully deficient situation approximates the total delivered (absorbed) dose in the absence of CYP3A and P-gp. The functional contribution of CYP3A or P-gp is to reduce that amount, and the relative contributions of the two can be determined by the extent of that reduction. The AUC in the fully proficient (wild-type) state, on the other hand, reflects the amount of drug that has escaped (first-pass) clearance rather than the amount of drug that was cleared by the combined actions of CYP3A and P-gp. This is therefore not the right starting point to assess the separate and combined actions of CYP3A and P-gp.

When we postulate that there is positional synergism between CYP3A and P-gp, the contribution of CYP3A alone is much smaller than in the presence of P-gp (Table 1, row "Synergistic"). Thus, the AUC in P-gp(−/−) mice could be 4-fold reduced (from 100 to 25) relative to the fully deficient mice as a result of the addition of CYP3A alone, and the AUC in Cyp3a(−/−) mice 5-fold reduced (from 100 to 20) as a result of P-gp addition. However, when both systems are present at the same time, the CYP3A contribution to reducing the AUC is boosted from 4- to 20-fold, and the overall effect on the AUC in wild-type mice is a 100-fold reduction (5 × 20). This is clearly mechanistic synergy: the predicted impact of adding both CYP3A and P-gp to the double-deficient mice would have been a 20-fold reduction in AUC (5 × 4) in wild-type mice, but the actual reduction is 100-fold.

And herein lies the catch: most people, when looking for synergy, will tend to start reasoning from the wild-type (fully proficient) situation and then see what happens when subsequently CYP3A, P-gp, or both are removed (or inhibited). In this approach, when looking at the data in the same row in Table 1, one will observe that the combined impact of removing both CYP3A and P-gp is actually much less than the expected (25 × 20 =) 500-fold increase in AUC, with a 100-fold increase observed, and come to the wrong conclusion that there is no synergy, and probably even some form of antagonism, between CYP3A and P-gp.

Matters get worse when there is actual functional competition (or antagonism) between the two systems, as presented in the row "Competitive" in Table 1. In this case, we have assumed that the contribution of each of the detoxifying systems to lowering the AUC is much reduced in the presence of the other system because they are competing for the same drug substrate (as is actually predicted by various mathematical models of the P-gp-CYP3A interplay in the enterocyte). For instance, as postulated in Table 1, addition to the double-deficient mice of CYP3A by itself can reduce the AUC by 25-fold. and addition of P-gp, by 20-fold, but because of competition, the combination of the two systems reduces the AUC by only 100-fold, instead of the expected 500-fold (25 × 20). However, when one looks at these data starting from the wild-type situation, one will observe that the removal (or inhibition) of each system separately has a much smaller impact on the AUC (4- or 5-fold) than the combined removal (100-fold, whereas only a 20-fold effect would have been expected). Many people will then erroneously conclude that there is positional synergism between P-gp and CYP3A, whereas they are in fact looking at positional antagonism. We thus feel that it is essential that, when one wants to obtain insight into the mechanistic basis of collaboration (and potential synergy or competition) between detoxifying systems, one starts reasoning from the situation without these systems, and then observes what is happening when the systems are added, either alone or in combination.

There is of course one very important point underlying this confusion: traditionally in pharmacology, people have been accustomed to assessing synergy by looking at what happens when two or more (usually inhibiting) drugs are combined—in which case they are studying synergy between the investigated drugs rather than synergy between the affected physiological targets of the drugs. In the CYP3A–P-gp interplay, then, it is tempting to consider what happens when CYP3A, P-gp, or both are inhibited using specific inhibitors (i.e., drugs) of each system. In that case, in a situation in which the detoxifying systems are functionally competitive (Table 1, row "Competitive"), they would correctly conclude that the inhibitors have a synergistic effect on the process studied (increase in AUC in combination drug treatment higher than expected). However, as explained above, in the case of P-gp and CYP3A interplay, this is indicative of an antagonistic (or competitive) mechanistic functioning of the two detoxifying systems involved rather than of a synergistic functioning. It is crucial, therefore, always to define whether one is interested in studying synergism between drugs, or a mechanistic synergistic interaction between the physiological targets. We therefore feel that one should always define exactly what is meant by the term synergy in each and every publication in which this term is used, and use it consistently throughout.

We note in passing that the above considerations are not only relevant for the CYP3A-P-gp interplay in the enterocyte. Specifically, also the interaction between P-gp and BCRP as drug-efflux transporters at the blood-

brain barrier has been subject to an analogous confusion as to what a "synergistic" increase in brain penetration means when both systems are simultaneously removed or inhibited (Lagas et al., 2009; Polli et al., 2009; Agarwal et al., 2010). What is often observed is that the loss of only one or the other of the transporters gives a small or even unnoticeable effect on brain drug levels, whereas the simultaneous loss of both proteins results in a disproportionately large increase in brain penetration. One will readily see that this situation is analogous to the data in the row "Competitive" in Table 1, and we think that such results are thus indicative of a competitive functioning of P-gp and BCRP in the blood-brain barrier rather than of a mechanistically synergistic activity between both transporters. It is noteworthy that Kodaira et al. (2010) showed that upon careful pharmacokinetic modeling, many apparently "synergistic" effects of P-gp and BCRP in reducing brain penetration of drugs could be readily explained by the additive activity of P-gp and BCRP in the blood-brain barrier. The appearance of synergism was mainly a consequence of the low efflux clearance of the brain in the absence of both transporters compared with the efflux clearances mediated by either of the transporters alone, or in combination.

B. In Vitro Studies

Several in vitro studies have addressed the interplay between CYP3A and P-gp (reviewed in Benet et al., 2003; Christians et al., 2005; Knight et al., 2006; Benet, 2009). The majority of these studies was done using Caco-2 cells that are known to express P-gp and in which CYP3A4 can either be induced or transfected. The cells are usually studied as polarized monolayers in transwell plates, allowing separate sampling of the apical, basolateral, and cellular drug and metabolite concentrations and assessment of transepithelial transport processes. For example, Gan et al. (1996) studied the in vitro transport of the dual CYP3A and P-gp substrate cyclosporine A using Caco-2 cell monolayers. They found significantly more cyclosporine A metabolite (M17) formation during the apical-to-basolateral transport of cyclosporine A compared with the basolateral-to-apical transport. This suggested that efflux by P-gp results in increased cyclosporine A metabolism during apical to basolateral transport. They also found that the amount of M17 accumulating on the apical side was much greater than that on the basolateral side during the apical-to-basolateral transport of cyclosporine A. This was attributed to P-gp–mediated efflux of M17 to the apical side. It must be noted, however, that CYP3A substrates that are not a substrate for P-gp have a tendency to be excreted more at the apical side. This could be due to the localization of CYP3A at the apical side of the cell as well as the greater surface area of the apical microvilli, allowing more extensive diffusion at this pole of the cell (Watkins, 1997). A role for other apical efflux transporters (e.g., ABCG2, ABCC2) in apical excretion of such metabolites cannot be excluded either.

The extraction ratio (ER) is often used as a measure for the extent of intestinal or enterocyte metabolism, and refers to the fraction of drug that is metabolized relative to the amount of parent drug that reaches the receiving compartment (Fisher et al., 1999):

$$ER = \frac{\Sigma Metabolites_{A+B+C}}{\Sigma Metabolites_{A+B+C} + \Sigma parent_{rec}} \cdot 100\%$$

Where A, B, and C represent apical, basolateral, and cellular amounts of metabolites, respectively, and rec represents the amount of parent drug recovered in the receiving compartment. Thus, in principle, the higher the ER, the higher the fraction of drug that has been metabolized. In a modification of this approach, Cummins et al. (2002) used a definition of ER (ER*) in which the intracellular amount of parent drug is also included in the denominator:

$$ER^* = \frac{\Sigma Metabolites_{A+B+C}}{\Sigma Metabolites_{A+B+C} + \Sigma parent_{rec+C}} \cdot 100\%$$

For apical-to-basolateral drug translocation, which is the relevant situation for drug absorption across the enterocyte, the receiving compartment is basolateral, but the ER can also be applied to translocation from basolateral to apical.

The cysteine protease inhibitor K77 is a dual CYP3A4 and P-gp substrate. This compound was investigated in CYP3A4-expressing Caco-2 cells along with felodipine, which is also a substrate of CYP3A4 but not of P-gp (Cummins et al., 2002). The ER* in the absorptive (apical-to-basolateral) direction for K77 was found to be reduced from 33 to 14% when the P-gp inhibitor GG918 was added to the cells. In contrast, the absorptive ER* of felodipine remained unaffected when GG918 was added to the system. Analogous, albeit quantitatively smaller, effects were reported for the dual CYP3A/P-gp substrate sirolimus (Cummins et al., 2004). These results led the authors to conclude that P-gp activity in enterocytes increases the CYP3A-mediated metabolism of K77 as well as of sirolimus. In a further extension of these studies, an in situ single-pass intestinal perfusion study was performed in rats, using K77 again as a probe substrate and midazolam as a non–P-gp substrate control (Cummins et al., 2003). Inhibition of P-gp with GG918 resulted in a significant decrease of the fraction K77 metabolized (from 95 to 85%), but the simultaneous decrease in ER (from 49 to 37%) was not statistically significant.

In apparent contrast with these findings, Mouly et al. (2004) found in CYP3A4-expressing Caco-2 cells that inhibition of P-gp led to a considerable increase in the total formation of the CYP3A4-mediated metabolite of the shared P-gp and CYP3A substrate saquinavir. This was attributed to the increased cellular content of

saquinavir as a result of the reduced efflux when P-gp was inhibited. Still, the ER was significantly decreased when P-gp was inhibited (Mouly et al., 2004; Knight et al., 2006), primarily because of the substantial increase in the basolateral amount of parent drug upon P-gp inhibition. Retrospective analysis of the in vitro K77 data of Cummins et al. (2002) indicates that there was likewise a substantial increase (~50%) in total K77 metabolite formation upon inhibition of P-gp. Thus, both studies yielded qualitatively similar results, but different aspects of the outcomes (changes in ER or ER* versus total amount of metabolite formation) were emphasized by the respective authors. In addition, in the in situ intestinal perfusion experiment, total K77 metabolite formation was increased by P-gp inhibition (Cummins et al., 2003). It seems, therefore, that upon inhibition of P-gp, overall metabolite formation is increased, but the ER or ER* is decreased. The increased amount of metabolite formation that is consistently found experimentally upon P-gp inhibition seems to be at odds with the purported increased efficiency of metabolism due to P-gp activity.

Indeed, the use of the ER as a means to obtain insight into the mechanistic interplay between CYP3A and P-gp in the enterocyte has been criticized by a number of groups, who argued that it may yield incorrect conclusions (Knight et al., 2006; Pang et al., 2009). This has been discussed in detail by Knight et al. (2006), who indicated that the ER can be used to determine only how much parent drug is metabolized relative to the amount of parent drug appearing in the receiver (usually basolateral) compartment but not to determine how exactly efflux affects metabolism. The ER (and ER*) is heavily dependent on the amount of parent drug appearing in the receiving compartment, a parameter that is often strongly dependent on P-gp activity in itself, irrespective of its influence on metabolism. Being in the denominator of the ER and ER* definitions, increased amounts of parent drug in the basolateral compartment as a result of P-gp inhibition will immediately result in a decrease in the ER values. The same applies to the intracellular parent drug amount that is added in the denominator of the ER* and that is likewise often strongly increased upon P-gp inhibition. ER and ER* values are therefore strongly biased to decrease upon inhibition of P-gp. Similar or even increased amounts of overall metabolite formation can therefore still result in drastically reduced ERs when P-gp is inhibited, simply because much more parent compound passes the cell layer. In fact, in most Caco-2 studies, the absolute amount of metabolites formed increased rather than decreased upon P-gp inhibition (Knight et al., 2006). Only because the amount of parent drug in the basolateral compartment increased faster, the ERs decreased.

In this respect, a further important limitation of the Caco-2 system as applied so far is that no clear assessments were made of both the separate contributions of P-gp or CYP3A alone on the ER and overall metabolite formation, because no specific inhibitors of CYP3A were used. To properly understand the interaction between P-gp and CYP3A, one needs to compare all four situations: no CYP3A or P-gp, P-gp alone, CYP3A alone, and CYP3A and P-gp together. With modern (transfection/transduction) techniques, this should now be possible. With such a complete comparison panel, it may be enough simply to analyze the relative amount of unchanged drug appearing in the basolateral compartment under the four different conditions to assess the net impact of the interaction between P-gp and CYP3A, thus avoiding the use of the somewhat contentious ERs.

Collectively, these considerations do not in themselves invalidate the possible existence of a true synergistic interaction between CYP3A and P-gp, but they illustrate the difficulties in establishing it experimentally with in vitro models.

C. In Silico Studies

In addition to in vitro approaches, several in silico studies have focused on the interplay between CYP3A and P-gp. For example, after developing and applying a theoretical model for drug absorption across the intestinal epithelium, Ito et al. (1999) found a synergistic (i.e., disproportionately large) increase in the fraction of drug absorbed into blood when CYP3A and P-gp were both inhibited relative to when each was inhibited alone. It is noteworthy, however, that this disproportionate increase does not demonstrate positional synergism between CYP3A and P-gp but rather the opposite (see section V.A.1): the simulated effect of adding the two detoxifying systems together to a fully deficient situation was much smaller than would have been expected from the effects of adding the systems separately. This model therefore suggests an antagonistic interaction between P-gp and CYP3A in the enterocyte rather than a synergistic interaction. It is worth noting that the model of Ito et al. (1999) did not incorporate the option that CYP3A activity might get saturated over the range of drug concentrations simulated, so possible positional synergy due to (prevention of) CYP3A saturation could not be assessed with this model. As correctly outlined by Garmire and Hunt (2008), true CYP3A-P-gp (positional) synergy occurs when the decrease in absorption due to the combined function of CYP3A and P-gp (relative to absorption in the absence of CYP3A and Pgp) is greater than the sum of the separate effects and reduced absorption when each is functioning alone. Ito et al. (1999) found the opposite effect.

Garmire et al. (2007) and Garmire and Hunt (2008) recently developed an independent in silico intestinal device to address the intestinal interplay between CYP3A and P-gp. It is noteworthy that results obtained with this model were not in support of a functional synergistic relationship between P-gp and CYP3A but rather found an additive or even a small antagonistic

effect between the proteins when looking at the amount of drug effectively absorbed (Garmire and Hunt, 2008). Perhaps not surprisingly, in this model, the ER* was found to be marginally increased with increased P-gp activity, but overall total metabolite formation was markedly diminished. In addition, this study did not specifically address the possibility that CYP3A might become saturated (only one dose level was simulated), but the model set-up would probably allow for this option. We note in passing that Garmire and Hunt (2008) also fell into the synergism trap once (see section V.A.1) and failed to realize that the results of Ito et al. (1999) are perfectly in line with their own results, spending a long paragraph on potential reasons why there might be a (nonexistent) discrepancy between their studies.

In contrast to the above in silico studies, the in silico models of Tam et al. (2003), Pang et al. (2009), and Fan et al. (2010), developed for studying P-gp-CYP3A interplay in Caco-2 cells, did specifically address as well the option that there may be saturation of CYP3A metabolizing activity. In case there was no saturation of CYP3A metabolism, this model, like the two described above, found that P-gp activity actually decreased the rate of total metabolite formation. It is noteworthy that even the ER*, which is intrinsically most biased to yielding an increase upon activation of P-gp, was found not to increase with increasing P-gp activity in these models and even yielded a modest decrease. However, if there was the possibility of saturation of CYP3A, under certain conditions in which P-gp-mediated drug efflux could lead to desaturation of CYP3A activity, P-gp activity did result in an increase in the ER* (Pang et al., 2009).

Finally, mixed in vitro, in situ perfusion, and in silico studies on jejunal epithelium (Johnson et al., 2001, 2003a,b) initially led to the suggestion that P-gp activity would lead to an increase in the cellular mean residence time (MRT) of drug in the absorptive direction, thus increasing its effective exposure to CYP3A activity and hence metabolism (Johnson et al., 2001). However, the validity of applying the MRT concept in these models to assess increased exposure of drug to CYP3A was questioned by Tam et al. (2003) and Pang et al. (2009) on theoretical grounds, because cellular AUC for drug was in fact decreased by P-gp activity in these models, despite a simultaneous increase in MRT. Indeed, later in silico calculations by the group of Porter (Johnson et al., 2003b) confirmed that the intestinal ER* could not be increased by increasing P-gp activity unless there was a nonlinear behavior of drug metabolism (such as saturable CYP3A activity).

Collectively, all the reviewed in silico studies therefore indicate that the simultaneous activity of P-gp and CYP3A is unlikely to result in a positional synergistic effect on drug metabolism in the enterocyte, and may even cause some antagonistic effect—that is, unless there is the possibility of saturation of CYP3A activity. All those in silico studies that did address the latter aspect indicated that this CYP3A (de-)saturation could result in a true positional synergy between P-gp and CYP3A. It seems likely, therefore, that those in vitro studies that did show a synergistic effect between P-gp and CYP3A (albeit assessed with the most optimistic parameters ER or ER*) covered conditions in which saturation of CYP3A was playing a role. The modest antagonistic effect between P-gp and CYP3A that is predicted by several of the in silico models has been interpreted as indicating competition between these two detoxifying systems for the intracellular drug.

To our knowledge, there are no in silico models and simulation studies yet available that show that enterocyte P-gp and CYP3A activity in themselves can lead to a positional synergy in first-pass metabolism, in the absence of saturable CYP3A activity. We think it is reasonable that researchers that still adhere to such a concept (e.g., Benet, 2009) should, in the face of the substantial literature indicating the contrary, provide a solid, mathematically defined mechanistic basis (model) for it.

We would like to emphasize that this is not a trivial point. If positional synergy can only occur when there is potential saturation of CYP3A activity, this puts a severe restriction on the number of situations (drugs, dosages) in which positional synergy can play a role: intracellular enterocyte drug concentrations must be close to the K_m of CYP3A (i.e., below in the presence of P-gp activity and well above without P-gp activity). On the other hand, if positional synergy could also occur independently of saturation of CYP3A, it would potentially apply to all drugs that are shared P-gp and CYP3A substrates and at most dosage levels. The impact of the synergy would then depend mainly on the relative impact of P-gp and CYP3A on intestinal disposition of the drug (and at the dosage) in question. Of course, the latter restriction will also apply to the situation in which CYP3A saturation is necessary for positional synergy.

VI. Novel Mouse Models to Study the In Vivo Interplay between CYP3A and P-glycoprotein

A. Aims and Background of the In Vivo Studies

We have two main aims with studying the in vivo interplay between CYP3A and P-gp:

- To obtain basic mechanistic insight into this interaction and establish its potential relevance for the oral bioavailability of various drugs.
- To establish the extent to which it may be possible to boost the oral bioavailability of drugs by simultaneously inhibiting P-gp and CYP3A and determine the potential opportunities and risks involved with this strategy.

Ultimately, something can only be judged when it is tested. The positional synergy hypothesis, although academically interesting, will have real-life value only

when it can at times have a substantial impact on the intestinal first-pass metabolism of a drug, resulting in marked differences in systemic drug levels. The only way to test this is in vivo in intact organisms in either preclinical or clinical studies. We can foresee two main approaches, neither of them ideal.

- Apply in vivo-specific inhibitors for CYP3A, P-gp, or both and study their separate and combined effects on the oral pharmacokinetics of shared P-gp and CYP3A substrate drugs. The main problem with this approach is that one can never be certain that the inhibitors used are truly specific, especially because they will need to be applied at relatively high doses to obtain complete inhibition of each system. Chances are high that other detoxifying systems (including drug uptake or efflux transporters, drug-metabolizing and -conjugating enzymes) that also affect the drug disposition will be changed as well.
- Use gene knockout animals. Advantage of this model is that one can be assured of the complete absence of the activity of the knocked-out genes. The potential main problem with this model is that we know that, upon inactivation of important and broad-specificity detoxifying proteins such as P-gp and CYP3A, there can be substantial changes in the expression of other detoxifying systems. Such changes can result from altered disposition in the knockout strains of food-derived xenobiotics and possibly some endogenous compounds that are substrates of CYP3A and/or P-gp. These compounds may activate one or more of the various xenobiotic nuclear receptors (e.g., PXR, CAR), resulting in substantial changes in expression of detoxifying systems. Some of these may also affect the pharmacokinetics of probe drugs that are used to test various hypotheses (e.g., van Waterschoot et al., 2008, 2009c). Experiments should therefore be carefully designed to minimize the risk of substantial interference of the gene expression changes with the questions to be addressed.

The successful generation of Cyp3a(−/−) mice has allowed us to generate a combination knockout mouse strain that lacks both CYP3A and P-gp. Although Cyp3a/P-gp(−/−) mice are deficient for two important detoxifying systems, they are viable, fertile, and without marked spontaneous abnormalities and are therefore amenable for various lines of experimentation to address the interaction between P-gp and CYP3A. Similar to Cyp3a(−/−) mice, however, several other detoxifying systems were up-regulated in liver and intestine of Cyp3a/P-gp(−/−) mice (van Waterschoot et al., 2009a).

B. Docetaxel Studies Do Not Reveal In Vivo Synergy between P-gp and CYP3A

To obtain more insight into the in vivo interplay between CYP3A and P-gp, we performed a pharmacokinetic study with the dual CYP3A/P-gp substrate docetaxel in Cyp3aP-gp(−/−) mice (van Waterschoot et al., 2009a). The primary question addressed was whether the simultaneous activity of CYP3A and P-gp resulted in an additive, a positional synergistic, or possibly an antagonistic effect on the effective plasma levels of docetaxel. Docetaxel (at 10 mg/kg) was given both orally and intravenously, and plasma levels of docetaxel were measured. The selection of the mouse models, the probe drug docetaxel, and the dose level (10 mg/kg) applied were based on the following considerations:

1. The availability of four genetically defined mouse models (with P-gp and CYP3A (wild-type), without P-gp, without CYP3A, or without either) allows a direct, systematic comparison of all four situations, which is necessary to assess the separate and combined impact of these proteins on oral availability as a measure of the efficiency of intestinal first-pass metabolism.
2. Previous experiments showed the oral pharmacokinetics of docetaxel to be strongly affected by both deficiency in P-gp (Bardelmeijer et al., 2002) and deficiency in mouse CYP3A (van Herwaarden et al., 2007). Given the inevitable background noise in in vivo pharmacokinetic studies (especially oral), a marked impact of each system is a prerequisite to be able to uncover functional interactions between P-gp and CYP3A.
3. The CYP3A-P-gp synergy hypothesis primarily concerns intestinal first-pass metabolism. Because CYP3A and P-gp elsewhere in the body (primarily liver) may also affect in vivo pharmacokinetics of docetaxel, it is preferable to test a situation in which intestinal metabolism is far more prominent than hepatic metabolism. We have previously demonstrated that intestinal CYP3A activity has a far (approximately 8-fold) higher impact on oral docetaxel availability (also at 10 mg/kg) than hepatic CYP3A activity, whereas the inverse is true for intravenously administered docetaxel (van Herwaarden et al., 2007).
4. CYP3A and P-gp should be the main determining factors for the oral pharmacokinetics of docetaxel at the applied dose. If there were very prominent alternative detoxification systems for docetaxel, possible alterations in their function in knockout animals (e.g., due to changes in gene expression) might affect the plasma and metabolite levels of docetaxel and potentially confound a clean interpretation of the results. As shown below, we found that when CYP3A and P-gp were both removed, the oral AUC was at least 70-fold higher than in wild-type mice, illustrating that alternative detoxification systems had only a marginal impact at that high exposure level.

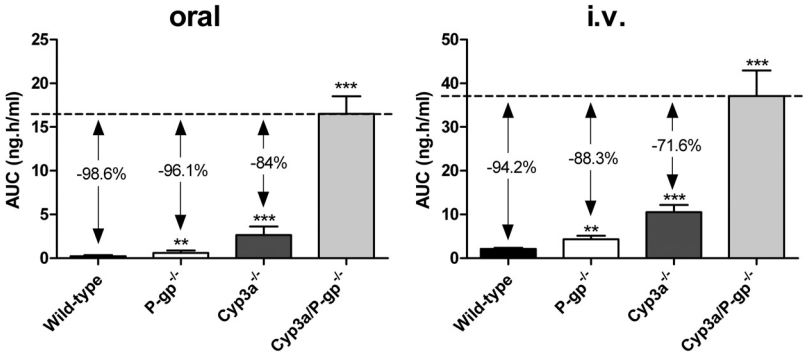

FIG. 2. Systemic exposure ($AUC_{0-\infty}$) of docetaxel after oral or intravenous (i.v.) administration (10 mg/kg) to wild-type, P-gp(−/−), Cyp3a(−/−), and Cyp3a/P-gp(−/−) mice. Data are shown as the mean concentration, and error bars represent the S.D. (n = 5–8 per time point) (van Waterschoot et al., 2009a).

However, expression of a number of detoxifying genes, including *Cyp2cs*, *Oatps*, and *Mrp3*, was significantly up-regulated in liver (>2-fold as assessed by microarray) and some also in small intestine of Cyp3a/P-gp(−/−) compared with wild-type mice. In view of the marginal residual oral docetaxel clearance in the combination knockout mice, it seems unlikely that any of these systems could mediate substantial metabolic clearance of oral docetaxel. Nevertheless, it is difficult to completely exclude this possibility. In addition, there may be alternative detoxifying systems that contribute significantly to docetaxel clearance at low exposure levels but are saturated when overall exposure levels rise considerably because of CYP3A and P-gp deficiency, resulting in a reduced clearance contribution (see below). In view of such possible confounders, one should probably try to give meaning only to effects that are quite pronounced (i.e., at least 2-fold higher or lower than expected) and then still with due caution.

5. Docetaxel, in addition to other taxanes such as paclitaxel, is a drug for which our institute is interested in developing oral (long-term) clinical application regimens by using inhibitors of CYP3A and/or P-gp, aiming to ameliorate the very poor oral availability of these compounds. Better insight into the principles governing this poor oral availability will be crucial for designing optimal modulation strategies.

When docetaxel was given orally to Cyp3a/P-gp(−/−) mice, a dramatic increase in systemic exposure (AUC) of more than 72-fold was observed compared with wild-type mice (Figs. 2 and 3). It is noteworthy that this effect seemed disproportionately large compared with the single Cyp3a (11.5-fold) or P-gp (2.8-fold) knockouts (i.e., approximately 2.2-fold more than predicted when assuming mere additive effects of the simultaneous removal of CYP3A and P-gp) (van Waterschoot et al., 2009a). This more-than-additive effect of depleting both detoxifying systems illustrates how big an impact CYP3A and P-gp together can have on reducing the systemic exposure of oral docetaxel. It is worth noting that the fold increase in systemic exposure seen in Cyp3a/P-gp(−/−) versus wild-type mice was much less striking after intravenous than after oral administration (17- versus 72-fold), in line with the importance of intestinal metabolism in limiting docetaxel oral bioavailability (van Herwaarden et al., 2007). As such, the oral docetaxel bioavailability was increased from ~10% in wild-type mice to ~45% in the Cyp3a/P-gp(−/−)

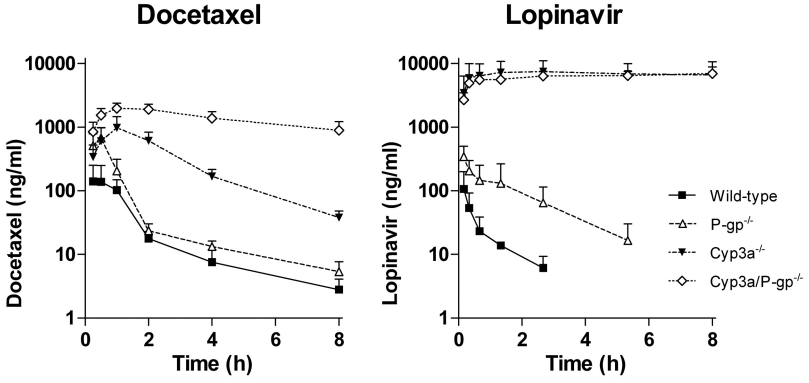

FIG. 3. Plasma concentration-time curves of docetaxel and lopinavir in wild-type, P-gp(−/−), Cyp3a(−/−), and Cyp3a/P-gp(−/−) mice after oral administration of docetaxel (10 mg/kg) or lopinavir (25 mg/kg). Data are shown as the mean concentration, and error bars represent the S.D. (n = 4–8 per time point) (van Waterschoot et al., 2009a, 2010).

strain. It is noteworthy that the absolute systemic exposure in Cyp3a/P-gp(−/−) mice after oral docetaxel administration was 7.6-fold higher than that in wild-type mice after intravenous administration (Fig. 2). This further illustrates the pronounced impact of the simultaneous loss of CYP3A and P-gp activity on oral docetaxel availability.

The disproportionate increase in docetaxel exposure when CYP3A and P-gp are both absent clearly demonstrates highly effective collaboration but not positional synergism between CYP3A and P-gp. A basic implication of positional synergism is that CYP3A and/or P-gp would function more efficiently in the presence of the activity of the other system than in its absence. As explained before (see section V.A.1), the proper way of assessing this is to compare the impact of CYP3A or P-gp alone or in combination, relative to a fully deficient situation [Cyp3a/P-gp(−/−)]. In Fig. 2, we have plotted the docetaxel AUC results, taking the AUC in Cyp3a/P-gp(−/−) mice as 100%. Compared with the Cyp3a/P-gp(−/−) situation, the activity of CYP3A alone [i.e., in P-gp(−/−) mice] can reduce the oral docetaxel systemic exposure by 96.1% (to 3.9%), and that of P-gp alone [i.e., in Cyp3a(−/−) mice] by 84% (to 16%), respectively (Fig. 2). Based on these percentages, the theoretically predicted combined effect of CYP3A and P-gp on the systemic exposure is a reduction to 0.62% (3.9% of 16%). This is fairly close to the 1.4% of the AUC of the Cyp3a/P-gp(−/−) we observed experimentally for the wild-type mice (Fig. 2). In the case of true positional synergism, this experimental percentage for the wild-type clearly should have been (much) lower than 0.62%.

Because the combined effect of P-gp and CYP3A in reducing the oral docetaxel AUC was even more than 2-fold smaller than expected from the separate P-gp and Cyp3a knockout strains (reduction to 1.4 instead of 0.62%), one could even argue that there might be some functionally antagonistic (or competitive) interplay between P-gp and CYP3A. However, in view of the size of the effect (just over 2-fold), the caveats indicated above, and the substantial standard deviation in the oral AUC data (van Waterschoot et al., 2009a), we prefer not to give too much meaning yet to this observation without further experimental substantiation. Assuming there is no significant difference between 0.62 and 1.4%, the data would suggest that each of the two systems functions with roughly equal efficiency, independent of the presence or absence of the other system. It is noteworthy, however, that there is no indication whatsoever in these data that there is positional synergy between P-gp and CYP3A. In addition, for intravenous docetaxel administration, there was no indication for functional synergism between CYP3A and P-gp [predicted value for the wild-type mice based on the single knockout data: 11.7% of 28.4% = 3.3%, fairly close to but again somewhat below the experimental value of 5.8% seen in wild-type mice (Fig. 2)].

We want to note that detailed analysis of the intravenous pharmacokinetic and metabolite data for docetaxel (van Waterschoot et al., 2009a) suggests that there is also an unidentified (i.e., not CYP3A- or P-gp–mediated) metabolic clearance pathway for docetaxel that accounts for approximately 35% of intravenous docetaxel clearance in the wild-type mice. In the Cyp3a(−/−) mice, the clearance contribution of this unidentified pathway is reduced by approximately 5-fold, corresponding roughly to the 5-fold increase in plasma AUC. This shift might suggest that there has been down-regulation of the gene(s) responsible for this docetaxel clearance pathway in the Cyp3a(−/−) mice. However, in microarray analysis of changes in hepatic expression of candidate detoxifying protein genes, all genes with expression changes were found to be up-regulated in Cyp3a(−/−) mice except for the cholesterol-catabolizing enzyme Cyp7b1, which is unlikely to affect docetaxel (van Waterschoot et al., 2009a). We therefore consider it more likely that this unidentified pathway represents a docetaxel-metabolizing pathway that is already saturated in the wild-type mice. A 5-fold increase in plasma AUC as a result of the Cyp3a knockout will then automatically result in a 5-fold reduction in the overall clearance contribution of this pathway: the absolute amount of docetaxel metabolized by this saturated pathway stays the same, but because of the 5-fold higher AUC, its relative clearance contribution drops 5-fold. Whether this presumably hepatic clearance pathway would significantly affect the oral docetaxel clearance is uncertain. Nevertheless, this finding illustrates that there can be unforeseen pitfalls in the pharmacokinetic analysis of knockout strains, particularly when large changes in plasma AUCs are involved. This is also one of the reasons for us to prefer only to give interpretation to relatively large (i.e., >2-fold) changes in pharmacokinetic parameters in these Cyp3a knockout strains.

The most obvious explanation for the observed lack of in vivo positional synergy between CYP3A and P-gp for oral docetaxel is that intestinal CYP3A [which is the most important determinant of oral docetaxel exposure (van Herwaarden et al., 2007)] does not reach saturation even in the absence of P-gp activity. In other words, the enterocyte intracellular drug concentration remains well below the effective K_m of CYP3A. As explained before, when the intracellular kinetics of metabolism remains linear, at best an additive collaboration between P-gp and CYP3A is expected to become apparent. A more general prediction therefore is that positional synergism may be found only in those cases in which the K_m of CYP3A for a certain drug is low relative to intra-enterocyte drug concentration reached upon oral drug administration (obviously, intestinal P-gp activity should also not be saturated to reveal positional synergy). In view of the very broad specificity of CYP3A (and the correspondingly relatively high K_m values for the vast majority of substrates), this might be a relatively rare occurrence.

The other main argument proposed for positional synergism between intestinal P-gp and CYP3A revolves around the repeated access to CYP3A that P-gp would provide by extruding the drug from the cell and allowing uptake in the next cell, where it can again be exposed to CYP3A activity. As explained above, a defined mathematical model to support that this process per se would result in overall more efficient intestinal first-pass metabolism is, to our knowledge, still lacking. Because this conception should also work when CYP3A is not saturated, the lack of positional synergy experimentally observed with oral docetaxel seems at odds with the assumption that this process has a significant impact in vivo.

C. Highly Increased and Qualitatively Altered Toxicity of Oral Docetaxel in Cyp3a/P-gp(−/−) Mice

It is noteworthy that the disproportionate increase in docetaxel exposure also resulted in lethal toxicity of a modest single oral dose of docetaxel (at 10 mg/kg) in Cyp3a/P-gp(−/−) mice. Although we observed no signs of toxicity in wild-type, P-gp(−/−), or Cyp3a(−/−) mice, we encountered severe toxicities in Cyp3a/P-gp(−/−) mice, especially in those tissues with rapidly dividing cells (intestinal, hematopoietic, and spermatogenic), consistent with the antimitotic action of docetaxel. This toxicity resulted in a rapid deterioration of the animals and death within 4 days. Apart from the increased toxicity in small intestine and bone marrow, the qualitative emergence of additional toxicities in colon and testis was especially striking. Among the toxicities observed, the severe intestinal toxicity was probably the most important cause of the early death of the Cyp3a/P-gp(−/−) mice. This suggests a qualitative shift in the type of toxicity, because hematotoxicity is typically dose-limiting for docetaxel in mice, whereas intestinal toxicity has not previously been observed in safety studies with intravenous docetaxel in this species (Bissery et al., 1995; Lavelle et al., 1995). In contrast to mice, humans already show some intestinal toxicity (diarrhea), even with intravenous docetaxel (Baker et al., 2006). The possible applicability of combined CYP3A and P-gp inhibition to greatly improve docetaxel oral bioavailability could well be limited by a possible further shift toward intestinal toxicity in humans. This should therefore be monitored very carefully in clinical trials of this strategy.

D. A Biological Perspective on Positional Synergy between CYP3A and P-gp

The docetaxel toxicity findings above illustrate an essential aspect of P-gp and CYP3A functioning: the main biological function of broad-specificity detoxifying proteins such as CYP3A and P-gp is protection of the organism from a plethora of xenobiotic toxins that are produced by plants, bacteria, fungi, and certain prey animals and that can be erratically or systematically highly abundant in the diet and the intestinal contents of animals. It is therefore important to realize that pronounced positional synergism between CYP3A and P-gp, although conceptually appealing, from a biological point of view would create a highly vulnerable situation: disruption or extensive inhibition by dietary compounds (for example, grapefruit juice can lead to extensive inhibition of intestinal CYP3A) of just one of the participating systems would lead to a near-complete functional collapse of the overall detoxification mechanism (see Table 1). This would conflict with the biological need for robust protection from xenobiotic toxins. In the docetaxel example described above, even the single inhibition of P-gp might lead to extreme sensitivity to docetaxel toxicity if there had been a pronounced positional synergism between CYP3A and P-gp. Instead, the situation that we observed for docetaxel of additive and perhaps even slightly antagonistic activity between P-gp and CYP3A means that when one of the detoxifying systems has been inactivated, there is still a quite efficient alternative protective mechanism (albeit not as efficient as the combination). Only when both systems are simultaneously inactivated (undoubtedly a much rarer occurrence in a natural situation) does a severe increase in sensitivity to the toxin become obvious. Thus, with respect to docetaxel detoxification, it is fortunate that CYP3A and P-gp have independent but partly overlapping and efficiently cooperating functions. In a broader perspective, it therefore seems biologically undesirable for there to be many xenobiotic toxins that would be subject to a strong positional synergy between relevant collaborating detoxification systems. There would probably be significant evolutionary pressure against developing such a sensitive and unstable protection mechanism. Instead, there would be positive selection for those animals that develop a broad array of partially redundant and functionally overlapping detoxification mechanisms.

E. Lopinavir: A Case of True but Inverted Synergism

As indicated above, lopinavir is an excellent substrate for CYP3A. In vitro studies have also demonstrated that lopinavir is transported by P-gp (Woodahl et al., 2005; Agarwal et al., 2007; van Waterschoot et al., 2010). We have investigated the individual and combined impact of CYP3A and P-gp on the oral pharmacokinetics of lopinavir in P-gp- and Cyp3a-deficient mouse models. Although there was a very substantial increase in lopinavir exposure (plasma AUC) in P-gp(−/−) mice (9-fold versus wild-type), this effect was dwarfed by the more than 2000-fold increase in exposure observed in Cyp3a(−/−) mice (Fig. 3). It is noteworthy, however, that no further increase in systemic exposure was observed when CYP3A and P-gp were both absent, in strong contrast to the docetaxel results (Fig. 3). It seems therefore that the function of P-gp is noticeable only in the presence of CYP3A activity. This actually suggests that, for lopinavir, CYP3A prevents saturation of P-gp rather than the

other way around. A crucial aspect here, of course, is the huge impact of CYP3A by itself on lopinavir pharmacokinetics: a >2000-fold increase of effective oral AUC (most likely reflecting similar changes in lopinavir concentrations in various tissues, including intestine and liver) is much more likely to result in saturation of other detoxifying proteins (such as P-gp) than the 12-fold increase in oral AUC that we observed for docetaxel upon CYP3A inactivation (van Waterschoot et al., 2009a). Even though P-gp is positioned before CYP3A in the intestinal wall, it can still be easily imagined how CYP3A might prevent saturation of intestinal P-gp. Very high accumulation of intracellular lopinavir in enterocytes as a result of complete absence of intestinal CYP3A-mediated metabolism will rapidly saturate the efflux capacity of intestinal P-gp and thus obliterate its impact on overall intestinal absorption of the drug.

We note that the situation for lopinavir seems to be an example of true functional synergism between CYP3A and P-gp: the impact of P-gp on reducing oral lopinavir AUC was undetectable in the absence of CYP3A but 9-fold in its presence. However, the synergistic relationship is inverse to what was originally proposed, in the sense that here CYP3A prevents saturation of P-gp activity rather than the other way around. This situation is also not critically dependent on the relative position of P-gp toward CYP3A and the flow of drug because it occurs in the intestine. One can easily imagine also that in the liver, a huge increase of lopinavir levels because of CYP3A absence would saturate biliary excretion of lopinavir through P-gp, thus making the overall contribution of P-gp–mediated hepatobiliary excretion to lopinavir clearance undetectable.

It would not surprise us if situations similar to that for lopinavir might emerge for other shared CYP3A and P-gp substrate drugs that are similarly profoundly affected by CYP3A activity. Note that the impact of P-gp by itself on the oral AUC of drugs rarely surpasses a 10-fold effect, as measured in P-gp(−/−) mice, and is often much lower. This makes it less likely that one will see a similarly profound effect of P-gp activity on CYP3A impact as applies to the inverse impact for lopinavir. These data clearly illustrate how 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.

VII. Perspectives and Concluding Remarks

The importance of intestinal metabolism has long been a matter of debate, but recent studies with tissue-specific CYP3A4 transgenic mice in conjunction with earlier convincing observations in humans have provided unequivocal evidence that the impact of intestinal CYP3A4-dependent metabolism can even exceed that of hepatic metabolism after oral drug administration. In addition, studies performed with an intestinal P450 reductase knockout mouse model have indicated the importance of intestinal metabolism (Zhang et al., 2009). It has been hypothesized that the functional interplay between CYP3A and P-gp might be why intestinal metabolism is so efficient. However, although only a few drugs have been tested so far, little in vivo evidence for true positional synergy between CYP3A and P-gp in intact organisms is currently available. Moreover, also for drugs that are not P-gp substrates such as triazolam, intestinal CYP3A-dependent metabolism can be more significant than hepatic metabolism after oral administration (van Waterschoot et al., 2009b). This illustrates that functional CYP3A/P-gp interplay is not essential for efficient intestinal metabolism.

Nevertheless, there can be little doubt that CYP3A and P-gp can cooperate efficiently in reducing the oral availability of shared substrate drugs. As illustrated by the in vitro, in silico, and in vivo studies discussed here, each of these systems can have a marked impact on the amount of parent drug appearing in the circulation after oral administration; when both systems are functioning together, the combined impact on oral availability can be profound. The same applies to their combined effect on protection of the organism from toxicity of orally administered compounds.

However, this efficient cooperation is not necessarily dependent on synergistic functioning of CYP3A and P-gp. This is perhaps best illustrated by the in vivo docetaxel studies, in which there was either an additive or (perhaps) even a small antagonistic effect of the combination of CYP3A and P-gp activities on the oral docetaxel AUC but certainly not a synergistic effect. However, losing both activities at the same time had profound effects on the docetaxel oral AUC and on docetaxel toxicity effects. Thus, positional synergism is not required for a profound impact of CYP3A and P-gp on oral drug pharmacokinetics and toxicology.

These findings imply that if one wants to boost the oral availability of a shared P-gp and CYP3A substrate drug, it can be very worthwhile to try and simultaneously inhibit both CYP3A and P-gp activity, because this may result in a dramatic increase in effective systemic drug levels. Unsurprisingly, proper caution should here be exercised with respect to potentially equally dramatic increases in toxicity of the drug. These findings vindicate the early proposal that intestinal CYP3A and P-gp can form a concerted barrier for drug absorption and that simultaneous inhibition of CYP3A and P-gp might be a very efficient way to boost oral availability of shared substrate drugs (Wacher et al., 1995, 1996).

Even though true (positional) synergistic activity between CYP3A and P-gp is not required for a marked impact of the combination of these proteins on intestinal first-pass metabolism, the question of whether it will occur often in (pre)clinical practice remains. So far, only two candidate drugs have been tested in the knockout

mouse models: docetaxel, yielding no synergistic effect, and lopinavir, yielding an inverse synergistic effect. We think it will be worthwhile to test a much broader range of drugs in the mouse models to assess how often synergistic, additive, or antagonistic interplay will occur. Based on our data so far, the available in silico models, and theoretical biological considerations, we expect that a true positional synergy situation will not occur very frequently. In the absence of convincing mathematical models that prove that P-gp–mediated recycling of drug in the enterocyte is in and of itself enough to enhance metabolism, it seems most likely that only specific conditions of intraenterocyte drug concentrations and saturation conditions (K_m) for CYP3A can lead to positional synergistic effects. Such specific restrictions will severely limit the number of shared P-gp and CYP3A drugs, and the specific dosages, that meet all the necessary requirements for positional synergy to occur. However, these are just theoretical considerations. Only further experimentation can yield answers to these questions.

We would like to point out that if wide-spread positional synergy were to occur for many drugs, a practical implication would be that it often would be sufficient to pharmacologically inhibit only one of the participating proteins (P-gp or CYP3A) to achieve a great gain in oral availability. The additional gain of also inhibiting the other system would then be very limited. If, however, positional synergy is a relatively rare occurrence—and we think this is the more likely situation—it will often be necessary to simultaneously inhibit both CYP3A and P-gp to obtain a substantial gain in oral availability. This consideration will need to be taken into account in current efforts to improve the oral availability of effective (anticancer) drugs that suffer from poor oral bioavailability because they are shared substrates of P-gp and CYP3A.

Even though we feel that mouse knockout and transgenic models can be extremely useful in addressing various pharmacokinetic and toxicological questions, including ways to pharmacologically modulate transporter or metabolic activities, it is also clear that they are not always ideal. This is primarily taking into consideration the potentially extensive changes in expression of alternative detoxification systems, especially in the *Cyp3a* knockout mice and their derivatives. In interpreting pharmacokinetic data obtained with these mouse models, one should therefore always carefully assess whether the drug in question might be substantially affected by one or more of these up- or down-regulated alternative detoxifying pathways. If feasible, it may be helpful to perform complete mass balance studies in these mice, as these may help in identifying alterations in unidentified drug clearance pathways. It may in general be advisable to try and interpret only relatively substantial (>2-fold) changes in the *Cyp3a* and related knockout mouse models, because it is then easier to spot potentially relevant confounders.

Drug-drug interactions are a major problem in clinical practice, and it is clear that CYP3A has an important role in many of them. It is often difficult to dissect whether drug-drug interactions take place primarily at the level of the intestine, liver, or both. By investigating drugs in hepatic- and intestinal-specific CYP3A4-Tg mice, more insight into the relative contribution to drug-drug interactions of each organ can be obtained. Because for some drugs intestinal metabolism seems to be more significant than hepatic metabolism after oral drug administration, one would expect that for these drugs, the intestine would also be the most important site for drug-drug interactions. However, a rapid absorption of a CYP3A inhibitor into the portal blood combined with a high accumulation of this inhibitor into the liver could still result in a situation in which the liver is the most relevant organ for the drug-drug interaction, even though the primary contribution of the intestine to metabolism surpasses that of the liver. For example, as discussed above, intestinal CYP3A4-dependent metabolism of oral triazolam is more important than hepatic. However, when triazolam was used as a probe to study drug-drug interactions in CYP3A4-transgenic mice, stimulation of triazolam metabolism by gefitinib was only observed in mouse strains that had CYP3A4 expression in the liver, not in strains with only intestinal CYP3A4 (van Waterschoot et al., 2009b). Thus, even when intestinal metabolism is relatively more important than hepatic metabolism, the liver can still be the most relevant organ for a drug-drug interaction.

Humanized mice are not humans, and there are clear limitations in using these models, especially for quantitative predictions of drug exposure in humans. However, many fundamental insights obtained with these mice are likely to be relevant for the human situation as well. For example, studies with tissue-specific CYP3A4-transgenic mice have revealed that intestinal CYP3A4 can regulate the expression of a wide variety of detoxifying systems in the liver. As such, long-term specific inhibition of intestinal CYP3A4 activity by, for example, grapefruit juice (Lown et al., 1997) could result in higher levels of detoxifying systems (including CYP3A) in the liver. In addition, people with high intestinal CYP3A levels could have lower levels of hepatic detoxifying systems and vice versa. This implies that intestinal and hepatic expression levels of CYP3A (but also of other detoxifying systems) do not always correlate and may at times even be inversely correlated. Indeed, several clinical studies have indicated that persons with low intestinal CYP3A activity have relatively high hepatic CYP3A activity (Lown et al., 1994; Gorski et al., 2003; Mouly et al., 2005). Although more evidence has to be provided in favor of such an inverse relationship, it could have important implications for the prediction of drug exposure and possibly drug-drug interactions.

CYP3A and P-gp are two important detoxifying systems that protect us from many potentially harmful

xenobiotics, but their activity also strongly limits the absorption of a wide variety of drugs. Although many drugs are substrate for both CYP3A and P-gp, only limited in vivo information is available on how their combined activity limits oral drug bioavailability. Strategies to simultaneously inhibit CYP3A and P-gp can be an efficient way to improve the oral drug bioavailability and potentially to reduce inter- and intrapatient differences in drug exposure. However, despite these interesting opportunities it should be noted that there are also potential risks of the combined inhibition of CYP3A and P-gp. The oral administration route, but also the significantly altered plasma and tissue pharmacokinetics upon CYP3A and P-gp inhibition, can give rise to toxicities that have not been previously encountered. Indeed, as demonstrated by the docetaxel toxicity studies in Cyp3a/P-gp(−/−) mice, not only hematotoxicity but also severe, even lethal, intestinal toxicity was found (van Waterschoot et al., 2009a). This toxicity was not observed in previous safety studies in which wild-type mice received the drug intravenously (Lavelle et al., 1995). Intestinal toxicity should therefore receive special attention during clinical trials that focus on improving docetaxel oral bioavailability by inhibiting CYP3A and MDR1 and could well limit the applicability of combined CYP3A/P-gp inhibition to improve docetaxel oral bioavailability.

Our understanding of the functional interplay between CYP3A and P-gp still has to mature; needless to say, further research is warranted. Based on our current knowledge, predicting the impact of combined CYP3A/P-gp inhibition for a certain drug based only on in vitro data seems to be extremely difficult, even if the impacts of individual CYP3A and P-gp inhibition are both known. It will therefore be of great interest to investigate more dual CYP3A/P-gp substrates in Cyp3a/P-gp(−/−) mice to unravel any possible common behavioral patterns.

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Authorship Contributions

Wrote or contributed to the writing of the manuscript: van Waterschoot and Schinkel.

References

Agarwal S, Pal D, and Mitra AK (2007) Both P-gp and MRP2 mediate transport of lopinavir, a protease inhibitor. *Int J Pharm* **339:**139–147.

Agarwal S, Sane R, Gallardo JL, Ohlfest JR, and Elmquist WF (2010) Distribution of gefitinib to the brain is limited by P-glycoprotein (ABCB1) and breast cancer resistance protein (ABCG2)-mediated active efflux. *J Pharmacol Exp Ther* **334:** 147–155.

Aller SG, Yu J, Ward A, Weng Y, Chittaboina S, Zhuo R, Harrell PM, Trinh YT, Zhang Q, Urbatsch IL, and Chang G (2009) Structure of P-glycoprotein reveals a molecular basis for poly-specific drug binding. *Science* **323:**1718–1722.

Andersson T, Miners JO, Veronese ME, and Birkett DJ (1994) Diazepam metabolism by human liver microsomes is mediated by both S-mephenytoin hydroxylase and CYP3A isoforms. *Br J Clin Pharmacol* **38:**131–137.

Baker SD, Sparreboom A, and Verweij J (2006) Clinical pharmacokinetics of docetaxel : recent developments. *Clin Pharmacokinet* **45:**235–252.

Bardelmeijer HA, Ouwehand M, Buckle T, Huisman MT, Schellens JH, Beijnen JH, and van Tellingen O (2002) Low systemic exposure of oral docetaxel in mice resulting from extensive first-pass metabolism is boosted by ritonavir. *Cancer Res* **62:**6158–6164.

Benet LZ (2009) The drug transporter-metabolism alliance: uncovering and defining the interplay. *Mol Pharm* **6:**1631–1643.

Benet LZ and Cummins CL (2001) The drug efflux-metabolism alliance: biochemical aspects. *Adv Drug Deliv Rev* **50:**S3–S11.

Benet LZ, Cummins CL, and Wu CY (2003) Transporter-enzyme interactions: implications for predicting drug-drug interactions from in vitro data. *Curr Drug Metab* **4:**393–398.

Bissery MC, Nohynek G, Sanderink GJ, and Lavelle F (1995) Docetaxel (Taxotere): a review of preclinical and clinical experience. Part I: Preclinical experience. *Anticancer Drugs* **6:**339–355, 363–368.

Bodin K, Bretillon L, Aden Y, Bertilsson L, Broomé U, Einarsson C, and Diczfalusy U (2001) Antiepileptic drugs increase plasma levels of 4beta-hydroxycholesterol in humans: evidence for involvement of cytochrome p450 3A4. *J Biol Chem* **276:** 38685–38689.

Borst P and Elferink RO (2002) Mammalian ABC transporters in health and disease. *Annu Rev Biochem* **71:**537–592.

Christians U, Schmitz V, and Haschke M (2005) Functional interactions between P-glycoprotein and CYP3A in drug metabolism. *Expert Opin Drug Metab Toxicol* **1:**641–654.

Cummins CL, Jacobsen W, and Benet LZ (2002) Unmasking the dynamic interplay between intestinal P-glycoprotein and CYP3A4. *J Pharmacol Exp Ther* **300:**1036–1045.

Cummins CL, Salphati L, Reid MJ, and Benet LZ (2003) In vivo modulation of intestinal CYP3A metabolism by P-glycoprotein: studies using the rat single-pass intestinal perfusion model. *J Pharmacol Exp Ther* **305:**306–314.

Cummins CL, Jacobsen W, Christians U, and Benet LZ (2004) CYP3A4-transfected Caco-2 cells as a tool for understanding biochemical absorption barriers: studies with sirolimus and midazolam. *J Pharmacol Exp Ther* **308:**143–155.

Deo AK and Bandiera SM (2008) Biotransformation of lithocholic acid by rat hepatic microsomes: metabolite analysis by liquid chromatography/mass spectrometry. *Drug Metab Dispos* **36:**442–451.

Dresser GK, and Bailey DG (2003) The effects of fruit juices on drug disposition: a new model for drug interactions. *Eur J Clin Invest* **33 (Suppl 2):**10–16.

Dresser GK, Spence JD, and Bailey DG (2000) Pharmacokinetic-pharmacodynamic consequences and clinical relevance of cytochrome P450 3A4 inhibition. *Clin Pharmacokinet* **38:**41–57.

Ekroos M and Sjögren T (2006) Structural basis for ligand promiscuity in cytochrome P450 3A4. *Proc Natl Acad Sci USA* **103:**13682–13687.

Fan J, Maeng HJ, and Pang KS (2010) Interplay of transporters and enzymes in the Caco-2 cell monolayer: I. Effect of altered apical secretion. *Biopharm Drug Dispos* **31:**215–227.

Fisher JM, Wrighton SA, Watkins PB, Schmiedlin-Ren P, Calamia JC, Shen DD, Kunze KL, and Thummel KE (1999) First-pass midazolam metabolism catalyzed by 1alpha,25-dihydroxy vitamin D3-modified Caco-2 cell monolayers. *J Pharmacol Exp Ther* **289:**1134–1142.

Gan LS, Moseley MA, Khosla B, Augustijns PF, Bradshaw TP, Hendren RW, and Thakker DR (1996) CYP3A-like cytochrome P450-mediated metabolism and polarized efflux of cyclosporin A in Caco-2 cells. *Drug Metab Dispos* **24:**344–349.

Garmire LX, Garmire DG, and Hunt CA (2007) An in silico transwell device for the study of drug transport and drug-drug interactions. *Pharm Res* **24:**2171–2186.

Garmire LX and Hunt CA (2008) In silico methods for unraveling the mechanistic complexities of intestinal absorption: metabolism-efflux transport interactions. *Drug Metab Dispos* **36:**1414–1424.

Gorski JC, Vannaprasaht S, Hamman MA, Ambrosius WT, Bruce MA, Haehner-Daniels B, and Hall SD (2003) The effect of age, sex, and rifampin administration on intestinal and hepatic cytochrome P450 3A activity. *Clin Pharmacol Ther* **74:**275–287.

Guengerich FP (1999) Cytochrome P-450 3A4: regulation and role in drug metabolism. *Annu Rev Pharmacol Toxicol* **39:**1–17.

Gupta RP, He YA, Patrick KS, Halpert JR, and Bell NH (2005) CYP3A4 is a vitamin D-24- and 25-hydroxylase: analysis of structure function by site-directed mutagenesis. *J Clin Endocrinol Metab* **90:**1210–1219.

Harlow GR and Halpert JR (1998) Analysis of human cytochrome P450 3A4 cooperativity: construction and characterization of a site-directed mutant that displays hyperbolic steroid hydroxylation kinetics. *Proc Natl Acad Sci USA* **95:**6636–6641.

Harmsen S, Meijerman I, Beijnen JH, and Schellens JH (2007) The role of nuclear receptors in pharmacokinetic drug-drug interactions in oncology. *Cancer Treat Rev* **33:**369–380.

Honig PK, Wortham DC, Zamani K, Conner DP, Mullin JC, and Cantilena LR (1993) Terfenadine-ketoconazole interaction. Pharmacokinetic and electrocardiographic consequences. *JAMA* **269:**1513–1518.

Hutzler JM and Tracy TS (2002) Atypical kinetic profiles in drug metabolism reactions. *Drug Metab Dispos* **30:**355–362.

Ito K, Kusuhara H, and Sugiyama Y (1999) Effects of intestinal CYP3A4 and P-glycoprotein on oral drug absorption—theoretical approach. *Pharm Res* **16:**225–231.

Johnson BM, Charman WN, and Porter CJ (2001) The impact of P-glycoprotein efflux on enterocyte residence time and enterocyte-based metabolism of verapamil. *J Pharm Pharmacol* **53:**1611–1619.

Johnson BM, Chen W, Borchardt RT, Charman WN, and Porter CJ (2003a) A kinetic evaluation of the absorption, efflux, and metabolism of verapamil in the autoperfused rat jejunum. *J Pharmacol Exp Ther* **305:**151–158.

Johnson BM, Charman WN, and Porter CJ (2003b) Application of compartmental

modeling to an examination of in vitro intestinal permeability data: assessing the impact of tissue uptake, P-glycoprotein, and CYP3A. *Drug Metab Dispos* **31:**1151–1160.
Jones SA, Moore LB, Shenk JL, Wisely GB, Hamilton GA, McKee DD, Tomkinson NC, LeCluyse EL, Lambert MH, Willson TM, Kliewer SA, and Moore JT (2000) The pregnane X receptor: a promiscuous xenobiotic receptor that has diverged during evolution. *Mol Endocrinol* **14:**27–39.
Kirby B, Kharasch ED, Thummel KT, Narang VS, Hoffer CJ, and Unadkat JD (2006) Simultaneous measurement of in vivo P-glycoprotein and cytochrome P450 3A activities. *J Clin Pharmacol* **46:**1313–1319.
Kivistö KT, Niemi M, and Fromm MF (2004) Functional interaction of intestinal CYP3A4 and P-glycoprotein. *Fundam Clin Pharmacol* **18:**621–626.
Knight B, Troutman M, and Thakker DR (2006) Deconvoluting the effects of P-glycoprotein on intestinal CYP3A: a major challenge. *Curr Opin Pharmacol* **6:**528–532.
Kodaira H, Kusuhara H, Ushiki J, Fuse E, and Sugiyama Y (2010) Kinetic analysis of the cooperation of P-glycoprotein (P-gp/Abcb1) and breast cancer resistance protein (Bcrp/Abcg2) in limiting the brain and testis penetration of erlotinib, flavopiridol, and mitoxantrone. *J Pharmacol Exp Ther* **333:**788–796.
Kronbach T, Mathys D, Umeno M, Gonzalez FJ, and Meyer UA (1989) Oxidation of midazolam and triazolam by human liver cytochrome P450IIIA4. *Mol Pharmacol* **36:**89–96.
Kumar GN, Dykstra J, Roberts EM, Jayanti VK, Hickman D, Uchic J, Yao Y, Surber B, Thomas S, and Granneman GR (1999) Potent inhibition of the cytochrome P-450 3A-mediated human liver microsomal metabolism of a novel HIV protease inhibitor by ritonavir: A positive drug-drug interaction. *Drug Metab Dispos* **27:**902–908.
Kuppens IE, Bosch TM, van Maanen MJ, Rosing H, Fitzpatrick A, Beijnen JH, and Schellens JH (2005) Oral bioavailability of docetaxel in combination with OC144–093 (ONT-093). *Cancer Chemother Pharmacol* **55:**72–78.
Lagas JS, van Waterschoot RA, van Tilburg VA, Hillebrand MJ, Lankheet N, Rosing H, Beijnen JH, and Schinkel AH (2009) Brain accumulation of dasatinib is restricted by P-glycoprotein (ABCB1) and breast cancer resistance protein (ABCG2) and can be enhanced by elacridar treatment. *Clin Cancer Res* **15:**2344–2351.
Lamba JK, Lin YS, Schuetz EG, and Thummel KE (2002) Genetic contribution to variable human CYP3A-mediated metabolism. *Adv Drug Deliv Rev* **54:**1271–1294.
Lavelle F, Bissery MC, Combeau C, Riou JF, Vrignaud P, and Andre S (1995) Preclinical evaluation of docetaxel (Taxotere). *Semin Oncol* **22:**3–16.
Lown KS, Bailey DG, Fontana RJ, Janardan SK, Adair CH, Fortlage LA, Brown MB, Guo W, and Watkins PB (1997) Grapefruit juice increases felodipine oral availability in humans by decreasing intestinal CYP3A protein expression. *J Clin Invest* **99:**2545–2553.
Lown KS, Kolars JC, Thummel KE, Barnett JL, Kunze KL, Wrighton SA, and Watkins PB (1994) Interpatient heterogeneity in expression of CYP3A4 and CYP3A5 in small bowel. Lack of prediction by the erythromycin breath test. *Drug Metab Dispos* **22:**947–955.
Makishima M, Lu TT, Xie W, Whitfield GK, Domoto H, Evans RM, Haussler MR, and Mangelsdorf DJ (2002) Vitamin D receptor as an intestinal bile acid sensor. *Science* **296:**1313–1316.
Malingré MM, Richel DJ, Beijnen JH, Rosing H, Koopman FJ, Ten Bokkel Huinink WW, Schot ME, and Schellens JH (2001) Coadministration of cyclosporine strongly enhances the oral bioavailability of docetaxel. *J Clin Oncol* **19:**1160–1166.
Mandlekar S, Hong JL, and Kong AN (2006) Modulation of metabolic enzymes by dietary phytochemicals: a review of mechanisms underlying beneficial versus unfavorable effects. *Curr Drug Metab* **7:**661–675.
Moore LB, Goodwin B, Jones SA, Wisely GB, Serabjit-Singh CJ, Willson TM, Collins JL, and Kliewer SA (2000a) St. John's wort induces hepatic drug metabolism through activation of the pregnane X receptor. *Proc Natl Acad Sci USA* **97:**7500–7502.
Moore LB, Parks DJ, Jones SA, Bledsoe RK, Consler TG, Stimmel JB, Goodwin B, Liddle C, Blanchard SG, Willson TM, Collins JL, and Kliewer SA (2000b) Orphan nuclear receptors constitutive androstane receptor and pregnane X receptor share xenobiotic and steroid ligands. *J Biol Chem* **275:**15122–15127.
Mouly SJ, Matheny C, Paine MF, Smith G, Lamba J, Lamba V, Pusek SN, Schuetz EG, Stewart PW, and Watkins PB (2005) Variation in oral clearance of saquinavir is predicted by CYP3A5*1 genotype but not by enterocyte content of cytochrome P450 3A5. *Clin Pharmacol Ther* **78:**605–618.
Mouly SJ, Paine MF, and Watkins PB (2004) Contributions of CYP3A4, P-glycoprotein, and serum protein binding to the intestinal first-pass extraction of saquinavir. *J Pharmacol Exp Ther* **308:**941–948.
Oostendorp RL, Huitema A, Rosing H, Jansen RS, Ter Heine R, Keessen M, Beijnen JH, and Schellens JH (2009) Coadministration of ritonavir strongly enhances the apparent oral bioavailability of docetaxel in patients with solid tumors. *Clin Cancer Res* **15:**4228–4233.
Paine MF and Oberlies NH (2007) Clinical relevance of the small intestine as an organ of drug elimination: drug-fruit juice interactions. *Expert Opin Drug Metab Toxicol* **3:**67–80.
Pal D and Mitra AK (2006) MDR- and CYP3A4-mediated drug-herbal interactions. *Life Sci* **78:**2131–2145.
Pang KS, Maeng HJ, and Fan J (2009) Interplay of transporters and enzymes in drug and metabolite processing. *Mol Pharm* **6:**1734–1755.
Perloff MD, von Moltke LL, Court MH, Kotegawa T, Shader RI, and Greenblatt DJ (2000) Midazolam and triazolam biotransformation in mouse and human liver microsomes: relative contribution of CYP3A and CYP2C isoforms. *J Pharmacol Exp Ther* **292:**618–628.
Polli JW, Olson KL, Chism JP, John-Williams LS, Yeager RL, Woodard SM, Otto V, Castellino S, and Demby VE (2009) An unexpected synergist role of P-glycoprotein and breast cancer resistance protein on the central nervous system penetration of the tyrosine kinase inhibitor lapatinib (N-{3-chloro-4-[(3-fluorobenzyl)oxy]phenyl}-6-[5-(([2-(methylsulfonyl)ethyl]amino)methyl)-2-furyl]-4-quinazolinamine; GW572016). *Drug Metab Dispos* **37:**439–442.
Schenkman JB (1992) Steroid metabolism by constitutive cytochromes P450. *J Steroid Biochem Mol Biol* **43:**1023–1030.
Schinkel AH and Jonker JW (2003) Mammalian drug efflux transporters of the ATP binding cassette (ABC) family: an overview. *Adv Drug Deliv Rev* **55:**3–29.
Schuetz JD, Schuetz EG, Thottassery JV, Guzelian PS, Strom S, and Sun D (1996) Identification of a novel dexamethasone responsive enhancer in the human CYP3A5 gene and its activation in human and rat liver cells. *Mol Pharmacol* **49:**63–72.
Schwarz UI, Büschel B, and Kirch W (2003) Unwanted pregnancy on self-medication with St John's wort despite hormonal contraception. *Br J Clin Pharmacol* **55:**112–113.
Staudinger JL, Goodwin B, Jones SA, Hawkins-Brown D, MacKenzie KI, LaTour A, Liu Y, Klaassen CD, Brown KK, Reinhard J, Willson TM, Koller BH, and Kliewer SA (2001) The nuclear receptor PXR is a lithocholic acid sensor that protects against liver toxicity. *Proc Natl Acad Sci USA* **98:**3369–3374.
Szakács G, Paterson JK, Ludwig JA, Booth-Genthe C, and Gottesman MM (2006) Targeting multidrug resistance in cancer. *Nat Rev Drug Discov* **5:**219–234.
Tam D, Sun H, and Pang KS (2003) Influence of P-glycoprotein, transfer clearances, and drug binding on intestinal metabolism in Caco-2 cell monolayers or membrane preparations: a theoretical analysis. *Drug Metab Dispos* **31:**1214–1226.
Tang W and Stearns RA (2001) Heterotropic cooperativity of cytochrome P450 3A4 and potential drug-drug interactions. *Curr Drug Metab* **2:**185–198.
Thummel KE and Wilkinson GR (1998) In vitro and in vivo drug interactions involving human CYP3A. *Annu Rev Pharmacol Toxicol* **38:**389–430.
Ueng YF, Kuwabara T, Chun YJ, and Guengerich FP (1997) Cooperativity in oxidations catalyzed by cytochrome P450 3A4. *Biochemistry* **36:**370–381.
Urquhart BL, Tirona RG, and Kim RB (2007) Nuclear receptors and the regulation of drug-metabolizing enzymes and drug transporters: implications for interindividual variability in response to drugs. *J Clin Pharmacol* **47:**566–578.
van Herwaarden AE, Wagenaar E, van der Kruijssen CM, van Waterschoot RA, Smit JW, Song JY, van der Valk MA, van Tellingen O, van der Hoorn JW, Rosing H, Beijnen JH, and Schinkel AH (2007) Knockout of cytochrome P450 3A yields new mouse models for understanding xenobiotic metabolism. *J Clin Invest* **117:**3583–3592.
van Waterschoot RA, Lagas JS, Wagenaar E, van der Kruijssen CM, van Herwaarden AE, Song JY, Rooswinkel RW, van Tellingen O, Rosing H, Beijnen JH, and Schinkel AH (2009a) Absence of both cytochrome P450 3A and P-glycoprotein dramatically increases docetaxel oral bioavailability and risk of intestinal toxicity. *Cancer Res* **69:**8996–9002.
van Waterschoot RA, Rooswinkel RW, Sparidans RW, van Herwaarden AE, Beijnen JH, and Schinkel AH (2009b) Inhibition and stimulation of intestinal and hepatic CYP3A activity: studies in humanized CYP3A4 transgenic mice using triazolam. *Drug Metab Dispos* **37:**2305–2313.
van Waterschoot RA, Rooswinkel RW, Wagenaar E, van der Kruijssen CM, van Herwaarden AE, and Schinkel AH (2009c) Intestinal cytochrome P450 3A plays an important role in the regulation of detoxifying systems in the liver. *FASEB J* **23:**224–231.
van Waterschoot RA, ter Heine R, Wagenaar E, van der Kruijssen CM, Rooswinkel RW, Huitema AD, Beijnen JH, and Schinkel AH (2010) Effects of cytochrome P450 3A (CYP3A) and the drug transporters P-glycoprotein (MDR1/ABCB1) and MRP2 (ABCC2) on the pharmacokinetics of lopinavir. *Br J Pharmacol* **160:**1224–1233.
van Waterschoot RA, van Herwaarden AE, Lagas JS, Sparidans RW, Wagenaar E, van der Kruijssen CM, Goldstein JA, Zeldin DC, Beijnen JH, and Schinkel AH (2008) Midazolam metabolism in cytochrome P450 3A knockout mice can be attributed to up-regulated CYP2C enzymes. *Mol Pharmacol* **73:**1029–1036.
Wacher VJ, Wu CY, and Benet LZ (1995) Overlapping substrate specificities and tissue distribution of cytochrome P450 3A and P-glycoprotein: implications for drug delivery and activity in cancer chemotherapy. *Mol Carcinog* **13:**129–134.
Wacher VJ, Salphati L, and Benet LZ (1996) Active secretion and enterocytic drug metabolism barriers to drug absorption. *Adv Drug Deliv Rev* **20:**99–112.
Watkins PB (1997) The barrier function of CYP3A4 and P-glycoprotein in the small bowel. *Adv Drug Deliv Rev* **27:**161–170.
Wei P, Zhang J, Dowhan DH, Han Y, and Moore DD (2002) Specific and overlapping functions of the nuclear hormone receptors CAR and PXR in xenobiotic response. *Pharmacogenomics J* **2:**117–126.
Wienkers LC and Heath TG (2005) Predicting in vivo drug interactions from in vitro drug discovery data. *Nat Rev Drug Discov* **4:**825–833.
Williams JA, Ring BJ, Cantrell VE, Jones DR, Eckstein J, Ruterbories K, Hamman MA, Hall SD, and Wrighton SA (2002) Comparative metabolic capabilities of CYP3A4, CYP3A5, and CYP3A7. *Drug Metab Dispos* **30:**883–891.
Williams PA, Cosme J, Vinkovic DM, Ward A, Angove HC, Day PJ, Vonrhein C, Tickle IJ, and Jhoti H (2004) Crystal structures of human cytochrome P450 3A4 bound to metyrapone and progesterone. *Science* **305:**683–686.
Willson TM and Kliewer SA (2002) PXR, CAR and drug metabolism. *Nat Rev Drug Discov* **1:**259–266.
Woodahl EL, Yang Z, Bui T, Shen DD, and Ho RJ (2005) MDR1 G1199A polymorphism alters permeability of HIV protease inhibitors across P-glycoprotein-expressing epithelial cells. *AIDS* **19:**1617–1625.
Wrighton SA, Brian WR, Sari MA, Iwasaki M, Guengerich FP, Raucy JL, Molowa DT, and Vandenbranden M (1990) Studies on the expression and metabolic capabilities of human liver cytochrome P450IIIA5 (HLp3). *Mol Pharmacol* **38:**207–213.
Xie W, Barwick JL, Downes M, Blumberg B, Simon CM, Nelson MC, Neuschwander-Tetri BA, Brunt EM, Guzelian PS, and Evans RM (2000) Humanized xenobiotic response in mice expressing nuclear receptor SXR. *Nature* **406:**435–439.
Xie W, Radominska-Pandya A, Shi Y, Simon CM, Nelson MC, Ong ES, Waxman DJ, and Evans RM (2001) An essential role for nuclear receptors SXR/PXR in detoxification of cholestatic bile acids. *Proc Natl Acad Sci USA* **98:**3375–3380.
Xu Y, Hashizume T, Shuhart MC, Davis CL, Nelson WL, Sakaki T, Kalhorn TF, Watkins PB, Schuetz EG, and Thummel KE (2006) Intestinal and hepatic CYP3A4

catalyze hydroxylation of 1α,25-dihydroxyvitamin D3: implications for drug-induced osteomalacia. *Mol Pharmacol* **69:**56–65.
Yano JK, Wester MR, Schoch GA, Griffin KJ, Stout CD, and Johnson EF (2004) The structure of human microsomal cytochrome P450 3A4 determined by X-ray crystallography to 2.05-A resolution. *J Biol Chem* **279:**38091–38094.
Zhang QY, Fang C, Zhang J, Dunbar D, Kaminsky L, and Ding X (2009) An intestinal epithelium-specific cytochrome P450 reductase-knockout mouse model: direct evidence for a role of intestinal cytochromes P450 in first-pass clearance of oral nifedipine. *Drug Metab Dispos* **37:**651–657.
Zhou S, Yung Chan S, Cher Goh B, Chan E, Duan W, Huang M, and McLeod HL (2005) Mechanism-based inhibition of cytochrome P450 3A4 by therapeutic drugs. *Clin Pharmacokinet* **44:**279–304.