Interrelationship Between Substrates and Inhibitors of Human CYP3A and P-Glycoprotein

Richard B. Kim,[1,2] Christoph Wandel,[1] Brenda Leake,[1] Mirjana Cvetkovic,[1] Martin F. Fromm,[1] Peter J. Dempsey,[1] Mark M. Roden,[1] Frank Belas,[1] Ajai K. Chaudhary,[1] Dan M. Roden,[1] Alastair J. J. Wood,[1] and Grant R. Wilkinson[1]

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Purpose. CYP3A and P-gp both function to reduce the intracellular concentration of drug substrates, one by metabolism and the other by transmembrane efflux. Moreover, it has been serendipitously noted that the two proteins have many common substrates and inhibitors. In order to test this notion more fully, systematic studies were undertaken to determine the P-gp-mediated transport and inhibitory characteristics of prototypical CYP substrates.

Methods. L-MDR1, LLC-PK1, and Caco-2 cells were used to evaluate established CYP substrates as potential P-gp substrates and inhibitors *in vitro*, and *mdr1a* deficient mice were used to assess the *in vivo* relevance of P-gp-mediated transport.

Results. Some (terfenadine, erythromycin and lovastatin) but not all (nifedipine and midazolam) CYP3A substrates were found to be P-gp substrates. Except for debrisoquine, none of the prototypical substrates of other common human CYP isoforms were transported by P-gp. Studies in *mdr1a* disrupted mice confirmed that erythromycin was a P-gp substrate but the CYP3A-inhibitor ketoconazole was not. In addition, CYP3A substrates and inhibitors varied widely in their ability to inhibit the P-gp-mediated transport of digoxin.

Conclusions. These results indicate that the overlap in substrate specificities of CYP3A and P-gp appears to be fortuitous rather than indicative of a more fundamental relationship.

KEY WORDS: CYP3A4; P-glycoprotein; drug transport; cytochrome P450.

INTRODUCTION

Interactions between concomitantly administered drugs are frequent and may have important clinical consequences. Because of this, mechanistic understanding of such interactions is important since it provides a basis for rationally addressing the situation and may avoid similar situations with other drugs. Much attention has focussed on the inhibition of drug metabolism mediated by, for example, the cytochrome P450 (CYP) superfamily of monooxygenases (1). Members of the CYP3A subfamily are particularly important in this regard since they are involved in the metabolism of many commonly used drugs and are localized in both the intestine and the liver (2). Accordingly, CYP3A is a major determinant of first-pass metabolism following oral drug administration of its substrates and also the drug's subsequent rate of removal from the body, and inhibition of this process results in increased drug levels (2,3). There is, however, increasing interest in the potential role and importance of impaired membrane transport in certain drug interactions. For example, it is now recognized that many interactions resulting in increased digoxin serum levels are caused by inhibition of P-glycoprotein (P-gp)-mediated transport (4). Significantly, P-gp is co-localized in a polarized fashion at the apical membrane of cells that also express CYP3A, for example, enterocytes in the small intestine, hepatocytes, and proximal tubular cells in the kidney, where it functions as an efflux pump to remove intracellular drug (5). Accordingly, inhibition of P-gp activity may increase the extent of drug absorption from the intestinal lumen into enterocytes and reduce elimination by biliary/renal excretion, and/or secretion into the lower intestinal tract, all of which would result in increased systemic drug levels (6).

It has been postulated that CYP3A and P-gp not only function in a complementary fashion to reduce systemic exposure to drugs but there is also considerable overlap in the drugs which interact with the two proteins (7). Much of the supportive evidence for this notion is based on studies of the multi-drug resistance (MDR) phenomenon whereby chronic exposure of tumor cells to a variety of cancer chemotherapeutic agents results in an over-expression of P-gp and an associated reduction in intracellular drug accumulation (5). Numerous agents have been identified *in vitro* which reverse this effect by inhibiting the transporter's activity (8,9), including many drugs which are known to be CYP3A substrates (e.g., cancer chemotherapeutic agents, calcium channel antagonists, immunosuppressant agents like cyclosporine, antiarrythmic agents, and azole antifungal agents). Also, other compounds, like steroids, which also undergo CYP3A-mediated metabolism, have been shown to be P-gp substrates (10). However, it is now apparent that P-gp inhibitors are not necessarily substrates for the transporter and vice-versa (11). Moreover, for a number of prototypical CYP3A substrates, their interaction with P-gp as potential substrates has not yet been defined. It is, therefore, currently difficult to assess the relative importance of CYP3A and P-gp in a drug's overall disposition profile and also the role of inhibited metabolism and/or transport in a specific drug interaction. Accordingly, a systematic study was undertaken to determine the P-gp-mediated transport and inhibitory characteristics of prototypical CYP3A substrates as well as those of drugs commonly used to characterize the catalytic activity of other CYP isoforms.

MATERIALS AND METHODS

Materials

[^{3}H]-Digoxin (15 Ci/mmol), [^{14}C]-caffeine (53 mCi/mmol) and [^{14}C]-erythromycin (54 mCi/mmol) were supplied by DuPont-New England Nuclear (Boston, MA), whereas [^{14}C]-mephenytoin was obtained from Amersham Life Sciences (Arlington Heights, IL). [^{14}C]-Lovastatin (53 mCi/mmol), [^{14}C]-terfenadine (52 mCi/mmol) and [^{14}C]-ketoconazole (14.2

[1] Departments of Medicine and Pharmacology, Vanderbilt University School of Medicine, Nashville, Tennessee 37232-6602.

[2] To whom correspondence should be addressed. (e-mail: richard.kim@mcmail.vanderbilt.edu)

ABBREVIATIONS: CYP, cytochrome P450; MDR, multidrug resistance; P-gp, P-glycoprotein; PBS, phosphate-buffered saline; BSA, bovine serum albumin; DNS, normal donkey serum; FBS, fetal bovine serum; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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mCi/mmol) were gifts from Merck & Co Inc (Rahway, NJ), Hoechst Marion Roussel Inc (Kansas City, MO), and Janssen Research Foundation (Beerse, Belgium), respectively. Radiopurity of all compounds was greater than 98% as measured by either TLC or HPLC. Midazolam, 1'-hydroxymidazolam and debrisoquine were provided by Hoffmann La-Roche (Nutley, NJ) and S-mephenytoin and PSC-833 by Novartis (Basel, Switzerland). All other chemical and reagents, unless stated otherwise, were obtained from Sigma-Aldrich Research (St. Louis, MO) and were of the highest quality available.

Immunofluorescence and Confocal Microscopy of P-Glycoprotein Expression

Indirect immunofluorescence and laser scanning confocal microscopy were performed as previously described with some modification (12). Cells cultured on Transwell™ filters (Costar, Cambridge, MA) for 4 days were washed three times with ice cold PBS (BioWhittaker, Walkersville, MD) and then were fixed with 2% paraformaldehyde for 30 min at 4°C. MRK-16 monoclonal antibody (Kamiya Biomedical Company, Thousand Oaks, CA) to the ectodomain of P-glycoprotein was diluted to 2 μg/ml in PBS-BSA (1%) containing 5% normal donkey serum (PBS-BSA-NDS) and added simultaneously to both apical and basolateral compartments. Following incubation for 30 min at 4°C, cells were washed in PBS-BSA and incubated with Cy3-conjugated donkey anti-mouse IgG diluted 1:400 in PBS-BSA-NDS for 30 min at 4°C. The cells were washed again, the filters excised and mounted in Vectashield mounting medium (Vector Laboratories, Burlingame, CA), and then viewed with a Zeiss Axiophot microscope (Thornwood, NY). Laser scanning confocal microscopy was performed using a Zeiss LSM 4 confocal microscope.

Transport in Cultured LLC-PK1, L-MDR1, and Caco-2 Cells

L-MDR1 and LLC-PK1 cells were provided by Drs. A. H. Schinkel (Netherlands Cancer Institute, The Netherlands) and E. G. Schuetz (St. Jude's Children's Research Hospital, Memphis TN), and Caco-2 cells were from Dr. R. J. Coffey (Vanderbilt University, Nashville, TN). Cells were plated on Transwell™ (Costar) filters and grown under identical conditions as described previsously (13). About 1–2 hr prior to the start of the transport experiments, the medium in each compartment was replaced with a serum free-medium (Optimem, Gibco BRL). Then, the medium in each compartment was replaced with 700 μl serum-free medium (Optimem), with or without drug (radiolabeled or unlabeled). The amount of the drug appearing in the opposite compartment after 1, 2, 3, and 4 hr was measured in 25 μl aliquots taken from each compartment.

Inhibition of P-gp mediated transport by Caco-2 cells was determined in a similar manner after the addition of the putative inhibitor to both the apical and basal compartments, and using radiolabeled digoxin as the P-gp substrate. Complete inhibition of P-gp mediated transport would be expected to result in the loss of digoxin's basal-to-apical (B → A) *versus* apical-to-basal (A → B) transport difference. Accordingly, percentage inhibition was estimated by Eq. 1.

$$\text{Degree of inhibition} = \left[1 - \left[\frac{i_{B\to A} - i_{A\to B}}{a_{B\to A} - a_{A\to B}}\right]\right] \times 100\% \quad (1)$$

where i and a are the percentages of digoxin transport in the presence and absence of the putative inhibitor, according to the direction of transport. Values estimated at each time point were averaged since digoxin transport appeared to be linear with respect to time. Control digoxin transport in the absence of any inhibitor (2 wells/plate) were included on every plate (12 wells). Data shown represent results obtained from studies carried out on at least 3 preparations on different days.

Drug Analyses

Aliquots (25 μl) of the compartmental buffer solution containing radiolabeled drug were analyzed by liquid scintillation counting (1219 Rackbeta LSC, LKB-Wallace, Gaithersburg MD), after the addition of 5 ml Scintiverse BD™ (Fisher Scientific, Fairlawn, NJ). Nifedipine concentrations were determined by an HPLC-based procedure (14) and similar methods were used for tolbutamide (15) and chlorzoxazone (16). Gas chromatography/electron capture, negative chemical ionization mass spectrometric analysis was used to determine debrisoquine levels (17). Midazolam and 1-hydroxymidazolam concentrations were measured using a recently described high performance liquid chromatographic, mass spectrometric/mass spectrometric procedure (18).

Determination of Tissue Distribution in *mdr1a* (+/+) and (−/−) Mice

Male *mdr1a* (−/−) mice, (FVB/TacfBR-[KO]mdr1aN7), 6–12 weeks of age and genetically matched male *mdr1a* (+/+) mice (FVB/NTtacfBR) were obtained from Taconic (Germantown, NY). Radiolabeled ketoconazole (2 mg/kg), or erythromycin (0.75 mg/kg) dissolved in 20% ethanol/0.9% saline were given orally using gastric lavage to separate groups of 3 mice—the total volume given was 2.1 ml/kg. After 4 hr, the animals were anesthetized with isoflurane (IsoFlo, Abbott Laboratories, IL) and blood and tissue samples were obtained, and tissue radioactivity determined as outlined previously (13). The protocols for the animal experiments were approved by Vanderbilt University Animal Care Committee, and the mice were cared for in accordance with the USPHS policy for the Care and Use of Laboratory Animals.

RESULTS

P-gp immunofluorescence detected using MRK16 antibody indicated marked P-gp expression in L-MDR1 cells and a much lower amount in Caco-2 cells (Fig. 1). Moreover, scanning laser confocal microscopy showed that such expression was localized in the apical membrane (Fig. 1). By contrast, P-gp was not detectable in LLC-PK1 cells. In addition, studies with L-MDR1 and Caco-2 cells using digoxin—a prototypical P-gp substrate—demonstrated that cellular translocation was markedly polarized (Figs. 2 and 4); transport being more extensive in the basolateral to apical direction than the reverse. On the other hand, in the LLC-PK1 parental cell line this vectorial transport difference was markedly reduced (Fig. 2) and the

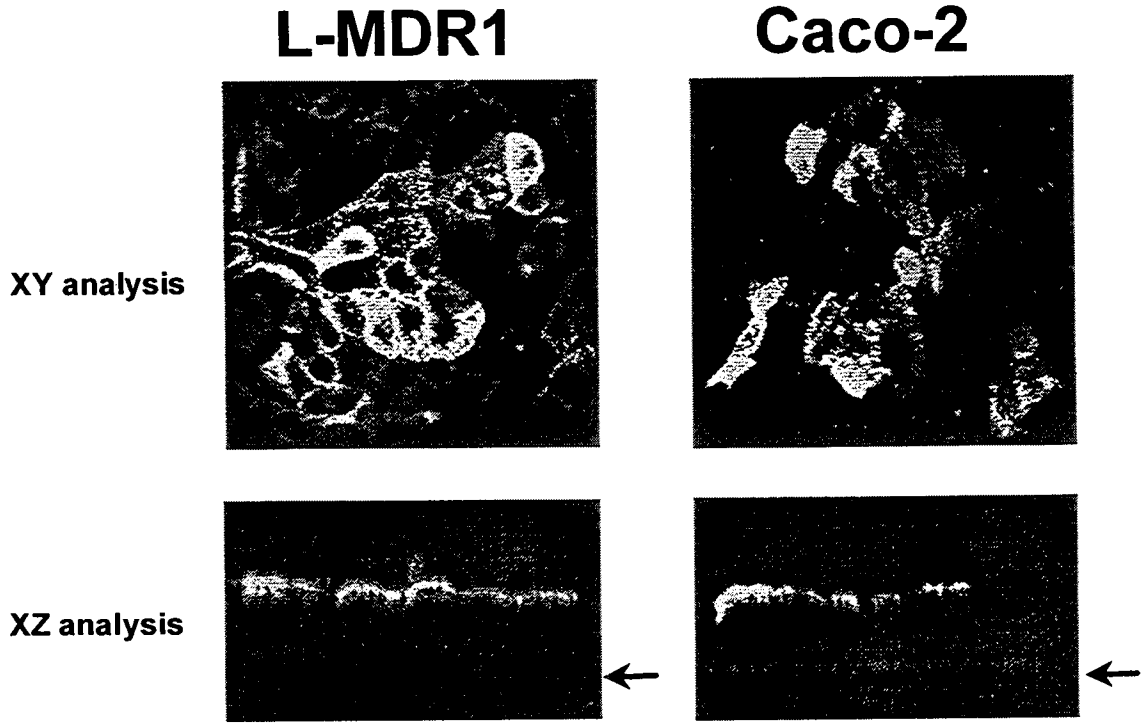

Fig. 1. Preferential localization of P-glycoprotein to the apical membrane domain in polarized L-MDR1 and Caco-2 cells. XY analysis (horizontal focal section) through the apical membrane of cell monolayer. XZ analysis (vertical section) through cell monolayer. Arrows indicate the basal surface of the monolayer which is contact with the Transwell™ filter.

addition of quinidine (10 to 100 μM) and PSC-833 (10 μM) to Caco-2 cells completely inhibited the phenomenon (Table 1). Accordingly, it was concluded that the cell lines were suitable for demonstrating P-gp mediated transport and its inhibition.

Evaluation of the transport of six established CYP3A substrates separated the drugs into three different classes. Both terfenadine and lovastatin demonstrated polarized cellular translocation in L-MDR1 cells that was less pronounced than with digoxin but was essentially absent in LLCPK1 cells, consistent with these drugs being P-gp substrates (Fig. 2). Pronounced polarized transport was also observed in L-MDR1 cells with erythromycin (data not shown). The potent CYP3A inhibitor ketoconazole also showed modest basal to apical *versus* apical to basal transport differences in both L-MDR1 and LLC-PK1 cells and also Caco-2 cells (Fig. 3). Moreover, the addition of PSC-833 (10 μM) failed to eliminate the polarized transport differences in Caco-2 cells (Fig. 3), consistent with ketoconazole not being a P-gp substrate. By contrast, the transport of nifedipine and midazolam did not show any difference in vectorial transport with respect to apical *versus* basal delivery; moreover, the results in L-MDR1 cells were similar to those in LLC-PK1 cells (Fig. 2). Thus, these drugs did not appear to be substrates for P-gp. Of the several drugs commonly used as prototypical substrates of CYP isoforms other than CYP3A (caffeine - CYP1A2, tolbutamide - CYP2C9, S-mephenytoin - CYP2C19, debrisoquine - CYP2D6, and chlorzoxazone - CYP2E1), only debrisoquine showed evidence of polarized transport, and this was relatively modest (Fig. 2). Neither the LLC-PK1 nor L-MDR1 cells appeared to have any significant endogenous CYP activity since metabolite formation was not detected during the incubation period with midazolam, tolbutamide, chlorzoxazone, or debrisoquine.

The ability of the drugs described above and additional compounds to inhibit P-gp-mediated transport was determined in Caco-2 cells at two drug concentrations (10 and 100 μM) and using digoxin as the prototypical substrate. A range of inhibitory effects were noted which appeared to be concentration-dependent (Table 1, Fig. 4). Polarized transport of digoxin was essentially absent in the presence of PSC-833, quinidine, verapamil and ketoconazole and terfenadine even at the lower drug concentration studied (Table 1). Extensive inhibition (>75%) also occurred at the higher concentration with erythromycin, tamoxifen, amiodarone and midazolam but, in general, this was considerably less pronounced at the lower concentration level (Table 1). Other CYP3A substrates, including cortisol and nifedipine, and certain metabolites had less pronounced inhibitory effects (Table 1). By contrast, prototypical substrates of CYP isoforms other than CYP3A exhibited only modest inhibition even at the highest studied concentration (Table 1).

Comparative *in vivo* tissue distribution studies of erythromycin and ketoconazole were undertaken in *mdr1a* (+/+) and *mdr1a* (−/−) mice. After oral administration of radiolabeled erythromycin, levels of total radioactivity measured 4 hr later in most tissues, including blood, were about 8-fold higher (data not shown) in the *mdr1a* "knockout" mice compared to their wild-type counterpart. The major exception was the brain where the level was nearly 15-fold greater than that in *mdr1a* (−/−) mice. By contrast, with ketoconazole, no significant differences in the levels of radioactivity in the blood and other tissues were noted between *mdr1a* (+/+) and *mdr1a* (−/−) mice (Table 2).

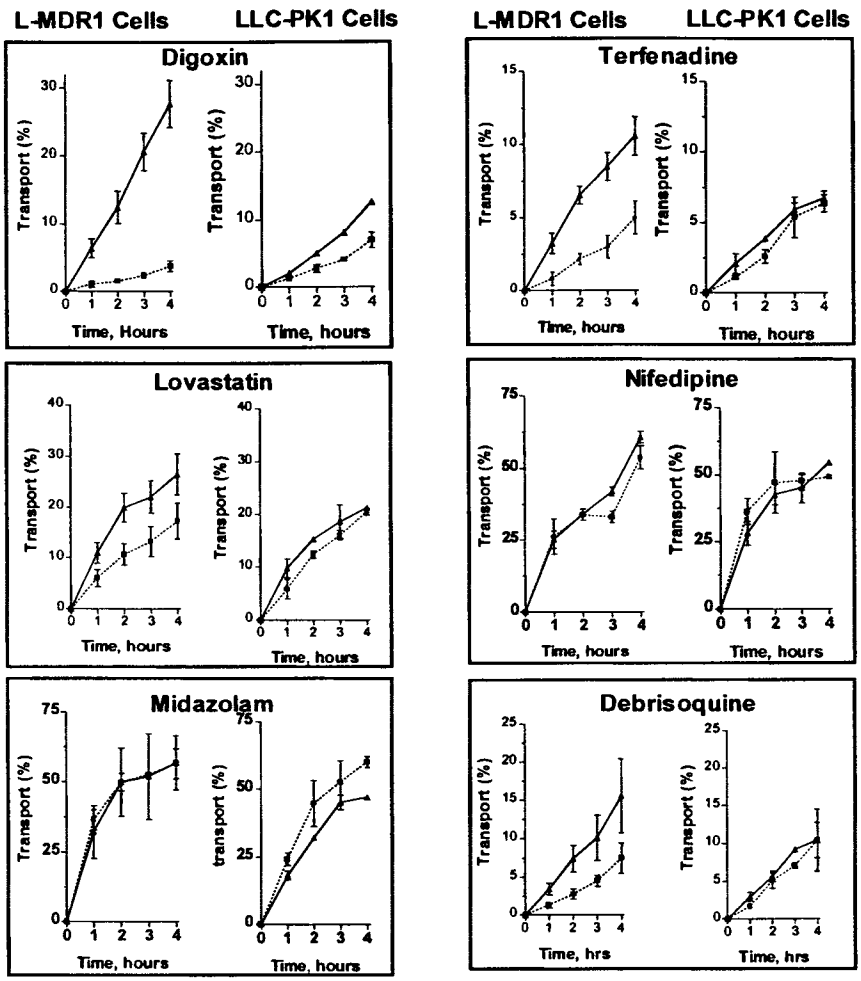

Fig. 2. Transepithelial transport across L-MDR1 (left subpanels) and LLC-PK1 (right subpanels) cell culture monolayers: [^{3}H]-digoxin (5 μM), and CYP3A substrates; [^{14}C]-terfenadine (5 μM), [^{14}C]-lovastatin (5 μM), nifedipine (50 μM), midazolam (3 μM), and debrisoquine (50 μM). Translocation from basal to apical compartments (▲ symbol and solid line); translocation from apical to basal compartments, (■ symbol and dotted line). Data are mean ± SE from 3 or more experiments.

DISCUSSION

Drugs that have been characterized as either P-gp substrates or inhibitors exhibit wide variability in their chemical structures (8,9). Similarly the substrate and inhibitor specificities of CYP3A are extremely broad (1,2,19). Therefore, it is not too surprising that overlap has been noted between drugs that interact with both proteins (7). This study provides evidence indicating an important role for P-glycoprotein in the disposition of some CYP3A substrates, but not others. Furthermore, it illustrates an overall strategy for assessing the potential ability of a compound to not only inhibit but also to be transported by P-gp, and to evaluate the role of the transporter in the *in vivo* disposition of the drug. Importantly, the data indicates that several *in vitro* and *in vivo* approaches are necessary in order to obtain reliable assessment. The role of P-gp in mediating the transport of prototypical CYP3A substrates was initially assessed *in vitro* by showing that their translocation across polarized cells is dependent on the direction of vectorial movement, i.e., basal to apical transport is greater than that of apical to basal. Indeed, confocal immunofluorescence microscopy revealed marked apical expression of P-gp in both L-MDR1 and Caco-2 cells (Fig. 1). Intestine-derived Caco-2 cells constitutively express P-gp and potentially other transporters. Accordingly, a finding of polarized cellular translocation may, in certain circumstances, not only reflect the involvement of P-gp. By contrast, the porcine kidney-derived L-MDR1 cell only differs from the parental LLC-PK1 line by the manipulated overexpression of P-gp, thus, data interpretation is not confounded by the potential presence of other transporters. In addition, the absence of metabolism mediated by CYP2C9, CYP2D6, CYP2E1, and CYP3A in LLC-PK1 and also L-MDR1 cells indicates that this factor is not a potentially confounding variable in the use of these cells.

Collectively, the data obtained with the polarized cells demonstrate that the speculated overlap between CYP3A and P-gp drug substrates is incomplete and does not reflect a more fundamental inter-relationship. For example, neither nifedipine nor midazolam, which have been extensively used to estimate

Table 1. Effects of Various CYP Substrates and Inhibitors (10 μM and 100 μM) on [^{3}H]-Digoxin (5 μM) Transport in Caco-2 Cells

CYP isoform	Substrate (S) inhibitor (I) metabolite (M)	% inhibition of P-gp transport (Mean ± SE)	
		10 μM	100 μM
CYP3A4	terfenadine (S)	112 ± 4.1***	—
	quinidine (S)	104 ± 4.0***	103 ± 4.7***
	PSC-833 (S/I)	103 ± 3.2***	—
	ketoconazole (I)	100 ± 8.2***	100 ± 11***
	verapamil (S)	90 ± 12**	103 ± 2.7***
	amiodarone (S)	72 ± 9.8**	78 ± 8.7**
	lovastatin (S)	62 ± 12**	65 ± 8.3**
	erythromycin (S/I)	50 ± 6.0**	101 ± 9***
	midazolam (S)	47 ± 15*	74 ± 7.2***
	tamoxifen (S)	36 ± 7.4*	86 ± 1.0***
	nifedipine (S)	36 ± 5.9**	56 ± 6.5**
	1-hydroxymidazolam (M)	17 ± 8.1	63 ± 6.3***
	6β-hydroxycortisol (M)	13 ± 3.3	57 ± 16*
	cortisol (S)	0.6 ± 1.8	56 ± 15*
CYP1A2	caffeine (S)	13 ± 8.9	36 ± 11*
CYP2C9	tolbutamide (S)	18 ± 10	14 ± 9.8
CYP2C19	S-mephenytoin (S)	3 ± 12	42 ± 13**
CYP2D6	debrisoquine (S)	16 ± 5.7	21 ± 13
CYP2E1	chlorzoxazone (S)	19 ± 4.1*	24 ± 5.9*

Note: Statistical difference was assessed by a two-sided Student's t-test, with $p < 0.05$ as the limit of significance. Each experiment was performed on at least 3 different experimental days.

* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.

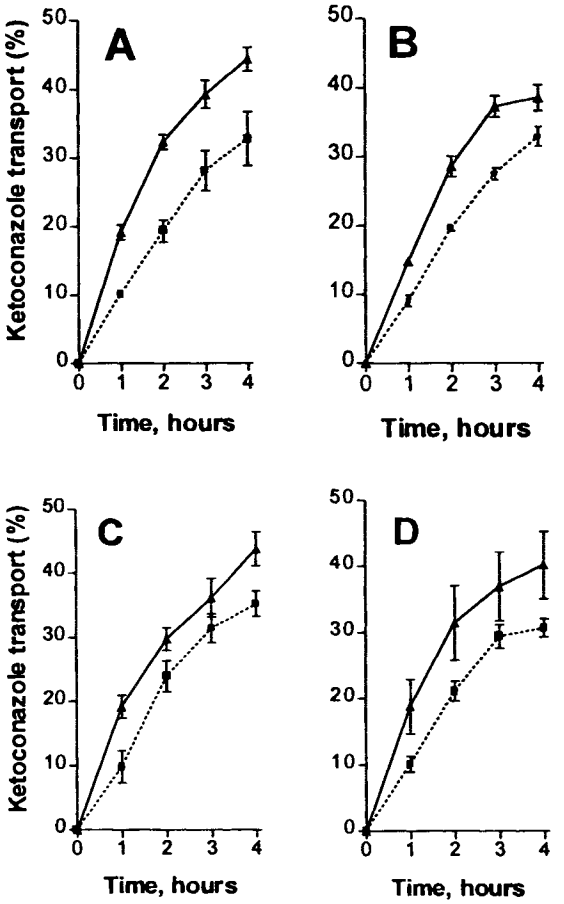

Fig. 3. Transepithelial transport of [^{14}C]-ketoconazole (5 μM) in L-MDR1 (Panels A), LLC-PK1 (Panel B), and Caco-2 cells in the absence of PSC-833 (panel C) and presence of 10 μM PSC-833 (Panel D). Translocation from basal to apical compartments, (▲ symbol and solid line); translocation from apical to basal compartments, (■ symbol and dotted line). Data are mean ± SE from 3 or more experiments.

CYP3A catalytic activity both *in vitro* (20,21) and *in vivo* (22,23), were found to be P-gp substrates. In the case of midazolam, this finding is consistent with the drug's rapid sedative effect indicative of ready transport across the blood-brain barrier - a site where P-gp expression and efflux function are particularly high (24). These experimental observations are important, since they substantiate use of these drugs as CYP3A probes, especially *in vivo*; that is, their oral bioavailability and elimination only reflect drug metabolism that is not confounded by any P-gp transport. The same situation appears to apply to other drugs commonly used as prototypical substrates for other CYP isoforms with the exception of debrisoquine and CYP2D6. By contrast, CYP3A substrates such as terfenadine (25) and lovastatin (26) were found to have transport characteristics consistent with them being P-gp substrates. However, it must be noted that the magnitude of polarized transport in L-MDR1 cells was not as marked as with digoxin suggesting that terfenadine and lovastatin interacted less readily with P-gp.

The observed transport characteristics of erythromycin in the L-MDR1 cells as well as its impaired elimination in *mdr1a* (-/-) mice, indicated by the 8-fold increase in the 4 hr blood level and the increased brain/blood concentration ratio compared to syngeneic animals, are similar to recently reported findings with this drug(27). Erythromycin, and possibly its metabolites, appear to be P-gp substrates, consistent with the finding that the level of erythromycin in human bile is nearly 10-fold greater than that in plasma(28). In turn, these findings may cast doubts on the use of erythromycin as an *in vivo* probe that only reflects CYP3A activity(29). Interestingly, transport of the potent CYP3A inhibitor ketoconazole was enhanced in basal-to-apical direction in all tested cell lines, and was not altered by the addition of the potent P-gp inhibitor PSC-833 (Fig. 3). Furthermore, ketoconazole's disposition in *mdr1a* (-/-) mice, was not statistically significant from that in wildtype mice. This indicates that ketoconazole is unlikely to be a substrate for P-gp and also suggests the likelihood of other efflux transporters being involved in ketoconazole cellular transport.

In conclusion, the present data suggests that prediction of a drug's functional interaction with P-gp solely on the basis of its interaction with CYP3A, and *vice-versa* is impossible. Some drugs will indeed be substrates and inhibitors of both proteins, but others will not. Each particular situation will have to be determined on a case-by-case basis, especially when interpreting possible mechanisms of interaction between two drugs.

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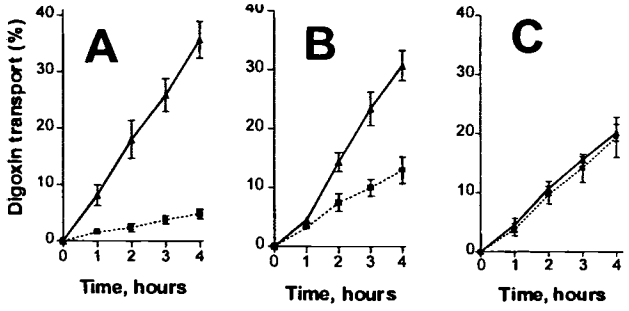

Fig. 4. Transepithelial transport of [^{3}H]-digoxin (5 μM) across a Caco-2 cell culture monolayer in the absence (Panel A), or presence of 10 μM erythromycin (Panel B) or 10 μM verapamil (Panel C). Translocation from basal to apical compartments, (▲ symbol and solid line); translocation from apical to basal compartments, ■ symbol and dotted line). Data are mean ± SE from 3 or more experiments.

Table 2. Tissue Levels of Radioactivity (ng/g tissue) in *mdr1a* (+/+) and (−/−) Mice at 4 hr After Oral Injection of [^{14}C]-Ketoconazole (2 mg/kg)

Tissue	*mdr1a* (+/+)	*mdr1a* (−/−)	Ratio (−/−)/(+/+)
Ketoconazole			
Plasma	41 ± 5.5	65 ± 22	1.6
Brain	26 ± 7.8	36 ± 5.7	1.4
Heart	56 ± 16	55 ± 4.7	1.0
Lung	100 ± 9.0	75 ± 23	0.8
Liver	237 ± 70	536 ± 124	2.3
Spleen	45 ± 5.7	75 ± 22	1.7
Kidney	111 ± 12	193 ± 49	1.7
Small intestine	719 ± 170	490 ± 167	0.7
Colon	45 ± 7.3	151 ± 25*	3.4

Note: Statistical difference in radioactivity between the two groups of mice (3 per group) was assessed by a two-sided Student's t-test, with $p < 0.05$ as the limit of significance (* $p < 0.05$).

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