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P-glycoprotein: A major determinant of rifampicin-inducible expression of cytochrome P4503A in mice and humans

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ABSTRACT **The P-glycoprotein (Pgp) efflux pump can influence the hepatocellular concentration of xenobiotics that are modulators and substrates of cytochrome P4503A (CYP3A). We tested the hypothesis that Pgp is a determinant of drug-inducible expression of CYP3A. The magnitude of CYP3A induction by rifampicin was compared in the human parental colon carcinoma cell line LS 180/WT (wild type) and in two derivative clones overexpressing the human multidrug resistance gene *MDR1* (also designated *PGY1*) because of either drug selection (LS 180/ADR) or transfection with MDR1 cDNA (LS 180/MDR). In both MDR1 cDNA-overexpressing clones, rifampicin induction of CYP3A mRNA and protein was decreased and required greater rifampicin concentrations compared with parental cells. The role of Pgp in regulation of CYP3A expression *in vivo* was analyzed in mice carrying a targeted disruption of the *mdr1a* mouse gene. Oral treatment with increasing doses of rifampicin resulted in elevated drug levels in the livers of *mdr1a* (−/−) mice compared with *mdr1a* (+/+) mice at all doses. Consistent with the enhanced accumulation of rifampicin in *mdr1a* (−/−) mice, lower doses of rifampicin were required for induction of CYP3A proteins, and the magnitude of CYP3A induction was greater at all doses of rifampicin in *mdr1a* (−/−) mice compared with *mdr1a* (+/+) mice. We conclude that Pgp-mediated transport is a critical element influencing the CYP3A inductive response.**

Human variation in biological response to many drugs is largely influenced by the interindividual differences in expression of cytochromes P450 (1). The most prominent of the genes expressing cytochromes P450 is the *CYP3A* gene family, encoding cytochromes P4503A (CYP3As), which constitute 30% and 70% of the total cytochromes P450 in most human livers and intestines, respectively (1, 2). Cytochromes P4503A catalyze a remarkable number of oxidation reactions of clinically important drugs. CYP3A substrates include terfenadine, cyclosporin A, midazolam, nifedipine, verapamil (3, 4), taxol, and the epipodophyllotoxins (5, 6) and endogenous hormones such as cortisol (7). Importantly, the expression of CYP3A protein and mRNA is highly inducible after treatment with many agents, including glucocorticoids, phenobarbital-like anticonvulsants, and antibiotics like rifampicin (8).

There is significant variation among individuals in expression of hepatic CYP3A proteins as well as in the clearance of many CYP3A-metabolized drugs (6). This variation extends to the magnitude of an individual's response to CYP3A inducers. For example, there is a 10-fold variation among individuals in the extent to which rifampicin can induce CYP3A4 expression in humans (9, 10). The clinical significance of wide variations in CYP3A are realized as major effects on drug efficacy, drug toxicity, and, hence, therapeutic outcome. The risks of adverse drug reactions related to large differences in CYP3A levels are particularly important for substrates with narrow therapeutic indices (e.g., cyclosporin A, chemotherapeutics). Therefore, it is important to elucidate the factors that regulate variability in *CYP3A* gene expression.

Because induction of CYP3A is dose-dependent (11), a primary determinant of CYP3A induction might be the intracellular concentration of inducer. Individual differences in the intracellular concentration of steroids and xenobiotics could influence variation in xenobiotic-inducible expression of CYP3A. Transport of xenobiotics and steroids out of the cell is one critical determinant of the intracellular concentration of xenobiotics and, hence, drug action. A drug efflux protein, P-glycoprotein (Pgp), which transports or interacts with many modulators of CYP3A [including glucocorticoids (12), nifedipine, ketoconazole, verapamil, and macrolide antibiotics like erythromycin (13)] is the product of the multidrug-resistance gene *MDR1* (also designated *PGY1*) (14). In humans only the *MDR1* gene product, Pgp, transports drugs (14), while in rodents the drug transport function is shared between *mdr1a* and *mdr1b* (15), with *mdr1a* being the more abundant protein in liver and intestine (16). There is substantial evidence that the amount of Pgp can influence the intracellular concentration of drugs. When Pgp is overexpressed (such as in the multidrug-resistant phenotype) it can completely prevent intracellular accumulation of drugs that are substrates (17). In contrast, mice with a targeted disruption of the *mdr1a* gene display 2- to 100-fold higher accumulation of Pgp substrates in many tissues (e.g., brain, gastrointestinal tract, liver) (18, 19). Because the amount of Pgp can influence the intracellular concentration of xenobiotic modulators of CYP3A expression, we tested the hypothesis that Pgp influences drug-inducible CYP3A expression. Drug induction of CYP3A was analyzed in two model systems, an *in vitro* system (LS 180 cells) in which human *MDR1* product Pgp is overexpressed and an *in vivo* system that lacks *mdr1a* Pgp (mice with targeted disruption of the *mdr1a* gene).

MATERIALS AND METHODS

Mice and Treatments. FVB control mice [*mdr1a* (+/+)] and *mdr1a* (−/−) mice (18) in a pure FVB background were obtained from Taconic Farms. Twenty-four hours before sacrifice, 12-week old female *mdr1a* (+/+) and (−/−) mice were treated by oral gavage with rifampicin (Sigma) (0.01 ml/g of body weight) dissolved in normal saline containing 10% (vol/vol) dimethyl sulfoxide (DMSO) or with vehicle alone, and solid food was removed. Blood was obtained from 24-week-old female *mdr1a* (−/−) and (+/+) mice treated as above.

Cell Cultures. The parental cell line designated LS 180/WT (wild type) (American Type Culture Collection) was derived from a human colon adenocarcinoma (20). LS 180/AD50 cells were previously derived from LS 180/WT by stepwise selec-

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Abbreviations: Pgp, P-glycoprotein; CYP3A, cytochrome P4503A; MDR, multidrug resistance; NEO, neomycin resistance; WT, wild type.

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tion in adriamycin (21) and have 12-fold overexpression of *MDR1* compared with LS 180/WT (21). LS 180/NEO (neomycin resistance) and LS 180/MDR (multidrug resistance) were derived by transfection of pSV2neo or co-transfection of pSV2neo and pHaMDR and selection with G418 (600 μg/ml). Overexpression of Pgp in LS 180/MDR cells (compared to LS 180/NEO cells) was determined by immunoblot analysis of PgP protein (see below).

Immunoblot Analysis. *LS 180 cells.* Lysates were prepared and, for immunodetection of Pgp, 50 μg of lysate was assayed by immunoblot with primary polyclonal rabbit anti-*mdr* (Ab-1) IgG (Oncogene Science) and developed with the enhanced chemiluminescence (ECL) detection system (Amersham) (22). For immunodetection of CYP3A4 and CYP3A5, 100 μg of lysate was assayed by immunoblot with monoclonal antibody to human CYP3A proteins (22, 23).

Mouse microsomes. Liver microsomes were prepared (11) and 10 μg was analyzed on immunoblots by using either monoclonal antibodies Ig8 (24) or K03 (23) raised against rat CYP3A1 or human CYP3A4, respectively. The relative amounts of Pgp and CYP3A proteins were determined by densitometric analysis.

Midazolam Hydroxylase Assay. Midazolam 1′- and 4-hydroxylation activities were measured as described (6) at a substrate concentration of 60 μM.

Northern Blot Analysis. Total RNA was extracted from LS 180 cells (25), and 10 μg was analyzed by Northern blot analysis (25). The β-actin cDNA was obtained from Lola Reid (Albert Einstein Medical Center, Bronx, NY). The pHFLA-A cDNA (for CYP3A) was isolated in this laboratory and represents nucleotides 458 to 846 of CYP3A7 cDNA (26) that under normal stringency conditions will recognize all human CYP3A mRNAs.

Cellular Uptake of [^{3}H]Vinblastine in LS 180/AD50 Cells. Intracellular accumulation of [^{3}H]vinblastine (2.8 Ci/mmol; Moravek Biochemicals, La Brae, CA; 1 Ci = 37 GBq) was assayed in LS 180/AD50 cells as described (27).

Assay of Rifampicin Concentrations. Two-tenths of a milliliter of internal standard papaverine·HCl (Acros, Pittsburgh) dissolved in methanol at 20 μg/ml and 0.8 ml of 0.5 M Sorensen's buffer (pH 7.2) were added to 0.2 ml of liver homogenate (1:1 wt/vol) in 0.15 M KCl or plasma containing 3.3 mg of ascorbic acid per ml; the mixture was extracted with two aliquots of chloroform that were combined, evaporated to dryness under nitrogen, and reconstituted in 0.1 ml of mobile phase, of which 0.05 ml was injected onto the HPLC (28). Mobile phase [methanol/0.05 M ammonium formate, pH 7.3, 65:35 (vol/vol)] was pumped isocratically at 1.6 ml/min through a Bondclone 10 C_{18} 300 × 3.9 mm column (Phenomenex, Torrance, CA), and UV detection was with a Spectromonitor-D (LDC, Riviera Beach, FL) detector at 342 nm.

RESULTS

To determine the role of Pgp in human *CYP3A4* gene expression we used the human colon adenocarcinoma cell lines LS 180/WT and LS 180/AD50 that express different amounts of Pgp (21). These LS 180/WT and LS 180/AD50 are unique because they were identified as human replicating cell lines that maintain expression of CYP3A4 and CYP3A5 (22) and because a number of drugs, including rifampicin, can induce CYP3A in these cells (22). In initial experiments we compared [^{3}H]vinblastine uptake into LS 180/AD50 cells in the presence or absence of another Pgp substrate (verapamil) or in the presence of the putative substrate rifampicin. Accumulation of [^{3}H]vinblastine was enhanced 100% and 34% over control cultures by verapamil and rifampicin, respectively (not shown), and suggests that rifampicin is a substrate for *MDR1*-encoded Pgp.

Next, we compared CYP3A induction by rifampicin at various concentrations in LS 180/AD50 and LS 180/WT cells. Induction of CYP3A mRNA was 5- to 6-fold greater (Fig. 1) at 1.0 and 5 μM rifampicin, and induction of CYP3A protein was 1.7- to 3.3-fold greater (Fig. 2) at 1.0 and 10 μM rifampicin, respectively, in the LS 180/WT compared with the LS 180/AD50. Similarly, induction of CYP3A mRNA by 10 μM reserpine, a CYP3A inducer (22) and a modulator and putative Pgp substrate (29), was 10-fold greater in the LS 180/WT cells compared with LS 180/AD50 cells. Hence, in the cells overexpressing Pgp, there was decreased induction of CYP3A compared with the LS 180/WT parent cells, despite identical extracellular concentrations of drug.

Because it could be argued that cells selected by drug exposure to overexpress Pgp may have undergone additional changes that might influence induction of CYP3A4, we derived a cell line with only a singular change—stable overexpression of *MDR1* Pgp. LS 180/WT cells were stably transfected with either pSV2neo to generate LS 180/NEO or with pSV2neo plus an expression vector for human Pgp to generate LS

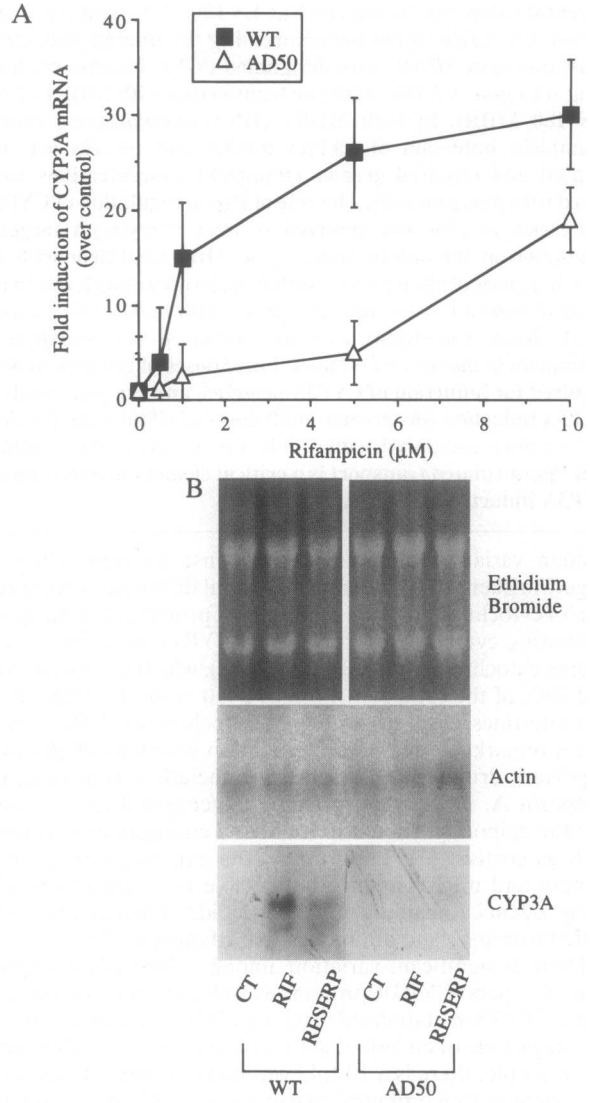

FIG. 1. RNA (Northern) blot analysis of drug induction of CYP3A in LS 180/WT and LS 180/AD50 cells. (*A*) Cells were treated in parallel for 48 hr with indicated doses of rifampicin and 10 μg of total RNA from untreated and treated cells analyzed by Northern blot followed by densitometric quantitation of the CYP3A mRNAs. Each point represents the mean ± SD of three separate determinations. (*B*) A representative Northern blot filter of 10 μg of total RNA from untreated control cells (lanes CT) or cells treated with 5 μM rifampicin (lanes RIF) or reserpine (lanes RESERP) and stained with ethidium bromide or hybridized with cDNAs for β-actin or human CYP3A.

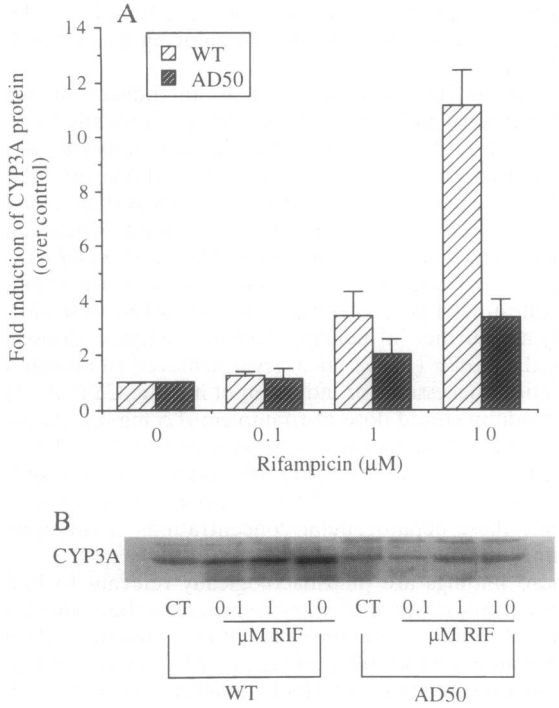

FIG. 2. Immunoblot analysis of rifampicin induction of CYP3A in LS 180/WT and LS 180/AD50 cells. (*A*) Cells were treated as in the Fig. 1 legend, and lysates were analyzed by quantitative immunoblot. Each bar represents the mean ± SD of three separate determinations. (*B*) Representative immunoblot.

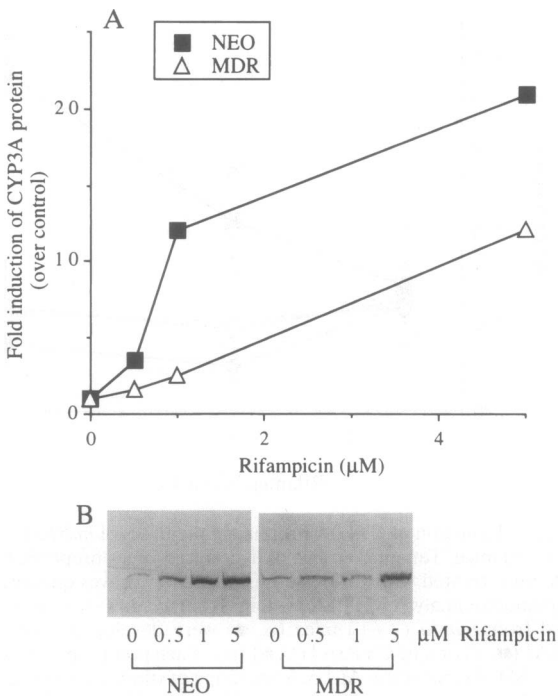

FIG. 3. Immunoblot analysis of rifampicin induction of CYP3A in LS 180/NEO and LS 180/MDR cells. (*A*) Cells were treated and analyzed as in the Fig. 2 legend. Each point represents the average of two separate determinations. (*B*) Representative immunoblot.

180/MDR (5.6-fold overexpression of Pgp). Rifampicin readily induced CYP3A4 in LS 180/NEO cells, whereas LS 180/MDR cells demonstrated a shift to the right in the dose–response curve for CYP3A4 induction by rifampicin (Fig. 3). The estimated concentration associated with 10-fold induction of CYP3A was 0.8 μM in the LS 180/NEO versus 4.2 μM in the LS 180/MDR cells (Fig. 3). These results are consistent with Pgp decreasing the accumulation of drug inducer in these cells.

There are four proteins in mouse liver that immunoreact with CYP3A antibodies (Fig. 4). On SDS/PAGE one CYP3A doublet selectively immunoreacts with monoclonal anti-CYP3A1, and the other doublet (faster migrating) immunoreacts with monoclonal anti-CYP3A4 (Fig. 4). The identification of these CYP3A proteins is unclear because proteins corresponding to mouse CYP3A cDNAs [CYP3A11 (30), CYP3A13 (31), and CYP3A16 (31)] have not been purified. However, anti-CYP3A1 polyclonal antiserum that has been immunoabsorbed against rat liver microsomes from 3-methylcholanthrene-treated male rats and then against microsomes from untreated females reacted with these same four proteins (24) (not shown), further supporting their identification as CYP3A proteins. The dose–response for rifampicin induction of these CYP3A proteins was compared in the *mdr1a* (−/−) and (+/+) mice. *Mdr1a* (−/−) mice responded to as little as 1.5 mg of rifampicin per kg of body weight with a 1.4-fold increase in hepatic anti-CYP3A1 immunoreactive proteins (Fig. 5). Induction of these CYP3A proteins further increased with increasing dose levels of rifampicin in *mdr1a* (−/−) mice (to a maximum of 3.8-fold). In contrast, rifampicin did not induce these same CYP3A proteins in *mdr1a* (+/+) littermates at the three concentrations shown in Fig. 5 but at a higher dose (6.5 mg/kg) increased these CYP3A proteins about 1.5-fold (not shown). There was no effect of rifampicin at any dose on expression of the anti-CYP3A4 reactive proteins. To determine the pharmacological impact of rifampicin induction on CYP3A-dependent drug metabolism, we determined the rate of formation of 4-hydroxymidazolam [a catalytic activity specific to CYP3A (6)] in these microsomes. The median rate of formation of 4-hydroxymidazolam was significantly greater in *mdr1a* (−/−) mice than in (+/+) mice over all rifampicin doses ($P < 0.05$) (Table 1).

To determine if *mdr1a* Pgp in the (+/+) mice was affecting rifampicin disposition, we measured rifampicin levels in the plasma and liver from *mdr1a* (+/+) and (−/−) mice by HPLC. Table 1 indicates that the most striking difference in liver was observed at the lowest administered dose of rifampicin (1.5 mg/kg) with an 11.3-fold higher hepatocellular content of rifampicin in *mdr1a* (−/−) mice. Induction of CYP3A correlated with hepatic rifampicin concentrations (Fig. 5), but induction was not detected until the tissue level of rifampicin exceeded 3.42 μg/500 mg of tissue. While this was readily achieved in the *mdr1a* (−/−) mice at a dose of 1.5 mg/kg, the (+/+) mice only achieved this tissue concentration at 3.3 times the administered dose (5 mg/kg). Moreover, for the *mdr1a* (−/−) mice, the hepatic concentration of rifampicin was highly correlated with midazolam 1′- (not shown) and 4-hydroxylation (r^2 = 0.981 and 0.664, respectively), while there was no correlation (r^2 = 0.011 and 0.397, respectively) in *mdr1a* (+/+) mice. The plasma levels of rifampicin ranged from 3.3- to 7-fold greater in *mdr1a* (−/−) versus (+/+) mice, consistent with intestinal Pgp contributing to the oral bioavailability of rifampicin.

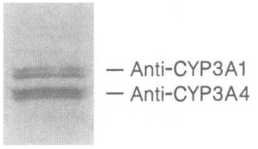

FIG. 4. Immunoreactivity of mouse liver microsomes with anti-CYP3A antibodies. Ten micrograms of mouse liver microsomes was developed sequentially, first with anti-CYP3A1 monoclonal antibody (which immunoreacts exclusively with the upper doublet) followed by anti-CYP3A4 monoclonal antibody (which immunoreacts exclusively with the lower doublet).

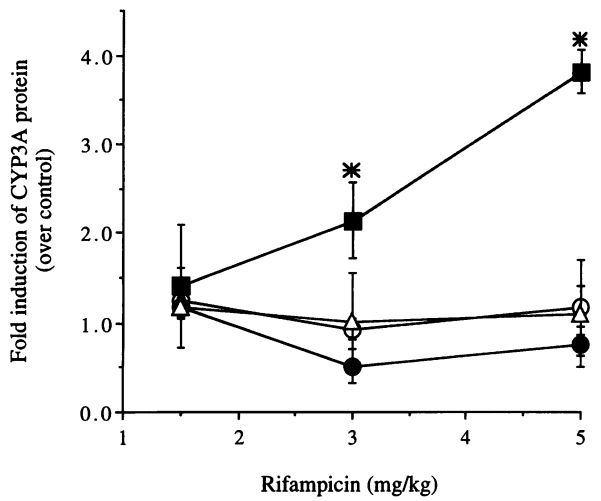

FIG. 5. Induction of CYP3A proteins in the livers of *mdr1a* (−/−) and (+/+) mice. Ten micrograms of liver microsomes prepared from female mice treated with various doses of rifampicin was quantitated by immunoblot analysis. CYP3A proteins from *mdr1a* (−/−) mice (■, ○) and from *mdr1a* (+/+) mice (△, ●) were developed with anti-CYP3A1 (■, △) or anti-CYP3A4 (○, ●) IgG. Each point represents the mean ± SD of three mice. At 1.5, 3, and 5 mg of rifampicin per kg, the *P* values for the *t* test were $P = 0.64$, 0.049, and 0.008, respectively; the asterisks designate those points where induction of CYP3A in *mdr1a* (−/−) mice was significantly different from *mdr1a* (+/+) mice.

DISCUSSION

Despite the obvious importance of Pgp-mediated transport in regulating the intracellular concentration of many xenobiotics that are recognized inducers of CYP3A, this is the first study to systematically test the hypothesis that Pgp has a role in modulating expression of CYP3A. The results of this study clearly indicate that human *MDR1*-encoded Pgp in the cell line LS 180 and its derivative clones, and the mouse *mdr1a*-encoded Pgp in intact mice limits the accumulation of rifampicin in cells and tissues, and thus a pharmacological action of the intracellular concentration of rifampicin—namely, the extent to which rifampicin can induce CYP3A.

These results indicate that the hepatic concentration of rifampicin can be influenced by mouse *mdr1a* Pgp. Indeed, 24 hr after a single oral administration of 1.5 or 3 mg of rifampicin per kg, the hepatic concentration of drug was 11.3- and 6.0-fold higher, respectively in the *mdr1a* (−/−) compared with *mdr1a* (+/+) mice. However, rifampicin does not further accumulate in *mdr1a* (−/−) mice at 5 mg of drug per kg. Similarly, while the *mdr1a* (+/+) mice accumulate rifampicin, this is not in direct proportion to the dose. In both *mdr1a* (+/+) and (−/−) animals, the diminished accumulation of rifampicin at higher doses is probably due to rifampicin inducing its own metabolism (32).

Rifampicin has been reported as an inducer of hepatic CYP3A in mice and rabbits treated intraperitoneally for 4 days at doses of 50 mg/kg (33). In the current study the animals were treated for just 24 hr with a single oral dose of 5–1.5 mg of rifampicin per kg. This, in part, explains why there is so little induction of CYP3A in the *mdr1a* (+/+) mice. Moreover, the elevated levels of rifampicin in the plasma of *mdr1a* (−/−) versus (+/+) mice following oral treatment are consistent with intestinal Pgp contributing to rifampicin oral bioavailability and further influencing hepatic rifampicin disposition. Thus, the *mdr1a* (+/+) mice never achieved rifampicin concentrations necessary for induction of hepatic CYP3A. At the lowest administered dose of rifampicin, 1.5 mg/kg, the *mdr1a* (−/−) mice demonstrated approximately 3.4 ± 0.7 μg of rifampicin/500 mg of liver 24 hr after administration, and only a modest induction of CYP3A. *Mdr1a* (+/+) mice only attained these hepatocellular concentrations of rifampicin at 3.3 times this dose.

These findings are pharmacologically relevant to humans because (*i*) the doses of rifampicin chosen for the *in vivo* studies in the *mdr1a* mice are in the range of the standard oral dosing regimen in humans (300–600 mg per 70-kg man); and (*ii*) we have documented large interindividual variation in the content of Pgp between human livers (34). Thus, it is likely that transport of rifampicin by Pgp is an important element in drug activity, and that variation in human expression of Pgp contributes to individual variability in rifampicin disposition. Moreover, our results demonstrating that rifampicin is a substrate for Pgp suggests that this transporter may be a locus for drug–drug interactions between rifampicin and many coadministered drugs, such as cyclosporin A, which are also Pgp substrates (35, 36). It must also be considered that because Pgp can influence the intracellular concentration of CYP3A substrates, such as cyclosporin A and digoxin (19), Pgp may also affect substrate availability to CYP3A and thus, the extent of CYP3A metabolites formed within the cell.

While we have only explored the influence of Pgp on CYP3A, we would predict that Pgp has a more generalized role in regulating expression of drug-metabolizing enzymes. Indeed, rifampicin is not a selective inducer of CYP3A in human liver but induces other phase I and II enzymes in human liver *in vivo*, including the polymorphic 4′-hydroxylation of *S*-mephenytoin (37), CYP2E1 (38), and the human UDP-glucuronosyltransferase that glucuronidates paracetamol (39).

The factors responsible for human variation in CYP3A expression are under intense study because this variation is believed to influence drug response for up to one-third of all drugs and may also contribute to interindividual differences in

Table 1. Tissue levels of rifampicin and midazolam hydroxylation in liver microsomes from female *mdr1a* (+/+) and (−/−) mice 24 hr after oral gavage of various doses

Dose, mg/kg	Genotype	Rifampicin,* μg per 500 mg of liver	Ratio,† (−/−)/(+/+)	Rifampicin, μg/ml of plasma‡	Midazolam 4-hydroxylation‡
1.5	+/+	0.3 ± 0.3	11.3§	0.03 ± 0.02	31.1 ± 5.4
	−/−	3.4 ± 0.7		0.21 ± 0.14	45.5 ± 8.0
3	+/+	1.9 ± 0.5	6.0¶	0.24 ± 0.20	39.8 ± 10.3
	−/−	11.3 ± 4.8		0.79 ± 0.54	53.2 ± 5.5
5	+/+	3.8 ± 0.7	2.5‖	0.57 ± 0.90	35.9 ± 0.8
	−/−	9.3 ± 1.4		2.15 ± 1.16	41.8 ± 6.3

*Values are the mean of 3–4 animals/group ± SD.

†*t* test for independent samples was used to test the hypothesis of equality of means at each dose level between the two genotypes: §$P = 0.0001$; ¶$P = 0.012$; ‖$P = 0.0001$.

‡The Wilcoxon matched pairs test was used to compare the median values of rifampicin concentration ($P = 0.144$) and midazolam 4-hydroxylation ($P = 0.043$) between *mdr1a* (+/+) and (−/−) mice at each dose. The median formation of 4-hydroxymidazolam in untreated *mdr1a* (−/−) and (+/+) mice was 33.45 and 31.35 nmol/mg per hr, respectively.

health effects resulting from exposure to CYP3A-metabolized carcinogens in the environment (40). In part, interindividual variation in CYP3A is due to large individual differences in xenobiotic [e.g., rifampicin (9, 10)] induction of CYP3A4. The extent to which drugs, like rifampicin, can up-regulate CYP3A is of therapeutic importance because it is coadministered with so many drugs that are CYP3A substrates and thus contributes to increased or decreased effectiveness of these drug therapies as well as adverse side effects (35, 41). Our findings show that the extent to which CYP3A can be induced by the prototypical inducer, rifampicin, depends on expression of the Pgp drug transporter and suggests that the magnitude to which CYP3A can be induced by other Pgp substrates, including glucocorticoids and imidazoles, may also be influenced by Pgp.

We conclude that the *mdr1a* (−/−) mice should prove to be a valuable model in which to assess the contribution of Pgp to (*i*) the pharmacokinetic variation in the disposition of important drugs and environmental chemicals, and (*ii*) xenobiotic inducible expression of CYP3A and other drug-metabolizing enzymes and thus, the extent to which Pgp contributes to individual variability in response to these chemicals.

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