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ACCELERATED COMMUNICATION

Mdr1 Limits CYP3A Metabolism in Vivo

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ABSTRACT

We determined whether the drug efflux protein P-glycoprotein (Pgp) could influence the extent of CYP3A-mediated metabolism of erythromycin, a widely used model substrate for CYP3A. We compared CYP3A metabolism of erythromycin (a Pgp substrate) using the erythromycin breath test in mice proficient and deficient of *mdr1* drug transporters. We first injected *mdr1*(+/+) mice with [^{14}C]*N*-methyl erythromycin and measured the rate of appearance of $^{14}CO_2$ in the breath as a measure of hepatic CYP3A activity. Animals treated with CYP3A inducers or inhibitor showed accelerated or diminished $^{14}CO_2$ in the breath, respectively. The erythromycin breath test was next administered to *mdr1a*(−/−) and *mdr1a*/*1b*(+/+) and (−/−) mice. These animals had equivalent levels of immunoreactive CYP3A and CYP3A activity as measured by erythromycin *N*-demethylase activity in liver microsomes. Nevertheless, the rate of $^{14}CO_2$ appearance in the breath showed no relationship with these measurements of CYP3A, but changed proportionally to expression of mdr1. The average breath test $^{14}CO_2$ area under the curves were 1.9- and 1.5-fold greater in *mdr1a*/*1b*(−/−) and *mdr1a*(−/−) mice, respectively, compared with (+/+) mice, and CER_{max} was 2-fold greater in *mdr1a*/*1b*(−/−) compared with (+/+) mice. We conclude that Pgp, by limiting intracellular substrate availability can be an important determinant of CYP3A metabolism of numerous medications that are substrates for CYP3A and Pgp.

Factors that contribute to interindividual variation in drug disposition influence drug toxicity, drug efficacy, and hence, therapeutic outcome. There is at least a 10-fold human variation in the systemic clearance of erythromycin (Watkins et al., 1985, 1990) and cyclosporin A (CsA). CYP3A is the exclusive P-450 involved in the *N*-demethylation of erythromycin, and the major CYP catalyzing the formation of CsA metabolites. Moreover, CYP3A has been estimated to metabolize 60% of all drugs (Wrighton et al., 1996). Because CYP3A expression varies 10- to 40-fold between humans (Watkins et al., 1985; Shimada and Guengerich, 1989), it has been proposed that differences in the clearance of drugs such as erythromycin and CsA can be explained by variation in hepatic CYP3A.

The "erythromycin breath test" (ERMBT) (Watkins et al., 1989; Watkins, 1991) was developed to phenotype the differences in hepatic CYP3A4 activity between humans. The ERMBT relies on the assumption that following i.v. administration of [^{14}C]*N*-methyl erythromycin, CYP3A4 in the liver limits the rate of erythromycin *N*-demethylation. CYP3A4 demethylates [^{14}C]*N*-methyl erythromycin, and at least half the radiolabeled carbon appears almost instantly in the breath as $^{14}CO_2$ (Watkins, 1991, 1994). Thus, the rate of production of breath $^{14}CO_2$ following i.v. [^{14}C]*N*-methyl erythromycin has been proposed as a standard assay for measuring hepatic CYP3A4 activity (Watkins et al., 1989; Watkins, 1994). The ERMBT also relies on the assumption that other process, such as cellular efflux of substrate, are not limiting.

However, it is now appreciated that P-glycoprotein (Pgp)-mediated transport of drugs out of cells is important in influencing intracellular drug concentration, and hence drug action. Pgp, the product of the mutidrug resistance gene *MDR1* is abundantly expressed in liver and intestine (Van der Bliek et al., 1987) and effluxes many clinically important

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ABBREVIATIONS: CsA, cyclosporin A; Pgp, P-glycoprotein; ERMBT, erythromycin breath test; CER_{max}, the maximum $^{14}CO_2$ exhalation rate; CYP, cytochrome P-450; PCN, 3β-hydroxy-20-oxopregn-5-ene-16 α-carbonitrile; TAO, troleandomycin; DEX, dexamethasone; AUC, area under curve.

drugs. Indeed, we have shown that Pgp transports erythromycin and determines tissue levels of erythromycin in vivo (Schuetz et al., 1998).

Because Pgp and CYP3A are colocalized in intestine and liver and because Pgp can influence the disposition of drugs that are inducers and substrates of CYP3A, we speculated that there was a functional relationship between the Pgp transporter and CYP3A. Indeed, we demonstrated in animals and cells that Pgp directly influences the intrahepatic concentration of a CYP3A inducer, rifampin, and hence a pharmacological action of this drug in the cell, namely the extent of CYP3A induction (Schuetz et al., 1996). We have also shown that, under some circumstances, Pgp influences basal expression of CYP3A (Schuetz et al., 2000).

Based on our previous studies showing erythromycin as a Pgp substrate (Schuetz et al., 1998), we hypothesized that Pgp could limit the extent of CYP3A metabolism of erythromycin. To test this idea, we compared CYP3A metabolism of erthryomycin in vivo (using the ERMBT), in the presence or absence of Pgp using *mdr1a1b*(+/+) and *mdr1a* and *mdr1a/1b*(−/−) mice. Because the hepatic content of CYP3A was similar in *mdr1a*, *mdr1a/1b*(−/−) and (+/+) mice, variation in the ERMBT between these mice cannot be attributed to CYP3A, but is likely due to differences in Pgp. According to our hypothesis, the extent to which CYP3A can generate metabolites would be related to the expression of Pgp. The results from this investigation demonstrate that Pgp influences the rate and extent of CYP3A-mediated metabolism of erythromycin in liver in vivo.

Materials and Methods

Chemicals. Dexamethasone (DEX), erythromycin, troleandomycin (TAO), DL-isocitric acid, isocitric dehydrogenase, and NADP were purchased from Sigma Chemical Company (St. Louis, MO). 3β-Hydroxy-20-oxopregn-5-ene-16 α-carbonitrile (PCN) was obtained from the Upjohn Company (Kalamazoo, MI). [^{14}C]*N*-methyl erythromycin (54.0 mCi/mmol) was a gift from Dr. Paul Watkins (University of North Carolina).

Mice and Treatments. Male *mdr1a/b*(+/+), *mdr1a*(−/−), and *mdr1a/b*(−/−) mice were purchased from Taconic Farms (Germantown, NY) and housed in the St. Jude Children's Research Hospital animal facility for a minimum of 3 weeks quarantine before use. DEX (300 mg/kg/day for 3 days), PCN (300 mg/kg/day for 3 days), or 0.9% NaCl were administered i.p. An additional group of mice was treated with dexamethasone (DEX; 300 mg/kg) for three days and then TAO (500 mg/kg) i.p. 2 h before the erythromycin breath test.

Erythromycin Breath Test. The ERMBT was performed as described (Watkins et al., 1989; Watkins, 1991). Briefly, mice were anesthetized with metofane and injected i.v. with [^{14}C]*N*-methyl erythromycin (1.0 μCi/100 g b.wt.) in 2.5% dextrose. The mice were then placed in a water-sealed polyurethane breath chamber with the air continuously drawn through a vapor trap (acetone and dry ice), then bubbled through a solution of acidic methanol (360 ml of methanol + 40 ml of 3 N HCl), and then through three gas washing bottles. The solutions to trap CO_2 were prepared by mixing 540 ml of methanol, 820 ml of toluene, and 100 ml of Emulsifier-safe (Packard Bioscience Company, Meriden, CT) for 20 min under nitrogen. Phenethylamine (540 ml) (Aldrich Chemical Company, Inc., Milwaukee, WI) was then added with stirring (under nitrogen) for an additional 15 min. The three washing bottles in series each contained 30 ml of the mixture with 2 ml of toluene (first collection vessel containing 40 ml of mixture with 4 ml of toluene). Collection of breath was obtained at 5- or 20-min intervals over 30 min or over 4 h, respectively. After collection of breath, duplicate 4-ml samples from each point were analyzed for ^{14}C content by scintillation counting. The values were used to calculate the total $^{14}CO_2$ exhaled during the collection interval (Watkins et al., 1989; Watkins, 1991). After the final breath sample, animals were anesthetized with metofane and blood obtained by cardiac puncture into heparinized tubes. Livers were removed, washed with cold phosphate-buffered saline, and frozen in liquid nitrogen. The tissue was stored at −70°C until used for preparation of microsomes and analysis of radioactivity. Tissue radioactivity was measured as previously described (Schuetz et al., 1998).

Immunoblot Analysis. Mouse liver microsomes were prepared (Schuetz et al., 2000) and 5 or 20 μg of protein was separated on 10% slab polyacrylamide gels and immunoblotted with polyclonal anti-CYP3A1 IgG followed by anti-goat IgG coupled with peroxidase and developed with diaminobenzidine tetrahydrochloride and hydrogen peroxide. CYP levels were quantified by comparing the densitometric values of a standard curve of purified rat CYP3A to the values obtained for microsomal samples of individual *mdr1a/b*(+/+), *mdr1a*(−/−), and *mdr1a/b*(−/−) mice livers analyzed on the same blots using the public domain NIH Image 1.62 program software (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/).

Erythromycin *N*-Demethylation Assay. [^{14}C]*N*-Methyl erythromycin *N*-demethylation was investigated as described (Riley and Howbrook, 1998). Incubations consisted of 200 μl of microsomal protein (83.6 μg), erythromycin (~0.1 μCi [^{14}C]*N*-methyl erythromycin and 12.5 to 150 μg of nonradiolabeled erythromycin) and a NADPH regenerating system (7.5 mM isocitric acid, 5 mM magnesium sulfate, 1 mM $NADP^+$, and 0.1 U of isocitrate dehydrogenase) were incubated at 37°C in a shaking water bath for 15 or 20 min, and the reaction was stopped by placing tubes on ice and adding 50 μl of 10% (v/v) trichloroacetic acid and centrifuged at 15,000*g* for 10 min. Control incubations contained identical reagents but incubated on ice for 15 or 20 min. The supernatant was then applied to preconditioned (2 ml of methanol, 2 ml of water) Envi-Card solid-phase extraction columns (250-mg bed volume, Supelco, Sigma-Aldrich Co.). [^{14}C]Erythromycin was retained on the column. [^{14}C]HCHO was eluted with 2 volumes of water (2 × 500 μl), and eluted radioactivity quantified by liquid scintillation counting.

Pharmacokinetics. Data were analyzed using noncompartmental pharmacokinetic analysis. CER_{max}, the maximum $^{14}CO_2$ exhalation rate, was determined from direct inspection of the exhalation rate versus time data. K_{breath}, the terminal rate constant for the decline in the CO_2 production rate, was calculated from the terminal slope of a plot of percentage of injected dose/min versus time from 100 to 240 min. The fraction of each dose converted to $^{14}CO_2$ (in units of percentage of injected dose) was calculated as the area under the $^{14}CO_2$ exhalation rate versus time curve using the linear trapezoid rule.

Statistics. Four-hour data were analyzed using two-way ANOVA and 30-min data by using one-way ANOVA.

Results

Contribution of Mouse Liver CYP3A to Erythromycin *N*-Demethylase Activity. We first determined whether CYP3A exclusively catalyzes erythromycin *N*-demethylation in mouse liver microsomes using an in vitro radiometric assay (Riley and Howbrook, 1998). This was necessary because there can be significant differences between CYP3As, e.g., even between CYP3A4 and CYP3A5, in the rate of product formation (Gillam et al., 1995). The evidence was strong that mouse liver CYP3A is the major enzyme catalyzing erythromycin *N*-demethylation. First, liver microsomes from mice treated with the CYP3A inducer DEX showed a significant increase in erythromycin *N*-demethylase activity over that in untreated control mice (see Fig. 4). Second, CYP3A

activity in mouse liver microsomes (Fig. 1) was inhibited by preincubation with the CYP3A-specific inhibitor TAO (67%), ketoconazole (94%), or antibody to CYP3A (91%). By contrast, this activity was not inhibited by nonimmune IgG and was somewhat stimulated (Fig. 1), a result previously seen for other CYP450 reactions (Guengerich and Mason, 1979). Because the activity was almost totally inhibited by anti-CYP3A IgG, these results implicate mouse liver CYP3A as the predominant mouse liver enzyme catalyzing the *N*-demethylation of erythromycin.

The ERMBT Is Influenced by Inducers and Inhibitors of CYP3A in Mice. We used a procedure previously validated for administering the ERMBT to rats (Watkins et al., 1989; Watkins, 1991). Mice were pretreated with either NaCl or inducers of CYP3A, i.e., DEX or PCN. One additional group was pretreated with DEX and administered the CYP3A inhibitor TAO 2 h before the ERMBT. *Mdr1*(+/+) mice were then administered i.v. a trace dose of [^{14}C]*N*-methyl erythromycin, immediately placed in the breath test chamber, and the rate of production of $^{14}CO_2$ over time in the breath was measured by trapping the $^{14}CO_2$. The mouse ERMBT bore all signatures of CYP3A being rate-limiting in the reaction. CYP3A inducers DEX and PCN increased the maximum rate of exhalation of $^{14}CO_2$ in the breath (CER_{max}) by 1.5- and 1.7-fold, respectively, compared to NaCl-treated mice (Fig. 2A). AUC_{0-240} was also increased 1.5- to 1.8-fold by CYP3A inducer treatment (Table 1). Treatment of DEX-pretreated mice with the CYP3A inhibitor TAO for 2 h before the ERMBT decreased the rate of $^{14}CO_2$ exhalation in the breath to one-third of that observed in DEX-treated animals and one-half that observed in NaCl-treated mice (Fig. 2A, Table 1). The accelerated rate of $^{14}CO_2$ production in the breath of DEX- and PCN-treated compared with control mice disappeared after 1 to 2 h, suggesting there was accelerated depletion of the trace dose of substrate in these treated mice during the ERMBT. In rats, the ERMBT pharmacokinetic parameters were better approximated in inducer-treated animals when pharmacological doses of erythromycin were administered with [^{14}C]*N*-methyl erythromycin (Watkins et al., 1989). Unfortunately, i.v. pharmacological doses of erythromycin proved fatal to untreated *mdr1/1b*(+/+) mice. Nevertheless, the induction and inhibition of the mouse ERMBT by CYP3A inducers and inhibitors bore the characteristics of a CYP3A catalyzed reaction.

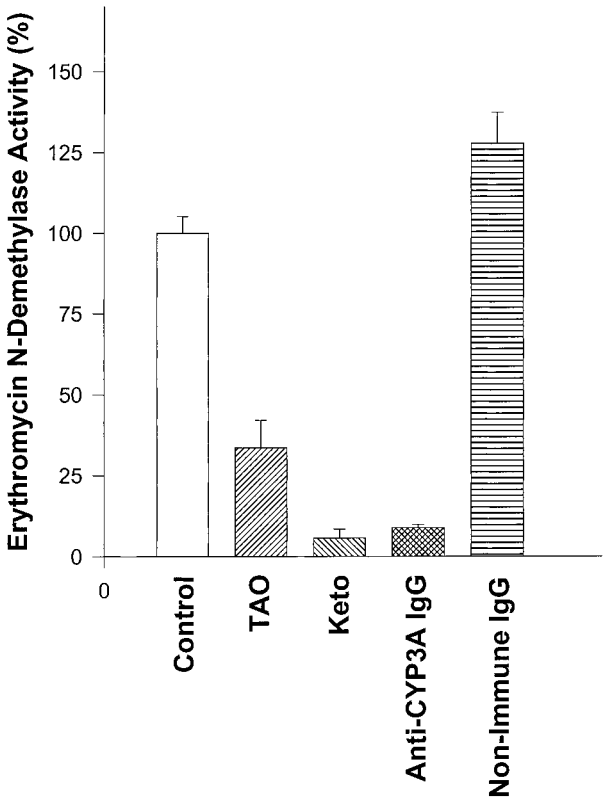

Fig. 1. Effect of CYP3A inducers or inhibitors on erythromycin *N*-demethylase activity in mouse liver microsomes. Erythromycin *N*-demethylase activity was measured in liver microsomes from *mdr1a/1b*(+/+) control mice treated with NaCl (200 μl of 0.9%/mouse/day) for 3 days. Activities are expressed relative to the activity in control liver microsomes assigned a value of 100%. Some microsomes were preincubated with TAO (200 or 100 μM), ketoconazole (100 μM), polyclonal goat anti-CYP3A1 IgG (5 mg of IgG/mg of protein) or nonimmune (N-IgG) (5 mg of IgG/mg of protein). Data are depicted as means ± S.D. (n = at least two experiments performed in quadruplicate).

Mdr1 Genotype Is Directly Correlated with the ERMBT. To directly examine the role of Pgp in the ERMBT, we administered i.v. [^{14}C]*N*-methyl erythromycin to *mdr1a*(−/−), *mdr1a/1b*(−/−) and (+/+) mice. The percentage of the i.v. [^{14}C]*N*-methyl erythromycin dose converted to $^{14}CO_2$ (as measured by AUC_{0-240}) was markedly increased in animals lacking the mdr1 transporters (Fig. 2B) with a rank order of *mdr1a/1b*(−/−) > *mdr1a*(−/−) > (+/+) mice (Table 1, Fig. 2B) indicating that both *mdr1a* and *mdr1b* in liver affected the breath test results. These differences in ERMBT AUC in *mdr1a* and *mdr1a/1b*(−/−) compared to (+/+) mice were statistically significant ($P < .0001$) (Table 1). The CER_{max} was also 2-fold greater in the *mdr1a/1b*(−/−) mice compared to the (+/+) mice. However, K_{breath} (the terminal rate constant for the decline in the CO_2 production rate) was not different among the *mdr1* genotypes.

In humans, the ERMBT is administered over 20 or 60 min after i.v. [^{14}C]*N*-methyl erythromycin (Watkins et al., 1989; Kinirons et al., 1999). To determine whether the rate of erythromycin demethylation is greater in the animals lacking Pgp at an earlier time point, we administered the ERMBT to mice over 0 to 30 min after i.v. [^{14}C]*N*-methyl erythromycin. As observed in studies conducted over 4 h, the AUC_{0-30} were significantly different between animal groups. The AUC_{0-30} min was 1.6-fold higher in *mdr1a*(−/−) mice, compared to (+/+) mice. Similarly, the CER_{max} values were 1.67-fold higher in the *mdr1a*(−/−) mice compared to (+/+) mice.

We next compared the impact of *mdr1* gene disruption on [^{14}C]-erythromycin (reflecting both erythromycin and the CYP3A cleaved radiolabeled ^{14}C atom) tissue concentrations at 30 min and 4 h and compared these values to the percentage of the dose converted to $^{14}CO_2$ (AUC) (Table 2). The 1.5-fold increase in hepatic and plasma radioactivity in *mdr1a*(−/−) mice was proportional to the 1.5-fold increase in the $^{14}CO_2$ AUC in these same mice. Thus, Pgp membrane transport processes were influencing the hepatic concentration of the CYP3A substrate erythromycin and its subsequent rate of CYP3A-mediated demethylation.

We recently demonstrated, in *mdr1*(−/−) mice housed in the United States, that hepatic CYP3A expression is similar or even lower to that than in *mdr1a*(+/+) mice (Schuetz et al., 2000). However, we also demonstrated that environmen-

tal and/or physiological challenges to the *mdr1*(−/−) mice can alter CYP expression. Therefore, we quantitated the hepatic content of CYP3A on immunoblots of liver microsomes from mice administered the ERMBT. The specific content of immunoreactive CYP3A in the *mdr1a/1b, mdr1a*(−/−) mice was similar (or even lower) than that in *mdr1a/1b*(+/+) mice (Fig. 3, Table 1).

Next we compared in the identical mice the in vivo ERMBT results to the in vitro immunocontent of hepatic CYP3A and associated erythromycin *N*-demethylase activity in their liver microsomes. In control, PCN-, and DEX-treated *mdr1*(+/+) mice there was excellent correlation between the AUC_{0-240} and the hepatic content of CYP3A ($r^2 = 0.932$) (Fig. 4A) and the liver microsomal erythromycin demethylase activity ($r^2 = 0.841$) (Fig. 4C). Thus, under these conditions CYP3A is limiting the ERMBT. However, using *mdr1a*, *mdr1a/1b*(−/−) and (+/+) mice we found no correlation between the in vivo ERMBT results and in vitro hepatic CYP3A immunocontent ($r^2 = 0.057$) (Fig. 4B) or the microsomal erythromycin demethylase activity in the corresponding liver microsomes ($r^2 = 0.092$) (Fig. 4D).

Discussion

Drug transport across biological membranes is a critical determinant of drug action. With the growing recognition that many CYP3A substrates are also Pgp substrates, the role of Pgp in CYP3A metabolism must be analyzed. Thus, the focus of this research was to test the hypothesis that Pgp transport influenced CYP3A metabolism. We used the

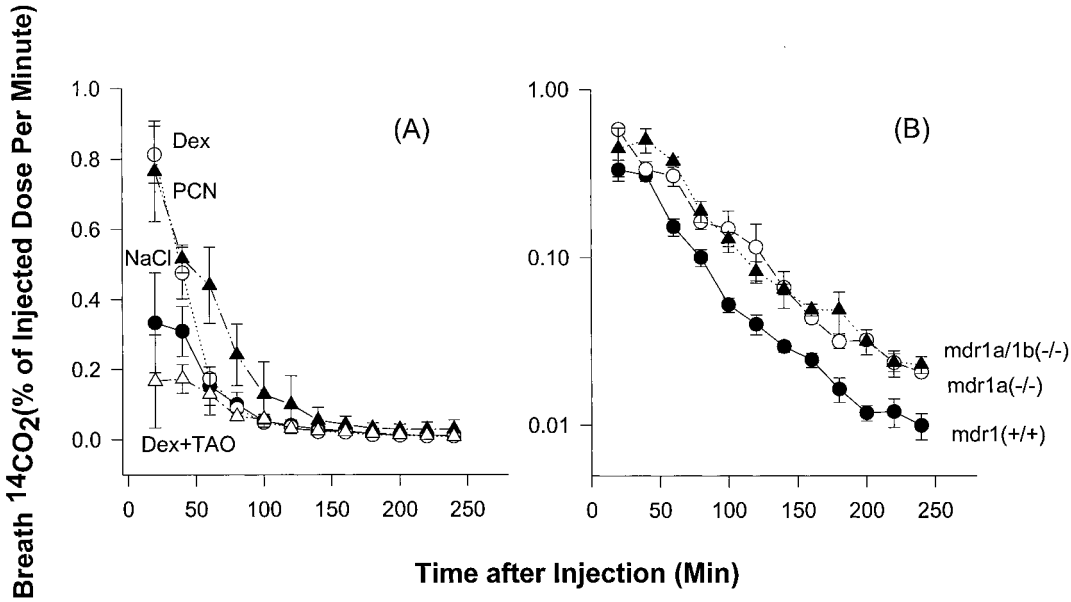

Fig. 2. Elimination of $^{14}CO_2$ in the breath of mice following i.v. [^{14}C]*N*-methyl erythromycin. A, *mdr1*(+/+) mice were injected i.p. with NaCl (●, 200 μl of 0.9%/mouse/day) or with CYP3A inducers DEX (▲, 300 mg/kg/day), or PCN (○, 300 mg/kg/day) for 3 days. In one group, mice treated i.p. with DEX (300 mg/kg/day) for 3 days were injected i.p. with TAO (△, 500 mg/kg) 2 h before the ERMBT. B, elimination of $^{14}CO_2$ in the breath of *mdr1a/b*(+/+), *mdr1a*(−/−), and *mdr1a/b*(−/−) mice administered the ERMBT. For the ERMBT each mouse was given trace [^{14}C]*N*-methyl erythromycin (0.1 μCi/100 g b.wt.). $^{14}CO_2$ in breath was determined at 20-min intervals. Data are depicted as means ± S.D. ($n \geq 3$).

TABLE 1

Pharmacokinetics of breath $^{14}CO_2$ after injection of [^{14}C]*N*-methyl erythromycin

Mice were injected with trace amounts of [^{14}C]*N*-methyl erythromycin, and the rate of elimination of $^{14}CO_2$ in the breath was calculated at 20-min intervals over 4 h or, for the 30-min ERMBT, at 5-min intervals over 30 min, and pharmacokinetic parameters determined in the indicated number of mice. Note: Statistical difference of breath $^{14}CO_2$ AUC_{0-240} in various mice after i.v. [^{14}C]*N*-methyl erythromycin assessed by two-way and one-way ANOVA were as follows: $^{14}CO_2$ AUC_{0-240} in *mdr1a/1b*(+/+) control mice was significantly different from results in mice treated with DEX, PCN, or DEX + TAO was $P = .0001$. The $^{14}CO_2$ AUC_{0-240} in *mdr1a/1b*(+/+) was significantly different from that in either *mdr1a*(−/−) or *mdr1a/1b*(−/−) mice ($P = .0001$). The $^{14}CO_2$ AUC_{0-30} in *mdr1a/1b*(+/+) was significantly different from that in *mdr1a*(−/−) mice ($P = .0329$).

	Genotype	CER_{max}	K_{breath}	AUC_{0-240}	CYP3A
		%ID/min	*1/min*	*%ID/min*	*pmol/mg*
Treatment					
Control ($n = 9$)	(+/+)	0.47 ± 0.05	0.0128 ± 0.001	22.7 ± 0.7	31.9 ± 8.6
Dexamethasone ($n = 3$)	(+/+)	0.69 ± 0.08	0.0118 ± 0.002	40.0 ± 1.1	268.6 ± 30.5
PCN ($n = 3$)	(+/+)	0.81 ± 0.09	0.0126 ± 0.002	34.3 ± 1.2	200.2 ± 37.9
DEX + TAO ($n = 3$)	(+/+)	0.23 ± 0.09	0.0124 ± 0.002	14.4 ± 1.2	312.3 ± 14.7
4-h ERMBT					
Mdr1 ($n = 4$)	(+/+)	0.47 ± 0.05	0.0128 ± 0.001	22.2 ± 0.9	29.8 ± 3.1
Mdr1a ($n = 3$)	(−/−)	0.51 ± 0.09	0.0137 ± 0.002	34.2 ± 2.6	24.3 ± 5.7
Mdr1a/1b ($n = 3$)	(−/−)	1.00 ± 0.09	0.0123 ± 0.002	42.3 ± 1.2	28.9 ± 6.6
30-min ERMBT					
Mdr1 ($n = 4$)	(+/+)	0.58 ± 0.15		9.67 ± 1.6[a]	27.1 ± 1.7
Mdr1a ($n = 3$)	(−/−)	0.95 ± 0.13		15.4 ± 1.4[a]	22.0 ± 9.5

ID, injected dose.
[a] AUC_{0-30}.

ERMBT to test this idea assuming the two major determinants of the ERMBT are the activity of CYP3A and cellular bioavailability of erythromycin. The latter idea is based on our previous findings that Pgp affects the tissue disposition of erythromycin (Schuetz et al., 1998). We predicted that Pgp, by limiting the hepatic erythromycin concentration, would influence the amount of CYP3A-generated metabolites. This hypothesis is based on the mathematical relationship describing the rate of conversion of erythromycin to the *N*-demethylated metabolite as $v = V_{max}[S]/K_m + [S]$. We know from our studies that *mdr1a*(−/−) and *mdr1a/1b*(+/+) and (−/−) mice have the same amount of hepatic CYP3A protein and activity (V_{max}/K_m, intrinsic clearance, is likely the same). Thus, any difference in the rate of erythromycin metabolism between these mice will be dependent on the intracellular concentration of the substrate and, thus, Pgp.

Although there have been several reports in the literature that suggest Pgp might influence CYP3A-dependent metabolism in vivo, these in vitro studies have largely used Caco-2 cells, which are problematic because of low levels of CYP3A4 and expression of multiple drug efflux transporters (Gan et al., 1996; Raeissi et al., 1999;Hochman et al., 2000). To determine whether the ERMBT was influenced by Pgp, we used mice genetically modified to express either one or both mdr1 transporters. Our results indicate that Pgp and CYP3A are functionally associated because the extent to which CYP3A metabolized erythromycin, as measured by the breath $^{14}CO_2$ AUC, was related to the gene dose of the *mdr1* alleles in these mice. These results support the idea that CYP3A is not the single rate-limiting determinant of the ERMBT, but that hepatic Pgp is also rate limiting in the conversion of [^{14}C]*N*-methyl erythromycin to $^{14}CO_2$ in the breath and that the ERMBT is measuring both hepatic Pgp and CYP3A. This finding emphasizes the critical role that Pgp plays in hepatic drug elimination even for drugs that are extensively metabolized. These findings extend our understanding of the interactions between CYP3A4 and Pgp expression in drug metabolism.

TABLE 2

Ratio of radioactivity (ng/g of tissue) and AUC [^{14}C]CO_2 in *mdr1a* (−/−), *mdr1a/1b*(−/−) mice compared to *mdr1a*(+/+) mice following [^{14}C]*N*-methyl erythromycin injection

The values from the (+/+) mice were assigned the number 1.0.

	Mdr1a(+/+) (n = 4)	Ratio *mdr1a*(−/−):(+/+) (n = 3)	Ratio *mdr1a/1b*(−/−):(+/+) (n = 3)
4-h ERMBT			
Plasma radioactivity	1 ± 0.66	1.50 ± 0.67	3.09 ± 0.65
Liver radioactivity	1 ± 0.17	1.41 ± 0.15	1.80 ± 0.14
AUC_{0-240}	1 ± 0.04	1.54 ± 0.07	1.91 ± 0.03
30-min ERMBT	(n = 4)	(n = 3)	
Liver radioactivity	1 ± 0.19	1.44 ± 0.17	
AUC_{0-30}	1 ± 0.09	1.59 ± 0.16	

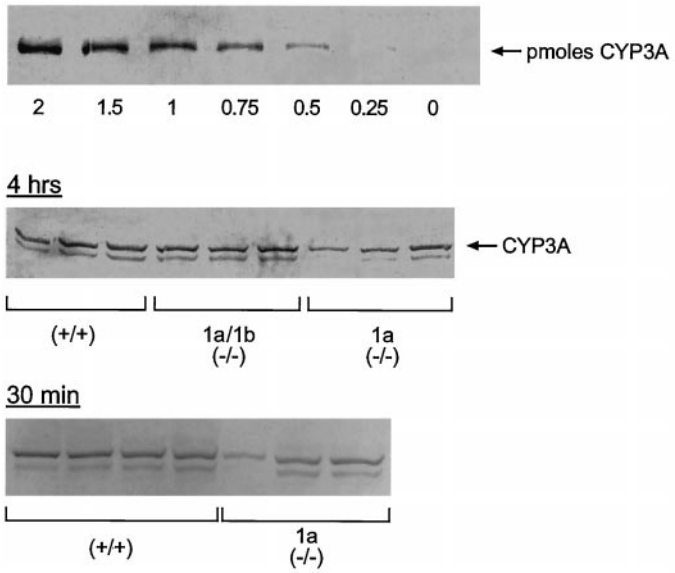

Fig. 3. Immunoquantitation of CYP3A in the livers of mice given the ERMBT. Liver microsomes were prepared from animals who received the ERMBT over 30 min or 4 h and 20 μg resolved for 2 h on 10% polyacrylamide gel electrophoresis. The gel was stopped, and purified CYP3A1 (0.25 to 2 pmol) was loaded in duplicate and the gel run for an additional 2 h, blotted to nitrocellulose and developed with anti-CYP3A IgG. The concentration of CYP3A proteins was calculated in the mouse liver microsomes from interpolation with the integrated density of the duplicate CYP3A standard curves.

The 1.5- to 1.8-fold induction of CER_{max} following DEX or PCN was less than the 6- to 8-fold induction of hepatic CYP3A protein (Table 1) that was observed following these inducers. It is possible that DEX induction of liver Pgp decreases the amount of methyl erythromycin available to CYP3A, resulting in a smaller increase in the rate of $^{14}CO_2$ exhalation in the breath of DEX-pretreated mice. Indeed the hepatic concentration of [^{14}C]*N*-methyl erythromycin was greater in control versus DEX-treated mice (70.3 ± 11.6 versus 40.8 ± 9.9 pmol [^{14}C]*N*-methyl erythromycin/g of liver, respectively), and DEX can increase Pgp levels in rodent liver (Salphati and Benet, 1998).

There are two possible explanations for the observed changes in AUC: 1) altered clearance of the metabolite (^{14}HCHO) or 2) an increased proportion of erythromycin that is converted to $^{14}CO_2$. The terminal rate constant for the decline in the CO_2 production rate (K_{breath}) did not change. In fact, K_{breath} did not differ between any of treatment groups, including animals treated with DEX, PCN, or TAO. One might expect K_{breath} to change with changes in hepatic CYP3A activity. However, this would only be true if the rate constant for erythromycin metabolism by CYP3A is less than the rate constant describing CO_2 production from formaldehyde and if demethylation were a major pathway for erythromycin elimination in mice. The fact that K_{breath} did not differ between any of our treatment groups might indicate that the rate of erythromycin metabolism by CYP3A is significantly faster than CO_2 production from formaldehyde, and that K_{breath} is a poor measure of CYP3A activity in mice. Alternatively, if CO_2 production from formaldehyde is not rate limiting, K_{breath} reflects elimination of erythromycin from the blood and that is relatively unchanged by any of the treatments in mice. Thus, it appears reasonable to conclude that the increased $^{14}CO_2$ AUC in mice lacking Pgp is due to a greater fraction of labeled erythromycin being metabolized. Because the amount of CYP3A protein and associated catalytic activity is indistinguishable between the *mdr1* genotypes, the differences in $^{14}CO_2$ AUC_{0-240} and AUC_{0-30} in *mdr1a/1b* and *mdr1a*(−/−) compared to (+/+) mice indicates Pgp is working to control the amount of drug available for metabolism.

There is evidence that Pgp similarly influences the extent of CYP3A-mediated metabolism of erythromycin in humans. The ERMBT, has consistently found that women have higher

CYP3A activity than men (Austin et al., 1980). However, immunoquantitation of CYP3A and analysis of CYP3A-mediated activity in liver microsomes from males and females has failed to identify a gender difference in CYP3A expression or activity (Shimada et al., 1994). Although a number of explanations are possible (e.g., volume of distribution), our report (Schuetz et al., 1995) of a gender difference in the hepatic expression of human Pgp (men have a significantly higher content of Pgp then women) could explain the in vitro/in vivo discrepancy in the ERMBT. Since men have higher Pgp then women, men would effectively attain lower intrahepatic levels of erythromycin, generate less erythromycin metabolite, have a lower erythromycin breath test, and thus an apparent lower level of hepatic CYP3A compared with women. This idea is supported by evidence that clearance of i.v. midazolam, a CYP3A substrate, but non-Pgp substrate (Kim et al., 1999), did not correlate with the ERMBT in measuring hepatic CYP3A activity and shows no gender difference (Kinirons et al., 1999).

Our study differs significantly from others examining the influence of Pgp on drug pharmacokinetics. Two recent studies reported that *mdr1* genotype does not effect CYP3A-mediated metabolism of midazolam, testosterone 6β-hydroxylation, nifedipine oxidation, or biotransformation of ivermectin or CsA (Kwei et al., 1999; Perloff et al., 1999). However, these studies were either performed in liver microsomes that do not contain Pgp, or only determined parent drug. Two other studies have examined metabolites of drugs in *mdr1a*(+/+) and (−/−) mice (van Asperen et al., 1996; 1999). These reports found higher accumulation of doxorubicin and vinblastine metabolites in the livers of *mdr1a*(−/−) compared to (+/+) mice 4 to 24 h after i.v. dosing (van Asperen et al., 1996, 1999). However, although the increased amount of these metabolites would be consistent with an increase in the rate and extent of drug metabolism, it is impossible to distinguish in these kinds of studies to what extent either increased metabolism, decreased further metabolism, or decreased efflux of metabolite contributes to the total amount of hepatic metabolite. In contrast, the ERMBT suffers from none of the aforementioned problems. Indeed, with the ERMBT CYP3A-mediated *N*-demethylation of [^{14}C]*N*-methyl erythromycin results in approximately half the radiolabeled carbon atoms appearing almost immediately in the breath as $^{14}CO_2$ (Watkins, 1991). Thus, the ERMBT offers the unique advantage of being a "real time" metabolism assay, allowing instantaneous measurement of hepatic CYP3A activity in the liver, and providing a better test for a functional relationship between Pgp and CYP3A.

Our identification of multiple levels of interaction between CYP3A and Pgp (Schuetz et al., 1996, 2000) demonstrates that the overall pharmacology of shared Pgp/CYP3A substrates needs to be reevaluated from the context of a role for Pgp. An additional implication of this study is that because Pgp transport exerts greater control over CYP3A metabolism than previously realized, induction or inhibition Pgp will also have consequences to CYP3A-mediated metabolism. Thus, there needs to be stronger emphasis on understanding the influence of Pgp to drug metabolism. In addition, although CYP3A metabolism of erythromycin in the liver is substrate limited, because the kinetics of interaction of any substrate with Pgp and CYP3A4 will be different it will be necessary to compare on a substrate-by-substrate basis (and at higher doses) whether Pgp membrane transport processes limit CYP3A4-mediated metabolism. However, it is not feasible or practical to test the interactions of most Pgp and CYP3A substrates using the *mdr1*(−/−) mice, in part due to the

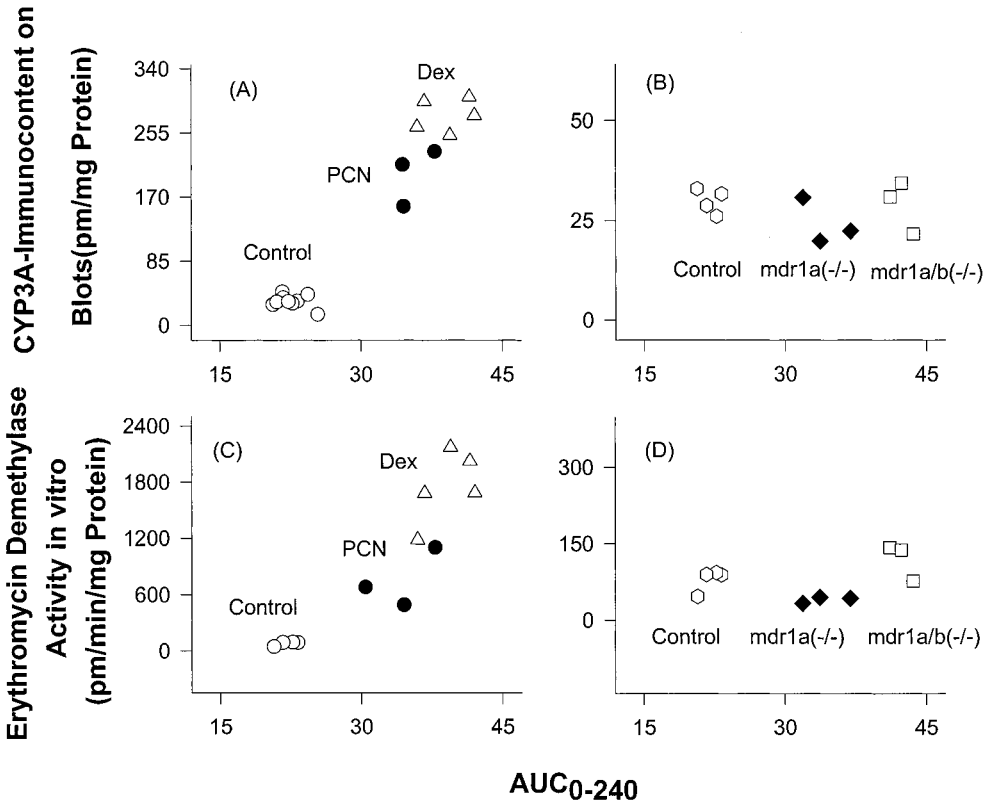

Fig. 4. Relationship between the in vivo erythromycin *N*-demethylation activity (ERMBT) and in vitro measures of CYP3A in liver microsomes from the identical mice. The AUC_{0-240} following i.v. [^{14}C]*N*-methyl erythromycin was compared with the liver microsomal content of CYP3A measured in the *mdr1*(+/+) animals treated with CYP3A inducers (A) and mice lacking the *mdr1a* and *mdr1a/1b* Pgp transporters (B). Each point represents a single mouse (○, control; ●, PCN; △, DEX). The values obtained from *mdr1a/b*(+/+) (○, control), *mdr1a*(−/−) (◆), and *mdr1a/b*(−/−) (□) mice. The AUC_{0-240} following i.v. [^{14}C]*N*-methyl erythromycin was compared with the erythromycin demethylase activity measured in liver microsomes of the *mdr1* (+/+) animals treated with CYP3A inducers (Table 1) (C) and mice lacking the mdr1a and *mdr1a/1b* transporters (Table 1) (D). Each point represents a single mouse (○, control; ●, PCN; △, DEX). The values obtained from *mdr1a/b*(+/+) (○, control), *mdr1a*(−/−) (◆), and *mdr1a/b*(−/−) (□) mice.

issues discussed above. However, we have recently generated a cellular system (Brimer et al., 2000) to model the interactions of human MDR1/Pgp and CYP3A4, which should allow a rapid assessment of whether Pgp influences the rate and extent of CYP3A4-mediated metabolism of other drugs.

A broader implication of this work is that Pgp could limit metabolism by other CYPs. For example, although CYP3A metabolizes the Pgp substrate taxol, the principal taxol biotransformation product in humans is generated by another CYP (Harris et al., 1994). Thus, Pgp membrane transport controlling the rate of CYP3A metabolism is likely to be a paradigm for additional Pgp-drug metabolism interactions.

The finding that the ERMBT measures both Pgp and CYP3A has several clinical implications. First, it is possible that, in the future, the ERMBT could be used simultaneously with some measure of plasma erythromycin to determine hepatic Pgp levels in humans. Second, for drugs, like erythromycin, that are substrates for both CYP3A4 and Pgp (e.g., CsA, indinavir) (Schinkel et al., 1995; Kim et al., 1998, 1999), the ERMBT may be the preferred assay to use for predicting their systemic clearances. Indeed, the ERMBT predicts the steady-state trough blood levels of CsA, a CYP3A4 and Pgp substrate (Watkins, 1994). Thus, our results also suggest that the previously described human variation in CsA clearance, explained solely by hepatic CYP3A4 content (as measured by the ERMBT), may also be due to human variation in hepatic Pgp. We conclude that Pgp, by limiting substrate concentration, is also an important determinant of the extent of CYP3A metabolites formed in the cell. Therefore, it is likely that individual variation in the hepatic content of Pgp and CYP3A contributes to the overall interindividual variation seen in the disposition and therapeutic efficacy of shared substrates.

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