Rifampin Induces the in Vitro Oxidative Metabolism, but Not the in Vivo Clearance of Diclofenac in Rhesus Monkeys

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ABSTRACT:

Effects of rifampin on in vitro oxidative metabolism and in vivo pharmacokinetics of diclofenac (DF), a prototypic CYP2C9 marker substrate, were investigated in rhesus monkeys. In monkey hepatocytes, rifampin markedly induced DF 4'-hydroxylase activity, with values for EC50 of 0.2 to 0.4 μM and Emax of 2- to 5-fold over control. However, pretreatment with rifampin did not alter the pharmacokinetics of DF obtained after either i.v. or intrahepatic portal vein (i.pv.) administration of DF to monkeys. At the dose studied, plasma concentrations of rifampin reached 10 μM, far exceeding the in vitro EC50 values. Under similar treatment conditions, rifampin was previously shown to induce midazolam (MDZ) 1'-hydroxylation in rhesus monkey hepatocytes (EC50 and Emax values ~0.2 μM and ~2- to 3-fold, respectively), and markedly affected the in vivo pharmacokinetics of MDZ (>10-fold decreases in the i.pv. MDZ systemic exposure and its hepatic availability, Fh) in this animal species. In monkey liver microsomes, DF underwent, predominantly, glucuronidation, and, modestly, oxidation; the intrinsic clearance (CLint = Vmax/Km) value for the glucuronidation pathway accounted for >95% (versus about 75% in human liver microsomes) of the total (glucuronidation + hydroxylation) intrinsic clearance value. In monkey hepatocytes, the hydroxylation also was a minor component (~10%) relative to the glucuronidation, supporting the liver microsomal finding. Collectively, our results suggest that the oxidative metabolism is not the major in vivo clearance mechanism of DF in either untreated or rifampin-treated monkeys and, conceivably, also in humans, raising a question about the utility of DF as an in vivo CYP2C9 probe.

Induction of drug-metabolizing enzymes, especially the cytochrome P450 (P450) superfamily, by some drug molecules is a well known phenomenon and generally is undesirable since it can cause profound clinical effects, either by reducing therapeutic efficacy of drugs or enhancing toxicity from toxic or reactive metabolites (Thummel and Wilkinson, 1998). Accordingly, the potential for new chemical entities to cause P450 induction is usually assessed during lead optimization and identification in early drug discovery processes (Weaver, 2001; Worboys and Carlile, 2001). Currently, measurement of enzyme activities in cultured hepatocytes is the accepted and recommended method for studying P450 induction (LeCluyse, 2001; Bjornsson et al., 2003). However, systematic and quantitative extrapolations of such in vitro enzyme induction data to in vivo situations have not been extensively studied, and studies to date, including our recent investigation on in vitro-in vivo drug interactions in rhesus monkeys (Prueksaritanont et al., 2006), have been limited to CYP3A, the most abundant of all the human isoforms.

In a quest to expand the database, we subsequently evaluated a relationship between in vitro-in vivo induction of CYP2C9 activity by rifampin, using diclofenac (DF) as a functional probe and the rhesus monkey as an animal model. Rifampin is a known human CYP2C9 inducer (Bjornsson et al., 2003; Parkinson et al., 2004). DF has been commonly used as a probe substrate for measuring in vitro and in vivo activity of CYP2C9 in humans (Tucker et al., 2001; Bjornsson et al., 2003). The rhesus monkey, which has recently been demonstrated to be a good animal model for studying CYP3A-mediated interactions in humans (Prueksaritanont et al., 2006), was selected as an animal model, based on several similarities between rhesus and human CYP3A and 2C isoforms (Tang et al., 2005). This article describes apparently conflicting in vitro-in vivo results obtained from these studies, as well as results obtained subsequently, to help explain the observed discrepancies. The latter studies included in vitro metabolism of DF in monkey liver microsomes and hepatocytes.

Materials and Methods

Materials. DF, 4'-hydroxy (4'-OH) DF, midazolam (MDZ), 1'-hydroxy midazolam, diazepam, and rifampin were obtained from Sigma (St. Louis, MO). All other reagents were of analytical or HPLC grade. Rhesus monkey and human liver microsomes (pooled from 10–20 individuals) were purchased from Xenotech (Kansas City, KS). Fresh rhesus monkey hepatocytes were prepared in-house according to the method of Moldeus et al. (1978).

In Vitro Induction Studies. The study was conducted using rhesus monkey hepatocytes (n = 3) as described previously (Prueksaritanont et al., 2005, 2006). In brief, hepatocyte cultures were treated, in triplicate for each treatment, for 2 days with culture media containing various concentrations of rifampin or vehicle control (dimethyl sulfoxide, 0.1% v/v). At the end of the treatment (48 h), DF 4'-hydroxylase activities were measured, in triplicate, by incubating DF (250 μM) with rhesus hepatocytes in 10 mM HEPES buffer, at 37°C, 95% humidity, and 5% CO2, for 20 min. Samples from each well were transferred to a 96-well plate containing an equal volume of acetonitrile, and...
stored at 4°C until analysis by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) for DF and 4'-OH DF (Kumar et al., 2002).

**In Vivo Studies.** All studies were reviewed and approved by the Merck Research Laboratories Institutional Animal Care and Use Committee. The in vivo studies were carried out in a crossover fashion, with at least a 2- to 3-week washout period. Male rhesus monkeys \( (n = 4, \text{ body weight} = 4–7 \text{ kg}) \) were pretreated orally with either vehicle (PEG400) or rifampin (18 mg/kg, in PEG400), once daily for 5 days. On the morning of day 5, DF was administered via a cannulated intraperitoneal portal vein (i.p.v.) at 0.1 mg/kg/day for 4 h to monkeys, and blood samples were collected via a saphenous or femoral vein at predose, and at 60, 120, 150, 180, 200, 220, 240 (end of i.p.v. infusion), 270, 300, 360, and 420 min after DF administration. Plasma samples were separated immediately at 10°C and kept frozen at −20°C. Additional studies also were conducted by i.v. administration of DF (0.1 mg/kg for 4 h) via a cephalic vein on day 5 in animals pretreated with the vehicle PEG400 and rifampin (18 mg/kg orally in PEG400) for 5 days.

**In Vitro Metabolism Studies.** Studies to investigate the acyl glucuronidation pathway were conducted using rhesus monkey liver microsomes incubated with various concentrations of DF in the presence of UDPGA, as described previously (Kumar et al., 2002). For comparison purpose, a parallel study also was conducted using human liver microsomes. In brief, incubations (0.2-ml final volume) consisted of liver microsomes (0.05–0.1 mg/ml) previously preincubated with alamethicin (25 μg/mg of microsomal protein) for 15 min, potassium phosphate buffer (100 mM pH 7.4), MgCl2 (10 mM), UDPGA (2 mM), and DF (0.5–150 μM final concentrations). After an incubation time of 5 to 10 min, the reaction was quenched with 0.4 ml of acetonitrile containing 3% formic acid, and the supernatant was separated for analysis by LC-MS/MS (Kumar et al., 2002). Studies on kinetics of the oxidative metabolism of DF also were conducted in monkey and human liver microsomes, using conditions described previously by Kumar et al. (2002).

Subsequent metabolism studies were conducted using rhesus monkey hepatocytes (0.5 × 10^6 cells/ml), incubated with 5 μM DF, in 10 mM HEPES buffer with a final incubation volume of 0.2 ml. The concentration of 5 μM is below \( K_{m} \) values for both the glucuronidation and hydroxylation of DF estimated from the aforementioned liver microsomal studies. After incubation at 37°C, 95% humidity, and 5% CO₂, the reaction was quenched, at various incubation times, with 0.2 ml of acetonitrile containing 3% formic acid, and the supernatant was separated for analysis by LC-MS/MS (Kumar et al., 2002).

**Analytical Procedures.** Concentrations of DF and rifampin in plasma were analyzed using LC-MS/MS. Plasma samples were spiked with the respective internal standard (tolbutamide for DF and diazepam for rifampin), and the analytes were quantitated by LC-MS/MS in selective reaction monitoring experiments in the positive ionization mode were performed using an AB/MDS SCIEX API 3000 tandem mass spectrometry. The concentration of the analyte in the unknown samples was determined by interpolation from the standard curve. For each analyte, standard curves showed satisfactory linearity and precision (<15% coefficient of variation).

**Data Analysis.** For the liver microsomal studies, apparent \( K_{m} \) and \( V_{max} \) values were estimated using a nonlinear regression program (Enfit from Biosoft, Ferguson, MO). The intrinsic clearance \( (C_{l-intr}) \) estimates were calculated by dividing \( V_{max} \) by \( K_{m} \). For the hepatocytes studies, the \( C_{l-intr} \) values were calculated by dividing initial metabolite formation rates obtained during the first 10-min incubation (nmol/min/10^6 cells) by the substrate concentration used (5 μM), which is below the \( K_{m} \) values determined for the glucuronidation and oxidation reactions.

The concentration of rifampin producing a 50% increase in DF 4'-hydroxylase or MDZ 1'-hydroxylase activity (EC_{50}) was determined using nonlinear regression analysis (PCNONLIN; Scientific Consulting, Cary, NC), as described previously (Pruksarsrianton et al., 2006).

The area under the plasma concentration-time profile (AUC_{o-last}) was calculated from time 0 to the last detectable sampling time using the trapezoidal rule. The apparent terminal half-life \( (t_{1/2}) \) was estimated by dividing 0.693 by the elimination rate constant determined using least-squares regression analysis of the log-linear portion of the DF plasma concentration-time data. Plasma clearance \( (CL) \) values for DF were calculated as the i.v. dose divided by their corresponding AUC from time 0 to infinity (AUC_{0-last}). Hepatic availability \( (F_{Hep}) \) was estimated by dividing AUC obtained after i.v. administration to that obtained by i.v. administration. Volume of distribution at steady state \( (V_{ss}) \) were calculated using conventional moment analysis as i.v. dose multiplied by the first moment of the plasma concentration-time profile (AUC) and divided by \( (AUC_{0-last})^{2} \). The peak plasma concentration \( (C_{max}) \) was determined by observation.

**Results**

**In Vitro Induction Studies.** In rhesus monkey hepatocytes, rifampin markedly induced the formation of 4'-OH DF in hepatocyte preparations from three different individuals. Results are mean of triplicate determinations. DF 4'-hydroxylase activities were determined using 250 μM DF. Rhesus monkey hepatocytes were treated with vehicle (dimethyl sulfoxide, control) or rifampin for 48 h before enzyme activity determinations.

**In Vivo Studies.** Upon i.v. administration of DF (0.1 mg/kg/h),...
plasma concentrations of DF appeared to reach a steady-state level of about 0.8 μM at about 2 h postdose and declined relatively quickly after the infusion stopped (Fig. 2A), with $t_{1/2}$ of about 1 to 2 h (Table 1). The CL of DF was moderate, with values of 10 ml/min/kg (Table 1). Treatment with rifampin (18 mg/kg) for 5 days did not significantly affect the i.v. plasma profiles (Fig. 2A) or pharmacokinetic parameters of DF; values for AUC, $V_{max}$, CL, and $t_{1/2}$ of DF were comparable between the control and rifampin-treated animals (Table 1). Effects of rifampin also were determined after i.pv. administration of DF to monkeys; this route of administration provides advantages of maximizing the magnitude of interactions resulting from changes in both hepatic first pass and systemic clearance (Wilkinson, 1987; Prueksaritanont et al., 2006). As shown in Fig. 2B and Table 1, rifampin also minimally affected the i.pv. plasma levels of DF and its corresponding pharmacokinetic parameters; plasma levels of DF, although significantly lower than those observed after i.v. administration, were comparable between the control and rifampin-treated animals. The hepatic availability, $F_{hep}$, of DF was estimated to be about 30% in monkeys with or without rifampin pretreatment (Table 1).

In rifampin-pretreated monkeys, rifampin plasma concentrations were maintained above 5 μM over a period of 6 h after the last dose (5th dosing day); values for $C_{max}$ were about 16 and 10 μM, and AUC$_{0-last}$ Values were 67 and 42 μM·h in the animals given i.v. and i.pv. administration of DF, respectively (Table 1).

**In Vitro Metabolism Studies.** Liver microsomal studies showed that DF underwent extensive glucuronidation in monkeys (Fig. 3A), with values for $V_{max}$, $K_{m}$, and $CL_{int}$ ($V_{max}$/$K_{m}$) of 7.5 nmol/min/mg, 12.2 μM, and 613 μl/min/mg, respectively. In contrast, the formation of 4′-OH DF was relatively minor (Fig. 3A); the $V_{max}$, $K_{m}$, and $CL_{int}$ values were 1.2 nmol/min/mg, 72.2 μM, and 16 μl/min/mg, respectively. Accordingly, in monkey liver microsomes, the glucuronidation pathway represented about 95% of the total intrinsic clearances. In a parallel study using human liver microsomes, the glucuronidation pathway accounted for about 75% of the total intrinsic clearance. Values for the $V_{max}$, $K_{m}$, and $CL_{int}$ were 11.1 nmol/min/mg, 21.5 μM, and 517 μl/min/mg for the glucuronidation, and 1.9 nmol/min/mg, 10.3 μM, and 184 μl/min/mg for the oxidation pathways, respectively. These human liver microsomal results were in a range comparable to those reported previously by Kumar et al. (2002).

In rhesus monkey hepatocytes, formation of DF glucuronide was much higher than that of 4′-OH DF over the entire 1-h incubation period (Fig. 3B). During the first 10 min of incubation, the sum of 4′-OH DF and DF glucuronide formed was approximately equal to the total DF consumption (Fig. 3B), suggesting minimal formation of other metabolites, including secondary products. The discrepancy between the combined DF glucuronide + 4′-OH DF formation and total consumption during the subsequent period (Fig. 3B) was suggestive of significant formation of secondary or sequential metabolites, probably to glucuronide and glutathione products of the hydroxyl DF (Tang et al., 1999; Kumar et al., 2002). The $CL_{int}$ values were approximately 10-fold higher than those observed using monkey liver microsomes.

**TABLE 1**

Pharmacokinetic parameters of DF after i.v. or i.pv. administration of DF (0.1 mg/kg/h for 4 h) to rhesus monkeys pretreated with vehicle (PEG400) or rifampin (18 mg/kg p.o.) for 5 days.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Pharmacokinetic Parameters</th>
<th>Vehicle (Control)</th>
<th>Rifampin (18 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>i.v. Dose</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DF</td>
<td>AUC$_{0-last}$ (μM·h)</td>
<td>2.69 ± 0.58</td>
<td>2.22 ± 0.32</td>
</tr>
<tr>
<td></td>
<td>AUC$_{0-inf}$ (μM·h)</td>
<td>2.74 ± 0.54</td>
<td>2.27 ± 0.36</td>
</tr>
<tr>
<td></td>
<td>$V_{max}$ (l/min/kg)</td>
<td>8.5 ± 1.4</td>
<td>10.2 ± 1.7</td>
</tr>
<tr>
<td></td>
<td>$K_{m}$ (l/kg)</td>
<td>0.32 ± 0.05</td>
<td>0.29 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>$t_{1/2}$ (h)</td>
<td>1.2 ± 0.7</td>
<td>1.2 ± 0.5</td>
</tr>
<tr>
<td>Rifampin</td>
<td>AUC$_{0-last}$ (μM·h)</td>
<td>67.0 ± 41.4</td>
<td>61.6 ± 10.6</td>
</tr>
<tr>
<td></td>
<td>$C_{max}$ (μM)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>i.pv. Dose</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DF</td>
<td>AUC$_{0-last}$ (μM·h)</td>
<td>0.69 ± 0.21</td>
<td>0.63 ± 0.19</td>
</tr>
<tr>
<td></td>
<td>AUC$_{0-inf}$ (μM·h)</td>
<td>0.73 ± 0.23</td>
<td>0.67 ± 0.21</td>
</tr>
<tr>
<td></td>
<td>$F_{ce}$</td>
<td>0.27 ± 0.09</td>
<td>0.31 ± 0.13</td>
</tr>
<tr>
<td></td>
<td>$t_{1/2}$ (h)</td>
<td>1.8 ± 1.3</td>
<td>1.6 ± 0.6</td>
</tr>
<tr>
<td>Rifampin</td>
<td>AUC$_{0-last}$ (μM·h)</td>
<td>42.4 ± 21.8</td>
<td>9.8 ± 4.7</td>
</tr>
</tbody>
</table>
exceeded its in vitro EC50 estimate for the induction of DF after a therapeutic dose in humans (Sanofi-Aventis, 2003) and well pathways -hydroxylation, and of CYP3A to the minor 5-hydroxylation/H11032 suggested the major contribution of CYP2C to the major oxidative monkey and human liver microsomes, including immunoinhibition, probe. Comparative in vitro metabolism studies of DF in rhesus induction observed in vitro by rifampin, using DF as a functional tocytes. Approximately 95% of the total metabolism of DF in rhesus monkey hepatocytes were comparable to the respective values ob- E and mean values for the induction of 4'-OH DF formation in rhesus monkey hepatocytes (A), and time profiles of DF metabolism (DF disappearance and 1.6 and 43.2 ± 6.0 µl/min/10⁶ cells, respectively. Thus, like the liver microsomal finding, the oxidative metabolism accounted for about 5%, and the glucuronidation represented approximately 95% of the total metabolism of DF in rhesus monkey hepatocytes.

Discussion

In this study, we examined the in vivo consequence of CYP2C9 induction observed in vitro by rifampin, using DF as a functional probe. Comparative in vitro metabolism studies of DF in rhesus monkey and human liver microsomes, including immunoinhibition, chemical inhibition, and activities of recombinant P450s, consistently suggested the major contribution of CYP2C to the major oxidative pathway -hydroxylation, and of CYP3A to the minor 5-hydroxylation in both species (Leemann et al., 1993; Shen et al., 1999; Tang et al., 2005). However, we observed an apparent disconnect between the in vitro and in vivo induction of DF metabolism in rhesus monkeys. In vitro, rifampin was a potent inducer of rhesus CYP2C; both EC₅₀ and Eₘ₅₀ values for the induction of 4'-OH DF formation in rhesus monkey hepatocytes were comparable to the respective values ob- served in this species for MDZ 1'-hydroxylation activity, a CYP3A marker (Prueksaritanont et al., 2006). In contrast to this in vitro observation, subsequent pharmacokinetic interaction studies in rhesus monkeys revealed that rifampin was not an inducer of DF clearance in vivo.

This apparent lack of in vivo effect of rifampin on the DF pharmaco-kinetics in the present monkey study is not likely due to an inadequate systemic exposure to rifampin. At the dose used in our in vivo studies, Cₘ₅₀ values for rifampin were similar to those reported after a therapeutic dose in humans (Sanofi-Aventis, 2003) and well exceeded its in vitro EC₅₀ estimate for the induction of DF 4'-hydroxylase activity in monkey hepatocytes. In addition, rifampin, at comparable plasma levels, has been shown to cause a marked decrease (>10-fold) in the MDZ systemic exposure after i.p.v. administration of MDZ to monkeys (Prueksaritanont et al., 2006), and after its oral administration to humans (Niemi et al., 2003). Rifampin is equally potent as an inducer of CYP3A-mediated MDZ 1'-hydroxylase activity in both rhesus monkey and human hepatocytes (Prueksaritanont et al., 2006).

Not only the present negative in vivo interaction results between DF and rifampin, but also the earlier positive observations between rifampin and MDZ ruled out the possibility that the CYP3A-mediated oxidative metabolism is a major determinant of the in vivo clearance of DF in monkeys, as suggested by Tang et al. (1999). In contrast to earlier in vivo metabolism data which seemed to suggest that the oxidative metabolism of DF might account for the majority of in vivo clearance (Stierlin and Faigle, 1979; Stierlin et al., 1979), Kumar et al. (2002) have recently proposed that the direct glucuronidation of DF is a more important component to the in vivo clearance than the oxidation pathway in rats, dogs, and humans. This proposal was made based primarily on 1) in vitro liver microsomal metabolism data, which showed much higher microsomal intrinsic clearance values for the acyl glucuronide (~70–90%) than the 4'-hydroxylation pathways, 2) a good agreement between in vitro and in vivo clearance data, in untreated subjects, when both the in vitro oxidation and direct glucu- ronidation were considered, and 3) in vitro liver microsomal data, which showed that the glucuronide of DF could be further metabolized via oxidation, presumably leading to the in vivo formation of its 4'-hydroxy derivative, which is eventually excreted to bile and urine. However, this proposal has not been corroborated by clinical drug interaction studies between DF and CYP2C9 inducers or inhibitors. Before our studies, there was no report on the glucuronidation of DF in monkeys.

Our in vitro metabolism findings in both the liver microsomal and hepatocyte systems revealed that direct glucuronidation was indeed the major (~90%), and hydroxylation, the minor (~5%), metabolic pathway of DF in control rhesus monkeys. If the findings in vitro are scalable to in vivo, one would anticipate that the pharmacokinetics of DF in monkeys would not be affected appreciably by changes in its
hydroxylation rate. This suggestion, which was in complete agreement with the present in vivo rifampin-DI interaction observations, was derived based on our preliminary study, which showed a modest increase (<30%) in DI glucuronide formation in rhesus monkey hepatocytes treated with 10 μM rifampin (data not shown), and an assumption that the intrinsic clearance value for the hydroxylation pathway was increased by 2- to 5-fold with rifampin treatment (i.e., from 5 of 95 units to 10 of 105 units to 25 of 120 units), based on the present in vitro Emax value of ~2- to 5-fold obtained for the 4'-OH DI formation in monkey hepatocytes.

In humans, the oxidative metabolism of DI, albeit more substantial and catalyzed by relatively higher affinity enzyme than in monkeys, also is probably a minor contributor (~25% based on the liver microsomal data) to its in vivo clearance. Similarly, it is expected that any changes in the DI hydroxylation rate, yielding <50% of its contributions to the total metabolic rate, would not have substantial impacts on the in vivo clearance of DI in humans. In this regard, there have been no clinical reports on the pharmacokinetic interactions of DI with known potent CYP2C9 inhibitors or inducers, including rifampin. In human hepatocytes, rifampin is an inducer of DI 4'-hydroxylase activity; a mean increase in the activity of about 2-fold over control has been reported in the presence of 20 μM rifampin (Parkinson et al., 2004), and a comparable magnitude of increase also was obtained with 10 μM rifampin in our preliminary study (data not shown). By analogy, rifampin may also elicit modest effects on the DI pharmacokinetics, via induction of CYP2C9, in humans, as was the case in monkeys. It is worth noting that possible differences in the magnitude of drug interaction between DI and other potent enzyme inhibitors/inducers exist between monkeys and humans, based on the quantitative differences in the kinetics of DI metabolism observed in this study between the two species, and considering potential species differences, in general, in enzyme induction and inhibition reported in the literature.

In conclusion, using the rhesus monkey as an animal model, we showed that in contrast to significant induction of the in vitro oxidative metabolism, the in vivo pharmacokinetics of DI were not sensitive to rifampin treatment. Subsequent in vitro metabolism studies suggested that this apparent in vitro-in vivo discrepancy was due probably to the fact that the CYP2C-mediated oxidative metabolism of DI is not the major determinant for its in vivo clearance in monkeys, both untreated and pretreated with rifampin. Our data also substantiated a previous suggestion made based on an in vitro finding in rat, dog, and human liver microsomes (Kumar et al., 2002) that the direct glucuronidation of DI instead is a more important determinant for the in vivo clearance of DI in both animals and humans, thus raising a question on the utility of DI as a useful probe for measuring CYP2C9 activity in vivo.

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