APPLICATION OF RAT HEPATOCYTE CULTURE TO PREDICT IN VIVO METABOLIC AUTO-INDUCTION: STUDIES WITH DFP, A CYCLOOXYGENASE-2 INHIBITOR

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ABSTRACT:

The drug candidate DFP [5,5-dimethyl-3-(2-isopropoxy)-4-(4-methanesulfonylphenyl)-2(5H)-furanone] is a selective cyclooxygenase-2 inhibitor under evaluation for analgesic and anti-inflammatory therapy. The in vitro metabolic pathways (rat microsomes) involve hydroxylation of the isopropyl side chain at either of two positions, the methyl or the methine, thus producing a hydroxylated metabolite (DFHP) or a dealkylated metabolite (DFH). DFH formation was the major pathway. Using hepatic microsomes from rats treated with agents that induce specific CYP isozymes, it was shown that the dexamethasone-inducible rat CYP3A isozyme(s) play a major role in DFH formation. The roles of CYP3A1 and -3A2 were confirmed with genetically engineered rat CYP enzymes. The potential for induction of rat CYP3A by DFP was evaluated by incubating DFP in rat hepatocyte cultures and measuring the CYP3A levels. Both CYP3A immunoreactive protein and enzyme activity were induced in a dose-dependent manner. The induction was confirmed in vivo by dosing rats with DFP at 100 mg/kg for 4 days. Microsomes prepared from the excised livers showed that DFP gave ~55% of the induction observed with dexamethasone, as determined by Western blot. In vitro metabolic auto-induction of DFP was assessed by measuring the metabolism of DFP in hepatocytes treated with DFP. DFH formation was significantly enhanced in the DFP-treated cells. In vivo, treating rats with DFP at doses of 10 to 100 mg/(kg·day) for 13 weeks indicated that DFP induced its own metabolism. The Cmax and plasma drug area under the curve values during the thirteenth week were significantly lower than that on the first day, and the effect was dose-dependent.

The cytochrome P450 (CYP) enzymes often play an important role in the biotransformations of drugs, and the oxidations they effect often govern the clearance of a given drug. Some CYP enzymes are inducible by drugs and other xenobiotics. The CYP enzyme levels and activities will vary in vivo depending on whether the enzymes’ expression is at basal or induced levels. This phenomenon was first recognized decades ago and has been the subject of numerous reviews (for example, Conney, 1967; Okey, 1990; Whitlock and Denison, 1995). If a drug causes induction of the CYP enzymes responsible for its own metabolism, it may increase its own clearance. This will lower the levels of circulating drug and reduce therapeutic efficacy. This phenomenon is classically referred to as “tolerance” or metabolic auto-induction and was first observed with such drugs as barbiturates, rifampin, phenytoin, and warfarin (Remmer and Merker, 1963; Park and Breckenridge, 1981; Okey, 1990).

The CYP enzyme that plays the most important role in human drug metabolism is CYP3A4. This enzyme contributes approximately 30% to the total CYP content of the liver (Shimada et al., 1994) and is estimated to be responsible for metabolism of >60% of drugs currently on the market (Choletton et al., 1992). CYP3A4 is also a highly inducible enzyme (Molowa et al., 1986), and numerous examples of drugs that cause clinically relevant CYP3A4 induction have been reported, including the antibiotic rifampin and the anticonvulsants phenobarbitone, phenytoin, and carbamazepine (review article by McInnes and Brodie, 1988). Rifampin causes auto-induction because CYP3A4, which it induces, is also responsible for its clearance (Accella, 1978).

The prediction of in vivo phenomena in humans may be attempted with in vitro experiments. To establish valid in vitro models, reliable in vitro-in vivo correlations must be established with laboratory animal models. Rat orthologs of human CYP3A4 are CYP3A1, -3A2, and -3A3, of which the latter two are inducible (Huss et al., 1999; Komori and Oda, 1994). Induction studies in the rat may be used as a model for human, even though species differences are known (Wrighton et al., 1985; Kocarek et al., 1995). Recently, the application of rat hepatocyte culture for predicting CYP induction has become refined and validated (for example, see Silva et al., 1998). There is a strong correlation between induction of rat CYP3A in vitro and in...
vivo, as indicated by Western blot measurement of protein (Silva et al., 1998). The induction of CYP3A enzyme activity also correlates, and this can be demonstrated using an activity measurement such as testosterone 6b-hydroxylation, which is primarily a rat CYP3A process (Sonderfan et al., 1987). This activity can be compared between that observed in hepatocyte culture and in incubations with hepatic microsomes prepared from rats dosed with drugs (Sonderfan et al., 1987; Li et al., 1995; Kostrubsky et al., 1999).

Appreciation of the fact that metabolism of probe substrates can be observed in hepatocyte culture, coupled with the knowledge that CYP induction in the cultured hepatocyte model correlates well with in vivo induction, suggested that the two could be combined. One could, in principle, treat cells with a drug and then probe the cells with the same compound to observe whether the metabolism profile had changed. For example, enhanced metabolism would indicate auto-induction. It could then be predicted that auto-induction would occur in vivo, giving rise to altered pharmacokinetic parameters such as lower maximum plasma concentration ($C_{\text{max}}$) and area under the plasma drug concentration versus time curve (AUC) values.

A recent drug candidate, DFP [5,5-dimethyl-3-(2-propoxy)-4-(4-methanesulfonylphenyl)-2(5H)-furaneone] (Fig. 1; Leblanc et al., 1999), from our cycloxygenase-2 discovery program afforded an opportunity to explore this methodology. The in vitro oxidative rat biortransformations of DFP were characterized, and the CYP enzyme responsible for the major pathway was identified. In vitro CYP induction and auto-induction were evaluated using the cultured rat hepatocyte model. These data were compared with those generated in vivo, including measurement of CYP induction in liver tissue and generation of pharmacokinetic data over a 13-week dosing regimen.

Materials and Methods

Chemicals and Reagents. DFP, DFH [5,5-dimethyl-3-(2-hydroxy)-4-(4-methanesulfonylphenyl)-2(5H)-furaneone], DFHP [5,5-dimethyl-3-(2’-hydroxyisopropoxy)-4-(4-methanesulfonylphenyl)-2(5H)-furaneone], and the internal standard (Fig. 1) were synthesized at Merck Frosst (Quebec, Canada) (Bellely et al., 1997; Charet et al., 1999; Leblanc et al., 1999). Standard chemicals and reagents were purchased from Sigma Chemical Co. (St. Louis, MO) unless specified. Testosterone and 6b-hydroxytestosterone were obtained from Steraloids (Wilton, NH). Matrigel was purchased from Collaborative (Bedford, MA), methylcellulose from Dow Chemical (Midland, MI), and Methocel A4C Premium from Colorcon (Bougival, France).

Hepatic and Recombinant Enzymes. Male Sprague-Dawley rats (Charles River, St. Constant, Quebec, Canada) weighing 250 to 300 g were used. Rats were housed as previously described (Nicoll-Griffith et al., 1995) and were given food and water ad libitum. Dexamethasone induction was achieved by intraperitoneal injection of 50 mg/kg in corn oil for 4 days. The rats were sacrificed ±24 h after the last dose and the livers harvested after exsanguination. The microsomal fractions were prepared from fresh rat liver tissue according to standard procedures (Lu and Levin, 1972) and stored at −80°C until use. Protein determination was conducted according to the method of Lowry et al. (1951) using bovine serum albumin as the standard. Hi5 insect cell microsomes containing recombinant rat CYP enzymes, coexpressed cytochrome P450 reductase, and cytochrome b5 (for CYP3A enzymes) were obtained from GENTEST Corporation (Woburn, MA).

Incubations with Hepatic Microsomal Fractions. Incubations of 50 μM DFP (methanol stock solution, final concentration 0.5%) were performed in 100 mM phosphate buffer containing 20 mM glucose 6-phosphate, 2 mM NADP, 2 mM magnesium chloride, and 1 mg of microsomal protein in a total volume of 300 μl. There was a 2-min preincubation step at 37°C before the reaction was started by the addition of 2 units of glucose-6-phosphate dehydrogenase. Control incubations contained no NADPH-generating system, and blank incubations contained no DFP. After 1 h, the incubations were quenched by the addition of 500 μl of acetonitrile and mixed by vortex. The precipitated proteins were separated by centrifugation for 10 min at 13,000 rpm in an Eppendorf (VWR Canlab, Ville Montre; Royal, Quebec, Canada) centrifuge 5415C. The supernatant was diluted 1:1 with 20 mM ammonium acetate (pH ~6.7) before HPLC analysis.

HPLC Conditions for Analysis of DFP Incubations. The HPLC system consisted of a Waters 600S controller, a Waters 717 plus autosampler, and a Waters 996 photodiode array detector (Milford, MA). The data were collected and processed by Waters Millennium version 2.15 software. Gradient mobile phase programming was used with a flow rate of 1 ml/min on a Zorbax Rx C18 column (4.6 mm × 15 cm; Agilent, Kirkland, Quebec, Canada). Eluant A was 20 mM ammonium acetate (no pH adjustment), and eluant B was methanol. A linear gradient was run from 85 to 15% A over 25 min. UV detection was conducted with a Waters model 996 photodiode array detector, and the chromatograms were plotted at 290 nm (for DFP and DFHP) and 350 nm (for DFH) using Millennium version 2.1 software. The retention times of DFP, DFH, and DFHP were 9.6, 13.7, and 17.6 min, respectively. Quantification of DFH and DFHP was accomplished by comparison of peak areas to appropriate external calibration curves. The limits of detection were approximately 0.005 μM for DFH and 0.02 μM for DFHP.

Characterization of Metabolites by LC/MS. HPLC/atmospheric pressure chemical ionization (APCI)-MS data were generated using a system consisting of a Waters 600 MS pump, Waters 919 autosampler, 3.9-× 150-mm Waters Novapak C18 HPLC column, a Waters 995 UV-diode array detector, and a TSQ-7000 mass spectrometer (Thermo Finnigam, San Jose, CA). Eluant A was methanol containing 20 mM ammonium acetate, and eluant B was water containing 20 mM ammonium acetate that was not pH-adjusted. The flow rate was 1 ml/min, and a linear gradient was used from 20 to 70% A over 25 min. Under these conditions, synthetic standards of DFH, DFHP, and DFP eluted at 10.5, 14.9, and 20.0 min, respectively. A single wavelength of the UV-diode array detector could be monitored and recorded and was, therefore, set to 280 nm. The mass spectrometer was operated in APCI mode, using a sheath gas pressure of 60 psi and vaporizer and capillary temperatures of 500 and 180°C, respectively. Full scan mass spectra were obtained from 200 to 700 Da at a rate of 1 scan/s.

Incubations with Recombinant CYP Enzymes. DFP was incubated in a manner analogous to that described above for hepatic microsomal incubations at 50 μM (0.5% methanol final volume) with 50 pmol of rat recombinant enzymes CYP2D1, -3A1, and -3A2. After 15 min, the incubations were quenched and analyzed as described above. Hi5 insect cell culture microsomes and incubations containing no NADPH were run as negative controls. Analysis was conducted by HPLC/UV.

CYP3A Induction in Rat Hepatocytes (in Vitro). This complete procedure was conducted as previously described by Silva et al. (1998). Briefly, rat hepatocytes were isolated and cultured on Matrigel-coated dishes (60-mm
plates) under Williams’ E medium containing 10−7 M dexamethasone and 10−6 M insulin. The 48-h-old primary hepatocyte cultures were treated for 2 days with DFP at concentrations of 2, 10, or 50 μM, and the CYP3A protein induction was determined by Western blot analysis of the hepatocyte microsomal pellet, according to standard procedures. The induction is presented as a percentage of the positive control, dexamethasone, which was dosed at 10 μM.

**CYP3A Induction in the Rat (in Vivo).** Male Sprague-Dawley rats (200–250 g) were obtained from Charles River. They were housed as previously described (Nicoll-Griffith et al., 1995) and were fed Teklad (Madison, WI) rodent diet (W) 8604. The rats (n = 4) were orally dosed with DFP at 100 mg/(kg·day) in 0.5% Methocel for 4 days and sacrificed. The positive control rats were treated with ip. injections of dexamethasone (50 mg/kg/day) for 3 days, as described above. A 100,000g pellet (microsomal fraction) was prepared from the excised livers, and the CYP3A was measured by Western blot, as previously described (Silva et al., 1998). Induction is reported as a percentage of the positive control, dexamethasone (Silva et al., 1998).

**Metabolic Auto-Induction in Rat Hepatocytes (in Vitro).** Rat hepatocytes were cultured in 24-well plates (Nunc Brand Products, Canadian Life Technologies, Burlington, Ontario, Canada) on Matrigel similarly to previously described procedures (Silva et al., 1998). The cell density was 0.6 × 106, and the volume of medium (Williams’ E medium containing 10−7 M dexamethasone and 10−6 M insulin) was 500 μl per well. The cells (triplicate wells) were treated with DFP (2, 10, or 50 μM), dexamethasone (10 μM), and NBP (10 μM), or vehicle (acetone 0.5% v/v of medium) for 2 days, with the medium and treatment replaced daily. The medium was removed and the cells washed three times with Krebs-Henseleit buffer containing 12.5 mM HEPES (pH 7.4, 37°C). A fourth aliquot of Krebs-Henseleit buffer was placed on the cells for the next step. The cells were then probed with testosterone (250 μM for 30 min), DFP (50 μM for 1 h), or vehicle (0.5% acetone final volume) to obtain blank samples. The media were analyzed by HPLC/UV as described to determine the quantities of metabolites formed.

**HPLC Conditions for Analysis of Testosterone and 6β-Hydroxysteroid terone.** Gradient mobile phase programming was used with a flow rate of 1 ml/min on a Supelcosil LC18 column (15 × 4.6, 3 μm) (Supelco, Inc., Bellefonte, PA). Eluant A was 39:60:1 methanol-water-acetonitrile. Eluant B was 80:18:2 methanol-water-acetonitrile. A linear gradient was run from 90 to 350 nm, DFP (50 μM for 1 h), or vehicle (0.5% acetone final volume) to obtain blank samples. The media were analyzed by HPLC/UV as described to determine the quantities of metabolites formed.

**Rat Studies to Obtain Plasma Levels for Day 1.** Male Sprague-Dawley rats (Charles River, Raleigh, NC) weighing ~240 to 330 g were used. The rats were housed in individual steel cages, fed Purina Certified Rodent Chow, and given free access to drinking water. Four groups of 15 rats each received 0, 10, 30, or 100 mg/kg/day DFP suspended in 0.5% acetonitrile for 4 days, with the medium and treatment replaced daily. The medium was removed and the cells washed three times with Krebs-Henseleit buffer containing 12.5 mM HEPES (pH 7.4, 37°C) for each time point. A fourth aliquot of Krebs-Henseleit buffer was placed on the cells for the next step. The cells were then probed with testosterone (250 μM for 30 min), DFP (50 μM for 1 h), or vehicle (0.5% acetone final volume) to obtain blank samples. The media were analyzed by HPLC/UV as described to determine the quantities of metabolites formed.

**Results**

Incubations of DFP (Fig. 1) with rat NADPH-fortified microsomal protein yielded two oxidative metabolites (Fig. 2). Characterization of the metabolites by HPLC/APCI (photodiode array detector) indicated that M1 had a different chromophore as indicated by the UV spectrum (Fig. 3), whereas that of M2 was identical to DFP (data not shown). M2 and DFP have a λmax of ~290 nm, but M1 has a λmax at ~350 nm.

Microsomal incubations and synthetic standards of DFP, DFHP, and DFP were examined using HPLC/APCI in both positive- (data not shown) and negative-ion mode (Fig. 4). In positive-ion mode, a single metabolite was observed for microsomal incubations at 14.9 min (M2); producing a pseudomolecular ion at 358 Da (M + NH4)+, while DFP eluted at 20.0 min and produced a pseudomolecular ion at 342 Da (M + NH4)+. Although an earlier peak was observed on the UV trace at 387 nm (M1), no positive-ion mass spectrum could be obtained. In positive-ion APCI, synthetic standards of DFHP and DFP produced (M + NH4)+ ions at 358 and 342 Da, respectively. The synthetic standard of DFH did not produce a mass spectrum in positive-ion mode. In negative-ion mode APCI examination of microsomal incubations, metabolites M1 and M2 were observed at 10.5 and 14.9 min, both of which produced major ions at 281 Da (Fig. 4). M1 also produced a minor ion at 341 Da, while M2 produced smaller ions at 339 and 399 Da. The negative-ion APCI mass spectra for DFP contained a strong ion at 281 Da and a smaller ion at 323 Da. Synthetic standards of the dealkylated metabolite, DFH, and the hydroxylated metabolite, DFHP, (Fig. 1) were found to have identical HPLC retention times, negative-ion APCI mass spectra, and UV spectra as M1 and M2, respectively.

Using appropriate reference standards for calibration curves, it was determined that the formation of DFH was about twice that of DFHP in untreated rat microsomal incubations of DFP (50 μM). The formation of DFH was increased 8-fold with microsomes prepared from dexamethasone-treated rats, whereas the enhancement of DFHP formation was approximately 1.6-fold (Table 1 and Fig. 2). Quantitative Western blot analysis of the induced microsomes from dexamethasone-treated rats indicated that the level of CYP3A was approximately 10-fold higher than those from untreated rats (Silva et al., 1998). Incubations with microsomes prepared from β-naphthoflavone-treated rats showed no enhancement of M1 or M2 formation (Table 1). Western blot analysis of these microsomes had confirmed the induction of CYP1A1/2 (Nicoll-Griffith et al., 1993). Incubations with recombinant rat CYP3A1 and -3A2 showed formation of DFH (Table 1), as determined by HPLC/UV, whereas M2 could not be detected. The rate of DFH formation was approximately 3 times greater with CYP3A1 than with CYP3A2. The measured rate for CYP3A1 was 0.23 pmol/(min·μmol CYP). The formation of 6β-hydroxytestosterone from testosterone was reported to be 9.4 and 16.4 pmol/(min·μmol CYP) for these lots of CYP3A1 and -3A2, respectively (C. Crespi, personal communication). Neither metabolite was formed with recombinant rat CYP2D1 enzyme.

In vitro and in vivo induction results are expressed as a percentage of the positive control, dexamethasone, as previously described (Silva et al., 1998). In vitro induction of CYP3A immunoreactive protein was measured by quantitative Western blot, and enzyme activity was assessed using either testosterone or DFP as the probe substrate (Table
2). The negative control, βNF, showed no induction of CYP3A. DFP caused a dose-dependent induction of CYP3A protein as measured by both Western blot and enzyme activity (Table 2). At 2 μM the induction was negligible, but at higher concentrations (10 and 50 μM) it was marked. In vivo in the rat, after oral dosing with DFP at 100 mg/(kg•day) for 4 days, CYP3A induction was observed in the excised livers. By Western blot analysis the induction was 55 ± 7% (± S.D., n = 4) of that observed for the positive control, dexamethasone (data not shown).

After a single oral dose of DFP at 10, 30, or 100 mg/kg, the C_{max} levels of DFP were ~20, 40, and 80 μM in rat plasma (Fig. 5). The C_{max} levels of DFP were lower in all dose groups after 13 weeks of treatment compared with day 1 (Fig. 6a). At a dose of 10 mg/kg DFP, the AUC was not different (Fig. 6b). However, at 30 and 100 mg/kg, the AUC was dramatically reduced after 13 weeks of treatment.

Discussion

The first objective of this study was to ascertain the metabolic pathways of DFP. The second was to determine whether the oxidative biotransformation pathways were catalyzed by inducible CYP enzymes. Finally, studies were conducted to verify whether DFP could induce its own metabolism in vitro and in vivo in the rat.

Incubations with NADPH-fortified rat microsomal enzymes yielded two metabolites (Fig. 2). The positive-ion mass spectrum of M2 contained a strong ion at 358 Da (M + NH₄)⁺. This corresponds to an M + 16 molecular weight increment compared with DFP [(M + NH₄)⁺ at 342 Da]. M1 was not observed in positive-ion mode. In negative-ion mode, M1, M2, and DFP all produced a strong ion at 281 Da, consistent with fragmentation to the enolate anion of DFH (Fig. 4). Thus, the enolate fragment was identical for M1, M2, and DFP. Since the chromophore of M2 was the same as the parent, it was proposed that this metabolite involved oxidation of the terminal methyl of the isopropyl group (Fig. 1). The other metabolite, M1, had a different chromophore (Fig. 3), suggesting loss of the isopropyl side chain (Fig. 1). This metabolite could result from hydroxylation of the methine and loss of the side chain as acetone, as shown in Scheme 1.

Fig. 2. Reverse-phase HPLC chromatograms of incubations of DFP with hepatic microsomes from untreated (a) and dexamethasone-treated (b) rats. M1, DFH; M2, DFHP. See Materials and Methods for conditions.

Fig. 3. UV spectra of DFP and DFH as determined by photodiode array detector. See Materials and Methods for chromatographic conditions.
The synthetic standards of DFH and DFHP were identical to the metabolites by chromatographic and spectrometric analysis. DFH (M1) has a different UV spectrum than DFP (M2) (Fig. 3) because the dealkylated material has a hydroxyl group that is probably deprotonated at pH 7.0. The enolate anion would be stabilized because of conjugation with the methylsulfonylphenyl ring. For quantitative analysis, the detection of DFH was conducted at 350 nm because the sensitivity was about 4 times higher than at 290 nm.

When DFP was incubated with microsomes from dexamethasone-treated rats, in which CYP3A2 and -3A23 are induced (Huss et al., 1999), the formation of DFH was enhanced approximately 8-fold compared with untreated rat (Fig. 2, Table 1). The increase in DFHP formation was less than 2-fold. Therefore, in dexamethasone (DEX)-induced microsomes, CYP3A is largely responsible for the formation of DFH. Further experiments with recombinant rat CYP3A1 and -3A2 confirmed that DFH is formed by CYP3A isozymes. Under the conditions used, the relative rate of metabolism by CYP3A1 versus -3A2 was 3:1. According to the manufacturer, the relative rate of testosterone 6β-hydroxylation was 0.66:1. In view of the high homology between rat CYP3A1 and -3A23 (97% deduced amino acid sequence; Komori and Oda, 1994), it is likely that DFP is a substrate for both CYP3A2 and -3A23 in dexamethasone-induced rat microsomal incubations. Studies with chemical or antibody inhibitors could not be conducted because well characterized rat-specific CYP3A inhibitors were not available. Studies with microsomes from βNF-

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**TABLE 1**

Rates of DFH and DFHP formation in microsomal incubations

<table>
<thead>
<tr>
<th>Microsome Source</th>
<th>Rate of DFH Production</th>
<th>Rate of DFHP Production</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated rat</td>
<td>0.30</td>
<td>0.15</td>
</tr>
<tr>
<td>DEX-induced rat</td>
<td>2.55</td>
<td>0.25</td>
</tr>
<tr>
<td>BNF-induced rat</td>
<td>0.20</td>
<td>0.13</td>
</tr>
<tr>
<td>Control</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>Rat CYP2D1</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>Rat CYP3A1</td>
<td>0.23 pmol/(min · pmol CYP)</td>
<td>N.D.</td>
</tr>
<tr>
<td>Rat CYP3A2</td>
<td>0.07 pmol/(min · pmol CYP)</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

N.D., not detected. Considering the limits of detection, this corresponds to <0.0025 pmol/(min · pmol of CYP) DFH and <0.01 pmol/(min · pmol of CYP) DFHP.

**TABLE 2**

Percentage of induction of CYP3A in rat hepatocyte culture as compared with dexamethasone

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Western Blot 6β-Hydroxytestosterone Formation</th>
<th>DFH Formation</th>
</tr>
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<tr>
<td>50 μM DFP</td>
<td>137 (±29) 78.4 (±3.1) 86.3 (±5.2)</td>
<td></td>
</tr>
<tr>
<td>10 μM DFP</td>
<td>74 (±14) 47.1 (±1.9) 43.4 (±2.7)</td>
<td></td>
</tr>
<tr>
<td>2 μM DFP</td>
<td>36 (±9) 6.1 (±1.4) 4.4 (±0.2)</td>
<td></td>
</tr>
<tr>
<td>10 μM βNF</td>
<td>0 (±5) −5.1 (±0.4) −1.4 (±0.1)</td>
<td></td>
</tr>
</tbody>
</table>

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The synthetic standards of DFH and DFHP were identical to the metabolites by chromatographic and spectrometric analysis.

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treated rats showed no enhancement of either DFH or DFHP formation. NF induces the CYP1A family, suggesting that the CYP1A enzymes are not involved in either of these oxidations. Incubations with recombinant rat CYP2D1 gave neither metabolic product. Although it is possible that other CYPs play a role in the formation of DFH, the data indicate that, at least, CYP3A is involved. The enzyme responsible for DFHP formation could not be determined, but is apparently not primarily CYP1A, -2D1, or -3A.

In rat hepatocyte culture, CYP3A levels were induced by DFP in a dose-dependent manner, as measured by Western blot (Table 2). For rat studies, 10 \( \mu \)M DEX defines the maximum induction response, and the induction of a new drug is compared with that of DEX (Silva et al., 1998). This helps to correct for the variability observed with the rat hepatocyte model. At 10 \( \mu \)M DFP, the immunoreactive CYP3A protein induction was \( \sim 74\% \) of DEX, whereas at 50 \( \mu \)M it was even greater than DEX (Table 2). This indicates that DFP is a marked inducer of rat CYP3A in vitro. In vivo studies with the rat confirmed the in vitro findings. The induction by DFP was about 55% of that observed with dexamethasone. The agreement between the in vitro and in vivo induction is consistent with previously reported data (Silva et al., 1998).

In rat hepatocyte culture, the induction of CYP3A was also measured by protein activity using testosterone or DFP as probe substrates. The formation of 6\( \beta \)-hydroxysterosterone from testosterone is a known marker for CYP3A activity, although there is some contribution from CYP1A (Sonderfan et al., 1987). From the studies presented above, it was presumed that the turnover of DFP to DFH is primarily catalyzed by CYP3A. Concentrations of the probe substrates were selected as close to the solubility limits as possible so that maximal turnover would be measured. Incubation times were selected so that less than 15% of the substrate was consumed.

In a typical experiment, after the hepatocytes had been treated with DEX, the 6\( \beta \)-hydroxylase activity was induced 7.4-fold and after 50 \( \mu \)M DFP treatment, 6.0-fold (data not shown). With DFP as the probe, induction was 9.9- and 8.6-fold for dexamethasone and DFP, respectively (data not shown). As shown in Table 2, the induction by DFP was dose-dependent. At the highest dose of 50 \( \mu \)M DFP, the induction was comparable with that of DEX (\( \sim 85\% \)). Both activity probes (testosterone and DFP) gave very similar results. This experiment shows that treatment of hepatocytes with DFP will induce the CYP3A-mediated metabolism of DFP to DFH. Thus, metabolic auto-induction is demonstrated in vitro, and the data are consistent with the hypothesis that it is caused by CYP3A induction.

Interestingly, the relative amounts of induction of CYP3A as measured by Western blot or by activity differ (Table 2), with Western blot giving higher values. Western blot analysis detects immunoreactive protein and will, therefore, represent the total of both active and inactive apoprotein. The induction process may result in some apoprotein formation, which is detected by Western blot but not by the activity assays.

In vivo rat studies conducted over 13 weeks confirmed that auto-induction occurs in vivo. At the top dose of 100 mg/kg, the \( C_{\text{max}} \) was reduced by about 50%, and the AUC was reduced by \( \sim 80\% \) (Fig. 6). Separate in vivo studies in the rat demonstrated that DFP is not excreted as the parent drug (Halpin et al., 1998). The major in vivo metabolite is a derivative of DFH, suggesting that the process leading to dealkylation is the most important oxidative pathway in vivo. Because no parent drug is excreted, oxidation is required to effect clearance of DFP and is presumably the rate-limiting step in its elimination. This is the pathway affected by CYP3A induction, and therefore the mechanism of auto-induction observed both in vitro and in vivo is consistent with involvement of the inducible CYP3A isozymes.

The in vitro DFP doses of 2 to 50 \( \mu \)M used for the induction fell
within the range of in vivo blood levels (Fig. 5), but it is not clear that these are the physiologically relevant concentrations for induction. At the lowest dose of 10 mg/kg the \( C_{\text{max}} \) was \( \sim 20 \) \( \mu \)M, and in vitro experiments demonstrated that this should cause significant induction of CYP3A, because 10 \( \mu \)M caused \( \sim 45\% \) induction (Table 2). However, even in vivo though the \( C_{\text{max}} \) was reduced after 13 weeks of dosing, the AUC was unaltered, indicating that the auto-induction was not significant. If the concentrations in vitro reflect those in vivo, one would have expected a more pronounced effect at this dosage.

Plasma protein binding of DFP is approximately 60\% (Halpin et al., 1998), indicating that the free fraction of the \( C_{\text{max}} \) concentrations are only \( \sim 12, 24, \) and 48 \( \mu \)M at the three doses of 10, 30, and 100 mg/kg. The hepatocytes were cultured in serum-free medium, suggesting that there should be a greater free fraction than in vivo. However, this is complicated by the fact that healthy hepatocytes synthesize and secrete albumin into the medium, and thus the free fraction in vitro may be lower than the nominally added concentrations. Even with these factors taken into consideration, induction in vivo is lower than expected from in vitro results.

Recent findings about the molecular basis for the induction of CYP3A have shown that gene expression occurs via receptor-driven mechanisms. The process involves binding of a drug to the pregnane X receptor, which then forms a dimer with the 9-cis-retinoic acid receptor. The dimer binds to a response element in the CYP3A promoter, initiating the induction of mRNA (Kliewer et al., 1998; Lehmann et al., 1998). In the case of DFP, it is impossible to know the actual concentrations of drug presented to the pregnane X receptor.

There are several possible explanations why the in vivo induction is less, for a given concentration, than the in vitro data would suggest. Possibly, this discrepancy lies in the temporal exposure of the hepatocytes to DFP in vivo and in vitro. Following in vivo treatment, peak drug levels are reached within 1 to 4 h but then decrease rapidly, such that (at the 10-mg/kg dose, at least) the hepatocytes may have been exposed to effective concentrations of the drug only briefly each day. By contrast, following dosing in vitro, the hepatocytes may be exposed to effective concentrations of the drug for a longer period of time. This difference in exposure time may contribute to the lower induction observed in vivo, as compared with in vitro. Other factors such as cell permeability, transcellular transport, plasma versus intracellular hepatocyte concentration, and distribution of DFP within the hepatocyte could also play roles. These are further confounding factors that make it difficult to extrapolate from in vitro to in vivo.

Regardless of these discrepancies and uncertainties, the use of in vitro models such as this one still have a place at the “discovery” stage in drug development, especially since numerous compounds need to be evaluated and often little compound is available. Furthermore, with the species differences known for induction (Wrighton et al., 1985; Kocarek et al., 1995), cultured human hepatocyte cell models remain the best way to predict the human induction response (for example, Pichard et al., 1990; Donato et al., 1995; Silva et al., 1998; Kostrubsky et al., 1999). To this end, the rat study described herein is useful to understand and validate the correlation between in vitro and in vivo auto-induction. This will aid the interpretation of analogous human hepatocyte auto-induction studies and allow us to better predict this phenomenon in humans.

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