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INDUCTION OF CYTOCHROME P450 3A4 IN PRIMARY HUMAN HEPATOCYTES AND ACTIVATION OF THE HUMAN PREGNANE X RECEPTOR BY TAMOXIFEN AND 4-HYDROXYTAMOXIFEN

PANKAJ B. DESAI, SRIKANTH C. NALLANI, RUCHA S. SANE, LINDA B. MOORE, BRYAN J. GOODWIN, DONNA J. BUCKLEY, AND ARTHUR R. BUCKLEY

Division of Pharmaceutical Sciences, College of Pharmacy, and Department of Molecular and Cellular Physiology, University of Cincinnati Medical Center, Cincinnati, Ohio (P.B.D., S.C.N., R.S.S., D.J.B., A.R.B.); and Departments of High-Throughput Biology and Systems Research, GlaxoSmithKline Inc., Research Triangle Park, North Carolina (L.B.M., B.J.G.)

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ABSTRACT:
Tamoxifen is a widely utilized antiestrogen in the treatment and chemoprevention of breast cancer. Clinical studies document that tamoxifen administration markedly enhances the systemic elimination of other drugs. Additionally, tamoxifen enhances its own clearance following repeated dosing. The mechanisms that underlie these clinically important events remain unresolved. Here, we report that tamoxifen and its metabolite 4-hydroxytamoxifen markedly induce cytochrome P450 3A4, a drug-metabolizing enzyme of central importance, in primary cultures of human hepatocytes. Tamoxifen and 4-hydroxytamoxifen (1–10 μM) significantly increased the CYP3A4 expression and activity (measured as the rate of testosterone 6β-hydroxylation). Maximal induction was achieved at the 5 μM level. At this level, tamoxifen and 4-hydroxytamoxifen caused a 1.5- to 3.3-fold (mean, 2.1-fold) and 3.4- to 17-fold (mean, 7.5-fold) increase in the CYP3A4 activity, respectively. In comparison, rifampicin treatment resulted in a 6- to 16-fold (mean, 10.5-fold) increase. We also observed corresponding increase in the CYP3A4 immunoreactive protein and mRNA levels. Furthermore, tamoxifen and 4-hydroxytamoxifen efficaciously activated the human pregnane X receptor (hPXR; also known as the steroid xenobiotic receptor), a key regulator of CYP3A4 expression. The efficacy of tamoxifen and 4-hydroxytamoxifen relative to rifampicin for hPXR activation was ~30 and 60%, respectively. Our results indicate that the mechanism of tamoxifen-mediated alteration in drug clearance pathways in humans may involve CYP3A4 induction by the parent drug and/or its metabolite. Furthermore, the CYP3A4 induction may be a result of hPXR activation. These findings have important implications for optimizing the use of tamoxifen and in the development of newer antiestrogens.

Tamoxifen, a nonsteroidal triphenylethylene, is currently the endocrine therapeutic agent of choice for all stages of breast cancer. It was also recently approved for use as a chemopreventive agent in women with high risk of contracting this disease in the future. Despite its well documented beneficial effects, tamoxifen use is associated with several major problems including serious drug-drug interactions. Several clinical trials indicate that tamoxifen reduces the plasma levels of coadministered compounds. In this regard, recently completed clinical trials indicate that tamoxifen reduced the plasma levels of aromatase inhibitors letrozole and anastrozole by 37 and 27%, respectively (Dowsett et al., 1999; ATAC Trialists’ Group, 2001). Such drug-drug interactions are of special concern with tamoxifen since numerous women are required to take tamoxifen daily for an extended time period and as such are likely to be simultaneously exposed to many drugs and nutraceuticals. Another consequence of tamoxifen-induced changes in drug disposition is that tamoxifen pharmacokinetics exhibit time-dependent changes. There are marked differences in the single dose versus steady-state (when multiple doses have been taken) pharmacokinetics of tamoxifen (Etienne et al., 1989). The relative abundance of tamoxifen (as a fraction of the combined levels of tamoxifen and its metabolites) is lower at steady state compared with that following single dose. This is accompanied with increased relative abundance of tamoxifen metabolites at steady state. The extent of tamoxifen conversion to its metabolites has serious implications for its efficacy and toxicity since some of its metabolites are potent antiestrogens whereas some others are impli-
cated as causative in the process of endometrial carcinogenesis associated with long-term tamoxifen use (Clarke et al., 2001). Taken together, these clinical studies suggest that tamoxifen increases its own systemic clearance as well as that of other drugs in humans.

The mechanisms that underlie the above-indicated tamoxifen-mediated changes in drug clearance in humans are poorly understood. In humans, tamoxifen is extensively metabolized to several active and inactive products primarily by the cytochrome P450 enzyme CYP2C8/9 (Crewe et al., 1997). Tamoxifen metabolites include 4-hydroxytamoxifen, which is a potent antiestrogen (~100-fold more active than tamoxifen), CYP3A4 is a drug-metabolizing enzyme of central importance since it participates in the metabolism of numerous xenobiotics. Induction of CYP3A4 activity by xenobiotics has profound clinical implications. For example, known P450 inducers such as rifampicin, phenobarbital, dexamethasone, and hyperforin (the active ingredient in St. John’s Wort) enhance the clearance of coadministered drugs, thereby reducing their efficacy (Pichard et al., 1990; Moore et al., 2000; Relling et al., 2000). Also, an enzyme inducer may serve as a substrate for the induced enzyme, stimulating its own metabolism. Such compounds exhibit altered pharmacokinetics under the conditions of a repeated drug administration schedule, requiring careful optimization of drug dosing regimens.

Recent studies have identified the human pregnane X receptor (hPXR, also known as steroid xenobiotic receptor) as a key transcriptional regulator of the CYP3A4 gene (Bertilsson et al., 1998; Blumberg et al., 1998; Lehmann et al., 1998). It is activated by a diverse array of xenobiotics, most notably rifampicin, phenobarbital, clotrimazole, RU486 (mifepristone), and hyperforin (Bertilsson et al., 1998; Blumberg et al., 1998; Lehmann et al., 1998; Jones et al., 2000). As a heterodimer with the 9-cis retinoic acid X receptor, hPXR binds its cognate recognition elements within the 5′-flanking region of the CYP3A4 gene. The CYP3A4 promoter harbors an everted repeat (ER6) of the AT/G/T/TCA hexad, which serves as a binding site for hPXR-9-cis retinoic acid X receptor heterodimers. In transient transfection experiments, heterologous reporter gene constructs containing multimerized copies of this element are activated in a hPXR-dependent manner (Goodwin et al., 1999). In this study, we show that tamoxifen and 4-hydroxytamoxifen markedly induce CYP3A4 activity and expression in primary human hepatocytes. 4-Hydroxytamoxifen in particular appears to have induction magnitude comparable with that of rifampicin. Furthermore, both antiestrogens efficaciously activated hPXR in cell-based reporter assays.

### Materials and Methods

**Chemicals and Reagents.**Tamoxifen, 4-hydroxytamoxifen, rifampicin, phenobarbital, testosterone, and 6β-hydroxytestosterone were purchased from Sigma-Aldrich (St. Louis, MO).

**Hepatocyte Culture and Drug Treatment.** Human hepatocytes, isolated from lobes of liver from five separate donors, were provided by Dr. Stephen Strom, Department of Pathology, University of Pittsburgh (Pittsburgh, PA), under the auspices of the liver tissue procurement and distribution system (LTPADS). Table 1 summarizes the medical history of the donors and the medications they received prior to organ donation. Hepatocytes were grown in Williams’ E medium (BioWhittaker, Walkersville, MD) as described previously (Kostrubsky et al., 1998). For the determination of CYP3A4 activity and immunoreactive protein levels, hepatocytes were plated in collagen-coated six-well plates (1 × 10^6 cells/well). In parallel, cells were plated in T-25 cm² flasks for Northern blot analysis of CYP3A4-specific mRNA. Drug solutions (1000×) were prepared in DMSO and diluted prior to use. Forty-eight hours after isolation and plating, hepatocytes were treated with vehicle, which contained the same amount of DMSO (0.1%), tamoxifen and 4-hydroxytamoxifen (1–10 μM), rifampicin (10 μM), or phenobarbital (2 mM). Previously reported studies from our laboratory and those of others (Kostrubsky et al., 1998; Nallani et al., 2001) have shown that DMSO at levels twice as high as those used here do not alter the expression of CYP3A4 enzymes. Drug-containing medium was removed 72 h later, and the cells were then incubated for 30 min in drug-free medium to facilitate drug elimination. Cells were then washed with buffer and exposed to testosterone-containing (250 μM) medium. The medium was then collected for HPLC analysis, and the cells processed for protein isolation.

**Measurement of CYP3A Activity.** The rate of testosterone conversion to 6β-hydroxytestosterone, a reaction catalyzed by CYP3A4 (and by CYP3A5), by intact hepatocytes was used to assess the enzyme activity. 6β-hydroxytestosterone levels were measured using a HPLC method published previously (Waxman et al., 1983).

**Immunodetection of CYP3A4 Protein.** Protein fractions (3 μg) were resolved employing SDS-polyacrylamide gel electrophoresis (12% acrylamide) and transferred to nitrocellulose membranes. The membranes were then blocked with 3% bovine serum albumin in phosphate-buffered saline supplemented with 0.1% Tween 20 for 45 min and then treated with primary anti-CYP3A4 antibody (Gentest, Woburn, MA), which cross-reacts with CYP3A5, followed by horseradish peroxidase-conjugated anti-mouse secondary antibody. The protein bands were visualized using enhanced chemiluminescence detection (Amersham Biosciences, Piscataway, NJ) and quantitated by photodensitometry.

**Northern Blot Analysis of CYP3A4 mRNA.** Total cellular RNA was isolated using TRIzol (Invitrogen, Carlsbad, CA). A 10-μg aliquot was fractionated by electrophoresis in 1% agarose gels containing formaldehyde (2.2 M) and transferred onto a nylon membrane (Millipore Corp., Bedford, MA). Equal loading per lane was verified by ethidium bromide staining of 18S and 28S rRNA, which was visualized and photographed under UV illumination. The membranes were hybridized as described earlier (Church and Gilbert, 1984) with CYP3A4 cDNA probe (780-bp base pair; Oxford Biomedical Research, Inc., Oxford, MI) labeled with [α-32P]dCTP (PerkinElmer Life Sciences, Boston, MA) using the random primer method.

**Transient Transfection Assays.** Human liver-derived HuH7 cells (20,000 per well) were inoculated into a 96-well plate in Dulbecco’s modified Eagle’s medium/F12 nutrient mixture supplemented with 10% charcoal/dextran-treated fetal bovine serum (HyClone Laboratories Inc., Logan, UT) and transfected 24 h later with LipofectAMINE Plus reagent (Invitrogen). Transfection mixes contained 8 ng of XREM-CYP3A4-luciferase reporter gene construct (Goodwin et al., 1999), 2 ng of hPXR expression vector pSG5-ATG-hPXR.
Results and Discussion

We first examined the influence of tamoxifen and 4-hydroxytamoxifen on the CYP3A4 activity (6β-hydroxytestosterone formed per minute per milligram of protein) as well as CYP3A4 immunoprotein and mRNA levels in drug-treated hepatocytes, relative to the untreated controls. The net increase in the testosterone 6β-hydroxylase activity in the hepatocytes treated with the antiestrogens and well known CYP3A4 inducers, rifampicin and phenobarbital, are tabulated in Table 2. Both tamoxifen and 4-hydroxytamoxifen significantly ($p < 0.05$, $n = 4$) increased the CYP3A4 activity at concentrations ranging from 1 to 10 μM. The maximal response was generally observed at antiestrogen levels of 5 μM. At this level, tamoxifen and 4-hydroxytamoxifen caused a 1.5- to 3.3-fold (mean, 2.1-fold) and 3.4- to 17-fold (mean, 5.3-fold) increase in the CYP3A4 activity, respectively. The differences in the response between 5 and 10 μM were not statistically significant ($p > 0.05$). In comparison, rifampicin treatment resulted in a 6- to 16-fold (mean, 10.5-fold) increase. The effect of the antiestrogens on the amount of CYP3A4-specific immunoreactive protein and mRNA levels is shown in Fig. 1. The fold increase relative to untreated control is tabulated in Table 2. In general, a good agreement was observed between the CYP3A4 activity and immunoreactive protein levels ($R^2 = 0.84$; plot not shown) and between the CYP3A4 mRNA and immunoreactive protein levels ($R^2 = 0.86$; plot not shown).

It is important to note that CYP3A5 is also capable of metabolizing testosterone to 6β-hydroxytestosterone and the antibody used for immunodetection of CYP3A4 cross-reacts with CYP3A5. Generally, however, the expression of CYP3A5 relative to CYP3A4 is low (except when expressed polymorphically), and the rate of testosterone β-hydroxylation is much slower for CYP3A4 than CYP3A5 (Wrighton and Thummel, 2000). Therefore, it is possible that our assessment of the activity and expression of CYP3A4 may include a potential, albeit minor, contribution by CYP3A5.

We next evaluated whether these compounds activated hPXR in HuH7 cells cotransfected with a hPXR expression vector and a reporter plasmid containing CYP3A4 proximal promoter region (bases −362 to +53) linked to the distal XREM region. In parallel transfection experiments, we compared the hPXR activation profiles of the antiestrogens and rifampicin (Fig. 2). Tamoxifen and 4-hydroxytamoxifen (5 μM each) were ~30 and 60%, respectively, as efficacious as rifampicin in hPXR activation.

It is apparent from the above results that tamoxifen and 4-hydroxytamoxifen markedly induce CYP3A4, resulting in significant increase in the enzymatic activity. Here, two observations are noteworthy. First, the CYP3A4 induction in human hepatocytes by tamoxifen is comparable with other established inducers including hyperforin and paclitaxel (Kostrubsky et al., 1998; Moore et al., 2000). Second, the magnitude of CYP3A4 induction by 4-hydroxytamoxifen is comparable with that of rifampicin, one of the most potent CYP3A4 inducers. These results provide important insight into mechanisms by which tamoxifen administration results in altered drug elimination pathways in humans. Although previous studies have shown that tamoxifen induces P450 enzymes in rats (White et al., 1993), to our knowledge the influence of tamoxifen on human CYP3A4 expression has not been reported. In fact, the lack of this information has been indicated as a significant deficiency in recent publications that documented tamoxifen interactions with the aromatase inhibitors (Dowsett et al., 1999; Ingle et al., 1999; ATAC Trialists’ Group, 2001). It is

Table 2. Both tamoxifen and 4-hydroxytamoxifen significantly ($p < 0.05$, $n = 4$) increased the testosterone 6β-hydroxylase activity, immunoreactive protein, and mRNA levels in hepatocytes treated with tamoxifen, 4-hydroxytamoxifen, rifampicin, and phenobarbital, relative to untreated control.

<table>
<thead>
<tr>
<th>CYP3A4 activity*</th>
<th>10 μM</th>
<th>Phenobarbital*</th>
<th>2 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tamoxifen</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYP3A4 activity</td>
<td>1 μM</td>
<td>5 μM</td>
<td>10 μM</td>
</tr>
<tr>
<td>10.54 ± 1.89</td>
<td>8.95 ± 2.97</td>
<td>1.30 ± 0.15</td>
<td>2.12 ± 0.77</td>
</tr>
<tr>
<td>CYP3A4 protein levels</td>
<td>4 μM</td>
<td>3.3 ± 0.37</td>
<td>4.8 ± 1.63</td>
</tr>
<tr>
<td>CYP3A4 mRNA levels</td>
<td>2 μM</td>
<td>13.46 ± 6.77</td>
<td>12.01 ± 5.3</td>
</tr>
</tbody>
</table>

*Rifampicin and phenobarbital were employed as positive controls for CYP3A4 induction.

Fig. 1. Effect of tamoxifen and 4-hydroxytamoxifen on the CYP3A4 immunoreactive protein and mRNA levels. A, representative Western blots depicting immunoreactive CYP3A4 protein levels in human hepatocytes treated for 72 h with tamoxifen (Tam, 1–10 μM), 4-hydroxytamoxifen (4-HT, 1–10 μM), phenobarbital (PB, 2 mM), rifampicin (Rif, 10 μM), and vehicle-treated controls (C) are shown. Protein fractions (3 μg) were resolved employing SDS-polyacrylamide gel electrophoresis (12% acrylamide) and transferred to nitrocellulose membranes. The immunoreactive CYP3A4 protein levels in various treatments were identified using a monoclonal anti-human CYP3A4 primary antibody and visualized using a horseradish peroxidase-linked secondary anti-mouse antibody by an enhanced chemiluminescence reaction.
well known that the induction of P450 enzymes is species-specific. For instance, although rifampicin is a potent P450 inducer in humans, it is a poor inducer of these enzymes in rodents. In fact, tamoxifen also exhibits interspecies variability in enzyme induction, and as such, it is not an inducer of CYP3A enzymes in mice (White et al., 1993). Therefore, the observations made in this study regarding tamoxifen-mediated CYP3A4 induction in human hepatocytes could not have been extrapolated from the animal studies. Accordingly, this study serves to provide clinically relevant information. Moreover, our novel finding includes the observation that 4-hydroxytamoxifen is a potent CYP3A3 inducer.

The extrapolation of data from our in vitro study to the in vivo situation should be done with a consideration of several factors. At a typically used tamoxifen regimen (20 mg b.i.d.), the steady-state tamoxifen plasma concentrations range between 0.1 and 1 μM. Furthermore, tamoxifen is highly bound to plasma proteins (>90%). In the case of 4-hydroxytamoxifen, a minor metabolite, the plasma concentrations are only in the range of 0.01 to 0.1 μM. However, both these compounds are highly lipophilic with extensive tissue distribution. The apparent distribution volume of tamoxifen is about 50 to 60 l/kg, which indicates that most of the administered drug (99.9%) is present in peripheral compartments (Lien et al., 1989). In humans, the liver uptake is particularly high with the hepatic levels of these compounds being ~60-fold higher than that in serum (Lien et al., 1991). This suggests that tamoxifen has much higher affinity for hepatic tissue than for the plasma proteins. Given that the equilibrium of drug binding to plasma proteins and tissues is dynamic in nature for drugs with high partitioning into lipids, the overall process favors tissue binding and accumulation. Therefore, it is conceivable that the intrahepatic tamoxifen levels achieved in vivo may be in the range used in our study. Also, since the tamoxifen elimination half-life in humans is extremely long (~7 days), the relative fluctuations between peak and trough levels during steady state is minimal. An important point to consider here is that tamoxifen is required to be taken continuously for many years. Therefore, in vivo exposure of hepatocytes to tamoxifen and 4-hydroxytamoxifen occurs for an extremely long time period. As such, it is likely that tamoxifen and 4-hydroxytamoxifen may induce CYP3A4 at concentrations lower than those used here. Finally, it is noteworthy that frequently 40-mg b.i.d. doses of tamoxifen are used and clinical trials employing high doses of tamoxifen (120 mg/m² b.i.d.) for the treatment of brain tumor are also in progress (Ducharme et al., 1997). In these studies, the plasma tamoxifen levels ranged from ~1 to 8 μM.

Tamoxifen and 4-hydroxytamoxifen are well known mixed antagonists or partial agonists of estrogen receptors, which are complex ligand-induced transcriptional factors belonging to the hormone nuclear receptor superfamily. In this study, we show that these agents also interact with hPXR (NR1I2), a recently cloned member of this family (Bertilsson et al., 1998; Blumberg et al., 1998). We used cell-based reporter assays to demonstrate that antiestrogens activate hPXR. In general, there was good agreement between the CYP3A4 induction and hPXR activation, which suggests that CYP3A4 induction may be a result of hPXR activation by the antiestrogens. Further studies are in progress to assess whether the antiestrogens are bona fide ligands for hPXR. In addition to hPXR, other nuclear receptors such as the constitutive androstane receptor (NR1I3) may also play a role in CYP3A4 regulation (Xie et al., 2000). The identification of hPXR and/or other receptors as key transcriptional regulators of CYP3A4 expression by tamoxifen and 4-hydroxytamoxifen has important implications for the development of newer antiestrogens. High-throughput screening methods based on nuclear receptor binding and activation can be utilized to evaluate the potential of a range of investigational compounds to induce CYP3A4. These methods are likely to accelerate the development of antiestrogens, which is a global priority since these compounds target a myriad of female-specific diseases, including breast cancer.

References


FIG. 2. Activation of hPXR by tamoxifen and 4-hydroxytamoxifen.

HuH7 cells were transiently transfected with the pSG5-hPXR ATG expression plasmids and the XREM-CYP3A4-luciferase reporter plasmid. Cell extracts were subsequently assayed for luciferase activity and normalized to secreted placentald alkaline phosphatase activity used as a control for transfection efficiency (mean ± S.E.M., n = 3). Fold increase in the reporter activity following treatment with tamoxifen (white bar), 4-hydroxytamoxifen (black bar), and rifampicin (gray bar) are shown.
receptor: a promiscuous xenobiotic receptor that has diverged during evolution. Mol Endocrinol 14:27–39.


