Effect of Tamoxifen on the Enzymatic Activity of Human Cytochrome CYP2B6

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Received December 3, 2001; accepted February 19, 2002 This article is available online at http://jpet.aspetjournals.org

ABSTRACT

Tamoxifen is primarily used in the treatment of breast cancer. It has been approved as a chemopreventive agent for individuals at high risk for this disease. Tamoxifen is metabolized to a number of different products by cytochrome P450 enzymes. The effect of tamoxifen on the enzymatic activity of bacterial P450s has been studied. The 7-ethoxy-4-(trifluoromethyl)coumarin O-deethylation activity of purified P450 was inactivated by tamoxifen in a time- and concentration-dependent manner. Enzymatic activity was lost only in samples that were incubated with both tamoxifen and NADPH. The inactivation was characterized by a K_d of 0.9 μM, a k_inact of 0.02 min⁻¹, and a t_1/2 of 34 min. The loss in the 7-ethoxy-4-(trifluoromethyl)coumarin O-deethylation activity did not result in a similar percentage loss in the reduced carbon monoxide spectrum, suggesting that the heme moiety was not the major site of modification. The activity of CYP2B6 was not recovered after removal of free tamoxifen using spin column gel filtration. The loss in activity seemed to be due to a modification of the CYP2B6 and not reductase because adding fresh reductase back to the inactivated samples did not restore enzymatic activity. A reconstituted system containing purified CYP2B6, NADPH-reductase, and NADPH-generating system was found to catalyze tamoxifen metabolism to 4-OH-tamoxifen, 4’-OH-tamoxifen, and N-desmethyl-tamoxifen as analyzed by high-performance liquid chromatography analysis. Preliminary studies showed that tamoxifen had no effect on the activities of CYP1B1 and CYP3A4, whereas CYP2D6 and CYP2C9 exhibited a 25% loss in enzymatic activity.

Cytochromes P450 are hemoproteins that catalyze the metabolism of a diverse group of xenobiotics (drugs, environmental pollutants, pesticides, and herbicides) and several biologically active endogenous compounds (steroids, hormones, and fatty acids) (Gonzalez, 1989; Porter and Coon, 1991). Cytochromes P450 are characterized by an absorbance maximum at 450 nm in the reduced CO-bound state (Porter and Coon, 1991; Oritz de Montellano, 1996). The catalytic mechanism of all P450 enzymes seems to involve the incorporation of one atom of molecular oxygen into the substrate with the concomitant formation of a molecule of water from the second oxygen (Gonzalez, 1989; Porter and Coon, 1991). With the exception of a low-resolution structure of modified CYP2C5 (Williams et al., 2000), little three-dimensional structural information concerning the critical amino acid residues in the active site of mammalian P450s is available. Therefore, other techniques, such as site-directed mutagenesis (Johnson et al., 1992; Hanna et al., 1998), mechanism-based inactivation (Kent et al., 2001), photoaffinity labeling, and comparison with crystal structures of bacterial P450s have been used to identify important residues involved in substrate binding and catalysis.

Tamoxifen, Z-[1-[4-(2-dimethylaminoethoxy)phenyl]-1,2-diphenyl-1-butene], is a nonsteroidal antiestrogen (Fig. 1) that is used in the treatment of all stages of hormone-dependent breast cancer (Furr and Jordan, 1984). The Food and Drug Administration also has approved the use of tamoxifen as a chemopreventive agent in women who are at risk for developing breast cancer (Fisher et al., 1998). Tamoxifen is metabolized by human P450s 3A4, 2C9, 2B6, 2C8, 2C19, 2D6, 1A1, 1A2, and 2A6 (Jacolot et al., 1991; Crewe et al., 1997; Dehal and Kupfer, 1997, 1999) and is also an effective inducer of P450s 2B2, 2B1, and 3A in rat liver at doses comparable with the therapeutic doses used in humans (Emile et al., 1995). Tamoxifen is a known liver carcinogen in rats (Williams et al., 1993) and tamoxifen-DNA adducts have been detected in rats administered tamoxifen (Han and Liehr, 1992). Epidemiological evidence showed a link between increased risk of developing endometrial cancer and tamoxifen therapy in humans (Fisher et al., 1994; van Leeuwen et al., 1994; Stearns and Gelman, 1998) and tamoxifen-
DNA adducts have been identified in the endometria of women treated with tamoxifen (Ravindernath et al., 2000). Extensive hepatic metabolism of tamoxifen has been described and the major metabolites formed are tamoxifen-N-oxide, N-desmethyl-tam, and 4-OH-tam (Fig. 1) (Fromson et al., 1973; Bates et al., 1982). Numerous studies have shown that metabolic activation of tamoxifen was a prerequisite for the generation of adducts of tamoxifen with DNA and protein (Adam et al., 1980; Mani and Kupfer, 1991) and that the activation was the result of oxidative metabolism of tamoxifen by cytochrome P450 (Mani and Kupfer, 1991; Pathak and Bodell, 1994; White et al., 1995).

Hellmold et al. (1998) demonstrated the occurrence of differential expression of P450 enzymes in human breast tissue samples. Similar expression levels of CYP1A1, 1B1, 2A6, 2B6, 2E1, 2C, 3A, and aromatase were seen in control epithelial samples and reduction mammmoplasty samples of human breast tissue, whereas increased expression of only CYP2B6 was seen in carcinoma samples (Hellmold et al., 1998). This increased expression of CYP2B6 in the human breast cancer tissues could confer vulnerability to damage through bioactivation of compounds metabolized by this enzyme. Thus, the current study was aimed at characterizing the effect of tamoxifen on the activity of CYP2B6 in a reconstituted system and identifying the metabolites formed during the process. We observed that tamoxifen inactivated CYP2B6 in a mechanism-based manner. We also report the results of our studies involving the metabolism of tamoxifen by human CYP2B6 using HPLC analysis.

**Experimental Procedures**

**Materials.** Tamoxifen (citrate salt), DLPC, bovine serum albumin, NADPH, catalase, sodium dithionite, Sephadex G-50, and HEPES were purchased from Sigma-Aldrich (St. Louis, MO). 7-EFC and 7-ethoxyresorufin were obtained from Molecular Probes (Eugene, OR). HPLC-grade methanol and acetonitrile were purchased from Mallinckrodt (Chesterfield, MO). Slide-A-Lyzer cassettes were from Pierce Chemical (Rockford, IL). N,N-Didesmethyletozifemien hydrochloride was a generous gift from Orion Farmos Pharmaceuticals (Turku, Finland). 3,4-diOH-tam, 4-OH-tam, a,4-dihydroxytamoxifen, a-OH-tam, and metabolite E were a gift from Dr. Judy Bolton (University of Illinois, Chicago, IL).

**Purification of Enzymes.** P450 NADPH-reductase was expressed in *Escherichia coli* Top3 cells and expression and purification was carried out as described previously (Hanna et al., 1998). CYP2B6 was expressed in *E. coli* MV1304 cells and purified as described previously (Hanna et al., 2000). P450s 3A4, 1B1, 2C9, and 2D6 coexpressed with reductase were expressed in *E. coli* and assayed in the bacterial membrane preparation (provided by Lesley McLaughlin, LINK at the Biomedical Research Center, Dundee, Scotland). P450s 3A4, 2D6, and 1B1 were expressed and purified as described previously (Gillam et al., 1993, 1995; Shimada et al., 1998).

**Time- and Concentration-Dependent Inactivation of CYP2B6 7-EFC Activity by Tamoxifen.** CYP2B6 was reconstituted with reductase and lipid at 4°C for 45 min. The primary reaction mixture contained 0.9 μM CYP2B6, 0.18 μM NADPH-reductase, 0.9 μM cytochrome b₅, 200 μg of DLPC, 2200 units of catalase, and 50 mM potassium phosphate buffer, pH 7.4, in a total volume of 0.75 ml. The samples then received increasing concentrations of tamoxifen in DMSO (5–80 μM) or DMSO (in the control sample). After the reaction mixtures were allowed to equilibrate at 37°C for 3 min, the reactions were initiated by the addition of 1.2 mM NADPH (primary mixture). Aliquots (12 μl, 14 pmol of CYP2B6) were removed at 0, 2, 5, 10, and 21 min and added to a secondary reaction mixture containing 1 mM NADPH, 100 μM 7-EFC, and 40 μg/ml bovine serum albumin in 50 mM potassium phosphate buffer, pH 7.4, in a volume of 988 μl. The secondary reaction was allowed to proceed at 37°C for 10 min and was then stopped with 334 μl of cold acetonitrile. The amount of 7-hydroxy-4-trifluoromethylcoumarin formed was determined spectrophotometrically on an SLM-Amino model SPF-500C spectrofluorometer with excitation at 410 nm and emission at 510 nm. In some cases 10 mM glutathione was added to the primary incubation mixtures together with 6.7 μM tamoxifen.

**Effect of Tamoxifen on CYP2B6 Heme.** CYP2B6 was reconstituted with reductase and lipid as described above and incubated in the presence of 6.7 μM tamoxifen. Inactivation was initiated by adding 1.2 mM NADPH and the reaction was allowed to proceed for 20 min and then stopped with 900 μl of quench buffer (50 mM potassium phosphate, pH 7.7, 40% glycerol, and 0.6% tertigol Nonidet P-40). The samples were then bubbled with CO for 60 s and the sodium dithionite reduced CO spectrum was recorded between 400 and 500 nm on a DW2 UV/VIS spectrophotometer equipped with an OLIS operating system (On Line Instruments Systems, Bogart, GA).

**Irreversibility of CYP2B6 Inactivation by Tamoxifen.** CYP2B6 was reconstituted as described above and inactivated with 6.7 μM tamoxifen and 1.2 mM NADPH. Control samples were incubated with tamoxifen but without NADPH. After 10 min at 37°C, 400 μl of the control and the inactivated samples were applied separately to two 5.5-ml G-50 spin columns saturated with 500 μl of 1 mg of DLPC/ml and washed extensively with 50 mM potassium phosphate buffer, pH 7.4. The columns were centrifuged as described by Fry et al. (1978). The P450-containing filtrate was collected and analyzed for enzymatic activity with 7-EFC, as described above, in the presence or absence of fresh reductase. Statistical analysis was performed using the Student’s t test.

**Protection of CYP2B6 from Inactivation by Coincubation with an Alternate Substrate.** Protection from tamoxifen-dependent inactivation of CYP2B6 was investigated by incubating reconstituted CYP2B6 together with tamoxifen and 7-EFC at a molar ratio of 1:5 (tamoxifen/7-EFC) in the primary reaction mixture. At the indicated time points, aliquots of the primary reaction mixture were transferred into the secondary reaction mixture and assayed spectrophotometrically as described above.

**Partition Ratio.** Reconstituted samples containing 0.5 μM CYP2B6 were incubated with 0 to 320 μM tamoxifen and 1.2 mM...
NADPH. The reactions were allowed to go to completion by incubating for 30 min at 37°C. Aliquots of the primary reaction mixtures were then transferred into secondary reaction mixtures and assayed for 7-EFC activity, as described above.

**Effect of Tamoxifen on Activities of Other P450s.** The effect of tamoxifen on the activities of CYP3A4 and CYP1B1 was determined by measuring testosterone-6β-hydroxylation and 7-EFC O-dealkylation, respectively. For CYP3A4, the primary reaction mixtures contained 6 μM tamoxifen together with 1 mM NADPH. At the indicated times, 50 μl of the reaction mixture (24 pmol of CYP3A4) was transferred into 950 μl of 50 mM HEPES buffer, pH 7.5, containing 200 μM testosterone and 100 μM NADPH and incubated for 20 min at 37°C. The reaction was quenched by adding 1 ml of ethyl acetate. The reaction mixture was extracted three times with ethyl acetate and the testosterone metabolites were extracted into the organic phase. The organic phases were pooled and dried under nitrogen. The samples were redissolved in 65% methanol, injected onto a C18 (4.9 × 25 cm) reverse phase column and eluted isocratically with a mobile phase of 65% methanol at a flow rate of 1 ml/min. The eluate was monitored at 254 nm and 6β-hydroxylation activity was quantitated. For CYP1B1, 25 μl of the primary reaction mixture (7.5 pmol of CYP1B1) containing 6 μM tamoxifen and 1 mM NADPH was transferred into 975 μl of a secondary mixture containing 100 μM 7-ethoxyresorufin, 1 mM NADPH, and 50 mM MgCl2 in 50 mM Tris-HCl buffer, pH 7.4. The reaction was allowed to proceed for 5 min at 37°C. Reaction mixtures were stopped by the addition of 750 μl of cold methanol and the activity was assayed spectrofluorometrically on an SLM-Aminco SPF-500C spectrofluorometer with excitation at 522 nm and emission at 586 nm (slit width 4).

The activities of CYP2C9 and 2D6 were also determined using the 7-EFC assay. For CYP2C9, the primary reaction mixture contained 6 μM tamoxifen (dissolved in DMSO) and 1.2 mM NADPH. Aliquots (240 μl, 54 pmol of CYP2C9) were transferred into 250 μl of a secondary reaction mixture containing 7-EFC and incubated as described above. The reactions were stopped with 0.1 M Tris buffer, pH 9.0, containing 30% acetonitrile. CYP2D6 assays were carried out in the same manner except that tamoxifen was dissolved in acetonitrile.

**Tamoxifen Metabolism by P450s.** Standard reaction mixtures for the metabolism of tamoxifen included 1.8 nmol of purified CYP2B6, 1.8 nmol of reductase, 200 μg of DLPC, 2000 units of catalase in 50 mM potassium phosphate buffer, pH 7.4, and 200 μM tamoxifen in DMSO. The incubations were carried out under reduced light to preserve the integrity of the metabolites. Incubation mixtures (3.2 ml) were initiated with an NADPH-regenerating system to avoid light to preserve the integrity of the metabolites. Incubation mixtures (3.2 ml) were initiated with an NADPH-regenerating system containing 1 mM NADP+, 2.5 mM glucose-6-phosphate, and 0.5 units/ml of glucose-6-phosphate dehydrogenase. The samples were incubated at 37°C for 90 min. The reactions were terminated by the addition of 1 ml of tert-butyl-methyl ether. N,N-Diemethyl-toremifene hydrochloride (100 μM of a 15 μM stock solution) was added as an internal standard to the CYP2B6 samples. The reaction mixtures containing tamoxifen and its metabolites were extracted twice with tert-butyl-methyl ether and the organic phases were combined and evaporated under nitrogen.

Tamoxifen metabolism by CYP3A4 was used as a control. CYP3A4 (0.5 nmol) was reconstituted with 20 μg of a mixture (1:1:1) of L-a-dilauroyl phosphocholine and L-a-dioleoyl-sn-glycero-3-phosphocholines and phosphatidyl serine, 1 nmol of NADPH-reductase, 0.5 nmol of cytochrome b5, 500 units of catalase, 2 mM GSH, 30 mM MgCl2, 0.5 mM EDTA, 165 μM tamoxifen, and 20% glycerol in a final volume of 1 ml of 50 mM HEPES buffer, pH 7.5. Incubations were initiated by the addition of the NADPH-regenerating system and the reactions were terminated, a small amount of internal standard was added, and the incubation mixtures extracted with ethyl acetate, as described above.

**HPLC Analysis of Tamoxifen Metabolites.** The dried, extracted metabolites were reconstituted in 100 μl of helium-purged acetonitrile, and 40 μl was injected onto a 3.9 × 150-mm Waters Symmetry C8 reverse phase column. HPLC was performed using a Shimadzu HPLC system, consisting of an LC-10AD pump, SPD-10A UV-Vis variable wavelength detector, SCL-10A system controller, and an SIL-10AXL autoinjector. Metabolites were eluted using a linear gradient of the second gradient step to optimize peak separation. Initial conditions were (v/v) ammonium acetate (solvent A) and acetonitrile (solvent B), 95:5, followed by a linear gradient to 20% B from 0 to 4 min then to 40% B over the next 20 min and to 65% B by 60 min. The percentage of B was maintained at 65% for 10 min and then lowered to 5% B over the next 10 min. Metabolites were detected by their absorbance at 280 nm. Under these conditions the retention times of tamoxifen and its main metabolites were as follows: α,4-dihydroxytamoxifen, 26.1 min; α-hydroxytamoxifen, 33.8 min; 3,4-dihydroxy-tamoxifen, 37.0 min; E-4-hydroxytamoxifen, 43.8 min; Z-4-hydroxytamoxifen, 44.9 min; 4’-hydroxytamoxifen, 46.7 min; N,N-didesmethyltamoxifen, 52.7 min; N-desmethyltamoxifen, 55.7 min; tamoxifen-N-oxide, 59.5; tamoxifen, 59.6 min; and metabolite E isomers, 61.1/63.9 min. Quantification of the CYP2B6 metabolites was performed with reference to standard curves prepared using authentic metabolites after correction for recovery of the internal standard (N,N-diemethyl toremifene hydrochloride).

**Results**

**Time- and Concentration-Dependent Inactivation of 7-EFC Activity of CYP2B6 by Tamoxifen.** In the presence of tamoxifen and NADPH, a time- and concentration-dependent inactivation of the 7-EFC O-deethylation activity of CYP2B6 was observed (Fig. 1). Pseudo first-order kinetics was observed for concentrations of tamoxifen between 5 and 80 μM. Control samples incubated without tamoxifen but with NADPH showed a loss of approximately 25% activity over a period of 20 min. The presence of cytochrome b5 did not seem to affect the kinetic parameters for the inactivation of CYP2B6 by tamoxifen. The activity of the enzyme increased 3-fold in presence of b5 but the inactivation rate remained the same in the presence or absence of b5. The kinetic constants describing the inactivation of CYP2B6 by tamoxifen were determined from the inset in Fig. 1, which was derived from the double reciprocal plot of the rate of inactivation as a function of the concentration of the inactivator. The concentration required for half-maximal inactivation (K_i) at 37°C was found to be 0.9 μM; the k_{inact}, the maximal rate of inactivation at a saturating concentration of tamoxifen was 0.02 min⁻¹, and the t_{1/2} was 34 min.

Incubation of 5 to 40 μM 4-OH-tam with purified CYP2B6 in the reconstituted system resulted in a time-dependent loss of activity with up to 60% of the loss after incubation for 10 min in the primary reaction. A time-dependent inhibition was also seen with 4-OH-tam. Kinetic constants were not determined because very low concentrations of 4-OH-tam (at or below the CYP2B6 concentrations) were sufficient to inhibit CYP2B6. Similar results were seen with the following metabolites of tamoxifen: 3,4-diOH-tam, metabolite E, α-OH-tam, and α,4-dihydroxytamoxifen (data not shown).

**Effect of Tamoxifen on CYP2B6 Heme.** The 7-EFC O-deethylation activity of CYP2B6 in the reconstituted system containing purified CYP2B6, NADPH-reductase, cytochrome b5, and lipid decreased by 73% after incubation with 80 μM tamoxifen for 10 min in the presence of NADPH (Table 1). In spite of this loss in enzymatic activity, most of
Irreversibility of CYP2B6 inactivation by tamoxifen

**TABLE 2**

<table>
<thead>
<tr>
<th>Sample</th>
<th>% Activity Remaining</th>
<th>% P450 Remaining</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 min</td>
<td>20 min</td>
</tr>
<tr>
<td>−Tam, −NADPH</td>
<td>100</td>
<td>93 ± 2</td>
</tr>
<tr>
<td>+Tam, −NADPH</td>
<td>80 ± 2</td>
<td>71 ± 4</td>
</tr>
<tr>
<td>+Tam, +NADPH</td>
<td>79 ± 7</td>
<td>27 ± 4</td>
</tr>
</tbody>
</table>

the spectrum of the reduced CO complex of the P450 was retained. In control samples incubated with tamoxifen but without NADPH, the activity loss was approximately 25%. This was presumably because of competition or inactivation by tamoxifen that was carried over into the secondary reaction mixture. The concentration of tamoxifen in the secondary assay mixture was 1 μM. In control samples incubated with NADPH and without tamoxifen, the activity loss was about 20%. In control samples that received neither tamoxifen nor NADPH, there was a minimal loss in both activity and reduced CO spectrum.

**Irreversibility of CYP2B6 Inactivation by Tamoxifen.** Table 2 shows the activity of the noninactivated and tamoxifen-inactivated CYP2B6 samples in comparison with samples where free tamoxifen had been removed by gel filtration spin columns. Under these conditions greater than 90% of the initial amount of tamoxifen in the sample was removed when samples were analyzed by HPLC before and after treatment (data not shown). After dilution of the column-treated samples into the 7-EFC-containing assay buffer the residual tamoxifen concentration was 0.02 μM or 45-fold below the Kᵢ for inactivation of CYP2B6 and would therefore not be expected to interfere with the assay. Enzymatic activity of the tamoxifen-inactivated enzyme was not restored after filtration, suggesting that the inactivation was not due to competition but was irreversible under these conditions. Similarly, addition of fresh reductase back to the inactivated samples did not result in a recovery in CYP2B6 activity. There was no statistical significance between the activities of the samples incubated with reductase compared with samples not supplemented with reductase. The slightly greater loss in enzymatic activity of the samples after gel filtration may reflect an inability to immediately stop the inactivation reaction.

**Protection against CYP2B6 Inactivation by Coincubation with an Alternate Substrate.** Addition of an alternate substrate, 7-EFC, in the primary incubation mixture together with tamoxifen slowed the rate of CYP2B6 inactivation, thus protecting the enzyme from tamoxifen-dependent inactivation. As seen in Fig. 2, incubation of CYP2B6 in the presence of 7-EFC and tamoxifen at a molar ratio of 5:1 reduced the rate of inactivation by nearly 50%.

**Partition Ratio.** The number of tamoxifen molecules required to inactivate one molecule of CYP2B6 was estimated from the data shown in Fig. 3. Increasing molar ratios of tamoxifen were added to a solution containing 0.5 μM CYP2B6 in the reconstituted system. The samples were incubated until no further decrease in the 7-EFC activity was observed. The percentage of activity remaining was plotted as a function of the ratio of the inactivator to enzyme concentration. The partition ratio (defined as the turnover number − 1) was then extrapolated from the intercept between the linear regression line of the lower inactivator concentrations and the straight line obtained from the higher ratios of inactivator. In this way, a partition ratio of 76 was determined.

**Effect of Tamoxifen on Activities of Other P450s.** The effect of tamoxifen on P450s 3A4, 1B1, 2C9, and 2D6 was determined as described under Experimental Procedures. The 7-EFC O-deethylation activity of membrane-bound CYP2C9 and 2D6 was inhibited in a time-dependent manner as seen in Fig. 4, A and B, respectively. Approximately 40 and 28% of the enzymatic activity of CYP2D6 and 2C9, respectively, was lost with 6 μM tamoxifen when assayed for 30 min. No further increase in the rate of inactivation was observed for either CYP2D6 or 2C9 when the concentration of tamoxifen was increased to 96 μM (data not shown). The testosterone-6β-hydroxylation activity of CYP3A4 was not inhibited by incubation with 6 or 96 μM tamoxifen in the reconstituted system (data not shown). The 7-ethoxy-O-dealkylation activity of CYP1B1 was also not inhibited by 6 or 96 μM tamoxifen (data not shown).

**Metabolism of Tamoxifen by CYP2B6.** Metabolism of tamoxifen by CYP2B6 in the reconstituted system was exam-
ined using reverse phase HPLC with UV detection of the metabolites at 280 nm (Fig. 5). In the presence of NADPH, N-desmethyl-tam (peak a, 55.8 min) was the major product. In addition, three minor products, 4-OH-tam (peak b, 44.2 min), 4'-OH-tam (peak c, 46.9 min), and one unidentified minor metabolite (peak d), were observed (Fig. 5A). The production of 4'-OH-tam was previously shown with human cytochrome P450 enzymes (Crewe et al., 1997). The identity of the products was determined from their retention times compared with the retention times of the authentic standards. A minor peak eluted at the same retention time as N-desmethyl-tam in the $t=0$ sample (Fig. 5B). Product peaks b to d were not seen in samples to which tamoxifen was not added (Fig. 5C). The results in Table 3 show that the major product was N-desmethyl-tam and that it accounted for more than 50% of the total amount of metabolites formed. Approximately 10 to 15% of the total amount of tamoxifen present in the incubation mixture was recovered in the form of three major metabolites observed (Table 3). The initial rate of formation of the N-desmethyl-tam could not be calculated because CYP2B6 was inactivated over the course of the incubation. Control incubations of tamoxifen and CYP3A4 in the presence of NADPH showed the formation of N-desmethyl-tam, 4-OH-tam, and $\alpha$-OH-tam (Fig. 5D). A negligible amount of 4'-OH-tam was also formed.

**Discussion**

The data presented herein demonstrate that tamoxifen is a mechanism-based inactivator of CYP2B6 in the reconstituted system. The experimental criteria that were used to establish mechanism-based inactivation were time-, NADPH-, and concentration-dependent inactivation, substrate protection, and irreversibility. The estimated turnover number of 77 (76 product molecules generated for each inactivation event) was higher than observed for another mechanism-based inactivator of CYP2B6 (Kent et al., 2001) and is consistent with the slow rate of inactivation of CYP2B6 by tamoxifen ($k_{\text{inact}} = 0.02; t_{1/2} = 34$ min). In contrast, the $K_i$ for the inactivation of CYP2B6 by tamoxifen was low (0.7 $\mu$M) and indicative of a relatively high affinity of tamoxifen for CYP2B6. Although a loss of 73% of the enzymatic activity was observed in 20 min in samples incubated with both NADPH and tamoxifen, a minimal loss in the reduced CO binding spectrum was seen (<10%). This observation suggested that the inactivation was not due to heme destruction but rather to a modification of the apoprotein. Additional evidence that the heme moiety was not destroyed came from HPLC analysis where the heme was monitored at 405 nm. Virtually no difference in the heme peak was observed between the control and tamoxifen-inactivated CYP2B6 (data not shown). No recovery of the activity was seen when free tamoxifen was removed from the inactivated samples, suggesting that the inactivation was irreversible under these conditions. Addition of fresh reductase to the tamoxifen-inactivated samples did not slow the rates of the inactivation reaction did not slow the rates of the inacti-
vation, suggesting that the reactive intermediate did not leave the active site of CYP2B6 (data not shown). Quinones and hydroxyquinones have been shown to inhibit via a redox cycling mechanism (Fan et al., 2000). Our observations suggest that reductase was unaffected and that such a redox cycling mechanism does not apply in the loss in activity of CYP2B6 after incubation with tamoxifen and NADPH.

Competition by the alternate substrate 7-EFC during the incubation with tamoxifen and NADPH significantly slowed the rate of tamoxifen-dependent loss in CYP2B6 activity. This suggested that the inactivation event occurred at the active site of CYP2B6.

The metabolism of tamoxifen by CYP2B6 generated four metabolites. N-desmethyl-tam was the predominant product generated by CYP2B6 in the reconstituted system. Other products included 4-OH-tam and 4'-OH-tam. The unidentified minor peak d could be a secondary metabolite of tamoxifen or an isomer of a primary metabolite. The level of this metabolite was too low for further structural identification.

Interestingly, α-OH-tam was not generated by CYP2B6. This was in contrast to the metabolism of tamoxifen by 3A4 as shown in Fig. 5D, where in addition to N-desmethyl-tam, 4-OH-tam, 4'-OH-tam, and a large α-OH-tam peak was seen.

Previous studies have shown that 4-OH-tam exhibited a much greater affinity for the estrogen receptor than tamoxifen (Furr and Jordan, 1984). In addition, tamoxifen and 4-OH-tam have been shown to produce identical patterns of adducts by ^32^P postlabeling and higher levels of adduct were reported when 4-OH-tam was used compared with tamoxifen (Pathak et al., 1995). Although the reactive metabolite responsible for the inactivation of CYP2B6 has not yet been identified, formation of a catechol from the activated 4-OH-tam has been shown (Fan et al., 2000; Zhang et al., 2000). Studies by Dehal and Kupfer (1999) have demonstrated that a catechol metabolite of tamoxifen participated in the covalent binding to proteins from microsomes of phenobarbital-

![Fig 5A](image)

![Fig 5B](image)

![Fig 5C](image)

![Fig 5D](image)

**Fig. 5.** HPLC profiles of the metabolites formed by the CYP2B6 reconstituted system. Incubations were carried out with 200 μM tamoxifen with the NADPH-regenerating system for 0 min (B) or 90 min (A) or in the absence of tamoxifen incubated with the NADPH-regenerating system (C). Peaks a, b, and c represent N-desmethyl-tam, 4-OH-tam, and 4'-OH-tam, respectively. Peak d was an unidentified minor metabolite. The internal standard (IS) eluted at 49.7 min. The data are representative of four separate incubations. The HPLC profile of the metabolites formed by the CYP3A4-reconstituted system after incubation with 200 μM tamoxifen in the presence of NADPH is seen in D. Peaks a and e represent N-desmethyl-tam and α-OH-tam, respectively, and these data are representative of two separate experiments.

**TABLE 3**

Tamoxifen metabolites generated by CYP2B6

CYP2B6 (1.8 nmol) was reconstituted and incubated with 200 μM tamoxifen. The reactions were initiated using the NADPH-regenerating system. The samples were incubated at 37°C for 90 min and the metabolites were extracted with ethyl acetate. The organic phases were dried under nitrogen, dissolved in acetonitrile, and injected into C8 reverse phase column. The data shown represent the mean and standard deviation from four separate experiments.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Retention Time</th>
<th>Concentration</th>
</tr>
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<tbody>
<tr>
<td>4-OH-Tam</td>
<td>44.3</td>
<td>0.4 ± 0.2</td>
</tr>
<tr>
<td>4'-OH-Tam</td>
<td>46.9</td>
<td>1.8 ± 0.5</td>
</tr>
<tr>
<td>N-Desmethyl-tam</td>
<td>55.7</td>
<td>16.5 ± 3.9</td>
</tr>
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</table>

1. Sridar et al.  
induced rats, suggesting an involvement of the 2B family of cytochromes. In subsequent studies, a 3,4-diOH-tam product derived from incubation mixtures containing microsomes from rats induced with dexamethasone was isolated (Zhang et al., 2000). The 3,4-diOH-tam was readily oxidized to the reactive o-quinone that could be responsible for the alkylation of cellular molecules. The primary precursor of 3,4-diOH-tam seems to be the 4-hydroxytamoxifen metabolite of tamoxifen (Dehal and Kupfer, 1996; Dehal and Kupfer, 1999; Fan et al., 2000). Although 3,4-diOH-tam was not observed as one of the metabolites generated by CYP2B6 in the reconstituted system, 4-OH-tam was produced. It is conceivable that the levels of the 3,4-dihydroxy-product were below the limit of detection but if produced by CYP2B6 could become further oxidized to the reactive o-quinone that could be responsible for alkylation of the 2B6 apoprotein. Furthermore, the covalent binding of [14C]tamoxifen to microsomal proteins has been shown to have a statistically significant correlation between the concentrations of CYP2B6 and CYP3A4 as determined by immunological techniques. Characterization of the tissue distribution of the cytochrome P450 enzymes has shown a greater abundance of CYP2B6 and CYP3A4 in human breast tumors compared with nontumor epithelial and reduction mammoplasty samples (Hellmold et al., 1998). It is not clear whether the increased levels of expression of CYP2B6 in these tumor samples occurred because CYP2B6 is involved in tumorigenesis or whether the elevated levels are a protective response of the affected tissue. It may be that the beneficial effects of tamoxifen on estrogen receptor-negative tumors may be due to the inactivation of CYP2B6 by tamoxifen. Wiseman and Lewis (1996) have presented evidence from molecular modeling that tamoxifen is able to fit in the putative active site of CYP2B6. These findings invite speculations that a reactive intermediate derived from 4-OH-tam could be the active species involved in covalent binding to the CYP2B6 apoprotein. CYP2B6 is generally found at low levels in human livers and may therefore not be a significant contributor in the liver to the generation of the putative precarcinogenic tamoxifen metabolites such as 4-OH-tam. However, CYP2B6 has also been shown to be inducible by phenobarbital (Madan et al., 1996; Ekins and Wrighton, 1999). Interestingly, tamoxifen has been shown to induce the expression of P450s 2B1, 2B2, and 3A in rats at doses comparable with the therapeutic doses administered in humans (Emile et al., 1995). It is not known whether tamoxifen therapy changes the levels of CYP2B expression in humans. Recently, it was shown that certain drugs are exclusively metabolized by CYP2B6 (Ekins and Wrighton, 1999). It is therefore conceivable that deleterious side effects may be encountered if tamoxifen is coadministered with a drug that is metabolized by CYP2B6 only. Our findings that P450s 2C9 and 2D6 were also inactivated by tamoxifen add further concern to multiple

Scheme 1. Proposed scheme for the metabolism of tamoxifen by CYP2B6 leading to N-desmethyl-tam, 4-OH-tam, and 4'-OH-tam. Further metabolism of 4-OH-tam may lead to the formation of a catechol that could then react with the CYP2B6 apoprotein.

![Scheme 1](image-url)
drug therapies that exclusively require any of these three P450s for clearance.

In conclusion, data presented herein show that tamoxifen inactivates the enzyme CYP2B6 and that the primary metabolites formed during this process are N-desmethyl-tam, 4-OH-tam, and 4-OH-tam. Preliminary observations with 4-OH-tam (and other metabolites) suggested that these metabolites can also inactivate CYP2B6 when incubated in the presence of NADPH. This ability of 4-OH-tam to inactivate CYP2B6 required further metabolism and could occur at levels below the enzyme concentration, indicating that 4-OH-tam is not the reactive intermediate but may be the immediate precursor to this intermediate. Previously, Dehal and Kupfer (1999) have identified a catechol, 3,4-diOH-tam, derived from 4-OH-tam metabolism, which was able to bind to microsomal proteins. A similar mechanism may lead to the inactivation of CYP2B6 (Scheme 1). Current studies are aimed at identifying the reactive intermediate responsible for the inactivation of CYP2B6.

Acknowledgments

We thank Dr. Judy Bolton for providing the standards for the tamoxifen metabolites and the metabolites of tamoxifen that were used as substrates. Special thanks to Hsia-Lien Lin for the preparation of cytochrome b5.

References


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