Comprehensive Evaluation of Tamoxifen Sequential Biotransformation by the Human Cytochrome P450 System in Vitro: Prominent Roles for CYP3A and CYP2D6

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ABSTRACT

We performed comprehensive kinetic, inhibition, and correlation analyses in human liver microsomes and experiments in expressed human cytochromes P450 (P450s) to identify primary and secondary metabolic routes of tamoxifen (TAM) and the P450s catalyzing these reactions at therapeutically relevant concentrations. N-Desmethyl-TAM formation catalyzed by CYP3A4/5 was quantitatively the major primary metabolite of TAM; 4-hydroxy-TAM formation catalyzed by CYP2D6 (and other P450s) represents a minor route. Other minor primary metabolites include α-, β-, and 4'-hydroxy-TAM and one unidentified metabolite (M-I) and were primarily catalyzed by CYP3A4, CYP3A5, CYP2B6/2C19, and CYP3A4, respectively. TAM secondary metabolism was examined using N-desmethyl- and 4-hydroxy-TAM as intermediate substrates. N-Desmethyl-TAM was predominantly biotransformed to α-hydroxy N-desmethyl-, N-didesmethyl-, and 4-hydroxy N-desmethyl-TAM (endoxifen), whereas 4-hydroxy-TAM was converted to 3,4-dihydroxy-TAM and endoxifen. Except for the biotransformation of N-desmethyl-TAM to endoxifen, which was exclusively catalyzed by CYP2D6, all other routes of N-desmethyl- and 4-hydroxy-TAM biotransformation were catalyzed predominantly by the CYP3A subfamily. TAM and its primary metabolites undergo extensive oxidation, principally by CYP3A and CYP2D6 to metabolites that exhibit a range of pharmacological effects. Variable activity of these P450s, brought about by genetic polymorphisms and drug interactions, may alter the balance of TAM effects in vivo.

The selective estrogen receptor modulator tamoxifen (TAM) was first approved in 1977 by the U.S. Food and Drug Administration for the treatment of women with metastatic breast cancer and in ensuing years for adjuvant treatment of breast cancer (Osborne, 1998). Currently, TAM is an established hormonal treatment for all stages of estrogen receptor (ER)-positive breast cancer (Early Breast Cancer Trialists’ Collaborative Group, 1998) and is widely used as a chemopreventive agent in women at risk for developing the disease (Fisher et al., 1998). Ancillary benefits of TAM include amelioration of cardiovascular and bone disease in women (Osborne, 1998). However, there is wide interindividual variability in the clinical efficacy and side effects of TAM: some patients may be refractory to TAM, and many develop resistance with ongoing treatment (Osborne, 1998; Clarke et al., 2001) and a significant proportion of patients experience side effects that include hot flashes, thromboembolic events, and gynecologic complications (Osborne, 1998). The mechanisms underlying variable response to TAM have been the subject of intense study but remain obscure. Since there is compelling evidence that TAM is converted to antiestrogenic metabolites that are more potent than TAM itself, one hypothesis is that altered patterns of metabolism of TAM and/or its primary metabolites might contribute to interindividual variability.

Theories about TAM metabolism and response have been linked since Jordan et al. demonstrated that high first-pass metabolism of TAM results in a significant increase in its activity and characterized the first active primary metabolite, 4-hydroxy-TAM (Jordan, et al. 1977; Jordan, 1982). This metabolite has been shown to possess a high affinity for ERs and 30- to 100-fold more potency than TAM in suppressing estrogen-dependent cell proliferation (Borgna and Rochefort, 1981; Robertson et al., 1982; Coezy et al., 1982; Jordan, 1982). Thus, TAM has been referred to as a pro-drug that requires activation to exert its effects. Recently, we have

ABBREVIATIONS: TAM, tamoxifen; ER, estrogen receptor; endoxifen, 4-hydroxy-N-desmethyl-tamoxifen; P450, cytochrome P450; HPLC, high-performance liquid chromatography; HLM, human liver microsome; CLint, intrinsic clearances; TEPA, N,N′,N″-triethylene phosphoramide.
demonstrated that 4-hydroxy-N-desmethyl-TAM (endoxifen), a secondary metabolite of TAM, exhibits potency similar to 4-hydroxy-TAM with respect to ER binding affinity, suppression of estrogen-dependent cell growth, and gene expression (Stearns et al., 2003; Johnson et al., 2004; Lim et al., 2004).

In vitro and in vivo human studies have demonstrated that TAM undergoes extensive oxidation predominantly by the cytochrome P450 (P450) system to several primary and secondary metabolites (Lonning et al., 1992; Poon et al., 1993; White, 2003). Clinical studies suggest that the plasma concentrations of TAM and its metabolites vary widely among patients (Lonning et al., 1992; Stearns et al., 2003). It is likely that variable activity of those P450s involved alter the pattern of metabolism of TAM, leading to differences in systemic exposure of TAM and/or one or more of its active metabolites.

Several studies have attempted to define the primary metabolism of TAM in humans. Demethylation of the aminothoxy side chain to N-desmethyl-TAM appears to be the main route of TAM metabolism (Lonning et al., 1992; Stearns et al., 2003). 4-Hydroxy-TAM is a relatively minor metabolite, but it has been studied by a number of investigators because it is a more potent antiestrogen than TAM (Jordan et al., 1977; Borgna and Rochefort, 1981; Coezy et al., 1982; Robertson et al., 1982). Other human primary metabolites that include α-hydroxy-TAM and TAM N-oxide have been identified (Lonning et al., 1992; White, 2003). Published reports implicate multiple P450s in TAM N-demethylation, 4-hydroxylation, and α-hydroxylation (e.g., Jacolot et al., 1991; Crewe et al., 1997, 2002; Dehal and Kupfer, 1997). Despite the conduct of extensive in vitro studies to characterize TAM primary metabolism, critical evaluation of the published literature highlights the lack of a thorough quantitative understanding that will allow better prediction of TAM clearance or production of its active antiestrogens or toxic metabolites in vivo.

There is evidence that the primary metabolites of TAM are further oxidized by the human P450 system to a variety of important metabolites (Lonning et al., 1992; Dehal and Kupfer, 1999; Stearns et al., 2003; White, 2003; Coller et al., 2004). The potential significance of TAM secondary metabolism was suggested by recent studies that identified endoxifen as a potent antiestrogen metabolite of TAM (Stearns et al., 2003; Johnson et al., 2004; Lim et al., 2004). Since the plasma concentration of this metabolite in breast cancer patients has been reported to be much higher (over 6-fold) than that of 4-hydroxy-TAM (Lien et al., 1990; Stearns et al., 2003), it may also play an important role in vivo. Despite this, little information is available regarding TAM sequential metabolism to its secondary metabolites. Recently, we reported an association between CYP2D6 genotype and endoxifen formation in breast cancer patients (Stearns et al., 2003), but the contribution of this and/or other enzymes to its production has not been subject to careful study. Several secondary metabolites other than endoxifen have been identified in humans. Knowledge of sequential metabolic pathways leading to these metabolites and identification of the specific P450s involved remains essential to a full understanding of the human metabolism of this important drug.

In the present study, we carried out a comprehensive series of kinetic, correlation, and inhibition studies in human liver microsomal preparation and expressed P450s to characterize the metabolism of TAM to both primary and secondary metabolites.

Materials and Methods

Chemicals. Endoxifen was synthesized from [Z]-4-hydroxy-TAM (98% pure from Sigma-Aldrich, St. Louis, MO) as described in our recent publications (Stearns et al., 2003; Johnson et al., 2004). Z-TAM, Z-4-hydroxy-TAM, propranolol, troleandomycin, ketocazol, diethyldithiocarbamate, quinidine sulfate, glucose 6-phosphate, glucose-6-phosphate dehydrogenase, and β-NADP⁺ were purchased from Sigma-Aldrich. For initial metabolite identification, N-desmethyl-TAM, N-didesmethyl-TAM, and TAM N-oxide that were kindly provided by Dr. Irving W. Wainer (Laboratory of Clinical Investigation, Intramural Research Program, National Institute on Aging, National Institutes of Health, Baltimore, MD) were used. For actual incubation experiments, N-desmethyl-TAM HCl and α-hydroxy-TAM were purchased from Toronto Research Chemicals Inc. (Toronto, ON, Canada), or N-desmethyl-TAM was synthesized from [Z]-tamoxifen (98% pure from Sigma-Aldrich) using the same protocol as described elsewhere (Stearns et al., 2003). ThioTEPA was purchased from the U.S. Pharmacopoeia Convention (Rockville, MD). Sulfaphenazole and furafylline were purchased from Ultrafine Pharmaceuticals (Manchester, UK). Metabolite E [trans-1(4-hydroxyphenyl)-1,2-diphenylbut-1-ene] was a generous gift from Dr. Michael D. Johnson (Department of Oncology, Lombardi Comprehensive Cancer Center, Georgetown University Medical Center, Washington, DC). 4'-Hydroxy-TAM was a generous gift from Dr. Martin Lennard (Section of Molecular Pharmacology and Pharmacogenetics, University of Sheffield, Sheffield, UK). 3-Hydroxy-TAM was kindly provided by Dr. Stephen D. Hall and Dr. David R. Jones (Division of Clinical Pharmacology, Indiana University School of Medicine, Indianapolis, IN). Omeprazole was a generous gift from Dr. Tommy Anderson (Clinical Pharmacology, Astra Hässle AB, Mölndal, Sweden). All other reagents were of HPLC grade.

Human Liver Microsomes (HLMs) and Expressed P450s. Most of the HLMs used in this study and Baculovirus insect cell expressed human liver 450 isoforms (1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4, and 3A5) (with reductase) were purchased from BD Biosciences (Franklin Lakes, NJ). Some HLMs were prepared from human liver tissues that were medically unsuitable for liver transplantation by differential centrifugation using standard procedures. Microsomal pellets were resuspended in a reaction buffer to a protein concentration of 10 mg/ml (stock). Protein concentrations were determined by the Bradford method (Bradford, 1976), using bovine serum albumin as a standard. All microsomal samples were kept at −80°C until use.

Incubation Conditions. Pilot experiments were performed in HLMs using TAM, N-desmethyl-TAM, and 4-hydroxy-TAM as substrates to identify TAM primary and secondary metabolites and to optimize conditions for incubation and HPLC analysis. Each substrate (1 mg/ml) was prepared in methanol and serially diluted with methanol to the required concentration. Prior to metabolic incubation, the methanol was evaporated to dryness under reduced pressure using a Speedvac SC110 Model RHR40-12 (Savant Instruments, Holbrook, NY). Duplicate mixtures of 10 μM of each substrate reconstituted in a phosphate reaction buffer (pH 7.4) and a NADPH-generating system (1.3 mM β-NADP+, 3.3 mM glucose 6-phosphate, 3.3 mM MgCl₂, and 0.4 U/ml glucose 6-phosphate dehydrogenase) were preincubated at 37°C for 5 min before the reaction was initiated by the addition of HLMs, 0.5 mg protein/ml (final volume, 250 μl; all values are final concentrations). After a 60-min incubation period, the reaction was terminated by addition of cold acetoniile (100 μl) and vortex mixing. Samples were centrifuged at 14,000 rpm for 5 min in an Eppendorf model 5415C centrifuge (Brinkmann Instruments, Westbury, NY) after addition of an internal standard (20 μl of 100 μg/ml propranolol). An aliquot of supernatant was injected into the HPLC system. Negative control incubations were run in parallel.
and included no incubation, no substrate, no cofactor, or no HLMs (bovine serum albumin was used instead). All experiments were performed under dim light and in brown Eppendorf tubes to minimize photodegradation of TAM and its metabolites.

**HPLC Analysis.** Metabolites in microsomal incubates of TAM and $N$-desmethyl- and 4-hydroxy-TAM were measured using an HPLC method developed previously by our group (Lee et al., 2003, Stearns et al., 2003) with slight modification. Briefly, the incubation sample was injected onto a semipermeable surface cyano guard loading column (1.0–0.46 cm i.d.; 5 μm, 100 Å) and washed with 1 mL/min deionized water followed by a mobile phase composed of 35% acetonitrile in 20 mM potassium phosphate buffer (pH 3) at a flow rate of 1 mL/min. The duration of washing off the guard column with water and the mobile phase was reduced from 4 min in the previous publication (Lee et al., 2003) to below 2 min in the present study. This modification significantly improved the sensitivity of the assay by increasing the yield of metabolites produced in small quantities in HLMs. Using the mobile phase, the eluant was directed (switched) through a Rexchrom cyano analytical C$_{18}$ column (250–4.6 cm i.d.; 5 μm, 100 Å; Regis Chemical, Morton Grove, IL) guarded with a Rexchrom 10–3 mm C18 guard column (Regis Chemical) to an ICT Beam Boost postcolumn photoreactor supplied with a 5-m reaction coil and a 254 UV lamp (Advanced Separation Technologies, Whippany, NJ) where the photoreaction converts tamoxifen and its metabolites to highly fluorescent phenanthrene derivatives. HPLC instruments were controlled by CLASS-VP version 7.1.1 SP1 Chromatographic Software (Shimadzu Scientific Instruments Inc., Columbia, MD) and included a Shimadzu Solvent Delivery Module SCL-10A VP, Autoinjector SIL-10A VP, Spectrofluorometric detector RF-10A XL (set at an excitation wavelength of 256 nm and emission wavelength of 380 nm) and System Controller SCL-10A VP (Shimadzu, Kyoto, Japan).

**Identification of Metabolites.** Using the incubation and HPLC conditions described above, we observed several well separated fluorescent HPLC peaks in the incubation mixture consisting of the substrate (TAM, $N$-desmethyl- or 4-hydroxy-TAM), a NADPH-generating system, and HLMs, but not in the negative control experiments. The identity of each metabolite peak was confirmed as follows. First, the retention times of the peaks formed in microsomal incubates were compared with peaks of authentic TAM primary or secondary metabolite standards when available. The retention times of the reference metabolites were tested after direct injection or after adding them to incubation mixture that did not contain active microsomes. Second, selected ion recording scans of the microsomal metabolites were performed with the help of liquid chromatography-mass spectrometry. Metabolites were separated using a Rexchrom cyano analytical C$_{18}$ column (250–4.6 cm i.d.; 5 μm, 100 Å; Regis Chemical) guarded with a Rexchrom 10–3 mm C18 guard column (Regis Chemical) and a mobile phase that consisted of 50 mM ammonium acetate/methanol/acetonitrile (50:25:25, v/v/v). The flow rate was 0.2 mL/min. The mass spectrometer (PE/SciEx API100) was operated in positive ion mode and tuned for optimal resolution over the mass range by PE/SciEx proprietary LC2Tune program software. The exact conditions were: capillary voltage, 5300 V; orifice voltage, 30 V; and mass range of 150 to 450. The mass spectrometric data obtained in microsomal incubates were compared with the molecular masses of reference metabolites run in parallel or with the molecular masses of metabolite profiles of tamoxifen previously reported in humans (Lonnning et al., 1992; Poon et al., 1993; White, 2003). Additional information on whether a metabolite peak represents primary or secondary metabolite was obtained by comparing the peaks of TAM microsomal incubates with those obtained from incubation of its primary metabolites: $N$-desmethyl-, 4-hydroxy-, or $\alpha$-hydroxy-TAM.

**Linear Conditions for Incubation.** The duration of incubation and the protein concentrations used above were useful for initial experiments aimed at identification of TAM primary and secondary metabolites, but they may not represent linear conditions. Several investigators have used long duration of incubation, probably to offset low assay sensitivity for metabolites that are normally made at a slower rate. Given that TAM undergoes sequential metabolism, another duration of incubation may increase the likelihood of secondary metabolism, which then may preclude precise estimation of kinetic parameters and of contribution of specific metabolic routes to the overall clearance or activation of the drug. The need for optimal microsomal protein concentrations during TAM metabolism studies has been emphasized elsewhere (Crewe et al., 1997). To determine conditions that are linear for time of incubation and protein concentration, 10 μM each substrate (TAM, $N$-desmethyl-TAM or 4-hydroxy-TAM) was incubated in HLMs (0–2 mg protein/ml) and a NADPH-generating system at 37°C across a range of incubation times (0–90 min). Further processing of the samples was the same as the procedures described above (see *Incubation Conditions and HPLC Analysis*). Based on the results obtained, a 10-min incubation and a final microsomal protein concentration of 0.1 mg/ml represent conditions that are linear and minimize secondary metabolism of all the substrates and were used in the subsequent experiments.

The concentration of each metabolite was measured by comparing the metabolite peak to a standard curve obtained using known concentrations of the respective authentic metabolite standard. Authentic metabolite standards were not available to us for few metabolites identified (M-1, 3,4-dihydroxy-TAM, and $\alpha$-hydroxy $N$-desmethyl-TAM), and these metabolites were quantified using standard curves obtained from a metabolite standard that had close structural similarity and HPLC properties with the metabolite in question. The limitation of this approach is that the fluorescent intensity between the metabolite and the compound used to quantify it may be different as a result of altered chromophore. As a result, although the $K_{m}$ values for the formation of these metabolites could be estimated appropriately, actual formation rates of the metabolites could not be estimated precisely. Thus, the formation rates presented for these metabolites as picomoles per minute per milligram of protein (or picomoles per minute per picomoles of P450) should be viewed more appropriately as apparent velocities (arbitrary unit per minute per milligram of protein or picomoles of P450) where an arbitrary unit = 1000 $\times$ (metabolite AUC/internal standard AUC)/slope of the standard curve.

**Kinetic Analyses in HLMs.** Full kinetic analyses for the metabolism of TAM and its primary metabolites, $N$-desmethyl- and 4-hydroxy-TAM, were determined in characterized HLMs ($n = 3–4$). A range of substrate concentrations (0–100 μM) was incubated for 10 min with a protein concentration of 0.1 mg protein/ml and a NADPH-generating system. Further processing of the samples and HPLC assay were the same as described above.

**Correlation Analyses in HLMs.** To determine the correlation between the metabolism of TAM, $N$-desmethyl- or 4-hydroxy-TAM, and the activity of individual P450 isoforms in a panel of characterized HLMs ($n = 10–11$), the respective substrate (10 μM) was incubated at 37°C for 10 min with HLMs (0.1 mg protein/ml) and a NADPH-generating system. Information on the activity of each P450 isoform, measured by specific reaction marker, and on P450 contents was provided by the supplier of the HLMs studied (see http://www.gentest.com) and has been detailed in our previous publication (Ward et al., 2003).

**Inhibition Experiments.** Formation rates of metabolites from TAM and $N$-desmethyl- and 4-hydroxy-TAM (10 μM) were evaluated in the absence (control) and presence of known P450 isozyme-specific inhibitors. The inhibitors used were 10 μM furafylline (CYP1A2), 50 μM thioTEPA (CYP2B6), 20 μM sulfaphenazole (CYP2C9), 5 μM omeprazole (CYP2C19), 1 μM quinidine (CYP2D6), 50 μM diethyl-dithiocarbamate (CYP2E1), and 1 μM ketoconazole and 50 μM troleandomycin (CYP3A4/5). For competitive inhibitors, each substrate was prewarmed for 5 min at 37°C with or without the inhibitor and with a NADPH-generating system. HLMs (0.1 mg protein/ml) were added to initiate the reaction and incubated at 37°C for 10 min. For mechanism-based inhibitors (troleandomycin, furafylline, and thio-
TEPA), the inhibitors and controls were first preincubated in the presence of a NADPH-generating system and HLMs at 37°C for 15 min before the reaction was initiated by addition of the respective substrate. The inhibitors were studied at concentrations chosen to be selective for the respective P450 isoforms (Ward et al., 2003). Inhibited formation rates of metabolites were compared with those of control (with no inhibitor present), and results are expressed as a percentage of control activity.

**Expressed Human P450s.** To further probe the specific isoforms involved in tamoxifen primary and secondary metabolism, 10 μM of each substrate (TAM or N-desmethyl- or 4-hydroxy-TAM) was incubated with microsomes from expressed human CYP 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4, and CYP3A5 (52–104 pmol P450/ml in phosphate reaction buffer, pH 7.4) at 37°C for 10 min (final incubation volume = 250 μl). All other conditions were the same as those described for HLMs. For selected P450 isoforms, kinetic parameters were estimated by incubating 0 to 100 μM of the substrates with expressed P450s (52–104 pmol of P450/ml) and a NADPH-generating system for 10 min at 37°C.

**Data Analysis.** Apparent kinetic constants were estimated by nonlinear regression analysis using WinNonlin Software Version 3.1 (Pharsight, Mountain View, CA). Formation rates (V) of metabolites versus substrate concentrations (C) were fit to a single-site (V = V_{max}/(1 + K_{m}C + C)) or two-site inhibition (V = V_{max}(1 + K_{m}C + C)) equation. The model that fit best the data was selected based on visual inspection of the Edie-Hofstee plots, the dispersion of residuals, and standard errors of the parameter estimates. In vitro intrinsic clearances (CL_{int}) were given as V_{max}/C_{p} (μl/min/mg of protein; l/min/mg of protein). Pearson’s correlation or linear regression tests were performed using GraphPad Prism Software version 3.1 (GraphPad Software Inc., San Diego, CA) to calculate correlation coefficients.

**Results**

**Tamoxifen Oxidation to Its Primary Metabolites in HLMs and Expressed P450s**

A series of experiments were performed in HLMs and expressed P450s to characterize TAM oxidation to its primary metabolites in conditions that minimize secondary metabolism.

**Identification of TAM Primary Metabolites in HLMs.** HPLC traces of tamoxifen and its metabolites from in vitro incubation of TAM with HLMs showed several metabolite peaks that depended on a NADPH, duration of incubation, and microsomal protein and substrate concentrations (data not shown). The metabolite peaks at the retention times of 47, 31, 30, 29, and 18.3 min, respectively, were identified as N-desmethyl-, 4’-hydroxy-, 3-hydroxy-, 4-hydroxy-, and α-hydroxy-TAM. 4’-Hydroxy-TAM has been previously detected in the rat (Ruenitz et al., 1984), and a recent study reported that expressed human P450s catalyze its formation (Crewe et al., 2002), but this is the first evidence that this metabolite is formed in HLMs. To our knowledge, 3-hydroxy-TAM was not reported as primary metabolite of TAM in humans. Two other metabolite peaks were noted at retention times of 15.5 and 16.8 min. The metabolite peak at 15.5 min had properties consistent with a secondary metabolite, and subsequent experiments (see below) showed that it concurs with α-hydroxy N-desmethyl-TAM. The metabolite at 16.8 min (designated as M-I) had properties that are consistent with a primary metabolite, but its identity remains unknown. Other primary metabolites that appeared at retention times > 60 min such as TAM N-oxide and metabolite E [trans-(14-hydroxyphenyl)]1,2-diphenylbut-1-ene) or those that were formed at very slow rates were not further characterized.

Secondary metabolites that include N-idesmethyl-TAM (retention time, 45 min) and endoxifen (retention time, 25 min) were also noted when TAM was incubated for 60 min. However, the secondary metabolites of TAM were not observed at 10 min of incubation.

**Kinetic Analyses of TAM Primary Metabolism in HLMs.** Several in vitro studies have suggested involvement of multiple P450 isoforms in TAM primary metabolism, but extrapolations to clinical conditions have been limited in part by the lack of appropriate kinetic analyses to help identify (and estimate the precise contributions of) the P450s that are responsible at therapeutically relevant concentrations. We have addressed this issue by conducting comprehensive kinetic analyses of TAM primary metabolism in three HLMs characterized for their P450 activity in incubation conditions that minimize subsequent metabolism of the primary metabolites formed. The kinetic parameters for the metabolism of TAM to N-desmethyl- and 4-hydroxy-TAM illustrated in Table 1 were estimated by fitting formation rates versus TAM concentrations to a two-site binding equation with use of a nonlinear regression analysis (see Data Analysis). Accordingly, the Edie-Hofstee plots of these data (formation rates versus rates/substrate concentration) showed biphasic kinetics (data not shown). Our data suggest the participation of at least two enzymatic activities: high affinity (low capacity) (K_{m1} and V_{max1}) and low affinity (high capacity) (K_{m2} and V_{max2}). The high-affinity (low-capacity) system with average K_{m} values <3 μM and a >10-fold higher V_{max}/K_{m} than that of V_{max}/K_{m2} (Table 1) is probably relevant at therapeutic concentrations.

**Table 1**

<table>
<thead>
<tr>
<th>HLMs</th>
<th>TAM to N-Desmethyl-TAM</th>
<th>TAM to 4-Hydroxy-TAM</th>
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<tbody>
<tr>
<td></td>
<td>V_{max1}</td>
<td>K_{m1}</td>
</tr>
<tr>
<td>HG06</td>
<td>33.3</td>
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</tr>
<tr>
<td>HG23</td>
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<tr>
<td>Mean</td>
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<td>2.2</td>
</tr>
<tr>
<td>± S.D.</td>
<td>69.2</td>
<td>1.1</td>
</tr>
</tbody>
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| V_{max}, pmol/min/mg of protein; K_{m}, μM; and V_{max}/K_{m}, μl/min/mg of protein. |
doses of the drug. The mean steady-state plasma concentrations of TAM following therapeutic doses (20–40 mg/day orally) (Lonning et al., 1992; Stearns et al., 2003) or even at high doses (120 mg/m² to treat brain tumors) (Ducharme et al., 1997) do not exceed 2 and 8 μM, respectively. There is evidence that the concentrations of TAM and its metabolites are notably higher in human liver tissue relative to plasma, in some cases up to 60-fold (Lien et al., 1991). Even then, given the high (over 98%) plasma protein (albumin) binding of TAM (Lien et al., 1989), the unbound TAM concentration is unlikely to exceed 10 μM.

N-Desmethyl- and 4-hydroxy-TAM have been described in the literature as major and minor metabolites of TAM, respectively (Lonning et al., 1992), but the relative contribution of these two metabolites to the overall oxidation of TAM has not been fully clarified. On the basis of the average in vitro CLint (Table 1), N-desmethyl-TAM formation by the high-affinity component was 10.7-fold higher than that of 4-hydroxy-TAM. Assuming that little secondary metabolism occurs during the 10-min incubation used in our study and that the contributions of other metabolic routes are minimal, our data suggest that N-desmethyl- and 4-hydroxy-TAM formation accounts for approximately 92 and 7% of primary TAM oxidation, respectively.

The human liver microsomal preparations listed in Table 1 were precharacterized with respect to the activity of individual P450s. For example, the activity of CYP2D6 in the HLMs listed in Table 1 was 135 and 24.5 pmol/min/mg protein in HG23 and HG112, respectively (and undetectable in HG06), whereas that of CYP3A in HG112, HG23, and HG06 was 17,205, 3320, and 3000 pmol/min/mg protein, respectively. We specifically focused on CYP3A and CYP2D6 because there is evidence that CYP3A and CYP2D6, respectively, are important catalysts of TAM N-demethylation (Jacolot et al., 1991; Coller et al., 2004) and 4-hydroxylation (Crewe et al., 1997; Dehal and Kupfer, 1997). The relationship between the activity of individual P450s in these HLMs and CLint (Table 1) was tested using linear regression analysis to obtain preliminary information on the P450s catalyzing the low- and high-affinity component. Our data show that the CLint for the formation of 4-hydroxy-TAM by the high-affinity component was significantly related to CYP2D6 activity (P = 0.0015), whereas the low-affinity system was predicted by the activity of CYP2B6 (P = 0.013) and to some extent by CYP2C19 (P = 0.053) and CYP3A (P = 0.061). The CLint for N-desmethyl-TAM formation by the high-affinity catalyst in the two HLMs (HG23 and HG112) was related with the activity of CYP3A. However, a higher than expected CLint was observed in HG06. Since both expressed CYP3A4 and CYP3A5 catalyze TAM N-demethylation at equivalent rate (described below), the discrepancy we observed in HG06 could stem from the fact that a high CYP3A5 level in HG06 might have not been captured by testosterone 6β-hydroxylation, a reaction marker that was used to measure CYP3A activity in these HLMs, but which is known to be preferentially catalyzed by CYP3A4 (Williams et al., 2002). N-Desmethyl-TAM formation by the low-affinity system was better predicted by CYP1A2 activity.

The kinetic parameters for the formation of 3-hydroxy-, 4’-hydroxy-, and α-hydroxy-TAM (and apparent values for M-I formation) in HLMs are shown in Table 2. Formation rates versus TAM concentrations were best fit to a single-site binding equation in all cases except those of α-hydroxy-TAM, which was best described by a substrate inhibition model with substrate inhibition constant (Km) of 221 μM. Although all these metabolites were formed when TAM was incubated with the HLMs tested, we were unable to reliably estimate kinetic parameters for the formation of α-hydroxy-TAM in HG06 and HG23 and for the formation of 4’-hydroxy-TAM in HG06.

Correlation of TAM Primary Metabolism with P450s in a Panel of HLMs. The mean ± S.D. apparent formation rates expressed in picomoles per minute per milligram of protein (range) of M-I, α-hydroxy-, 4-hydroxy-, 3-hydroxy-, 4’-hydroxy-, and N-desmethyl-TAM from 10 μM TAM in 10 characterized HLMs were 3.1 ± 2.2 (1.1–7.8), 0.8 ± 1.7 (0–5.3), 2.3 ± 0.9 (0.9–4.3), 1.0 ± 0.9 (0–3.1), 0.9 ± 0.9 (0–2.8), and 79.0 ± 34.8 (54.2–165.0), respectively. The formation rate of N-desmethyl-TAM in these HLMs was 39.2 ± 19.8-fold (range, 13.8–68.4-fold) higher than that of 4-hydroxy-TAM and 10.4 ± 4.2-fold (range, 6.6–15.1-fold) higher than the summation of all other TAM primary metabolites. The data regarding formation rates of M-I should be interpreted carefully because the identity of this metabolite was not known. Nevertheless, these results and the kinetic analysis presented in Table 1 suggest that the N-demethylation pathway accounts for the majority of TAM oxidation and that the contribution of all other pathways is probably minimal.

The formation rates of α-hydroxy-, 4’-hydroxy-, N-desmethyl-TAM, and M-I correlated significantly with the activity of CYP3A (r = 0.76–0.96; P = 0.01–0.0001) and CYP2B6 (r = 0.64–0.81; P = 0.044–0.004). The activity of other P450s showed no correlation with any of these metabolites (data not shown). Whether CYP3A and CYP2B6 are both involved in catalyzing these reactions is not apparent from these data because we noted a significant correlation between the activity of CYP3A and CYP2B6 (r = 0.80; P = 0.006) in the panel of HLMs studied. Inhibition experiments in HLMs and data from expressed P450s (described below) suggest that, except 4’-hydroxy-TAM formation that was cat-

### Table 2

<table>
<thead>
<tr>
<th>Metabolites</th>
<th>HG112</th>
<th>HG23</th>
<th>HG06</th>
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<tbody>
<tr>
<td>Vmax (pmol/min/mg of protein)</td>
<td>Kmax (μM)</td>
<td>Vmax/Kmax</td>
<td>Vmax (pmol/min/mg of protein)</td>
</tr>
<tr>
<td>M-I</td>
<td>11.9</td>
<td>5.2</td>
<td>2.3</td>
</tr>
<tr>
<td>α-Hydroxy-TAM</td>
<td>6.5</td>
<td>1.5</td>
<td>4.5</td>
</tr>
<tr>
<td>3-Hydroxy-TAM</td>
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<td>4.5</td>
<td>0.5</td>
</tr>
<tr>
<td>4’-Hydroxy-TAM</td>
<td>5.0</td>
<td>4.0</td>
<td>1.3</td>
</tr>
</tbody>
</table>

Vmax, pmol/min/mg of protein; Kmax, μM; and Vmax/Kmax, μmol/min/mg of protein; NE, not estimated.
alyzed in part by CYP2B6, CYP3A (but not CYP2B6) was the principal catalyst of α-hydroxy-TAM, N-desmethyl-TAM, and M-I. Formation rates of 3-hydroxy- and 4-hydroxy-TAM showed no correlation with the activity of any of the P450s tested (data not shown), except a nonsignificant trend for the formation of 4-hydroxy-TAM with the activity of CYP2D6 \( (r = 0.56; P = 0.09) \) and CYP2C9 \( (r = 0.45; P = 0.20) \).

**Inhibition of TAM Primary Metabolism in HLMs.** The effect of P450 isoform-specific chemical inhibitors on TAM primary metabolism in HLMs \( (n = 4) \) was tested to identify the P450s involved. The main findings (data not shown) could be summarized as follows. First, ketoconazole \( (1 \mu M) \) and troleandomycin \( (50 \mu M) \) inhibited TAM N-demethylation by an average of 62% \( (\text{range}, 28-90\%) \) and 78% \( (\text{range}, 61-90.2\%) \), respectively; sulfaphenazole \( (20 \mu M) \) also showed inhibition in three HLMs by an average of ~31% \( (\text{range}, 24-35\%) \), but it had no effect in one HLM (HG112). Second, quinidine \( (1 \mu M) \) partially inhibited (by an average of 47.7%; range, 32–56%) 4-hydroxy-TAM formation in three HLMs (HK23, HG23, and HG112), whereas this inhibition was minimal \( (\text{by} < 12\%) \) in another HLM (HL2); ketoconazole and troleandomycin, although having negligible effect on 4-hydroxy-TAM formation in the three HLMs inhibitable by quinidine, were moderate inhibitors in HL2 \( (\text{by} > 45\%) \), suggesting a contribution of CYP3A in this specific HLM. Third, ketoconazole \( (1 \mu M) \) was a potent inhibitor of TAM metabolism to M-I \( (\text{by} 58\%) \), α-hydroxy-TAM \( (\text{by} 77\%) \), and 3-hydroxy-TAM \( (\text{to an undetectable level}) \). Fourth, thioTEPA \( (50 \mu M) \) and omeprazole \( (5 \mu M) \) decreased 4'- and 3-hydroxy-TAM formation by ~39 and ~26%, respectively. The effect of other inhibitors on TAM primary metabolism was negligible (data not shown).

**TAM Primary Metabolism by Expressed Human P450s P450s.** To further probe the specific P450 involved, TAM metabolism to its primary metabolites was determined in a panel of expressed P450s. TAM metabolism to M-I was catalyzed at the highest rate by CYP3A4 (with small contribution of CYP3A5, CYP2B6, CYP2C8, and CYP2D6), to α-hydroxy-TAM by CYP3A4 (with contribution of CYP3A5), to 4-hydroxy-TAM by CYP2D6 (other isoforms that showed activity include CYP2C19 > CYP2B6 > CYP2C9 > CYP3A4), to 3-hydroxy-TAM by CYP3A5 (with small activity of CYP2B6 and CYP3A4), to 4'-hydroxy-TAM by CYP2B6 (and CYP2C19), and to N-desmethyl-TAM by multiple P450s \( (\text{CYP2D6} > \text{CYP2C19} > \text{CYP3A5} \equiv \text{CYP3A4} > \text{CYP1A2} = \text{CYP2B6}) \).

Full kinetic analyses were performed for selected P450s that showed significant activity toward formation of TAM primary metabolites. The \( K_m \) values obtained by fitting formation rates versus substrate concentrations to a single-site enzyme model were compared with those values obtained in HLMs (Tables 1 and 2). The \( K_m \) values derived for the formation of N-desmethyl-TAM by CYP2D6, CYP3A4, and CYP3A5 \( (11.6, 12.6, \text{and} 19.4 \mu M) \), respectively, were close to the \( K_m \) values obtained in HLMs by the high-affinity system. CYP2D6, consistent with previous reports \( (\text{Crewe et al., 2002}) \), catalyzes TAM N-demethylation at the highest rate \( (\text{CL}_{\text{int}} \text{ for CYP2D6, CYP3A4, and CYP3A5 was 0.33, 0.12, and 0.08 ml/min/pmol P450, respectively}) \), but it appears that CYP3A4 and CYP3A5 are the main catalysts at lower concentrations because no important role could be confirmed for CYP2D6 from the inhibition and correlation data in HLMs.

Of note, a marked increase of TAM N-demethylation has been reported when healthy volunteers were pretreated with rifampin \( (\text{Kivistö et al., 1998}) \), a drug known to induce CYP3A and other P450s \( (\text{but not CYP2D6}) \). In systems that consist of multiple P450s \( (\text{e.g., HLMs and in vivo in humans}) \), the contribution of CYP2D6 in TAM N-demethylation appears to be minimal. Although expressed CYP2B6 shows catalytic activity toward TAM N-demethylation, the small inhibitory effect \( (<20\%) \) of thioTEPA \( (\text{a relatively selective CYP2B6 inhibitor}) \) and the high \( K_m \) value derived from expressed CYP2B6 \( (126 \mu M) \) makes it unlikely that this isofrom represents the high-affinity system. The significant correlation between TAM N-demethylation and CYP2B6 activity in a panel of HLMs may stem from a significant correlation between CYP2B6 and CYP3A activity in these HLMs \( (\text{Ward et al., 2003}) \).

Consistent with earlier reports \( (\text{Crewe et al., 1997, 2002}) \), we found significant activity of CYP2C9, CYP2C19, and CYP1A2 in TAM N-demethylation, but the high \( K_m \) values derived from CYP2C9 \( (118 \mu M) \) and CYP2C19 \( (39.4 \mu M) \) and the fact that the inhibition of this reaction by specific inhibitors of these enzymes was small in HLMs suggest that these enzymes play a minimal role in TAM N-demethylation in vivo.

Although the \( K_m \) values for 4-hydroxy-TAM formation by CYP2B6, 2C9, 2C19, 2D6, CYP3A4, and 3A5 were <10 \( \mu M \) \( (\text{range}, 4.9-9.8 \mu M) \); CL_{\text{int}} \text{ (microliters per minute per picomole of P450) was relatively higher for CYP2C19 (0.09), CYP2D6 (0.08), and CYP2B6 (0.014) compared with the CL_{\text{int}} \text{ obtained in CYP3A5, CYP3A4, or CYP2C9 (<0.003) (data not shown). The kinetic parameters for the formation of other TAM primary metabolites were also calculated (data not shown) and essentially confirmed that CYP3A4 was the main enzyme responsible for the metabolism of TAM to M-I and α-hydroxy-TAM, CYP3A5 was the main enzyme responsible for TAM 3-hydroxylation, and CYP2B6 and CYP2C19 were important catalysts of TAM 4'-hydroxylation.**

**Summary of TAM Primary Metabolism in HLMs and Expressed P450s.** Based on the data described above in HLMs and expressed P450s, the proposed human routes of TAM biotransformation to its primary metabolites and the specific P450s catalyzing them are summarized in Fig. 1. In this part of the study, we identified additional TAM primary metabolites. We carried out comprehensive kinetic analyses, which, together with other approaches, allowed the identification (and estimate contribution) of the metabolic pathways and the specific P450s involved at therapeutically relevant concentrations of TAM. N-Demethylation of TAM by CYP3A is the major metabolic route of TAM primary metabolism, whereas 4-hydroxy-TAM, which represents minor route, was consistently formed in all HLMs. We also provided the scientific groundwork required for subsequent studies of TAM secondary metabolism.
Identification of Secondary Metabolites of TAM in HLMs. Evidence from the literature suggests that TAM undergoes sequential metabolism to several secondary metabolites, including a recently identified active antiestrogen metabolite, endoxifen (Stearns et al., 2003; Johnson et al., 2004). In the present study, we noted secondary metabolites when TAM was incubated with HLMs for 60 min. Here, we tested sequential metabolism of TAM to endoxifen and other secondary metabolites using \( N \)-desmethyl- and 4-hydroxy-TAM as substrates.

Figure 2 shows HPLC traces of human liver microsomal incubations of \( N \)-desmethyl-TAM (Fig. 2A). Three major and two minor (M-II and M-III) metabolite peaks whose formation depended on the P450 system were observed in the microsomal incubations of \( N \)-desmethyl-TAM (Fig. 2A). The peaks at retention times of 38.2, 24.5, and 14.7 min (Fig. 2A) were consistent with \( N \)-didesmethyl-TAM, endoxifen, and \( \beta \)-hydroxy \( N \)-desmethyl-TAM, respectively.

Another possible route for the formation of this metabolite could be \( \alpha \)-hydroxylation of TAM followed by \( N \)-demethylation. We tested this possibility by incubating \( \alpha \)-hydroxy-TAM in HLMs. Indeed, our data provide evidence that \( \alpha \)-hydroxy-TAM undergoes \( N \)-demethylation to \( \alpha \)-hydroxy \( N \)-desmethyl-TAM (data not shown). The metabolite peaks at 15.5 (M-II) and 25.5 (M-III) min were formed at relatively low rate in the HLMs used (Fig. 2A) under the HPLC conditions tested, and no further attempt was made to characterize their identity.

As shown in Fig. 2B, 3,4-dihydroxy-TAM (retention time, \( \sim 19.9 \) min) and endoxifen (retention time, \( \sim 25 \) min) were the main metabolites of 4-hydroxy-TAM. We noted that endoxifen is formed when TAM or \( N \)-desmethyl- or 4-hydroxy-TAM were used as substrates. Together, our data demonstrate that TAM undergoes sequential metabolism that includes step-wise \( N \)-demethylation (e.g., to \( N \)-didesmethyl-TAM), hydroxylation (e.g., to 3,4-hydroxy-TAM), or \( N \)-demethylation followed by hydroxylation or vice versa (e.g., to endoxifen and \( \alpha \)-hydroxy \( N \)-desmethyl-TAM).

Kinetic Analysis of TAM Secondary Metabolism in HLMs. The kinetics for the metabolism of \( N \)-desmethyl-TAM to \( \beta \)-hydroxy \( N \)-desmethyl-TAM, endoxifen, and \( N \)-didesmethyl-TAM, and of 4-hydroxy-TAM to 3,4-dihydroxy-TAM and endoxifen were determined in different HLMs (\( n = 3–4 \)) that had been characterized for the activity of individual P450 isoforms. Our pilot data indicated that CYP3A and CYP2D6 might be important in TAM secondary metabolism. Thus, HLMs with high and low CYP3A activity and high and low CYP2D6 activity were deliberately selected for the kinetic analyses experiments. The activity of CYP3A (picomoles per minute per milligram of protein) was higher in HG112 (17205) \( \gg \) HG23 (3300) \( > \) HG06 (3000) \( > \) HG93.
The activity of CYP2D6 was highest in HG23 (135) >>> HG93 (47) > HG112 (24.5) >>> HG06 (ndetectable). The kinetic parameters for N-desmethyl-TAM metabolism were summarized in Table 3. Kinetic parameters for α-hydroxy N-desmethyl-TAM formation (Fig. 3A) and endoxifen (Fig. 3B) were obtained by fitting the data to a single-site binding equation. The Edie-Hofstea plots (data not shown) were characterized by monophasic kinetics. N-Didesmethyl-TAM formation on the other hand was best described by biphasic kinetics in two HLMs (HG112 and HG23) (Fig. 3C), with high- (Km1 and Vmax1) and low- (Km2, in millimolar range and Vmax2) affinity components. The in vitro Clint for the high-affinity component was 20- and 3-fold higher than the low-affinity system in HG112 and HG23, respectively. The kinetics of this metabolite in HG06 exhibited monophasic characteristics, and it appears that the high-affinity component is absent in this microsomal preparation (Km value in HG06 was 22- and 10-fold higher, respectively, than those estimated in HG112 and HG23 for the high-affinity component).

The kinetic parameters for the metabolism of 4-hydroxy-TAM listed in Table 4 were estimated by fitting formation rates versus 4-hydroxy-TAM concentrations to a single-site binding equation 3,4-dihydroxy-TAM and endoxifen in HG06 and HG93) or to a two-site binding equation (endoxifen in HG23, and endoxifen and 3,4-dihydroxy-TAM in HG112). The Clint for endoxifen formation by the high-affinity component in HG112 (7.1 µmol/min/mg protein) and HG23 (11.8 µmol/min/mg protein), respectively, was 32- and 9.6-fold higher than by the low-affinity component (2.24 and 1.23 µmol/min/mg protein in HG112 and HG23, respectively). Similarly, the Clint for the formation of 3,4-dihydroxy-TAM in HG112 by the high-affinity system (59.53 µmol/min/mg protein) was 56.9-fold higher than by the low-affinity system (1.05 µmol/min/mg protein).

Representative plots of formation rates of metabolites versus substrate concentrations are shown in Fig. 3 where the data form two HLMs with high and low CYP3A activity (Fig. A, C, D, and E) or high and low CYP2D6 activity (Fig. 3B) are displayed. Visual inspection of these kinetic curves suggest that the metabolism of N-desmethyl-TAM to α-hydroxy N-desmethyl-TAM (Fig. 3A) and of 4-hydroxy-TAM to 3,4-dihydroxy-TAM and endoxifen (Fig. 3, D and E) is exclusively dependent on CYP3A activity, whereas CYP2D6 appears to be the main enzyme mediating the formation of endoxifen from N-desmethyl-TAM (Fig. 3B). The formation of N-desmethyl-TAM was also dependent on CYP3A activity, but to a lesser extent (Fig. 3C). To obtain pilot information as to which isoforms might be involved at therapeutically relevant substrate concentrations, we examined the relationship between the in vitro Clint values obtained for the high-affinity component and the activity of P450 isoforms in the HLMs. The Clint for N-desmethyl-TAM metabolism to α-hydroxy N-desmethyl-TAM and the Clint for 4-hydroxy-TAM metabolism to 3,4-dihydroxy-TAM and endoxifen correlated with the activity of CYP3A (data not shown). CYP3A activity in HG112 was 10.4-fold higher than in HG93, and there was a ~14- to 15-fold higher Clint for the metabolism of 4-hydroxy-TAM to its metabolites (Table 4). The formation of endoxifen from N-desmethyl-TAM was dependent on CYP2D6. Consistent with the involvement of CYP2D6 in endoxifen formation, HG06 (a phenotypically poor CYP2D6 metabolizer preparation) formed endoxifen at a very low rate (Table 3). The Clint derived for endoxifen in HG23 (5.42 µmol/min/mg protein), a microsomal preparation with high CYP2D6 activity, was over 80-fold higher than that obtained from HG06 (0.06 µmol/min/mg protein). We observed an increase in Km value for N-desmethyl-TAM formation with decreasing CYP3A activity (Table 3), suggesting that the higher affinity enzyme responsible for catalyzing this reaction might be CYP3A. Endoxifen is formed when both 4-hydroxy- and N-desmethyl-TAM are used as substrates. However, no relationship was observed between the Clint for endoxifen formation from 4-hydroxy-TAM and the Clint for endoxifen formation from N-desmethyl-TAM (r² = 0.05; P = 0.85), further supporting the involvement of different enzymes in 4-hydroxylation of N-desmethyl-TAM and N-demethylation of 4-hydroxy-TAM to endoxifen. Together, our data point to an important role of CYP3A in all metabolic routes of N-desmethyl- and 4-hydroxy-TAM, except in N-desmethyl-TAM 4-hydroxylation, which seems to be dependent on CYP2D6 activity.

**Correlation Analysis of TAM Secondary Metabolism in HLMs.** The rate of metabolism of N-desmethyl- and 4-hydroxy-TAM was determined in a panel of characterized HLMs. The average ± S.D. (range) formation rates of α-hydroxy N-desmethyl-TAM, endoxifen, and N-didesmethyl-TAM from N-desmethyl-TAM (10 µM) in 11 HLMs were 33.5 ± 18.1 (range, 11–74.4), 7.9 ± 5.5 (range, 1.6–18.7), and 27.0 ± 11.7 (12.7–53.5) pmol/min/mg protein, respectively. Formation rates of α-hydroxy N-desmethyl-TAM correlated significantly with that of N-desmethyl-TAM (r² = 0.63; P = 0.004), but formation rates of endoxifen did not show any correlation with those of α-hydroxy N-desmethyl- or N-didesmethyl-TAM (r² < 0.1; P > 0.35). The average ± S.D. (range)

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**TABLE 3**

Kinetic parameters for the metabolism of N-desmethyl-TAM to α-hydroxy N-desmethyl-TAM, endoxifen, and N-didesmethyl-TAM in HLMs

Since endoxifen was formed very slowly in HG06 (undetectable at substrate concentrations ≤ 25 µM), the kinetic parameters estimated from a single-site binding equation were overestimated and are not listed in the table; only mean values from HG23 and HG112 are presented. Data for the formation of N-didesmethyl-TAM were characterized by biphasic kinetics in HG23 and HG112 (low and high affinity) and by monophasic kinetics in HG06. The low affinity Km values were in millimolar ranges, and the kinetic data that represent the high affinity system (Vmax, Km, and Vmax/Km) are presented here.

<table>
<thead>
<tr>
<th>HLMs</th>
<th>α-hydroxy N-Desmethyl-TAMa</th>
<th>Endoxifenb</th>
<th>N-Desmethyl-TAM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vmax (pmol/min/mg protein)</td>
<td>Km (µM)</td>
<td>Vmax/Km</td>
</tr>
<tr>
<td>HG06</td>
<td>36.0 ± 5.5</td>
<td>6.2</td>
<td>6.2 ± 0.6</td>
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<tr>
<td>HG23</td>
<td>19.7 ± 5.9</td>
<td>3.4</td>
<td>3.4 ± 0.3</td>
</tr>
<tr>
<td>HG112</td>
<td>94.3 ± 4.7</td>
<td>20.2</td>
<td>20.2 ± 1.4</td>
</tr>
<tr>
<td>Mean</td>
<td>50.0 ± 5.5</td>
<td>9.9</td>
<td>9.9 ± 0.7</td>
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Vmax, pmol/min/mg of protein; Km, µM; and Vmax/Km, µmol/min/mg of protein.

a Formulation rates (apparent) vs. substrate concentrations were best fit to a one-site binding equation using a nonlinear regression analysis (see Data Analysis).
formation rates of 3,4-dihydroxy-TAM and endoxifen from 4-hydroxy-TAM were 23.4/11006 18.1 (range, 9.3–69.8) and 40.2/11006 29.8 (range, 14.4–112.5) pmol/min/mg protein, respectively. A significant correlation was noted between 3,4-dihydroxy-TAM and endoxifen formation rates in the HLMs tested (r² 0.89; P < 0.0001).

Preliminary analyses of the data in Tables 3 and 4 suggested that CYP2D6 and CYP3A might be important to the metabolism of N-desmethyl- and 4-hydroxy-TAM. We arbitrarily classified the HLMs as high (106.4/11006 19.7 pmol/min/mg protein; n = 5) and low (20.6/11006 16.2 pmol/min/mg protein; n = 6) CYP2D6 activity HLMs and high (9730/11006 4063 pmol/min/mg protein; n = 5) and low (3123/11006 1045 pmol/min/mg protein; n = 5) CYP3A activity. The rate of endoxifen formation from N-desmethyl-TAM in the HLMs with high CYP2D6 activity was significantly higher (12.8/11006 3.8 pmol/min/mg protein) than those in HLMs with low CYP2D6 activity (3.7/11006 1.8 pmol/min/mg protein) (P < 0.003). Formation rates of α-hydroxy N-desmethyl-TAM and N-didesmethyl-TAM from N-desmethyl-TAM, and of 3,4-dihydroxy-TAM and endoxifen from 4-hydroxy-TAM in HLMs with high CYP3A activity were significantly higher (40.3/11006 19.9, 34.5/11006 10.3, 33.7/11006 21.3, and 58.9/11006 33 pmol/min/mg protein, respectively) than those values obtained in HLMs

### TABLE 4

<table>
<thead>
<tr>
<th>HLMs</th>
<th>3,4-Dihydroxy-TAM</th>
<th>Endoxifen</th>
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<tr>
<td></td>
<td>Vₘₐₓ</td>
<td>Kₘ</td>
</tr>
<tr>
<td>HG112</td>
<td>144</td>
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</tr>
<tr>
<td>HG23</td>
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<td>ND</td>
</tr>
<tr>
<td>HG06</td>
<td>41</td>
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</tr>
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<td>±S.D.</td>
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</tr>
</tbody>
</table>

Vₘₐₓ, pmol/min/mg of protein; Kₘ, μM; and Vₘₐₓ/Kₘ, μl/min/mg of protein.

formation rates of 3,4-dihydroxy-TAM and endoxifen from 4-hydroxy-TAM were 23.4 ± 18.1 (range, 9.3–69.8) and 40.2 ± 29.8 (range, 14.4–112.5) pmol/min/mg protein, respectively. A significant correlation was noted between 3,4-dihydroxy-TAM and endoxifen formation rates in the HLMs tested (r² = 0.89; P < 0.0001).

Preliminary analyses of the data in Tables 3 and 4 suggested that CYP2D6 and CYP3A might be important to the metabolism of N-desmethyl- and 4-hydroxy-TAM. We arbitrarily classified the HLMs as high (106.4 ± 19.7 pmol/min/mg protein; n = 5) and low (20.6 ± 16.2 pmol/min/mg protein; n = 6) CYP2D6 activity HLMs and high (9730 ± 4063 pmol/min/mg protein; n = 5) and low (3123 ± 1045 pmol/min/mg protein; n = 5) CYP3A activity. The rate of endoxifen formation from N-desmethyl-TAM in the HLMs with high CYP2D6 activity was significantly higher (12.8 ± 3.8 pmol/min/mg protein) than those in HLMs with low CYP2D6 activity (3.7 ± 1.8 pmol/min/mg protein) (P = 0.003). Formation rates of α-hydroxy N-desmethyl-TAM and N-didesmethyl-TAM from N-desmethyl-TAM, and of 3,4-dihydroxy-TAM and endoxifen from 4-hydroxy-TAM in HLMs with high CYP3A activity were significantly higher (40.3 ± 19.9, 34.5 ± 10.3, 33.7 ± 21.3, and 58.9 ± 33 pmol/min/mg protein, respectively) than those values obtained in HLMs.
with low CYP3A activity (25.4 ± 13.2, 17.9 ± 4.6, 15.1 ± 8.6, and 21.5 ± 6.3 pmol/min/mg protein, respectively) (P < 0.05).

When correlation analyses were performed across the panel of HLMs tested, CYP3A correlated significantly with rates of N-desmethyl-TAM metabolism to α-hydroxy N-desmethyl-TAM (Fig. 4A) and N-didesmethyl-TAM (Fig. 4C) and 4-hydroxy-TAM metabolism to 3,4-dihydroxy-TAM (Fig. 4D) and endoxifen (Fig. 4E). There was a statistically significant correlation between endoxifen formation from N-desmethyl-TAM and CYP2D6 activity (Fig. 4B). In addition, rates of α-hydroxy N-desmethyl-TAM and N-didesmethyl-TAM formation correlated significantly with the activity of CYP2B6 (r² = 0.58 and 0.61; P = 0.01 and 0.007, respectively) and with total P450 content (r² = 0.54 and 0.44; P = 0.016 and 0.038, respectively) (data not shown). Similarly, rates of 4-hydroxy-TAM metabolism to 3,4-dihydroxy-TAM and endoxifen showed significant correlation with the activity of CYP2B6 (r² = 0.49 and 0.61; P = 0.024 and 0.007, respectively). Despite the statistically significant correlation between the activity of CYP2B6 and formation rates of most secondary metabolites, these data alone do not confirm that CYP2B6 is important catalyst of these reactions because, as described above, there is a significant correlation between the activity of CYP2B6 and CYP3A. P450s other than those mentioned here showed no statistically significant correlation with any of the metabolic routes of N-desmethyl- and 4-hydroxy-TAM.

**Inhibition of TAM Secondary Metabolism in HLMs.**

The inhibitory effects of isoform-specific inhibitors on the metabolism of N-desmethyltamoxifen (N-desmethyl-TAM) and 4-hydroxytamoxifen (4-hydroxy-TAM) in a panel of different HLMs. Upper panel, correlation between formation rates from N-desmethyl-TAM of α-hydroxy N-desmethyl-TAM and CYP3A activity (A), endoxifen and CYP2D6 activity (B), and N-didesmethyl-TAM and CYP3A activity (C). Lower panel, correlation between formation rates of 3,4-dihydroxy-TAM (D) and of endoxifen (E) from 4-hydroxy-TAM and CYP3A activity. Each point represents average of duplicate incubations.

**Fig. 4.** Correlation between the activity of P450 isoforms and the metabolism of N-desmethyltamoxifen (N-desmethyl-TAM) and 4-hydroxytamoxifen (4-hydroxy-TAM) in a panel of different HLMs. Upper panel, correlation between formation rates from N-desmethyl-TAM of α-hydroxy N-desmethyl-TAM and CYP3A activity (A), endoxifen and CYP2D6 activity (B), and N-didesmethyl-TAM and CYP3A activity (C). Lower panel, correlation between formation rates of 3,4-dihydroxy-TAM (D) and of endoxifen (E) from 4-hydroxy-TAM and CYP3A activity. Each point represents average of duplicate incubations.

**Fig. 5.** Inhibition of N-desmethyltamoxifen (N-desmethyl-TAM) and 4-hydroxytamoxifen (4-hydroxy-TAM) metabolism by P450 isoform-specific inhibitors in HLMs. The isoform-specific inhibitors used were 20 μM furafylline (FURA, CYP1A2), 50 μM thioTEPA (CYP2B6), 20 μM sulfaphenazole (SPZ, 2C9), 10 μM omeprazole (OMP, 2C19), 1 μM quinidine (QUIN, 2D6), and 1 μM ketoconazole (KETO) and 50 μM troleandomycin (TAO, 3A). Rates of metabolite formation during incubation with the inhibitors are presented as percentage of control (without inhibitor) activity. Upper panel, inhibition of N-desmethyl-TAM metabolism in HLMs to α-hydroxy N-desmethyl-TAM (A), endoxifen (B), and N-didesmethyl-TAM (C). Lower panel, inhibition of 4-hydroxy-TAM metabolism to 3,4-dihydroxy-TAM (D) and endoxifen (E). Data are average of duplicate incubation measurements.
N-desmethyl-TAM (by ~56–70%; Fig. 5C) and 4-hydroxy-TAM metabolism to 3,4-dihydroxy-TAM (by >88%; Fig. 5D) and endoxifen (by >84%; Fig. 5E). The formation of endoxifen from N-desmethyl-TAM was almost completely inhibited by quinidine (Fig. 5B). Other inhibitors showed only mild to moderate inhibitory effect on the metabolism of N-desmethyl- or 4-hydroxy-TAM.

**TAM Secondary Metabolism by Expressed P450 Isoforms.** The metabolism of N-desmethyl- and 4-hydroxy-TAM by a panel of expressed human P450s is summarized in Fig. 7. N-Desmethyl-TAM metabolism to α-hydroxy N-desmethyl-TAM was catalyzed at the highest rate by CYP3A4 and CYP2D6, whereas other P450s had less activity (Fig. 6A); to endoxifen by CYP2D6 (Fig. 6B); and to N-desmethyl-TAM by CYP2D6 (Fig. 6C), with participation of CYP3A4, CYP3A5, CYP2C19, CYP2C9, CYP2B6, and CYP1A2. The metabolism of 4-hydroxy-TAM to 3,4-dihydroxy-TAM (Fig. 6D) and endoxifen (Fig. 6E), respectively, was catalyzed by CYP3A5 and CYP2D6 at the highest rates.

**Discussion**

We report a systematic characterization of TAM sequential metabolism in HLMs and expressed P450s, including complete kinetic analyses. The data obtained may serve as a basis to predict and estimate the contribution of those metabolic pathways and P450s relevant to TAM clearance and its conversion to pharmacologically active metabolites in vivo at therapeutic concentrations.

**TAM Biotransformation to Its Primary Metabolites.** A thorough quantitative understanding of the primary metabolism of TAM is critical because the rate of primary metabolism determines the clearance of the parent drug, the production of more active antiestrogens (e.g., 4-hydroxy-TAM) or more toxic metabolites (e.g., α-hydroxy-TAM), and the rate at which secondary metabolites that include potent antiestrogens (e.g., endoxifen) or more toxic compounds are formed. We identified six primary metabolites of TAM and the specific P450s involved in metabolism to them (Fig. 1). Our data, consistent with clinical studies (Lonning et al., 1992; Stearns et al., 2003), demonstrate that the liver is an efficient catalyst of N-desmethyl-TAM formation, accounting for the majority of TAM oxidation. Our results confirm previous reports (Jacolot et al., 1991; Crewe et al., 1997, 2002) that TAM N-demethylation is catalyzed by the CYP3A subfamily and provide the first evidence that both CYP3A4 and CYP3A5 are catalysts of this reaction at low TAM concentrations.

The hydroxylated metabolites, which represent minor routes, are unlikely to play a significant role in TAM clear-

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**Fig. 6.** Metabolism of N-desmethyltamoxifen (N-desmethyl-TAM) and 4-hydroxytamoxifen (4-hydroxy-TAM) by a panel of recombinant human P450 isoforms. Upper panel, metabolism of N-desmethyl-TAM to α-hydroxy N-desmethyl-TAM (A), endoxifen (B), and N-desmethyl-TAM (C). Lower panel, metabolism of 4-hydroxy-TAM to 3,4-dihydroxy-TAM (D) and endoxifen (E). Data are presented as average picomoles product per minute per picomole P450 of duplicate incubation.
ance, but the possibility that these metabolites contribute to its activity/toxicity in vivo cannot be ruled out. TAM 4-hydroxylation is the most studied in this respect because it has been shown that 4-hydroxy-TAM is approximately 30- to 100-fold more potent antiestrogen than TAM (Borgna and Rochefort, 1981; Coezy et al., 1982; Jordan, 1982; Robertson et al., 1982) and because it is believed to be on the path to a reactive intermediate formation that binds covalently to proteins (Dehal and Kupfer, 1999). For these reasons, several investigators have attempted to identify P450s responsible for formation of 4-hydroxy-TAM, with varying results (e.g., Crewe et al., 1997, 2002; Dehal and Kupfer, 1997; Coller et al., 2002). We conducted comprehensive studies in multiple HLMs and expressed P450s. Our data suggest that TAM 4-hydroxylation can be predominantly catalyzed by CYP2D6 in some HLMs with high CYP2D6 activity. However, in conditions with diminished (or absent) CYP2D6 activity, it seems that other isoforms become important. These data are consistent with the involvement of multiple P450s and with our clinical data that show relatively little interpatient variability of 4-hydroxy-TAM in the plasma of breast cancer patients taking tamoxifen (Stearns et al., 2003). α-Hydroxy-TAM and its sulfated metabolites have been implicated in TAM-induced toxicity in vitro and animal studies (White, 2003). The present data and previous reports (White, 2003; Crewe et al., 1997, 2002; Dehal and Kupfer, 1997; Coller et al., 2002). We conducted comprehensive studies in multiple HLMs and expressed P450s. Our data suggest that TAM 4-hydroxylation can be predominantly catalyzed by CYP2D6 in some HLMs with high CYP2D6 activity. However, in conditions with diminished (or absent) CYP2D6 activity, it seems that other isoforms become important. These data are consistent with the involvement of multiple P450s and with our clinical data that show relatively little interpatient variability of 4-hydroxy-TAM in the plasma of breast cancer patients taking tamoxifen (Stearns et al., 2003). α-Hydroxy-TAM and its sulfated metabolites have been implicated in TAM-induced toxicity in vitro and animal studies (White, 2003). The present data and previous reports (White, 2003; Crewe et al., 1997, 2002; Dehal and Kupfer, 1997; Coller et al., 2004) suggest that CYP3A4 and CYP3A5 are important catalysts of TAM α-hydroxylation, raising the possibility that high CYP3A activity in patients may enhance TAM-induced toxicity. We have also provided the first evidence that 4’- and 3-hydroxy-TAM are formed in HLMs and that CYP2B6 (and probably CYP2C19) and CYP3A5, respectively, might be the principal catalysts of these reactions. The relevance of 4’- and 3-hydroxy-TAM to the multiple effects of the drug and their abundance in plasma of patients remains to be determined. Limited studies available in the literature suggest that these metabolites might have higher affinity for the estrogen receptor than TAM (Ruenitz et al., 1982; Roos et al., 1983).

**TAM Sequential Biotransformation to Its Secondary Metabolites.** We describe here the first comprehensive in vitro characterization of TAM metabolism to its secondary metabolites, using its primary metabolites as intermediaries (Fig. 7). We have demonstrated that N-desmethyl-TAM and 4-hydroxy-TAM, like TAM, undergo extensive oxidation by the P450 system to a number of metabolites: α-hydroxy N-desmethyl-TAM, endoxifen, and N-didesmethyl-TAM were the major N-desmethyl-TAM metabolites; and 3,4-dihydroxy-TAM and endoxifen were the principal metabolites of 4-hydroxy-TAM. Our previous (Stearns et al., 2003) and current works have demonstrated that endoxifen is formed primarily from N-desmethyl-TAM, but we have shown herein that endoxifen is also formed from 4-hydroxy-TAM.

Several lines of evidence obtained from a variety of experimental approaches clearly show a prominent role of CYP3A in TAM secondary metabolism: we have shown for the first time that the metabolism of N-desmethyl-TAM to α-hydroxy N-desmethyl-TAM and of 4-hydroxy-TAM to endoxifen is predominantly catalyzed by CYP3A, and we have confirmed CYP3A as the principal catalyst of N-desmethyl-TAM metabolism to N-didesmethyl-TAM and of 4-hydroxy-TAM metabolism to 3,4-dihydroxy-TAM, consistent with other reports (Dehal and Kupfer, 1999; Coller et al., 2004). These findings may have important implications. 3,4-Dihydroxy-TAM is probably further oxidized to a reactive intermediate that covalently binds to proteins (Dehal and Kupfer, 1999). N-Didesmethyl-TAM appears to be highly concentrated in the liver and is a potent inhibitor of certain P450s that include CYP3A (Comoglio et al., 1996), and this metabolite may mediate drug interactions involving TAM, or it may modify TAM-induced toxicity by inhibiting formation of P450-catalyzed reactive and toxic metabolites of TAM (Comoglio et al., 1996). Little is known regarding α-hydroxy N-desmethyl-TAM, but, like α-hydroxy-TAM (White, 2003), it might participate in TAM-induced genotoxicity. It is known that the expression of CYP3A4/5 is highly variable largely due to interindividual differences in the response to various environmental exposures (coadministered drugs, herbal medicines, nutritional supplements) and as the result of genetic polymorphisms that code for CYP3A4/5 proteins. Given the major role played by CYP3A in catalyzing a number of pr-
mary and secondary metabolic pathways of TAM, we would expect that interindividual differences in CYP3A activity alters TAM activation/detoxification patterns and its efficacy/toxicity in vivo.

Our recent clinical study showed that the plasma concentrations of endoxifen in breast cancer patients exhibit large interindividual variation (Stearns et al., 2003). Although endoxifen was formed from both N-desmethyl- and 4-hydroxy-TAM, the P450s involved in its formation depended on the substrate used: CYP2D6 when N-desmethyl-TAM was used as a substrate and CYP3A when 4-hydroxy-TAM was used as a substrate. Despite the efficient conversion of 4-hydroxy-TAM to endoxifen in vitro, the quantitative contribution of this route is likely small. Instead, the 4-hydroxylation of N-desmethyl-TAM by CYP2D6 appears to be the major source of endoxifen production in vivo. First, consistent with our in vitro data, we have clinical evidence that endoxifen plasma concentrations in breast cancer patients receiving TAM (20 mg/day) are significantly reduced by coprescription of paroxetine (a known inhibitor of CYP2D6) and in patients who carry variant alleles of CYP2D6 (Stearns et al., 2003).

Second, the steady-state plasma N-desmethyl-TAM concentrations after therapeutic doses of TAM are much higher (on the average >70-fold) than those of 4-hydroxy-TAM (Lonning et al., 1992; Stearns et al., 2003), essentially in agreement with our in vitro findings that approximately 90% of TAM oxidation is accounted for by N-desmethyl-TAM formation. Whether other factors, such as altered production of the precursor (N-desmethyl-TAM) from TAM by CYP3A or altered elimination of endoxifen itself due to conjugation by phase II enzymes, contribute to the interpatient variability in endoxifen production in vivo remains to be tested.

Endoxifen is of particular interest to us for a number of reasons. Although this metabolite was identified in human bile in 1988 (Lien et al., 1988) and subsequently in other human biological fluids including plasma (Lien et al., 1989, 1990, 1991), its biological significance was not clear until recently. A series of in vitro studies conducted by our group suggest that endoxifen exhibits similar potency to 4-hydroxy-TAM in terms of its ability to bind with estrogen receptors, in suppressing estrogen-dependent growth of human breast cancer cell lines (Stearns et al., 2003; Johnson et al., 2004), and in global estrogen-dependent gene expression (Lim et al., 2004). There is evidence that endoxifen is much more abundant in plasma of breast cancer patients than 4-hydroxy-TAM (Lien et al., 1990; Stearns et al., 2003). Thus, we speculate that a significant part of TAM pharmacological activity in vivo may be due to the conversion of TAM to active metabolites, most notably endoxifen. The metabolic activity of CYP2D6 exhibits high interindividual variability, mainly due to the polymorphism of the CYP2D6 gene (over 80 alleles and allele variants have been described, many of which result in loss of enzyme function) (Zanger et al., 2004) but also due to several drugs that potently inhibit the activity of CYP2D6 and mimic the inactivating polymorphisms of the enzyme. It follows that variations in endoxifen concentrations that result from CYP2D6 polymorphisms and drug interactions may influence antitumoral efficacy and side effects of TAM. It is interesting to note that plasma TAM concentrations have been reported to be poor predictors of therapeutic outcome (Bratherton et al., 1984), but that they are also poor predictors of endoxifen concentrations in breast cancer patients (Y. Jin, Z. Desta, V. Stearns, B. Ward, H. Ho, J. Lee, T. Skaar, A. M. Storniolo, L. Li, A. Araba, et al., unpublished data). Further research is warranted to relate endoxifen plasma concentrations with validated clinical outcomes.

In conclusion, we provide a systematic and comprehensive characterization of TAM biotransformation to an array of primary and secondary metabolites. Our data make it clear that TAM metabolism is complex and that multiple approaches as opposed to a single approach are necessary to appropriately identify the metabolic routes of TAM and the P450s responsible at therapeutically relevant concentrations. Overall, CYP3A and CYP2D6 were identified as major enzymes involved in the principal TAM sequential metabolic routes (Figs. 1 and 7). Despite the recent introduction of aromatase inhibitor drugs for the treatment of breast cancer (Baum et al., 2002), TAM remains the endocrine treatment of choice for ER-positive tumors in premenopausal women and as a chemopreventive in women at high risk for the disease. Given that TAM and its metabolites exhibit different pharmacological activities that contribute to the overall therapeutic effect, tissue selectivity, and toxicity, our data should allow improved understanding of the mechanisms and factors that control TAM clearance, activation, and effects in vivo. They also form the basis for a variety of future studies designed to determine the effects of genetic polymorphisms and drug interactions on TAM response and toxicity.

References
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