INHIBITION AND ACTIVATION OF THE HUMAN LIVER MICROSOMAL AND HUMAN CYTOCHROME P450 3A4 METABOLISM OF TESTOSTERONE BY DEPLOYMENT-RELATED CHEMICALS

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ABSTRACT:

Cytochrome P450 (P450) enzymes are major catalysts involved in the metabolism of xenobiotics and endogenous substrates such as testosterone (TST). Major TST metabolites formed by human liver microsomes include 6β-hydroxytestosterone (6β-OHTST), 2β-hydroxytestosterone (2β-OHTST), and 15β-hydroxytestosterone (15β-OHTST). A screen of 16 cDNA-expressed human P450 isoforms demonstrated that 94% of all TST metabolites are produced by members of the CYP3A subfamily with 6β-OHTST accounting for 86% of all TST metabolites. Similar K_m values were observed for production of 6β-, 2β-, and 15β-OHTST with human liver microsomes (HLM) and CYP3A4. However, V_max and Clint were significantly higher for 6β-OHTST than 2β-OHTST (~18-fold) and 15β-OHTST (~40-fold). Preincubation of HLM with a variety of ligands, including chemicals used in military deployments, resulted in varying levels of inhibition or activation of TST metabolism. The greatest inhibition of TST metabolism in HLM was following preincubation with organophosphorus compounds, including chlorpyrifos, phorate, and fonofos, with up to 80% inhibition noticed for several metabolites including 6β-OHTST. Preincubation of CYP3A4 with chlorpyrifos, but not chlorpyrifos-oxon, resulted in 98% inhibition of TST metabolism. Phorate and fonofos also inhibited the production of most primary metabolites of CYP3A4. Kinetic analysis indicated that chlorpyrifos was one of the most potent inhibitors of major TST metabolites followed by fonofos and phorate. Chlorpyrifos, fonofos, and phorate inhibited major TST metabolites non-competitively and irreversibly. Conversely, preincubation of CYP3A4 with pyrdostigmine bromide increased metabolite levels of 6β-OHTST and 2β-OHTST. Preincubation of human aromatase (CYP19) with the test chemicals had no effect on the production of the endogenous estrogen, 17β-estradiol.

The cytochrome P450 (P450^3) monooxygenase system is comprised of a superfamily of heme-containing enzymes, expressed in many mammalian tissues with the highest levels found in liver, and capable of catalyzing the metabolism of a wide range of both endogenous and exogenous substrates (Nelson et al., 1996). Human CYP3A4 is one of the most important and most abundant drug-metabolizing P450 isoforms in human liver microsomes and accounts for approximately 40% of the total P450 in human liver microsomes (Lehmann et al., 1998). CYP3A4 not only metabolizes xenobiotics but is also responsible for the metabolism of endogenous compounds, such as steroid hormones. Human CYP3A4 plays an important role in the metabolism of testosterone (TST), androstenedione (AD), and progesterone (Waxman et al., 1988). Direct and indirect approaches have been employed to show that isoforms belonging to the CYP3A subfamily are the major contributors to 6β-hydroxylation of testosterone as well as the production of several minor metabolites (Waxman et al., 1988, 1991; Yamazaki and Shimada, 1997).

In the human male, TST is the major circulating androgen. TST is essential for the development and maintenance of specific reproductive tissues as well as for other characteristic male properties such as control of spermatogenesis, retention of nitrogen, promotion of muscle strength, hair growth, bone density, and many aspects of sexually dimorphic behavior (Nieschlag and Behre, 1998; Wilson et al., 1998). Maintaining hormonal balance relies upon a number of variables including rate of hormone synthesis, interactions among hormones, and rates of secretion, transport, and metabolism. P450s are a major controlling element in the maintenance of proper steroid hormone levels in mammalian systems. Exposure to foreign compounds can exert changes in endocrine function both directly (hormone agonists or antagonists) or indirectly (altering circulating levels of hormones by influencing rates of hormone synthesis or metabolism) that can severely affect steroid hormone action (Wilson and LeBlanc, 1998). Steroids such as TST are hydroxylated by P450 in a regioselective and stereoselective manner (Waxman et al., 1988). It follows that perturbation of the P450 system by xenobiotics may in turn affect the subsequent metabolism and disposition of TST. Perturbations in TST metabolism may affect levels of circulating TST with possible reproductive and other consequences, including further modulation of the expression of some P450 proteins.
Following the Gulf War some veterans reported illnesses which may have been the result of chemical exposures. Some studies of these veterans have concluded that significant correlations between perceived illnesses and chemical use exist (Haley and Kurt, 1997). The reported chemical exposures included the insect repellent N,N-diethyl-m-toluamide (DEET), insecticides such as permethrin and chlorpyrifos to protect against insect borne diseases and the neuroprotective agent pyridostigmine bromide to protect against possible nerve gas attack. It has been reported that chlorpyrifos and DEET are metabolized by human P450s (Tang et al., 2001; Usmani et al., 2002) and that interactions of Gulf War related chemicals can inhibit or induce the P450s involved in their metabolism (Usmani et al., 2002). Other studies have reported that interaction of Gulf War related chemicals could produce greater than additive toxicity in rats and mice (Chaney et al., 1997; McCain et al., 1997), increased neurotoxicity in hens associated with increased inhibition of brain acetylcholinesterase and Neurotoxicity Target Esterase (Abou-Donia et al., 1996a,b), and neurobehavioral deficit associated with significant inhibition of brainstem acetylcholinesterase activities in rats (Abou-Donia et al., 2001). However, no studies have been carried out to examine the induction or acetylcholinesterase activities in rats (Abou-Donia et al., 2001). How- ever, no studies have been carried out to examine the induction or inhibition potential of these or related compounds on human P450-mediated metabolism of steroid hormones, such as TST. An understand- ing of how Gulf War related chemicals affects the metabolism of TST could aid in the evaluation of the possible role that these chemicals may play in deployment-related illnesses.

The main objectives of present study were to identify human liver P450 isoforms responsible for TST metabolism and the products of their activity using an improved HPLC method, to study the effects of various deployment-related chemicals on the metabolism of TST using HLM and CYP3A4, and to study the effects of the test compounds on human aromatase (CYP19).

Materials and Methods

**Chemicals.** DEET, chlorpyrifos, chlorpyrifos-oxon, phorate, fonofos, deltamethrin, fipronil, imidacloprid, and permethrin (isomeric mix 78% trans-20% cis) were purchased from Chem Service (West Chester, PA). Pyridostigmine bromide was purchased from Roche Diagnostics (Indianapolis, IN), 6a-, 15β-, 15α-, 7α-, 6β-, 16α-, 16β-, 2α-, 2β-, 11β-OHTST, 11-ketotestosterone (11-KTST), 11β-hydroxyandrostenedione (11β-OHAD), AD, and 4-hydroxyandrostenedione (4-OHAD) were purchased from Steraloids (Newport, RI). HPLC grade water, methanol, acetonitrile, and tetrahydrofuran were purchased from Fisher Scientific (Pittsburgh, PA). TST, 17β-estradiol, and all other chemicals were purchased, if not specified, from Sigma-Aldrich (St. Louis, MO).

**Human Liver Microsomes and Human P450 Isoforms.** Pooled human liver microsomes (HLM) (pooled from 21 donors) and human P450 isoforms expressed in baculovirus infected insect cells (S9) (BTI-TN-5B1–4), CYP1A1, 1A2, 2B6, 3A4, 3A5, 3A7, 4A11, 2B6, 2C8, 2A6, 2C9*1 (Arg144), 2C9*2 (Cys44), 2C9*3 (Leu49), 2C18, 2C19, 2D6*1 (Val374), 2E1, and human aromatase (CYP19) were purchased from BD Gentest Corporation.

**In Vitro TST Metabolism.** Metabolite activity assays for human P450 isoforms were performed by incubation of TST (final concentrations, 250 μM) with an NADPH-regenerating system (0.25 mM NADP, 2.5 mM glucose 6-phosphate, and 2 U/ml glucose-6-phosphate dehydrogenase) in specific buffers recommended by the supplier (BD Gentest Corporation). For CYP1A1, 1A2, 2E1, 2C8, 2D6*1 (Val374), 3A4, 3A5, 3A7, 2B6, 2C18, 2C19, and an insect cell control, a 100 mM potassium phosphate buffer with 3.3 mM MgCl2 (pH 7.4) was used. For 2C9*1 (Arg144), 2C9*2 (Cys44), 2C9*3 (Leu49), 4A11, and 2A6, a 100 mM Tris-Cl buffer with 3.3 mM MgCl2 (pH 7.5) was used. After preincubation at 37°C for 5 min, the reactions were initiated by the addition of ice-cold P450 isoforms (final P450 contents 50 pmol/ml) for 30 min at 37°C. The controls were performed under identical conditions with the insect cell control.

Enzyme kinetic assays for HLM and CYP3A4 were performed by incubation of serial concentrations of TST (final concentrations, 9.375–500 μM) with HLM (final protein concentration, 1 mg/ml) or CYP3A4 (final concentration, 50 pmol/ml) in 100 mM potassium phosphate buffer (pH 7.4 at 37°C) containing 3.3 mM MgCl2. After preincubation at 37°C for 5 min, the reactions were initiated by the addition of ice-cold HLM or CYP3A4 for 10 min.

The effects of test chemicals on TST metabolism were examined in HLM and CYP3A4 after preincubation with test compounds. The HLM (final protein concentration, 1 mg/ml) or CYP3A4 (final concentration, 50 pmol/ml) were incubated with individual test compounds (final concentration, 100 μM), NADPH-generating system, and 100 mM potassium phosphate buffer with 3.3 mM MgCl2, pH 7.4, for 5 min at 37°C before adding TST (final concentration, 100 μM). Reactions were terminated and analyzed as described above. With selected concentration levels based on the range finding assay, using chlorpyrifos, fonofos, and phorate inhibition on TST major metabolites was investigated. For Michaelis-Menten plots, chlorpyrifos (2 μM), fonofos (5 μM), and phorate (30 μM) were incubated with CYP3A4 (final concentration, 50 pmol/ml), NADPH-generating system, and 100 mM potassium phosphate buffer with 3.3 mM MgCl2, pH 7.4, for 5 min at 37°C before adding TST (final concentration, 100 μM).

To determine (inhibition constant) Ki, values of chlorpyrifos (1–8 μM), fono- fos (1–25 μM), and phorate (10–100 μM) were incubated for 5 min at 37°C with CYP3A4 (final concentration, 50 pmol/ml), NADPH-generating system, and 100 mM potassium phosphate buffer with 3.3 mM MgCl2, pH 7.4, prior to adding TST (final concentrations, 50, 100, or 200 μM). Ki values were calculated from Dixon plots.

Since cytochrome b5 (b5) is not coexpressed with CYP3A4 as supplied by BD Biosciences (San Jose, CA), a comparison of CYP3A5 metabolism of TST was made using 10 pmol 3A5 with and without addition of 20 pmol b5. Human aromatase (CYP19) catalyzes the conversion of TST to estradiol. To study the effects of the test chemicals on this conversion, test compounds (final concentration, 200 μM) or a well known competitive inhibitor, 4-OHAD (final concentration, 200 μM) were incubated with CYP19 (final concentration, 50 pmol/ml), NADPH-generating system, and 100 mM potassium phosphate buffer with 3.3 mM MgCl2, pH 7.4, for 5 min at 37°C before adding TST (final concentration, 100 μM). The reaction was terminated after an additional 10 min, and supernatant was analyzed for 17β-estradiol concentration by HPLC.

All assays were conducted in triplicate. All reactions were terminated by the addition of an equal volume of methanol and vortexing. After 10-min centrif-

<table>
<thead>
<tr>
<th>Common Name</th>
<th>Chemical Name</th>
<th>Retention Time</th>
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<tbody>
<tr>
<td>6α-Hydroxytestosterone</td>
<td>4-Androsten-6α,17β-diol-3-one</td>
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</tr>
<tr>
<td>15β-Hydroxytestosterone</td>
<td>4-Androsten-15β,17β-diol-3-one</td>
<td>15.11</td>
</tr>
<tr>
<td>15α-Hydroxytestosterone</td>
<td>4-Androsten-15α,17β-diol-3-one</td>
<td>15.53</td>
</tr>
<tr>
<td>7α-Hydroxytestosterone</td>
<td>4-Androsten-7α,17β-diol-3-one</td>
<td>15.81</td>
</tr>
<tr>
<td>6β-Hydroxytestosterone</td>
<td>4-Androsten-6β,17β-diol-3-one</td>
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<td>16α-Hydroxytestosterone</td>
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<tr>
<td>11-Ketotestosterone</td>
<td>4-Androsten-17β-ol-3,11-dione</td>
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<td>11β-Hydroxyandrostenedione</td>
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<td>2β-Hydroxytestosterone</td>
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<td>Testosterone</td>
<td>4-Androsten-17β-ol-3-one</td>
<td>28.90</td>
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Uglation at 15,000 rpm in a microcentrifuge, the supernatants were analyzed for TST metabolite concentrations by HPLC. The protein concentrations and incubation times used in the assays were found to be in the linear range in preliminary experiments. No metabolites were detected when incubations were carried out in the absence of an NADPH-generating system.

Analysis of Metabolites by HPLC. Metabolites were analyzed using a Shimadzu HPLC system (Kyoto, Japan) used in this study consisted of one pump (LC-10AT VP), a four-position selection proportioning valve (FCV-10AL VP), a degasser (DUG-14A), a Shimadzu autoinjector (SIL-10AD VP), and a Shimadzu UV/Vis detector (SPD-10AV VP). All system components were controlled through the Shimadzu powerline firmware. Data were collected via a Shimadzu HPLC system controller (SCL-10A VP) and analyzed using CLASS-VP 4.3 software. A reverse phase HPLC method was modified based on the HPLC method of Purdon and Lehman-McKeeman (1997), for the separation of TST metabolites. The mobile phase for pump A was 5% tetrahydrofuran, 95% water, for pump B 100% methanol. A gradient system was made in methanol and 50-60% B, 10 to 22 min (60–65% B), 22 to 28 min (65–80% B), 28 to 30 min (80–90% B), 30 to 32 min (90% B), 32 to 34 min (90–30% B), and 34 to 36 min (30% B). The flow rate was 0.5 ml/min. Metabolites were separated by a Prodigy column [Prodigy 3 μ, 150 × 4.6 mm, ODS (3), 100A; Phenomenex, Rancho Palos Verdes, CA] and detected at 247 nm. A summary of the retention times of TST and 14 TST metabolites are presented in Table 1. The limits of detection for most of TST metabolites were approximately 0.04 μM except for 6β-OH-TST (0.15 μM) and 4-OHAD (0.30 μM). Standards of TST metabolites were made in methanol and 50-μl standard or sample injected on HPLC. Concentrations of metabolites were obtained by extrapolation of peak height from a standard curve. Percentages of individual metabolites are expressed on the basis of the total metabolites produced by the isof orm or preparation in question.

For 17β-estradiol, the mobile phase was 60% H2O and 40% acetonitrile. TST and 17β-estradiol were eluted isocratically at a flow rate of 1.0 ml/min for 15 min, separated by a Prodigy column [Prodigy 3 μ, 150 × 4.6 mm, ODS (3), 100A, Phenomenex, Rancho Palos Verdes, CA] and detected at 200 nm. The retention time of 17β-estradiol and TST was 10.4 and 11.3 min, respectively.

### TABLE 2

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<th>Isof orms</th>
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<th>15β-OHTST</th>
<th>6β-OHTST</th>
<th>16α-OHTST</th>
<th>11-KT</th>
<th>16β-OHTST</th>
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<th>AD</th>
<th>4-OHAD</th>
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<td>2A6</td>
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<td>0.14 ± 0.01</td>
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<td>NDA</td>
<td>0.53 ± 0.05</td>
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<td>3A4</td>
<td>0.22 ± 0.02</td>
<td>3.18 ± 0.11</td>
<td>157.7 ± 6.00</td>
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<td>1.04 ± 0.04</td>
<td>0.44 ± 0.01</td>
<td>1.70 ± 0.02</td>
<td>0.19 ± 0.01</td>
<td>7.05 ± 0.23</td>
<td>0.27 ± 0.01</td>
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<td>3A5</td>
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<td>12.4 ± 1.57</td>
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<td>3A7</td>
<td>0.09 ± 0.01</td>
<td>0.15 ± 0.02</td>
<td>3.89 ± 0.34</td>
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<td>0.79 ± 0.05</td>
<td>3.05 ± 0.24</td>
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<td>0.06 ± 0.00</td>
<td>1.40 ± 0.08</td>
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</table>

NDA, no detectable activity; no metabolite was formed with 2C9+3.

Fig. 1. Effects of deployment-related chemicals on the rate of testosterone metabolism by pooled human liver microsomes.

Specific activities were expressed as nanomole products formed per nanomole P450 per minute. *, statistically significantly different when compared with respective control (P < 0.01).
The limit of detection for 17β-estradiol was approximately 0.10 μM. Concentrations of metabolites were obtained by extrapolation of peak height from a standard curve.

Data Analysis and Statistics. The apparent $K_m$ and $V_{max}$ parameters were calculated using nonlinear regression analysis program (Prism, GraphPad software Inc., San Diego, CA), and the $K_i$ values were estimated by nonlinear regression analysis from the Dixon plot (Segel, 1975) using SigmaPlot Enzyme Kinetics Module (Chicago, IL). Significant differences between data sets were determined by one-way analysis of variance, and multiple comparisons were performed with the Dunnett’s method using a JMP 4.0.2, SAS program (SAS, 1989).

Results

Four major metabolites were formed after incubation of TST with pooled HLM: 6β-, 2β-, 15β-OHTST, and 4-OHAD as well as seven minor metabolites (Fig. 1). Among 16 different human P450 isoforms screened, only 2C9*3 (Leu 359) had no detectable activity toward TST (Table 2). All other P450 isoforms were active in generating one or more than one TST metabolites, although the extent of metabolism and the ratios of metabolites varied widely among isoforms. In this comparison of metabolite production by equal quantities of each isoform, CYP3A4, 3A5, and 3A7 were most active in TST metabolism among all the P450 isoforms tested (93.5% of the metabolites produced by all isoforms). Among members of the CYP3A subfamily, CYP3A4 produced the highest amount of total TST metabolites (88.5%) compared with 3A5 (6.9%) and 3A7 (4.6%). 6β-OHTST, the most prominent TST metabolite, accounts for 86% of all TST metabolites. Among the CYP3A subfamily, CYP3A4 produced the highest amount of 6β-OHTST (90.6%) compared with 3A5 (7.1%) and 3A7 (2.2%). Other major TST metabolites formed by CYP3A4 were 15β-, 2β-OHTST, and 4-OHAD, whereas 6α-, 16β-, 11β-, 2α-OHTST, 11-KTST, and AD were minor metabolites. Among the P450 isoforms tested, CYP3A5 and 3A7 were significantly more important in forming the major TST metabolites than most of the others, but their activity was 10- to 20-fold less than that of CYP3A4. Interestingly, CYP3A7 produced 16 times more 2α-OHTST than CYP3A4. CYP1A1 is involved in the oxidation of TST at the 6β-position (3.0 nmol/nmol isoform/min), whereas CYP1A2 oxidized TST poorly at the 6β-position (0.6 nmol/nmol isoform/min). As can be observed in Table 2, the other P450 isoforms tested generally produced small amounts of one or more TST metabolites. CYP2C19 metabolized TST to AD more actively.
than any other isoform tested, whereas it catalyzed the formation of 6α-, 6β-, 16α-, 16β-, and 2β-OHTST poorly.

HLM and CYP3A4 displayed similar $K_m$ values for 6β-, 2β-, and 15β-OHTST (Table 3). $V_{max}$ and intrinsic clearance rate ($CL_{int} (V_{max}/K_m)$) for 6β-OHTST was significantly higher than 2β-OHTST (~18-fold) and 15β-OHTST (~40-fold), respectively.

The effects of various deployment-related chemicals on TST metabolism were investigated by preincubating them with pooled HLM (Fig. 1). Preincubation of pooled HLM with chlorpyrifos, phorate, and fonofos resulted in significant inhibition of 6β-, 2β-, 15β-OHTST, 11-KTST, 11β-OHAD, and 4-OHAD. Preincubation of pooled HLM with DEET, chlorpyrifos-oxon, phorate, imidacloprid, and deltamethrin in some cases caused small but significant increases in the production of some TST metabolites by HLM.

Preincubation of CYP3A4 with a variety of chemicals resulted in varying levels of activation and inhibition of TST metabolism (Fig. 2). The greatest inhibition of TST metabolism was observed for the organophosphorus compound chlorpyrifos with up to 98% inhibition of major (6β-, 2β-, 15β-OHTST, and 4-OHAD) and several minor (11-KTST, 16β-OHTST, 11β-OHAD, and AD) TST metabolites. However, chlorpyrifos-oxon, an active metabolite of chlorpyrifos, has no inhibitory effect on the major TST metabolites. Two other organophosphorus compounds, phorate and fonofos, also significantly inhibited formation of several TST metabolites including 6β-, 2β-, 15β-OHTST, 11-KTST, 11β-OHAD, AD, and 4-OHAD. In contrast, preincubation of CYP3A4 with pyridostigmine bromide resulted in the production of small but significantly greater levels of the 6β- and 2β-OHTST metabolites. Some other TST metabolites were also significantly increased by preincubation of CYP3A4 with chlorpyrifos-oxon, phorate, and fonofos.

To investigate the type of inhibition of CYP3A4 by chlorpyrifos, fonofos, and phorate on major TST metabolites, chlorpyrifos (2 μM), fonofos (5 μM), and phorate (30 μM) were preincubated for 5 min before adding the varying concentrations of TST. Michaelis-Menten plots showed that the $V_{max}$ values were significantly reduced without affecting $K_m$ values, indicative of a noncompetitive inhibition of major TST metabolites by chlorpyrifos (Fig. 3). Similar results were obtained with fonofos and phorate (data were not shown). Further investigation of noncompetitive reversible or nonreversible inhibition data revealed that the inhibition is nonreversible (Fig. 4).

The $K_i$, an indicator of inhibitor affinity to target enzyme, was calculated by Dixon plot (Table 4; Fig. 5). Chlorpyrifos was the most
potent inhibitor of major TST metabolites with $K_i$ values ranges from 2.0, 3.6, and 3.7 μM for 6β-, 2β-, 15β-OHTST, respectively. Fonofos was the second best inhibitor with $K_i$ values ranging from 5.8, 10.1, and 6.3 μM for 6β-, 2β-, 15β-OHTST, respectively. Phorate $K_i$ values ranged from 34.1, 42.9, and 33.8 μM for 6β-, 2β-, 15β-OHTST, respectively.

We investigated the possibility that $b_5$ may stimulate CYP3A5 catalytic activity by incubating $b_5$ (20 pmol) and CYP3A5 (10 pmol), which, in the preparations used, does not have $b_5$ coexpressed, with 250 μM of TST for 10 min. Addition of $b_5$ resulted in a more than 2-fold increase in TST 6β- and 2β-OHTST activity.

The possibility that conversion of TST to estradiol, which is catalyzed by aromatase (CYP19), could be inhibited by the test compounds was also investigated. Preincubation of human aromatase (CYP19) with various chemicals (chlorpyrifos, chlorpyrifos-oxon, permethrin, pyridostigmine bromide, DEET, phorate, fonofos, fipronil, imidacloprid, and deltamethrin) had no significant effect on the production of estradiol (data not shown). However, incubation with 4-OHAD, a well known competitive aromatase inhibitor, resulted in 90% inhibition of the aromatase enzyme activity.

### Discussion

P450-dependent hydroxylation appears to be a major pathway of oxidative metabolism of TST in mammalian liver. Studies carried out using human P450 isoforms provide further insight into the range of TST hydroxylation reactions that can be catalyzed by human P450 enzymes. Our isoform data corroborates earlier findings (Waxman et al., 1988, 1991; Yamazaki and Shimada, 1997) that CYP3A4 is one of the major isoforms responsible for TST metabolism, and 6β-OHTST is the major TST metabolite. Greater than 82% of the TST metabolites are formed by CYP3A4, and 87% of the major 6β-OHTST metabolite is formed by CYP3A4. The mean metabolic intrinsic clearance rates, as estimated by $V_{max}/K_{cat}$, also indicated that 6β-OHTST is the major metabolite of TST. Interestingly, CYP3A4 also metabolized TST to 4-OHAD, a potent inhibitor of extrahematic aromatase (CYP19). It has been reported that 4-OHAD was able to inhibit 90% of the aromatase activity at a concentration of 1 μM (Mak et al., 1999). The physiological significance or consequence of this reaction is unclear and will require further investigation. Our results indicate that CYP1A1 and 1A2 were able to metabolize TST to 6β-OHTST, however, activity of CYP1A1 was much higher (4.7-fold) than CYP1A2. Consistent with a previous report (Yamazaki and Shimada, 1997), our data also indicated that CYP2C19 catalyzed oxidation of TST to form AD as a major TST metabolite. However, CYP2C18, which has 81% amino acid sequence identity to CYP2C19, exhibited distinctly poor hydroxylation activity in comparison with CYP2C19. Furthermore, our data indicated that CYP2D6*1, 4A11, and 2A6 metabolized TST to form AD but not as actively as CYP2C19. Guengerich et al. (2002) characterized the affinity of CYP2D6 for testosterone.

Endogenous steroids, such as TST, always exist in vivo, and considerable amounts of these steroids are metabolized by the P450s expressed in the human liver, where foreign compounds are mainly metabolized. If xenobiotics substantially affect TST metabolism, it may alter the rate of TST metabolism, which may ultimately disrupt TST homeostasis. Preincubation of pooled HLM with organophosphorus compounds, such as chlorpyrifos, phorate, and fonofos, resulted in the extensive inhibition of major and some minor TST metabolism.
metabolites. Chlorpyrifos, fonofos, and phorate inhibited major TST metabolites noncompetitively and irreversibly, and it is clear that organophosphorus compounds are some of the most potent inhibitors of the CYP3A4-dependent oxidation of TST yet described. Organophosphorus pesticides, such as chlorpyrifos, phorate, and fonofos are activated by a P450-catalyzed desulfuration reaction (Fukuto, 1990). The sulfur atom released from these pesticides in this reaction is highly reactive and is believed to bind immediately to the heme iron of P450 and inhibit its activity (Neal, 1980). On the other hand, enzyme stimulation is a process by which direct addition of one chemical to an enzyme stimulates the rate of reaction of the substrate (Guengerich, 1997). Our data indicated that some compounds, such as pyridostigmine bromide, DEET, chlorpyrifos-oxon, phorate, imidacloprid, and deltamethrin may stimulate the production of some of the TST metabolites.

Several studies, including this, have shown that CYP3A4 is the major P450 involved in the metabolism of TST in human liver microsomes (Waxman et al., 1988, 1991; Yamazaki and Shimada, 1997). Either inhibition or induction can modulate the activity of an enzyme; P450s may exhibit stimulation or inhibition in the presence of certain xenobiotic compounds (Guengerich, 1997; Szklarz and Halpert, 1998). It has been suggested that CYP3A4 is an allosteric enzyme, even though the identity of the allosteric site is not known (Shimada and Guengerich 1989; Lee et al., 1995). In addition, it is known that CYP3A4 is a polar solvent molecule and even more than one substrate (Shou et al., 1994). Inhibition may, in some interactions, be more serious than enzyme induction since inhibition happens more rapidly, not taking time to develop, as with induction (Guengerich, 1997). Preincubation of CYP3A4 with chlorpyrifos resulted in almost complete inhibition of major TST metabolites. The K_i value indicated that chlorpyrifos is one of the most potent inhibitors yet shown for the production of major TST metabolites. This inhibition was not due to inhibition by the metabolite, chlorpyrifos-oxon, since the latter had no inhibitory effect on the production of the major TST metabolites. Phorate and fonofos also inhibited the production of major and some minor metabolites of TST. The K_i value indicated that fonofos was a much better inhibitor of major TST metabolites than phorate. The possibility exists that inhibition of CYP3A4 may lead to higher levels of TST and may alter hormonal properties. However, in vivo studies are necessary to understand the impact of these changes. Preincubation with pyridostigmine bromide resulted in higher production of 6β- and 2β-OH-TST, suggesting stimulation of CYP3A4. Preincubation with chlorpyrifos-oxon, phorate, and fonofos with CYP3A4 also resulted in activation of the production of some TST metabolites. A number of in vivo studies in rodents have shown that organochlorine pesticides increased the overall rate of TST metabolism (Cassidy et al., 1994; Wilson and LeBlanc, 1998; Dai et al., 2001).

Several studies have demonstrated that simultaneous expression of CYP3A4 and P450 reductase in bacterial or baculovirus-based insect cell membranes can produce high catalytic activity for TST 6β-octanolysis (Guengerich and Johnson 1997; Shaw et al., 1997), although addition of b5 to the system can enhance the reaction rates (Yamazaki et al., 1999). In contrast to the CYP3A4 used in these experiments, cytochrome b5 was not coexpressed in CYP3A5. A comparison of CYP3A5 with and without the addition of exogenous b5 demonstrated a 2-fold increase in the activity of 6β- and 2β-octanolysis in the presence of b5.

Human aromatase (CYP19), an extrahepatic P450, catalyzes the conversion of TST via three hydroxylation steps to estradiol. Inhibitors of aromatase currently in use have received considerable attention as treatments for postmenopausal breast cancer and other estrogen-dependent diseases (Bordie et al., 1999). Endocrine disruptors are hormone mimics that modify hormonal action in humans. Currently, inhibitors of human aromatase have been identified as potential endocrine disruptors or environmental toxicants (Mak et al., 1999). The chemicals used in this study have no significant effect on the activity of aromatase.

In conclusion, the hydroxylation of TST by P450 isoforms indicates important functions for these enzymes other than detoxification of xenobiotics. The present study provided further insight into the range of TST hydroxylation reactions that can be catalyzed by different human P450 isoforms. The deployment-related chemicals used in this study, including pesticides, caused a marked modification of P450-mediated TST metabolism in vitro. Organophosphorus pesticides were very potent inhibitors of the production of the primary metabolites of CYP3A4 and inhibited major TST metabolites noncompetitively and irreversibly. Addition of b5 to CYP3A5 increased the catalytic activity of this enzyme. Preincubation of the test chemicals had no effect on the production of estradiol from TST.

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