EVALUATION OF THE CONTRIBUTION OF CYTOCHROME P450 3A4 TO HUMAN LIVER MICROSOMAL BUPROPION HYDROXYLATION

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ABSTRACT:

The purpose of this investigation was to evaluate the role of cytochrome P450 (CYP) 3A4 in human liver microsomal bupropion (BUP) hydroxylation. Across the BUP concentration range of 0.075 to 12 mM, cDNA-expressed CYP3A4 demonstrated BUP hydroxylase activity only when incubated with concentrations ≥ 4 mM. When assayed at 12 mM BUP, cDNA-expressed CYP3A4 catalyzed BUP hydroxylation at a 30-fold lower rate than cDNA-expressed CYP2B6 (0.2 versus 7 pmol/min/pmol of P450). Among a panel of 16 human liver microsomes (HLMs), BUP hydroxylase activity varied 80-fold when assayed at 500 μM and did not strongly correlate with testosterone 6β-hydroxylase activity when assayed at 250 μM testosterone ($r^2 = 0.39$), nor with CYP3A4 protein expression. A se-

lective CYP3A4 inhibitor, troleandomycin (TAO), did not significantly alter rates of BUP hydroxylation when assayed in a moderate activity HLM at 10 to 2000 μM BUP, as reflected by a similarity in the kinetic parameters of BUP hydroxylation in the absence or presence of TAO. In addition, the same range of TAO concentrations (0.025–100 μM) that inhibited testosterone 6β-hydroxylation in a concentration-dependent manner (46–81%) in pooled HLMs produced negligible inhibition (7%) of BUP hydroxylation when assayed at 500 μM BUP. These results suggest that CYP3A4 does not significantly catalyze BUP hydroxylation. Furthermore, these results complement recent data supporting selectivity of BUP hydroxylation for CYP2B6 at 500 μM BUP.

Bupropion (BUP)[1] is a second-generation antidepressant agent that is also used in the management of smoking cessation. This drug undergoes extensive hepatic metabolism in humans via oxidative and reductive pathways (Fig. 1). A primary route of BUP metabolism includes methyl-hydroxylation of its *t*-butyl substituent to form hydroxybupropion (HBUP), a major constituent in human plasma following oral administration of BUP (Welch et al., 1987). HBUP exhibits more potent antidepressant activity compared with other BUP metabolites, as measured by prevention of tetrabenazine-induced sedation in rodents. In addition, HBUP possesses greater toxicity potential compared with BUP and its other metabolites, as evidenced by lower LD_{50} values in mice (Schroeder, 1983). After standard oral doses of BUP (300–450 mg/day), human plasma and cerebrospinal

[1] Abbreviations used are: BUP, bupropion; HBUP, hydroxybupropion; AUC, area under the plasma concentration-time curve; CYP or P450, cytochrome P450; HLM, human liver microsome; TAO, troleandomycin; HPLC, high-performance liquid chromatography.

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fluid concentrations of HBUP are approximately 4- and 6-fold greater than those of parent drug, respectively (Welch et al., 1987; Golden et al., 1988; Ketter et al., 1995; Hsyu et al., 1998). In addition, HBUP systemic exposure is characterized by a 10- to 16-fold greater AUC than BUP (Welch et al., 1987; DeVane et al., 1990; Ketter et al., 1995; Hsyu et al., 1998). Limited data suggest a relationship between BUP and HBUP plasma concentrations and antidepressant efficacy (Preskorn, 1983; Golden et al., 1988).

Clinical pharmacokinetic studies have demonstrated 3- to 10-fold interindividual differences in HBUP C_{max} and AUC (Laizure et al., 1985; DeVane et al., 1990; Ketter et al., 1995). Interindividual differences in the expression and activity of drug-metabolizing isozyme(s) responsible for BUP hydroxylation could potentially result in variable HBUP systemic exposure, leading to clinically important differences in the overall therapeutic and toxic effects of BUP. CYP3A4 has been postulated to catalyze BUP hydroxylation in vivo because carbamazepine, a recognized substrate and inducer of CYP3A4, was shown to decrease BUP yet increase HBUP C_{max} and AUC in subjects receiving concurrent carbamazepine and BUP therapy (Ketter et al., 1995). In addition, case reports have documented increased concentrations of sodium valproate in subjects receiving concomitant BUP (Popli et al., 1995), perhaps due to competitive inhibition of CYP3A4-mediated valproate metabolism by BUP.

In a prior in vitro study reported in abstract form, CYP3A4 demonstrated the second highest rate of BUP hydroxylation among a panel of cDNA-expressed P450 isozymes (Wurm et al., 1996). Although the rate of HBUP formation by cDNA-expressed CYP3A4 was at least 40-fold lower than by CYP2B6, the contribution of CYP3A4 to BUP hydroxylation in human liver could still be significant due to the
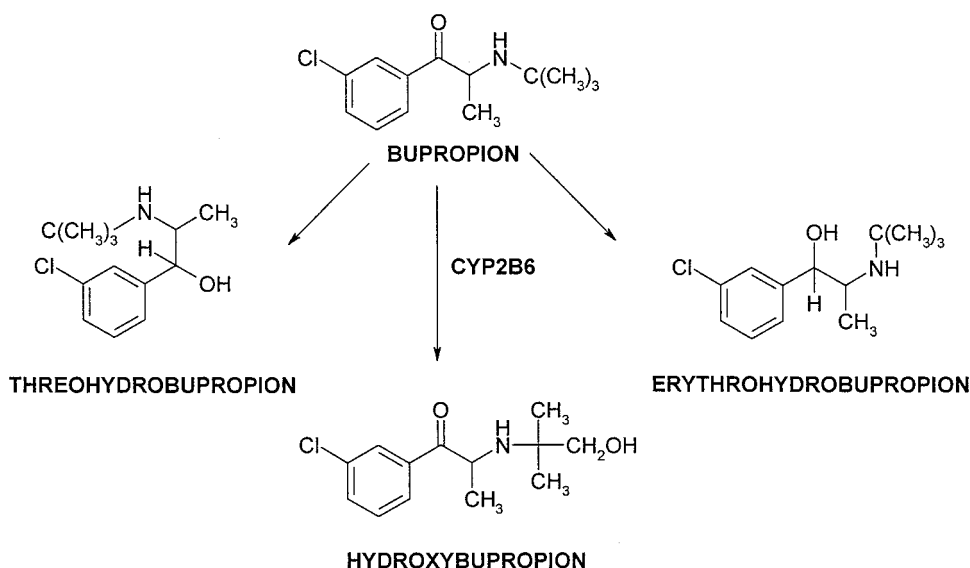
FIG. 1. *Bupropion and its major metabolites in humans.*

estimated 30- to 40-fold relative greater abundance of CYP3A4 compared with CYP2B6 (Shimada et al., 1994). Notably, Wurm et al. (1996) observed an increased contribution of CYP3A4 to the overall rate of BUP hydroxylation by cDNA-expressed enzymes when the BUP concentration was reduced from 100 to 10 μM, a substrate concentration near the range of BUP plasma concentrations encountered clinically (0.5–1 μM). This finding raises the possibility that CYP3A4 may serve as a low K_m, high-affinity catalyst of BUP hydroxylation at substrate concentrations encountered clinically.

Recently, this laboratory has shown that BUP hydroxylation is selectively catalyzed by CYP2B6 in HLMs when assayed at a substrate concentration of 500 μM (Faucette et al., 2000). However, this finding does not preclude the contribution of other P450 isozymes to BUP hydroxylation at lower substrate concentrations achieved in vivo. Notably, BUP hydroxylation has been characterized by biphasic kinetics in HLMs with absent or reduced CYP2B6 protein expression, suggesting the contribution of additional isozymes with relatively greater hepatic expression to HBUP formation (Faucette et al., 2000). The potential contribution of isozymes other than CYP2B6 to BUP hydroxylation in vivo is supported by the unimodal population distribution of HBUP formation despite evidence that CYP2B6 expression is absent or low in some human livers (Mimura et al., 1993; Shimada et al., 1994; Edwards et al., 1998). Thus, assessment of other potential catalysts of BUP hydroxylation appears to be warranted.

The purpose of the current study was to investigate the role of CYP3A4 in human liver microsomal BUP hydroxylation because of early suggestions of its involvement in BUP hydroxylation in vivo and its greater level of hepatic expression compared with CYP2B6. Assessment of the relative contribution and the kinetic parameters of CYP3A4-mediated BUP hydroxylation in vitro should provide an estimate of the potential for CYP3A4 to contribute to BUP hydroxylation in vivo. The present results demonstrate that CYP3A4 does not catalyze appreciable rates of HBUP formation at substrate concentrations that are likely encountered in vivo.

Materials and Methods

Chemical and Biological Reagents. BUP hydrochloride, triprolidine hydrochloride, NADP$^+$, glucose 6-phosphate, glucose-6-phosphate dehydrogenase, magnesium chloride, EDTA, triethylamine, TAO, and cortisol were purchased from Sigma (St. Louis, MO). HBUP was obtained from Glaxo Wellcome, Inc. (Research Triangle, NC). Testosterone and 6β-hydroxytestosterone were purchased from Steraloids, Inc. (Newport, RI). Formic acid was obtained from Fischer Scientific (Fair Lawn, NJ), and HPLC-grade acetonitrile and methanol were purchased from Mallinckrodt Baker, Inc. (Paris, KY). All other chemicals were of the highest grade commercially available. Dr. Bing-fang Yan (University of Rhode Island, Kingston, RI) graciously provided polyclonal antibodies raised against human CYP3A4 peptide.

Human Liver Microsomes and cDNA-Expressed Human P450s. A phenotyped bank of 16 individual (20 mg/ml, product no. H0500, version 4) and a pool of 15 (20 mg/ml, category no. HO610, lot 042099A) HLMs were obtained from XenoTech, LLC (Kansas City, KS). The spectrally determined P450 content of these HLM preparations, expressed as picomoles of P450 per milligram of protein, was provided by the supplier as follows: 395 (pooled HLM), 544 (HLM 2), 291 (HLM 7), 580 (HLM 11), 302 (HLM 13), 356 (HLM 14), 355 (HLM 15), 633 (HLM 16), 733 (HLM 17), 424 (HLM 18), 351 (HLM 19), 345 (HLM 20), 500 (HLM 21), 478 (HLM 22), 645 (HLM 23), 276 (HLM 24), and 444 (HLM 25). In addition, one HLM preparation (HLN-006) used in kinetic experiments was prepared by differential centrifugation of a frozen sample of human liver obtained from the human liver bank in the Department of Drug Delivery and Disposition at the University of North Carolina. The protein concentration of this HLM preparation was determined with a commercially available kit containing Coumassie protein assay reagent and bovine serum albumin as standard, according to instructions provided by the supplier (Pierce, Rockford, IL).

Microsomes prepared from baculovirus-infected insect cells (SUPERSOMES) expressing cDNAs for CYP2B6 or CYP3A4 and NADPH-P450 oxidoreductase were purchased from GENTEST (Woburn, MA). The CYP2B6 and CYP3A4 contents of each cDNA-expressed preparation were 133 and 1053 pmol of P450/mg of protein, respectively, as determined spectrophotometrically by the supplier. Microsomes containing baculovirus vector only served as controls for experiments with cDNA-expressed enzymes.

Microsomal Assays. Rates of HBUP and 6β-hydroxytestosterone formation were determined in duplicate with microsomes prepared from baculovirus infected-insect cells expressing cDNAs for CYP2B6 or CYP3A4 and/or with pooled or individual HLMs from the HLM bank. Preliminary experiments in pooled HLMs and insect cell-derived microsomes were conducted to identify microsomal protein amounts and incubation times resulting in linear rates of HBUP and 6β-hydroxytestosterone formation.

Microsomal assays were conducted with 0.1 or 0.2 mg of HLMs or 50 pmol of cDNA-expressed CYP2B6 or CYP3A4, BUP (0.01–12 mM) or testosterone (250 μM), 50 mM potassium phosphate buffer (pH 7.4), 1 mM EDTA, 3 mM magnesium chloride, 1 mM NADP$^+$, 5 mM glucose 6-phosphate, and 1 U/ml glucose-6-phosphate dehydrogenase. Total incubation volumes for the BUP and testosterone assays were 0.2 and 0.25 ml, respectively. BUP and testosterone stock solutions were prepared in methanol and added to individual

reaction mixtures in volumes not exceeding 1% of total incubation volumes. To aid solubility, HCl (0.01 N) was also used to prepare stock solutions consisting of greater than 75 mM BUP.

Reactions in HLMs were initiated at 37°C by the addition of NADPH-regenerating system (or substrate for chemical inhibition experiments) and proceeded for 12.5 or 30 min for the testosterone and BUP assays, respectively, before the termination of reactions with 100 μl of a mixture containing ice-cold acetonitrile and internal standard (cortisol for testosterone assay and triprolidine for bupropion assay). On completion of the reaction, incubation mixtures were vortexed and centrifuged at 3000 rpm for 5 min to remove precipitated protein. The amount of 6β-hydroxytestosterone or HBUP in the resulting supernatant fraction was determined by reverse-phase HPLC. Similar procedures were followed for incubations with insect cell-derived microsomes, except that reactions were initiated by the addition of ice-cold microsomes rather than the NADPH-regenerating system.

Inhibition Experiments. Bupropion hydroxylase and testosterone 6β-hydroxylase activities were determined in pooled HLMs assayed at 500 μM BUP and 250 μM testosterone, respectively, in the presence of varying TAO concentrations (0.025, 0.05, 0.1, 2.5, 10, 25, 50, and 100 μM). Determination of the extent of CYP3A4 inhibition of testosterone 6β-hydroxylation was performed as a positive control. Incubation mixtures containing HLMs (0.2 mg), buffer, and TAO (dissolved in dimethyl sulfoxide) were preincubated for 15 or 30 min in the presence of the NADPH-regenerating system before the initiation of BUP hydroxylation or testosterone 6β-hydroxylation by the addition of the appropriate substrate. Control incubations containing all components of the test incubations, including 1% dimethyl sulfoxide, but not TAO, were performed in parallel. Rates of BUP hydroxylation in the presence of TAO were expressed as the percentage of control activity.

Enzyme Kinetic Analysis. The kinetics of BUP hydroxylation for HLN-006 was examined over the BUP concentration range of 10 to 2000 μM, in the absence or presence of 2.5 μM TAO. This concentration of TAO was selected on the basis of a preliminary experiment that demonstrated greater than 80% inhibition of testosterone 6β-hydroxylation when assayed in the presence of 2.5 μM TAO. An expanded range of BUP concentrations (0.075–12 mM) was used for analysis of rates of HBUP formation by cDNA-expressed CYP3A4 because initial experiments with cDNA-expressed CYP3A4 demonstrated low rates of BUP hydroxylation when assayed at 2000 μM BUP. Initial estimates of kinetic parameters for BUP hydroxylation were obtained by visual inspection of Michaelis-Menten and Eadie-Hofstee plots generated from raw concentration-rate data. Using initial parameter estimates, experimental data were fit to a one-component Michaelis-Menten model and/or to the Hill equation via iterative nonlinear regression using WinNonlin 1.1 software (Scientific Consulting, Inc., Apex, NC). The reported values of apparent K_m, V_{max}, and n were estimated from the fitted data. The appropriateness of fit of experimental data to each kinetic model was ascertained by examining the sum of squares of residuals, the size of the coefficients of variations and standard errors of parameter estimates, and Akaike's information criterion.

HPLC Analysis. Quantitation of HBUP and triprolidine (internal standard) was accomplished by a previously described reverse-phase HPLC assay with UV detection (Faucette et al., 2000). The HPLC system for quantitation of 6β-hydroxytestosterone and cortisol (internal standard) consisted of a Shimadzu model SCL-10A system controller, model CTO-10A oven, model LC-10AD pump, model SIL-10A autoinjector, and model SPD-10AV UV detector set at 248 nm. 6β-Hydroxytestosterone and cortisol peaks were separated and resolved at 40°C on a 5-μm Waters Symmetry C$_{18}$ column (15 × 0.39 cm) preceded by a Waters Symmetry guard column (2 × 0.39 cm; Millipore Corp., Milford, MA). Mobile phases A [90% formic acid (0.1%) and triethylamine (0.25%); 10% acetonitrile] and B [40% formic acid (0.1%) and triethylamine (0.25%); 60% acetonitrile] were pumped over a gradient at a flow rate of 1 ml/min. Retention times for 6β-hydroxytestosterone and cortisol were 4.3 and 5.5 min, respectively.

Calibration standards of HBUP and 6β-hydroxytestosterone were prepared by the addition of a known amount of metabolite to microsomal incubation mixture components. HBUP concentrations and 6β-hydroxytestosterone picomoles were calculated from peak height or area ratios, respectively, using least-squares linear regression, with weighting by the reciprocal of the squared standard concentrations. Interday coefficients of variation ranged from 13 to 22% for HBUP and from 6 to 28% for 6β-hydroxytestosterone standards.
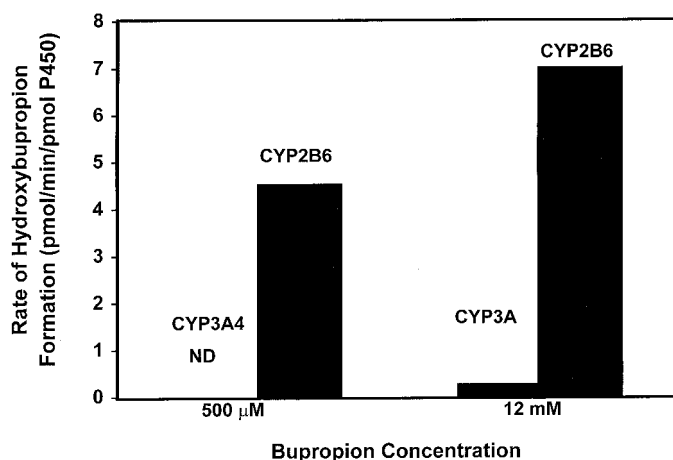

FIG. 2. *Evaluation of BUP hydroxylation by cDNA-expressed CYP2B6 and CYP3A4.*

Rates of BUP hydroxylation were assessed in microsomes isolated from baculovirus-infected insect cells expressing cDNA for NADPH-P450 oxidoreductase and CYP2B6 or CYP3A4. CDNA-expressed CYP2B6 and CYP3A4 (50 pmol) were assayed for BUP hydroxylase activity at 500 and 12 mM BUP. Control incubations were also conducted with insect cell-derived microsomes transfected with only baculovirus vector. Data are expressed as picomoles per minute per picomole of P450 and represent the mean of duplicate determinations. ND, nondetectable.

Lower limits of quantification were 20 ng/ml for HBUP and 180 pmol for 6β-hydroxytestosterone.

Immunoblotting Analysis. Immunodetection of CYP3A4 protein in the HLM bank preparations was accomplished by Western blotting according to previously described methods (Parkinson and Gemzik, 1991). Microsomal protein (5 μg) was denatured and resolved by SDS-polyacrylamide gel electrophoresis on a HSI SE600 electrophoresis unit (Hoefer Scientific Instruments, San Francisco, CA). Following the electrophoretic run, microsomal protein was transferred to nitrocellulose membranes according to previously published methods (Towbin et al., 1979). Membranes were initially probed with specific polyclonal antibodies against human CYP3A4 peptide, followed by alkaline phosphatase-conjugated goat anti-rabbit IgG secondary antibody. CYP3A4 protein was visualized using 5-bromo-4-chloroindolyl-phosphatase/nitrobenzotetrazolium.

Statistical Analysis. Statistical analyses were performed with JMP version 3.2 software (SAS Institute, Inc., Cary, NC). The correlation of BUP hydroxylase activity with testosterone 6β-hydroxylase activity was assessed by linear regression analysis. Significance of the correlation was determined by an F test. The acceptance limit for statistical significance was set at $\alpha = 0.05$.

Results

Comparison of Rates of BUP Hydroxylation by cDNA-Expressed CYP2B6 and CYP3A4. Microsomes from baculovirus-infected cells containing cDNA-expressed CYP2B6 and CYP3A4 were screened for BUP hydroxylase activity at 500 μM and 12 mM BUP. These substrate concentrations were selected on the basis of preliminary kinetic experiments to achieve saturation of both isozymes, irrespective of their relative affinity for BUP. cDNA-expressed CYP2B6, but not CYP3A4, catalyzed detectable HBUP formation at 500 μM BUP (4.5 pmol/min/pmol of P450), whereas both cDNA-expressed enzymes catalyzed BUP hydroxylation at 12 mM BUP (Fig. 2). However, at the higher substrate concentration, cDNA-expressed CYP2B6 catalyzed BUP hydroxylation at a 30-fold higher rate than cDNA-expressed CYP3A4 (7.0 versus 0.2 pmol/min/pmol of P450). The ability of CYP3A4 to hydroxylate BUP at 12 mM but not at 500 μM suggests that CYP3A4 is a low- rather than high-affinity catalyst of the reaction.

Correlation Analysis of BUP Hydroxylase and Testosterone 6β-Hydroxylase Activities and CYP2B6 and CYP3A4 Protein
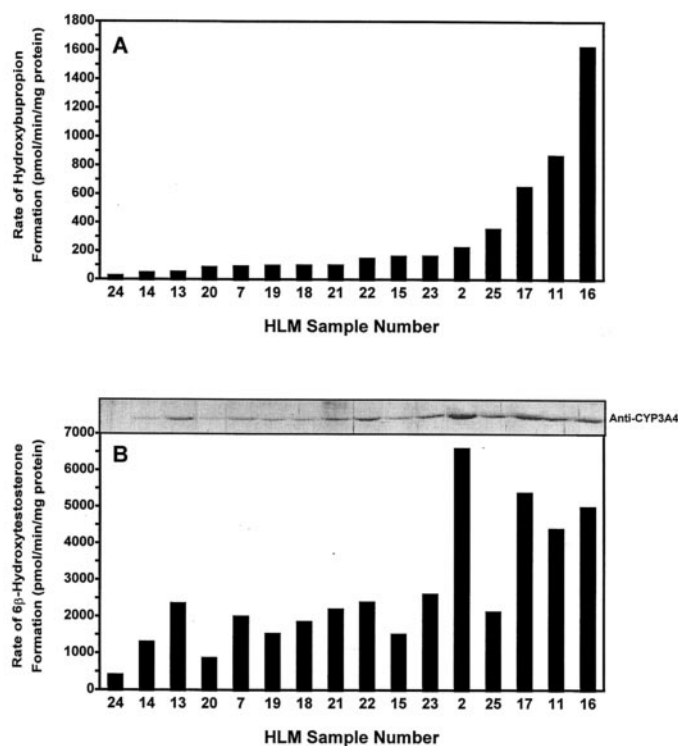
FIG. 3. *Relationship between rates of BUP hydroxylation and testosterone 6β-hydroxylation and CYP3A4 protein expression among HLM bank (n = 16).*

A and B, BUP hydroxylase (A) and testosterone 6β-hydroxylase (B) activities were determined in duplicate at BUP and testosterone concentrations of 500 and 250 μM, respectively, as described under *Materials and Methods*. Data represent the mean of the duplicate activities. B, Western immunoblotting procedures were performed as described under *Materials and Methods*, using polyclonal anti-CYP3A4 antibodies for primary detection and alkaline phosphatase-conjugated goat anti-rabbit antibody for secondary detection. Lane 1, HLM 24; lane 2, HLM 14; lane 3, HLM 13; lane 4, HLM 20; lane 5, HLM 7; lane 6, HLM 19; lane 7, HLM 18; lane 8, HLM 21; lane 9, HLM 22; lane 10, HLM 15; lane 11, HLM 23; lane 12, HLM 2; lane 13, HLM 25; lane 14, HLM 17; lane 15, HLM 11; lane 16, HLM 16.

Expression in HLMs. To examine the relative contributions of CYP2B6 and CYP3A4 to the overall rate of BUP hydroxylation when these enzymes are differentially expressed in human liver, a panel of 16 HLMs was assayed for BUP hydroxylase and testosterone 6β-hydroxylase activities at BUP and testosterone concentrations of 500 and 250 μM, respectively. The rationale for selection of 500 μM BUP for HLM assays was based on K_m estimates from preliminary kinetic experiments. A testosterone concentration of approximately 5 times the reported K_m for CYP3A4-mediated testosterone 6β-hydroxylation (47 μM; Waxman et al., 1983) was chosen to ensure substrate saturation of CYP3A4, thereby maximizing the potential contribution of this isozyme to HLM-catalyzed BUP hydroxylation.

Rates of BUP hydroxylation varied approximately 80-fold (20–1623 pmol/min/mg of protein; Fig. 3A), and rates of testosterone 6β-hydroxylation varied approximately 17-fold (384–6579 pmol/min/mg of protein; Fig. 3B) among the HLM bank when assayed at 500 μM BUP and 250 μM testosterone, respectively. A weak correlation was observed between BUP hydroxylase and testosterone 6β-hydroxylase activities among the HLM bank ($r^2 = 0.39$, $p < 0.001$; Fig. 4). Although these two catalytic activities did not strongly correlate, the relative rank orders of BUP hydroxylase and testosterone 6β-hydroxylase activities were similar for individual HLMs. The rank order of the two catalytic activities was discordant for four of the 16 HLMs (2, 13, 15, and 25), where discordance was arbitrarily defined
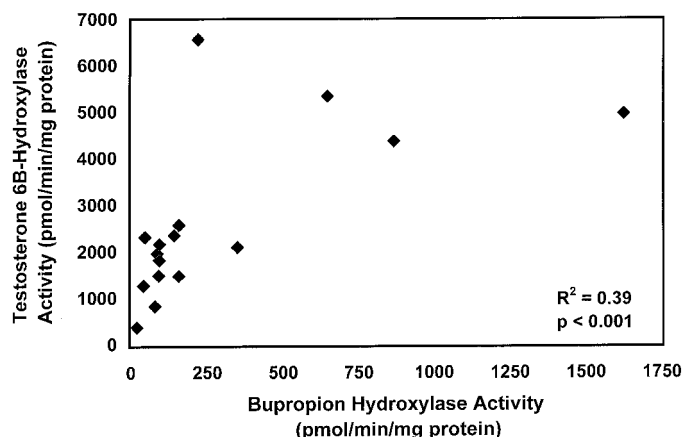

FIG. 4. *Correlation between BUP hydroxylase and testosterone 6β-hydroxylase activities in HLMs (n = 16).*

BUP hydroxylase and testosterone 6β-hydroxylase activities were determined as described under *Materials and Methods*. The correlation of the two activities was evaluated by linear regression analysis. An *F* test was used to determine the statistical significance of the correlation.

as a difference of five or greater in the relative rank order. When these discordant HLMs were removed from correlative analysis, an improved correlation between BUP hydroxylase and testosterone 6β-hydroxylase activities was observed ($r^2 = 0.69$; data not shown).

Previously characterized polyclonal antibodies against human CYP2B6 and CYP3A4 were used for immunodetection of the respective proteins in the HLM bank preparations. As previously reported, CYP2B6 blotting intensity varied 100-fold among the 15 of 16 HLMs with detectable levels of CYP2B6 and significantly correlated with BUP hydroxylase activity assayed at 500 μM ($r^2 = 0.99$, $p < 0.001$; Faucette et al., 2000). Immunodetectable levels of CYP3A4 protein are shown in Fig. 3B. On a qualitative basis, CYP3A4 blotting intensity correlated with testosterone 6β-hydroxylase activity but not with BUP hydroxylase activity (Fig. 3, A and B).

Kinetic Analyses of BUP Hydroxylation by HLMs and cDNA-Expressed CYP3A4. The kinetics of BUP hydroxylation by cDNA-expressed CYP3A4 was evaluated for comparison to previously reported kinetic parameters for BUP hydroxylation in HLMs and insect cell-derived microsomes containing cDNA-expressed CYP2B6 (Faucette et al., 2000). cDNA-expressed CYP3A4 failed to exhibit BUP hydroxylase activity when incubated with BUP concentrations less than 4 mM (data not shown). In addition, saturable rates of BUP hydroxylation were not achieved over the substrate concentration range of 0.075 to 12 mM. BUP concentrations greater than 12 mM could not be evaluated due to solubility limitations. Failure to attain saturable rates of HBUP formation with cDNA-expressed CYP3A4 prohibited reliable fitting of concentration-rate of formation data to any kinetic model tested; therefore, accurate determination of kinetic parameters for CYP3A4-catalyzed BUP hydroxylation was not possible. Based on the assumption that saturable kinetics is attained at substrate concentrations approximately 3 times the apparent K_m for a particular metabolic reaction, the apparent K_m for CYP3A4-mediated BUP hydroxylation was estimated to be greater than 4 mM. This K_m value is approximately 25-fold greater than the previously determined apparent K_m estimate for BUP hydroxylation with HLMs (130 μM) and cDNA-expressed CYP2B6 (156 μM; Faucette et al., 2000). Overall, these results suggest that CYP3A4 is a low-affinity, high K_m catalyst of BUP hydroxylation that is unlikely to significantly contribute to the reaction at lower substrate concentrations encountered clinically.
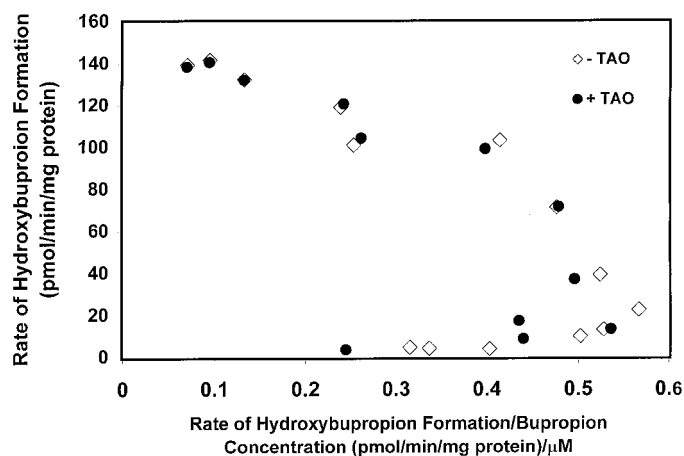
FIG. 5. *Kinetic analysis of HLM-catalyzed BUP hydroxylation in the absence or presence of TAO.*

Rates of BUP hydroxylation in HLN-006 were determined in duplicate at 10 to 2000 μM BUP in the absence or presence of 2.5 μM TAO. Weighted concentration-rate data were fit to the Hill equation using nonlinear regression. Kinetic parameters were estimated from the fitted data. The kinetics of BUP hydroxylation was characterized by a K_m of 150 μM in the absence of TAO and 153 μM in its presence.

In a recent report, BUP hydroxylation was characterized by sigmoidal kinetic behavior in the majority of HLM bank preparations selected for kinetic analysis (Faucette et al., 2000). Even though initial kinetic studies suggested that CYP3A4 is a nonsignificant contributor to BUP hydroxylation at lower substrate concentrations, it was postulated that CYP3A4 might mediate the previously observed allosteric activation of BUP hydroxylation in HLMs because of extensive evidence supporting allosteric kinetics for the metabolism of many CYP3A4 substrates (Shaw et al., 1997; Ueng et al., 1997; Shou et al., 1999). To test the possibility of CYP3A4-mediated allosteric activation of BUP hydroxylation, the kinetics of BUP hydroxylation was examined in HLN-006 in the absence or presence of TAO (2.5 μM), a selective CYP3A4 chemical inhibitor. In a preliminary inhibitory experiment, 2.5 μM TAO inhibited testosterone 6β-hydroxylation by greater than 80% when assayed in HLN-006 at 250 μM testosterone. In the ensuing kinetic experiment using HLN-006, rates of BUP hydroxylation in the presence of TAO (2.5 μM) were comparable to those in the absence of TAO at each of the tested BUP concentrations (10–2000 μM; Eadie-Hofstee plot of kinetic data shown in Fig. 5). The apparent K_m and V_{max} (CV%) for BUP hydroxylation in HLN-006, estimated by fitting experimental data to the Hill equation, were 150 μM (8.8%) and 143 pmol/min/mg of protein (3.3%) in the absence of TAO and 153 μM (8.5%) and 142 pmol/min/mg of protein (3.3%) in the presence of TAO. The characteristic hook indicative of allosteric activation remained present on the Eadie-Hofstee plot of kinetic data despite the presence of TAO (Fig. 5), providing additional evidence that CYP3A4 does not mediate the sigmoidal kinetics of BUP hydroxylation in HLMs.

Effect of TAO on Bupropion Hydroxylase and Testosterone 6β-Hydroxylase Activities in HLMs. The relative contribution of CYP3A4 to BUP hydroxylation in human liver was further explored by examining the effects of multiple TAO concentrations (0.025, 0.05, 0.1, 2.5, 10, 25, 50, and 100 μM) on BUP hydroxylation assayed at 500 μM BUP in pooled HLMs. In a previous experiment, these same concentrations were tested for their ability to inhibit testosterone 6β-hydroxylation when assayed at 250 μM testosterone. As expected, this reaction was inhibited in a concentration-dependent manner in the presence of TAO. The IC$_{50}$ for TAO inhibition of testosterone 6β-
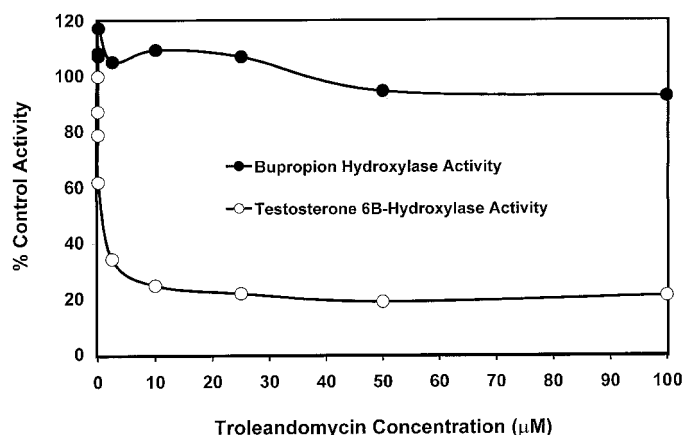

FIG. 6. *Inhibition of testosterone 6β-hydroxylation, but not BUP hydroxylation, by TAO in pooled HLMs.*

TAO (0, 0.025, 0.05, 0.1, 2.5, 10, 25, 50, and 100) was preincubated with pooled HLMs, buffer, and NADPH-regenerating system for 30 min before addition of substrate (250 μM testosterone or 500 μM BUP). BUP hydroxylase and testosterone 6β-hydroxylase activities in the presence of TAO were determined in duplicate and expressed as the percentage of control activities.

hydroxylation, obtained by fitting experimental data to an inhibitory sigmoidal E_{max} model, was 4.6 μM. Maximum inhibition of testosterone 6β-hydroxylation (81%) was observed with 50 μM TAO (Fig. 6). In contrast, negligible inhibition (7%) of BUP hydroxylase activity was observed at the highest TAO concentration tested (100 μM). Interestingly, rates of HBUP formation were higher in the presence of most concentrations of TAO than in their absence (105–118% of control; Fig. 6). Inhibitory profiles for BUP hydroxylation and testosterone 6β-hydroxylation were comparable when the preincubation time allowed for irreversible complexion of CYP3A4 by TAO was 15 or 30 min (data not shown).

Discussion

Until recently, the identity of the primary isozyme responsible for BUP hydroxylation has remained speculative. In a previous in vivo study examining the pharmacokinetics of BUP and its metabolites in the absence or presence of carbamazepine, Ketter et al. (1995) postulated that BUP hydroxylation is mediated by CYP3A4 upon observing substantially decreased BUP, but increased HBUP plasma concentrations, in subjects receiving dual therapy with BUP and carbamazepine. This postulation was based on the assumption that carbamazepine selectively induced CYP3A4, resulting in enhanced conversion of BUP to HBUP. Consequences of CYP3A4-mediated BUP hydroxylation would include the risk of drug interactions with numerous other psychotropic agents whose metabolism is primarily mediated by CYP3A4 (von Moltke et al., 1995) and interindividual variability in antidepressant efficacy and toxicity due to possible differences in CYP3A4-dependent formation of HBUP. Because of prior evidence suggesting the involvement of CYP3A4 in BUP hydroxylation in vivo and the demonstrated capacity of CYP3A4 to metabolize a significant number of therapeutic agents, the present study evaluated the ability of CYP3A4 to catalyze BUP hydroxylation in human liver using a variety of in vitro techniques.

The results of this study demonstrate that CYP3A4 does not significantly contribute to the overall rate of BUP hydroxylation in human liver. The compilation of evidence supporting this conclusion includes the following: 1) the inability of cDNA-expressed CYP3A4 to catalyze BUP hydroxylation at substrate concentrations of 0.075 to 4 mM, 2) the ability of cDNA-expressed CYP2B6 to catalyze the reaction at a higher rate than cDNA-expressed CYP3A4 when as-

sessed at 12 mM BUP, 3) the lack of strong correlation between catalytic and immunochemical markers of CYP3A4 (testosterone 6β-hydroxylase activity and CYP3A4 protein expression) and BUP hydroxylase activity assayed at 500 μM BUP, 4) the kinetic parameters of BUP hydroxylation assessed over the substrate concentration range of 10 to 2000 μM were essentially unchanged in the presence of TAO, and 5) the lack of inhibition of BUP hydroxylation when assayed at 500 μM BUP in pooled HLMs in the presence of various TAO concentrations.

Although the above-mentioned evidence suggests that CYP3A4 is unlikely to significantly contribute to HBUP formation at clinically relevant substrate concentrations, cDNA-expressed CYP3A4 was capable of mediating the formation of HBUP from BUP at very high substrate concentrations ($\geq$4 mM). However, kinetic studies with cDNA-expressed CYP3A4 estimated that the K_m for CYP3A4-catalyzed BUP hydroxylation was at least 1000-fold greater than measured BUP concentrations in human plasma ($\sim$0.5–1 μM). These findings suggest that BUP binding to the catalytic site of CYP3A4 is characterized by relatively low affinity, and similar to binding of BUP to CYP2E1, which has been previously identified as a high K_m catalyst of BUP hydroxylation (Faucette et al., 2000; Hesse et al., 2000). Thus, the contribution of CYP3A4 to BUP hydroxylation in vivo, like that of CYP2E1, is unlikely to be significant at lower substrate concentrations encountered clinically. This postulation is further supported by results from another laboratory that demonstrated absence of HBUP formation by cDNA-expressed CYP3A4 when assayed at substrate concentrations of 50 and 500 μM (Hesse et al., 2000). These in vitro results are consistent with an in vivo study that reported no significant alterations in the pharmacokinetic parameters for bupropion and hydroxybupropion upon concurrent administration of cimetidine, a nonselective CYP3A4 inhibitor (Kustra et al., 1999).

Our data showing that CYP3A4 can catalyze BUP hydroxylation at higher, but not lower, substrate concentrations conflicts with data previously presented in abstract form by Wurm et al. (1996). These authors noted an increased CYP3A4 contribution to the overall rate of HBUP formation by a panel of cDNA-expressed enzymes when the substrate concentration was reduced from 100 μM to a lower concentration (10 μM) that potentially could be achieved in vivo. The discrepancy between the two laboratories in the presumed contribution of CYP3A4 to BUP hydroxylation at lower substrate concentrations could reflect differences in cDNA expression systems and analytical techniques used by each laboratory. For example, this laboratory used baculovirus-infected insect cells for expression of recombinant enzymes and HPLC with UV detection for HBUP quantification, whereas the laboratory of Wurm et al. (1996) used human β-lymphoblastoid cells and HPLC with mass spectrometry detection. However, since our UV detection system allowed quantification of HBUP concentrations as low as 20 ng/ml, which is lower than human plasma concentrations of HBUP observed up to 72 h after standard oral doses of BUP (DeVane et al., 1990; Hsyu et al., 1998), sensitivity differences in detection systems are unlikely to account for the different results obtained by each laboratory. In contrast, both laboratories were similar in finding that cDNA-expressed CYP2B6 catalyzed BUP hydroxylation at a greater rate than CYP3A4, regardless whether lower or higher BUP concentrations were used. When comparing rates of BUP hydroxylation by cDNA-expressed CYP2B6 and CYP3A4, Wurm et al. (1996) demonstrated 46- and 92-fold higher rates for cDNA-expressed CYP2B6 when assayed at 10 and 100 μM BUP, respectively. Likewise, the present study demonstrated 30-fold greater rates of HBUP formation at 12 mM BUP for cDNA-expressed CYP2B6 compared with cDNA-expressed CYP3A4. These data complement our recent study, which failed to reveal a catalyst of BUP

hydroxylation with higher affinity (lower K_m) than CYP2B6 and demonstrated that CYP2B6 selectively catalyzes the reaction when assayed at 500 μM BUP (Faucette et al., 2000).

Although our data did not support a strong correlation between BUP hydroxylase activity and CYP3A4 blotting density, a weak correlation was observed between BUP hydroxylase and testosterone 6β-hydroxylase (r^2 = 0.39) activities among the HLM bank. In addition, the rank order of these activities was similar in the majority of HLM bank preparations, suggesting the possibility of coordinate regulation of CYP2B6 and CYP3A4 catalytic activities. When four HLMs with discordant rank orders of activities were omitted from correlation analysis, a stronger relationship between BUP hydroxylase and testosterone 6β-hydroxylase activities was noted (r^2 increased from 0.39 to 0.69). Significant relationships between other CYP2B6 and CYP3A4 probes have been previously documented, including S-mephenytoin N-demethylation and nifedipine oxidation (r = 0.74; Heyn et al., 1996), immunoquantified CYP2B6 protein levels and midazolam 1'-hydroxylation (r^2 = 0.63; Ekins et al., 1998), and immunoquantified CYP2B6 and CYP3A4 levels (r^2 = 0.58; Ekins et al., 1998).

Because some HLM preparations in this study were known to be isolated from donor livers exposed to inducing agents before harvest, the potential relationship between CYP2B6 and CYP3A4 expression and catalytic activity could be explained by concomitant induction of these enzymes by the same drug. Prototypical inducers of CYP3A4 protein and catalytic activity that have been shown to increase CYP2B6 protein expression in human hepatocyte cultures include phenytoin (LeCluyse et al., 1996), rifampin (Chang et al., 1997), phenobarbital (Chang et al., 1997), dexamethasone (Chang et al., 1997), and carbamazepine (S. Faucette, manuscript in preparation). In fact, carbamazepine induction of CYP2B6 activity in addition to that of CYP3A4 could account for the altered pharmacokinetic profiles of BUP and HBUP reported in subjects administered both carbamazepine and BUP (Ketter et al., 1995). Preliminary data from this laboratory suggest a similar pattern of induction of both BUP hydroxylase and testosterone 6β-hydroxylase activities and CYP2B6 and CYP3A4 immunoreactive proteins in microsomes isolated from cultured human hepatocytes exposed to carbamazepine (S. Faucette, manuscript in preparation). Induction of both CYP2B6 and CYP3A4 by similar drugs could reflect shared regulation of transcriptional activation of the respective genes by receptor-dependent mechanisms. For example, phenobarbital inducibility of CYP2B6 and CYP3A4 may be conferred by transactivation of human CYP2B6 and CYP3A4 genes by the same orphan nuclear receptor. Recent studies have demonstrated constitutively activated receptor-mediated transactivation of a phenobarbital-responsive enhancer module in the 5'-flanking region of the mouse *CYP2B10* (Honkakoski et al., 1998) and human *CYP2B6* genes (Sueyoshi et al., 1999), as well as a steroid/rifampicin-response element in the human *CYP3A4* gene. Alternatively, coinduction of CYP2B6 and CYP3A4 could represent the ability of inducers to activate multiple nuclear receptors. Phenobarbital, for example, has been shown to activate both the pregnane X receptor (Lehmann et al., 1998) and constitutively activated receptor (Sueyoshi et al., 1999).

In summary, in vitro data demonstrate that CYP3A4 does not contribute to the overall rate of BUP hydroxylation in human liver at concentrations near those encountered clinically. At higher substrate concentrations, CYP3A4 is capable of BUP hydroxylation, although at significantly lower rates than CYP2B6. Despite the lack of significant involvement of CYP3A4 in BUP hydroxylation, a correlation trend between BUP hydroxylation and testosterone 6β-hydroxylation was noted, perhaps reflecting shared regulation of CYP2B6 and CYP3A4 induction. Further evaluation of the potential coregulation of

inducible CYP2B6 and CYP3A4 catalytic activities and protein expression appears warranted.

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