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HUMAN LIVER CARBAMAZEPINE METABOLISM

ROLE OF CYP3A4 AND CYP2C8 IN 10,11-EPOXIDE FORMATION

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Abstract—A number of drugs inhibit the metabolism of carbamazepine catalyzed by cytochrome P450, sometimes resulting in carbamazepine intoxication. However, there is little information available concerning the identity of the specific isoforms of P450 responsible for the metabolism of this drug. This study addressed the role of CYP3A4 in the formation of carbamazepine-10,11-epoxide, the major metabolite of carbamazepine. Results of the study showed that: (1) purified CYP3A4 catalyzed 10,11-epoxidation; (2) cDNA-expressed CYP3A4 catalyzed 10,11-epoxidation (V_{max} = 1730 pmol/min/nmol P450, K_m = 442 μM); (3) the rate of 10,11-epoxidation correlated with CYP3A4 content in microsomes from sixteen human livers ($r^2 = 0.57$, $P < 0.001$); (4) triacetyloleandomycin and anti-CYP3A4 IgG reduced 10,11-epoxidation to 31 ± 6% (sixteen livers) and 43 ± 2% (four livers) of control rates, respectively; and (5) microsomal 10,11-epoxidation but not phenol formation was activated 2- to 3-fold by α-naphthoflavone and progesterone and by carbamazepine itself (substrate activation). These findings indicate that CYP3A4 is the principal catalyst of 10,11-epoxide formation in human liver. Experiments utilizing a panel of P450 isoform selective inhibitors also suggested a minor involvement of CYP2C8 in liver microsomal 10,11-epoxidation. Epoxidation by CYP2C8 was confirmed in incubations of carbamazepine with cDNA-expressed CYP2C8. The role of CYP3A4 in the major pathway of carbamazepine elimination is consistent with the number of inhibitory drug interactions associated with its clinical use, interactions that result from a perturbation of CYP3A4 catalytic activity.

Key words: cytochrome P450; CYP3A4; CYP2C8; carbamazepine; drug metabolism; human liver microsomes

Several difficulties associated with clinical use of the anticonvulsant carbamazepine are related to metabolism of the drug by cytochrome P450. Carbamazepine dose selection and adjustments are complicated by autoinduction of cytochrome P450 clearance processes during chronic administration, and by heteroinduction during coadministration with other anticonvulsants such as phenobarbital or phenytoin [1]. Conversely, a number of drugs, including verapamil, propoxyphene, erythromycin, triacetyloleandomycin, stiripentol, and danazol, inhibit the metabolism of carbamazepine by cytochrome P450, sometimes resulting in pronounced increases in carbamazepine plasma levels and intoxication [2–4]. The inhibitory interactions are particularly hazardous because such interactions are usually unexpected and are only discovered accidentally when a patient receiving carbamazepine becomes intoxicated. Finally, metabolism by cytochrome P450 complicates the usefulness of plasma carbamazepine concentrations for therapeutic monitoring due to the concomitant accumulation in plasma of the pharmacologically active metabolite, carbamazepine-10,11-epoxide. Despite the impact of cytochrome P450 on the clinical pharmacology and toxicology of carbamazepine, there is little information concerning the identity of the cytochrome P450 isoforms responsible for metabolism of this drug.

The interactions of macrolide antibiotics with carbamazepine have been described in numerous patient case reports [5–7], and have also been observed in controlled studies involving healthy volunteers [8–10]. Effects of the macrolides on carbamazepine disposition include inhibition of carbamazepine total clearance and the partial clearance to the 10,11-epoxide, diminished plasma levels of the epoxide, and elevations in carbamazepine plasma levels, occasionally resulting in serious intoxication. Triacetyloleandomycin and erythromycin have the most pronounced effects on carbamazepine clearance and plasma levels, but josamycin, miocamycin, clarithromycin, and flurithromycin also inhibit the *in vivo* elimination of carbamazepine [11]. The recognized involvement of CYP3A4‖ in macrolide antibiotic interactions with other drugs [12, 13] suggests not only that this form of cytochrome P450 may be involved in the carbamazepine interactions, but also that CYP3A4 may be the principal enzyme governing metabolism of carbamazepine in humans. The objective of this

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‖ Abbreviations: CYP, cytochrome P450; HL-, banked human liver number; and IgG, immunoglobulin G.

study was to evaluate the contribution of CYP3A4 and other P450 isoforms to carbamazepine metabolism *in vitro*, using microsomes prepared from a bank of twenty-five different human livers, a purified preparation of CYP3A4*, and cDNA-expressed cytochrome P450 isoforms.†

MATERIALS AND METHODS

Chemicals. Carbamazepine, carbamazepine-10, 11-epoxide, 2-hydroxycarbamazepine, and 3-hydroxy-carbamazepine were gifts of the Ciba–Geigy Corp. (Summit, NJ). Stable-labeled [1-^{15}N,2-^{13}C] carbamazepine, [1-^{15}N,2-^{13}C]carbamazepine-10,11-epoxide, and d_4-carbamazepine-10,11-epoxide were synthesized as described previously [18]. Triacetyloleandomycin, progesterone, quinidine sulfate, diethyldithiocarbamate, α-naphthoflavone, and NADPH (tetrasodium salt, type I) were purchased from the Sigma Chemical Co. (St. Louis, MO). (*S*)-Mephenytoin and sulfaphenazole were gifts from Dr. William F. Trager, Department of Medicinal Chemistry, University of Washington (Seattle, WA). Furafylline was a gift from Dr. Kent Kunze, Department of Medicinal Chemistry, University of Washington (Seattle, WA). Tolbutamide was a gift from Dr. Edward Antal (Upjohn Co., Kalamazoo, MI). 2-Methylcarbamazepine was purchased from the Aldrich Chemical Co. (Milwaukee, WI).

Human liver microsomal incubations. Whole human livers were obtained from organ donors through the Solid Organ Transplant Program at the University of Washington Medical Center and the Northwest Organ Procurement Agency (Seattle, WA). Tissue was cut into approximately 10 g pieces, immersed in liquid nitrogen, and stored at −70°. Microsomes were prepared from liver homogenates by differential centrifugation [19] and stored until further use (−70°) as suspensions in 100 mM phosphate buffer, pH 7.4. Microsomal protein concentrations were determined by the method of Lowry *et al.* [20]. Total microsomal cytochrome P450 contents were determined from the reduced minus oxidized carbon monoxide difference spectra [21], and contents of microsomal CYP3A4 (CYP3A3 + CYP3A4) were quantitated by western blot analysis [22] using specific rabbit polyclonal antibody, prepared against human CYP3A4, for primary immunodetection and band quantitation by densitometry (Bio Image, Millipore Corp., Ann Arbor, MI).

Microsomal incubations with carbamazepine (0–180 μM) were performed in 100 mM phosphate buffer at pH 7.4. Carbamazepine was preincubated with microsomes at 37° for 2–5 min prior to the addition of NADPH. Reactions were terminated 30 min after the addition of NADPH by rapidly vortexing each 1-mL incubation with 5 mL *t*-butyl methyl ether. The incubate concentration of NADPH was 1 mM, and microsomal protein was 1 mg/mL. Chemical inhibitors or activators of cytochrome P450, when included, were added during the 2- to 5-min preincubation period. Methanol was used to solubilize triacetyloleandomycin, α-naphthoflavone, and progesterone, and was present in incubations containing those compounds (and in corresponding control incubations) at final concentrations (v/v) of 0.1, 1.0, and 1.0 %, respectively. Inhibitory effects of antibodies were evaluated by preincubating IgG with microsomes at 37° for 30 min, followed by the addition of carbamazepine, NADPH, and other incubation components as described above. Other pertinent details (e.g. inhibitor concentrations) of the incubation procedure outlined above are noted in the presentation of results from individual studies. Human liver microsomal incubations with labeled carbamazepine were performed as above except that all or part of the unlabeled carbamazepine was replaced with [^{13}C,^{15}N]-carbamazepine.

Reconstituted CYP3A4 incubations. CYP3A4 was purified from human liver microsomes as described previously [22]. The enzyme was reconstituted according to the method described by Halvorson *et al.* [23] for CYP3A1. Final incubation conditions were: 0.2 nmol CYP3A4; 0.4 nmol rabbit cytochrome P450 reductase (purified as described [24]); 0.2 nmol rabbit cytochrome b_5 (purified as described [24]); 200 μg rat liver microsomal lipid, prepared as described by Folch *et al.* [25]; 1 mM NADPH; sufficient 100 mM sodium phosphate buffer, pH 7.4, to yield a volume of 1 mL. The incubation procedure with carbamazepine was the same as described above for liver microsomes, except that the incubation time was 15 rather than 30 min in order to optimize epoxide formation under analytical and linearity constraints.

cDNA-expressed P450 incubations. HepG2 cells were infected with a recombinant vaccinia virus containing human cDNA for one of the following: P450 1A2, 2C8, 2C9, 2E1, 2B7, 3A4, or 3A5, as described earlier [26]. Control HepG2 cells were infected with wild-type vaccinia virus. Microsomes were prepared from the transfected HepG2 cell lysates (provided by the laboratory of Dr. Kenneth R. Korzekwa, Laboratory of Molecular Carcinogenesis, NCI). In activity screening experiments, 0.9 to 4.6 mg of microsomal protein were incubated with 150 μM carbamazepine, 1 mM NADPH and 100 mM potassium phosphate buffer (pH 7.4) in a final volume of 1 mL. Following a 30 min incubation period at 37°, the reaction was terminated by placing the tubes in a dry ice and acetone bath. Samples remained frozen at −70° until assayed. *t*-Butyl methyl ether (5 mL) was added to the frozen

* Although CYP3A4 and CYP3A3 are distinct gene products in human liver, they are highly homologous and indistinguishable in the experiments (except for cDNA-expressed CYP3A4 incubations) employed in this study. Only CYP3A4 is referred to in this manuscript since it appears to be the predominant isoform in human liver [14]. Given our inability to differentiate between the two isoforms, results attributed to CYP3A4 may also be attributed to CYP3A3.

† Results from these studies have been presented, in part, at the Annual Meeting of the American Epilepsy Society (1990) [15], and the 6th and 7th Annual Meetings of the American Association of Pharmaceutical Scientists (1991–1992) [16, 17].

incubates just prior to sample preparation for HPLC analysis.

The procedure for kinetic studies with expressed enzyme was similar except that carbamazepine was added in 5 μL of methanol, resulting in a final methanol concentration of 0.5% in all samples. This allowed incubation concentrations of carbamazepine to range from 0 to 1000 μM. Control incubations containing the two lowest concentrations of carbamazepine (50 and 100 μM) were also performed in the absence of methanol to ensure that the solvent did not alter the 10,11-epoxide formation velocity in the expressed enzyme incubations.

HPLC analysis. After termination of microsome or reconstituted enzyme reactions containing unlabeled carbamazepine, the internal standard, 2-methylcarbamazepine (1125 ng in 100 μL methanol), was added to each sample. Samples were shaken for 10 min and centrifuged at 250 *g* for 10 min, and the *t*-butyl methyl ether was recovered for analysis. Following evaporation of the ether, samples were reconstituted in HPLC mobile phase and analyzed for metabolites of carbamazepine. Analysis for carbamazepine epoxide was performed at 50° using a Zorbax C_8 column (5 μm; 4.6 × 250 mm) and a mobile phase consisting of methanol and water (45:55, v/v) at a flow rate of 1.5 mL/min. Under these conditions, carbamazepine epoxide (5.4 min retention time) was resolved from 2- and 3-hydroxycarbamazepine (4.9 and 6.2 min, respectively). UV detection of the epoxide was accomplished at 210 nm. Quantitative analyses for the phenolic metabolites were performed at 40° using a Rainin C_{18} column (5 μm; 4.6 × 250 mm) and a mobile phase consisting of acetonitrile and water (28:72, v/v) at a flow rate of 2 mL/min. UV detection of the phenols was selectively performed at 290 nm, a wavelength of negligible absorbance for the 10,11-epoxide. Retention times for 2- and 3-hydroxycarbamazepine were 3.7 and 4.4 min, respectively. Recovery of metabolites in the extraction procedure was essentially complete (greater than 90%), and the assay showed excellent linearity and precision (CV less than 5%) at epoxide concentrations greater than 35 ng/mL and phenol concentrations greater than 10 ng/mL.

After termination of microsomal reactions containing [^{13}C,^{15}N]-carbamazepine or combinations of unlabeled and [^{13}C,^{15}N]-carbamazepine, the internal standard d_4-carbamazepine-10,11-epoxide was added to each sample. Samples were analyzed for ^{13}C,^{15}N-labeled and unlabeled carbamazepine-10,11-epoxide by LC/MS as described previously [18].

Statistical analyses. Comparisons of mean results were performed using Cochran's approximation of a two-sample *t*-test for independent samples with unequal variances. A one-sample *t*-test was used to test the null hypothesis: *r* (regression correlation coefficient) = 0.

RESULTS

Human liver microsomal carbamazepine-10,11-epoxide formation. Figure 1 depicts a typical profile of metabolite formation rate as a function of carbamazepine concentration in human liver microsomes. Carbamazepine concentrations in Fig. 1 include the range of plasma concentrations observed in patients receiving the drug (15–60 μM). Data for 2- and 3-hydroxycarbamazepine formation were fit separately to a single enzyme Michaelis–Menten equation (BMDP Statistical Software; University of California Press, Berkeley, CA). Respective K_m and V_{max} estimates were 349 ± 27 μM and 30.5 ± 1.7 pmol/min/mg for 2-hydroxycarbamazepine, and 338 ± 20 μM and 75.6 ± 3.2 pmol/min/mg for 3-hydroxycarbamazepine.

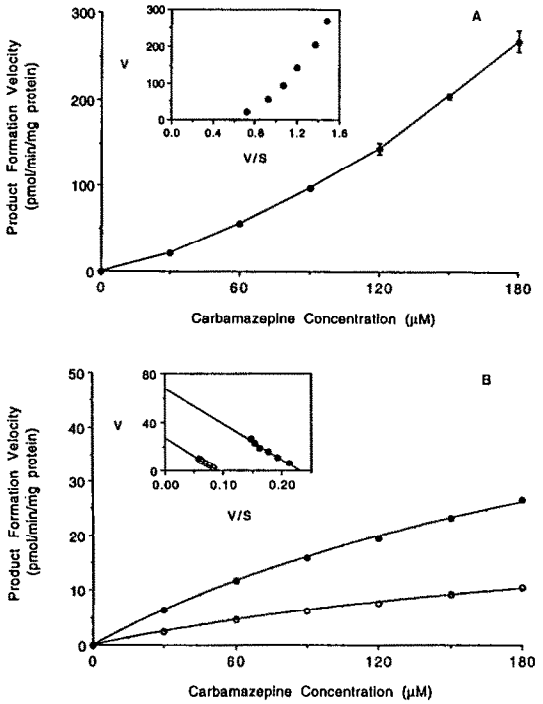

Fig. 1. Carbamazepine metabolism catalyzed by human liver microsomes. The formation rates of (A) carbamazepine-10,11-epoxide, and (B) 3-hydroxycarbamazepine (●) and 2-hydroxycarbamazepine (○) were measured in incubations of carbamazepine with microsomes from a representative liver (HL-105). Each data point is the mean result from three separate incubations ±1 SD. Insets show the data plotted according to the Eadie–Hofstee equation.

The microsomal 10,11-epoxide formation rate diverged from the phenol formation rates and accelerated disproportionately as the carbamazepine concentration was increased. This unusual nonlinearity is inconsistent with simple Michaelis–Menten enzyme kinetics (Fig. 1A), and is not explained by secondary metabolism of the epoxide (<5% of epoxide formed was metabolized further) or nonspecific binding or partitioning of carbamazepine (microsomal free fraction by ultrafiltration and equilibrium dialysis was 90% and was concentration independent). Co-incubation of ^{13}C,^{15}N-labeled and unlabeled carbamazepine with liver microsomes confirmed that carbamazepine itself activates 10,11-

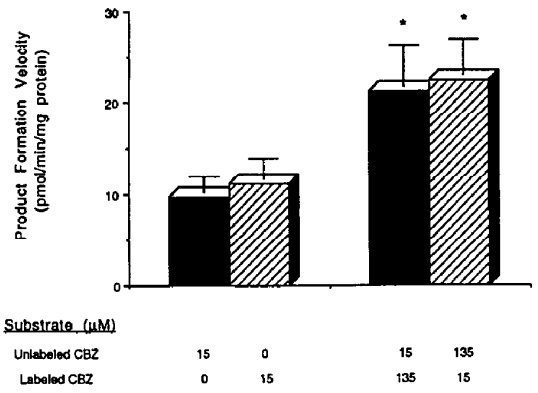

Fig. 2. Auto-activation of carbamazepine-10,11-epoxide formation. Microsomes from a representative liver (HL-105) were incubated with mixtures of unlabeled and stable-labeled carbamazepine, as indicated. Product formation velocities were determined for unlabeled 10,11-epoxide (closed bars) and labeled 10,11-epoxide (striped bars) in the absence and presence of the alternative isotopic form. Each bar represents a mean result from four separate incubations ±1 SD. Key: (*) denotes a velocity that was significantly different from the respective control, $P < 0.02$.

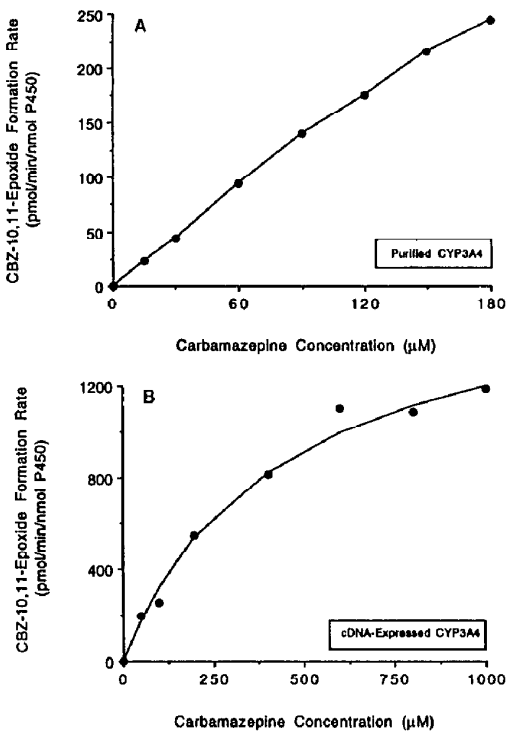

Fig. 3. Carbamazepine-10,11-epoxide formation catalyzed by purified and cDNA-expressed CYP3A4. Incubations with carbamazepine (CBZ) contained (A) 200 pmol CYP3A4, 400 pmol cytochrome P450 reductase, 200 pmol cytochrome b_5, and 200 μg microsomal lipid, or (B) microsomes isolated from HepG2 cells infected with recombinant vaccinia containing CYP3A4 cDNA. Results from duplicate incubations are presented for each carbamazepine concentration. The solid line in Fig. 3A was obtained by connecting mean data points. The data in Fig. 3B were fit by nonlinear regression (solid line) to obtain kinetic constants for a single enzyme system: K_m = 442 μM, V_{max} = 1730 pmol/nmol P450/min.

epoxide formation. As shown in Fig. 2, the rate of 10,11-epoxide formation from 15 μM unlabeled carbamazepine was approximately doubled in the presence of the 135 μM $^{13}C,^{15}N$-labeled carbamazepine. A similar result was also obtained when 135 μM unlabeled carbamazepine was added to incubations with 15 μM labeled carbamazepine. Under classic Michaelis–Menten kinetics, the velocity of 10,11-epoxide formation from 15 μM carbamazepine would either remain unaffected by the addition of the alternatively labeled substrate or be inhibited if the concentration of the alternative substrate approached the enzyme K_m for carbamazepine.

Other molecules were found also to activate carbamazepine-10,11-epoxidation. Microsomes from four different human livers (HL-105, 106, 109 and 116) co-incubated with carbamazepine (50 μM) and either α-naphthoflavone (10 μM) or progesterone (10 μM) resulted in a 10,11-epoxidation rate that was 2- to 3-fold higher than the control rate (33.8 ± 6.4 pmol/min/mg for control vs 74.7 ± 11.2 or 76.7 ± 10.1 pmol/min/mg for α-naphthoflavone or progesterone, respectively), while phenol formation was unchanged. Both α-naphthoflavone [27] and progesterone [28] have been reported to activate cytochrome P450 enzymes in the 3A subfamily.

Role of CYP3A4 in carbamazepine-10,11-epoxide formation. Purified reconstituted CYP3A4 catalyzed carbamazepine-10,11-epoxidation (Fig. 3A), but phenol formation was not detectable. Complete characterization of epoxidation kinetics (V_{max} and K_m) was not possible since the maximum experimental substrate concentration was limited to the aqueous solubility of carbamazepine, a concentration that proved not to saturate the enzyme. The disproportionate increase in the rate of epoxidation with increasing carbamazepine concentration (as seen in microsomes, Fig. 1) was not observed with the reconstituted enzyme.

Incubations with cDNA-expressed CYP3A4 cell lysates were performed to confirm the observations made with purified CYP3A4 (formation of carbamazepine-10,11-epoxide and lack of autoactivation). In addition, carbamazepine was dissolved in methanol to achieve a higher range of substrate concentrations in the incubations. In this experiment, the rate of formation of carbamazepine-10,11-epoxide was well described by simple Michaelis–Menten kinetics for a single enzyme with no evidence of autoactivation (Fig. 3B). The rates of carbamazepine-10,11-epoxide formation from incubations with 50 and 100 μM carbamazepine were identical to the respective rates achieved in the absence of 0.5% methanol (data not shown). cDNA-expressed CYP3A4 did not catalyze 2- or 3-hydroxycarbamazepine formation. Further, sequential metabolism of carbamazepine-10,11-epoxide to its transdihydrodiol metabolite was negligible. The

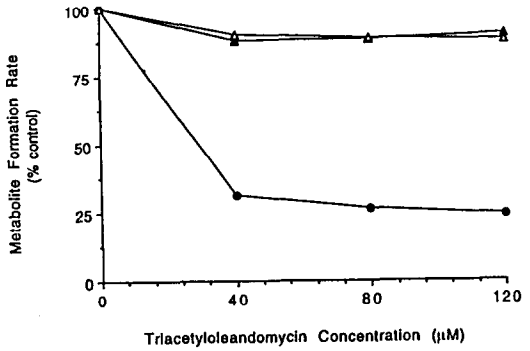

Fig. 4. Inhibition of carbamazepine-10,11-epoxide formation by triacetyloleandomycin. Microsomes from a representative liver (HL-105) were incubated with 0, 40, 80, or 120 μM triacetyloleandomycin, 100 μM carbamazepine, and 0.1% methanol final concentration. Metabolite formation velocities for 10,11-epoxide (●) and 2-hydroxy (▲) and 3-hydroxy (△) were measured. Each data point represents the mean result from three separate incubations. Respective control formation rates in the absence and presence of methanol were 122 and 119 pmol/min/mg for the 10,11-epoxide, 5.5 and 4.0 pmol/min/mg for 2-hydroxy, and 20.3 and 16.4 pmol/min/mg for 3-hydroxy metabolites.

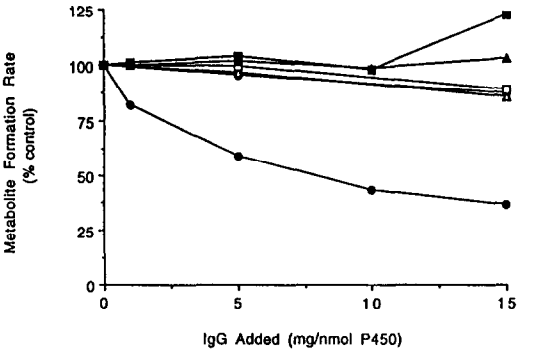

Fig. 5. Inhibition of carbamazepine-10,11-epoxide formation by anti-CYP3A4 IgG. Microsomes from a representative liver (HL-105) were preincubated for 30 min with 0, 1, 5, 10, or 15 mg of anti-CYP3A4 (closed symbols) or 5 or 15 mg pre-immune (open symbols) IgG protein/nmol total P450, and then incubated with 100 μM carbamazepine. The rates of carbamazepine-10,11-epoxide (circles), 2-hydroxycarbamazepine (squares), and 3-hydroxycarbamazepine (triangles) formation were measured; the respective formation rates for incubations without antibody were 95.1, 6.5 and 19.3 pmol/min/mg protein. Each data point is the mean result from duplicate incubations.

10,11-epoxidation data were fit to the Michaelis–Menten equation (BMDP Statistical Software; University of California Press, Berkeley, CA) to obtain the following kinetic constants: K_m = 442 μM, V_{max} = 1730 pmol/nmol P450/min.

Addition of the selective CYP3A inhibitor triacetyloleandomycin (40–120 μM) to microsomal incubations reduced the rate of carbamazepine-10,11-epoxidation to one-third of control values, while carbamazepine phenol formation was essentially unchanged (Fig. 4). Similar results were obtained in an antibody titration study, which showed selective concentration-dependent inhibition of epoxidation by anti-CYP3A4 IgG, but not pre-immune IgG, following pretreatment of microsomes with the respective IgG (Fig. 5). The selective inhibitory effects of triacetyloleandomycin and anti-CYP3A4 IgG towards 10,11-epoxidation were confirmed in microsomes prepared from multiple human livers. In sixteen different liver microsomal preparations incubated with a "therapeutic" carbamazepine concentration of 50 μM, triacetyloleandomycin (100 μM) reduced the 10,11-epoxidation rate to an average of 31 ± 6% of the control formation rate (range: 20–40% of control value), while formation rates for 2- and 3-hydroxycarbamazepine in the presence of triacetyloleandomycin were 100 ± 10 and 87 ± 7% of control formation rates, respectively. In four different liver microsomal preparations incubated with 50 μM carbamazepine, anti-CYP3A4 (15 mg IgG/nmol P450) reduced the 10,11-epoxidation rate to 43 ± 2% of the control formation rate (range: 41–45% of control value), while formation rates of 2- and 3-hydroxycarbamazepine in the presence of anti-CYP3A4 IgG were 98 ± 8 and 90 ± 7% of control formation rates, respectively.

The rate of carbamazepine-10,11-epoxide formation in sixteen different human liver microsomal preparations incubated with 50 μM carbamazepine (therapeutic concentration) correlated (r^2 = 0.57, P < 0.001) with the microsomal contents of CYP3A4, as measured by relative immunoblot band density (Fig. 6). The significant positive intercept and r^2 value of 0.57 are suggestive of a contribution from other P450 isoforms to the formation of

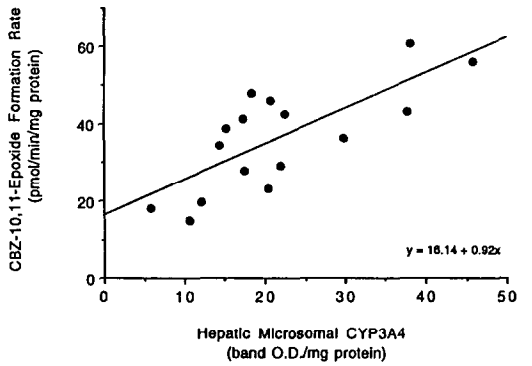

Fig. 6. Correlation between CYP3A4 content and carbamazepine-10,11-epoxide formation rate in human liver microsomes. Microsomes from sixteen different human livers were analyzed for CYP3A4 content by western blot analysis. In a separate experiment, microsomes from the same livers were incubated with 50 μM carbamazepine, and carbamazepine-10,11-epoxide formation was measured. The rate of 10,11-epoxide formation was positively and significantly correlated with CYP3A4 content, expressed as immunoblot band optical density (O.D.)/mg protein, r^2 = 0.57, P < 0.001.

Table 1. Drug treatment and history of liver donors

Liver	Age (yr)	Sex	Significant drug history	Pathology
HL-101	13	F	None	None
HL-103	15	F	Trandate	None
HL-104	32	M	Alcohol abuse	Fatty liver
HL-105	21	M	None	None
HL-106	45	F	Synthroid	None
HL-107	28	M	None	None
HL-108	42	F	None	None
HL-109	46	M	Alcohol abuse, Inderal	Fatty liver
HL-110	14	M	None	None
HL-111	28	M	None	None
HL-113	9	F	None	None
HL-114	19	M	None	None
HL-115	52	F	Dexamethasone, phenytoin, atenolol	None
HL-116	31	M	Alcohol abuse	None
HL-118	25	M	Alcohol abuse	Fatty liver
HL-119	24	M	Dexamethasone	None
HL-120	45	F	Prozac, Sinequan, lithium	Fatty liver
HL-121	59	F	Synthroid	Fatty liver
HL-122	50	F	Dexamethasone, phenytoin	None
HL-123	15	M	Phenytoin	None
HL-124	51	F	None	Fibrosis
HL-125	33	M	Dexamethasone, phenytoin	Acute liver injury
HL-126	10	F	None	Fatty liver
HL-127	38	M	None	None
HL-128	51	M	Alcohol abuse	Fatty liver
HL-130	49	M	Alcohol abuse, chronic insulin	Fatty liver

HL-101 and HL-103 through HL-119 were used for the sixteen liver carbamazepine-10,11-epoxide vs CYP3A correlation. HL-101 and HL-104 through HL-130 were used for the twenty-five liver correlation.

carbamazepine-10,11-epoxide. A similar correlation coefficient ($r^2 = 0.63$, $P < 0.001$) and y-intercept were obtained from an analysis of microsomes from an expanded panel of twenty-five livers incubated with a higher (180 μM) carbamazepine concentration. Medical records from the respective donors of the livers used in the latter experiment indicate that five of the livers were exposed to known CYP3A inducers (phenytoin and dexamethasone) during a 3- to 7-day period immediately preceding organ procurement (Table 1). The mean rate of carbamazepine-10,11-epoxide formation for the five drug-exposed livers was 1.9-fold higher (500 ± 107 pmol/mg protein/min) than the mean rate for the other twenty livers (261 ± 112 pmol/mg protein/min), $P < 0.001$. However, the similar correlation coefficients in the two experiments indicate that CYP3A4 is the predominant enzyme responsible for hepatic carbamazepine-10,11-epoxidation, regardless of induction status.

Among both the sixteen liver and twenty-five liver panels screened, formation rates of 2- and 3-hydroxycarbamazepine showed no significant correlation to microsomal CYP3A4 content ($r^2 < 0.1$).

Involvement of other P450 isoforms in 10,11-epoxide formation. Further analysis of results from incubations of microsomes from different livers revealed that the degree of activation of carbamazepine-10,11-epoxide formation for any given microsomal preparation was variable. Table 2 shows that among the four microsomal preparations examined, the percent inhibition of epoxidation by triacetyloleandomycin and percent activation by α-naphthoflavone and progesterone appeared to cosegregate. Thus, the liver sample (HL-106) in which epoxidation was inhibited most extensively (80% inhibition) also showed the greatest degree of activation (178 and 173% increase from control). Likewise, the liver sample (HL-109) least susceptible to inhibition (60%) was also least susceptible to activation (59 and 67% increase from control). These results are consistent with the presence of at least one P450 isoform alternative to CYP3A4 that contributes to epoxidation, which is present at a proportionally higher level in HL-109 than the other three livers examined and which is insensitive to triacetyloleandomycin, α-naphthoflavone, and progesterone. The existence of alternative P450 isoforms that contribute to carbamazepine-10,11-epoxidation may explain two other observations: (1) 20–40% of 10,11-epoxidation in every liver sample appeared resistant to inhibition by triacetyloleandomycin and anti-CYP3A4 IgG; (2) anti-CYP3A4 IgG inhibition was enhanced (Table 3) when carbamazepine was co-incubated with the CYP3A activator α-naphthoflavone (activation of CYP3A4 decreases the percent contribution of alternative enzyme(s) to total 10,11-epoxidation, and thereby increases the effectiveness of anti-CYP3A4 inhibition).

A series of selective and non-selective inhibitors

Table 2. Cosegregation between the percent inhibition by triacetyloleandomycin and the magnitude of carbamazepine-10,11-epoxide activation by α-naphthoflavone or progesterone

Liver	Modification of carbamazepine-10,11-epoxide formation rate* (% change from control)		
	Triacetyloleandomycin	Progesterone	α-Naphthoflavone
HL-109	(−) 60 ± 1	(+) 67 ± 2	(+) 59 ± 2
HL-105	(−) 69 ± 0.4	(+) 146 ± 5	(+) 144 ± 35
HL-116	(−) 73 ± 0.2	(+) 144 ± 2	(+) 127 ± 13
HL-106	(−) 80 ± 1	(+) 173 ± 11	(+) 178 ± 12

* Microsomes were incubated with 50 μM carbamazepine and either nothing (control), 100 μM triacetyloleandomycin, 10 μM α-naphthoflavone, or 10 μM progesterone. Values are expressed as means ± 1 SD of triplicate incubations. Control 10,11-epoxide formation rates were 60.7 (HL-109), 36.3 (HL-105), 41.2 (HL-116), and 43.0 (HL-106) pmol/min/mg for the experiment with triacetyloleandomycin, and 42.4 (HL-109), 26.9 (HL-105), 33.2 (HL-116), and 32.5 (HL-106) pmol/min/mg for experiments with progesterone and α-naphthoflavone.

Table 3. Reciprocal effects of anti-CYP3A4 IgG and α-naphthoflavone (α-NF) on carbamazepine-10,11-epoxide formation*

Liver	% Change by anti-CYP3A4		% Change by α-NF	
	−α-NF	+α-NF	−anti-CYP3A4	+anti-CYP3A4
HL-103	(−) 49	(−) 57	(+) 115 ± 5	(+) 82
HL-105	(−) 54	(−) 71	(+) 176 ± 5	(+) 75

* Microsomes were incubated with 50 μM carbamazepine and either nothing (control), 10 μM α-NF, 15 mg anti-CYP3A4 IgG/nmol P450, or both α-NF + anti-CYP3A4 IgG. Data are expressed as an average value from duplicate incubations when anti-CYP3A4 was included, or a mean ± 1 SD of quadruplicate incubations when anti-CYP3A4 was not included. Control 10,11-epoxide formation rates were 17.6 ± 0.2 (HL-103) and 18.1 ± 0.4 (HL-105) pmol/min/mg.

Table 4. Effect of selective and non-selective cytochrome P450 inhibitors on carbamazepine-10,11-epoxide formation

Inhibitor	Carbamazepine-10,11-epoxide formation rate* (% control)
Triacetyloleandomycin (100 μM)	35
Furafylline (20 μM)	133
Quinidine (5 μM)	101
Diethyldithiocarbamate (100 μM)	155
Sulfaphenazole (1.3 μM)	108
(*S*)-Mephenytoin (300 μM)	105
Tolbutamide (800 μM)	77

* Microsomes from HL-105 were incubated with 100 μM carbamazepine ± inhibitor. Data are expressed as a mean value from two to three incubations. The control 10,11-epoxide formation rate was 115 pmol/min/mg protein.

of cytochrome P450 were used to determine the contribution of non-CYP3A4 isoforms to the 10,11-epoxidation of carbamazepine. Results from this experiment are reported in Table 4. The selective inhibitors quinidine, 5 μM (CYP2D6 [29]), sulfaphenazole, 1.3 μM (CYP2C9/10 [30]), diethyldithiocarbamate, 100 μM (CYP2E1 [31], and furafylline, 20 μM (CYP1A2 [32]) had little or no inhibitory effect on 10,11-epoxide formation. (*S*)-Mephenytoin, likewise, had no effect at a con-

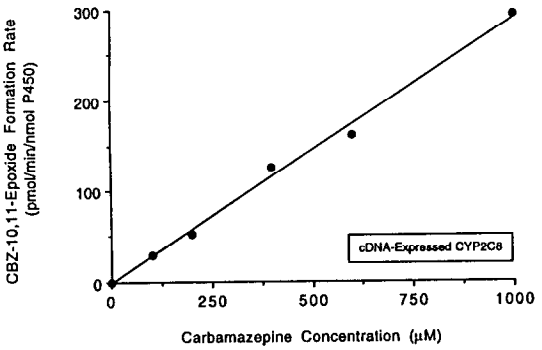

Fig. 7. cDNA-expressed CYP2C8 catalyzed carbamazepine-10,11-epoxide formation. Microsomes were isolated from HepG2 cells infected with recombinant vaccinia containing CYP2C8 cDNA and incubated with 100–1000 μM carbamazepine. The rate of 10,11-epoxide formation was measured. Each data point represents the mean result of duplicate incubations. The data were fit by linear regression (solid line), $r^2 = 0.995$.

centration of 300 μM (three times the reported K_m concentration of (*S*)-mephenytoin in human liver microsomes [33]), suggesting that 10,11-epoxide formation is not catalyzed significantly by the mephenytoin hydroxylase.

The non-selective CYP2C8/9/10 substrate tolbutamide inhibited carbamazepine-10,11-epoxidation, but to a more modest degree than triacetyloleandomycin. Co-incubations of 800 μM tolbutamide with 100 μM carbamazepine reduced the 10,11-epoxide formation rate to 77% of control (Table 4). Since sulfaphenazole, the more selective CYP2C9/10 competitive inhibitor, did not affect carbamazepine metabolism (Table 4), the results with tolbutamide suggest that CYP2C8 also contributes to carbamazepine-10,11-epoxide formation.

To pursue the involvement of CYP2C8 and other P450 isoforms in carbamazepine-10,11-epoxidation, catalytic experiments with individual cDNA-expressed P450 isoforms were performed. The lysate system containing CYP2C8 catalyzed the reaction with a velocity of 17–300 pmol/min/nmol P450 across a 100–1000 μM carbamazepine concentration range (Fig. 7). The reaction showed no evidence of autoactivation and was not saturated at the highest substrate concentration examined. Incubations with cDNA-expressed CYP1A2, 2C9, 2E1, 2B7, and 3A5 did not produce detectable levels of carbamazepine-10,11-epoxide.

DISCUSSION

Prior to this investigation, only indirect evidence existed concerning the identity of the cytochrome P450 isoforms responsible for carbamazepine metabolism in humans. Eichelbaum *et al.* [1] reported that the partial clearance of carbamazepine to the 10,11-epoxide did not correlate with the debrisoquine phenotype, suggesting little or no involvement of CYP2D6 in 10,11-epoxide formation. The lack of effect of quinidine, a potent inhibitor of CYP2D6, on carbamazepine microsomal oxidative metabolism in the present study confirms the conclusion of Eichelbaum. Faigle and Feldmann [34] concluded that a cytochrome P450 in the 2B or 2C subfamily is responsible for biotransformation of carbamazepine to the 10,11-epoxide because those enzymes, as well as the partial clearance of carbamazepine to the epoxide, are inducible by phenobarbital and phenytoin, two anticonvulsants commonly coadministered with carbamazepine. However, other more direct evidence to support the 2B/2C hypothesis is lacking. The present investigation focussed on the role of CYP3A4 in carbamazepine metabolism in light of the pronounced clinical interactions of carbamazepine with macrolide antibiotics [2, 11], compounds known to both inhibit and induce CYP3A4 [12, 35, 36].

Several pieces of evidence from the present study confirm that CYP3A4 plays a major but selective role in the oxidative biotransformation of carbamazepine: (1) purified CYP3A4 catalyzed formation of the 10,11-epoxide but not the phenolic 2- or 3-hydroxylated metabolites of carbamazepine; (2) cDNA-expressed CYP3A4 catalyzed 10,11-epoxide formation with a K_m of 442 μM, consistent with the absence of saturable carbamazepine kinetics at therapeutic concentrations; (3) formation of the 10,11-epoxide but not the phenols correlated with CYP3A4 content in microsomal preparations from a panel of sixteen and twenty-five human livers; (4) triacetyloleandomycin and anti-CYP3A4 IgG each inhibited the majority of 10,11-epoxide formation but had little or no effect on phenol formation in the human liver microsomes; and (5) two activators of CYP3A4, progesterone and α-naphthoflavone, increased formation of the 10,11-epoxide 2- to 3-fold, but had no effect on phenol formation. Taken together, these results indicate that CYP3A4 is the principal enzyme responsible for biotransformation of carbamazepine to the 10,11-epoxide, accounting for at least 60–80% of this metabolic pathway.

Additional experiments with a panel of P450 inhibitors and substrates revealed the possible involvement of CYP2C8 but not CYP2C9/10 in 10,11-epoxide formation. Tolbutamide, but not sulfaphenazole, inhibited the reaction, although to a much lesser degree than triacetyloleandomycin. Tolbutamide is a substrate for CYP2C8 and CYP2C9/10 but with different K_m values: CYP2C8, 650 μM; CYP2C9/10, 72–132 μM [37]. Since the fraction of 10,11-epoxide formation not accounted for by CYP3A4 was less than 40%, the extent of inhibition observed with 800 μM tolbutamide (23%) is consistent with a competitive mechanism and the reported K_m of CYP2C8 for tolbutamide.

Incubations with cDNA-expressed CYP2C8 confirmed its catalytic activity towards carbamazepine. Assuming that the K_m of CYP2C8 and CYP3A4 for carbamazepine is 442 μM or greater, the relative intrinsic clearances for each P450 isoform can be calculated from the product formation velocities at non-saturating, therapeutic carbamazepine concentrations. Intrinsic clearances (V_{max}/K_m) of 233 and 17.5 μL/hr/nmol P450 were obtained for CYP3A4 and CYP2C8, respectively, further sup-

porting the major (CYP3A4) and minor (CYP2C8) role of these P450 isoforms in carbamazepine-10,11-epoxidation. It is likely that CYP3A4 dominates 10,11-epoxide formation in epileptic patients receiving chronic treatment with enzyme-inducing anticonvulsants, because CYP3A4 is highly induced by the phenobarbital-type inducing agents [12, 38], whereas there is no evidence in the literature to date for the induction of CYP2C8 under similar treatments.

The absence of carbamazepine-10,11-epoxide formation in incubations with cDNA-expressed CYP3A5 is unusual, although a number of good CYP3A4 substrates, such as erythromycin, quinidine, and 17α-ethynylestradiol, have also been reported to be relatively poorer substrates for CYP3A5 [39]. It is possible that CYP3A5 catalyzes 10,11-epoxide formation but at a rate that, in our experiment, produced metabolite concentrations below the limit of detection.

Eichelbaum *et al.* [1] previously deduced that the principal cytochrome P450 enzyme-catalyzing formation of the 10,11-epoxide must be different from the enzyme involved in the formation of 2- and 3-hydroxycarbamazepine. This conclusion was based on the differing susceptibilities of the epoxide and phenol pathways to auto- and heteroinduction in a clinical study. The results of the present investigation support this hypothesis in that inhibition with triacetyloleandomycin or anti-CYP3A4 IgG, or activation with progesterone or α-naphthoflavone, had pronounced effects on the *in vitro* epoxidation of carbamazepine but had little or no effect on phenol formation. The identity of the principal enzyme controlling carbamazepine phenol formation remains unknown.

Preincubation of liver microsomes with anti-CYP3A4 IgG impaired the activation of 10,11-epoxide formation by α-naphthoflavone (Table 3), confirming that CYP3A4 is involved in the activation process. This conclusion is consistent with other literature reports that P450 isoforms in the 3A subfamily are susceptible to *in vitro* activation [40–43]. It is likely that carbamazepine itself also activates CYP3A4, as indicated by the unusual nonlinear relationship between 10,11-epoxide formation rate and carbamazepine substrate concentration in the microsomal incubations (Fig. 1). Epoxide formation but not phenol formation increased more than proportionally to carbamazepine concentration, an effect not explained by a change in the carbamazepine free fraction (which was concentration independent). Rather, the disproportional increase in the rate of epoxide formation was apparently due to autoactivation of CYP3A4 by the substrate carbamazepine. This conclusion is based on the LC/MS results indicating that 10,11-epoxide derived from 15 μM carbamazepine (unlabeled or stable-labeled) was increased 2-fold by co-incubation with the alternatively labeled carbamazepine at 135 μM. The unusual nonlinear relationship between 10,11-epoxide formation rate and carbamazepine substrate concentration was observed previously in human liver microsomes (including enzyme-induced microsomes) but the phenomenon was not discussed (see Tybring *et al.* [44], Fig. 2). Similar to our results with carbamazepine, the 2-hydroxylation of estradiol by human liver microsomal CYP3A4 was reported recently to undergo both auto- and heteroactivation [43].

The lack of autoactivation in either the purified or cDNA-expressed CYP3A4 systems is unexplained. Unexplored factors such as the ratio of cytochrome b_5:CYP3A4:cytochrome P450 reductase, or the lipid membrane composition and orientation of the P450 protein complex, may be critical for manifestation of the effect. It is also possible that a second metabolite of carbamazepine, such as 2- or 3-hydroxycarbamazepine, exerts the activating effect on 10,11-epoxide formation in microsomal incubations, and that its formation is catalyzed by a non-CYP3A4 isoform. Thus, isolated CYP3A4 preparations that do not generate the second metabolite do not exhibit the activation phenomenon. If this hypothesis is correct, the activating species must have a high affinity for CYP3A4 (or co-enzymes if implicated), since the concentration of the hydroxy carbamazepine metabolites generated in our microsomal incubations did not exceed 1 μM. Activation of microsomal 10,11-epoxide formation by progesterone, α-naphthoflavone, and by carbamazepine itself raises the question as to whether these phenomena (hetero- and autoactivation) may occur *in vivo* as well. It is notable that carbamazepine clearance and the ratio of 10,11-epoxide to carbamazepine plasma are both increased during pregnancy [45], a condition when circulating levels of the activator progesterone are elevated.

In summary, the present investigation revealed that CYP3A4 is the principal enzyme and that CYP2C8 is a minor enzyme governing biotransformation of carbamazepine to the 10,11-epoxide, while an alternative enzyme(s) catalyzes formation of the phenolic carbamazepine metabolites. Because 10,11-epoxidation is the single most important elimination pathway for carbamazepine, accounting for up to two-thirds of the total clearance [1, 34], it is likely that the numerous drug interactions involving induction or inhibition of carbamazepine clearance can be largely or entirely explained through effects on CYP3A4. Finally, unrecognized drug interactions (e.g. with new chemical entities) that lead to induction or inhibition of carbamazepine clearance can probably be predicted simply by knowing the effects of the concomitant drug on CYP3A4 activity.

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