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Influence of Some Novel *N*-Substituted Azoles and Pyridines on Rat Hepatic CYP3A Activity

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ABSTRACT. A series of *N*-substituted heteroaromatic compounds structurally related to clotrimazole was synthesized, and the effects of these compounds on ethosuximide clearance in rats were determined as a measure of their abilities to induce cytochrome P4503A (CYP3A) activity. Ethosuximide clearance and *in vitro* erythromycin *N*-demethylase activity were shown to correlate. In this series, imidazole or other related heteroaromatic "head groups" were linked to triphenylmethane or other phenylmethane derivatives. Within the series, it was found that 1-triphenylmethane-substituted imidazoles elicited the greatest increase in CYP3A activity, and that among the triphenylmethyl-substituted imidazoles, the highest activities were achieved by the substitution of F- or Cl- in either the meta or para position of one of the phenyl rings. Diphenylmethyl-substituted pyridine was effectively devoid of activity. Compounds eliciting the largest increase in CYP3A activity (viz. 1-[(3-fluorophenyl)diphenylmethyl]imidazole, 1-[(4-fluorophenyl)diphenylmethyl]imidazole, and 1-[tri-(4-fluorophenyl)methyl]imidazole) produced little or no increase in ethoxyresorufin *O*-dealkylase (EROD) activity (i.e. CYP1A), whereas benzylimidazole, which elicited only a small increase in CYP3A activity, produced an almost 9-fold increase in CYP1A activity. For a series of eleven compounds exhibiting a wide range of influence on CYP3A activity, a positive correlation was found between ethosuximide clearance and hepatic CYP3A mRNA levels. BIOCHEM PHARMACOL **55**;11:1881–1892, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. cytochrome P4503A; CYP3A; enzyme induction; ethosuximide; triphenylmethylimadazole; structure–activity relationships

Drugs and other xenobiotics are oxidatively metabolized by the family of CYP¶. It is now appreciated that drug interactions often arise from specific inhibition or induction of CYP isozymes that alters the rate of metabolism of other drug molecules. Because many of the commonly prescribed drugs are metabolized by the CYP3A isoform, understanding the structure–activity relationship for both the inhibition and the induction of this cytochrome is key to the prediction of many drug interactions. Many *N*-substituted azoles have the ability to inhibit and/or induce hepatic microsomal mixed-function oxidases [1–5]. Some *N*-substituted imidazoles have been characterized as high magnitude inducers of rat hepatic CYP such as *N*-benzylimidazole [6], which elicited a three-fold increase in CYP content, a two-fold increase in erythromycin-*O*-demethylase activity, a two-fold increase in ethylmorphine *O*-demethylase activity, a 56-fold increase in ethoxyresorufin deethylase activity, and a 16-fold increase in pentoxyresorufin *O*-dealkylase activity. A series of *N*-substituted imidazoles and related pyridines were compared for their ability to induce rat hepatic CYP after single doses [7]. Matsuura *et al.* [7] found that the imidazole structure, itself, was important for CYP induction; that triazole could be substituted for imidazole; and that the chlorine atom in clotrimazole was not important for CYP induction. However, these authors and others [8] have commented on the ability of imidazoles and other agents to induce multiple isoforms of CYP. Hostetler *et al.* [9] have demonstrated that clotrimazole is a relatively selective inducer of CYP3A in rats. The induction of CYP3A was several orders of magnitude greater than the induction of CYP2B.

In view of the marked induction of CYP3A by clotrimazole [9–11], and because the SARs for imidazoles regarding CYP induction have thus far been based largely on the measurement of hepatic CYP content [7], we synthesized other selective inducers of CYP3A, using clotrimazole as a lead compound, and characterized the effects of this series with specific regard to the increase of CYP3A activity after

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¶ *Abbreviations:* CYP, cytochrome P450; EF-1α, elongation factor-1 alpha; EROD, ethoxyresorufin *O*-dealkylase; SAR, structure–activity relationship; THF, tetrahydrofuran.

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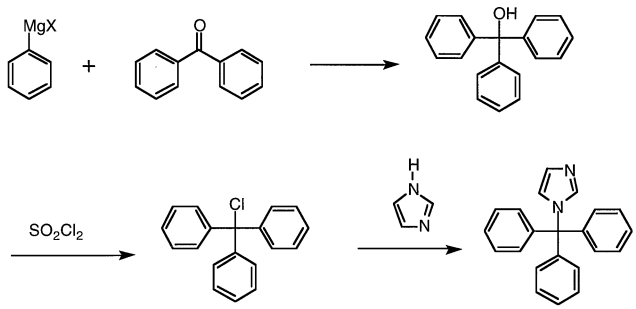

SCHEME 1. Synthesis of 1-triphenylmethyl-1*H*-imidazoles.

subchronic (8-day) treatment. This approach is empirical of necessity, since the mechanism of CYP3A induction is as yet unknown, and the biochemical nature of the macromolecular receptor for CYP3A inducers has not been described yet [12].

MATERIALS AND METHODS

Materials

1-Benzylimidazole (CDD 3500), 4-benzylpyridine (CDD 3513), 3-benzylpyridine (CDD 3515), and 2-benzylpyridine (CDD 3529) were purchased from the Aldrich Chemical Co. Clotrimazole (1-[(2-chlorophenyl)diphenylmethyl]imidazole) was purchased from the Sigma Chemical Co. Purities ranged from 97% to greater than 99%. 1-Triphenylmethylimidazole (CDD 3501) was synthesized using method A and crystallized from ethanol: m.p. 222–224° (reported [13], 221–223°). 1-Triphenylmethylpyrazole (CDD 3502), 1-triphenylmethyl-1,2,4-triazole (CDD 3504), and 1-diphenylmethylimidazole (CDD 3510) were prepared by the procedure of Claramunt *et al.* [14]. 1-Triphenylmethylpyrrole (CDD 3503) was prepared from triphenylmethylamine using the procedure of Chadwick and Hodgson [15]. Compounds CDD 3508, 3522, 3523, 3524, 3530, 3532, 3535, 3536, 3537, 3538, and 3540 were synthesized as described by Buechel *et al.* [16]. The physical properties (m.p. and ^{1}H NMR) determined for these compounds were in agreement with that reported for the compound in the literature cited. The purity of tested compounds was established by obtaining combustion analysis (C, H, and N). In all cases, analysis obtained agreed with calculated compositions to within ±0.4%.

General Methods (Chemistry)

1-Triphenylmethyl-1*H*-imidazoles (see Scheme 1) were prepared by treating a triphenylchloromethane or other alkyl halide with an imidazole or a related heterocycle (Method A). The triphenylchloromethanes were synthesized by treating triphenylmethanols with thionyl chloride (Method B). A variety of substituted triphenylmethanols were available commercially, or were readily prepared from commercially available benzophenones using the Grignard reaction. Phenylmagnesium halides or substituted phenylmagnesium halides were added to benzophenone or to one of a variety of commercially available substituted benzophenones producing the mono-, di-, and trisubstituted triphenylmethanols required for the study (Methods C and D).

Melting points were determined in open capillary tubes using an Electrothermal model IA9100 apparatus and are uncorrected. ^{1}H NMR spectra were determined at 300 MHz in $CDCl_3$ unless otherwise noted. Chemical shifts are reported in parts per million downfield from internal tetramethylsilane. Microanalyses were performed by Atlantic Microlabs Inc. Analyses indicated by the symbol of the elements were within ±0.4% of theoretical values. THF was dried by distillation over sodium and benzophenone.

General Procedure for the Synthesis of 1-Triphenylmethyl-Substituted Azoles (Method A)

The heterocycle (15 mmol), a triphenylchloromethane (15 mmol), and triethylamine (15 mmol) were heated at reflux in 100 mL of acetonitrile for 2–7 hr. Solvent was removed *in vacuo*, 100 mL of water was added, and the product was extracted into chloroform (two portions of 50 mL each). Extracts were washed with water and dried ($MgSO_4$), the solvent was evaporated, and the product was purified by crystallization, resulting in isolated yields ranging between 50 and 80% of theory.

General Procedure for the Conversion of Triphenylmethanols to Triphenylchloromethanes (Method B)

The tertiary alcohol (25 mmol), thionyl chloride (7 g, 60 mmol), and dry THF (100 mL) were heated at reflux for 1 hr. Solvent was evaporated *in vacuo*, 30 mL of fresh THF was added to dissolve the residue, and the solvent was evaporated again. The resulting triphenylchloromethanes were somewhat unstable and were neither purified nor characterized, but used immediately in a subsequent reaction.

General Procedure for Conversion of a Substituted Benzophenone to an (Aryl)diphenylmethanol (Method C)

A 500-mL three-neck flask was fitted with a condenser, a nitrogen inlet, and a magnetic stirrer and charged with the benzophenone (35.71 mmol) and 120 mL of dry THF. The mixture was stirred under an atmosphere of dry N_2 as a 3.0 M solution of phenyl magnesium bromide in THF (12.2 mL, 36.6 mmol, Aldrich) was added dropwise from a pressure-equalized dropping funnel. The mixture was heated at reflux for 1 hr and, after the solution had cooled to room temperature, solvent was removed *in vacuo*. The residue was treated with 1% H_2SO_4 (100 mL), stirred for 30 min, and extracted twice with 100-mL portions of chloroform. The extract was washed with 100 mL of water, dried over anhydrous $MgSO_4$, and filtered. The solvent was

TABLE 1. Synthesis of substituted azoles*†

Compound No.	Compound name	Method of synthesis	Crystallization solvent	m.p. (°)
3505	4-Nitro-1-triphenylmethylimidazole‡	A	Acetonitrile	210–211
3506	4-Amino-1-triphenylmethylimidazole	Hydrogenation§	Methanol	235–236
3511	1-(Triphenylmethyl)-2-methylimidazole	A	Acetonitrile	215–216
3512	1-Triphenylmethylbenzimidazole	A	Cyclohexane	180–181
3526	1-[α-(4-Pyridyl)-α-phenylbenzyl]imidazole	B and A	Hexane	211–212
3527	1-[(5-Pyrimidyl)diphenylmethyl]imidazole	C‖, B, and A	Acetonitrile	213–214
3531	1-Methyl-1-(triphenylmethyl)imidazole	A	Acetonitrile	220–221
3533	1-[(3-Methoxyphenyl)diphenylmethyl]imidazole	D, B, and A	Hexane	115–117
3534	1-(9-Phenyl-9-fluorenyl)imidazole	B and A	Hexane	197–198
3539	1-[(2-Naphthyl)diphenylmethyl]imidazole	D, B, and A	Hexane	155–157
3541	1-[(1-Naphthyl)diphenylmethyl]imidazole	D, B, and A	Hexane	171
3542	1(11-Phenyl-11-dibenzosuberenyl)imidazole	D	Hexane	222–224
3543	1-[Di(4-fluorophenyl)phenylmethyl]imidazole	D, B, and A	Hexane	135–138

*Combustion analysis for C, H, and N was performed for all new target compounds. In all cases, analyses agree with calculated compositions to within ±0.4%.
†Each compound was characterized by ^{1}H NMR, and the spectrum that was obtained supported the structure assigned.
‡Structure was confirmed by x-ray crystallographic analysis [17].
§CDD 3506 was hydrogenated using 10% palladium on carbon in ethanol solution for 2 hr under 20 psi H_2.
‖In this case, a lithium reagent was formed from *n*-butyllithium and 5-bromopyrimidine at −95°, followed after 30 min by the addition of benzophenone.

evaporated, and the oily residue was crystallized. The structure and purity of the alcohols were verified by ^{1}H NMR and TLC.

Alternate General Procedure for Conversion of a Substituted Benzophenone to an (Aryl)diphenylmethanol (Method D)

A 500-mL three-neck flask fitted with a reflux condenser, a magnetic stirrer, and a nitrogen inlet was charged with magnesium turnings (1.4 g, 58 mmol), the desired aryl halide (59 mmol), and 100 mL of dry THF. The mixture was maintained under an atmosphere of N_2 and heated at reflux until all magnesium disappeared (2 hr). A solution of benzophenone or the desired substituted benzophenone (58 mmol) dissolved in 30 mL of dry THF was added dropwise to the resulting gray mixture of Grignard reagent, and after the addition was complete, was heated at reflux for 1 hr. After cooling to room temperature, solvent was evaporated, and 100 mL of 1% H_2SO_4 was added. The mixture was stirred for 30 min and extracted twice with 100-mL portions of chloroform. The organic extract was washed with 100 mL of water, dried over anhydrous $MgSO_4$, and filtered. Solvent was evaporated *in vacuo* producing a residue, which was further purified. The structure and purity of the alcohols were verified by ^{1}H NMR and TLC. Compounds synthesized according to the General Procedures are listed in Table 1.

1-*(2-Phenylethyl)imidazole (CDD* 3507*)*

Imidazole (4.0 g, 58.75 mmol) and 1-chloro-2-phenylethane (4.2 g, 29.9 mmol, Aldrich) were heated at reflux for 3 hr. Water (100 mL) was added, and the mixture extracted twice with 100-mL portions of chloroform. The organic phase was washed with water (100 mL) and dried over anhydrous $MgSO_4$. After evaporating solvent *in vacuo*, a yellow oil was obtained and purified by Kugelrohr distillation at 100° (5 mm Hg) to produce 2.5 g (48.5%) of a colorless liquid: ^{1}H NMR d 7.15–7.23 (4H, m), 6.98–7.02 (3H, m), 6.78 (1H, s), 4.09 (2H, t, J = 7.1 Hz), 2.97 (2H, t, J = 7.1 Hz). Anal. ($C_{11}H_{12}N_2$) C, H, N.

1-*(1-Phenylethyl)imidazole (CDD* 3514*)*

1-Chloro-1-phenylethane (6.3 g, 44.8 mmol) and imidazole (6.0 g, 88.1 mmol) were heated at 100° for 3 hr. After cooling, 100 mL of chloroform was added, the organic phase was washed twice with 100-mL portions of water, and then dried ($MgSO_4$). The solvent was removed and the product was purified by column chromatography (silica gel/chloroform), yielding 3.2 g (41%) of a colorless oil: ^{1}H NMR d 7.54 (1H, s), 7.32–7.26 (3H, m), 7.11–7.09 (2H, m), 7.03 (1H, s), 6.89 (1H, s), 5.29 (1H, q, J = 7.0 Hz), 1.79 (3H, d, J = 7.0 Hz). Anal. ($C_{11}H_{12}N_2$) C, H, N.

1-*Triphenylmethyl-2-methyl-2-imidazoline (CDD* 3520*)*

A mixture of 2-methyl-2-imidazoline (3.0 g, 35.66 mmol), triphenylchloromethane (10.0 g, 35.87 mmol) and 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU, 5.55 g, 36.46 mmol) in 30 mL of dimethylformamide was stirred overnight at room temperature. The mixture was poured into 100 mL of ice water, and the precipitate was collected. The solids were dissolved in 100 mL of chloroform, dried ($MgSO_4$), and filtered. The product was purified by column chromatography (silica gel/10% CH_3OH in $CHCl_3$) and crystallized from chloroform/cyclohexane to yield 2.4 g (21%) of white crystals; m.p. 189–190°. ^{1}H NMR d 7.33–7.27 (15H, m), 3.61 (2H, t, J = 9.6 Hz), 3.37 (2H, t, J = 9.5 Hz), 1.31 (3H, s). Anal. ($C_{23}H_{22}N$) C, H, N.

(4-Pyridyl)diphenylmethane (CDD 3521)

A mixture of α-(4-pyridyl)benzhydrol (Aldrich) (3.0 g, 11.5 mmol) and sodium borohydride (4.32 g, 114.0 mmol) was added in portions over 10 min to 100 mL of trifluoroacetic acid (TFA) at 0° under a dry nitrogen stream. After stirring the mixture for 30 min at 0°, TFA was evaporated *in vacuo*. Water (100 mL) was added, and the pH was adjusted to 9 by adding KOH. The aqueous solution was extracted twice with 100-mL portions of chloroform, and the organic extracts were dried ($MgSO_4$) and filtered. After evaporating the solvent, the oily residue was crystallized from toluene yielding 1.52 g (54%) of white crystals: m.p. 125–126°; ^{1}H NMR d 8.52 (2H, d, J = 4.2 Hz), 7.35–7.28 (6H, m), 7.12–7.05 (6H, m). Anal. ($C_{18}H_{15}N$) C, H, N.

Animals

Male Sprague–Dawley rats (Harlan Sprague–Dawley) weighing between 140 and 270 g were used in groups of 3–6. All rats had free access to standard chow and water. Animals were maintained in the vivarium at 24° on a 12-hr light–dark cycle.

Treatments

The compounds were either administered by gavage or mixed into the diet. All compounds given by gavage were suspended in a 1% methylcellulose suspension to a final concentration of 10% (w/v), and administered on a weight basis (100 mg/kg) daily for 8 days. The sole exception was CDD 3540, which was administered in a dose of 50 mg/kg/day. Compounds administered in the diet were incorporated at 0.1% (w/w) into Purina rodent chow supplemented with 0.25% cholic acid and 0.75% cholesterol. For CDD 3540, the supplemented amount was 0.05%. Based on the average daily consumption, these doses approximated 100 mg/kg/day for all compounds except CDD 3540, which was ingested in daily doses approximating 50 mg/kg/day. Supplemented diets were consumed for 8 days.

Ethosuximide Clearance

Ethosuximide (Sigma) was prepared as a 3.5% (w/v) solution dissolved in normal saline (0.9% sodium chloride solution), and was administered in a single dose of 35 mg/kg via tail vein injection 24 hr following the eighth compound dose. Comment on this 24-hr interval is warranted here, since Ritter and Franklin had established that a 48-hr interval following the last dose of clotrimazole was required in order to maximize its inductive effect, since residual hepatic clotrimazole can function as an inhibitor of CYP activity [18]. Thus, a 48-hr interval was recommended [18] to preclude the possibility that the net effect of clotrimazole on CYP activity might represent a mixture of both inductive and inhibitory effects. It must be noted, however that Ritter and Franklin used a 3-day treatment period [18], whereas we used an 8-day treatment period. We have established that with a 24-hr interval following an 8-day treatment with clotrimazole, one measures the identical increase in CYP3A activity (ethosuximide clearance) compared with a 3-day clotrimazole period followed by a 48-hr interval. Compared with a mean control ethosuximide clearance of 0.063 L/hr/kg (±0.01), various clotrimazole treatment schedules produced the following ethosuximide clearances: A 3-day treatment followed by a 24-hr interval prior to the administration of ethosuximide elicited an ethosuximide clearance of 0.073 L/hr/kg (±0.003). A 3-day treatment followed by a 48-hr interval elicited an ethosuximide clearance of 0.171 L/hr/kg (±0.046). An 8-day treatment followed by a 24-hr interval elicited an ethosuximide clearance of 0.171 L/hr/kg (±0.029). The latter two values for ethosuximide clearance were significantly different from the control value ($P < 0.01$).

Rats were anesthetized with CO_2 for approximately 20 sec prior to ethosuximide injection. Whole blood was collected by cardiac puncture under CO_2 anesthesia exactly 8 hr after ethosuximide infusion. Animals were killed by CO_2 and cervical dislocation. Plasma was separated from EDTA-anticoagulated whole blood at room temperature using a bench-top centrifuge (3500 *g* × 5 min). Plasma for ethosuximide analysis was stored unpreserved at −20° for up to 5 days. Ethosuximide concentrations in plasma were analyzed by fluorescence polarization immunoassay using a TDx analyzer (Abbott Laboratories) and commercially available reagents. The assay exhibited a coefficient of variation <5% and a detection limit of 0.5 mg/L. Ethosuximide clearance (CL) was estimated from plasma concentration measurements in samples acquired from blood drawn 8 hr after ethosuximide administration according to the following equation:

$$CL = [\ln(D/V) - \ln C_t] \cdot (V/t)$$

where D is the dose of ethosuximide (35 mg/kg), t is the sampling time (8 hr), C_t is the concentration of ethosuximide, and V is the volume of distribution of ethosuximide, which is set at 0.73 L/kg. The strategy for estimating ethosuximide clearance from a single plasma concentration measurement has been described several times [10, 19, 20]. An 8-hr postinfusion sampling time was used instead of the originally reported 24-hr [10] sampling time, since the total clearance of ethosuximide is significantly slower subsequent to urethane anesthesia versus CO_2 anesthesia [21], i.e. urethane slows ethosuximide clearance whereas CO_2 does not. Ethosuximide clearance is an index of CYP3A activity in rats [10].

Enzyme Assays

Excised livers were rinsed in chilled 1.15% KCl, and then prepared as 25% homogenates in 0.1 M of potassium phosphate buffer (pH 7.4). A Brinkmann Polytron® ho-

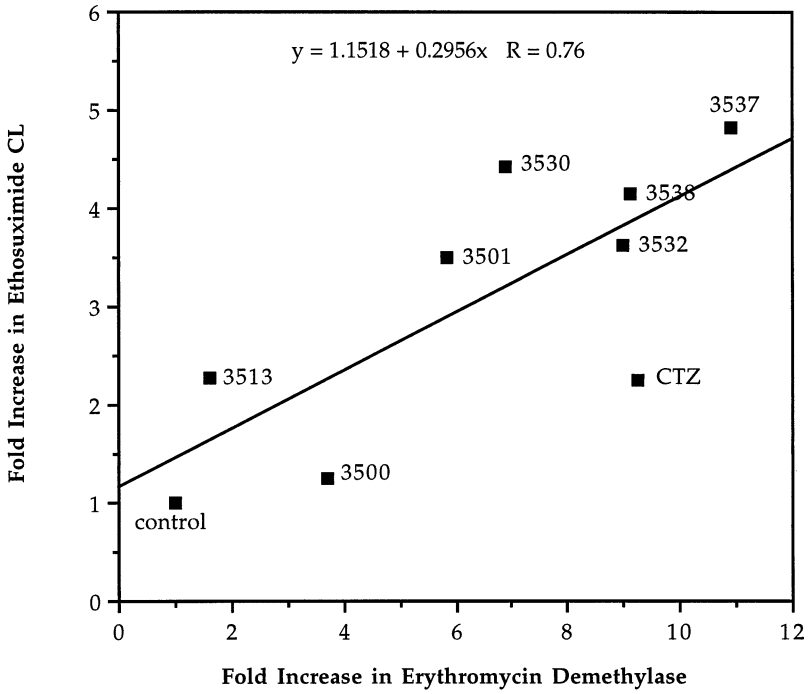

FIG. 1. Correlation between induced increases in hepatic erythromycin *N*-demethylase activity measured *in vitro* and the increase in ethosuximide clearance (CL) measured *in vivo*. Each plotting symbol depicts the effect of a different compound. After an 8-day treatment, the *in vivo* clearance of ethosuximide was measured, the animal was killed, and hepatic erythromycin *N*-demethylase activity was measured.

mogenizer was used in the preparation of homogenates. Homogenates were centrifuged at 10,000 *g* for 20 min at 4° using a Beckman J2-21M/E centrifuge to remove nuclei and mitochondria. The supernatant (3 mL) was centrifuged at 100,000 *g* for 60 min at 4° using a Beckman L-60 preparative ultracentrifuge. Microsomal pellets were resuspended in 8 mL of 0.1 M of erythromycin dissolved in 0.1 M of potassium phosphate buffer or were suspended in the buffer with no erythromycin added. Protein was measured by the method of Lowry *et al.* [22]. Erythromycin demethylase activity was measured by the formation of formaldehyde using the method of Nash [23].

EROD was measured spectrofluorometrically using a Turner model 430 spectrofluorometer. A 300-μL suspension of microsomes (1 mg of protein/mL in 0.1 M of potassium phosphate buffer, pH 7.4) was diluted with 2.985 mL of phosphate buffer and 15 μL of ethoxyresorufin (1 mM) dissolved in DMSO. The mixture was placed in a cuvette and allowed to equilibrate for 60 sec at 37°. The reaction was started by the addition of 15 μL of 50 mM of NADPH. The rate of resorufin formation, which was linear for at least 10 min, was measured at excitation/emission wavelengths of 530 and 585 nm, respectively, 4 min after the start of the reaction.

RNA Materials and Methods

RNA was prepared from approximately 0.25 g of frozen liver essentially by the method of Chomczynski and Sacchi [24]. RNAzol A solution was prepared by mixing equal volumes of solution D (4 M of guanidinium thiocyanate, 25 mM of sodium citrate, pH 7, 0.5% Sarcosyl, 0.1 M of 2-mercaptoethanol, 0.02 M of sodium acetate, pH 4) and water-saturated phenol. After addition of 2 mL of RNAzol A, the tissue was homogenized immediately for *ca.* 30 sec with a Polytron homogenizer, Kinematica AG (PT1200). One hundred microliters of chloroform was added to 1 mL of homogenate, transferred to a new tube, and shaken vigorously for 15 sec. The homogenate was incubated on ice for 15 min, and centrifuged at 15,000 *g* for 15 min at 4°. The aqueous phase containing the RNA was transferred to a new tube, mixed vigorously with 500 μL of phenol: chloroform (5:1), and centrifuged at 15,000 *g* for 5 min at 4°. The RNA was precipitated by mixing the aqueous phase with an equal volume of isopropyl alcohol, and incubating at −70° for at least 15 min. The precipitate was collected by centrifugation at 15,000 *g* for 20 min at 4°, and the supernatant was removed. The resulting RNA pellet was washed by addition of 0.5 mL of 70% ethanol followed by centrifugation at 15,000 *g* for 5 min. The supernatant was removed completely, and the RNA pellet was air dried and resuspended in 200 μL of water treated with 0.1% diethylpyrocarbonate.

DNA oligonucleotides, antisense to the mRNAs of CYP3A [25], and EF-1α [26] were used as probes for Northern blot hybridization. Oligonucleotides were labeled at the 5′ end with [γ-^{33}p]ATP (2000 Ci/mmol, Amersham Life Science) in a reaction catalyzed by T4 polynucleotide kinase [27].

Statistics

Multiple comparison *t*-tests with a Bonferroni adjustment were used to assess the significance of mean differences for treatments versus controls and treatments versus clotrimazole. Differences were considered significant at $P <$

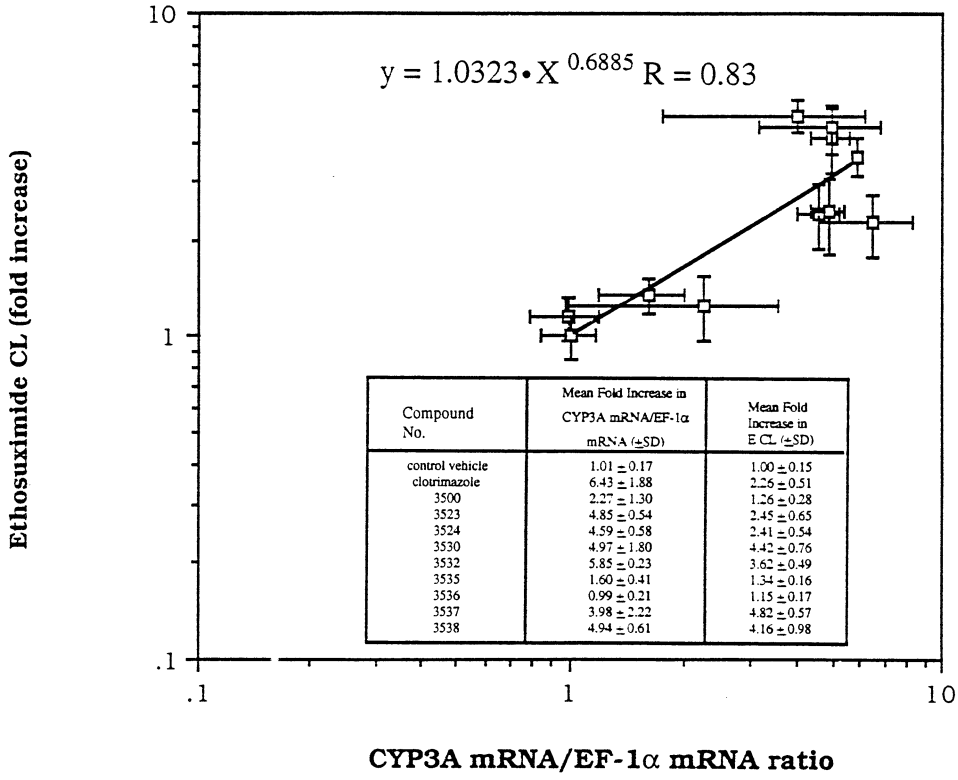

FIG. 2. Induced increase in *in vivo* ethosuximide clearance (CL) as a function of increased hepatic CYP3A mRNA. Each plotting symbol depicts the mean (±SD) effect of a different compound, each of which is shown in the inset table (N = 6). The *in vivo* clearance of ethosuximide was measured after an 8-day treatment with the specified compound. Animals were killed, and hepatic CYP3A mRNA levels were measured. Data are plotted logarithmically for ease of visualization. with details given in the tabular form inset. These data were analyzed statistically in Table 2.

0.05. Sequences and coordinates of probes were as follows: CYP3A (+201 to +157) 5′-CCCAGGAATCCCCTGTT TCTTGAAAAGTCCATGTGTGCGGGTCCC-3′, EF-1α (+420 to +361) 5′-GATACCAGCTTCAAATTCACCA ACACCAGCAGCAACAATCAGGACAGCACAGTC AGCCTG-3′.

Northern blot hybridization assays were performed as follows: RNA samples were prepared for electrophoresis as described [28]. For compound analysis, 10 μg of hepatic RNA was analyzed per animal. RNA samples were fractionated through 1.5% agarose gels containing 1× MOPS buffer [0.62 M of formaldehyde, 20 mM of MOPS (3-[N-morpholino]propanesulfonic acid), 5 mM of sodium acetate, 1 mM of EDTA, pH 7.0]. After electrophoresis, the RNA was transferred to a charged nylon membrane (Amersham Life Science, Hybond-N^+) by capillary action resulting from an ascending flow of 20× SSC buffer (3.0 M of NaCl, 0.3 M of sodium citrate, pH 7.0), and fixed by UV cross-linking at 120 mJ/cm^2 in a SpectroLinker (XL-1000 UV Crosslinker, Spectronics Corp.)

RNA blots were hybridized with 10 million cpm of the radiolabeled probe per 100 cm^2 membrane in a rate-enhanced hybridization buffer (Rapid-hyb buffer, Amersham) at 65° for *ca.* 18 hr. The blots were washed by incubating in 100 mL of 0.5% SSC for 5 min at 65°, followed by three 30-min washes at 60°. Following exposure to the blots, storage phosphor screens were scanned, and bands were quantitated by a Molecular Dynamics PhosphorImager. For reprobing, the CYP3A probe was removed by heating the membrane to 90° in 0.1% SDS and cooling to room temperature. Hybridization with the EF-1α probe was performed as described above. Bands of interest were quantitated using ImageQuant software provided with the Molecular Dynamics PhosphorImager. A numerical value was obtained for each band representing the amount of radioactivity associated with CYP3A and EF1α mRNAs. The amount of hybridization to CYP3A was divided by the value for EF-1α in the same sample to obtain a ratio, normalized for variations in sample preparation. EF-1α was

TABLE 2. Two-by-two contingency table comparing magnitude of treatment-induced increases in ethosuximide clearance and CYP3A mRNA levels

	Number of compounds increasing ethosuximide clearance ≥ ×2	Number of compounds increasing ethosuximide clearance < ×2
Number of compounds increasing CYP3A mRNA ≥ ×3	7	0
Number of compounds increasing CYP3A mRNA < ×3	0	4

$\chi^2 = 11.0$ (df = 1), $P < 0.001$.

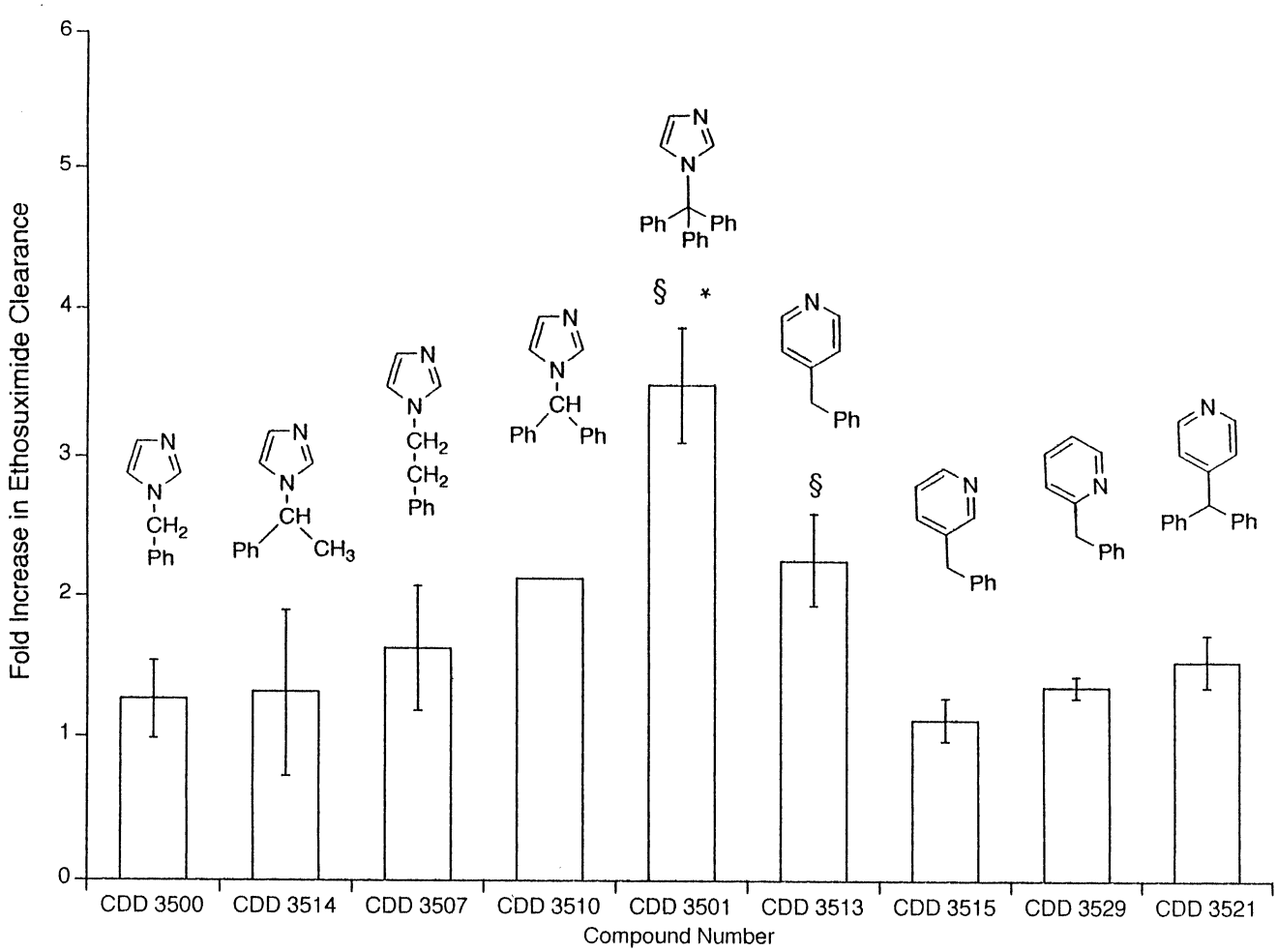

FIG. 3. Basic structural requirements for induction of ethosuximide clearance. Rats were treated with test compounds for 8 days, and ethosuximide was injected via the tail vein 24 hr after the last treatment. Clearance was estimated as described under Materials and Methods. The highest magnitude induction for the compounds shown was observed for triphenylmethylimidazole (CDD 3501). Each value is the mean ± SD of 4–6 rats, except for CDD 3510. Due to chronic toxicity of this compound, only a single variate is depicted. For those compounds that raised ethosuximide clearance above control values, *P* values for multiple *t*-test comparisons were calculated. Appearing at the top of a bar, the symbol § denotes $P < 0.01$ as compared with control. *P* values for multiple *t*-test comparisons were also calculated for the fold-increase in ethosuximide clearance compared to that induced by clotrimazole (CTZ) (see Fig. 5). An asterisk (*) denotes $P < 0.01$ compared with CTZ.

chosen as the internal control, because its expression in adult mammalian tissues is constitutive [29, 30].

RESULTS

Through the use of selective CYP inducers and inhibitors we have shown previously that the *in vivo* rate of ethosuximide clearance serves as a surrogate measure of CYP3A activity in rats [10]. *In vitro* experiments with microsomes enriched in CYP3A1/2 (clotrimazole treatment), CYP2E1 (isoniazid treatment), or CYP1A (β-naphthoflavone treatment) and immunoinhibition experiments have established that ethosuximide is oxidized almost entirely by CYP3A1/2 [31]. The data reported herein further support that contention. When eight different compounds were tested for their ability to induce erythromycin demethylase activity measured *in vitro* in hepatic microsomes and by ethosuximide clearance *in vivo*, a positive correlation between the two parameters was shown (see Fig. 1). In addition, compounds that induced ethosuximide clearance also induced high levels of CYP3A mRNA (Fig. 2). The compounds tested appeared to stratify into two groups such that a three-fold or more increase in the hepatic content of CYP3A mRNA appeared to be required before a significant (i.e. ~two-fold or more) increase in ethosuximide clearance occurred (Table 2). Compounds failing to cause a three-fold or greater increase in CYP3A mRNA failed to cause a significant increase (i.e. ~two-fold or more) in CYP3A activity as measured by ethosuximide clearance. Taken together, these results plus earlier *in vivo* [10] and *in vitro* findings [31] support the contention that ethosuximide clearance is a measure of CYP3A activity in rats.

The effects of a series of heteroaromatic compounds on CYP3A activity are depicted in Figs. 3–6. We used as a

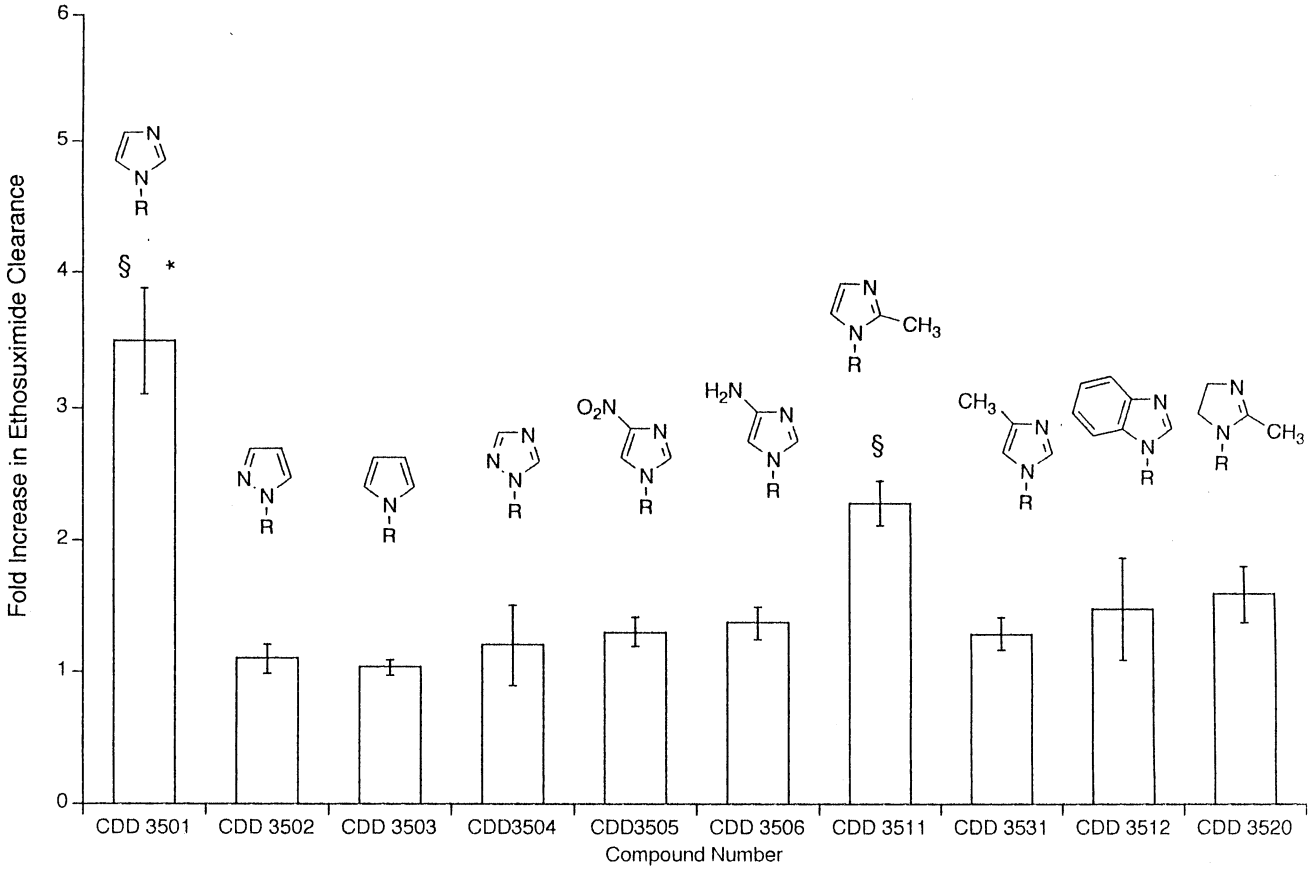

FIG. 4. Structural requirements of the heteroaromatic group on the induction of ethosuximide clearance *in vivo*. Each compound contained the heteroaromatic "head group" shown attached to triphenylmethane (R = $-CPh_3$). Experimental conditions and methods were identical to those depicted in the legend of Fig. 3. The highest magnitude induction for the compounds shown was observed for triphenylmethylimidazole (CDD 3501). For those compounds that raised ethosuximide clearance above control values, *P* values for multiple *t*-test comparisons were calculated. Appearing at the top of a bar, the symbol § denotes $P < 0.01$ as compared with control. *P* values for multiple *t*-test comparisons were also calculated for the fold-increase in ethosuximide clearance compared with that induced by clotrimazole (CTZ) (Fig. 5). An asterisk (*) denotes $P < 0.01$ compared with CTZ. Values are means ± SD, N = 4–6.

starting point the ability of benzylimidazole to function as a high magnitude inducer of cytochrome P450. 1-Benzylimidazole (CDD 3500) was a relatively poor inducer of CYP3A activity as measured by ethosuximide clearance (Fig. 3). Methylation (CDD 3514) or increasing chain length (CDD 3507) did not increase CYP3A activity significantly. Addition of a second phenyl substituent (CDD 3510) led to increased CYP3A activity relative to CDD 3500, but also produced nonspecific hepatotoxicity (Fig. 3). Addition of a third benzene ring (CDD 3501) produced significant additional activity, measured as a 3.5-fold increase in ethosuximide clearance (Fig. 3). 3-Benzylpyridine (CDD 3515) failed to induce CYP3A activity, and 2-benzylpyridine (CDD 3529) was only a weak inducer. However, 4-benzylpyridine (CDD 3513) was significantly active relative to benzylimidazole. Introduction of an additional aryl substituent to 4-benzylpyridine (see CDD 3521) caused a significant loss of activity in contrast to the result of addition of a second aryl substituent to 1-benzylimidazole (CDD 3500; Fig. 3).

We evaluated the effects of the substitution of various heteroaromatic groups on the ability of the compound to induce CYP3A activity. Each compound contained triphenylmethane [R = $-C(Ph)_3$] which, as shown in Fig. 3, was most effective when combined with imidazole (CDD 3501). Although a variety of five-membered heteroaromatic compounds and substituted imidazoles were evaluated, modification of the 1-imidazole invariably led to a significant loss of activity when compared with the induction of CYP3A activity elicited by CDD 3501 (Fig. 4). However, compound 3511 containing the 2-methyl-substituted imidazole still possessed the ability to increase ethosuximide clearance by about two-fold.

The effects of the introduction of a single substituent into one of the phenyl rings of triphenylmethylimidazole (CDD 3501) are depicted in Fig. 5. The substituents considered were: $-OCH_3$, $-CH_3$, $-F$, and $-Cl$. Each halogenated compound exhibited marked activity. All ortho-substituted compounds showed comparable activity; however, the inductive activity was considerably less than the unsubstituted compound CDD 3501 (see Fig. 4). Introduction of methoxyl or methyl substituents into the

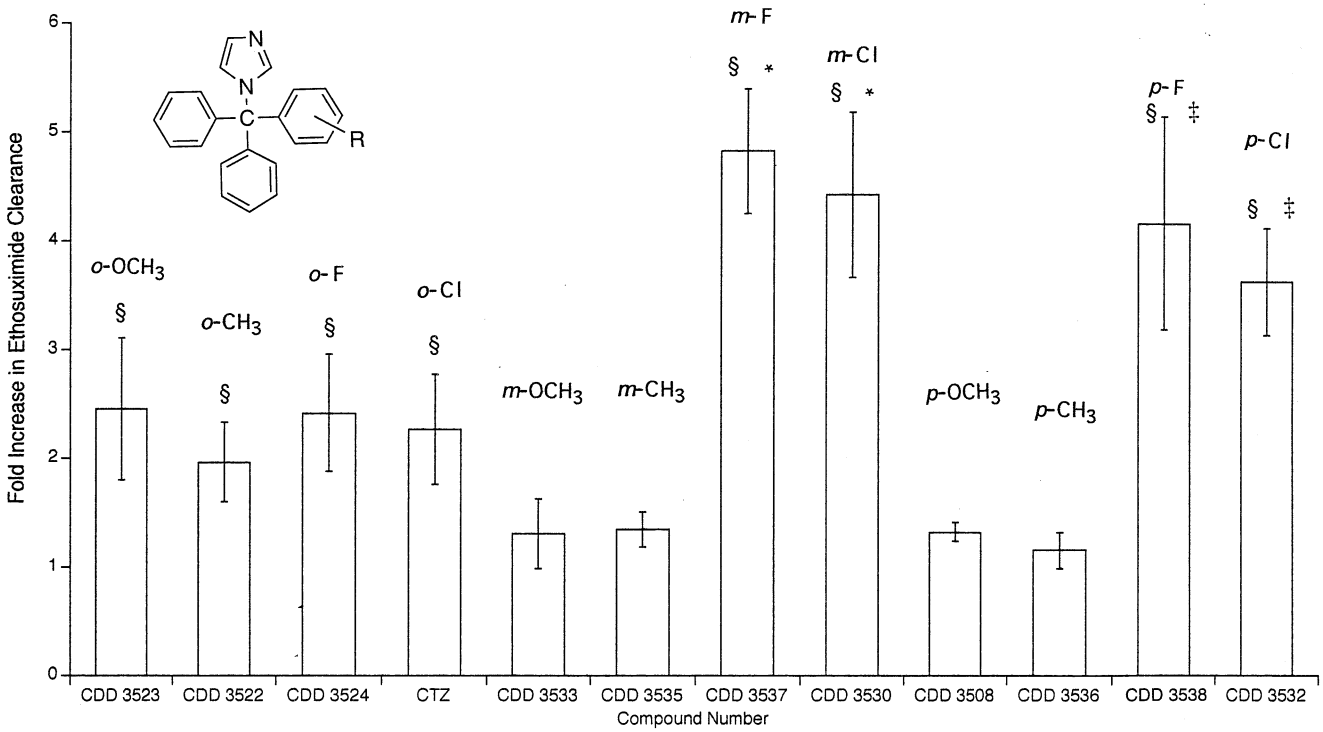

FIG. 5. Effect of the introduction of a substituent into one of the phenyl rings of triphenylmethylimidazole (CDD 3501) on the ability of the compound to increase ethosuximide clearance *in vivo*. Experimental conditions and methods were identical to those depicted in the legend of Fig. 3. The highest magnitude induction was associated with the introduction of halogenated substituents in either the meta or para positions of the phenyl ring. For those compounds that raised ethosuximide clearance above control values, *P* values for multiple *t*-test comparisons were calculated. Appearing at the top of a bar, the symbol § denotes $P < 0.01$ as compared with control. *P* values for multiple *t*-test comparisons were also calculated for the fold increase in ethosuximide clearance compared with that induced by clotrimazole (CTZ). An asterisk (*) denotes $P < 0.01$ compared with CTZ; a double dagger (‡) denotes $P < 0.05$ compared with CTZ. Values are means ± SD, $N = 4–6$.

meta or para positions led to loss of activity, whereas halogenation at meta and para positions produced highly active compounds. One of these compounds (CDD 3537), bearing the meta-fluoro substituent, exhibited apparently higher activity than the unsubstituted CDD 3501 (Fig. 5).

In Fig. 6 we considered the introduction of further modification of the triphenylmethyl substituent of CDD 3501. Introduction of a nitrogen into one of the aryl rings (CDD 3526) resulted in a loss of activity. Introduction of two nitrogens led to total loss of activity (CDD 3527). Although mono-halogenation produced highly active compounds (see Fig. 5), dihalogenation (CDD 3543) and trihalogenation (CDD 3540) led to a partial loss of activity compared with the monohalogenated compounds. Replacement of a phenyl ring with the naphthyl ring (CDD 3539 and CDD 3541) led to marked losses of activity. Directly connecting two of the phenyl rings (CDD 3534) yielded a highly active compound comparable to CDD 3501. Connecting the aryl rings with an ethylene bridge (CDD 3542), however, led to a partial loss of activity.

The issue of selectivity of induction was investigated for eight of the compounds synthesized by measuring changes in the *in vitro* activity of hepatic EROD subsequent to 8-day treatments as described in Materials and Methods. Mean values for EROD activity in vehicle-treated (i.e. control) animals ranged from 0.24 to 0.52 nmol/mg/min. Table 3 shows the mean fold increase (relative to respective control experiment) in EROD activity (i.e. treated value/control value) for each of eight compounds.

Those compounds that elicited the largest increases in CYP3A activity (viz. CDD 3537, 3538, and 3540) produced little or no increase in EROD activity. In contrast, *N*-benzylimidazole (CDD 3500), which produced a relatively small increase in CYP3A activity, elicited almost a nine-fold increase in EROD activity.

DISCUSSION

We synthesized a series of compounds in which we covalently linked either imidazole or other heteroaromatic "head groups" to triphenylmethane or other phenylmethane derivatives, and evaluated each compound in the series for its ability to induce hepatic CYP3A activity in rats. Ethosuximide clearance was used as a measure of CYP3A activity, as it has been shown to be a CYP3A substrate [10], its *in vivo* clearance correlates with the *in vitro* activity of hepatic erythromycin *N*-demethylase, a standard marker enzyme for CYP3A activity, and substances that are capable of substantially increasing hepatic CYP3A mRNA content also increase ethosuximide clearance. More recent *in vitro* findings within our laboratory, including immuno-inhibi-

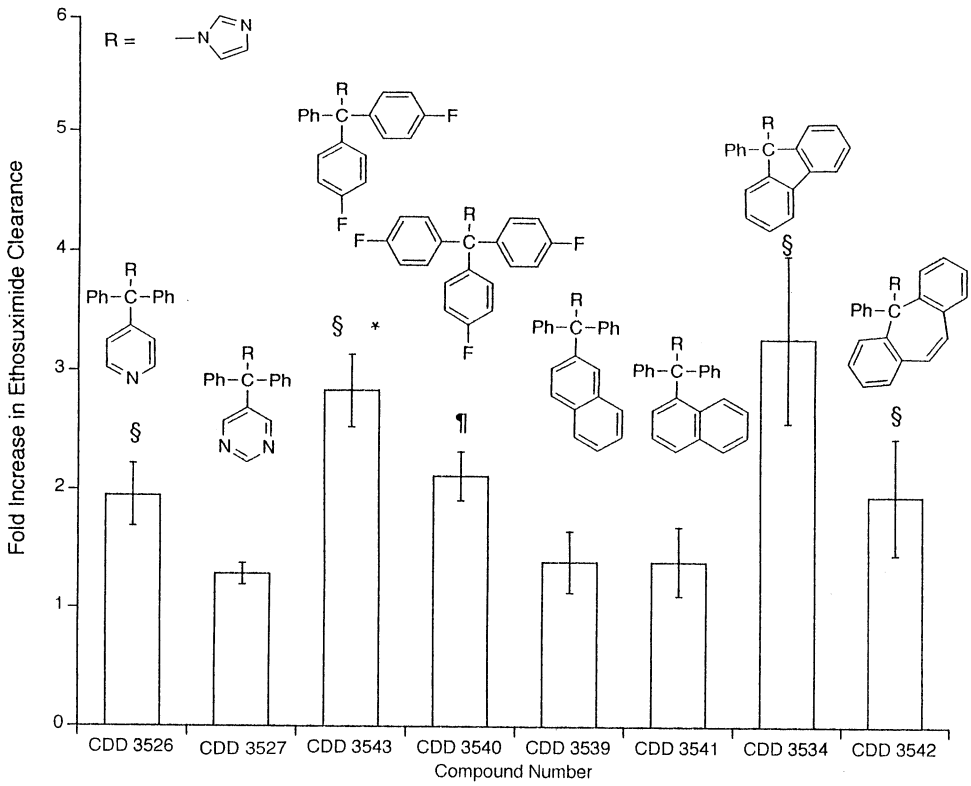

FIG. 6. Effects of modification of the triphenylmethyl group on the ability of compounds to induce ethosuximide clearance *in vivo*. Each compound contained an imidazole "head group" linked via the N1 to the substituted phenylmethane shown on the figure. Experimental conditions and methods were identical to those depicted in the legend of Fig. 3. The highest magnitude induction was observed for CDD 3534, the compound in which two of the phenyl rings are covalently linked. For those compounds that raised ethosuximide clearance above control values, *P* values for multiple *t*-test comparisons were calculated. Appearing at the top of a bar, the symbol § denotes $P < 0.01$ as compared with control; ¶ denotes $P < 0.05$ compared with control. *P* values for multiple *t*-test comparisons were also calculated for the fold-increase in ethosuximide clearance compared with that induced by clotrimazole (CTZ) (Fig. 5). An asterisk (*) denotes $P < 0.01$ compared with CTZ. Values are means ± SD, $N = 4–6$.

tory studies, confirm that only CYP3A plays a significant and predominant role in the oxidation of ethosuximide, and that ethosuximide is hydroxylated primarily on the ethyl side chain [31]. Several other putative probes for CYP3A activity have been used by others, including erythromycin [32, 33], dapsone [33], and midazolam [34, 35]. Unfortunately, there may not be a single ideal probe for CYP3A activity. For example, reliable correspondence in the characterization of CYP3A activity by various probes is less than satisfactory [36–38].

Within this series we found that triphenylmethane-(trityl-) substituted imidazoles elicited the greatest increase in CYP3A activity, and that the highest inductive effects were achieved by the substitution of F- or Cl- in either the meta or para position of one of the phenyl rings. Since we evaluated the inducibility of a specific subfamily of the cytochromes P450, our findings are somewhat at variance with those of Matsuura *et al.* [7] whose SAR analysis was to a large extent based on measures of total hepatic cytochrome P450 content. For example, Matsuura *et al.* reported inductive equivalency between *N*-benzylimidazole, diphenylmethylimidazole, and triphenylmethylimidazole, whereas we found a decreasing order of induction of CYP3A activity as follows: triphenylmethylimidazole was at least three times as effective as *N*-benzylimidazole. Diphenylimidazole was intermediate between the two; however, in our hands it was also extremely toxic, and its inductive ranking is effectively precluded, because only a single animal survived the 8-day treatment. We found that triphenylmethyl-substituted triazole was ineffective in increasing CYP3A activity, whereas Matsuura *et al.* reported that triphenylmethyltriazole with an ortho-substituted Cl- on one of the phenyl rings (i.e. the triazole analogue of clotrimazole) increased the hepatic content of cytochrome P450 to the same extent as clotrimazole. On the other hand, our findings pertaining to triphenylmethyl-substituted pyridine were similar to those of Matsuura *et al.*, who reported that this compound failed to increase hepatic cytochrome P450 content. It must be pointed out that our treatments extended for 8 days rather than for a single dose [7], and that we used Sprague–Dawley rather than Wistar rats [7]. Though there are quantitative differences between

TABLE 3. Fold increase in EROD activity*

Compound	Fold increase in EROD activity†
Clotrimazole	1.79 ± 0.41
CDD 3500	8.94 ± 3.43
CDD 3501	1.48 ± 0.48
CDD 3513	1.72 ± 0.13
CDD 3530	0.82 ± 0.09
CDD 3532	1.34 ± 0.50
CDD 3537	0.86 ± 0.06
CDD 3538	0.73 ± 2.47
CDD 3540	1.24 ± 0.20

*EROD activity was measured as the rate of formation of resorufin (nmol/mg/min).

†Fold increase represents the ratio of activity in compound-treated rats to that in control (vehicle-treated) rats. Mean values for EROD activity in vehicle-treated animals ranged from 0.24 to 0.52 nmol/mg/min. Values are means ± SD, $N = 3$.

our findings and those of Franklin [11], there are some areas of broad agreement. Franklin reported that 1-benzylimidazole produced both substantial increases in EROD and CYP3A activity, whereas we found that 1-benzylimidazole produced a substantial increase only in EROD activity. However, we found the extent of the benzylimidazole-induced increase (or fold increase) in EROD activity was about seven times greater than for the benzylimidazole-induced increase in CYP3A activity. Franklin reported that the effect of 1-benzylimidazole on EROD activity was about 13 times greater than the effect on CYP3A activity. Thus, the relative effects of 1-benzylimidazole on EROD activity compared with CYP3A activity were broadly similar in both studies. The reason for the actual difference in the extent of induced EROD activity is likely a reflection of our higher control EROD values compared with those of Franklin. Differences in the benzylimidazole effect on CYP3A activity may simply reflect the differences in the measures of CYP3A activity. We used *in vivo* ethosuximide clearance, whereas Franklin used *in vitro* erythromycin *N*-demethylase. These substrates may be processed, in part, by different CYP3A isoforms.

Clotrimazole and 1-tritylimidazole (CDD 3501) were the only other imidazoles that both we and Franklin evaluated on both EROD and CYP3A [11]. Our findings were qualitatively the same in that both of those imidazoles produced substantial increases in CYP3A activity and only marginal increases in EROD activity.

The inductive effects of this series of substituted heteroaromatic compounds were evaluated in rats receiving daily treatments by gavage. To that extent we are not in a position to offer comment on the structural requirements for binding to a hepatic receptor. No hepatocyte receptor governing the induction of CYP3A activity has, as yet, been convincingly demonstrated to exist, though there is preliminary evidence that CYP3A induction may be regulated by the interaction of inducers and a microsomal protein [12], though the protein is probably not CYP3A1 [12]. Moreover, the structural features that we describe as being important for maximizing the expression of CYP3A activity in the rat may exert their influence at one or more of several levels including absorption across the gastrointestinal epithelium, the rate of oxidative biotransformation of the compound itself, as well as "receptor" binding. Structural contributions to each of these processes can only be deduced with additional *in vitro* assays.

In conclusion, we have evaluated the SAR characteristics involved in the expression of induced CYP3A activity in rats, and found that maximum induction is elicited by tritylimidazoles with no substitutions on the imidazole "head group" and with either Cl- or F- substituted at either the meta or para position of one of the phenyl rings.

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