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INHIBITION AND METABOLITE COMPLEXATION OF RAT HEPATIC MICROSOMAL CYTOCHROME P450 BY TRICYCLIC ANTIDEPRESSANTS

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Abstract—Administration of imipramine (IMIP) and other tricyclic antidepressants to humans and experimental animals has been associated with inhibition of hepatic cytochrome P450 (P450)-mediated drug oxidation. This study investigated the capacity of several structurally related tricyclic antidepressants to inhibit microsomal P450 activity *in vitro*. It was found that IMIP, desipramine (DES), amitriptyline (AMIT) and nortriptyline (NOR) were poor inhibitors of P450 activity unless they were preincubated with microsomes and NADPH prior to transfer to flasks containing substrate. Thus, subsequent experiments characterized the time-dependent intensification of inhibition produced by the drugs. Preincubation of the *N*-methylaminoalkyl agents DES and NOR (200 μM) with NADPH-supplemented microsomes for 30 min led to an approximate 30% decrease in spectrally apparent P450 content; the *N,N*-dimethylaminoalkyl drugs IMIP and AMIT did not significantly decrease apparent P450 content. Analysis of optical difference spectra of microsomes during NADPH-mediated metabolism of these drugs revealed a prominent increase in absorbance at 454 nm with DES and NOR but not IMIP or AMIT. Monospecific antibodies to the male-specific P450 2C11 and, to a lesser extent, P450 3A2 were effective in preventing the formation of the DES metabolite 454 nm-Soret peak. In addition, the 454 nm absorbance was not produced by the incubation of DES with NADPH-fortified hepatic microsomes from adult female or immature male rats. Studies with the steroid substrate testosterone, which undergoes P450-specific positional hydroxylation, indicated that P450 2C11-mediated 2α- and 16α-hydroxylation were most susceptible to the time-dependent intensification of inhibition produced by DES (8.5 and 7.0 min preincubation required for loss of 50% activity, respectively) and NOR (4.0 and 4.0 min for loss of 50% of both activities). The 6β- (P450 3A2) and 7α-hydroxylase (P450 2A1) pathways were somewhat less susceptible to inhibition than 2α- and 16α-hydroxylation. These findings suggest that DES and NOR form a metabolite intermediate (MI)-complex, characterized by a Soret region absorbance maximum near 454 nm in the optical difference spectrum, with microsomal P450 in male rat liver *in vitro*. Studies with the steroid substrate testosterone as well as immunoinhibition experiments are consistent with the proposition that this MI complex forms principally with the male-specific enzymes P450 2C11 and 3A2. Although a human orthologue of P450 2C11 has not yet been identified, P450s of the 3A subfamily are quantitatively important enzymes in human liver. MI complexation of such enzymes could be a feasible underlying mechanism for certain clinically important drug interactions involving tricyclic antidepressants.

Tricyclic antidepressants, such as imipramine (IMIP†), are substrates and inhibitors of the hepatic microsomal cytochrome P450 (P450) monooxygenase system [1–3]. Patients that receive these drugs are also likely to receive other therapeutic agents in concurrent therapy including benzodiazepine anxiolytics and major tranquilizers; therapy is likely to be continued for long periods. Many of these agents have been associated with adverse effects, e.g. cholestasis produced by phenothiazine tranquilizers [4]. Therefore, interactions that effectively decrease the metabolic clearance and increase serum concentrations of the drugs could well contribute to toxicity. Prediction of toxic consequences, however, would be improved if the P450s inhibited by tricyclic antidepressants were identified. This would facilitate the selection of therapeutic alternatives that undergo less extensive metabolic oxidation by those P450s that are subject to inhibition. Thus, the present study assessed the capacity of IMIP and amitriptyline (AMIT) and their major N-monodemethylated metabolites desipramine (DES) and nortriptyline (NOR) to inhibit quantitatively important P450 enzyme activities in rat liver. As shown in Fig. 1, IMIP and DES are substituted dibenz[*b,f*]azepines and AMIT and NOR are based on the dibenzo[*a,d*]cycloheptenylidene ring system.

It is now accepted that many alkylamine-substituted drugs are able to generate MI complexes with P450s *in vivo* [5–8]. Once inhibited in this fashion, the P450s are non-functional and new enzyme must be synthesized to overcome the defect in metabolic oxidation. Since the four tricyclic antidepressants contain either the *N,N*-dimethylamino- or *N*-methylamino side chain, the present study also evaluated MI complexation in rat liver as a possible contributory mechanism to P450 inhibition.

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† Abbreviations: IMIP, imipramine; DES, desipramine; AMIT, amitriptyline; NOR, nortriptyline; MI complex, metabolite intermediate-complex; P450, cytochrome P450.

MATERIALS AND METHODS

Chemicals. [^{14}C]Testosterone (sp. act. 59 mCi/

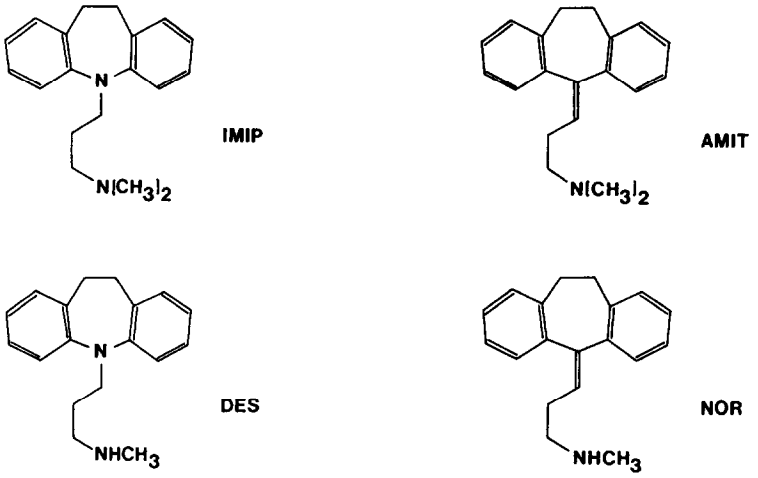

Fig. 1. Structures of the tricyclic antidepressants investigated in this study.

mmol) was purchased from Amersham Australia (North Ryde, NSW, Australia). Androstenedione, testosterone, and 16α-hydroxytestosterone standards were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.), 6β- and 7α-hydroxytestosterones were from Steraloids (Wilton, NH, U.S.A.) and 2α-hydroxytestosterone was from the MRC Steroid Reference Collection (Queen Mary's College, London, U.K.). IMIP, AMIT, DES and NOR were purchased from Sigma and biochemicals were obtained from Sigma or Boehringer-Mannheim (Sydney, Australia). Analytical reagent grade solvents and other chemicals were from Ajax (Sydney, Australia).

Animals and preparation of microsomal fraction. Mature male Wistar rats (10 weeks of age) were generally used, although immature male rats (1 week of age) and mature female rats (10 weeks of age) were also used in some experiments. They were held in wire cages under constant temperature and lighting (12 hr light–dark cycle) and were killed under ether anesthesia. Washed hepatic microsomes were prepared by standard differential ultracentrifugation and the final microsomal pellets obtained were resuspended in 50 mM potassium phosphate, pH 7.4, containing 20% glycerol and 1 mM EDTA, snap frozen in liquid nitrogen and stored at −70° until used in experiments [9].

Enzyme assays. Microsomal protein was estimated by the Lowry procedure using bovine serum albumin as standard [10]. Microsomal P450 content was determined as described by Omura and Sato [11].

Testosterone hydroxylation was estimated as before [12]. Incubations (0.4 mL, 2.5 min, 37°) contained the steroid substrate (0.18 μCi; 50 μM final concentration), microsomal protein (0.15 mg) and an NADPH generating system (1 mM NADP, 4 mM glucose 6-phosphate and 1 U glucose 6-phosphate dehydrogenase); drugs were added to incubations in microlitre volumes of distilled water. Metabolites were extracted with chloroform. In other experiments, the reaction components excluding the steroid, were incubated at 37° for varying periods and then removed to tubes containing the substrate ([^{14}C]testosterone). The reaction was then continued for the usual period of 2.5 min, the products were extracted as described and applied to TLC plates (Merck silica gel 60 F_{254} type, heated for 15 min at 100° before use). The plates were developed sequentially in the solvent systems dichloromethane: acetone, 4:1 and chloroform: ethyl acetate: ethanol, 4:1:0.7.

Preparation of anti-P450 antiserum. Anti-P450 2C11 and anti-P450 3A1 IgG were isolated from the serum of rabbits that received either P450 2C11 [13] or P450 3A1 [14] using an established immunization regimen [13]; preimmune IgG was isolated from non-immunized rabbit serum. The IgG fractions were recycled overnight through columns of cholate-solubilized microsomes from adult female rat liver that had been coupled to *n*-octylamino-Sepharose 4B [15]. Anti-P450 2C11 IgG was male-specific (and did not recognize an antigen in female rat hepatic microsomes), and preferentially inhibited androst-4-ene-3,17-dione 16α-hydroxylation activity. Anti-P450 3A was male-specific and preferentially inhibited steroid 6β-hydroxylation, although 7α-hydroxylation (catalysed by the minor enzyme P450 2A1) was also inhibited. A similar finding has been reported previously by Waxman *et al.* [15].

Detection of MI complexes between P450 and tricyclic antidepressants. MI complexes (200 μM drug) were generated in hepatic microsomes (1 mg/mL) following the addition of NADPH (1 mM). Microsomes were divided between cuvettes in a Cary 2300 spectrophotometer, a baseline of zero absorbance was established and MI complexation (reflected by the absorbance maximum at 454 nm) was monitored continuously. In experiments where the effect of IgG fractions on MI complexation was observed, the IgG was incorporated with the microsomes (at a ratio of 8 mg IgG/mg microsomal protein) for 30 min prior to NADPH addition.

Statistics. Differences between means from two and four treatment groups were detected using the Student's *t*-test and Dunnett's test, respectively.

Table 1. *In vitro* formation of P450–MI complexes from tricyclic antidepressants in rat hepatic microsomes

Drug	P450 content (% of control) (nmol/mg protein)	Initial rate of MI complexation ($\Delta A_{454-490nm}$ absorbance/min/mg protein)
None (control)	0.90 ± 0.05 (100)	—
IMIP (200 μM)	0.97 ± 0.05 (108)	ND†
DES (200 μM)	0.63 ± 0.02* (70)	$8 \pm 1 \times 10^{-3}$
AMIT (200 μM)	0.93 ± 0.04 (103)	ND†
NOR (200 μM)	0.60 ± 0.04* (67)	$9 \pm 3 \times 10^{-3}$

Data are means ± SD of triplicate observations.
* Significant difference from control, $P < 0.025$.
† ND, not determined due to slow rate of initial MI complexation ($<0.2 \times 10^{-3}$ absorbance/min/mg protein).

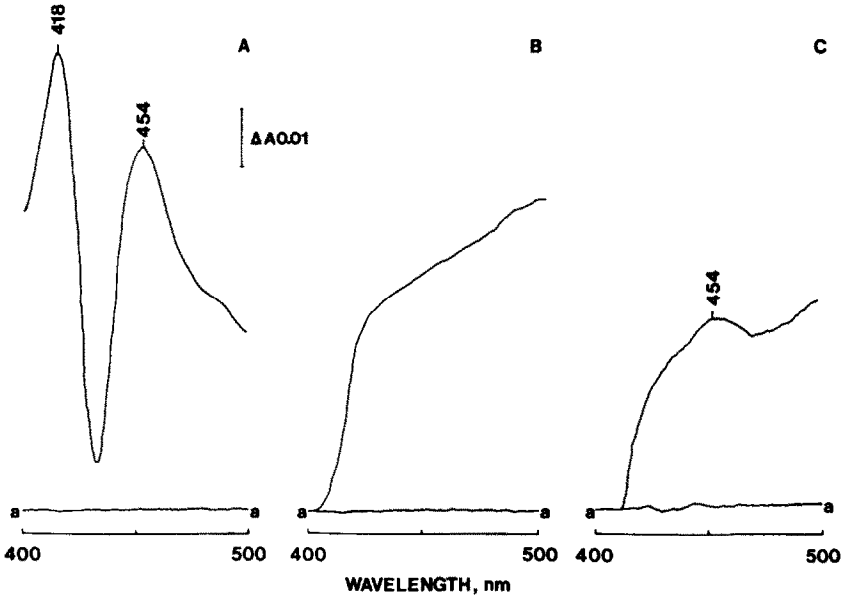

Fig. 2. MI complexation by DES of P450 in hepatic microsomes (1 mg protein/mL) from adult male rats. (A) Complex formation in the presence of preimmune IgG, (B) anti P-450 2C11 and (C) anti-P450 3A, respectively (8 mg IgG/mg protein). Optical difference spectra were obtained at 37° 20 min after NADPH addition (final concentration 1 mM).

RESULTS

MI complexation of rat liver microsomal P450 by tricyclic antidepressants

Incubation of the four tricyclic antidepressant drugs (200 μM for 30 min) with hepatic microsomes from untreated male rats and NADPH produced varying effects on apparent P450 content. The *N,N*-dimethyl analogues IMIP and AMIT did not decrease P450 content measurably but DES and NOR, the *N*-monodesmethyl metabolites of IMIP and AMIT respectively, produced approximate 30% decreases in total P450 (Table 1). Data from the direct measurement of MI complex formation at 454 nm in the optical difference spectrum concurred with these observations (Table 1). Thus, the initial rates of complexation produced by DES and NOR were $8 \pm 1 \times 10^{-3}$ and $9 \pm 3 \times 10^{-3}$ absorbance units/mg protein. In contrast, initial rates produced by IMIP and AMIT were too slow to estimate with precision.

Immunoinhibition of MI complexation by DES was effected with anti-P450 2C11 and anti-P450 3A. As shown in Fig. 2, the magnitude of the 454 nm MI complex peak was decreased approximately 80% by anti-P450 3A and almost completely with anti-P450 2C11.

MI complexation by DES in microsomes from immature male (1 week of age) and mature female (10 weeks of age) rat liver was less extensive than that in microsomes from mature male rat liver. In immature male rat hepatic microsomes the complex peak at 454 nm was absent whereas, in adult female rat liver, the absorbance difference between the wavelength pair 454 and 490 nm was 0.0042

Table 2. *In vitro* inhibition of testosterone hydroxylation pathways in male rat liver microsomes by tricyclic antidepressant drugs

Drug	IC_{50} (μM) versus hydroxytestosterone metabolite formation 2α-	6β-	7α-	16α-
No preincubation				
Imipramine	73	*	†	*
Desipramine	*	*	†	*
Amitriptyline	48	91	†	53
Nortriptyline	*	*	†	*
After preincubation				
Imipramine	7.0 (10)†	*	*	6.6 (>15)
Desipramine	2.9 (>34)	100 (>1)	25 (>4)	1.7 (>59)
Amitriptyline	4.1 (12)	24 (4)	48 (>2)	4.7 (11)
Nortriptyline	1.6 (>63)	9.1 (>11)	40 (>2.5)	1.5 (>67)

IC_{50} values were estimated from semilogarithmic plots of concentration versus per cent uninhibited activity. Values were derived from duplicate incubations.

* Signifies that less than 50% inhibition was observed at a concentration of 100 μM drug.

† IC_{50} values not determined since enhancement of the activity was observed at drug concentrations up to 10 μM.

‡ Numbers in parenthesis indicate the fold increase in inhibition potency produced by a 20 min preincubation of drug with microsomes before transfer to flasks containing substrate (testosterone).

absorbance units/mg protein (compared with 0.010 absorbance units/mg protein in adult male liver; data not shown).

Inhibition of microsomal testosterone hydroxylation by tricyclic antidepressants

Initial experiments assessed the capacities of the four drugs to inhibit the microsomal P450-dependent positional hydroxylation of testosterone. IC_{50} values were determined for each of the four drugs against the four major pathways of testosterone hydroxylation (at the 2α, 6β, 7α and 16α positions of the steroid). From Table 2 it is apparent that, without preincubation, P450 2C11-dependent testosterone 2α- and 16α-hydroxylation was susceptible to inhibition by AMIT (IC_{50} 48 and 53 μM). The only other inhibitory interactions were IMIP against testosterone 2α-hydroxylation and AMIT against 6β-hydroxylase activity. Importantly, in the absence of preincubation, NOR and DES were poor inhibitors of all pathways.

Preincubation of the drugs for 20 min with NADPH-supplemented microsomes from untreated rat liver markedly enhanced the observed extent of inhibition (Table 2). Particularly noteworthy was the enhanced inhibition by DES and NOR of the reactions mediated by P450 2C11. Thus, after the preincubation step, DES exhibited IC_{50} values of 2.9 and 1.7 μM against testosterone 2α- and 16α-hydroxylation and NOR exhibited IC_{50} values of 1.6 and 1.5 μM (Table 2). AMIT and IMIP were also more potent as inhibitors of these pathways after the preincubation step. The 6β- and 7α-hydroxylations of testosterone were also somewhat more susceptible to inhibition by metabolites rather than the parent drugs.

The preincubation studies were supplemented with additional experiments that assessed the time-dependent intensification of the inhibition of steroid hydroxylation (typified by the experiments shown in Fig. 3). Pseudo-first order analysis of the inhibition data gave rise to the results summarized in Table 3. This analysis emphasized the inhibitory potency of DES and NOR. Thus, the preincubation time required for a two-fold enhancement of inhibition of P450 2C11 activity was only 4.0 min for NOR (against 2α- and 16α-hydroxylation) and 8.5 min (against 2α-hydroxylation) or 7.0 min (against 16α-hydroxylation) for DES. AMIT and IMIP were, in contrast, relatively ineffective and at least 20 min was required for 50% enhancement of inhibition.

DISCUSSION

The body of evidence is increasing that many alkylamine-substituted drugs undergo hepatic microsomal P450-mediated metabolism to reactive species that form a tight complex with the enzyme. Although this phenomenon has been recognized for some time in the case of alkylamine derivatives that have no therapeutic utility (e.g. SKF 525-A [5]), more recent studies have indicated that drugs such as triacetyloleandomycin [6], erythromycin [16] and orphenadrine [7] also generate inhibitory MI complexes with P450s. It has emerged that MI complexation due to orphenadrine exhibits a previously unexpected preference for specific P450s [8, 17]. That is, pharmacokinetic interactions elicited by alkylamine drugs are due in part to MI complexation of relatively few P450s. Thus, triacetyloleandomycin-mediated MI complexation and induction of P450 is reportedly specific for members of the 3A subfamily in both human and rodent liver [14, 18]. Pharmacokinetic interactions have been documented between macrolide antibiotics and agents such as warfarin [19], carbamazepine [20] and alfentanil [21]. Although care should be taken in the interpretation of the significance of MI complexation to the observed pharmacokinetic interaction, its potential importance should be

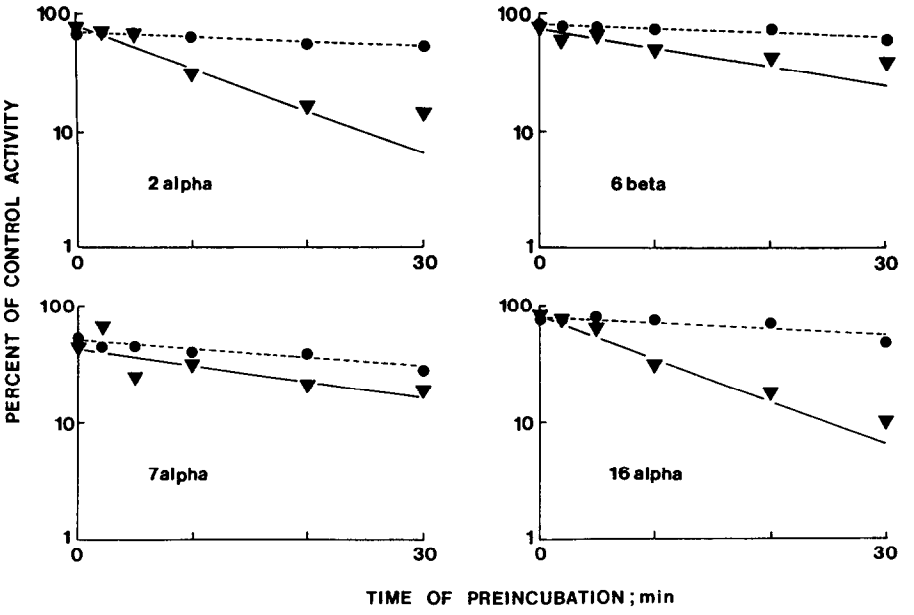

Fig. 3. Time-dependent intensification of the inhibition of testosterone hydroxylation pathways after preincubation of DES (▼) or IMIP (●) with NADPH-supplemented microsomal fractions. Drug concentrations were 50 μM.

Table 3. Pseudo-first order kinetic analysis of the time-dependent intensification of inhibition of microsomal steroid hydroxylation by tricyclic antidepressants

Drug	Hydroxytestosterone metabolite			
	2α-	6β-	7α-	16α-
	Time for loss of 50% activity (min)			
IMIP (50 μM)	>20	>20	>20	>20
DES (50 μM)	8.5	17	19	7.0
AMIT (50 μM)	18	>20	>20	14
NOR (50 μM)	4.0	10	10	4.0

Values were determined from semilogarithmic plots of preincubation time versus log (per cent activity remaining). Plots were constructed using data derived from preincubation times of 0, 2, 5, 10, 20 and 30 min. All incubations were performed in duplicate and the data have been corrected for changes in activity observed in the absence of added drug (<15% in all cases).

considered since complexation gives rise to long-lived inhibition of drug oxidation.

Several lines of evidence suggest that the principal male-specific P450s 2C11 and 3A2 are implicated in MI complexation by the tricyclic antidepressant DES. First, in preincubation studies to generate the MI complex it was found that testosterone 2α- and 16α-hydroxylation activities, which are catalysed primarily by P450 2C11 [15], were more susceptible to inhibition than other pathways (Tables 2 and 3). P450 3A2-mediated testosterone 6β-hydroxylation [15] was also susceptible to inhibition by DES and NOR. These studies served to underline that metabolism of the drug to a reactive species is a prerequisite for potent inhibition. Second, antisera to the P450s 2C11 and 3A2 effectively decreased the extent of MI complexation produced in normal incubations with DES and, third, in microsomes from immature male and adult female rat liver, MI complexation was much less pronounced than in adult male rat liver. This experiment was conducted since it has been demonstrated that 2C11 and 3A2 are not expressed in either adult female or immature male liver [15, 22, 23].

It should be noted that, since steroid 7α-hydroxylation also exhibited time-dependent intensification of inhibition by DES and NOR, that the minor P450 form 2A1 may undergo MI complexation by these drugs. However, immunoinhibition of MI complexation with an antibody to this enzyme would be required to assess this possibility.

In the present study, it was noted that MI complexation appeared to be most efficient with the *N*-methylamino-substituted tricyclic antidepressants DES and NOR. The *N,N*-dimethylamino analogues IMIP and AMIT were considerably less effective. Previous work with alkylamines such as orphenadrine [7] and amphetamines [24] has suggested this possibility so that it appears that N-dealkylation of dialkylamino compounds is an obligatory step in the ultimate generation of alkylamine MI complexes. Indeed, it has been suggested that the nitrosoalkyl analogue is the species involved in the complexation process and that this derivative is formed by sequential steps including oxidative N-dealkylation, *N*-hydroxylamine formation and oxidation [7, 25]. In preliminary experiments we measured formaldehyde production from both IMIP and AMIT and found that it was quite low (1.50 nmol/min/mg protein; data not shown). Thus, formation of DES and NOR from IMIP and AMIT, respectively, would be no faster than this rate. Formaldehyde was produced from DES and NOR at an even slower rate (0.27 and 0.31 nmol/min/mg protein; not shown). It is therefore possible that the small extent of P450 complexation produced by IMIP and AMIT could be due to the relatively slow rate of N-demethylation to DES and NOR, since these agents appear more proximate to the eventual MI complex forming species (presumably the nitroso metabolite).

However, it would appear that the increases in inhibition potency observed after the preincubation step are not completely attributable to complex-generating metabolites. IMIP and AMIT did not generate significant quantities of MI complexes in hepatic microsomes but, from preincubation studies, the potency of both drugs against reactions catalysed by P450 2C11 was increased at least 10-fold. Other reports have established that IMIP is converted to a number of metabolites in P450 reactions [1, 26]. Thus, it is possible that some of these metabolites are significantly inhibitory toward P450 reactions but do not form MI complexes.

IMIP and DES are dibenzazepine derivatives whereas AMIT and NOR are substituted dibenzocycloheptenylidenes. As depicted in Fig. 1, the essential structural difference between the two pairs of drugs is restricted to the atoms involved in the attachment of the alkylaminoalkyl side chain to the tricyclic nucleus. However, this distinction would not be expected to give rise to a markedly different spatial configuration about the junction of the side chain and the ring system. It may have been expected that the side chain in IMIP and AMIT would have been more flexible but this is unlikely to be due to restricted rotation about the σ-bonds of the side chain atom adjacent to the tricyclic ring system. Similar steric hindrance would be observed in the side chain of the dibenzocycloheptadienylidenes AMIT and NOR. Certainly, the structural dissimilarities between DES and NOR did not give rise to significant differences in the propensity of the drugs to inhibit particular P450s; inhibition and MI complexation characteristics were similar for both compounds.

The present study adds to the growing number of drugs that generate reactive metabolites that interact with P450 enzymes. MI complexation can be considered as a form of mechanism-based inhibition so that observed selectivity may well be attributed to the involvement of specific P450s in catalysis of the final oxidation reaction [27]. To our knowledge, agents that preferentially inactivate or MI complex with P450 2C11 have not yet been described. Thus, DES or NOR may constitute useful probes, that could be used in conjunction with other chemicals, to characterize the involvement of P450s in toxicological or enzymic analyses.

A protein orthologous to P450 2C11 has not been identified in human liver but P450s 3A appear to be major catalysts with a range of drug substrates in microsomes of several species; at least four closely related 3A proteins have been described in human liver [28]. In addition, the polymorphic debrisoquine 4-hydroxylase, P450 2D6, is involved in the hydroxylation of the tricyclic antidepressants in this report [26, 29]. It remains to be investigated whether agents such as DES exhibit enzyme-specific MI complexation of these or other human P450s. However, it will also be important to assess the capacity of agents such as DES and NOR to elicit MI complexation of P450s *in vivo* in order to establish the significance of the present findings.

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