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Metabolite Intermediate Complexation of Microsomal Cytochrome P450 2C11 in Male Rat Liver by Nortriptyline

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SUMMARY

Antidepressant drugs that contain alkylaminoalkyl substituents have been associated with serious pharmacokinetic interactions in humans that may be related to the inhibition of cytochrome P450 (P450) enzymes. In this study, the propensity of the tricyclic antidepressant nortriptyline (NOR) to inhibit individual microsomal P450 enzymes in rat liver was investigated to provide a mechanistic explanation for these pharmacokinetic interactions. Enzyme kinetic studies revealed that NOR inhibited steroid 2α-, 6β, 7α-, and 16α-hydroxylation in untreated rat liver with K_m/K_i ratios of 0.53, 0.59, 0.25, and 0.29, respectively. When the drug was preincubated with microsomes and NADPH before testosterone hydroxylation was conducted, marked increases in the K_m/K_i ratios were observed (to 8.8, 3.9, 0.62, and 13, respectively). Thus, enzymic oxidation of NOR enhanced its inhibition capacity against P450 activities. Indeed, the altered K_m/K_i ratios indicate 17-, 6.6-, 2.5-, and 47-fold increases in inhibition of the four pathways of testosterone hydroxylation after the biotransformation of NOR to its metabolites. From these experiments it was apparent that testosterone 2α- and 16α-hydroxylations, catalyzed predominantly by P450 2C11, were subject to the most pronounced increase in inhibition. Under these conditions, the apparent content of microsomal P450 was decreased, thus suggesting the formation of a NOR metabolite intermediate (MI) complex with the cytochrome. Further, optical difference spectroscopy of NADPH-supported metabolism of NOR in microsomes and in a reconstituted system incorporating purified P450 2C11 indicated the appearance of an absorbance peak near 454 nm, similar to those produced by triacetyloleandomycin, SKF 525-A, and orphenadrine. Formation of this absorbance peak in microsomes was inhibited by an antibody raised against the male-specific P450 2C11. Because oxidative metabolism of NOR to inhibitory products would not necessarily involve MI complexation, additional experiments were undertaken in which NOR-related free metabolites produced in microsomal incubations were removed on Sep-Pak mini-C_{18} columns before estimation of testosterone hydroxylation. The principal finding from this experiment was that P450 3A2-dependent steroid 6β-hydroxylase activity was inhibited to a much lesser extent after removal of unbound NOR metabolites on Sep-Pak columns (25% inhibition after Sep-Pak extraction, compared with 82% inhibition observed when all NOR metabolites were present during subsequent testosterone hydroxylation); inhibition of P450 2C11-mediated 2α- and 16α-hydroxylation was not noticeably different after Sep-Pak treatment. Thus, from the present experiments, it seems clear that the principal male-specific P450 enzyme, 2C11, is primarily involved in the formation of an MI complex with the tricyclic antidepressant NOR. Indeed, most of the inhibition of this enzyme by oxidized products of NOR appears to be mediated by MI complexation. In contrast, P450 3A2 appears to be only partially complexed by a NOR metabolite, so that the major inhibitory effect of NOR on this enzyme is mediated by a reversible process. Preincubation of NOR with NADPH-supplemented human microsomal fractions produced somewhat variable effects on apparent P450 content (ranging from no change to decreases on the order of 8–12%). Thus, it is conceivable that MI complexation of P450s in human liver could provide at least part of the mechanism for observed adverse pharmacokinetic interactions in humans receiving NOR or related drugs.

P450s participate in the mixed-function oxidation of endogenous and exogenous substances such as steroids, prostaglandins, pesticides, carcinogens, and drugs (1–4). Generally, the action of P450s is to enhance the hydrophilicity of organic molecules, thus facilitating their elimination. Numerous P450s exist in liver and have been distinguished on the basis of primary structure (amino acid and/or nucleotide sequence) (5), substrate specificity, and differential susceptibility to inhibitors (6, 7).

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The existence of multiple P450s provides a straightforward explanation for the previously described broad substrate specificity of the microsomal oxidase system. However, over the last decade it has been determined that steroids such as testosterone undergo positional hydroxylations that are catalyzed to a large extent by individual P450s (8, 9). Thus, the principal enzymes involved in testosterone 2α-, 6β-, 7α-, and 16α-hydroxylation in male rat liver are the P450s 2C11, 3A2, 2A1, and 2C11, respectively (8). To some degree quantitatively minor P450s may participate in these and other positional hydroxylations (9, 10).

A large number of drugs and other chemical compounds have

ABBREVIATIONS: P450, cytochrome P450; AMIT, amitriptyline; MI, metabolite intermediate; NOR, nortriptyline.

been identified as inhibitors of P450s, but the propensity of such agents to elicit P450 enzyme-specific or enzyme-preferred inhibition has been investigated on relatively few occasions. Clearly, the identification of therapeutic agents that are associated with potent inhibition of one or a few P450s is of great significance. A pharmacokinetic interaction is likely when one drug that is a substrate for a particular P450 is coadministered with another drug that is a potent inhibitor of the same enzyme. Thus, detailed information on inhibition processes involving therapeutic agents is as valuable as the analogous information on P450 substrate specificity.

Several years ago it was recognized that certain agents, including piperonyl butoxide, the methylenedioxyphenyl insecticide synergist, and structurally similar compounds such as safrole and isosafrole, underwent P450-mediated oxidation to metabolites that sequestered the cytochrome in an inactive state (11, 12). Incubation of microsomes with these agents and NADPH led to a decrease in apparent P450 content and some oxidase activities; inhibition was nonuniform (11, 13). It was also found that the classical P450 inhibitor SKF 525-A generated similar MI complexes (14). Because the alkylamine moiety found in SKF 525-A is also present in a large number of therapeutic substances, the formation of analogous MI complexes could give rise to long-lived inhibition of mixed-function oxidase activity. Importantly, because the complexation process involves a catalytic event, it appears that inhibition by this mechanism is somewhat more specific for certain P450s in liver.

Tricyclic antidepressants such as AMIT and NOR (Fig. 1) contain alkylaminoalkyl side chains and have been associated with inhibition of drug elimination (15, 16). The present study investigated the specificity of inhibition of P450 enzymes in male rat hepatic microsomes in relation to MI complexation. Kinetic analysis of the inhibition of testosterone hydroxylation was combined with immunoinhibition experiments and studies in reconstituted systems to establish the involvement of the male-specific P450 2C11 in MI complex formation with NOR.

Materials and Methods

Chemicals. NOR HCl, AMIT HCl, DEAE-Sephacel, and biochemicals were purchased from Sigma Chemical Co (St. Louis, MO). [^{14}C] Testosterone (specific activity, 59 mCi/mmol) was obtained from Amersham Australia, NSW. Hydroxytestosterone standards were purchased from Sigma, Steraloids (Wilton, NH), or the MRC Steroid Reference Collection (Queen Mary's College, London, England). Reagents for electrophoresis, DEAE-Affigel Blue, and Bio-Gel HT were from Bio-Rad Laboratories (Richmond, CA). CNBr-activated Sepharose 4B was obtained from Pharmacia (Sydney, Australia). Analytical grade solvents and miscellaneous chemicals were purchased from Ajax Chemicals (Sydney, Australia).

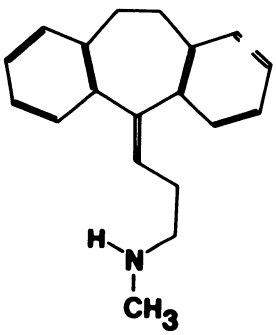

Fig. 1. Structure of the tricyclic antidepressant NOR.

Preparation of hepatic microsomal fractions. Male Wistar rats (approximately 250 g) were used in these experiments and were held in cages under constant temperature and lighting (12-hr light-dark cycle). Animals were killed under ether anesthesia and washed hepatic microsomes were isolated by differential ultracentrifugation (13); microsomal fractions were resuspended in 50 mM potassium phosphate, pH 7.4, that contained 20% glycerol and 1 mM EDTA, placed in liquid nitrogen, and held at −70° until required for experiments.

Human liver samples were obtained as unwanted tissue from donor or recipient livers during liver transplantation or surgical resection. All samples were obtained directly from the operating theater and were frozen immediately. Microsomal fractions from individual liver samples were prepared and stored as described above for rat hepatic microsomes.

Purification of microsomal enzymes. P450 2C11 was isolated from sodium cholate-solubilized male rat hepatic microsomes as described previously (17). Briefly, the solubilized microsomes were chromatographed sequentially on *n*-octylamino-Sepharose 4B, DEAE-Sephacel at pH 7.25, Bio-Gel HT at pH 7.25, and CM-Trisacryl-M at pH 6.5. Detergent was removed on hydroxylapatite. The final preparation was apparently homogeneous by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and contained 14 nmol of P450 2C11/mg of protein. NADPH-P450-reductase was purified to electrophoretic homogeneity from male rat hepatic microsomes essentially as described by Yasukochi and Masters (18). Detergent was removed on Extracti-gel D (Pierce Chemical Co., Rockford, IL). The preparation reduced 49 μmol of cytochrome *c*/min/mg of protein.

Preparation of anti-P450 antiserum. Anti-P450 2C11 IgG was isolated from serum of rabbits that had been immunized with purified P450 2C11 as outlined previously (17); preimmune IgG was isolated from nonimmunized rabbit serum. The IgG fractions from the DEAE-Affigel Blue column were recycled overnight through columns of cholate-solubilized microsomes, from adult female rat liver, that had been coupled to CNBr-activated Sepharose 4B (19). Immunoblotting indicated that the anti-P450 2C11 IgG was male specific; the preparation recognized a single polypeptide in male rat hepatic microsomes and gave no reaction in female rat liver (17). The antibody also preferentially inhibited androst-4-ene-3,17-dione 16α-hydroxylation activity; 87% inhibition was produced by a ratio of 5 mg of IgG/mg of microsomal protein. In contrast, 18% and 23% inhibition of steroid 6β- and 16β-hydroxylation, respectively, was noted and there was slight stimulation of steroid 7α-hydroxylation.

Testosterone hydroxylation and its inhibition by NOR. Testosterone hydroxylation reactions were conducted at 37° for 2.5 min in the presence of 0.15 mg of microsomal protein and were initiated by the addition of 1 mM NADPH. The usual concentration of [^{14}C] testosterone was 50 μM (0.18 μCi/0.4-ml incubation). The reactions were terminated by removal to ice and addition of 5 ml of chloroform. After extraction, centrifugation, and separation, the organic phase was removed and evaporated under N_2. The residue was applied in a small quantity of chloroform to thin layer chromatography plates (Merck silica gel 60 F_{254} type, dried at 100° before use), which were developed in the solvent system dichloromethane/acetone (4:1), air dried, and then developed in the system chloroform/ethyl acetate/ethanol (4:1:0.7) (20). Radioactive metabolites were identified by autoradiography (Hyperfilm-MP; Amersham Australia, Sydney, Australia) for approximately 60 hr. Quantification of metabolite formation was achieved by scintillation counting (ACS II; Amersham).

The kinetics of inhibition of testosterone hydroxylation by NOR were investigated in several experiments. Direct inhibition was assessed under the incubation conditions described above, using varying concentrations of substrate (5–100 μM and NOR (0–100 μM). In other experiments, NOR (0–25 μM) was preincubated with microsomes and NADPH before transfer of the reaction components to substrate (5–100 μM); an additional aliquot of NADPH (0.2 mM) was added. Thus,

reactive NOR metabolites were generated before addition of the substrate testosterone. Both sets of experiments were performed in triplicate.

Data were analyzed according to the graphical methods of Hanes-Woolf (S/V versus S) [for determination of the K_m (Michaelis constant) and V_{max} (maximal reaction velocity)], Lineweaver-Burk ($1/V$ versus $1/S$), and Dixon ($1/V$ versus I). Appropriate replots (slopes of the line in the Dixon plots versus I) were used to characterize the type of inhibition and to determine K_i (the equilibrium dissociation constant for the enzyme-inhibitor complex) values. It has been shown that, in the Dixon replot described, the slope of the line of best fit is equal to $K_m \cdot (K_i\ V_{max})^{-1}$ (21). In competitive inhibition the line of best fit passes through the origin but in other types of inhibition the y-intercept is $(\alpha K_i V_{max})^{-1}$, where α is the factor by which the inhibitor alters the affinity of the enzyme for its substrate; in simple inhibition $\alpha = 1$. Throughout these studies, the K_m and K_i values have been determined from each separate kinetic experiment and the K_m/K_i ratio has been used as an index of relative inhibition potency.

Finally, varying concentrations of NOR (1, 5, or 10 μM) were preincubated with microsomes and NADPH for either 0, 5, 10, 20, or 30 min before transfer to vials containing testosterone. Thus, time-dependent changes of testosterone hydroxylation were determined.

Significance of MI complex formation in inhibition of P450 enzymes by NOR. In these experiments, the possibility that NOR may elicit some of its inhibitory effects by reversible inhibition (apart from MI complex formation) was investigated. Thus, NOR (25 μM) was incubated with microsomes and NADPH as described and then passed through a Sep-Pak mini-C_{18} cartridge before transfer to vials containing testosterone. Steroid hydroxylation was continued for 2.5 min in the presence of additional NADPH (0.2 mM).

In another approach, NOR (25 μM) was preincubated with microsomes and NADPH for 20 min, and then potassium ferricyanide (25 μM) was added to oxidize the sample and dissociate any MI complex formed in the microsomes. The mixture was passed through Sep-Pak cartridges and transferred to vials containing testosterone as described. The effectiveness of Sep-Pak for removal of NOR was established by passing a mixture of NOR (25 μM) and microsomes through Sep-Pak cartridges before NADPH addition.

MI complex formation in a reconstituted system incorporating purified P450 2C11 and in microsomes. NADPH-P450 reductase (0.2 nmol) and P450 2C11 (0.1 nmol) were added to sonicated dilauroyl phosphatidylcholine (40 μg) in 0.1 M potassium phosphate buffer, pH 7.4, that contained 20% glycerol and 1 mM EDTA (final volume, 0.45 ml). The mixture was divided between two cuvettes (0.2 ml, 5-mm path length) and a base line of equal light absorbance was established in a Cary 2300 spectrophotometer at 37°. NOR (35 or 200 μM) or AMIT (35 or 200 μM) was added to the sample cuvette and NADPH (1 mM) was added to both cuvettes to initiate the reaction. The optical difference spectrum was scanned repetitively between the wavelength pair of 380 and 500 nm and the change in the absorbance difference between 454 and 490 nm was plotted as a function of time.

A similar procedure was followed in immunoinhibition experiments in microsomes. Varying amounts of anti-P450 2C11 or preimmune IgG (in the ratio 1–10 mg of IgG/mg of protein) were added to hepatic microsomes from untreated male rats (1 mg/ml). After 20 min, the mixture was divided between two cuvettes and MI complex formation due to NOR (100 μM) was monitored as described above.

The relationship between NOR concentration and the initial rate and extent of MI complex formation was also determined in microsomal fractions from untreated male rat liver. In these experiments, the NOR concentration was varied over the range of 0.2 to 100 μM and MI complexation was initiated and monitored as described above.

Similar experiments were conducted in microsomal fractions from human liver (1 mg of protein/ml). P450 content was estimated in eight individual microsomal fractions that had been incubated for 20 min with NADPH (1 mM) in the presence or absence of NOR (250 μM). In two of these samples time-dependent changes in the optical difference spectrum of the microsomal fractions were monitored for the formation of P450-NOR MI complexes.

Other enzyme assays. Microsomal protein was estimated by the method of Lowry *et al.* (22), with bovine serum albumin as standard. P450 content in hepatic microsomes was determined as described by Omura and Sato (23).

Statistics. Differences between means from two or more than two treatment groups were detected using the Student's t test or one-way analysis of variance in conjunction with the Student-Newman-Keuls test, respectively.

Results

Kinetics of inhibition of microsomal testosterone hydroxylation by NOR. Consistent with previous reports (20), the four principal hydroxylations of testosterone occurred at the 2α-, 6β-, 7α- and 16α-positions. Michaelis constants were determined from Hanes-Woolf plots and were found to be 11 ± 3, 26 ± 4, 2.0 ± 0.3 and 17 ± 3 μM (mean ± standard error), respectively, whereas maximal reaction velocities were 2.05 ± 0.47, 2.74 ± 0.59, 0.36 ± 0.07, and 2.97 ± 0.64 nmol/min/mg of protein, respectively. Inclusion of NOR in incubations led to slight inhibition of the positional hydroxylation of testosterone, which was more significant at low substrate concentrations. Inhibition appeared to be competitive in nature, and the Dixon replot analysis led to K_i estimates of 25 ± 11, 45 ± 4, 5.3 ± 1.3, and 58 ± 9 μM for NOR as an inhibitor of testosterone 2α-, 6β-,7α-, and 16α-hydroxylation (kinetics of inhibition of 2α-hydroxylation shown in Fig. 2), respectively in microsomes. The analysis, therefore, yielded values of 0.53 ± 0.09, 0.59 ± 0.07, 0.25 ± 0.01, and 0.29 ± 0.01 for the K_m/K_i ratios against the four pathways of testosterone hydroxylation.

Data obtained from studies in which NOR was preincubated with NADPH and microsomes before the measurement of testosterone hydroxylation revealed significant increases in inhibition potency against all four hydroxylation pathways (as reflected by the respective K_m/K_i ratios of 8.8 ± 1.4, 3.9 ± 1.0, 0.62 ± 0.05, and 13 ± 2) (Fig. 3). Thus, after the 20-min preincubation step, the inhibition potency of NOR against testosterone 2α-, 6β-, 7α- and 16α-hydroxylation (kinetics of inhibition of 2α-hydroxylation shown in Fig. 4) was enhanced 17-, 6.6-, 2.5-, and 45-fold, respectively. The kinetics observed after the preincubation step could be described best as apparent linear noncompetitive (mixed).

In view of the findings from preincubation studies, the phenomenon of potentiation of inhibition was investigated further

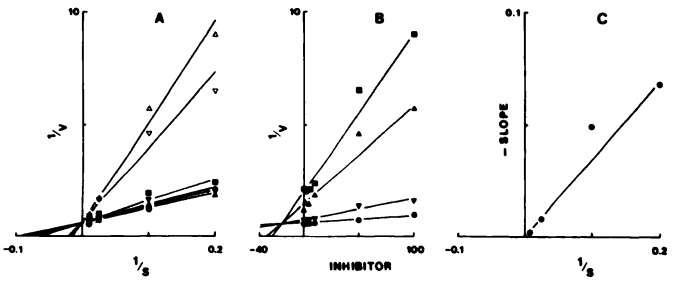

Fig. 2. Kinetics of the inhibition of microsomal P450 2C11-mediated testosterone 2α-hydroxylation by NOR in the absence of preincubation. A, Lineweaver-Burk plots at a range of NOR concentrations: ●, 0 μM; ▼, 1 μM; ▲, 5 μM; ■, 10 μM; ▽, 50 μM; △, 100 μM. B, Dixon plots at a range of testosterone concentrations: ●, 5 μM; ▼, 10 μM; ▲, 40 μM; and ■, 100 μM. C, Primary replot of Dixon plot slopes versus reciprocal testosterone concentration. Units are as follows: substrate and inhibitor concentration, μM; V, nmol/min/mg of protein.

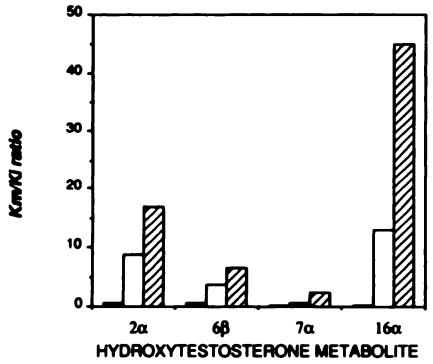

Fig. 3. Potentiation of the inhibition of P450 enzyme activity by NOR. K_m/K_i ratios were determined for each pathway of testosterone hydroxylation with (□) and without (■) the preincubation procedure described in Materials and Methods. The fold increase in the inhibition potency of NOR produced by preincubation is also indicated (▨).

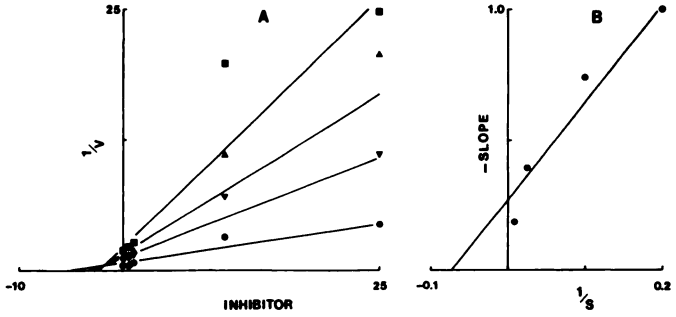

Fig. 4. Kinetics of the inhibition of microsomal P450 2C11-mediated testosterone 2α-hydroxylation by NOR after a 20-min preincubation step. A, Dixon plots at a range of testosterone concentrations: ●, 5 μM; ▼, 10 μM; ▲, 40 μM; ■, 100 μM. B, Primary replot of Dixon plot slopes versus reciprocal testosterone concentration. Units are as follows: substrate and inhibitor concentration, μM; V, nmol/min/mg of protein.

in time-course experiments. The data in Fig. 5 and Table 1 were derived from experiments in which NOR, NADPH, and microsomes were preincubated for varying periods up to 30 min before determination of testosterone hydroxylation. Semilogarithmic plots of percentage of remaining activity versus time were constructed and the times for loss of 50% of the initial activity were determined. Pseudo-first-order kinetic rate constants were calculated from the relationship $k = \ln 2 \cdot (\text{half-time})^{-1}$. Again, it is clear that testosterone 2α- and 16α-hydroxylations are considerably more susceptible to time-dependent increases in inhibition than are the other two positional hydroxylations of the steroid. Approximately 4 min was required for loss of 50% of the initial 2α- and 16α-hydroxylation activity in microsomes during preincubation with 5 or 10 μM NOR; some inhibition of testosterone hydroxylation in the absence of preincubation was apparent. Somewhat longer half-times were noted with 1 μM NOR. These experiments also verified that testosterone 6β- and 7α-hydroxylations were less susceptible to inhibition by NOR.

Characterization of NOR MI complex formation in untreated male rat liver. NOR is metabolized to a reactive species that binds tightly to microsomal P450 in rat liver, as indicated by the absorbance maximum near 454 nm in the optical difference spectrum of NADPH-supplemented microsomes containing the drug. From the data in Fig. 6, the process is concentration dependent. Studies were undertaken with a range of NOR concentrations between 0.2 and 100 μM, and the optimal initial rate and extent of MI complex formation appeared to be attained with 10 μM drug (4.1×10^{-3} absorbance units/min/mg of protein and 1.77×10^{-2} absorbance units/mg of protein). Application of the extinction coefficient of 65 mM^{-1} cm^{-1} (at 455 nm) reported by Franklin (24) for the analogous complex produced by oxidative metabolism of amphetamines suggests that 0.27–0.30 nmol of P450 (approximately 30% of the total) is complexed in male rat liver by NOR.

From the inhibition data obtained with NOR, it appeared likely that P450 2C11 may be preferentially involved in the MI complexation process. Addition to microsomes of an antibody raised against highly purified P450 2C11 decreased the rate of microsomal MI complex formation (Fig. 7). Thus, a ratio of 1 mg of anti-P450 2C11 IgG/mg of microsomal protein decreased the initial rate of complex formation to 48% of control. Higher ratios, of 5 and 10 mg of IgG/mg of protein, elicited 81% and 86% inhibition of complex formation rates, respectively; 10 mg of preimmune IgG/mg of protein produced only a 20% decrease in the rate of MI complexation. In accord with these immunoinhibition studies, it was found that an active reconstituted system containing highly purified P450 2C11 converted NOR (but not its *N,N*-dimethylamino analogue AMIT) to a 454-nm-absorbing metabolite (Fig. 8). With 35 and 200 μM NOR, similar initial rates of MI complex formation were measured (4.7 and 5.1×10^{-3} absorbance units/min/nmol of P450, respectively).

Contribution of MI complexation to inhibition of microsomal P450 by NOR. Preincubation of NOR with NADPH and microsomes generates reactive metabolites of the drug but it is possible that some of these metabolites may not be involved in MI complexation. As part of the present study the importance of MI complexation to the overall process of inhibition of P450-mediated drug oxidation by NOR was investigated (Table 2). It was noted that passage of incubations (after a 20-min preincubation) containing reactive NOR metabolites through Sep-Pak mini-C_{18} column led to inhibition of testosterone positional hydroxylations, 2α- (84% inhibition), 6β- (25%), 7α- (54%), and 16α- (82%). In the absence of the Sep-Pak extraction process 87%, 82%, 67%, and 92% inhibitions of the respective pathways were observed. Thus, it is evident that the degree of inhibition was similar, with the exception of the 6β-hydroxylation pathway, i.e., 25% inhibition when free NOR metabolites were removed from the incubate and 82% when they were not.

Experiment 2 of Table 2 indicates that the restoration of inhibited testosterone hydroxylations occurred after treatment of the reaction mixtures with ferricyanide. The exception was testosterone 7α-hydroxylation, which remained inhibited despite the ferricyanide treatment. The reason for this is not evident from the present data. Experiment 3 confirmed the effectiveness of the Sep-Pak procedure in removing the drug from the reaction.

***In vitro* formation of a NOR MI complex in human hepatic microsomes.** In eight human liver microsomal fractions, preincubation with NOR (250 μM) and NADPH produced perceptible changes in P450 content (HL1-8; Table 3). The P450 contents of these fractions were in the range of 0.33 to 0.62 nmol/mg of protein and NOR was found to produce decreases (by 0.01–0.05 nmol/mg of protein, representing 2.6–12.1% decreases) in six of the fractions. Repetitive scanning of the optical difference spectrum of two of the human fractions (HL3 and HL7; Table 3) after addition of NOR (250 μM) and

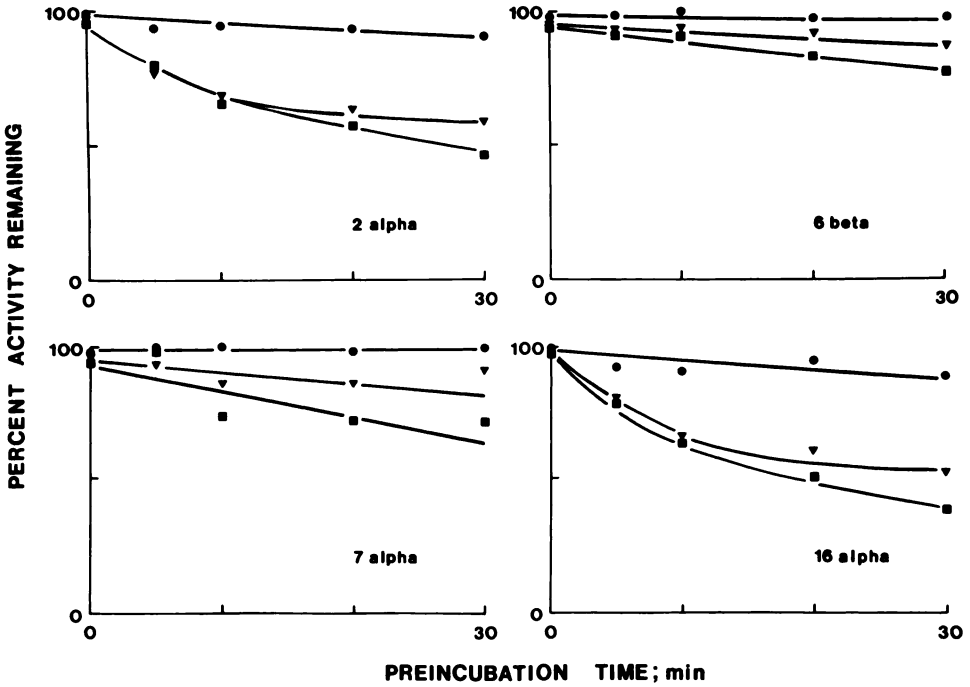

Fig. 5. Pseudo-first-order (semilogarithmic) plots of the effect of duration of preincubation between NOR- and NADPH-supplemented rat hepatic microsomes on residual testosterone hydroxylation activities. NOR concentrations: ●, 1 μM; ▼, 5 μM; ■, 10 μM.

TABLE 1

Time-dependent intensification of inhibition of P450-mediated testosterone hydroxylation by NOR in male rat hepatic microsomes

Formation of testosterone metabolites was determined after 0, 5, 10, 20, or 30 min of preincubation between NOR, microsomes, and NADPH before transfer to vials containing the steroid substrate. Each experiment was performed in duplicate and values were determined from the linear portions of plots of log (percentage of uninhibited activity) versus time.

Nortriptyline concentration	Time for loss of 50% activity (pseudo-first-order rate constant) for hydroxytestosterone metabolite formation[a]			
	2α-	6β-	7α-	16α-
μM	min(min^{-1})			
1.0	63 (0.011)	Slow[b]	Slow	40 (0.018)
5.0	3.7 (0.19)	42 (0.017)	78 (0.009)	4.0 (0.17)
10.0	4.8 (0.14)	26 (0.027)	58 (0.012)	3.8 (0.19)

[a] Time required for a 50% decrease in metabolite formation (min) and pseudo-first-order rate constant for the process (min^{-1}).

[b] Reaction too slow for determination of half-time or rate constant (>80 min required for loss of 50% activity).

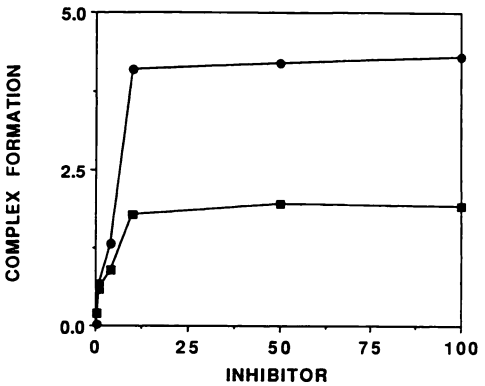

Fig. 6. Concentration-dependent formation of the NOR MI complex with P450 in male rat hepatic microsomes. ●, Initial rate of complex formation ($A_{454-490\ nm}$/mg of protein/min × 10^3); ■, magnitude of complex formation ($A_{454-490\ nm}$/mg of protein × 10^2). The unit of inhibitor (NOR) concentration is μM.

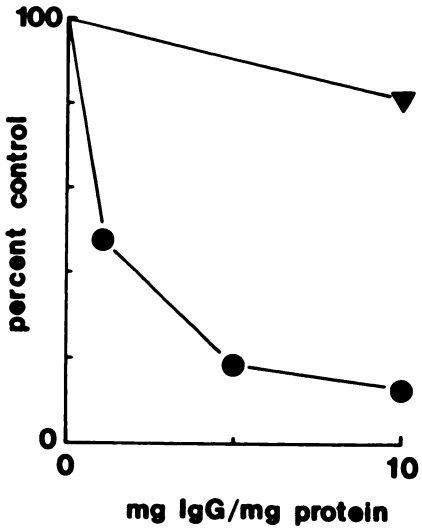

Fig. 7. Effect of an IgG preparation raised against P450 2C11 on NOR MI complex formation in male rat hepatic microsomes. (●) Anti-P450 2 CII IgC; (▼) preimmune IgG.

NADPH led to the detection of a small absorbance peak around 455 nm (data not shown), similar to that produced in male rat hepatic microsomes.

Discussion

Many alkylamine derivatives are converted during microsomal P450-mediated metabolism to reactive species that produce inhibitory complexes with the cytochrome. Since early reports of this phenomenon with SKF 525-A (14), it has been demonstrated that amphetamines (24), macrolide antibiotics such as erythromycin (25) and triacetyloleandomycin (26), and antihistamines such as diphenhydramine (27) and orphenadrine (28, 29) also elicit P450 complexation. Recent evidence also suggests that pharmacokinetic drug interactions of clinical significance may be due in part to MI complexation of relatively few P450s.

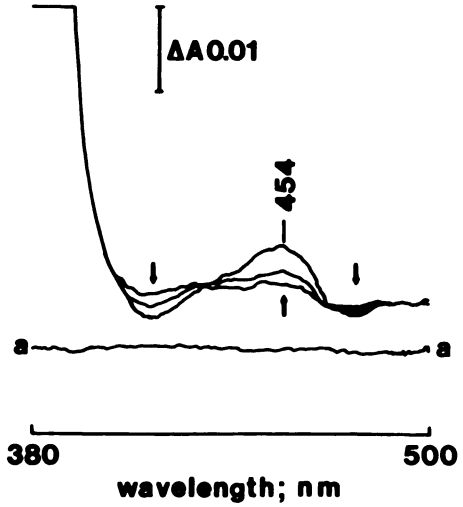

Fig. 8. Optical difference spectrum of the interaction between a NOR metabolite and purified P450 2C11 in an active reconstituted system (drug concentration, 200 μM). *Arrows*, direction in which the spectrum changed with time after NADPH addition. Scans were recorded at 1.5, 3.0, and 10.0 min after NADPH addition. *a*, base line of zero light absorbance.

That is, as a result of the requirement for a catalytic event in the formation of the metabolite involved in complexation, the process appears to be relatively selective for some P450s. For example, orphenadrine-mediated MI complexation seems to be restricted to P450 2B1/2 and 2C6 in rat liver (29, 30). It is noteworthy that multiple doses of orphenadrine in humans lead to accumulation of the drug in the body (31). Because sequestration of P450 by reactive metabolites would give rise to protracted inhibition of drug oxidation, the potential importance of MI complexation in observed pharmacokinetic interactions should be considered.

There are several lines of evidence that suggest that the principal male-specific P450, 2C11, is involved preferentially in MI complexation by the tricyclic antidepressant NOR. In preincubation studies to generate the MI complex, it was found that testosterone 2α- and 16α-hydroxylation activities, which are catalyzed primarily by P450 2C11, were more susceptible to inhibition than were testosterone 6β- and 7α-hydroxylation, which are catalyzed extensively by the P450s 3A2 and 2A1, respectively. Indeed, 17- and 45-fold increases in inhibitory effectiveness (K_m/K_i ratio) were observed when NOR was preincubated with NADPH and microsomes before transfer of the reaction mixture to the substrate (testosterone). There was also an apparent shift in the nature of inhibition from competitive to linear mixed after preincubation. It should be added, however, that a decrease in free enzyme (in this case P450) would have occurred during MI complexation in NADPH-supplemented microsomes. This process would be expected to give rise to an apparent noncompetitive component of inhibition, reflected, in the general scheme depicted in Fig. 9, as an increase in the concentration of P450-NOR and P450-NOR-testosterone, so that less P450 is available for substrate oxidation. In an active reconstituted system that incorporated highly purified P450 2C11, the initial rate of formation of the 454-nm peak was similar to that measured in male rat hepatic microsomes. Confirmation of the involvement of P450 2C11 in MI complexation by NOR was obtained from studies with an antibody raised against the protein. Anti-P450 2C11 decreased the extent of MI complexation observed in normal incubations with NOR.

Although P450 3A2-mediated testosterone 6β-hydroxylation was also susceptible to inhibition by NOR, it was notable that the extent of inhibition observed after removal of free NOR metabolites from the reaction mixture (on a Sep-Pak mini-C_{18} column) was considerably lower than that observed when these species were left in the reaction mixture. Other reports have established that the structurally similar imipramine is converted to a number of metabolites in hepatic microsomes (32–34). It is, therefore, possible that some of these metabolites are significantly inhibitory toward P450 reactions but do not form MI complexes.

The testosterone 7α-hydroxylase, P450 2A1, also exhibited time-dependent intensification of inhibition by NOR, suggesting that this enzyme may also be a target for MI complexation by the drug. Interestingly, complete restoration of 7α-hydroxylase activity was not observed in the experiment indicated in Table 2, in which ferricyanide was used to dissociate the MI

TABLE 2

Significance of MI complexation in inhibition of P450 activities by NOR *in vitro*

Three experiments were conducted. In experiment 1 NOR (25 μM) was preincubated with microsomes (0.15 mg of protein) and NADPH (1 mM) for 20 min, passed through a Sep-Pak mini-C_{18} column, and transferred to vials containing testosterone (50 μM) and an additional aliquot of NADPH (0.2 mM). The metabolism of testosterone proceeded for 2.5 min, after which the products were extracted, separated, and quantified as described in Materials and Methods. In experiment 2 NOR was preincubated with NADPH and microsomes as described for experiment 1, except that potassium ferricyanide (25 μM) was added to the incubation, to dissociate any alkylamine MI complex, before passage through a Sep-Pak column. In experiment 3 NOR (25 μM) and microsomes were passed through a Sep-Pak column before NADPH addition in order to demonstrate the capacity of the procedure to remove the drug from the incubation. Data are mean ± standard error of separate estimates; each experiment was performed in triplicate. Values in parentheses (experiment 1) are percentage of control means.

Experiment	Nortriptyline	Hydroxytestosterone metabolite formation			
		2α-	6β-	7α-	16α-
		nmol/min/mg of protein			
1	−	1.91 ± 0.07	1.75 ± 0.10	0.68 ± 0.11	2.58 ± 0.08
	+	0.31 ± 0.02[a](15)	1.32 ± 0.66[b](75)	0.31 ± 0.04[c](46)	0.46 ± 0.01[a](18)
2	−	1.71 ± 0.08	1.64 ± 0.12	0.69 ± 0.07	2.18 ± 0.08
	+	2.20 ± 0.05[a]	1.63 ± 0.03	0.23 ± 0.04[c]	2.34 ± 0.15
3	−	1.59 ± 0.07	1.68 ± 0.06	0.64 ± 0.06	2.20 ± 0.03
	+	1.68 ± 0.10	1.75 ± 0.06	0.80 ± 0.03	2.22 ± 0.10

[a] Significant difference from corresponding control mean, $p < 0.001$.
[b] $p < 0.025$.
[c] $p < 0.005$.

TABLE 3

Effect of incubation of NOR with NADPH and human hepatic microsomal fractions on apparent P450 content

Hepatic microsomes (1 mg/ml) from individual human livers were incubated with NOR (250 μM) and NADPH (1 mM) for 20 min and P450 content (NOR present) was estimated as described in Materials and Methods. Control incubations (20 min) included NADPH but the drug substrate was omitted (NOR absent).

Human liver sample	P450 content		
	NOR absent	NOR present	Change
	nmol/mg of protein		
HL1	0.50	0.51	+0.01 (+2.0)[a]
HL2	0.58	0.53	−0.05 (−8.6)
HL3	0.39	0.35	−0.04 (−10.3)
HL4	0.47	0.43	−0.04 (−8.5)
HL5	0.45	0.45	0 (0)
HL6	0.38	0.37	−0.01 (−2.6)
HL7	0.33	0.29	−0.04 (−12.1)
HL8	0.62	0.60	−0.02 (−3.2)

[a] Values in parentheses are percentage of change.

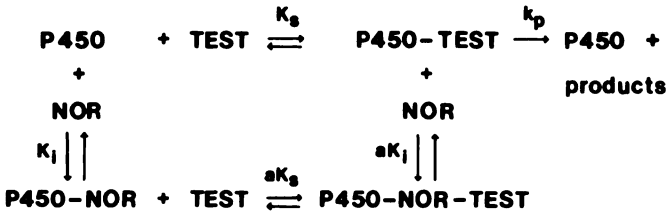

Fig. 9. Scheme of the equilibria between enzyme (P450), substrate [testosterone (*TEST*)], and inhibitor (NOR) in rat hepatic microsomes. aK_s, substrate constant modified by the presence of inhibitor; aK_i, P450-inhibitor constant modified by substrate. K_s, dissociation constant of P450-substrate complex; K_i, dissociation constant of P450-inhibitor complex; k_p, rate constant for break down of P450-substrate complex.

complex. It is possible that this may reflect the inability of ferricyanide to oxidize the P450 2A1-NOR MI complex, or it could reflect a conformational or other change in the structure of P450 2A1 that results in hypoactivity.

In this study MI complexation in the reconstituted system incorporating P450 2C11 was more efficient with the *N*-methylamino-substituted drug NOR than with the *N,N*-dimethylamino-substituted analogue AMIT. Previous studies with alkylamines related to orphenadrine and amphetamine are consistent with these findings (27, 35). Indeed, it has been suggested that *N*-dealkylation is necessary for the generation of the proximate metabolite, considered to be the nitrosoalkane species, that is involved in MI complexation (28, 36).

Agents with a preference for inactivation or MI complexation of P450 2C11 have not yet been described, although it has been suggested that SKF 525-A may generate an MI complex with this enzyme (37). Two recent reports have indirectly implicated MI complexation of P450 2C11 as a possible mechanism to account for the greater inhibition potency of cimetidine *in vivo* than is normally exhibited in rat hepatic microsomes *in vitro* (38, 39). Presumably, P450-preferred MI complexation reflects the capacity of the target enzyme to generate the reactive intermediary metabolite. Further work is now required to account for the structural features within drug substrates that promote MI complexation of specific P450s.

Tricyclic antidepressants such as NOR undergo biotransformation to a number of metabolites in humans. Many recent studies, however, have centered on the association between debrisoquine hydroxylation phenotype and the 2-hydroxylation of desipramine (40) and imipramine (34) and the 10-hydroxylation of NOR (41) and AMIT (42). All reactions appear to be catalyzed extensively by human hepatic P450 2D6. However, it is likely that other P450s participate in the oxidation of tricyclic antidepressant drugs in humans, because other metabolites have been isolated from microsomal incubations (33). The present findings suggest that up to 12% of the total P450 present in some microsomal fractions may be involved in an MI complex with a metabolite of NOR. Thus, it seems appropriate to consider inhibition, and perhaps MI complexation, of P450 enzymes as a possible underlying mechanism of the documented pharmacokinetic interactions involving NOR or other tricyclic antidepressant agents (43).

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