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Selectivity in the Inhibition of Mammalian Cytochromes P-450 by Chemical Agents*

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I. Introduction

A. The P-450 Reaction Cycle

P-450 is the term used to describe members of the hemoprotein superfamily that catalyses the oxidative biotransformation of lipophilic substrates to more polar metabolites (44, 97). P-450s are found in the endoplasmic reticulum or mitochondrial fractions of many tissues and function in either catabolic pathways or in the biosynthesis of molecules of physiological importance (70). In the endoplasmic reticulum (which gives rise to the microsomal fraction) P-450 activity is also dependent upon a flavoprotein, reduced nicotinamide adenine dinucleotide phosphate‡ (NADPH)-P-450-reductase, that functions in the transfer of electrons from NADPH to the P-450-substrate complex. On the other hand, mitochondrial P-450 systems include another essential component which is a nonheme iron protein. In the adrenal mitochondria this protein is termed adrenodoxin. The non-

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‡ Abbreviations: NADPH, reduced nicotinamide adenine dinucleotide phosphate; MFO, mixed-function oxidase; SKF 525-A, 2-diethylaminoethyl-2,2-diphenyl-*n*-pentanoate; MI, metabolite intermediate complex with cytochrome P-450; CAP, chloramphenicol; AIA, allylisopropylacetamide; ABT, 1-aminobenzotriazole; AD, androst-4-ene-3,17-dione; 20-OH monyne, (20*R*)-20-(1-hexynyl)-pregn-5-en-3β,20-diol; 20-OH diyne, (20*R*)-20-(1,5-hexadiynyl)-pregn-5-en-3β,20-diol; TMS, (20*S*)-20-(2-trimethylsilylethyl)-pregn-5-en-3β,20-diol; PB, phenobarbital; 22-ABC, 22-amino-23,24-bisnor-5-cholen-3β-ol.

heme iron protein shuttles between the flavoprotein reductase and the P-450 enzyme and facilitates electron transfer (112).

The P-450 reaction cycle is widely considered to proceed as shown in fig. 1. Briefly, initial binding of a substrate molecule (RH) to the ferric form of the cytochrome results in the formation of a binary complex and a shift in the spin equilibrium of the ferric enzyme from the low- to high-spin state. Some evidence has been presented that suggests that this configuration more readily accepts an electron from the flavoprotein reductase to form the ferrous P-450-substrate complex (8). However, not all P-450s exhibit a relationship between high-spin content and reduction rate (7, 41, 58). Indeed, it has been proposed that several factors, including the nature of the P-450 substrate, the topography of the enzyme/substrate complex, and the potentials of oxidisable atoms each play a role in the regulation of reduction rate (41). Molecular oxygen binds to the ferrous P-450-substrate complex to form the ferrous dioxygen complex which is then reduced by a second electron from the P-450 reductase (or perhaps, in some cases, from reduced nicotinamide adenine dinucleotide via cytochrome b_5 and its reductase). Dioxygen bond cleavage in the reduced ferrous dioxygen complex results in the insertion of one atom of oxygen into the substrate, reduction of the other oxygen atom to water, and restoration of the ferric hemoprotein (99). The precise nature of the active oxidant species remains unclear. The P-450 cycle may also be uncoupled, i.e., the oxygen bound to P-450 may be reduced to hydrogen peroxide or superoxide, in the absence of true substrate molecules (109).

B. Historical Perspective

Each P-450 hemoprotein consists of two components: an iron protoporphyrin IX heme moiety and a single polypeptide chain or apoprotein. The iron atom of the heme prosthetic group is located at the center of the protoporphyrin ring and is bonded to the ring system via its four pyrrolic nitrogens. The fifth (axial) ligand is a sulfur atom from a cysteinyl residue of the apoprotein. It is this structural feature that gives rise to the facile interconversion of the low-/high-spin states of ferric P-450, as well as to the unusual Soret absorption of the ferrous/P-450-carbonyl complex at 450 nm (approximately 30 nm longer in wavelength than that commonly observed for hemoproteins). Maintenance of the thiolate fifth ligand is essential for catalytic activity. The low-spin state of P-450 probably has a water molecule at the sixth ligand position, whereas the high-spin cytochrome (substrate-bound) is pentacoordinated (149). The interconversion of the low-/high-spin states may be observed by electron spin resonance spectroscopy or, more readily, by optical difference spectroscopy (61, 124). Because the high- and low-spin forms exhibit spectral maxima near 386 and 417 nm, respectively, optical difference spectroscopy in the visible region, upon addition of substances to microsomal fractions or purified P-450s, reveals spin state changes that can be correlated with effects on catalytic activity.

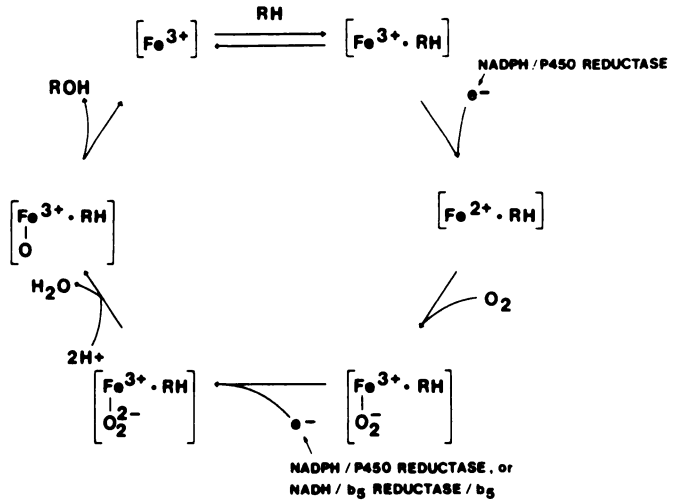

FIG. 1. The cytochrome P-450 reaction cycle. Ferricytochrome P-450 is represented by Fe^{3+}. RH, P-450 substrate; ROH, the product of oxidation.

C. P-450 Nomenclature

During the last decade several groups have isolated and characterised P-450 enzymes. As is often the case in such a field of intense activity, several terminologies have evolved in parallel that describe apparently identical proteins. Recent efforts have included a unifying nomenclature, based on amino acid sequence alignment data, that is considered to reflect evolutionary relationships within the P-450 gene superfamily across species. P-450s are considered to be in the same family if they share greater than a 40% sequence identity. Subfamilies are constituted by P-450s that share at least 60% sequence identity (a detailed description of this nomenclature is presented in ref. 97).

D. Hepatic Forms of P-450

Tissue expression and regulation is an identifying characteristic of each form of P-450. The liver has the highest total P-450 concentration of any organ. Changes in the relative concentrations of P-450s can be effected by different drug treatments and by hormonal manipulation. Distinct P-450s usually exhibit broad, but characteristic, substrate specificities (23). A list of hepatic forms of P-450, their synonyms, and typical inducers, as well as principal reactions catalysed by the enzymes, is given in table 1. In a number of cases these reactions are catalysed by only one isoform of P-450, and it is this property that can be exploited in studies of preferential inhibition of the function of specific P-450 enzymes.

E. Extrahepatic P-450s and Their Involvement in Physiological Processes

P-450 and its associated mixed-function oxidase (MFO) activity have been described in many extrahe-

TABLE 1
*Hepatic forms of P-450**

Subfamily/protein	Synonyms	Inducer	Principal reactions catalysed
IA			
IA1	Rat P-450c, rabbit form 6, human P_1	βNF, 3-MC, ACLR, TCDD	7-Ethoxyresorufin-O-deethylation
IA2	Rat P-450d, rabbit form 4, human P_3	Isosafrole, 3-MC, βNF, TCDD, ACLR	N-Hydroxylation of IQ and MeIQ
IIA			
IIA1	Rat P-450a, rat UT-F	βNF, PB	Androstenedione and testosterone, 7α-hydroxylation
IIA2	Rat RLM2	Noninducible	Testosterone 15α-hydroxylation
IIB			
IIB1	Rat P-450b, rat PB-B	PB, ACLR	Androstenedione 16β-hydroxylation, pentoxyresorufin O-depentylation, 7,12-Dimethylbenz[a]anthracene 7-methyl hydroxylation
IIB2	Rat P-450e, rat PB-D	PB	7,12-Dimethylbenz[a]anthracene 12-methyl hydroxylation
IIB4	Rabbit LM2, rabbit form 2	PB	Benzphetamine N-demethylation
IIC			
IIC5	Rabbit form 1	PB	Progesterone 21-hydroxylation
IIC6	Rat P-450k, rat PB-C	PB	Progesterone 21-hydroxylation, *S*-warfarin 7-hydroxylation
IIC9	Human MP-A	?	Mephenytoin 4′-hydroxylation
IIC11	Rat P-450h, rat UT-A	Noninducible	Androstenedione 16α-hydroxylation, testosterone 16α- and 2α-hydroxylation
IIC12	Rat P-450i, rat UT-I	Noninducible	5α-Androstane-3α,17β-diol-3,17-disulfate 15β-hydroxylation
IID	Rat UT-H, rat db1, db2, human db1	Noninducible	Debrisoquine 4-hydroxylation
IIE			
IIE1	Rat P-450j, $P\text{-}450_{ac}$, rabbit 3a, human form j	Ethanol, ether, acetone, dimethyl sulfoxide	N-Nitrosodimethylamine N-demethylation
IIIA			
IIIA1	Rat PCN_1, rat P-450p, rat PCNa	TAO, pregnenolone 16α-carbonitrile	TAO complex-formation, testosterone 2β- and 15β-hydroxylation
IIIA2	Rat PCN_2, rat P-450 PB-1, rat PCNc	PB	Testosterone-2β- and 6β-hydroxylation
IIIA4	Human nf	PB, TAO, dexamethasone	Testosterone, androstenedione, and progesterone 6β-hydroxylation
IIIA6	Rabbit 3c	Rifampicin	Erythromycin N-demethylation
IVA	P-452, $P\text{-}450_{LA\omega}$	Clofibrate and other hypolipidemic agents	ω- and ω-1 Hydroxylation of fatty acids and prostaglandins

* Sources were as follows: refs. 21, 22, 39, 44, 62, 72, 97, 118, 121, 135, 137, 143, 145, and 147. Abbreviations: βNF, β-naphthoflavone; 3-MC, 3-methylcholanthrene; ACLR, aroclor 1254; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; PB, phenobarbital; TAO, triacetyloleandomycin; IQ, 2-amino-3-methylimidazo [4,5-*f*] quinoline; MeIQ, 2-amino-3,5-dimethylimidazo [4,5-*f*] quinoline.

patic tissues including brain, adrenal, kidney, testis, ovary, lung, and skin. These activities often play critical roles in steroidogenesis, fatty acid metabolism, and, in some cases, xenobiotic biotransformation. A number of important extrahepatic P-450s and their characteristic reactions, as well as tissue localisation, are listed in table 2.

It is clear that the lung and skin are organs that are likely to receive exposure to environmental chemicals and toxins. Little is known regarding the expression of individual P-450s in dermal tissues or, indeed, the extent to which multiplicity exists. Several P-450s have been isolated from lung tissues, although it now seems clear that the same P-450s may be expressed either in lung or in liver. It has been demonstrated that the lung-specific P-450 form 5 is active in aromatic amine oxidation and medium-chain fatty acid ω-hydroxylation (122). Another form, which is regulated by progesterone, shares greater than 70% sequence identity with P-450 IVA1 and is active in prostaglandin A and E ω- and ω-1 hydroxylation (81).

P-450s in adrenal, testicular, ovarian, and placental mitochondria and microsomes are active in steroidogenesis. Cholesterol is converted by $P\text{-}450_{scc}$ (side-chain cleavage) to pregnenolone that can, in turn, be metabolised further to mineralocorticoids and glucocorticoids by adrenal P-450s that catalyse 17α-, 21-, and/or 11β-hydroxylation (83). Alternately, pregnenolone (a C_{21} ste-

TABLE 2
*Extrahepatic forms of P-450**

Subfamily/protein	Synonyms	Tissue	Reactions catalysed
IVB1	Lung P-450, form 5 rat (liver), form 5 rabbit	Lung	Aromatic amine oxidation, medium chain fatty acid ω-hydroxylation
XIA	$P\text{-}450_{scc}$	Adrenal (mitochondrion)	Cholesterol side-chain cleavage
XIB	$P\text{-}450_{11\beta}$	Adrenal (mitochondrion)	Deoxycorticosterone 11β-hydroxylation
XVIIIA1	$P\text{-}450_{17\alpha}$	Testis and adrenal (microsomes)	Progesterone and pregnenolone, 17α-hydroxylation
XIXA1	$P\text{-}450_{arom}$	Placenta and ovary (microsomes)	Aromatization of testosterone and androstenedione
XXIA1	$P\text{-}450_{c21}$	Adrenal (microsomes)	Progesterone and 17-hydroxyprogesterone 21-hydroxylation

* Sources were as follows: refs. 38, 39, 79, 81, 83, and 97.

roid) is converted to the C_{19} steroid androst-4-ene-3,17-dione (AD) by $P\text{-}450_{17\alpha}$ and P-450 17/20-lyase (probably the same enzyme as $P\text{-}450_{17\alpha}$) in testicular, ovarian, or placental microsomes. This steroid serves as the common precusor of androgens and estrogens (following the action of aromatase which is another P-450 enzyme and is present in high concentration in the ovary and the placenta).

In general terms, the use of inhibitors of extrahepatic P-450 reactions has been low, but the identification of apparently specific chemical inhibitors (especially in the case of aromatase) may have utility in the development of therapeutic agents.

II. The Nature of P-450 Enzymes

A. Inhibition of Drug Metabolism

Many inhibitors of oxidative drug metabolism have been described (85, 136). The nature of the P-450 reaction cycle clearly shows a number of potential points of interaction with inhibitors of drug oxidation. For example, certain inhibitors exert their effects on P-450-dependent MFO activity by interfering with substrate binding or electron transport and control of the P-450 spin state. On the other hand, other inhibitors rely on the catalytic function of the P-450 cycle, and metabolism of the inhibitor is a prerequisite. Certain organic compounds including 2-diethylaminoethyl-2,2-diphenyl-*n*-pentanoate (SKF 525-A) and piperonyl butoxide (fig. 2) have been widely used for many years as "inhibitors of oxidative drug metabolism." However, recent findings suggest that both compounds are not uniformly potent against the activity of all P-450s (86, 87). Consequently, negative findings with such agents may lead to the erroneous conclusion that P-450 is not involved in a particular toxicological process. The identification of specific chemicals that are inhibitors of individual P-450 enzymes will facilitate the correct interpretation of toxicological phenomena.

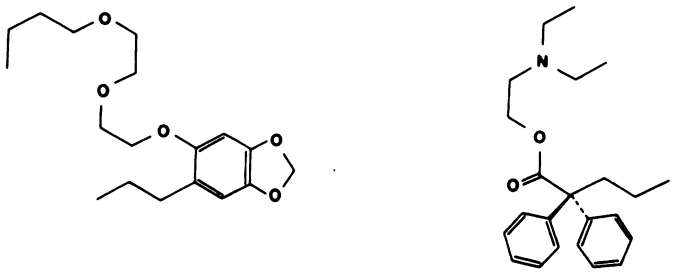

FIG. 2. Structures of the classical inhibitors of oxidative drug metabolism (left) piperonyl butoxide and (right) SKF 525-A.

B. Induction of Drug Metabolism

Normal liver contains a number of distinct P-450s, but the in vivo exposure of mammals to exogenous chemicals often results in the de novo synthesis of P-450 molecules (23). As a consequence, the relative proportion of individual P-450 enzymes is altered so that the disposition of drugs and other P-450 substrates may proceed along pathways that are different from normal. In early studies, two classes of P-450-inducing agents were identified: barbiturates and polycyclic aromatic hydorcarbons (97, 133). More recently, the glucocorticoids, alcohol, ketones, isoniazid, and many other xenobiotics have been shown to induce individual members of the P-450 superfamily (40, 119, 143). Isolation and characterisation of P-450s from various sources have led to the recognition of the multiplicity of the P-450 system of enzymes as well as to the subtle regulatory processes that are involved in their expression. The cellular expression of polycyclic aromatic hydrocarbon-inducible P-450s is regulated by a cytosolic receptor (the *Ah* receptor) that interacts with the polycyclic aromatic hydrocarbon, is translocated to the nucleus where it interacts with the nuclear DNA, and stimulates transcription (16, 31, 60, 117). It has also been suggested that the glucocorticoid receptor may be involved in the regulation of another member of the P-450 superfamily (P-450 IIIA1) (126, 127), whereas all efforts to date have failed to uncover a receptor for phenobarbital (PB)-inducible P-450s (2, 34). Physiological regulatory processes, involving sex hormones and

thyroid hormone, are now established for the hepatic expression of certain constitutive (those present in relatively high concentration in untreated rat liver) forms of P-450 (5, 142, 144, 146). A more detailed treatment of the multiplicity of P-450s, in relation to catalytic activities, is presented in section III.

C. Enhancement of Drug Metabolism

The in vitro activation of rates of MFO substrate oxidation has been observed on numerous occasions and is termed enhancement. Acetone, butanone, metyrapone, and ethyl isocyanide have all been reported to enhance microsomal drug oxidase activities, but the mechanism has remained elusive (3). More recently, the kinetics of α-naphthoflavone-mediated enhancement of rabbit liver P-450 IIB1 and P-450 IIC5 activities have been evaluated in a mechanistic sense, and it appears that this agent exerts its effect on P-450 activity by an allosteric phenomenon (128). Chemical compounds that elicit P-450 form-specific enhancement would be useful tools in the elucidation of the catalytic functioning of individual P-450s, but, to date, very little information in this regard is available from the literature. A detailed discussion of enhancement of MFO activity is beyond the scope of this review.

III. Inhibition of Activities Mediated by Specific P-450 Enzymes

A. Mechanisms of Inhibition of P-450

1. Reversible inhibition. The polysubstrate nature of P-450s is responsible for the large number of documented interactions associated with inhibition of drug oxidation and drug biotransformation. The majority of such interactions are no doubt due to the coadministration of two or more agents that undergo biotransformations catalysed by the same forms of P-450. In mechanistic terms, such interactions arise as a result of competition at the P-450 active center and probably involve only the first step of the P-450 reaction cycle (fig. 1) (85). However, such interactions are usually of a transient nature and long-term problems are seldom encountered. This is the case even in situations in which the inhibition is quite potent, for example, that associated with some of the antimycotic imidazoles. Thus, after the inhibitory agent has been eliminated from the organism, the normal function of the P-450 enzymes continues.

2. Metabolite intermediate complexation. A number of drugs and other foreign compounds are converted by P-450 from relatively noninhibitory species to more effective MFO inhibitors. The formation of such metabolite intermediates that sequester the P-450 in a catalytically nonfunctional state has been associated with a limited range of chemical structures, for example, the dioxole/dioxolane functionality (25, 150), certain hydrazines (57, 84), and a number of alkylamine derivatives (73, 110). This process relies on at least one cycle of the P-450 catalytic process. After formation, the complex between P-450 and the metabolite intermediate (MI complex) is quite stable. In the case of the benzodioxoles (also known as methylenedioxyphenyl compounds), which include the insecticide synergist piperonyl butoxide (fig. 2), and former food flavoring agents such as isosafrole, the ferric and ferrous forms of the MI complex are both quite stable; the ferric form is, however, dissociable by lipophilic compounds including MFO substrates (32, 91). Dissociation, or displacement, of the benzodioxole metabolite-P-450 complex results in the reactivation of MFO activity (32, 88). It is of interest that the nature of the benzodioxole side-chain substituent is an important determinant of (*a*) the extent of MI complex formation of P-450 (87, 91), (*b*) the generation of carbon monoxide and other metabolites that are probably independent of MI complexation (150), and (*c*) the capacity of the derivative to elicit P-450 induction and MI complex formation in vivo (fig. 3) (90, 91). Dioxolane MI complexes are transient and decompose soon after formation (26).

Alkylamine MI complexes, unlike the benzodioxole analogs, are stable only in the ferrous state. Oxidation of hepatic microsomal fractions in vitro from animals treated with such agents in vivo results in P-450 and MFO reactivation (86, 110).

Although the catalytic function of P-450s involved in MI complexation can be readily restored in vitro, it is likely that the complex in vivo would be subject to the normal catabolic processes involving heme and apoprotein turnover. It is for this reason that MI complex-forming agents are associated with abnormal drug pharmacokinetics for longer periods than reversible inhibitors.

3. Autocatalytic inactivation. The autocatalytic inactivators of P-450, including olefins and acetylenes (103, 105), cyclopropylamines (54), and dihydropyridines (101) (fig. 4), are converted to radical intermediates by P-450 that alkylate the enzyme and inactivate its function.

Inactivation of drug oxidation occurs after the in vivo administration of autocatalytic or suicide substrates of P-450 (14). Again, prolonged depression of the MFO system is anticipated under these conditions because de

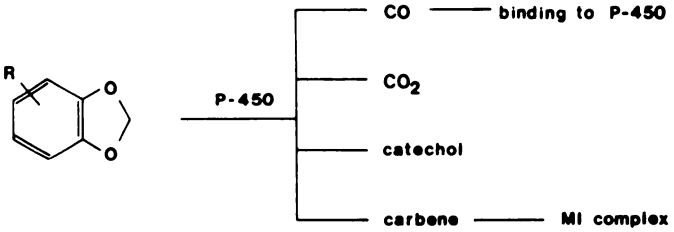

FIG. 3. Alternate pathways of P-450-mediated metabolism of methylenedioxyphenyl compounds. When the substituent R is an electron-withdrawing group, carbon monoxide production is enhanced and MI complex formation is diminished (150). When R is electron donating or is a long-chain alkyl group, MI complex formation is increased and stabilised (87, 91, 150).

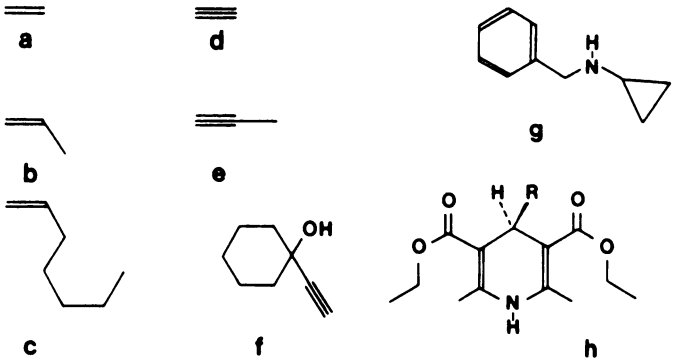

FIG. 4. Structures of chemicals that inactivate P-450 during oxidation. a, ethylene; b, propene; c, hept-1-ene; d, acetylene; e, propyne; f, 1-ethynyl-cyclohexanol; g, N-cyclopropylbenzylamine; h, general structure of 4-substituted 2,6-dimethyl-3,5-bis(ethoxycarbonyl)-1,4-dihydropyridine (only the 4-alkyl compounds are inhibitory).

novo synthesis of P-450s must occur for the restoration of drug-metabolising capacity.

Other chemical agents have been reported to elicit autocatalytic destruction of P-450. These include the antibiotic chloramphenicol (CAP) (48, 52, 53), and structurally related compounds (134), and thionosulfur agents such as parathion and malathion (30, 98). Rather than heme destruction, these chemicals exert their inhibitory effect on MFO activity by P-450 apoprotein modification.

Apart from reversible inhibition, MI complexation, and suicide substrate processing, a number of alternate means of eliciting inhibition of drug metabolism are feasible. These include depletion of cofactor supply (which could occur in vivo in abnormal nutrition states), xenobiotic down-regulation of P-450s that are normally expressed in high concentration in untreated animals, or agents affecting heme or apoprotein biosynthesis. Although such phenomena have been reported in the literature, they are beyond the scope of this review which is concerned primarily with inhibition of P-450 reactions at the enzyme-active site.

B. Development of Agents That Are Preferential Inhibitors of Specific P-450s

To date the majority of studies dealing with inhibition of P-450-mediated MFO activity has concentrated on existing molecules, usually drugs, food additives, or environmental chemicals that are associated with specific toxicological problems. Fewer investigators have described structure-activity relationships between the nature of chemical substitution around a basic nucleus associated with MFO inhibition. Clearly, such studies are necessary to facilitate the design of optimal probes, but an additional important factor is the investigation of the selectivity of the inhibition process. A potent inhibitor of P-450-associated activity that has minimal selectivity is likely to elicit a wide range of biological effects that could be deleterious to the organism. For example, ketoconazole is an antifungal imidazole drug that reportedly inhibits many P-450 reactions including testicular steroidogenesis (64), vitamin D homeostasis (56), most hepatic biotransformations (92), and adrenal corticosteroid production (96). To fully delineate the involvement of individual P-450s in toxicological processes, such as the production of reactive metabolites, it is also necessary to have available chemical probes with a high degree of inhibition selectivity. Without such probes it is difficult to obtain the detailed mechanistic information that could be gained from such experiments.

The relatively recent assignment of particular oxidative reactions as indicators of the activity of different P-450s has facilitated the design and development of specific inhibitors. In the following sections the information that is available in this regard is summarised.

1. Chloramphenicol (CAP), 21-halosteroids, and analogs. Early studies documented the capacity of the antibacterial agent CAP to inhibit microsomal drug oxidation in mice (33). More recently, CAP has been shown to produce mechanism-based inactivation of P-450 following oxidative dechlorination of the dichloroacetamido substituent to an oxamyl moiety (45) (fig. 5). This reactive species apparently binds to a lysine residue in the P-450-active site, but there is no loss of heme. Rather, it appears that the capacity of the cytochrome to accept electrons from the reductase is impaired (52). This phenomenon is not uniform for all P-450s, and studies of the selectivity of the process revealed that the rat hepatic P-450s IIC6, IIC11, and IIB1 are susceptible to inactivation by CAP, whereas IA1, IA2, and IVA are resistant to inactivation (48). Structural modification of the CAP nucleus (fig. 6) led to compounds that were able to elicit inactivation of specific P-450s: (*a*) N-(2-phenethyl)dichloroacetamide (k_{inact} = 0.40 min^{-1}) and N-(2-*p*-nitrophenethyl)-dichloroacetamide (k_{inact} = 0.39 min^{-1}) were quite potent inactivators of P-450 IA1 activity compared with other analogs (47, 134). (*b*) The most efficient inactivator of P-450 IIIA1/2-mediated androstenedione 6β-hydroxylation was N-(1,2-diphenethyl)dichloroacetamide (k_{inact} = 0.32 min^{-1}). (*c*) N-(2,2-diphenethyl)dichloroacetamide was a potent inactivator of P-450 IIB1 activity (k_{inact} = 0.72 min^{-1}). (*d*) α,α-dichlorotoluene was found to be a selective inactivator of IIB1 activity (k_{inact} of 0.35 min^{-1}).

The potency of N-(2-*p*-chlorophenethyl)dichloroacetamide as an inhibitor of P-450 IA1 activity is interesting in light of the report by Parkinson et al. regarding 2-bromo-4′-nitroacetophenone (fig. 6). This compound was a selective inhibitor of P-450 IA1 despite the fact that 10 individual P-450s were alkylated by the compound (109). This interesting finding was attributed to the presence of a cysteinyl residue (amino acid 292) in P-450 IA1 that, upon alkylation, leads to uncoupling of the reaction cycle and enhanced formation of hydrogen peroxide and a concomitant decrease in substrate oxidation.

The finding that CAP analogs serve as mechanism-

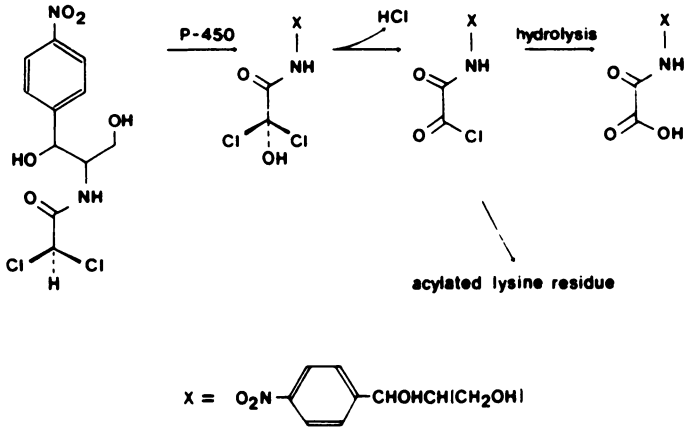

FIG. 5. P-450-dependent oxidation of CAP to an oxamyl derivative that either undergoes hydrolysis or acylates a lysine residue in the P-450 active center.

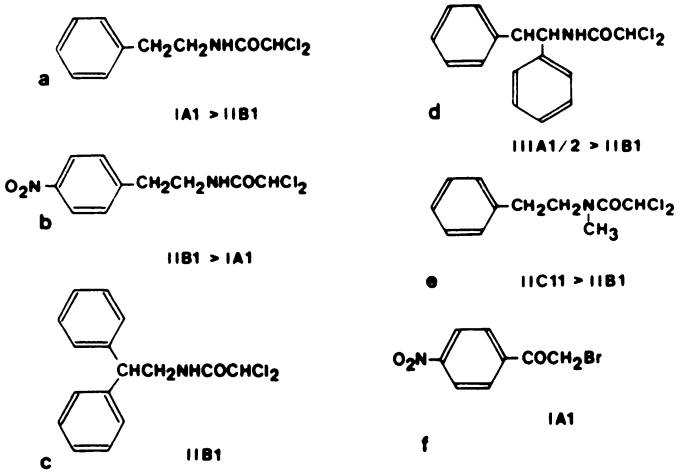

FIG. 6. Dichloromethyl and bromomethyl compounds that elicit covalent modification of P-450 protein. a, N-(2-phenethyl) dichloroacetamide; b, N-(4′-nitro-2-phenethyl)dichloroacetamide; c, N-(2,2-diphenylethyl)dichloroacetamide; d, N-(1,2-diphenylethyl)-dichloroacetamide; e, N-(methyl)-N-(2-phenethyl)dichloroacetamide; f, 2-bromo-4′-nitroacetophenone. The gene designations of the P-450 isoforms that are most susceptible to inactivation by each agent are indicated.

based inactivators of P-450 led to the development of a number of 21-halo derivatives of progesterone and pregnenolone (fig. 7). Several general points have emerged from studies with these compounds (49–51). First, the 21-dichloro- and 21-chlorofluorosteroids were effective inactivators of rabbit liver progesterone 21-hydroxylation (P-450 IIC5 activity), whereas the 21-difluorosteroid was an inhibitor of 21-hydroxylation but did not elicit a time-dependent increase in the loss of activity. Presumably, this reflects the fact that the fluoro substituent is a poor leaving group (as fluoride). Because nucleophilic substitution of the halosteroid is unfavorable, alkylation of an active site amino acid residue is unlikely and inactivation does not occur. Second, the 21-dichloro- and 21-chlorofluorosteroids also inactivated progesterone 16α-hydroxylase activity in rabbit liver and progesterone 6β-and 21-hydroxylases in PB-induced rat liver. Taken together, these findings are consistent with the assertion that the 21-dichlorosteroid could well be a useful starting point in the development of more specific inactivators of P-450 enzymes.

2. Olefins and acetylenes as mechanism-based inactivators of hepatic P-450. Many simple olefins and acetylenes, as well as complex molecules containing side-chains with terminal sites of unsaturation (some examples are shown in fig. 8), have been shown to inactivate MFO enzymes and to produce porphyria. The elegant studies of Ortiz de Montellano and coworkers have clearly demonstrated the mechanisms of the inactivation processes associated with various olefinic and acetylenic compounds (18, 19, 99, 100, 103, 105, 106, 129). It has been established that, during the normal functioning of the P-450 reaction cycle, a limited number of unsaturated substrate molecules are converted to radical species that alkylate the P-450 heme moiety. Iron is lost from the heme and abnormal N-alkylated porphyrins are produced. Gan et al. (37) suggested that 1-ethynylpyrene, a pyrene nucleus substituted with an ethynyl group and designed to be a mechanism-based inactivator of P-450c (also termed IA1), does not alkylate the P-450 heme. Instead, this compound appears to exert its inhibitory effect by protein modification.

The propensity of different P-450s to undergo inactivation by such compounds has been evaluated in some studies. Allylisopropylacetamide (AIA; fig. 8a) the classical suicide substrate of P-450, is now recognised as an effective inactivator of P-450s IIB1 and IIIA1, with the P-450s IIC6 and IIC11 somewhat less susceptible (14).

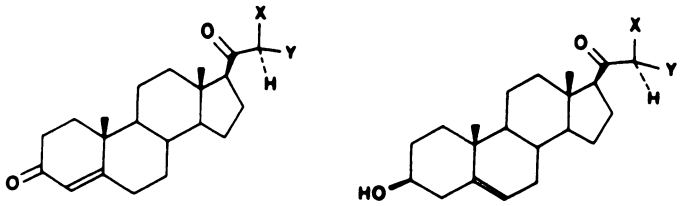

FIG. 7. General structures of 21-dihaloprogesterone (left) and 21-dihalopregnenolone (right). X and Y are halogens.

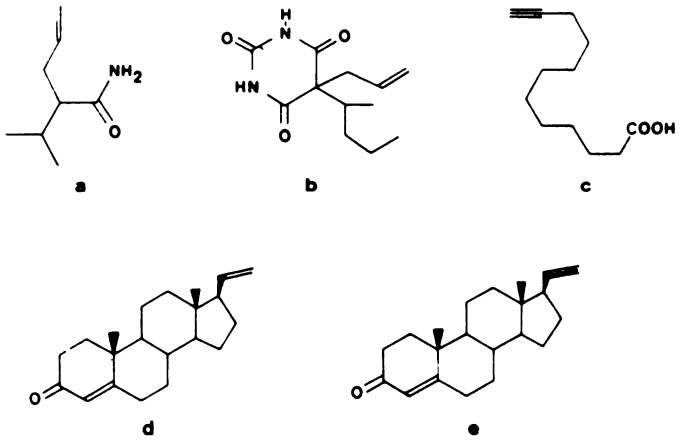

FIG. 8. Inactivators of P-450 enzymes. a, AIA; b, secobarbital; c, 11-dodecynoic acid; d, 17β-vinylandrost-4-ene-3-one; e, 17β-ethynylandrost-4-ene-3-one.

In vivo heme supplementation of AIA-deactivated P-450s restores IIIA1 and IIB1 activities and, to a lesser extent, P-450s IIC11 and IIC6 (13). In contrast to these findings with AIA it has also been reported that the hypnotic secobarbital (fig. 8b) is a selective inactivator of P-450 IIB1 and that the activity of the enzyme is not restored after heme supplementation (74). Substitution of steroids with unsaturated functional groups reportedly results in potent inhibitors of P-450 pathways. The steroid nucleus would appear to be a useful starting point for the development of specific P-450 inhibitors because steroids are excellent substrates for these enzymes. The data of Halpert et al. (49) demonstrate the potency of 17β-vinylandrost-4-ene-3-one and 17β-ethynylandrost-5-ene-3-one (fig. 8, d and e) as inactivators of rat hepatic P-450 IIIA1/2-dependent 6β-hydroxylation (k_{inact} = 0.23 min^{-1}) and IIC6-dependent 21-hydroxylation (k_{inact} = 0.20 min^{-1}) of progesterone, respectively.

A similar approach has been taken by CaJacob et al. (18) and Ortiz de Montellano et al. (19) in which incorporation of a terminal acetylene functionality into the lauric acid structure (fig. 8c) resulted in effective inhibitors of those P-450s involved in fatty acid ω- and ω-1 hydroxylation. The compounds were active in vivo and in vitro, suggesting the potential for future development of these basic structures as therapeutic agents.

It should be added that it has been demonstrated recently that a range of agents that elicit mechanism-based inactivation of P-450 also generate adducts in which the modified P-450 heme is covalently attached to P-450 apoprotein (27, 28, 42, 108). This pathway appears to be quantitatively significant with as much as 50% of the heme loss produced by AIA attributable to heme-protein adducts (28). The propensity of different P-450s to undergo heme-protein adduct formation with olefinic and acetylenic inactivators has not yet been assessed.

3. 1-Aminobenzotriazole (ABT) and derivatives. Metabolism of ABT produces benzyne which adds across two pyrrole nitrogens of the P-450 porphyrin structure (104) (fig. 9). Early experiments established the potency of ABT in that up to 80% of total P-450 is lost after in vivo administration to rats. As is also the case with AIA, induction of P-450 has been reported. An interesting observation that has been made is that certain MFO activities appear to recover more rapidly than others from the destructive effects of ABT (102). It therefore appears that inactivators may be useful tools to investigate the regulation and relative heme requirements of individual P-450s.

Mathews and Bend (80) prepared two analogs of ABT that were structurally similar to benzphetamine. Both analogs had greater selectivity than the parent ABT as inhibitors of rabbit lung P-450s. Benzphetamine N-demethylation is catalysed by lung P-450 form 2 and was extensively inactivated by the ABT analogs [1-(N-benzylamino)-benzotriazole and 1-(N-α-methylbenzylamino)-benzotriazole; fig. 10], whereas form 5 activity was not affected. An important point to emerge from their study was that the two ABT analogs were actively taken up by lung and accumulated so that only the pulmonary, and not the hepatic, activity was inactivated. The clear possibility that emerges from this study is that structural modification of P-450 inactivators could lead not only to enzyme-specific inhibitors but also to organ-specific inactivators.

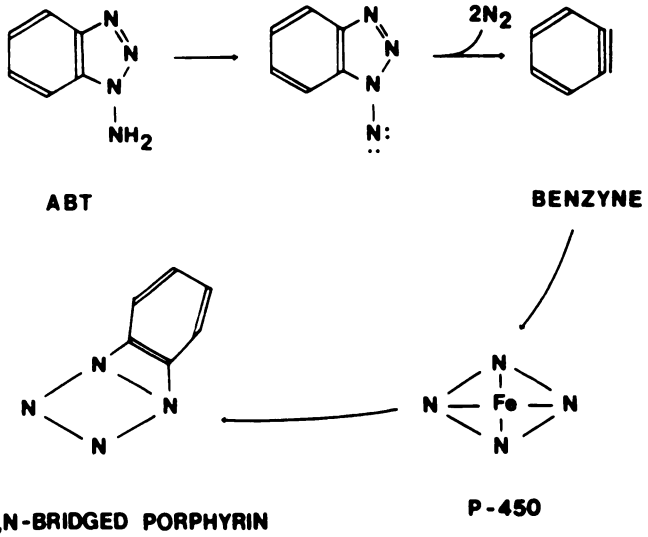

FIG. 9. Formation of benzyne during oxidation of ABT and structure of the resultant N,N-bridged porphyrin.

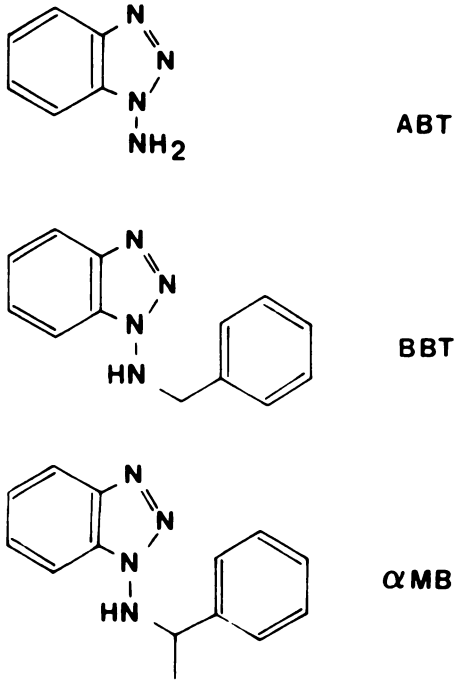

FIG. 10. Structures of ABT analogues. BBT, 1-(N-benzylamino)-benzotriazole; αMB, 1-(N-α-methylbenzylamino)-benzotriazole.

4. Thionosulfur compounds. Thionosulfur compounds have been well documented for their capacity to inhibit MFO activity in vitro and in vivo. The mechanism of inhibition in the case of many of these compounds is

considered to involve the covalent attachment of atomic sulfur to the P-450-active site (30, 98) and perhaps also to the formation of heme-protein adducts, as has been reported for parathion (42). Thus, thionosulfur compounds constitute another class of mechanism-based inhibitors of P-450. A recent report revealed that the thioether diallyl sulfide is a potent and specific inhibitor of the alcohol-inducible P-450 IIE1 that is active as the high-affinity N-nitrosodimethylamine N-demethylase in rat liver (15). In vivo administration of this agent also down-regulated the expression of P-450 IIE1 in rat liver. The relative importance of P-450 inactivation due either to metabolism of the allyl substituent or to covalent binding of atomic sulfur remains to be assessed. Indeed, these forms of direct inhibition must be distinguished from down-regulation of the hepatic content of P-450 IIE1 for the potential value of such compounds to be realised. Nevertheless, diallyl sulfide may be a starting point for the development of P-450 IIE1-specific oxidase inhibitors.

5. Cyclopropylamines. The first systematic study of MFO inhibition by cyclopropylamine derivatives was reported by Hanzlik et al. in 1979 (54). Inhibition potency was enhanced following the preincubation of the cyclopropylamine with NADPH-fortified hepatic microsomes. Therefore, this class of MFO inhibitors was added to the list of documented P-450 inactivators. Unlike the olefins and related agents there was no evidence of N-alkylprotoporphyrin adducts with cyclopropylamines. The mechanism of inactivation of P-450 was initially considered to involve a protonated cyclopropylidiene Schiff base intermediate. The subsequent recognition that the 1-methylcyclopropylamine compound also inactivated P-450 (55, 75), but could not generate an analogous intermediate, led to the suggestion that the reactive metabolite is an aminium ion that undergoes ring expansion to the substituted azetidine that becomes covalently attached to the P-450 enzyme (12, 55) (fig. 11a). These definitive studies have established the mechanism of action of cyclopropylamine inactivators, but the capacity of such compounds to elicit inhibition of specific P-450s has not been evaluated. Several lines of evidence, however, suggest that cyclopropylamines may inhibit P-450 IIB1 preferentially: (*a*) biphenyl 4-hydroxylation in aroclor 1254-induced microsomes was usually more susceptible than the 2-hydroxylation pathway to inhibition by cyclopropylamines (138); (*b*) P-450 IIB1 appears to play a major role in the metabolism of cyclopropylamines to destructive intermediates (12); and (*c*) mechanism-based inactivators are generally potent inhibitors of those enzymes that generate the reactive intermediates (113).

A recent report is consistent with the assertion that cyclopropylamine-substituted P-450 substrates may prove to be potent inhibitors of oxidative pathways mediated by specific P-450s. 17β-(Cyclopropylamino)-androst-5-ene-3β-ol (fig. 11b) was found to be a mechanism-based inhibitor of human testicular P-450$_{17\alpha}$ but was devoid of destructive activity against adrenal P-450$_{scc}$ and P-450$_{c21}$ (4).

6. Mechanism-based inactivators of steroidogenic P-450s. Although the action of inhibitors of aromatase activity such as aminoglutethimide (fig. 12) have been studied and described for many years (123), a relatively recent advance has been the evolution of suicide inhibitors of this unusual P-450 enzyme. Aminoglutethimide does not possess the required selectivity to be an especially valuable therapeutic agent, but mechanism-based inactivators, which are generally analogs of the aromatase substrate AD, appear quite selective and potent. Such compounds include 4-acetoxy- and 4-hydroxyAD, 1,4,6-androstatriene-3,17-dione (17), and the 10β-unsaturated analogs of AD (77) (fig. 12). 4-HydroxyAD has been shown to be an effective agent for the reduction of the size of mammary tumors in rats. In this regard, 4-hydroxyAD has superior potency to tamoxifen and aminoglutethimide (17).

Other potent inhibitors of aromatase have been described that do not elicit inactivation of the enzyme. These include analogs of AD that have the C19 methyl group replaced with a thiirane or oxirane moiety (65) (fig. 13, a and b). However, the specificity of these agents is presently unknown, and it is possible that drug interactions due to the interference of these compounds with hepatic P-450 function could occur. Such interactions, of course, limit the potential utility of thiirane- or oxirane-substituted androgens as therapeutic compounds.

In an interesting study, Osawa et al. (107) noted that the stereochemistry of the 6-bromo substituent in 6-bromoAD was an important determinant of the mechanism and potency of inhibition of human placenta P-450 aromatase activity (107). Thus, the 6α-bromo derivative was a potent competitive inhibitor of the activity (K_i =

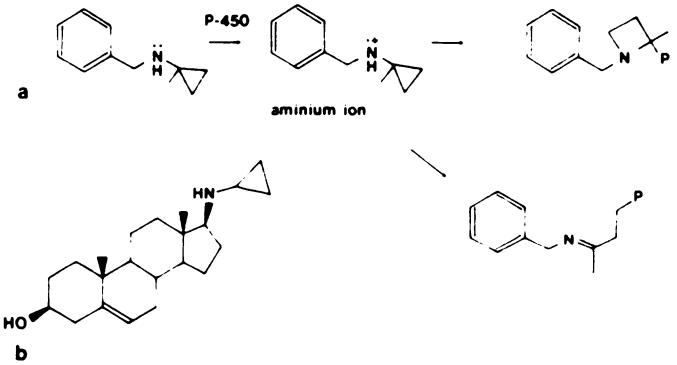

FIG. 11. a, Reaction scheme showing the postulated mechanism of aminium ion formation from N-(1-methylcyclopropyl)benzylamine and covalent attachment of the ring-expanded azetidine to P-450 (P). The alternate pathway involving opening of the cyclopropyl ring and adduction to P-450 is not considered to be sufficiently stable to hydrolysis to be responsible for P-450 inactivation (12, 55). b, Structure of 17β-(cyclopropylamino)-androst-5-ene-3β-ol, a mechanism-based inactivator of the testicular P-450$_{17\alpha}$.

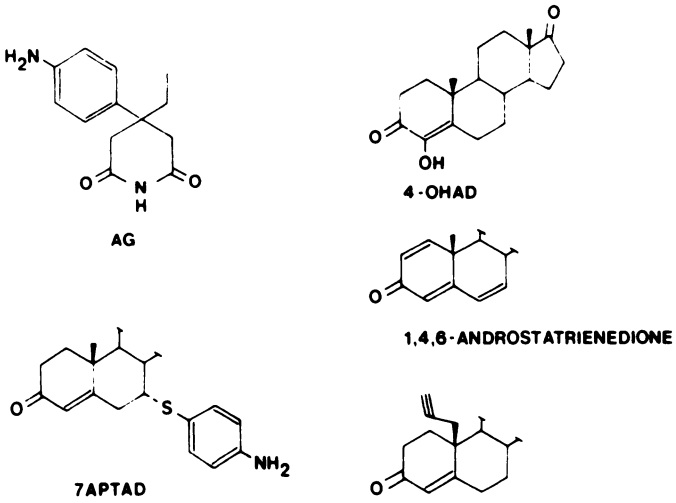

FIG. 12. Aromatase inhibitors. AG, aminoglutethimide; 4-OHAD, 4-hydroxyandrost-4-ene-3,17-dione; 7-APTAD, 7α-(4′-aminophenylthio)-androst-4-ene-3,17-dione.

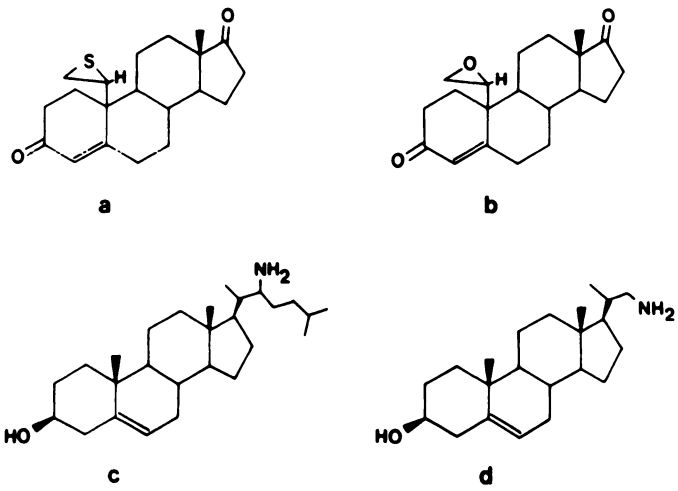

FIG. 13. Inhibitors of aromatase and side-chain cleavage P-450s. Structures a and b are the 10β-thiiranyl- and oxiranyl-derivatives of AD which have been evaluated as aromatase inhibitors. Structures c and d are 22-aminocholesterol and 22-ABC which are side-chain cleavage inhibitors.

3.4 nM), whereas the 6β-epimer elicited mechanism-based inactivation of the enzyme. 6β-BromoAD did not appear to produce substantial inhibition of rabbit hepatic microsomal P-450s. Again, this compound possesses features that could lead to the design of effective and selective P-450 aromatase inhibitors.

There have been reports of inactivation of steroidogenic P-450 activities by acetylenic steroids and related compounds. Interestingly, and in contrast to the situation with hepatic P-450s, such compounds are not considered to produce heme alkylation and abnormal porphyrins with steroidogenic P-450s. Instead, Nagahisa et al. (95) suggested the production of an oxiranyl intermediate that alkylates the P-450 apoprotein. If this assertion is correct, then it is possible that acetylenic steroids may exert their destructive effects by mechanisms that are dependent upon the specific P-450 in question. Danazol is a 17α-ethynyl steroid derivative that decreases testicular P-450 and associated 17α-hydroxylase/17–20-lyase activity after in vivo administration to rats (9). Although not yet explored, the mechanism of this effect could well involve suicide processing of the synthetic steroid to a destructive metabolite.

Spironolactone is a steroidal diuretic that possesses a 7α-acetylthio substituent and undergoes deacetylation to the 7α-thiosteroid which elicits potent destruction of P-450s from hepatic and extrahepatic sources (29, 82). A similar compound, 7α-(4′-amino)phenylthioAD (fig. 12), has been used as an aromatase inhibitor (1). Although 7α-(4′-amino)phenylthioAD appears to be quite effective in this regard, the problems of nonselectivity associated with spironolactone suggest that 7α-(4′-amino)-phenylthioAD should be screened as a potential inhibitor of other P-450s apart from aromatase.

Using isolated adrenal cells, Krueger et al. (68) screened a series of cholesterol analogs as inhibitors of $P\text{-}450_{scc}$ activity. These authors found that the acetylenic derivatives (20*R*)-20-(1-hexynyl)-pregn-5-en-3β,20-diol (20-OH monyne) and (20*R*)-20-(1,5-hexadiynyl)-pregn-5-en-3β,20-diol (20-OH diyne) were mechanism-based inhibitors of $P\text{-}450_{scc}$ activity but exerted their inhibition through protein modification rather than heme alkylation. Both 20-OH monyne and 20-OH diyne appeared selective in their inhibition because these compounds did not inhibit $P\text{-}450_{17\alpha}$, $P\text{-}450_{c21}$, or $P\text{-}450_{11\beta}$ activities (the functional properties of these enzymes are indicated in table 2). However, 20-OH monyne and 20-OH diyne were not as effective in inhibiting $P\text{-}450_{scc}$ activity as the trimethylsilylethyl derivative (20*S*)-20-(2-trimethylsilylethyl)-pregn-5-en-3β-20-diol (TMS). Although all three compounds exhibited similar affinities for $P\text{-}450_{scc}$ (TMS, K_d = 0.8 μM; 20-OH monyne and 20-OH diyne, K_d = 0.4 μM), TMS caused up to 50% greater inhibition of $P\text{-}450_{scc}$ activity in isolated adrenal cells. The authors suggested that the discrepancy between the inhibitory effects of TMS and the acetylenic derivatives is due to the latter being converted, in the cell, to less inhibitory compounds.

The development of chemical inhibitors of aromatase or other steroidogenic P-450s has highlighted the therapeutic benefits that can arise from such an approach. However, many of the compounds have not been assessed for the incidence of drug interactions resulting from the inhibition of other P450s.

7. Alkylamines. It is now well recognised that a number of alkylamine derivatives are converted by P-450 to reactive intermediates that sequester the cytochrome in a catalytically inactive complexed state. The proximate complexing metabolite is considered to be the nitroso species that is involved in a quasicovalent ligand interaction with the P-450 heme iron (76). One of the first alkylamines shown to elicit MI complexation was SKF

525-A (10, 125), but recent studies have indicated the lack of selectivity of this compound as a P-450 inhibitor (86). Thus, catalytic data suggest that SKF 525-A generates a complex with the P-450s IIB1, IIC11, and IIIA1/2 in vivo, whereas P-450 IIA1-dependent steroid 7α-hydroxylation was not complexed by this agent.

Other alkylamines are metabolised to reactive intermediates that generate complexes with specific P-450s. For example, macrolide antibiotics like triacetyloleandomycin and erythromycin seem to be exclusive MI complexation agents for the glucocorticoid-inducible P-450 (P-450 IIIA1) (46). Human microsomal fractions are also able to generate the complex (111) and consequently this interaction is now held to be responsible for prolonged inhibition of the elimination of coadministered drugs such as cyclosporin A (78) and carbamazepine (139).

Studies from this laboratory have shown the induction and MI complexation of P-450 IIB1 in rat liver by the antiparkinsonian agent orphenadrine (114) (fig. 14). The drug also generated an MI complex in vitro in microsomes from PB-induced rat liver and appeared to be a selective inhibitor of P-450 IIB1 activities. Thus, orphenadrine was a potent inhibitor (K_i = 30 μM) of P-450 IIB1-mediated AD 16β-hydroxylase activity (K_m = 200 μM) in PB-induced rat hepatic microsomes. More recently, we have noted the inhibition of progesterone 21-hydroxylation (K_i = 150 μM) catalysed by the constitutive and PB-inducible P-450 IIC6 (K_m = 8 μM) in rat liver (unpublished observations). Orphenadrine, however, is a far more potent inhibitor/complexation agent toward IIB1 (K_m/K_i ratio = 6.7) than IIC6 (ratio = 0.053). Diphenhydramine, an antihistamine that is structurally very similar to orphenadrine, is also an efficient MI complexation agent of P-450 IIB1 in vitro and in vivo in rats. The related alkylamines, tripelennamine and pyrilamine, were less able to generate a complex with P-450, so that the existence of the alkylamine moiety does not necessarily result in complexation (unpublished observations). Indeed, it appears that bulky lipophilic alkylamines such as tamoxifen and hydrophilic alkylamines with other structural features that may interact with P-450 (e.g., the nitrogen atom of the pyridine system of pyrilamine) are not able to elicit MI complexes (115). These observations suggest that, to generate MI complexes, metabolism of the alkylamine to a nitroso derivative must be a major transformation reaction. Alternate sites of metabolism within a particular drug structure detract from MI complexation.

The structural features that give rise to MI complexation characteristics of the various alkylamines need to be established. The MI complex is only stable in the ferrous oxidation state, and no evidence is available to suggest that the nitroso products of alkylamine metabolism can leave the active site of the P-450 in which they are formed. Thus, it is apparent that the P-450s that

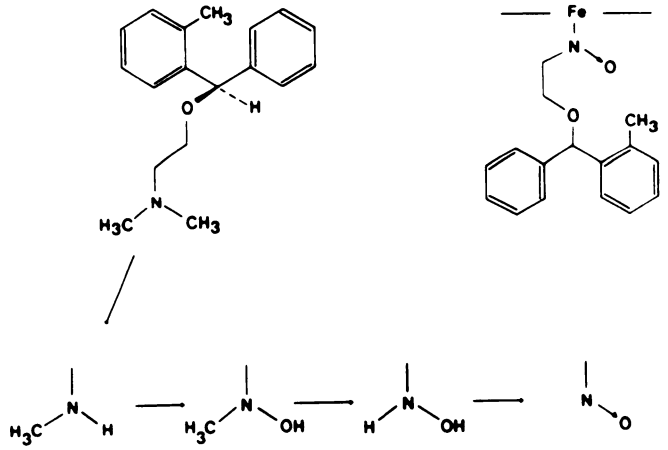

FIG. 14. Oxidative metabolism of orphenadrine to the nitroso analogue that generates an MI complex with P-450 (the structure of the orphenadrine metabolite-ligating species is shown at the top right).

undergo complexation by the various alkylamine agents are those in which the hydroxylamine precursor metabolite undergoes oxidation to the nitroso species. It may well be, however, that P-450s not subject to MI complexation are involved in earlier steps in the pathway leading to P-450 sequestration. Certainly, we have observed that a reconstituted system incorporating highly purified P-450 IIB1 does not generate an MI complex with orphenadrine (unpublished data). The inference that we have drawn from this finding is that P-450 IIB1 catalyses at least the final step in MI complex formation but clearly can not catalyse all of the reactions necessary for MI complexation to occur. Elucidation of the structural requirements for MI complexation of various P-450s by alkylamine agents will facilitate the development of selective MI complexation agents. In addition, because the alkylamino group is common to many active drug molecules, detailed knowledge of the P-450s involved in MI complexation will enable the prediction of serious pharmacokinetic interactions with other drugs.

8. Methylenedioxyphenyl compounds. The insecticide synergist piperonyl butoxide has been used as an inhibitor of microsomal MFO activity for many years. It is a standard tool for the elucidation of the role of P-450 in toxicological processes. Part of the inhibitory action of piperonyl butoxide and related methylenedioxyphenyl compounds is due to the formation of an inhibitory MI complex with P-450. There is now evidence that certain forms of P-450 are preferentially complexed by reactive metabolites of methylenedioxyphenyl compounds. Isosafrole generates an MI complex with the PB-inducible P-450 IIB1 in vivo (93) and in vitro (89), and the polycyclic aromatic hydrocarbon-inducible P-450 IA2 in vivo (120, 141), but does not elicit MI complexation of P-450s IA1, IIC11, or IIA1 (93). Because steroid 6β-hydroxylation is reactivated after the dissociation of the isosafrole metabolite from its complex in isosafrole-induced rat liver, one of the P-450 IIIA subfamily must also be complexed (93). However, in vitro studies revealed the

TABLE 3
Inhibitors with specificity toward P-450 enzymes

Protein/subfamily	Inhibitor	Mechanism of inhibition	References
IA1	α-Naphthoflavone*	Reversible	62, 71, 136
	Ellipticine and 9-hydroxyellipticine*	Reversible	59, 71, 148
	2-Bromo-4′-nitroacetophenone	Protein alkylation	109
IIB1	Secobarbital	Heme/protein alkylation	74
	Orphenadrine†	MI complexation	114
	Diphenhydramine	MI complexation	Unpublished data
	α,α-Dichlorotoluene	Heme/protein alkylation	47
IIB4	1-(N-Benzylamino)- and 1-(N-α-methyl-benzylamino)-benzotriazole	Inactivation	80
IIC5/IIC6	17β-Vinyl- and 17β-ethynylandrost-4-ene-3-one	Inactivation	49
	22-Amino-23,24-bisnor-5-cholen-3β-ol (in hepatic tissue)	Reversible	63
IID	Ajamalicine	Reversible	36
IIE1	Diallyl sulfide	Protein alkylation and covalent binding of atomic sulfur	15
IIIA1	Triacetyloleandomycin and erythromycin	MI complexation	46
IIIA4	17α-Ethynylestradiol	Inactivation	43
IVA	Terminal acetylenic fatty acids	Inactivation	18, 19
XIA	(20*S*)-20-(2-Trimethylsilylethyl)-pregn-5-en-3β,20-diol	Protein alkylation	68
	(20*R*)-20-Phenylpregn-5-en-3β,20-diol	Reversible	140
	(22*R*)-22-Aminocholesterol	Reversible	94
XVIIIA1	17β-(Cyclopropylamino)-androst-5-ene-3β-ol	Inactivation	4
XIXA1	4-Acetoxy-, 4-hydroxyandrost-4-ene-3,17-dione and 1,4,6-androstatriene-3,17-dione	Inactivation	17, 77

* P-450 IA2 is also inhibited by these compounds.
† P-450 IIC6 is also subject to MI complexation with orphenadrine.

absence of MI complexation of these P-450s (89). Therefore, either complexation of P-450 IIIA members occurs only in vivo and not in vitro or was not observed in vitro due to the absence of the P-450 IIIA member from the microsomal fraction involved in complexation.

An interesting observation was reported by Dahl and Brezinski (25) who found the methylenedioxyphenyl compound piperonal to be an inhibitor of nasal hexamethylphosphoramide N-demethylase activity. Piperonal has been shown on numerous occasions to be an ineffective inhibitor of hepatic P-450 activity because it is readily oxidised to piperonylic acid and eliminated. The differential susceptibility of the nasal and hepatic activities to inhibition suggests that organ specificity may be an additional complication in the design of P-450-specific or P-450-preferred MI complexation agents.

9. Inhibitors of fatty acid oxidation. P-450s active in fatty acid oxidation are present in many tissues. It has been observed that the hydrophilic imidazoles dazmegrel and dazoxiben are inhibitors of the platelet P-450 thromboxane synthetase (35) but are poor inhibitors of hepatic P-450s that are usually inhibited by imidazoles with greater hydrophobicity (88). Terminal acetylenic fatty acids are documented inhibitors of the leukotriene B_4 ω-hydroxylase of human polymorphonuclear leukocytes (129) and the hepatic lauric acid ω- and ω-1 hydroxylases (106). Xenobiotic hydroxylase activities were uninhibited by these compounds, again indicating the value of mechanism-based inactivators as pharmacological tools.

A series of substituted eicosatetraenoic acids inhibited hepatic microsomal arachidonic acid metabolism (to prostacyclin) (20). The aziridine-containing analog was an effective inhibitor of ω- and ω-1 hydroxylation, but the capacity of these agents to modulate other P-450s involved in hepatic xenobiotic oxidation needs to be estimated for any specificity of P-450 inhibition to be apparent.

10. Reversible inhibitors of specific P-450 enzymes. Many compounds that reversibly inhibit cytochrome P-450 have been described (85, 99, 136). However, few of these compounds exhibit selectivity in the inhibition of P-450 enzymes. Two classes of reversible inhibitors that have been utilised successfully as selective probes are the ellipticines and benzoflavones (62, 71). Both these chemical classes show preference for the inhibition of P-450 IA subfamily catalytic activity. 7,8-Benzoflavone (α-naphthoflavone) has been shown to be a potent inhibitor of reconstituted rabbit liver P-450 IA2 biphenyl 4-hydroxylation (IC_{50} = 27 nM) and 7-ethoxyresorufin-O-deethylation (IC_{50} = 10 nM) (62). In contrast, P-450 IIB4-dependent activity was only inhibited to a minor extent at an α-naphthoflavone concentration of 240 μM. Similarly, ellipticine and 9-hydroxyellipticine have been shown to exhibit preference for the inhibition of P-450 IA1/2 activity (59, 148).

By introducing inhibitory groups such as the primary amino function into the steroidal nucleus, several au-

thors have successfully produced highly potent, and sometimes selective, reversible inhibitors of P-450. Using this approach, Sheets and Vickery (130) synthesised a cholesterol analog, 22-amino-23,24 bisnor-5-cholen-3β-ol (22-ABC; fig. 13d) which exhibited potent inhibition of reconstituted P-450$_{scc}$ (IC_{50} = 0.1 μM). However, despite its apparent high affinity for P-450$_{scc}$, 22-ABC was shown to be rather nonspecific, with reports indicating potent inhibition of both P-450 IIC5 (K_i = 0.1 nM) (63) and P-450$_{17\alpha}$ activities (132). In an attempt to mimic the nonpolar side-chain of cholesterol, Vickery and Kellis (140) introduced a terminal hydrophobic phenyl substituent in the C20 position of cholesterol to produce (20*R*)-20-phenyl-5-pregnene-3β-20-diol. This cholesterol analog was a potent inhibitor (K_i = 30 nM) of bovine P-450$_{scc}$ activity (K_m = 100 μM) and, unlike 22-ABC, did not inhibit P-450$_{17\alpha}$ or P-450$_{c21}$ activities. By varying the side-chain length of a series of alkylamine-substituted cholesterol analogs, Sheets and Vickery (131) observed potent inhibition when the terminal amino group was in either the 22- or 23-position. Indeed, the analog 23-amino-24-nor-5-cholen-3β-ol was found to be a potent inhibitor (IC_{50} <500 nM) of P-450$_{scc}$ activity in bovine adrenal and placental tissue, human adrenal (neonate and adult) and placental tissue, and rat adrenal and testicular tissue (66). As with 22-ABC, the short side-chain of 23-amino-24-nor-5-cholen-3β-ol may enable this compound to be coordinated into the active site and to inhibit the activity of other P-450 enzymes.

In an attempt to increase the specificity for P-450$_{scc}$ inhibition Nagahisa et al. (94) synthesised a cholesterol analog with an amino group projecting from C22 of the side-chain. This analog, (22*R*)-22-aminocholesterol (fig. 13c), exhibited potent inhibition (K_i = 30 nM) of reconstituted bovine P-450$_{scc}$ activity (K_m = 50 μM). Although its potential to inhibit other P-450s was not examined, by maintaining the full cholesterol side-chain it would be expected that this analog would be more specific in its inhibition than shorter chain analogs such as 22-ABC.

11. Inhibition of human P-450s. The pharmacological and toxicological consequences of inhibition of human drug-metabolising enzymes is well recognised. However, until recent advances in the identification of the multiplicity of the human liver P-450 system, many studies were simply descriptive. There are a number of chemical agents that are now known to inhibit specific human P-450s. One of the commonly used probes is sulfaphenazole, which appears to be a selective inhibitor of tolbutamide hydroxylase activity (K_i = 0.22 μM) (6). Similarly, quinidine is a potent inhibitor of debrisoquine 4-hydroxylase (IC_{50} = 3.6 μM) compared to its stereoisomer quinine (223 μM) (67). Much interest has surrounded the debrisoquine 4-hydroxylase enzyme because this is a P-450 subject to genetic polymorphism. The inhibition of this activity in a poor metaboliser subject could lead to significant problems in drug therapy. Fonne-Pfister and Meyer (36) recently observed the potent inhibition of P-450 IID6 (the debrisoquine and bufuralol hydroxylase) in human liver. Phenylcyclopropylamine (refer to section IIIB5) was apparently a noncompetitive inhibitor with a K_i = 31 μM but, in view of the established P-450 inactivation properties of this compound, the mode of inhibition should be reassessed. In the same study a number of alkaloids were found to be potent inhibitors of P-450 IID6 activity. These included α-lobeline (K_i = 123 nM) and, in particular, ajmalicine (K_i = 3.3 nM). Again, other P-450 reactions were not screened, so the selectivity of these inhibitors for P-450 IID6 can not be assessed. Another point to emerge from this study is the finding that diphenhydramine is a potent inhibitor of P-450 IID6 in human liver. Because this drug is known to generate an MI complex in rat liver, future studies could evaluate the effect of preincubation of diphenhydramine and NADPH-supplemented human liver microsomes on its potency as an inhibitor of P-450 IID6.

The anticancer agent teniposide, which is chemically similar to podophyllotoxin, was found to be an inhibitor of mephenytoin 4-hydroxylation catalysed by P-450$_{MP}$ but was not an inhibitor of P-450 IID6-dependent bufuralol 1′-hydroxylation (116).

Bocker and Guengerich (11) reported the inhibition of P-450$_{NF}$ (a major P-450 present in human liver and which is also termed P-450 IIIA4) by 4-alkyl and arylalkyl dihydropyridines that are structurally similar to nifedipine. These agents had previously been reported to inactivate rat hepatic P-450s (now known to involve the P-450s IIC11, IIC6, and members of the IIIA subfamily preferentially) (24). In similar fashion, P-450 IIIA4 is inactivated by 4-alkyl and arylalkyl derivatives during oxidative metabolism to the pyridine. Thus, it appears that an alkyl radical is lost from the 4-position of the dihydropyridine. The inhibition is mechanism based but not suicidal because the carbon radical can be trapped outside the P-450-active site and may travel to other P-450s. At this stage it is not clear which P-450s are involved in this process. Apparently, P-450s involved in phenacetin O-deethylation and hexobarbital 3′-hydroxylation are also susceptible to inhibition to inhibition by 4-alkyldihydropyridines. Another mechanism-based inactivator of P-450 IIIA4 appears to be 17α-ethynylestradiol which is able to inhibit its own metabolism (2-hydroxylation of 17α-ethynylestradiol is catalysed by P-450 IIIA4) (43). The recognition that these processes occur in human liver serves to validate the relevance of many animal studies and facilitates our understanding of drug interactions in man.

4-Ethyl-2,6-dimethyl-3,5-bis(ethoxycarbonyl)-1,4-dihydropyridine (fig. 4h, R = ethyl) reportedly generates heme-protein covalent adducts during oxidative metabolism in hepatic microsomes from PB-induced rats (24, 108). Future studies may well be directed toward the

identification of the P-450s involved in the heme-protein adduction process and whether the process is relevant to the human situation.

C. Significance of Inhibition of Pathways Catalysed by Distinct P-450s

Compared to studies obtained in hepatic microsomes from experimental animals, studies of the inhibition of individual human microsomal P-450s by drugs and other chemicals are in their infancy. Nevertheless, interesting data have already been documented that will ultimately identify the capacity of chemicals to modulate particular P-450 reactions. The value of these studies lies in the fact that detailed mechanistic information will be provided to account for drug interactions and toxicity at a molecular level. Ultimately, it may be possible to design therapeutic agents that are analogs of the inhibitory compounds but which have a decreased capacity to elicit inhibition. This is one practical consequence of the development of probes with high (or low) affinity for particular P-450s.

Another use for probes that inhibit specific P-450 enzymes is in the mapping of active sites. The elegant studies of Ortiz de Montellano and coworkers (69, 100) have utilised the capacity of certain alkenes to generate regiospecific N-alkylporphyrin adducts to obtain information relating to substrate orientation and the location of the P-450 apoprotein-active site. Similar studies with MI complexation agents could also yield valuable information, as could the quantitation of relationships between chemical structure and inhibition potency toward different P-450s.

Eventually, it should be possible to delineate the differences between active sites and catalytic properties of P-450s. Furthermore, the properties of inhibitor probes that result in modulation of oxidase activity may also be defined. The development of such selective probes will be of great value in the understanding of toxicological processes and for the elucidation of the specific oxidative enzymes involved in biotransformation pathways such as those leading to the activation of xenobiotics to toxic intermediates.

IV. Summary and Future Trends

The relatively recent assignment of a number of hepatic and extrahepatic activities to the function of individual P-450s has led to the development of inhibitor probes that exhibit some degree of specificity for different P-450s. These probes will have great utility in the understanding of the P-450-active site topology and the catalytic function of different P-450s and will facilitate the elucidation of the mechanisms of toxic interactions. Eventually, therapeutic agents could be produced that have the required biological effects, without unrequired side-effects and toxicity.

Mechanism-based inactivators and MI complex-forming agents appear to have the greatest value in the development of P-450-selective inhibitors. Because these compounds rely upon the catalytic function of one (or a relative few) P-450(s), the design of appropriate probes with the desired sensitivity has a greater chance of success. It is less likely that reversible inhibitors, especially those containing functions such as an imidazole ring or amino grouping, will have the requisite selectivity to be useful as drugs without side-effects or as specific biochemical probes.

A current list of chemical agents that are preferential inhibitors of specific P-450 enzymes is given in table 3. These compounds constitute a useful starting point for the design, synthesis, and development of inhibitors of individual P-450s. Studies to date have clearly revealed interesting structure inhibition trends. For example, Halpert and coworkers (47, 49, 134) have successfully manipulated the propensity of dichloromethyl compounds to inactivate different rat and rabbit P-450s. The MI complexation selectivity of alkylamines appears to be similarly dependent upon the physicochemical nature of the remainder of the molecule. Future studies will need to address these factors so that the design of rational drug therapy and the development of agents of practical value in biotransformation studies will eventually be realised.

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