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STRUCTURAL BASIS OF SELECTIVE CYTOCHROME P450 INHIBITION

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ABSTRACT

Isoform-selective cytochrome P450 inhibitors have greatly facilitated the characterization of the catalytic specificities and pharmacological and toxicological significance of individual P450 enzymes in experimental animals and humans. Recent advances in elucidating the enzymatic determinants of P450 substrate specificity now make it possible to explore how complementary properties of inhibitors and their target enzymes dictate inhibitor selectivity. A thorough understanding of the basis of specificity should lead to the rational design of a new generation of structure-based cytochrome P450 inhibitors for use as probes and modulators of P450 function in vivo.

INTRODUCTION

Cytochromes P450

Cytochromes P450 constitute a superfamily of hemoproteins that play a pivotal role in the metabolism of a wide variety of xenobiotics and endogenous compounds. Cytochromes P450 are classified based on structure into gene families and subfamilies, with members of the same family exhibiting at least 40% amino acid sequence identity and members of the same subfamily ≥55% identity (1). Three gene families (*CYP1, CYP2,* and *CYP3*) are thought to be responsible for most hepatic xenobiotic metabolism (2). Despite the fact that

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many of these enzymes exhibit partially overlapping substrate specificities, under many conditions a single P450 may be exclusively or primarily responsible for the detoxification or bioactivation of a particular compound. Accordingly, the phenotype of an individual human or experimental animal with respect to the amounts of particular P450 forms expressed in a given organ can determine the metabolism and pharmacological activity or toxicity of a compound. Detailed knowledge of the substrate specificities and regulation of cytochromes P450 is therefore crucial for predicting and/or rationalizing species, strain, and individual differences in xenobiotic metabolism as well as metabolic interactions between compounds (2–7).

The Scope of This Review

The emergence in recent years of a battery of isoform-selective chemical inhibitors that can be used in vitro and in vivo in experimental animals and humans has greatly facilitated the identification of individual cytochromes P450 responsible for specific bioactivation and detoxification reactions (8). Many of these inhibitors are mechanism based and owe their selectivity to metabolism by the target enzyme (8–13). Several excellent recent reviews have addressed how specific classes of compounds such as olefins and acetylenes, as well as nitrogen-, sulfur-, and halogen-based compounds, cause P450 inhibition and/or inactivation (11–14). Very recent advances in our understanding of the basis of P450 substrate specificity (15) now make it possible to begin to explore how complementary properties of the inhibitors and the enzymes dictate inhibitor selectivity (8). Therefore, a major focus of this review is the chemical and enzymatic determinants of selectivity of P450 inhibition and inactivation. Ultimately, a thorough understanding of the basis of specificity should lead to the rational design of a new generation of structure-based cytochrome P450 inhibitors. Such compounds would be invaluable not only as diagnostic tools but also as therapeutic agents for modulation of human drug metabolism (3, 16).

DETERMINATION OF P450 SUBSTRATE SPECIFICITY

General

In recent years a general strategy for determining the catalytic activities of a cytochrome P450 has emerged (5, 17, 18). This involves (*a*) correlation of the activity under consideration with known marker activities for individual P450s or immunochemically determined levels of individual P450s, (*b*) selective inhibition or stimulation, (*c*) immunoinhibition, (*d*) enzyme purification and reconstitution, and (*e*) heterologous expression. Each approach has certain inherent advantages and disadvantages. For example, chemical inducers can

be used to address the pharmacological or toxicological consequences of increased metabolism of a compound by a subset of cytochromes P450. However, lack of selectivity is often a problem, and identification of inducible enzymes responsible for catalyzing a particular reaction does not necessarily define the P450s involved in the constitutive state. In contrast, antibodies are often highly specific but may not always be readily available and are restricted to in vitro use. Purification and reconstitution may be the only approach available if a known cytochrome P450 is not involved in catalyzing the reaction of interest; however, isolating a new P450 can be labor intensive, and reconstitution is not always straightforward (19). Demonstration of substrate metabolism following heterologous expression of a cDNA indicates unambiguously that a P450 of defined sequence can catalyze a particular reaction, but rates may be difficult to interpret unless careful kinetic analysis (K_m and V_{max}) is performed (18, 20). Therefore, definitive assessment of the role of an individual P450 in a given metabolic pathway can only be accomplished by using the entire battery of experimental approaches (5, 8, 17).

Use of Inhibitors for Determining Substrate Specificity

Chemical inhibitors have proven valuable for attributing specific metabolic reactions to individual cytochromes P450. These compounds are simple to use and readily available. In addition, chemical inhibitors can be used with intact cells and in vivo, thus making it possible to link a particular cytochrome P450 with a specific toxicological or pharmacological response (21). Until recently the major drawback with most available cytochrome P450 inhibitors was poorly defined or incomplete selectivity. However, during the past five years a number of laboratories have devoted their efforts to identifying and/or designing compounds of sufficient selectivity to target individual P450 forms preferentially (8, 11, 13). Many of these inhibitors are mechanism based and require metabolism by the target enzyme into intermediates or products that inactivate the enzyme. Because all or some fraction of the enzyme is destroyed, residual activity should be the same regardless of the substrate tested. In contrast, with a competitive inhibitor, the activity toward a particular substrate will depend on the K_m and K_i and the concentrations of the inhibitor and substrate.

The high degree of selectivity often obtainable with mechanism-based inactivators makes them particularly attractive for in vivo applications (11–13). A further advantage of mechanism-based inactivators in vivo is that the enzyme inhibition persists after the free compound has been cleared. A reversible inhibitor, by contrast, must be present continuously (12, 13). However, even compounds of high selectivity require cautious use. In particular, the dosing regimen and time frame of the experiment may be crucial in vivo, and acute inhibition and/or inactivation of one P450 may be accompanied by subsequent induction of that or another P450 form (21, 22).

OTHER USES OF P450 INHIBITORS

Although oxygen transfer by cytochromes P450 was first thought to occur by means of a concerted two-electron mechanism, it is now generally accepted that radicals are transient intermediates in most P450-catalyzed oxidations (23–25). Key evidence of sequential one-electron transfer reactions in substrate oxidation has been obtained in studies of mechanism-based inactivators, including substituted olefins (26), cycloalkylamines (27, 28), and 4-alkyl-1,4-dihydropyridines (29). Another promising use of irreversible inhibitors is as active-site-directed reagents for identifying functionally important amino acid residues (30–34).

Recent advances in protein biochemistry and mass spectrometry have made possible identification of active-site-modified peptides using mechanism-based inactivators or photoaffinity labels. Inhibitors have also been used in chemical models of acute hepatic porphyria (35) and for studying cytochrome P450 turnover (36–38). For these purposes, selectivity is not necessarily an advantage. Selectivity is of paramount importance, however, when inhibitors are used to address the physiological role of cytochrome P450–catalyzed metabolism of endogenous compounds such as prostaglandins (39, 40) and arachidonic acid (41).

GENERAL MECHANISMS OF CYTOCHROME P450 INHIBITION

P450 Catalytic Cycle

The catalytic mechanism of cytochromes P450 consists of at least seven discrete steps (24, 25): 1. substrate binding to the ferric form of the enzyme; 2. reduction to the ferrous form by NADPH–cytochrome P450 reductase; 3. binding of molecular oxygen; 4. introduction of a second electron from P450 reductase and/or cytochrome b_5; 5. dioxygen bond cleavage, releasing water and forming the active oxidizing species; 6. substrate oxygenation; and 7. product release. Impairment of any one of these steps can lead to inhibition of monooxygenase activity. Such inhibition may be reversible (competitive, noncompetitive, uncompetitive, mixed) or irreversible depending on the interaction between the inhibitor and the enzyme and the particular step(s) in the catalytic cycle that are blocked. For example, any two alternate substrates for the same enzyme will exhibit competitive inhibition. The clinical relevance of such competition in terms of drug-drug interactions in humans depends on the relative affinities of the enzyme for the two compounds, the concentrations achieved at the active site after therapeutic doses, the role of the cytochrome P450 in the overall elimination of the two compounds, and the therapeutic

index (3). Such competitive interactions may be particularly relevant in the case of human P450 3A enzymes, which have broad substrate specificities (3). In general, however, the most potent and/or selective cytochrome P450 inhibitors rely on an additional mode of interaction with the enzymes.

Reversible Inhibition by Nitrogen Heterocycles

Among the most potent and potentially selective reversible inhibitors of cytochromes P450 are nitrogen heterocycles, including imidazoles, quinolines, pyridines, and ellipticines (3, 14, 16, 42). These compounds rely on hydrophobic interactions with the P450 protein moiety as well as specific interactions between the lone electron pair on the nitrogen and the heme iron. Such coordination with ferric cytochrome P450 results in a spectrum with the Soret band shifted to 420–425 nm, or a Type II spectrum in the difference mode (43). Binding to ferrous cytochrome P450 can also occur, characterized by a spectral complex centered around 445–450 nm (42). The capacity of nitrogen heterocycles to bind with high affinity to both the oxidized and reduced forms of cytochrome P450, and to both the protein and heme moieties, confers the ability to interfere not only with substrate but also with oxygen binding. This accounts for the potent inhibition by such compounds of hepatic and extrahepatic cytochromes P450. The ability of the compounds to discriminate among different cytochromes P450 depends on the tightness of the fit to the active site and whether the binding orientation allows coordination of the nitrogen with the heme iron (14, 16). An example of a useful and selective heterocyclic inhibitor of human cytochrome P450 2D6 is quinidine (3, 44), whereas numerous drug-drug interactions can be attributed to inhibition of human cytochrome P450 3A4 by ketoconazole (45).

Metabolite Intermediate Complexes

The selectivity of an enzyme inhibitor can be enhanced greatly by including a step involving catalysis by the target enzyme in the mechanism of inhibition. This is especially true in the case of the cytochrome P450 superfamily, with its multiplicity of related enzymes with similar binding sites. Incorporating a latent functional group into the inhibitor restricts catalysis-dependent inhibition to those cytochromes P450 that are capable of binding the compound, with the group in question located in appropriate proximity to the iron-bound active oxygen. Such inhibition can be reversible, involving the formation of metabolite intermediates that sequester the P450 in a catalytically nonfunctional state (13, 42). Chemical structures associated with metabolite intermediate complexes include alkylamines, hydrazines, and methylenedioxybenzene derivatives (13, 14, 42, 46–48).

Among the best-studied alkylamines are the macrolide antibiotics and am-

phetamine derivatives. These compounds are converted through multiple P450-dependent steps to nitroso derivatives that bind with high affinity to the reduced heme iron of cytochromes P450 and prevent oxygen binding. Although the complex with the ferrous enzyme is often so stable that the protein can be isolated with the metabolite intermediate complex intact (49), oxidation to the ferric state results in rapid decomplexation and restoration of enzyme activity (50). This provides a dual-pronged approach to using such inhibitors: One can monitor either a decrease in activity toward a particular substrate following complexation of selected cytochromes P450 or an increase in activity following decomplexation.

Among the alkylamines, the macrolide antibiotic triacetyloleandomycin has found the most widespread use as a selective inhibitor of cytochrome P450 3A enzymes in several species, including humans (17). A number of drug-drug interactions involving the commonly used antibiotic erythromycin may also involve metabolite intermediate complex formation with P450 3A enzymes (3, 14). In contrast to the alkylamines, methylenedioxybenzenes are converted to metabolites (possibly carbenes) that form a complex with both the ferrous and ferric form of cytochromes P450 (13, 42). The complex with the ferric form is dissociable by lipophilic compounds, resulting in reactivation of monooxygenase activity (51). Cytochrome P450 inhibition by methylenedioxybenzene compounds has been utilized extensively in the development of insecticide synergists (42).

Mechanism-Based Inactivators

The most selective cytochrome P450 inhibitors generally fall into the class known as mechanism-based inactivators. These are substrates for the target enzyme and are converted to reactive species, which interact with the enzyme and cause loss of function (9–12). As the term "inactivation" implies, modification of the enzyme or its prosthetic group is covalent, and the loss of function irreversible. The kinetics of the inactivation process can be described by the minimal scheme shown in Figure 1. Mechanism-based inactivation is a time-dependent, pseudo-first-order process and obeys saturation kinetics. Therefore, just as V_{max} and K_m characterize a normal enzyme catalyzed reaction, inactivation can be characterized by a maximal rate constant (k_{inact}) and by an inhibitor constant (K_I). A marked difference in either constant between two different cytochromes P450 can result in selective inactivation. The rate constant for inactivation reflects not only the rate of conversion of the inhibitor to reactive intermediates, but also the partition ratio. This ratio is a measure of the efficiency with which the reactive species inactivates the enzyme, as opposed to rearranging to stable products (9, 10, 12). Selectivity based on a difference in the maximal rate constants for inactivation is most desirable, but

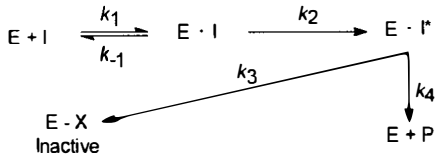

Figure 1 Minimal kinetic scheme for mechanism-based inactivation. The inactivator binds reversibly to the enzyme to produce an EI complex, governed by a dissociation constant (k_{-1}/k_1). In the presence of NADPH and NADPH–cytochrome P450 reductase, the compound is converted with a rate constant k_2 to a reactive intermediate (EI*). The reactive species may either covalently modify the enzyme (E–X) with a rate constant k_3 or dissociate from the active site with a rate constant k_4 to yield a stable product P. The partition ratio (k_4/k_3) designates the number of times product is released from the enzyme per inactivation event.

in the case of a large difference in K_I between two enzymes, a high level of selectivity may be attained at the appropriate inhibitor concentration in vitro or the appropriate dose in vivo (52, 53).

The covalent modification of cytochromes P450 by mechanism-based inactivators can occur by three different processes. The most thoroughly studied involves alkylation of a pyrrole nitrogen in the prosthetic heme group by reactive intermediates formed from such compounds as olefins, acetylenes, and dihydropyridines (11, 12, 22). Cytochrome P450 is the major consumer of heme in hepatocytes, and alkylation of P450 heme often results in perturbation of hepatic heme metabolism and induction of porphyria (8, 22, 54). A second mechanism involves the generation of intermediates that activate the heme moiety into reactive species that bind to the protein moiety (55). In the case of cumene hydroperoxide-mediated inactivation of cytochrome P450 2B1, the site of attachment of the activated heme group has been localized to a peptide that includes Cys-436, the conserved residue that acts as the fifth ligand to the heme iron (31). Covalent binding of heme to the protein moiety of cytochromes P450 appears to brand them for proteolytic degradation (36–38).

The third mechanism of P450 inactivation involves alkylation or acylation of the protein moiety. The best-studied examples of this include chloramphenicol (30, 56) and certain acetylenic fatty acids (57) and polycyclic aromatic hydrocarbons (32–34, 58–60). Chloramphenicol is converted to an oxamyl chloride that inactivates cytochrome P450 2B1 by modifying one or more lysine residues in the protein and preventing electron transfer from NADPH-cytochrome P450 reductase (30). 2-Ethynylnaphthalene is converted by cytochromes P450 2B1 and 2B4 to a ketene which modifies an active-site peptide that includes Thr-302 (33, 34), a highly conserved residue thought to play a role in oxygen activation (61).

A compound may modify a P450 by more than one mechanism, as has been described for cytochrome P450 2B1 inactivation by a number of chloramphenicol analogues (62) or by secobarbital (63). Compounds that act solely by formation of *N*-alkyl- or arylporphyrins and leave the protein moiety intact may allow functional repair of the cytochrome P450 upon acceptance of fresh heme (64), whereas modification of the protein moiety by the inactivator or by heme fragments precludes functional restoration (8, 36, 37).

The multiplicity of mechanisms of P450 inactivation has intriguing consequences. For example, inactivation by 3,5-dicarbethoxy-2,6-dimethyl-4-ethyl-1,4-dihydropyridine of rat liver P450 2C6 and 2C11 occurs mainly through *N*-ethylation of the heme moiety, whereas P450 3A is destroyed mainly by prosthetic heme alkylation of the apocytochrome (36). Although both 2C6 and 2C11 are inactivated by the same general mechanism, only the latter enzyme appears to exhibit increased susceptibility to proteolysis. The increased proteolysis of 2C11 but not of 3A can be blocked by exogenous hemin. Thus, the fates of three different cytochromes P450 following inactivation by a single compound may be very different, resulting in differences in the duration of enzyme inhibition in vivo (8, 36). 10-Undecynoic acid is another example of a single inhibitor that inactivates two cytochromes P450 by different mechanisms. This compound inactivates rat liver P450 2B1 by heme *N*-alkylation but inactivates 4A1 by protein acylation (57). Apparently, enzymatic constraints in P450 4A1 cause activated oxygen to be delivered to the thermodynamically disfavored terminal carbon of the acetylenic group, leading to ketene formation and protein modification rather than heme destruction. Very recent results suggest that high-affinity binding or precise orientation of the substrate at the active site of the enzyme is required for a ketene to be trapped by P450 1A1 or 2B1 prior to hydrolysis (59, 60). In this context it is interesting to note that among a series of *N*-alkyl dichloroacetamides, protein acylation is favored by long-chain alkyl groups and heme destruction by a methyl substituent (62). Ultimately, determination of the molecular basis for the differential inactivation mechanisms and/or processing of various cytochromes P450 should provide invaluable information about the active site (60).

DETERMINATION OF INHIBITOR SELECTIVITY

In Vitro Methods

Until the mid 1980s, knowledge of the selectivity of P450 inhibitors lagged far behind knowledge of the multiplicity and substrate specificity of the cyto-

chromes P450 themselves. In fact, a report in 1985 on the selectivity of chloramphenicol in rat liver was the first example of a systematic effort to characterize the inhibition of as many as nine different cytochromes P450 by a single compound (65). Since then, however, advances in protein biochemistry and recombinant DNA technology have made P450 characterization accessible to many laboratories, and the importance of inhibitors as an integral part of the characterization of existing and new enzymes is now well recognized. Consequently, in recent years, knowledge about the selectivity of cytochrome P450 inhibitors and inactivators has increased dramatically, and a number of highly selective compounds have been identified in experimental animals and humans.

Three methods have been employed to characterize the P450 isoform selectivity of inhibitors in vitro: 1. diagnostic reactions for monitoring the activity of individual cytochrome P450 forms in animal and human microsomal preparations (3–8, 17, 21, 41, 51, 52, 64–76); 2. purified enzymes (32–34, 40, 57, 58, 62, 63, 65, 71–73); and 3. heterologous expression systems (76–86).

Diagnostic marker activities in intact liver microsomes provide a convenient means of assessing the inhibition and/or inactivation of multiple cytochromes P450 simultaneously using a single substrate. With irreversible inhibitors this can be accomplished following in vivo administration of the compound or by monitoring the time and NADPH-dependent loss of various activities in vitro. The steroid hydroxylase assays pioneered by Waxman (67) have proven ideal for monitoring the inactivation of a number of rat and rabbit hepatic cytochromes P450 (66, 71–74). Simple methodology for such monitoring has been described recently (74). It is important to emphasize that a particular marker activity may only be valid in conjunction with the appropriate source of liver microsomes. For example, steroid 6β-hydroxylase activity is a convenient marker for P450 3A2 in liver microsomes from untreated, adult male rats but mainly reflects 3A1 in liver microsomes from dexamethasone-treated adult female rats (74, 87). Another useful substrate in human and rat liver microsomes is warfarin (18, 64, 65, 69, 73), although this assay is currently used in relatively few laboratories. A complementary approach, this one involving multiple substrates that are converted to the same product by various cytochromes P450, uses alkoxyresorufin derivatives (41, 52, 53, 70, 75, 88–90). With this approach as well, it is important to use the substrates with the appropriate microsomes. 7-Ethoxyresorufin *O*-deethylase is a selective marker for P450 1A1 in liver microsomes from 3-methylcholanthrene-treated rats but mainly reflects 1A2 in samples from untreated rats (91). The same issue arises with human liver microsomes; in those cases where more than one enzyme contributes to a particular activity under various circumstances, the relative expression levels may be important in determining the validity of using any given sample to monitor a particular enzyme (20).

Purified enzymes and heterologous expression systems provide an excellent complement to diagnostic marker activities in intact microsomes. Since most markers of P450 2B1 also reflect a contribution from P450 2B2, reconstituted systems can be used to determine unambiguously whether both enzymes are susceptible to an inhibitor (73). The same substrates used in microsomes lend themselves to use in reconstituted systems. However, since only a single enzyme is being tested at a time, any substrate for which a sensitive assay is available, such as 7-ethoxycoumarin, can be used (73). In those cases where a particular enzyme is difficult to purify to homogeneity, heterologous expression systems provide a number of advantages. In fact, studies of enzyme inactivation circumvent the inherent difficulties of variable expression in many systems, such as COS cells. This is because the rate constant for inactivation at a particular inhibitor concentration is an intrinsic property of a given enzyme and is unaffected by the expression level (77).

Finally, an important issue is the dynamic relationship between studies of P450 substrate specificity and studies of inhibitor selectivity. Judicious use of selective inhibitors is an integral part of any systematic characterization of P450 specificity. In turn, once a particular reaction has been attributed to an individual P450, this reaction serves as a ready marker for studies of enzyme inhibition. The recent literature contains several examples of how inhibitors have led to a reexamination of the use of particular marker activities as indicative of a single enzyme (49, 69). Likewise, as particular marker activities have been revealed as less specific than originally thought, reexamination of inhibitor selectivity may be in order (74, 92). In addition, even today, few laboratories have at their disposal the entire battery of experimental tools required for complete characterization of the selectivity of an inhibitor in a particular organ or species. Therefore, claims of selectivity must always be judged according to how many potential target enzymes have actually been investigated (86).

In Vitro–In Vivo Correlations

An important question is to what extent inhibitor selectivity assessed in vitro also applies in vivo. Extensive experience in this laboratory over the past ten years has revealed no differences in selectivity between (*a*) microsomes harvested from animals treated in vivo with mechanism-based inactivators and (*b*) microsomes treated in vitro, provided that care is taken in the in vitro studies to distinguish inactivation from reversible inhibition. The latter is dependent on the presence of the compound and will rarely manifest itself in washed liver microsomes from an animal treated in vivo. For example, in vitro studies of the chloramphenicol analogue *N*-(2-*p*-nitrophenethyl)chlorofluoroacetamide revealed inhibition of cytochromes P450 2B1, 2C6, 2C11, and 3A2 but inactivation of only 2B1. An observation consistent with these results is

that only P450 2B1 activity was decreased in liver microsomes from phenobarbital-treated rats given the inhibitor in vivo (73, 74). On the other hand, the extent of inactivation in vivo may not always be complete. In the same study (73) it was found that only two thirds of the P450 2B1 could be inactivated in vivo, even after multiple doses. These and other results suggest the presence of a population of hepatocytes that may not be exposed to the inhibitor in vivo (73, 93).

The possibility of unexpected biological effects of inhibitors in vivo is also important. Induction of porphyrias by compounds that alter P450 heme has already been discussed. However, compounds that modify the protein moiety of cytochromes P450 may also lead to untoward effects, such as hepatotoxicity, owing to the production of autoantibodies to the modified cytochromes P450 (85, 94). Our limited understanding at present of the factors that predispose to autoantibody formation precludes a prediction of which P450 inactivators are likely to cause such effects in vivo.

APPROACHES FOR IDENTIFYING SELECTIVE INHIBITORS

Initial reports of selective cytochrome P450 inhibition generally encompassed only a limited or ill-defined number of enzymes (reviewed in 42). More recently, the availability of rigorous methodology for defining selectivity has prompted a number of laboratories to develop strategies for identifying or designing selective inhibitors. Three basic approaches have been followed: 1. careful characterization of already known compounds; 2. enhancement of the selectivity of existing inhibitors by suitable structural alterations; and 3. introduction of appropriate functional groups into substrates, such as fatty acids and steroids, known to be metabolized in a specific fashion by the target enzymes. Specific examples of these approaches, as well as their advantages and disadvantages, are described below.

Known Compounds

Most known compounds that exhibit a high degree of P450 isoform selectivity cause mechanism-based inactivation or give rise to metabolite intermediate complexes. Examples include furafylline for human P450 1A2 (76, 95), chloramphenicol for dog P450 2B11 (96), phencyclidine for rabbit P450 2B4 (97), diethyldithiocarbamate for human P450 2E1 (17) and 2A6 (98), and triacetyloleandomycin and gestodene for human liver P450 3A4 and 3A5 (17, 86, 99). Useful reversible inhibitors of human cytochromes P450 include 7,8-benzoflavone for P450 1A1 and 1A2 (86, 100), sulfaphenazole for P450 2C9 and 2C10 (18, 84), and quinidine for P450 2D6 (44). It is noteworthy that all selective inhibitors of human cytochromes P450 listed in recent compilations

are known synthetic or naturally occurring compounds, rather than compounds designed for the express purpose of inhibiting a particular P450 form (6, 8, 17).

Refinement of Existing Inhibitors

When no compound of sufficient selectivity is available for a particular P450, refinement of the structure of known inhibitors may be fruitful. The author's laboratory has employed such an empirical approach using chloramphenicol as a starting point. Although chloramphenicol inactivates a number of constitutive and phenobarbital-inducible rat liver cytochromes P450, the most rapidly inactivated is 2B1 (66). By replacing the propanediol side chain of chloramphenicol with an ethyl group and substituting a chlorofluoroacetamido for the dichloracetamido group, the analogue *N*-(2-*p*-nitrophenethyl) chlorofluoroacetamide was synthesized. This compound retains the ability to inactivate P450 2B1 but no longer inactivates 2C6, 2C11, 3A1, or 3A2 (73). Other compounds that lend themselves to an empirical approach to inhibitor design include 4-alkyl dihydropyridine derivatives (70) and aryl acetylenes (60). A prerequisite for the successful use of such a strategy is generally the facile synthesis of a large number of analogues (60, 62, 73).

Rational De Novo Design

Perhaps the most attractive way to design selective inhibitors is to introduce appropriate functional groups into substrates that are normally metabolized in a specific fashion by the target enzyme. Successful application of this approach has often involved the synthesis of mechanism-based inactivators in the form of fatty acid and steroid derivatives containing ethynyl, vinyl, dihalomethyl, or cyclopropyl groups in the appropriate position. Selective compounds identified in this manner include terminal acetylenic fatty acid derivatives for rat liver P450 4A1 (57) and rabbit lung 4A4 (39, 40), pregn-4,20-diene-3-one for rat liver P450 2C6 (72), 21-chloro-21-fluoropregnenolone for rabbit liver P450 2C5 (72), and 17β-(cyclopropylamino)-androst-5-en-3β-ol for human testicular P450 17 (101). The major drawbacks of this approach are 1. The target enzyme may not always exhibit the expected activity toward the derivatized substrate, rendering it ineffective as an inactivator; and 2. Nontarget enzymes may exhibit unexpected activity toward the compound, rendering it less selective than anticipated. The author's laboratory has evaluated a series of pregnenolone and progesterone derivatives as potential selective inactivators of the rat (2C6) and rabbit (2C5) hepatic progesterone 21-hydroxylases. The compounds contained a vinyl, ethynyl, difluoromethylketo, chlorofluoromethylketo, or dichloromethylketo group in place of the normal 17β-methylketo substitutent. In each species only one of the ten compounds inactivated the target enzyme without causing adventitious inactivation of other

hepatic progesterone hydroxylases that do not normally hydroxylate the 17β side chain (72). It should also be recognized that appropriate specific substrates for derivatization are not available for many hepatic cytochromes P450, such that most of the successful examples of this approach involve cytochromes P450 involved in steroid hormone biosynthesis (101–103). An alternate strategy involves designing compounds that include molecular features for enzyme inactivation and mimicry of known selective substrates. Examples include *N*-aralkyl derivatives of 1-aminobenzotriazole as mimics of benzophetamine for pulmonary cytochromes P450 2B (41, 52, 53) and 4-allyloxymethamphetamine for rat P450 2D (104). The rapidly increasing knowledge of the substrate specificities of cytochromes P450 will undoubtedly result in identification of additional substrates that can serve as the basis for rational design of selective inhibitors.

STRUCTURAL BASIS OF SUBSTRATE SPECIFICITY

Relatively minor structural alterations in a substrate or inhibitor can have a dramatic effect on P450 activity or specificity [cf 7-ethoxy vs methoxyresorufin (90); testosterone vs androstenedione (67, 79); and *N*-substituted dichloroacetamides vs chlorofluoroacetamides (66, 73)]. Equally minor changes in the cognate substrate contact residues in the enzyme should therefore also have a dramatic effect. Recent advances in site-directed mutagenesis and heterologous expression have made it possible to investigate in a systematic fashion the role of enzyme structure in P450 substrate specificity (15). Numerous recent examples have confirmed the dramatic impact of substitutions such as Val → Ala (105–106), Gly → Ala (107), Ile → Leu (84, 108), and Ser → Thr (83). This has prompted an intense effort to identify substrate contact residues and to understand the molecular basis for the alterations in substrate specificity that accompany particular side-chain replacements.

In the absence of a three-dimensional structure for a mammalian cytochrome P450, several different strategies have been used to target residues for site-directed mutagenesis. Such approaches include sequence comparisons of highly structurally related but functionally distinct cytochromes P450, including allelic variants of the same enzyme (83, 84, 105, 107–112); comparative sequence alignments of multiple cytochromes P450 within a subfamily (79, 81, 106, 112, 113) or family (114); analogy with the bacterial enzymes of known three-dimensional structure P450 101 (61) and P450 102 (115); and molecular modeling (116–118). Progress has been especially rapid with cytochromes P450 from family 2. A recent model based on comparative sequence alignments and analogy with P450 101 proposed the existence of six substrate recognition sites (SRSs) for this family (114). All amino acid residues and chimeric fragments identified to date as critical for the substrate specificity of

Table 1 Selected site-directed cytochrome P450 mutants

SRS	Mutants[a]	2B1 position	P450 101 residue[b]
1	2A4 A117V, 2B1 I114A, 2C4 V113A	114	F98
2	2A4 L209N, 2A5 F209L/N, 2B1 F206L	206	T185
3	1A2 K250L	233	
4	2B1 I290D	290	
	1A2 E318D/A, 19 D309A	301	D251
5	2C9 I359L	360	
	2A4 L365M	362	
	2B1 V363A	363	V295
	2C3 S364T	366	
	2B1 V367A	367	D297
	2D1 I380F	370	
6	2B1 G478A	478	V396

[a] Mutants are designated by the abbreviated name for the enzyme followed by the single letter code for the amino acid replaced, the position in the sequence, and the new residue.
[b] Adapted from References 114 and 118. The residues indicated play a role in P450 101 activity or specificity.

P450 2 forms fall within or near the putative SRSs (Table 1). A brief overview of representative findings with cytochromes P450 from the 2A, 2B, and 2C subfamilies is given below. This emphasis on selected P450 2 enzymes is not meant to diminish the importance of elegant mutagenesis studies performed on many other cytochromes P450, including P450 101 (119), P450 1A2 (120, 121), and P450 19 (116, 122), to name a few.

Mouse P450 2A Enzymes

The first report that a single amino acid substitution is sufficient to confer a new catalytic activity involved mouse P450 2A4 and 2A5 (109). These two enzymes differ in only 11 positions in the amino acid sequence; however, P450 2A4 is a steroid 15α-hydroxylase, whereas P450 2A5 is a coumarin 7-hydroxylase. The Phe-209 → Leu substitution in P450 2A5 confers near 2A4-like steroid 15α-hydroxylase activity with retention of approximately one third of the coumarin 7-hydroxylase activity. Each of three substitutions, Ala-117 → Val, Leu-209 → Phe, and Leu-365 → Met, confers some coumarin 7-hydroxylase activity on P450 2A4. To convert P450 2A5 to a fully active steroid 15α-hydroxylase devoid of coumarin 7-hydroxylase activity, simultaneous substitutions at positions 117, 209, and 365 are required.

Residue 209 appears to be the most critical determinant of substrate specificity of mouse P450 2A4 and 2A5, and subsequent studies have focused on the importance of different amino acid residues at this position. In P450 2A5

large hydrophobic residues at position 209 favor a low K_m and K_d for coumarin and a high V_{max}. It has been suggested that residue 209 resides in a pocket that binds to the 2-keto group of coumarin and directs its 7-position toward the P450 active oxygen species (80). The Phe-209 → Asn mutant of P450 2A5 acquires 15α-hydroxylase activity toward 11β-hydroxysteroids such as corticosterone (123). The presence of Asn-209 in a recently discovered mouse testosterone 7α-hydroxylase also controls binding of 11β-hydroxysteroids to this enzyme (81). Molecular modeling based on the structure of P450 101 is consistent with the positioning of residue 209 in the vicinity of C-11 on the steroid molecule (123).

P450 2B Enzymes

A major focus of the author's laboratory has been the structural basis of substrate specificity of P450 2B enzymes from rats, rabbits, and dogs. Targeting of residues for site-directed mutagenesis has been greatly facilitated by the discovery of allelic variants of P450 2B1 (107) and 2B2 (110). This discovery has led to the identification of Ile-114 and Gly-478 as critical determinants of the stereo- and regioselectivity of P450 2B1 toward androstenedione and testosterone (77–79, 107, 110). Simultaneous substitution of both residues converts P450 2B1 from an androgen 16-hydroxylase to a 15α-hydroxylase (78). Comparison of various 2B enzymes within the putative SRSs has also led to identification of Phe-206, Ile-290, Val-363, and Val-367 in P450 2B1 as important determinants of substrate specificity (79; JR Halpert & Y-A He, unpublished observations). Residues 114 and 206 in P450 2B1 map to residues 117 and 209, respectively, in the mouse 2A enzymes. The simultaneous substitutions Ile-114 → Ala and Phe-206 → Leu in P450 2B1 yield the 2A4 residues and confer the progesterone 15α-hydroxylase activity characteristic of 2A4 (124).

Studies with rabbit P450 2B4 and 2B5 and dog 2B11 have confirmed the importance of residues 114, 290, 363, and 367 in at least one of these 2B enzymes (112, 113). In addition, the effects of the particular side chains at these sites appear to be conserved among the various 2B enzymes. For example, mutations at positions 114, 290, or 363 that convert the 2B1 to the 2B11 residue alter the steroid hydroxylase profile to make it more like 2B11 (79), and vice versa (113). Moreover, a Val-367 → Ala substitution confers androgen 6β-hydroxylase activity on 2B1 and 2B4 (79, 112). As with the mouse 2A enzymes, the size of key hydrophobic residues is a major determinant of substrate specificity (78, 79).

P450 2C Enzymes

Studies of rabbit P450 2C4 and 2C5, which differ by 24 amino acid residues, were the first to indicate the importance of residue 113 in P450 2C enzymes

(corresponding to 114 in 2B1 and 117 in mouse 2A) for substrate specificity. An Ala at this position is crucial for low K_m 21-hydroxylase activity of 2C4 or 2C5 (15, 105) and also confers progesterone 21-hydroxylase activity on P450 2C1 (106). More recent studies of position 113 in P450 2C2 have revealed the importance of hydrophobicity for rates of progesterone and lauric acid hydroxylation, with the structural requirements being more stringent for the rigid steroid than for the more flexible fatty acid substrate (125). Whereas the substitutions at position 113 in the rabbit 2C enzymes appear primarily to affect rates rather than regioselectivity of progesterone hydroxylation, position 364 is a crucial determinant of regioselectivity (82, 83). Two naturally occurring variants of P450 2C3 exist, which differ at five amino acid positions. The variant 2C3v is a high-efficiency progesterone 6β- and 16α-hydroxylase, whereas 2C3 catalyzes only 16α-hydroxylation, and with lower affinity. The major determinant of the 6β-hydroxylase activity is Thr-364, which maps close to residue 365 in the mouse 2A enzymes and to residues 363 and 367 in the 2B enzymes. Another key P450 2C residue that maps close to this region is Ile-359 in 2C9. A Leu at this position converts the enzyme from an (*S*)-warfarin 7-hydroxylase to an (*R*)-warfarin 4′-hydroxylase (108) and also decreases phenytoin hydroxylase activity (84).

STRUCTURAL BASIS OF INHIBITOR SELECTIVITY

Many of the same studies that have yielded insights into amino acid residues in P450 family 2 enzymes important for substrate specificity have also revealed residues crucial for enzyme inhibition or inactivation (77–83, 107). Such information has also emerged from studies of P450s from other families, including P450 1A2 (120, 121) and P450 19 (122). With reversible inhibitors or ligands, profound effects on the affinity, mode, and enantioselectivity of binding have been reported (80, 81, 83, 120–122). With mechanism-based inactivators of P450 2B1, a number of point mutants refractory to reversible inhibition and/or inactivation have been observed (77–79, 107). In one case, inability of a mutant to metabolize the inhibitor has been shown to be responsible for the lack of enzyme inactivation (107). However, examples of point mutations leading to alterations in partition ratios or even in the mode of enzyme inactivation (heme vs protein modification) will undoubtedly emerge. The SRS model provides a convenient means to summarize current information on enzymatic determinants of P450 inhibition and/or inactivation. Key examples of the effects of single amino acid substitutions on susceptibility to various compounds have been chosen with the goal of illustrating general principles, as well as possibilities for future study. Together with recent advances in molecular modeling of P450 enzymes and inhibitors (116–118, 126), these studies set the stage for structure-based inhibitor design.

Substitution of Ile-114 in P450 2B1 with Ala renders the enzyme much less susceptible to reversible inhibition and inactivation by chloramphenicol (78). A Val → Ala substitution at the corresponding position in P450 2C3v confers progesterone 21-hydroxylase activity and enhances the maximal rate constant for inactivation by 21-chloro-21-fluoropregnenolone 2.5-fold (PA Klekotka, TH Richardson, EF Johnson & JR Halpert, unpublished observations). Thus, in both a rat P450 2B and a rabbit P450 2C enzyme, a minor change in the size of the same hydrophobic residue in SRS-1 affects enzyme inhibition and inactivation as well as substrate specificity. This residue is located in a highly variable loop (15), which may provide a convenient target for design of selective inhibitors and inactivators.

Residue 209 (SRS-2) in mouse P450 2A enzymes is a key determinant of inhibition by various coumarin derivatives and steroids. Phe-209 in 2A5 is optimal for inhibition by 2-coumaranone, whereas the K_i value increases more than 20-fold with the Val mutant and more than 150-fold with the Ala mutant (80). These changes in affinity for the inhibitor parallel the changes in binding of the substrate coumarin. The presence of Asn at position 209 in mouse $P450_{7\alpha}$ is critical for inhibition by the 11β-hydroxysteroid corticosterone, and a Leu-209 → Asn substitution in P450 2A4 confers susceptibility to corticosterone (81). However, deoxycorticosterone inhibits both P450 2A4 and $P450_{7\alpha}$ regardless of whether Asn or Leu is present at position 209. The results strongly suggest a critical interaction between residue 209 and the C-11 position on the steroid in determining enzyme inhibition. Conversion of the corresponding residue Phe-206 in 2B1 to Leu confers some of the enzymatic properties of P450 2A4, but no inhibitors have yet been tested (124).

Key residues in SRS-3 and SRS-4 of rat P450 1A2 have been shown to be important determinants of the enantioselectivity and mode of binding of nitrogenous compounds. A Lys-250 → Leu substitution (SRS-3) increases threefold the preference for binding of (*R*)-(+) as opposed to (*S*)-(−)-1-(1-naphthyl)-ethylamine (121). An even greater increase in the *R/S* ratio (12-fold) is observed with a Glu-318 → Ala substitution (SRS-4). Another substitution at this position (Glu-318 → Asp) decreases the dissociation constant of P450 1A2 for 2-phenylimidazole seven-fold and confers the ability to bind the compound through coordination of an imidazole nitrogen with the heme iron (120). Substitution of the corresponding Asp-309 with Ala in P450 19 has a differential effect on inhibition by two important compounds, rendering the enzyme much less susceptible to 4-hydroxyandrostenedione and much more susceptible to aminoglutethimide (122). Glu-318 in P450 1A2 and Asp-309 in P450 19 align to Asp-251 in P450 101 and Glu-267 in P450 102, which are thought to facilitate oxygen activation along with the adjacent conserved Thr residue (61, 115). Since virtually all mammalian P450s have either an Asp or Glu preceding the conserved Thr, it is unclear whether the dramatic effects on

enzyme inhibition of mutations at this position can be harnessed to design selective inhibitors.

SRS-5, on the other hand, is highly variable among P450 family 2 enzymes and contains several residues that have a dramatic effect on enzyme inhibition or inactivation. Replacement of Val-363 or Val-367 with Ala in P450 2B1 suppresses susceptibility to inactivation by the P450 2B1–selective chloramphenicol analogue *N*-(2-*p*-nitrophenethyl)chlorofluoroacetamide, whereas the Val-367 → Ala substitution also prevents inactivation by chloramphenicol itself (79). Val-363 in the P450 2B enzymes corresponds to Val-295 in P450 101, a known substrate contact residue (61, 119), and to Ala-328 in P450 102, which lines the hydrophobic pocket (115). Residues 363 and 367 are highly variable among 2B enzymes and may be promising sites for selective inhibitor action. Another interesting residue is position 364 in rabbit P450 2C3 and 2C3v, where a Ser → Thr substitution underlies the difference between the two variants in sensitivity to 16α-methylprogesterone. This residue aligns to position 366 in the 2B enzymes, again confirming the crucial importance of this hypervariable region for substrate specificity and inhibition.

SRS-6 contains Gly-478 in P450 2B1, which was the first residue definitively shown to dictate susceptibility of a cytochrome P450 to mechanism-based inactivation (77, 107). The presence of an Ala at this position is responsible for the lack of inactivation by *N*-(2-*p*-nitrophenethyl)chlorofluoroacetamide of the P450 2B1 variant in Wistar Munich rats. The resistance to inactivation results from an inability to catalyze the oxidative dehalogenation reaction required for reactive metabolite formation (107). The Gly-478 → Ala substitution also confers protection against the L-*threo*-diastereomer of chloramphenicol (77). Substituting an even larger residue suppresses inactivation by chloramphenicol as well. Residue 478 aligns with Val-396 in P450 101, which along with the adjacent Ile-395 is thought to control substrate binding and/or access (61). These two positions exhibit considerable variability among P450 2 enzymes and offer another promising target for selective inhibition/inactivation.

CONCLUSIONS

Major recent advances in the identification and design of isoform-selective cytochrome P450 inhibitors and inactivators have greatly facilitated the characterization of the catalytic specificities and pharmacological and toxicological significance of individual P450 enzymes in experimental animals and humans. It is now possible to rationalize many clinically relevant drug-drug interactions in terms of the substrate and inhibitor specificities of individual human P450 forms. Appropriate use of inhibitors requires knowledge of their mechanisms as well as their specificities. For example, the efficacy and selectivity of

mechanism-based inactivators are highly dependent on the treatment regimen. Thus, if used in vitro without prior activation, the compounds may be nonselective or ineffective, whereas following preincubation in the presence of NADPH and removal of unmetabolized inhibitor, a high degree of specificity may be obtained. Proper in vivo use of chemical inhibitors requires even more caution. Agents that destroy cytochrome P450 heme may impair normal heme biosynthetic pathways, and the duration of irreversible inhibition obtained in vivo depends on whether the inactivated enzyme can be restored functionally or must be replenished through de novo synthesis. Such recovery is a function of the precise mechanism by which enzyme inactivation occurs and can be both compound and isoform dependent. Major recent breakthroughs regarding the enzymatic determinants of substrate specificity and inhibitor action portend an era of structure-based design of isoform-selective cytochrome P450 inhibitors and inactivators that offer great promise as probes and modulators of cytochrome P450 function in vivo. The next five years should reveal whether selective inhibitors of xenobiotic metabolizing cytochromes P450 can be used therapeutically the way inhibitors of steroidogenic P450 enzymes are used to treat cancer and various endocrine disorders.

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