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Inhibition of Cytochrome P450 Enzymes

Maria Almira Correia and Paul R. Ortiz de Montellano

1. Introduction

Three steps in the catalytic cycle of cytochrome P450 (P450, CYP; see Chapters 5 and 6) are particularly vulnerable to inhibition: (a) the binding of substrates, (b) the binding of molecular oxygen subsequent to the first electron transfer, and (c) the catalytic step in which the substrate is actually oxidized. Only inhibitors that act at one of these three steps will be considered in this chapter. Inhibitors that act at other steps in the catalytic cycle, such as agents that interfere with the electron supply to the hemoprotein by accepting electrons directly from P450 reductase[1–3], are not discussed here.

P450 inhibitors can be divided into three mechanistically distinct classes: Agents that (a) bind reversibly, (b) form quasi-irreversible complexes with the heme iron atom, and (c) bind irreversibly to the protein or the heme moiety, or accelerate the degradation and/or oxidative fragmentation of the prosthetic heme. Agents that interfere in the catalytic cycle prior to the actual oxidative event are largely reversible competitive or noncompetitive inhibitors. Those that act during or subsequent to the oxygen transfer step are generally irreversible or quasi-irreversible inhibitors and often fall into the category of mechanism-based (or suicide) inactivators. Extensive lists of P450 inhibitors are available in various reviews[4–12]. This chapter focuses on the mechanisms of inactivation; thus, most of the chapter is devoted to the discussion of agents that require P450 catalysis to fulfill their inhibitory potential. The mechanisms of reversible competitive and noncompetitive inhibitors, despite their practical importance, are relatively straightforward and are discussed more briefly.

2. Reversible Inhibitors

Reversible inhibitors compete with substrates for occupancy of the active site and include agents that (a) bind to hydrophobic regions of the active site, (b) coordinate to the heme iron atom, or (c) enter into specific hydrogen bonding or ionic interactions with active-site residues[4–10]. The first mechanism, in which the inhibitor simply competes for binding to lipophilic domains of the active site, is often responsible for the inhibition observed when two substrates compete for oxidation by a single P450 isoform. A clear example of such an interaction is provided by the mutual *in vitro* and *in vivo* inhibition of benzene and toluene metabolism[13]. This form of inhibition, which is optimal when the inhibitory compound is bound tightly but is a poor substrate, is usually not highly effective but can

Maria Almira Correia • Department of Cellular and Molecular Pharmacology, Department of Pharmaceutical Chemistry, Department of Biopharmaceutical Sciences and the Liver Center, University of California, San Francisco, CA. **Paul R. Ortiz de Montellano** • Department of Pharmaceutical Chemistry, and Department of Cellular and Molecular Pharmacology, University of California, San Francisco, CA.

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cause physiologically relevant metabolic changes and clinically significant interactions[14].

2.1. Coordination to Ferric Heme

The binding of a strong sixth ligand to the pentacoordinated heme iron atom of a P450 enzyme, or displacement of a weak ligand in a P450 hexacoordinated state by a strong ligand, causes a shift of the iron from the high- to the low-spin state. This shift is characterized by a "type II" binding spectrum with a Soret maximum at 425–435 nm and a trough at 390–405 nm[15–18]. The change in the redox potential of the enzyme associated with this spin state change makes its reduction by P450 reductase more difficult (see Chapter 5)[18, 19]. This change in reduction potential, as much as physical occupation of the sixth coordination site, is responsible for the inhibition associated with the binding of strong iron ligands.

Cyanide and other ionic ligands bind preferentially, albeit weakly, to the ferric state of a P450 enzyme[20, 21]. The three positive charges of the iron are matched in the ferric hemoprotein by the three negative charges of its permanent ligands (i.e., the two porphyrin nitrogens and the thiolate ion), but in the reduced state there is a charge imbalance of three ligand negative charges but only two ferrous iron positive charges. The cyanide ion, a negatively charged species, binds more readily to the neutral (ferric) than the negative (ferrous) enzyme. Indeed, cyanide binds more weakly to ferric P450 than to ferric myoglobin because the P450 thiolate ligand places a higher electron density on the iron than does the imidazole ligand of myoglobin[22]. The chelation of ionic ligands is disfavored, in addition, by the lipophilic nature of the P450 active site[23].

P450 enzymes are inhibited by nitric oxide (NO), a molecule of great interest because of its role in diverse physiological and pathological processes. Inhibition initially involves reversible coordination of the nitrogen to the iron but a subsequent time-dependent, irreversible inactivation of the enzyme by an undefined mechanism has been reported[24–26]. Inhibition by endogenous NO of P450 enzymes involved in endogenous substrate metabolism, including eicosanoid formation and sterol metabolism, may have physiological consequences[27, 28].

2.2. Coordination to Ferrous Heme

In its simplest form, inhibition through coordination to the heme iron is exemplified by carbon monoxide, a neutral ligand that binds exclusively to the ferrous (reduced) form of P450. The binding of carbon monoxide involves donation of electrons from the carbon to the iron through a σ-bond as well as back-donation of electrons from the occupied ferrous iron d-orbitals to the empty antibonding π-orbitals of the ligand[29]. P450 enzymes (P450, CYP) are so named because their carbon monoxide complexes have spectroscopic absorption maxima at approximately 450 nm[30]. Early studies with model ferroporphyrins, which indicated that only those with a thiolate ligand *trans* to the carbon monoxide yielded the 450-nm absorption, provided key evidence for the presence of a thiolate fifth ligand in P450.[31] Inhibition by carbon monoxide is a signature of P450-catalyzed processes, although the sensitivities of different P450 isoforms to carbon monoxide differ[32] and a few P450-catalyzed reactions are resistant to inhibition by carbon monoxide[33–35]. In particular, the sensitivity of aromatase[36, 37] and $P450_{SCC}$[38] to inhibition by carbon monoxide decreases drastically as the enzymes traverse the conformational and ligand states inherent in their multistep catalytic sequences. Among the human liver P450 isoforms, the susceptibility of different families to carbon monoxide inhibition appears to decrease in the order 2D > 2C > 3A[32].

2.3. Heme Coordination and Lipophilic Binding

Agents that simultaneously bind to lipophilic regions of the active site and to the heme iron atom (Figure 7.1) are inherently more effective P450 inhibitors than agents that only exploit one of these binding interactions. The effectiveness of these agents as P450 inhibitors is governed both by their hydrophobic character and the strength of the bond between their heteroatomic lone pair and the heme iron. Agents such as alcohols, ethers, ketones, lactones, and other structures in which an oxygen atom of the ligand coordinates to the iron, or which act by stabilizing the coordination of the distal water ligand, are relatively poorly bound and are

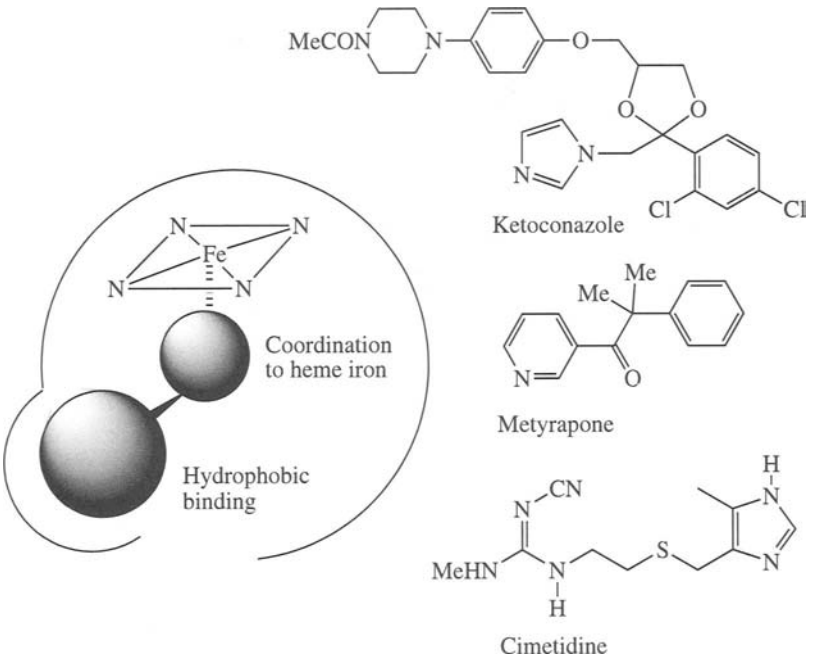


Figure 7.1. Schematic illustration of the binding of an inhibitor to a P450 active site by both coordinating to the heme-iron atom and interacting with the surrounding protein residues. The structures of three agents that inhibit P450 by binding in this manner are shown.

weak inhibitors[17, 38–43]. The Soret band of such complexes is found at approximately 415 nm[14, 17]. In contrast, agents that interact strongly with both the protein and the heme iron atom are often highly effective reversible inhibitors[4–10]. As already noted, the binding of such inhibitors yields a "type II" difference spectrum with a Soret maximum at 430 nm[15, 17, 44, 45]. The structures of these powerful inhibitors usually incorporate nitrogen-containing aliphatic or aromatic functions.

Pyridine, imidazole, and triazole derivatives have proven particularly useful in P450 inhibitors[4]. Metyrapone (Figure 7.1), one of the first P450 inhibitors to be widely employed, first gained prominence as an inhibitor of 11β-hydroxylase, the enzyme that catalyzes the final step in cortisol biosynthesis[46]. This activity led to its use in the diagnosis and treatment of hypercortisolism (Cushing's syndrome) and other hormonal disorders[47]. The determinants of the inhibitory potency of metyrapone and other nitrogen heterocycles are valid for most reversible inhibitors: (a) the intrinsic affinity of the ligand nitrogen electron pair for the heme iron, (b) the degree to which the intrinsic affinity of the ligand for the iron is moderated by steric interactions with substituents on the inhibitor[48, 49], (c) the lipophilicity of the nonligating portion of the inhibitor[23, 50], and, naturally, (d) the congruence between the geometry of the inhibitor and that of the active site. The synergism that results from binding simultaneously through lipophilic interactions and coordination with the heme iron is illustrated by the fact that imidazole and benzene individually are weak inhibitors, but when coupled together as in phenylimidazole they produce a powerful inhibitor[48]. Optimization of these structural features has made possible the therapeutic exploitation of azole compounds such as ketoconazole (Figure 7.1) as antifungal and cancer chemotherapeutic agents[5]. On the other hand, similar features in cimetidine are responsible for the drug–drug interactions that result from the inhibition of the metabolism of co-administered drugs. This

inadvertent side effect instigated the search for non-imidazole containing H2-antagonists devoid of this undesirable feature[40, 51]. Improvement of the potency and particularly specificity of metyrapone, aminoglutethimide, and other classical inhibitors by structural modification continues to be of therapeutic interest. This consideration has led to the development and clinical testing of, for example, pyridylaminoglutethimide, CGS16949A {4-(5,6,7,8-tetra-hydroimidazo[1,5α]pyridin-5-yl) benzonitrile}, CGS18320B bis-(*p*-cyanophenyl) imidazo-1-yl-methane hemisuccinate and R-76713 [6-(4-chlorophenyl)1H (1,2,4 triazol-1-yl)-methyl]-1-methyl-1H-benzotriazole as aromatase inhibitors (see Section 5.2)[5]. Likewise, efforts to improve the properties of ketoconazole have fostered the design and use in antifungal therapy of sterol 14α-demethylase inhibitors such as miconazole, fluconazole, saperconazole, and terconazole (see Section 5.3)[5].

3. Catalysis-Dependent Inhibition

Multiple classes of compounds are now known that undergo P450-catalyzed activation to reactive intermediates that irreversibly or quasi-irreversibly inactivate the enzyme responsible for their activation. This irreversible inactivation by a catalytically generated species is superimposed on the reversible inhibition associated with competitive binding of the parent agent to the ferric enzyme. Mechanism-based[52, 53] (catalysis-dependent) inactivators can be highly enzyme-specific because (a) the compound must first bind reversibly to the enzyme and must satisfy all the constraints imposed on normal substrates of the enzyme, (b) it must be acceptable as a substrate and thus undergo catalytic activation and, finally, (c) the resulting reactive intermediate must irreversibly alter the enzyme and permanently remove it from the catalytic pool. Four general classes of mechanism-based irreversible P450 inactivators are known: (a) agents that bind covalently to the protein, (b) agents that quasi-irreversibly bind to the prosthetic heme iron atom, (c) agents that alkylate or arylate the porphyrin framework of the heme, and (d) agents that degrade the prosthetic heme to products that can, in some cases, themselves modify the protein. However, this mechanistic classification is not rigid, as in the course of its P450, metabolism, a compound may simultaneously partition into two or more of these inhibitory trajectories.

3.1. Covalent Binding to the Protein

Agents that are oxidatively activated and inactivate the enzyme by covalently binding to it include (a) diverse sulfur compounds (e.g., carbon disulfide[54–56], parathion[57, 58], diethyldithiocarbamate[59], isothiocyanates[60], thioureas[61], thiophenes[62], tienilic acid[62, 63], and mercaptosteroids[64–71], (b) halogenated structures such as chloramphenicol[72–75], *N*-monosubstituted dichloroacetamides[76], and *N*-(2-*p*-nitrophenethyl)dichloroacetamide[77], (c) alkyl and aryl olefins and acetylenes[78–84] such as 10-undecynoic acid[78, 83], 10-dodecynoic acid[84], 1-ethynylpyrene[81, 83], 17β-ethynylprogesterone[85, 86], 17α-ethynylestradiol[86–91], gestodene[92], 1- and 2-ethynylnaphthalene[79, 80, 82, 93], 7-ethynylcoumarin (7-EC)[94], mifepristone[95, 96], and secobarbital[97], (d) furanocoumarins such as 8-methoxypsoralen (8-MOP, methoxsalen)[98–106], 6′,7′-dihydroxybergamottin (6′,7′-DHB)[107–109], and the furano-pyridine, L-754,394[110–112] and (e) compounds such as carbamazepine (CBZ) and tamoxifen that are hydroxylated to catechol metabolites[113–116]. 2-Phenylphenanthridinone, which inhibits CYP1A1, is a member of a distinct class of mechanism-based inactivating agents that is thought to bind covalently to the protein[117]. The details of the mechanisms by which a few of these compounds inactivate P450 remain unclear, but much is now known about the mechanisms of activation of many of these inhibitors and, in some instances, about the sites on the P450 enzymes which they covalently modify.

3.1.1. Sulfur and Halogenated Compounds

Incubation of liver microsomes with [^{35}S]parathion results in radiolabeling of the protein but no radiolabeling occurs when the parathion ethyl groups are labeled with ^{14}C[57, 58]. Ninety percent of the ^{35}S-label, bound covalently to the microsomal proteins is immunoprecipitated

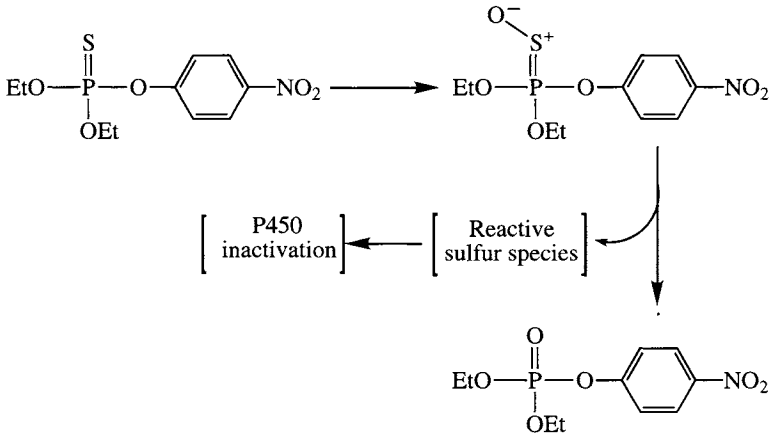


Figure 7.2. The activation of parathion to species that destroy cytochrome P450 is proposed to involve oxidation of the thiophosphonate to reactive sulfur species that bind to protein residues. The reactive species can be envisioned to be a sulfur atom with six electrons or HSOH.

by anti-P450 antibodies. Approximately 75% of the P450 prosthetic heme is degraded to unknown products in incubations with parathion, but ~4 nmol of radiolabeled sulfur are bound covalently to the protein for each nanomole of heme chromophore that is lost. The bulk (50%–75%) of the radiolabeled sulfur is removed from the protein by treatment with cyanide or dithiothreitol, which suggests that most of the sulfur label is present in the form of hydrodisulfides (RSSH), but the enzyme is not reactivated by these treatments. The binding of multiple equivalents of labeled sulfur indicates that catalytic sulfur activation persists despite covalent attachment of the sulfur to the protein until the heme itself is damaged[57] or is released from the protein as a consequence of protein damage. The oxidative mechanism in Figure 7.2 is suggested by (a) the covalent binding of sulfur, (b) the oxidation of sulfur compounds to S-oxides, and (c) the formation of metabolites in which the sulfur is replaced by an oxygen. More recent studies reveal that parathion competitively inhibits certain rat liver P450 enzymes (i.e., CYP2B1 and -2C6) at low concentrations, but at higher concentrations inactivates CYP2A1, -2A2, -2C11, -3A2, and -3A4 (but not CYP2B1 or -2C6) *in vitro* but not *in vivo*[118–121]. Analogous *in vitro* studies with human liver microsomes reveal that CYP3A4 is the principal isoform that is inactivated, CYP2C9 and -1A2 are minor forms that are also inactivated, whereas CYP2E1 is not inactivated[118–121]. While P450 heme destruction unequivocally occurs *in vitro* in incubations of parathion with NADPH-supplemented rat and human liver microsomes or purified recombinant P450 enzymes, the *in vivo* relevance of these findings remains to be established[122]. It appears that the concentrations required for *in vitro* P450 destruction are considerably higher than the concentrations that cause death through inhibition of acetylcholinesterase[122, 123].

Tienilic acid (Figure 7.3), a substituted thiophene, is oxidized by CYP2C9 and -2C10 to a product that irreversibly inactivates these enzymes [the values for half-maximal inactivation (K_I) = 4.3 μM, the maximal rate constant for inactivation (k_{inact}) = 0.22 min^{-1}, and partition ratio = 11.6][62, 63]. Tienilic acid is oxidized by these P450 enzymes to 5-hydroxytienilic acid and a product that is covalently bound to the P450 protein. Covalent labeling of the protein is partially prevented by glutathione (GSH) but GSH does not protect the enzyme from inactivation or loss of the heme chromophore. In the presence of GSH, approximately 0.9 equivalents of label are covalently bound to the protein before catalytic activity is suppressed. The results are explained by the formation of a thiophene sulfoxide that reacts with water to give the isolated metabolite or with a protein nucleophile to inactivate the enzyme[123]. The

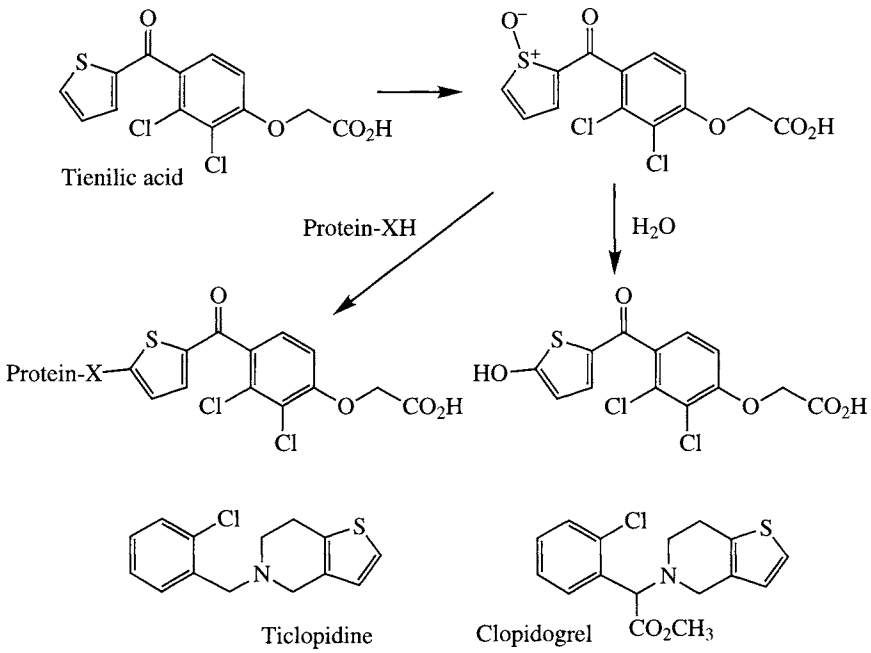


Figure 7.3. The mechanism proposed for P450 bioactivation of the thiophene in tienilic acid to a reactive species that binds covalently to the protein (the protein nucleophile is denoted by Protein-XH). The structures of two other thiophene-containing drugs, ticlopidine and clopidogrel, that might be similarly activated are also shown.

CYP2C9 site that is modified by tienilic acid has not been identified, but HPLC/electrospray mass spectrometric (ESI–MS) analysis of the tienilic acid-modified and native proteins reveals the presence of mono- and diadducted CYP2C9 species with molecular masses of 55,923 ± 1.1 and 56,273 ± 4.4 Da, respectively, which corresponds to mass shifts of 344.4 ± 1.1 and 694 ± 4.2 Da[123]. Diadduct formation is abolished by the inclusion of GSH (10 mM), suggesting that the diadduct may result from modification of a second residue outside the active site. The mass shift in the CYP2C9 monoadduct is consistent with the binding of a monohydroxylated tienilic acid obtained either by thiophene ring oxidation or formation of a sulfoxide that resists dehydration[123].

Ticlopidine (Figure 7.3), another substituted thiophene that is clinically utilized as an anti-platelet aggregation agent, is reportedly the first selective mechanism-based inactivator of CYP2C19[124]. This inactivation apparently occurs during S-oxidation of the thiophene moiety. The failure of GSH (5 mM) to inhibit ticlopidine-mediated CYP2C19 inactivation suggests that it occurs within the active site of the enzyme. The inactivation follows classical mechanism-based inactivation criteria with the following kinetic parameters: $t_{1/2max}$ = 3.4 min, k_{inact} = 3.2 × 10^{-3} s^{-1}, K_I = 97 μM, k_{inact}/K_I = 37 L mol^{-1} s^{-1}, and partition ratio = 26[124]. *In vitro* inhibition studies with functionally competent yeast-expressed recombinant human liver CYP1A2, -2C8, -2C9, -2C18, -2C19, -2D6, and -3A4 yielded IC_{50} (μM) values of 75 ± 15, 100 ± 20, >200, 100 ± 15, 10 ± 5, 10 ± 3, and 50 ± 10, respectively[124]. These findings establish that ticlopidine efficiently inhibits CYP2C19 and -2D6. Furthermore, within the human liver CYP2C subfamily, ticlopidine is a relatively selective mechanism-based inactivator of CYP2C19[124], although it is not quite as potent as tienilic acid is in CYP2C9 inactivation. These findings rationalize the clinical observation that ticlopidine inhibits the clearance of phenytoin, another CYP2C19 substrate[125–128]. However, recent studies with an extensive battery of recombinant human P450 enzymes in Supersomes™ indicate that

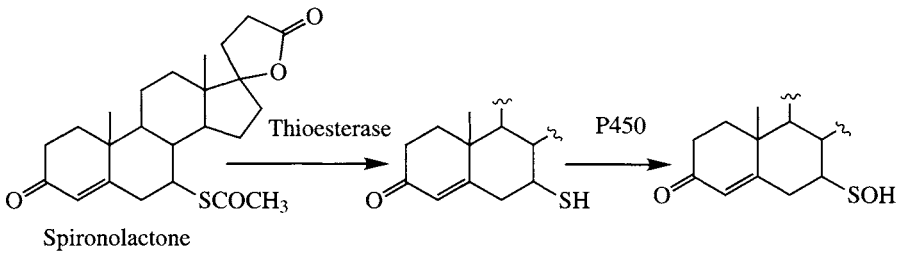


Figure 7.4. The activation of spironolactone to products that inactivate sterol 17α-hydroxylase by covalent attachment to the protein, and hepatic P450 3A by degradation of the heme group, occurs during oxidation of the free thiol group unmasked by the action of a thiolesterase.

CYP2B6 is even more effectively inactivated than CYP2C19 not only by ticlopidine, but also by clopidogrel, a related thienopyridine antiplatelet aggregating agent[129]. These observations suggest that clinically relevant drug–drug interactions with these two agents may occur with substrates of not only CYP2C19 but also CYP2B6 and -2D6.

The aldosterone antagonist spironolactone (Figure 7.4), which is used as a diuretic and antihypertensive[130], inactivates P450 enzymes in both the liver and steroidogenic tissues[64–71], including the adrenal steroid 17α-hydroxylase[64, 65, 70, 71] and members of the hepatic CYP3A and CYP2C subfamilies[67, 68]. Spironolactone-mediated hepatic P450 inactivation requires hydrolysis of the thioester function to give the free thiol that[67] is subsequently oxidized to a species that reacts with the protein and/or the heme[66]. Hepatic P450 inactivation involves fragmentation of the prosthetic heme to products that bind covalently to the protein (see Section 3.4)[66, 67]. In contrast, inactivation of the adrenal P450 appears to result from covalent binding of the thiosteroid itself to the protein[64, 65, 68, 69]. A role for thiol oxidation in enzyme inactivation is supported by the fact that rat hepatic microsomes enriched in CYP3A enzymes oxidize the thiol group (–SH) to the sulfinic ($-SO_2H$), and sulfonic ($-SO_3H$) acids[67], and give rise to a disulfide adduct with GSH[71]. However, formation of the disulfide adduct with GSH is catalyzed, at least in part, by a flavin monooxygenase[71]. Two reactive intermediates that can arise by thiol oxidation are the sulfhydryl radical (–S•) and the sulfenic acid (–SOH), either or both of which could be involved in P450 inactivation. One possibility is that fragmentation of the heme results from its reaction with the sulfhydryl radical, whereas protein modification involves reaction of the sulfenic acid metabolite with amino acid side chains (Figure 7.4).

Three other sulfur-containing drugs are noteworthy in that they are also bioactivated by human liver CYP3A4 and result in inactivation of the enzyme. The first is the oral antidiabetic drug troglitazone (Figure 7.5)[131], which was recently withdrawn from the US market due to its association with clinically severe hepatotoxicity[132, 133]. As revealed by structural analyses of its GSH-adducts, this 2,4-thiazolidinedione is activated by two distinct metabolic routes, one involving oxidation of the substituted chromane ring system to a reactive *o*-quinone methide, and the other involving a novel oxidative cleavage of the thiazolidinedione ring to highly electrophilic α-ketoisocyanate and sulfenic acid intermediates[131].

The second drug is the selective estrogen receptor modulating drug, raloxifene (Figure 7.5)[134], which is used for the treatment of postmenopausal osteoporosis. The *in vitro* bioactivation of raloxifene by human liver microsomal CYP3A4 results in mechanism-based inactivation of this enzyme. The inactivation is attenuated by the CYP3A4-selective inhibitor ketoconazole (10 μM), is quenched minimally (~15%) by GSH (5 mM), and is characterized by K_I, k_{inact}, and partition ratio values of 9.9 μM, 0.16 min^{-1}, and 1.8, respectively[134]. Indeed, raloxifene, albeit a slower CYP3A4 inactivator, is more potent than gestodene, which exhibits K_I, k_{inact} and partition ratio values of 46 μM, 0.39 min^{-1}, and 9, respectively[92]. CYP2D6 is also capable of bioactivating raloxifene at half the rate of CYP3A4, but the CYP2D6 contribution to human liver microsomal raloxifene activation is minimal as judged by

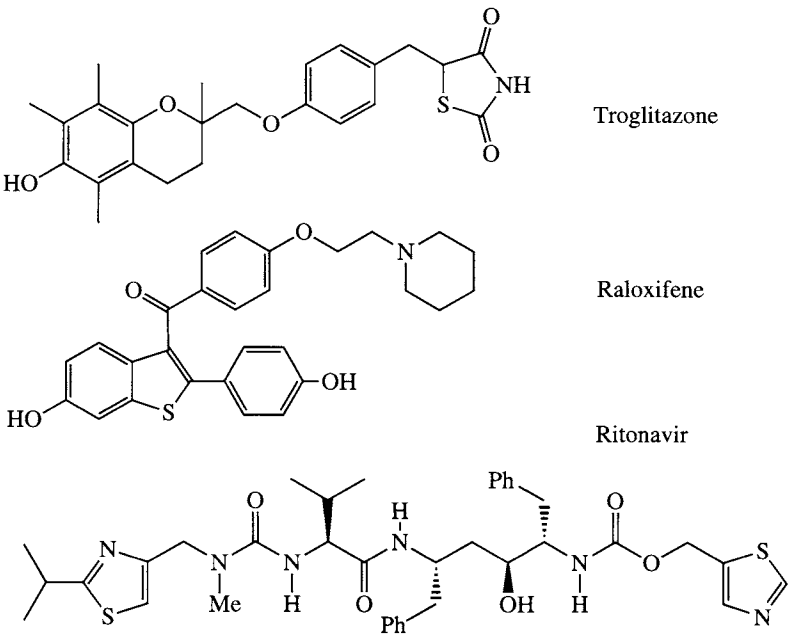


Figure 7.5. Structures of sulfur-containing compounds that inactivate P450 enzymes by mechanisms that have not been elucidated but may involve oxidation of a sulfur atom.

the failure of quinidine to significantly inhibit the process at concentrations known to inhibit CYP2D6[134]. The mechanism of this inactivation has not yet been explored, but could involve oxidative activation of the phenolic or thiophene functions.

Ritonavir (Figure 7.5), an inhibitor of the HIV-1 protease, is another example of a potent, sulfur-based inactivator of CYP3A4/CYP3A5, although it is also a CYP2D6 inhibitor[135–137]. Its inhibitory potency depends on the presence of both the 5-thiazolyl and 2-(1-methylethyl) thiazolyl groups. It has been proposed that it is oxidized by CYP3A to a chemically reactive fragment containing the 2-(1-methylethyl)thiazolyl group that causes enzyme inactivation[137].

Other noteworthy sulfur-containing mechanism-based inactivators include thiocyanates such as phenethyl isothiocyanate[138–140], benzylisothiocyanate[137–143], *tert*-butylisothiocyanate[144]; diallyl sulfide, and diallylsulfone derivatives[145–147], 1,2-dithiole-3-thione[148], oltipraz, and its derivatives[148], sulforaphane[148], and thiosteroids[149].

Chloramphenicol was among the first chlorinated mechanism-based P450 inactivators shown to act through irreversible protein modification[72–75]. Not only does the binding of [^{14}C] chloramphenicol to the apoprotein correlate with the loss of CYP2B1-dependent ethoxycoumarin *O*-deethylase activity, but proteolytic digestion of the inactivated P450 has been shown to yield a single [^{14}C]-modified amino acid[72–75]. Lysine and the chloramphenicol fragment in Figure 7.6 were released by hydrolysis of the modified amino acid. These results indicate that chloramphenicol is converted to an oxamyl chloride intermediate that either acylates a critical lysine, probably at the protein surface, or is hydrolyzed to the oxamic acid. Acylation of the lysine apparently inhibits electron transfer from P450 reductase to the CYP2B1 heme because the inactivated enzyme still catalyzes ethoxycoumarin *O*-deethylation in the presence of cumene hydroperoxide or iodosobenzene[75]. Furthermore, the unimpaired ethoxycoumarin *O*-deethylation supported by activated oxygen donors suggests that chloramphenicol is not covalently bound within the substrate binding-site.

The P450 isoform selectivity of inactivation by chloramphenicol and several of its analogs

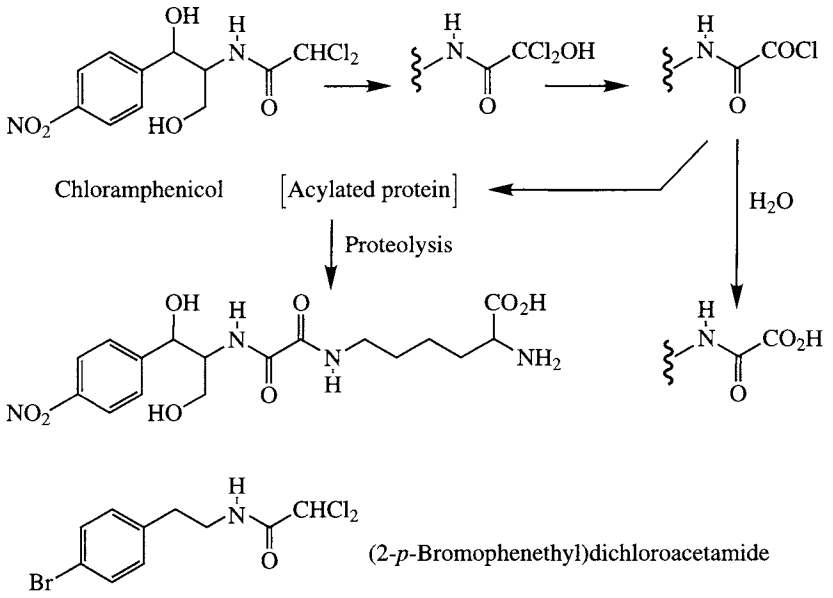


Figure 7.6. The inactivation of P450 by chloramphenicol involves oxidation of the dichloromethyl group to an acyl chloride that reacts with an amino group of the protein. A potent inactivating agent related to chloramphenicol is (2-*p*-bromophenethyl)dichloroacetamide.

has been reported[150]. Using four regiospecific androstenedione hydroxylases as functional markers, chloramphenicol was found to inactivate rat liver microsomal CYP2B1 > -3A > -2C11 > -2A1[150]. The P450 selectivity of the chloramphenicol analogs as mechanism-based inactivators was determined by at the least three structural features: (a) the number of halogen atoms, (b) the presence of a *para*-nitro group on the phenyl ring, and (c) substitutions on the ethyl side chain. Thus the analog *N*-(2-phenethyl)dichloroacetamide reversibly inhibited but did not inactivate CYP3A enzymes, in contrast to *N*-(2-*p*-nitrophenethyl)- and *N*-(1,2-diphenethyl)dichloroacetamide, both of which rapidly did so[150]. The incorporation of a *para*-nitro or -bromo substituent on the phenyl ring, or a second phenyl at the 1- or 2-position of the phenethyl side chain, yielded compounds that selectively inactivated CYP2B1 over CYP2C11, -2C6, or -2A1[77]. Thus, *N*-(2-*p*-bromophenethyl)-dichloroacetamide (Figure 7.6) and *N*-(2-*p*-nitrophenethyl)dichloroacetamide were the two most effective and selective CYP2B1 inactivators both *in vitro* and *in vivo*[77].

21-Chloropregnenolone, 21,21-dichloropregnenolone, and 21,21-dichloroprogesterone have also been probed for their selectivity of P450 inactivation using regiospecific and/or stereoselective progesterone or androstenedione hydroxylases as functional probes[151, 152]. The findings reveal that 21,21-dichloropregnenolone and 21,21-dichloroprogesterone are comparably effective in the inactivation of rat liver microsomal CYP3A enzymes, with k_{inact} values of ≈ 0.1 min^{-1} for both substrate probes, while 21,21-dichloroprogesterone even more efficiently inactivated CYP2C6 (k_{inact} = ~ 0.2 min^{-1})[151]. 21,21-Dichloropregnenolone was also a very efficient mechanism-based inactivator of rabbit liver CYP2C5 but not of rabbit adrenal CYP21[152]. On the other hand, CYP21 was rapidly inactivated by 21,21-dichloroprogesterone[152]. Thus, replacement of a methyl group that is normally oxidized by a P450 enzyme with a dichloromethyl moiety may be a valuable strategy in the design of specific P450 inhibitors.

3.1.2. Olefins and Acetylenes

As discussed in detail below (Section 3.2), compounds containing an olefinic bond, ranging from compounds as simple as ethylene to more complex structures such as allylisopropylacetamide (AIA),

allylisopropylcarbamide (sedormid), aprobarbital, allobarbital, and secobarbital, can inactivate P450 enzymes by *N*-alkylating the porphyrin group of the prosthetic heme[153–156]. However, studies with secobarbital show that it completely inactivates CYP2B1 but only causes partial loss of the heme chromophore[97, 157, 158]. Isolation of the *N*-alkylated porphyrins (see Section 3.3.1) and of the modified CYP2B1 protein reveal that the compound partitions between heme *N*-alkylation, CYP2B1 protein modification, and formation of an epoxide metabolite in a ratio of 0.8 : 0.2 : 59, respectively[97, 157, 158]. The *in situ* presence of the heme adduct in the CYP2B1 active site is spectrally confirmed by its typical ~445 nm absorption maximum,[158] a feature characteristic of iron-complexed *N*-modified porphyrins[159]. The *N*-modified porphyrins have been isolated both as the parent adducts and as the corresponding dimethyl esters and identified by mass spectrometry (MH^+ 816.9 and 845.8 Da, respectively) as adducts of protoporphyrin IX and hydroxysecobarbital (Figure 7.7)[157]. The CYP2B1 peptide modified by the drug has also been isolated and shown to be comprised of residues 277–323, residues that by sequence analogy to $P450_{cam}$ correspond to the distal I helix[157, 160–163]. Similar structural analysis after further digestion of this CYP2B1 peptide has narrowed down the region of secobarbital modification to residues G_{299}-S_{304}[158]. Although the precise residue that is modified remains to be identified, these findings are consistent with modification of the protein within the active site. In view of this, it is not surprising that specific mutations in the CYP2B1 putative substrate-recognition sites (SRS) 2, 4, 5, and 6, but not SRS-1, attenuate secobarbital-mediated inactivation. The SRS-mutations T302S and V363L markedly reduced CYP2B1 heme-modification and a V367A (SRS-5) mutation most markedly impaired protein modification[158].

Terminal acetylenes, like terminal olefins, alkylate the P450 prosthetic heme (see Section 3.3.2), but compounds such as 10-undecynoic acid, 1-ethynylpyrene, 17β-ethynylprogesterone, 17α-ethynylestradiol (EE), and 9- and 2-ethynylnaphthalene have been shown to inactivate P450s primarily by binding covalently to the protein with only partial loss of the heme group[78–83, 90, 91]. Thus, near stoichiometric binding of 2-ethynylnaphthalene and 1-ethynylpyrene to CYP1A1 and -1A2, of EE to CYP3A4, and of 10-undecynoic acid to rat liver CYP4A1 (ω-hydroxylase) has been observed[78–83, 87–91]. The isolation of the terminal acid metabolites from the incubations of 10-undecynoic acid (Figure 7.8) and

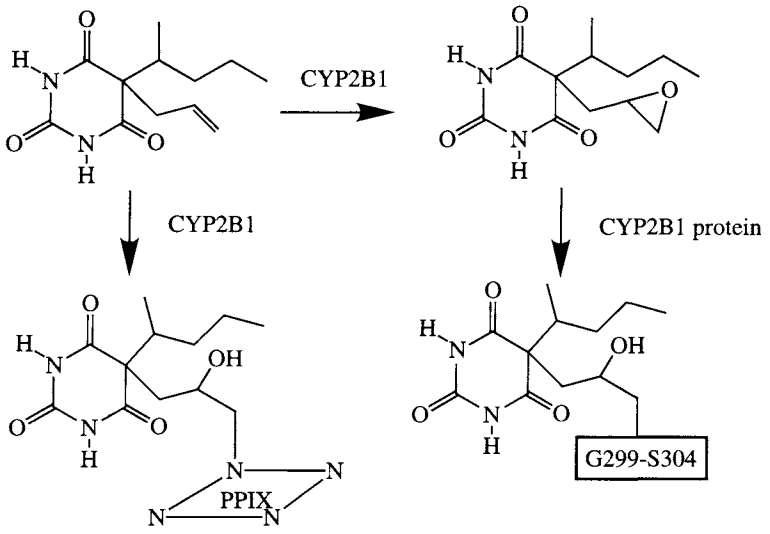


Figure 7.7. The oxidation of secobarbital by CYP2B1 results in both alkylation of one of the residues between Gly299 and Ser304 and *N*-alkylation of the heme group as well as the generation of an epoxide.

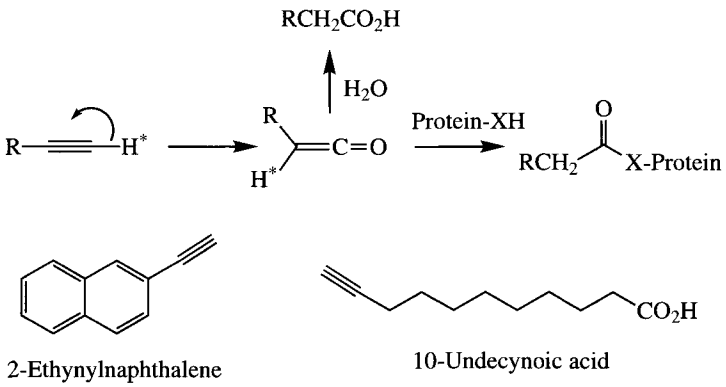


Figure 7.8. The oxidation of terminal acetylenes, and even some internal acetylenes, results in the formation of ketene intermediates that react with water to give the carboxylic acids (see Chapter 6). It appears that the ketenes also react with active-site residues, inactivating the P450 enzyme that forms them. The hydrogen that undergoes a 1,2-migration during the oxidation reaction is indicated by a star. The structures of 2-ethynylnaphthalene and 10-undecynoic acid, both of which inactivate P450 enzymes, at least in part by this mechanism, are shown.

1-ethynylpyrene strongly supports a mechanism in which oxygen transfer from the P450 heme to the terminal carbon of the triple bond triggers migration of the terminal hydrogen to the adjacent carbon (Figure 7.8). The reactive ketene generated by this hydrogen shift mechanism, which is supported by deuterium labeling experiments, either acylates the protein or is hydrolyzed to the carboxylic acid metabolite[83]. The corresponding ketene metabolites have been similarly invoked in the acylation of bovine adrenal CYP21 by 17β-ethynylprogesterone[85, 86] and of CYP1A2, -2B1, and -2B4 by 2-ethynylnaphthalene (Figure 7.8)[80, 82, 93]. In accord with this postulate, CYP2B1 oxidizes 2-ethynylnapthalene to 2-(2-napthyl)acetic acid, presumably via the same ketene intermediate as is postulated to modify the protein. The mechanism-based inactivation of CYP2B1 by 2-ethynylnaphthalene is characterized by a $K_I = 0.08$ μM, $k_{inact} = 0.83\ min^{-1}$, and a partition ratio of approximately 4–5 moles of acid formed per inactivation event[82].

In contrast, CYP2B1-catalyzed addition of the activated oxygen to the internal rather than terminal triple bond carbon of phenylacetylene results in heme *N*-alkylation rather than protein acylation (see below)[83, 164]. Predominant inactivation of CYP2B1 by phenylacetylene via heme alkylation[164] and by 2-ethynylnaphthalene via protein acylation[80, 82, 93] sheds some light on the influence exerted by the fit of the inhibitor within the active site as a determinant of the inactivation mechanism. Both of these terminal aryl acetylenes yield ketene metabolites but only that from 2-ethynylnaphthalene acylates the CYP2B1 protein. Protein acylation by 2-ethynylnaphthalene demonstrates that the failure of phenylacetylene to acylate the CYP2B1 protein is not due to the absence of appropriate nucleophilic residues. Furthermore, the formation of phenylacetic acid in the oxidation of phenylacetylene confirms that the ketene is formed. The differential inactivation of CYP2B1 by 2-ethynylnaphthalene and phenylacetylene suggests, in fact, that: (a) 2-ethynylnaphthalene, unlike phenylacetylene, is bound in a manner that prevents delivery of the ferryl oxygen to the internal carbon and (b) heme alkylation by phenylacetylene is sufficiently efficient relative to protein acylation by the phenylketene metabolite that the enzyme is inactivated before protein acylation becomes significant. These differences presumably stem from differential binding affinities and/or orientations of the two agents within the CYP2B1 active site.

Peptide mapping and amino acid sequence analysis of radiolabeled peptides indicate that 2-ethynylnaphthalene binds to amino acid residues 67–78 and 175–184, respectively, of rat and rabbit CYP1A2[80]. However, the residue that is actually modified and the precise nature of the covalent link to the inhibitor have escaped definition due to the instability of the adducts.

Alignment of the modified peptides with the sequence of $P450_{cam}$ (CYP101) suggests that the CYP1A2 peptides correspond to helices A and D of $P450_{cam}$ (see Chapter 3). Thus, the rat CYP1A2 peptide may include residues from the substrate-binding regions[160–163]. The 2-ethynylnaphthalene-modified peptides from CYP2B1 and CYP2B4 have also been characterized and proposed to be adducts of 2-naphthylacetic acid with a peptide residue[82, 93]. Digestion of CYP2B1 after its oxidation of radiolabeled 2-ethynylnapthalene yields a radiolabeled peptide (ISLLSLFFAGTETSSTTLRYGFLLM) that includes residues 290–314 of the protein. An analogous peptide (E_{273}–M_{314}) is obtained with CYP2B4. The two modified peptides correspond in sequence to the highly conserved distal I helix of $P450_{cam}$ (CYP101) that contacts both the substrate and the heme group[160–163]. The specific residue modified in each peptide has not been identified but several nucleophilic residues, including serine, threonine, and tyrosine, are present. If the protein nucleophile is the hydroxyl group of such a residue, the resulting adduct would be an ester. Indeed, recent studies with a CYP2B4 T302A mutant reveal that the k_{inact} of 2-ethynylnaphthalene is decreased from 0.20 ± 0.05 min^{-1} for the wild type to 0.05 ± 0.01 min^{-1} in the mutant, suggesting that T302 is at least one acylated residue[165].

7-Ethynylcoumarin (7-EC) (Figure 7.9)[94] is a rationally designed mechanism-based inactivator with the ethynyl moiety at the 7-position of coumarin where, by analogy to 7-ethoxycoumarin and 7-ethoxy-4-(trifluoromethyl) coumarin, it should be readily oxidized by CYP2B enzymes[166, 167]. In comparative assays, 7-EC (100 μM) only marginally (15%–20%) inactivated human liver microsomal CYP2A6, but markedly (>90%) inactivated purified CYP2B1[94]. This inactivation left the heme and its thiolate ligand unscathed, as the electronic absorption of the reduced-CO complex of the inactivated enzyme was little affected. The inactivation by 7-EC was unaffected by the presence of nucleophiles such as GSH and NaCN, of the iron-chelator deferoxamine, or of superoxide dismutase or catalase and conformed to all the other established criteria for a mechanism-based inactivation. It exhibited a K_I of 25 ± 2 μM, a k_{inact} at 30°C of 0.39 ± 0.01 min^{-1}, a partition ratio of 25, and a half-life ($t_{1/2}$) of 1.8 min. ESI–LC/MS of the dialysed intact native and 7-EC-inactivated CYP2B1 yielded masses of 55,899 ± 1 Da and 56,084 ± 3 Da, respectively, for the two proteins[94]. This corresponds to a mass difference of 185 Da (0.005% variability in mass assignment), and indicates that the entire 7-EC molecule together with an oxygen atom was covalently bound to CYP2B1 in a 1 : 1 stoichiometry. The precedents set by other arylacetylenes suggest that the protein adduct involves addition of an active-site nucleophilic residue to the activated acetylene. Two mechanisms have been proposed for the acylation: One that is mediated by a ketene metabolite and a second involving attack on a putative oxirene intermediate[94]. In view of the fact that oxirenes are only hypothetical species due their practically barrierless conversion to ketenes, the protein modification is almost certainly mediated by the ketene.

17α-Ethynylestradiol (EE) (Figure 7.9) has long been known to inactivate rat and human liver P450 enzymes in a mechanism-based fashion[87–89]. Its inactivation was shown to result from P450-dependent metabolic activation of its acetylenic moiety to a species that alkylates a heme pyrrole nitrogen. More recently, EE has been shown to also modify the CYP3A4 protein in a reconstituted enzyme system with k_{inact} = 0.04 min^{-1}, K_I = 18 μM, $t_{1/2}$ = 16 min, and a partition ratio of ~50[90]. Loss of activity was paralleled by some loss of CO-dependent chromophore. A net stoichiometry of 1.3 nmol of EE-metabolite bound/nmol CYP3A4 was observed with [^{3}H]EE as the substrate. HPLC analysis yielded several NADPH-dependent [^{3}H]EE-labeled fractions, the predominant one of which eluted at 31 min. ESIMS analysis in the negative ion mode of this peak fraction yielded a mass $(M\text{-}H)^-$ of 479 Da. Although the chemical structure of this EE-related species has not been definitively characterized, the authors believe it to be an EE-adducted monopyrrolic heme fragment[90]. EE thus appears to modify both the heme and protein moieties of CYP3A4, whereas only the heme moieties of CYP2B1 and -2B6 are susceptible to EE-modification.

The monoamine oxidase (MAO) inhibitor deprenyl (Figure 7.9) is a propargylamine whose terminal acetylene reacts irreversibly with the MAO flavin moiety[168]. Deprenyl also inactivates CYP2B1 relatively selectively with K_I = 1.05 μM, k_{inact} = 0.23 min^{-1}, and partition ratio = 2[169]. However, although there is a loss of

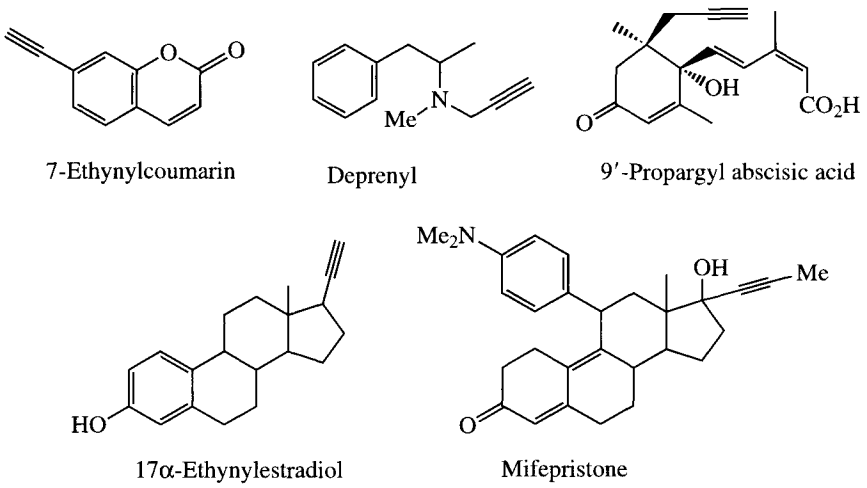


Figure 7.9. The structures of acetylenes that have been shown to inactivate P450 enzymes. In all cases except perhaps that of mifepristone the reactive intermediate appears to arise by oxidation of the triple bond.

spectrally detectable P450 chromophore, no significant change is observed in the 405 nm heme absorbance. The complete loss of CYP2B1 function, however, suggests protein modification following activation of the terminal acetylene. Analogs of deprenyl such as (*R*)-*N*-(2-heptyl)-*N*-methyl-propargylamine and (*R*)-*N*-(2-heptyl)-propargylamine have been tested for their P450 isoform inactivation selectivity[170]. Of these, *(R)*-*N*-(2-heptyl)-propargylamine was the more potent, with K_I = 0.5 μM and k_{inact} = 0.36 min^{-1} [170].

A specific inhibitor of the P450 enzyme that degrades the phytohormone abscisic acid has been constructed by replacing the methyl group of abscisic acid that is normally oxidized with acetylenic moieties[171]. The most active of the inactivating agents is 9′-propargyl abscisic acid (Figure 7.9), which *in vivo* exhibits a greater inhibition of *Arabidopsis thaliana* seed germination than abscisic acid itself.

3.1.3. Other P450 Protein Modifying Inactivators

The recreationally abused psychotomimetic amine phencyclidine [1-(1-phenylcyclohexyl) piperidine; PCP] (Figure 7.10) is another mechanism-based inactivator that inactivates CYP2B1, -2B4, and -2B6 relatively selectively via protein modification[172, 173]. Incubations of radiolabeled PCP with NADPH-supplemented liver microsomal preparations from phenobarbital-pretreated rabbits demonstrated a time- and concentration-dependent loss of ketamine and benzphetamine *N*-demethylase activity as well as irreversible binding of the radiolabeled amine to microsomal protein[174–176]. Inhibition of both of these events by cyanide (NaCN), but not GSH, led to the proposal that α-oxidation yielded a PCP iminium species that was responsible for the inactivation. This iminium derivative could directly bind to proteins, but apparently could also undergo further NADPH-dependent metabolism by P450, resulting in inactivation of those enzymes[176–179]. This second oxidation was proposed to convert the PCP iminium ion to an allyl alcohol via a reactive, electrophilic 2,3-dihydropyridinium intermediate [1-(1-phenylcyclohexyl)-2,3-dihydro-4-pyridone] that reacted irreversibly with the protein[178]. *In vitro* studies with five rabbit liver P450 isoforms confirmed that the inactivation was indeed mechanism-based, highly selective for CYP2B4, and involved parallel loss of the CYP2B4 heme chromophore and function[179]. However, parallel studies of PCP and its iminium metabolite revealed that the latter was less selective than the parent compound and inactivated P450 3b (a constitutive rabbit liver isoform) as well[179]. More recent *in vivo* studies have shown that CYP2D is also markedly (>74%) inactivated by PCP in uninduced

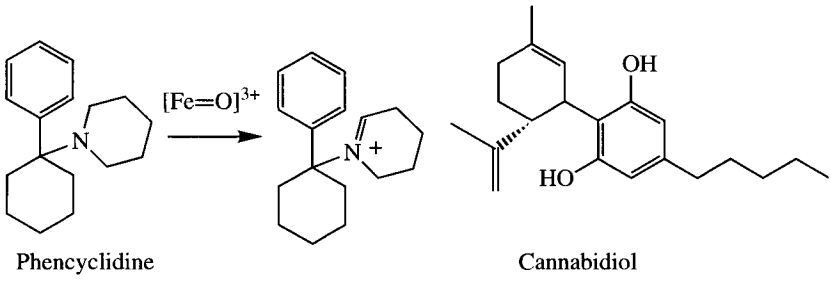


Figure 7.10. Phencyclidine is oxidized to an iminium species whose role as an intermediate in the inactivation of P450 enzymes is unclear. The structure of cannabidiol (CBD), which also inactivates P450 enzymes by an undefined mechanism, is also shown.

rats[180]. Comparative *in vitro* studies of PCP analogs containing a five [PCPY; 1-(1-phenylcyclohexyl) pyrrolidine]- or six [PCHMI; 1-(1-phenylcyclohexyl) hexamethyleneimine]-membered heterocyclic ring indicated that the rates and $t_{1/2}$ of inactivation and heme loss were comparable for PCPY and PCP, both of which were 10-fold higher than those of PCHMI even though the latter was metabolized to a greater extent[181]. No mechanism-based inactivation was observed with either phenylcyclohexylamine or the diethylamino analog of PCP, suggesting that the substituted nitrogen is necessary and must be incorporated into a heterocyclic ring. The observation of prosthetic heme loss and its sensitivity to cyanide ion reinforced the notion that the iminium ion is involved in the PCP-mediated inactivation[181].

HPLC-analyses of heme extracts from PB-pretreated rabbit liver microsomes, incubated with or without PCP, identified PCP-modified heme fractions proposed to be *N*-modified porphyrins, but the nature of these porphyrins was not established[179]. It is therefore unclear whether heme modification or degradation was responsible for the loss of P450 activity or simply coincidental with it. However, a substantial discrepancy in the partition ratio obtained for PCP-mediated functional inactivation and that for heme loss suggested that this enzyme inactivation might also entail protein modification.

Protein modification might indeed be primarily responsible for the PCP-mediated inactivation of CYP2B1 and -2B6, as no loss of heme chromophore in its ferric or CO-bound form was detected[172, 173]. Studies with CYP2B1- and CYP2B6-selective probes (7-ethoxycoumarin *O*-deethylase and 7-ethoxy-4-(trifluoromethyl) coumarin *O*-deethylase, respectively) yielded K_I values of 3.8 and 10 μM, k_{inact} values of 0.12 min^{-1} and 0.01 min^{-1}, respectively, and partition ratios of ~45 for both processes. More importantly, contrary to previous reports, not only was CN (up to 1 mM) incapable of inhibiting either inactivation event, but little inactivation was observed when the synthetic amino enone [1-(1-phenylcyclohexyl)-2,3-dihydro-4-pyridone], the putative reactive electrophilic 2,3-dihydropyridinium intermediate, was used as the inactivating substrate[172, 173, 182]. Studies with [^{3}H]PCP revealed a molar stoichiometry of PCP binding to CYP2B1 and CYP2B6 protein of 4 : 1 and 5.5 : 1, respectively. GSH (10 mM) did not decrease the rate of PCP-mediated inactivation but reduced the binding of [^{3}H]PCP to CYP2B6 protein to the expected 1 : 1 molar stoichiometry. Consistent with these findings, LC–MS analyses of the PCP–P450 adduct in the ESI–LC/MS mode yielded a mass difference of 244 ± 5 Da between PCP-inactivated CYP2B1 and the wild-type protein, confirming the formation of a 1 : 1 PCP–CYP2B1 adduct. Similar studies of PCP-inactivated CYP2B4 gave a mass difference of 261 ± 2 Da, consistent with the binding of one molecule of monohydroxylated PCP to the CYP2B4 protein. Similar analyses of PCP–CYP2B6 adducts were less conclusive due to inconsistent results[173]. Collectively, the recent findings argue for protein modification at the CYP2B active site as the major mode of inactivation. Although a reactive electrophilic inactivating species is involved, the PCP-iminium ion may not be its precursor.

Modification of the P450 protein is also observed with cannabidiol (CBD) (Figure 7.10), a

major constituent of marijuana. CBD inactivates mouse P450 isoforms 2C and 3A via a mechanism that involves stoichiometric covalent binding of the inhibitor and loss of its CBD oxidase activity[183, 184]. Limited structure-activity studies show that a free phenolic group in the resorcinol moiety of CBD is essential for inactivation[183]. Mass spectrometric analyses of the concomitantly generated CBD metabolite trapped as a GSH-adduct led to identification of CBD-hydroxyquinone as the inactivating species[184], a finding consistent with the electrophilic reactivity and P450 inactivating potential of this compound[185]. HPLC-peptide mapping, microEdman degradation, and mass spectrometric analyses of the CBD-modified peptides obtained by proteolytic digestion of CBD-inactivated CYP3A11 identified the peptide region spanning residues A_{344}–K_{379} and G_{426}–K_{454} as the loci of CBD modification[184]. These regions correspond to the CYP3A11 active site SRS-5 in the K-region and the heme-binding Cys_{443}-region/helix L domain[160–163], fulfilling an essential criterion of a mechanism-based inactivation process[184]. Interestingly, both peptides contain Cys residues that could react with the CBD-hydroxyquinone. Analogous studies with CYP3A4 have identified similar protein adducts, albeit at a lower level than those detected with the mouse ortholog (L.M. Bornheim, personal communication). Although Δ^9- and Δ^8-tetrahydrocannabinol (THC), the other major and minor psychoactive components of marijuana, respectively, are efficiently metabolized by CYP3A and -2C, they are not inactivating agents. In contrast, unsaturated Δ^8-THC-enyl and -ynyl analogs that are also present in the complex marijuana extracts have been found to be selective mechanism-based inactivating agents *in vitro* but not *in vivo*[186].

Other classes of compounds that inactivate human and rat liver microsomal P450 enzymes via protein modification are known. One such class is represented by the furanocoumarins, natural constituents of foods such as celery, parsley, figs, parsnips, and grapefruit juice[187]. Among these compounds, the photoactive linear furanocoumarin, 8-MOP (methoxsalen) is a particularly potent P450 inactivator. Evidence that 8-MOP was activated by P450 enzymes to an electrophilic product that bound covalently to the protein[98–102] was obtained from incubations with rodent liver microsomes. Inclusion of cysteine or GSH in these incubations did not prevent P450 inactivation but suppressed nonspecific covalent binding. The residual covalent binding of 8-MOP to microsomal protein was nearly stoichiometric with the loss of microsomal P450[101, 102]. This finding, together with the observation of a type I spectrum in incubations of rat liver microsomes with 8-MOP in the presence but not absence of NADPH[101], implicated an active-site directed inactivation mechanism. Furthermore, because cysteine markedly quenches the covalent binding of the label from [^{3}H]-methoxy- but not [4-^{14}C]-ring-labeled methoxypsoralen, the reactive intermediate is likely to reflect oxidation of the furan ring[102]. This inference is supported by the finding that trioxsalen (trimethylpsoralen), in which the furan ring bears a methyl substituent, does not destroy P450. Methyl-substituted furans, however, can also inactivate P450 enzymes, as illustrated by the inactivation of human liver enzymes observed in incubations with (*R*)-(+)-menthofuran[103].

8-MOP is a potent inactivator of human liver CYP2A6 and rat liver CYP2B1, but also inactivates CYP2B2, -1A, -3A, and -2C11[98, 106, 188]. Of all the furanocoumarins tested, 8-MOP was the most potent inactivator of CYP2B1 with a K_I, k_{inact}, and partition ratio of 2.9 μM, 0.34 min^{-1}, and 1.3, respectively[188]. SDS-PAGE and/or HPLC analyses of the components from incubations of purified CYP2B1 with [^{14}C-]8-MOP indicated that the radiolabel bound to the protein rather than to the heme with a stoichiometry of 0.7 : 1. This [^{14}C-]8-MOP-binding was unaffected by GSH or methoxylamine (MOA), suggesting that covalent binding occurred within the active site where it was inaccessible to external nucleophiles. LC–ESIMS analyses of the [^{14}C-]8-MOP-modified CYP2B1 revealed a mass shift of 237.9 ± 9.6 Da over that of the native enzyme. Analogous studies of 5-MOP- and psoralen-modified CYP2B1 gave mass shifts of 240 ± 6.2, and 204 ± 11.8 Da, respectively[188]. These results indicate that a single psoralen molecule is covalently bound to the protein and are consistent with CYP2B1-catalyzed oxidation of the 8-MOP furan ring to the 8-MOP furanoepoxide (MW, 232.2 Da) followed by reaction of the protein with the epoxide or a cationic species derived from it.

Another notable furanocoumarin, 6′,7′-DHB, that is derived from the processing of grapefruit juice and is one of its common constituents,

is now a well-recognized mechanism-based inactivator of CYP3A4[107–109, 189]. Inactivation of intestinal CYP3A4 after oral grapefruit juice intake has been clinically associated with increased bioavailability of several CYP3A4 drug substrates. Numerous reports exist of clinically relevant adverse drug–drug interactions between grapefruit juice and CYP3A4 substrates, in some instances resulting in withdrawal of the drug in question[189]. Studies with 6′,7′-DHB indicate that it is activated to a reactive species that irreversibly modifies the CYP3A4 protein with $K_I = 59\ \mu M$ and $k_{inact} = 0.16\ min^{-1}$ [107–109], resulting in accelerated hepatic degradation of the protein (see below; Malhotra and Watkins, personal communication).

The furanopyridine L-754,394, *N*-[2(*R*)-hydroxy-1(*S*)-indanyl]-5-[2(*S*)-((1,1-dimethylethyl)amino)carbonyl]-4-[(furo[2,3-*b*]pyridin-5-yl)methylpiperazin-1-yl]-4(*S*)-hydroxy-2(*R*)-(phenylmethyl)pentenamide, is also a potent mechanism-based inactivator of human liver microsomal CYP3A4 but is less selective, as it also inactivates human CYP2D6[110–112, 190]. Its K_I, k_{inact}, and partition ratio for human liver microsomal CYP3A4 are reportedly 7.5 μM, 1.62 min^{-1}, and 1.35, respectively[111]. Structural characterization of the concomitantly generated L-754,394 metabolites revealed that the mechanism of inactivation probably entails CYP3A4-dependent oxidation of the furan ring to the corresponding epoxide and/or γ-ketoenal that binds to the protein within the active site. Accordingly, neither the corresponding dihydrofuran derivative nor the analog that lacks the furan ring is active as a CYP3A4 irreversible inhibitor. Indeed, Tricine-SDS-PAGE, HPLC-peptide mapping, and MALDI-TOF-MS analyses of L-754,394-bound CYP3A4 showed that the inhibitor bound to the I-helix peptide I_{257}–M_{317}[112]. The chemical instability of this adduct under the acidic conditions required for these analyses precluded identification of the active-site residue actually modified. In view of the markedly higher stability of the adducts with GSH, *N*-acetylcysteine (NAC), and MOA, and of *O*-linked heterodimers of the hydroxylated/parent furano- compounds, the instability led the authors to propose an ester linkage between activated L-754,394 and CYP3A4. The authors proposed E307 as the most plausible target[112].

Two other drugs of note that have been reported to inactivate P450 by protein modification are tamoxifen and CBZ. Tamoxifen (*Z*-{1-[4-(2-dimethyl-aminoethoxy)phenyl]-1,2-diphenyl-1-butene}) (Figure 7.11), a nonsteroidal antiestrogen used in hormone-dependent breast cancer chemotherapy, inactivates CYP2B6 (but not CYP1B1 and CYP3A4) with $K_I = 0.9\ \mu M$, $k_{inact} = 0.02\ min^{-1}$, and $t_{1/2} = 34$ min. CYP2D6 and CYP2C9 were also partially (25%) inactivated[114–116, 191]. The ultimate inactivating species remains to be identified, but the sequential metabolism of tamoxifen to 4-hydroxytamoxifen, and then to 3,4-dihydroxytamoxifen-*o*-quinone, a known reactive and carcinogenic species[191], suggested it might be responsible for the inactivation[115]. This proposal has been challenged by studies showing that incubation with 3-hydroxy or 4-hydroxytamoxifen reversibly inhibits CYP3A4 but does not inactivate it[116]. A mechanism-based inactivation by tamoxifen and its *N*-desmethyltamoxifen metabolite (instead of 3- or 4-hydroxytamoxifen) involving the formation of a metabolic intermediate (MI) complex was proposed as an alternative explanation[116].

CBZ (Figure 7.11), a widely used anticonvulsant, is known to be metabolized to several reactive species, including the arene oxide, 9-acridine carboxaldehyde, and an iminoquinone metabolite[192–196]. Clinically, CBZ is often associated with idiosyncratic hypersensitivity syndromes[197–199]. CBZ-treated patients often exhibit anti-CYP3A4 autoantibodies, suggesting that covalent binding of CBZ to CYP3A4 leads to the immunoproteosomal formation of antigenic CBZ-modified peptides[198, 199]. CBZ is indeed an excellent CYP3A4 substrate that is metabolized predominantly to the CBZ 10,11-epoxide, a reaction that has been used as a CYP3A4 functional marker[200]. The incubation of [^{3}H]CBZ with CYP3A4 results in irreversible binding of [^{3}H]CBZ to the protein with a stoichiometry of 1.58 ± 0.15 pmol [^{3}H]CBZ bound/pmol CYP3A4. In the presence of GSH (4 mM) this stoichiometry is reduced to 1.09. However, no concentration (0–1 mM) nor time-dependent CYP3A4 inactivation was detected in these incubations[201]. In the absence of other substrates, CBZ, if anything, protected from NADPH-dependent oxidative uncoupling[201]. Given these findings, one can speculate that the serum anti-CYP3A4 autoantibodies detected in CBZ-dependent hypersensitivity might arise in patients whose CBZ-CYP3A4 adducts remain

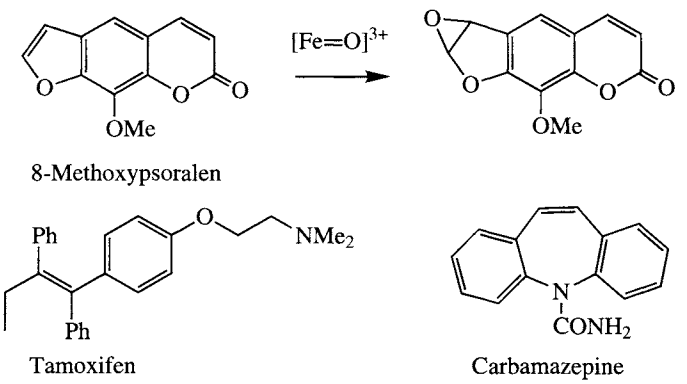


Figure 7.11. 8-Methoxypsoralen (8-MOP) is oxidized to an epoxide, and either the epoxide or a ring-opened product derived from it is responsible for inactivation of P450. The structures of two drugs, tamoxifen and carbamazepine (CBZ), that inactivate P450 by unknown mechanisms are also shown.

stable, possibly because of low intrahepatic GSH levels, and are therefore available for antigenic processing[199, 201].

Collectively, the results convincingly establish that the reactive species formed by P450 enzymes can alkylate, acylate, or otherwise modify the protein skeleton, resulting in the loss of catalytic activity. It is likely, furthermore, that protein modification has gone undetected in some instances where inactivation has been attributed to heme modification. Conversely, the data on parathion and CBZ suggests that heme destruction is required for enzyme inactivation in some instances where protein modification clearly occurs. Furthermore, the fact that CYP2B1 is inactivated by secobarbital by both heme alkylation and protein modification, by phenylacetylene predominantly by heme alkylation, and by 2-ethynylnaphthalene predominantly by protein acylation, underscores the critical role of active-site–substrate interactions in dictating the mode of inactivation. Active-site–inhibitor interactions presumably also explain why *N*-phenyl or *N*-octyl 2,2-dichloroacetamides predominantly inactivate CYP2B1 via protein acylation whereas the corresponding *N*-hexyl, *N*-butyl, or *N*-methyl dichloroacetamides do so via heme destruction[76, 77]. Similar factors presumably also explain the differential modes of inactivation observed with terminal acetylenes (e.g., *N*-alkylation of CYP2B1 by phenylacetylene vs protein acylation of CYP4A1 by 10-undecynoic acid)[78, 83].

3.2. Quasi-Irreversible Coordination to the Prosthetic Heme

This section focuses on inhibitors that are transformed by P450 enzymes into metabolic intermediate (MI) products that coordinate so tightly to the heme iron atom that they can be displaced only under unique experimental conditions. The two major classes of these inhibitors are compounds with a dioxymethylene function and nitrogen compounds, usually amines that are converted *in situ* to nitroso metabolites. A related mechanism is also partially responsible for the inhibition of P450 by 1,1-disubstituted hydrazines and acyl hydrazines. The anaerobic reductive coordination of halocarbons to the heme iron atom is discussed in Section 3.4 because the reaction is linked to destruction of the heme.

3.2.1. Methylenedioxy Compounds

P450 enzymes oxidize aryl and alkyl methylenedioxy compounds, some of which are used as insecticide synergists[202, 203], to species that coordinate tightly to their heme iron atom[204]. The time, NADPH, oxygen, and concentration dependence of the reaction, as well as the finding that NADPH and oxygen can be replaced by cumene hydroperoxide, confirm that the inhibitory species is unmasked by the catalytic action of the P450

enzymes[202, 205, 206]. The resulting ferrous complex is characterized by a difference absorption spectrum with maxima at 427 and 455 nm, whereas the ferric complex exhibits a single absorption maximum at 437 nm[202, 207]. The ferrous peaks at 427 and 455 nm are due to distinct complexes, although their structural interrelationship is obscure[204]. The ferrous complex can be isolated intact from animals treated with isosafrole, demonstrating its stability, but the less stable ferric complex can be disrupted by incubation with lipophilic compounds with concomitant regeneration of the catalytically active enzyme[208, 209]. The ferrous complex is unaffected by incubation with lipophilic compounds but can be disrupted by irradiation at 400–500 nm[210, 211]. Structure activity studies of 4-alkoxy-1,2-methylenedioxybenzene reveal that the size and lipophilicity of the alkoxy group is an important determinant of the complex stability: alkyl chains of 1–3 carbons yield unstable complexes whereas those with longer alkyl groups are stable[212, 213]. As in the case of reversible inhibitors (Section 2.3), the ferrous complex is stabilized by concurrent binding interactions of the ligand with the lipophilic active site[213]. The ferrous to ferric transition weakens the complex, indicating that the activated species, like carbon monoxide, only strongly coordinates to the ferrous iron.

The above results are consistent with the catalysis-dependent generation of a carbene–iron complex (Figure 7.12). The synthesis and characterization of model carbene complexes provides supporting evidence for a carbene complex[214, 215]. The structural analogy between a carbene and carbon monoxide provides a ready explanation for the unusual 455-nm absorption maximum of the complexes. As already noted, a different complex is responsible for the absorption maximum at

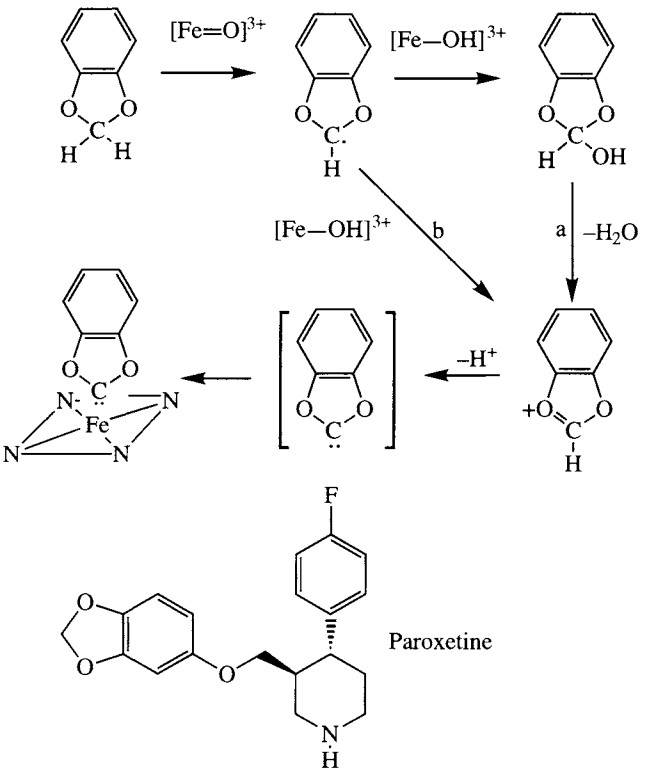


Figure 7.12. The quasi-irreversible inactivation of P450 enzymes by methylenedioxy compounds involves oxidation of the methylene bridge to a species that forms a tight, but reversible, complex with the heme iron atom. The coordinating species is probably a carbene, as shown. Paroxetine is an example of a drug that inhibits P450 by this mechanism.

427 nm, perhaps a carbene complex in which the *trans* ligand, as in P420, is not a thiolate[216]. A carbene complex also provides a ready rationale for the incorporation of oxygen from the medium into the carbon monoxide metabolite formed from the dioxymethylene bridge carbon (see below), and the observation that carbon monoxide formation is enhanced by electron-withdrawing substituents[217]. Water addition to the iron-coordinated carbene produces an iron-coordinated anion that should readily decompose into the observed catechol and carbon monoxide metabolites. A different but undefined mechanism is required to explain the incorporation of an atom of molecular oxygen into a fraction of the carbon monoxide[217].

The link between the dioxymethylene function and P450 inhibition, the requirement for catalytic activation of the inhibitor, and the fact that the dioxymethylene group is oxidized implicate this function in the inhibitory events. An inhibitory role has been postulated for free radical[218], carbocation[219], and carbanion[210] intermediates, but formation of the carbene from the bridge-hydroxylated metabolite or from its radical precursor is most consistent with the results (Figure 7.12). Substituents other than an alkoxy group on the dioxymethylene group suppress complex formation[203, 204, 220]. The retention of activity with an alkoxy substituent is understandable because *O*-dealkylation of the substituent provides an independent route to the bridge-hydroxylated precursor of the carbene[211]. The oxidation of aryldioxymethylenes to catechols, carbon monoxide, carbon dioxide, and formic acid is consistent with hydroxylation of the methylenedioxy bridge[203, 217, 221–223], as is the observation that deuterium substitution on the dioxymethylene carbon decreases the rate of formation of carbon monoxide (k_H/k_D = 1.7–2.0). The observation of a similar isotope effect on the insecticide synergizing *in vivo* activity of these compounds clearly links the formation of carbon monoxide with complex formation and P450 inhibition[224].

Three mechanisms can be envisioned for oxidation of the dioxymethylene bridge to the iron-coordinated carbene. In one mechanism, elimination of a molecule of water after hydroxylation of the dioxymethylene bridge yields an acidic oxonium ion that upon deprotonation gives the carbene (Figure 7.12, path a). In a second mechanism, formation of the oxonium species could precede formation of the bridge-hydroxylated metabolite if the radical formed in the hydroxylation reaction is oxidized by the ferryl species before the oxygen rebound occurs (Figure 7.12, path b). Finally, the same radical intermediate could bind to the iron of the $[Fe–OH]^{3+}$ catalytic intermediate[214]. Deprotonation and intramolecular transfer of the oxygen from the iron to the carbon would give the bridge-hydroxylated metabolite that could then decompose to the carbene complex as in the first mechanism.

Whatever the precise mechanism, *in vitro* experiments with purified CYP2D6 indicate that the formation of MI complexes with a telltale spectroscopic signature at 456 nm may be responsible for the clinical reports of potent CYP2D6 inhibition by paroxetine (Figure 7.12), a serotonin reuptake inhibitor[225–231]. The formation of a carbene complex is supported by the fact that paroxetine is metabolized by CYP2D6 via demethylenation of the methylenedioxy group to a catechol and formic acid[228, 232]. K_I and k_{inact} values of 6.6 ± 2.7 μM and 0.25 ± 0.09 min^{-1}, respectively, have been calculated for the paroxetine-mediated inhibition of human liver microsomal CYP2D6-dependent dextromethorphan *O*-demethylation[231].

Additional methylenedioxyphenyl compounds have been synthesized and their human isoform selectivity as mechanism-based inactivators evaluated[233]. Their inactivating potential depends on the side-chain structure, with bulky side chains such as 1,4-benzothiazine inactivating some P450 enzymes but not others[233].

3.2.2. Amines

Alkyl and aromatic amines, including the MAO inhibitor clorgyline[234], and a number of clinically useful amine antibiotics such as troleandomycin (TAO) (Figure 7.13) and erythromycin, belong to a second large class of agents that form quasi-irreversible (MI) P450 complexes[4, 235–240]. These amines are oxidized to intermediates that coordinate tightly to the ferrous heme and give rise to a spectrum with an absorbance maximum at 445–455 nm[235]. Complex formation requires a primary amine but secondary and tertiary amines, as in the case of TAO, can give P450 complexes if they are first *N*-dealkylated to the primary amines. The complexes from aromatic amines differ from

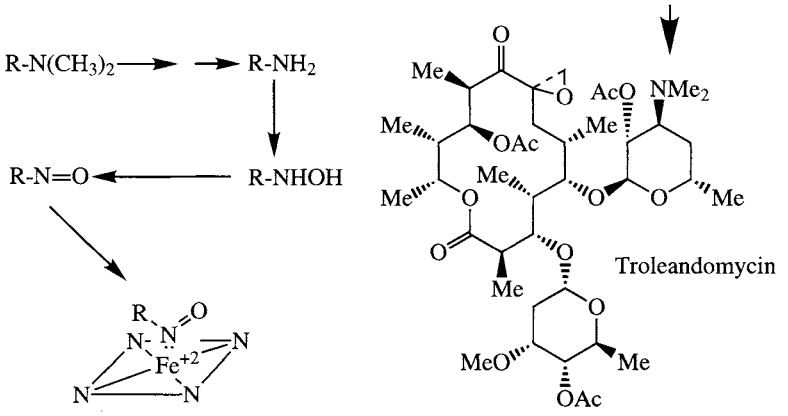


Figure 7.13. The spectroscopically detectable metabolic intermediate (MI) heme complexes formed when some primary amines are oxidized by P450 enzymes involve oxidation of the nitrogen to a nitroso species that coordinates to the iron. The primary amine function can be unmasked by *N*-demethylation reactions, as is the case in the inhibition of CYP3A enzymes by troleandomycin (TAO) (AcO– in the structure represents $CH_3CO_2^-$). The arrow shows the nitrogen that is involved in the reaction in TAO.

those from alkyl amines in that they are unstable to reduction by dithionite[238]. The normal competitive inhibition associated with the binding of amines does not, of course, depend on catalytic oxidation of the inhibitor, but catalytic activation is required for formation of the tight, quasi-irreversible complexes[234, 238, 239]. It is likely that the primary amines are first hydroxylated because the same complexes are obtained with the corresponding hydroxylamines[240], but the coordination requires further oxidation and thus involves a function beyond the hydroxylamine[238, 240]. In fact, the moiety that chelates to the iron appears to be the nitroso function obtained by two-electron oxidation of the hydroxylamine (Figure 7.13)[239–241]. As hydroxylamines readily autooxidize, the final oxidative step may not always require catalytic participation of the enzyme[242]. The coordination of a nitroso function is consistent with the observation that apparently identical complexes are obtained by reduction of nitro compounds[243]. The crystal structure of a complex between a nitroso compound and a model iron porphyrin shows, as expected, that the nitrogen rather than the oxygen of the nitroso group is bound to the iron[214].

It is noteworthy that the *in vivo* complexation of TAO to the heme of CYP3A enzymes stabilizes them and prolongs their half-lives in hepatocytes[244–246]. A consequence of this is that the concentration of the protein in the cell increases, an example of "induction" through protein stabilization. It remains unclear whether the protein levels are elevated because of a substrate-induced conformational stabilization or because the formation of a heme complex suppresses normal damage to the protein associated with the reactive O_2 species produced through uncoupled turnover of the enzyme. This latter possibility is the most likely, given that inhibition of P450 reductase[247, 248] or conditional deletion of the reductase[249], which suppresses catalytic turnover, also results in enzyme stabilization.

3.2.3. 1,1-Disubstituted and Acyl Hydrazines

P450 enzymes oxidize 1,1-disubstituted, but not monosubstituted, hydrazines (see Section 3.3.4) to products that coordinate tightly to the heme iron atom. The complexes, which are characterized by a ferric absorption maximum at ~438 nm and a ferrous maximum at 449 nm, are formed in a time-, NADPH-, and oxygen-dependent manner[250]. The oxidation of isoniazid and other acyl hydrazines by liver microsomes yields a transient complex with a similar absorption maximum at 449 nm[251, 252]. However, the isoniazid complex dissociates on addition of

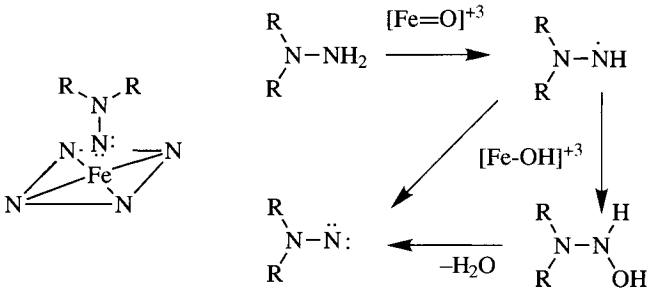


Figure 7.14. The nitrene–iron structure proposed for the complexes formed during the metabolism of 1,1-dialkylhydrazines and possible mechanisms for formation of the nitrene.

ferricyanide and thus is only stable in the ferrous state[253]. Model studies indicate that 1,1-dialkylhydrazines are oxidized to disubstituted nitrenes that form end-on complexes with the iron of metalloporphyrins. The nitrene complexes formed in the reactions of 1-amino-2,2,6,6-tetramethylpiperidine and several iron tetraarylporphyrins have been characterized by NMR, Mössbauer, and X-ray methods[254, 255]. The P450 complexes generated during the metabolism of 1,1-disubstituted hydrazines, and possibly acyl hydrazines, are therefore likely to be aminonitrene–iron complexes (Figure 7.14). Oxidation of the dialkylhydrazines to aminonitrenes is easily rationalized by initial hydroxylation of the hydrazine or, more probably, by stepwise electron removal from the hydrazine (Figure 7.14).

3.3. Covalent Binding to the Prosthetic Heme

P450 is often irreversibly inactivated via covalent attachment of the catalytically activated inhibitor, or a fragment of it, to the heme group. A heme alkylation mechanism has been unambiguously demonstrated, in many instances, by evidence of equivalent activity and heme loss and the isolation and structural characterization of the modified hemes. It must be noted that in the absence of explicit evidence for heme adduct formation, an equimolar loss of enzyme content and heme does not unambiguously establish that heme alkylation is responsible for enzyme inactivation because alternative mechanisms exist for the catalysis-dependent destruction of the heme (see Section 3.4). It is also possible for a heme adduct to be generated that is either reversible or too unstable to be isolated. Unfortunately, the quantitative correlation of heme adduct formation with enzyme inactivation is technically difficult. Without such data, it is difficult to exclude the possibility that the enzyme is also inactivated by mechanisms such as protein modification even when heme alkylation is conclusively demonstrated.

3.3.1. Terminal Olefins

The P450-catalyzed epoxidation of terminal olefins is often associated with *N*-alkylation of its prosthetic heme and inactivation of the enzyme (see Figure 7.7)[153, 156, 256]. Early studies with 2-isopropyl-4-pentenamide (AIA) and 5-allyl-substituted barbiturates such as secobarbital[153, 154], established that the oxidative metabolism of homoallylic amides results in: (a) comparable loss of P450 and heme content, (b) the accumulation of "green pigments" identified as abnormal porphyrins, and (c) derangement of the heme biosynthetic pathway.

The only structural requirement for prosthetic heme alkylation by olefins is a monosubstituted double bond. Accordingly, ethylene, but not ethane, is able to destroy the P450 heme while 3-hexene, cyclohexene, and 2-methyl-l-heptene are inactive[153, 156]. Even monosubstituted olefins fail to inactivate the enzyme if they are not substrates for the enzyme, if the double bond is not the site of catalytic oxidation, or if the double bond is a part a of a conjugated system[156]. Thus, the oxidation of styrene by a model iron porphyrin showed that heme alkylation only occured once in

ten thousand turnovers[256], in contrast to the ratio of less than 300 turnovers per alkylation even that is commonly observed with unconjugated terminal olefins[7]. These observations suggest that alkylation of the heme by olefins is compromised by steric constraints and/or by the presence of substituents that can delocalize charge or electron density from the double bond.

Spectroscopic methods have unambiguously established the structures of *N*-alkylated porphyrins isolated from the livers of rats treated with diverse olefins, including ethylene, propene, octene, fluroxene, 2,2-diethyl-4-pentenamide, 2-isopropyl-4-pentenamide, and vinyl fluoride[7, 257–260]. Analogous products are probably formed in the inactivation of P450 by other olefins, such as in the inactivation of CYP2E1 by the garlic components diallyl sulfide and diallylsulfone[261–264], but the resulting adducts have not been isolated. The terminal carbon of the double bond is bound to a porphyrin nitrogen and the internal carbon of the olefin bears a hydroxyl group in the structures of all the olefin adducts determined so far (Figures 7.7 and 7.15[154, 258]). The oxygen in the ethylene and 2-isopropyl-4-pentenamide adducts, which has been shown by ^{18}O studies to derive from molecular oxygen, is presumed to be the catalytically activated oxygen[257, 258]. The structure of the adduct is consistent with addition of the porphyrin nitrogen to the epoxide metabolite of the olefin, but this possibility is precluded by the following findings: (a) the enzyme is refractory to inactivation by the epoxides of olefins that destroy the enzyme[153, 156, 258], (b) *cis*-addition of the nitrogen and oxygen to the double bond is inconsistent with the *trans* sterochemistry expected for the addition of a nucleophile to an epoxide[258], (c) the nitrogen reacts with the terminal rather than internal carbon of vinyl ethers although the internal (oxygen-substituted) carbon is more reactive in the corresponding epoxides[259], and (d) the pyrrole nitrogens are weak nucleophiles and do not react with epoxides even under harsh chemical conditions. These considerations and the requirement for enzyme turnover indicate that catalytic oxygen transfer to the double bond initiates enzyme inactivation but it does not result from reaction with the epoxide metabolite.

Ethylene, propene, and octene, all linear olefins, only detectably alkylate pyrrole ring D of the prosthetic heme of the phenobarbital-inducible rat liver P450 enzymes (Figure 7.15), but heme alkylation by the more "globular" olefins 2-isopropyl-4-pentenamide and 2,2-diethyl-4-pentenamide is less regiospecific[257, 260]. The regiochemistry and stereochemistry of heme alkylation by *trans*-[1-^{2}H]-1-octene has established that the olefin stereochemistry is preserved

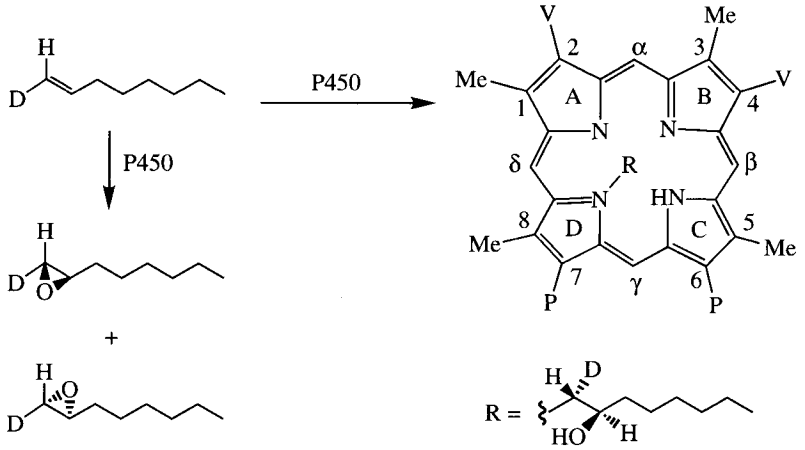


Figure 7.15. The oxidation of *trans*-1-[1-^{2}H]octene by rat liver microsomes yields both the epoxide metabolite and the indicated *N*-alkyl heme adduct, the structure of which has been unambiguously established. The heme substituents are: Me=CH_3, V=CH=CH_2, P=$CH_2CH_2CO_2H$. The peripheral pyrrole carbons and the *meso* carbons of the porphyrin are labeled.

during heme alkylation. Furthermore, heme alkylation only occurs when the oxygen is delivered to the *re* face of the double bond even though stereochemical analysis of the epoxide metabolite shows that the oxygen is delivered almost equally to *both* faces of the π-bond[258]. P450 heme alkylation is thus a highly regio- and stereospecific process.

Chemical models have successfully reproduced autocatalytic heme alkylation and have confirmed and extended the mechanistic information provided by enzymatic studies[262-269]. Thus, iron porphyrins can oxidize terminal olefins to species that alkylate the porphyrin nitrogens and give the same types of adducts as are obtained biologically[262-265]. In the models, as in the biological process, the oxygen is added to the olefin from the same side as the porphyrin nitrogen, the nitrogen is not alkylated by epoxide or aldehyde metabolites, and the reaction is subject to steric interference by substituents on the olefin[262-264]. However, in the model systems, heme alkylation can occur with disubstituted olefins, and binding of the porphyrin nitrogen to the internal carbon and the hydroxyl group to the terminal carbon of monosubstituted olefins occurs to a limited extent[262-266]. As a case in point, spectroscopic studies suggest that an *N*-alkylated porphyrin is transiently formed in the oxidation of norbornene by an iron porphyrin[267, 268]. The finding that formation of the adduct appears to be reversible led to the suggestion that this reversibility may mask the biological formation of secondary *N*-alkyl adducts[266, 269, 270]. Reversible *N*-alkyl adduct formation has not been generally detected with P450, although evidence for the reversible *N*-alkylation of the heme of a CYP2E1 T303A mutant by *tert*-butyl acetylene has recently been reported[271]. On the other hand, it has been known for some time that the catalytic oxidation of terminal olefins by chloroperoxidase and H_2O_2 results in reversible *N*-alkylation of its heme group, with up to 80% recovery of the enzyme activity over several hours at 25°C[272].

Several mechanisms can be envisaged for heme alkylation that are consistent with the experimental data, none of which involves a concerted transfer of the oxygen to the π-bond. Subsequent to possible formation of a charge transfer complex between the ferryl species and the olefin π-bond, addition of the oxygen to the π-bond could give a transient carbon radical that alkylates the heme, closes to the epoxide, or transfers the unpaired electron to the heme to give a cation that alkylates the heme. The partitioning between metabolite formation and heme alkylation may be determined, in part, by the regiochemistry (i.e., inner or outer carbon) of oxygen addition to the π-bond. The P450-catalyzed oxidation of olefins can also be explained by initial addition of the oxoiron complex to the π-bond to give one of the two possible metallacyclobutane intermediates. The ratio of epoxide formation to heme alkylation might then reflect the relative proportion of the metallacyclobutane with the oxygen bound to the internal carbon vs that with the oxygen bound to the terminal carbon. However, the heme alkylation details, the parameters that govern partitioning between epoxidation and heme alkylation, and the relationship between the mechanism of heme alkylation vs epoxide formation remain to be clarified. The recent formulation by Shaik of a two-state oxidation mechanism, in which spin state pairing of electrons in the transition state determines whether oxygen transfer follows a virtually concerted pathway or occurs stepwise, provides a highly attractive rationale for the observation of both heme alkylation and epoxide formation pathways in the turnover of a single substrate[273]. The two-state hypothesis is treated in detail in Chapter 2 and in less detail in Chapter 6.

3.3.2. Acetylenes

P450-catalyzed oxidation of terminal acetylenes to substituted acetic acids (Chapter 6) is more prone to result in heme alkylation than the oxidation of terminal olefins. The structure–activity relationships for the acetylene reaction are similar to those for terminal olefins, except that there are fewer instances in which the reaction does not result in enzyme inactivation. For example, P450 is inactivated by phenylacetylene but not detectably by styrene[164], and P450 is inactivated by internal acetylenes, albeit without heme adduct formation, but not by internal olefins[156, 274]. Catalytic oxidation of the acetylenic function is required for enzyme inactivation and terminal acetylenes give heme adducts analogous to those obtained with terminal olefins[258, 259]. The salient difference in the adducts obtained with acetylenes and olefins

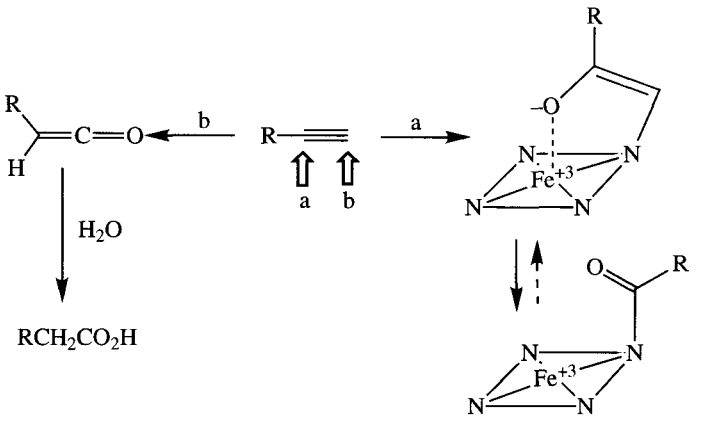


Figure 7.16. The P450-catalyzed oxidation of a terminal acetylene partitions between formation of the ketene and heme alkylation. Which of these events occur is determined by the carbon to which the activated oxygen is added: addition to the internal carbon (a) results in heme alkylation, and to the terminal carbon (b) yields the ketene. In the absence of the iron, the enol adduct tautomerizes to the ketone.

is that addition of the hydroxyl group and the porphyrin nitrogen across the triple bond produces an *enol* that eventually tautomerizes to a ketone. A second difference is that in the adducts of phenobarbital-inducible rat liver P450 enzymes the alkylation occurs almost exclusively on the nitrogen of pyrrole ring A whereas linear olefins primarily alkylate the nitrogen of pyrrole ring D. A topographical rationale has been provided to explain this difference in alkylation regiochemistry.

The mechanisms proposed for P450 inactivation by terminal olefins can be applied to inactivation by terminal acetylenes if one keeps in mind that all the reaction intermediates bear an additional double bond. Triple bond oxidation is furthermore unique in that the carbon to which the oxygen is *initially* bound is revealed by the products, whereas this information is lost in olefin epoxidation because the oxygen is bound to both carbons in the epoxide metabolite. Thus, in all the heme adducts with acetylenes that have been sufficiently well characterized, the oxygen is bound to the internal carbon, whereas all the metabolites are derived from the ketene obtained by addition of the oxygen to the terminal carbon. As already mentioned, the ketene or a closely related species can also inactivate the enzyme by reacting with the protein[78, 81]. This difference in oxygen addition regiochemistry, in conjunction with the observation of a large isotope effect on metabolite formation but not heme alkylation in the metabolism of terminally deuterated phenylacetylene, indicates that the commitment to either metabolite formation or heme alkylation occurs as the oxygen transfer step is initiated (Figure 7.16)[164]. The factors that determine to which triple bond carbon the oxygen is transferred, and therefore whether heme or protein modification occurs, are unclear. However, the observation that 1-ethynylpyrene and phenylacetylene inactivate CYP1A2 by, respectively, protein and heme modification demonstrates that the reaction regiochemistry is governed by substrate–enzyme interactions and not simply by intrinsic differences between P450 enzymes[83]. Indeed, such specific substrate/inactivator-active sit fit may account for the relatively selective inactivation of CYP3A4 by gestodene (K_I = 46 μM, k_{inact} = 0.4 min^{-1}, and partition ratio of ~9)[92].

To gain insight into these reactivity determinants, a series of aryl and arylalkyl acetylenes varying in the size and shape of the aromatic ring system, the placement of the carbon–carbon triple bond, the length of the alkyl side chains, and/or the presence of a terminal hydrogen or methyl group have been examined as inhibitors/inactivators of human and rat liver CYP1A1, -1A2, and -2B1/2B2[79–83, 275]. The findings reveal that all these features can influence the potency, type, and selectivity of P450 inhibition. Accordingly, the arylacetylenic compounds with the larger pyrene, phenanthrene, or biphenyl rings

appear to inactivate the CYP1A isoforms, whereas 2-ethynylnaphthalene, 4-phenyl-1-butyne, 1-phenyl-1-propyne, and 5-phenyl-1-pentyne are selective CYP2B1 inactivators[79–83, 275]. On the other hand, the 9-ethynyl- and 9-propynylphenanthrene isomers reversibly inhibit the CYP1A isoforms, but are among the most effective mechanism-based inactivators of CYP2B1/2B2[275].

The length of the alkyl side chain was an important reactivity determinant among the arylalkyl acetylenes[275]. Thus, while phenylacetylene is a reversible CYP2B1/2B2 inhibitor, analogues with three or four methylene groups (5-phenyl-1-pentyne and 6-phenyl-1-hexyne, respectively) are among the most potent prototype CYP2B1/2B2 inactivators. Replacement of the terminal hydrogen with a methyl, giving disubstituted acetylenes, results in reduced CYP2B and increased CYP1A inactivation. Thus, 2-(1-propynyl)phenanthrene, 4-ethynylbiphenyl, and 4-(1-propynyl)biphenyl are very effective inactivators of both rat liver CYP1A1 and -1A2, whereas 1-(1-propynyl)pyrene, 2-ethynylphenanthrene, 3-ethynylphenanthrene, 3-(1-propynyl) phenanthrene, 2-(1-propynyl)naphthalene, and 6-phenyl-2-hexyne are effective inactivators of CYP1A1 but not CYP1A2[275]. Furthermore, replacement of the terminal acetylenic hydrogen with a methyl enhanced the mechanism-based inactivation of both CYP1A1 and -1A2, or converted a reversible inhibitor into an effective inactivator, as exemplified by 1-ethynylpyrene and 1-(1-propynyl)pyrene, 2-ethynylphenanthrene and 2-propynylphenanthrene, 3-ethynylphenanthrene and 3-propynylphenanthrene, and 6-phenyl-1-hexyne and 6-phenyl-2-hexyne[275]. Indeed, 2-propynylphenanthrene and 4-propynylbiphenyl (4PBi) are among the more selective inhibitors of rat liver CYP1A and human liver CYP1A2 enzymes.

In contrast, 4PBi fails to inactivate human liver microsomal CYP2E1, -2C9/10, -3A4 or -2C19. The identification of 2-biphenylylpropionic acid from the CYP1A1- and -1A2-catalyzed metabolism of 4PBi links this mechanism-based inactivation with that of terminal acetylenes, as it involves a 1,2-shift of the terminal methyl to give a ketene intermediate[275]. The importance of the 1,2 methyl shift and the resulting ketene in P450 inactivation by internal acetylenes such as 4PBi is underscored by the finding that P450 enzymes such as CYP2B1, which do not oxidize 4PBi to 2-biphenylylpropionic acid, are refractory to inactivation.

There are other documented examples of mechanism-based P450 inactivation by methyl-substituted (i.e., internal) acetylenes[84, 94]. A clinically relevant internal acetylene that potently and selectively inactivates human liver CYP3A4 is the antiprogestin drug mifepristone [RU486; (11β,17β)-11-[4-(dimethylamino)-phenyl]-17-hydroxy-17-(1-propynyl)-estra-4,9-dien-3-one] (Figure 7.9)[95, 276], a drug used for medical abortion in the first trimester of pregnancy[277]. K_I and k_{inact} values of 4.7 μM and 0.089 min^{-1} place mifepristone among the most potent CYP3A4 inactivators[95]. Although the activities of CYP1A, -2B, and -2D6 enzymes were also inhibited *in vitro*, this inhibition, unlike that of CYP3A4 and -3A2, was reversed when mifepristone was removed by dialysis. Inactivation with [^{3}H]mifepristone showed that the drug binds covalently to the CYP3A4 protein with a stoichiometry of 1.02 ± 0.15 mol per mol of protein[95]. *In vitro* studies with CYP3A4 and -3A5, the other major adult human liver CYP3A isoform, indicate that the latter, although capable of metabolizing the drug, is not subject to mifepristone-mediated inactivation[96]. Mifepristone may be a useful probe with which to distinguish these two CYP3A isoforms. The acetylenic moiety of mifepristone is thought to also be activated to a ketene, although the expected propionic acid metabolites have not been detected with either enzyme. However, LC-MS of mifepristone metabolites revealed that although both enzymes generate the *N,N'*-didemethylated and *N*-monodemethylated products, only CYP3A4 hydroxylates the terminal methyl group. Thus, the susceptibility of CYP3A4 but not CYP3A5 to inactivation may be due to the ability of the first but not the second to oxidize the acetylenic moiety of mifepristone[96]. Although not considered in the publications, it is very possible that the inactivation observed with mifepristone does not reflect oxidation of the triple bond at all but rather oxidation of the terminal methyl to an aldehyde, giving an α,β-unsaturated aldehyde that adds to the protein as a Michael acceptor.

Not surprisingly, the acetylenic function has been exploited in the design and synthesis of P450 isoform-selective or -specific irreversible inhibitors, including inhibitors of $P450_{scc}$, aromatase, prostaglandin ω-hydroxylase[278], and the

P450 enzymes that oxidize saturated fatty acids, arachidonic acid, and leukotriene B_4 (Section 5.5). It may also play a role in the alterations of oxidative metabolism observed in individuals treated with ethynyl sterols such as gestodene and 17α-EE[87–92].

Strategies to convert selective P450 substrates to suicide inactivators by the incorporation of a suitable activatable function at the position oxidized are not always successful. For instance, the introduction of an acetylenic moiety into the chemical template *N*-(3,5-dichloro-4-pyridyl)-3-(cyclopentyloxy)-4-methoxybenzamide (DCMB), a CYP2B6 functional marker, to yield *N*-(3,5-dichloro-4-pyridyl)-4-methoxy-3-(prop-2-ynyloxy)benzamide gave a mechanism-based agent that, based on a correlation of activity and ferrous–CO chromophore loss, probably inactivated CYP2B6 via heme modification[279]. However, this inactivation was not very selective as other human liver CYP2C isoforms were also inactivated, indicating that the catalytic selectivity for CYP2B6 resides in the *O*-alkyl chain of the parent DCMB molecule.

3.3.3. Dihydropyridines and Dihydroquinolines

The administration of 3,5-bis(carbethoxy)-2,4,6-trimethyl-1,4-dihydropyridine (DDC)[280–284] perturbs heme biosynthesis and causes a loss of hepatic P450 content, both of which have been traced to *N*-methylation of the P450 prosthetic heme[285–290]. Substitution of the dihydropyridine at position 4 with a primary, unconjugated moiety (methyl, ethyl, propyl, *sec*-butyl, nonyl), but not an aryl (phenyl), secondary (isopropyl), or conjugated (benzyl) group results in *N*-alkylation of the heme[287, 291–294]. 4-Aryl-substituted dihydropyridines do not inactivate the enzyme, whereas those bearing secondary or conjugated substituents inactivate the enzyme but do not yield detectable *N*-alkyl heme adducts[291–294]. Dihydropyridines with simple 4-alkyl groups *N*-alkylate the heme of certain P450 isoforms, but inactivation of others occurs by a mechanism that appears to involve heme degradation to fragments that irreversibly modify the protein (Section 3.4).

The mechanisms of enzyme inactivation and heme destruction by analogs that do not give identifiable heme adducts remain unclear, but the mechanism of analogues that alkylate the heme nitrogen is understood better. The adducts consist of protoporphyrin IX with the 4-alkyl group of the parent substrate covalently attached to one of its nitrogen atoms (Figure 7.17)[285, 286, 288, 289]. Different nitrogens are alkylated in different P450 enzymes[290, 295], and the dihydropyridines cause isoform-selective inactivation[295, 296]. The catalytic turnover of 4-alkyl-1,4-dihydropyridines thus leads to transfer of the 4-alkyl group to a nitrogen of the heme. The following observations further elucidate the nature of the enzyme inactivation: (a) the dihydropyridines are oxidized to the pyridines with partial loss of 4-alkyl but not 4-aryl groups[293, 294], (b) the *N*-methyl or *N*-ethyl derivatives of 4-alkyldihydropyridines still inactivate P450 enzymes but inactivation may follow *N*-dealkylation because with those substrates *N*-dealkylation is faster than dihydropyridine aromatization[293], (c) no primary isotope effect is observed on enzyme inactivation when the hydrogen at position 4 is replaced by deuterium[293], (d) the heme adducts that are formed are chiral and therefore are generated within the active site[297], and (e) free radicals have been detected with a spin trap in incubations of a 4-ethyldihydropyridine with hepatic microsomes[286]. However, studies with deferoxamine-washed microsomes suggest metal-catalyzed oxidation of the dihydropyridine accounts for most if not all of the spin-trapped radical[298]. In any case, as neither GSH nor the radical trap prevent enzyme inactivation, the radicals detected in the medium appear not to be involved in heme alkylation. Conversely, if radicals are formed within the active site, they are not readily detected in the medium. In view of these results, it is highly likely that electron abstraction from the dihydropyridine produces a radical cation that aromatizes either by extruding the 4-alkyl group as a radical or by directly transferring the alkyl group to the heme. Although the 4-alkyl group may be directly trapped by the porphyrin nitrogen, model studies suggest that it first adds to the iron to form an alkyl–iron complex from which it migrates to the porphyrin nitrogen. The absence of detectable heme adducts in the P450 inactivation by the 4-isopropyl and 4-benzyl analogs is consistent with such a mechanism, not only because the iron–nitrogen shift is sensitive to steric effects[299], but also because the more oxidizable secondary or benzylic moieties may be converted

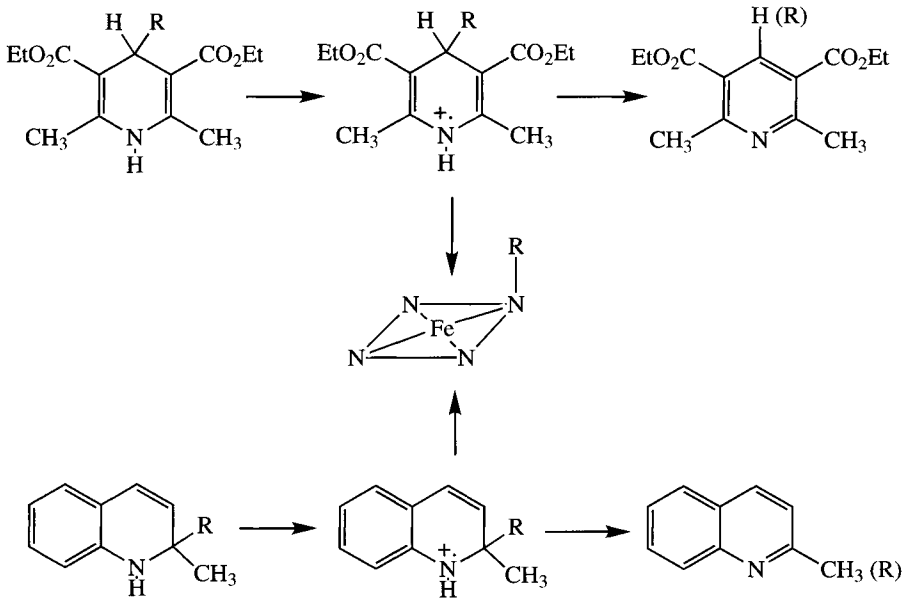


Figure 7.17. Oxidation of 4-alkyl-1,4-dihydropyridines and 2,2-dialkyl-1,2-dihydroquinolines is proposed to yield cation radical intermediates that aromatize by either losing a second electron and a proton or an alkyl radical that adds to a nitrogen of the prosthetic heme group.

to the corresponding cations by electron loss to the iron in preference to undergoing the oxidative iron–nitrogen shift.

The precedent set by the P450-catalyzed oxidation of dihydropyridines to radical cations that aromatize by radical extrusion has led to the observation of comparable processes in related structures. Thus, 2,2-dialkyl-1,2-dihydroquinolines are also oxidized by P450 enzymes to species that *N*-alkylate the heme and inactivate the enzymes. In these reactions, the 2-alkyl substituent of the dihydroquinoline is bound to a nitrogen of protoporphyrin IX, presumably by a mechanism similar to that proposed for the 4-alkyldihydropyridines (Figure 7.17)[300].

3.3.4. Alkyl- and Arylhydrazines and Hydrazones

The P450-destructive mechanism of phenelzine, an alkylhydrazine, is relatively well defined. It causes an approximately equimolar loss of enzyme and heme when incubated with hepatic microsomes[301], and these losses are accompanied by the generation of a heme adduct identified as *N*-(2-phenylethyl)protoporphyrin IX (Figure 7.18)[302]. The generation of products in a microsomal system that implicate the 2-phenylethyl radical as a central MI suggests a role for the 2-phenylethyl radical in this enzyme inactivation[303]. Spin-trapping experiments confirm that the 2-phenylethyl radical is generated in the incubations but the bulk of the radical that is spin trapped is formed by transition metal, rather than P450-catalyzed reactions[302, 304]. It appears from these results that phenelzine is converted to the 2-phenylethyl radical within the P450 active site where it is captured by the heme to give the *N*-(2-phenylethyl) adduct (Figure 7.18). The 2-phenylethyl radical could react directly with the porphyrin nitrogens, but by analogy to the reactions of hemoproteins with arylhydrazines (see below) it is likely that the alkyl radical is trapped by reaction with the heme iron to give an unstable alkyl–iron complex that subsequently rearranges to the isolated *N*-alkyl heme adduct.

A complex with an absorbance maximum at 480 nm is generated in the reaction of P450 with phenylhydrazines and *N*-phenylhydrazones.

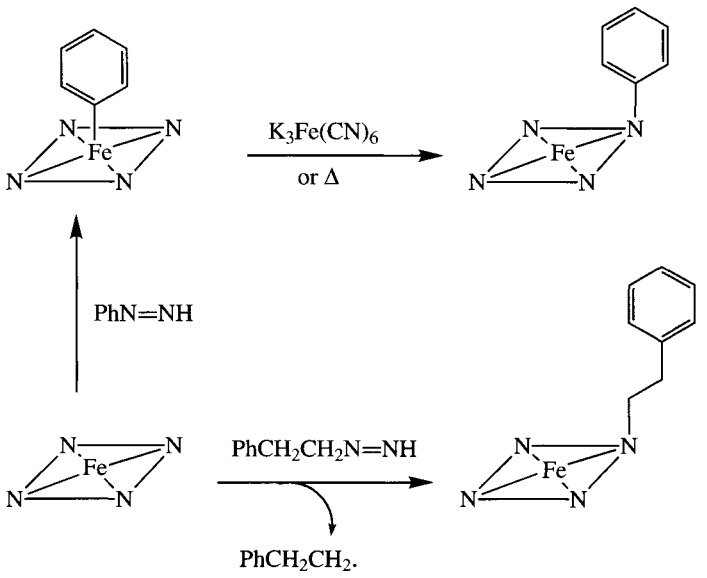


Figure 7.18. The oxidation of phenelzine ($PhCH_2CH_2NHNH_2$) and phenylhydrazine ($PhNHNH_2$) by P450 produces carbon radical products that bind to the prosthetic heme group. In the case of phenylhydrazine, the phenyl radical binds first to the iron atom to give a complex that subsequently rearranges under oxidative conditions to the *N*-phenyl adduct. It is not known if phenelzine initially forms a similar but less stable carbon–iron intermediate.

Formation of this complex inactivates the enzyme and precedes irreversible destruction of its prosthetic heme[305–308]. The reactions of myoglobin, hemoglobin, and catalase with phenylhydrazine yield a similar complex, in these cases with an absorbance maximum at ~430 nm due to the difference between the proximal thiolate and histidine ligation[309–313]. As revealed by X-ray crystallography, the myoglobin and CYP101 ($P450_{cam}$) structures are σ-aryl–iron complexes with the phenyl group bound end-on to the iron (Figure 7.18)[308, 314]. Extraction of the heme complex from CYP101 under oxidative conditions, yields a roughly equal mixture of the four possible *N*-phenylprotoporphyrin IX regioisomers, as found with the complexes from myoglobin, hemoglobin, and catalase[308, 315]. The intact phenyl–iron heme complex, which has been characterized by absorption and NMR spectroscopy, is obtained if the prosthetic group is extracted under *anaerobic* conditions[308, 312]. Exposure of the anaerobically extracted phenyl–iron heme complex to oxygen or other oxidizing agents under acidic conditions results in migration of the phenyl from the iron to the porphyrin nitrogens (Figure 7.18)[312]. This migration is sensitive to steric effects because the aryl moiety in aryl-iron complexes obtained from *ortho*-substituted phenylhydrazines does not undergo the oxidative shift[299]. Arylhydrazines, including phenyl-, 2-naphthyl-, and *p*-biphenylhydrazine, are known to be P450 substrates[316]. Migration of the phenyl group from the iron to the porphyrin nitrogens can be induced to occur within the undenatured active site by the addition of ferricyanide to the intact P450 complexes[317–319]. The distribution of the four *N*-aryl protoporphyrin IX regioisomers produced by this method reflects the active-site topology and varies widely from enzyme to enzyme. The migration of aryl groups within the active sites of P450 enzymes provides a tool for the determination of their active-site topology because the regioselectivity of the migration is controlled by the degree to which the active site is sterically unencumbered above each of the four pyrrole ring nitrogens[316–322]. The *in situ* shift of the aryl moiety from the iron to the porphyrin nitrogens occurs readily with most, but not all, of the P450 iron–aryl complexes, but does not occur with the complexes of proteins

such as myoglobin that have an imidazole as the fifth iron ligand.

The likelihood that alkyl radicals, like their aryl counterparts, bind to the iron before shifting to the nitrogen is supported by the observation that the type II complexes formed between alkyldiazenes and P450 in the absence of oxygen are converted, in the presence of limited amounts of oxygen, to complexes with an absorption maximum at ~480 nm characteristic of iron–carbon σ-bonded complexes[306, 323]. Furthermore, alkyl diazene-iron tetraphenylporphyrin complexes can be prepared under anaerobic conditions[324]. However, the alkyl–iron complexes are much less stable and less well characterized than the aryl–iron complexes and their involvement in heme *N*-alkylation reactions remains to be demonstrated.

In addition, some aryl hydrazines, notably dihydralazine, also inactivate P450 via protein modification in a mechanism-based process[325]. Thus, dihydralazine has been shown to inactivate rat liver microsomal CYP1A2, -2C11 and -3A, but not CYP2B1 or -1A1[325], and also inactivates human liver CYP1A2 and -3A4, but not CYP2C9[326]. This inactivation appears to involve irreversible binding of a reactive metabolite to the P450 protein in a process that is not affected by co-incubation with GSH (5 mM)[326]. The generation of irreversible dihydralazine–protein adducts *in vivo* and their subsequent immunoproteasomal processing into antigenic P450 peptides could possibly account for the detection of CYP1A2-reactive anti-liver microsomal (anti-LM) autoantibodies, in the sera of patients with dihydralazine-induced immunoallergic hepatitis[327, 328].

3.3.5. Other N–N Functions

The P450 heme is *N*-alkylated or *N*-arylated by reactive intermediates formed when it oxidizes 1-aminoaryltriazoles, 2,3-bis(carbethoxy)-2,3-diazabicyclo[2.2.0]hex-5-ene, and the sydnones.

The oxidation of 1-aminobenzotriazole (ABT) by chemical reagents yields benzyne, an exceedingly reactive species, and two molecules of nitrogen[329]. The finding that benzyne, or its equivalent, is bound across two of the nitrogens of the prosthetic heme isolated from inactivated P450 enzymes suggests that the enzyme-catalyzed oxidation of ABT follows the same reaction trajectory[330, 331]. The benzyne may add directly to the two nitrogens, generating an *N,N*-bridged species that autooxidizes to the isolated bridged porphyrin, or may first bind to the iron and a nitrogen of the heme and subsequently rearrange to the *N,N*-bridged species (Figure 7.19). Introduction of small substituents on the phenyl ring or on the exocyclic nitrogen of ABT, or replacement of the phenyl framework with alternative aryl moieties, does not impair destructive activity[331–333]. It is not known whether the oxidation of ABT to benzyne proceeds via hydroxylation of the exocyclic nitrogen or electron abstraction to give a radical or radical cation (Figure 7.20), but a notable similarity exists between the activation mechanism proposed for ABT and other 1,1-disubstituted hydrazines (Figure 7.14).

ABT inactivates a wide variety of P450 enzymes without detectable toxic effects[332–338]. Thus, ABT administration to guinea-pigs inactivates both adrenal steroidogenic- and xenobiotic-metabolizing P450 isoforms[337, 338]. The inactivation of steroidogenic enzymes is apparently indirect and due to the generation of an extra-adrenal ABT metabolite(s), as these guinea-pig adrenal P450 isoforms (unlike their hepatic and adrenal xenobiotic metabolizing counterparts) are not susceptible to direct ABT-mediated inactivation in reconstituted *in vitro* systems[337, 338]. P450 isoform and tissue selectivity is conveyed by placing substituents on the exocyclic nitrogen of the aminotriazole function[332, 333, 339–341]. Furthermore, it is noteworthy that certain ABT analogs [*N*-benzyl-, *N*-(α-methylbenzyl)-, or *N*-(α-ethylbenzyl)-1-ABT] also inactivate phenobarbital-inducible hepatic P450 enzymes (other than the CYP2B4/CYP2B1 orthologs) via the formation of MI complexes rather than by heme modification[342]. Overall, ABT is a highly effective agent for the *in vivo* inactivation of a variety of P450 enzymes in plants[343, 344], insects[345], and animals[334–342, 346–348].

Cyclobutadiene, which can be envisioned as a rectangular structure with a singlet electronic state or a square structure with a triplet electronic state, is formed upon chemical oxidation of 2,3-diazabicyclo[2.2.0]hex-5-ene[349]. Bis(carbethoxy)-2,3-diazabicyclo-[2.2.0]hex-5-ene (DDBCH), a precursor of the above compound, is a mechanism-based irreversible inhibitor of P450 that exploits the basic reactivity of the parent bicyclic system[350]. The bis(carbethoxy) derivative was employed for the enzymatic studies because the

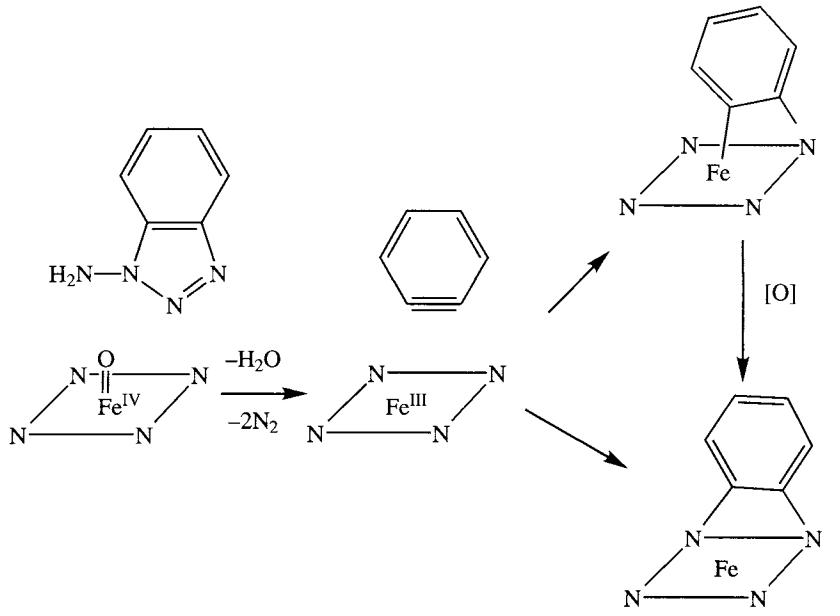


Figure 7.19. Two alternative mechanisms for addition of the benzyne released from 1-aminobenzotriazole (ABT) to the heme. The heme porphyrin framework is represented by a square of nitrogens, each of which represents one of the four nitrogens of the porphyrin.

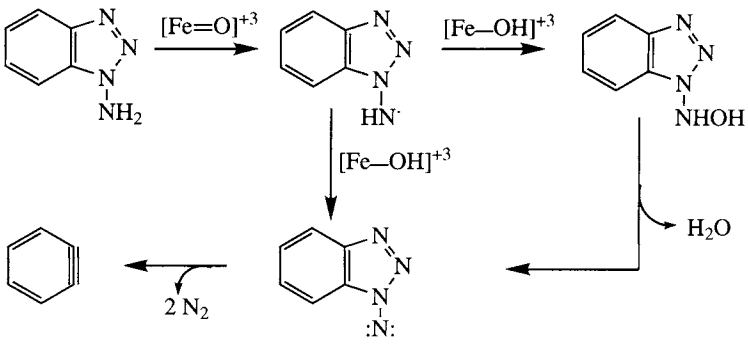


Figure 7.20. Mechanistic alternatives for the P450-catalyzed generation of benzyne from 1-aminobenzotriazole.

parent bicylic hydrazine autooxidizes too readily to be biologically useful. The heme of the P450 enzyme is converted into the *N*-2-cyclobutenyl derivative during the inactivation reaction (Figure 7.21). The secondary, allylic, carbon–nitrogen bond in this adduct makes it much less stable than other adducts, which bear the primary, unactivated, *N*-alkyl groups. The failure of internal olefins and acetylenes to detectably alkylate the prosthetic heme suggests, in fact, that secondary carbons are generally too sterically encumbered to react with the heme moiety. Although the precise nature of the reactive species remains undefined, the generation of the 2-cyclobutenyl adduct implies the involvement of cyclobutadiene itself, or of a closely related

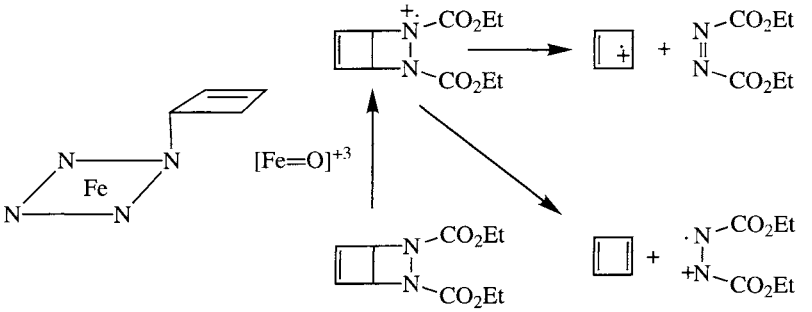


Figure 7.21. Possible mechanisms for the oxidative generation of cyclobutadienoid species that alkylate the prosthetic heme group of P450. The heme of P450 is abbreviated as indicated in Figure 7.19.

species, in heme alkylation. The structure of the adduct is readily rationalized if cyclobutadiene adds to a porphyrin nitrogen to give a transient, probably anionic intermediate, that is neutralized by a proton from the medium. The transient intermediate could be stabilized by formation of a carbon–iron bond with the carbon that is eventually protonated. Electron abstraction from DDBCH can lead to the observed adduct by pathways that depend on whether the cyclobutadiene is generated as a neutral, cationic, or anionic species (Figure 7.21).

The accumulation of a fluorescent hepatic pigment in dogs and rats administered a sydnone derivative[351] led to the discovery that the sydnone is catalytically activated by P450 enzymes to a species that alkylates the prosthetic heme[352]. The heme adduct isolated from rats treated with the sydnone has been identified as *N*-vinylprotoporphyrin IX (Figure 7.22). This finding suggests that the sydnone is first activated by hydroxylation of the electron-rich zwitterionic carbon, followed by ring opening and elimination of the carboxylic fragment to give the diazo species (Figure 7.22). A similar mechanism explains the oxidation of sydnones by simple chemical reagents[353]. The diazoalkane then reacts with the heme, possibly via an initial carbene complex, to give a nitrogen–iron bridged intermediate. The formation of just such a bridged nitrogen–iron complex has been observed in model porphyrin systems[354, 355]. The negative charge on the carbon in the bridged intermediate finally eliminates the thiophenyl moiety and generates the *N*-vinyl adduct[352]. The mechanism is further clarified by the fact that P450 inactivation by 3-(2-phenylethyl)-4-methylsydnone produces both *N*-(2-phenylethyl)- and *N*-(2-phenylethenyl) protoporphyrin IX[356]. These results are most consistent with oxidation of the sydnone to the (2-phenylethyl)diazonium cation that reacts with the heme in two different ways. In one mechanism, deprotonation of the diazonium intermediate, as discussed above, results in a carbene-like addition. In the absence of a β-leaving group, the resulting carbanion intermediate is oxidized to a cation that is deprotonated to introduce the double bond into the *N*-alkyl group[356]. In the second mechanism, reduction of the diazo intermediate prior to deprotonation yields a phenyldiazenyl radical that is trapped by a porphyrin nitrogen atom in the same manner as the 2-phenylethyl radical produced by the oxidation of phenylethyldiazene (Figure 7.18)[302]. The validity of this mechanism is strengthened by the fact that the 2-phenylethyl adduct obtained from the 1,1-dideuterated substrate retains both deuteriums and therefore arises by a mechanism that does not involve deprotonation of the diazo intermediate[356].

The *in vivo* administration of diethylnitrosamine (Et_2N–N=O) to mice reportedly generates an alkylated porphyrin that was tentatively identified by mass spectrometry as *N*-(2-hydroxyethyl)protoporphyrin IX[357]. *In vitro* studies with rabbit liver microsomes and purified P450 enzymes have independently shown that P450 oxidizes diethylnitrosamine to ethylene[358]. Although not actually demonstrated, the proposed *N*-(2-hydroxyethyl) porphyrin adduct is likely to be derived from P450 enzymes inactivated by the ethylene metabolically generated from diethylnitrosamine (see Section 3.3.1).

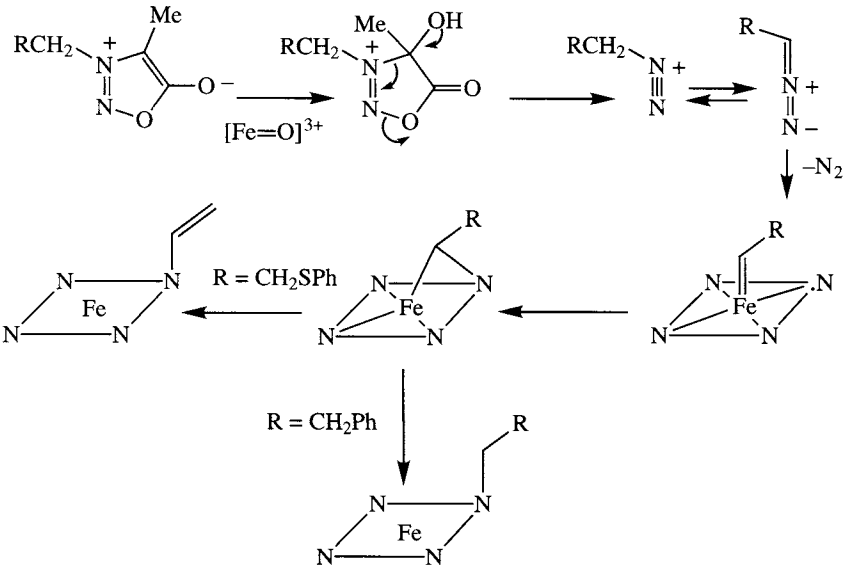


Figure 7.22. Mechanism proposed for the oxidation of sydnones to reactive intermediates that add to the prosthetic heme group of P450. The structures of the *N*-alkylporphyrins isolated from rats treated with the 3-(2-phenylthioethyl)- and 3-(2-phenylethyl) sydnones are shown.

3.3.6. Other Functionalities

The herbicide 1-[4-(3-acetyl-2,4,6-trimethylphenyl)-2,6-cyclohexanedionyl]-*O*-ethyl propionaldehyde oxime (ATMP) (Figure 7.23) causes hepatic protoporphyria in the mouse, albeit not in other species, and this derangement of the porphyrin biosynthetic pathway appears to be linked to P450 inactivation via a heme modification mechanism[359]. A pigment tentatively identified as *N*-methylprotoporphyrin IX by HPLC analysis has been isolated from mice treated with ATMP, and its formation has been shown to be decreased by pretreatment with the P450 inhibitors SKF 525A and piperonyl butoxide, consistent with a P450-dependent process[359]. Replacement of the ethyl on the oxime carbon with a propyl group suppresses the porphyrinogenic activity of the analog, presumably by preventing heme alkylation. Nothing further is known about the mechanism by which ATMP mediates the P450-dependent formation of an *N*-alkyl (possibly *N*-methyl) protoporphyrin IX adduct.

Treatment of mice with griseofulvin (Figure 7.23), an agent long known to cause hepatic porphyrias, has been reported to cause the catalysis-dependent destruction of P450 and the formation of a green pigment[360]. In order to characterize the pigment, the *N*-alkyl group was transferred to an amine in a copper-mediated reaction and the alkyl amine was analyzed by mass spectrometry. The results suggested that *N*-methylprotoporphyrin IX was a minor product and a porphyrin with most of the griseofulvin structure bound to the nitrogen of pyrrole ring C of the heme was the major product[361–363]. The NMR spectrum of the latter adduct confirms that it is an *N*-alkylated porphyrin with the griseofulvin structure attached to the nitrogen of either pyrrole ring C or D[364]. The process appears to be species-specific because, at most, only very low levels of pigment have been observed with rats or chicken embryos[365–367]. It is difficult to postulate a mechanism for the formation of *N*-methyl heme from griseofulvin, particularly as an identical pigment is reportedly present in lower amounts in the livers of control mice[365, 366]. The presence of an endogenous *N*-alkylporphyrin in mice would clearly be of high interest, but more definitive evidence on its origin is required before the significance of these results can be evaluated. It is to be

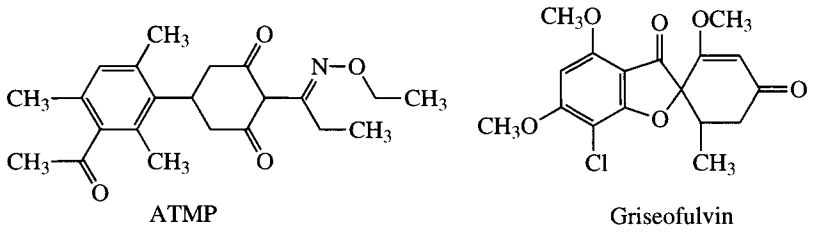


Figure 7.23. The structures of two compounds that cause P450 inactivation. In the case of griseofulvin, the inactivation appears to involve heme *N*-alkylation, but the detailed mechanism of inactivation by ATMP is not known.

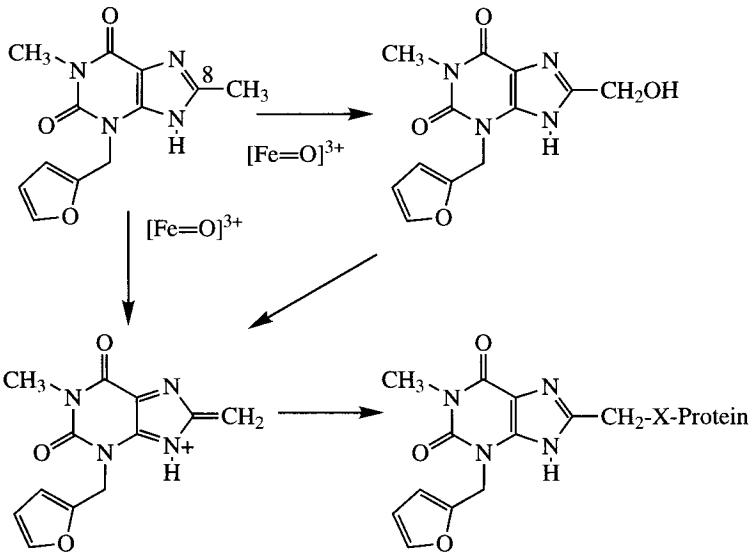


Figure 7.24. The oxidation of furafylline is proposed to yield a chemically reactive intermediate that binds to a protein residue (denoted by Protein-X).

noted that a mechanism is also not obvious for attachment of the griseofulvin structure to the porphyrin nitrogen via a mechanism-based process.

Furafylline (Figure 7.24), a potent selective inhibitor of human CYP1A2, causes time- and NADPH-dependent inactivation of this enzyme[368, 369]. CYP1A1, -2A6, -2B6, -2C9, -2C19, -2D6, -3A4, and -2E1 are not similarly inactivated, although the evidence suggests that other enzymes can be inhibited[369]. Clinical studies confirm that furafylline can almost completely suppress *in vivo* human CYP1A2 function[370, 371]. This loss of function is paralleled by a similar loss of the heme chromophore, with K_I = 23 μM and k_{inact} = 0.87 min^{-1}, and a partition ratio of 3–6 substrate molecules oxidized per enzyme molecule inactivated[368]. Oxidation of the C-8 methyl in the inactivation process is suggested by the fact that deuterium substitution on the C-8 methyl gives an isotope effect of ~2.0 on k_{inact} but not on K_I, and the observation that inactivation activity is suppressed when the methyl is removed[368].

Recent mechanistic studies confirm that the oxidation of the 8-methyl of furafylline yields the corresponding 8′-carbinols and/or results in covalent binding of the agent to the CYP1A2 protein (Figure 7.24)[372]. This result suggests that the oxidation of furafylline converts it to a two-electron oxidized electrophilic intermediate such as the exocyclic 8-methyleneimidazolenine or

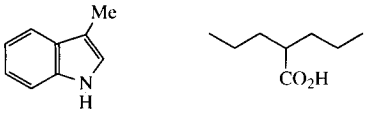
imidazomethide, and that this intermediate is trapped by a nucleophilic side chain within the CYP1A2 active site[372]. Consistent with this, neither GSH nor cyanide significantly impair either covalent binding or inactivation. Although the identity of the alkylated active-site residue remains undetermined, studies of furafylline docked in a homology model of the CYP1A2 active site suggest that the interactions of the furan moiety with the protein indeed favor oxidation of the 8-methyl group[372–374]. Ancillary evidence that the 8-methyl and not the furan moiety of furafylline is critical for CYP1A2 inactivation is provided by comparable findings (k_{inact} = 0.89 min^{-1}, partition ratio = 7.6, equivalent covalent binding to CYP1A2) with cyclohexylline, in which the furan is replaced by a cyclohexyl[372]. Supportive evidence for the proposal that the imidazole N^7-hydrogen is lost during CYP1A2 inactivation is provided by the inactivity of the corresponding N^7-methylated analogs as CYP1A2 inactivators[372].

Analogous P450-catalyzed dehydrogenations have been invoked in the mechanism-based inactivation of select P450 isoforms by the pulmonary toxin 3-methylindole[375], the anticonvulsant valproic acid (see Chapter 6)[376], and the leukotriene inhibitor zafirlukast (Figure 7.25)[377].

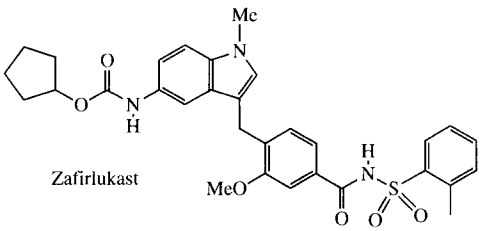
The mechanism of P450 inactivation is unclear for some classes of agents. As an example, CYP2E1 is inactivated by 3-amino-1,2,4-triazole in a time- and NADPH-dependent manner but the inactivation is not associated with covalent binding of the radiolabeled agent to the protein, the formation of P420, or loss of the heme[378]. Similarly, the inactivation of CYP3A4 by delavirdine (1-[3-[(1-methylethyl)amino]-2-pyridinyl]-4-[[5-[(methylsulfonyl)amino]-1H-indol-2-yl] carbonyl]-piperazine), a potent inhibitor of HIV-1 reverse transcriptase, also reportedly adheres to the criteria for a mechanism-based inactivation, but the chemical details of the process remain to be elucidated[379].

3.4. Modification of the P450 Protein by Heme Fragments

During the catalytic oxidation of some substrates, certain P450 enzymes (i.e. CYP3A, CYP2E) undergo a mechanism-based inactivation process in which fragments of the heme are irreversibly bound to the protein. Examples of such inactivators include CCl_4[380–382], spironolactone (see Figure 7.4)[67, 68], 3,5-dicarbethoxy-2,6-dimethyl-4-ethyl-1,4-dihydropyridine (DDEP), and its 4-isopropyl and 4-isobutyl analogs (see also Figure 7.17)[383–385]. The features that predispose an enzyme to cross-linking of heme fragments to the protein remain unclear. Studies with the above substrates suggest that the generation of free radical products is important, but per se is not sufficient because not all the P450 enzymes that produce radicals undergo such inactivation. Thus,



3-Methylindole Valproic acid



Zafirlukast

Figure 7.25. Structures of three agents that cause irreversible inactivation of cytochrome P450.

during the one-electron oxidation of DDEP, CYP2C6 and -2C11 undergo heme *N*-ethylation, whereas the CYP3A enzymes predominantly incur cross-linking of heme fragments to the protein[293, 384]. Furthermore, studies with DDEP and its analogs with a secondary carbon attached to the 4-position (4-isopropyl and 4-isobutyl) reveal that the reaction outcome is largely dictated by the P450 active-site structure rather than by the inability of the inactivator to *N*-alkylate the heme[385]. Thus, DDEP, which can form *N*-ethyl porphyrins, and the 4-isopropyl and 4-isobutyl analogs that cannot, exhibit comparable extents of heme destruction and heme fragment cross-linking. Cross-linking of heme fragments to the protein is thus not merely the result of a defective or inefficient heme *N*-alkylation process. The fact that spironolactone inactivates hepatic CYP3A enzymes via heme fragment cross-linking[66, 67] but inactivates adrenal P450 enzymes by direct protein modification[70] further demonstrates that the binding of heme fragments to the protein is isoform-specific. Conceivably, the propensity of the CYP3A enzymes to undergo heme fragment cross-linking is related to their unusually large and, given their ability to accommodate large substrates such as cyclosporin, macrolide antibiotics, and FK506, as well as small substrates such as DDEP, highly flexible active sites. Their active sites may therefore exhibit an unusual degree of substrate mobility and/or water content (Chapter 10). Regardless of the mechanism, it is clear that CYP3A active sites are particularly susceptible to inactivation by heme fragment cross-linking. A related process is mediated by peroxides such as H_2O_2 and cumene hydroperoxide that partially degrade the prosthetic heme to soluble monopyrrole and dipyrrole fragments[383, 386–388]. In these reactions, the bulk of the heme is fragmented to products that irreversibly bind to the protein[382–391].

Myoglobin and hemoglobin have been employed as models in efforts to elucidate this unusual heme degradation process, but it now appears that these hemoproteins are not good models for the P450 reaction[392–395]. The H_2O_2-mediated oxidation of myoglobin results in the covalent binding of its heme via either the α or β-*meso* carbon or one of the vinyl groups to Tyr103[392]. In contrast, the reduction of CCl_4 or $CBrCl_3$ by myoglobin results in covalent attachment of the heme via one of its vinyl groups to His93[394, 395]. In both of these processes, as well as during the hemoglobin-mediated reduction of $CBrCl_3$[396] the cross-linked heme retains its Soret absorption maximum (at ~405 nm) and is thus bound to the protein without substantial structural disruption of its chromophore. Furthermore, γ-*meso* alkylated heme adducts without appreciable heme-protein cross-linking are observed during the myoglobin-mediated oxidative metabolism of alkylhydrazines[393]. In contrast, the cross-linking of heme fragments to protein observed in the P450 reactions with H_2O_2, cumene hydroperoxide, DDEP, and spironolactone involves complete loss of the heme chromophore and therefore major structural disruption of the heme skeleton[66, 383, 386–391]. This heme degradation also occurs if the cumene hydroperoxide-mediated inactivation is carried out under anaerobic conditions, albeit at a considerably slower rate, implying a role for molecular O_2 in this process[389, 390].

Attempts to elucidate this process have focused on cumene hydroperoxide-inactivated [^{14}C]-heme-labeled CYP3A23, -3A4, and -2B1[389, 390]. Proteolytic digestion with lysyl endopeptidase-C of the [^{14}C]-heme-modified P450enzymes, coupled with HPLC-mapping of the [^{14}C]-heme-modified peptides, Tricine-SDS-PAGE, electroblotting, microEdman degradation/amino acid sequencing, and electrospray ionization mass spectrometry (ESIMS), have located the specific sites modified by the heme fragments within the active sites of the P450 enzymes[389, 390]. Specifically, the labeled peptide in CYP3A23 encompasses residues 287–330, and in CYP2B1 residues 434–466. Sequence alignment of CYP3A23 and -2B1 with bacterial CYP101, -102, -107 and -108 reveal that the [^{14}C]-heme-fragment-modified peptide in CYP3A23 corresponds to the bacterial I-helix[160–163]. This domain contains the conserved Thr, which in the crystal structure of CYP101 is known to interact both with the substrate and the heme-bound O_2 and to be part of the active site (Chapter 3)[160–163]. On the other hand, the labeled CYP2B1 region corresponds to the bacterial L-helix that provides the conserved Cys thiolate ligand, and thus is also within the active site. However, until recently, the structure of the attached heme-derived fragments remained uncharacterized, largely because of their highly labile nature under the experimental conditions

required for isolation and structural analysis of the modified P450 peptides.

Optimization of the methodology combined with the larger peptide amounts available through the use of recombinant [^{14}C]-heme-labeled CYP3A4, enabled the structural characterization not only of the CYP3A4 peptide targets, but also of the protein modifying heme-fragments[390]. The combined structural analyses identified three major heme-modified CYP3A4 peptides comprised of residues 354–371, 372–386, and 429–450. Sequence alignments and homology modeling of CYP3A4 reveal that the 354–371 and 429–450 peptides correspond to the K-region and helix L/Cys region respectively, of P450. Several residues in these peptides are within 5 Å of the heme and thus within striking distance. Differential LC–ESMS analyses of the native and heme-modified peptide fragments provided molecular masses of ≈302, 314, and 197 for the heme-modifying species, corresponding to the deformylated and formylated A–D/B–C ring dipyrroles and the monopyrrole 2-formyl hematinic acid[390]. The precise amino acid residues modified in these peptides remain to be identified. Nevertheless, these findings suggest that the peroxidative inactivation of CYP3A4 (and presumably other P450 enzymes) involves rupture of its tetrapyrrolic skeleton along its α-γ and/or β–δ axes to yield reactive heme fragments that modify residues in their immediate proximity.

It is instructive that in this process, HCOOH (rather than CO) is the major product, and that together with minor amounts of CO and CO_2, it stoichiometrically accounts for the oxidative loss of two heme *meso*-carbons[386, 387, 397–399]. In contrast, peroxidative heme degradation in model systems yields considerably larger quantities of soluble dipyrrolic species [hydroxylated and nonhydroxylated propentdyopents and HCOOH] as major products[387, 397–400]. Approximately 15%–20% of the prosthetic heme loss after NADPH-induced oxidative uncoupling can be traced to soluble mono- and dipyrrolic products in incubations of purified CYP2B1[386]. However, this fraction drops to ~2.5% (comprised largely of hematinic acid, with traces of methylvinylmaleimide and propentdyopents) in CYP3A-enriched rat liver microsomal incubations with DDEP or cumene hydroperoxide[383]. Accordingly, in liver microsomal or purified P450 incubations, the bulk of the heme-derived species appear to irreversibly modify the CYP3A proteins.

The chemical nature of the heme fragment–protein adduct remains to be elucidated. In principle, it could entail a Schiff-base between a protein NH_2-group and the formyl group of a hydroxydipyrrolic fragment. Alternatively, it could involve attack by a suitable nucleophilic protein moiety on the 2-formylated dipyrrole. The possibility of a protein adduct with the heme vinyl also exists, even though no vinyl-modified dipyrrolic species have been detected in model heme degradation systems.

Both *in vivo* and *in vitro*, cross-linking of heme fragments to the CYP2E1 and -3A proteins targets them for proteasomal degradation by the 20*S* or ubiquitin-dependent 26*S* species[383, 401–405]. Low basal levels of microsomal heme fragment-cross-linked P450 proteins detected after [^{14}C]labeling of the P450 heme moiety *in vivo* indicate that P450 heme-modification probably occurs physiologically, possibly as a result of futile oxidative cycling of the enzymes. Furthermore, the extent of this cross-linking is increased considerably after CYP3A is induced by dexamethasone and phenobarbital[385, 406]. Since this endogenous post-translational modification targets the P450 proteins for proteolytic degradation, it could serve as a determinant of their normal physiological turnover. Not surprisingly, suppression of P450 futile oxidative cycling by blocking the P450 heme iron with TAO or isosafrole, or interrupting the electron flow through chemical or genetic impairment of P450 reductase, results in protein stabilization and consequent "induction" of CYP1A2, -2E1, and -3A[246–249].

3.5. Other Modes of P450 Heme Degradation and Protein Denaturation

The inactivation of CYP2B4 by aldehydes such as citral (an α,β unsaturated terpenoid aldehyde), and other aromatic aldehydes (cinnamaldehyde, benzaldehyde, and 3-phenylpropionaldehyde) is accompanied by bleaching of the heme chromophore that is not prevented by catalase, superoxide dismutase, epoxide hydrolase, GSH, or ascorbic acid[407, 408]. The corresponding k_{inact} values revealed that saturated aldehydes are generally

more inhibitory than their α,β unsaturated counterparts, and primary aldehydes are more potent inactivators than the structurally related secondary and tertiary aldehydes[408]. Studies with wild-type CYP2B4 and its T302A mutant, including measurements of the deuterium isotope effects, rates of inactivation, and rates of product formation suggest that aldehyde-mediated CYP2B4 inactivation involves deformylation of the aldehyde. In the inactivation of CYP2B4 by 3-phenylpropionaldehyde, a heme adduct is formed with a molecular weight equal to that of native heme plus 104 mass units, in agreement with loss of the carbonyl group from the original aldehyde. P450 inactivation by aldehydes has been proposed to involve homolytic cleavage of a peroxyhemiacetal intermediate to yield formic acid and an alkyl radical that adds to the heme moiety[408]. The heme adduct obtained in similar reactions of the F87G mutant of CYP108 (P450BM3) has been fully characterized by NMR and has been shown to involve addition of the decarbonylated substrate radical specifically to the γ-*meso* position of the heme group (Figure 7.26)[409]. The resulting heme-modified enzyme could be reduced in the presence of NADPH and lauric acid but was not able to actually oxidize the lauric acid.

Interestingly, incubation of CYP2B4 with artificial oxidants and aldehydes yielded different heme adducts. 3-Phenylpropionaldehyde yielded an adduct with a mass equal to that of native heme plus a phenylethyl group (Figure 7.26). The adduct was proposed to involve reaction of the hydroperoxy catalytic intermediate with the aldehyde to give a peroxyhemiacetal that fragmented to yield an alkyl radical[410]. In contrast, with *m*-chloroperbenzoic acid, 3-phenylpropionaldehyde yielded a phenylpropionyl-modified heme adduct purportedly generated from the reaction of the heme with the corresponding carbonyl radical (Figure 7.27). In this reaction, homolytic oxygen–oxygen bond cleavage of *m*-chloroperbenzoic acid itself also generated a chlorobenzoyloxy–heme adduct[410].

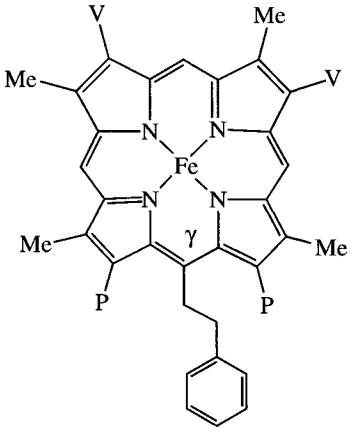


Figure 7.26. Structure of the heme adduct isolated from P450 inactivated by 3-phenylpropionaldehyde.

Similar studies with *trans*-4-hydroxy-2-nonenal (HNE, a cytotoxic byproduct of biological membrane lipid peroxidation), indicate that it is also metabolically activated by CYP2B1 and -2B4 to a reactive species that binds irreversibly to their prosthetic heme[411]. Unlike the mechanism-based inactivation by aromatic aldehydes, structural analyses of the corresponding heme adduct (MW 770) revealed that the reaction proceeds without deformylation and involves an acyl carbon radical that partitions between addition to the heme and formation of the carboxylic acid[411]. Together these findings suggest that the P450-mediated metabolic activation of aldehydes is a versatile process wherein the enzyme may be inactivated via mechanistically diverse heme modifications.

It is to be noted that P450 enzymes are sometimes inactivated by mechanisms that involve destruction of the prosthetic heme without the detectable formation of heme adducts. In some instances, these reactions result in binding of heme fragments to the protein (Section 3.4), but in most instances the incidence of heme-protein cross-linking has not been investigated. The destructive mechanisms of most peroxides[386–388], halocarbons (CCl_4)[380, 381], internal acetylenes (3-hexyne)[274], allenes (1,1-dimethylallene)[412], cyclopropylamines (*N*-methyl-*N*-benzylcyclopropylamine)[413, 414], benzothiadiazoles such as 5,6-dichloro-1,2,3-benzothiadiazole[415], and methyl thieno[3.2-*d*][1,2,3]-thidiazole-6-carboxylate[416], phenolic compounds such as the anti-inflammatory drug diclofenac[417, 418], rhapontigenin[419], and resveratrol[420], the HIV-1 reverse transcriptase inhibitor delavirdine[379, 421], and the D4 dopamine receptor antagonist SCH66712[422] (Figure 7.28) remain poorly characterized. Hypothetical mechanisms can be formulated

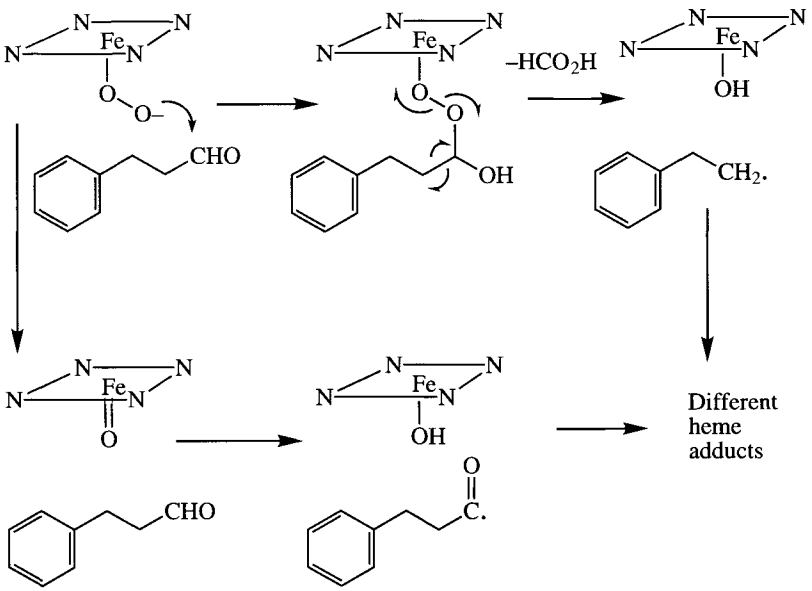


Figure 7.27. The inactivation of P450 enzymes by aldehydes appears to involve free radical intermediates, one of which retains the carbonyl group and one which does not. These radicals add to the heme group.

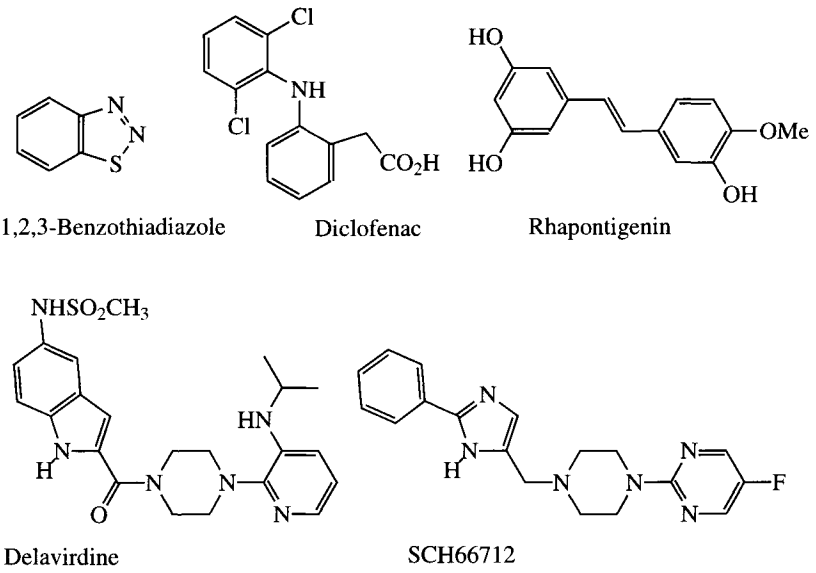


Figure 7.28. Structures of other classes of P450 mechanism-based inactivating agents.

for these reactions but experimental evidence to support the mechanisms is not available.

The P450 destruction mediated by halocarbons was once believed to stem from the secondary action of the lipid peroxides that are concomitantly formed, but it is now evident that substances like CCl_4 can destroy the heme group directly[380, 381, 423–426]. The associated cross-linking of heme fragments to the protein suggests that a radical species ($CCl_3^{\bullet}$ or $CCl_3O_2^{\bullet}$) may be responsible for heme destruction[381]. On the other hand, the catalytic reduction of halocarbons, including CCl_4, produces semistable complexes with Soret maxima in the 450–500 nm range[425–431]. Studies with model iron porphyrins, including the detailed characterization of a dichlorocarbene–metalloporphyrin complex[432], suggest that the long-wavelength Soret bands are due to ferrous halocarbene–heme complexes. This hypothesis is supported by the finding that carbon monoxide is formed in the reductive metabolism of CCl_4 by P450. This reaction is likely to occur by a mechanism similar to that for the generation of carbon monoxide from methylenedioxyphenyl complexes (Section 3.2.1)[433]. In fact, porphyrin dichlorocarbene–iron complexes do react with water to give carbon monoxide and with primary amines to give isonitriles[433, 434]. Studies with halothane suggest that it is also possible to form complexes in which the halocarbon is σ-bonded to ferric heme iron atom[435–437].

The links between formation of an iron–alkyl complex and irreversible destruction of the heme moiety have not been forged, but model studies with diaryl- and carbethoxy-substituted carbene complexes suggest that the halogenated carbenes may shift to form a bond with a nitrogen of the porphyrin[438–441]. The resulting *N*-haloalkyl adduct are likely to undergo water-dependent hydrolysis and might therefore not be detected by the methods used to isolate other *N*-alkyl porphyrins. However, the formation of alternative reactive species that attack the protein or the heme cannot be ruled out.

High (1–5 mM) concentrations of indomethacin and other nonsteroidal anti-inflammatory agents reportedly denature P450 enzymes because of their surfactant properties[442]. The loss of P450 content seen when indomethacin is added to liver microsomes is paralleled by essentially stoichiometric appearance of a P420 peak. Although it is likely that other agents cause P450 denaturation, it is unlikely that the process is physiologically relevant because of the high drug concentrations that are required.

4. P450 Enzyme Specificity

The isoform-specific inhibition of P450 enzymes is a promising avenue for the development of therapeutic, insecticidal, and herbicidal agents, as well as for investigation of the structures, mechanisms, and biological roles of individual P450 enzymes. The biosynthetic P450 enzymes have been the primary focus of efforts to develop isoform-specific P450 inhibitors because (a) they are better targets for specific inhibitors because of their high substrate specificity and (b) there is high practical utility for such inhibitors. In contrast, the broad, overlapping, specificities of xenobiotic metabolizing P450 isoforms makes the design of isoform-specific rather than -selective inhibitors more difficult[443, 444]. Selective inhibitors of P450 enzymes, as illustrated by the amphetamines[235, 240], TAO[236, 237,244–246], secobarbital[97, 157], gestodene[92], furafylline[368, 372], 1-ethynylpyrene[81, 83], and 2,3′,4,5′-tetramethoxystilbene[445] are fairly common (see Appendix). Caution is required in evaluating claims of inhibitor selectivity or specificity, as they are limited by the range of P450 enzymes actually examined. Only in the case where an inhibitor has been tested with all the known P450 isoforms in an organism can it be truly said to be specific, at least in that organism. The claim for specificity of inhibitors tested against only two or three isoforms is necessarily limited.

5. Inhibitors of Biosynthetic Enzymes

Several comprehensive review articles have discussed the potential clinical relevance and applications of inhibitors of biosynthetic P450 enzymes[5, 446–448]. The following discussion will therefore be limited to an illustration of the strategies employed in the design and development of the currently available and/or prospective inhibitors and their mechanistic diversity.

5.1. $P450_{scc}$

A single P450 enzyme ($P450_{scc}$, CYP11A) catalyzes the three oxidative steps required to cleave the side chain of cholesterol. A rational approach to the development of $P450_{scc}$ inhibitors was based on the incorporation of amino[449–452] and thiol[453] functions on the cholesterol side chain at positions that favor their coordination to the prosthetic heme iron (Figure 7.29), yielding potent reversible inhibitors (K_I = 25–700 nM). Thus, replacement of the first hydroxyl group catalytically inserted into the cholesterol side chain with an amine function yields (22*R*)-22-aminocholesterol, one of the most potent $P450_{scc}$ inhibitors[452]. The stereochemistry of this insertion is critical for inhibition since (22*S*)-22-aminocholesterol, binds to $P450_{scc}$ ~1,000 times more weakly (K_I = 13 μM) even though the amino function is located on the correct carbon.

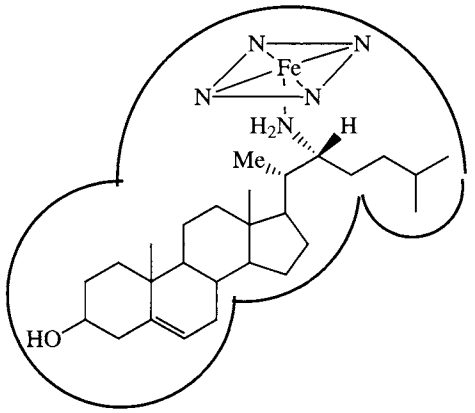


Figure 7.29. Reversible inhibitors of biosynthetic enzymes are usually constructed by incorporating a nitrogen or other coordinating atom into a lipophilic structure with high affinity for the protein active site (see Figure 7.1). An inhibitor of $P450_{scc}$ was thus obtained by placing an amino group at the position normally occupied by the first hydroxyl group added to the cholesterol side chain by $P450_{scc}$.

Various mechanism-based, irreversible inhibitors of $P450_{scc}$, such as analogs of pregnenediol with an acetylenic group grafted into their side chain, have been developed (Figure 7.30)[454–456]. Although this enzyme inactivation results in destruction of the heme chromophore, no alkylated heme was detected. Replacement of the side-chain carbons beyond C-23 in 20-hydroxycholesterol by a trimethylsilyl group also results in a $P450_{scc}$ mechanism-based inactivator (Figure 7.30)[457]. Model studies have demonstrated that 1-substituted 3-trimethylsilyl-1-propanol is oxidized by chemical reagents to ethylene, the trimethylsilyl radical, and an aldehyde[458]:

$$RCH(OH)CH_2CH_2Si(CH_3)_3 \rightarrow RCHO + CH_2{=}CH_2 + (CH_3)_3Si^{\bullet}$$

If the chemical model is relevant, $P450_{scc}$ may be inactivated by reaction of the enzyme with the trimethylsilyl radical or, less likely, from oxidation of the ethylene produced in the initial catalytic turnover. An interesting variant of a mechanism-based inhibitor is provided by (20*S*)-22-*nor*-22-thiacholesterol, in which the sulfur that replaces the carbon at position 22 is oxidized by $P450_{scc}$ to a sulfoxide that is a potent but not an irreversible inhibitor of the enzyme[457, 459, 460].

5.2. Aromatase

Aromatase, through a three-step catalytic transformation, controls the conversion of androgens to estrogens. Competitive and mechanism-based inhibitors of aromatase have been clinically exploited in the treatment of estrogen-dependent

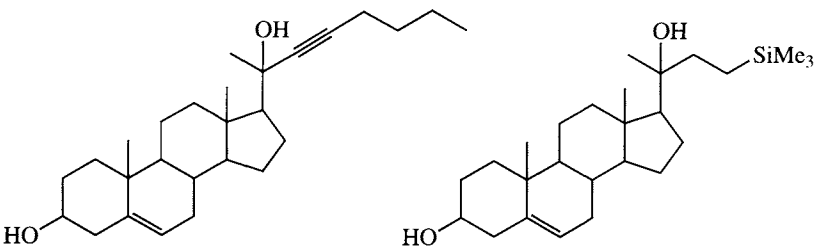


Figure 7.30. Two mechanism-based inhibitors of cytochrome $P450_{scc}$.

mammary tumors[5, 461–466] and benign prostatic hyperplasia[467, 468], and have some promise in the control of coronary heart disease[469]. Indeed, some of the more promising newer agents are in clinical trials[463–466]. Aminoglutethimide, an inhibitor of aromatase, has been used to treat hormone-dependent metastatic breast carcinoma, but its poor specificity and the incidence of side effects, primarily from inhibition of $P450_{scc}$, has compromised its utility[470–472]. Replacement of the aminophenyl group in aminoglutethimide (Figure 7.31) by a pyridine moiety affords [pyridoaminoglutethimide (3-ethyl-3-(4-pyridyl) piperidine-2,6-dione)], an agent that inhibits aromatase but not $P450_{scc}$[472, 473]. The enhanced specificity for aromatase may reflect a differential positioning of the pyridyl nitrogen within the two active sites that enables it to coordinate with the heme of aromatase but not of $P450_{scc}$. Interestingly, an aminoglutethimide analog with a nitrogen at the opposite end is a more potent $P450_{scc}$ inhibitor than glutethimide but has little or no activity against aromatase[473]. These findings suggest that the 19-methyl and carbon 22 of the sterol side chain are separated by a distance roughly equal to the length of the aminoglutethimide structure. It is therefore tempting to speculate that aminoglutethimide binds within the active sites of $P450_{scc}$ and $P450_{arom}$ in approximately the same orientation as the normal sterol substrate, and that the location of the nitrogen in the two inhibitors dictates their differential enzyme selectivity.

Potent inhibitors have also been developed that use an imidazole or related functions to coordinate to the aromatase iron atom. The most promising of these for the treatment of breast cancer are fadrozole, {4-(5,6,7,8-tetrahydroimidazo[1,5α]-pyridin-5-yl)benzonitrile monochloride} (CGS16949; Figure 7.31), and its congener [bis-(*p*-cyanophenyl)-imidazo-1-yl-methane hemisuccinate][474–478]. Both of these agents selectively inhibit aromatase rather than of $P450_{scc}$, P45021, or P45011B[475, 478]. Fadrozole does inhibit aldosterone production (18-hydroxylase activity) in rats, but this is much less pronounced with its congener[474]. Phase I clinical trials of fadrozole indicated that it is a potent inhibitor of estrogen biosynthesis in postmenopausal women with advanced breast cancer[474–478]. In addition to being well tolerated by patients, even at the maximally effective dose, it did not significantly alter cortisol, androstenedione, testosterone or aldosterone levels[474–478].

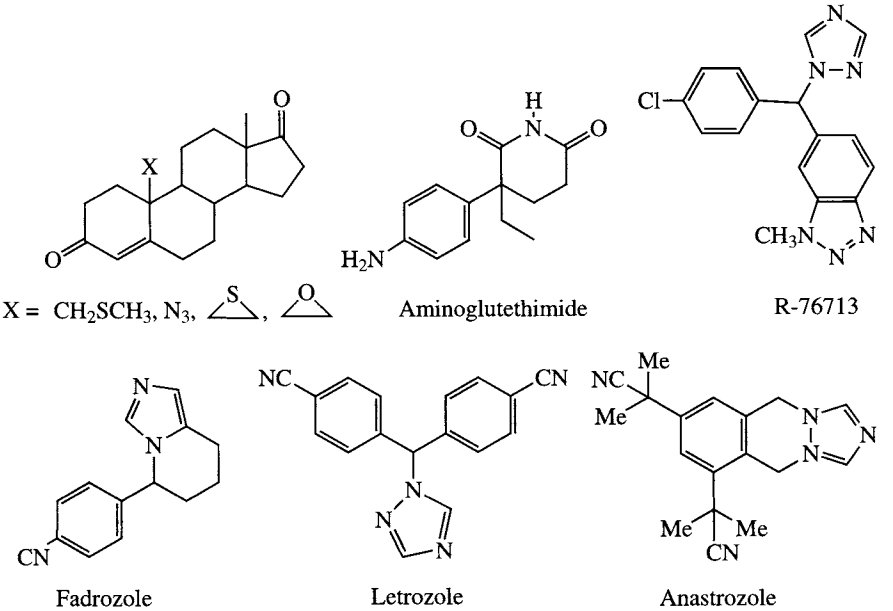


Figure 7.31. Structures of some competitive inhibitors of aromatase. In each of these compounds, a heteroatom is placed so that it can coordinate to the heme iron atom.

However, fadrozole may now be surpassed by letrozole (CGS20267 or Femara; Figure 7.31), an advanced nonsteroidal aromatase inhibitor, which appears to be more potent and effective than fadrozole in the treatment of postmenopausal women with advanced breast cancer[479, 480].

6[(4-Chlorophenyl)(1H-1,2,4-triazol-1-yl) methyl]-1-methyl-1H-benzotriazole, (R76713; Figure 7.31), is a relatively selective and very potent inhibitor of human placental aromatase[481–483]. Its (+) enantiomer has a lower IC_{50} value than the (−) enantiomer when assayed against human placental aromatase and exhibits no appreciable inhibition of other steroidogenic enzymes or liver microsomal P450 enzymes at concentrations up to 1,000-times the aromatase IC_{50}[482, 483].

Several other nonsteroidal compounds have been developed as novel and selective aromatase inhibitors, including 4-(4′-aminobenzyl)-2-oxazolidinones[484], 7-(alpha-azolylbenzyl)-1H-indoles and indolines of which 1-ethyl-7-[(imidazol-1-yl) (4-chlorophenyl)methyl]-1H-indole 12c exhibited the most promising potency[485], 4-imidazolylflavans[486], and anastrozole (Arimidex; Figure 7.31)[487]. Of these anastrozole has recently been approved in the United States and several other countries for the adjuvant treatment of postmenopausal women with hormone receptor-positive early breast cancer[488, 489].

Powerful competitive steroid inhibitors of aromatase have been synthesized by replacing the C-19 methyl with sulfur- or nitrogen-containing functions that can coordinate to the heme iron (e.g., Figure 7.31)[490–495]. The 19-methyl substituents include the following: CH_3SCH_2[490–492], $CH_3SCH_2CH_2$[493], $HSCH_2$[491, 494], $RSSCH_2$ (Org-30958, R = ethyl is best)[494], SH[491], NH_2, and NH_2CH_2[496]. Oxygen (oxiranyl), sulfur (thiiranyl), and nitrogen (aziridinyl) three-membered rings have also been used to replace the 19-methyl group (Figure 7.32)[495–501], the best of the resulting steroids exhibiting K_I values in the 1 nM range. The epoxide, episulfide, and aziridine functions inhibit the enzyme by stereoselective coordination of the heteroatom to the iron atom but, despite their reactivity, apparently do not inactivate the enzyme. However, the steroids in which the 19-methyl group is replaced by a sulfhydryl or $HSCH_2$ group (Figure 7.32) are irreversible mechanism-based inhibitors rather than simple competitive inhibitors[491].

The strategies developed for the inactivation of hepatic P450 enzymes have also been exploited

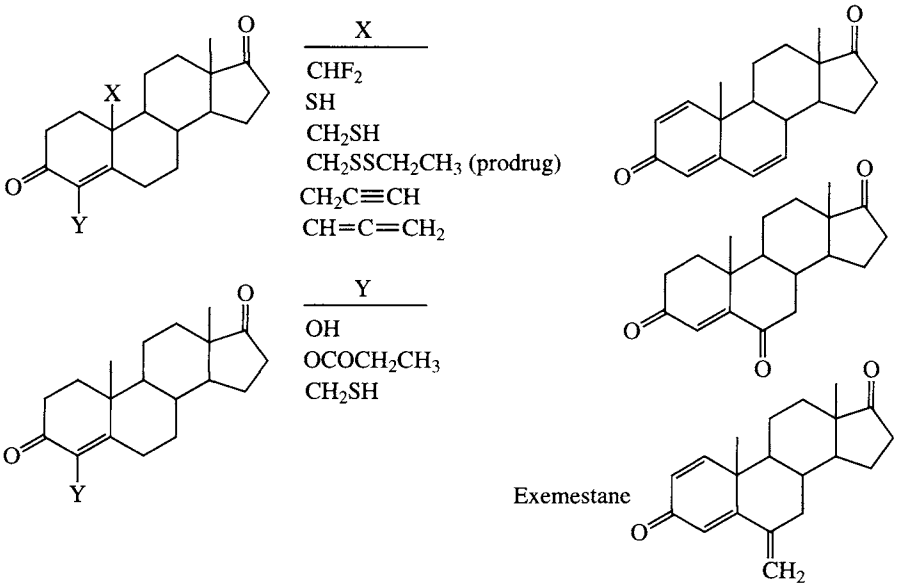


Figure 7.32. Structures of a selection of mechanism-based inactivators of aromatase.

in the design and synthesis of mechanism-based aromatase inactivators. Substitution of the normally hydroxylated methyl group (C-19) with a propargylic or allenic moiety (Figure 7.32) converts the sterol into an irreversible aromatase inhibitor[502–507]. The details of aromatase inactivation by these acetylenic and allenic agents remain unclear, but it is likely that they are activated to intermediates that react with either the heme or the protein (see Sections 3.1 and 3.3.2). Replacement of the C-19 methyl with a difluoromethyl also yields a mechanism-based inactivator that must be activated by C-19 hydroxylation (Figure 7.32)[506–511] as tritium release from the tritium-labeled difluoromethyl derivative is required for enzyme inactivation[512]. It is likely that the difluoromethylalcohol thus produced decomposes to the acyl fluoride that irreversibly binds to a protein nucleophile.

The 19-substituted analog of androst-4-ene-3, 17 dione steroid inhibitors, Org-30958 [19-(ethyldithio)androst-4-ene-3,17-dione], has been assessed in Phase I clinical trials for estrogen-dependent breast cancer chemotherapy[494]. The ethyldithio substitution apparently renders the steroid more stable extracellularly than the free thiol Org-30365 (19-mercapto-androst-4-ene-3, 17-dione), resulting *in vivo* in animal models in an 8-fold greater aromatase inhibitory activity than either 4-OHA or SH-489. Its *in vivo* potency requires intracellular reduction of the disulfide to release the 19-mercapto analog Org-30365, a more potent mechanism-based human placental aromatase inactivator[468] than 4-OHA or SH489[494].

Clinically effective mechanism-based aromatase inactivators can also be obtained by introducing substituents at the 4- or 6-positions of the sterol skeleton. 4-Acetoxy- and 4-hydroxy-4-androstene-3,17-dione (4-OHA) (Figure 7.32) irreversibly inactivate placental aromatase by catalysis-dependent mechanisms involving the 19-methyl[513, 514]. A possible mechanism for inhibition of aromatase by the 4-substituted analogs, as illustrated by 4-OHA, is shown in Figure 7.33. 4-OHA is used for the treatment of estrogen-dependent breast cancer[462, 515]. Of a series of $\Delta^{1,2}$, $\Delta^{4,5}$, and $\Delta^{6,7}$ analogs evaluated as prospective aromatase inhibitors in preclinical trials, FC 24928 (4-aminoandrostan-1,4,6-triene-3,17-dione) is the most promising candidate because it inactivates human placental aromatase activity as potently as 4-OHA and FCE-24304 (6-methylene-androstan-1,4,-diene-3,17-dione) but, unlike both these compounds, it has little intrinsic androgenic activity and does not affect 5α-reductase or $P450_{scc}$[516–518].

Conjugation of the 4-hydroxyandrostene nucleus as in 1,4,6 androstatriene-3,17-dione (ATD), conveys aromatase inhibitory and marked tumor regression activities (~80%)[519, 520]. On the other hand, the introduction of a C_1-methyl into 1,4-androstadiene-3,17-dione as in Atamestane (1-methylandrosta-1,4-diene-3,17-dione, SH-489), apparently enhances its affinity (K_I ~2 nM vs K_I of 29 nM for 4-OHA) for the human placental aromatase while slowing its inactivation of the enzyme, thereby reducing the production of estrogenic products[518, 521]. The compound along with its 1,2 methylene-substituted congeners has been evaluated in Phase I clinical trials for possible therapy of estrogen-dependent conditions such as breast cancer and benign prostatic hypertrophy. Additional steroidal agents explored for their aromatase suicide inactivation include androst-5-ene-7,17-dione and its 19-hydroxy derivative[522].

Turnover-dependent irreversible inactivation of the enzyme via protein modification is also achieved by introducing a 6-keto group into the steroid skeleton (Figure 7.33)[523–525]. Monitoring the $^3H:^{14}C$ ratio in studies with the C-19 double-labeled inhibitor indicates that the C-19 methyl, one of the C-19 hydrogens and, from a separate double label experiment, the 1β-hydrogen, are retained in the covalently bound species[525]. These findings do not define the underlying inactivation mechanism but appear to exclude the involvement of C-19 demethylation and aromatization, although normal aromatization is possible because 6-oxoestrone and 6-oxoestradiol are concurrently formed.

Exemestane, 6-methylene-androsta-1,4-diene-3,17-dione (Figure 7.32), is an aromatase inhibitor with an IC_{50} for inhibition of human placental aromatase comparable to that of 4-OHA[516, 517]. The K_I (nM) and $t_{1/2}$ (min) values for the inactivation processes were 26 ± 1.4 and 29.0 ± 7.5, and 13.9 ± 0.7 and 2.1 ± 0.2, for Exemestane and 4-OHA, respectively. In spite of its relatively slow inactivation of aromatase, Exemestane is a more potent agent in experimental animals[516], and also much more effectively causes regression of

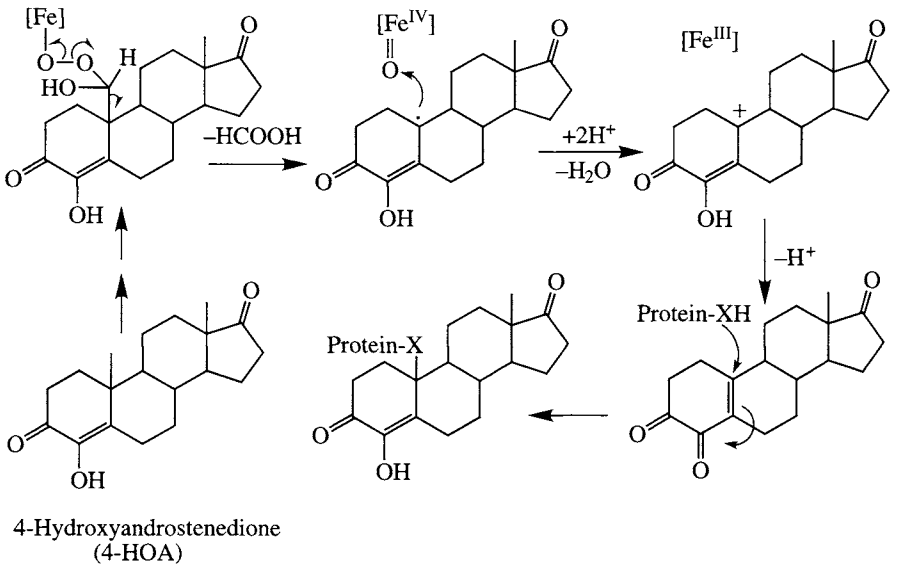


Figure 7.33. A possible mechanism for the inactivation of aromatase by 4-OHA.

chemically induced mammary tumors in rat than MDL18962 or SH-489[518]. Because of its oral effectiveness and potent irreversible aromatase inhibition, Exemestane has been recently approved and introduced into the global market under the name Aromasin[526–531].

MDL18,962 10-(2-propynyl)estr-4-ene-3,17-dione is one of the most potent of the mechanism-based inactivators of aromatase, with K_I values of the order of 3–4 nM, and $t_{1/2}$ of 9 and 6 min, respectively, for the human and baboon placental aromatases[503, 504, 506, 508, 532]. Phase I clinical trials and preclinical findings indicate that MDL18,962 is indeed effective in lowering estrogen levels[533].

The time-dependent inactivation of aromatase by 10-hydroperoxy-4-estrene-3,17-dione, reportedly a mechanism-based inactivator of aromatase[534, 535], is inhibited by NADPH or an alternative substrate and is partially reversed by dithiothreitol. Other high affinity 10-substituted analogs, such as the mechanism-based inhibitor 10-β-mercaptoestr-4-ene-3,17-dione, and the competitive inhibitors (19*R*)-10-oxiranyl and 10-thiiranyl-estr-4-ene-3,17-diones and 10-β,-$MeSCH_2$-estr-4-ene-3,17-dione, have been reported[491, 492, 494, 536, 537]. C-10, C-2 hydroxyethyl bridged steroids synthesized as stable carbon analogs of the 2β-hydroxylated 19-oxoandrostenedione, a putative intermediate in the aromatization reaction, have been found to be potent competitive inhibitors of the human placental aromatase[538]. In contrast, related halohydrin analogs are potent mechanism-based aromatase inhibitors[538].

Finally, 4β,5β-epoxyandrostenedione as well as its 19-hydroxy and 19-oxo derivatives have been examined as aromatase inhibitors. The epoxides were found to be weak competitive inhibitors, whereas the 19-hydroxy and 19-oxo derivatives were largely ineffective[539].

5.3. Lanosterol 14-Demethylation

The 14-demethylation of lanosterol is a key step in the biosynthesis of cholesterol. The preferential inhibition of the P450 enzyme that catalyzes this reaction by a number of substituted imidazoles, pyridines, pyrimidines, and other lipophilic heterocycles has been exploited in the construction of clinically important antifungal agents[540–542]. The antifungal action following the inhibition of 14-demethylase is thought to result from the accumulation of 14-methyl sterols in the membranes of susceptible fungi that cause deleterious changes in their membrane permeability[543–545].

Miconazole (Figure 7.34), fluconazole (Figure 7.34), ketoconazole (Figure 7.1), and

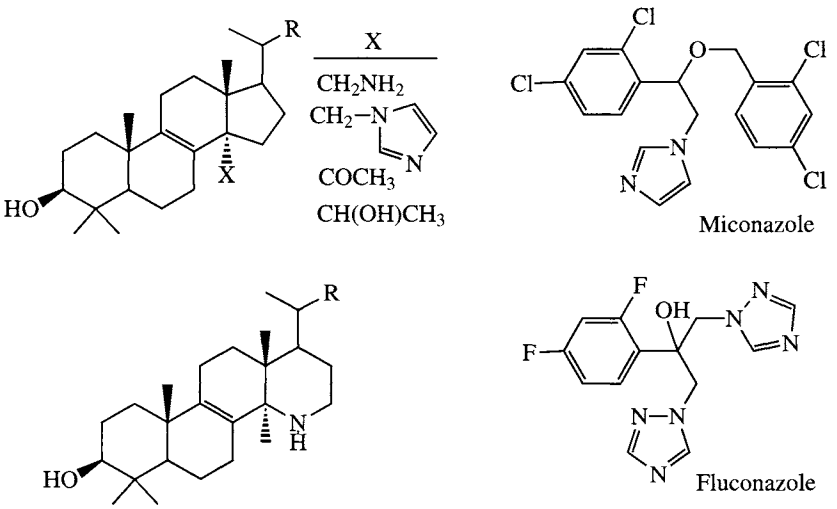


Figure 7.34. Competitive inhibitors of lanosterol 14-demethylase. The group R on the sterols is either $C(CH_3)CH_2CH_2CH_2C(CH_3)_2$ or a close variant. Fluconazole and miconazole are clinically employed as antifungal agents.

related azole structures inhibit the fungal 14-demethylase activity at extremely low (nM) concentrations[546, 547], but only inhibit the 14-demethylase activity of the mammalian host at higher (up to 100 μM) concentrations[548, 549]. Low doses of ketoconazole, however, appear to inhibit not only the C_{17-20} lyase, but also human hepatic CYP3A4, and thus this agent is not very clinically useful[550, 551]. As expected, the substituted imidazoles and other nitrogen-based heterocyclic antifungal agents coordinate with the P450 heme iron to yield typical type II binding spectra[552].

Potent isoprenoid-containing imidazole antifungal agents, such as AFK-108 (1-[2-(2,4-dichlorophenyl)-2-((2*E*)-3,7-dimethylocta-2,6-dienyloxy) ethyl]-1H-imidazole), have also been developed whose geranyl moiety specifically interacts with the sterol side-chain recognition region within the 14α-demethylase active site. Unfortunately, this compound and its farnesyl derivative inhibited not only the rat liver counterpart but also other hepatic drug metabolizing P450 enzymes, placing their practical utility in question[553].

Structural considerations have also prompted the development of 14α-methyl-15-aza-D-homosterols (Figure 7.34), which exhibit relatively high *in vitro* fungistatic potencies and high fungicidal activities as well as respectable *in vivo* efficacies in a murine model of candidiasis[554]. Introduction of an oxime functionality (=NOH) at position 15 of the sterol group, also results in an inhibitor with a very similar geometry of the nitrogen as that in the 15-azahomosterols[555]. Similarly, 7-oxo-24,25-dihydrolanosterols, which uncouple the catalytic turnover of CYP51 by competitively blocking transfer of a second electron to the oxyferrocomplex, have also been considered as prospective inhibitors[556]. Furthermore, 15-, 32-, and 15,21-oxylanosterol analogs have been examined as mechanism-based inactivators of lanosterol demethylase. Of these (32*S*)-vinyllanost-8-en-3β,32-diol is a potent time-dependent inactivator ($k_{inact}/K_I = 0.36\ min^{-1}\ \mu M^{-1}$), while the (32*R*)-vinyllanost-8-en-3β,32-diol functions solely as a competitive demethylase inhibitor[557]. Replacement of the 15-hydrogen normally lost during catalysis by a fluorine yields 15α-fluorolanost-7-en-3β-ol which is oxidized by the enzyme to the 14-aldehyde but proceeds no further due to the fluorine substitution[558]. The compound is therefore a "prodrug" inhibitor that requires metabolic activation of its latent form rather than a true mechanism-based inactivating agent.

Steroid analogs with amino and imidazole functions attached to the 14-methyl carbon have

been developed as inhibitors of lanosterol 14α-demethylation (Figure 7.34)[559]. Steroids with a 14-(1-hydroxyethyl), 14-(1-oxoethyl), or 14-carboxyl group are competitive inhibitors of the enzyme (Figure 7.34)[560–562]. Lanosterol analogs in which the 14α-methyl group has been replaced by a vinyl, ethynyl, allyl, propargyl, 1-hydroxypropargyl, 1-ketopropargyl, difluoromethyl, epoxide, or episulfide moiety have been synthesized as potential mechanism-based inhibitors of lanosterol 14α-demethylase[562–565]. Although the ethynyl sterols are clearly mechanism-based inactivators of the enzyme[565], most of these compounds act as simple competitive inhibitors (e.g., the epoxide and episulfide analogs). Finally, because of their cholesterol lowering potential, 14α-demethylase inhibitors have also been considered as hypolipidemic agents[566].

5.4. Other Biosynthetic Sterol Hydroxylases

A current strategy for the development of new drugs for the treatment of prostatic cancer is the development of inhibitors of CYP17-dependent androgen biosynthesis. Thus, as in the case of other steroidogenic enzymes, an intense search has been undertaken for reversible and irreversible CYP17 inhibitors[567]. The resulting agents include substituted imidazoles, pyridines, pyrimidines, and other lipophilic heterocycles as the enabling moiety[567–581]. Of these, abiraterone (17-(3-pyridyl)androsta-5,16-dien-3β-ol), a potent inhibitor (IC_{50}, 4 nM) of the hydroxylase activity of human cytochrome CYP17, has been employed clinically[571]. Its potency is apparently due to activation of the 16,17-double bond that is required for irreversible binding of these pyridyl steroids to CYP17[571]. Analogs such as [17-(5-pyrimidyl)androsta-5,16-diene-3β-ol] and its 3-acetyl derivative, which are even more potent CYP17 inhibitors in rats than abiraterone, are particularly promising[580]. With testicular microsomal CYP17, these compounds apparently exhibit the dual characteristics of a type II binding spectrum and noncompetitive inhibition[581].

Another mechanism-based inactivator of both the 17-hydroxylase and C-17/C-20 lyase activities of CYP17 is 17β-(cyclopropylamino)-androst-5-en-3β-ol[582]. This compound reportedly does not inhibit $P450_{scc}$ or the sterol 21-hydroxylase. In contrast, sterols with a 17-difluoromethyl group selectively inactivate the C-21 hydroxylase, whereas sterols with a 17-dichloromethyl, -vinyl, or -ethynyl function inactivate both the C17- and C21-hydroxylases[86]. More recently, 20-fluoro-17(20)-pregnenolone derivatives were designed as enol mimics of pregnenolone. All of the targeted, novel fluoroolefins were found to be potent inhibitors of C-17(20) lyase[579].

Similar strategies have been used to develop inhibitors that target the C-18 hydroxylation involved in the biosynthesis of aldosterone[583–586]. Thus, aldosterone analogs with C-18 iodomethyl, chloromethyl, allyl, propargyl, vinyl, and methylthiomethyl functionalities have been synthesized and some have been found to irreversibly inactivate the enzyme. An active site-directed 18-acetylenic deoxycorticosterone [21-hydroxy-13(-2-)propynyl)-18-nor-preg-4-ene-3,20-dione, MDL19,347] is a promising inactivator of the rat and rhesus monkey adrenal corticosterone 18-hydroxylase ($K_I \approx 38$ nM; $t_{1/2}$, 4.6 min) that also effectively reduces plasma aldosterone levels *in vivo*[586]. This compound appears to be a relatively selective inhibitor of aldosterone biosynthesis, as it fails to inhibit the 11β-hydroxylation of corticosterone and DOC[586]. Rationally developed inhibitors such as these may be useful in the management of conditions such as hypertension, hypokalemia, and edema that are associated with hyperaldosteronism.

5.5. Fatty Acid and Leukotriene Monooxygenases

Microsomal P450 enzymes of the CYP2C and 4A subfamilies in tissues such as the liver, lung, kidney, peripheral vasculature, and polymorphonuclear leukocyte oxidize a variety of fatty acids, including arachidonic acid and its derivatives, to physiologically active metabolites. Some of these metabolites are well recognized as biologically important regulators of renal, pulmonary, and cardiac function and vascular tone[587–595]. Two general classes of such vasoactive metabolites are known to be produced in vascular and extravascular tissues: EETs generated by CYP2C epoxygenases, and HETEs products hydroxylated at the ω- and ω-1 positions

by CYP4A[590–596]. Furthermore, with the exception of 20-HETE, all the other arachidonic acid products occur as stereo- and regioisomers that vary considerably in their biological activities and potencies[592, 593]. Thus, 5,6-, 8,9-, 11,12-, and 14,15-EETs are potent vasodilators, specially in various capillary beds, that act through activation of K^+-channels in vascular smooth muscle cells. In contrast, 12(*R*)- and 20-HETEs are potent vasoconstrictors. 20-HETE is a particularly potent cerebral and renal microvessel vasoconstrictor, as well as a mediator of other important physiological processes (reviewed in Chapter 11). The altered production of EETs and 20-HETE in various genetic and experimental models of pathological diseases has led to their consideration as plausible causative factors. Thus, not surprisingly, the P450 enzymes responsible for their biosynthesis have been singled out as key targets not only for defining the pathological roles of these metabolites, but also as possible sites for pharmacological intervention in the treatment of these conditions[592–598]. In this context, both the previously existing and new agents have been exploited as reversible or irreversible inhibitors of these enzymes.

Accordingly, two reversible inhibitors of CYP4A enzymes have been assessed as probes of 20-HETE involvement in various physiological processes with varying degrees of success: the not so selective antifungal agent miconazole (Section 2.3)[599–601], and the rationally designed and thus more selective, *N*-methylsulfonyl-12,12-dibromododec-11-enamide (DDMS; Figure 7.35) and its acid analog, 12,12-dibromo-dodec-11-enoic (DBDD)[602–608]. DDMS has been found to be an effective acute and chronic blocker of 20-HETE formation *in vivo* in rats[602–604]. More recently an even more potent and selective inhibitor of 20-HETE formation, HET0016 (*N*-hydroxy-*N'*-(4-butyl-2-methylphenyl)formamidine) (Figure 7.35) has been discovered through combinatorial chemistry, with an IC_{50} of 35 ± 4 nM[608]. Because of its high selectivity, it holds considerable promise as a probe of 20-HETE dependent function.

Several mechanism-based irreversible inhibitors of P450-dependent arachidonic acid metabolism have also been developed. For instance, in 11-dodecynoic acid, the terminal acetylenic analog of lauric acid, and 10-undecynoic acid have been shown to inactivate hepatic CYP4A lauric acid ω-hydroxylases while minimally altering the spectrophotometrically detectable hepatic P450 content or function[78, 609, 610].

The high susceptibility of these acetylenic fatty acid inactivators to β-oxidation, however, compromises their *in vivo* utility and prompted the development of 10-undecynyl sulfate (10-SUYS), the acetylenic analog of 10-undecynoic acid in which the carboxyl group is replaced by a sulfate (Figure 7.35)[610]. The sulfate is not susceptible to β-oxidation and thus prolongs the biological lifetime, reduces the toxicity, and enables the use of sulfate agent as an *in vivo* mechanism-based

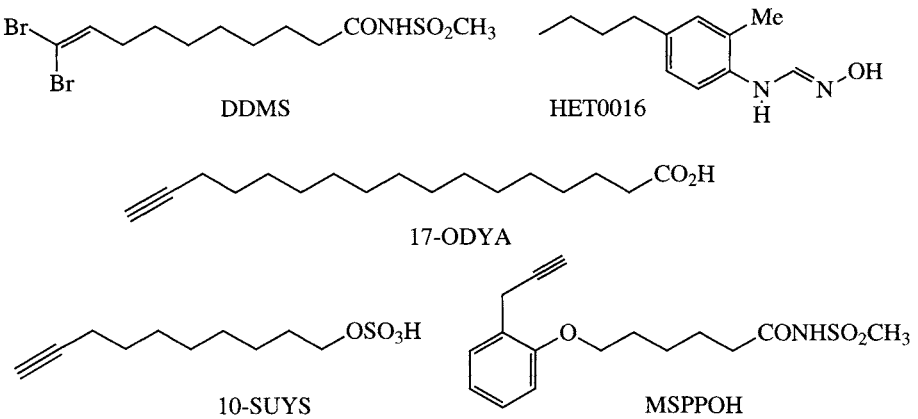


Figure 7.35. Reversible and mechanism-based inhibitors of enzymes involved in the metabolism of fatty acids and related endogenous substrates.

CYP4A inactivator[610]. Administration of a single dose of 10-SUYS, a potent and selective mechanism-based CYP4A inactivator, acutely reduced the mean arterial blood pressure as well as the urinary 20-HETE excretion in spontaneously hypertensive rats, consistent with the inactivation of renal 20-HETE formation[611]. These findings thus suggest that 20-HETE could play an important role in blood pressure regulation in hypertensive states and that the inhibition of its synthesis in these conditions may be of therapeutic benefit[611].

CYP4A and CYP2C isoforms are also inactivated by the acetylenic fatty acid analog, 17-octadecynoic acid (17-ODYA; Figure 7.35), in a mechanism-based process[612–614]. 17-ODYA has been particularly useful *in vivo* to probe the involvement of these metabolites in physiological and/or pathological processes[597, 598, 603, 605–609, 612–618]. Its use has helped elucidate the specific role of 20-HETE in the myogenic activation and hypoxic dilation of skeletal muscle resistance arteries as well as the vasodilatory effects of NO in the renal microcirculation[603, 606, 607, 618].

The acetylenic fatty acids 15-hexadecynoic (15-HDYA) and 17-ODYA have also been explored as modulators of leukotriene B_4 (LTB_4), an important and clinically relevant inflammatory mediator, and its physiologically active ω-hydroxylated metabolite[619–622]. Both 15-HDYA and 17-ODYA inactivated the polymorphonuclear leukocytic LTB_4 ω-hydroxylase in whole cells and cell lysates[622]. In contrast, the shorter-chain acid, 10-undecynoic acid was much less effective, while the saturated analogs of 15-HDYA and 17-ODYA were inactive. 15-HDYA and 17-ODYA also inactivate pulmonary prostaglandin ω-hydroxylases[623].

The mechanism-based inactivator 1-ABT (Section 3.3.5), which is not very selective and inactivates multiple P450 enzymes, including those responsible for the synthesis of both EETs and 20-HETE, has also been used, alone or in conjunction with other inhibitors, to assess the role of these metabolites in skeletal muscle angiogenesis induced by electrical stimulation as well as in the renal and vasoconstrictor actions of angiotensin II[348, 602, 608, 624].

Mechanism-based inhibitors of EET formation have also been used as probes of the physiological roles of these metabolites. One such agent is *N*-methylsulfonyl-6-(2-propargyloxyphenyl)hexanamide (MSPPOH; Figure 7.35)[605, 625–627].

Finally, since fatty acid hydroxylations at the ω, ω-1, ω-2, ω-3, and ω-4 positions also occur in plants and bacteria[628, 629], acetylenic fatty acids have also been employed to examine whether these hydroxylases are mechanistically similar to their mammalian counterparts and/or to identify the role of specific plant and bacterial P450 enzymes[629, 630]. Thus, midchain and terminal acetylenes such as 10-dodecynoic acid have been used as probes of plant lauric acid ω−hydroxylases[84, 629]. Similarly, 17-ODYA has been shown to inactivate $P450_{BM-3}$ (CYP108), an enzyme that hydroxylates fatty acids at positions other than the terminal (ω) carbon, through a heme alkylation mechanism[630].

6. Summary

Our knowledge of the structure and function of P450 enzymes has greatly expanded over the past three decades, as has our appreciation of the chemical functionalities that they accept as substrates and of the moieties that can inhibit or terminate their catalytic action. As a result, multiple routes are now available for the inhibition or inactivation of P450 enzymes that target either the protein and/or the heme. This knowledge has been exploited in the design and construction of both reversible and irreversible inhibitors of increasing potency, specificity, and potential practical utility. One area of continuing growth is that of reversible inhibitors that, by simultaneously conforming to the predominantly lipophilic contours of the P450 active site and providing a nitrogen that coordinates to the heme iron atom, have achieved enhanced potency and specificity. These agents include the clinically relevant azole antifungal and cancer chemotherapeutic drugs (Section 5).

The high promise of irreversible P450 inactivating agents has not yet culminated in agents of large-scale practical utility. Progress in the field has continued to provide instruction on approaches for the design of mechanism-based inhibitors that inactivate a P450 enzyme with high specificity and negligible release of reactive metabolites into the medium, whether the inactivation involves modification of the protein or the

heme. High specificity is provided by the requirement not only for binding within the P450 active site but also for the more rigorous test of compatibility with catalytic turnover by the target enzyme. Potency results from the irreversible nature of the inhibition, which means that relatively high concentrations of the agent need not be maintained throughout the period of inhibition. However, the involvement of reactive intermediates such as radicals and ketenes in the inactivation requires that the inactivation process be highly efficient and occur without leakage of reactive functions into the medium. As our increased understanding of mechanism-based inhibition makes this feasible, this remains an attractive and highly promising approach to novel therapeutic agents and other commercial applications.

On the negative side, the inhibition of P450 enzymes has increasingly been recognized as a key locus of clinically relevant drug–drug interactions and other pharmacological effects. One needs only to consider the consequences of the lethal inhibition of CYP3A4 in patients treated with agents like terfenadine to appreciate the general concern in the pharmaceutical industry with the subject of P450 inhibition. Our growing understanding of the structural and chemical basis of P450 inhibition should greatly improve our ability to rapidly elucidate their nature when and if they do occur and, of course, to avoid their occurrence in the later stages of drug design.

Finally, on a more heuristic level, the past few years have led to the development of an increasing repertoire of isoform-selective inhibitors that can be deployed in basic studies of the physiological function of individual P450 enzymes. One area to which such inhibitors have contributed is to elucidation of the specific roles of the products of P450-catalyzed arachidonic acid oxidation. A toolbox of highly isoform-specific inhibitors covering the full range of human and rodent P450 enzymes, each with pharmacokinetic properties and a low toxicological profile that makes it suitable for *in vivo* use, would be a panacea but at this time is nothing more than a possibility.

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