Biochemical Pharmacology, Vol. 44, No. 11, pp. 2089–2098, 1992.
Printed in Great Britain.

0006-2952/92 $5.00 + 0.00
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COMMENTARY

SPECULATIONS ON THE SUBSTRATE STRUCTURE–ACTIVITY RELATIONSHIP (SSAR) OF CYTOCHROME P450 ENZYMES

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Specificity and selectivity of substrates of P450 as a function of apoprotein structure?

The oxidation of many drugs and other chemicals is catalysed by cytochrome P450 enzymes (EC 1.14.14.1, non-specific monoxygenase). The catalytic mechanism of these enzymes has been rationalised on theoretical grounds by Guengerich and Macdonald [1], although full experimental proof has yet to be obtained. In essence once oxygen activation has occurred, oxidation of the substrate proceeds by abstraction of a hydrogen atom or an electron from the substrate and oxygen rebound (radical recombination). This mechanism includes hydroxylation at carbon and nitrogen and also dealkylation of amines and ethers. What is unclear, however, is why P450 enzymes metabolising exogenous substrates should show selectivity or otherwise towards substrates. Guengerich [2] states that the chemistry in different reactions is thought to be invariant, and the key influence on catalytic activity is the apoprotein. In addition, he draws attention to the type of reaction (epoxidation, N-demethylation) being a function of the fit of the substrate (or transition state) with the protein. Theoretical studies on substrates [3], however, suggest that the apoprotein alone does not explain the selectivity of the enzyme, and it is probably a function of both binding characteristics of the enzyme and the ease at which various groups or functions within a molecule undergo oxidation. It is the purpose of this review to present a hypothesis on the major P450 forms of human liver and how the two functions of binding and chemical lability vary in their importance across the various enzymes, using P4502D and P4503A as examples. As previously outlined by Smith [4] P4502D substrate binding is governed by an ion-pair formation between the basic nitrogen of substrates, whereas P4503A binding is predominantly hydrophobic. These two have been chosen as being two of the most important isoenzymes involved in the metabolism of drugs by humans. In many cases these isoenzymes are present in similar forms in animals [4]. Only where the data are unique to humans are the precise isoforms such as P4502D6 identified.

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Selectivity of P4502D

It can be rationalised that potent selective inhibitors of an enzyme match closely the dimensions of the active site. This approach has been adopted by Strobl *et al.* [5, 6] who have overlapped a series of inhibitors of cytochrome P4502D6 (the human P4502D enzyme) and established the following structural requirements: an extended hydrophobic region, a positively charged, basic nitrogen, and groups with negative potential, and the ability to accept hydrogen bonds, positioned 5–7Å from the nitrogen atom. Table 1 lists the inhibitors of P4502D6 with K_i values of 10 μM or less. Some of these are also substrates. All of these compounds largely fit the above criteria. The most potent inhibitor of P4502D6 is quinidine. Its stereoisomer quinine can also be considered as a selective potent inhibitor. Specificity depends on species with quinine the more potent inhibitor in rats and quinidine the more potent inhibitor in humans. Inhibition of P4502D is

Table 1. Inhibitors of cytochrome P4502D6 with K_i values of 10 μM or less

	K_i (μM)	pK_a*	Ref.
Oxprenolol	10	9.3	8
Perazine	8.4		5
Fluvoxamine	8.2		9
Propranolol	6.7	9.4	8
Bufuralol	4.8	9.0	8
Citalopram	5.1	~9.5	8
Amitriptyline	4.0	9.4	9
Chlorpromazine	3.8	~9.5	5
Ajmalicine	2.6		5
Ajmaline	2.5		5
Desmethylimipramine	2.3	~10.0	9
Clomipramine	2.2	9.4	9
Levomepromazine	1.0	9.4	9
Sertraline	0.7		9
Fluoxetine	0.6		9
Thioridazine	0.5		9
Yohimbine	0.4	7.6	5
Paroxetine	0.15		9
Budipine	0.1		12
Quinidine	0.03	7.9	9

* pK_a values in Tables 1 and 2 were obtained from the MedChem computer program (Dr A. Leo, Pomona College Medicinal Chemistry Project, Claremont, CA 91711, U.S.A.).

Table 2. Substrates of cytochrome P4502D and their pK_a values

	pK_a	Ref.
Dextromethorphan (O-demethylation)	8.3	10
Debrisoquine	~12.9	4
Propranolol	9.6	4
Metoprolol	9.6	4
Bufuralol	9.0	4
Timolol	9.0	4
Propafenone	~9.0	4
Encainide	~10.0	4
Flecainide	~11.0	4
Methoxyphenamine	10.4	4
Codeine (O-demethylation)	8.1	4, 10
Ethylmorphine (O-demethylation)	8.1	4
Thebaine (O-demethylation)	8.1	13
Sparteine	12	4
Indoramin	7.7	14
Minaprine	7.4	15
Thioridazine	9.5	16
Bunitrolol	~9.6	17
Imipramine	~9.5	4
Desmethylimipramine	~10.0	4
Lidocaine (3-hydroxylation)	7.9	18
Reduced haloperidol (hydroxyl oxidation)	8.7	19
Brofaromine		20
Mexiletine	8.4	21
Paroxetine		22

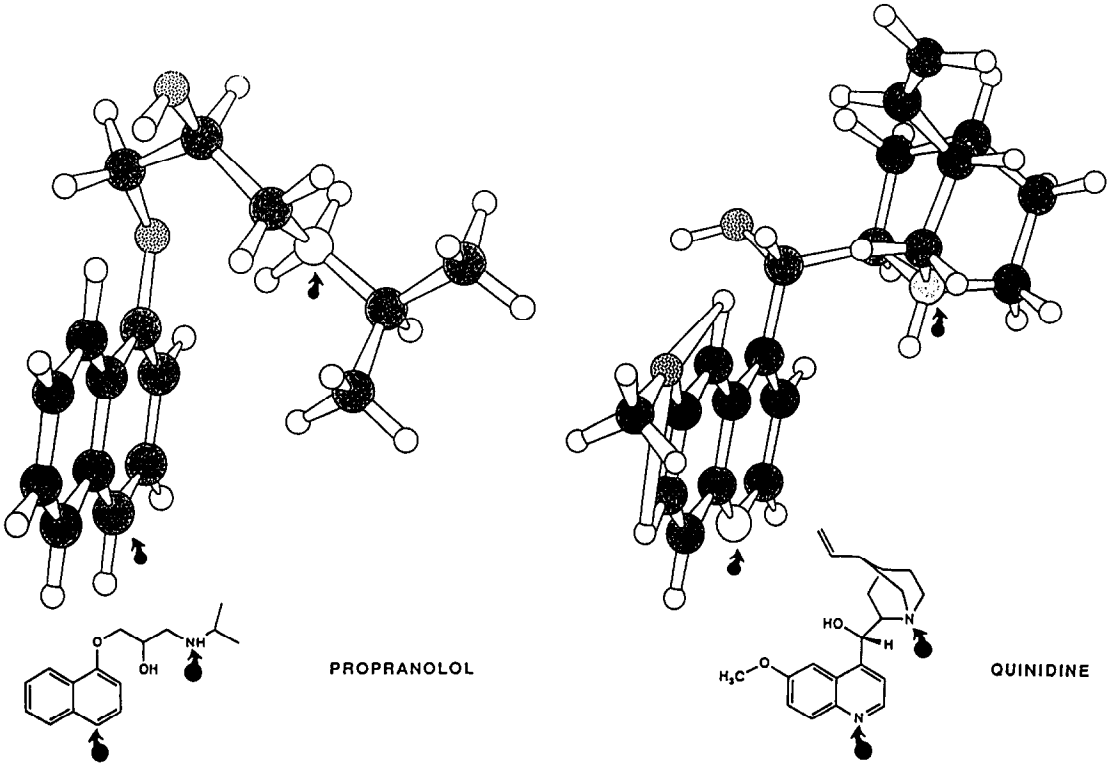

Fig. 1. Structures of propranolol and quinidine. Propranolol is a substrate for P4502D6 and quinidine is a potent, selective reversible inhibitor. The structures are superimposable such that when the ionised nitrogens overlap, the quinoline nitrogen of quinidine occupies the same region of space as the C-4 position of propranolol.

accomplished with K_i values in the nanomolar range (Table 1), indicating the tight fit of quinidine/quinine to the enzyme.

Considerations similar to the above can also be written for the substrates of P4502D enzymes, that of a basic nitrogen mainly ionised at physiological pH, and a hydrophobic region (Table 2). The importance of ionisation is indicated by all the substrates having pK_a values of 7.4 (minaprine) or above. Several substrates have pK_a values of 10 or even greater (e.g. encainide, flecainide and debrisoquine). This dependence on ion pair formation as a major determinant of substrate/enzyme binding, rather than simple hydrophobic forces, explains why debrisoquine, with a log $D_{7.4}$ value of −3, is a substrate. Substrates, however, possess chemical functions capable of P450 oxidation 5–7Å from the basic nitrogen.

The overlap of properties between that of inhibitor and that of substrate is illustrated by a consideration of quinidine and a typical P4502D substrate, propranolol (Fig. 1). Quinidine has a basic nitrogen in the quinucleodine ring (pK_a 7.9) which will be highly ionised at physiological pH. Structural overlay of quinidine and propranolol (in which the basic nitrogen is present in the alkylamine side chain, pK_a 9.6), with the ionisable nitrogens superimposed, indicates that the quinoline nitrogen occupies the same region in space as the C-4 position of propranolol, the position of hydroxylation. It can be assumed, therefore, that either the quinoline nitrogen (pK_a 4.2) is metabolically inert or is involved in a ligand interaction with the heme function of the enzyme. With propranolol the C-4 position is available for hydroxylation.

Regioselectivity of oxidation is consistent for almost all P4502D substrates. Oxidation occurs in the hydrophobic region 5–7Å from the basic nitrogen. The distance of 5–7Å is actually not a critical one. More accurately the fit to the site is governed by the position of the anionic binding site and its distance to the heme–oxygen complex. A template defining the stereochemical requirements of the active site has been proposed [7] using X-ray crystallographic coordinates of the location of the C-5 atom of camphor in relation to the heme in cytochrome $P450_{CAM}$. Known substrates were used to identify allowed conformers. The anionic site was fixed, allowing the basic nitrogen-anion distance to be 2.5–4.5Å. Some clues to the anionic site were also revealed with the suggestion that it is provided by the side chain of Asp 301.

Providing the functionality is in a favourable position (i.e. distanced from the basic nitrogen) for the regioselectivity of the enzyme, P4502D will catalyse a full spectrum of reactions [4, 18] including aromatic hydroxylation (propranolol), aliphatic hydroxylation (metoprolol), N-dealkylation (amiflamine) and even hydroxyl oxidation (reduced haloperidol). Consideration of a single substrate such as mexiletine [21] with multiple metabolites illustrates the effect of binding to the anionic site

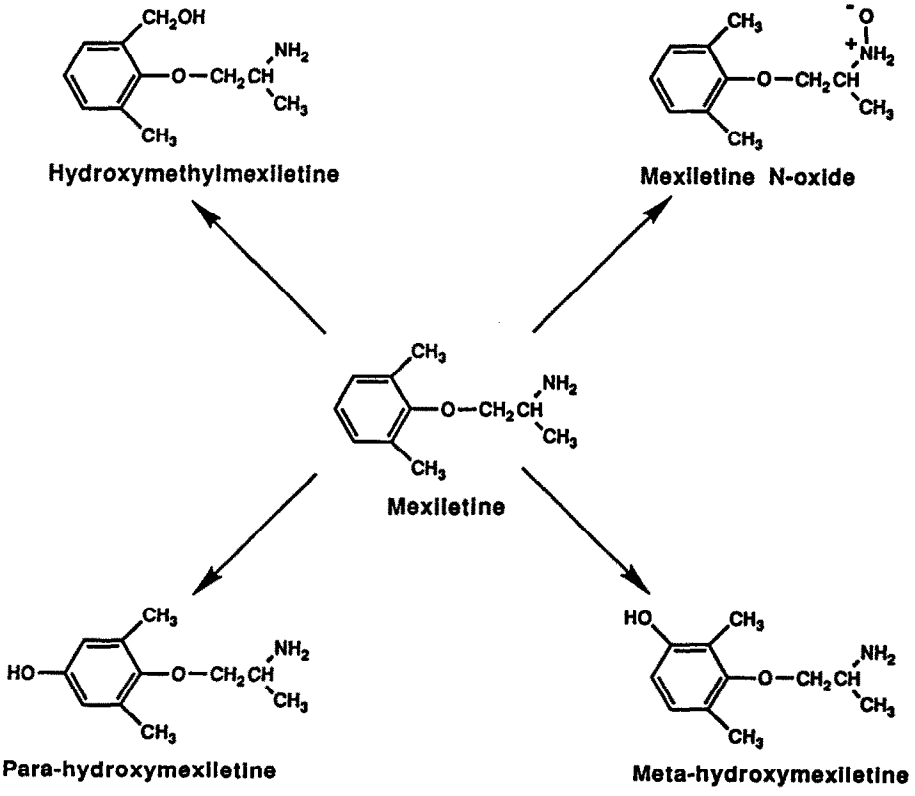

Fig. 2. Metabolic pathway of mexiletine. P4502D catalyses the formation of hydroxymethylmexiletine, *p*-hydroxymexiletine and *m*-hydroxymexiletine, but not mexiletine *N*-oxide.

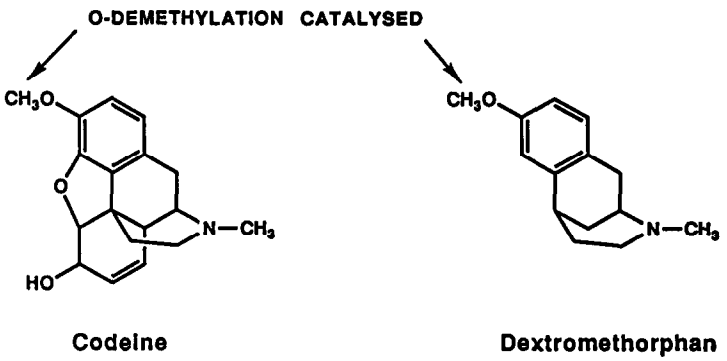

Fig. 3. Structures of codeine and dextromethorphan. P4502D catalyses the O-demethylation of these substrates but not the N-demethylation. The N-demethylation is catalysed by P4503A.

and the resultant regioselectivity of oxidation. Mexiletine is metabolised to hydroxymethylmexiletine, *m*-hydroxymexiletine and *p*-hydroxymexiletine by the P4502D enzyme, the metabolism occurring in the hydrophobic region distanced from the basic nitrogen atom (Fig. 2). A further metabolite *N*-hydroxymexiletine (i.e. oxidation of the basic nitrogen) is formed by a separate (P450) enzyme.

Similar observations on the regioselectivity of P4502D can be drawn from studies on codeine and dextromethorphan [10] (Fig. 3) where the O-demethylation reaction is catalysed and imipramine [11] where the aromatic 2-hydroxylation is catalysed. In these examples, and all others known to date, such reactions as N-dealkylation of basic (pK_a above 7, hence ionised at physiological pH) nitrogens are not catalysed at an appreciable rate by the P4502D enzyme.

Selectivity of P4503A

In contrast to P4502D no such structural overlap is possible for selective inhibitors of P4503A. Those deemed potent (low micromolar K_i values) are in most cases substrates for the enzyme. Moreover, those inhibitors deemed selective such as triacetylolandeomycin require high concentrations (100 μM) and metabolic activation to achieve inhibition. This suggests that compounds bound to the active site of P4503A are not constrained to the same defined substrate structure–activity relationship (SSAR) as with a P450 such as P4502D. Similarly, P4503A shows no apparent selectivity for substrates, and the diversity of structure in its substrate range (e.g. steroids, cyclosporin and lovastatin; Table 3) has been commented on before [4]. All the compounds are lipophilic, again in contrast to P4502D substrates, with log $D_{7.4}$ values ranging from 0.4 (codeine) upwards. With many of the substrates either aliphatic oxidation or N-dealkylation are the major routes catalysed, albeit in widely differing, steric positions in the molecules. In considering the various substrates, closely, the enzyme shows surprising selectivity for chemical substituent. For

Table 3. Substrates of cytochrome P4503A

	Ref.
Nifedipine (aromatisation)	4
Cyclosporin (N-demethylation and methyl hydroxylation)	4
Erythromycin (N-demethylation)	4
Steroids (6β-hydroxylation)	4
Midazolam (methyl hydroxylation)	4
Ethynyl estradiol (aromatic hydroxylation)	4
Dihydroergotamine (hydroxylation of proline ring)	4
Ethylmorphine (N-demethylation)	4
Codeine (N-demethylation)	10
Diazepam (C_3-hydroxylation)	4
Dextromethorphan (N-demethylation)	10
Quinidine (N-oxidation and 3-hydroxylation)	23
Lidocaine (N-deethylation)	4
Diltiazem (N-deethylation)	24
Tamoxifen (N-demethylation)	25
Lovastatin	26
Verapamil (N-demethylation and N-dealkylation)	27
Δ^1-Tetrahydrocannabinol (6β-hydroxylation)	28
Amiodarone (N-deethylation)	29
Cocaine (N-demethylation)	30
FK-506 (O-demethylation)	31
Dapsone (N-oxidation)	32

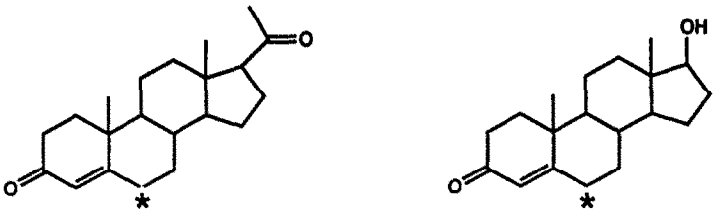

Fig. 4. Structures of progesterone and testosterone indicating the major hydroxylation position by P4503A.

instance, P4503A is the principal enzyme involved in the metabolism of steroids to 6β-hydroxyl products. Consideration of the 6β-position shows it is the only allylic position in steroid molecules such as testosterone and progesterone (Fig. 4).

Aliphatic carbon hydroxylation proceeds by hydrogen atom abstraction, rather than electron transfer, followed by radical recombination [33]. Energy requirements for breaking the carbon hydrogen bond are approximately 420 kJ/mol for a simple primary alkyl carbon in contrast to 340–360 kJ/mol for the equivalent allylic or benzylic position. In addition to steroids, allylic positions are the major site of oxidation catalysed by P4503A in such structurally unrelated compounds as cyclosporin, quinidine, Δ^1-tetrahydrocannabinol and lovastatin (Table 3, Fig. 5).

Catalysis by P450 can proceed either by abstracting hydrogen atoms as in aliphatic carbon hydroxylation or electrons where the circumstances are more favourable. N-Dealkylation of aliphatic tertiary amines proceeds by an initial one-electron oxidation of nitrogen [1]. This step can be considered as chemically facile due to the low $E_{1/2}$ for one-electron oxidation of these functions. Many substrates of cytochrome P4503A are metabolised by N-dealkylation reactions including erythromycin, ethylmorphine, lidocaine, diltiazem, cocaine, amiodarone, verapamil and tamoxifen (Table 3, Fig. 6). Again the diversity of structure is remarkable, the only common feature being the reaction catalysed.

This analysis suggests that, unlike the highly constrained binding of substrate to the cytochrome P4502D enzyme, substrates of cytochrome P4503A have more freedom to find "catalytically competent" orientations in the active site. This loss of regioselectivity parallels the change in substrate binding mechanism from the ion-pair formed with the enzyme for P4502D substrates to the reliance on solely hydrophobic interactions. Such mobility differs from the conventional view of substrate binding for an enzyme. Recent studies on cytochrome $P450_{CAM}$ by Raag and Poulos [34] however, support this idea. The natural substrate camphor (Fig. 7) hydrogen bonds to Tyr 96 and is metabolised to a single hydroxylated product. Camphane and thiocamphor (Fig. 7) do not hydrogen bond to the enzyme and are hydroxylated in multiple positions albeit at a slower rate. The finding is interpreted as the lack of substrate–enzyme hydrogen bond allowing the substrate greater mobility in the active site. This mobility is further emphasised by the X-ray crystal structure of the $P450_{CAM}$/thiocamphor complex, demonstrating that thiocamphor is bound in a position unrelated to its site of metabolism. This orientation, with the site of hydroxylation, carbons 5 and 6, near the Tyr 96 rather than the heme iron, indicates that the substrate spends significant time in non-productive positions. Since cytochrome P450s are, in general, slow enzymes, the kinetics of such flexible binding is reflected in the overall catalytic rate. That P450 will attack the chemically most favourable position, given such substrate mobility, is illustrated also by $P450_{CAM}$ [34]. Adamantane, (Fig. 7), like camphane and thiocamphor, cannot hydrogen bond to the enzyme. However, it forms a single hydroxylated product at the tertiary carbon center. Energy requirements for breaking the tertiary carbon hydrogen bond are 10–15 kJ/mol lower than that required for a secondary carbon. Evidence for flexibility in binding position with P4503A is provided by compounds which form multiple metabolites all catalysed by the enzyme. For instance, P4503A is responsible for the metabolism of cyclosporin to its three primary metabolites which are formed at regions up to five peptide units apart in this cyclic decapeptide [35]. This lack of regioselectivity is in marked contrast to that described above for the P4502D enzyme. Clearly, chemical lability by itself does not explain the selectivity of the P4503A enzyme. The selectivity must be due in part to other structural features which limit the accessibility of chemically "vulnerable" positions. For instance, lovastatin has two allylic positions. It is interesting that with a compound like cyclosporin, X-ray crystal analysis suggests a fairly globular rigid compact structure with the C_8 alkylene side chain, on which hydroxylation occurs, folded into the ply of the β-pleated sheet [36]. However, in apolar solutions, perhaps closer to the environment of the active site of P4503A than the environment to produce the crystal, NMR studies show this side chain to stand out, proboscis like, making the allylic position highly accessible in this complex molecule.

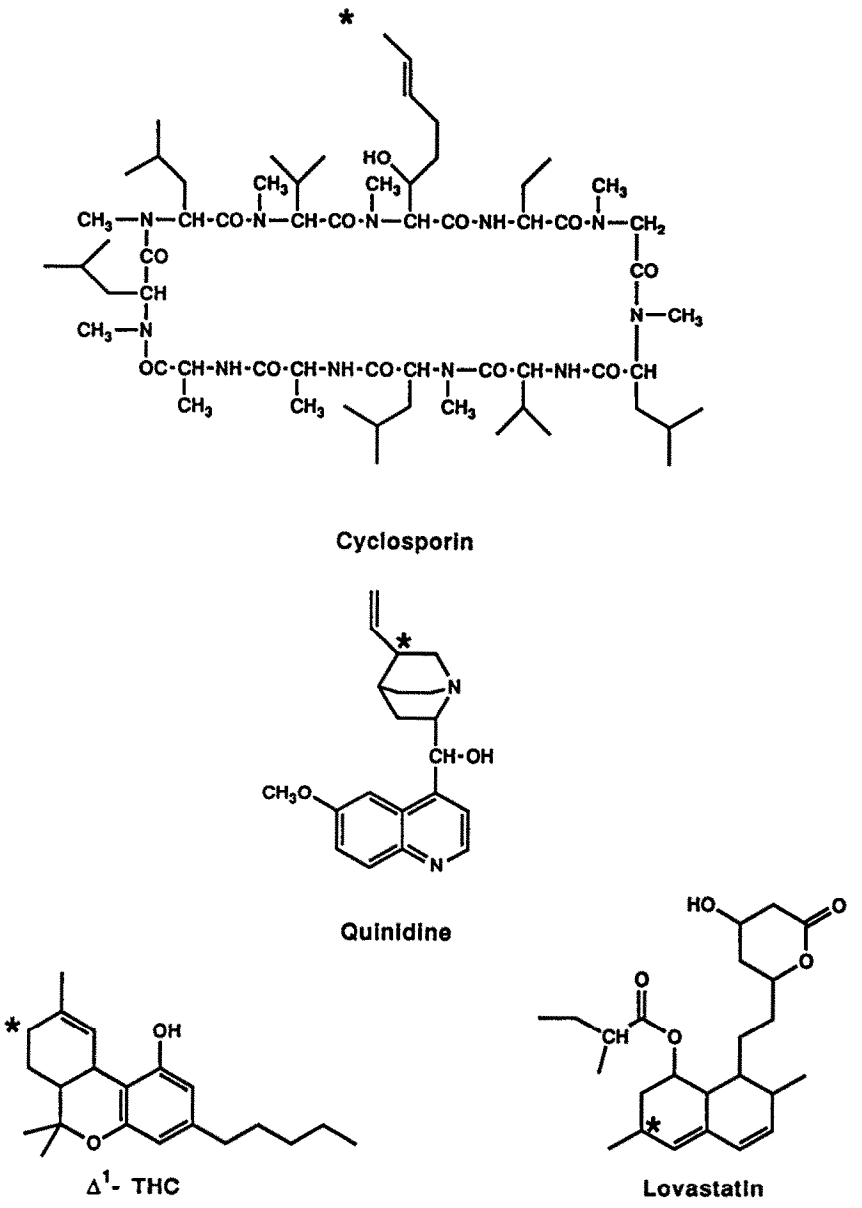

Fig. 5. Structures of cyclosporin, quinidine, Δ^1-tetrahydrocannabinol (Δ^1-THC) and lovastatin indicating the major hydroxylation position by P4503A.

Site of metabolic attack and relationship to rate

The initial step in the metabolic reaction may not be rate-limiting. For instance, hydrogen atom abstraction from a benzylic position rather than an aromatic ring is an easier first step based on energy requirements for hydrogen abstraction (360 compared with 464 kJ/mol). As such, the regioselectivity of hydroxylation will be defined. Recent experiments by Hanzlik and Ling [37] demonstrate, however, that as deuterium substitution with toluene shifts the hydroxylation from benzylic to aromatic ring so the reaction proceeds with a faster rate. This indicates that overall rate is a complex product of subsequent steps. Thus, calculation of the ease of the initial hydrogen or electron abstraction does not allow the reaction rate to be calculated for a chemical; however, it may be a strong indicator of likely metabolism sites for enzymes such as P4503A.

Summary

This brief review attempts to define the SSAR of two families of cytochrome P450. With P4502D catalytic competence is achieved by tight ionic binding which gives the enzyme high regioselectivity. In contrast P4503A achieves catalytic competence

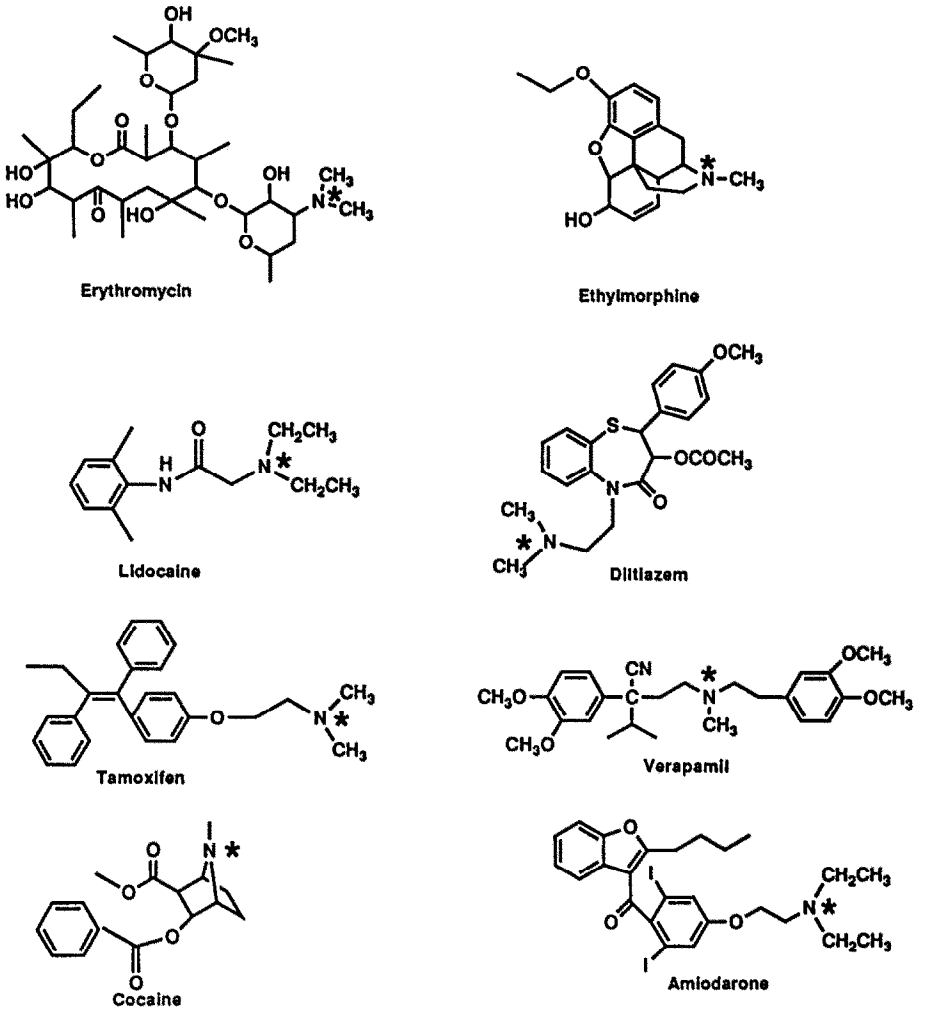

Fig. 6. Structures of erythromycin, ethylmorphine, lidocaine, diltiazem, tamoxifen, verapamil, cocaine and amiodarone indicating the position of N-dealkylation by P4503A.

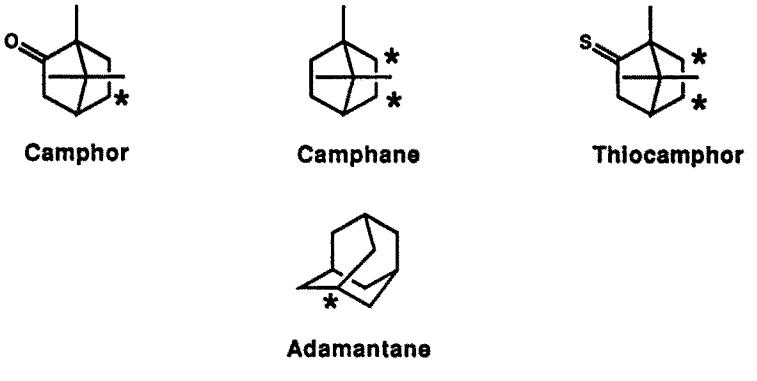

Fig. 7. Structures of camphor, camphane, thiocamphor and adamantane indicating the position of hydroxylation by $P450_{CAM}$.

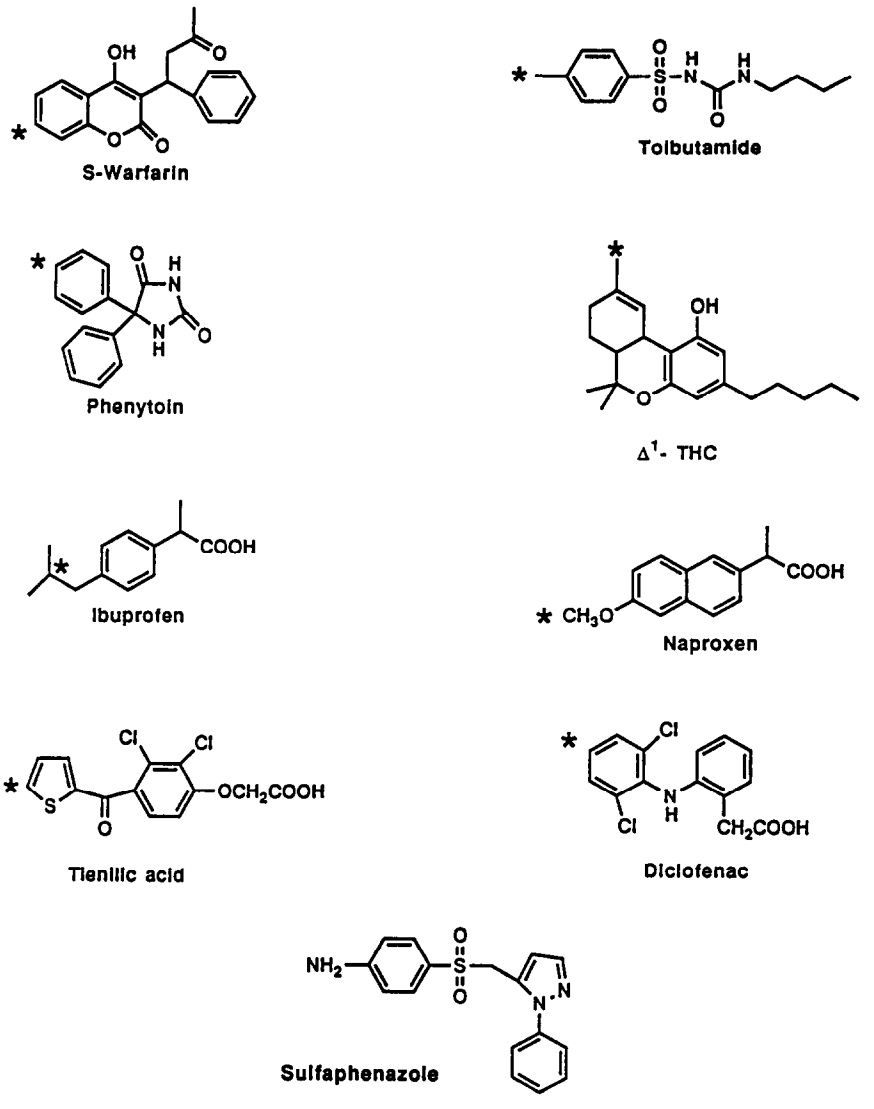

Fig. 8. Structures of phenytoin, tolbutamide, tienilic acid, naproxen, ibuprofen, diclofenac, *S*-warfarin and Δ^1-tetrahydrocannabinol indicating the position of metabolism (hydroxylation) by P4502C9, and the selective inhibitor sulphaphenazole.

by a flexible binding site relying on hydrophobic forces that allow chemically vulnerable sites to be the principal sites of metabolism. In general, the different binding mechanism should be reflected in the enzyme, such that substrates of P4502D should have lower K_m values than substrates of P4503A. Thus, routes of metabolism catalysed by P4502D may be saturated at substrate concentrations lower than routes catalysed by P4503A.

The apparent differences between P4502D and P4503A in terms of substrate specificity bring into question what relationships govern other families of cytochrome P450. Our analysis of data suggests that the other principal form involved, generally, in the metabolism of pharmaceuticals in humans is P4502C9 (possibly 2C8 and 2C10). The enzyme is responsible for the metabolism of phenytoin, tolbutamide, tienilic acid [4], naproxen, ibuprofen, diclofenac [38], the 7-hydroxylation of *S*-warfarin [39] and the 7-hydroxylation of Δ^1-tetrahydrocannabinol [40]. These compounds all have areas of strong hydrogen bond [4] forming potential (Fig. 8), all distanced 5–10Å from the site of metabolism. Moreover the carboxylic acid function of naproxen, ibuprofen and diclofenac (pK_a 4.5) and the sulfonylurea of tolbutamide (pK_a 5.4) render the compounds ionized at physiological pH. The ionised group is positioned 7–11Å from the site of metabolism. It is likely, therefore, that hydrogen bonding and possibly ion-pair interactions play a major role in determining the SSAR of the P4502C isoenzymes. These interactions would suggest that the P4502C enzymes are analogous

to P4502D rather than P4503A. In this regard it is noteworthy that P4502C9 is selectively and potently inhibited by sulfaphenazole (IC_{50} of 0.6 μM), a compound that is structurally related (Fig. 8) to the substrates in terms of potential hydrogen bonding regions [4, 41].

Simplistically we suggest that the SSAR of the various P450 enzymes ranges from the highly selective enzymes dealing with endogenous substrates, through the enzymes metabolising exogenous substrates with narrow substrate structure requirements such as P4502D to P4503A with its broad substrate structure range. It would seem logical that animals and humans would evolve such combinations of isoenzymes to deal with the vast array of exogenous xenobiotics.

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