Oxidation of Dihydropyridine Calcium Channel Blockers and Analogues by Human Liver Cytochrome P-450 IIIA4

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A series of 21 different 4-substituted 2,6-dimethyl-3-(alkoxycarbonyl)-1,4-dihydropyridines was considered with regard to oxidation to pyridine derivatives by human liver microsomal cytochrome P-450 (P-450). Antibodies raised against P-450 IIIA4 inhibited the microsomal oxidation of nifedipine and felodipine to the same extent, as did cimetidine and the mechanism-based inactivator gestodene. Gestodene was $\sim 10^3$ times more effective an inhibitor than cimetidine, on a molar basis. When rates of oxidation of the 1,4-dihydropyridines were compared to each other in different human liver microsomal preparations, all were highly correlated with each other with the exceptions of a derivative devoid of a substituent at the 4-position and an N^1-CH_3 derivative. A P-450 IIIA4 cDNA clone was expressed in yeast and the partially purified protein was used in reconstituted systems containing NADPH-cytochrome P-450 reductase and cytochrome b_5. This system catalyzed the oxidation of all of the 1,4-dihydropyridines except the two for which poor correlation was seen in the liver microsomes. Principal component analysis supported the view that most of these reactions were catalyzed by the same enzyme in the yeast P-450 IIIA4 preparation and in the different human liver microsomal preparations, or by a closely related enzyme showing nearly identical properties of catalytic specificity and regulation. The results indicate that the enzyme P-450 IIIA4 is probably the major human catalyst involved in the formal dehydrogenation of most but not all 1,4-dihydropyridine drugs.

Nifedipine (1a) is a prototype for the dihydropyridine calcium channel blockers, and numerous useful derivatives exist. Usually only the parent compound is active—most of the metabolic steps involve reactions catalyzed by cytochrome P-450 (P-450) enzymes (Scheme I). P-450 enzymes have been shown to catalyze pyridine formation,[1-4] methyl hydroxylation (often accompanied by lactone formation involving anchimeric assistance),[5] and various modes of side-chain oxidation, including the oxidative cleavage of R_2 (Scheme I), a reaction that has been demonstrated both in vitro[5] and in vivo.[6] Of these reactions, the most prominent is usually pyridine formation. In the case of 1a essentially all of the metabolism may be attributed to a pathway involving initial pyridine formation and then subsequent oxidations.[7] This paradigm also applies to many of the other simple dihydropyridines. With more complex substitution patterns such as those seen in nimodipine (6a) and nicardipine (7a), extensive oxidation occurs at sites other than the ring and some of the major products still retain the dihydropyridine ring.[8,9] In many instances the clearance of dihydropyridine drugs shows a first-pass effect. The area under the plasma concentration vs time curve varies considerably among individuals and appears to be distributed with a continuous modality.[10,11]

Human P-450 IIIA4 has been characterized as the major nifedipine oxidase.[2,12] Immunoinhibition experiments with human liver microsomal preparations indicated that other dihydropyridines are probably oxidized by the same or related enzymes.[3] However, recent studies in several laboratories have suggested that as many as four closely related genes are found in the human P-450 CYP3A family,[13] and another one of these, P-450 IIIA5, has been shown to be a catalyst of 1a oxidation.[18,21]

A question of interest is whether the single enzyme P-450 IIIA4 can catalyze the oxidation of many or all of the Hantzsch dihydropyridine esters. This question has considerable significance in that the level of the enzyme appears to vary widely among individuals[2,10,15,25] and is inducible by barbiturates and other compounds.[15,26] In-

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(13) Several related sequences have been reported to be coded for by genes in the so-called human P-450 IIIA family,[14] including P-450 IIIA3 (HLp),[15,16] P-450 IIIA4 (P-450$_{NF}$, P-450 hPCN1),[2,12,17,18] P-450 IIIA5 (P-450hPCN3),[18-21] and P-450 IIIA7 (P-450 HLFa).[22,23] The anti-P-450 IIIA preparation used in this work would undoubtedly react with all of these proteins when they are present in mixtures and is therefore termed anti-P-450 IIIA, although it was raised against P-450 IIIA4. For further discussion of the regulation of catalytic activities of these gene products, see the references cited.[12,18-21,24]
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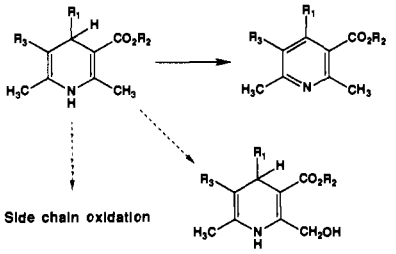
Scheme I. Generalized Scheme for Metabolism of Dihydropyridine Drugs[a]



[a] The predominant initial pathway of pyridine formation is indicated with a solid arrow.

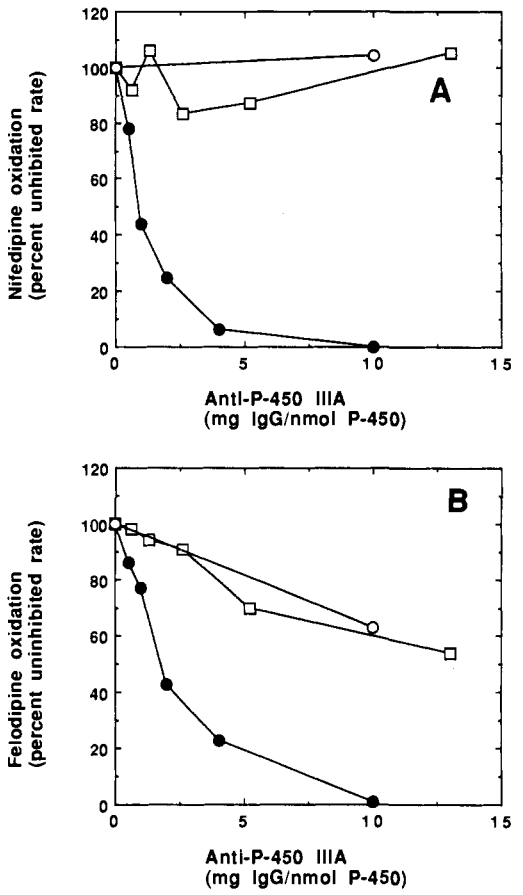


Figure 1. Inhibition of oxidation of nifedipine and felodipine in human liver microsomes by anti-P-450 IIIA. Results are shown for the effects of a preimmune antibody (○), anti-P-450$_{MP-1}$ (□), and anti-P-450 IIIA (●): A, nifedipine oxidation, B, felodipine oxidation. The microsomal preparation was HL 110 and the uninhibited rates were 5.4 and 1.0 nmol of product formed/min per mg of protein for nifedipine and felodipine, respectively.

deed, the inducibility of the enzyme can play a role in some undesired drug interactions.[25] The catalytic specificity of

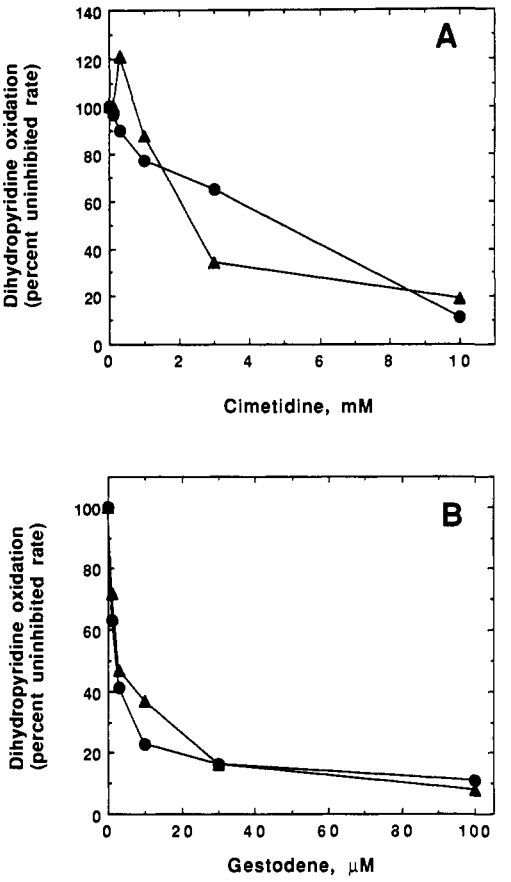


Figure 2. Inhibition of oxidation of nifedipine and felodipine in human liver microsomes by gestodene and cimetidine. In the case of gestodene, microsomes were first incubated with gestodene and NADPH as described in the Experiment Section. Results (means of duplicate experiments) are shown for nifedipine oxidation (●) and felodipine oxidation (▲): A, cimetidine, B, gestodene. In part B, the microsomal samples were preincubated with NADPH and gestodene for 20 min at 37 °C prior to the addition of dihydropyridine. The microsomal sample was HL 110 and the uninhibited rates were 5.4 and 1.0 nmol of product formed/min per mg of protein for nifedipine and felodipine, respectively.

P-450 IIIA4 has been reconsidered with a set of previously used dihyropyridines[3] and new ones using several approaches, including comparison of activities among different liver samples and a protein synthesized in yeast from a defined cDNA clone, with principal component (PC) analysis of the activities.

Results and Discussion

Inhibition of Nifedipine (1a) and Felodipine (12a) Oxidation by Antibodies and Inhibitors. The selective inhibition of oxidation of many dihydropyridines by antibodies specific for P-450 IIIA enzymes has already been demonstrated,[3] suggesting that the oxidation of many dihydropyridines to pyridines is catalyzed primarily by P-450 IIIA4 or a related protein. Specifically, such studies have been reported for compounds **1a**–**9a**, **11a**, **13a**, **14a**, and **16a**[3] (Table I). In view of interests in the metabolism of felodipine (**12a**), its oxidation was considered in further detail. Anti-P-450 IIIA selectively inhibited the human liver microsomal oxidation of both **1a** and **12a**, and neither

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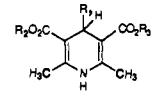
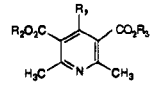
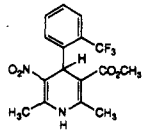
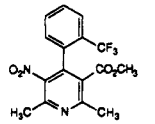
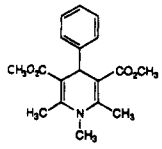
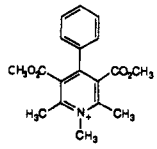
Table I. Structures of Dihydropyridines and HPLC Conditions

no.	R_1	R_2	R_3	HPLC ref	trivial name
					
	a	**b**			
1	2-NO_2-C_6H_4	CH_3	CH_3	2	nifedipine
2	3-NO_2-C_6H_4	CH_3	C_2H_5	3	nitrendipine
3	3-NO_2-4-Cl-C_6H_3	CH_3	CH_3	3	
4	3-NO_2-C_6H_4	$CH_2CH_2CH(CH_3)_2$	$CH_2CH_2OCH(CH_3)_2$	3	niludipine
5	2-NO_2-C_6H_4	$CH_2CH(CH_3)_2$	CH_3	3	nisoldipine
6	3-NO_2-C_6H_4	$CH(CH_3)_2$	$CH_2CH_2OCH_3$	3	nimodipine
7	3-NO_2-C_6H_4	CH_3	$CH_2CH_2N(CH_3)CH_2C_6H_5$	27	nicardipine
8	C_6H_5	CH_3	CH_3	3	
9	C_6H_5	C_2H_5	C_2H_5	3	
10	1-naphthalenyl	CH_3	CH_3	4	
11	3-Cl-C_6H_4	CH_3	CH_3	3	
12	2,3-Cl_2-C_6H_3	CH_3	C_2H_5	a	felodipine
13	2,3,5,6-F_4-C_6H	CH_3	CH_3	3	
14	4-CF_3-C_6H_4	CH_3	CH_3	3	
15	2-OCH_3-C_6H_4	C_2H_5	C_2H_5	b	
16	3-CN-C_6H_4	CH_3	CH_3	3	
17	H	CH_3	CH_3	4	
18	C_2H_5	C_2H_5	C_2H_5	c	
					
	a	**b**			
	19 (+)			4	R 4407 (+K8644)
	20 (-)			4	R 5417 (-K8644)
					
	'21a	**21b**		4	

[a] Octadecasilyl (C18) column, Zorbax, 4.6 × 150 mm, 5 μm, CH_3OH/H_2O 73:27 (v/v), flow rate 2.5 mL min^{-1}; t_R **12b** 3.8 min, t_R **12a** 5.0 min. [b] Octadecasilyl (C18) column, Zorbax, 4.6 × 150 mm, 5 μm, 5 mM potassium phosphate buffer (pH 2.2) containing 0.1% $(C_2H_5)_4N^+Cl^-$ (w/v) and 45% CH_3CN, flow 3.0 mL min^{-1}; t_R **15b** 5.6 min, t_R **15a** 6.6 min. [c] Octadecasilyl (C18) column, Zorbax, 4.6 × 150 mm, 5 μm, 6 mM potassium phosphate buffer (pH 2.2) containing 0.12% $(C_2H_5)_4N^+Cl^-$ (w/v) and 36% CH_3CN at a flow rate of 3.0 mL min^{-1}; t_R **17b** (4-H) 8.5 min and t_R **18b** (4-C_2H_5) 9.3 min—the CH_3CN concentration was raised to 56% after 10 min to elute residual 18a.

a preimmune antibody nor anti-P-450_{MP-1} [an antibody raised against an (*S*)-mephenytoin 4′-hydroxylase][28] specifically blocked either reaction (Figure 1).

Nifedipine oxidation can be inhibited by cimetidine both in vitro[29] and in vivo,[30] and P-450 IIIA4 appears to be one of the cytochrome P-450 enzymes most sensitive to this compound.[29] Oxidations of **1a** and **12a** were inhibited by cimetidine in a similar manner (Figure 2A). Recently the progestogen gestodene has been shown to be an effective mechanism-based inhibitor of the in vitro oxidation of nifedipine and 17α-ethynylestradiol[31]— in vivo studies also indicate inhibition of 17α-ethylnylestradiol 2-hydroxylation.[32,33] After preinbuation with microsomes in the presence of NADPH, gestodene was a potent inactivator of the oxidation of both **1a** and **12a** (Figure 2B).

Principal Component Analysis of Oxidation Activities in Different Microsomal Preparations. One approach to determining if two reactions are catalyzed by the same enzyme involves comparison of the rates of the two reactions in a series of tissue samples varying in activity.[34-36] A series of liver samples prepared from different individuals was used for such a purpose—such an analysis works best when a reasonable variation exists in the range of activities.

Overall, a total of 24 reaction rate constants for the oxidation of the 21 different dihydropyridine derivatives were determined in 10 different human liver microsomal preparations and in a yeast recombinant P-450 IIIA4 preparation. A P-450 IIIA4 cDNA was expressed in a yeast vector system and the protein was partially purified in two steps (>50% homogeneous as judged by band intensity following electrophoresis and staining).[37,38] One of the

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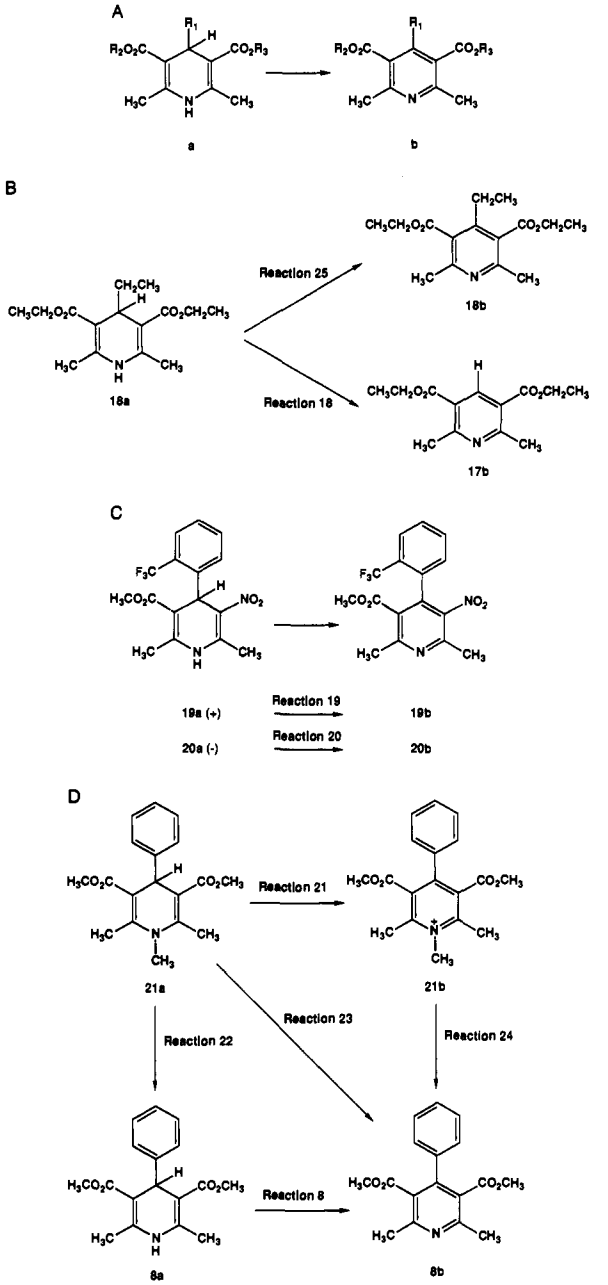
Scheme II. Reactions under Consideration. (A) Reactions (**a** → **b**) with Compounds **1–17**, (B) Reactions with Compound **18**, (C) Reactions with Compounds **19** and **20**, (D) Reactions with compound **21**



difficulties encountered in working with this particular purified cytochrome P-450, whether purified from liver or yeast, is that the rate of reduction of the ferric enzyme is slow and, therefore, apparent turnover numbers for overall catalytic function are also somewhat lower than expected.[37] The rate constants for product formation (nanomole of product formed/minute per milligram of protein or nanomole of product formed minute per nanomole of P-450 for the yeast system) were determined as described and are listed in Table II.

The reactions of interest are shown in Scheme II. For compounds **1a–17a**, **19a**, and **20a** only the rate of the oxidation of the dihydropyridine to the corresponding pyridine was measured; it is a major reaction detected in each case. Relatively small amounts of other polar and un-

Table II. Measured Reaction Rate Constants for Reactions 1–25 in 10 Human Liver Preparations[a] and the Yeast P-450 IIIA4 Preparation[b]

enzyme sample	rate constant																								
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
HL 100	0.4	0.3	1.2	0.03	1.0	0.1	0.01	0.1	0.2	0.12	0.08	0.10	0.07	0.23	0.13	0.8	3.3	0.04	0.16	0.04	<0.02	2.8	7.6	0.03	0.03
HL 105	2.2	3.5	8.5	0.26	5.4	0.5	0.09	2.8	2.0	0.63	2.38	0.58	0.43	1.92	1.73	5.0	3.4	0.04	1.06	0.52	0.31	5.8	8.3	0.03	0.16
HL 106	0.3	0.4	1.2	0.01	0.9	0.1	0.01	0.1	0.1	0.09	0.08	0.10	0.38	0.04	0.27	0.7	4.0	0.01	0.11	0.04	<0.02	<0.1	1.0	0.04	0.03
HL 107	2.1	4.0	6.8	0.47	5.4	0.3	0.12	2.9	2.2	0.54	2.32	0.51	0.82	1.19	0.51	4.0	1.8	0.02	1.22	0.58	0.06	1.0	2.2	0.02	0.10
HL 108	0.4	0.8	1.2	0.02	1.6	0.1	0.10	0.1	0.1	0.12	0.08	0.14	0.08	0.17	1.28	0.8	3.9	0.01	0.18	0.06	<0.02	<0.1	0.9	0.03	0.02
HL 110	5.9	6.2	14.3	0.76	7.7	2.0	0.21	6.4	4.6	1.55	2.53	1.07	1.33	1.69	3.43	11.1	2.3	0.05	2.67	1.90	0.87	10.8	6.7	0.05	0.16
HL 112	0.8	1.6	3.9	0.06	3.2	0.1	0.07	0.2	1.4	<0.05	1.35	0.28	0.28	0.46	0.71	2.3	4.5	0.05	0.50	0.19	<0.02	2.2	5.8	0.03	0.07
HL 115	3.3	6.6	7.4	1.01	7.4	0.6	0.19	3.6	1.8	2.10	2.54	0.83	0.80	1.38	3.00	6.2	4.4	0.06	2.27	0.64	0.06	5.3	4.9	0.03	0.19
HL 118	0.3	0.6	0.9	0.02	0.9	0.1	0.01	0.1	0.1	0.11	0.08	0.09	0.32	0.28	0.22	0.7	0.5	0.01	0.06	0.05	<0.02	<0.1	0.9	0.03	0.03
HL 129	0.6	0.6	1.2	0.03	1.5	0.1	0.01	0.1	0.2	0.65	0.08	0.15	0.18	0.27	0.36	0.9	2.1	0.01	0.23	0.10	<0.02	0.3	1.1	0.03	0.04
yeast P-450 IIIA4	1.4	2.6	2.1	0.6	2.1	0.6	0.4	0.4	0.7	0.5	1.5	0.8	1.2	1.1	1.3	1.9	<0.05	0.2	0.4	0.4	<0.05	<0.05	<0.05	<0.05	0.4

[a] In nmol of product formed/min per mg of protein. [b] In nmol of product formed/min per nmol of P-450.

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identified products, as judged by A_{254} measurement, were observed in the cases of **2a**, **7a**, **8a**, **15a**, and **16a**—with **3a**, **4a**, **9a**, **12a**, and **14a** the *apparent* amounts of these products were approximately half those of the pyridines formed, even with the recombinant yeast P-450 IIIA4. These are apparently not products arising from ester cleavage of the pyridines, at least in the cases where the monoacids were available as standards. For compound **18a** ring oxidation gives two different pyridines, **17b** (4-H) and **18b** (4-ethyl), and both rate constants were measured. For compound **21a** four reaction rate constants for the formation of the three products (**21b**, **8a**, and **8b**) were measured (including reaction 8). The rate constants for reaction 21 are excluded from all calculations since 6 out of 11 reaction rate constants are missing, i.e., the formation of compound **21b** was less than the limit of detection under the conditions used.

Similar compounds are likely to undergo the same reactions; i.e., be transformed by the same enzyme to the same kind of products. Therefore, a subset of the structurally most closely related (most similar) compounds in Table I is selected as a reference set of compounds to which other compounds are compared. These are all 4-phenyl-1,4-dihydropyridine-3,5-dicarboxylates without additional functional groups in the ester groups in the 3- and 5-positions. The reference set consists of compounds **1a–6a** and **8a–16a**. They are all metabolized by a single uniform type reaction, i.e., the oxidation by cytochrome P-450 to the corresponding 4-phenylpyridines (see reactions 1–6, 8–16, 19, and 20, respectively, in Scheme I) (the formation of other products, even by the same enzyme, is considered a different set and does not interfere with the analysis). The reaction rate constants for these 17 reactions then constitute a reference set of reactions to which other reaction rate constants are compared. It is assumed *a priori* that the 10 liver preparations all behave in a similar way; i.e., that they oxidize the reference set of compounds in the same way and that there is no difference in the mechanism by which they operate. Differences in reaction rate constants among the various liver samples merely reflect different biological entities. However, the reaction rate constants should have the same order in all liver samples, if a single species is catalyzing the reaction in all cases. Thus the 15 reaction rate cosntants for the reference set of reactions measured in the 10 liver preparations form a reference class to which other objects, i.e. liver preparations or the yeast P-450 IIIA4, can be compared.

PC analysis[39] with the reference set of livers as objects and the reference set of reaction rate constants as columns gives one highly significant component according to cross validation that explains 89% of the sum of squares. The **P** loadings for all the 15 reaction rate constants are all almost the same (0.23–0.28). The residual standard deviation for the reference set is 0.37. The distance for yeast P-450 IIIA4 to the reference class is 0.53, which is only 1.4 standard deviations. Thus it is well-justified to conclude that the yeast preparation behaves as the reference set of liver samples with respect to the reference class. However, for each single reaction rate constant the calculated value (the value predicted from the reference set) deviates more from the measured values than for members of the reference set. The high significance of the PC analysis and the similar **P** loadings for all the 15 reaction rate constants

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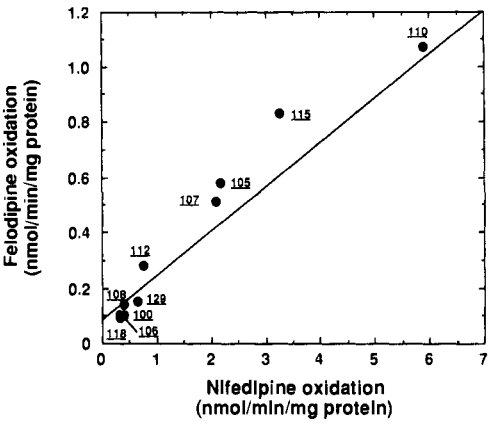

Figure 3. Correlation of rates of oxidation of nifedipine and felodipine in different human liver microsomal samples. The correlation coefficient (r) was 0.97 for the liver samples. The rates measured with each sample are designated with each liver sample number.

Table III. Calculated Distance from the Reference Class of Reactions to the Reactions 7, 17, 18, 19, 20, 22, 23, 24 and 25, Measured Reaction Rate Constants, and Calculated Values for the Reaction Rate Constants in the Yeast Preparation

reaction	calcd distance	measured rate constant	calcd rate constant
7	0.19	0.37	0.64
17	3.9	<0.05	2.9
18	0.31	0.2	0.59
19	0.26	0.4	0.88
20	0.18	0.4	0.73
22	2.5	<0.05	1.9
23	5.7	<0.05	3.0
24	0.34	<0.05	0.61
25	0.19	0.4	0.65

indicate that there is a high degree of correlation between the reaction rates in the various preparations. This point is illustrated in Figure 3, where the reaction rate constants for the oxidation of felodipine are plotted vs those of nifedipine. All of the reactions examined showed generally high correlations among their rates when considered in terms of simple linear regression (i.e., $r \geq 0.7$ and $p < 0.05$) except 17, 21, 22, and 23 (although reaction 23, a combination of a demethylation and dehydrogenation, showed correlation with several of the other reactions).

Generally, it can be concluded that the protein P-450 IIIA4 made by the yeast behaves in a similar way to the enzyme that performs the oxidation of the dihydropyridines to the pyridines in the liver samples. Moreover, other systems present in the liver preparations do not seem to contribute significantly to the oxidation, or if they do, they behave like P-450 IIIA4.

Reaction rate constants for the reactions 7, 17, 18–20, 22–25 were excluded from the reference set of reactions since they describe the transformation of compounds that were considered structurally different from the reference set of compounds. In order to investigate the relationship between these nine reactions and the reference set of reactions, the original data matrix is transposed so that the reactions become the objects and the livers and the yeast preparation become the columns. The yeast is included in the reference set according to the previous PC analysis. PC analysis of the new data matrix gives one highly significant component according to cross validation that explains 85% of the sum of squares. The **P** loadings for all the 10 liver preparations and the yeast preparation are almost all the same (0.27–0.31). The residual standard deviation for the reference set is 0.42. The distances for those reaction rate constants outside the reference set are

calculated and shown in Table III. These distances should be compared to 0.41. Thus it can be assumed that the reactions 7, 18, 19, 20, and 25 behave, overall, as the reference set of reactions. This might imply that these reactions are performed either by P-450 IIIA4 itself or other enzyme systems in the liver preparation that behave similarly to it.

Reactions 22 and 24 are N-demethylations (see Scheme I) and reaction 23 also involves such a step. Thus they are somewhat mechanistically different from the oxidation of a dihydropyridine ring to an aromatic ring, even if an aminium radical is a common intermediate, as proposed for reaction 22.[4] Reactions 22 and 23 are well outside the reference class according to the PC analysis while 24 should belong to it. From Table III it is seen that these three reactions are predicted to be relatively fast in the yeast P-450 IIIA4 preparation, but they do not occur at a measurable rate. This indicates that these three reactions are apparently performed in the livers by some other system than P-450 IIIA4.

Reaction 17 involves the oxidation of dihydropyridines without a substituent in the 4-position. From Table III it is seen that reaction 17 was not catalyzed by the yeast enzyme. Thus, another enzyme would appear to catalyze the reaction (the reaction is NADPH-dependent but it is unknown if this is due to a P-450 or not).

Conclusions

4-Alkyldihydropyridines are mechanism-based P-450 inactivators, with the destruction of heme related to the formation of alkyl radicals.[2,40,41] The previous work with several 4-alkyl inactivators and the studies with 4-aryl derivatives done here would argue that most 4-alkyldihydropyridines should be mechanism-based inactivators of P-450 IIIA4. It should be pointed out that even the recombinant yeast P-450 IIIA4 (apparently devoid of other P-450s) produced some more polar products as well as the pyridines (reactions 2–4, 7–9, 12–16). The identities of these products have not been ascertained, but they do not appear to be carboxylic acids.[5] At this time it must be concluded that P-450 IIIA4 is able to oxidize other portions of some of these molecules as well as the dihydropyridine ring. The conclusion is reached that most 4-substituted dihydropyridines are substrates for ring oxidation by P-450 IIIA4, and compelling evidence for an oxidation mechanism involving odd-electron processes (electron/proton/electron transfer) has been presented elsewhere.[3,4,40-44]

Recently this mechanism of dihydropyridine oxidation has been questioned by another group,[45] who suggest that the release of an ethyl radical (from 18a, reaction 18) is due to adventitious iron present under in vitro assay conditions. While it is certainly possible to oxidize dihydropyridines by various types of nonenzymatic radical systems, the results presented in Table II indicate that reactions 18 and 25 appear to be P-450-catalyzed, as judged by the correlation and demonstration of the reaction with the recombinant enzyme. Sugiyama et al.[46] have found that ratios of reactions 18 and 25 (with 18a as substrate) vary among different types of rat liver microsomal preparations (and presumably different P-450s). Further, we found no effect of adding EDTA (2 mM) or catalase (1600 units mL^{-1}) or treating buffer with Chelex 100 on the rate of oxidation of 1a (resulls not presented). Thus, although nonenzymatic dihydropyridine oxidation can occur, we do not feel that it is a significant process and that P-450 catalysis is rather obligatory.

Most dihydropyridine calcium blockers appear to be oxidized to pyridines (and additional products in some cases) by P-450 IIIA4, as judged by these studies involving human liver microsomes and recombinant P-450 IIIA4. Reactions 17 and 21–24 do not really fit with all of the others. Other studies indicate that P-450 IIIA4 is a major P-450 enzyme in human liver[21,46] and that the enzyme is inducible by barbiturates and other compounds.[15,25,26]

Studies utilizing selective oligonucleotides have indicated that the levels of mRNA coding for the related P-450 IIIA3 and P-450 IIIA7 proteins are very low if present at all in most adult human livers[22,24,47] and these enzymes should not contribute significantly to hepatic dihydropyridine oxidation. P-450 IIIA5 can oxidize 1a,[18,21] but the rate is considerably lower than that of P-450 IIIA4.[21] This protein has been detected in 29% of the liver samples examined and, when detected, is present only at levels of ≤25% of any other P-450 IIIA family proteins.[21] Specifically, P-450 IIIA5 was undetectable in the liver samples HL 105 and HL 107 used in this study (Table II, Figure 3), two samples with relatively high activity toward dihydropyridines. We previously concluded that P-450 IIIA5 contributes relatively little to the vast majority of the compounds known to be metabolized by the P-450 IIIA family.[21] Although P-450 IIIA3, P-450 IIIA4, and P-450 IIIA7 do not appear to contribute extensively to hepatic dihydropyridine oxidation, our results cannot rule out the possibility that another uncharacterized but closely related P-450 IIIA4 gene product exists with considerable similarity regarding catalytic specificity and regulation and contributes to dihydropyridine oxidation. The possibility also exists that some of the other P-450 IIIA enzymes may be more readily expressed than P-450 IIIA4 in extrahepatic tissues.[47] Nevertheless, P-450 IIIA4 can catalyze all of the reactions considered here and in the most simple paradigm it can account for the bulk of the activity.

A variety of experiments, including cDNA-directed expression work, indicate that P-450 IIIA4 is a major catalyst in the oxidation of testosterone, androstenedione, progesterone, cortisol, dehydroepiandrostane-3-sulfate, 17β-estradiol, 17α-ethynylestradiol, quinidine, erythromycin, troleandomycin, gestodene, warfarin, dapsone, lovastatin, lidocaine, aflatoxins B_1 and G_1, sterigmatocystin, senecionine, 6-aminochrysene, and at least three bay-region dihydrodiol epoxide derivatives of carcinogenic polycyclic hydrocarbons[2,18,25,35,36,48-54] and the reduction of 1,6-di-

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nitropyrene.[55] The lack of a substituent at C-4 appears to make a dihydropyridine a less "favorable" substrate for P-450 IIIA4. Most side chains do not block the ring oxidation, but in the case of nicardipine (**7a**), oxidation on the side chain seems to be preferred.[9,56] The approaches described here may be of use in making predictions about the in vivo disposition of other dihydropyridines and potential substrates for P-450 IIIA4.

Experimental Section

Chemicals. The dihydropyridine derivatives used are listed in Table I—dihydropyridines are all denoted **a** and the corresponding pyridines **b**. Compounds **1a**, **7a**, and cimetidine were purchased from Sigma Chemical Co., St. Louis, MO; **1a** was recrystallized (in amber glass) from hot C_2H_5OH before use.[42] Compounds **12a** and **12b** were synthesized at AB Hässle (Mölndal, Sweden). Compounds **2a**, **4a**, **5a**, **6a**, **19a**, and **20a** were obtained from Dr. A. Scriabine, Miles Laboratories, New Haven, CT. Compounds **3a**, **11a**, **13a**, **14a**, and **16a** were gifts of Dr. D. J. Triggle, State University of Buffalo, Buffalo, NY.[57] Gestodene (13-ethyl-17β-hydroxyl-18,19-dinor-17α-pregna-4,15-dien-20-yn-3-one) was a gift of Dr. H. Kuhl, Johann-Wolfgang-Goethe Universität, Frankfurt, Germany. All other compounds except **7b** were prepared previously in this laboratory and characterized: **1b**;[42] **2b**, **3b**, **4b**, **5b**, **6b**, **8a**, **8b**, **9a**, **9b**, **11b**, **13b**, **14b**, **16b**, **17a**, **17b**, **18a**, and **18b**;[3] **10a**, **10b**, **19b** (same as **20b**), **21a**, and **21b**;[4] and **15a** and **15b**.[5] **7b** was synthesized from **7a** by HNO_3 oxidation in 83% yield,[58] purified by high-performance liquid chromatography (HPLC) using a 10 × 250 mm octadecasilyl column (5 μm, Beckman, San Ramon, CA) with a 1:1 (v/v) mixture of CH_3OH and H_2O, recovered as the (hygroscopic) HCl salt from $(C_2H_5)_2O$: positive-ion fast atom bombardment mass spectrometry (glycerol matrix): *m/z* (relative abundance) 478 (M + H^+, 65), 462 (M − 15, 20), 148 (64), 134 (23), 91 (100); UV (CH_3OH, λ_{max} from derivative spectra) ϵ_{276} 8.0 mM^{-1} cm^{-1}, ϵ_{224} 21.0 mM^{-1} cm^{-1}, ϵ_{208} 33.0 mM^{-1} cm^{-1}; ^1H NMR (C^2HCl_3) δ 2.14 (3 H, s, NCH_3), 2.41 (2 H, t, CH_2CH_2N), 2.61 (6 H, s, CCH_3), 3.42 (2 H, s, CH_2phenyl), 3.57 (3 H, s, CO_2CH_3), 4.10 (2 H, t, $CO_2CH_2CH_2$), and phenyl protons at 7.28 (5 H, m), 7.39 (1 H, t), 7.57 (1 H, t), 8.14 (1 H, s), 8.20 (1 H, m) (the triplets centered at δ 2.41 and 4.10 were clearly coupled to each other, J = 6.1 Hz).

Enzyme Preparations. Human liver samples were obtained (from organ donors who met with accidental deaths) through Tennessee Donor Services, Nashville, TN. The processing of these samples and preparation of microsomes are described elsewhere.[59] The samples included those designated (HL indicates human liver): HL 39, HL 100, HL 105, HL 106, HL 107, HL 108, HL 110, HL 112, HL 115, HL 118, and HL 129, several of which have been utilized and cited in other studies.[24,25,35-37,50,60,61]

The cDNA clone NF-25,[17] corresponding to P-450 IIIA4,[14] was inserted at a *Hin*dIII site between the alcohol dehydrogenase promoter and terminator in the vector pAAH5, as described in detail elsewhere.[37,38] The vector was used to transform *Saccharomyces cerevisiae* AH22, and yeast microsomes were used for the partial purification of P-450 IIIA4.[37,38] In all of the studies described here, enzymatic oxidation systems were reconstituted by mixing components as described by Halvorson et al.[62] for rat P-450 IIIA1, except at pH 7.7. Specifically, each incubation included 100 pmol of P-450 IIIA4, 400 pmol of rabbit liver NADPH-P-450 reductase,[63] 100 pmol of human liver cytochrome b_5,[28] 200 μg of human liver HL 39 lipid extract, 50 μg of Emulgen 911, 5 μmol of $MgCl_2$, 1 μmol of EDTA, 40 nmol of substrate, and 100 μmol of potassium phosphate (pH 7.7) in a final volume of 500 μL.

Cytochrome P-450 measurements were made as described elsewhere[64] and protein estimation was done by using the Pierce bicinchoninic (BCA) assay (Pierce Chemical Co., Rockford, IL).

Antibodies. Antisera were raised in rabbits against human liver P-450 IIIA4 ($P\text{-}450_{NF}$) and human $P\text{-}450_{MP\text{-}1}$, and immunoglobulin G (IgG) fractions were prepared (also from preimmune sera).[65] The characterization of such antibody preparations has been published in detail elsewhere.[2,28]

Dihydropyridine Oxidation Assays. All manipulations with compounds containing nitro groups were done in amber glass because of sensitivity to light. In general, incubations included 0.5 mg of human liver microsomal protein or the system reconstituted with yeast P-450 IIIA4 described above. In the case of human liver microsomes, a 0.1 M potassium phosphate buffer (pH 7.7) was used with 200 μM substrate.[2] Incubations were done for 10 min (37 °C), and extraction and preparation methods for HPLC analysis are described in detail elsewhere.[2-5] Zorbax HPLC columns, used for all assays, were purchased from Mac-Modd (Chadds Ford, PA). HPLC conditions are presented in Table I—the UV absorbance detector was set at 254 or 270 nm.

Data Analysis. For the overviews of the data matrices formed by a group or class of objects, principal component analysis (PC analysis) is used.[39] This treatment decomposes the data matrix **x** into its mean vector **X**, a product of a score matrix **T** and a loading matrix **P** plus a residual **E** so that $\mathbf{x} = 1 \times \mathbf{X} + \mathbf{T} \times \mathbf{P} + \mathbf{E}$. The variables **x** (reaction rate constants) are scaled to unit variance for each set (column or part of a column) of data analyzed. The standard deviation of the residuals **E** is calculated and it is used as a measure of how well the objects are described by the principal component(s).

The significance of each component is calculated by cross validation. Only principal components that are significant according to this test are used for describing a class of objects.

For objects not initially included in the analysis T values can be calculated, as then can the distance to the class vector. If this distance is less than 2.0 standard deviations, the object is considered to be a member of the class. The PC analysis used is able to handle a small amount of missing data in the matrix provided that they are randomly distributed.[39]

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Registry No. **1a**, 21829-25-4; **1b**, 67035-22-7; **2a**, 39562-70-4; **2b**, 89267-41-4; **3a**, 51384-20-4; **3b**, 103026-76-2; **4a**, 22609-73-0; **4b**, 133349-25-4; **5a**, 63675-72-9; **5b**, 103026-83-1; **6a**, 66085-59-4; **6b**, 85677-93-6; **7a**, 55985-32-5; **7b**, 59875-58-0; **8a**, 70677-78-0; **8b**, 77234-00-5; **9a**, 1165-06-6; **9b**, 1539-44-2; **10a**, 50672-60-1; **10b**, 133349-26-5; **11a**, 73257-45-1; **11b**, 103026-82-0; **12a**, 72509-76-3; **12b**, 96382-71-7; **13a**, 103026-85-3; **13b**, 103026-81-9; **14a**, 103026-84-2; **14b**, 103026-80-8; **15a**, 42972-42-9; **15b**, 115722-00-4; **16a**, 32947-20-9; **16b**, 103026-77-3; **17a**, 17438-14-1; **17b**, 27525-74-2; **18a**, 1153-66-8; **18b**, 1153-67-9; **19a**, 98791-67-4; **19b**, 115700-40-8; **20a**, 98625-26-4; **21a**, 70008-26-3; **21b**, 133349-27-6; cytochrome P450, 9035-51-2.

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