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CYP3A4 ACTIVE SITE VOLUME MODIFICATION BY MUTAGENESIS OF LEUCINE 211

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ABSTRACT:

The leucine 211 → phenylalanine (L211F) and leucine 211 → tyrosine (L211Y) mutant forms of cytochrome P450 3A4 have been generated by site-directed mutagenesis and expressed functionally in *Escherichia coli*. Substrate binding affinities (S_{50} values) for testosterone and 7-benzyloxy-4-trifluoromethylcoumarin (BFC) were similar for the mutants and wild-type CYP3A4 (49 and 21 μM for L211F, 35 and 20 μM for L211Y, and 33 and 20 μM for the wild type, respectively). For erythromycin, however, the K_m values determined for the L211F and L211Y mutants were 2.4- and 10.5-fold higher than for the wild type. Furthermore, IC_{50} values for the inhibition of testosterone 6β-hydroxylation by erythromycin and troleandomycin for L211F were 2.4- and 3.7-fold higher, and those for L211Y were 3.4- and 9.2-fold higher than those measured for the wild type. Conversely, small inhibitors, such as diazepam, exhibited no significant difference in IC_{50} values between the wild type and the L211F and L211Y mutants. It is proposed that large substrates bound in the catalytic center of CYP3A4 with molecular volumes greater than ~600 Å^3 were less well accommodated in the altered active sites, resulting in lower association energies and increased IC_{50} values.

Cytochrome P450 3A4 (CYP3A4) is known to metabolize a very large variety of compounds varying in molecular weight from lidocaine (mol. wt. = 234) to cyclosporin A (mol. wt. = 1203). It is thought that this is achieved by having a large, hydrophobic active site, which can accommodate a diverse range of compounds. As a result, CYP3A4 binding interactions are dominated by the lipophilicity of the drug molecule involved, there being a strong correlation between the octanol partition coefficient (log $D_{7.4}$) and CYP3A4 K_i (Ishigami et al., 2001; Riley et al., 2001). In the drug development process, potential drug-drug interactions or high oxidative clearance due to interactions with some cytochromes P450 can be ameliorated by minor chemical modifications. For instance, when the carbon chain length of imipramine was reduced by one methylene unit, the inhibition of CYP2D6 bufuralol hydroxylation was reduced 27-fold (Halliday et al., 1997). Such changes are unlikely to attenuate CYP3A4 substrate binding or metabolism.

The most distant amino acids from the catalytic center of CYP3A4 that have a role in substrate binding are leucine 210, leucine 211, and aspartic acid 214 (Harlow and Halpert, 1997, 1998). Modeling and amino acid alignment studies have proposed that these amino acids occupy positions in the F-helix, remote from the heme iron of the CYP3A4 active site (Szklarz and Halpert, 1997). Mutagenesis studies have demonstrated that replacement of leucine 210 or leucine 211 with alanine reduced, but did not abolish, the activation effect of α-naphthoflavone on progesterone and testosterone hydroxylase activities (Harlow and Halpert, 1997). Further mutagenesis studies, in which leucine 211 was replaced with phenylalanine and aspartic acid 214 replaced with glutamic acid, displayed hyperbolic rather than sigmoidal substrate-saturation kinetics (Harlow and Halpert, 1998). From these studies, it has been proposed that the L211F mutation constricted the active site, limiting the simultaneous binding of more than one substrate molecule to the CYP3A4 active site as required for allosteric enhancement. This study has used the L211F and L211Y mutant forms of CYP3A4 to examine substrate occupancy of the CYP3A4 active site and shows that the CYP3A4 active site can be constricted to a volume of approximately 600 Å^3 without adversely affecting metabolic activity toward testosterone, BFC,[1] or diazepam.

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Materials and Methods

All reagents and fine chemicals were obtained from Sigma-Aldrich (Poole, Dorset, UK), with the following exceptions: isopropyl β-D-thiogalactoside (Invitrogen, Carlsbad, CA); aprotinin, ampicillin, and leupeptin (Roche Applied Science, Indianapolis, IN); *N*-methyl-(^{14}C)-erythromycin (PerkinElmer Life Sciences, Boston, MA); and 7-benzyloxy-4-trifluoromethylcoumarin and 7-hydroxy-4-trifluoromethylcoumarin (GENTEST, Woburn, MA). Enzymes for DNA manipulation were obtained from Promega (Madison, WI).

Expression of CYP3A4 and Cytochrome P450 Reductase in *Escherichia coli*. Mutagenesis reactions were performed following the single-stranded method of Kunkel (1985). The reverse complementary primers 5′-AAAATCAAATCTAAAGAGCTTCTTGGTGTTTTC-3′ and 5′-AAAATCAAATCTGTAGAGCTTCTTGGTGTTTTC-3′ introduced the L211F and L211Y mutations, respectively. Transformant clones were checked for mutagenesis by manual dideoxy chain termination sequencing using the T7 sequenase kit (Amersham Biosciences, Piscataway, NJ). Protein expression and quantitation

[1] Abbreviations used are: BFC, 7-benzyloxy-4-trifluoromethylcoumarin; P450, cytochrome P450.

experiments were carried out as described previously (Fowler et al., 2000). Briefly, expression experiments were carried out as described by Blake et al. (1996) and Pritchard et al. (1997, 1998), using the expression vectors pB35 and pB212. Protein yields were in the range of 50 to 200 nmol of P450 per liter of bacterial cell culture. *E. coli* membranes containing CYP3A4 and human cytochrome P450 reductase were prepared by the method of Blake et al. (1996) and resuspended in detergent-free 1× TSE buffer (50 mM Tris acetate, pH 7.6, 250 mM sucrose, and 0.25 mM EDTA). The cytochrome *c* (horse heart; Sigma) reductase activity was determined spectroscopically by the method of Vermilion and Coon (1974), monitoring at 550 nm with an extinction coefficient change of 21,400 M^{-1} cm^{-1}. This indicated P450 reductase concentrations to be greater than three times that of CYP3A4. All experiments were carried out using *E. coli* membrane suspensions in aqueous buffers in the absence of lipid, detergent, or cytochrome b_5 and without further purification or reconstitution steps. Turnover rates measured in this study were generally lower than those reported by other laboratories using insect cell-expressed or purified and reconstituted CYP3A4, due to the nonoptimized nature of the *E. coli* membrane preparations.

Diazepam, Testosterone, Erythromycin, and BFC Metabolism. Diazepam, testosterone, and erythromycin substrate saturation kinetics and inhibition experiments were carried out as described previously (Fowler et al., 2000). BFC kinetics were determined as follows: incubations containing BFC in methanol (1% v/v), CYP3A4 (150 pmol), and NADPH (1 mM final) were made up with deionized water and 2× HEPES buffer (final concentrations 50 mM HEPES/20 mM $MgCl_2$; pH 7.4) to total incubation volumes of 200 μl. Enzyme and substrate were preincubated for 5 min before initiation by addition of NADPH. Generation of the fluorescent metabolite, 7-hydroxy-4-trifluoromethylcoumarin, was measured over 30 min using a Spectrofluor Plus (TECAN, Durham, NC) instrument equipped with 405-nm excitation and 535-nm emission filters. Initial metabolism rates (typically the mean rate over the first 6 to 9 min was used, where the reaction rate was linear) were calibrated against the authentic metabolite standards.

Inhibition assay incubations contained 20 μM BFC, 150 pmol of P450, and 1 mM NADPH in 50 mM HEPES/20 mM $MgCl_2$ buffer, to which test compound in acetonitrile/pure acetonitrile (2% v/v) was added. Incubations were preincubated for 5 min at 37°C before initiation by NADPH addition. Presence of the additional 2% v/v acetonitrile reduced turnover rates by ~10%, similar to the effects of acetonitrile observed for CYP3A4 by others (Chauret et al., 1998; Busby et al., 1999). Fluorescent product generation was measured as above.

Data Treatment. Nonlinear regression analysis was performed using the program Origin 5.0 (MicroCal Software, Inc., Northampton, MA) with either the Michaelis-Menten or Hill equations fitted to the substrate saturation data, as appropriate to the enzyme/substrate combination. A simple inhibition effect model was fitted to the inhibition data. K_i values were estimated using the relationship $K_i = IC_{50}/(1 + [S/S_{50}])$, where S was the substrate concentration used in the assay, S_{50} was the half-saturation substrate concentration, and IC_{50} was the inhibitor concentration, which resulted in 50% inhibition of enzyme activity. More complex estimates of K_i to take account of the sigmoidal nature of some of the substrate-saturation profiles, have not been used in this analysis (Leff and Dougall, 1993). Molecular volumes were calculated using Cerius II (Advanced Visual Systems and Molecular Simulations, Waltham, MA) with an initial geometry optimization followed by calculation of the Van der Waals volume.

Results

Enzyme Kinetics. Kinetic parameters for testosterone 6β-hydroxylase, diazepam *N*-demethylase, diazepam 3-hydroxylase, erythromycin *N*-demethylase, and BFC debenzylase activities were determined and are shown in Fig. 1 and Table 1. S_{50} values for all three enzyme forms were similar for testosterone and BFC, whereas the S_{50} values for erythromycin of the L211F and L211Y mutants were 2.4- and 10-fold higher than that of the wild type, respectively. The wild type and L211F showed similar S_{50} values for diazepam, whereas the values for the L211Y mutant were unexpectedly lower. For the three smaller substrates, testosterone, diazepam, and BFC, the L211F mutant displayed the highest V_{max} values, followed by the wild type. The L211Y mutant showed the lowest V_{max} values. For erythromycin, this order was partially reversed, with the wild type exhibiting the highest V_{max} followed by the L211F and L211Y mutants, respectively.

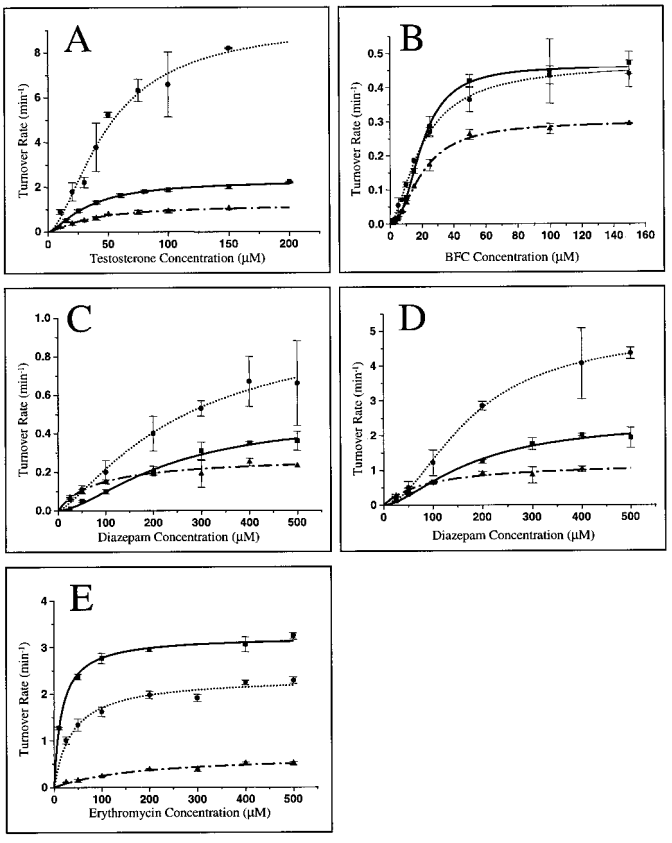

FIG. 1. *Substrate saturation profiles for testosterone 6β-hydroxylase (A), BFC O-debenzylase (B), diazepam N-demethylase (C), diazepam 3-hydroxylase (D), and erythromycin N*-demethylase (E) activities of CYP3A4 wild type (solid lines, filled squares), L211F (dotted lines, filled circles), and L211Y (dashed lines, filled triangles) mutants.

Values plotted are means of three determinations with error bars showing one standard deviation. Turnover rates are in picomoles of product per minute per picomole of P450.

Inhibition. Figure 2 shows the inhibition profiles generated using diazepam, bromocriptine, erythromycin, and troleandomycin. The K_i values estimated from these inhibition profiles are shown in Tables 2 and 3 for inhibition of testosterone 6β-hydroxylase and BFC debenzylase activities, respectively. K_i values estimated using the two different substrates for the three different enzyme forms were generally in agreement. However, differences were seen between the values estimated for progesterone and vinblastine between the two substrate probes. This may have been a reflection of the difference in character of the substrate probes used. In general, BFC showed greater sensitivity than testosterone to the effect of the site-directed mutations on inhibitor K_i values, compared with the wild type.

In Tables 2 and 3, the competitive substrates have been listed in order of molecular weight. It can be seen that, for the smaller competitive inhibitors, diazepam, nifedipine, diltiazem, progesterone, haloperidol, verapamil, and bromocriptine, the K_i values for the L211F and L211Y mutants were within 2.5-fold of the wild type both for testosterone and BFC inhibition. The leucine 211 mutant enzyme K_i values for both substrates were significantly greater than those of the wild type for erythromycin, vinblastine, troleandomycin, and cyclosporin A.

Correlation of K_i and Measured log $D_{7.4}$. K_i values for testoster-

TABLE 1

Oxidation kinetics for testosterone, diazepam, BFC, and erythromycin

S_{50} values quoted are in micromolar concentration, V_{max} values are in picomoles of product per minute per picomole of P450. Values are determined from profiles shown in Fig. 1.

Reaction	S_{50}			V_{max}			Hill Coefficient		
	Wild Type	L211F	L211Y	Wild Type	L211F	L211Y	Wild Type	L211F	L211Y
Testosterone 6β-hydroxylase	33 ± 2	49 ± 5	35 ± 5	2.3 ± 0.1	9.3 ± 0.7	1.2 ± 0.1	1.5 ± 0.1	1.7 ± 0.2	1.3 ± 0.2
Diazepam 3-hydroxylase	176 ± 33	214 ± 115	66 ± 19	2.4 ± 0.3	4.9 ± 1.6	1.1 ± 0.1	1.6 ± 0.3	1.5 ± 0.6	1.2 ± 0.4
Diazepam *N*-demethylase	217 ± 52	287 ± 228	78 ± 36	0.5 ± 0.1	1 ± 0.5	0.3 ± 0.1	1.7 ± 0.4	1.2 ± 0.4	1.1 ± 0.4
BFC debenzylase	20 ± 0.6	21 ± 1	20 ± 1	0.46 ± 0.1	0.47 ± 0.01	0.30 ± 0.01	2.4 ± 0.1	1.5 ± 0.1	1.9 ± 0.2
Erythromycin *N*-demethylase	17 ± 1	41 ± 3	178 ± 27	3.24 ± 0.04	2.43 ± 0.05	0.69 ± 0.04			

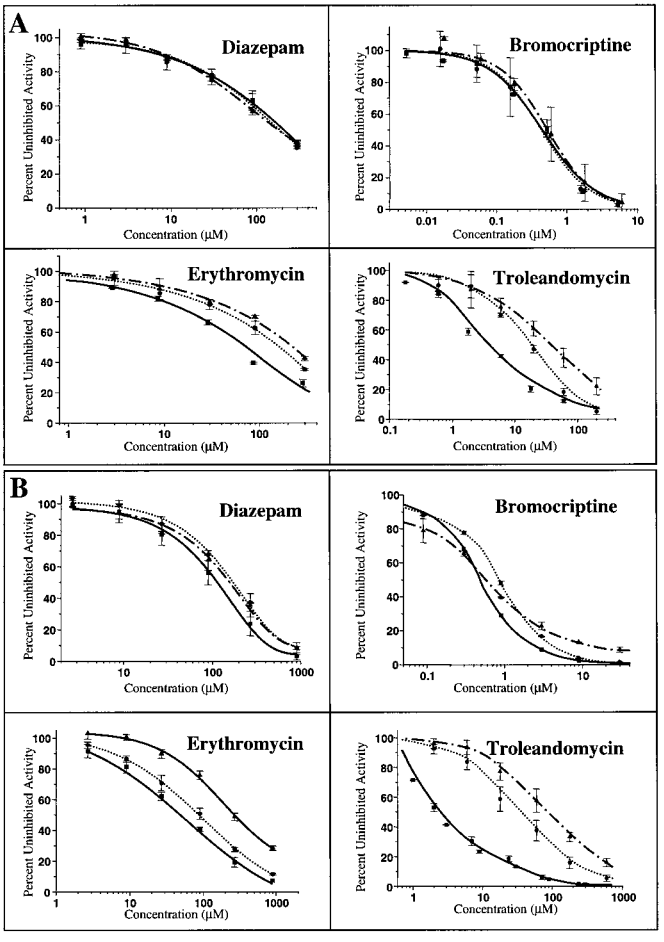

FIG. 2. *Inhibition of testosterone 6β-hydroxylase (A) and BFC O-debenzylase (B) activities by various inhibitors.*

Inhibition profiles for CYP3A4 wild type (solid lines, filled squares), L211F (dotted lines, filled circles), and L211Y (dashed lines, filled triangles) mutants. Values plotted are means of three determinations with error bars showing one standard deviation. Values are expressed as the percentage of uninhibited activity.

one and BFC inhibition have been plotted against measured log $D_{7.4}$ values previously determined (Riley et al., 2001) in Fig. 5. All three enzyme forms showed a high degree of correlation between the K_i values estimated in these studies and the octanol partition coefficients.

Discussion

In this study, the effect of CYP3A4 amino acid substitutions at positions in the active site thought to be remote from the heme have been investigated. A variety of substrate probes and inhibitors have been used to characterize the effects of the L211F and L211Y mutations and ascertain whether the effects seen were general or substrate-dependent.

The less conservative amino acid substitution, leucine → tyrosine, had a significant effect in attenuating overall enzyme activity, whereas the L211F mutation generated an enzyme form at least as active as the wild type in carrying out a variety of CYP3A4-mediated biotransformations. These findings were consistent with those of Khan and Halpert (2000), who found that the L211F and wild type had similar 7-hexoxycoumarin metabolism activities whereas the L211Y mutant activity was about 3-fold lower.

Of the reactions studied, only erythromycin showed true Michaelis-Menten hyperbolic kinetics, indicating that although it was possible for more than one molecule of testosterone, diazepam, or BFC to bind to the active site simultaneously, only a single molecule of erythromycin was probably accommodated. Better fitting of the substrate saturation profiles for testosterone, BFC, and diazepam was achieved by introducing an element of sigmoidicity in all three enzyme forms. The Hill coefficients for the L211F and L211Y mutants were lower than those determined for the wild type, as might be expected from the results of Harlow and Halpert (1998) who demonstrated that the L211F mutation reduced the sigmoidicity of steroid hydroxylase kinetics for CYP3A4. These studies have demonstrated the reduction of sigmoidicity for the kinetics of CYP3A4 leucine 211 mutants applied to benzodiazepines and 7-hydroxycoumarin derivatives as well as steroids.

As an indirect measure of active site binding/competition during catalysis, inhibition of metabolism has been used as a tool to investigate changes in active site association. Testosterone and BFC were selected as chemically dissimilar substrates that showed very similar S_{50} values for the wild type, L211F, and L211Y mutants and had S_{50} values well below the aqueous substrate solubility limits.

The strong relationship between estimated K_i and measured log $D_{7.4}$ (Fig. 5) for all three enzyme forms indicated that lipophilicity was still the dominant factor in determining active site association of a variety of diverse molecules. In these correlations, the L211Y mutant exhibited the lowest correlation coefficients ($r^2 = 0.85$ for BFC; $r^2 = 0.84$ for testosterone). The hydroxyl group of the tyrosine introduced in the L211Y mutant could interact with the inhibitor molecules differentially, decreasing the r^2 of the lipophilicity-IC_{50} correlation. However, the major reason for the reduction in r^2 value was the increase in erythromycin and troleandomycin IC_{50} values relative to the other inhibitors tested. Despite the increase in erythromycin and troleandomycin IC_{50} values, the correlation with lipophilicity was strongest for the L211F mutant ($r^2 = 0.97$ for both BFC and testosterone). Introduction of a phenylalanine residue at position 211 thus appeared to enhance the dependence of compound binding to CYP3A4 of log $D_{7.4}$.

The relationship between molecular size and the difference in K_i values between the wild type and the L211F and L211Y mutants is represented graphically in Fig. 3. Here the effect of the leucine → phenylalanine/tyrosine substitutions on enzyme inhibition by large molecules was dramatic (see Fig. 4 for structures of competitive

TABLE 2

Inhibition of testosterone 6β-hydroxylase activity

Calculated molecular volumes are in Å^3, molecular weights in grams per mole, and estimated K_i values are in micromolar concentrations. Sample profiles from this data set are shown in Fig. 2.

Compound	Calculated Molecular Volume	Molecular Weight	Estimated K_i			K_i Ratio	
			Wild Type	L211F	L211Y	L211F/WT	L211Y/WT
Diazepam	242	285	75 ± 1	90 ± 1	76	1.2	1.0
Nifedipine	292	346	3.1 ± 0.3	3.9 ± 0.3	2.3 ± 0.6	1.3	0.7
Diltiazem	298	415	7.4 ± 0.9	19 ± 1	8.3 ± 1.1	2.5	1.1
Progesterone	312	315	6.0 ± 0.5	6.9 ± 0.9	1.3 ± 0.8	1.2	0.2
Haloperidol	332	376	16 ± 1	18 ± 1	14 ± 5	1.1	0.9
Verapamil	437	455	16 ± 1	13 ± 4	13 ± 1	0.83	0.8
Bromcriptine	551	657	0.17 ± 0.01	0.31 ± 0.03	0.27 ± 0.05	1.8	1.6
Erythromycin	681	734	31 ± 1	92 ± 6	109 ± 1	3.0	3.5
Vinblastine	742	811	10 ± 0.4	94 ± 14	191	9.1	18.0
Troleadomycin	751	814	1.9 ± 0.01	8.6 ± 0.7	18 ± 2	4.5	9.6
Cyclosporin A	164	1201	0.39 ± 0.001	3.4 ± 0.1	3.6 ± 0.3	8.8	9.4

TABLE 3

Inhibition of BFC debenzylase activity

Calculated molecular volumes are in Å^3, molecular weights in grams per mole, and estimated K_i values are in micromolar concentration. Sample profiles from this data set are shown in Fig. 2.

Compound	Calculated Molecular Volume	Molecular Weight	Estimated K_i			K_i Ratio	
			Wild Type (WT)	L211F	L211Y	L211F/WT	L211Y/WT
Diazepam	242	285	80 ± 23	71 ± 7	75 ± 5	0.9	0.9
Nifedipine	292	346	3.0 ± 1.6	7 ± 2	3.4 ± 0.8	2.2	1.1
Diltiazem	298	451	18 ± 4	33 ± 3	32 ± 3	1.8	1.8
Progesterone	312	315	55 ± 12	57 ± 2	61 ± 9	1.0	1.1
Haloperidol	332	376	9.0 ± 0.8	10 ± 1	23 ± 5	1.1	2.5
Verapamil	437	455	12 ± 2	14 ± 2	25 ± 3	1.2	2.1
Bromocriptine	551	657	0.31 ± 0.06	0.45 ± 0.05	0.41 ± 0.10	1.5	1.3
Erythromycin	681	734	21 ± 0.6	42 ± 5	147 ± 11	2.0	7.1
Vinblastine	742	811	1.5 ± 0.4	18 ± 1	35 ± 4	12.0	23.0
Troleadomycin	751	814	1.1 ± 0.3	13 ± 3	46 ± 3	13.0	43.0
Cyclosporin A	1164	1201	0.14 ± 0.04	1.5 ± 0.2	3.2 ± 0.8	11.0	23.0

inhibitors tested in this study). For both the L211F and L211Y mutants, vinblastine, troleandomycin, and to a lesser extent, erythromycin were above the threshold size where K_i values are many times greater than determined for the wild type. These effects were more pronounced for the L211Y mutant, where the estimated K_i value for troleandomycin inhibition of BFC metabolism was 43-fold higher than the wild type. The molecular weight at which these effects became apparent was somewhere between bromocriptine (mol. wt. = 657; molecular volume = 551 Å^3) and erythromycin (mol. wt. = 734; molecular volume = 681 Å^3). Thus, the threshold molecular volume at which differential inhibition was seen was ~600 Å^3.

On these plots, cyclosporin A was a notable outlier, having a very large molecular volume but not giving the fold increase in L211F and L211Y IC_{50} values expected for such a large molecule. As the largest increases in IC_{50} for the L211F mutant were 9- to 13-fold, and these changes were comparable to those observed for cyclosporin A, it could be inferred that there was a maximum K_i increase, which could be generated by the L211F mutation, of around 10-fold. However, for the L211Y mutant, vinblastine and troleandomycin showed (for testosterone and BFC, respectively) almost double the fold increase in K_i values (relative to wild-type CYP3A4) to those generated by cyclosporin A. Therefore, for the L211Y mutant at least, cyclosporin A did not elicit the greatest increase in K_i of any test compound despite being the largest compound used in this study. Cyclosporin A can be metabolized at several different positions by CYP3A4 (Kronbach et al., 1988), and it has been postulated that cyclosporin A may be

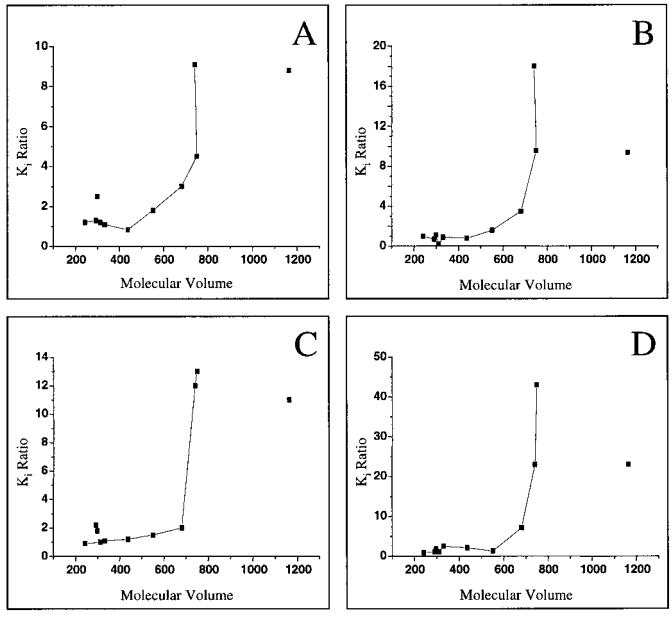

FIG. 3. *Estimated K_i ratios of L211F (A and C) and L211Y (B and D) mutants to wild-type CYP3A4 plotted against calculated molecular volumes (in Å^3) for inhibition of testosterone 6β-hydroxylase activity (A and B) and BFC debenzylase activity (C and D).*

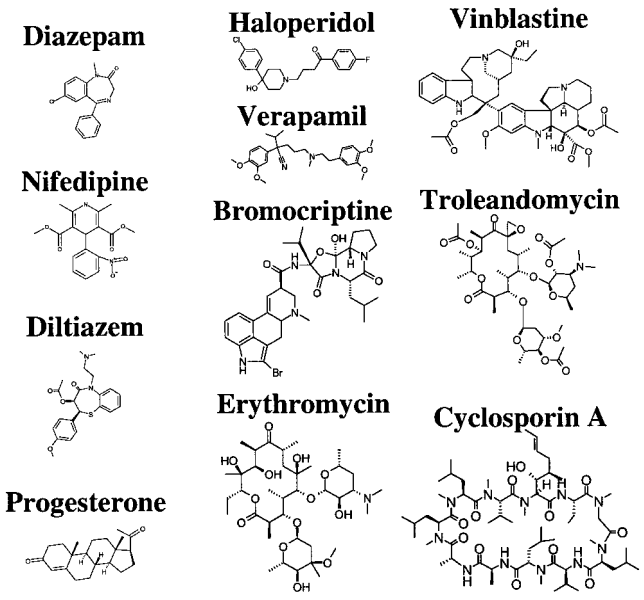

FIG. 4. *Chemical structures of inhibitors used in this study.*

flexible enough to allow part of the macrocycle to occupy the CYP3A4 active site. Comparison of the estimated K_i values for cyclosporin A for the L211F and L211Y mutants and both substrates shows that they are, in general, similar to those determined for troleandomycin and vinblastine. The calculated molecular volumes of troleandomycin and vinblastine are ~750 Å^3, about 60% of that calculated for cyclosporin A. Thus, if the difference in L211F or L211Y mutant and wild type K_i values for a compound were indicative of the volume of compound inserted into the enzyme active sites, just over half of the cyclosporin A molecule could be expected to be bound within the CYP3A4 active site cavity. Such a hypothesis is, however, only likely to be proven by the generation of a CYP3A4 crystal structure in which cyclosporin A is bound.

In conclusion, this study has demonstrated that the L211F and L211Y mutations not only reduced the sigmoidal character of testosterone and BFC substrate saturation kinetics, but also that the mutations differentially affected the inhibitors in a molecular volume-dependent manner. The competitive inhibition of testosterone and BFC oxidation by compounds with molecular volumes less than ~600 Å^3 was unaffected, or only marginally affected, by the substitution of leucine 211 with phenylalanine or tyrosine. Changes in inhibition potencies of up to 43-fold were observed for compounds of molecular volume greater than ~600 Å^3 in the L211F and L211Y mutants.

It is concluded that these mutations have reduced the CYP3A4 active site volume in a region remote from the catalytic center to create enzymes with effective active site volumes of ~600 Å^3. The ability of vinblastine, troleandomycin, and cyclosporin A to bind to the smaller active site of the mutants was reduced but not abolished. Similarly, the allosteric character of testosterone and BFC substrate-saturation kinetics were reduced, but not abolished, by these mutations. It is likely that the CYP3A4 active site is flexible enough to accommodate substrate molecules with molecular volumes up to 750 Å^3 or simultaneous binding of more than one smaller molecule. However, in the L211F and L211Y mutants, the amount of strain placed upon the enzyme when accommodating compounds of molecular volume >600 Å^3 may have been increased, making such interactions less favorable.

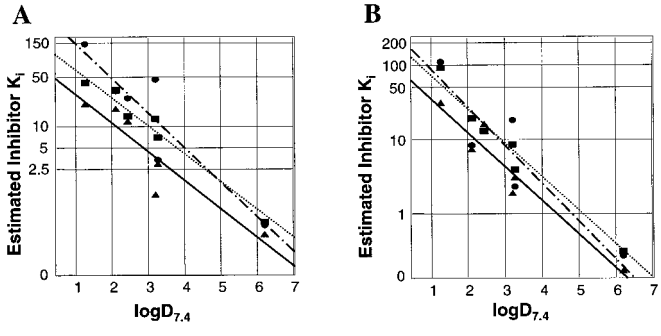

FIG. 5. *Correlation of estimated inhibitor K_i and measured log $D_{7.4}$ for inhibition of BFC debenzylase activity (A) and testosterone 6β-hydroxylase activity (B) by a variety of inhibitors.*

Correlation coefficients for BFC and testosterone inhibition were 0.86 and 0.94 for wild-type CYP3A4 (triangles, solid regression line), 0.97 and 0.97 for L211F (squares, dashed regression line), and 0.85 and 0.84 for L211Y (circles, dashed regression line).

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