

# Residues Glutamate 216 and Aspartate 301 Are Key Determinants of Substrate Specificity and Product Regioselectivity in Cytochrome P450 2D6\*

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Cytochrome P450 2D6 (CYP2D6) metabolizes a wide range of therapeutic drugs. CYP2D6 substrates typically contain a basic nitrogen atom, and the active-site residue Asp-301 has been implicated in substrate recognition through electrostatic interactions. Our recent computational models point to a predominantly structural role for Asp-301 in loop positioning (Kirton, S. B., Kemp, C. A., Tomkinson, N. P., St.-Gallay, S., and Sutcliffe, M. J. (2002) *Proteins* 49, 216–231) and suggest a second acidic residue, Glu-216, as a key determinant in the binding of basic substrates. We have evaluated the role of Glu-216 in substrate recognition, along with Asp-301, by site-directed mutagenesis. Reversal of the Glu-216 charge to Lys or substitution with neutral residues (Gln, Phe, or Leu) greatly decreased the affinity ( $K_m$  values increased 10–100-fold) for the classical basic nitrogen-containing substrates bupropion and dextromethorphan. Altered binding was also manifested in significant differences in regioselectivity with respect to dextromethorphan, producing enzymes with no preference for *N*-demethylation versus *O*-demethylation (E216K and E216F). Neutralization of Asp-301 to Gln and Asn had similarly profound effects on substrate binding and regioselectivity. Intriguingly, removal of the negative charge from either 216 or 301 produced enzymes (E216A, E216K, and D301Q) with elevated levels (50–75-fold) of catalytic activity toward diclofenac, a carboxylate-containing CYP2C9 substrate that lacks a basic nitrogen atom. Activity was increased still further (>1000-fold) upon neutralization of both residues (E216Q/D301Q). The kinetic parameters for diclofenac ( $K_m$  108  $\mu$ M,  $k_{cat}$  5 min<sup>-1</sup>) along with nifedipine ( $K_m$  28  $\mu$ M,  $k_{cat}$  2 min<sup>-1</sup>) and tolbutamide ( $K_m$  315  $\mu$ M,  $k_{cat}$  1 min<sup>-1</sup>), which are not normally substrates for CYP2D6, were within an order of magnitude of those observed with CYP3A4 or CYP2C9. Neutralizing both Glu-216 and Asp-301 thus effectively alters substrate recognition illustrating the central role of the negative charges provided by both residues in defining the specificity of CYP2D6 toward substrates containing a basic nitrogen.

Cytochromes P450 are a superfamily of heme-containing enzymes responsible for the oxidative metabolism of an extremely wide variety of substrates. Human cytochrome P450 2D6 (CYP2D6)<sup>1</sup> is one of the most important members of this family due to its central role in the metabolism of many drugs in common clinical use (1), such as opioids, antidepressants, neuroleptics, and various cardiac medications. CYP2D6 is polymorphic, giving rise to wide interindividual and ethnic differences in drug metabolism (2, 3). Inheritance of the defective gene results in the “poor metabolizer” phenotype that results in impaired drug oxidation reactions (4) and may be linked to altered disease susceptibility (5, 6). P450-drug and drug-drug interactions involving CYP2D6 ligands are a prime consideration in the development of new drugs, emphasizing the importance of a detailed understanding of the factors that govern the substrate specificity of this enzyme.

CYP2D6 substrates are structurally diverse, but several key structural features have been identified (reviewed in Ref. 7). These include a basic nitrogen atom 5–10 Å from the site of metabolism that is present in the majority of CYP2D6 substrates, although it is far from being a universal requirement. For example, CYP2D6 can metabolize progesterone (8) and pregnenolone (9), and Guengerich *et al.* have recently described a spiroisopropylamine as a high affinity substrate of CYP2D6, which lacks a basic nitrogen (10). Furthermore, the enzyme can catalyze *N*-dealkylation reactions of substrates such as deprenyl (11), amitriptyline (12), methamphetamine (13), the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (14), and dextromethorphan (15), a commonly used antitussive.

An ionic interaction between a negatively charged carboxylate group in the active site of the enzyme and the basic nitrogen atom of substrates has been proposed to be the key determinant of the specificity of CYP2D6 reactions (16). A number of structural models have pointed to aspartate 301 in the I helix being the specific residue involved (4, 16–18) (see Fig. 1). This is supported by mutagenesis studies that showed that substitution of Asp-301 by neutral residues leads to a marked reduction in catalytic activity against “classical” CYP2D6 substrates (17, 19, 20), although it has little effect on activity against a substrate lacking a basic nitrogen (10). However, it is not clear if the carboxylate of this residue interacts directly with the basic nitrogen of the substrate within the active site as generally assumed, or whether it has a structural role, perhaps in the positioning of the B'-C loop in the active site (21), and is

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<sup>1</sup> The abbreviations used are: CYP2D6, cytochrome P450 2D6; HPLC, high pressure liquid chromatography; 3MM, 3-methoxymorphinan.

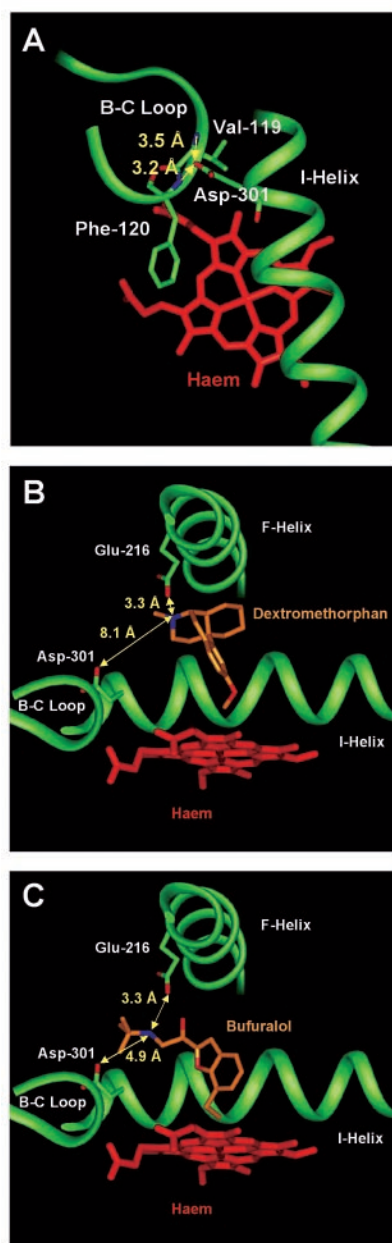


FIG. 1. The active site region of our models of CYP2D6. A, the hydrogen bonds between the carboxyl of Asp-301 and the backbone amides of Val-119 and Phe-120. B, the docked position of dextromethorphan. C, the docked position of bufuralol.

therefore an indirect determinant of substrate specificity (20).

Our recent modeling studies (21), which incorporate information from the recently determined crystal structure of rabbit 2C5 (22), indicate that a second acidic residue, Glu-216, is in a position where it may play a role in the binding of the basic nitrogen of CYP2D6 substrates (Fig. 1). We now report site directed mutagenesis studies of Glu-216 and Asp-301 aimed at clarifying their respective roles in CYP2D6 substrate specificity and regioselectivity.

#### MATERIALS AND METHODS

##### Chemicals

Terrific Broth (TB; 12g/liter bacto-tryptone, 24g/liter bacto-yeast extract, and 4ml/liter glycerol), chloramphenicol,  $\delta$ -aminolevulinic acid, dithiothreitol, glucose 6-phosphate, NADP<sup>+</sup>, phenylmethylsulfonyl fluoride, sodium dithionite, cytochrome c, dextromethorphan, diclofenac, tolbutamide, nifedipine, and testosterone were all purchased from Sigma. Ampicillin was obtained from Beecham Research (Welwyn Gar-

den City, UK), and isopropyl  $\beta$ -D-thiogalactopyranoside from Melford Laboratories (Ipswich, UK). Glucose 6-phosphate dehydrogenase (type VII) was from Roche Molecular Biochemicals. HPLC grade solvents were purchased from Rathburn Chemicals (Walkerburn, UK), and HPLC columns from Agilent (Crawford Scientific, UK). DNA modifying enzymes were obtained from Invitrogen and Promega. Dextrorphan, 3-methoxymorphinan, 6 $\beta$ -hydroxy-testosterone, bufuralol, 1'-hydroxy bufuralol, nifedipine N-oxide, hydroxy-tolbutamide, and 4'-hydroxydiclofenac were purchased from Ultra Fine Chemicals (Manchester, UK). All other chemicals were from BDH (Poole, UK). Library-efficient competent *Escherichia coli* JM109 were purchased from Promega.

##### Mutagenesis

The isolation of the cDNAs and construction of expression plasmids ompA 2D6(His<sub>6</sub>) (pB81) and pJR7 (human P450 reductase) have been described elsewhere (23–25). Site-directed mutagenesis was performed using the single-stranded DNA template method (26) using pB81 as a template, the *dut- ung-E. coli* strain CJ236, and the mutating oligonucleotide. A list of the oligonucleotides used with the mutated nucleotides underlined is as follows: E216Q, 3'-cag aaa gcc cga ctg ctc ctt cag tcc; E216D, 3'-cag aaa gcc cga gtc ctc ctt cag tcc; E216F, 3'-cag aaa gcc cga gaa ctc ctt cag tcc; E216A, 3'-cag aaa gcc cga cgc ctc ctt cag tcc; E216K, 3'-cag aaa gcc cga ctt ctc ctt cag tcc; D301E, 3'-ggc aga gaa cag ctc agc cac cac tat; D301Q, 3'-ggc aga gaa cag ctg agc cac cac tat; D301N', 3'-ggc aga gaa cag gtt agc cac cac tat and D301A, 3'-ggc aga gaa cag ggc agc cac cac tat. Note, all oligonucleotides are reverse complement sequences and written in the direction 3' to 5'. The presence of the desired mutations was confirmed by automated DNA sequencing.

##### Coexpression of the P450s and P450 Reductase in *E. coli*

Expression was carried out essentially as previously described (25). Briefly, pB81 and pB81-mutant plasmids were co-transfected with pJR7 into the *E. coli* strain JM109. Cultures were grown in TB at 30 °C until the A<sub>600</sub> reached >0.8, whereupon the heme precursor  $\delta$ -aminolevulinic acid was added to a final concentration of 1 mM. Induction was initiated with the addition of isopropyl-1-thio- $\beta$ -D-galactopyranoside to a final concentration of 1 mM. Cultures were grown until the appearance of P450 in the CO reduced spectra of whole cells (usually 24 h), at which point cells were harvested. Sphaeroplasts were prepared and sonicated, and the membrane fraction pelleted by ultracentrifugation at 100,000  $\times$  g. Membranes were resuspended in TSE buffer (50 mM Tris, pH 7.6, 250 mM sucrose, 10% glycerol), and the P450 content determined by P450(Fe<sup>2+</sup>)-CO versus P450(Fe<sup>3+</sup>) difference spectra. P450 reductase activity was estimated by NADPH-dependent cytochrome c reduction (27). Membranes were stored at -70 °C until required.

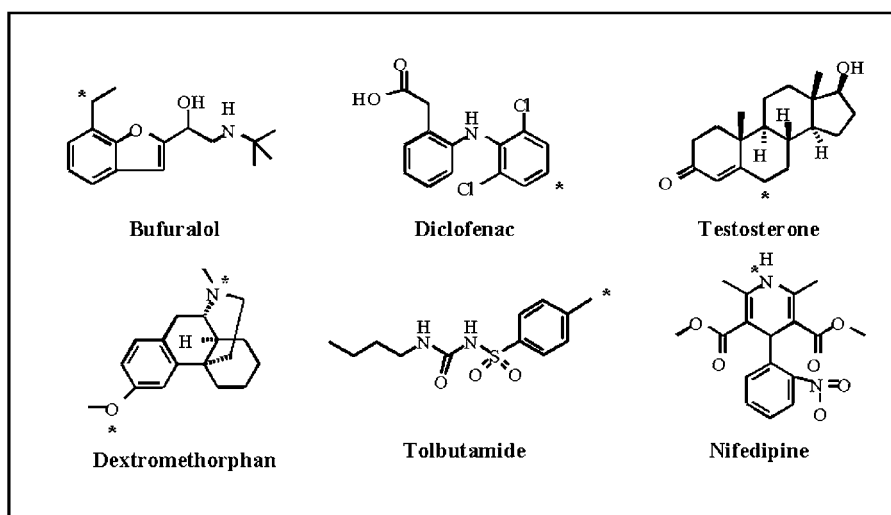
##### Enzyme Assays

All reactions were carried out in triplicate at 37 °C with shaking. HPLC analysis was carried out using a Hewlett Packard 1100 HPLC and Chemstation software.

**Bufuralol 1'-Hydroxylation**—Reactions were carried out in 50 mM potassium phosphate, pH 7.4, 0–800  $\mu$ M bufuralol, 10 pmol P450, and an NADPH-generating system (5 mM glucose 6-phosphate, 1 unit of glucose 6-phosphate dehydrogenase, 1 mM NADP<sup>+</sup>) in a total volume of 300  $\mu$ L. After 3 min of pre-incubation at 37 °C, reactions were initiated with the addition of the NADPH-generating system and incubated for a further 6 min before stopping with 15  $\mu$ L of 60% perchloric acid. Samples were incubated on ice for 10 min before centrifugation at 16,100  $\times$  g for 10 min to remove particulate material. Routinely, 100- $\mu$ L aliquots of the reaction supernatant were injected onto HPLC. Metabolites were separated using a Hypersil ODS column (5  $\mu$ m; 125  $\times$  4.0 mm), flow rate, 1.0 mL/min. A step gradient was applied using 100 mM ammonium acetate, pH 5 (Buffer A) and acetonitrile (Buffer B). The gradient profile was 0 min = 73% A:27% B; 11.3 min = 60% A:40% B; 12.3 min = 49% A:51% B; and 13.3 min = 73% A:27% B (all v/v). The fluorescent metabolite 1'-hydroxy bufuralol was detected using  $\lambda_{\text{ex}}$  = 252;  $\lambda_{\text{em}}$  = 302 and quantitated with reference to an authentic standard.

**Dextromethorphan O- and N-Demethylation**—Assays were carried out in 50 mM potassium phosphate, pH 7.4, 0–2000  $\mu$ M dextromethorphan (for  $K_m$  determination), or 100  $\mu$ M dextromethorphan (for the regioselectivity studies), 10 pmol P450, and an NADPH-generating system in a total volume of 200  $\mu$ L. After a 3-min pre-incubation at 37 °C, reactions were initiated by the addition of the NADPH-generating system and were incubated a further 6 min before stopping with 100  $\mu$ L of ice-cold methanol and 5  $\mu$ L of 60% perchloric acid. Samples were incubated on ice for 10 min before centrifugation to remove particulate material. Metabolites were separated by HPLC using a Hypersil C18

FIG. 2. Structures of probe substrates used in this study. Sites of metabolism investigated are indicated with an asterisk.



BDS column (5  $\mu$ m; 250  $\times$  4.6 mm) with a flow rate of 1 ml/min. Isocratic separation was used with mobile phases of 100 mM ammonium acetate, pH 5, and acetonitrile mixed at ratios of 78%:22% (v/v) respectively for the separation of dextromethorphan and 68%:32% (v/v) for 3-methoxymorphinan. Metabolites were detected by fluorescence ( $\lambda_{\text{ex}}$  = 270;  $\lambda_{\text{em}}$  = 312) and quantitated with reference to authentic standards.

**Testosterone 6 $\beta$ -Hydroxylation**—Assays were carried out in 50 mM Hepes, pH 7.4, 400  $\mu$ M testosterone, 30 pmol P450, and an NADPH-generating system in a total volume of 200  $\mu$ l. After a 3-min pre-incubation at 37  $^{\circ}$ C, reactions were initiated by the addition of the NADPH-generating system and incubated for 3 h before stopping with 100  $\mu$ l of ice-cold methanol and 5  $\mu$ l of 60% perchloric acid. Samples were incubated on ice for 10 min before centrifugation to remove particulate material. Metabolites were separated by HPLC using a Hyper-sil ODS (5  $\mu$ m; 250  $\times$  4.6 mm) column with a flow rate of 1 ml/min. Step gradient separation using acetonitrile (Buffer A), water (Buffer B), and methanol (Buffer C) was used. The gradient profile was: 0 min = 2.5% A:45% B:52.5% C; 8 min = 2.5% A:25% B:72.5% C; 11.5 min = 2.5% A:45% B:52.5% C (all v/v/v). The run was terminated after 15 min. Products were detected at 240 nm and quantified with respect to an authentic 6 $\beta$ -hydroxytestosterone standard.

**Diclofenac 4'-Hydroxylation**—Assays were carried out in 100 mM Tris-HCl, pH 7.4, 500  $\mu$ M diclofenac (for the specific activity determination) or 0–750  $\mu$ M diclofenac (for kinetic analysis of the double mutant), 10 pmol P450, and an NADPH-generating system in a total volume of 200  $\mu$ l. Following a 3-min pre-incubation at 37  $^{\circ}$ C, reactions were initiated by the addition of the NADPH-generating system and incubated for 90 min (for specific activities) or 4 min (for kinetics) before termination by the addition of 200  $\mu$ l of ice-cold methanol. Samples were incubated on ice for 10 min before centrifugation to remove particulate material. Metabolites were separated by HPLC using a Hyper-sil ODS column (5  $\mu$ m; 250  $\times$  4.6 mm) run isocratically at 1 ml/min with mobile phase of 20 mM potassium phosphate, pH 7, and acetonitrile (77%:23% (v/v), respectively) and detected at 280 nm. 4'-Hydroxy diclofenac formation was quantified using an authentic standard. A minor peak corresponding to a 5'-hydroxy metabolite was seen in some cases but not quantified.

**Tolbutamide 4-Methylhydroxylation**—Assays were carried out in 50 mM potassium phosphate, pH 7.4, 0–2000  $\mu$ M tolbutamide, 10 pmol P450, and an NADPH-generating system in a total volume of 250  $\mu$ l. Following a 3-min pre-incubation at 37  $^{\circ}$ C, reactions were initiated by the addition of the NADPH-generating system and incubated for 6 min before termination by the addition of 50  $\mu$ l of 10% trichloroacetic acid. Samples were incubated on ice for 10 min before centrifugation to remove particulate material. Metabolites were separated by HPLC using a Hyper-sil ODS column (5  $\mu$ m; 250  $\times$  4.6 mm). Step gradient separation using acetonitrile (Buffer A), 10 mM sodium acetate, pH 4.3, (Buffer B) was used. The gradient profile was: 0 min = 32% A:68% B; 11.5 min = 49% A:51% B; 12.5 min = 32% A:68% B (all v/v), and the metabolite was detected at 230 nm. Tolbutamide 4-methylhydroxylation formation was quantitated using an authentic standard.

**Nifedipine N-Oxidation**—Reactions were carried out in 100 mM potassium phosphate, pH 7.85, 0–1000  $\mu$ M nifedipine, 10 pmol P450, and an NADPH-generating system in a total volume of 200  $\mu$ l. After a 3-min

pre-incubation at 37  $^{\circ}$ C, reactions were started by the addition of the NADPH-generating system, and samples were incubated for 10 min before stopping with 100  $\mu$ l of ice-cold methanol. Samples were incubated on ice for 10 min before centrifugation at 16,100  $\times$  g for 10 min to remove particulate material. Aliquots of the supernatant were injected onto HPLC. Metabolites were separated using a Hyper-sil ODS column (5  $\mu$ m; 125  $\times$  4.0 mm) and run isocratically at 1.0 ml/min using a mobile phase of water:methanol:acetonitrile (45:25:30) (v/v/v). The oxidized nifedipine metabolite was detected at 254 nm and quantitated with reference to an authentic standard.

#### Modeling

The models of CYP2D6 were produced, and the dockings of substrates performed as described previously (21). In brief, the models of CYP2D6 were produced using comparative modeling (Modeler; Ref. 28) with five structural templates—P450s cam, terp, eryF, BM3, and 2C5. The substrates were then docked into the active site of CYP2D6 using GOLD (29).

#### RESULTS

**Protein Expression**—A series of CYP2D6 mutants were constructed in which the acidic amino acid residues Glu-216 or Asp-301 were replaced with acidic, basic, and uncharged residues. Additionally both charged residues were neutralized in the double mutant E216Q/D310Q. Levels of expression of the mutant P450s ranged from 150–645 nmol/liter *E. coli* culture compared with 300 nmol/liter for wild type CYP2D6. P450 reductase levels ranged between 289 and 467 nmol of cytochrome *c* reduced/min/mg of *E. coli* membrane. The D301A mutant failed to form spectroscopically detectable P450, and the D301N mutant was found to be less stable than the other mutant isoforms in the presence of sodium dithionite during P450(Fe<sup>2+</sup>)-CO versus P450(Fe<sup>2+</sup>) difference spectroscopy. Thus, mutations to the Asp-301 residue generally had a destabilizing effect on the enzyme, in agreement with previous findings (20).

**Metabolism of Typical CYP2D6 Substrates**—Kinetic analysis was carried out on the mutant P450s co-expressed in *E. coli* with human cytochrome P450 reductase using bufuralol and dextromethorphan, two prototypical CYP2D6 substrates that each contain a basic nitrogen (Fig. 2). The kinetic parameters for bufuralol 1'-hydroxylation and dextromethorphan O-demethylation are shown in Table I. Conservative replacements of Glu-216 or Asp-301 with Asp and Glu, respectively, produced small (2–6-fold) increases in  $K_m$  values and had negligible effects on  $k_{\text{cat}}$  for both substrates. However, replacement of either of the negatively charged side chains with a neutral group had much larger effects, particularly on the  $K_m$  values. Substitution of Asp-301 by asparagine or glutamine led to a



TABLE I  
Kinetic parameters for bufuralol 1'-hydroxylation and dextromethorphan O-demethylation

Assays were performed in triplicate as described under Materials and Methods. The error values shown are the standard deviations calculated from fitting the Michaelis-Menten equation to the data.

| CYP2D6      | Bufuralol 1'-hydroxylation |                | Dextromethorphan O-demethylation |                |
|-------------|----------------------------|----------------|----------------------------------|----------------|
|             | $K_M$                      | $k_{cat}$      | $K_M$                            | $k_{cat}$      |
|             | $\mu M$                    | $min^{-1}$     | $\mu M$                          | $min^{-1}$     |
| Wild type   | $1.1 \pm 0.04$             | $2.1 \pm 0.01$ | $2.6 \pm 0.3$                    | $2.0 \pm 0.04$ |
| E216D       | $6 \pm 0.2$                | $1.9 \pm 0.02$ | $13 \pm 1$                       | $2.2 \pm 0.04$ |
| E216Q       | $188 \pm 5$                | $3.1 \pm 0.03$ | $51 \pm 3$                       | $0.4 \pm 0.01$ |
| E216A       | $162 \pm 5$                | $3.6 \pm 0.04$ | $63 \pm 11$                      | $1.3 \pm 0.06$ |
| E216F       | $117 \pm 5$                | $3.0 \pm 0.04$ | $30 \pm 3$                       | $0.9 \pm 0.03$ |
| E216K       | $187 \pm 7$                | $3.3 \pm 0.05$ | $312 \pm 24$                     | $0.2 \pm 0.01$ |
| D301E       | $2.0 \pm 0.1$              | $1.9 \pm 0.02$ | $11 \pm 1$                       | $1.4 \pm 0.05$ |
| D301N       | $160 \pm 6$                | $2.0 \pm 0.03$ | $3598 \pm 166$                   | $3.5 \pm 0.06$ |
| D301Q       | $142 \pm 6$                | $3.2 \pm 0.04$ | $200 \pm 10$                     | $1.4 \pm 0.03$ |
| E216Q/D301Q | $522 \pm 29$               | $1.4 \pm 0.03$ | $438 \pm 49$                     | $7.6 \pm 0.53$ |

130- to 145-fold increase in  $K_m$  values for bufuralol; for dextromethorphan, the increase was 80-fold with the D301Q mutant but as much as 1400-fold for D301N. For both substrates, the effects on  $k_{cat}$  were modest, ranging from a 30% decrease to a 70% increase.

The role of Glu-216 has not hitherto been established experimentally. Substitution of this residue by glutamine, alanine, or phenylalanine had rather similar effects to the Asp-301 substitution as far as bufuralol 1'-hydroxylation is concerned;  $K_m$  was increased by 100- to 170-fold, and  $k_{cat}$  increased by 40–70%, there being little effect of the bulk of the neutral side chain at this position. For dextromethorphan O-demethylation, the effects on  $K_m$  were smaller (10–25-fold increase), but  $k_{cat}$  was decreased by as much as 5-fold in the E216Q mutant.

Substitution of both acidic residues by glutamine in E216Q/D301Q led to larger increases in  $K_m$  values for both bufuralol and dextromethorphan than observed for the individual substitutions. However, the effects on the  $k_{cat}$  values were quite different. Both the individual E216Q and D301Q substitutions led to an ~50% increase in  $k_{cat}$  for bufuralol, but the double mutant showed a 40% decrease in  $k_{cat}$  relative to the wild type (less than half the rate of either individual mutant). An analogous effect but in the opposite direction was seen for dextromethorphan, where the individual substitutions lead to a decrease in  $k_{cat}$  by as much as 5-fold, while the double mutant shows an ~4-fold increase.

A clear difference between the two substrates is also seen in the effects of replacing the negative charge at position 216 by a positive charge in the E216K mutant. Such a mutation might be expected to show the largest reduction in binding affinity for nitrogenous substrates through repulsion of the basic nitrogen atom by the positive side chain. For bufuralol, this mutant shows kinetic constants indistinguishable from those of the neutral E216Q mutant. For dextromethorphan, by contrast, there is a much greater increase in  $K_m$  values (120-fold as opposed to 11-fold) and a greater decrease in  $k_{cat}$  values (10-fold versus 5-fold). Dextromethorphan, the more rigid substrate experiences a larger differential effect.

**Regiospecificity of Dextromethorphan Dealkylation**—To gain further information on the effect of mutations on the positioning of substrate in the active site, changes in the ratios of dextromethorphan metabolites were analyzed. The major pathway of dextromethorphan metabolism by CYP2D6 is O-demethylation, with the formation of dextrorphan. However, CYP2D6 can also catalyze demethylation at the basic nitrogen to produce 3-methoxymorphinan (3MM) in a concentration-dependent manner (15). To probe any changes in regioselectivity produced by the mutations, the ratio of the two activities was measured at 0.1 mM dextromethorphan. The wild type enzyme and the conservative mutants E216D and D301E produced

similar dextrorphan/3MM ratios of ~8:1, indicating a strong preference for the O-demethylation pathway (Fig. 3). However, this ratio was substantially decreased following removal of either of the negative charges, consistent with a decreased preference for binding the basic nitrogen close to Glu-216 and Asp-301, distant from the heme iron. Dextrorphan:3MM ratios as low as 1:1 were observed, for the E216F, E216K, D301N, D301Q, and E216Q/D301Q mutants.

**Metabolism of Novel Substrates**—If the interaction of the two acidic residues of CYP2D6 with the substrate is a key determinant of the substrate specificity of the enzyme, specifically of its preference for substrates containing a basic nitrogen, the mutants in which one or both of these residues have been mutated might be expected to show a broader substrate specificity. As an initial test of this, we measured the ability of the mutants to oxidize two atypical CYP2D6 substrates lacking a basic nitrogen. The compounds used were testosterone (uncharged; a CYP3A4 substrate) and diclofenac (negatively charged; a CYP2C9 substrate) (Fig. 2). Measured rates of formation of 6 $\beta$ -hydroxytestosterone and 4'-hydroxy diclofenac, the products formed from these substrates by CYP3A4 and CYP2C9, respectively, are shown in Table II.

The wild type enzyme did catalyze the hydroxylation of these two atypical substrates, though at rates  $\geq 100$ -fold less than the rates observed with bufuralol or dextromethorphan. Most of the mutants showed only modest increases (up to 4-fold) in the rate of formation of 6 $\beta$ -hydroxy-testosterone compared with wild type; the largest effect was seen for the E216F mutant, which showed a 10-fold increase. These relatively modest effects most probably reflect the steric constraints on the binding of the bulky testosterone molecule as discussed earlier (23).

Removal of the carboxyl groups from residues 216 and 301 had larger effects on the diclofenac 4'-hydroxylase activity. The wild type, E216D, and D301E enzymes produced only barely detectable quantities of 4'-hydroxy diclofenac (Table II), but a number of other mutants showed significant increases in the rate of product formation. Mutants E216Q, E216F, and D301N produced rates ~5-, 10-, and 22-fold higher than the wild type enzyme, respectively, while the turnover rates of the E216A, E216K, and D301Q derivatives were increased 50- to 75-fold. The double mutant E216Q/D301Q produced the highest diclofenac 4'-hydroxylase activity of 3.84 min<sup>-1</sup>, around 1000-fold higher than wild type; interestingly, its testosterone 6 $\beta$ -hydroxylase activity was increased only 2-fold over wild type. The rate of formation of 4'-hydroxy diclofenac was not significantly greater with the E216K mutant than with E216A, suggesting that the carboxylate group of the substrate is not positioned near this residue.

Since neutralization of both Glu-216 and Asp-301 in the double mutant produced the highest levels of diclofenac activ-

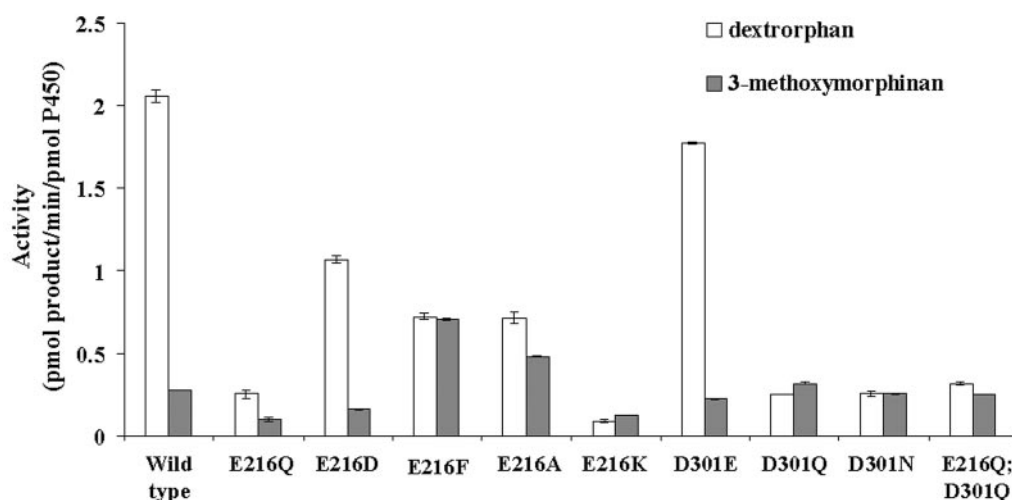


FIG. 3. Relative amounts of dextrophan and 3-methoxymorphinan produced from dextromethorphan by the action of wild type and mutant CYP2D6. Assays were performed using 100  $\mu\text{M}$  dextromethorphan as described under "Materials and Methods." The error bars represent the average deviation from duplicate incubations.

TABLE II  
Specific activities of *E. coli* membranes expressing wild type and mutant CYP2D6 for testosterone and diclofenac

| Mutant      | Catalytic activity <sup>a</sup>                   |                                       |
|-------------|---|---------------------------------------|
|             | Testosterone<br>(6 $\beta$ -hydroxy-testosterone) | Diclofenac<br>(4'-hydroxy-diclofenac) |
|             | $\text{min}^{-1}$                                 |                                       |
| Wild type   | $0.013 \pm 0.002$                                 | 0.003                                 |
| E216D       | $0.014 \pm 0.001$ (1)                             | ND                                    |
| E216Q       | $0.050 \pm 0.006$ (4)                             | $0.015 \pm 0.006$ (5)                 |
| E216F       | $0.146 \pm 0.003$ (11)                            | $0.031 \pm 0.003$ (10)                |
| E216A       | $0.040 \pm 0.007$ (3)                             | $0.147 \pm 0.005$ (49)                |
| E216K       | $0.014 \pm 0.001$ (1)                             | $0.194 \pm 0.020$ (65)                |
| D301E       | $0.032 \pm 0.002$ (2)                             | $0.006 \pm 0.001$ (2)                 |
| D301Q       | $0.018 \pm 0.002$ (1.5)                           | $0.226 \pm 0.004$ (75)                |
| D301N       | $0.019 \pm 0.001$ (1.5)                           | $0.067 \pm 0.015$ (22)                |
| E216Q/D301Q | $0.027 \pm 0.001$ (2)                             | $3.84 \pm 0.23$ (1280)                |
| CYP2C9      |   | $24 \pm 2^b$                          |
| CYP3A4      | $2.3 \pm 0.1^c$                                   |                                       |

<sup>a</sup> Assays were analysed as described under "Materials and Methods," using substrate concentrations of 400  $\mu\text{M}$  for testosterone and 500  $\mu\text{M}$  for diclofenac. Values are the mean  $\pm$  standard deviation of triplicate determinations. Figures in parentheses are the fold increase over wild type CYP2D6 values. ND is no detectable product.

<sup>b</sup> Data from (31).

<sup>c</sup> Data from (30).

ity, the kinetic parameters of this mutant were determined for diclofenac, tolbutamide, a CYP2C9 substrate which lacks a carboxylate group, and nifedipine, a non-steroidal CYP3A4 substrate (see Fig. 2 for structures). As shown in Table III, the  $k_{\text{cat}}$  values for each of these three compounds, which are not normally metabolized to a significant extent by CYP2D6, ranged between 1–5  $\text{min}^{-1}$ , indicating that the mutations have substantially altered the specificity of the enzyme. Although the E216Q/D301Q mutant showed a 2-fold increase in testosterone 6 $\beta$ -hydroxylase activity compared with the wild type enzyme (Table II), this is still 1000-fold lower than that observed for than CYP3A4 expressed in an equivalent system (30). By contrast, the *N*-oxidation of nifedipine by the E216Q/D301Q mutant is described by kinetic parameters much closer to values previously reported for CYP3A4 ( $K_m$  28 versus 9  $\mu\text{M}$ ,  $k_{\text{cat}}$  2 versus 6.5  $\text{min}^{-1}$ ; Ref. 27). For the two CYP2C9 substrates, the  $k_{\text{cat}}$  values measured with the CYP2D6 E216Q/D301Q mutant are only a factor of 2–5 lower than those reported for CYP2C9 itself (31, 32), and the  $K_m$  value for tolbutamide is comparable to that reported for CYP2C9 purified from human liver (32). However, it should be noted that

the  $K_m$  value for diclofenac is significantly higher (36-fold) than the average reported values (33) and that the catalytic efficiency ( $k_{\text{cat}}/K_m$ ) much lower (around 1%) than that of CYP2C9.

## DISCUSSION

CYP2D6 shows a clear, though not exclusive, preference for the metabolism of basic substrates that contain a nitrogen atom protonated at physiological pH. A common hypothesis has been that this may be due to electrostatic interactions of the protonated atom with the carboxylate group of Asp-301 (4, 16–20). Several studies have pointed to a possible role for a second carboxylate group, that of Glu-216, as a binding determinant, principally to explain the metabolism of the larger substrates with a basic nitrogen atom  $\geq 10$  Å from the site of oxidation (34–37). The proposed role for Glu-216 is consistent with our recent modeling study of CYP2D6 (21). This model also suggests a structural role for Asp-301, through the formation of a hydrogen bond with a residue in the flexible B'-C loop. This latter hypothesis is consistent with the fact that we and others (20) find that mutations to Asp-301 generally result in a less stable protein. It should be noted, however, that these mutations do not lead to a marked decrease in the  $k_{\text{cat}}$  of the enzyme toward substrates having a basic nitrogen or to the specific activity toward atypical substrates (Tables I and II); this suggests that any structural changes produced by mutation of this residue are unlikely to be gross.

Comparative analysis of the alignment of P450 sequences has shown that Asp-301 is present in a number of P450s that do not metabolize basic substrates, such as members of the 2C family that are known to hydroxylate steroids (21). Glu-216 is also not sufficient by itself to confer a preference for basic substrates as it is present without Asp-301 in P450s that do not metabolize basic substrates, such as the CYP73A family that preferentially metabolizes cinnamic acid (21). However, basic substrates are metabolized by all four known P450s that contain a residue equivalent to both Glu-216 and Asp-301; CYP2D6, CYP2D14 (bovine), CYP2D4 (rat), and CYP2J1 (rabbit). Thus indicating strongly that if both Asp-301 and Glu-216 are present, basic substrates appear to be preferred and both residues are important for the metabolism of these substrates.

It is clear from the results presented here, that removal of the negative charge at either position 216 or position 301 significantly increases the  $K_m$  values for the classical CYP2D6 substrates bufuralol and dextromethorphan (Table I). This shows clearly that the role of Glu-216 is thus not limited to

TABLE III  
Kinetic parameters of CYP2D6 E216Q/D301Q acting on atypical CYP2D6 substrates

Assays were performed in triplicate as described under Materials and Methods. The error values shown are standard deviations calculated from fitting the Michaelis-Menten equation to the data.

| Enzyme             | Diclofenac (4'-hydroxylation) |              | Tolbutamide (4-methyl-hydroxylation) |             | Nifedipine  |               |
|--------------------|-------------------------------|--------------|--------------------------------------|-------------|-------------|---------------|
|                    | $K_M$                         | $k_{cat}$    | $K_M$                                | $k_{cat}$   | $K_M$       | $k_{cat}$     |
|                    | $\mu M$                       | $min^{-1}$   | $\mu M$                              | $min^{-1}$  | $\mu M$     | $min^{-1}$    |
| CYP2D6 E216Q/D301Q | $108 \pm 10$                  | $5 \pm 0.10$ | $315 \pm 54$                         | $1 \pm 0.1$ | $28 \pm 3$  | $2 \pm 0.1$   |
| CYP2C9             | $3^a$                         | $22^a$       | $178-407^b$                          | $3-7^b$     |             |               |
| CYP3A4             |                               |              |                                      |             | $9 \pm 4^c$ | $7 \pm 0.5^c$ |

<sup>a</sup> Data from Ref. 33. The data represents the mean values calculated from a range of kinetic data.

<sup>b</sup> Data from Ref. 32.

<sup>c</sup> Data from Ref. 30.

substrates in which the basic nitrogen is relatively remote from the site of oxidation. It is apparent that the relative importance of these two residues is different for the two substrates studied; the  $K_m$  for bufuralol is similarly affected by the neutralization of either Glu-216 or Asp-301, whereas for dextromethorphan the substitution of Asp-301 clearly has a larger effect than that of Glu-216. The models of the CYP2D6-bufuralol and CYP2D6-dextromethorphan complexes (Fig. 1) give insight into a possible explanation for this. The basic nitrogen of both bufuralol and dextromethorphan is predicted (Fig. 1) to lie close to the negatively charged carboxylate group of Glu-216, but relatively distant from that of Asp-301. This in turn suggests the existence of an ionic interaction between Glu-216 and the basic nitrogen—an interaction that is removed when Glu-216 is replaced by anything other than Asp, hence the ~100-fold increase in  $K_m$ .

The models suggest that the role of Asp-301 is also electrostatic in nature but that this interaction is weaker than that with Glu-216. Despite this weaker interaction, the effect on  $K_m$  of mutating Asp-301 is at least as great as when Glu-216 is mutated. The models provide a possible explanation of this apparent paradox; they suggest that Asp-301 is responsible for maintaining the integrity of the active site *via* a hydrogen bond with the backbone amides of two residues in the B-C loop, Val-119 and Phe-120. Given this scenario, it might be expected that the mutation D301N would behave similarly to wild type; the modeling (consistent with the experimental  $K_m$ ) suggests that this might not be the case because the  $\beta$  amide is repelled by the backbone amide of Phe-120, and therefore in the D301N mutant the B-C loop, also known as substrate recognition site 1 (SRS 1), adopts a different conformation to that in the wild type enzyme. The D301Q mutant has an extra degree of freedom with respect to D301N, and modeling suggest that this substitution can be accommodated without the same need for structural rearrangement of the B-C loop as with D301N. The relative rigidity of dextromethorphan, and thus its decreased ability to adapt to changes in the active site, could explain why mutation of Asp-301 has a greater effect on the binding of dextromethorphan than on that of bufuralol. It is notable that the  $k_{cat}$  values are relatively unchanged while the  $K_m$  values are markedly increased. This suggests that, although these substrates bind much more weakly, they bind in a position and orientation appropriate for catalysis.

Glu-216 and Asp-301 also play an important role in the regiospecificity of CYP2D6 toward dextromethorphan. The wild type enzyme shows a strong (8-fold) preference for O-demethylation over N-demethylation, but this is essentially abolished by mutation of either or both of these residues to neutral side chains. These observations are consistent with the idea that the negatively charged "patch" in the active site created by these two residues (notwithstanding the structural rearrangement we suggest could result from removal of the

negative charge on Asp-301) is the principal determinant of the regiospecificity of CYP2D6 toward dextromethorphan and probably also toward other substrates that contain a basic nitrogen. While there are thus marked effects of both these negatively charged residues on the binding of basic substrates, it is also clear that for some neutral substrates, such as the spirosulphonamide studied by Guengerich *et al.* (10), the position of the bound substrate and/or its binding interactions with other residues in the active site are such that mutation of Asp-301 has little effect on binding.

A striking demonstration of a different aspect of the importance of Glu-216 and Asp-301 in determining the substrate specificity of CYP2D6 is afforded by the activity of the E216Q/D301Q mutant toward a number of compounds that are not normally metabolized to a significant extent by this enzyme. The double mutation increased the rate of 4'-hydroxylation of diclofenac 1000-fold to a  $k_{cat}$  value within a factor of five of that of CYP2C9, which plays the dominant role in diclofenac metabolism in man (38). Similar comparisons showed that CYP2D6 E216Q/D301Q metabolizes tolbutamide almost as well as CYP2C9 and nifedipine almost as well as CYP3A4 (although its rate of metabolism of testosterone fell well short of that of CYP3A4). These observations indicate that the size and shape of the CYP2D6 active site is such as to allow it to bind a wide range of compounds. (This range does not apparently extend to molecules the size of testosterone; we have earlier shown that testosterone binding is facilitated by the substitution of Phe-483 by a smaller residue (23)). The binding site of CYP2D6 is thus intrinsically rather promiscuous in nature, and the two residues Asp-301 and Glu-216, while they are obviously not the only factor, do play a crucial role in defining the CYP2D6 substrate specificity not simply by favoring the binding of basic substrates but also by discriminating against acidic substrates. It will be important to take into account this dual role of these two key residues in further development of predictive models of the specificity of CYP2D6.

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#### REFERENCES

1. Jones, B. C., Tyman, C. A., and Smith, D. A. (1997) *Xenobiotica* **27**, 1025–1037
2. Mahgoub, A., Idle, J. R., Dring, L. G., Lancaster, R., and Smith, R. L. (1977) *Lancet* **2**, 584–586
3. Eichelbaum, M., Spannbrucker, N., Steincke, B., and Dengler, H. J. (1979) *Eur. J. Clin. Pharmacol.* **16**, 183–187
4. Koymans, L., Vermeulen, N. P., van Acker, S. A., te Koppele, J. M., Heykants, J. J., Lavrijsen, K., Meuldermans, W., and Donne-Op den Kelder, G. M. (1992) *Chem. Res. Toxicol.* **5**, 211–219
5. Smith, G., Stanley, L. A., Sim, E., Starnge, R. C., and Wolf, C. R. (1995) *Cancer Survey* **25**, 27–65
6. McCann, S. J., Pond, S. M., James, K. M., and Le Couteur, D. G. (1997) *J. Neurol. Sci.* **153**, 50–53

7. Ekins, S., de Groot, M. J., and Jones, J. P. (2001) *Drug Metab. Dispos.* **29**, 936–944
8. Niwa, T., Yabusaki, Y., Honma, K., Matsuo, N., Tatsuta, K., Ishibashi, F., and Katagiri, M. (1998) *Xenobiotica* **28**, 539–547
9. Hiroi, T., Kishimoto, W., Chow, T., Imaoka, S., Igarashi, T., and Funae, Y. (2001) *Endocrinology* **142**, 3901–3908
10. Guengerich, F. P., Miller, G. P., Hanna, I. M., Martin, M. V., Léger, S., Black, C., Chauret, N., Silva, J. M., Trimble, L. A., Yergey, J. A., and Nicoll-Griffith, D. A. (2002) *Biochemistry* **41**, 11025–11034
11. Grace, J. M., Kinter, M. T., and Macdonald, T. L. (1994) *Chem. Res. Toxicol.* **7**, 286–290
12. Ghahramani, P., Ellis, S. W., Lennard, M. S., Ramsay, L. E., and Tucker, G. T. (1997) *Br. J. Clin. Pharmacol.* **43**, 137–144
13. Lin, L. Y., Di Stefano, E. W., Schmitz, D. A., Hsu, L., Ellis, S. W., Lennard, M. S., Tucker, G. T., and Cho, A. K. (1997) *Drug Metab. Dispos.* **25**, 1059–1064
14. Modi, S., Gilham, D. E., Sutcliffe, M. J., Lian, L. Y., Primrose, W. U., Wolf, C. R., and Roberts, G. C. (1997) *Biochemistry* **36**, 4461–4470
15. Yu, A., Dong, H., Lang, D., and Haining, R. L. (2001) *Drug Metab. Dispos.* **29**, 1362–1365
16. Islam, S. A., Wolf, C. R., Lennard, M. S., and Sternberg, M. J. (1991) *Carcinogenesis* **12**, 2211–2219
17. Ellis, S. W., Hayhurst, G. P., Smith, G., Lightfoot, T., Wong, M. M., Simula, A. P., Ackland, M. J., Sternberg, M. J., Lennard, M. S., Tucker, G. T., *et al.* (1995) *J. Biol. Chem.* **270**, 29055–29058
18. Modi, S., Paine, M. J., Sutcliffe, M. J., Lian, L. Y., Primrose, W. U., Wolf, C. R., and Roberts, G. C. (1996) *Biochemistry* **35**, 4540–4550
19. Mackman, R., Tschirret-Guth, R. A., Smith, G., Hayhurst, G. P., Ellis, S. W., Lennard, M. S., Tucker, G. T., Wolf, C. R., and Ortiz de Montellano, P. R. (1996) *Arch. Biochem. Biophys.* **331**, 134–140
20. Hanna, I. H., Kim, M. S., and Guengerich, F. P. (2001) *Arch. Biochem. Biophys.* **393**, 255–261
21. Kirtton, S. B., Kemp, C. A., Tomkinson, N. P., St.-Gallay, S., and Sutcliffe, M. J. (2002) *Proteins* **49**, 216–231
22. Williams, P. A., Cosme, J., Sridhar, V., Johnson, E. F., and McRee, D. E. (2000) *J. Inorg. Biochem.* **81**, 183–190
23. Smith, G., Modi, S., Pillai, L., Lian, L. Y., Sutcliffe, M. J., Pritchard, M. P., Friedberg, T., Roberts, G. C., and Wolf, C. R. (1998) *Biochem. J.* **331**, 783–792
24. Pritchard, M. P., Glancey, M. J., Blake, J. A., Gilham, D. E., Burchell, B., Wolf, C. R., and Friedberg, T. (1998) *Pharmacogenetics* **8**, 33–42
25. Pritchard, M. P., Ossetian, R., Li, D. N., Henderson, C. J., Burchell, B., Wolf, C. R., and Friedberg, T. (1997) *Arch. Biochem. Biophys.* **345**, 342–354
26. Kunkel, T. A., Roberts, J. D., and Zakour, R. A. (1987) *Methods Enzymol.* **154**, 367–382
27. Strobel, H. W., and Dignam, J. D. (1978) *Methods Enzymol.* **52**, 89–96
28. Sali, A., and Blundell, T. L. (1993) *J. Mol. Biol.* **234**, 779–815
29. Jones, G., Willett, P., Glen, R. C., Leach, A. R., and Taylor, R. (1997) *J. Mol. Biol.* **267**, 727–748
30. Fowler, S. M., Riley, R. J., Pritchard, M. P., Sutcliffe, M. J., Friedberg, T., and Wolf, C. R. (2000) *Biochemistry* **39**, 4406–4414
31. Mancy, A., Antignac, M., Minoletti, C., Dijols, S., Mouries, V., Duong, N. T., Battioni, P., Dansette, P. M., and Mansuy, D. (1999) *Biochemistry* **38**, 14264–14270
32. Lasker, J. M., Wester, M. R., Aramsombatdee, E., and Raucy, J. L. (1998) *Arch. Biochem. Biophys.* **353**, 16–28
33. Lee, C. R., Goldstein, J. A., and Pieper, J. A. (2002) *Pharmacogenetics* **12**, 251–263
34. de Groot, M. J., Ackland, M. J., Horne, V. A., Alex, A. A., and Jones, B. C. (1999) *J. Med. Chem.* **42**, 4062–4070
35. Lewis, D. F., Eddershaw, P. J., Goldfarb, P. S., and Tarbit, M. H. (1997) *Xenobiotica* **27**, 319–339
36. Lewis, D. (1998) *Xenobiotica* **28**, 617–661
37. Venhorst, J., Onderwater, R. C., Meerman, J. H., Commandeur, J. N., and Vermeulen, N. P. (2000) *Drug. Metab. Dispos.* **28**, 1524–1532
38. Yamazaki, H., Inoue, K., Chiba, K., Ozawa, N., Kawai, T., Suzuki, Y., Goldstein, J. A., Guengerich, F. P., and Shimada, T. (1998) *Biochem. Pharmacol.* **56**, 243–251