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A Combination of Mutations in the *CYP2D6*17* (*CYP2D6Z*) Allele Causes Alterations in Enzyme Function

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SUMMARY

In many black African populations, the capacity for CYP2D6-dependent drug metabolism is generally reduced. A specific variant of the *CYP2D6* gene (*CYP2D6*17*) that carries three functional mutations (T107I, R296C, and S486T) has been found to be present in Zimbabwean subjects with impaired CYP2D6-dependent hydroxylase activity. To evaluate whether the *CYP2D6*17* allele was the major cause behind the decreased rate of drug metabolism and to examine the role of the different mutations, CYP2D6 cDNAs containing all eight combinations of the mutations were created. Expression of the cDNAs in COS-1 cells revealed that the CYP2D6 17 enzyme displayed only 20% of the wild-type (CYP2D6 1) activity, whereas the T107I substitution on its own had no significant effect on enzyme function. Expression in yeast showed that the three possible single amino-acid mutant CYP2D6 variants all had properties similar to CYP2D6 1 when the kinetics of bufuralol hydroxylation was examined. However, enzymes containing both the T107I and R296C mutations exhibited a more than 5-fold higher K_m for bufuralol than the wild-type enzyme, whereas the S486T mutation was of little importance. In contrast, when codeine was used as a substrate, the T107I substitution alone was sufficient to cause a significant increase in the apparent K_m, indicating a differential effect for this substitution depending on the CYP2D6 substrate. In conclusion, the *CYP2D6*17* allele represents the first human cytochrome P450 polymorphic variant in which a combination of substitutions is required to alter the enzyme's catalytic properties and is the first case in which a decreased CYP2D6 activity, as monitored *in vivo,* has been documented to be caused by an enzyme with altered affinity for CYP2D6 substrates.

The human P450 2D6 (CYP2D6; debrisoquine hydroxylase) catalyzes the oxidative metabolism of several important classes of drugs, including tricyclic antidepressants and some of the novel selective serotonin reuptake inhibitors, most antipsychotic agents, antiarrhythmic drugs, β-adrenergic antagonists, and codeine (1–3). The CYP2D6-dependent conversion of codeine to morphine has been shown to be required for the analgesic effect of codeine (4). Moreover, this enzyme has been implicated to be involved in the pathogenesis of several cancer forms and other diseases (5, 6).

A tremendous variation in CYP2D6 enzyme activity has been shown among individuals, mainly because of genetic polymorphism of the *CYP2D6* gene. Historically speaking, the population has been divided into two phenotypes (7): extensive metabolizers (EMs), who exhibit CYP2D6 activity, and PMs, who lack CYP2D6 activity. In addition, ultrarapid metabolizers have been identified who carry multiple copies of active *CYP2D6* genes (8). In Caucasian populations, the prevalence of PMs is 5–10% (9), the majority of whom (90–95%) are homozygous for any of the known defective *CYP2D6* alleles or combinations thereof (10). Alleles that cause reduced but not abolished activity, *CYP2D6*9*[1] [C; (11)] and *CYP2D6*10* [J, Ch_1, Ch_2; (12)], have also been found. Here, the reduced rate of drug metabolism seen *in vivo* is explained by mutations in the *CYP2D6* gene that cause a less stable enzyme.

Besides interindividual differences in CYP2D6-dependent drug metabolism, there are also remarkable interethnic differences. In this respect, essentially no PMs are seen in the Asian (13) and black African populations (14 and references therein), but the capacity for CYP2D6-dependent drug metabolism is generally diminished. Among Asians, this is caused by the frequent distribution of the *CYP2D6*10B* allele encoding the unstable CYP2D6 10 enzyme (12).

The *CYP2D6*17* (*CYP2D6Z*) allele, common among black Zimbabweans, has been described recently (15). This gene contains four mutations, of which one is silent, thus yielding the three amino acid exchanges T107I, R296C, and S486T.

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[1] In this report we have used the new nomenclature system for *CYP2D6* alleles (10).

ABBREVIATIONS: P450, cytochrome P450; PM, poor metabolizer; reductase, NADPH cytochrome P450 reductase; HPLC, high performance liquid chromatography; SRS, substrate recognition site.

The distribution of this allele correlates well with decreased CYP2D6 activity seen *in vivo* measured as metabolic ratio of the probe drug debrisoquine. We considered it of interest to study whether the CYP2D6 17 enzyme exhibited altered catalytic properties and to investigate the role of the different mutations in this respect using two different heterologous expression systems, mammalian COS-1 cells and yeast cells. The data indicate that CYP2D6 17 exhibits an altered substrate affinity for CYP2D6 substrates and that a combination of the T107I and R296C amino-acid substitutions are required for altered catalytic properties of the enzyme toward hydroxylation of bufuralol.

Experimental Procedures

Site-Directed Mutagenesis and Expression Vectors

Mutant CYP2D6 cDNAs that contained the C1111T, C2938T, and G4268C mutations (numbering based on genomic DNA) were generated with the USE mutagenesis kit (Pharmacia Biotech, Uppsala, Sweden), using a wild-type CYP2D6 cDNA subcloned into a pBluescript KS vector (Stratagene, LaJolla, CA), and mutagenic primers 5′-GTG CCC ATC ATC CAG ATC CTG-3′, 5′-GAG AAC CTG TGC ATA GTG GT-3′, and 5′-CCT GGT GAC CCC ATC CCC CT-3′ respectively (mutations underlined). The reactions were carried out according to the manufacturer's recommendations; the incorporation of the mutations were confirmed by DNA sequencing. Using the restriction enzymes *Bss*HII and *Tth*111 I, eight constructs that contained all the possible combinations of these three mutations were created. The variant cDNAs were subsequently subcloned into the V60 (pYeDP60) yeast expression vector (16) and into the mammalian cell expression vector pCMV4 (17) using the restriction enzymes *Bam*HI and *Kpn*I.

Expression of Mutated cDNAs in COS-1 Cells

COS-1 cells were transfected with the pCMV4–2D6 plasmids described above as reported previously (12). After a 50-hr incubation, cells for Western blot analysis and bufuralol 1′-hydroxylase activity measurements were harvested in 100 mM sodium phosphate buffer, pH 7.4, sonicated for 20 × 1 sec, and then centrifuged at 10,000 × *g* for 10 min at 4°.

Quantification of CYP2D6 mRNA Levels, Apoprotein Levels, and Catalytic Activities in COS-1 Cells

Total RNA was prepared from transfected COS-1 cells using Trizol Reagent (Life Technologies, Rockville, MD). Northern Blot analysis was carried out with 7 μg of total RNA using ^{32}P-labeled CYP2D6 cDNA and β-actin cDNA as probes. For apoprotein quantification, cell supernatant (10,000 × *g*) corresponding to 1 μg of protein was subjected to sodium dodecyl sulfate gel electrophoresis using 8.7% polyacrylamide gels. The proteins were transferred to a nitrocellulose filter (BioRad, Hemel Hempstead, UK), incubated with the monoclonal mouse-anti-2D6 antibody 114/2 and subsequently a horseradish-peroxidase linked goat anti-mouse antibody (DAKO AS, Glostrup, Denmark). The enhanced chemiluminescence method (Amersham, Buckinghamshire, UK) was used to visualize the proteins. Quantification of the Northern and Western blot results was done using a personal densitometer (Molecular Dynamics, Sunnyvale, CA).

Cell supernatant corresponding to 100 μg of protein was incubated at 37° in 100 mM sodium phosphate buffer, pH 7.4, with 100 μM (+)-bufuralol, 75 μg NADPH, and 200 pmol of rat P450 reductase [purified as described previously (18)] in a total volume of 75 μl for 30 min; the reaction was terminated by the addition of 7.5 μl of 70% perchloric acid. The samples were centrifuged at 15,000 × *g* for 5 min and the supernatants subjected to reversed-phase HPLC as described previously (19). Linearity of the reaction for at least 30 min was established.

Expression of Mutated cDNAs in Yeast Cells

Saccharomyces cerevisiae strain W(R), a strain that has been genetically engineered to overexpress the yeast reductase (Yred) (20), was transfected with the above described V60–2D6-plasmids. Expression of CYP2D6 was carried out essentially as described previously (21). Briefly, precultures of transfected yeast cells were grown in SGI medium (l g/liter Casamino acids, 7 g/liter yeast nitrogen base, 20 g/liter glucose, 20 mg/liter tryptophan) to a density of approximately 30 × 10^6 cells/ml, and diluted to 2.5 × 10^6 cells/ml in YPGE medium (10 g/liter yeast extract, 10 g/liter BactoPeptone, 5 g/liter glucose, 2% ethanol). The cells were then grown for another 22–24 hr. Galactose was subsequently added to a final concentration of 2% (w/v) to induce the galactose-driven promoter. After a 16-hr incubation, the cells were harvested by centrifugation. After mechanical disruption of the cell walls, yeast cell microsomes were prepared by polyethylene glycol precipitation of the 20,000 × *g* supernatants, essentially as described previously (22).

Assay of CYP2D6 Holoprotein and Reductase

Microsomal protein concentration (23), reductase levels (24), and total P450 content estimated from the CO-induced spectrum of dithionite-reduced yeast microsomes (25) were determined using previously described methods.

Kinetic analysis of CYP2D6 Activity in Yeast Microsomes

Bufuralol 1′-hydroxylation. Yeast microsomes corresponding to 150 μg of protein were incubated at 37° in 100 mM sodium phosphate buffer, pH 7.4, with 0–40 μM (+)-bufuralol and 120 μg of NADPH in a total volume of 300 μl. The reactions were terminated by the addition of 30 μl of 70% perchloric acid, and linearity with time was ensured in all cases. The samples were subsequently centrifuged at 15,000 × *g* for 5 min and the supernatants subjected to a reversed-phase HPLC system essentially as described by Kronbach *et al.* (19). 1′-Hydroxybufuralol was used as an external standard. Analysis of the pH dependency of the reactions was carried out under half-saturated enzyme conditions [2 μM and 10 μM of (+)-bufuralol for the CYP2D6 1 and CYP2D6 17 variants, respectively] in 100 mM sodium phosphate or 100 mM Tris·HCl buffers with a range of different pH values.

Codeine *O*-demethylation. Yeast microsomes corresponding to 200 μg of protein were incubated at 37° in 100 mM sodium phosphate buffer, pH 7.4, with 0–6 mM codeine and 120 μg of NADPH in a total volume of 100 μl. The reactions were terminated by the addition of 25 μl of acetonitrile, and the samples were subsequently centrifuged at 15,000 × *g* for 10 min. The supernatants were transferred to fresh tubes and extractions were done according to Ladona et al. (26). After evaporation under a steam of N_2, the remaining residues were dissolved in 50 μl of mobile phase and subjected to a reversed-phase HPLC system with an electrochemical detection method. The mobile phase consisted of 5% acetonitrile, 8% methanol, 0.5 mM EDTA, and 70 mM KH_2PO_4. Morphine was used as an external standard.

Data Analysis

Apparent K_m and V_{max} parameters from the kinetic studies were determined from Lineweaver-Burke plots. Student's *t* test was used for intergroup comparisons.

Results

The three functional mutations present in the *CYP2D6*17* allele were introduced using site-directed mutagenesis in all eight combinations into a CYP2D6 cDNA. Four of the different CYP2D6 cDNAs [wild-type (CYP2D6 1), T107I, R296C + S486T, and T107I + R296C + S486T (CYP2D6 17)] were

subcloned into the pCMV4 expression vector and expressed in COS-1 cells. Cells transfected with an empty pCMV4 vector were used as a negative control. The CYP2D6 mRNA expression was measured by Northern blot analysis and CYP2D6 apoprotein levels were quantified by Western blot analysis. The CYP2D6-dependent catalytic activities were determined by measuring NADPH-dependent 1′-hydroxylation of bufuralol in 10,000 × *g* supernatants of transfected cells to which purified reductase had been added.

All cDNA variants examined, except for the pCMV4 vector alone, yielded CYP2D6 mRNA and immunodetectable CYP2D6 apoprotein. No major difference in mRNA levels was seen between cells transfected with different cDNAs (Fig. 1A). Introduction of the T107I substitution alone yielded higher CYP2D6 levels, whereas the combination of the R296C and S486T substitutions with or without the T107I exchange resulted in decreased CYP2D6 levels. This is indicative of a slightly more unstable enzyme (Fig. 1B), although a decreased translation efficiency cannot be excluded. Determination of the catalytic activity using bufuralol as a substrate revealed that the mutant enzyme having the R296C and S486T substitutions (corresponding to CYP2D6 2) exhibited somewhat reduced activity compared with CYP2D6 1, which is in agreement with recent *in vivo* data presented by Sachse *et al.* (27), whereas CYP2D6 17 had only 20% of the hydroxylase activity seen using CYP2D6 1. In contrast, the T107I substitution alone did not significantly alter the bufuralol hydroxylase activity (Fig. 1C).

To further study the mechanism by which the three amino acid substitutions interact, all different cDNAs were subcloned into the V60 yeast expression vector (16), where the expression is driven by a galactose-inducible promoter. These were subsequently expressed in *S. cerevisiae* W(R), a strain genetically engineered to overexpress the yeast reductase (Yred) (20). Because the *Yred* gene is also under the control of a galactose-inducible promoter, the microsomal levels of Yred were measured as an indicator of induction (Table 1).

All plasmids except for the V60 vector alone yielded immunodetectable apoprotein (Fig. 2) and had similar expression of reductase, indicating a similar extent of galactose-dependent induction in all cases. The CYP2D6 levels were determined spectrally from the reduced CO-bound form. There was a tendency, although not significant, that enzymes that contained the R296C substitution yielded somewhat lower expression levels (Table 1), consistent with the COS-1 cell expression data.

Kinetic studies using bufuralol were carried out in microsomes isolated from yeast expressing all eight different cDNAs. These experiments revealed that all the corresponding enzymes had similar apparent V_{max} for bufuralol 1′-hydroxylation (Fig. 3B). When the three amino acid substitutions present in the CYP2D6 17 mutant (T107I, R296C, and S486T) were introduced one by one, no significant effect on the enzyme properties was observed. However, when the amino acid substitutions were introduced in various combinations, it was found that both the T107I and R296C substitutions were required for an enzyme with altered kinetic properties of bufuralol (Fig. 3A). The CYP2D6 17 enzyme exhibited a 5-fold higher apparent K_m compared with CYP2D6 1.

Also the kinetics of codeine *O*-demethylation were analyzed in microsomes isolated from yeast transfected with either CYP2D6 1 (wt), CYP2D6 2 (R296C and S486T) or CYP2D6 17 (T107I, R296C, and S486T) cDNA as well as with the plasmid encoding only the T107I exchange. The apparent K_m for codeine was 5- to 10-fold higher for CYP2D6 17 compared with CYP2D6 1 (Fig. 4). In contrast to the finding with bufuralol, the T107I substitution alone caused a significant increase in K_m. In addition, the V_{max} for codeine *O*-demethylation catalyzed by CYP2D6 2 was significantly higher than for the other CYP2D6 enzyme variants.

To further analyze the molecular mechanism behind the differences between CYP2D6 1 and CYP2D6 17, the effect of pH on 1′-hydroxylation of bufuralol was examined in the yeast microsomes. The pH optimum was significantly higher for CYP2D6 1 compared with CYP2D6 17 (7.41 ± 0.11 versus 7.22 ± 0.05, $n = 4$, $p = 0.01$). As shown in Fig. 5, there was a statistically significant difference at higher pH values, and CYP2D6 1 was active throughout a wider pH range. This difference indicates an important role for charged amino acid(s) in the active site.

Discussion

The results from this study show that the decreased CYP2D6 activity seen in individuals with the *CYP2D6*17* allele is caused by an enzyme with a reduced affinity for some of the CYP2D6 substrates. It was found unexpectedly that a combination of the T107I and R296C substitutions was needed to alter the kinetic properties of the CYP2D6 17 variant, because no significant effect was seen on bufuralol 1′-hydroxylation when they were introduced one by one. In contrast, examination of the rate of *O*-demethylation of codeine revealed a significant change in the apparent K_m when only the T107I substitution was introduced. Also, in the COS-1 expression system, there was a combined effect of the substitutions, affecting the enzyme activity.

The *CYP2D6*17* allele is frequently distributed among Zimbabweans [allele frequency of 34.0% (15)], Ethiopians [allele frequency of 9.0% (28)], and other African populations. Thus, more than 10% of the Zimbabwean population is homozygous for this allele, with important implications for pharmacological treatment with CYP2D6 substrates.

It seems that the C1111T mutation (yielding the T107I substitution) is always linked to the C2938T (R296C) and G4268C (S486T) mutations on the same allele (15). It cannot be excluded, however, that there are alleles that only contain the C1111T mutation or alleles where this mutation is linked to other mutations in the *CYP2D6* gene. This possibility is interesting because the T107I substitution had a significant effect on the apparent K_m of codeine *O*-demethylation, whereas no effect was seen when bufuralol was used as a substrate, a result consistent with the fact that these two substrates are structurally unrelated.

It is noteworthy that the correlation between the phenotypes obtained using different probe drugs is generally lower in black African than in other populations. The correlation coefficient between the metabolic ratio for debrisoquine and metoprolol has been shown to be only 0.16–0.67 in studies carried out on black African subjects (14, 29–31), compared with 0.81 in a Caucasian population (32). Future studies are needed to evaluate whether the distribution of the *CYP2D6*17* allele, creating an enzyme with altered substrate specificity, can explain this lack of correlation.

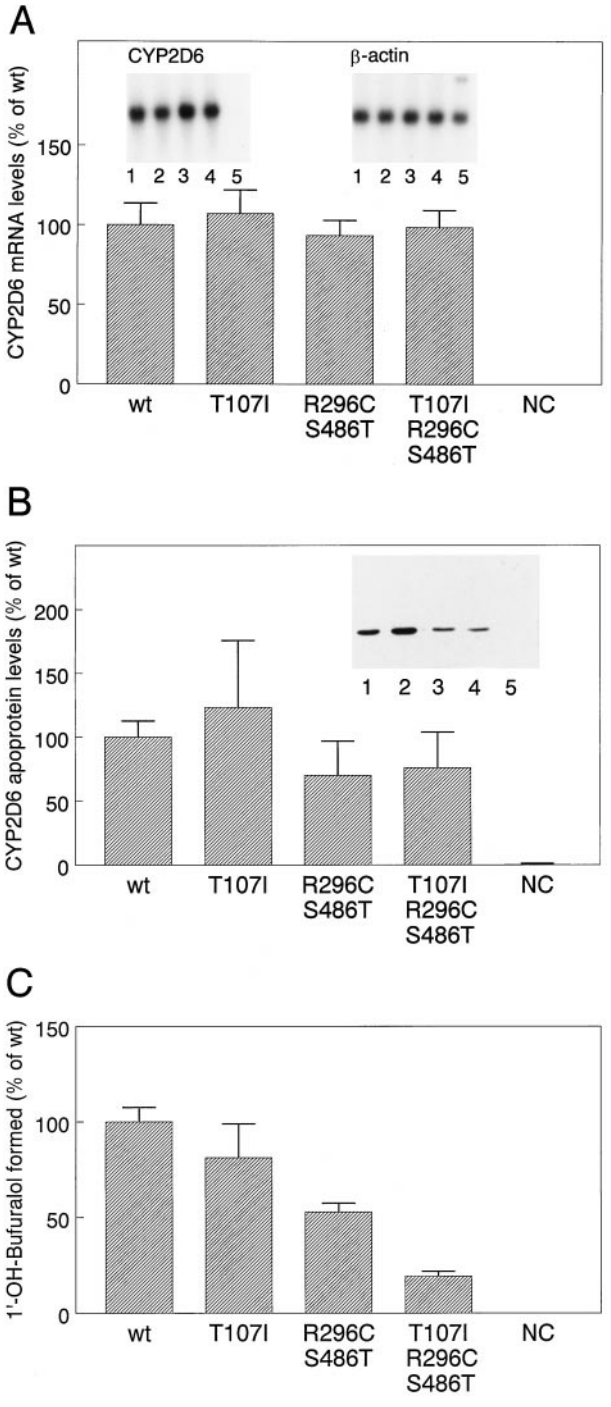

Fig. 1. Expression of different CYP2D6 cDNAs in COS-1 cells. A, Northern blot analysis. CYP2D6 and β-actin mRNA levels were measured in cells transfected with the different plasmids, and the ratio between CYP2D6 mRNA and β-actin mRNA levels was calculated. *Inset,* representative Northern blot analysis. *Lane 1*, wild-type; *lane 2*, T107I; *lane 3*, R296C+S486T; *lane 4*, T107I+R296C+S486T; *lane 5*, negative control. B, Western blot analysis with densitometric quantification of the CYP2D6 apoprotein levels. *Inset,* representative Western blot analysis of the amount of CYP2D6 in the cell supernatants. *Lane 1*, wild-type; *lane 2*, T107I; *lane 3*, R296C+S486T; *lane 4*, T107I+R296C+S486T; *lane 5*, negative control. C, Rate of bufuralol 1′-hydroxylation activity as measured in 10,000 × *g* supernatants. Data are expressed as a percentage of the level/activity seen in cells transfected with CYP2D6*1 cDNA. The results represent data (mean ± standard deviation) from two (A) or three (B and C) experiments performed in duplicate. Plasmids from two independent plasmid preparations were used for transfection. *wt*, wild-type.

TABLE 1

Characterization of yeast microsomes obtained from the S. cerevisiae P450 reductase overexpressing strain W(R) transfected with CYP2D6 cDNAs containing various combinations of mutations. The data represent mean ± standard deviation of experiments from three independent batches of transfected cells.

Amino acid substitutions	CYP2D6 holoprotein level[a]	Reductase level
	pmol/mg	
None	11.6 ± 5.4	1660 ± 350
T107I	10.8 ± 6.8	1520 ± 500
R296C	9.7 ± 4.1	1490 ± 540
S486T	11.0 ± 3.9	1710 ± 470
T107I + R296C	8.0 ± 2.7	1530 ± 300
T107I + S486T	8.8 ± 3.4	1520 ± 490
R296C + S486T	7.2 ± 2.3	1640 ± 480
T107I + R296C + S486T	6.1 ± 3.6	1530 ± 320

[a] The endogenous P450 levels obtained in microsomes from yeast transfected with the empty V60 vector (0.6 ± 0.6 pmol/mg) have been subtracted from the levels obtained in the microsomes from CYP2D6 expressing yeast cells.

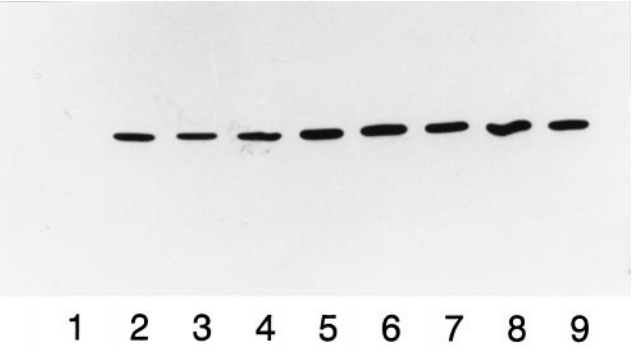

Fig. 2. Western blot analysis of CYP2D6 variants expressed in *S. cerevisiae*. *Lane 1*, negative control; *lane 2*, wild-type; *lane 3*, R269C+S486T; *lane 4*, T107I; *lane 5*, R296C; *lane 6*, S486T; *lane 7*, T107I+R296C; *lane 8*, T107I+S486T; *lane 9*, T107I+R296C+S486T.

In the absence of a crystal structure for CYP2D6 or any other mammalian P450s, homology building models based on the bacterial P450 structures have been used for the prediction of residue positions in the three-dimensional structure of the enzyme (33–35). By structurally aligning these three bacterial P450s, an extended multiple sequence alignment was made, including representatives from the major mammalian P450 families (36). According to this model, the Thr107 residue is located in the B′ helix, a region of the molecule known to be involved in substrate binding. Gotoh (37) defined six SRSs for the CYP2 family, and Thr107 is localized in SRS 1, thereby implicating an important function for this residue in substrate binding. Modi *et al.* (34) have combined homology modeling techniques and NMR studies to build a model of the CYP2D6 active site and its interaction with codeine. From this model, it is apparent that Thr107 is involved in the binding of codeine and that the substitution of the hydrophilic residue threonine to a hydrophobic isoleucine is likely to affect the substrate binding. This is compatible with the experimental data here presented, because the T107I CYP2D6 variant exhibited reduced affinity for codeine but not for bufuralol. This model also shows that Asp301 is involved in the binding of the basic nitrogen found in codeine and all other CYP2D6 substrates (38). In support of this, Ellis *et al.* (39) have studied the importance of this residue using site-directed mutagenesis and shown that an acidic

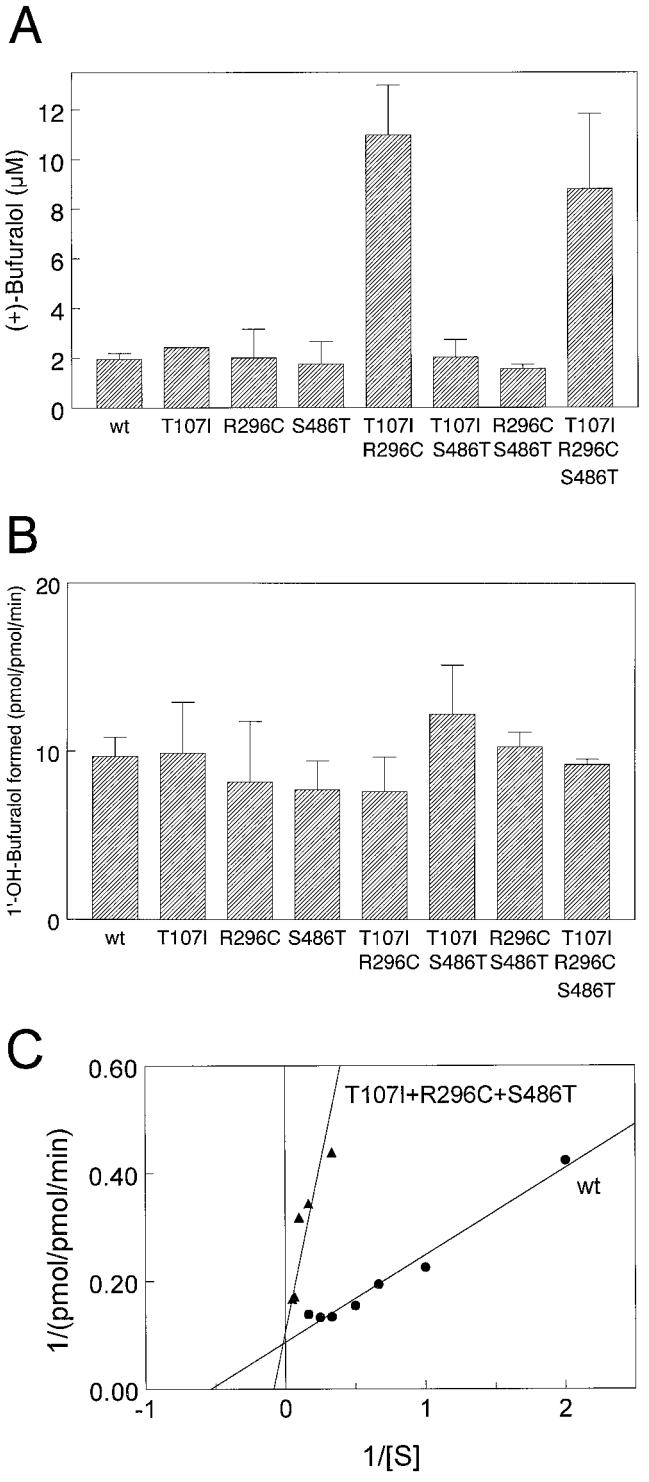

Fig. 3. Kinetic analysis of bufuralol 1′-hydroxylation in yeast microsomes obtained from *S. cerevisiae* strain W(R) transfected with CYP2D6 cDNA containing various combinations of mutations. Apparent K_m (A) and V_{max} (B) for the reaction are shown. The data represent mean ± standard deviation from at least three independent experiments carried out in different batches of transfected yeast cells. C, Representative Lineweaver-Burke plot for the CYP2D6 1 (wild-type; ●) and CYP2D6 17 (T107I+R296C+S486T; ▲) variants. *wt,* wild-type.

amino acid is required at this position, because activities were diminished in all of the mutants except where aspartic acid was replaced with glutamic acid.

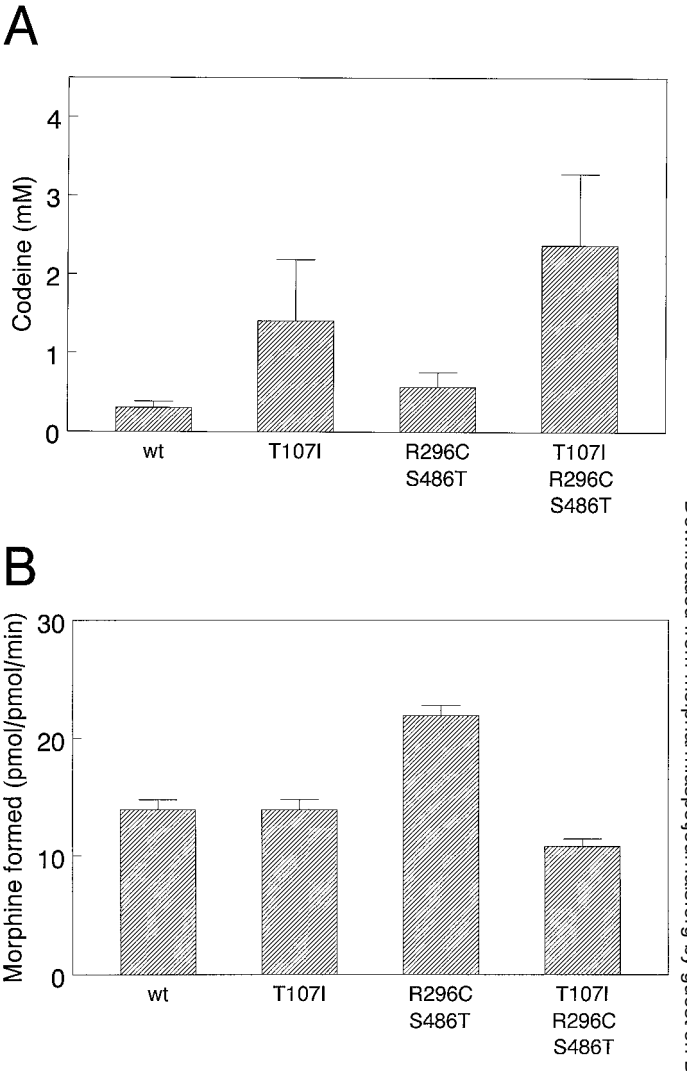

Fig. 4. Kinetic analysis of codeine *O*-demethylation in yeast microsomes obtained from *S. cerevisiae* strain W(R) transfected with CYP2D6 cDNA containing various combinations of mutations. Apparent K_m (A) and V_{max} (B) for the reaction are shown. The data represent mean ± standard deviation from three independent experiments carried out in different batches of transfected yeast cells. *wt,* wild-type.

The Arg296 residue is located in the I helix near Asp301. This helix is the longest one found in P450s and is known to be involved not only in substrate binding but also in delivery of catalytic protons, with the central part of the helix coinciding with SRS 4. Because the CYP2D6 17 exhibited a more acidic pH-optimum than the wild-type enzyme, it can be speculated that the R296C substitution affects the pK_a of some residue(s) involved in substrate contact or electron transfer. The β-pleated sheet region of the enzyme where the Ser486 residue is located is less well conserved between bacterial and mammalian P450s and predictions are difficult to make.

Based on this structural information, it is evident that at least both Thr107 and Arg296 are located near the CYP2D6 active site. This is in agreement with the kinetic data, which shows that these two residues were necessary for the effect observed on the enzyme's catalytic properties. It must be pointed out, however, that the substitutions introduced at these positions are much more drastic than the conserved

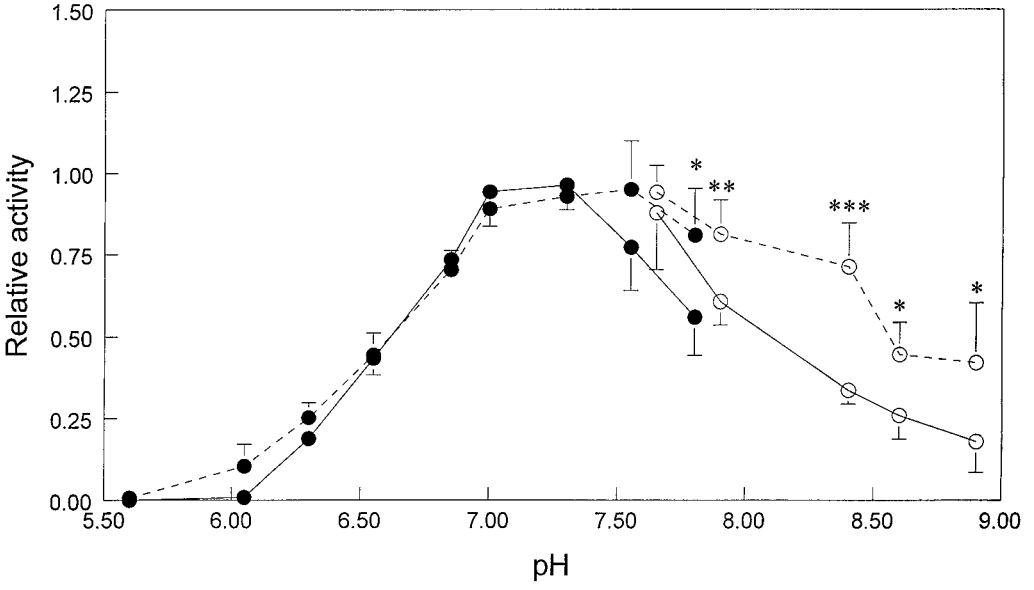

Fig. 5. Determination of pH dependence of bufuralol 1′-hydroxylation activity. Microsomes obtained from yeast expressing CYP2D6 1 (*dashed line*) or CYP2D6 17 (*solid line*) were incubated with bufuralol at half-saturated enzyme conditions at various pH values with 100 mM sodium phosphate (●) or 100 mM Tris·HCl (○) as buffer. Values are expressed as percentage of maximal activity and represent mean ± standard deviation of four independent experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

S486T substitution, and it is difficult to predict what conformational changes in the protein structure these substitutions will cause. The combined effect of the T107I and R296C substitutions on bufuralol kinetics remains partly unexplained, because no major effect was seen when these substitutions were introduced independently. High-resolution models of CYP2D6, and especially the active site structure, are needed to further understand this combined effect.

In conclusion, we have identified a molecular explanation for the decreased *in vivo* activity seen in individuals with the *CYP2D6*17* allele. This allele represents a unique case in which reduced CYP2D6 activity is caused by a decrease in substrate affinity; this allele is also interesting, in that a combination of substitutions is necessary to yield a P450 enzyme with altered activity. The CYP2D6 17 variant constitutes a challenging model for future structural studies and can provide important information concerning CYP2D6 active site structure-function relationships. The finding of the properties of CYP2D6 17 emphasizes the importance of the pronounced interethnic differences with respect to the *CYP2D6* gene and indicates interethnic differences in the metabolism of CYP2D6 substrates also from a qualitative point of view. This might be of importance with respect to drug efficacy and drug toxicity.

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References

1. Dahl, M. L., and L. Bertilsson. Genetically variable metabolism of antidepressants and neuroleptic drugs in man. *Pharmacogenetics* **3:**61–70 (1993).
2. Spina, E., and A. P. Caputi. Pharmacogenetic aspects in the metabolism of psychotropic drugs: pharmacokinetic and clinical implications. *Pharmacol. Res.* **29:**121–137 (1994).
3. Eichelbaum, M., and A. S. Gross. The genetic polymorphism of debrisoquine/sparteine metabolism–clinical aspects. *Pharmacol. Ther.* **46:**377–394 (1990).
4. Sindrup, S. H., and K. Brøsen. The pharmacogenetics of codeine hypoalgesia. *Pharmacogenetics* **5:**335–346 (1995).
5. Rannug, A., A. K. Alexandrie, I. Persson, and M. Ingelman-Sundberg. Genetic polymorphism of cytochromes P450 1A1, 2D6 and 2E1: regulation and toxicological significance. *J. Occup. Environ. Med.* **37:**25–36 (1995).
6. Raunio, H., K. Husgafvel-Pursiainen, S. Anttila, E. Hietanen, A. Hirvonen, and O. Pelkonen. Diagnosis of polymorphisms in carcinogen-activating and inactivating enzymes and cancer susceptibility–a review. *Gene* **159:** 113–121 (1995).
7. Mahgoub, A., J. R. Idle, L. G. Dring, R. Lancaster, and R. L. Smith. Polymorphic hydroxylation of debrisoquine in man. *Lancet* **2:**584–586 (1977).
8. Johansson, I., E. Lundqvist, L. Bertilsson, M. L. Dahl, F. Sjöqvist, and M. Ingelman-Sundberg. Inherited amplification of an active gene in the cytochrome P450 CYP2D locus as a cause of ultrarapid metabolism of debrisoquine. *Proc. Natl. Acad. Sci. USA* **90:**11825–11829 (1993).
9. Alván, G., P. Bechtel, L. Iselius, and U. Gundert-Remy. Hydroxylation polymorphisms of debrisoquine and mephenytoin in European populations. *Eur. J. Clin. Pharmacol.* **39:**533–537 (1990).
10. Daly, A. K., J. Brockmöller, F. Broly, M. Eichelbaum, W. E. Evans, F. J. Gonzalez, J. D. Huang, J. R. Idle, M. Ingelman-Sundberg, T. Ishizaki, E. Jacqz-Aigrain, U. A. Meyer, D. W. Nebert, V. M. Steen, C. R. Wolf, and U. M. Zanger. Nomenclature for human CYP2D6 alleles. *Pharmacogenetics* **6:**193–201 (1996).
11. Tyndale, R., T. Aoyama, F. Broly, T. Matsunaga, T. Inaba, W. Kalow, H. V. Gelboin, U. A. Meyer, and F. J. Gonzalez. Identification of a new variant CYP2D6 allele lacking the codon encoding Lys-281: possible association with the poor metabolizer phenotype. *Pharmacogenetics* **1:**26–32 (1991).
12. Johansson, I., M. Oscarson, Q. Y. Yue, L. Bertilsson, F. Sjöqvist, and M. Ingelman-Sundberg. Genetic analysis of the Chinese cytochrome P4502D locus: characterization of variant CYP2D6 genes present in subjects with diminished capacity for debrisoquine hydroxylation. *Mol. Pharmacol.* **46:** 452–459 (1994).
13. Bertilsson, L., Y. Q. Lou, Y. L. Du, Y. Liu, T. Y. Kuang, X. M. Liao, K. Y. Wang, J. Reviriego, L. Iselius, and F. Sjöqvist. Pronounced differences between native Chinese and Swedish populations in the polymorphic hydroxylations of debrisoquin and S-mephenytoin. *Clin. Pharmacol. Ther.* **51:**388–397 (1992).
14. Masimirembwa, C., J. Hasler, L. Bertilsson, I. Johansson, O. Ekberg, and M. Ingelman-Sundberg. Phenotype and genotype analysis of debrisoquine hydroxylase (CYP2D6) in a black Zimbabwean population—reduced enzyme activity and evaluation of metabolic correlation of CYP2D6. *Eur. J. Clin. Pharmacol.* **51:**117–122 (1996).
15. Masimirembwa, C., I. Persson, L. Bertilsson, J. Hasler, and M. Ingelman-Sundberg. a novel mutant variant of the CYP2D6 gene (CYP2D6*17) common in a black African population—association with diminished debrisoquine hydroxylase activity. *Br. J. Clin. Pharmacol.* **42:**713–719 (1996).
16. Urban, P., C. Cullin, and D. Pompon. Maximizing the expression of mammalian cytochrome P-450 monooxygenase activities in yeast cells. *Biochimie* **72:**463–472 (1990).
17. Andersson, S., D. L. Davis, H. Dahlbäck, H. Jörnvall, and D. W. Russell.

Cloning, structure, and expression of the mitochondrial cytochrome P-450 sterol 26-hydroxylase, a bile acid biosynthetic enzyme. *J. Biol. Chem.* **264:**8222–8229 (1989).
18. Ingelman-Sundberg, M., and H. Glaumann. Incorporation of purified components of the rabbit liver microsomal hydroxylase system into phospholipid vesicles. *Biochim. Biophys. Acta* **599:**417–435 (1980).
19. Kronbach, T., D. Mathys, J. Gut, T. Catin, and U. A. Meyer. High-performance liquid chromatographic assays for bufuralol 1′-hydroxylase, debrisoquine 4-hydroxylase, and dextromethorphan *O*-demethylase in microsomes and purified cytochrome P-450 isozymes of human liver. *Anal. Biochem.* **162:**24–32 (1987).
20. Truan, G., C. Cullin, P. Reisdorf, P. Urban, and D. Pompon. Enhanced in vivo monooxygenase activities of mammalian P450s in engineered yeast cells producing high levels of NADPH-P450 reductase and human cytochrome b5. *Gene* **125:**49–55 (1993).
21. Bellamine, A., J. C. Gautier, P. Urban, and D. Pompon. Chimeras of the human cytochrome P450 1A family produced in yeast. Accumulation in microsomal membranes, enzyme kinetics and stability. *Eur. J. Biochem.* **225:**1005–1013 (1994).
22. Cullin, C., and D. Pompon. Synthesis of functional mouse cytochromes P-450 P1 and chimeric P-450 P3–1 in the yeast *Saccharomyces cerevisiae*. *Gene* **65:**203–217 (1988).
23. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurements with the Folin phenol reagent. *J. Biol. Chem.* **193:**265–275 (1951).
24. Yasukochi, Y., and B. S. Masters. Some properties of a detergent-solubilized NADPH-cytochrome c(cytochrome P-450) reductase purified by biospecific affinity chromatography. *J. Biol. hem.* **251:**5337–5344 (1976).
25. Omura, T., and R. Sato. The carbon monoxide-binding pigment of liver microsomes, I. Evidence of its hemoprotein nature. *J. Biol. Chem.* **239:** 2370–2378 (1964).
26. Ladona, M. G., B. Lindström, C. Thyr, P. Dun-Ren, and A. Rane. Differential foetal development of the *O*- and *N*-demethylation of codeine and dextromethorphan in man. *Br. J. Clin. Pharmacol.* **32:**295–302 (1991).
27. Sachse, C., J. Brockmöller, S. Bauer, and I. Roots. Cytochrome P450 2D6 variants in a Caucasian population: allele frequencies and phenotypic consequences. *Am. J. Hum. Genet.* **60:**284–295 (1997).
28. Aklillu, E., I. Persson, L. Bertilsson, I. Johansson, F. Rodrigues, and M. Ingelman-Sundberg. Frequent distribution of ultrarapid metabolizers of debrisoquine in an Ethiopian population carrying duplicated and multiduplicated functional CYP2D6 alleles. *J. Pharmacol. Exp. Ther.* **278:**441–446 (1996).
29. Iyun, A. O., M. S. Lennard, G. T. Tucker, and H. F. Woods. Metoprolol and debrisoquin metabolism in Nigerians: lack of evidence for polymorphic oxidation. *Clin. Pharmacol. Ther.* **40:**387–394 (1986).
30. Simooya, O. O., E. Njunju, A. R. Hodjegan, M. S. Lennard, and G. T. Tucker. Debrisoquine and metoprolol oxidation in Zambians: a population study. *Pharmacogenetics* **3:**205–208 (1993).
31. Lennard, M. S., A. O. Iyun, P. R. Jackson, G. T. Tucker, and H. F. Woods. Evidence for a dissociation in the control of sparteine, debrisoquine and metoprolol metabolism in Nigerians. *Pharmacogenetics* **2:**89–92 (1992).
32. McGourty, J. C., J. H. Silas, M. S. Lennard, G. T. Tucker, and H. F. Woods. Metoprolol metabolism and debrisoquine oxidation polymorphism–population and family studies. *Br. J. Clin. Pharmacol.* **20:**555–566 (1985).
33. Koymans, L. M., N. P. Vermeulen, A. Baarslag, and G. M. Donné-Op den Kelder. A preliminary 3D model for cytochrome P450 2D6 constructed by homology model building. *J. Comput. Aided Mol. Des.* **7:**281–289 (1993).
34. Modi, S., M. J. Paine, M. J. Sutcliffe, L. Y. Lian, W. U. Primrose, C. R. Wolf, and G. C. Roberts. A model for human cytochrome P450 2D6 based on homology modeling and NMR studies of substrate binding. *Biochemistry* **35:**4540–4550 (1996).
35. De Groot, M. J., N. P. E. Vermeulen, J. D. Kramer, F. A. A. van Acker, and G. M. Donné-Op den Kelder. A three-dimensional protein model for human cytochrome P450 2D6 based on the crystal structures of P450 101, P450 102, and P450 108. *Chem. Res. Toxicol.* **9:**1079–1091 (1996).
36. Hasemann, C. A., R. G. Kurumbail, S. S. Boddupalli, J. A. Peterson, and J. Deisenhofer. Structure and function of cytochromes P450: a comparative analysis of three crystal structures. *Structure* **3:**41–62 (1995).
37. Gotoh, O. Substrate recognition sites in cytochrome P450 family 2 (CYP2) proteins inferred from comparative analyses of amino acid and coding nucleotide sequences. *J. Biol. Chem.* **267:**83–90 (1992).
38. Koymans, L., N. P. Vermeulen, S. A. van Acker, J. M. te Koppele, J. J. Heykants, K. Lavrijsen, W. Meuldermans, and G. M. Donné-Op den Kelder. A predictive model for substrates of cytochrome P450-debrisoquine (2D6). *Chem. Res Toxicol.* **5:**211–219 (1992).
39. Ellis, S. W., G. P. Hayhurst, G. Smith, T. Lightfoot, M. M. Wong, A. P. Simula, M. J. Ackland, M. J. Sternberg, M. S. Lennard, G. T. Tucker, and C. R. Wolf. Evidence that aspartic acid 301 is a critical substrate-contact residue in the active site of cytochrome P450 2D6. *J. Biol. Chem.* **270:** 29055–29058 (1995).

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