EFFECTS OF G169R AND P34S SUBSTITUTIONS PRODUCED BY MUTATIONS OF CYP2D6*14 ON THE FUNCTIONAL PROPERTIES OF CYP2D6 EXPRESSED IN V79 CELLS

TOMOKO SHIRAISHI, MASAKIYO HOSOKAWA, KAORU KOBASHI, HITOSHI TAINAKA, YOSHIYUKI YAMURA, MIWO TAGUCHI, AND KAN CHIBA

Laboratory of Pharmacology and Toxicology, Graduate School of Pharmaceutical Sciences, Chiba University, Chiba-shi (T.S., M.H., K.K., H.T., Y.Y. M.T., K.C.); Third Biological Section, Department of First Forensic Science, National Research Institute of Police Sciences, Kashiwa-shi (T.S.), and Asahi Technoglass, Funabashi-shi, Chiba, Japan (H.T.)

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ABSTRACT:
CYP2D6 is a polymorphic enzyme that catalyzes the oxidation of various drugs. At least 40-mutant alleles of CYP2D6 have been reported. CYP2D6*14, which is one of them found in Asian populations, causes deficient activity of CYP2D6. Four amino acid substitutions, P34S, G169R, R296C, and S486T, are present in the protein encoded by CYP2D6*14 (CYP2D6 14). Among them, G169R is thought to be a definitive substitution because it is unique to CYP2D6 14. However, a previous study showed that the activity of G169R-substituted CYP2D6 was about 40% of wild-type CYP2D6, suggesting that a combination of G169R and other substitutions may be required to abolish the activity of CYP2D6. In the present study, we examined the effects of combined substitutions of G169R and P34S on the functional properties of CYP2D6 and compared them with those of a single substitution of G169R or P34S using a cDNA expression system of V79 cells. The results showed that a combined substitution of G169R and P34S reduced the activities of CYP2D6 to less than the detection limit of our analytical method for bufuralol 1-hydroxylation and dextromethorphan O-demethylation. However, these activities were not completely abolished by a single substitution of P34S or G169R. The findings suggest that simultaneous substitution of G169R and P34S is crucial for almost completely abolishing the activity of CYP2D6 at least in V79 cells, although whether the absence of metabolism is due to the absence of functional protein or catalytic incompetency remains unclear because the levels of CYP2D6 protein expressed in V79 cells were too low to be determined by difference CO-reduced spectra.

Cytochrome P450 2D6 (CYP2D6) catalyzes the oxidative metabolism of various clinically important drugs (Rendic and Di Carlo, 1997). Interindividual and interethnic differences have been seen in the metabolic activity of CYP2D6, mainly due to the polymorphism of CYP2D6 gene (Marez et al., 1997; Sachse et al., 1997; Griese et al., 1998). The percentage of poor metabolizers that have a deficiency of CYP2D6 activity varies from 5 to 10% in Caucasian populations (Gonzalez et al., 1988; Sachse et al., 1997), whereas it is less than 1% in Asian populations (Kubota et al., 2000). In Caucasian populations, CYP2D6*3, *4, *5, and *6 are mainly involved in the deficiency of CYP2D6 activity (Sachse et al., 1997), whereas CYP2D6*5 and *14 are mainly involved in the deficiency of CYP2D6 activity in Asian populations (Kubota et al., 2000; Nishida et al., 2000).

CYP2D6*14 is a mutant allele that was first found in a Chinese subject (Wang, 1992) and has since been found only in Asian populations (Wang et al., 1999; Kubota et al., 2000). The variant protein corresponding to CYP2D6*14 (CYP2D6 14) is thought to have little activity, because a Chinese subject carrying CYP2D6*5/*14 was shown to be the phenotype of a poor metabolizer (Wang et al., 1999). There are four amino acid substitutions in CYP2D6 14: P34S, G169R, R296C, and S486T (Daly et al., 1996; Wang et al., 1999). Among them, only G169R is unique to this variant protein (Human CYP Allele Nomenclature Committee, http://www.imm.ki.se/CYPalleles/cyp2d.htm). Therefore, G169R substitution was considered to be mainly responsible for the deficient activity of CYP2D6 14. However, a previous study using a Rat-1 cell cDNA expression system showed that substitution of P34S caused a dramatic decrease in CYP2D6 activity, whereas the decrease induced by G169R substitution was not as dramatic as that induced by substitution of P34S. Based on these findings, Wang et al. (1999) speculated that G169R is not a critical substitution but that its combination with P34S, R296C, and/or S486T may abolish the activity of CYP2D6 14. However, this possibility has not been studied by direct construction of variant CYP2D6 protein having combined substitutions of G169R and others.

Therefore, we constructed mutated cDNAs that yield CYP2D6 variant proteins substituted with G169R and/or P34S, expressed in V79 cells, and compared their metabolic properties for the prototype substrates of CYP2D6. R296C and S486T substitutions were not examined in the present study, since they are present in CYP2D6 2, and subjects with CYP2D6*2/*2 show similar metabolic activities to those of wild type for various substrates of CYP2D6 (Johansson et al., 1993; Dahl et al., 1995; Tateishi et al., 1999). On the other hand, P34S...
substitution has been shown to cause a significant reduction in CYP2D6 activity in cDNA expression systems (Kagimoto et al., 1990; Johansson et al., 1994; Fukuda et al., 2000).

Materials and Methods

Construction of Mammalian Cell Expression Vectors. A G1758A mutation for a Gly169-to-Arg169 change was introduced into wild-type CYP2D6 cDNA in a pUC19 plasmid vector using a transformer site-directed mutagenesis kit (BD Biosciences Clontech, Palo Alto, CA). The sequence of the oligonucleotide used for the mutation was 5′-CGCCAACACTCTAGGCCC-3′. The mutation was confirmed by sequencing, and the mutated fragment was subcloned into a pBluescript SK+ vector (Strategene, La Jolla, CA). CYP2D6/pTARGET and CYP2D6 G169R/pTARGET expression vectors were obtained by ligating the wild-type CYP2D6 and mutated CYP2D6 G169R cDNA to a pTARGET Mammalian Cell Expression Vector (Promega, Madison, WI), respectively.

A 562-bp DNA fragment containing a C100T mutation for a Pro34-to-Ser34 change was prepared by PCR1 methods using a human genomic DNA that showed the genotype of CYP2D6*10/*10 as a template. The nucleotide sequences of the primers used for the amplification were 5′-ATTCGGATCCTCCCCTGCTGGAATTCATGGGGCTAGAAGCACTGAGTCCAGGCTGCAGGAATTCATGGGGCTAGAAGCACTGAGTCCAGGCTGCAGGAATTCATGGGGCTAGAAGCACTG-3′ and 5′-AAGAGACCTTGGGGGAAGGGGCCC-3′. The obtained PCR product was subcloned into a pGEM-T vector (Promega) and digested by BamHI and Apal. CYP2D6 P34S/pTARGET and CYP2D6 P34S+G169R/pTARGET expression vectors were obtained by ligating the digested DNA fragment containing the C100T mutation into BamHI- and Apal-digested CYP2D6/pTARGET and CYP2D6 G169R/pTARGET, respectively. The nucleotide sequences of all the recombinant expression vectors were confirmed.

Mammalian Cell Culture and Expression of Recombinant Protein. Parental V79 cells (V79–4, CL93; American Type Cell Culture Collection, Manassas, VA) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin in 5% CO2 and 95% air at 37°C. The V79 cells were plated at 1 × 105 cells/100-mm plate. On the following day, recombinant expression vectors were transfected into V79 cells using LIPOFECTAMINE Reagent and Plus Reagent (Invitrogen, Carlsbad, CA) according to manufacturer’s recommendations. For negative control, V79 cells were transfected with an empty pTARGET vector. After two days of transfection, V79 cells were plated into a 100-mm plate with a medium containing the selective agent G418 (Invitrogen) at 800 µg/ml. Each medium was changed every 2 to 3 days, and the cells were cultured for at least 3 weeks to obtain stable expression colonies. After selection with G418, V79 cells were harvested and cell homogenate was prepared.

The amount of CYP2D6 protein expressed in V79 cells was measured by Western blot analysis. The V79 cell homogenates were electrophoresed on SDS-polyacrylamide gels and transferred onto a nitrocellulose sheet. CYP2D6 Western blot analysis. The V79 cell homogenates were electrophoresed on SDS-polyacrylamide gels and transferred onto a nitrocellulose sheet. CYP2D6 activity in cDNA expression systems (Kagimoto et al., 1990; Johansson et al., 1994; Fukuda et al., 2000).

Expression of Wild-Type and Mutated CYP2D6 in V79 Cells. The expression of CYP2D6 protein in V79 cells transfected with recombinant CYP2D6 cDNA was confirmed by Western blot analysis (Fig. 1A). There were no significant differences between CYP2D6 protein levels in V79 cells expressing wild-type (2D6WT) and G169R-substituted CYP2D6 (2D6G169R). On the other hand, CYP2D6 protein levels in V79 cells expressing P34S-substituted CYP2D6 (2D6P34S) and those expressing both G169R- and P34S-substituted CYP2D6 (2D6G169R/P34S) decreased to approximately 26% that of 2D6WT (Fig. 1B). No significant differences were found between mRNA levels of CYP2D6 in V79 cells transfected with wild-type and mutated CYP2D6 CDNA's (Fig. 2).

Enzyme Activities. Bufuralol 1'-hydroxylation activity of 2D6G169R (1.00 pmol/min/pmol CYP2D6) was slightly lower than that of 2D6WT, whereas 2D6P34S (0.05 pmol/min/pmol CYP2D6) was much lower than that of 2D6WT (1.23 pmol/min/pmol CYP2D6), which corresponds to 4% of 2D6WT (Fig. 3A). However, the activity of 2D6G169R/P34S was under the detection limit of our analytical method (Fig. 3A).

Similar results were also found for dextromethorphan O-demethylation activity (Fig. 3B). The activity of 2D6G169R (1.19 pmol/min/pmol CYP2D6) was not different, whereas that of 2D6P34S (0.37 pmol/min/pmol CYP2D6) was approximately one-third (32%) of that of 2D6WT (1.17 pmol/min/pmol CYP2D6). However, the activity of 2D6G169R/P34S was under the detection limit of the analytical method employed in the present study.

Kinetic Analysis of Bufuralol 1'-Hydroxylation. Kinetic parameters for bufuralol 1'-hydroxylation in V79 cells expressing wild-type

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1 Abbreviations used are: PCR, polymerase chain reaction; GAPDH, glyceraldehyde triphosphate dehydrogenase; HPLC, high-performance liquid chromatography.
and mutated CYP2D6 are shown in Table 1. While the \( K_m \) value was not affected, the \( V_{\text{max}} \) value slightly decreased in 2D6G169R. On the other hand, the \( K_m \) value and \( V_{\text{max}} \) value of 2D6P34S were 5.4-times higher and 7.2-times lower than those of 2D6WT, respectively, thus the \( V_{\text{max}}/K_m \) value of 2D6P34S was 40-times lower than that of 2D6WT. The kinetic parameters could not be determined for 2D6G169R/P34S because of the extremely low activity of bufuralol 1'-hydroxylation.

Discussion

The present study clearly showed that a combination of G169R and P34S substitutions diminishes the activities of CYP2D6 to less than the detection limit of our analytical method for bufuralol 1'-hydroxylation and dextromethorphan O-demethylation, respectively). The findings suggest that simultaneous substitution of G169R and P34S is crucial for almost completely abolishing the activity of CYP2D6 at least in a cDNA expression system using V79 cells.

P34S is a key substitution of CYP2D6 10 encoded by CYP2D6*10, which has been found to reduce but not abolish the activity of CYP2D6 by in vitro studies (Ramamoorthy et al., 2001; Senda et al., 2001; Shimada et al., 2001). In vivo studies have also shown that the metabolic capacity of homozygous CYP2D6*10 is between those of extensive and poor metabolizers for various substrates of CYP2D6, and homozygous CYP2D6*10 has therefore been categorized as an intermediate metabolizer (Wang et al., 1993; Dahl et al., 1995; Lai et al., 1995; Tseng et al., 1996). In addition, heterozygous subject with CYP2D6*10 and a defective allele (e.g., CYP2D6*5/*10) has also been categorized as an intermediate metabolizer (Griese et al., 1998). However, a heterozygous subject with CYP2D6*14 showed a metabolic ratio of debrisoquine of more than 12.6, indicating that the subject can be categorized as a poor metabolizer (Wang et al., 1999). Based on that finding, Wang et al. (1999) concluded that the suppressive effect of CYP2D6*14 on CYP2D6 activity is more pronounced than that of CYP2D6*10 and that CYP2D6*14 is a mutant allele causing defective activity of CYP2D6.

There are four amino acid substitutions (P34S, G169R, R296C, and S486T) in CYP2D6 14. Two of them (P34S and S486T) are overlapped with substitution in CYP2D6 10. Since S486T does not affect the suppressive effect of P34S on the activity of CYP2D6 (Fukuda et al., 2000; Tsuzuki et al., 2001), the pronounced effect of CYP2D6*14 appears to be derived from the combination of either G169R and P34S or R296C and P34S. The results of the present study support the former possibility. However, the possibility that interaction between R296C and P34S or among R296, S486T, and P34S plays a role in the pronounced effect of CYP2D6*14 cannot be ruled out, since CYP2D6*14 allele contains the substitutions R296C and S486T and...
has a significantly lower in vivo activity when compared with both *1 and *2 (Raimundo et al., 2000).

The precise mechanism underlying the combined effects of G169R and P34S could not be determined in the present study. However, based on three-dimensional models of bacterial P450 CYP102, G169R substitution of CYP2D6 corresponds to Ala135 of CYP102, which is located in the turn region between the D and E helices (Lewis et al., 1997; Lewis, 1998). Since Gly in a turn region is important for maintenance of a tertiary structure of a protein, it is assumed that substitution of Gly169 to Arg alters the conformation of the CYP2D6 protein. On the other hand, Pro34 of CYP2D6 is the first residue in the proline-rich region, and this region is followed by the N-terminal signal anchor region (Yamazaki et al., 1993), which is highly conserved in the mammalian CYP2 family (Yamazaki et al., 1993). Although the proline-rich region is far from the catalytic domain of CYP2D6 (Lewis, 1998) and not involved in the substrate-binding sites of CYP2D6 (Modi et al., 1996), P34S substitution has been reported to alter the catalytic properties of CYP2D6 for bufuralol 1'-hydroxylation, venlafaxine O-demethylation, and bunitrolol 4-hydroxylation (Fukuda et al., 2000; Tsuzuki et al., 2001). The present study also showed that the K_m value and V_max value of 2D6P34S for bufuralol 1'-hydroxylation were 5.4-times higher and 7.2-times lower than those of 2D6WT, respectively, thus the V_max/K_m value of 2D6P34S was 40-times lower than that of 2D6WT (Table 1). These findings suggest that the substitution of P34S, although it is not in a substrate-recognition site, affects the tertiary structure of CYP2D6 and reduces the activities of CYP2D6. Therefore, we are tempted to speculate that combined substitutions of G169R and P34S cause a more drastic change in the structure of CYP2D6 and reduces the activities of CYP2D6. Similarly, a combined substitution of G169R and P34S may deteriorate the incorporation of heme into the CYP2D6 apoprotein, although this possibility could not be assessed in the present study, since the levels of CYP2D6 protein expressed in V79 cells were too low to be determined by difference CO-reduced spectra.

Regarding the level of CYP2D6 protein, a combined substitution of G169R and P34S decreased it substantially in V79 cells (Fig. 1). However, the extent of decrease was not different from that caused by a single substitution of P34S (Fig. 1). This finding suggests that G169R substitution does not accelerate the decreasing effect of P34S substitution on the protein level of CYP2D6 reported previously (Johansson et al., 1994; Fukuda et al., 2000; Tsuzuki et al., 2001). The present study also showed that there were no major differences between the levels of CYP2D6 mRNA in V79 cells transfected with wild-type and mutated CYP2D6 cDNA (Fig. 1). This finding suggests that substitutions of P34S and G169R do not appear to affect the transcriptional efficiency of CYP2D6 cDNA introduced to V79 cells.

In conclusion, the present study showed that a combination of G169R and P34S substitutions reduced the activities of CYP2D6 to undetectable levels despite the fact that the activities of CYP2D6 were not completely abolished by the substitution of P34S. The findings suggest that simultaneous substitution of G169R and P34S is required.

### Table 1

<table>
<thead>
<tr>
<th>Substitution</th>
<th>K_m (µM)</th>
<th>V_max (pmoles/min/pmol 2D6)</th>
<th>V_max/K_m (µM⁻¹/min/pmol 2D6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>10.8</td>
<td>2.51</td>
<td>0.24</td>
</tr>
<tr>
<td>G169R</td>
<td>11.7</td>
<td>2.13</td>
<td>0.19</td>
</tr>
<tr>
<td>P34S</td>
<td>58.2</td>
<td>0.35</td>
<td>0.0060</td>
</tr>
<tr>
<td>G169R/P34S</td>
<td>—</td>
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*The protein levels of wild-type and substituted CYP2D6 determined here were those of the apoprotein and not of the native protein.

*Kinetic parameters could not be calculated because of lower activity for bufuralol 1'-hydroxylation.

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**Fig. 3.** Enzyme activities of V79 cells expressing wild-type CYP2D6 or CYP2D6 substituted with G169R and/or P34S.

A, bufuralol 1'-hydroxylation; B, dextromethorphan O-demethylation. The protein levels of wild-type and substituted CYP2D6 determined here were those of the apoprotein and not of the native protein. The data are means of two or three independent experiments. WT, wild type; N.D., not detected.
for the activity of CYP2D6 to be almost abolished, at least in V79 cells. This combined effect may be a factor responsible for the poor metabolism of debrisoquine found in a subject carrying CYP2D6*14 (Wang, 1992; Wang et al., 1999).

References
Fukuda T, Nishida Y, Imaoka S, Hiroi T, Naohara M, Funae Y, and Azuma J (2000) The metabolism of debrisoquine found in a subject carrying CYP2D6*14 alleles may be a factor responsible for the poor metabolism of debrisoquine. This combined effect may be a factor responsible for the poor metabolism of debrisoquine found in a subject carrying CYP2D6*14 (Wang, 1992; Wang et al., 1999).