

# Phenotypic analysis of human CYP2C9 polymorphisms using fluorine-substituted tolbutamide

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**SUMMARY** To investigate the effect of fluorine substitution on tolbutamide (TB) hydroxylation catalyzed by CYP2C9, the hydroxylation of TB and its fluorinated derivative 3'-fluoro-tolbutamide (3'-F-TB) by recombinant human CYP2C9\*1, CYP2C9\*2, and CYP2C9\*3 was analyzed. In general, fluorine substitution near the metabolic site may decrease enzymatic oxidation owing to its electron-withdrawing nature. Fluorine substitution reduced the Michaelis–Menten-derived  $K_m$  of 4'-hydroxylation of TB catalyzed by CYP2C9\*1 from 115 (TB) to 77 (3'-F-TB)  $\mu\text{M}$ . In the case of TB hydroxylation catalyzed by CYP2C9\*2, the  $K_m$  value of TB was also reduced by fluorine substitution from 129 to 88  $\mu\text{M}$ . The greatest effect of fluorine substitution on the  $K_m$  in TB hydroxylation was observed in the catalysis by CYP2C9\*3, in which the  $K_m$  value decreased from 287 to 117  $\mu\text{M}$ . When a mixture containing TB and 3'-F-TB was hydroxylated by CYP2C9, the hydroxylated metabolite ratio in CYP2C9\*3 was significantly increased compared with that in CYP2C9\*1 and CYP2C9\*2 ( $p < 0.01$ , Tukey–Kramer test). These results suggest that obtaining the metabolite profiles of fluorine-substituted analogs of the key substrate molecule may be useful as a new tool for phenotyping polymorphic CYP isoforms.

**Keywords** fluorine substitution, tolbutamide, CYP2C9 polymorphism, phenotyping

## 1. Introduction

CYP2C9 catalyzes the metabolism of several clinically used drugs, including S-warfarin, phenytoin, flurbiprofen, and tolbutamide (TB) (1-4). Human CYP2C9 has three major polymorphic isozymes: CYP2C9\*1 (wild-type), CYP2C9\*2 (Arg144Cys), and CYP2C9\*3 (Ile359Leu) (5). The genetic variability of CYP2C9 is dominated by the \*2 allele (11.7%) and \*3 allele (7%) in Europeans, whereas the major allele in Asian and African populations is \*3 (3.4% in East Asians and 11.3% in South Asians) (6,7). Generally, the difference in metabolic activity among the CYP2C9 isozymes may vary considerably. It is suggested that CYP2C9\*2 shows moderate activity and CYP2C9\*3 shows poor activity compared with that shown by CYP2C9\*1 in the metabolism of most CYP2C9 substrates (8). Additionally, CYP2C9\*3 has been reported to reduce the hydroxylation activity of TB and phenytoin (9,10).

Fluorine substitution of a hydrogen atom often exerts so-called mimic effects on interactions with biological molecules because the covalent and van der Waals' radii of the fluorine atom are similar to those of the hydrogen atom (11). However, the replacement of hydrogen with

fluorine changes not only the distribution of electron density within the molecule but also the electric repulsive/attractive interactions with intra/intermolecular environments, which may significantly affect enzyme–substrate and enzyme–inhibitor interactions. In our previous study, the inhibitory effects of fluorinated benzo[h]quinolines (BhQ) on drug metabolism by recombinant human CYP2C9 were analyzed, and the position-specific substitution by a fluorine atom was shown to alter the ability of BhQ to inhibit CYP2C9 (11). The present study aimed to determine whether fluorine substitution improves the decrease in metabolic activity catalyzed by CYP2C9 polymorphic isozymes. In this study, we compared the changes in hydroxylation metabolism by CYP2C9\*1, CYP2C9\*2, and CYP2C9\*3 using TB and 3'-fluoro-tolbutamide (3'-F-TB) as a fluorine-substituted TB analog (Figure 1).

## 2. Materials and Methods

### 2.1. Materials

Microsome preparations from baculovirus-infected insect cells coexpressing CYP2C9\*1, \*2, or \*3 with

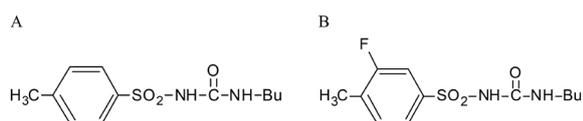


Figure 1. The chemical structures of (A) TB and (B) 3'-F-TB.

NADPH-CYP oxidoreductase were purchased from Gentest (Woburn, MA, USA); NADP, glucose-6-phosphate (G6P), and G6P dehydrogenase were obtained from Oriental Yeast (Tokyo, Japan); and TB, 4'-hydroxytolbutamide, and all other chemicals were obtained from Sigma-Aldrich (St. Louis, Mo, USA). The melting point was determined using a MP-500D (Yamato Scientific, Tokyo, Japan) micro melting point apparatus without correction. Mass spectra (electron impact ionization) were measured using an AX505HA spectrometer (JEOL, Tokyo, Japan).  $^1\text{H-NMR}$  spectra were recorded using a JNM-A500 spectrometer (JEOL) in  $\text{CDCl}_3$  using tetramethylsilane as an internal standard.

## 2.2. Synthesis of 3'-F-TB

4- $^{18}\text{F}$  fluorotolbutamide, a fluorine-substituted TB analog, is used in positron analysis as an imaging reagent for pancreatic islets of Langerhans. 4- $^{18}\text{F}$  fluorotolbutamide has previously been synthesized by a nucleophilic addition reaction of 4- $^{18}\text{F}$  fluorobenzensulfonamide and butyl isocyanate (12,13). Similarly, 3'-F-TB was synthesized from 3-fluoro-4-methylbenzonsulfonamide (3-F-4-Me-BSA) and butyl isocyanate. The melting point was 123-125°C.  $^1\text{H-NMR}$  (500 MHz,  $\text{CDCl}_3$ )  $\delta$ : 0.91 (m, 3H, H-4'), 1.25-1.39 (m, 2H, H-3'), 1.43-1.52 (m, 2H, H-2'), 2.35 (d, 3H,  $\text{CH}_3$ ), 3.13-3.22 (m, 2H, H-1'), 4.46 (br, 1H, 1-NH), 6.44 (br, 1H, 3-NH), 7.34 (t, 1H, H-5''), 7.55 (dd, 1H, H-2''), 7.61 (dd, 1H, H-6'');  $J_{\text{F-CH}_3} = 2.1$ ,  $J_{2'-\text{F}} = J_{5'-6''} = 8.7$ ,  $J_{2'-6''} = 2.0$  Hz. HR-MS  $m/z$ : 288.0942, Calcd for  $\text{C}_{12}\text{H}_{17}\text{FN}_2\text{O}_3\text{S}$ : 288.0944.

## 2.3. Determination of CYP2C9 activity by TB hydroxylation

The determination of TB hydroxylase activity catalyzed by CYP2C9 was performed according to previous reports with slight modifications (11,14). Briefly, the incubation mixture (50  $\mu\text{L}$  in a 1.5-mL microtube) contained 0.1 M potassium phosphate buffer (pH 7.4), 1.3 mM NADP, 3.3 mM G6P, 3.3 mM  $\text{MgCl}_2$ , 0.08 units of G6P dehydrogenase, TB, or 3'-F-TB, and 5.0 pmol CYP (approximately 8  $\mu\text{g}$  protein). After incubation at 37°C for 40 min, the resulting metabolites were extracted with 2.5 volumes of ethyl acetate, and the organic solvent layer was evaporated. The residue was dissolved in 30  $\mu\text{L}$  of a solution containing 0.01 M HCl and 20%  $\text{CH}_3\text{CN}$

and analyzed by HPLC. HPLC analysis was performed using a Shimadzu liquid chromatograph (Shimadzu, Kyoto, Japan) equipped with a Model LC-10A solvent delivery system (Shimadzu), Model SPD-10AV UV-vis spectrophotometric detector (Shimadzu), and Wakosil II 5C18RS (ODS) column (2 mm  $\times$  150 mm) (Fujifilm Wako Pure Chemical, Osaka, Japan). The solvent system consisted of 40%  $\text{CH}_3\text{CN-H}_2\text{O}$ . The flow rate was 0.15 mL/min. Hydroxylated TB (OH-TB) was quantified from the peak areas measured by ultraviolet absorption at 236 nm with reference to 10  $\mu\text{M}$  4'-hydroxytolbutamide as the standard sample. Hydroxylated 3'-F-TB (3'-F-OH-TB) was semi-quantitatively determined from the integrated area measured at 236 nm, assuming that the  $\epsilon$  value was the same as that of OH-TB. At least four independent experiments were performed.

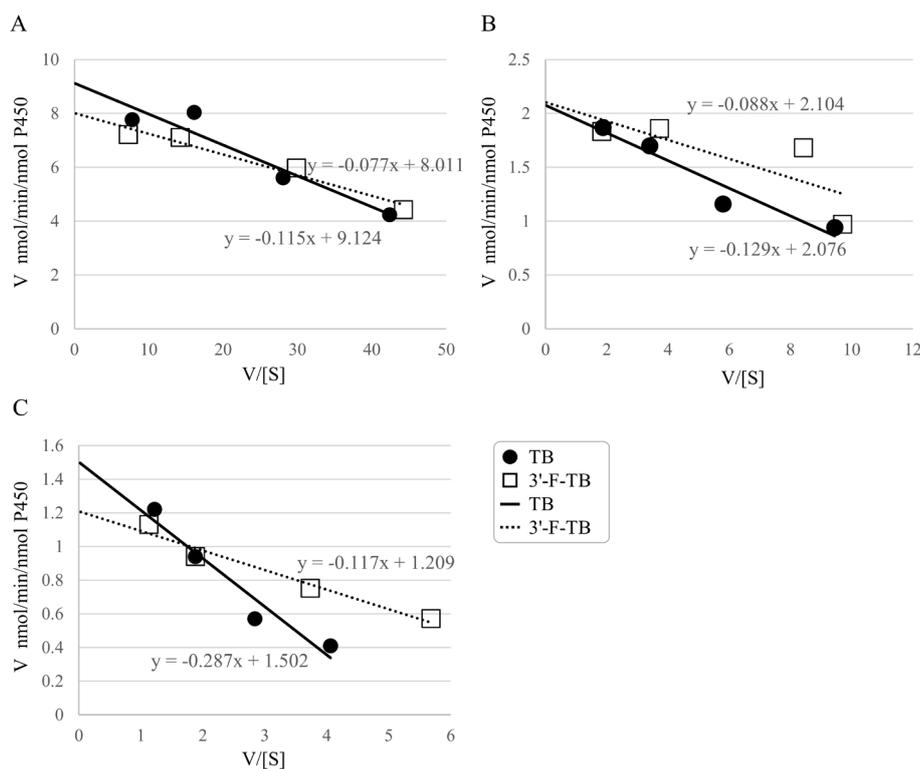
## 2.4. Statistical analysis

The ratio of the hydroxylated metabolites of TB and 3'-F-TB was statistically analyzed using one-way analysis of variance with the Tukey-Kramer test (SPSS Statistics version 24; IBM, Armonk, NY, USA). A  $p$  value of  $< 0.05$  was considered statistically significant.

## 3. Results and Discussion

Using TB and 3'-F-TB as substrates, the changes in hydroxylation metabolism by CYP2C9\*1, CYP2C9\*2, and CYP2C9\*3 were compared. To determine the metabolic rate, the peak area of the hydroxylation reactions catalyzed by CYP2C9 was measured by comparison with that of 10  $\mu\text{M}$  4'-hydroxytolbutamide as the standard sample. The kinetics of metabolite formation by CYP2C9 was assessed in quadruplicate, and the substrate concentration ranged from 0.1 to 1.0 mM.  $V_{\text{max}}$  and  $K_m$  were then calculated using Eadie-Hofstee plots (Figure 2 and Table 1). Fluorine substitution reduced the Michaelis-Menten-derived  $K_m$  of TB 4'-hydroxylation catalyzed by CYP2C9\*1 from 115 (TB) to 77 (3'-F-TB)  $\mu\text{M}$ . In the case of TB hydroxylation by CYP2C9\*2, the  $K_m$  value was also reduced from 129 to 88  $\mu\text{M}$  by fluorine substitution. The largest effect of fluorine substitution on the  $K_m$  value in TB hydroxylation was observed in the case of catalysis by CYP2C9\*3, in which the  $K_m$  decreased from 287 to 117  $\mu\text{M}$ .

The mixture containing equal amounts of TB and 3'-F-TB was quantified as the ratio of the hydroxylated metabolite by CYP2C9\*1, CYP2C9\*2, and CYP2C9\*3 (Table 2). The ratio of OH-TB compared with that of 3'-F-OH-TB in the combination metabolism of 100  $\mu\text{M}$  TB and 100  $\mu\text{M}$  3'-F-TB showed almost the same tendency as the ratio of  $K_m$  values, and the hydroxylated metabolite ratio (3'-F-OH-TB/OH-TB) obtained from the metabolism by CYP2C9\*3 was significantly higher than that of CYP2C9\*1 and CYP2C9\*2 ( $p < 0.01$ ,



**Figure 2.** Eadie–Hofstee plots for the hydroxylation of TB and 3'-F-TB by (A) CYP2C9\*1, (B) CYP2C9\*2, and (C) CYP2C9\*3. The hydroxylation catalyzed by CYP2C9 was quantified by comparison with 10  $\mu\text{M}$  4'-hydroxytolbutamide as the standard sample. Reactions were performed in quadruplicate, and the concentration of TB or 3'-F-TB ranged from 0.1 to 1.0 mM. Each point represents the mean of quadruplicate determinations. TB (black circles and solid lines) and 3'-F-TB (white squares and dotted lines).

**Table 1.** Hydroxylation metabolism parameters of TB and 3'-F-TB

Items	TB	3'-F-TB
CYP2C9*1		
$V_{\max}$ (nmol/min/nmol P450)	9.12	8.01
$K_m$ ( $\mu\text{M}$ )	115	77
$K_m$ ratio (TB/3'-F-TB)	1.49	
CYP2C9*2		
$V_{\max}$ (nmol/min/nmol P450)	2.08	2.10
$K_m$ ( $\mu\text{M}$ )	129	88
$K_m$ ratio (TB/3'-F-TB)	1.47	
CYP2C9*3		
$V_{\max}$ (nmol/min/nmol P450)	1.50	1.21
$K_m$ ( $\mu\text{M}$ )	287	117
$K_m$ ratio (TB/3'-F-TB)	2.45	

**Table 2.** Ratio of the hydroxylated metabolite (3'-F-OH-TB/OH-TB)

Items	3'-F-OH-TB/OH-TB
CYP2C9*1	1.73 $\pm$ 0.09
CYP2C9*2	1.67 $\pm$ 0.09
CYP2C9*3	2.28 $\pm$ 0.14 <sup>*,#</sup>

Data are expressed as the mean  $\pm$  S.D. (CYP2C9\*1;  $n = 7$ , CYP2C9\*2;  $n = 8$ , CYP2C9\*3;  $n = 8$ ). <sup>\*</sup> $p < 0.01$ , vs. CYP2C9\*1 by the Tukey–Kramer test. <sup>#</sup> $p < 0.01$ , vs. CYP2C9\*2 by the Tukey–Kramer test.

Tukey–Kramer test).

Many gene polymorphisms in CYP2C9 have been discovered, and it is well recognized that differences in genotypes cause differences in metabolic activity (15). It is important to determine the differences in metabolic activity among each isozyme to understand how they affect drug efficacy and safety, especially considering the possibility of serious side effects due to a reduction in the metabolic capacity of CYP2C9\*3. It has been reported that the human plasma area under the curve of OH-TB and the ratio of OH-TB/TB were approximately

twice as high in individuals with the wild-type CYP2C9 genotype than in heterozygous CYP2C9\*3 individuals (16). In fact, we showed that the  $K_m$  value of TB in CYP2C9\*1 was reduced compared with that of CYP2C9\*3 (Table 1). In this study, we compared the changes in hydroxylation metabolism by CYP2C9 polymorphisms using a fluorine-substituted TB analog. 3'-F-TB reduced the  $K_m$  and increased the production of hydroxylated metabolites compared with TB (Table 1). These results indicate that fluorine substitution increases the affinity for CYP2C9 and is susceptible to hydroxylation by CYP2C9. The effect of fluorine substitution on TB suggests that TB hydroxylation in humans with CYP2C9\*3 may be enhanced to the same extent as CYP2C9\*1.

Phenotypic analysis directly reflects various external environments, intrinsic factors, and genotypes

that affect CYP activity, unlike genetic analysis (17). Therefore, phenotypic analysis is considered to provide more useful information than genetic analysis in clinical practice. However, very few simple and quick phenotype analysis methods have been established (17). When the mixture containing TB and 3'-F-TB was hydroxylated by CYP2C9, the hydroxylated metabolite ratio (3'-F-OH-TB/OH-TB) in CYP2C9\*3 was significantly increased compared with that in CYP2C9\*1 and CYP2C9\*2 (Table 2). This result indicates that fluorine substitution in TB suppressed the decreased metabolic activity by CYP2C9\*3 and/or the metabolism of TB was reduced by CYP2C9\*3. It was reported that the inhibition of CYP2C9 by BhQ and its fluorinated derivatives varied with the position of the substituted fluorine (11). The effects of fluorine substitution on the inhibition of TB hydroxylation catalyzed by CYP2C9\*1 were different compared with the effects on the hydroxylation catalyzed by CYP2C9\*2 and CYP2C9\*3 (11). Additionally, fluorine substitution in quinoline and 4-methylquinoline affected their mutagenicity and the formation of hydroxylated metabolites (18,19). Fluorine substitution could alter the metabolic profiles of other substrates, such as phenytoin, in addition to TB, which may enhance their efficacy and reduce their side effects.

In conclusion, we suggest that obtaining the metabolite profiles of fluorine-substituted analogs of the key substrate molecule may be useful as a new tool for phenotyping polymorphic CYP isoforms. Future studies should develop probes that can differentiate between the various metabolic enzymes in human liver microsomes to estimate the clinical relevance at *in vitro* levels. The development of compounds with a large difference in the  $K_m$  ratio between CYP2C9\*1 and CYP2C9\*3 may contribute to a new method for phenotypic analysis.

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### References

1. Rettie AE, Korzekwa KR, Kunze KL, Lawrence RF, Eddy AC, Aoyama T, Gelboin HV, Gonzalez FJ, Trager WF. Hydroxylation of warfarin by human cDNA-expressed cytochrome P-450: a role for P-4502C9 in the etiology of (S)-warfarin-drug interactions. *Chem Res Toxicol.* 1992; 5:54-59.
2. Veronese ME, Mackenzie PI, Doecke CJ, McManus ME, Miners JO, Birkett DJ. Tolbutamide and phenytoin hydroxylations by cDNA-expressed human liver cytochrome P4502C9. *Biochem Biophys Res Commun.* 1991; 175:1112-1118.

3. Srivastava PK, Yun CH, Beaune PH, Ged C, Guengerich FP. Separation of human liver microsomal tolbutamide hydroxylase and (S)-mephenytoin 4'-hydroxylase cytochrome P-450 enzymes. *Mol Pharmacol.* 1991; 40:69-79.
4. Veronese ME, Miners JO, Randles D, Gregov D, Birkett DJ. Validation of the tolbutamide metabolic ratio for population screening with use of sulfaphenazole to produce model phenotypic poor metabolizers. *Clin Pharmacol Ther.* 1990; 47:403-411.
5. Lee CR, Goldstein JA, Pieper JA. Cytochrome P450 2C9 polymorphisms: a comprehensive review of the *in-vitro* and human data. *Pharmacogenetics.* 2002; 12:251-263.
6. Kirchheiner J, Brockmoller J. Clinical consequences of cytochrome P450 2C9 polymorphisms. *Clin Pharmacol Ther.* 2005; 77:1-16.
7. Zhou Y, Ingelman-Sundberg M, Lauschke VM. Worldwide distribution of cytochrome P450 alleles: A meta-analysis of population-scale sequencing projects. *Clin Pharmacol Ther.* 2017; 102:688-700.
8. Yasar U, Eliasson E, Forslund-Bergengren C, Tybring G, Gadd M, Sjoqvist F, Dahl ML. The role of CYP2C9 genotype in the metabolism of diclofenac *in vivo* and *in vitro*. *Eur J Clin Pharmacol.* 2001; 57:729-735.
9. Goldstein JA, de Morais SM. Biochemistry and molecular biology of the human CYP2C subfamily. *Pharmacogenetics.* 1994; 4:285-299.
10. Veronese ME, Doecke CJ, Mackenzie PI, McManus ME, Miners JO, Rees DL, Gasser R, Meyer UA, Birkett DJ. Site-directed mutation studies of human liver cytochrome P-450 isoenzymes in the CYP2C subfamily. *Biochem J.* 1993; 289 ( Pt 2):533-538.
11. Matsuyama N, Kato T, Kimura K, Mizutani T, Saeki K. Phenotype analysis of human cytochrome P450 2C9 polymorphism using a panel of fluorine substituted benzo[h]quinolines as inhibitor of tolbutamide hydroxylation. *J Health Sci.* 2006; 52:821-824.
12. Shiue GG, Schirmacher R, Shiue CY, Alavi AA. Synthesis of fluorine-18 labeled sulfonureas as  $\beta$ -cell imaging agents. *J Labelled Cpd Radiopharm.* 2001; 44:127-139.
13. Schirmacher R, Shiue GG, Shiue CY, Alavi AA. Synthesis of F-18 Labeled Tolbutamide as Beta-Cells Imaging Agent. <https://download.uni-mainz.de/fb09-kernchemie/jb1999/b6.pdf> (accessed August 10, 2020).
14. Relling MV, Aoyama T, Gonzalez FJ, Meyer UA. Tolbutamide and mephenytoin hydroxylation by human cytochrome P450s in the CYP2C subfamily. *J Pharmacol Exp Ther.* 1990; 252:442-447.
15. Daly AK, Rettie AE, Fowler DM, Miners JO. Pharmacogenomics of CYP2C9: Functional and Clinical Considerations. *J Pers Med.* 2017; 8:1.
16. Shon JH, Yoon YR, Kim KA, Lim YC, Lee KJ, Park JY, Cha IJ, Flockhart DA, Shin JG. Effects of CYP2C19 and CYP2C9 genetic polymorphisms on the disposition of and blood glucose lowering response to tolbutamide in humans. *Pharmacogenetics.* 2002; 12:111-119.
17. Streetman DS, Bertino JS Jr, Nafziger AN. Phenotyping of drug-metabolizing enzymes in adults: a review of *in-vivo* cytochrome P450 phenotyping probes. *Pharmacogenetics.* 2000; 10:187-216.
18. Kato T, Saeki K, Kawazoe Y, Hakura A. Effects of oligofluorine substitution on the mutagenicity of quinoline: a study with twelve fluoroquinoline derivatives. *Mutat Res.* 1999; 439:149-157.

19. Kato T, Hakura A, Mizutani T, Saeki K. Anti-mutagenic structural modification by fluorine-substitution in highly mutagenic 4-methylquinoline derivatives. *Mutat Res.* 2000; 465:173-182.

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