School of Pharmacy, Shujitsu University, Okayama, Japan

Contribution of the human cytochrome P450 2C subfamily to the metabolism of and the interactions with endogenous compounds including steroid hormones

T. Niwa*, S. Yasuda, Y. Yamamoto, M. Murakami, R. Ishii

Received August 22, 2021, accepted September 30, 3021

**Corresponding Author: Prof. Toshiro Niwa, Ph.D., School of Pharmacy, Shujitsu University, 1-6-1 Nishigawara, Naka-ku, Okayama 703-8516, Japan*
tniwa@shujitsu.ac.jp

Pharmazie 76: 611-613 (2021) *doi: 10.1691/ph.2021.1836*

The objectives of this study were as follows: 1) to compare the metabolic activities of endogenous compounds and their effects on dopamine formation and hydroxylation of steroid hormones, mediated by human cytochrome P450 (CYP), including CYP2C9.1 and CYP2C19, as well as the variants CYP2C9.2 (Arg144Cys) and CYP2C9.3 (Ile359Leu); and 2) to assess the effects of steroid hormones on the activities of CYP2C9.1, CYP2C9.2, and CYP2C19 to estimate the contribution of the CYP2C subfamily to metabolism and drug-drug interactions of endogenous compounds. Dopamine formation from *p*-tyramine and 6β- and 21- (for progesterone) hydroxylation of testosterone, cortisol, and progesterone by CYP2C9 variants, CYP2C19, CYP2D6, and CYP3A4 were determined using HPLC. The effects of steroid hormones such as testosterone, cortisol, and progesterone on tolbutamide methyl hydroxylation mediated by CYP2C subfamily members were investigated. Only CYP2D6 catalyzed dopamine formation. The 6β-hydroxylation activities of testosterone, cortisol, and progesterone catalyzed by CYP2C9 variants and CYP2D6 were less than 5% of those by CYP3A4. Although cortisol did not inhibit tolbutamide methyl hydroxylation catalyzed by CYP2C9.1, CYP2C9.2, or CYP2C19 and testosterone did not inhibit CYP2C19 activity, the reactions catalyzed by CY2C9.1 and CYP2C9.2 were inhibited by testosterone. The inhibition of progesterone by CYP2C19 was stronger than that by CYP2C9.1 and CYP2C9.2. CYP2C9.1 and CYP2C19 noncompetitively and competitively inhibited tolbutamide methyl hydroxylation with inhibition constants of 43.2 μM and 1.03 μM, respectively. Clinical interactions among endogenous compounds would vary within the CYP2C subfamily, although the contribution of the CYP2C subfamily may be of minor importance for dopamine formation and the detoxification (6β-hydroxylation) of endogenous steroid hormones.

1. Introduction

Cytochrome P450 (CYP or P450) enzymes are catalyzing the metabolism of both exogenous and endogenous compounds (Rendic 2002; Niwa et al. 2015). The CYP2C subfamily, especially CYP2C9 and CYP2C19, accounts for about one-fifth of all P450s in human livers. It is one of the most important P450 subfamilies because it metabolizes more than one-fifth of all therapeutic drugs (Shimada et al. 1994; Williams et al. 2004). Although CYP2C9 and CYP2C19 share 93% amino acid sequence identity, they have distinct substrate specificities (Niwa et al. 2002; Niwa and Yamazaki 2012). While CYP3A4 accounts for approximately 30% of all P450s in the human liver and metabolizes half of the clinically used medicines (Shimada et al. 1994; Williams et al. 2004), CYP2D6 is expressed in both the liver and the brain (McFayden et al. 1998). We recently demonstrated the effects of steroid hormones on the metabolic activities catalyzed by CYP3A subfamily members (CYP3A4, CYP3A5, and CYP3A7) (Niwa et al. 2020, 2021) and the contribution of brain CYP2D6 variants to dopamine formation from *p*-tyramine (Niwa et al. 2017). However, there are few reports on the contribution of the CYP2C subfamily to the biotransformation of and the interactions with endogenous compounds.

CYP2C9 is polymorphically expressed (The Pharmacogene Variation 2018). The *CYP2C9*2* allele is frequently observed in Caucasians, with 1–3% and 15–22% of the population being homozygous and heterozygous carriers, respectively, and the corresponding frequencies for *CYP2C9*3* are 0.4–1% and 14–15%, respectively (Scordo et al. 2001; Wang et al. 2009). *CYP2C9*2* and *CYP2C9*3* alleles had amino acid substitutions (Arg144Cys and Ile359Leu, respectively) (Wang et al. 2009). However, the effects of CYP2C9 polymorphisms on the metabolism of and interaction with endogenous compounds, including steroid hormones, are yet to be fully elucidated.

Tolbutamide is a typical substrate of CYP2C9 *in vitro* and a recommended clinical index substrate for studying *in vivo* drug interactions of CYP2C9 by the US Food and Drug Administration (FDA) (U.S. Food and Drug Administration 2020). On the other hand, tolubutamide methyl hydroxylation, a major metabolic pathway of the drug, is catalyzed by CYP2C9 and CYP2C19 (Niwa and Imagawa 2016b), although the CYP2C9 content is much higher than that of CYP2C19 in the human liver (Williams et al. 2004). CYP2C9.3 was previously found to have a higher Michaelis constant (K_m) and a lower maximal velocity (V_{max}) than CYP2C9.1 and CYP2C9.2 (Niwa et al. 2016b, 2014). Consequently, we compared the effects of typical endogenous steroid hormones such as progesterone (a corpus luteal hormone), testosterone (an androgen), and cortisol (a glucocorticoid) on the tolbutamide methyl hydroxylation and activities of CYP2C9 variants and CYP2C19 in order to predict drug interactions *in vivo*.

2. Investigations and results

First, the effect of P450s including the CYP2C subfamily on metabolic activities of endogenous compounds were assessed (Table 1). With regard to dopamine formation from *p*-tyramine,

Table 1: Metabolism of endogenous compounds by human hepatic P450s

	Metabolic activity (nmol/min/nmol P450)					
Metabolic activity	CYP3A4	CYP2D6	CYP2C9.1	CYP2C9.2	CYP2C9.3	CYP2C19
Dopamine formation from *p*-tyramine [200]	–	2.92	<0.5	–	–	<0.5
Testosterone 6β-hydroxylation [100]	181.2 (100)	–	2.1 (1.2)	2.0 (1.1)	2.4 (1.3)	2.2 (1.2)
Testosterone 6β-hydroxylation [500]	219.7 (100)	–	6.7 (3.0)	6.9 (3.1)	11.0 (5.0)	7.7 (3.5)
Cortisol 6β-hydroxylation [100]	3.67 (100)	–	<0.1	<0.1	<0.1	<0.1
Cortisol 6β-hydroxylation [500]	12.84 (100)	–	0.63 (4.9)	0.49 (3.8)	0.71 (5.5)	0.34 (2.7)
Progesterone 6β-hydroxylation [20]	38.16 (100)	0.31 (0.8)	<0.02	<0.02	<0.02	0.71 (1.9)
Progesterone 6β-hydroxylation [200]	57.90 (100)	0.99 (1.7)	0.32 (0.5)	0.40 (0.7)	0.48 (0.8)	3.10 (5.4)
Progesterone 21-hydroxylation [20]	0.13 (4.7)	0.12 (4.6)	<0.02	<0.02	<0.02	2.67 (100)
Progesterone 21-hydroxylation [200]	0.03 (2.2)	0.12 (8.3)	<0.02	<0.02	<0.02	1.48 (100)

Values in brackets and parentheses show substrate concentrations (μM) and percentages of the highest metabolic activities (%), respectively. The results were expressed as the means of duplicate or triplicate measurements.

only CYP2D6 catalyzed the reaction whereas the activities of CYP2C9.1 and CYP2C19 were not observed. The 6β-hydroxylation of testosterone, cortisol, and progesterone catalyzed by CYP2C9 variants, CYP2C19, and CYP2D6 were less than 5% of those catalyzed by CYP3A4. On the other hand, the highest activity in progesterone 21-hydroxylation was observed for CYP2C19, followed by CYP2D6 and CYP3A4, whereas the activities by CYP2C9 variants were not detected.

Next, the effects of endogenous steroid hormones on tolbutamide methyl hydroxylation catalyzed by CYP2C9.1, CYP2C9.2, and CYP2C19 were assessed (Table 2); however, the activity catalyzed by CYP2C9.3 was too low to estimate the exact inhibitory effect of the hormones. Although cortisol did not inhibit tolbutamide methyl hydroxylation catalyzed by CYP2C9.1, CYP2C9.2, or CYP2C19 while testosterone did not inhibit CYP2C19 activity, the reactions catalyzed by CY2C9.1 and CYP2C9.2 were inhibited by more than 50% by testosterone at a concentration of 100 μM. Progesterone at 10 μM inhibited the catalytic activity of CYP2C19 by 76%, and the inhibitory effect of progesterone on CYP2C19 was stronger than those on CYP2C9.1 and CYP2C9.2. Therefore, the inhibition pattern of progesterone and the magnitude of effect were further evaluated (Fig.). Interestingly, progesterone noncompetitively and competitively inhibited tolbutamide methyl hydroxylation catalyzed by CYP2C9.1 and CYP2C19 with K_i values of 43.2 and 1.03 μM, respectively.

Table 2: Effects of steroid hormones on tolbutamide methyl hydroxylation catalyzed by CYP2C9.1, CYP2C9.2, and CYP2C19

			% of control		
Steroid	P450	Conc.:	1 μM	10 μM	100 μM
Testosterone	CYP2C9.1		77.1 ± 10.3**	86.8 ± 6.1	46.4 ± 2.6**
	CYP2C9.2		100.3 ± 3.4	84.7 ± 13.4	42.3 ± 3.6**
	CYP2C19		98.4 ± 14.6	89.5 ± 15.4	86.7 ± 14.
Cortisol	CYP2C9.1		94.5 ± 13.9	107.0 ± 3.1	85.1 ± 1.3*
	CYP2C9.2		94.6 ± 1.3	93.9 ± 7.1	93.6 ± 5.0
	CYP2C19		91.2 ± 16.7	92.0 ± 3.3	91.0 ± 5.6
Progesterone	CYP2C9.1		92.2 ± 8.6	60.1 ± 7.8**	21.4 ± 4.9**
	CYP2C9.2		86.9 ± 3.8**	69.1 ± 6.3**	33.7 ± 1.0**
	CYP2C19		72.1 ± 5.8**	24.1 ± 0.1**	7.8 ± 0.9**

Results are expressed as the means ± S.D. of duplicate or triplicate measurements. * $p<0.05$, ** $p<0.01$ *vs* control.

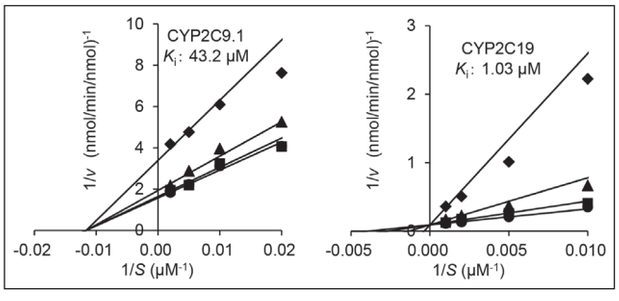

Fig.: Inhibitory effects of progesterone on tolbutamide hydroxylation activities mediated by CYP2C9.1 and CYP2C19. Progesterone concentrations for CYP2C9.1, ●: 0 μM, ▲: 2 μM, ■: 10 μM, ♦: 50 μM; for CYP2C19, ●: 0 μM, ▲: 0.5 μM, ■: 2 μM, ♦: 10 μM. The results were analyzed by nonlinear regression through duplicate or triplicate measurements at each substrate/inhibitor concentration.

3. Discussion

The present study demonstrated that dopamine formation from *p*-tyramine was not catalyzed by CYP2C9.1 and CYP2C19, whereas only CYP2D6 catalyzed this reaction. Since CYP2D6 but not the CYP2C subfamilies are present not only in the liver but also in the brain, and dopamine is not able to travel across the blood-brain barrier, CYP2D6 in the brain might be essential for dopamine biotransformation from *p*-tyramine in addition to its synthesis from dihydroxyphenylalanine (L-DOPA) (Hiroi et al. 1998). Although the 6β-hydroxylation activities of three steroid hormones by CYP2C9 variants, CYP2C19, and CYP2D6 were less than 5% of those for CYP3A4 (Table 1), the highest progesterone 21-hydroxylation (deoxycorticosterone formation) activity was observed for CYP2C19, suggesting that hepatic CYP2C19 might partially contribute to the biotransformation of deoxycorticosterone, a mineral corticoid secreted by the adrenal gland (Reddy 2006). However, the hydroxylation of steroid hormones catalyzed by CYP2C9.2 and CYP2C9.3 were similar to those by CYP2C9.1 (Table 1), suggesting that CYP2C9 polymorphisms had minor effects on endogenous hormone metabolism.

In the present study, progesterone inhibited tolbutamide methyl hydroxylation catalyzed by CYP2C9.1 and CYP2C19 noncompetitively and competitively, respectively. In addition, testosterone inhibited the activities catalyzed by CYP2C9.1 and CYP2C9.2, but not CYP2C19. We have previously reported that voriconazole, an azole antifungal, noncompetitively inhibited tolbutamide methyl hydroxylation catalyzed by CYP2C9.1 and CYP2C9.3, whereas fluconazole and voriconazole competitively inhibited its hydroxylation catalyzed by CYP2C9.2. Fluconazole also competitively inhibited tolbutamide methyl hydroxylation catalyzed by CYP2C9.1 (Niwa et al. 2016b). Thus, the inhibition type seems to differ by P450 enzymes, substrates, inhibitors, and/or other experimental conditions. Further investigation, for instance, using three-dimensional structural analysis such as molecular docking simulation (Niwa et al. 2019) would be required.

The K_i value of progesterone for CYP2C19 (1.03 μM) was much lower than that for CYP2C9.1 (43.2 μM). When the substrate concentration is lower than the K_m value, the degree of interaction (R, expressed as fold-change in AUC) can be expressed using the following equation, independent of the inhibition type, except in the case of uncompetitive inhibition (Ito et al. 1998):

$R = 1 + I_u/K_i$

where I_u is the unbound concentration of the inhibitor exposed to the active site of the enzyme. The plasma concentrations of testosterone, cortisol, and progesterone have been reported to be below 35 nM (Diver 2009), 46.2 ng/dL (1.3 μM) (Benfield et al. 2010), and 1.2 nM (Andréen et al. 2006) or 10.5 (range: 5.6–16.9) ng/mL (33 nM, range: 18–54 nM) (Delfs et al. 1994), respectively. They are much lower than the concentrations at which the inhibitory effects were observed in this study. However, reports on the distribution of these hormones in the human liver and brain are rare due to challenges in measuring their concentrations in humans. Further pharmacokinetic investigations, including the measurement of their distribution in the liver and brain, are required to assess the pharmacological effects of steroid hormones in these tissues.

In conclusion, the present study suggests that the substrate specificity and inhibition sensitivity for both extraneous substances and endogenous compounds differ between CYP2C9 variants and CYP2C19. The significance of the CYP2C subfamily in endogenous metabolism warrants further elucidation.

4. Experimental

4.1. Materials

CYP2C9.1, CYP2C9.2, CYP2C9.3, CYP2C19, CYP2D6, and CYP3A4, co-expressed with NADPH-P450 reductase but not cytochrome b_5 in recombinant *Escherichia coli* (Bactosomes, Easy CYP), were obtained from Cypex Ltd. (Dundee, UK). Tolbutamide, methyl hydroxylated tolbutamide, and 6β-hydroxytestosterone were purchased from Sigma-Aldrich (MO, USA), Tocris Bioscience (Bristol, UK), and Funakoshi Co., Ltd (Tokyo, Japan), respectively. Cortisol and deoxycorticosterone (21-hydroxyprogesterone) were purchased from Fujifilm Wako Pure Chemicals (Osaka, Japan). Testosterone and progesterone were obtained from Nacalai Tesque (Kyoto, Japan). *P*-tyramine, dopamine, 6β-hydroxyprogesterone, and 6β-hydroxycortisol were purchased from Tokyo Chemical Industry (Tokyo, Japan). All other reagents and organic solvents used were of the highest commercially available purity.

4.2. Determination of drug-metabolizing P450 activities

Tolbutamide methylhydroxylation, dopamine formation from *p*-tyramime, cortisol 6β-hydroxylation, testosterone 6β-hydroxylation, and 6β- and 21-hydroxylation of progesterone were determined as previously described (Hiroi et al. 1998; Niwa et al. 2005, 2014, 2017, 2019). To assess the effect on tolbutamide methyl hydroxylation, the incubation mixture consisted of human CYP2C9.1, CYP2C9.2, or CYP2C19 subfamily, 1 mM NADPH, 100 μM tolbutamide [close to the previously reported K_m (Niwa et al. 2014)], 1–100 μM steroid hormones, and 100 mM potassium phosphate buffer (pH 7.4) in a final volume of 500 μL. The reaction linearity with different concentrations and incubation times was confirmed for each P450 in preliminary experiments. All data were analyzed by performing duplicate or triplicate measurements. Inhibitory constants (K_i) were determined by Michaelis-Menten kinetics using nonlinear least squares regression by means of MULTI software (Yamaoka et al. 1981).

Acknowledgement: We would like to thank Editage (Cactus Communications, www.editage.com) for English language editing.

Conflict of interest: none declared

References

Andréen L, Spigset O, Andersson A, Nyberg S, Bäckström T (2006) Pharmacokinetics of progesterone and its metabolites allopregnanolone and pregnanolone after oral administration of low-dose progesterone. Maturitas 54: 238–244.

Benfield RD, Hortobágyi T, Tanner CJ, Swanson M, Heitkemper MM, Newton ER (2010) The effects of hydrotherapy on anxiety, pain, neuroendocrine responses, and contraction dynamics during labor. Biol Res Nurs 12: 28–36.

Delfs TM, Klein S, Fottrell P, Naether OG, Leidenberger FA, Zimmermann RC (1994) 24-Hour profiles of salivary progesterone. Fertil Steril 62: 960–966.

Diver M (2009) Laboratory measurement of testosterone. Front Horm Res 37: 21–31.

Hiroi T, Imaoka S, Funae Y. Dopamine formation from tyramine by CYP2D6 (1998) Biochem Biophys Res Commun 249: 838–843.

Ito K, Iwatsubo T, Kanamitsu S, Ueda K, Suzuki H, Sugiyama Y (1998) Prediction of pharmacokinetic alterations caused by drug-drug interactions: metabolic interaction in the liver. Pharmacol Rev 50: 387–412.

McFayden MC, Melvin WT, Murray GI (1998) Regional distribution of individual forms of cytochrome P450 mRNA in normal adult human brain. Biochem Pharmacol 55: 825–830.

Niwa T, Hata T (2016a) The effect of genetic polymorphism on the inhibition of azole antifungal agents against CYP2C9-mediated metabolism. J Pharm Sci 105: 1345–1348.

Niwa T, Imagawa Y (2016b) Substrate specificity of human cytochrome P450 (CYP) 2C subfamily and effect of azole antifungal agents on CYP2C8. J Pharm Pharm Sci 19: 423–429.

Niwa T, Imagawa Y, Yamazaki H (2014) Drug interactions between nine antifungal agents and drugs metabolized by human cytochromes P450. Curr Drug Metab, 15:651–679.

Niwa T, Inoue S, Shiraga T, Takagi A (2005) No inhibition of cytochrome P450 activities in human liver microsomes by sulpiride, an antipsychotic drug. Biol Pharm Bull 28: 188–191.

Niwa T, Kageyama A, Kishimoto K, Yabusaki Y, Ishibashi F, Katagiri M (2002) Amino acid residues affecting the activities of human cytochrome P450 2C9 and 2C19. Drug Metab Dispos 30: 931–936.

Niwa, T, Murayama, N, Imagawa, Y, Yamazaki, H (2015) Regioselective hydroxylation of steroid hormones by human cytochromes P450. Drug Metab Rev 47: 89–110.

Niwa T, Narita K, Okamoto A, Murayama N, Yamazaki H (2019) Comparison of steroid hormone hydroxylations by and docking to human cytochromes P450 3A4 and 3A5. J Pharm Pharm Sci 22: 332–339.

Niwa T, Okamoto A, Narita K, Toyota M, Kato K, Kobayashi K, Sasaki S (2020) Comparison of steroid hormone hydroxylation mediated by cytochrome P450 3A subfamilies. Arch Biochem Biophys 682: 108283.

Niwa T, Shizuku M, Yamano K (2017) Effect of genetic polymorphism on the inhibition of dopamine formation from *p*-tyramine catalyzed by brain cytochrome P450 2D6. Arch Biochem Biophys 620: 23–27.

Niwa T, Toyota M, Kawasaki H, Ishii R, Sasaki S (2021) Comparison of the stimulatory and inhibitory effects of steroid hormones and α-naphthoflavone on steroid hormone hydroxylation catalyzed by human cytochrome P450 3A subfamilies. Biol Pharm Bull 44: 579–584.

Niwa T, Yamazaki H (2012) Comparison of cytochrome P450 2C subfamily members in terms of drug oxidation rates and substrate inhibition. Curr Drug Metab 13: 1145–1159.

Reddy DS (2006) Physiological role of adrenal deoxycorticosterone-derived neuroactive steroids in stress-sensitive conditions. Neuroscience 138: 911–920.

Rendic S (2002) Summary of information on human CYP enzymes: human P450 metabolism data. Drug Metab Rev 34: 83–448.

Scordo MG, Aklillu E, Yasar U, Dahl ML, Spina E, Ingelman-Sundberg M (2001) Genetic polymorphism of cytochrome P450 2C9 in a Caucasian and a black African population. Br J Clin Pharmacol 52:447–450.

Shimada T, Yamazaki H, Mimura M, Inui Y, Guengerich FP (1994) Interindividual variations in human liver cytochrome P-450 enzymes involved in the oxidation of drugs, carcinogens and toxic chemicals: studies with liver microsomes of 30 Japanese and 30 Caucasians. J Pharmacol Exp Ther 270: 414–423.

The Pharmacogene Variation (PharmVar) Consortium. The Human Cytochrome P450 (CYP) Allele Nomenclature Database. Launched on March 20, 2018. PharmVar. Available at: https://www.pharmvar.org/gene/CYP2C9. Updated August 5, 2021. Accessed August 22, 2021.

U.S. Food and Drug Administration. Drug Development and Drug Interactions: Table of Substrates, Inhibitors and Inducers.
https://www.fda.gov/drugs/drug-interactions-labeling/drug-development-and-drug-interactions-table-substrates-inhibitors-and-inducers#table1. Updated March 10, 2020. Accessed August 22, 2021.

Yamaoka K, Tanigawara Y, Nakagawa T, Uno T (1981) A pharmacokinetic analysis program (MULTI) for microcomputer. J Pharmacobiodyn 4:879–885.

Wang B, Wang J, Huang SQ, Su HH, Zhou SF (2009) Genetic polymorphism of the human cytochrome P450 2C9 gene and its clinical significance. Curr Drug Metab 10: 781–834.

Williams JA, Hyland R, Jones BC, Smith DA, Hurst S, Goosen TC, Peterkin V, Koup JR, Ball SE (2004) Drug-drug interactions for UDP-glucuronosyltransferase substrates: a pharmacokinetic explanation for typically observed low exposure (AUCi/AUC) ratios. Drug Metab Dispos 32: 1201–1208.