Comparison of Steroid Hormone Hydroxylations by and Docking to Human Cytochromes P450 3A4 and 3A5

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ABSTRACT - Purpose: Hydroxylation activity at the 6β-position of steroid hormones (testosterone, progesterone, and cortisol) by human cytochromes P450 (P450 or CYP) 3A4 and CYP3A5 and their molecular docking energy values were compared to understand the catalytic properties of the major forms of human CYP3A, namely, CYP3A4 and CYP3A5. **Methods:** Testosterone, progesterone, and cortisol 6β-hydroxylation activities of recombinant CYP3A4 and CYP3A5 were determined by liquid chromatography. Docking simulations of these substrates to the heme moiety of reported crystal structures of CYP3A4 (Protein Data Bank code ITQN) and CYP3A5 (6MJM) were conducted. **Results:** Michaelis constants (K_m) for CYP3A5-mediated 6β-hydroxylation of testosterone and progesterone were approximately twice those for CYP3A4, whereas the value for cortisol 6β-hydroxylation mediated by CYP3A5 was similar to the value for that by CYP3A4. Maximal velocities (V_{max}) of the three steroid hormones 6β-hydroxylation catalyzed by CYP3A5 were 30%-63% of those by CYP3A4. Thus, V_{max}/K_m values of these hormones for CYP3A5 resulted in 22%-31% of those for CYP3A4. The differences in the docking energies between CYP3A4 and CYP3A5 for steroid hormones were slightly correlated to the logarithm of CYP3A5/CYP3A4 ratios for K_m values (substrate affinity). **Conclusions:** The V_{max}, rather than K_m values, for CYP3A5-mediated 6β-hydroxylation of three steroid hormones were different from those for CYP3A4. Molecular docking simulations could partially explain the differences in the accessibility of substrates to the heme moiety of human CYP3A molecules, resulting in the enzymatic affinity of CYP3A4 and CYP3A5.

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INTRODUCTION

Cytochrome P450 (P450 or CYP) 3A is the most important human P450 subfamily, due to its high abundance in the human hepatic microsomes, which accounts for approximately 30% of total P450s enzymes (1, 2). Furthermore, CYP3A enzymes are responsible for 50% of all pharmaceuticals oxidation (3). Inducible CYP3A4 is generally recognized as the predominant CYP3A form expressed in human livers and intestines. On the other hand, polymorphically expressed CYP3A5 contributes as much as 50% of hepatic CYP3A in a third of Caucasians and about half of African–Americans (4). It was thought that the substrate specificity of CYP3A5 is similar to that of CYP3A4, because CYP3A4 and CYP3A5 are 83% homologous in terms of amino acid sequences. However, some

differences in catalytic properties have been reported (5, 6). We previously reviewed the reported values for (a) Michaelis-Menten constants (K_m), maximal velocities (V_{max}), and intrinsic clearance (CL$_{int}$, V_{max}/K_m) values for reactions mediated by CYP3A4 and/or CYP3A5, (b) inhibition constants (K_i) and 50% inhibitory concentrations (IC$_{50}$), and (c) maximum inactivation rate constants (k_{inact}), and demonstrated that the substrate specificities of CYP3A4 and CYP3A5 generally overlap, but some differences can be identified. (7, 8).

It is well established that some steroid hormones are metabolized by not only steroidogenic P450s, such as CYP11B1, CYP11B2, CYP17A1, CYP19A1,

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CYP27A1, but also by drug-metabolizing P450s including CYP2C, CYP2D, and the CYP3A subfamily (9, 10). In terms of hydroxylation at the C6β position, testosterone 6β-hydroxylation is predominantly catalyzed by the CYP3A subfamily (9, 10) and is recommended as a preferred reaction of CYP3A4/5 for *in vitro* experiments in the guidelines for new drug applications regarding *in vitro* studies of drug metabolism and drug–drug interactions regulated by the US Food and Drug Administration (FDA), European Medicines Agency (EMA), and Japanese Ministry of Health, Labour and Welfare (11-13). The ratio of 6β-hydroxycortisol to cortisol, a stress hormone, in plasma or urine has been used as an *in vivo* endogenous marker of CYP3A4/5 metabolic activity (14-16). Progesterone is hydroxylated by drug-metabolizing P450s including CYP2D6 and CYP3A4 at the C2β, C6α, C6β, C16α, and C21 positions, as well as by the steroidogenic CYP21A1 at the C21 position (9, 10). However, there are few reports that compare, in detail, the kinetic parameters for the hydroxylation of these steroid hormones mediated by CYP3A4 and CYP3A5.

We previously conducted docking simulation studies for substrates and inhibitors of P450 proteins, including CYP3A4 and CYP3A5, and demonstrated that some of the results were consistent with the enzymatic characteristics observed in *in vitro* studies, although the primary sequence of CYP3A5 was aligned with that of CYP3A4 using three-dimensional modeling software (17, 18).

In the present study, we compared the CYP3A4 and CYP3A5-mediated 6β-hydroxylation of three steroid hormones: testosterone, progesterone, and cortisol. In addition, we carried out docking simulations for the binding of CYP3A4 and CYP3A5 with steroid hormones using a docking simulation program and compared the results with previously reported compounds (18). We report herein that the differences in accessibility of substrates to the heme of human CYP3A molecules resulted in the apparent enzymatic affinity of CYP3A4 and CYP3A5.

MATERIALS AND METHODS

Materials

CYP3A4 and CYP3A5 expressed in recombinant *Escherichia coli* (Bactosomes) were obtained from Cypex Ltd (Dundee, UK). Testosterone and cortisol were purchased from Nakarai Tesque (Kyoto, Japan) and Fujifilm Wako Pure Chemicals (Osaka, Japan), respectively. Metabolites 6β-hydroxytestosterone and 6β-hydroxycortisol were obtained from Sigma-Aldrich (St Louis, MO, USA). Progesterone and 6β-hydroxyprogesterone were purchased from Tokyo Chemical Industry (Tokyo, Japan) and Steraloids Inc. (Newport, RI USA), respectively. All other reagents and organic solvents used were of the highest purity commercially available.

Hydroxylation Activities of Steroid Hormones

Testosterone and progesterone 6β-hydroxylation activities were determined as described previously with minor modification (19-22). The incubation mixtures consisted of 10 pmol/mL CYP3A4 or CYP3A5, 1 mM NADPH, and 100 mM potassium phosphate buffer (pH 7.4) in a final volume of 0.5 mL. Incubation times were 5 min, with the exception of cortisol 6β-hydroxylation by CYP3A4 (10 min) or CYP3A5 (20 min). For the 6β-hydroxycortisol assay, the reaction was terminated by adding 3.5 mL of ethyl acetate. The organic phase (3 mL) was evaporated and the residue was immediately dissolved in 200 µL of 5 mM KH_2PO_4/methanol (75:25). HPLC analysis was performed by injecting 50 µL into an Inertsil ODS-3 column (150×4.6 mm I.D., GL Sciences Inc.), with the column temperature set at 40 °C. The UV-detector was set at 240 nm. Mobile phase A was 5 mM KH_2PO_4/methanol (75:25) and mobile phase B was 5 mM KH_2PO_4/methanol (20:80). A gradient elution at a flow rate of 1mL/min was performed as follows: 0-15 min 0% B, 15-16 min 0-100% B (linear gradient), 16-25 min 100% B, 25-26 min 100-0% B (linear gradient), and 26-40 min 35% B. Calibration curves were linear for 6β-hydroxycortisol concentrations ranging from 0.1 to 5 µM.

All data were analyzed using the average of triplicate determinations. In the preliminary experiments, the linearity of the reaction with P450

concentration and incubation time was confirmed for each CYP3A subfamily. K_m and V_{max} values were calculated by performing Michaelis-Menten kinetics using nonlinear least squares regression by means of MULTI (23).

Docking Simulation of Steroid Hormones with Human P450 Enzymes

The crystal structures of CYP3A4 (Protein Data Bank code ITQN) and CYP3A5 (6MJM) were used (24, 25). Prior to the docking simulations, the energies of the CYP3A4 and CYP3A5 structures were minimized using the CHARMM22 force field. Docking simulations were carried out for the binding of substrates to P450 enzymes using the MMFF94x force field distributed in the Molecular Operating Environment (MOE) ASEDock software (Chemical Computing Group, Montreal, Canada) (18). Twenty solutions were generated for each docking experiment and then ranked according to total interaction energy (U value).

Literature Search for Kinetic Parameters

For the correlation analysis, kinetic parameters such as K_m, V_{max}, and V_{max}/K_m for several compounds, including estradiol and estrone, were obtained from the literature (10, 18, 26). Correlation analysis was carried out using Prism (GraphPad Software, La Jolla, CA).

RESULTS

The kinetic parameters for the 6β-hydroxylation of three steroid hormones by CYP3A4 and CYP3A5 were estimated by fitting the values into the Michaelis-Menten plots (Table 1). The K_m values for CYP3A5-mediated 6β-hydroxylation of testosterone and progesterone were approximately twice those for CYP3A4, whereas the value for cortisol 6β-hydroxylation by CYP3A5 was similar to that of CYP3A4. The V_{max} values for CYP3A5-mediated 6β-hydroxylation of the three steroid hormones were 30-63% of those for CYP3A4. Thus, V_{max}/K_m values for the 6β-hydroxylation of these hormones by CYP3A5 were 22-31% of those for CYP3A4. For both CYP3A4 and CYP3A5, K_m values for progesterone were lowest, followed by testosterone. V_{max} values for testosterone were highest among the three steroid hormones, and V_{max}/K_m values for cortisol were lowest.

Molecular docking simulations were conducted on six steroid hormones with CYP3A4 and CYP3A5 (Table 2). For estrone, the K_m value for CYP3A5-mediated 4-hydroxylation is reported to be higher than that for CYP3A4 (10, 26). As such, ligand-P450 interaction energies (U values) were found to be lower for CYP3A4 than for CYP3A5. However, U values for CYP3A5 in other steroid hormones were lower than those for CYP3A4. In all cases, the docking distance was between 3.82 and 11.3Å. Several compounds, other than these steroid hormones, were investigated in order to confirm the present docking results.

Table 1. Kinetic parameters for steroid 6β-hydroxylation activities by recombinant human CYP3A4 and CYP3A5

Steroid	CYP3A4/5	K_m (μM)	V_{max} (nmol/min/nmol CYP3A)	V_{max}/K_m (mL/min/nmol CYP3A)
Testosterone	CYP3A4	63.8±10.5 (100)	125±7 (100)	1.96 (100)
	CYP3A5	119±24 (186)	51.1±4.1 (41)	0.43 (22)
Progesterone	CYP3A4	14.3±2.7 (100)	43.4±1.6 (100)	3.03 (100)
	CYP3A5	29.2±3.6 (204)	27.2±0.8 (63)	0.93 (31)
Cortisol	CYP3A4	358±37 (100)	12.2±0.5 (100)	0.034 (100)
	CYP3A5	413±136 (115)	3.6±0.5 (30)	0.0087 (26)

Kinetic parameters were determined by nonlinear regression analysis (mean ± standard deviation, n = 7 substrate concentrations in the range of 2–1,000 μM, in triplicate determinations). Values in parentheses are percentage of those for CYP3A4.

Vincristine, which is a typical substrate of CYP3A5 and has higher V_{max} and V_{max}/K_m values and a lower K_m value for reactions mediated by CYP3A5 than by CYP3A4 (27), could more closely dock to the heme region of CYP3A5 (10.8Å), with a slightly lower U value, than to that of CYP3A4 (18.5Å). β-Endosulfan, which has a lower K_m value and higher V_{max} and V_{max}/K_m values for reactions mediated by CYP3A5 than by CYP3A4 (28), had a lower U value for CYP3A5 than for CYP3A4. On the other hand, midazolam 1'- and 4-hydroxylations which have higher K_m and V_{max} values for reactions catalyzed by CYP3A4 than by CYP3A5 (8, 18), had a lower U value for CYP3A4 than that for CYP3A5.

We investigated the relationship between the ratios of mean K_m, V_{max}, and V_{max}/K_m values obtained in the present study and previously reviewed for CYP3A4 and CYP3A5 (18), and the differences between the U values for these P450s (Fig. 1). The difference in U values between CYP3A5 and CYP3A5 were slightly correlated with the logarithm of CYP3A5/CYP3A4 ratios for K_m ($r = 0.62$, $p = 0.14$, Fig. 1A) and V_{max} values ($r=0.64$, $p = 0.12$, Fig. 1B), but not with the logarithm of CYP3A5/CYP3A4 ratios for V_{max}/K_m values (Fig. 1C). U: interaction energy in kcal/mol. Seven reactions of five steroid hormones as shown in Table 2, except for DHEA, were investigated. Dotted lines show the regression curve between the logarithm of the CYP3A5/CYP3A4 ratios of K_m, V_{max}, and V_{max}/K_m values and the differences between U values for CYP3A4 and CYP3A5 for steroid hormones.

DISCUSSION

Testosterone 6β-hydroxylation is recommended as a preferred reaction of CYP3A4/5 for *in vitro* experiments (11-13) and the 6β-hydroxycortisol/cortisol ratio is used as an *in vivo* endogenous marker of CYP3A4/5 metabolic activities (14-16). In addition, cortisol, a stress hormone, is known to be one of the many hormones responsible for the physiological changes involved in response to stress, as well as a glucocorticoid hormone that is produced by the adrenal glands and released for a variety of reasons (29). Progesterone 6β-hydroxylation is catalyzed by drug-metabolizing P450s, including CYP3A4 and CYP2D6 (9, 10).

Table 2. Docking simulation results of steroid hormones with respect to human CYP3A4 and CYP3A5

Compound	Reaction	U value (kcal/mol)		Distance (Å)	
		CYP3A4	CYP3A5	CYP3A4	CYP3A5
Testosterone	6β-Hydroxylation	-26.3	-38.5	5.03	7.36
Progesterone	6β-Hydroxylation	-11.8	-34.7	10.7	11.3
Cortisol	6β-Hydroxylation	-27.0	-44.7	6.65	6.99
DHEA	7β-Hydroxylation	-27.6	-39.0	8.31	8.22
	16α-Hydroxylation	-30.4	-41.8	5.68	6.03
Estradiol	2-Hydroxylation	-5.7 (18)	-18.8	9.27	7.24
	4-Hydroxylation	-5.7 (18)	-18.8	9.21	7.62
Estrone	2-Hydroxylation	-38.0	-20.7	4.04	7.65
	4-Hydroxylation	-38.0	-20.7	3.82	8.81
Vincristine	M1 formation	-17.8 (18)	-20.6	18.5	10.8
β-Endosulfan	Sulfate formation	-7.17 (18)	-21.1	5.77	9.92
Midazolam	1'-Hydroxylation	-52.3 (18)	-26.0	4.64	4.71
	4-Hydroxylation	-52.3 (18)	-24.0	5.31	4.52

DHEA: dehydroepiandrosterone.

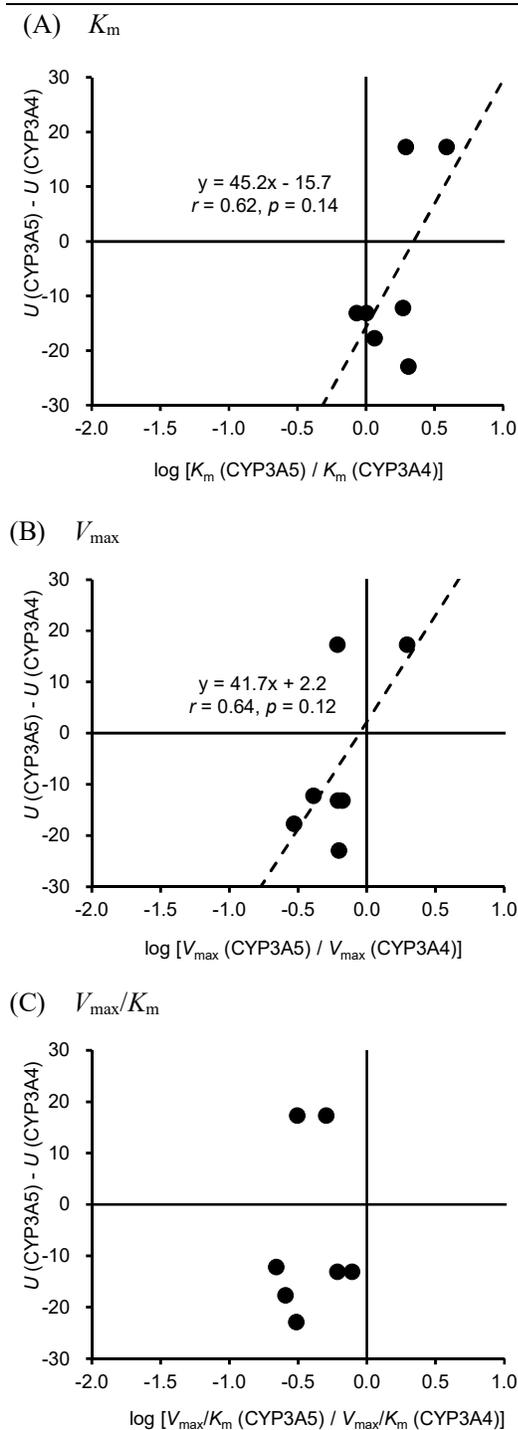(A) K_m

(B) V_{max}

(C) V_{max}/K_m

Figure 1. Relationship between the ratios of K_m (A), V_{max} (B), and V_{max}/K_m (C), and the differences between docking energy values for CYP3A4 and CYP3A5. U: interaction energy in kcal/mol. Seven reactions of five steroid hormones as shown in Table 2, except for DHEA, were investigated. Dotted lines show the regression curve between the logarithm of the CYP3A5/CYP3A4 ratios of K_m, V_{max}, and V_{max}/K_m values and the differences between U values for CYP3A4 and CYP3A5 for steroid hormones.

Although K_m and V_{max} values for testosterone 6β-hydroxylation by recombinant CYP3A4 and/or CYP3A5 have already been reported as 24-110 µM (for CYP3A4) and 44-400 µM (for CYP3A5) for K_m values and 3.8-3600 nmol/min/nmol P450 (for CYP3A4) and 2.2-1600 nmol/min /P450 (for CYP3A5) for V_{max} values (7, 10, 30-36), the values obtained in the present study (K_m: 63.8 µM for CYP3A4 and 119 µM for CYP3A5, V_{max}: 125 nmol/min/nmol P450 for CYP3A4 and 51.1 nmol/min/nmol P450 for CYP3A5) were similar to the medians of the reported values. In the present study, K_m values for 6β-hydroxylation of three steroid hormones (testosterone, progesterone, and cortisol) mediated by CYP3A5 were at most twice those for the CYP3A4-mediated reactions, whereas V_{max} values for the hydroxylation catalyzed by CYP3A5 were 30-63% of those for CYP3A4. Therefore, V_{max}/K_m values, which correspond to intrinsic clearance, for the hydroxylation of these hormones by CYP3A5 were 22-31% of those for CYP3A4. These results suggest that testosterone 6β-hydroxylation activity in human liver microsomes for *in vitro* studies of drug-drug interactions would depend on the CYP3A4 level rather than CYP3A5. Thus, the addition of other substrates, such as midazolam, might be necessary for the assessment of CYP3A4/5 activity as recommended by FDA and PMDA (11, 13). Furthermore, the effect of CYP3A5 polymorphism on the plasma or urine ratio of 6β-hydroxycortisol/cortisol *in vivo* as a marker for stress might be of minor importance. Further clinical studies are necessary to elucidate the problem.

We previously carried out docking simulations for the binding of CYP3A4 and CYP3A5 with 13 substrates using docking simulation program MOE and found that the docking energy ratio between CYP3A5 and CYP3A4 were not significantly correlated with the CYP3A5/CYP3A4 ratios of V_{max} and V_{max}/K_m values, nor with molecular weight, volume, or log P values of substrates. Furthermore, neither the differences in the enzyme source (baculovirus infected insect cells or *Escherichia coli* engineered to express P450s) nor the supplementation or co-expression of cytochrome b_5 were correlated with the K_m, V_{max}, and V_{max}/K_m values for CYP3A4 or CYP3A5 (18). In our

previous study, the primary sequence of CYP3A5 was aligned with that of CYP3A4 using three-dimensional modeling software (18), while in the present study, we used the recently reported crystal structures of CYP3A5 (Protein Data Bank code 6MJM) (25). Vincristine, a typical substrate of CYP3A5, but not of CYP3A4, has higher V_{max} and V_{max}/K_m values and a lower K_m value for reactions mediated by CYP3A5 than by CYP3A4 (27). In the present study, vincristine was shown to dock closer to the heme region of CYP3A5 (10.81Å) with a lower U value than to that of CYP3A4 (18.50Å); the findings were similar to the previous *in silico* analysis (18). In the case of midazolam, for which K_m value for CYP3A5 was higher than for CYP3A4, the docking energies for CYP3A4 were lower than those for CYP3A5. Although U values for CYP3A5 in steroid hormones, with the exception of estrone, were lower than those for CYP3A4, the difference in K_m values as well as V_{max} values, but not V_{max}/K_m values and docking results, between CYP3A4 and CYP3A5 for steroid hormones were slightly corelated (Fig. 2). However, because it's highly unlikely that the reaction with higher V_{max} values would have higher U values, we suggest that molecular docking simulations could partially explain the differences in substrate affinity (the K_m values), as demonstrated in the previous study (18). As CYP3A4 and CYP3A5 are 83% homologous in terms of amino acid sequences, the most plausible explanation for the differences between CYP3A4 and CYP3A5 is that conformational changes, caused by amino acid differences, lead to altered accessibility of substrates and inhibitors to the heme iron of CYP3A4 and CYP3A5. However, it would be difficult to determine the substrates for which K_m and V_{max} values for CYP3A5 are different from those for CYP3A4, by using the differences in U values (18). Further quantitative analytical studies with additional substrates and inhibitors, including those with a preference for either CYP3A4 or CYP3A5, are required. In the future, more advanced simulations such as molecular dynamics simulations are of interest, in order to explain the induced-fit effects caused by different ligands binding.

In conclusion, V_{max} values, rather than K_m values, for 6β-hydroxylation of three steroid hormones by CYP3A5 were different from those for CYP3A4, and molecular docking simulations might partially explain the differences in the affinity of substances for CYP3A4 and CYP3A5, based on the accessibility of substrates to the heme moiety of CYP3A molecules.

ACKNOWLEDGEMENTS

DECLARATION OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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