Direct Interaction between Substrates and Endogenous Steroids in the Active Site May Change the Activity of Cytochrome P450 3A4

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ABSTRACT: CYP3A4 exhibits unusual kinetic characteristics that result from the metabolism of multiple substrate including endogenous steroids and some drugs that coexist at the active site. To clarify the mechanism of the effect of endogenous steroids on the drug metabolism, the interaction between substrates, nevirapine (NVP) and carbamazepine (CBZ), and endogenous steroids was investigated by theoretical calculations. When the activities of NVP 2-hydroxylation and CBZ 10,11-epoxidation by expressed CYP3A4 were measured in the presence of steroids, NVP 2-hydroxylation was found to be remarkably increased by aldosterone and inhibited by estradiol. CBZ 10,11-epoxidation was increased by androstenedione. Three-dimensional computer modeling has shown that the active site of CYP3A4 is especially large, permitting access of two substrate molecules. The interactions between NVP and aldosterone and between CBZ and androstenedione were estimated by theoretical calculations assuming the substrate and steroids to be present in the active site at the same time. It was shown that NVP or CBZ would be stably fixed close to the oxygen atom at the sixth ligand of heme by interaction with steroids, suggesting that NVP and CBZ may be hydroxylated more easily due to the interaction with steroids. Estradiol was also expected to interact with NVP via a π/π interaction between a benzene ring, in which the NVP hydroxylation site is located, and a benzene ring of estradiol, suggested to inhibit the reaction. From these results, interactions between the substrate and endogenous steroids in the active site may change the activity of CYP3A4.

The cytochrome P450 (CYP)[1] enzymes play a clinically important role in drug metabolism. CYP3A4 is known to be expressed at the highest level in human liver (*1*, *2*) and is the most important enzyme involved in the metabolism of pharmaceuticals (*3*). CYP3A4 is also capable of metabolizing endogenous steroids such as cortisol (*4*), testosterone (*5*), 17β-estradiol (*6*), and progesterone (*7*). A previous report has shown that some kinds of endogenous steroids activate and/or inhibit CYP3A4-mediated drug metabolism by human liver microsomes (*8*). As these endogenous steroids are always present in vivo, it is important to clarify the mechanism of the activation and inhibition of CYP3A4-mediated drug metabolism by endogenous steroids.

Most CYP oxidations show hyperbolic saturation kinetics and competitive inhibition between substrates. Therefore, some pharmacokinetic properties can be predicted by standard Michaelis−Menten kinetic analysis. However, some CYP reactions show unusual enzyme kinetics, and most such enzymes identified so far are associated with oxidation by the CYP3A enzymes. The unusual kinetic characteristics of the CYP3A enzymes include four categories: activation, autoactivation, partial inhibition, and substrate inhibition. Activation is an increased reaction velocity in the presence of another compound (*9*, *10*). Sigmoidal saturation kinetics is considered to be caused by autoactivation because the activator is the substrate itself (*11*, *12*). For partial inhibition, saturating concentrations of inhibition do not completely inhibit substrate metabolism. Substrate inhibition occurs when an increase in substrate concentration beyond a certain value results in a decrease in the rate of metabolism. To explain the unusual kinetics, some analytical models have advanced the hypothesis of two substrates coexisting in the active site of CYP3A4, demonstrating cooperativity. CYP3A4 shows homotropic cooperativity toward a number of substrates (*10*, *12*−*14*) and heterotropic cooperativity, which shows activation upon the addition of a second compound, such as flavonoids or steroid hormones (*8*, *12*, *15*−*19*). Although multisite kinetic models were developed in detail in order to better understand the interaction between substrates and substrates/effectors of CYP3A4 (*17*, *18*, *20*), all of these models assume the combined effects of allosteric modulation, steric effects, and/or the electronic characteristics of the enzyme.

The three-dimensional structure of CYP3A4 was modeled on the basis of crystallographic coordinates of four bacterial P450s: P450 BM-3, P450cam, P450terp, and P450eryF (*21*).

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[1] Abbreviations: CYP, cytochrome P450; NVP, nevirapine; CBZ, carbamazepine; DHEA, dehydroepiandrosterone.

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Especially, the structure of the active site of CYP3A4 was modeled by that of P450eryF because the substrate for P450eryF was large (*22*). Actually, crystal structures of P450eryF contained two molecules of androstenedione or 9-aminophenanthrene in the active site (*23*). Thus, a homology model of CYP3A4 (*21*) suggested that the active site of the enzyme is very large and probably capable of accommodating more than one substrate at a time. The simultaneous presence of two different substrates in the active site has been used to explain the partial competitive inhibition of testosterone metabolism caused by erythromycin (*24*). Studies using flavonoids as heterotropic stimulators of polycyclic aromatic hydrocarbon metabolism have suggested that the effector and substrate are probably both present in the active site at the same time (*25*).

A better understanding of the variability associated with CYP3A both in vitro and in vivo is required to fully understand the role of CYP3A in drug metabolism and thus improve the current capabilities for predicting drug metabolism by this enzyme. However, the current understanding of this enzyme is considered insufficient to predict interactions among substrates, effectors, and CYP3A4, because the demonstrated kinetic models for cooperativity require unestablished assumptions to calculate K_m and V_{max}. To explain the inconsistencies in clarifying the interactions among substrates, effectors, and CYP3A4, we suggested another possibility, a direct interaction between substrates and substrates/effectors in the active site of CYP3A4 using nevirapine (NVP) and carbamazepine (CBZ) as substrates and endogenous steroids as effectors in this study.

EXPERIMENTAL PROCEDURES

Chemicals. CBZ and CBZ 10,11-epoxide were generous gifts from Novartis Pharma Co. (Tokyo, Japan). NVP and 2-hydoroxy-NVP were synthesized by Boehringer Ingelheim Pharma Co. (Ingelheim, Germany). Pregnenolone sulfate (5-pregnen-3β-ol-20-one sulfate), *d*-aldosterone (4-pregnen-18-al-11β,21-diol-3,20-dione), DHEA (5-androsten-3β-ol-17-one), androstenedione (4-androstene-3,17-dione), testosterone (4-androsten-17β-ol-3-one), estrone [1,3,5(10)-estrarien-3-ol-17-one], and β-estradiol [1,3,5(10)-estratriene-3,17β-diol] were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals used were of the highest grade commercially available.

CYP3A4 Expression in Baculovirus. The full-length cDNAs encoding human CYP3A4 and P450 reductase were isolated from a human adult liver cDNA library (*26*). The entire coding region of each cDNA was inserted into baculovirus shuttle vectors, p2Bac (*Bsi*wI/*Not*I for CYP3A4 and *Bam*HI/*Hin*dIII for P450 reductase), and then cotransfected into SF-9 cells with viral DNA. Recombinant virus was constructed according to the instructions provided by the manufacturer (GIBCO, Gaithersburg, MD). The TN-5 cells were infected with the recombinant viruses in the presence of hemin (2 μg/mL). After 2−3 days, TN-5 cells were harvested, and microsomes were prepared by two centrifugations (*27*). The CYP3A4 content was determined from the CO difference spectrum (*28*), and the levels of CYP3A4 and P450 reductase enzymes were about 100 pmol/mg of microsomal protein. The reductase activity was measured by the method of Phillips and Langdon (*29*). The protein concentration was determined according to the method of Lowry et al. (*30*) using bovine serum albumin as the standard.

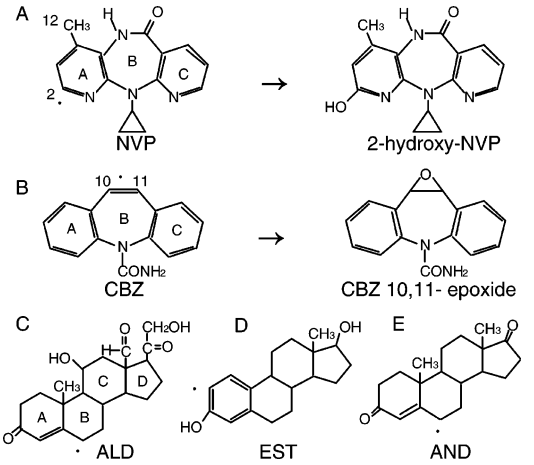


FIGURE 1: Metabolic pathway of nevirapine and carbamazepine and structures of endogeneous steroids. (A) Hydroxylation of nevirapine (NVP) by CYP3A4. (B) Carbamazepine (CBZ) 10,11-epoxidation by CYP3A4. (C) Aldosterone (ALD). (D) Estradiol (EST). (E) Androstenedione (AND). Arrows show the major hydroxylation point by CYP3A4.

Assay of NVP 2-Hydroxylase Activity and CBZ 10,11-Epoxidase Activity. The major metabolites by CYP3A4 are shown in Figure 1A,B. Analysis of both NVP 2-hydroxylation and CBZ 10,11-epoxide was carried out according to the method described previously (*8*, *31*). The incubation mixture consisted of 10 pmol of microsomal CYP3A4 protein, 100 mM potassium phosphate buffer (pH 7.4), 0.1 mM EDTA, an NADPH-generating system (0.33 mM $NADP^+$, 8 mM glucose 6-phosphate, 0.1 unit of glucose 6-phosphate dehydrogenase, 6 mM $MgCl_2$), substrate (100 μM), and a steroid (100 μM) as an effector in a final volume of 500 μL.

Mathematical Derivation and Analysis. Kinetic parameters for NVP 2-hydroxylation were determined from the Michaelis−Menten equation. Parameters for CBZ 10,11-epoxidation were determined by the modified two-site equation ($V_{max1} = 0$) (*15*, *32*): $V = (V_{max2}S^2/K_{m1}K_{m2})/(1 + S/K_{m1} + S^2/K_{m1}K_{m2})$. In this model, the binding of substrate to site 1 facilitates binding to site 2, the sole site responsible for product formation. The values were adjusted by iteration of the calculation until the best data fit was obtained using the Levenberg−Marquardt (*33*) nonlinear least-squares algorithm by the Pro Fit program version 5.5 (QuantumSoft, Zurich, Switzerland).

Theoretical Calculations. (*1*) *Construction of the Model for Calculation.* The three-dimensional structure of CYP3A4 was constructed by Szklarz and Halpert (*21*). The structure was modeled on the basis of the crystallographic coordinates of four bacterial P450s: P450 BM-3, P450cam, P450terp, and P450eryF. The coordinates of the model were then calculated using a consensus strategy, and the final structure was optimized in the presence of water. This structure was used for our investigation. The model for molecular mechanics (MM) calculations was constructed as follows: (1) Substrate and steroid were placed into the active site of CYP3A4. In constructing the ternary complexes, we considered that the oxidation site of substrate must interact with the oxygen atom of heme. (2) Water model molecules (TIP3P

model) (*34*) were generated around the enzyme by the Monte Carlo method (*35*). The thickness of the water molecules was 11 Å, and there were about 6000 water molecules in the model. After MM calculations, the interaction between substrate and steroid was first investigated, and the interaction between substrate/steroid and amino acids in CYP3A4 was secondarily investigated.

(*2*) *Computational Details.* The computational program package used for MM calculations was AMBER 6 (*36*). An all-atom force field was applied to the model structure (*37*).

Point charges on heme, substrates, and steroids for use in MM calculations were determined by the RESP program including AMBER 6. Electrostatic potentials and coordinates for use of the input of RESP were determined by quantum chemical calculations at the ab initio Hartree−Fock level. The basis set used was 6-31G**. The program package used for calculations was Gaussian 98 (*38*). For point charges on the other residues, the standard AMBER residue database was utilized. For vdW parameters, the AMBER force field parameter file (parm94. dat) was utilized. The model of the substrate−steroid−enzyme ternary complex described in the previous section was fully minimized under the conditions shown above. The minimized structures are shown in Figures 4−6.

Density functional theory (DFT) calculations were also performed to estimate stabilization energies on substrate−steroid interaction. The basis set used was 6-31G**. The exchange functional was Becke's three-parameter functional (*39*), and the correlation functional was the Lee−Yang−Parr formula (*40*). The models for calculations were constructed as follows. Substrate and steroid were extracted from the energy-minimized structure described above, and three models were made from the extracted structure: i.e., a substrate−steroid complex model, a substrate model, and a steroid model. Potential energy calculations were performed for these models, and stabilization energies, ΔE, were calculated from the equation:

$$\Delta E = \text{PE (substrate)} + \text{PE (steroid)} - \text{PE (substrate−steroid)}$$

where PE (substrate−steroid), PE (substrate), and PE (steroid) are potential energies for the substrate−steroid complex, substrate, and steroid, respectively. The values are shown in Figures 4−6. The program package used was Gaussian 98 (*38*).

RESULTS

Effect of Endogenous Steroids on NVP 2-Hydroxylation and CBZ 10,11-Epoxidation by Expressed CYP3A4. To investigate the effects of endogenous steroids on drug metabolism by expressed CYP3A4, various kinds of steroids were added to the reaction mixture. Figure 2 shows the effect of endogenous steroids on NVP 2-hydroxylase and CBZ 10,11-epoxidase activities. The activity of NVP 2-hydroxylase was activated 1.8-fold by the addition of aldosterone and inhibited 50% and 74% by estradiol and estrone, respectively. The activity of CBZ 10,11-epoxidase was activated 3.0-, 2.3-, and 1.8-fold by androstenedione, DHEA, and testosterone, respectively. Pregnenolone sulfate decreased the activity by 30% from the control level.

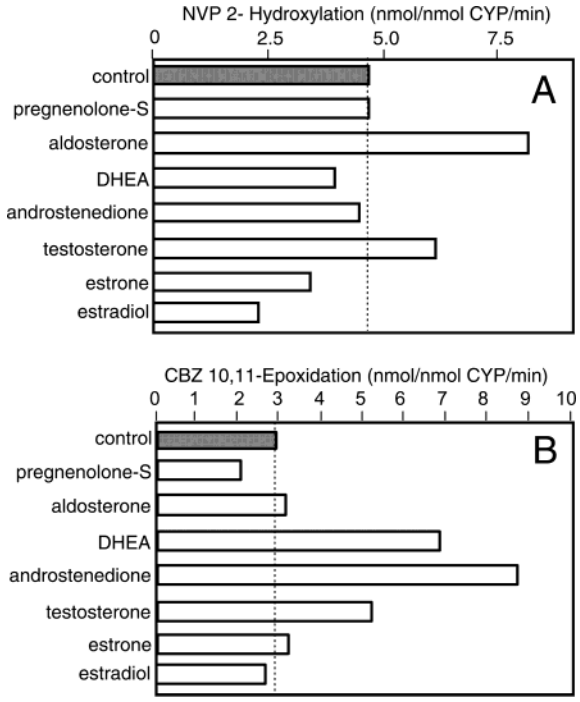


FIGURE 2: Effect of steroids on the activities of NVP hydroxylation and CBZ 10,11-epoxidation by expressed CYP3A4. (A) 2-Hydroxylation of NVP. (B) CBZ 10,11-epoxidation. Both reactions were measured in the presence of added P450 reductase and cytochrome b_5 as described in Experimental Procedures. Control means the addition of only organic solvent without endogenous steroid with the total concentration of solvent less than 2% (v/v). The concentration of steroids and substrates used in this experiment was 100 μM. Each value represents the mean of duplicate determinations.

We observed three kinds endogenous steroids: aldosterone which was the strongest activator of NVP 2-hydroxylation, androstenedione which was the strongest activator of CBZ 10,11-epoxidation, and estradiol which was the strongest inhibitor of NVP 2-hydroxylation. Then, the dose effect of aldosterone, androstenedione, and estradiol was determined in order to compare the effects of these steroids on both activities (Figure 3). Aldosterone increased and estradiol decreased the NVP 2-hydroxylase activity in a dose-dependent manner up to 200 μM. Androstenedione did not have a strong effect on this activity, while it increased the formation of CBZ 10,11-epoxide. An especially low dose of androstenedione (~50 μM) had a strong effect. Aldosterone and estradiol did not affect CBZ 10,11-epoxidation at any concentration.

Kinetic Analysis of the Endogenous Steroid Effect on NVP 2-Hydroxylation and CBZ 10,11-Epoxidation by Expressed CYP3A4. The kinetics of the effects of aldosterone, androstenedione, and estradiol on NVP 2-hydroxylation and CBZ 10,11-epoxidation were analyzed in order to elucidate the mechanisms of the steroid-induced stimulation or suppression of CYP3A4 activities. The K_m and V_{max} for NVP 2-hydroxylation calculated by the Michaelis−Menten equation increased in the presence of aldosterone, and the V_{max}/K_m was 2.6-fold higher than in the control (Table 1). In contrast, the V_{max}/K_m was 36% of the control in the presence of estradiol because of an increase in the K_m. The presence of androstenedione increased both the K_m and V_{max}, resulting in almost the same V_{max}/K_m as the control.

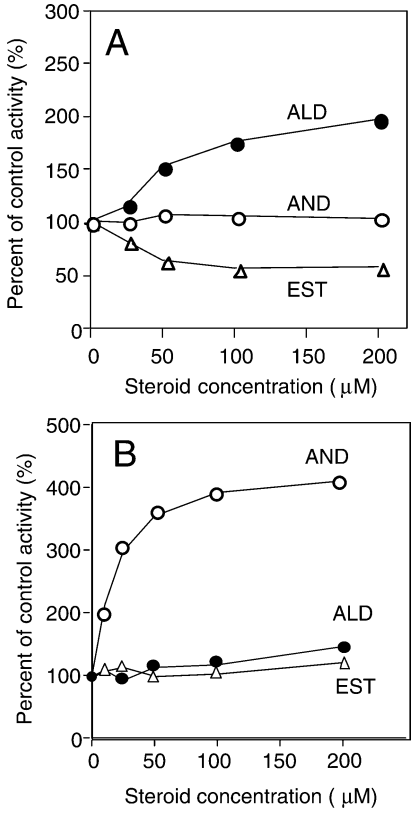


FIGURE 3: Concentration dependence of the effect of aldosterone, androstenedione, and estradiol on NVP hydroxylation and CBZ epoxidation by expressed CYP3A4. (A) 2-Hydroxylation of NVP. (B) CBZ 10,11-epoxidation. Both reactions were measured in the presence of extra added P450 reductase and cytochrome b_5 as described in Experimental Procedures. Aldosterone (ALD), estradiol (EST), and androstenedione (AND) were added at the concentrations specified in the figure. Control activity of NVP 2-hydroxylation was 4.6 nmol (nmol of CYP)$^{-1}$ min^{-1}. Control activity of CBZ 10,11-epoxidation was 1.4 nmol (nmol of CYP)$^{-1}$ min^{-1}. Control means the addition of only organic solvent without endogenous steroid with the total concentration of solvent less than 2% (v/v). The concentration of substrates used in this experiment was 100 μM. Each value represents the mean of duplicate determinations.

Table 1: Kinetic Parameters of NVP 2-Hydroxylation by Expressed CYP3A4[a]

	K_m	V_{max}	V_{max}/K_m
NVP only	36.9	4.1	0.11
+aldosterone	51.8	15.2	0.29
+androstenedione	121.0	14.5	0.12
+estradiol	112.2	4.3	0.04

[a] The reactions were performed in the absence or presence of 100 μM steroids. Units are as follows: K_m, μM; V_{max}, nmol (nmol of CYP)$^{-1}$ min^{-1}.

As is well-known, the kinetic character of CBZ 10,11-epoxidation was shown to be sigmoid in the absence of steroids, judging from Eadie−Hofstee plots (data not shown). The sigmoidal curve changes to a Michaelis−Menten-type curve by the addition of aldosterone, androstenedione, or estradiol. When the kinetic parameters were calculated by the Michaelis−Menten equation and the modified two-site equation as shown in Table 2, the values of K_{m1} decreased markedly in the presence of these steroids (K_{m1} was 2.3, 8.6, and 1.1 μM in the presence of aldosterone, androstenedione, and estradiol, respectively). The parameters in the presence of steroids could also be calculated by the Michaelis−Menten equation, and K_m and V_{max} were found to be almost the same as K_{m2} and V_{max2} when calculated using the two-site equation. Androstenedione decreased to 46% of K_{m2} and increased to more than three times the value of V_{max}/K_m (V_{max2}/K_{m2}), indicating that efficiency of CBZ metabolism is increased by the presence of androstenedione. The V_{max}/K_m was not changed by the presence of aldosterone or estradiol, although the pattern of the reaction curve was changed.

Table 2: Kinetic Parameters of CBZ 10,11-Epoxidation by Expressed CYP3A4[a]

	K_m	V_{max}	V_{max}/K_m
CBZ only	259.7[b]	7.3[b]	0.03[b]
+aldosterone	386.6[c]	8.9[c]	0.02[c]
+androstenedione	132.8[c]	13.0[c]	0.10[c]
+estradiol	230.3[c]	6.3[c]	0.03[c]

[a] The reactions were performed in the absence or presence of 100 μM steroids. Units are as follows: K_m, μM; V_{max}, nmol (nmol of CYP)$^{-1}$ min^{-1}. [b] Analyzed by the modified two-site equation ($V_{max1} = 0$). $V_{max} = (V_{max2}S^2/K_{m1}K_{m2})/(1 + S/K_{m1} + S^2/K_{m1}K_{m2})$ (*15*). K_m and V_{max} in the table were fitted to K_{m2} and V_{max2} in the equation, respectively. [c] Analyzed by the Michaelis−Menten equation. $V = V_{max}/[1 + (K_m/S)]$.

Direct Interaction between NVP and Steroids in the Active Site of CYP3A4. The structures of aldosterone (Figure 1C), estradiol (Figure 1D), and androstenedione (Figure 1E) are quite similar, but they have different effects on CYP3A4. Furthermore, the reason these steroids change the patterns of the reaction curves cannot be fully explained by kinetic analysis. Therefore, the reaction mechanism in the presence of steroids was investigated by using the three-dimensional structure of CYP3A4.

As an active site of CYP3A4 is large as estimated by computer modeling (*21*), it was expected that more than two substrates could access the site simultaneously. Therefore, the possibility for a direct interaction between substrates and steroids in the active site of CYP3A4 was investigated by theoretical calculations. An energy-minimized structure of NVP and aldosterone-bound CYP3A4 is shown in Figure 4A. The hydroxyl group of Ser312 in CYP3A4 interacts with aldosterone through a hydrogen bond and with the carbonyl group and hydroxyl group of the D-ring of aldosterone binds to a peptide group in NVP through hydrogen bonds. From these interactions, NVP is stably held by aldosterone, and this might make NVP easier to hydroxylate.

When NVP and androstenedione were present at the same time, Ser312 could bind androstenedione in the same way as aldosterone. However, androstenedione dose not stabilize NVP as in the case of aldosterone. Although estradiol also interacts with Ser312, a benzene ring in estradiol interacts with a benzene ring (A-ring in Figure 1A) in NVP; i.e., this is considered to be a π/π interaction. Then, site 2 of NVP is possibly blocked to hydroxylate.

Next, the difference in the potential energy between the nonbinding and binding states of substrate and steroid was calculated as stabilization energy by the density functional theory (Figure 4). The stabilization energy of NVP plus aldosterone is the largest of all combinations. This indicates that the formation of a complex of NVP and aldosterone may be more stable during NVP 2-hydroxylation in the active site compared with other combinations or in the absence of the complex.

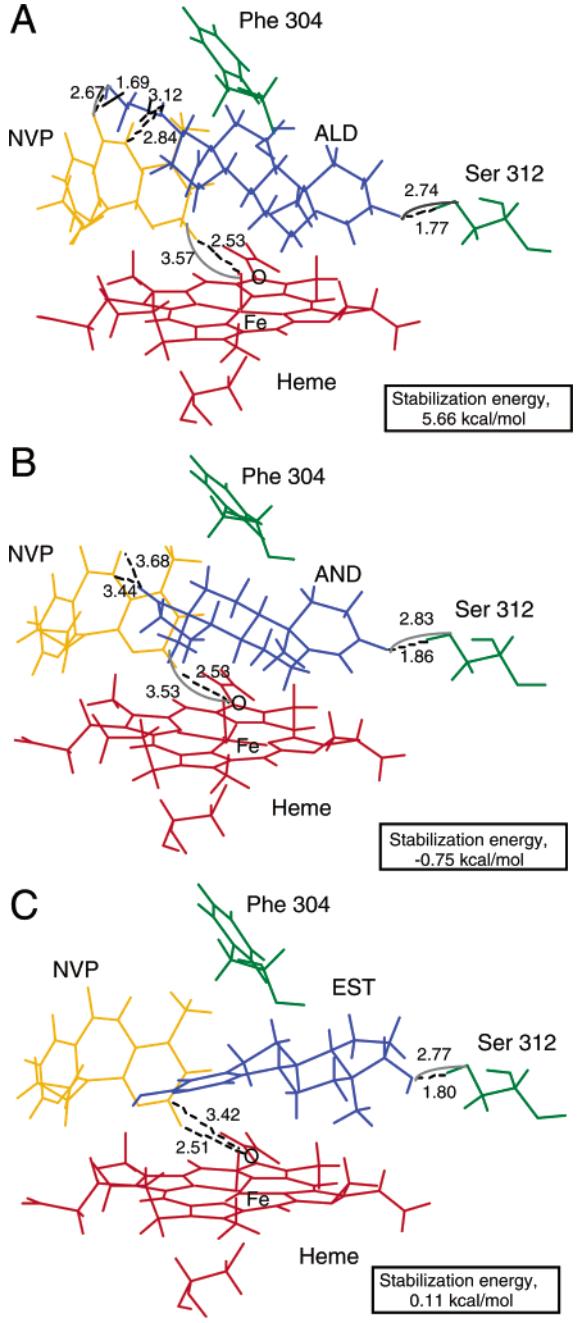


FIGURE 4: Proposed interaction between NVP and steroids in the active site of a CYP3A4 model. NVP docked into the active site of CYP3A4 in an orientation conducive to its 2-hydroxylation. (A) Interaction between NVP and aldosterone (ALD). (B) Interaction between NVP and androstenedione (AND). (C) Interaction between NVP and estradiol (EST). The heme group is shown in red. NVP is shown in yellow. AND, ALD, and EST are shown in blue. Phe304 and Ser312 are shown in green.

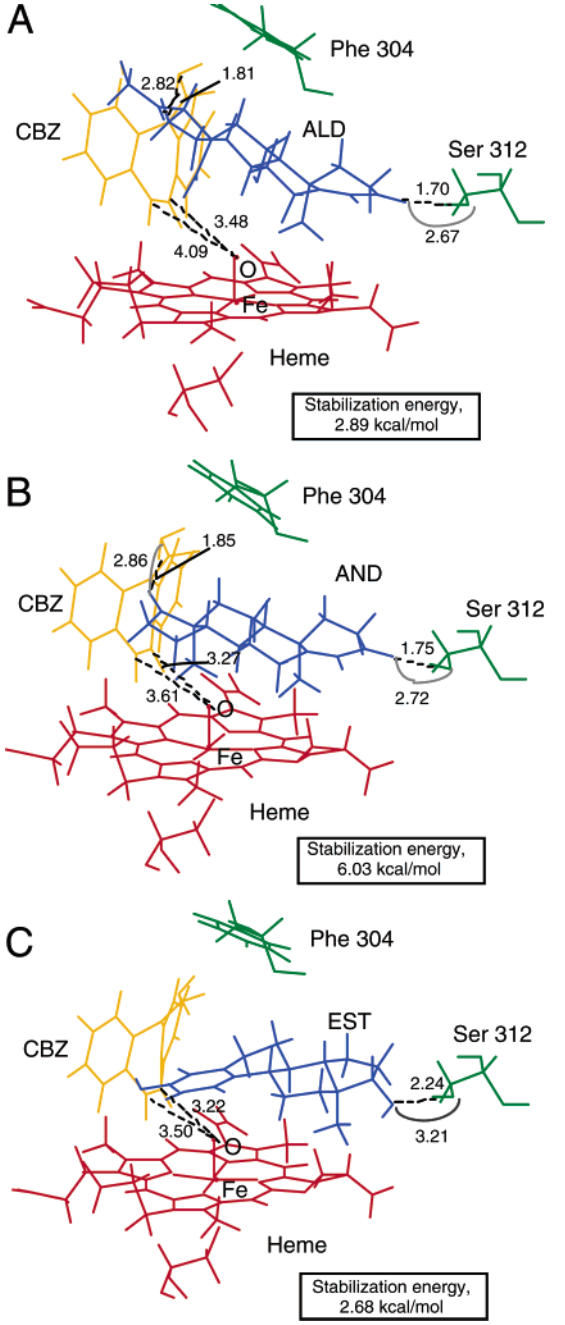


FIGURE 5: Proposed interaction between CBZ and steroids in the active site of a CYP3A4 model. CBZ docked into the active site of CYP3A4 in an orientation conducive to its 10,11-epoxidation. (A) Interaction between CBZ and aldosterone (ALD). (B) Interaction between CBZ and androstenedione (AND). (C) Interaction between CBZ and estradiol (EST). The heme group is shown in red. NVP is shown in yellow. AND, ALD, and EST are shown in blue. Phe304 and Ser312 are shown in green.

Direct Interaction between CBZ and Steroids in the Active Site of CYP3A4. The effects of steroids on CBZ were also analyzed by molecular mechanics and the density functional theory (Figure 5). If it is assumed that CBZ and androstenedione are present at the same time in CYP3A4, the hydroxyl group of Ser312 interacts with the carbonyl group in the A-ring of androstenedione through a hydrogen bond, and the carbamoyl group of CBZ binds the carbonyl group in the D-ring through a hydrogen bond. In this case, CBZ is stably fixed in the active site, and the epoxidation can be made easier by keeping the interaction between the oxygen atom in heme and the epoxidation site of CBZ.

When CBZ and aldosterone are present in the site at the same time, the distance between Ser312 and aldosterone is almost the same as in the case of androstenedione, but the interaction between oxygen in the heme and the epoxidation site of CBZ might be weak because the side chain of the D-ring is long. In the case of the interaction between CBZ and estradiol, the interaction might become weak because estradiol is not stably held by Ser312, resulting in estradiol becoming easily movable in the site. The stabilization energy

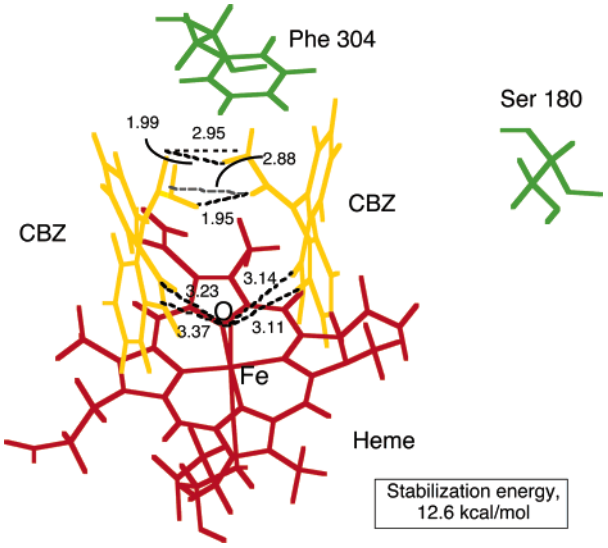


FIGURE 6: Proposed interaction between CBZ and CBZ in the active site of a CYP3A4 model. CBZ docked into the active site of CYP3A4 in an orientation conducive to its 10,11-epoxidation. The heme group is shown in red. NVP is shown in yellow. Phe304 and Ser312 are shown in green.

of CBZ plus androstenedione is larger than that of CBZ plus aldosterone or estradiol. From these results, androstenedione may work as an activator of CBZ 10,11-epoxidation.

Finally, the interaction between two CBZ molecules was calculated in the same way. Figure 6 shows that two CBZ molecules are fixed stably by the interaction of their carbamoyl groups produced through a hydrogen bond, indicating that CBZ is stabilized in the active site of the enzyme due to intermolecular interaction.

π/π Interaction between Substrates and Steroids in the Active Site of CYP3A4. The A-ring of estradiol is a benzene ring. The A- and C-rings of NVP and CBZ are also benzene rings (Figure 1). Then, we expected that there could be *π/π* interaction between the A-ring of estradiol and the A- or C-rings of substrates, and the proposed interactions between the A-ring of estradiol and the A-rings of NVP or CBZ showed the *π/π* interactions (Figures 4C and 5C). To clarify the strength of the *π/π* interactions, we measured the distance between indicated atoms using the Tsuzukis model (*41*) (Figure 7).

The proposed *π/π* interaction was the edge-to-face type, which is considered to be one of the most stable interactions in all kinds of *π/π* interactions. The distance between NVP and estradiol was shorter than that between CBZ and estradiol.

Interaction among Substrates, Endogenous Steroids, and Amino Acid in the Active Site in Mutant CYP3A4. To clarify whether Ser312 dominates substrate selectivity or turnover properties, the theoretical calculation with a mutant that the 312th of serine was changed to alanine was carried out (Figure 8). The three-dimensional structure of the mutant was almost the same with the wild type, and the I-helix was also maintained in the mutant. The hydrogen bond disappeared between three kinds of steroids (aldosterone, androstenedione, and estradiol) and the 312th amino acid because of Ala312 of the mutant. In addition, a hydrogen bond between endogenous steroids and another amino acid of the I-helix is not also observed. Although it is shown that substrates (NVP and CBZ) still interacted with three kinds

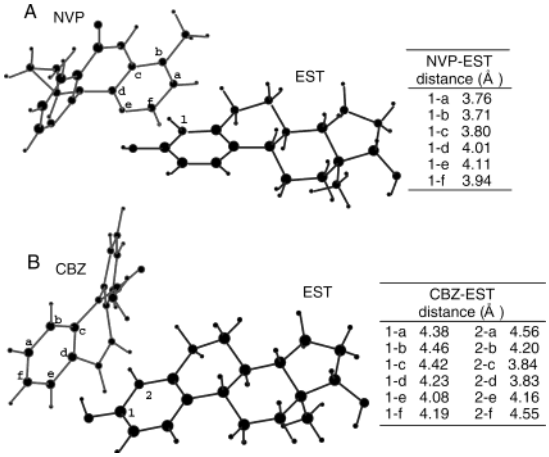


FIGURE 7: Proposed *π/π* interactions between substrates and estradiol. The distances between the A-ring of estradiol and the A-rings of NVP (A) or CBZ (B) were also shown. The *π/π* interaction between estradiol and NVP was based on the model D of Tsuzuki et al. (*41*). The *π/π* interaction between estradiol and CBZ was based on the model F (*41*).

of steroids in the active site of the mutant, the distances between substrates and the O atom was changed in the range from −0.07 to 0.40 Å. These distances between substrates and the O atom are not a big difference of the distances between the wild type and the mutant.

The *π/π* interaction between substrates and estradiol in the active site of the mutant was also calculated (Figure 9). The distance of the *π/π* interaction was almost the same as that in the wild type. From these results, it is expected that the mutant does not stably maintain the complex of substrates and endogenous steroids.

DISCUSSION

It was clarified that some kinds of endogenous steroids affect CBZ 10,11-epoxidation by expressed CYP3A4. Our previous report showed that endogenous steroids also have almost the same effects on the activity using human liver microsomes as the enzyme source (*8*). Although the ratio of activation and inhibition by steroids in the system using expressed CYP3A4 was somewhat lower than that observed in the system using human microsomes, it is possible that the steroids work on the enzyme more directly because the same results were obtained in an assay system containing a single enzyme.

There are special characteristics common to the structures of NVP and CBZ (Figure 1A,B). Both have three connected rings, including benzene rings and an −NHCO− group (peptide bound in NVP and carbamoyl group in CBZ). Endogenous steroids also have structures quite similar to one another. However, different steroids showed different effects on the activities of NVP 2-hydroxylation and CBZ 10,11-epoxidation by CYP3A4. From these data, it seemed difficult to arrive at a rule for the reactions catalyzed by CYP3A4. It is known that CYP3A4 has an open space that contains the active site (*21*). We then compared NVP hydroxylation, which shows Michaelis−Menten-type kinetics, with CBZ epoxidation, which shows non-Michaelis−Menten-type kinetics, by pharmacokinetic analysis and theoretical calculation.

The kinetics of NVP 2-hydroxylation were calculated by the Michaelis−Menten equation with and without steroids.

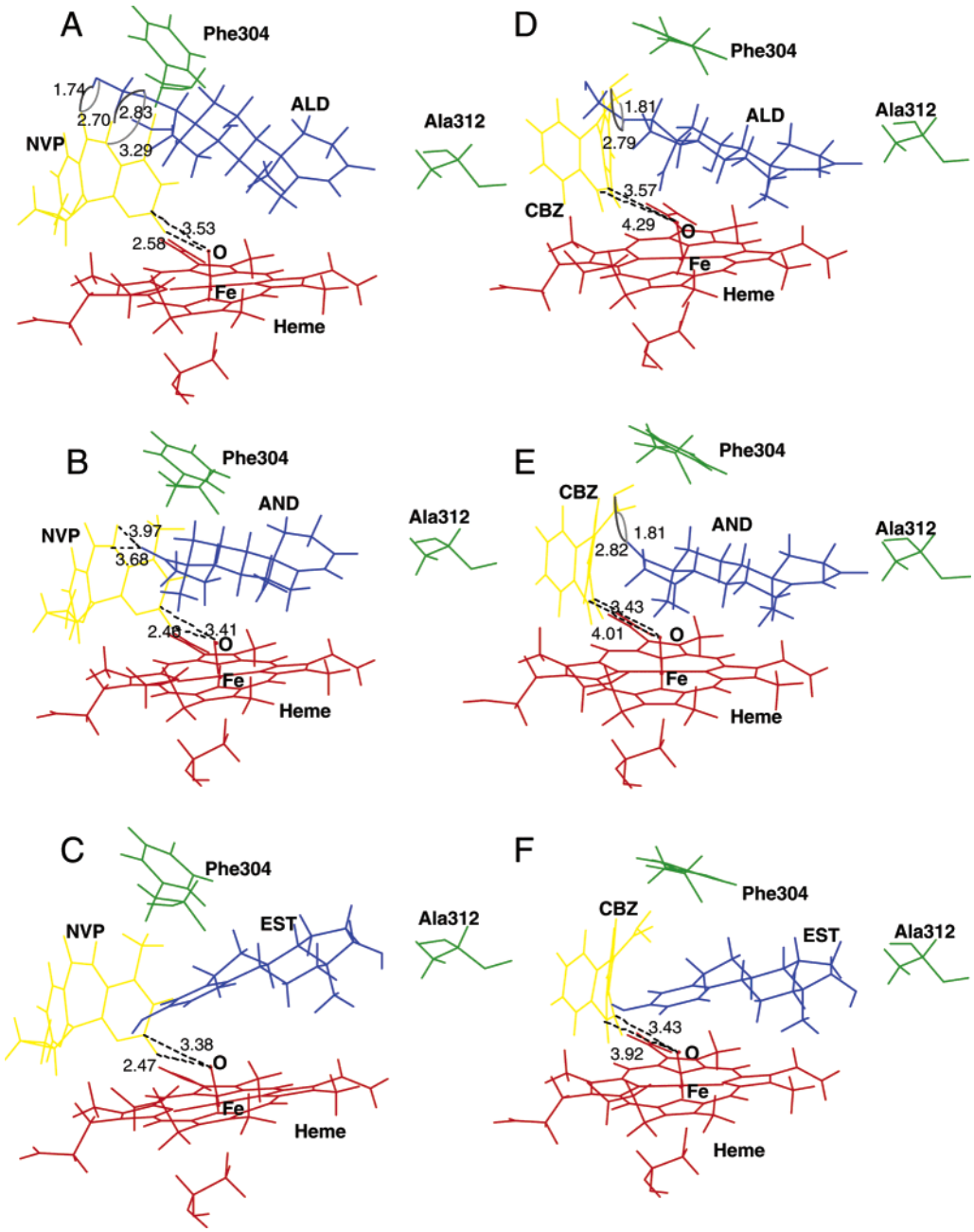
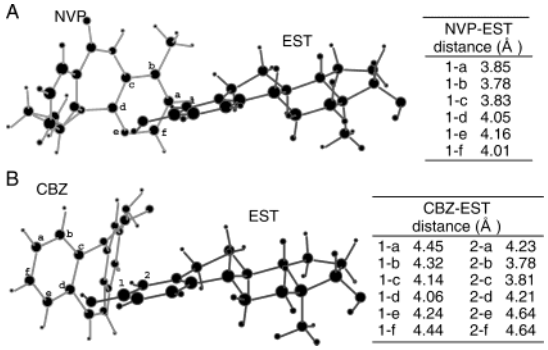


FIGURE 8: Proposed interaction between NVP/CBZ and steroids in the active site of a CYP3A4 mutant model. Ser312 of CYP3A4 was changed to Ala312 in the mutant model. NVP docked into the active site of the mutant CYP3A4 in an orientation conducive to its 2-hydroxylation. CBZ docked into the active site of the CYP3A4 mutant in an orientation conducive to its 10,11-epoxidation. (A) Interaction between NVP and aldosterone (ALD). (B) Interaction between NVP and androstenedione (AND). (C) Interaction between NVP and estradiol (EST). (D) Interaction between CBZ and ALD. (E) Interaction between CBZ and AND. (F) Interaction between CBZ and EST. The heme group is shown in red. NVP and CBZ are shown in yellow. AND, ALD, and EST are shown in blue. Phe304 and Ala312 are shown in green.

Aldosterone stimulated the activity by a slight decrease in the affinity but a great increase in the reaction velocity. On the other hand, estradiol inhibited by decreasing both affinity and reaction velocity and androstenedione showed no effect on the V_{max}/K_m value despite changing both the K_m and V_{max}. Why do these steroids, which have similar structures, show different effects? An investigation of the direct interaction between substrates and endogenous steroids by molecular mechanics and the density functional theory gave a possible answer to this question. The three kinds of steroids associate with Ser312 in CYP3A4 and can interact with NVP. The difference between the structures of aldosterone and androstenedione lies in the side chains derived from the D-ring. They contribute to the hydrogen bond with NVP. The size of the side chain in aldosterone is suggested to be suitable to form a complex between NVP and aldosterone that fits well in the active site. NVP in this complex is, therefore, assumed to fit stably. This may explain why the V_{max} increases even though the K_m increases a little in the presence of aldosterone. On the other hand, the D-ring of androstenedione includes a ketone. This oxygen in the ketone group could also contribute to the formation of a hydrogen bond with NVP, but an interaction with only one oxygen atom is considered to be too weak to retain NVP. Thus, it is



NVP-EST distance (Å)	
1-a	3.85
1-b	3.78
1-c	3.83
1-d	4.05
1-e	4.16
1-f	4.01

CBZ-EST distance (Å)			
1-a	4.45	2-a	4.23
1-b	4.32	2-b	3.78
1-c	4.14	2-c	3.81
1-d	4.06	2-d	4.21
1-e	4.24	2-e	4.64
1-f	4.44	2-f	4.64

FIGURE 9: Proposed π/π interactions between substrates and estradiol in the active site of the mutant CYP3A4. The distances between the A-ring of estradiol and the A-rings of NVP (A) or CBZ (B) were also shown. The π/π interaction between estradiol and NVP was based on the model D of Tsuzuki et al. (*41*). The π/π interaction between estradiol and CBZ was based on the model F (*41*).

considered that the interaction between androstenedione and NVP is weak compared with the case of aldosterone and NVP, and androstenedione could not stably maintain NVP. This may be a reason the value of K_m for NVP 2-hydroxylation was increased in the presence of androstenedione. Furthermore, if the affinity is weak, the velocity to release NVP from the androstenedione−NVP complex might be increased. That is, the value of V_{max} increased (Table 1). Taken together, it is possible to assume that androstenedione shows no effect on NVP 2-hydroxylation due to the increase of both K_m and V_{max} at the same time.

As CBZ 10,11-epoxidation in the absence of the steroids shows a sigmoid curve, kinetic analysis was done using the two-binding-site model. Why does CBZ 10,11-epoxidation show a sigmoid curve while NVP hydroxylation does not? A previous report explained the sigmoid kinetic curve of CBZ 10,11-epoxidation by the two-binding-site model as follows. The binding of CBZ to site 1 facilitates substrate binding to site 2, the sole site responsible for product formation, following a conformational change in the enzyme (homotropic cooperativity) (*10*, *12*−*14*). Our theoretical study clarifies that CBZ forms a dimer by a hydrogen bond in the active site (Figure 6), while NVP does not (unpublished data). It is expected that the frequency of the CBZ interaction may accompany an increase in CBZ concentration, causing an increase in the amount of dimer. Moreover, the epoxidation site of CBZ may be stabilized at high concentrations of CBZ. This might be one of the reasons that CBZ epoxidation shows a sigmoid curve.

In the presence of steroids, the K_{m1} value decreases violently and kinetic analysis can be done by the Michaelis−Menten equation. At this time, the K_{m2} values are almost the same as the K_m values, suggesting that the sigmoid curve is converted to a Michaelis−Menten-type curve. This can be explained by the two-binding-site model as site 1 being occupied by steroids because the affinities of these steroids is much higher than that of CBZ and the affinity of CBZ for site 2 is probably increased by an allosteric effect. All three kinds of steroids change the kinetic pattern from a sigmoid curve to a Michaelis−Menten-type curve; however, androstenedione increases CBZ epoxidation while aldosterone and estradiol do not. Why then does only androstenedione induce an increase in the affinity of CBZ for site 2? Because the carbamoyl group in CBZ and the ketone in androstenedione form a hydrogen bond, the epoxidation site is suggested to be stably maintained close to the oxygen in heme. Although aldosterone forms a hydrogen bond, the epoxidation site is far from the oxygen because the side chain is too long for binding.

It has been reported that P450eryF has an enlarged active site compared with the other bacterial enzymes according to the X-ray crystal structure of the P450eryF−6-deoxyerythronolide B complex. Then, the three-dimensional structure of P450eryF was used for the structure of the active site for CYP3A4 modeling. Recently, the X-ray crystal structure of P450eryF containing two molecules of androstenedione in the active site was reported (*23*). The two androstenedione molecules interacted with some kinds of amino acid and also had interactions with one another. The majority of the protein−steroid interactions occur with the A- and B-rings, whereas the closest steroid−steroid interactions occur between the C- and D-rings of each androstenedione. In the present study, it was shown that two compounds were also present in the active site at the same time. Furthermore, the A-ring and D-ring of androstenedione were demonstrated to interact with amino acid (Ser312) and substrates, respectively.

Our results also showed that NVP or CBZ interacted with estradiol by π/π interaction in the active site of CYP3A4. The proposed π/π interaction was the edge-to-face type. The atomic distance between NVP and estradiol was about 3.89 Å (Figure 7), and this is almost the same distance of model D reported by Tsuzuki et al. (*41*). From these data, it is considered that the π/π interaction between NVP and estradiol was stable. But, because the hydroxylation site was present in the A-ring of NVP, NVP 2-hydroxylation was inhibited by the π/π interaction. On the other hand, atomic distance between CBZ and estradiol was longer than the distance of the Tsuzukis model F. Then, it is likely that the π/π interaction between CBZ and estradiol was weak, resulting in noninhibition of CBZ epoxidation by estradiol.

Although the CBZ−CBZ homodimer showed the large stabilization energy, it is not assumed that the CBZ−CBZ homodimer existed in the assay system used in the present study. There are two reasons why we consider that the CBZ−steroid heterodimer is present more than the CBZ−CBZ homodimer in the assay. (1) The CBZ 10,11-epoxidation in the absence of steroids shows a sigmoid curve. The slant of the substrate−velocity curve changed from low to high over 100 μM CBZ concentration as shown previously (*42*). We used 100 μM CBZ and 100 μM steroids in this study. Then, it is reasonable to assume that CBZ would not tend to make the homodimer at this concentration. (2) The affinity of steroids is higher than that of CBZ. For example, K_m of androstenedione was 100.3 μM in the absence of CBZ and 97.8 μM in the presence of CBZ (unpublished data). The K_m of CBZ was 259.7 μM in the absence of androstenedione (Table 2). From these data, it is expected that androstenedione may enter the active site faster than CBZ when both 100 μM CBZ and androstenedione are present at the same time.

Molecular modeling of CYP3A4 shows that the core structure includes a very highly conserved element in the neighborhood of heme, such as the central and C-terminal end of the I-helix (*21*). The I-helix contains S312, and amino acids having a hydroxy group (serine, threonine, and tyrosine)

line up at both sides of S312. The hydroxy group of Thr309, Ser312, and Ser315 may face the heme because these amino acids lie on the α-helix. Then, it was expected that Thr309 or Ser315 may maintain the endogenous steroids instead of Ser312 when Ser312 was changed to Ala312 as a mutant. However, any amino acid did not maintain the endogenous steroids in the active site of the CYP3A4 mutant. It has been reported that Thr309 is present in the distance of 5 Å (*21*) and plays an important role in determining regioselectivity of diazepam (*43*) or midazolam oxidation by the experiments using mutants (*44*). When another substrate, triazolam, was used in the theoretical calculations, the complex of triazolam and an endogenous steroid was shown to be maintained by the interaction between the steroid and Ser315 of the enzyme (unpublished data). Thus, although Thr309, Ser315, or other amino acids having a hydroxy group may access to maintain the endogenous steroids, it is likely that the endogenous steroids predominantly interact with Ser312 in the cases of NVP and CBZ.

Recently, there have been many papers describing drug mutual interactions, and some reports describe side effects based on gender differences. The previous interpretation was that one chemical acts on amino acids in enzymes and changes their structure, resulting in a change in the activity. In this study, molecular mechanics calculation showed a direct interaction between two chemicals in the CYP3A4 active site. However, before any conclusion can be drawn, several limitations should be taken into account. First, the theoretical calculation was conducted under conditions disallowing a drastic conformational change of the enzyme. Second, general rules of interaction could not be found in the present study. This problem will, however, be resolved by repeated trials of molecular mechanics calculations. Finally, the relationship between pharmacokinetics and molecular mechanics calculations has remained unclear until now. Recently, the two attractive results have been reported; the one was that pyrene−pyrene complexes were observed to be present in the active site of CYP3A4 by fluorescence emission spectra (*45*), and the other was that two molecules of S-warfarin were expected to be present in the active site of CYP2C9 by a real crystal structure (*46*). These data strongly support our consideration. At this stage, although there are still some problems, we think that the direct interaction between two molecules may present one possibility for elucidating the side effects and drug interaction mechanism.

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