A Kinetic Model for the Metabolic Interaction of Two Substrates at the Active Site of Cytochrome P450 3A4*

In many cases, CYP3A4 exhibits unusual kinetic characteristics that result from the metabolism of multiple substrates that coexist at the active site. In the present study, we observed that α-naphthoflavone (α-NF) exhibited a differential effect on CYP3A4-mediated product formation as shown by an increase and decrease, respectively, of the carboxylic acid (P2) and ω-3-hydroxylated (P1) metabolites of losartan, while losartan was found to be an inhibitor of the formation of the 5,6-epoxide of α-NF. Thus, to address this problem, a kinetic model was developed on the assumption that CYP3A4 can accommodate two distinct and independent binding domains for the substrates within the active site, and the resulting velocity equations were employed to predict the kinetic parameters for all possible enzyme-substrate species. Our results indicate that the predicted values had a good fit with the experimental observations. Therefore, the kinetic constants can be used to adequately describe the nature of the metabolic interaction between the two substrates. Applications of the model provide some new insights into the mechanism of drug-drug interactions at the level of CYP3A4.

Multiple drug therapy is practiced widely either to treat a single medical disorder or to treat more than one simultaneous illness in the same patient (1). Thus, drug-drug interactions have become an important clinical issue due to the effects of one drug on the efficacy, toxicity, or disposition of another drug. The estimated incidence of clinically significant drug-drug interactions is as high as 20% in patients receiving multiple drugs (2). The consequences of drug-drug interactions often are detrimental to clinical therapeutics and frequently result in an alteration of blood drug levels, leading in severe cases to life-threatening adverse reactions. For example, terfenadine cardiotoxicity has been observed when the drug is coadministered with certain agents such as ketoconazole (3) and erythromycin (4). Furthermore, mortality has occurred in patients who were dosed concurrently with sorivudine and fluoropyrimidines (5). Oral contraceptive failures have been noted in several populations of women taking rifampin, griseofulvin, and anticonvulsants (6, 7). However, coadministration of multiple drugs also can be clinically useful. For instance, phenobarbital, which induces enzyme expression, has been beneficial in treating patients with unconjugated and neonatal hyperbilirubinemia (8). The majority of cases of drug-drug interactions are a result of pharmacokinetic (metabolic clearance of one or more drugs) or pharmacodynamic (antagonistic or additive drug effects) alterations. Although interacting agents can affect all aspects of drug disposition, including absorption, distribution, metabolism, and excretion through a variety of mechanisms (9), the most common drug interactions can be understood in terms of alterations in metabolism, which are associated primarily with changes in the activities of cytochromes P450, a gene superfamily consisting of at least 40 human isoenzymes (10). Currently, only families CYP1, -2, and -3 are thought to be important in drug metabolism (11, 12). Of the individual isozymes, CYP3A4, CYP2D6, and CYP2C9 appear to be responsible for the metabolism of the greatest number of drugs. Metabolism-based drug interactions are primarily mediated by the enhancement or inhibition of enzyme systems that govern the biotransformation of xenobiotics.

The kinetic parameters that describe enzyme-based drug interactions, e.g. $K_i$, $K_m$, $V_{max}$, can be estimated by in vitro techniques and appropriate kinetic models. These estimates then may be used to predict the in vivo pharmacokinetic and pharmacodynamic consequences associated with exposure to multiple drugs. Although the kinetic expressions for many types of drug interaction, such as competitive, noncompetitive, uncompetitive inhibition, and direct activation, have been described in detail (13), no kinetic model has yet been reported for a P450 enzyme that catalyzes the metabolism of two substrates coexisting at the active site and that differentiates the fate of one another through mutual inhibition or activation. Due to the complexity of such interactions, the resulting kinetics are not straightforward and hamper mechanistic interpretation of the interaction in question. Thus, kinetic models that adequately describe the interaction between multiple drugs that bind simultaneously to a single enzyme active site are needed to aid in the understanding of clinically observed drug-drug interactions and in an assessment of their clinical significance. To better understand multiple substrate/effector enzyme kinetics, the present study investigated the metabolic interaction between losartan and α-naphthoflavone (α-NF) modulated by CYP3A4. These results of investigations, in turn, were employed to conduct a kinetic model that was used to define the nature of the

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1 The abbreviations used are: α-NF, α-naphthoflavone; OR, oxi-doreductase; HPLC, high performance liquid chromatography; MS/MS, tandem mass spectrometry; RSS, residual sum of squares.
losartan-α-NF interaction and that may be applied to other metabolic interactions occurring at the active site of CYP3A4.

EXPERIMENTAL PROCEDURES

Materials—α-NF was purchased from Sigma. Losartan and its metabolites, namely the α-3-hydroxy (P1) and carboxylic acid derivatives (P2), were obtained from Merck. The purified human P450 oxidoreductase (OR) was kindly provided from Dr. James P. Hardwick (Northeastern Ohio University College of Medicine).

CYP3A4 Expression in Baculovirus—The full-length cDNAs encoding human CYP3A4 and CYP OR were provided from Dr. Frank J. Gonzalez (National Cancer Institute, Bethesda, MD). The entire coding region of each cDNA was inserted into baculovirus shuttle vectors, pBlueBac (XbaI/KpnI for CYP3A4 and EcoRI for OR) and then recombined separately into baculoviruses according to the manufacturer’s procedure (Invitrogen, Carlsbad, CA). CYP3A4 and OR proteins were expressed in Sf21 insect cells coinfected with the two separate recombinant viruses at a ratio of CYP3A4:OR = 5 of multiplicity of infection of virus in the presence of hemin (1 μg/ml) (14). Cells were harvested after 3 days, and microsomes were prepared by two different centrifugations (15). The CYP3A4 content was determined by the CO difference spectrum, and ~150 nmol of CYP3A4 in 500 ml cell culture was obtained. The specific activity of the CYP3A4 was measured by the testosterone 6-hydroxylase assay and found to be 42 nmol of product formed/min/nmol of CYP3A4 and its metabolites was detected at wavelengths of 250 nm for excitation and 370 nm for emission. The quantities of metabolites formed were determined from the individual fluorescence obtained at different excitation and emission wavelengths.

HPLC—HPLC analyses were performed on a Hewlett-Packard model 1100 liquid chromatograph equipped with both a diode array detector and an HP 1046 programmable fluorescence detector. Losartan and its metabolites were separated on a 2020 ODS column (4.6 mm x 20 cm, 5 μm; TLC, Springfield, VA) and eluted with a 22-min linear gradient from 30 to 45% acetonitrile in water containing 0.1% acetic acid at a flow rate of 1 ml/min. The retention times of P1, P2, and losartan were 9.1, 17.2, and 18.9 min, respectively. Fluorescence of metabolites was detected at wavelengths of 250 nm for excitation and 370 nm for emission. The quantities of metabolites formed were determined from the appropriate standard curves. α-NF and its metabolites were separated on a 2020 ODS column eluted with a 40-min linear gradient from 50 to 100% methanol in water and were detected by UV absorbance at 260 nm. The flow rate was 1 ml/min and the retention times of the 5,6-epoxide metabolite and α-NF itself were 21 and 30 min, respectively.

Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/-MS)—For liquid chromatography-MS/MS studies, chromatography was conducted on a Hewlett-Packard HP1050 gradient system. Separation was carried out on a Phenomenex Luna™ C18 column (2.0 mm x 25 cm, 5 μm) using a mobile phase consisting of 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B) and a flow rate of 0.2 ml/min. The HPLC system was interfaced to a Finnigan TSQ 7000 tandem mass spectrometer, which was operated using electrospray ionization in the positive ion mode. The capillary temperature was 200 °C, and the electrospray ionization voltage was maintained at 5.0 kV. MS/MS was based on collision-induced dissociation of ions entering the rf-only octapole region, where argon was used as the collision gas at a pressure of 1.7 millitorr. A combination of collision offset voltages ranging from ~35 eV to ~55 eV were used for all MS/MS analyses.

Experimental Hypothesis—Multiple Substrate Enzyme Kinetics—Based on the experimental findings of this study (see below), we observed that (i) α-NF modulated CYP3A4 activity by increasing the formation of the carboxylic acid derivative (P2) and decreasing the formation of α-3 hydroxylated derivative (P1), (ii) losartan was an inhibitor of CYP3A4-mediated formation of the 5,6-epoxide (Q) metabolite of α-NF (Fig. 1), and (iii) the apparent kinetic constants, e.g. Kd, and Vmax, describing the metabolism of the coexisting substrates as altered significantly by drug interaction in a manner that could not be explained simply by any of the Michaelis-Menten kinetic models (inhibition or activation) by an assumption of the one-binding domain at the active site. Thus, a kinetic model was devised to address the complexity of metabolism exhibited in this study. The model postulates that CYP3A4 contains two distinct and independent binding sites at the active site that can be occupied simultaneously by two substrate molecules (either the same or different molecular species). The binding orientations of substrate with enzyme are defined on the basis of the metabolic fates of each substrate as presented in Scheme I. The two occupied binding sites interact kinetically such that occupancy of the two sites results in changes in the rates of turnover of the two substrates and in the apparent Kd and Vmax values. Thus, velocity equations for the formation of each major product are derived according to the expressions shown below, and kinetic estimates that can truly reflect the relationship between substrate(s) and enzyme in any of substrate/effector-enzyme combinations are revealed. The model is based on the assumption of rapid equilibrium. Thus, all species in the reaction described by Scheme I equilibrate rapidly, meaning that the dissociation of each enzyme-substrate complex is much faster than its breakdown to product(s).
enzyme reaction forms two closed circles as indicated in Scheme I, the terms of $K_{m1}v_{m1}$ and $K_{m2}v_{m2}$ in the equations can be substituted by $K_{m1}P_1$ and $K_{m2}P_2$, respectively, and vice versa. Thus, the velocity equation for each product formation may be expressed in different ways.

Data Analysis—Values for all kinetic parameters were calculated by an iterative procedure based on appropriate initial estimates that best fitted the data using the Marquardt-Levenberg nonlinear least squares algorithm (17). The resulting equations were simplified by the program Mathematica 4 (Wolfram Research, Inc., Champaign, IL). The experimental data were fitted with the equations to generate the surface plots. Statistics of the data was performed using both the residual sum of squares (RSS) and $R^2$. All kinetic constant statistics were determined with Equation 1 (RSS = 0.0213, $R^2 = 0.992$).

RESULTS

Effect of $\alpha$-NF on the Metabolism of Losartan—Incubation of losartan with cDNA-expressed CYP3A4 in the presence of NADPH led to the formation of two major metabolites, namely P1 ($\alpha$-3-hydroxy losartan) and P2 (the carboxylic acid metabolite), which were identified by comparing the HPLC retention times and MS/MS spectra with authentic standards (Fig. 1). Time-dependent formation of products in the metabolism of losartan (20 $\mu$M) was shown to be linear up to at least 30 min of incubation at 37 °C, whereas less than 10% of the substrate was consumed during the experiment. The ratio of the concentration of substrate to that of CYP3A4 in the incubation usually was between 250 and 2,000. Thus, the enzyme-mediated reaction was considered to meet the requirements of both steady state and rapid equilibrium. The addition of $\alpha$-NF (11–100 $\mu$M) enhanced significantly the rate of formation of P2 in the metabolism of losartan by 6–11-fold (Fig. 3). However, $\alpha$-NF proved to be a potent inhibitor of the $\alpha$-3 hydroxylation of losartan (P1). When the $\alpha$-NF concentration was increased to $>25\mu M$, the P1 metabolite was no longer detected (Fig. 2). Surface plots that indicate the effect of $\alpha$-NF on the concomitant inhibition and activation of losartan metabolism are shown in Figs. 2 and 3, respectively.

Identification of $\alpha$-NF 5,6-Epoxide—A major metabolite (retention time = 17 min) of the CYP3A4-catalyzed metabolism of $\alpha$-NF that accounted for $>90\%$ of the HPLC peak area of total metabolites exhibited upon liquid chromatography-MS analysis an MH+ ion at $m/z$ 289, suggestive of the addition of an oxygen atom to $\alpha$-NF. Upon collision-induced dissociation, major fragment ions were observed at $m/z$ 103, 131, 143, and 187, respectively (Fig. 4). Although ion at $m/z$ 131 indicated that the isolated phenyl ring of the molecule was intact. The ions at $m/z$ 143, 187, and 131 suggested that the oxygen atom had been incorporated into the naphthalene moiety of the molecule. By comparing the HPLC profile and mass spectral information with previous reports (18–20), the metabolite was identified tentatively as $\alpha$-NF 5,6-epoxide.

Effect of Losartan on the Metabolism of $\alpha$-NF—In the metabolism of $\alpha$-NF by CYP3A4, the inclusion of losartan ($6.25–150\mu M$) was found to inhibit the formation of $\alpha$-NF 5,6-epoxide by up to 64%. In these experiments, inhibition of $\alpha$-NF metabolism increased in parallel with increases in losartan concentration, as shown in Fig. 5.

Kinetic Analysis—Full kinetic analyses were performed on the effect of $\alpha$-NF on losartan metabolism. The apparent kinetic parameters and statistics are given in Table I. The absence of $\alpha$-NF, two apparent $K_{m}$ values were determined by monitoring the formation of P1 ($K_{mP1(app)}$) and P2 ($K_{mP2(app)}$), these were 82 and 40.3 $\mu M$, respectively. The effect of $\alpha$-NF on losartan metabolism indicated that $\alpha$-NF at 1–150 $\mu M$ stimulated dramatically the formation of P2 with a marked increase in $V_{maxP2(app)}$ (up to 4.6-fold) and a rapid decrease in $K_{mP2(app)}$ (up to 4-fold). However, when the formation of metabolite P1 was quantified, $\alpha$-NF exhibited the opposite effect, showing an increase in $K_{mP1(app)}$ (4.2-fold) but a decrease in $V_{maxP1(app)}$. At $\alpha$-NF concentrations $>25\mu M$, formation of P1 was inhibited completely ($V_{P1} \rightarrow 0$). In the metabolism of $\alpha$-NF by CYP3A4, incorporation of losartan resulted in a considerable decrease in $V_{maxQ(app)}$ (35.7% of $V_{maxQ(app)}$ in the absence of losartan) and a slight increase in $K_{mQ(app)}$ (Table II).

These observed kinetic changes associated with the interaction of two substrates with the enzyme cannot be explained by simple Michaelis-Menten kinetics, in which all equations are based on the assumption of the one binding domain in the catalytic portion of the enzyme. Hence, introduction of a second binding domain in the active site is needed to explain the observed kinetics. Of the two proposed binding sites in CYP3A4, the model hypothesizes that the first site can be occupied by either losartan (S1) or $\alpha$-NF (F). The binding of
Losartan forms $S_1E$ and $S_1ES_2$ complexes for the breakdown to $P_1$, whereas the binding of $\alpha$-NF forms $FE$ and $FES_2$ for the production of $Q$ (Scheme I). Two substrates for the binding site are competitive, resulting in an inhibition, as reflected in part by an increase in $K_{mP1(app)}$ or $K_{mQ(app)}$ and decrease in $V_{maxP1(app)}$ or $V_{maxQ(app)}$. Meanwhile, the second site is occupied by losartan ($S_2$) to form $ES_2$, $FES_2$, and $S_1ES_2$ species for generating $P_2$ exclusively. Thus, the activation of losartan oxidation to $P_2$ by $\alpha$-NF clearly is attributed to $FES_2$, one of the three $P_2$-forming species, suggesting that the binding of $\alpha$-NF to this site changes the kinetic nature for the other site for losartan ($S_2$), allowing losartan to be metabolized readily to $P_2$ (probably an allosteric effect). Since the two binding sites are involved in the enzyme reaction and numerous substrate-enzyme complexes are formed, each complex possesses its own dissociation constant(s) and product-forming rate(s). In fact, the observed rate of formation of a particular metabolite represents the sum of the rates of all associated enzyme-substrate complexes generating that metabolite. For example, the net rate of $P_2$ production is accounted for by the rates of product formation from $ES_2$, $S_1ES_2$, and $FES_2$. Therefore, the resulting apparent $K_m$ and $V_{max}$ determined by the total rate of product formation (Table I and II) do not truly represent the kinetic characteristics of each individual substrate-enzyme complex. The model that describes the observed metabolic interaction leads to the solution of kinetic parameters in equilibria and provides a prediction of the potential in vitro drug-drug interaction via the enzyme-mediated reaction.

As seen in Table III, all kinetic parameters in the model were determined by the equations. $K_{S1}$, $K_{S2}$, and $K_F$ are the estimates of dissociation constants for three single-substrate-bound species, $S_1E$, $ES_2$, and $FE$, respectively, whereas $V_{maxP1}$, $V_{maxP2}$, and $V_{maxQ}$ are the maximum velocities for individual products formed at their specific sites. In contrast, the two binding sites had different $K$ and $V_{max}$ for losartan, namely $K_{S1}$ and $V_{maxP1}$ for $S_1E$, and $K_{S2}$ and $V_{maxP2}$ for $ES_2$. Thus, $K_{S1}$ (67–107 $\mu$M), $K_{S2}$ (25–41 $\mu$M), and $K_F$ (158–203 $\mu$M) were calculated by the three individual equations (Table III), and $V_{maxP1}$, $V_{maxP2}$, and $V_{maxQ}$ were determined to be 0.16, 0.007, and 4.1 min$^{-1}$, respectively. When both sites were occupied with losartan, $V_{max}$ values were changed by factors $a$ ($0.38$) for $S_1ES_2 \rightarrow P_1$ and $b$ ($0.43$) for $S_1ES_2 \rightarrow P_2$, leading to an inhibition by $aV_{maxP1} < V_{maxP1}$ and $bV_{maxP2} < V_{maxP2}$. In addition, $K_{m}$ values were shown to increase for both $K_{ES}$ (435 $\mu$M) and $K_{S2}$ (25–41 $\mu$M) for $ES_2$ and $S_1ES_2$ species, respectively, implying that the kinetic interaction of the two substrates at the active sites occurs. On the other hand, when $ES_2$ and $S_1ES_2$ were occupied/displaced with $\alpha$-NF, $V_{maxP2}$ for $FES_2 \rightarrow P_2$ increased substantially by a factor $d$ ($d = 8.3$, $dV_{maxP2} > V_{maxP2}$) with a little decrease in $K_{S2}$ for $FES_2 \rightarrow FE$ ($K_{FS} = 15.1$ $\mu$M $< K_{S2} = 25–40$ $\mu$M). In contrast, losartan decreased the $V_{max}$ for $FES_2 \rightarrow Q$ by a factor $g$ ($g = 0.29$, $gV_{maxQ} < V_{maxQ}$) but...
did not alter $K_m$ for FES$_\text{2}$ = ES$_\text{2}$ ($K_{\text{ES}} = 182–203 \mu\text{M} = K_F = 158–203 \mu\text{M}$). These results suggest that the presence of the two substrates (either the same or different molecular species) elicits changes in the metabolic profile of the enzyme as a result of several mechanisms of substrate interactions, e.g. competitive, cooperative, conformational, steric, and/or electronic effects that cause kinetic and metabolic multiplicity.

**DISCUSSION**

The clinical importance of drug-drug interactions is well recognized. Recent advances in this field have included the development of a better understanding of the mechanism of such interactions and the application of mechanistic information to the construction of experimental approaches for evaluating new and existing drugs for their potential to interact with therapeutic agents. Information on in vitro drug-drug interactions at the level of cytochrome P450 enzymes can be extremely useful in the evaluation of the potential of a new agent to cause drug interactions in the clinic. Such data derive from well defined kinetic models that describe precisely the consequences of a drug interaction at the enzyme level. However, not all drug interactions can be described adequately by such approaches, and there is a need to develop kinetic treatments that address unusual drug interactions of this type.

Cytochrome P450 enzymes are believed to be monomers that bear a single catalytic site with a common heme prosthetic group but with distinct apoprotein structures that determine their broad and overlapping substrate specificity. CYP3A4 is the major P450 enzyme present mainly in human liver, the content of which can vary 40-fold among individuals (12, 21, 22). The enzyme appears to be responsible for the oxidative metabolism of more than 50% of clinically used drugs, and drug interactions involving CYP3A4 substrates, inhibitors, and activators and/or inducers are more prevalent and complex than those of other members of the P450 family. Substrates for CYP3A4 substrates vary greatly in their physiochemical properties such as structure, molecular size and shape, lipophilicity, electronic characteristics, and kinetic interaction with enzyme protein. The fact that CYP3A4 can accommodate substrates of a relatively large size, e.g. cyclosporin ($M_r = 1201$), suggests that multiple small or intermediate-sized molecules might be able to coexist in the active site of this enzyme. Indeed, some evidence has been obtained to support this hypothesis based on kinetic studies and NMR data (14, 19, 23, 24). If an active site is capable of accommodating two substrates simultaneously, the resulting kinetic properties, e.g. binding affinity and catalytic ability, are likely to be affected differently from those observed with simple Michaelis-Menten inhibition and activation, both of which are derived from the one binding region in the active site. The unusual kinetics associated with two or more substrate interactions have been documented in a number of reports (14, 19, 23, 25, 26, 45–49). Thus, the two-site model described in the present study was developed in an attempt to address the questions related to the kinetics of complex of drug-drug interactions at the active site of CYP3A4.

Losartan, an angiotensin II receptor antagonist used for the treatment of hypertension (27), undergoes metabolism by CYP3A4 and CYP2C9 to two major products, namely an $\omega$-3 hydroxy losartan ($P_1$) and a carboxylic acid derivative ($P_2$), the latter originating via an aldehyde intermediate (28, 29). The carboxylic acid metabolite has been shown to be pharmacologically more active than the parent compound (30) and is believed to be largely responsible for the long duration of action of losartan (31). $\alpha$-NF, like other flavonoids, is a widely distributed phytochemical that is consumed regularly in the human diet (32). There is increasing interest in the ability of $\alpha$-NF to modulate cytochrome P450-mediated reactions (33, 34). Thus, $\alpha$-NF has been shown to increase the total oxidation of losartan by human liver microsomes and by CYP3A4 expressed in Escherichia coli (28). As demonstrated in the present study, a major product of this increased metabolism was the carboxylic acid, $P_2$. Enzyme activation refers to the process by which direct addition of one compound (the activator) to an enzyme enhances turnover of the substrate. The mechanism by which activation of cytochrome P450 takes place has not been explored extensively. However, it has been proposed that activation by agents such as $\alpha$-NF entails allosteric binding of an activator to the enzyme (20, 35, 36). There are many reports in the literature of direct activation of CYP3A4 in the in vitro oxidation of foreign compounds, e.g. $\alpha$-NF (19, 20, 37–39), carbamazepine (40), acetaminophen (39), steroids (35, 41), and benzodiazepines (14). In the oxidation of the carcinogen aflatoxin $B_1$ by CYP3A4 (25,
42), α-NF modulates catalysis in a regioselective fashion. Thus, α-NF inhibits the 3α-hydroxylation of aflatoxin B₁ but stimulates the 8,9-epoxidation reaction (22, 25). Since α-NF itself is known to be a substrate of CYP3A4, it must have access to the active site for metabolism. Interaction of two substrates according to the one-site model usually results in simple competitive, noncompetitive inhibition or activation. In the present study, however, the differential effect of α-NF on the values \( k_{m} \) and \( V_{max} \) associated with metabolism of losartan to the different products (P₁ and P₂), i.e. increase in \( K_{mP1(app)} \) with a decrease in \( V_{maxP2(app)} \) and decrease in \( K_{mP2(app)} \) with an increase in \( V_{maxP1(app)} \) cannot be explained by the one-site model. Hence, the presence of a second binding region in the active site must be considered to explain these unusual kinetics. According to such a model, α-NF can bind to one site that is associated with its metabolism and modulation of enzyme activity while simultaneously activating the second site for oxidation of losartan to P₂. If α-NF were to bind to an allosteric site independent of the active site, the kinetic parameters, e.g. \( K_{mP1(app)} \) and \( K_{mP2(app)} \) for losartan, should change to the same extent and in the same direction. The fact that this was not what was observed (Table I) is critical to the development of the model. In addition, the inhibitory effect of either of the two substrates on P₁ or Q production that gives rise to increased \( K_{m} \) and decreased \( V_{max} \) also is suggestive of displacement for one site by the two different substrates. In light of these considerations, an appropriate model that describes the observed kinetic changes was developed according to the following criteria: (a) α-NF can only bind to one defined site that is also occupied by losartan in the position required for 8,9-epoxidation (P₂), and both substrates can displace each other; (b) the binding of α-NF to the one site causes an allosteric effect on the other substrate-bound site required for the formation of losartan carboxylic acid (P₂), thereby altering active site geometry and oxidation efficiency reflected by a considerable decrease in \( K_{mP2(app)} \) and increase in \( V_{maxP2(app)} \) and (c) kinetic properties, e.g. binding affinity and reaction velocity, for the two-bound sites can be influenced by each other. These changes probably are due to the combined effects of allosteric modulation, steric effects, and/or electronic characteristics. Hence, the entire model was built to generate five possible enzyme-substrate complexes that are used to interpret all metabolic outcomes of drug interactions with the CYP3A4 enzyme that were observed in the present study.

Three velocity equations for P₁, P₂, and Q, respectively, assume rapid equilibria between free and bound enzyme species in the model (Equations 1–3). The terms of \( k_{1}, k_{2}, \) and \( k_{3} \) are the respective catalytic rate constants when the enzyme is occupied by a single substrate, whereas \( \alpha, \beta, \delta, \) and \( \gamma \) are the limiting factors that indicate the changes in the velocity of product formation at the one site when the other site is occupied. \( K \) values represent the dissociation constants of individual enzyme-substrate species during the rapid equilibrium or steady-state period in which the \( K \) value would be equivalent to \( K_{m} \), reflecting the binding affinity of substrate to enzyme.

Table III lists all calculated kinetic parameters. Since the model results in three product velocity equations, a set of three values each of \( K_{m1}, K_{m2}, \) and \( K_{p} \) has been obtained and were shown to be consistent, suggesting the three rate equations are valid for assessment of kinetic constants. In consideration of the losartan oxidation to P₂ in the presence of α-NF, losartan was found to have more affinity for the enzyme (\( K_{P2} \) = 15.1 μM) than that observed in the absence of α-NF (\( K_{S2} \) = 25–40 μM), whereas \( V_{max} \) for FES₂ = FE + P₂ (\( \delta V_{maxP2} = 0.058 \text{ min}^{-1}, \delta = 8.3 \)) was increased substantially with respect to \( V_{maxP2} \) (0.007 min⁻¹), implying that the addition of α-NF increases the amount of FES₂ and accelerates the P₂ formation by 8.3-fold. This suggests that α-NF binding to the one site changes the kinetic properties, exhibited by \( K_{PES} \) and factor \( \delta \), of the vacant site for losartan, which allows losartan to be metabolized readily. However, α-NF inhibited the conversion of losartan to P₁, leading to a significant increase in \( K_{mP1(app)} \) and decrease in \( V_{maxP1(app)} \). The differential effect of α-NF on the kinetic constants for losartan oxidation is due apparently to both competition between the two substrates for the one site and the allosteric effect of α-NF on the other site. Conversely, losartan has similar but opposite effects on the kinetic properties of α-NF, i.e. the \( K_{SP} \) is similar to \( K_{P2} \) but \( \gamma V_{maxP2} \) is only 29% of \( V_{maxQ} \) (\( \gamma = 0.29, \) Table III). Additionally, the binding affinity for α-NF (\( K_{aK(app)} \)) is decreased as the losartan concentration is increased (Table II). These results suggest that (i) α-NF competes with losartan for the one site (low affinity), which results in the decrease in turnover of both two substrates, and (ii) the changes in \( K \) and \( V_{max} \) values in the presence of both substrates are due to kinetic interactions within the active site, such as competition of the two substrates for the one site, and/or effects of one occupied site on the other occupied site, e.g. rigid and allosteric factors. However, since the \( K_{m} \) for S₁E = E (\( K_{S1} = 67–107 \mu M \)) and S₁ES₂ = ES₂ (\( K_{B} = 435 \mu M \)) are much

### Table III

| Equilibrium | Product | \( K \pm S.E. \) | Factor | \( V_{max} \pm S.E. \) | Equation |
|-------------|---------|----------------|--------|----------------|---------|
| \( S_{1}E \leftrightarrow E \) | P₁ | \( K_{s1} = 97 \pm 12^a \) | \( \alpha \) | \( 0.38 \pm 0.12 \) | \( V_{maxP1} = 0.16 \pm 0.04 \) |
| | | \( K_{s1} = 67 \pm 23 \) | | | |
| | | \( K_{s1} = 107 \pm 30 \) | | | |
| \( S_{1}ES_{2} \leftrightarrow ES_{2} \) | P₁ | \( K_{s4} = 435 \pm 108 \) | \( \beta \) | \( 0.43 \pm 0.21 \) | \( V_{maxP4} = 0.003 \pm 0.001 \) |
| | | \( K_{s4} = 25.1 \pm 5.6 \) | | | |
| | | \( K_{s4} = 40.5 \pm 1.1 \) | | | |
| | | \( K_{s4} = 36.1 \pm 3.2 \) | | | |
| \( S_{1}ES_{2} \leftrightarrow S_{1}E \) | P₂ | \( K_{s1} = 250 \pm 29 \) | \( \gamma \) | \( 0.29 \pm 0.11 \) | \( V_{maxQ} = 1.19 \) |
| | | \( K_{s1} = 150 \pm 45 \) | | | |
| | | \( K_{s1} = 203 \pm 30 \) | | | |
| FES₂ | P₂ | \( K_{B} = 182 \pm 44 \) | \( \delta \) | \( 8.3 \pm 2.1 \) | \( V_{maxP2} = 0.058 \) |
| | | \( K_{B} = 183 \pm 51 \) | | | |
| | | \( K_{B} = 203 \pm 89 \) | | | |

\( ^a \) Enzyme-substrate complexes and their equilibria shown in Scheme I.

\( ^b \) Product formed from the specific enzyme-substrate complex.

\( ^c \) \( K \), dissociation constant of each substrate-enzyme complex in the model, and the values were calculated by the equations shown above.

\( ^d \) Factor that determines the change in \( V_{max} \) for the first site when the second site is occupied.

\( ^e \) \( V_{max} \) values indicate that maximum velocity at the specific site of each product-forming complex in the model and were calculated by the equations, respectively. RSS and R² are 0.0213 and 0.992 for Equation 1, 0.101 and 0.988 for Equation 2, and 0.0153 and 0.990 for Equation 3, respectively.
greater than the $C_{\text{max}} (< 1 \mu M)$ in human (43), losartan as an inhibitor is unlikely to be relevant to drug-drug interaction in clinical pharmacokinetics (44).

The model also indicates that an interaction occurred when the two substrates (losartan and $\alpha$-NF) at the active site of CYP3A4 and for the differential effect of $\alpha$-NF on losartan oxidation. A key feature of this model is that it allows for the binding of both substrates simultaneously at the active site of the enzyme and describes in kinetic terms the nature of the interaction between these molecular species. It is hoped that application of this model will further aid in our understanding of the complex kinetics exhibited by CYP3A4 and provide an explanation for drug-drug interactions at the level of CYP3A4, which hitherto have not been available to kinetic evaluation.

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REFERENCES

1. Caranasos, G. J., Stewart, R. B., and Cluff, L. E. (1985) *Ann. Rev. Pharmacol. Toxicol.* 25, 67–96
2. Hardman, J. G., Limbird, L. E., Molinoff, P. B., Ruddon, R. W., and Gilman, A. G. (1996) in *The Pharmacological Basis of Therapeutics* (Gudman, L. S., and Gilman, A. G., eds). 9th Ed., McGraw-Hill Inc., New York
3. Honig, P. K., Wooley, R. L., Zamani, K., Conner, D. P., and Cantilena, L. R. (1992) *Clin. Pharmacol. Ther.* 52, 231–238
4. Koley, A. P., Buters, J. T. M., Robinson, R. C., Markowitz, A., and Friedman, F. K. (1997) *J. Biol. Chem.* 272, 3149–3152
5. Kerr, B. M., Thummel, K. E., Wurden, C. J., Klein, S. M., Kroetz, D. L., Goldberg, M. R., and Bjornsson, T. D. (1995) *Biochemistry* 34, 75–80
6. Harlow, G. R., and Halpert, J. R. (1998) *Proc. Natl. Acad. Sci. U.S.A.* 95, 6636–6641
7. Miller, D. M., Helms, S. E., and Brodell R. T. (1994) *Arch. Dermatol. 130*, 1098–1101
8. Hunter, J., and Chasseaud, L. F. (1976) *Prog. Drug Metab.* 8, 1198–1201
9. Kellis, J. T., and Vickery, L. E. (1984) *Arch. Biochem. Biophys.* 231, 238–239
10. Domanski, T. L., Hu, Y.-A., Harlow, G. R., and Halpert, J. R. (2000) *J. Pharmacol. Exp. Ther.* 293, 585–591
11. Wang, P. P., Beaufee, P., Kaminsky, L. S., Dannaan, G. A., Kadihoun, F. P., Larrey, D., and Guengerich, F. P. (1983) *Biochemistry* 22, 5375–5383
12. Ueng, Y. G., Kwabbara, T., Chun, Y. J., and Guengerich, F. P. (1997) *Biochemistry* 36, 370–381
13. Guengerich, F. P. (1988) *Mol. Pharmacol.* 33, 500–508
14. Hunter, J., and Chasseaud, L. F. (1976) *Prog. Drug Metab.* 8, 19–20
15. Honig, P. K., Wooley, R. L., Zamani, K., Conner, D. P., and Cantilena, L. R. (1995) *Drug Metab. Dispos.* 23, 207–215
16. Gang, P. C., Price, W. A., Jr., Chiu, A. T., Duncan, J. S., Vanni, R. A., Rettie, A. E., Gonzalez, F. J., and Tracy, T. S. (1998) *Biochemistry* 37, 4137–4147
17. Johnson, E. L., and Gilman, A. (1999) in *The Pharmacological Basis of Therapeutics* (Harbone, J. B., ed). pp. 619–652, Chapman & Hall, London
18. Caranasos, G. J., Stewart, R. B., and Cluff, L. E. (1985) *Ann. Rev. Pharmacol. Toxicol.* 25, 67–96
