

Inhibition of Human Cytochrome P450 3A4 by Cholesterol*[§]

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Raku Shinkyo and F. Peter Guengerich¹

From the Department of Biochemistry and Center in Molecular Toxicology, Vanderbilt University School of Medicine, Nashville, Tennessee 37232-0146

Cholesterol has been shown to be hydroxylated at the 4 β -position by cytochrome P450 3A4, and the reaction occurs *in vivo* (Bodin, K., Andersson, U., Rystedt, E., Ellis, E., Norlin, M., Pikuleva, I., Eggertsen, G., Björkhem, I., and Diczfalusy, U. (2002) *J. Biol. Chem.* 277, 31534–31540). If cholesterol is a substrate of P450 3A4, then it follows that it should also be an inhibitor, particularly in light of the high concentrations found in liver. Heme perturbation spectra indicated a K_d value of 8 μ M for the P450 3A4-cholesterol complex. Cholesterol inhibited the P450 3A4-catalyzed oxidations of nifedipine and quinidine, two prototypic substrates, in liver microsomes and a reconstituted enzyme system with $K_i \sim 10 \mu$ M in an apparently non-competitive manner. The concentration of cholesterol could be elevated 4–6-fold in cultured human hepatocytes by incubation with cholesterol; the level of P450 3A4 and cell viability were not altered under the conditions used. Nifedipine oxidation was inhibited when the cholesterol level was increased. We conclude that cholesterol is both a substrate and an inhibitor of P450 3A4, and a model is presented to explain the kinetic behavior. We propose that the endogenous cholesterol in hepatocytes should be considered in models of prediction of metabolism of drugs and steroids, even in the absence of changes in the concentrations of free cholesterol.

Cytochrome P450 enzymes are nearly ubiquitous in nature. These hemoproteins are involved in the metabolism of numerous steroids, drugs, carcinogens, and other substrates in mammals and, in addition, in many pathways of secondary metabolism and defense in plants and microorganisms (1). Humans have 57 CYP genes potentially coding for P450 enzymes (2). One of these, P450 3A4, is involved in the metabolism of approximately one-half of the drugs marketed today (of those known to be oxidized) (2–4). In addition to drugs, this enzyme also has roles in both the bioactivation and detoxication of chemical carcinogens, *e.g.* aflatoxin B₁ (2, 5, 6). P450 3A4 also has relatively high catalytic activities in the oxidation of a number of steroids, *e.g.* testosterone, progesterone, and 17 β -estradiol (7, 8). More recently, P450 3A4 has been shown to catalyze the 4 β -hydroxylation of cholesterol (9, 10), and the product has

been proposed to be an *in vivo* biomarker of the activity of P450 3A4 (and the related protein P450 3A5, which is generally expressed at lower levels) (11).

P450 3A4 has been described as displaying rather schizophrenic behavior toward some ligands, which has been difficult to understand (2). Evidence for cooperativity (both homotropic and heterotropic) was first described in 1993–1994 (12, 13). Subsequently, considerable work has provided insight into the phenomenon, although not all aspects have been clarified (reviewed in Refs. 2 and 14). A general conclusion is that multiple ligands can be accommodated and that this multiplicity somehow accounts for the cooperative behavior that is seen, *e.g.* due to forcing substrates into juxtapositions more (or less) conducive for catalysis. This multiple-ligand hypothesis is supported by several kinetic studies (13, 15–17) and by fluorescence measurements (18). The binding of testosterone is a relatively slow process and is best described in terms of two (or more?) molecules of testosterone, with a conformational change in the protein (19, 20). X-ray crystallographic studies have provided some evidence for a multiple-ligand model of P450 3A4, with some structures indicating progesterone and testosterone bound at a peripheral site (21, 22) and two molecules of ketoconazole in a P450 3A4 crystal (23).

Ligand competition is another issue with P450 3A4 and is the basis of many of the drug-drug interaction issues in pharmaceutical development (24). Another consideration, which has received less discussion, is competition between drugs and endogenous compounds, *e.g.* sterols. Testosterone, progesterone, and estrogens are known substrates (7, 8) but are generally present at only low concentrations in the tissues where P450 3A4 is highly expressed, *i.e.* liver and small intestine. However, cholesterol is present at high concentrations in liver. Even considering the low solubility of cholesterol and equilibrium with bound forms, the high concentration of cholesterol in the liver is a potential issue.

In consideration of the demonstrated 4 β -hydroxylation of cholesterol by P450 3A4 (9, 10), if cholesterol is a substrate of P450 3A4, then it follows that it should also be an inhibitor, which may be an issue in the context of the aforementioned high concentrations of cholesterol in liver (and brain). We considered this issue and have described the *in vitro* kinetics, which appear to follow non-competitive behavior but can be considered in other paradigms presented by Segel (25). The level of cholesterol in cultured human hepatocytes could be elevated by treatment with cholesterol. Elevation of the cholesterol level led to attenuated nifedipine oxidation activity, a marker for P450 3A4 (7). We conclude that consideration should be made for cholesterol inhibition of P450 3A4 in extrapolation to *in vivo*

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¹ To whom correspondence should be addressed: Dept. of Biochemistry, Vanderbilt University School of Medicine, 638 Robinson Research Bldg., 2200 Pierce Ave., Nashville, TN 37232-0146. Tel.: 615-322-2261; Fax: 615-322-3141; E-mail: f.guengerich@vanderbilt.edu.

situations, analogous to binding of drugs and steroids to plasma proteins.

EXPERIMENTAL PROCEDURES

Chemicals—Cholesterol, 17 α -ethynyl estradiol, 5-dimethylaminonaphthalene-1-sulfonyl (dansyl)² chloride, 4,4-dimethylaminopyridine, 2-hydroxypropyl- β -cyclodextrin (HP β CD), miconazole, nifedipine, quinidine, L- α -1,2-dioleoyl-*sn*-glycero-3-phosphocholine, L- α -1,2-dilauroyl-*sn*-glycero-3-phosphoserine, and Tween 20 were purchased from Sigma, and L- α -1,2-dilauroyl-*sn*-glycero-3-phosphocholine was purchased from Enzo Life Sciences, Inc. (Plymouth Meeting, PA). Nifedipine was recrystallized from hot C₂H₅OH in amber glass. 4 β - and 7 α -hydroxycholesterol were purchased from Steraloids (Newport, RI). 3S-Hydroxyquinidine and quinidine *N*-oxide were obtained from Toronto Research Chemicals (North York, Ontario, Canada). Quinine was purchased from New York Quinine and Chemical Works (New York, NY). HPLC-grade solvents were purchased from Fisher. Oxidized nifedipine (pyridine derivative product) was synthesized by HNO₃ oxidation as described previously (26).

Enzyme Preparations—Recombinant cytochrome P450 3A4 with a C-terminal His₅ tag (27) was expressed in *Escherichia coli* and purified as described (16). Rat NADPH-cytochrome P450 reductase (28) and human cytochrome *b*₅ (29) were expressed in *E. coli* and purified as described previously. Human liver samples (for preparation of microsomes) were from organ donors and obtained through Tennessee Donor Services (Nashville, TN) in accord with Vanderbilt Institution Review Board policies.

MS and Spectroscopy—A Thermo Finnigan TSQ Quantum mass spectrometer connected to a Waters ACQUITY UPLC[®] system was used for quantitation of dansylated compounds. UV-visible spectra were recorded with an Aminco DW-2/OLIS spectrophotometer (On-Line Instrument Systems, Bogart, GA).

Measurement of Cholesterol 4 β -Hydroxylation Activity with a P450 3A4-reconstituted System—A 5 \times P450 protein premixture was prepared with slight modification (30). Briefly, a 5 \times protein premixture including 2.5 μ M P450 3A4, 5 μ M NADPH-cytochrome P450 reductase, 2.5 μ M cytochrome *b*₅, 150 μ g/ml phospholipid mixture (1:1:1 (w/w/w) L- α -1,2-dioleoyl-*sn*-glycero-3-phosphocholine/L- α -1,2-dilauroyl-*sn*-glycero-3-phosphoserine/L- α -1,2-dilauroyl-*sn*-glycero-3-phosphocholine), 0.81 mM sodium CHAPS (0.50 mg/ml), and 3.0 mM GSH in 50 mM potassium HEPES buffer (pH 7.4) and a 5 \times buffer mixture including 12 mM GSH and 150 mM MgCl₂ in 200 mM potassium HEPES (pH 7.4) were prepared. A typical 500- μ l enzyme reaction mixture was prepared by mixing 100 μ l of 5 \times P450 3A4 protein premixture, an equal volume of 5 \times buffer mixture, 5 μ l of cholesterol solution in 310 mM HP β CD (45% (w/v)), and 220 μ l of H₂O. Incubations were initiated by the addition of 75 μ l of an NADPH-generating system (31). Reactions were quenched with 2.0 ml of CH₂Cl₂ including 150 pmol of 17 α -ethynyl estradiol (as the internal standard) and mixed

with a Vortex device. Following centrifugation (2000 \times *g*, 5 min), 1.4 ml of the organic layer was transferred and taken to dryness under an N₂ stream. A general method for derivatization with dansyl chloride was used with slight modification (32). Briefly, the samples were dissolved in 200 μ l of CH₂Cl₂ containing 2 mg of dansyl chloride, 0.5 mg of 4,4-dimethylaminopyridine, and 2 μ l of triethylamine and incubated at 65 $^{\circ}$ C for 1 h. The samples were dried under an N₂ stream and then dissolved in 100 μ l of CH₃CN for analysis.

Dansylated products were analyzed by LC-MS on a UPLC[®] system connected to a TSQ Quantum mass spectrometer using a Hypersil GOLD octadecylsilane column (2.1 \times 150 mm, 3 μ m; Thermo Scientific). LC conditions were as follows. Solvent A contained 95% H₂O, 5% CH₃CN, and 0.1% (v/v) HCO₂H, and solvent B contained 5% H₂O, 85% CH₃CN, 10% *tert*-butyl methyl ether, and 0.1% (v/v) HCO₂H. The column was maintained at the initial condition of 50% (v/v) solvent B for 0.5 min at a flow rate of 350 μ l/min, followed by a linear gradient increasing to 100% (v/v) solvent B over 2.0 min. This condition was maintained for 7.5 min and then returned to the initial condition over 0.05 min and maintained until the end of a 13-min run. The column temperature was maintained at 40 $^{\circ}$ C. The injection volume onto the column was 15 μ l. MS analyses were performed in the positive ion electrospray mode. Quantitation was based on multiple-reaction monitoring (dansylated 4 β -hydroxycholesterol, *m/z* 636 \rightarrow 252 and collision energy of 22 V; dansylated 17 α -ethynyl estradiol, *m/z* 530 \rightarrow 171 and collision energy of 40 V). The following (optimized) parameters were used for the detection of the analyte and the internal standard: N₂ sheath gas, 27 p.s.i.; N₂ auxiliary gas, 21 p.s.i.; spray voltage, 5.0 kV; capillary temperature, 270 $^{\circ}$ C; capillary offset, 35 V; tube lens voltage, 220 V; argon collision gas, 1.5 millitorr; scan time, 50 ms; Q3 scan width, 1 *m/z*; and Q1/Q3 peak widths at half-maximum, 0.7 *m/z*. The data were collected and quantified using Thermo Finnigan Xcalibur Version 1.0 software.

Measurement of Cholesterol 4 β -Hydroxylation Activity with Human Liver Microsomes—The amount of cholesterol in a set of 10 pooled human liver microsomes (seven males and three females) (31) was first quantified as described previously (33). Aliquots of 10 pooled human liver microsomal samples, including exactly 25 nmol of cholesterol, were added to 1.0 ml of reaction mixture containing different amounts of cholesterol (0–225 nmol) and 3.1 mM HP β CD in 50 mM potassium phosphate buffer (pH 7.4). The assays were conducted at 37 $^{\circ}$ C for 30 min. Incubations were initiated by the addition of an NADPH-generating system (31). Following extraction, the derivatization and quantitation steps were conducted as described above.

Inhibition Studies with Human Liver Microsomes and a P450 3A4-reconstituted System—Reaction mixtures with human liver microsomes included 0.10 mg/ml microsomal protein, 5.8 μ M endogenous cholesterol, 0–60 μ M exogenous cholesterol, and 3.1 mM HP β CD in 100 mM potassium phosphate buffer (pH 7.4). Incubation times were 30 min for nifedipine and 20 min for quinidine. Reaction mixtures with recombinant P450 3A4 included 0.10 μ M P450 3A4, 0.20 μ M NADPH-cytochrome P450 reductase, 0.10 μ M cytochrome *b*₅, 40 μ g/ml phospholipid mixture, 0.1 mg/ml CHAPS, 3.0 mM GSH, 30 mM MgCl₂, 0–60 μ M cholesterol, and 3.1 mM HP β CD in 50 mM potassium

² The abbreviations used are: dansyl, 5-dimethylaminonaphthalene-1-sulfonyl; HP β CD, 2-hydroxypropyl- β -cyclodextrin.

Cholesterol Inhibition of P450 3A4

HEPES buffer (pH 7.4). Incubation times were 10 min for nifedipine and 5 min for quinidine. A typical volume of enzyme reaction mixture was 500 μl , and the substrate concentrations ranged from 5 to 200 μM (nifedipine) and from 10 to 300 μM (quinidine). After a 3-min preincubation, enzyme reactions were initiated by the addition of 75 μl of an NADPH-generating system (31).

The nifedipine reactions were terminated by the addition of 200 μl of CH_3CN including 20 μM miconazole (as the internal standard), and the quinidine reactions were terminated by the addition of 20 μl of 5% (v/v) $\text{CF}_3\text{CO}_2\text{H}$ and 2 μl of a 100 μM quinine solution in $\text{C}_2\text{H}_5\text{OH}$ (as the internal standard). Samples were centrifuged at $12,000 \times g$ for 10 min, and the supernatants were collected for LC analyses.

Samples for nifedipine oxidation studies were analyzed with a UPLC[®] system connected to an ACQUITY UV detector using an ACQUITY UPLC[®] BEH C_{18} octadecylsilane column (2.1 \times 50 mm, 1.7 μm ; Waters). LC conditions were as follows. Solvent A contained 45% H_2O and 55% (v/v) CH_3OH , and solvent B contained 100% (v/v) CH_3OH . The column was maintained at an initial condition of 0% (v/v) solvent B for 2 min at a flow rate of 500 $\mu\text{l}/\text{min}$, followed by a linear gradient increasing to 100% (v/v) solvent B over 1.5 min. This condition was maintained for 1.5 min and then returned to the initial condition over 0.1 min and maintained until the end of a 7-min run. The column temperature was maintained at 40 $^\circ\text{C}$. The injection volume was 15 μl . Absorbance was monitored at 269 nm (oxidized nifedipine) and 230 nm (miconazole).

Quinidine oxidation samples were analyzed with a UPLC[®] system connected to an ACQUITY fluorescence detector using an ACQUITY UPLC[®] BEH C_{18} octadecylsilane column (2.1 \times 50 mm, 1.7 μm). LC conditions were as follows. Solvent A contained 90% H_2O , 10% CH_3CN , and 0.1% (v/v) $\text{CF}_3\text{CO}_2\text{H}$, and solvent B contained 10% H_2O , 90% CH_3CN , and 0.1% (v/v) $\text{CF}_3\text{CO}_2\text{H}$. The column was maintained at the initial condition of 0% (v/v) solvent B for 0.5 min at a flow rate of 350 $\mu\text{l}/\text{min}$, followed by a linear gradient increasing to 5% (v/v) solvent B over 3 min. After that, the percentage of solvent B was increased with a linear gradient to 100% over 1 min. This condition was maintained for 1.5 min and then returned to the initial condition over 0.1 min and maintained until the end of a 7.7-min run. The column temperature was maintained at 40 $^\circ\text{C}$. The injection volume was 7.5 μl . Fluorescence measurements were made at an excitation wavelength of 360 nm and an emission wavelength of 415 nm. The data collection and quantitative analysis were conducted with Waters MassLynx Version 4.1 and QuanLynx Version 4.1 software, respectively.

Hepatocyte Incubations—Cryopreserved human hepatocytes (lots Hu1126 and Hu4242) were purchased from Invitrogen, stored frozen in liquid N_2 until used, and thawed according to the supplier's recommended procedure. The cell number and viability were assessed using a trypan blue exclusion test. Cells were seeded at 0.9×10^5 cells/well in collagen type 1-precoated 96-well plates in culture medium composed of Williams' E medium including 10% (v/v) FBS. The cells were maintained in a humidified incubator at 37 $^\circ\text{C}$ (5% (v/v) CO_2) for 24 h. The medium was changed to FBS-free Hepato-STIM medium (BD Biosciences) including 200 μM cholesterol (0.31 mM $\text{HP}\beta\text{CD}$)

or 30 μM pravastatin to modify the cholesterol levels in the cells. After 24 h, the leakage of lactate dehydrogenase was measured with a cytotoxicity detection kit (lactate dehydrogenase; Roche Diagnostics). The cells were washed twice with 100 μl of prewarmed PBS (10 mM potassium phosphate buffer (pH 7.4) containing 150 mM NaCl), and P450 3A4 activities were measured using 200 μM nifedipine (in FBS-free Hepato-STIM medium) with a 30-min incubation. The media were collected and stored at -20°C until analysis.

Quantitation of Nifedipine Product in Media—The media (100 μl , following incubation with nifedipine) were mixed with 100 μl of CH_3CN containing 1 μM miconazole (as the internal standard). Samples were centrifuged at $12,000 \times g$ for 10 min, and the supernatants were collected and analyzed by LC-MS on a UPLC[®] system connected to a TSQ Quantum mass spectrometer using an L-column2 ODS (2.1 \times 150 mm, 5 μm ; Chemicals Evaluation and Research Institute, Saitama, Japan). LC conditions were as follows. Solvent A contained 95% H_2O , 5% CH_3CN , and 0.1% (v/v) HCO_2H , and solvent B contained 5% H_2O , 95% CH_3CN , and 0.1% (v/v) HCO_2H . The column was maintained at the initial condition of 20% (v/v) solvent B for 1.0 min at a flow rate of 350 $\mu\text{l}/\text{min}$, followed by a linear gradient increasing to 100% (v/v) solvent B over 3.5 min. This condition was maintained for 2.0 min and then returned to the initial condition over 0.1 min and maintained until the end of a 9-min run. The column temperature was maintained at 40 $^\circ\text{C}$. The injection volume onto the column was 10 μl . MS analyses were performed in the positive ion electrospray mode. Quantitation was based on multiple-reaction monitoring (oxidized nifedipine, m/z 345 \rightarrow 284 and collision energy of 26 V; miconazole, m/z 415 \rightarrow 159 and collision energy of 32 V). The following (optimized) parameters were used for the detection of the analyte and the internal standard: N_2 sheath gas, 40 p.s.i.; N_2 auxiliary gas, 15 p.s.i.; spray voltage, 5.0 kV; capillary temperature, 270 $^\circ\text{C}$; capillary offset, 12 V; tube lens voltage, 146 V; argon collision gas, 1.9 millitorr; scan time, 50 ms; Q3 scan width, 1 m/z ; and Q1/Q3 peak widths at half-maximum, 0.7 m/z . Data were collected and quantified using Thermo Finnigan Xcalibur Version 1.0 software.

Quantitation of Cholesterol in Hepatocytes—After treatment with 200 μM cholesterol or 30 μM pravastatin, cells were washed twice with 100 μl of prewarmed PBS buffer (pH 7.4) and collected with 200 μl of 0.25% (w/v) trypsin solution. The cell solutions were treated with 2.0 ml of CH_2Cl_2 including 500 pmol of 7 α -hydroxycholesterol (as the internal standard) and mixed with a Vortex device. Following derivatization and quantitation, steps were conducted as reported previously (33).

Immunoquantitation of P450 3A4 in Hepatocytes—After treatment with 200 μM cholesterol or 30 μM pravastatin, the cells were washed twice with 100 μl of prewarmed PBS buffer, and the proteins in the cells were collected with TRIzol[®] reagent (Invitrogen). The protein concentration was determined by a BCA assay (Pierce). The proteins extracted from hepatocytes (2–2.5 μg of protein/well in triplicate) were separated by 7.5% (w/v) SDS-PAGE (31, 34), along with standard samples containing 0–100 fmol of purified P450 3A4/well. The proteins were electrophoretically transferred to a polyvinylidene fluoride membrane (Bio-Rad). A primary rabbit serum including

polyclonal antibodies raised against human P450 3A4 (35) was used (125-fold dilution in PBS). The secondary antibody was goat anti-rabbit IRD800CW, which emits infrared light at 800 nm (detected using a LI-COR Odyssey instrument). The slope of the standard curve (based on the spiked samples) was used to quantify the amount of P450 3A4 in proteins extracted from hepatocytes.

Kinetic Analyses—The kinetic parameters were calculated from untransformed data using GraphPad Prism. The models used in this study were as follows (Equations 1 and 2),

$$v = (V_{\max}/(1 + [I]/K_i)) \times [S]/(K_m + [S]) \quad (\text{Eq. 1})$$

$$v = (V_{\max}/(1 + [I]^h/K_i^h)) \times [S]/(K_m + [S]) \quad (\text{Eq. 2})$$

where V_{\max} is the maximal formation rate (k_{cat} was used with purified P450 3A4), $[I]$ is the inhibitor concentration, K_i is the inhibitor constant, $[S]$ is the substrate concentration, K_m is the Michaelis constant, and h is a Hill coefficient. The Hill constant-incorporated non-competitive inhibition model (Equation 2) was applied for the analysis of nifedipine metabolism by human liver microsomes, and the usual non-competitive inhibition model (Equation 1) was applied for the rest of the analyses.

RESULTS

Cholesterol 4 β -Hydroxylation Activity with Human Liver Microsomes and a Cytochrome P450 3A4-reconstituted System—The cholesterol 4 β -hydroxylation activity of P450 3A4 was measured in the presence of 3.1 mM HP β CD. Cholesterol binding to HP β CD has been reported with a K_d value of 1.1 mM (36). Free cholesterol concentrations were calculated using the K_d value of the cholesterol-HP β CD complex and a simple two-state binding model (*i.e.* cholesterol + HP β CD \rightleftharpoons cholesterol-HP β CD). These free cholesterol concentrations were used for further kinetic analyses.

The calculated V_{\max} and $K_{m(\text{cholesterol})}$ values in human liver microsomes were 1.0 ± 0.2 pmol/min/mg of protein and 27 ± 9 μM , respectively (Fig. 1A). The calculated k_{cat} and $K_{m(\text{cholesterol})}$ values for the reconstituted system were 2.2 ± 0.2 pmol/min/nmol of P450 and 6.4 ± 2.1 μM , respectively (Fig. 1B), yielding an estimated catalytic efficiency of $3.4 \times 10^2 \text{ M}^{-1} \text{ min}^{-1}$ ($6 \text{ M}^{-1} \text{ s}^{-1}$).

Cholesterol Binding to P450 3A4—Binding of cholesterol to P450 3A4 induced a substrate-type (“Type I”) difference spectrum (Fig. 2, *inset*) (37). The calculated ΔA_{max} /nmol of P450 and K_d values (hyperbolic binding model) were 0.005 ± 0.001 and 8.3 ± 1.0 μM , respectively (Fig. 2).

Inhibitory Effect of Cholesterol on P450 3A4 Activity in Human Liver Microsomes and a Reconstituted System—Line-weaver-Burk plots for nifedipine metabolism in human liver microsomes (with varying cholesterol concentrations) indicated that the inhibition type of cholesterol was apparently non-competitive (supplemental Fig. S1). However, the plots of nifedipine concentrations *versus* nifedipine oxidation rates in human liver microsomes could not be fit well to the basic non-competitive inhibition model (Equation 1). Instead of this model, the non-competitive model including an exponent (Hill coefficient) was used (Equation 2). This model provided a good

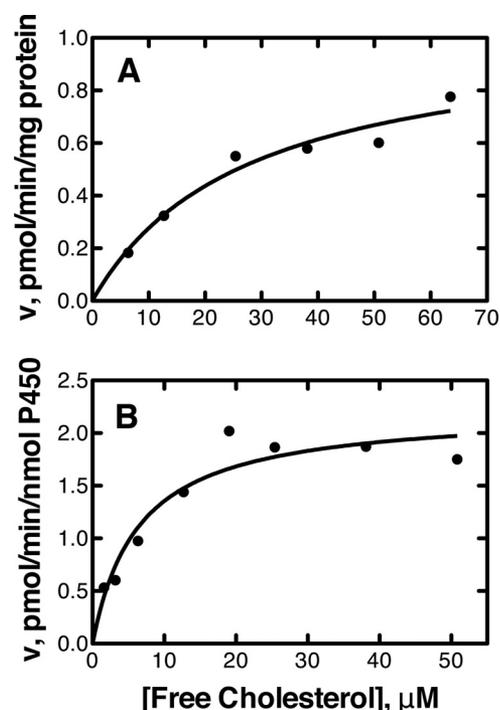


FIGURE 1. Rates of cholesterol 4 β -hydroxylation by human liver microsomes and recombinant P450 3A4. The resulting data points were fit to the Michaelis-Menten equation (GraphPad Prism software). A, microsomes ($K_m = 27 \pm 9$ μM and $V_{\max} = 1.0 \pm 0.2$ pmol/min/mg of protein); B, recombinant P450 3A4 ($K_m = 6.4 \pm 2.1$ μM and $k_{\text{cat}} = 2.2 \pm 0.2$ pmol/min/nmol of P450).

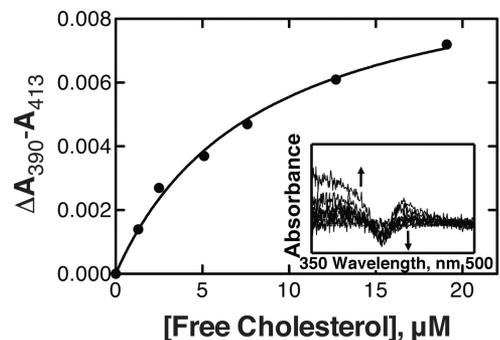


FIGURE 2. Binding of cholesterol to P450 3A4. Titration of 2.0 μM P450 3A4 was done with increasing concentrations of cholesterol (in the presence of 3.1 mM HP β CD). The plot (—) is a fit of the changes in absorbance (\bullet) *versus* the calculated free cholesterol concentration using a hyperbolic binding equation (GraphPad Prism software). The *inset* shows the spectral changes. $K_d = 8.3 \pm 1.0$ μM . Averaged absorbance values were used to analyze the data, *i.e.* A_{390} means the averaged value of absorbance data between 389 and 391 nm, and A_{413} means the averaged value of data between 412 and 414 nm.

fit to the curve, and the calculated K_i value and the Hill coefficient were 11.4 ± 0.3 μM and 2.8 ± 0.2 , respectively (Fig. 3A and Table 1). On the other hand, plots of nifedipine concentrations *versus* nifedipine oxidation rates in the P450 3A4-reconstituted system could be fit well to the basic non-competitive inhibition model (Equation 1), with a K_i value of 9.2 ± 0.3 μM (Fig. 4A, Table 1, and supplemental Fig. S2).

Cholesterol showed apparent non-competitive inhibition with quinidine 3S-hydroxylation activity in both human liver microsomes and the reconstituted system. The K_i values were calculated as 11.1 ± 1.0 and 9.1 ± 0.6 μM , respectively, with good fitting to the basic non-competitive inhibition model

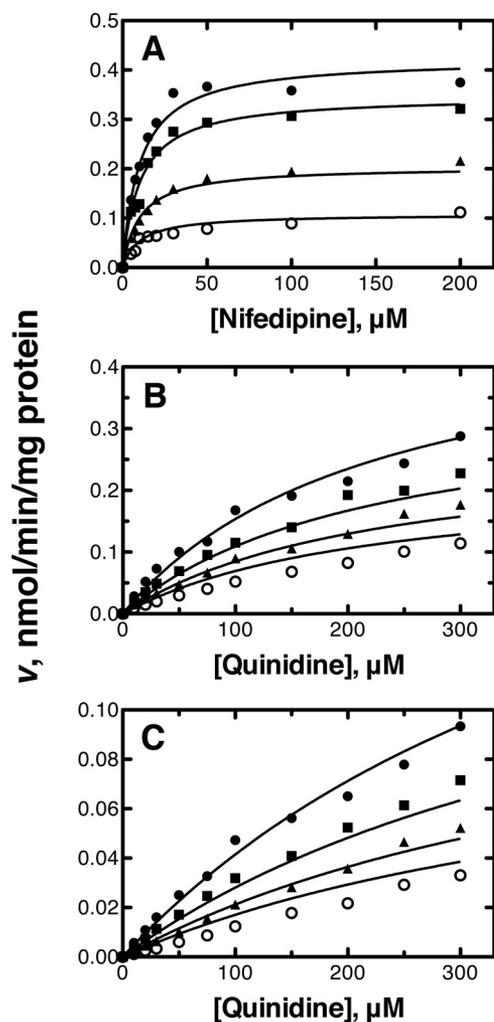


FIGURE 3. Inhibitory effects of cholesterol in human liver microsomes. A, nifedipine oxidation (5–200 μM); B, quinidine 3S-hydroxylation (10–300 μM); C, quinidine N-oxidation (10–300 μM). The data points represent means of duplicate determinations. The lines represent a simultaneous fit to the corresponding models defined under “Experimental Procedures” at free cholesterol concentrations of 1.5, 6.6, 11.7, and 16.8 μM . See Table 1 for K_i values.

TABLE 1
Calculated K_i values of cholesterol for activities of human P450 3A4

Enzyme source	K_i	Hill constant	Apparent inhibition type
	μM		
Nifedipine oxidation			
Human liver microsomes	11.4 ± 0.3^a	2.8 ± 0.2^a	Non-competitive
Reconstituted system	9.2 ± 0.3^b		Non-competitive
Quinidine 3S-hydroxylation			
Human liver microsomes	11.1 ± 1.0^b		Non-competitive
Reconstituted system	9.1 ± 0.6^b		Non-competitive
Quinidine N-oxidation			
Human liver microsomes	9.2 ± 0.7^b		Non-competitive
Reconstituted system	15.0 ± 0.8^b		Non-competitive

^a The K_i value was calculated using Equation 2 under “Experimental Procedures.”

^b The K_i value was calculated using Equation 1 under “Experimental Procedures.”

(Equation 1) (Figs. 3B and 4B, Table 1, and supplemental Figs. S1 and S2).

The inhibition of quinidine N-oxidation by cholesterol in both enzyme sources was also apparently non-competitive. The K_i values calculated with the basic non-competitive inhibition model (Equation 1) were 9.2 ± 0.7 and 15.0 ± 0.8 μM , respectively (Figs. 3C and 4C, Table 1, and supplemental Figs. S1 and S2).

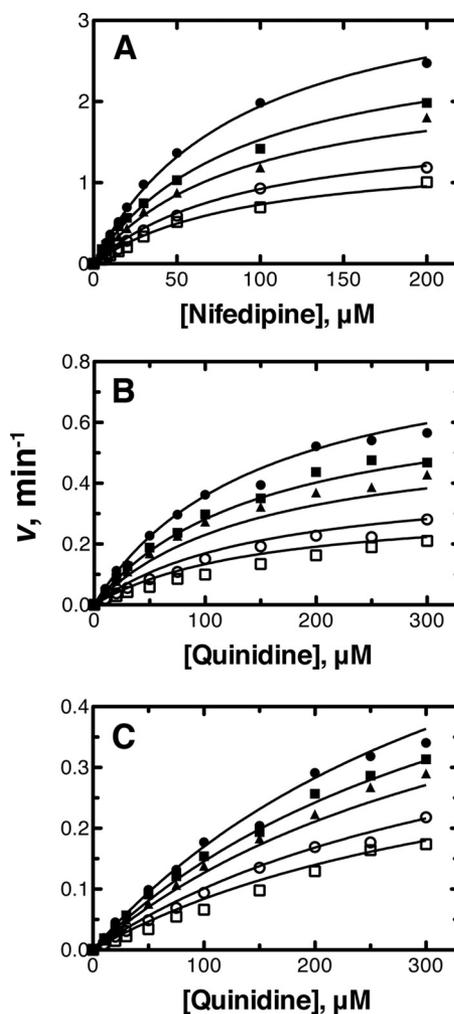


FIGURE 4. Inhibitory effects of cholesterol on recombinant P450 3A4. A, nifedipine oxidation (5–200 μM); B, quinidine 3S-hydroxylation (10–300 μM); C, quinidine N-oxidation (10–300 μM). The data points represent means of duplicate determinations. The lines represent a simultaneous fit to the corresponding models defined under “Experimental Procedures” at free cholesterol concentrations of 0, 2.5, 5.1, 10.2, and 15.3 μM . See Table 1 for K_i values.

Effect of Cholesterol on P450 3A4 Activity in Hepatocytes—Cholesterol concentrations were quantified in a preliminary study in hepatocytes (lot Hu1126) following treatment with 200 μM cholesterol or 30 μM pravastatin (supplemental Fig. S3). The cholesterol amount in the cells treated with 200 μM cholesterol was increased up to 48 h, and that in the cells treated with 30 μM pravastatin was decreased by up to 30% after 24 and 48 h. However, lactate dehydrogenase leakage from the cells after a 48-h treatment with either cholesterol or pravastatin was increased, although that after a 24-h treatment was not changed (data not shown). These results indicated that a 24-h treatment with cholesterol or pravastatin could modify the cholesterol amount in the cells without causing cytotoxicity. The P450 3A4 contents in human hepatocytes (lots Hu1126 and Hu4242) after a 24-h treatment with 200 μM cholesterol (or 30 μM pravastatin) were not significantly changed compared with those in the cells without any treatment (Table 2).

The nifedipine oxidation activities of human hepatocytes were measured after a 24-h treatment with 200 μM cholesterol.

TABLE 2
Cholesterol and P450 3A4 levels in human hepatocytes

Treatment	Cholesterol	P450 3A4
	<i>nmol/10⁶ cells</i>	<i>pmol/mg protein</i>
Hu1126 cells		
No treatment	8.7 ± 0.5	15.8 ± 2.6
200 μM cholesterol	36.1 ± 1.7	12.8 ± 0.6
Hu4242 cells		
No treatment	3.7 ± 0.8	46.6 ± 1.6
200 μM cholesterol	23.8 ± 2.2	42.7 ± 5.2

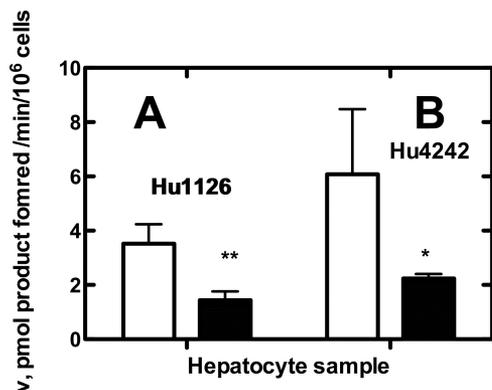


FIGURE 5. Effects of cholesterol levels on P450 3A4 catalytic activity in human hepatocytes. Human hepatocytes (lots Hu1126 and Hu4242) were treated with FBS-free Hepato-STIM medium (*open bars*; no treatment) or 200 μM cholesterol in FBS-free Hepato-STIM medium (*closed bars*) for 24 h. The catalytic activities of human hepatocytes for nifedipine (in FBS-free Hepato-STIM medium) were measured with a 30-min incubation. *A*, nifedipine oxidation in Hu1126 cells; *B*, nifedipine oxidation in Hu4242 cells. *Error bars* represent means ± S.D. of quadruplicate determinations. *, $p < 0.05$; **, $p < 0.005$.

Nifedipine oxidation activities in the cells (lot Hu1126) treated with 200 μM cholesterol were significantly decreased (59%) compared with the activities in the cells that received no treatment (Fig. 5A). Nifedipine oxidation activity in the Hu4242 cells treated with 200 μM cholesterol was significantly decreased (by 63%) (Fig. 5B).

DISCUSSION

The purpose of this study was to determine the inhibitory effects of cholesterol on cytochrome P450 3A4, the major human P450 in liver and small intestine, in light of reports that cholesterol is a substrate for 4β-hydroxylation by P450 3A4 (Fig. 1) (9–11). Cholesterol was shown to bind to P450 3A4 ($K_d = 8 \mu\text{M}$) (Fig. 2) and to inhibit prototypic P450 3A4 reactions with a K_i value of $\sim 10 \mu\text{M}$ (Figs. 3 and 4 and Table 1). These results, considered in the context of the known concentration of cholesterol in liver, suggest that cholesterol inhibition occurs in cells. Raising the level of cholesterol in cultured human hepatocytes resulted in the inhibition of nifedipine oxidation by P450 3A4 (Fig. 5).

Cholesterol inhibition studies were done with human liver microsomes and a purified enzyme system (reconstituted with NADPH-cytochrome P450 reductase and cytochrome b_5). Steroids (*e.g.* testosterone) and other hydrophobic substrates could not be utilized as substrates in these assays because they are bound to HPβCD, resulting in the inhibition of hydroxylation activity in the absence of cholesterol. Therefore, we used the drug nifedipine, the first P450 3A4 substrate identified (7). HPβCD did not inhibit nifedipine or quinidine oxidation activ-

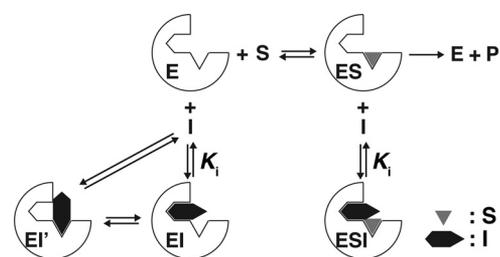


FIGURE 6. Proposed model for apparent non-competitive inhibition by cholesterol on P450 3A4 activities. *E*, P450 3A4; *I*, cholesterol; *P*, product; *S*, substrate; *EI*, P450 3A4-cholesterol complex; *EI'*, P450 3A4-cholesterol complex showing 4β-hydroxylation activity toward cholesterol; *ES*, P450 3A4-substrate complex, *ESI*, P450 3A4-substrate-cholesterol ternary complex.

ity. The inhibition of these activities by cholesterol appeared to be non-competitive, and the data were fit to a classic model (Equation 1) to obtain K_i values (Table 1). In the case of the results obtained with liver microsomes (Fig. 3 and [supplemental Fig. S1](#)), a poor fit was obtained but could be improved by the inclusion of an exponent (Hill factor) (Equation 2). However, we do not have a specific physical interpretation of this factor for inhibition, as in the case of Hill coefficients applied to cooperative phenomena observed with P450 3A4 substrates (2, 15, 38).

Although the simplistic view of non-competitive inhibition is that the inhibitor is interacting at a site distinct from that of the substrate (39), a number of possibilities exist (Fig. 6) (25), particularly in a case in which there is extensive evidence for the presence of multiple ligands in a large active site (15, 16, 23, 40). In the model shown in Fig. 6, the substrate is nifedipine or quinidine (*S*). Cholesterol is the inhibitor (*I*), but it is also a substrate (the *EI'* complex). However, in the configurations of *EI* and *ESI*, cholesterol (*I*) can block the oxidation of other substrates without being in the “*S*” site. This model comes from the classic text of Segel (41) and can explain the results observed here. Furthermore, the proposed proclivity for multiple modes of binding can explain the relatively weak absorbance changes observed when cholesterol binds to P450 (Fig. 2) in that only a small population of the modes of binding could result in H_2O displacement (as an iron ligand) and spin-state shift. The multiplicity of configurations would also be an issue contributing to the low rate of catalysis, *i.e.* 4β-hydroxylation (Fig. 1).

As in the general case of prediction of drug-drug interactions involving P450 and other enzymes, the K_i can be compared with the estimated free concentration of the inhibitor. A typical concentration of total cholesterol in human serum is $\sim 5 \text{ mM}$ (*i.e.* 20 mg/ml or 200 mg/dl). One estimate of the amount of unesterified cholesterol in human liver is 840 μmol, based only on the concentration of unesterified cholesterol in human liver microsomes ($\sim 56 \text{ nmol/mg}$ of protein) (33) multiplied by a typical recovery factor of 10 mg of microsomal protein/g of liver (42). For a liver (of 1.5 kg), this would give an “average” concentration of 560 μM. Our own work with human liver microsomes showed a mean value of 56 pmol of unesterified cholesterol/mg of microsomal protein or $\sim 100 \text{ nmol}$ of free (unesterified) cholesterol/nmol of (total) P450. Some of the cholesterol is bound to lipoprotein complexes, so the exact concentration of free cholesterol is not precisely known. Another approach to the estimation is to consider the solubility of cholesterol in buffer

Cholesterol Inhibition of P450 3A4

(5.2 μM) (43), although this value may not be appropriate in considering the overall equilibrium among various proteins. However, using a conservative value of 5 μM for an “effective” cholesterol concentration and a K_i value of 10 μM , Equation 1 would predict an increase in the “area under the curve” (integral) for an *in vivo* pharmacokinetic profile of 50% (*i.e.* 50% more exposure of the tissues to drug). If cholesterol were treated as an experimental drug candidate in development, this degree of inhibition would suggest caution. It is possible that the effective concentration of free cholesterol that P450 3A4 encounters is higher, given the ratio of 100 nmol of unesterified cholesterol/nmol of P450 in liver microsomal membranes. To put this in perspective, the decrease in nifedipine oxidation activity observed in the hepatocytes was $\sim 60\%$ (Fig. 5) after raising the cholesterol concentration. However, this experiment did not directly address the question of how much inhibition was produced by the basal level of cholesterol in the hepatocytes.

Use of an animal model to test the hypothesis that cholesterol inhibits P450 3A4 function in cells would not be trivial in that P450 3A4 is not present in mice, and mice contain seven subfamily 3A P450 enzymes, thus requiring a complex transgenic model. Accordingly, we designed experiments with cultured human hepatocytes and modulated the level of cholesterol in the cells. We considered reducing the level of cholesterol in the hepatocytes with the use of statin inhibitors of 3-hydroxymethylglutaryl-CoA reductase, but many of the statins are P450 3A4 substrates themselves and could not be used (44). Pravastatin is not a P450 3A4 substrate, but we were able to achieve an attenuation of only $\sim 30\%$ of the cholesterol level with this statin in the cells (supplemental Fig. S3), which is very significant clinically but in subsequent experiments was not sufficient to modify rates of nifedipine oxidation (at least at the level of statistical significance) (data not shown). Cholesterol levels in the hepatocytes could be elevated 4–6-fold by incubation with 200 μM cholesterol, concordant with the knowledge that hepatocytes are known to have active cholesterol uptake transporters (45).

Three individual lots of cultured human hepatocytes were used in this work. In one set, the viability decreased during the course of the experiment and was reversed by the presence of the added cholesterol, thus confounding any interpretation of results (data not shown). In the other two sets of experiments, nifedipine oxidation (a P450 3A4 marker) (7) was significantly decreased when the level of cholesterol increased (Fig. 5). Nifedipine oxidation is considered a classic and reliable marker of P450 3A4 (7); we have not extended these studies to other substrates.

In summary, we evaluated the inhibitory effect of cholesterol on P450 3A4 activities in human liver microsomes, a reconstituted system, and human hepatocytes. Cholesterol showed apparent non-competitive inhibition toward three oxidation reactions catalyzed by P450 3A4, with K_i values comparable with that of the clinically established typical P450 3A4 inhibitor verapamil (46). The increased cholesterol could cause attenuation of P450 3A4 activity in hepatocytes (Fig. 5), indicating that cholesterol in human liver could affect the metabolism of drugs

and other compounds by P450 3A4. This conclusion is relevant to the basal level of cholesterol as well as elevated levels.

REFERENCES

1. Ortiz de Montellano, P. R. (ed) (2005) *Cytochrome P450: Structure, Mechanism, and Biochemistry*, 3rd Ed., Plenum Press, New York
2. Guengerich, F. P. (2005) in *Cytochrome P450: Structure, Mechanism, and Biochemistry* (Ortiz de Montellano, P. R., ed), 3rd Ed., pp. 377–530, Plenum Press, New York
3. Williams, J. A., Hyland, R., Jones, B. C., Smith, D. A., Hurst, S., Goosen, T. C., Peterkin, V., Koup, J. R., and Ball, S. E. (2004) *Drug Metab. Dispos.* **32**, 1201–1208
4. Wienkers, L. C., and Heath, T. G. (2005) *Nat. Rev. Drug Discov.* **4**, 825–833
5. Shimada, T., and Guengerich, F. P. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 462–465
6. Ueng, Y. F., Shimada, T., Yamazaki, H., and Guengerich, F. P. (1995) *Chem. Res. Toxicol.* **8**, 218–225
7. Guengerich, F. P., Martin, M. V., Beaune, P. H., Kremers, P., Wolff, T., and Waxman, D. J. (1986) *J. Biol. Chem.* **261**, 5051–5060
8. Waxman, D. J., Attisano, C., Guengerich, F. P., and Lapenson, D. P. (1988) *Arch. Biochem. Biophys.* **263**, 424–436
9. Bodin, K., Bretillon, L., Aden, Y., Bertilsson, L., Broomé, U., Einarsson, C., and Diczfalusy, U. (2001) *J. Biol. Chem.* **276**, 38685–38689
10. Bodin, K., Andersson, U., Rystedt, E., Ellis, E., Norlin, M., Pikuleva, I., Eggertsen, G., Björkhem, I., and Diczfalusy, U. (2002) *J. Biol. Chem.* **277**, 31534–31540
11. Diczfalusy, U., Nylén, H., Elander, P., and Bertilsson, L. (2011) *Br. J. Clin. Pharmacol.* **71**, 183–189
12. Guengerich, F. P., Kim, B.-R., Gillam, E. M. J., and Shimada, T. (1994) in *Proceedings of the Eighth International Conference on Cytochrome P450: Biochemistry, Biophysics, and Molecular Biology* (Lechner, M. C., ed) pp. 97–101, John Libbey Eurotext, Paris
13. Shou, M., Grogan, J., Mancewicz, J. A., Krausz, K. W., Gonzalez, F. J., Gelboin, H. V., and Korzekwa, K. R. (1994) *Biochemistry* **33**, 6450–6455
14. Sligar, S. G., and Denisov, I. G. (2007) *Drug Metab. Rev.* **39**, 567–579
15. Ueng, Y. F., Kuwabara, T., Chun, Y. J., and Guengerich, F. P. (1997) *Biochemistry* **36**, 370–381
16. Hosea, N. A., Miller, G. P., and Guengerich, F. P. (2000) *Biochemistry* **39**, 5929–5939
17. Shou, M., Dai, R., Cui, D., Korzekwa, K. R., Baillie, T. A., and Rushmore, T. H. (2001) *J. Biol. Chem.* **276**, 2256–2262
18. Dabrowski, M. J., Schrag, M. L., Wienkers, L. C., and Atkins, W. M. (2002) *J. Am. Chem. Soc.* **124**, 11866–11867
19. Baas, B. J., Denisov, I. G., and Sligar, S. G. (2004) *Arch. Biochem. Biophys.* **430**, 218–228
20. Isin, E. M., and Guengerich, F. P. (2006) *J. Biol. Chem.* **281**, 9127–9136
21. Williams, P. A., Cosme, J., Vinkovic, D. M., Ward, A., Angove, H. C., Day, P. J., Vonnrhein, C., Tickle, I. J., and Jhoti, H. (2004) *Science* **305**, 683–686
22. He, Y.-A., Gajiwala, K. S., Wu, M., Parge, H., Burke, B., Lee, C. A., and Wester, M. R. (2006) *Proceedings of the Sixteenth International Symposium on Microsomes Drug Oxidations (MDO 2006)*, Budapest, Hungary, September 3–7, 2006
23. Ekroos, M., and Sjögren, T. (2006) *Proc. Natl. Acad. Sci. U.S.A.* **103**, 13682–13687
24. Guengerich, F. P. (2009) in *Inhibition of Drug Metabolizing Enzymes: Molecular and Biochemical Aspects* (Wienkers, L., and Pearson, P., eds) 2nd Ed., pp. 203–226, Marcel Dekker, New York
25. Segel, I. H. (1975) *Enzyme Kinetics: Behavior and Analysis of Rapid Equilibrium and Steady-state Enzyme Systems*, John Wiley & Sons, New York
26. Böcker, R. H., and Guengerich, F. P. (1986) *J. Med. Chem.* **29**, 1596–1603
27. Gillam, E. M., Baba, T., Kim, B. R., Ohmori, S., and Guengerich, F. P. (1993) *Arch. Biochem. Biophys.* **305**, 123–131
28. Hanna, I. H., Teiber, J. F., Kokones, K. L., and Hollenberg, P. F. (1998) *Arch. Biochem. Biophys.* **350**, 324–332
29. Guengerich, F. P. (2005) *Arch. Biochem. Biophys.* **440**, 204–211
30. Shaw, P. M., Hosea, N. A., Thompson, D. V., Lenius, J. M., and Guengerich, F. P. (1997) *Arch. Biochem. Biophys.* **348**, 107–115

31. Guengerich, F. P., and Bartleson, C. J. (2007) in *Principles and Methods of Toxicology* (Hayes, A. W., ed) 5th Ed., pp, 1981–2048, CRC Press, Boca Raton, FL
32. Tang, Z., and Guengerich, F. P. (2010) *Anal. Chem.* **82**, 7706–7712
33. Shinkyo, R., and Guengerich, F. P. (2011) *J. Biol. Chem.* **286**, 4632–4643
34. Laemmli, U. K. (1970) *Nature* **227**, 680–685
35. Soucek, P., Martin, M. V., Ueng, Y. F., and Guengerich, F. P. (1995) *Biochemistry* **34**, 16013–16021
36. Mast, N., and Pikuleva, I. A. (2005) *J. Lipid Res.* **46**, 1561–1568
37. Schenkman, J. B., Remmer, H., and Estabrook, R. W. (1967) *Mol. Pharmacol.* **3**, 113–123
38. Sohl, C. D., Isin, E. M., Eoff, R. L., Marsch, G. A., Stec, D. F., and Guengerich, F. P. (2008) *J. Biol. Chem.* **283**, 7293–7308
39. Dixon, M., and Webb, E. C. (1964) *Enzymes*, 2nd Ed., Longman's, Green and Co., London
40. Yano, J. K., Wester, M. R., Schoch, G. A., Griffin, K. J., Stout, C. D., and Johnson, E. F. (2004) *J. Biol. Chem.* **279**, 38091–38094
41. Segel, I. H. (1975) *Enzyme Kinetics: Behavior and Analysis of Rapid Equilibrium and Steady-state Enzyme Systems*, p. 109, John Wiley & Sons, New York
42. Guengerich, F. P. (1990) *Chem. Res. Toxicol.* **3**, 363–371
43. O'Neil, M. J. (2001) *Merck Index*, 13th Ed., p. 2223, Merck Research Laboratories, Whitehouse Station, NJ
44. Wang, R. W., Kari, P. H., Lu, A. Y., Thomas, P. E., Guengerich, F. P., and Vyas, K. P. (1991) *Arch. Biochem. Biophys.* **290**, 355–361
45. Garcia-Calvo, M., Lisnock, J., Bull, H. G., Hawes, B. E., Burnett, D. A., Braun, M. P., Crona, J. H., Davis, H. R., Jr., Dean, D. C., Detmers, P. A., Graziano, M. P., Hughes, M., Macintyre, D. E., Ogawa, A., O'Neill, K. A., Iyer, S. P., Shevell, D. E., Smith, M. M., Tang, Y. S., Makarewicz, A. M., Ujjainwalla, F., Altmann, S. W., Chapman, K. T., and Thornberry, N. A. (2005) *Proc. Natl. Acad. Sci. U.S.A.* **102**, 8132–8137
46. Ohno, Y., Hisaka, A., and Suzuki, H. (2007) *Clin. Pharmacokinet.* **46**, 681–696