# Mechanism-Based Inactivation of Cytochrome P450 3A4 by L-754,394†

Luke Koenigs Lightning,‡,§ Jeffrey P. Jones,|| Thomas Friedberg,⊥ Michael P. Pritchard,⊥ Magang Shou,○ Thomas H. Rushmore,○ and William F. Trager*,‡

*Department of Medicinal Chemistry, University of Washington, Box 357610, Seattle, Washington 98195, Department of Chemistry, Room 477 Fulmer Hall, Washington State University, Pullman, Washington 99164-4630, Biomedical Research Centre, Ninewells Hospital and Medical School, Dundee, UK, and Department of Drug Metabolism, WP-26A-2044, Merck Research Laboratories, West Point, Pennsylvania 19486*

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ABSTRACT: Mechanism-based inactivation of human liver P450 3A4 by L-754,394, a Merck compound synthesized as a potential HIV protease inhibitor, was investigated using recombinant P450 3A4. Enzyme inactivation was characterized by a small partition ratio (3.4 or 4.3 ± 0.4), i.e., the total number of metabolic events undergone by the inhibitor divided by the number of enzyme inactivating events, lack of reversibility upon extensive dialysis, no decrease in the characteristic 450-nm species relative to control, and covalent modification of the apoprotein. The major and minor products formed during the inactivation of P450 3A4 were the monohydroxylated and the dihydrodiol metabolites of L-754,394, respectively. L-754,394 that had been adducted to P450 3A4 was hydrolyzed under the conditions used for SDS-PAGE, $Ni^{2+}$ affinity chromatography, and proteolytic digestion. In addition, the modification was not stable to the acidic conditions of HPLC separation and CNBr digestion. The labile nature of the peptide adduct and the nonstoichiometric binding of the inactivating species to P450 3A4 precluded the direct identification of a covalently modified amino acid residue or the peptide to which it was attached. However, Tricine SDS-PAGE in combination with MALDI-TOF-MS and homology modeling, allowed I257-M317 to be tentatively identified as an active site peptide, while prior knowledge of the stability of N-, O-, and S-linked conjugates of activated furans implicates Glu307 as the active site amino acid that is labeled by L-754, 394.

Cytochrome P450 (P450)[1] 3A4, the major P450 isoform present in human liver, has been shown to be responsible for the oxidation of over 60% of clinically relevant drugs including: acetaminophen (*1*), cyclosporin A (*2*, *3*), lovastatin (*4*), erythromycin (*5*, *6*), nifedipine (*7*), and lidocaine (*8*, *9*). Because of its general importance in drug metabolism and carcinogen bioactivation, elucidation of the key structural elements responsible for substrate recognition leading to oxidation by P450 3A4 is of considerable interest. To date, the crystal structures of four bacterial P450s, namely, $P450_{CAM}$ (*10*), P450 BM3 (*11*), $P450_{TERP}$ (*12*), and $P450_{EryF}$ (*13*) have been solved; however, the crystal structure of a mammalian P450 is still lacking. Therefore, indirect methods must be used to gain active site structural information. Since tertiary structure appears to be conserved throughout the P450 superfamily, hypothetical three-dimensional molecular models of mammalian P450s have been constructed based on analogy to the known crystal structures of these bacterial P450s. This approach, while imperfect, allows insight to be gained into the relationship between P450 structure and function. This information should provide the foundation for the rational design of drugs and inhibitors and allow for potential in vivo metabolically based drug-drug interactions to be assessed and predicted. The inherent inaccuracies of these molecular models, however, require that the predictions be verified experimentally. Mechanism-based inactivators offer a promising approach to identify active site residues, since they are converted by P450 to reactive species that can cause the loss of enzyme activity by several different mechanisms, one of which involves covalent linkage to an active site residue(s) (*14*). This experimental approach, in turn, allows computer models of the active site to be challenged and verified.

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* To whom correspondence should be addressed: Department of Medicinal Chemistry, University of Washington, Box 357610, Seattle, Washington 98195. Telephone: (206) 543-9481. Fax: (206) 685-3252. E-mail: trager@u.washington.edu.

‡ Department of Medicinal Chemistry, University of Washington.

§ Present Address: School of Pharmacy, Rm. S-926, Department of Pharmaceutical Chemistry, University of CA, San Francisco, San Francisco, CA 94143-0446.

|| Department of Chemistry, Washington State University.

⊥ Biomedical Research Centre.

○ Merck Research Laboratories.

[1] Abbreviations: $b_5$, human cytochrome $b_5$; CID, collisionally induced dissociation; CNBr, cyanogen bromide; DLPC, L-α-dilauroylphosphatidylcholine; DLPS, L-α-dilaurylphosphatidylserine; DOPC, L-α-dioleoylphosphatidylcholine; EDTA, ethylenediamine-*N*,*N*,*N*′,*N*′-tetraacetic acid; GSH, reduced glutathione; HT, poly(His) tag; HPLC/ESI-MS, high-performance liquid chromatography electrospray ionization mass spectrometry; MALDI-TOF-MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; MBI, mechanism-based inactivator; MM, molecular mass; MOA, methoxylamine; NAC, *N*-acetylcysteine; NaCN, sodium cyanide; NADPH, reduced nicotinamide adenine dinucleotide phosphate; NADPH-GS, NADPH generating system; P450, cytochrome P450; P450 3A4, baculovirus-expressed P450 3A4; P450 3A4-HT, *Escherichia coli*-expressed poly-(His) tagged P450 3A4; P450 reductase, rat NADPH-cytochrome P450 reductase; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SOD, superoxide dismutase; TFA, $CF_3CO_2H$.; TSE buffer, 50 mM Tris-HCl (pH 7.6), 250 mM sucrose, and 0.25 mM EDTA.; Tris-HCl, tris(hydroxymethyl)aminomethane and HCl.

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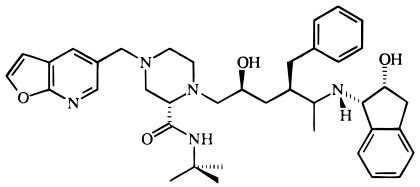

L-754,394

FIGURE 1: Chemical structure of L-754,394.

In addition to oxidizing a wide variety of substrates to nontoxic metabolites, P450 3A4 has been shown to convert several compounds, among them RU-486 (*15*) and bergamottin (*16*), to reactive species that can cause a loss of activity by covalently modifying the apoprotein. L-754,394 (Figure 1), was investigated by Merck as a potential HIV protease inhibitor. While it showed promising activity, it was not developed further because it also proved to be a potent MBI of human liver P450 3A4 activity both in vivo and in vitro (*17*, *18*). A $K_I$, $k_{inact}$, and partition ratio, of 7.5 $\mu$M, 1.62 $min^{-1}$, and 1.35, characterized P450 3A4 inactivation. Since the dihydrofuran derivative of L-754,394 failed to inactivate P450 3A4, the mechanism of L-754,394 mediated P450 inactivation was believed to involve the intact furan ring and proceed via initial epoxidation of the furanopyridine, followed by epoxide ring opening catalyzed by an active site nucleophile. The small partition ratio determined for the inactivation process suggested that the furanoepoxide was too reactive to diffuse from the active site of P450 3A4. For these reasons, it appeared that L-754,394 might serve as an ideal probe for identifying active site residues of human P450 3A4. Therefore, the inactivation of expressed human P450 3A4 by L-754,394 was further characterized using a combination of techniques, including mass spectrometry and sequence homology modeling, that allowed an active site residue to be tentatively identified.

## MATERIALS AND METHODS

*Materials.* L-754,394 and [$^{14}$C]-L-754,394 (48.9 Ci/mol) were generous gifts of Dr. Thomas Baillie, Merck Research Laboratories (West Point, PA). [$^{14}$C]-L-754,394 was purified by high-performance liquid chromatography (HPLC) to >99% radiochemical purity prior to use. L-α-dilaurylphosphatidylcholine (DLPC), L-α-dioleoylphosphatidylcholine (DOPC), L-α-dilaurylphosphatidylserine (DLPS), catalase, reduced glutathione (GSH), *N*-acetylcysteine (NAC), superoxide dismutase (SOD), imidazole, Triton X-100, sequencing grade trypsin, $\beta$-mercaptoethanol, and NADPH were purchased from Sigma (St. Louis, MO). Sodium cyanide (NaCN), $H_2O_2$, semicarbizide hydrochloride, and methoxylamine hydrochloride (MOA) were purchased from Aldrich (Milwaukee, WI). Centricon 30 concentrators were from Amicon (Bedford, MA) and Cytoscint ES liquid scintillation cocktail and hyamine hydroxide were from ICN (Costa Mesa, CA). Sep-Pak cartridges were from J. T. Baker, (Phillipsburg, NJ). HiTrap columns were from Pharmacia-Biotech (Piscataway, NJ). Cyanogen bromide (CNBr) was from Fluka (Ronkonkoma, NY). Extracti-Gel D columns and Slide-A-Lyzer dialysis cassettes were from Pierce (Rockford, IL). 10−20% Tricine SDS-gels were obtained from NOVEX (San Diego, CA). A POROS R1 perfusion column was from Perseptive Biosystems (Cambridge, MA). Human microsomal lipid was extracted from human liver microsomes as previously described (*19*). Peptide synthesis grade $CF_3CO_2H$ (TFA) was from Applied Biosystems (Foster City, CA). HPLC solvents were of the highest grade commercially available and were used as received. All other reagents were analytical grade.

*Enzymes.* P450 3A4 (P450 3A4) was expressed using a baculovirus insect cell system and purified to electrophoretic homogeneity as previously described (*20*). P450 3A4 with six histidines added to the C-terminus (P450 3A4-HT) was coexpressed with NADPH cytochrome P450 reductase in bacterial cultures as previously described for the P450 3A4 system lacking the poly(His) tag (*21*, *22*). P450 3A4-HT membranes were prepared by resuspending the bacterial cell pellets in TSE buffer (50 mM Tris-HCl (pH 7.6), 250 mM sucrose, and 0.25 mM EDTA). Recombinant rat NADPH cytochrome P450 oxidoreductase (P450 reductase) was expressed in and purified from bacterial cultures according to published procedures (*23*), with minor modifications. Human cytochrome $b_5$ ($b_5$) was expressed in and purified from bacterial cultures according to previously published procedures (*24*). Experimental data are presented as the average of duplicate determinations, which did not vary by greater than 10%. Each experiment was repeated at least twice and consistent results were obtained. Unless otherwise specified, incubations in which buffer alone (i.e., minus NADPH) was the final additive, served as controls.

*Instrumentation.* The HPLC interface to the electrospray ionization (ESI) mass spectrometer consisted of a Shimadzu LC10AD solvent delivery module and a Shimadzu SPD-10AV module UV−vis spectrophotometric detector monitoring absorbance at 214 nm (Shimadzu Scientific Instruments, Inc., Columbia, MD). A Digital DECpcLPx 466d2 computer, running Fisons Mass Lynx 0.1 software, controlled the Quattro II. Instrument settings were source temperature 100 °C; $N_2$ drying gas = 150 L/hr; nebulizing gas = 20 L/hr; probe voltage = 3.8 kV; cone voltage = 30 kV; probe position, on axis. Acquisition was carried out from *m*/*z* 100−1000 Da in 4.5 s in the CENTROID scanning mode, with unit resolution up to at least 1500 Da based on calibration and resolution optimization using poly(ethylene glycol) (PEG) 300/600/1000/1500. MS/MS spectra were acquired from *m*/*z* 100−1000 Da in 2.5 s using the CONTINUUM scanning mode. Argon was employed as the target gas for collisionally induced dissociation (CID) studies.

Matrix-assisted laser desorption ionization (MALDI) mass spectra were acquired using a delayed extraction MALDI time-of-flight (TOF) Mass Spectrometer (model Voyager DE, Perseptive Biosystems Inc., Framingham, MA) operated in the linear mode. Protein samples (5−10 pmol each) were dissolved in 40% acetonitrile containing 0.1% TFA. Each protein solution, 1.0 $\mu$L, was deposited in the sample well of a MALDI plate along with 1.0 $\mu$L of an aqueous solution, 10 mg/mL, of sinapinic acid matrix. Each sample well solution was then evaporated with a gentle stream of air and analyzed. Bovine serum albumin was used as an external mass standard.

*P450 3A4-HT Inactivation Assays.* Preliminary experiments were performed to determine appropriate amounts of P450 and L-754,394 to use in order to avoid substrate depletion. P450 3A4-HT membranes (50 pmol spectrally detectable P450) were preincubated with [$^{14}$C]-L-754,394 (5

nmol) and GSH (3 mM), $MgCl_2$ (30 mM), catalase (200 U), or $b_5$ (50 pmol) for 3 min at 37 °C. Ethanolic solutions of L-754,394 were added to each incubation, but in a manner such that the final concentration of ethanol in each was always less than 1%. The reaction was initiated by the addition of an NADPH-generating system (NADPH-GS) or TSE buffer, allowed to proceed for 45 min (final incubation volume, 100 $\mu$L), and terminated by the addition of 0.4 N $HClO_4$ (100 $\mu$L). The NADPH-GS consisted of (final concentrations) glucose 6-phosphate (10 mM), $NADP^+$ (0.5 mM), and 1 U yeast glucose 6-phosphate dehydrogenase (1 U/mL). Insoluble material was pelleted by centrifugation for 10 min at 2500 rpm (HNS II Centrifuge, International Equipment Company, Needham HTS, MA) and an aliquot (100 $\mu$L) of the supernatant was injected onto an HPLC equipped with a reverse-phase C8 column (Econosphere 5 $\mu$m, 150 mm × 4.6 mm). Variable wavelength detection at 214 nm (Hewlett-Packard 1046A programmable variable wavelength detector, Avondale, PA) and radiometric detection coupled to the HPLC was used to monitor [$^{14}$C]-L-754,394 metabolite formation. The HPLC operated at 1 mL/min in tandem with a radiometric detector (Packard Radiomatic 150TR Flow Scintillation Analyzer, Downers Grove, IL) operated at 3 mL/min that was equipped with Flo Scint II liquid scintillation cocktail. The HPLC solvent system consisted of buffer A (0.05% TFA) and buffer B (0.05% TFA in 95:5 acetonitrile:$H_2O$) and a stepwise gradient elution of 40−75% B from 0 to 20 min and 20−25 min at 75% B was used to separate L-754,394 and its metabolites.

*Partition Ratio Determinations.* A partition ratio is one measure of the efficiency of a MBI and is defined as the number of all metabolic events that the inactivator undergoes divided by the number of inactivating events (*25*). After preliminary experiments to determine appropriate amounts of P450 and L-754,394 to use to avoid substrate depletion, P450 3A4-HT membranes (200 pmol) were preincubated with [$^{14}$C]-L-754,394 (20 nmol) in the absence or presence of catalase (200 U), GSH (2 or 10 mM), NAC (2 or 10 mM), NaCN (1 mM), or MOA (2 or 10 mM) for 3 min at 37 °C in TSE buffer. The reaction was initiated by the addition of the NADPH-GS or TSE buffer (50 mM, pH 7.4), and allowed to proceed for 45 min (final incubation volume, 100 $\mu$L). A portion of the incubate (75 $\mu$L) was terminated by the addition of 0.4 N $HClO_4$ (125 $\mu$L) and set on ice. After centrifugation, an aliquot (100 $\mu$L) was injected onto the HPLC equipped with C8 column described above. The metabolites and parent compound were separated using the solvent system and stepwise gradient elution described above. Partition ratios for the L-754,394 mediated inactivation of P450 3A4-HT were calculated as the ratio of the amount of L-754,394 consumed (relative to the minus NADPH-GS control) to the amount of spectrally detectable P450 3A4-HT present in the incubation. Experiments involving nucleophilic trapping agents and reactive oxygen species scavengers were carried out in exactly the same manner.

*HPLC/ESI-MS Analysis of L-754,394 Metabolites.* P450 3A4-HT membranes (50 nmol of P450) were preincubated with L-754,394 (5 $\mu$mol) and $MgCl_2$ (30 mM) for 3 min at 37 °C in TSE buffer. The reaction was initiated by the addition of NADPH (1 mM) or TSE buffer and allowed to proceed for 60 min (final incubation volume, 5 mL). After centrifugation to remove insoluble material, the supernatant was applied to a reverse phase Sep-Pak cartridge (3 mL) that had previously been washed with methanol (6 mL), then followed by $H_2O$ (6 mL). The cartridge was washed with $H_2O$ (12 mL) and the metabolites and parent compound were eluted with methanol (3 mL). An aliquot (500 $\mu$L) of the methanol solution was injected onto an HPLC equipped with the C8 column described above and approximately 2% (50 $\mu$L) of the flow was directed to the ESI mass spectrometer using a splitter. Mass spectra were collected under the conditions described earlier in the *Instrumentation* section. The metabolites and parent compound were separated using the solvent system and stepwise gradient elution described above. The retention times of the suspected dihydrodiol metabolite, N-dealkylated metabolite, monohydroxylated metabolite, and L-754,394 were 8, 11, 13, and 22 min, respectively.

*Covalent Binding of [$^{14}$C]-L-754,394 to Protein Determined by SDS-PAGE.* The remaining portion of the P450 3A4-HT incubate described under *Partition Ratio Determinations* (25 $\mu$L) was terminated by the addition of SDS-PAGE sample buffer (35 $\mu$L). The sample was boiled for 3 min and the proteins were separated by SDS-PAGE (9%) and visualized by Coomassie blue staining. The destained gels were sliced into sections (5 mm) that were extracted into 75% hyamine hydroxide by incubation in glass scintillation vials for 3 h at 60 °C. After the sample was cooled to room temperature, Cytoscint ES liquid scintillation fluid (10 mL) was added to the samples. These samples were allowed to equilibrate in the dark for at least 12 h prior to liquid scintillation counting (Packard 2200CA Tricarb liquid scintillation counter, Downers Grove, IL).

*Effect of L-754,394 on P450 3A4-HT Spectral Content.* P450 3A4-HT membranes (1.2 nmol) were preincubated with catalase (1200 U) and $MgCl_2$ (30 mM) at 37 °C for 3 min in TSE buffer in the absence or presence of L-754,394 (60 nmol). The reaction was initiated by addition of the NADPH-GS or TSE buffer (final incubation volume, 0.6 mL). At 0, 2, 5, 10, and 20 min, an aliquot (100 $\mu$L) was removed and diluted 1:6.5 into chilled Solubilization buffer and set on ice. These mixtures were then assayed for cytochrome P450 spectral content (Hewlett-Packard 8451A Diode Array Spectrophotometer, Avondale, PA) using the method of Omura and Sato (*26*).

*Determination of [$^{14}$C]-L-754,394 Binding Stoichiometry to P450 3A4-HT.* Six incubation vials containing P450 3A4-HT membranes (50 pmol) were preincubated with [$^{14}$C]-L-754,394 (2 nmol), $MgCl_2$ (30 mM) for 3 min at 37 °C in TSE buffer. The reactions were initiated by the addition of the NADPH-GS (final incubation volume, 100 $\mu$L) to three of the vials while TSE buffer was added to the remaining three. The reactions were allowed to proceed for 60 min, then terminated by the addition of 0.5% (w/v) cholate (400 $\mu$L). Each protein mixture was dialyzed four times against 1000 volumes of 0.5% cholate in buffer (100 mM potassium phosphate buffer, pH 7.4) using a Slide-a-Lyzer dialysis cassette (0.5−3 mL). The total volume of each dialyzed sample was determined using a pipettor and 20% of each mixture was added to Cytoscint liquid scintillation fluid (10 mL). The amount of radioactivity present in each of these aliquots was determined using the liquid scintillation counter described above.

*Purified Reconstituted P450 3A4 Inactivation Assays.* P450 3A4 was reconstituted by combining purified P450 3A4,

P450 reductase, and $b_5$ in that order in a 1:2:1 molar ratio (50 pmol) and dialyzing the mixture against 100 volumes of buffer (100 mM potassium phosphate, pH 7.4) for at least 3 h at 4 °C. This initial dialysis step was performed in an attempt to remove any residual glycerol and/or detergent present in the purified protein samples. Freshly sonicated human microsomal lipid (125−1000 μg/nmol P450), DLPC (125−1000 μg/nmol), or DLPC/DLPS/DOPC (1:1:1, 125−1000 μg/nmol) was added and this mixture was allowed to reconstitute on ice for at least 1 h. [$^{14}$C]-L-754,394 (5 nmol) and catalase (200 U) were added to the reconstituted enzyme mixture and the reaction was initiated by the addition of NADPH or buffer (final incubation volume, 100 μL). The incubations were terminated and analyzed by HPLC-radiometric detection as described above.

*Covalent Binding of [$^{14}$C]-L-754,394 to P450 3A4 Determined by POROS HPLC.* Reconstituted P450 3A4 (83 pmol) was preincubated with [$^{14}$C]-L-754,394 (2 nmol), GSH (3 mM) and $MgCl_2$ (30 mM) for 3 min at 30 °C in potassium phosphate buffer (100 mM, pH 7.4). The reaction was initiated by the addition of NADPH (1 mM) and allowed to proceed for 60 min (final incubation volume, 100 μL). This solution was concentrated and washed at least 10 times with 2.5 mL ammonium bicarbonate buffer (100 mM, pH 8.1) using a Centricon 30 concentrator. An aliquot (25 μL) of the concentrated protein mixture was injected onto an HPLC equipped with a POROS R1 perfusion column (4.6 × 100 mm) operating at a flow rate of 3 mL/min. The solvent system consisted of buffer A and buffer B described above. The components of the reconstituted enzyme mixture were separated using a stepwise gradient elution of 20−50% buffer B from 0 to 5 min, hold at 50% buffer B from 5 to 6.5 min, and 50−85% buffer B from 6.5 to 8 min. The elution of the components was monitored by variable wavelength detection at 214 nm operated in tandem with radiometric detection as described above. Under these conditions, L-754,394 eluted at 2 min, heme eluted at 3.3 min, catalase eluted at 4.4 min, $b_5$ eluted at 5.0 min, P450 reductase eluted at 5.5 min, and P450 3A4 eluted at 8 min. The stoichiometry of binding was determined by collecting fractions (0.5 min, or approximately 1.5 mL) of the HPLC flow, followed by addition of Cytoscint liquid scintillation fluid (2.5 mL) to the samples, and liquid scintillation counting. The total amount of P450 3A4 injected was determined using variable wavelength detection at 214 nm to construct a standard curve comprised of known amounts of unmodified P450 3A4.

*Purification of P450 3A4-HT After Exposure to L-754,394.* P450 3A4-HT membranes (20 nmol) were preincubated with L-754,394 or [$^{14}$C]-L-754,394 (200 nmol) and $MgCl_2$ (30 mM) for 3 min at 37 °C in TSE buffer. The reaction was initiated with NADPH (1 mM), incubated for 45 min (final incubation volume, 5 mL), and terminated by the addition of 10% Triton X-100 (3 mL). The samples were stirred for 60 min at 4 °C after addition of binding buffer (22 mL, 50 mM potassium phosphate buffer (pH 7.4), 20% glycerol, and 1 mM β-mercaptoethanol). The solubilized membranes were centrifuged at 100000*g* for 35 min at 4 °C, and the supernatant was passed through a 0.45 μm filter to remove any insoluble material. The filtered solution was diluted 4-fold with binding buffer and loaded onto a HiTrap column (1 mL) preequilibrated with binding buffer + 0.5% Triton X-100. Loosely associated proteins were washed from the column with 5 column volumes of Wash buffer (binding buffer + 0.5% Triton X-100 + 25 mM imidazole). Elution buffer (binding buffer + 0.5% Triton X-100 + 250 mM imidazole) was used to elute purified P450 3A4-HT as a bright red fraction. Residual detergent was removed by passage of P450 3A4-HT through three 1 mL Extracti-Gel D columns. The detergent free purified P450 3A4-HT preparation was dialyzed against binding buffer (4 × 250 volumes).

*Chemical and Enzymatic Digestion of P450 3A4.* P450 3A4 (10 nmol) was reconstituted as described above in freshly sonicated human liver microsomal lipid (1.25 mg) and was preincubated with [$^{14}$C]-L-754,394 (500 nmol) for 3 min at 30 °C. The reaction was initiated by the addition of NADPH (final concentration, 1 mM) or buffer (100 mM potassium phosphate, pH 7.4) and allowed to proceed for 60 min (final incubation volume, 6 mL). The protein mixture was divided into six Centricon-30 microconcentrators, concentrated to approximately 100 μL, and washed three times with 2.5 mL buffer (100 mM ammonium bicarbonate buffer, pH 8.1). The enzyme mixture was incubated with trypsin (trypsin/protein ratio of 1:50 (w/w)) for 0−12 h at room temperature. Alternatively, the reconstituted P450 3A4 mixture was lyophilized to dryness, dissolved in approximately 5 μL of 70% TFA, and incubated with CNBr in a ratio of 1:100 (w/w) in the dark for 12 h at room temperature. The chemical cleavage reaction was terminated by 20-fold dilution with $H_2O$ and lyophilized to near dryness. In another set of experiments, P450 3A4 was purified from the other proteins in the reconstituted mixture after exposure to [$^{14}$C]-L-754,394 and NADPH using the POROS HPLC methodology described above. The POROS HPLC purified and inactivated P450 3A4 was then subjected to enzymatic or chemical cleavage.

The CNBr generated fragments were separated using a POROS HPLC column operated at 3 mL/min and a gradient elution of 5−100% B over 35 min with 50 μL of the flow directed to the Quattro II ESI mass spectrometer using a splitter. Fractions (0.5 min or approximately 1.5 mL) were collected and an aliquot (200 μL) from each sample was added to Cytoscint liquid scintillation fluid (2 mL) for determination of radioactivity. Fractions containing the most radioactivity were lyophilized to near dryness and analyzed by MALDI-TOF-MS.

In addition, Tricine SDS-PAGE was used to separate CNBr generated peptide fragments followed by liquid scintillation counting to detect radioactive gel slices. The chemical digest mixture was loaded onto a 10−20% Tricine gel and, after electrophoresis, the gel was stained, destained, and sliced, as described above for the determination of [$^{14}$C]-L-754,394 binding to P450 3A4-HT membranes by SDS−PAGE. The 1−2 mm gel slices were solubilized with hyamine hydroxide and analyzed for radioactivity by liquid scintillation counting as described above.

*Homology Model of L-754,394 Bound P450 3A4.* The alignments of $P450_{CAM}$ and P450 BM3 are those reported previously (*27*) and $P450_{EryF}$ was aligned with $P450_{CAM}$ and P450 BM3 as previously described (*13*). A three-dimensional homology model of P450 3A4 was constructed by alignment of the P450 3A4 sequence with the known structures of $P450_{CAM}$, P450 BM3, and $P450_{EryF}$ using the program Modeler (*28*). Since the furanopyridine ring is the known

Table 1: Optimization of P450 3A4-HT Activity in Bacterial Membranes

| incubation components | % L-754,394 consumed[a] | % P450 bound[b] |
|---|---|---|
| complete system[c] | 100 | 100 |
| (−) NADPH-GS | 0 | 0 |
| (−) 30 mM $MgCl_2$ | 83 | 83 |
| (+) 2 mM GSH | 87 | 95 |
| (+) 2 mM MOA | 63 | 71 |
| (+) 2 mM NAC | 100 | 105 |
| (+) 2 mM NaCN | 95 | 97 |
| (+) 200 U Catalase | 100 | 101 |
| (+) 50 pmol human cytochrome $b_5$ | 105 | ND[d] |

[a] The amount of [$^{14}$C]-L-754,394 consumed was determined using an HPLC-radiometric detection method. [b] The amount of covalently modified P450 3A4-HT was determined by SDS−PAGE followed by liquid scintillation counting. [c] The Complete system consisted of P450 3A4-HT membranes (50 pmol), [$^{14}$C]-L-754,394 (50 $\mu$M), $MgCl_2$ (30 mM) and the NADPH-GS in TSE buffer. [d] ND = not determined.

site of oxidation prior to inactivation, L-754,394 was docked into P450 3A4 by placing this ring over the heme iron. Molecular mechanics using the Tripos force field in Sybyl was used to relax the substrate in the active site. Molecular dynamics simulations were then conducted for 1000 × 1 fs steps at 100, 200, 300, and 500 °C. The structures displayed in Figure 8 a and b are the result of a molecular mechanics minimization of one of the low energy conformations of the 500 °C molecular dynamics simulation. For both the molecular mechanics and dynamics simulations, only the substrate and amino acid residues directly in contact with the substrate were allowed to move. This method was used to provide a low energy binding mode for this compound for oxidation at the furanopyridine ring. It is important to note that other positions of L-754,394 are oxidized and that oxidation at these positions most likely occurs from different binding orientations.

## RESULTS

*Inactivation of P450 3A4-HT by L-754,394.* P450 3A4-HT mediated [$^{14}$C]-L-754,394 turnover was optimal in the presence of $MgCl_2$ and exhibited no enhancement when $b_5$, in a 1:1 ratio to P450 3A4-HT, was added (Table 1). These results agree with previous reports using the nonpoly(His) tagged P450 3A4 membrane preparations (*21*). Upon completion of the incubations, extensive dialysis was used to remove noncovalently bound radioactivity and 173 ± 63 dpm and 1459 ± 63 dpm remained associated with the P450 3A4-HT in the absence and presence of the NADPH-GS, respectively. Thus, 11.8 pmol of radiolabel (109 dpm/pmol) remained associated with P450 3A4-HT compared to the predicted value of 10.9 pmol based on the amount of spectrally detectable P450 3A4-HT present. These results led to a calculated binding stoichiometry of 1.08 ± 0.5 between spectrally detectable P450 3A4-HT and the reactive intermediate generated from L-754,394. In contrast, if P450 3A4-HT was first separated from the other proteins in the incubation mixture by SDS-PAGE before determining the amount of L-754,394 that had become covalently bound, then a different result was obtained. When the SDS gel was sectioned into 5 mm slices after electrophoresis and the radioactivity present in each slice determined, only 59% of the radioactivity loaded onto the gel was found to be

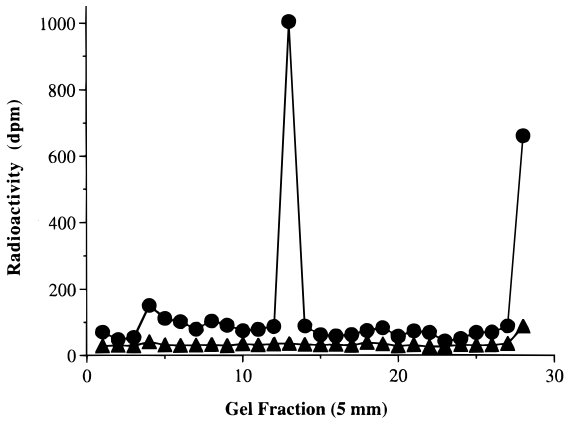


FIGURE 2: Covalent binding of [$^{14}$C]-L-754,394 to P450 3A4-HT. The amount of radioactivity associated with each gel slice after SDS-PAGE (9%) separation of the components of the P450 3A4-HT membranes that had been exposed to [$^{14}$C]-L-754,394 in the absence (s) or presence of NADPH (l). The gel was sliced into sections (5 mm) and each section was extracted and analyzed for radioactivity by liquid scintillation counting.

associated with the electrophoretic migration distance of purified P450 3A4. The balance of the loaded radioactivity (41%) was associated with the migratory front but not the heme or any of the other proteins present in the original mixture (Figure 2)

Covalent binding of activated [$^{14}$C]-L-754,394 to P450 3A4-HT, as determined by SDS-PAGE, was time dependent and was found to be maximal after a 30 min exposure time. The inactivation of P450 3A4-HT was dependent on the NADPH-GS and was not prevented by the addition of catalase, a reactive oxygen species scavenger (Table 1). Inclusion of even relatively low concentrations (2 mM) of various nucleophilic trapping agents led to a decrease in the amount of P450 3A4-HT bound due to a corresponding decrease in P450 3A4-HT activity (Table 1). As might be expected, this decrease was enhanced at higher concentrations (10 mM) of trapping agents. The decrease in P450 3A4 catalytic activity due to various nucleophilic trapping agents noted here was also observed in similar studies employing P450 2A6 expressed in a $\beta$-lymphoblastoid cell line (*29*). The reason for the decrease in activity is currently under investigation. Finally, a 20 min exposure of P450 3A4-HT membranes to the NADPH-GS in the absence or presence of L-754,394 caused a 47% decrease in the amount of spectrally detectable P450 3A4-HT.

*P450 3A4-HT Membrane Catalyzed L-754,394 Metabolite Formation.* Radiometric analysis of the incubation mixture after exposure of P450 3A4-HT membranes to [$^{14}$C]-L-754,-394 and the NADPH-GS revealed the presence of one major metabolite and several minor metabolites. HPLC/ESI-MS analysis indicated that the major metabolite possessed an $[M+H]^+$ ion = 670.5 corresponding to a monohydroxylated product (Figure 9, path A). CID of this metabolite produced a major fragment ion with a $m/z$ = 437.1, whereas the corresponding fragment ion observed for the parent compound possessed a $m/z$ = 421.1. There are several possible sites where monohydroxylation of L-754,394 could occur; however, comparison of the CID spectrum of the metabolite with that of L-754,394 ruled out hydroxylation at the furanopyridine ring, the methylene bridge separating the furanopyridine and piperidine rings, and the tertiary butyl

Table 2: Optimization of Purified Reconstituted P450 3A4 Activity

| incubation components | % L-754,394 consumed[a] |
|---|---|
| complete system[b] | 100 |
| 1:3:1 (P450 3A4:P450 reductase:$b_5$)[c] | 96 |
| 1:2:0 | 84 |
| (−) 875 $\mu$g/nmol Lipid | 95 |
| (−) Lipid, (+) DLPC:DLPS:DOPC (1:1:1, 1 mg/nmol) | 0 |
| (−) Lipid, (+) DPLC (100−1000 $\mu$g/nmol) | 0 |
| (−) 3 mM GSH | 93 |
| (−) 30 mM $MgCl_2$ | 93 |
| (−) 50 mM potassium phosphate buffer | 75 |
| (+) 100 mM potassium phosphate buffer | 65 |

[a] The amount of [$^{14}$C]-L-754,394 consumed was determined using an HPLC-radiometric detection method. [b] The complete system consisted of P450 3A4, P450 reductase, and $b_5$ reconstituted in a ratio of 1:2:1 and human microsomal lipid (1 mg/nmol), GSH (3 mM), $MgCl_2$ (30 mM) and NADPH (1 mM) in potassium phosphate buffer (100 mM, pH 7.4). [c] Various components were deleted from or added to the complete system prior to determining the % L-754,394 consumed.

group. No other fragments ions were detected in the MS/MS analysis of the monohydroxylated metabolite that permitted its further characterization. Large scale incubations with P450 3A4-HT membranes (50 nmol), L-754,394, and the NADPH-GS were performed to help identify minor metabolites. HPLC/ESI-MS analysis of the large-scale incubation mixtures suggested that L-754,394 dihydrodiol ($[M+H]^+ = 688.1$) (Figure 9, path C) and N-dealkylated L-754,394 ($[M+H]^+ = 524.1$) (Figure 9, path B) were possible metabolites. The partition ratio characterizing the L-754,394 mediated inactivation of P450 3A4-HT was calculated to be 4.3 ± 0.4 and 3.9 ± 0.4 based on total inactivator depletion and major metabolite (monohydroxylated L-754,394) formation, respectively.

*P450 3A4 Catalyzed L-754,394 Metabolite Formation.* In the presence of NADPH, P450 3A4 catalyzed the oxidation of L-754,394 when reconstituted in a 1:2:1 ratio of P450/P450 reductase/$b_5$ and 1 mg human microsomal lipid per nmol P450 in 100 mM potassium phosphate buffer (pH 7.4) (Table 2). Activity was further optimized by the inclusion of GSH (3 mM), which has been reported to enhance the binding interactions between P450 and P450 reductase (*30*), and $MgCl_2$ (30 mM). Various mixtures of commercially available lipids were unable to substitute for lipid purified from human liver microsomes in catalyzing L-754,394 oxidation and could not support P450 3A4-HT activity once purified from the P450 3A4-HT membrane preparations. The same L-754,394 metabolic profile was observed when either P450 3A4-HT membranes or reconstituted P450 3A4 was used as the enzyme source. A partition ratio of 3.4 ± 0.3 was calculated for the L-754,394 mediated inactivation of P450 3A4 based on total inactivator depletion and the assumption of a complete loss of reconstituted P450 3A4 activity.

*Purification of Covalently Modified P450 3A4-HT.* Exposure of the P450 3A4-HT membranes to 37 °C, NADPH, and L-754,394 changed the behavior of the poly(His)-tagged protein to purification relative to native P450 3A4-HT. This was also true for P450 3A4-HT exposed to only 37 °C and NADPH. More specifically, this P450 3A4-HT fraction differed from P450 3A4-HT that was not exposed to 37 °C and NADPH in that it (a) eluted with 25 mM imidazole in the presence of 250 mM NaCl (commonly included in the purification of poly(His)-tagged proteins in order to wash away loosely bound hydrophobic proteins from the $Ni^{2+}$ column), (b) eluted with 75 mM imidazole in the absence of NaCl, (c) precipitated on the column head when detergent was removed from the buffers, and (d) precipitated on the column head when cholate (0.2%) was substituted for Triton X-100 (0.5%) or Emulgen 911 (0.5%). It is unlikely that a simple conformational change in the protein induced by exposure of P450 3A4-HT to NADPH and 37 °C could account for all of the observed changes in the chromatographic properties of P450 3A4-HT, especially the decreased affinity of the poly(His)tag for the $Ni^{2+}$ column. However, a loss of some (or all) of the six histidines comprising the poly(His) tag, possibly due to proteolysis, in combination with a conformational change in the P450 3A4-HT protein under the conditions of the incubation might account for its altered chromatographic behavior (data not shown). Indeed, HPLC/ESI-MS analysis of P450 3A4-HT following incubation provided an ion envelope that corresponded to several species differing in MM. P450 3A4-HT that had not been exposed to NADPH and a 37 °C incubation temperature exhibited an ion envelope corresponding to only one molecular species (*20*). A protocol that was successful in purifying P450 3A4-HT from the membrane preparations after exposure to 37 °C and NADPH involved the use of 25 mM imidazole in the wash buffer (in the absence of NaCl) and Triton X-100 (0.5%) instead of Emulgen 911 (0.5%) as a detergent.

On the basis of previous experience purifying P450 3A4-HT from membrane preparations that had not been exposed to 37 °C and NADPH, a yield of approximately 50% could be expected after the $Ni^{2+}$ column. It was also assumed that approximately 10 nmol of purified P450 3A4-HT would be required for identification of a peptide and/or amino acid originating from P450 3A4-HT that had been covalently modified by a reactive intermediate of [$^{14}$C]-L-754,394. Therefore, a relatively large amount of P450 3A4-HT (20 nmol) was exposed to NADPH and [$^{14}$C]-L-754,394. A small portion of the incubation mixture was analyzed by SDS-PAGE and liquid scintillation counting to confirm that radioactivity was associated with P450 3A4-HT and ensure that covalent modification of P450 3A4-HT had occurred. After loading the solubilized, radiolabeled P450 3A4-HT membrane preparations, eluting loosely bound proteins, eluting the purified modified P450 3A4-HT protein (Figure 3), and removing the detergent, it was discovered that the radiolabel had been lost. Apparently, the limited time (approximately 2−3 h total) and conditions required to purify P450 3A4-HT from the solubilized membrane preparations, were sufficient to result in complete loss of radiolabel, presumably by hydrolysis.

*Covalent Modification of P450 3A4 Determined by HPLC.* Reconstituted P450 3A4 was exposed to [$^{14}$C]-L-754,394 in the absence or presence of NADPH followed by concentration of the incubation mixture. Residual noncovalently bound [$^{14}$C]-L-754,394 was removed by extensive washing of the concentrated protein mixture with buffer. POROS HPLC was used to separate the $b_5$, P450 reductase, and P450 3A4 as described above. Radiometric detection operating in tandem with POROS HPLC indicated that binding was specific for the apoprotein of P450 3A4 (Figure 4). No radioactivity was associated with the heme of P450 3A4. On the basis of the known specific content of the [$^{14}$C]-L-754,394 used in the

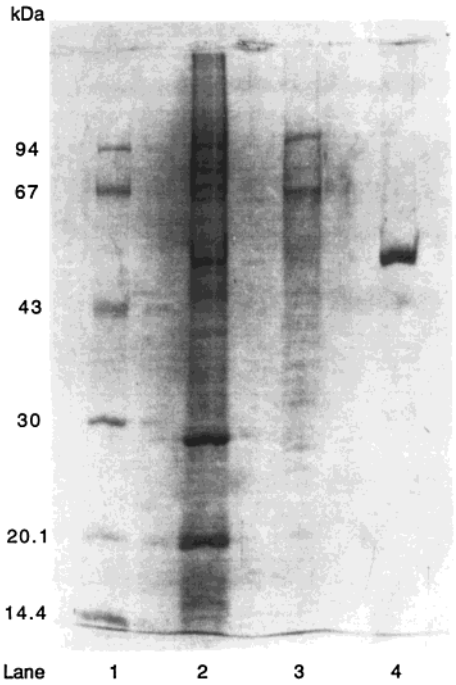


FIGURE 3: Purification of P450 3A4-HT. SDS-PAGE (9%) analysis of the purification of P450 3A4-HT from the membrane preparations using a HiTrap column after exposure to [$^{14}$C]-L-754,394 and NADPH. Lane 1 contains the MM markers, Lane 2 contains a sample of the *E. coli* cell paste, Lane 3 contains a sample of the 25 mM imidazole wash, and Lane 4 contains a sample of purified P450 3A4-HT. The gel was sliced into sections (5 mm) and each section was extracted and analyzed for radioactivity by liquid scintillation counting.

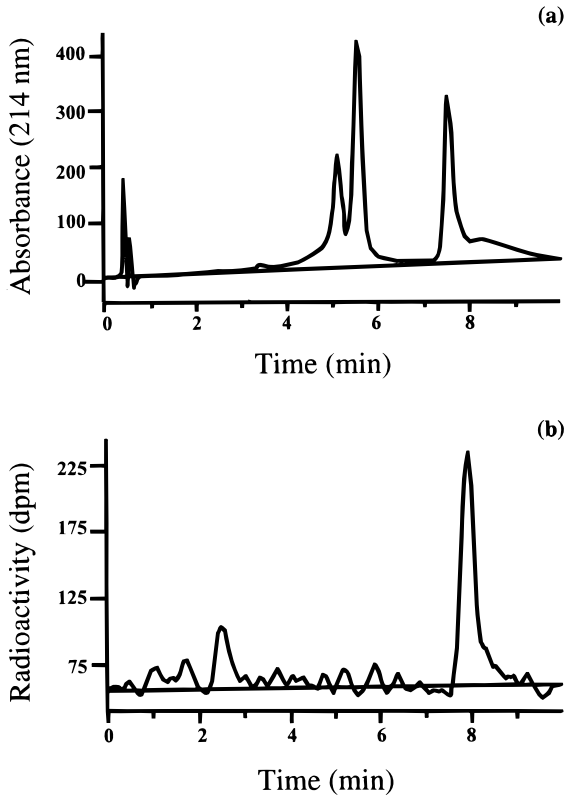


FIGURE 4: Covalent binding of [$^{14}$C]-L-754,394 to P450 3A4. POROS HPLC separation of the components of the reconstituted P450 3A4 system after exposure to [$^{14}$C]-L-754,394 and NADPH monitored by (a) variable wavelength detection at 214 nm and (b) radiometric detection. P450, P450 reductase and heme eluted at 7.8, 5.6, and 3.3 min, respectively.

experiments and a standard curve comprised of unlabeled P450 3A4, the amount of P450 3A4 associated with radioactivity corresponded to a binding stoichiometry of 0.4 $\pm$ 0.1:1 between the reactive intermediate of [$^{14}$C]-L-754,394 and P450 3A4. In previous investigations, it was demonstrated that ESI-MS operating in tandem with POROS HPLC could be used to identify tienilic acid adducts to intact P450 2C9 (*20*). Similar experiments were attempted with P450 3A4 after exposure to NADPH and L-754,394, however, the large amount of human microsomal lipid used in the incubation severely contaminated and/or suppressed the signal associated with modified P450 3A4. Because of its strict dependence on human microsomal lipid for activity, no further attempts to identify the L-754,394 adducts to intact P450 3A4 were made.

*Proteolytic Digestion of P450 3A4.* Subjecting covalently modified P450 3A4 to enzymatic digestion with trypsin resulted in hydrolysis of the radiolabel from the protein. This loss of the radiolabel has also been observed in similar experiments using MBIs of P450s 2A6 and 2B1.[2] In contrast, other experiments with MBIs of P450s that used CNBr mediated chemical cleavage of methionine residues, followed by Tricine SDS-PAGE, were successful in identifying covalently modified active site residues (*31*−*34*). Therefore, reconstituted P450 3A4 was exposed to NADPH and [$^{14}$C]-L-754,394, subjected to CNBr digest, and the resulting peptides were separated by Tricine SDS−PAGE (Figure 5). TFA was used in the CNBr chemical cleavage reactions instead of formic acid because of the ability of the latter acid to formylate amino acid residues (*35*, *36*). After solubilization of 2 mm gel slices, a radioactive fraction was observed at a migration distance that occurred between the 3.5 and 6.2 kDa molecular mass markers. Approximately 50% of the total amount of radiolabel associated with the protein was lost under the conditions of Tricine SDS-PAGE, which is in agreement with the results obtained using SDS-PAGE and the intact protein (Figure 2). Similarly, under the conditions used for POROS HPLC separation of the reconstituted proteins, approximately 50% of the radiolabel associated with the apoprotein of P450 3A4 is hydrolyzed to a product that has a retention time (5 min) expected for the L-754,394 dihydrodiol (Figure 6). For this reason, POROS HPLC was not used initially to separate P450 3A4 from the reconstituted enzyme mixture prior to CNBr cleavage and Tricine SDS-PAGE analysis.

In a subsequent experiment, reconstituted P450 3A4 that had been covalently modified by [$^{14}$C]-L-754,394 was first separated from the reconstituted mixture by POROS HPLC prior to CNBr digestion despite the loss of radiolabel inherent in the process. A CNBr digest time of 10 h was found to minimize subsequent loss (approximately 50%) of radiolabel as determined by POROS HPLC-radiometric analysis of the amount of radiolabeled peptide present in the resultant peptide mixture. Loss of radiolabel at this stage was most likely due to hydrolysis of the reactive intermediate of L-754,394 from the protein under the acidic conditions of the cleavage reaction. One major radiolabeled peptide fraction with a retention time of approximately 17.5 min was observed upon POROS HPLC separation of the CNBr

[2] Lightning, L. K., and Trager, W. F., unpublished observations.

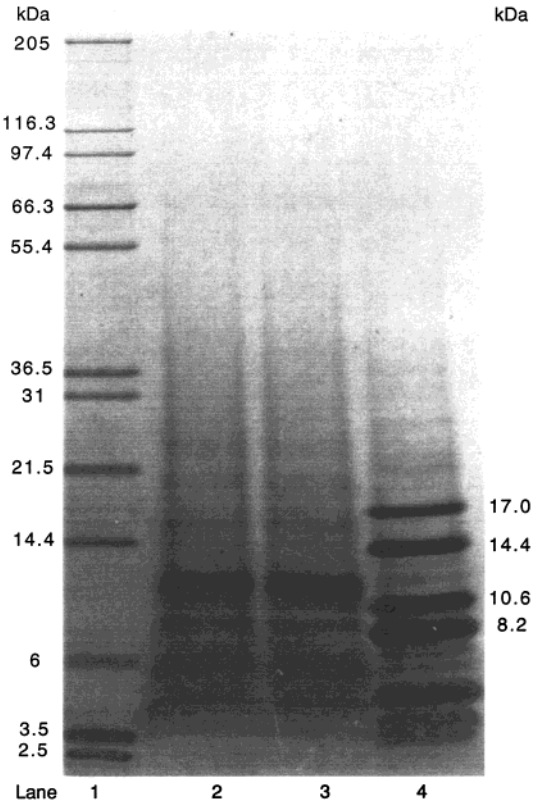


FIGURE 5: Separation of CNBr-generated peptides of P450 3A4 by Tricine SDS-PAGE. Tricine SDS-PAGE was used to separate CNBr generated peptides of reconstituted P450 3A4 that had been exposed to [$^{14}$C]-L-754,394 in the absence (Lane 2) or presence (Lane 3) of NADPH. Lanes 1 and 4 contain the MM markers. The gel was sliced into sections and each section was extracted and analyzed for radioactivity by liquid scintillation counting. The radioactivity was observed to be associated with a peptide that migrated between 3.5 and 6 kDa.

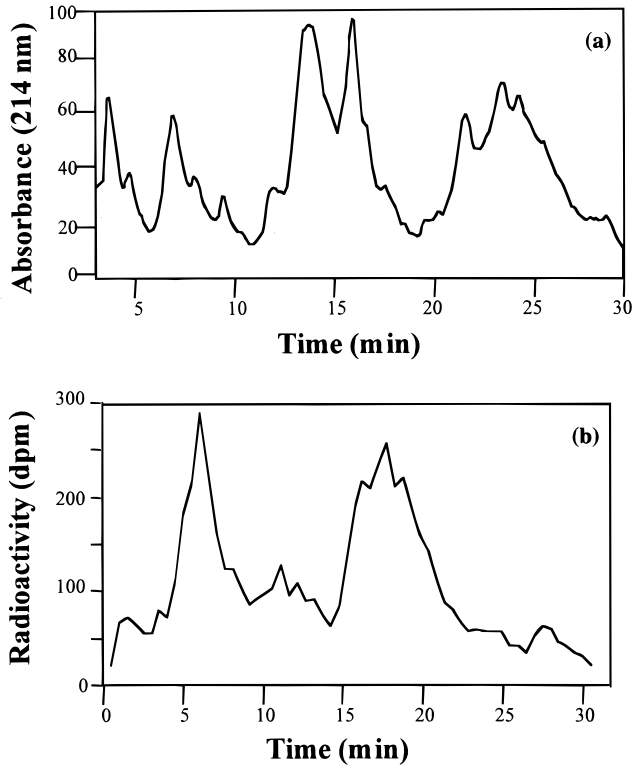


FIGURE 6: Separation of CNBr-generated peptides of P450 3A4 by POROS HPLC.The CNBr generated peptide fragments from HPLC purified P450 3A4 that had been exposed to [$^{14}$C]-L-754,-394 and NADPH were separated by POROS HPLC and their elution was monitored by (a) variable wavelength detection at 214 nm and (b) radiometric detection. L-754,394 dihydrodiol eluted at 5 min and a radiolabeled peptide eluted at 17 min. The peptide containing the major portion of the radioactivity was collected, lyophilized, and subjected to MALDI-MS (see Figure 7).

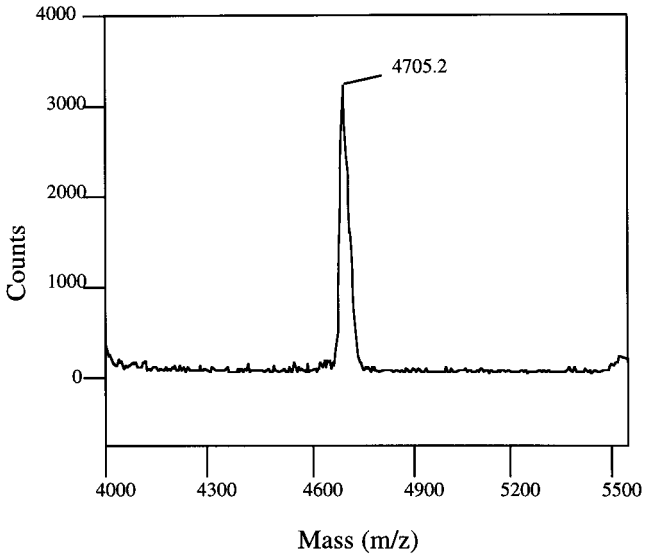


FIGURE 7: MALDI mass spectrum of the POROS HPLC purified peptide. Based on the known sequence of P450 3A4 and the predicted CNBr fragments, the labeled peptide was determined to be composed of residues I257-X317. Sequence homology with P450 enzymes of known crystal structure the I-helix (SRS-4) of P450 3A4 within this peptide.

generated fragments followed by liquid scintillation counting. The fractions containing radioactivity were pooled, lyophilized to near dryness, and subjected to MALDI-MS.

The mass spectrum resulting from MALDI revealed the presence of one major peak with an $[M+H]^+$ at a *m*/*z* 4705.2 ± 0.9 (Figure 7). The theoretical $[M+H]^+$ for the average mass of the I257-X317 peptide (where X denotes the homoserine lactone that would be formed after CNBr cleavage at methionine residues under acidic conditions) from P450 3A4 is 4703.2. Even if only incomplete cleavage of P450 3A4 had occurred, there are no other peptides that could produce this $[M+H]^+$ ion after CNBr cleavage. The theoretical average mass for the other peptides which could produce $[M+H]^+$ ions nearest the value observed in the MADLI mass spectrum are 4600.2 and 4937.0 for Y318-X357 and E353-X394, respectively. It is important to note that a peak corresponding to the [$^{14}$C]-L-754,394 adducted I257-X317 peptide was not detected by mass spectrometry. This result is not surprising given the instability of the radiolabel and the extensive procedures that were necessary in order to isolate the peptide for mass spectral analysis.

*Homology Model of L-754,394-Bound P450 3A4.* Docking of L-754,394 into a homology model for P450 3A4 by positioning the furan ring nearest the heme followed by molecular dynamics simulations indicated several contact regions between the substrate and enzyme (Figure 8), including the I helix (area shown in red) and the B′-helix, F-helix, beta 5 turn, and beta 4 sheet regions (areas shown in blue).

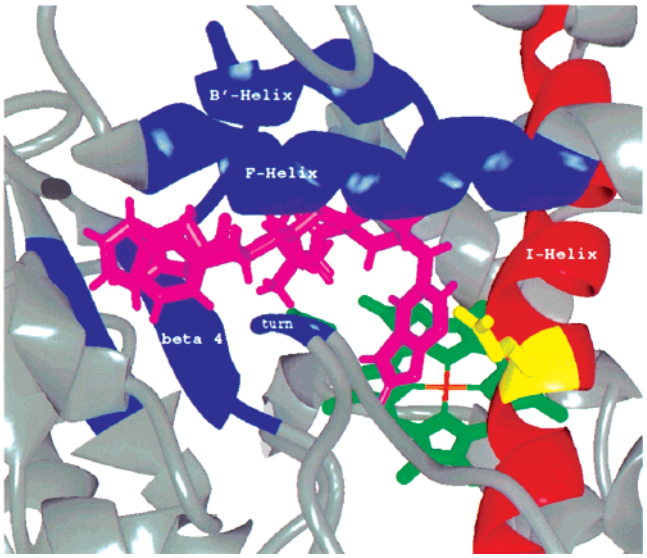


FIGURE 8: Computer-generated diagram of L-754,394 bound to the active site of P450 3A4 based on homology modeling using the known crystal structures of P450s CAM, BM3, and EryF. The heme is shown in green, the substrate in magenta, the I-helix in red, Glu-307 in yellow, and other substrate contact regions in blue.

## DISCUSSION

Previous investigations established that human liver microsomal P450 3A4 exhibits a relatively high affinity ($K_I$ = 7.5 $\mu$M) for L-754,394. It was also demonstrated that P450 3A4 could convert L-754,394 to a species that is a highly efficient ($k_{inact}$ = 1.62 $min^{-1}$ and partition ratio = 1.35) inactivator of the enzyme (*17*). In accord with these earlier findings, P450 3A4-HT membranes prepared from *Escherichia coli* bacterial cultures, were found to oxidize L-754,394 to a product(s) that leads to covalent modification of P450 3A4-HT (Table 1). The inactivation of P450 3A4 catalyzed by L-754,394 and NADPH resulted in loss of the characteristic reduced CO complex; however, the same amount of complex was lost in the absence of L-754,394. Our result using expressed P450 3A4 differs from earlier reports using liver microsomes (*17*, *18*) and is probably due to the inactivation of other P450s (possibly P450 2D6 in the case of human liver microsomes (*17*)) present in the previous in vitro experiments. Extensive dialysis, or SDS-PAGE separation of the P450 3A4-HT membrane proteins after incubation with [$^{14}$C]-L-754,394 and NADPH, revealed a binding stoichiometry of 1.08 ± 0.05 or 0.59 ± 0.09, respectively, between [$^{14}$C]-L-754,394 and P450 3A4-HT as determined by liquid scintillation counting (Figure 2). This suggests that approximately 40% of radiolabel associated with covalently modified P450 3A4 was unstable to the denaturing conditions of SDS-PAGE.

A significant portion of radioactivity was associated with the dye front of the gel relative to the minus NADPH control. Since no other portions of the gel were associated with radioactivity, it seems unlikely that the radioactivity migrating at the dye front was due to selective covalent modification of a low MM protein. Likely possibilities for the radioactive species migrating at the dye front include: (a) L-754,394 dihydrodiol (MM = 688 Da) formed as a result of hydrolysis of the bond between the activated form of L-754,394 and a peptide of P450 3A4 and/or (b) the heme of P450 3A4 that has been covalently modified by the

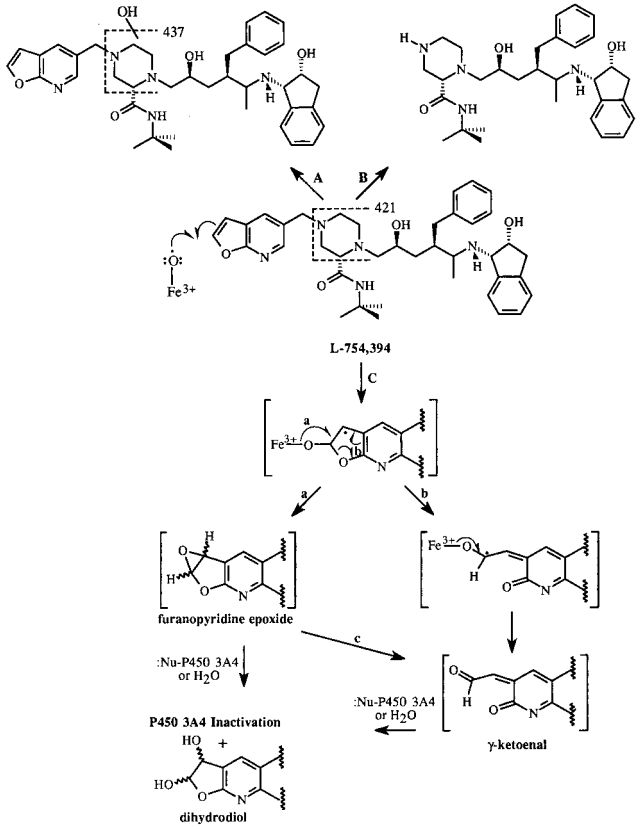


FIGURE 9: Postulated scheme for the mechanism-based inactivation of P450 3A4 by L-754,394 and for the formation of metabolites from L-754,394 by P540 3A4. MS/MS studies indicated that L-754,394 was monohydroxylated by P450 3A4 at a site other than the furanopyridine ring and 1,1-dimethylethylaminocarbonyl group. Production of the monohydroxylated, N-dealkylated, and dihydrodiol metabolite accounted for >90%, <5%, and <5%, respectively, of the total L-754,394 consumed.

reactive species of L-754,394 (MM approximately 1300 Da). The latter possibility could be discounted because POROS HPLC-radiometric analysis of the reconstituted P450 3A4 enzyme mixture after exposure to [$^{14}$C]-L-754,394, revealed no radioactivity associated with the heme of P450 3A4 (Figure 4). Thus, we conclude that the radioactive species migrating at the dye front is the L-754,394 dihydrodiol.

One major and several minor metabolites of L-754,394 were formed after incubation with P450 3A4-HT membranes. The minor metabolites account for the difference between the partition ratio values based on major metabolite formation (3.9 ± 0.4) and total substrate depletion (4.3 ± 0.4). The major metabolite possessed an $[M+H]^+$ ion at 670.1 corresponding to a monohydroxylated product (Figure 9, path A). Although the exact site of monohydroxylation could not be identified, it seems most likely, based on CID studies, that the metabolite is formed as a result of benzylic hydroxylation. In earlier studies of the metabolism of L-754,394, it was necessary to use large quantities (10 nmol) of monkey liver microsomes in order to obtain mass spectral information on the L-754,394 dihydrodiol and the conjugates formed with exogenous nucleophiles (e.g. GSH, NAC, and MOA) (*37*). Similarly, we found it necessary to use a large amount of human P450 3A4-HT (50 nmol) in order to detect the dihydrodiol metabolite of L-754,394 ($[M+H]^+$ = 688.1) (Figure 9, path C) by HPLC/ESI-MS. No conjugate formation was observed by radiometric detection or HPLC/ESI-

MS analysis when GSH, NAC, and MOA were included in the incubations with P450 3A4-HT and, similar to previous experiments with P450 2A6 (*29*), all were found to decrease the activity of P450 3A4-HT (Table 1). In addition, no conjugate formation was observed when the reconstituted baculovirus expressed P450 3A4 was used. These results demonstrate that very little (if any) of the reactive species of L-754,394 escapes the active site of P450 3A4.

The formation of dihydrodiols as an alternate pathway to P450 inactivation has been noted in studies of the furanocoumarin mediated inactivation of P450s 2A6 (*29*) and 2B1 (*38*). These metabolites could arise from L-754,394 via P450 3A4 mediated (a) furan epoxidation (Figure 9, path C, a), (b) ring opening to a $\gamma$-ketoenal intermediate (Figure 9, path C, b), and/or (c) furan epoxidation followed by ring opening to the $\gamma$-ketoenal intermediate (Figure 9, path C, c). In previous studies, $H_2{}^{18}O$ experiments were critical for establishing the identity of the reactive intermediate formed prior to formation of the dihydrodiol and, presumably, protein adduct. However, due to the large amount of P450 3A4-HT required to form enough L-754,394 dihydrodiol for HPLC/ESI-MS analysis and the limited supply of L-754,394, these experiments were not performed. Thus, a mechanism of inactivation that involves formation of the furanoepoxide and/or $\gamma$-ketoenal must be invoked (Figure 9, path C).

Because of the instability of the L-754,394 adduct of P450 3A4-HT to the conditions necessary for its purification from the bacterial membrane preparations and our inability to generate functional P450 3A4-HT in a reconstituted system another approach was needed. In previous investigations, we had been successful in reconstituting other P450s expressed using a baculovirus/insect cell system (*20*, *29*, *38*). Therefore, baculovirus expressed P450 3A4 was purified from the insect cell membranes and used in reconstitution experiments. By varying the components of the reconstituted system, P450 3A4 activity was optimized and, interestingly, an absolute requirement for human microsomal lipid was observed (Table 2). The metabolic profile of the reconstituted baculovirus P450 3A4 matched that of the P450 3A4-HT membrane preparations. However, in contrast to the P450 3A4-HT system a 1:1 binding stoichiometry between P450 3A4 and [$^{14}$C]-L-754,394 was not observed. The reason for the relatively low binding stoichiometry of 0.4:1 that was observed may be due to a nonoptimal reconstituted enzyme system. This could be explained in several ways: (a) two pools of P450 3A4 could be present, one that is catalytically active and one that is not. The catalytically inactive fraction may not be able to accept electrons because of lack of functional access to P450 reductase and/or $b_5$. If true, it may be an unavoidable consequence of using the reconstituted enzyme system, since several different components of the reconstituted system were varied to enhance P450 3A4 activity. (b) Nonionic detergents commonly used in the purification of P450 and its redox partners are known to be oxidized by P450 3A4 (*39*). Therefore, any residual detergent present in the reconstituted P450 3A4 mixture would be expected to inhibit L-754,394 metabolism. (c) P450 3A4 catalyzed metabolism of L-754,394 competes with "uncoupling pathways" such as generation of $H_2O_2$, superoxide anion, and $H_2O$ in the normal catalytic cycle as well as simple thermal inactivation (30 °C) of the proteins. Most microsomal P450s are poorly coupled enzymes that produce large amounts of $H_2O_2$ and $H_2O$ relative to oxidized substrate. Superoxide anion, $H_2O_2$, or $H_2O$ formation occurs by decomposition of the ferrous-dioxygen complex, ferric-peroxy intermediate, or ferryl-oxo species, respectively (*40*−*44*). These uncoupling processes could cause inactivation of the P450 by reacting with the heme or by decreasing the number of productive catalytic events (in this case, oxidation of L-754,394) during the lifetime of the enzyme at a given incubation temperature. Indeed, as judged by the decrease in the amount of reduced P450 that could bind CO, approximately 50% of the initial P450 was lost upon exposure to the NADPH generating system, presumably due to these abortive events. It seems likely that a combination of events (a), (b), and/or (c) is responsible for the low binding stoichiometry observed.

Covalently modified P450 3A4 was subjected to chemical and enzymatic digestion followed by Tricine SDS-PAGE and MALDI mass spectral analysis with the hope of identifying the peptide and the specific amino acid residue that had been adducted. Tricine SDS-PAGE separation of the peptides generated after CNBr cleavage followed by liquid scintillation counting of gel slices (Figure 5) allowed us to estimate the MM of the radiolabeled peptide. To avoid contamination from the other proteins in the reconstituted mixture, it was necessary to separate radiolabeled P450 3A4 from the other components of the reconstituted mixture prior to chemical digestion and mass spectral analysis. This type of analysis provided a much more accurate measurement of the MM of the modified peptide (Figure 7) than did Tricine SDS-PAGE. Based on the results from these experiments, it was concluded that the modified peptide was composed of residues I257-X317. This region encompasses the predicted locations of substrate recognition site 4 (SRS-4) and the I helix for P450 3A4 based on homology modeling with $P450_{CAM}$, P450 BM3, $P450_{TERP}$, and $P450_{EryF}$ (Figure 8, area shown in red). Substrate recognition sites are believed to be in close proximity to the substrate binding pocket (*45*). In particular, the highly conserved I-helix is commonly found to be the distal helix that (along with the proximal L helix) sandwiches the heme moiety in computer generated models and sequence alignments (*46*, *47*). Docking of progesterone into a P450 3A4 homology model in an orientation favorable for oxidation at the 6$\beta$-position also demonstrated that residues in the I-helix can participate in binding interactions with this substrate (*47*). Moreover, recent studies have demonstrated that mutation of P450 3A4 I-helix residues can affect the regiospecificity of progesterone hydroxylation and the stimulatory effect of α-naphthaflavone (*48*). Interestingly, the I-helix of other P450s has been identified as the site of adduction for several different MBIs (*31*−*33*, *49*, *50*). Other regions of contact between L-754,394 and the active site of P450 3A4 could be identified including: the B′-helix, F-helix, beta 5 turn, and beta 4 sheet (Figure 8, area shown in blue).

Although generation of the reactive intermediate from L-754,394 results in a nonreversible (not hydrolyzed by extensive dialysis) inactivation and covalent modification of P450 3A4-HT, the presumed covalently adducted active site peptide is unstable to the conditions that were used for its purification. The instability of the peptide adduct to the conditions of POROS HPLC, CNBr digest, and SDS-PAGE precluded detection of an $[M+H]^+$ ion corresponding to the

adducted peptide in the MALDI mass spectrum. Detection of the proposed modification by ESI-MS operating in tandem with the POROS HPLC also failed. This might be expected since only 40% of the reconstituted P450 3A4 is labeled after exposure to [$^{14}$C]-L-754,394 and NADPH, while approximately 50% of the radiolabel is lost at each step in the generation of the CNBr fragments. Thus, at best only 5−10% of the P450 3A4 present in the initial incubation would remain adducted by the time the sample was subjected to MALDI-MS. In contrast, GSH, NAC, MOA conjugates of the reactive intermediate formed from furanocoumarins or L-754,394, in addition to a 5-hydroxypsoralen/8-methoxypsoralen heterodimer, were all stable to acidic conditions and both long term (> 2 weeks) storage at 4 °C and short-term storage for (2−3 days) at room temperature (*29*, *38*). The stability of the GSH, NAC, and MOA conjugates and heterodimer suggests that bonds formed between the reactive intermediate of L-754,394 and Ser, Cys, Thr, and/or Lys residues should be covalent and resistant to hydrolysis. Taken together, these findings suggest that covalent modification of P450 3A4 by L-754,394 most likely occurs at an Asp or Glu residue(s). Covalent modification of these residues by the putative furanoepoxide and/or $\gamma$-ketoenal intermediate would result in formation of an ester bond, which would be expected to be susceptible to hydrolysis. It seems likely that an ester linkage would be stable as long as it is buried within the hydrophobic active site of P450 3A4. However, once the protein is denatured or begins to unfold and the label becomes exposed to an acidic aqueous environment, it is susceptible to rapid hydrolysis.

Consideration of the P450 3A4 homology model derived from the known three-dimensional structures of the bacterial P450 enzymes $P450_{CAM}$, P450 BM3, and $P450_{EryF}$ leads to the tentative identification of Glu307 as the likely site of covalent adduction. While the primary sequence of P450 3A4 has 25 aspartate and 30 glutamates, the homology model predicts that only Asp213, Asp216, Glu373, and Glu307 are in potential substrate recognition sites. Asp213 and Asp216 are on the surface of the protein and are probably solvated, whereas Glu373 is relatively far removed from the iron of the heme. The remaining amino acid, Glu307 is in the I-helix, (Figure 8, residue shown in yellow) and positioned to react with the activated species of L-754,394 almost as soon as it is formed.

In summary, we have tentatively identified an active site residue of human liver P450 3A4 using the MBI L-754,394 and a variety of analytical techniques. As demonstrated by the results from SDS-PAGE and POROS HPLC experiments, the adduct between the reactive species of L-754,394 and P450 3A4 was extremely labile and, therefore, it was necessary to use several different experimental approaches. These observations, in combination with mass spectral analysis of the peptide adduct, homology modeling of L-754,-394 bound P450 3A4, and prior knowledge of the stability of various N-, O-, and S-linked conjugates of activated furans, strongly suggests that the residue of P450 3A4 adducted by L-754,394 is Glu307.

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