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Mechanism-Based Inactivation of Cytochrome P450 3A4 by Mibefradil through Heme Destruction[S]

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ABSTRACT:

Mibefradil (Posicor) was developed as a calcium channel blocker for the treatment of chronic hypertension. The compound was withdrawn from the market in 1998 because of the potential for rhabdomyolysis, renal failure, or bradycardia when it was coadministered with other drugs. Mibefradil has previously been shown to be a potent reversible (IC_{50} = 0.3–2 μM) and mechanism-based (K_i = 2.3 μM; k_{inact} = 0.4 min^{-1}) inhibitor of CYP3A4-catalyzed statin metabolism. At present, the mechanism of CYP3A4 inactivation by mibefradil is not known. Mechanism-based inactivation experiments and spectral studies were used to examine the mechanism of CYP3A4 inactivation by mibefradil and its major metabolite, des-methoxyacetyl mibefradil (Ro 40-5966), in vitro. Both mibefradil and Ro 40-5966 were shown to exhibit type I binding characteristics (K_s = 0.69 ± 0.06 and 1.39 ± 0.04 μM, respectively) toward CYP3A4. Complete K_i/k_{inact} experiments were performed, revealing a rapid and irreversible decrease in CYP3A4-catalyzed 1′-hydroxymidazolam formation. Approximately 70% of CYP3A4 activity was lost in the first minute of incubation with mibefradil, and inactivation was nonlinear after 2 min. Ro 40-5966 also resulted in time-dependent inhibition of CYP3A4, albeit to a lesser extent than mibefradil. The decrease in CYP3A4 activity in the presence of mibefradil and NADPH was subsequently shown to have a good correlation with the time-dependent loss of CO binding, which, coupled with the lack of stable heme and/or apoprotein adducts, suggests heme destruction as the mechanism of inactivation of CYP3A4 by mibefradil.

Introduction

Mibefradil (Posicor) was introduced as a calcium-channel blocker for the treatment of hypertension and chronic angina (Veronese et al., 2003). The drug is cleared primarily through hepatic metabolism with the major metabolic routes including N-dealkylation, hydroxylation on the benzimidazole moiety, and cleavage of the ester side chain (Wiltshire et al., 1997a,b). Mibefradil was voluntarily withdrawn from the market in 1998 because of the potential for rhabdomyolysis, renal failure, or bradycardia when it was coadministered with other drugs (Paoletti et al., 2002). The measured plasma concentrations of drugs such as simvastatin, tacrolimus, cyclosporine, and digoxin exhibited a marked increase when coadministered with mibefradil, because of inhibition of either CYP3A or the cellular efflux transporter P-glycoprotein by mibefradil (Zhou et al., 2005). After withdrawal from the market, mibefradil was shown to be a potent mechanism-based inactivator of CYP3A4 (Prueksaritanont et al., 1999).

A number of important criteria exist for a compound to be classified as a mechanism-based inactivator of an enzyme (Silverman, 1988). More recently, the criteria have been modified to accommodate some of the unique features associated with the cytochromes P450 (P450), a superfamily of heme-containing enzymes capable of catalyzing the oxidation of a wide range of substrates. Simply stated, a P450 mechanism-based inactivator is any compound that is metabolically altered by the enzyme to a reactive intermediate that results in inactivation of the enzyme before leaving the active site (Hollenberg et al., 2008). Possible mechanisms of mechanism-based inactivation are through formation of a metabolite-intermediate complex, covalent modification of the protein, or modification of the heme prosthetic group (alkylation or bleaching). Well documented examples of P450 mechanism-based inactivators include raloxifene, troleandomycin, 1-aminobenzotriazole, and mifepristone (Ortiz de Montellano and Mathews, 1981; Lindstrom et al., 1993; Khan et al., 2002; Baer et al., 2007).

The aim of this current work was to extend previous time-dependent inactivation studies with mibefradil to elucidate a potential mechanism responsible for the observed CYP3A4 inactivation. In vitro experiments designed to determine whether inactivation of CYP3A4 proceeds through protein adduction or heme modification were performed. Trapping reagents were used in an attempt to determine the reactive intermediate responsible for the inactivation of CYP3A4. Finally, liquid chromatography-UV/mass spectrometry detection was used to probe for the formation of an isolable heme adduct and/or concurrent loss of intact heme.

Materials and Methods

Materials. Mibefradil, midazolam, tolbutamide, glutathione, potassium cyanide, methyl hydroxylamine, superoxide dismutase, catalase, dimethyl pyrroline *N*-oxide, magnesium chloride, sodium sulfate, acetonitrile, and methylene chloride were obtained from Sigma-Aldrich (St. Louis, MO). NADPH,

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ABBREVIATIONS: P450, cytochrome P450; Ro 40-5966, des-methoxyacetyl mibefradil; LC, liquid chromatography; MS, mass spectrometry; HPLC, high-performance liquid chromatography; amu, atomic mass units; NNC55-0396, (1*S*,2*S*)-2-(2-(*N*-[(3-benzimidazol-2-yl)propyl]-*N*-methylamino)ethyl)-6-fluoro-1,2,3,4-tetrahydro-1-isopropyl-2-naphthyl cyclopropanecarboxylate dihydrochloride; MI, metabolite-intermediate.

sodium dithionite, *N*-acetyl cysteine and *N*-acetyl lysine were purchased from Calbiochem (San Diego, CA). Slide-A-Lyzer mini-dialysis units were from Thermo Fisher Scientific (Waltham, MA). d_8-Protoporphyrin-IX was purchased from Frontier Scientific (Logan, UT). CYP3A4 Baculosomes were obtained from Invitrogen (Carlsbad, CA). Purified CYP3A4 was a generous gift from Dr. William Atkins (University of Washington, Seattle, WA).

Synthesis of Ro 40-5966 (Des-methoxyacetyl Mibefradil). The synthesis of the des-methoxyacetyl metabolite of mibefradil was performed as described previously (Bui et al., 2008; Wu et al., 2008). In brief, 100 mg of mibefradil was dissolved in ethanol-1 N NaOH (50:50) and stirred at room temperature for 4 h. Complete hydrolysis of the ester bond was confirmed by thin-layer chromatography and LC-UV/MS comparison to a mibefradil standard. After completion, the solvents were evaporated off under nitrogen, leaving a clear syrup residue that was reconstituted in CH_2Cl_2-water (50:50). The water layer was extracted two additional times with CH_2Cl_2, and the combined organic layers were dried over Na_2SO_4.

Time-Dependent Inhibition Assays. Both mibefradil and Ro 40-5966 were screened for time-dependent inhibition in CYP3A4 Baculosomes at 10 μM. In brief, the test compounds were preincubated with 5 pmol of CYP3A4, 3 mM $MgCl_2$, and 1 mM NADPH in 100 mM potassium phosphate buffer (pH 7.4) for 0, 1.5, 5, 10, and 15 min at 37°C. After the preincubation period, a 20-fold dilution was made into a secondary assay containing 25 μM midazolam, 3 mM $MgCl_2$, and 1 mM NADPH in potassium phosphate buffer. Secondary incubations were quenched after 5 min at 37°C with 2 volumes of acetonitrile containing 0.1 μM tolbutamide as an internal standard. Assays to determine the inactivator concentration at half the maximum inactivation rate (K_i) and inactivation rate constant (k_{inact}) parameters for mibefradil and Ro 40-5966 (0–8 μM) were performed in CYP3A4 Baculosomes (5 pmol, primary incubation). To assess the potential for rapid time-dependent inactivation in vitro, aliquots from the primary incubation were removed every 30 s and transferred to a secondary incubation containing midazolam (25 μM, final concentration). In addition, a more standard time-dependent inactivation protocol in which aliquots were removed at 2.5, 5, 10, and 15 min after the addition of NADPH (1 mM, final concentration) to the primary incubation was performed. Secondary incubations (20-fold dilution) were allowed to proceed for 5 min at 37°C and stopped with 2 volumes (v/v) of acetonitrile containing 0.1 μM tolbutamide as an internal standard. K_i and k_{inact} values were estimated by fitting the slope of the linear portion of each inhibitor concentration to a single binding site nonlinear regression in GraphPad Prism (version 5.01; GraphPad Software Inc., San Diego, CA).

Effect of Trapping Reagents. In an attempt to identify potential reactive intermediates involved in the inactivation of CYP3A4 by mibefradil, time-dependent inactivation experiments were repeated in the presence of various nucleophilic trapping reagents and reactive oxygen scavengers. The effect of glutathione, semicarbazide, potassium cyanide, methyl hydroxylamine, *N*-acetyl cysteine, *N*-acetyl lysine, superoxide dismutase, catalase, and dimethyl pyrroline *N*-oxide at 1 mM (final concentration) on the inactivation of CYP3A4 by mibefradil was assessed using a similar protocol to that previously described for mibefradil time-dependent inactivation experiments above.

Spectral Studies. Binding affinity studies using purified CYP3A4 together with metabolite-intermediate complex formation and carbon monoxide binding experiments in Baculosomes were conducted in an attempt to further elucidate the mechanism of inactivation. Binding spectra of mibefradil and Ro 40-5966 in purified CYP3A4 (1 μM, final concentration) were obtained using the difference spectra at substrate concentrations of 0 to 5 μM. To further confirm the binding mode of mibefradil, an absolute spectrum was recorded at substrate concentrations from 0 to 50 μM. Assessment of metabolite-intermediate complex formation was performed using 10 μM mibefradil (or 10 μM verapamil as a positive control) from 0 to 45 min. For CO binding experiments, sodium dithionite was added to samples containing CYP3A4 Baculosomes, buffer, and the appropriate amount of mibefradil or Ro 40-5966 (plus or minus NADPH). After a baseline scan was obtained, CO was bubbled through the sample cuvette for approximately 30 s. The sample was allowed to sit for 1 min before the UV spectrum from 400 to 500 nm was recorded. Dialysis procedures were performed using the Slide-A-Lyzer mini-dialysis unit as instructed by the manufacturer, except that dialysis was performed against 1 liter of potassium phosphate buffer (100 mM, pH 7.4).

Time-Dependent Loss of CO Binding/Enzyme Activity. To further elucidate the mechanism of CYP3A4 inactivation by mibefradil, the time-dependent loss of CO binding was compared with the loss of enzyme activity over a 5-min incubation. The primary incubation contained 1 pmol of CYP3A4, 3 mM $MgCl_2$, 100 mM potassium phosphate buffer (pH 7.4), and either mibefradil (10 μM), raloxifene (10 μM), or 1-aminobenzotriazole (50 μM). Aliquots were removed at 0, 1, 2, 3, 4, and 5 min after initiation with 1 mM NADPH and either measured for CO binding as described above or transferred to a secondary incubation containing midazolam (25 μM), 1 mM NADPH, 3 mM $MgCl_2$, and 100 mM potassium phosphate buffer. Secondary incubations were quenched after 5 min in a fashion similar to that for the time-dependent inhibition experiments described above and were subject to LC-tandem mass spectrometry analysis.

Assessment of Protein Adduct Formation. Purified CYP3A4, NADPH-P450 reductase, and cytochrome b_5 (1:2:1) were combined with 100 μg/ml CHAPS, 20 μg/ml lipids (dilauroylphosphatidylcholine, dioleoylphosphatidylcholine, and dilauroylphosphatidylserine, 1:1:1), 3 mM glutathione, 50 mM HEPES buffer (pH 7.4), and 30 mM magnesium chloride. Mibefradil (10 μM) was added, and the incubation was allowed to proceed at 37°C for 3 min before the addition of NADPH (1 mM, final concentration). Then the incubation was allowed to proceed for 5 min, at which point an aliquot was transferred to a Microcon centrifugal filter tube (30-kDa filter) and centrifuged for 20 min at 10,000 rpm. The resulting filtrate was analyzed as described below.

In brief, centrifugal filtrates from samples designed to assess the formation of CYP3A4 apoprotein adducts were subjected to mass spectrometry analysis using a LTQ Orbitrap Velos mass spectrometer (Thermo Fisher Scientific) coupled to an Accela 1250 HPLC system with a Leap CTC PAL autosampler (Leap Technologies, Carrboro, NC). Chromatographic separation was achieved on a Poros R2 column as described previously (Baer et al., 2007). LTQ Orbitrap parameters were set as follows: source temperature, 300°C; sheath gas, 50 arbitrary units; auxiliary gas, 5 arbitrary units; spray voltage, 4.2 kV; capillary temperature, 335°C; S-lens radiofrequency, 47%; resolution, 15,000; ion trap and Orbitrap maximum injection time, 500 ms; *m/z* scan range, 650 to 1850 Da. Mass spectra were averaged over the entire width of the peak and deconvoluted using ProMass (Novatia, Monmouth Junction, NJ).

HPLC-UV/Mass Spectrometry Analysis of Intact Heme. After inactivation of CYP3A4 by mibefradil (10 μM) in Baculosomes as described above, reactions were quenched with 1 N HCl and extracted into 2 volumes of CH_2Cl_2. The organic layer was evaporated to dryness, and the remaining residue was reconstituted in acetonitrile before analysis. Determination of heme loss via HPLC-UV/MS was achieved on an Agilent 1200 HPLC system using a 5-μm Zorbax 300 SB-C18 column (2.1 × 150 mm) coupled to a Thermo Fisher Scientific LTQ ion trap mass spectrometer. The mobile phase system consisted of 0.1% trifluoroacetic acid in water and 0.1% trifluoroacetic acid in acetonitrile. Linear 30-min gradients were used, and heme was monitored by LC/MS (*m/z* 616 amu) as well as by UV absorption at 398 nm. Full Q1 scans were also used to detect formation of any potential heme adducts (*m/z* 100–2000 amu). Formation of a CYP3A4 heme adduct with 1-aminobenzotriazole was monitored as a positive control. d_8-Protoporphyrin-IX (*m/z* 584 amu) was added to all samples as an internal standard before extraction.

Results

Spectral Binding. To characterize the interaction of mibefradil and its des-methoxyacetyl analog (Fig. 1) with CYP3A4, initial time-dependent inhibition screens and spectral binding studies were conducted with each compound. Difference spectra for each compound with purified CYP3A4 indicated a type I binding interaction with the enzyme (mibefradil, Fig. 2A; Ro 40-5966, Fig. 2B). Data from each difference spectrum were fit to a hyperbolic kinetics model and resulted in observed K_s values of 0.69 ± 0.06 and 1.39 ± 0.04 μM for mibefradil and Ro 40-5966, respectively (Fig. 2C). An absolute binding spectrum obtained for mibefradil confirmed the type I binding observed in the difference spectrum (Supplemental Fig. 1).

Inactivation of CYP3A4 by Mibefradil and Ro 40-5966. Initial time-dependent inhibition experiments at 10 μM in recombinant

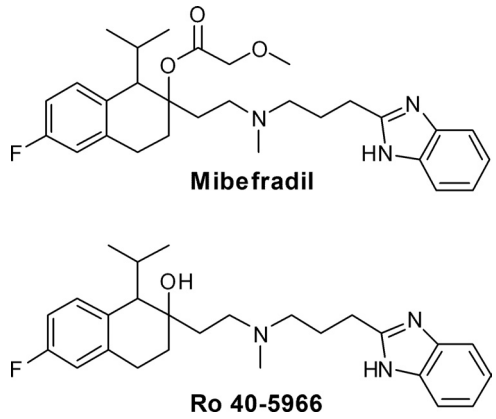

FIG. 1. Structures of mibefradil (top) and Ro 40-5966 (des-methoxyacetyl mibefradil) (bottom).

CYP3A4 indicated that mibefradil inactivated CYP3A4 more rapidly than Ro 40-5966. CYP3A4 was inactivated to less than 20% of control within the first 1.5 min by mibefradil (Fig. 3A). In contrast, approximately 60% activity remained after a 1.5-min preincubation with Ro 40-5966. Inactivation by both compounds deviated from linearity after approximately 2 min.

Additional kinetic assessment of the inactivation of CYP3A4 by mibefradil was performed by conducting K_i/k_{inact} experiments (Fig. 3B). Because of the rapid inactivation observed in the initial time-dependent inhibition assays, a protocol designed to fully capture the inactivation by mibefradil was used (initial preincubation for 30 s). After the rapid inactivation protocol, which included sampling of the primary incubation every 30 seconds, nonlinearity in the inactivation kinetics of mibefradil was observed after approximately 2 min for most of the concentrations of mibefradil tested. Using only the linear portion of the experimental data, a K_i of 0.28 μM was obtained with a corresponding k_{inact} of 0.59 min^{-1} (Fig. 3C). Inactivation parameters were also obtained for Ro 40-5966, although the observed inactivation was not nearly as rapid or complete as that observed for mibefradil. Inactivation experiments for Ro 40-5966 gave a K_i of 0.82 μM and a k_{inact} of 0.08 min^{-1}.

Effect of Nucleophilic Trapping Agents. To determine the possibility of an electrophilic reactive metabolite being involved in the inactivation of CYP3A4 by mibefradil, multiple nucleophilic trapping agents were used in subsequent time-dependent inactivation experiments. Inclusion of a final concentration of 1 mM glutathione or *N*-acetyl cysteine (hard electrophiles), *N*-acetyl lysine (soft electrophiles), semicarbazide or methyl hydroxylamine (aldehydes), potassium cyanide (imine-type intermediates), superoxide dismutase and catalase (reactive oxygen species), or dimethyl pyrroline *N*-oxide (radicals) in the incubations with CYP3A4 and mibefradil afforded no protection from inactivation by mibefradil (Fig. 4). In addition, no adducts of mibefradil with any of the nucleophilic trapping agents were observed.

Loss of CO Binding in the Presence of Mibefradil Plus NADPH. The ability of CYP3A4 to bind carbon monoxide upon preincubation with mibefradil was examined to gain a greater understanding of the mechanism of enzyme inactivation. After a 5-min preincubation of CYP3A4 with mibefradil and NADPH, a carbon monoxide binding spectrum of CYP3A4 was obtained. No CO binding was observed when CYP3A4 was incubated in the presence of mibefradil and NADPH (Fig. 5). Preincubation with Ro 40-5966 also afforded a loss of CO binding although, in concordance with results obtained from K_i/k_{inact} experiments, the loss was less dramatic than that observed with mibefradil. To determine the reversibility of mibefradil inactivation, dialysis against 1 liter of potassium phosphate buffer (100 mM, pH 7.4) was performed overnight. A reassessment of CO binding revealed no recovery after dialysis. Finally, to determine whether the inactivation of CYP3A4 by mibefradil was due to the formation of a metabolite-intermediate complex, the increase of absorbance at 455 nm was determined in the presence of mibefradil. Compared with the positive control verapamil, no increase in the absorbance at 455 nm was observed.

Correlation of Time-Dependent Loss of CO Binding and CYP3A4 Activity by Mibefradil. The correlation between the time-dependent loss of CO binding and CYP3A4 activity due to mibefradil was compared with that of raloxifene (CYP3A4 apoprotein adduct) and 1-aminobenzotriazole (heme adduct). No loss of CO binding occurred in the presence of raloxifene, although CYP3A4 activity decreased over time (Fig. 6A). The loss of CYP3A4 activity paralleled the loss of CO binding over time for 1-aminobenzotriazole (Fig. 6B). Preincubation of CYP3A4 with mibefradil resulted in a concurrent loss of enzyme activity and CO binding, as was observed for 1-aminobenzotriazole (Fig. 6C).

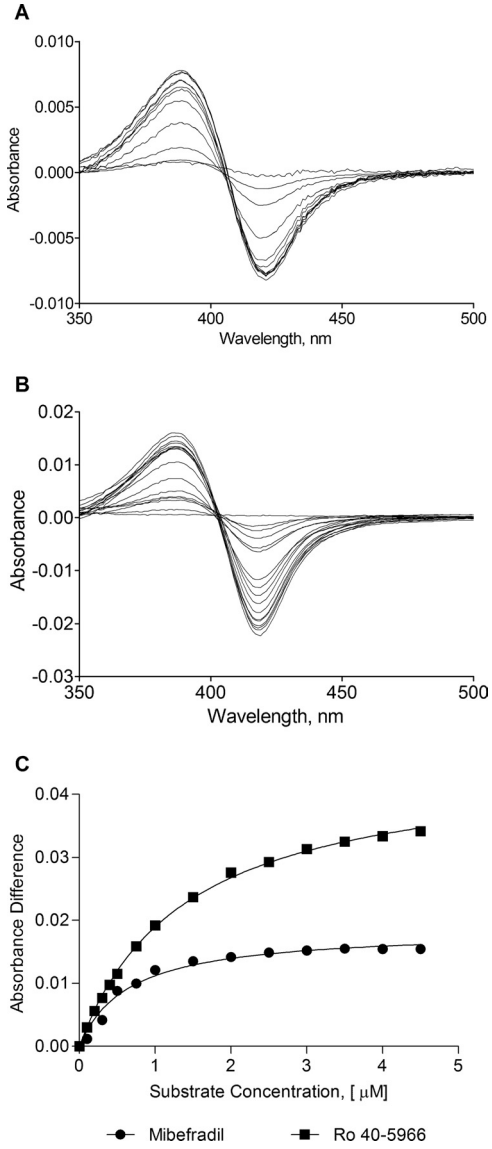

FIG. 2. Spectral binding affinity data for mibefradil (A) and Ro 40-5966 (B) with purified CYP3A4, indicating type I binding interactions with the enzyme. C, spectral binding affinity constants (K_s) of 0.69 and 1.39 μM for mibefradil and Ro 40-5966, respectively, were calculated using nonlinear regression analysis.

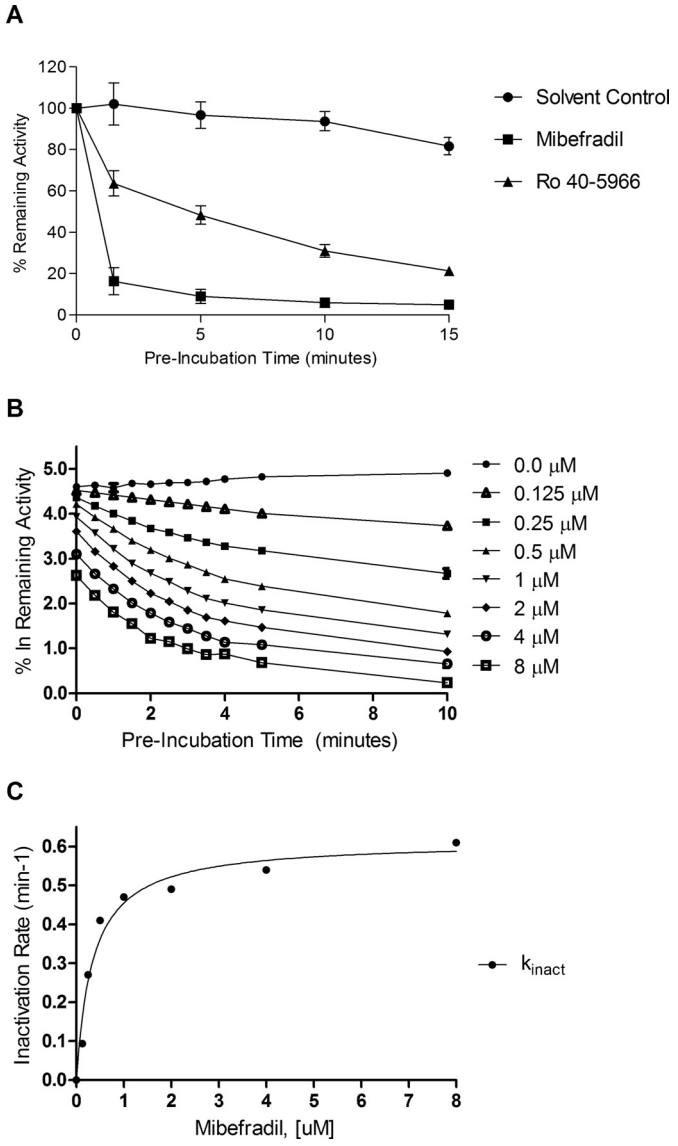

FIG. 3. A, time-dependent inhibition of CYP3A4 in the presence of NADPH and mibefradil or Ro 40-5966. The solvent control consisted of acetonitrile-dimethyl sulfoxide (9:1) and NADPH. B, K_i/k_{inact} experiment using rapid sampling from the primary incubation of mibefradil, CYP3A4, and NADPH. C, nonlinear regression analysis of time-dependent inhibition parameters for mibefradil (K_i = 0.28 μM; k_{inact} = 0.59 min^{-1}).

Assessment of Protein Adduct Formation by Mibefradil. The potential for mibefradil to cause time-dependent inactivation of CYP3A4 through the formation of an apoprotein adduct to CYP3A4 was examined using purified enzyme. Apoprotein adduction of raloxifene to Cys239 through diquinone methide formation was used as a positive control (Baer et al., 2007). Unlike incubations with CYP3A4 and raloxifene in which two ion envelopes were observed that upon deconvolution suggested an addition of 472 Da to the CYP3A4 apoprotein, no change to the CYP3A4 apoprotein was observed upon incubation with mibefradil and NADPH (Fig. 7).

Quantitative Measurement of Heme Loss. Finally, intact heme was extracted from incubations preincubated with and without mibefradil and directly measured by LC/UV/MS. Both preincubations were conducted in the presence of NADPH to account for any loss of heme in the control sample. Peak areas of intact heme (m/z = 616 amu; retention time 11.3 min) were compared with an internal standard (d_8-protoporphyrin IX: m/z = 584 amu; retention time 13.9 min).

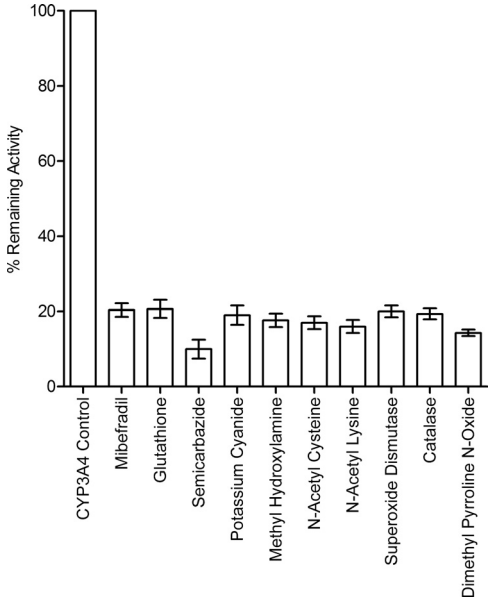

FIG. 4. Effect of various nucleophilic trapping agents on the inactivation of CYP3A4 by mibefradil. None of the trapping agents were able to block inactivation of CYP3A4 by mibefradil plus NADPH.

Preincubation with mibefradil and NADPH resulted in an approximate decrease of 70% in intact heme relative to the control incubation (Fig. 8). No isolable heme adducts were observed by UV and/or mass spectrometry. Preincubation of CYP3A4 with raloxifene and NADPH resulted in a negligible loss of intact heme, whereas inclusion of 1-aminobenzotriazole and NADPH as a positive control resulted in a 94% loss of measurable heme as well as the formation of the intact heme-benzyne adduct as indicated by the presence of a peak with m/z 692 (data not shown). The peak observed at 16.4 min was determined to be an impurity in the d_8-protoporphyrin IX standard.

Discussion

Mibefradil is a calcium channel blocker that was voluntarily withdrawn from the market in 1998 because of serious adverse events and potential drug interactions with HMG-CoA reductase inhibitors (Omar and Wilson, 2002; Williams and Feely, 2002). Subsequent in vitro metabolism studies revealed that mibefradil is a potent mechanism-based inactivator of CYP3A4 (Prueksaritanont et al., 1999). Mechanism-based inactivation of CYP3A4 has been observed for other calcium channel blockers, such as verapamil (Ma et al., 2000), which inhibit P450 activity through the formation of a metabolite-

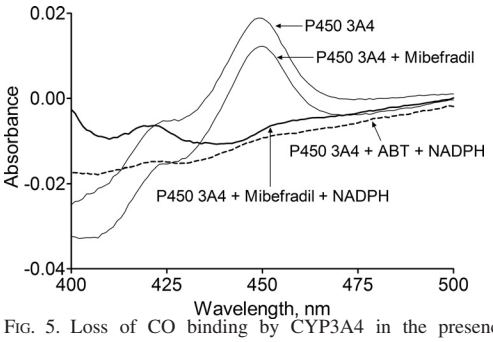

FIG. 5. Loss of CO binding by CYP3A4 in the presence of mibefradil plus NADPH, similar to the positive control, 1-aminobenzotriazole (ABT). Only a minimal decrease in CO binding was observed when NADPH was not included in the preincubation. No recovery of CO binding was observed when the sample was subject to dialysis for 12 h.

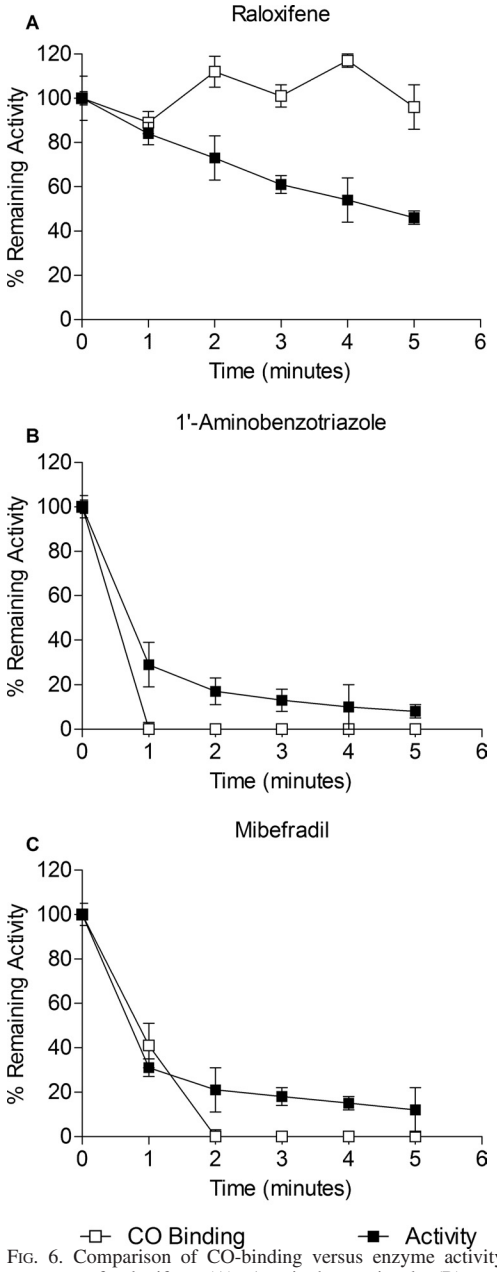

FIG. 6. Comparison of CO-binding versus enzyme activity for CYP3A4 in the presence of raloxifene (A), 1-aminobenzotriazole (B), and mibefradil (C). No correlation was observed for raloxifene (apoprotein adduct); however, loss of CO-binding trended with loss in enzyme activity for 1-aminobenzotriazole and mibefradil (heme modification).

intermediate complex. Early studies have shown that this is not the case for mibefradil, and the exact mechanism of CYP3A4 inactivation by mibefradil remains unclear. The goal of the present study was to elucidate the mechanism of CYP3A4 inactivation by mibefradil and determine the effects of various incubation parameters (preincubation time, trapping agents, and others) on the observed inactivation of CYP3A4.

With the use of an in vitro protocol designed to capture rapid time-dependent inactivation, it was observed that the majority of CYP3A4 inactivation by mibefradil occurs within the first 2 min of the incubation period, shortly after which the inactivation becomes nonlinear. The use of a rapid mechanism-based inactivation protocol was necessary because the application of a more standardized mechanism-based inactivation assay is not designed to capture this early deviation from linearity and can therefore result in underprediction of the potency of the inactivator (Ghanbari et al., 2006). Moreover, mechanistic protocols designed to capture the initial phases of time-dependent inactivation have been shown to often result in more potent K_i values (Yang et al., 2007), as is the case with mibefradil. As a result, using the current experimental conditions, the K_i for mibefradil decreased approximately 8-fold and the k_{inact} increased approximately 1.5-fold compared with previously reported values in human liver microsomes (Prueksaritanont et al., 1999). In addition, the observed k_{inact}/K_i ratio of 2143 ml $\cdot$ min^{-1} $\cdot$ μmol^{-1} under the current conditions suggests that CYP3A4 inactivation by mibefradil is approximately 12-fold more potent than reported previously, which places the compound among the most potent P450 inactivators observed to date (Obach et al., 2007).

Bui et al. (2008) investigated a hydrolysis-resistant analog of mibefradil [(1*S*,2*S*)-2-(2-(*N*-[(3-benzimidazol-2-yl)propyl]-*N*-methylamino)ethyl)-6-fluoro-1,2,3,4-tetrahydro-1-isopropyl-2-naphthyl cyclopropanecarboxylate dihydrochloride (NNC55-0396)], in which the methoxyacetyl side chain of mibefradil was replaced with a cyclopropylacetyl moiety and observed decreased time-dependent inhibition of CYP3A4. The previous study also demonstrated the inhibitory potential of Ro 40-5966 toward CYP3A4, although not with respect to time-dependent inhibition. In light of these results, we also conducted an initial time-dependent inhibition assessment of the major desmethoxy metabolite of mibefradil, Ro 40-5966. Our studies show that the hydrolysis product of mibefradil, Ro 40-5966, still results in time-dependent inhibition of CYP3A4, albeit to a lesser extent than mibefradil, which suggests that hydrolytic cleavage of the methoxyacetyl side chain of mibefradil is not the mechanism by which the compound inactivates CYP3A4. Furthermore, Ro 40-5966 binds in the same manner as mibefradil (type I) but with a lower affinity, indicating that the methoxyacetyl side chain may play a role in positioning mibefradil in an orientation in the active site of CYP3A4 that is highly conducive to rapid mechanism-based inactivation of the enzyme.

As mentioned previously, there are three primary routes of mechanism-based inactivation of P450 enzymes: through formation of a metabolite-intermediate complex with the heme iron, via covalent modification of the apoprotein, or through modification of the heme prosthetic group (e.g., alkylation or heme destruction) (Ernest et al., 2005; Blobaum, 2006). Based on the known sites of oxidation associated with CYP3A4 metabolism of mibefradil (Welker et al., 1998), multiple mechanisms of inactivation like the ones described above are possible. For instance, compounds containing tertiary amines are known to inactivate P450 enzymes through N-dealkylation to the secondary amine and subsequent MI complex formation, as observed for the calcium channel blocker diltiazem (Zhao et al., 2007). Although mibefradil does undergo N-dealkylation of the tertiary amine to a secondary amine, the lack of a concomitant increase in absorbance at 455 nm negates the possibility of MI complex formation being responsible for the observed inactivation of CYP3A4 by mibefradil.

Likewise, an alternative mechanism of inactivation can be proposed involving the tertiary amine whereby an apoprotein adduct is produced through formation of an iminium species, as has been noted for phencyclidine or nicotine (Murphy, 1973; Ward et al., 1982a,b). The lack of observable cyanide or *N*-acetyl cysteine adducts would seemingly rule out this pathway. The benzimidazole moiety of mibefradil also has the potential to be involved in the observed inactivation of CYP3A4. Hydroxylation at the 5-position of the benzene ring can produce inactivating species through quinone imine formation, which can subsequently form glutathione and/or P450 apoprotein adducts (Dalvie et al., 2006). In addition, hydroxylation of the benzimidazole

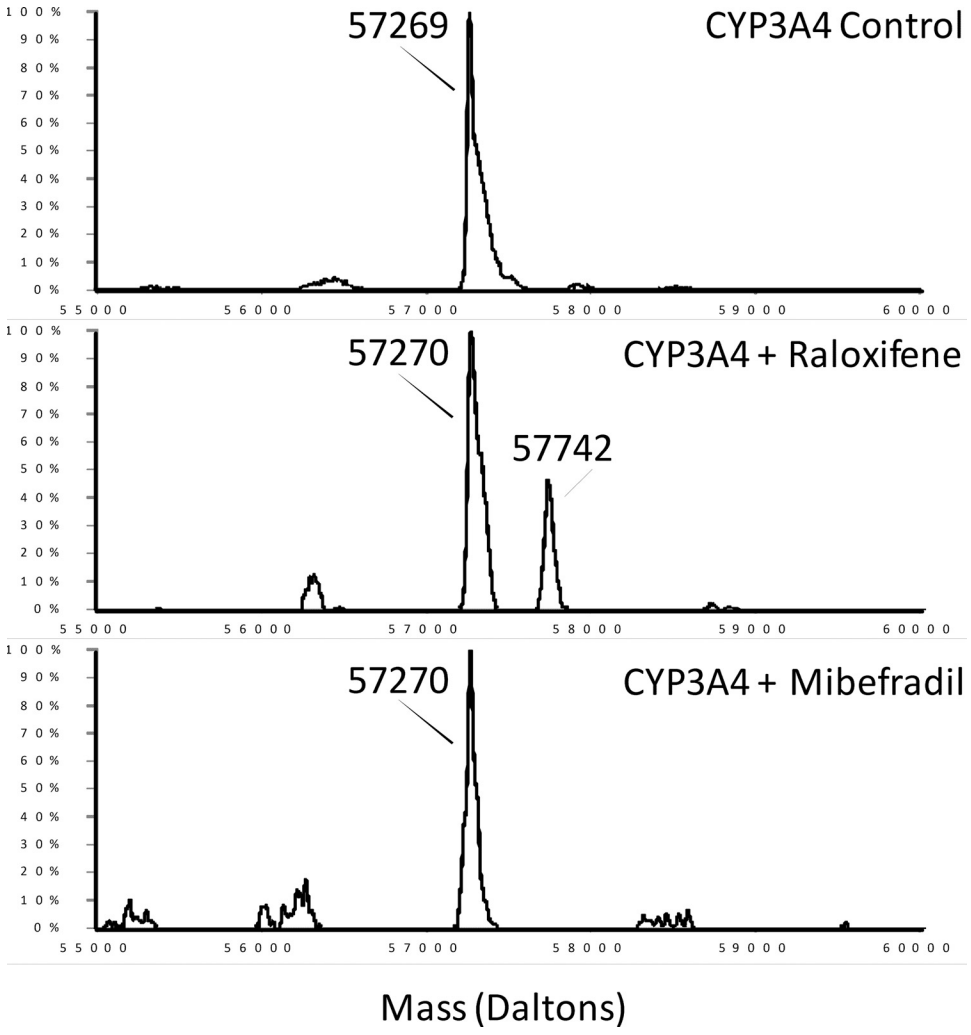

FIG. 7. Qualitative assessment of intact CYP3A4 apoprotein after incubation with mibefradil or raloxifene (positive control). No modification of the intact CYP3A4 apoprotein was observed in the presence of mibefradil plus NADPH.

could result in the formation of a highly reactive arene oxide intermediate, which could similarly form an apoprotein adduct and inactivate the enzyme (Delatour et al., 1984; Uetrecht, 2003). No glutathione adducts were observed from incubations of mibefradil and CYP3A4, arguing against adduction through a cysteine thiol. Supporting this observation is the ability of mibefradil to inactivate CYP3A5 (data not shown), which, because of the absence of Cys239, has been shown to not be susceptible to adduction by electrophiles

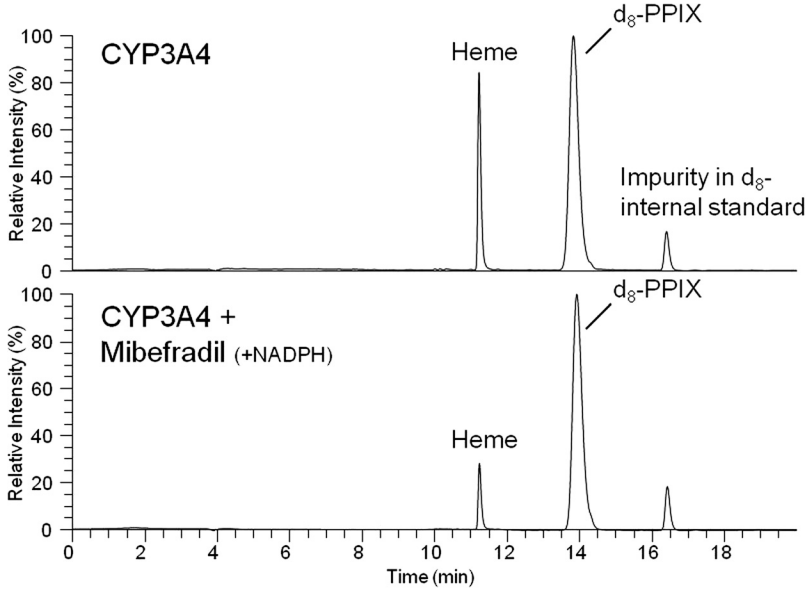

FIG. 8. Quantitative loss of heme after preincubation of CYP3A4 with mibefradil and NADPH. Approximately 70% of measurable heme was lost [relative to d_8-protoporphyrin-IX (d_8-PPIX) internal standard] after a 5-min preincubation with mibefradil (10 μM) and NADPH. The peak at 16.4 min was also observed as an impurity in the d_8-protoporphyrin-IX standard.

such as the reactive intermediate of raloxifene (Pearson et al., 2007). It is important to note that although the lack of any observable adducts with nucleophilic trapping agents may seem to preclude certain inactivation mechanisms, the low partition ratio of mibefradil against CYP3A4 may also reflect an inherent inability to trap a reactive intermediate for those inactivating species that do not escape the active site of the P450 enzyme. However, the negative data obtained with the nucleophilic trapping agents coupled with the lack of an observed change to the deconvoluted mass spectra of the intact CYP3A4 apoprotein indicate that inactivation of CYP3A4 by mibefradil does not occur through protein adduction.

To further discern the mechanism of CYP3A4 inactivation by mibefradil, results were compared with those obtained after preincubation of CYP3A4 with 1-aminobenzotriazole (heme adduct) or raloxifene (apoprotein adduct). A decrease in the ability to bind carbon monoxide coupled with a loss of intact heme is expected upon preincubation of CYP3A4 with compounds that result in modification of the heme moiety. On the other hand, inactivation of the enzyme through protein adduction should cause a minimal loss in CO binding and intact heme. Similar to what was observed for 1-aminobenzotriazole, time-dependent loss of carbon monoxide binding paralleled the loss of enzyme activity as would be expected for a modification to the heme prosthetic group. No correlation was observed between mibefradil and raloxifene in regard to loss of CO binding and enzyme activity. Further implication of heme destruction by mibefradil was afforded by spectral analysis. In addition to the loss of CO-binding by CYP3A4 in the presence of mibefradil and NADPH, the loss of enzyme activity is not paralleled by a marked increase in absorbance at 420 nm relative to control incubations, as would be observed if CYP3A4 inactivation by mibefradil resulted in a distortion of the iron–sulfur bond as could be caused by degradation of the protein. Furthermore, whereas loss of heme was evident from UV and LC-MS studies with both mibefradil and control experiments with 1-aminobenzotriazole, only the incubations with 1-aminobenzotriazole resulted in an observable intact heme adduct (benzyne intermediate, *m/z* 692 amu). No loss of heme was observed when raloxifene was used as a control compound in the preincubation step.

Compounds may inactivate P450 enzymes through destruction of the heme prosthetic group with no formation of stable or isolable heme adducts. The observed result is bleaching of the heme chromophore due to fragmentation of the heme moiety. Heme bleaching through destruction of the prosthetic heme has been attributed to compounds such as carbon tetrachloride and *N*-methyl-*N*-benzylcyclopropylamine, and phenol-containing compounds such as resveratrol and diclofenac (Guzelian and Swisher, 1979; Macdonald et al., 1982; Davies et al., 1986; Shen et al., 1997; Chang et al., 2001; Ortiz de Montellano and Correia, 2005). Mibefradil seems to fall into this latter category, resulting in a loss of heme with no identifiable heme adducts. This type of heme destruction has also been observed for P450 enzymes in the presence of hydrogen peroxide or cumene hydroperoxide (Schaefer et al., 1985; He et al., 1998). Heme cleavage at the meso carbons results in loss of the chromophore and the corresponding formation of both monopyrrolic and dipyrrolic heme fragments. This type of heme destruction can occur either along the α-γ and the β-δ axis of the protoporphyrin moiety, yielding multiple heme fragments (He et al., 1998). Alternative mechanisms of heme destruction include oxidation by ascorbic acid and oxygen (resulting in a mixture of bilverdin isomers) or degradation by heme oxygenase (forming only biliverdin IXα and releasing CO). No biliverdin isomers were observed after CYP3A4 inactivation by mibefradil.

In summary, mibefradil is a potent mechanism-based inactivator of CYP3A4 that results in rapid and irreversible inactivation of the enzyme. No MI complex or isolable heme and/or apoprotein adducts were observable after preincubation of CYP3A4 with mibefradil plus NADPH and nucleophilic trapping agents did not reduce the degree of CYP3A4 inactivation by mibefradil. Unlike other calcium channel blockers, the elimination of possible mechanisms of P450 inactivation combined with the marked loss of isolable heme suggests that mibefradil inactivates CYP3A4 through destruction of the heme moiety.

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Authorship Contributions

Participated in research design: Foti, Rock, Pearson, Wahlstrom, and Wienkers.

Conducted experiments: Foti, Rock, and Pearson.

Contributed new reagents or analytic tools: Foti.

Performed data analysis: Foti.

Wrote or contributed to the writing of the manuscript: Foti, Wahlstrom, and Wienkers.

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