Cytochrome P450 3A4-Mediated Oxidative Conversion of a Cyano to an Amide Group in the Metabolism of Pinacidil

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ABSTRACT: The conversion of nitriles to amides is generally considered to be a hydrolytic process that does not involve redox chemistry. We demonstrate here that cytochrome P450 (CYP) is responsible for the conversion of the cyano group of pinacidil to the corresponding amide. The reaction in human liver microsomes was NADPH-dependent and was nearly completely inhibited by an anti-CYP3A4 antibody. Incubations of pinacidil with recombinant CYP enzymes confirm that CYP3A4 is the principal catalyst of this reaction. The kinetics of pinacidil amide formation by CYP3A4 yielded an apparent K_m of 452 ± 33 μM and k_{cat} of 0.108 min^{-1} (k_{cat}/K_m = 0.238 $mM^{-1}\cdot min^{-1}$). Incubation of pinacidil with CYP3A4 in the presence of $^{18}O_2$ or $H_2{}^{18}O$ showed that the amide carbonyl oxygen derived exclusively from molecular oxygen. The CYP3A4-mediated reaction also was supported by hydrogen peroxide when incubations were carried out in the absence of cytochrome P450 reductase and NADPH. The reaction can be explained by a nucleophilic attack of a deprotonated ferric peroxide intermediate ($Fe^{3+}-O-O^-$) on the carbon atom of the −C≡N triple bond to form an Enz−Fe(III)−O−O−C(═NH)R intermediate, followed by cleavage of the O−O bond to give pinacidil amide. This nucleophilic addition of an $Fe^{3+}-O-O^-$ intermediate to a −C═N π-bond in a P450 system resembles the analogous reaction catalyzed by the nitric oxide synthases.

Cytochrome P450[1] enzymes catalyze a wide range of oxidative and reductive biotransformations. Thus, CYP-mediated reactions include carbon hydroxylation, π-bond oxidation, heteroatom oxidation, hydrocarbon desaturation, and halocarbon dehalogenation (*1*). The catalytic cycle of CYP enzymes involves multiple intermediates, and an electron transfer process is generally involved (*1*). CYP3A4 is the most abundant CYP enzyme in human liver and exhibits a broad substrate specificity (*2*).

Nitrile-containing compounds exist widely in plants and also are used in the chemical industry [e.g., nitrile herbicides (*3*)]. Nitrile hydratase from bacteria can catalyze the hydration of a nitrile to form the corresponding amide and is widely studied in environmental preservation (*3*) as well as in chemical biocatalysis (*4*). Some drugs also contain cyano functional groups, and even though the biotransformation of nitrile-containing drugs (e.g., cimetidine, pinacidil, and 1,3,4-thiadiazol-2-ylcyanamide) to the corresponding amides has been observed in vivo (*5*−*7*), very little is known about the individual enzymes responsible for this bioconversion in humans.

Pinacidil (Figure 1) is a potent orally active vasodilator used to treat hypertension (*8*). In addition to its antihypertensive activity, pinacidil also has influence on hair growth (*9*). Pinacidil belongs to a class of drugs called potassium channel openers. In rat studies, pinacidil amide (Figure 1) was detected in urine from rats dosed orally with pinacidil at 100 mg kg^{-1} day^{-1} for 2 days (*10*). In preliminary studies, we also observed that pinacidil amide was one of the metabolites formed in incubations of pinacidil with human hepatocytes, suggesting that human exposure to pinacidil amide may occur. In this report, we demonstrate that the conversion of the cyano group of pinacidil to the corresponding amide in human liver microsomes is catalyzed primarily by CYP3A4 via a novel mechanism.

MATERIALS AND METHODS

Materials. Pinacidil, NADPH, 1-ABT, and 1,2-didodecanoyl-*sn*-glycero-3-phosphocholine were purchased from Sigma-Aldrich Co. (St. Louis, MO). NADPH−cytochrome P450 reductase was obtained from Gentest Co. (Woburn, MA). The ^{18}O-labeled water (95 atom % excess ^{18}O) and $^{18}O_2$ (99 atom % excess ^{18}O) were from Isotec Inc. (Miamisburg, OH). All other reagents and solvents were obtained from either Fisher Scientific (Fair Lawn, NJ) or Sigma-Aldrich Co. (St. Louis, MO).

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[1] Abbreviations: 1-ABT, 1-aminobenzotriazole; CID, collision-induced dissociation; CYP, cytochrome P450; Enz, enzyme; ESI, electrospray ionization; His-CYP3A4, C-terminal $(His)_5$-tagged CYP3A4; IgG, immunoglobulin G; LC/MS/MS, liquid chromatography−tandem mass spectrometry; L-NHA, *N*-hydroxy-L-arginine; NOS, nitric oxide synthase; OR, NADPH−cytochrome P450 reductase; pinacidil, (±)-*N*-cyano-*N*-4-pyridinyl-*N*″-(1,2,2-trimethylpropyl)guanidine; pinacidil amide, *N*-aminocarbonyl-*N*-4-pyridinyl-*N*″-(1,2,2-trimethylpropyl)-guanidine; S, substrate.

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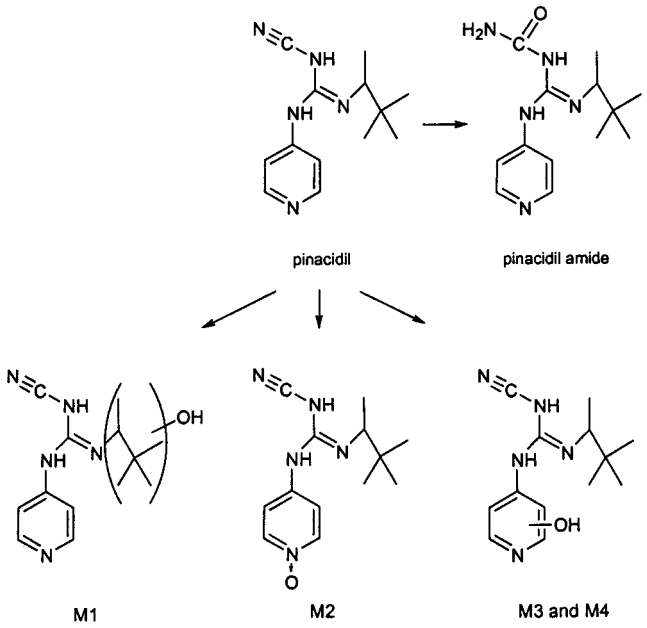

FIGURE 1: CYP3A4-catalyzed metabolism of pinacidil.

Microsomes from a baculovirus insect cell line containing individual human CYP1A2, -2A6, -2B6, -2C8, -2C9, -2C19, -2D6, -2E1, and -3A4 coexpressed with NADPH−cytochrome P450 reductase were prepared at Merck Research Laboratories (*11*). Pooled liver microsomal fractions were isolated from Sprague-Dawley rats (40 male animals) and humans (3 males and 2 females) by differential centrifugation (*12*, *13*). Monoclonal antibodies against human hepatic CYP3A4 or -2D6 were products of hybridomas derived from the fusion of myeloma cells and spleen cells from mice immunized with baculovirus-expressed CYP3A4 or -2D6 (*11*). The plasmid for His-CYP3A4 containing a C-terminal $(His)_5$ tag (*14*) was kindly provided by Dr. F. Peter Guengerich (Vanderbilt University).

Instrumentation. LC/MS/MS was carried out on either a Perkin-Elmer SCIEX API 3000 tandem mass spectrometer (Toronto, Canada) interfaced to an HPLC system consisting of a Perkin-Elmer Series 200 quaternary pump and a Series 200 autosampler (Norwark, CT) or a Finnigan LCQDeca mass spectrometer (San Jose, CA) interfaced to a HPLC system consisting of two Shimadzu LC-10AD pumps (Kyoto, Japan), a Shimadzu SIL-10AD auto injector, and a Finnigan UV6000LP photodiode array detector (San Jose, CA). LC/MS/MS experiments were carried out on a SCIEX API 3000 mass spectrometer using a heated nebulizer interface (for quantitation) with positive ion detection. The source temperature was set at 400 °C, the ion spray voltage at 5 kV, the focusing potential at 110 V, and the entrance potential at −10 V. The collision gas was nitrogen. LC/MS/MS experiments were performed on a LCQDeca mass spectrometer using an electrospray ionization probe (for metabolite identification) in positive ion mode. The heated capillary temperature was 220 °C, the normalized collision energy was 44%, the sheath gas flow rate was 60 units, and the auxiliary gas flow rate was 20 units. The ion spray voltage, the capillary voltage, and the tube lens offset were adjusted against pinacidil to achieve the maximum sensitivity.

For metabolite identification by LC/MS/MS, samples (75 μL) were loaded onto an Agilent Zorbax Rx-C8 column (Wilmington, DE, 4.6 × 250 mm, 5 μm) and were delivered at a flow rate of 1 mL/min. The HPLC eluents were monitored by a photodiode array UV detector (scanning from 200 to 700 nm) before being directed to a LCQDeca mass spectrometer with 1:5 split. The mobile phase consisted of solvent A (5 mM ammonium acetate in water−acetonitrile−acetic acid, 95:5:0.05) and solvent B (5 mM ammonium acetate in acetonitrile−water−acetic acid, 95:5:0.05). The HPLC runs were programmed by a linear increase from 0% to 100% of solvent B during a 30 min period. The MS/MS spectra were recorded by collision-induced dissociation of MH^+ species. The ^1H NMR spectrum of synthetic pinacidil amide was recorded with a Varian Inova600 spectrometer operating at 600 MHz. Chemical shifts (δ) are reported in parts per millions (ppm) downfield from tetramethylsilane, and coupling constants (J) are expressed in hertz (Hz) with multiplicity of the signals noted: s = singlet, d = doublet, m = multiplet, and br = broad.

Synthesis of Pinacidil Amide. Pinacidil (50 mg) was suspended in a mixture of glacial acetic acid (1 mL) and concentrated hydrochloric acid (0.1 mL) and was stirred at room temperature for 24 h. The mixture was concentrated in vacuo, and the residue was loaded onto a preparative C18 HPLC column. The flow was delivered at 3 mL/min with a linear gradient of 20% aqueous acetonitrile to 100% acetonitrile during a 15 min period. The fractions containing pinacidil amide were combined and evaporated in vacuo to give the final product (32 mg, 60%). LC/MS/MS *m/z* (relative abundance): 264 (MH^+, 3%), 247 (73%), 221 (100%), 204 (12%), 163 (3%), 120 (8%), 95 (25%). ^1H NMR (acetone-d_6): δ 9.81 (br, NH), 8.23 (2H, d, J = 6.6 Hz, ArH), 7.02 (2H, d, J = 6.6 Hz, ArH), 3.93 (1H, m, J = 6.6 Hz, −CH<), 3.41(br, NH), 1.17 (3H, d, J = 6.6 Hz, >CHCH_3), 0.96 [9H, s, $-C(CH_3)_3$].

Incubations of Pinacidil with Liver Microsomes in the Presence or Absence of 1-Aminobenzotriazole (1-ABT) or Anti-CYP Antibodies. A 1-ABT stock solution (50 mM in DMSO) was added to rat liver microsomes (1 mg of protein/mL) suspended in phosphate buffer (100 mM, pH 7.4) containing EDTA (1 mM) and $MgCl_2$ (0.1 mM) in a total volume of 0.5 mL, and the mixture was preincubated in the presence of NADPH (1.2 mM) at 37 °C for 10 min. The final concentration of 1-ABT was 0.1 mM. To the reaction mixture was added pinacidil (50 μM), and the resulting mixture was incubated for an additional 30 min. The reaction was quenched by addition of 0.5 mL of acetonitrile. The suspension then was sonicated for 5 min and was centrifuged at 14000 rpm for 10 min. The supernatant was loaded onto an HPLC column for LC/MS/MS analysis. In the immunoinhibition studies, human liver microsomes were preincubated with anti-CYP3A4 (2.5 mg of IgG/nmol of CYP) or anti-CYP2D6 IgG (7.0 mg of IgG/nmol of CYP) at room temperature for 15 min before addition of substrate and NADPH. The remaining procedures were the same as those described above for rat liver microsome incubations, except that the acetonitrile-quenched supernatants were evaporated to dryness under nitrogen at room temperature, and the residues were reconstituted in 300 μL of methanol. The samples then were loaded onto an HPLC column for LC/MS/MS analysis. The control experiments contained liver microsomes and pinacidil in the presence or absence of NADPH.

Incubations of Pinacidil with Microsomes Expressing Individual CYP Enzymes. A pinacidil stock solution (10 mM) in methanol was added to baculovirus insect cell microsomes coexpressing NADPH−cytochrome P450 reductase and individual human CYP enzymes (CYP1A2, -2A6, -2B6, -2C8, -2C9, -2C19, -2D6, -2E1, and -3A4) suspended in phosphate buffer (100 mM, pH 7.4) containing EDTA (1 mM) and $MgCl_2$ (0.1 mM). The mixture (50 μM pinacidil, 0.25 μM individual CYP enzymes in a final volume of 0.5 mL) was preincubated at room temperature for 5 min. NADPH (1.2 mM) in phosphate buffer (100 mM, pH 7.4) was added, and the mixture was incubated at 37 °C for an additional 30 min. The reaction was quenched by addition of 0.5 mL of acetonitrile containing cimetidine (final concentration of 83 ng/mL, internal standard). The suspension then was sonicated for 5 min and was centrifuged at 14000 rpm for 10 min. The supernatant was loaded onto an HPLC column for LC/MS/MS analysis. The control experiments contained baculovirus insect cell microsomes and pinacidil in the absence of NADPH. An additional control experiment was conducted in the presence of pinacidil (50 μM) and recombinant NADPH−cytochrome P450 reductase (2 μM) and NADPH (1.2 mM).

Kinetics Studies of Pinacidil Amide Formation in Incubations of Pinacidil with CYP3A4. Pinacidil stock solution (10−1000 mM) in methanol was added to baculovirus insect cell microsomes coexpressing human CYP3A4 and NADPH−cytochrome P450 reductase suspended in phosphate buffer (100 mM, pH 7.4) containing EDTA (1 mM) and $MgCl_2$ (0.1 mM). The mixture (0−5 mM pinacidil, 0.25 μM CYP3A4 in a final volume of 0.5 mL) was preincubated at room temperature for 5 min before addition of NADPH (1.2 mM) in phosphate buffer and was incubated at 37 °C for additional 30 min. The reaction was quenched by addition of 0.5 mL of acetonitrile containing cimetidine (final concentration of 83 ng/mL, internal standard). The suspension then was sonicated for 5 min and centrifuged at 14000 rpm for 10 min. The supernatants were loaded onto an HPLC column for LC/MS/MS analysis.

Quantitation of Pinacidil Amide. Aliquots (75 μL) from incubations of recombinant CYP enzymes or liver microsomes were loaded onto a Keystone Scientific (Wilmington, DE) Betasil C8 column (4.6 × 50 mm, 5 μm) and eluted at a flow rate of 1 mL/min. The mobile phase consisted of 5 mM ammonium acetate in methanol−H_2O (95:5). Quantification was carried out by selected reaction monitoring LC/MS/MS in which the transitions monitored were *m*/*z* 264.2 → 120.1 (pinacidil amide) and *m*/*z* 253.1 → 159.4 (cimetidine, internal standard). Standard curves of pinacidil amide were linear over a range of 10−1000 nM.

Quantitation of Pinacidil in CYP3A4 Incubations. Aliquots (100 μL) taken from incubations of recombinant CYP3A4 (0.25 μM) with pinacidil (50 μM) at different time points (0, 5, 10, 15, 20, 25, and 30 min) were mixed with cimetidine (25 ng, internal standard) and 8 M urea (1 mL) and applied to an Oasis C-18 MCX 96-well extraction plate, which was prewashed with methanol and water. The plate then was washed with water (1 mL) and eluted with 300 μL of 5 mM ammonium acetate in methanol−H_2O (95:5). The elutes (75 μL) were quantitated using the LC/MS/MS method described above. The transitions were monitored at *m*/*z* 246.2 → 120.1 (pinacidil) and *m*/*z* 253.1 → 159.4 (cimetidine, internal standard). Standard curves of pinacidil were linear over a range of 20−2000 nM. The experiments were conducted in duplicate.

Incubations of Pinacidil with CYP3A4 in the Presence of $H_2{}^{18}O$. The incubation (final volume of 0.5 mL) was conducted under the same conditions as used for the CYP/NADPH systems, except that the buffer contained $H_2{}^{18}O$−H_2O (1:1). The reaction was quenched by addition of 0.5 mL of acetonitrile. The resulting suspension then was sonicated for 5 min and centrifuged at 14000 rpm for 10 min before being subjected to LC/MS/MS analysis.

Incubations of Pinacidil with CYP3A4 under $^{18}O_2$. The incubation was conducted under the same conditions as used for the above CYP/NADPH systems, except that it was conducted under an atmosphere of $^{18}O_2$. The components, except pinacidil, were placed in a test tube connected to a vacuum manifold which permitted the introduction of either argon or $^{18}O_2$. The test tube was immersed in an ice bath and was evacuated eight times and filled with argon each time. After a final evacuation, the $^{18}O_2$ was introduced into the reaction mixture. The reaction was initiated by adding pinacidil stock solution in methanol via syringe injection and was kept at 37 °C for an additional 30 min. The reaction was quenched by adding an equal volume of acetonitrile via syringe injection, and sample processing and LC/MS/MS analysis procedures were conducted as described above.

Incubations of Pinacidil with Purified His-CYP3A4 in the Presence of H_2O_2. An expression plasmid for His-CYP3A4 (*14*) was transformed into *Escherichia coli* DH5α cells, and a single ampicillin-resistant colony was selected from an agar plate in Luria−Bertani medium containing 50 μg/mL ampicillin. His-CYP3A4 protein was expressed and purified using procedures described previously (*14*). The reaction was carried out in 0.25 mL of potassium phosphate buffer (100 mM) containing pinacidil (50 μM), His-CYP3A4 (1.2 μM), 1,2-didodecanoyl-*sn*-glycero-3-phosphocholine (30 μM), and $MgCl_2$ (0.1 mM). After preincubation at 37 °C for 5 min, the reaction was initiated by the addition of a H_2O_2 stock solution (200 mM in H_2O) to a final concentration of 2 mM. The incubation was conducted at 37 °C for an additional 20 min and was quenched by addition of 0.25 mL of acetonitrile. The remaining sample processing and LC/MS/MS quantitation of pinacidil amide were the same as those described above. Control experiments were conducted in the absence of His-CYP3A.

RESULTS

Formation of Pinacidil Amide in Liver Microsomal Preparations. The conversion of the cyano group of pinacidil to the amide was found to be NAPDH-dependent in both rat and human liver microsomes. In the complete system, the formation of pinacidil amide in rat liver microsomes occurred at a rate of 19.0 pmol·min^{-1}·(mg of protein)$^{-1}$ and was 98% inhibited by the mechanism-based CYP inhibitor 1-ABT (*15*, *16*). The formation of pinacidil amide in human liver microsomes occurred at a rate of 0.9 pmol·min^{-1}·(mg of protein)$^{-1}$ and was inhibited by 98% and 17% in the presence of anti-CYP3A4 and anti-CYP2D6 antibodies, respectively.

Incubations of Pinacidil with Recombinant CYP Enzymes. To unambiguously establish the role of CYP enzymes in the

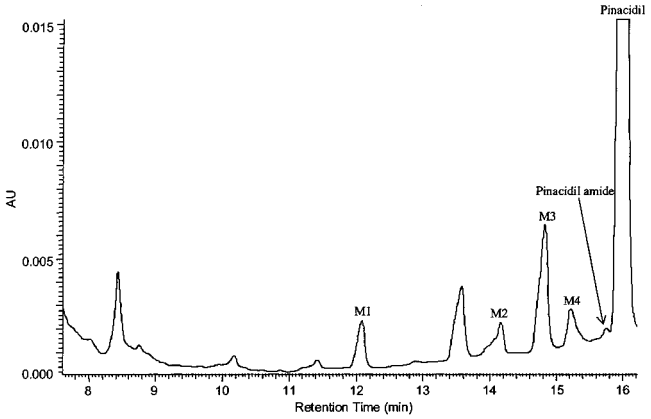

FIGURE 2: Representative HPLC chromatogram of pinacidil and its metabolites in CYP3A4 incubations.

formation of pinacidil amide, screening of different recombinant human CYP enzymes (CYP1A2, -2A6, -2B6, -2C8, -2C9, -2C19, -2D6, -2E1, and -3A4) showed that both CYP3A4 and CYP2D6 are capable of catalyzing the conversion of the cyano group of pinacidil to the amide. At a pinacidil concentration of 50 μM, the formation rates of pinacidil amide catalyzed by CYP3A4 (0.25 μM) and CYP2D6 (0.25 μM) were 0.012 and 0.001 nmol·min^{-1}·(nmol of CYP)$^{-1}$ in a period of 30 min, respectively. The formation of pinacidil amide in negative control experiments (in the presence of CYP and OR and absence of NADPH or in the presence of OR and NADPH and absence of CYP enzymes) was below the detection limit by LC/MS/MS.

In addition to pinacidil amide, four monooxygenated metabolites (M1−M4) were detected by LC/MS/MS analyses of incubations of pinacidil with CYP3A4 (Figure 2). On the basis of their ESI-MS/MS spectra, it was concluded that M1 was monohydroxylated on the trimethylpropyl group of pinacidil, M2 was a pyridine *N*-oxide derivative, and M3 and M4 were monohydroxylated on the pyridine ring. The molecular ions and major fragments of the metabolites from LC/MS/MS analyses were as follows: M1, 262 (MH^+), 220, 178, 136, 120, 95; M2, 262 (MH^+), 218, 143, 120, 95; M3, 262 (MH^+), 218, 178, 136, 127, 111; M4, 262 (MH^+), 218, 178, 136, 111. The proposed metabolic pathways for pinacidil in incubations with CYP3A4 are shown in Figure 1. On the basis of HPLC/UV analysis, the formation of monooxygenated metabolites M1−M4 predominated over that of pinacidil amide (Figure 2). The quantitative analysis of metabolic stability of pinacidil in CYP3A4 showed that pinacidil disappeared at a rate of 0.383 nmol·min^{-1}·(nmol of CYP3A4)$^{-1}$.

Kinetics of Pinacidil Amide Formation Catalyzed by CYP3A4. The formation of pinacidil amide by CYP3A4 (0.25 μM) was found to be linear over a 30 min period. Under those linear reaction conditions, the saturation curve of pinacidil amide formation was obtained over the substrate concentration range of 0−5 mM (Figure 3). The linearity of the Eadie−Hofstee plot suggested simple Michaelis−Menten kinetics, yielding an apparent K_m of 452 ± 33 μM and k_{cat} of 0.108 min^{-1} with the k_{cat} /K_m ratio of 0.238 mM^{-1}·min^{-1}.

^{18}O-Labeling Experiments. To elucidate the mechanism by which the cyano group of pinacidil was converted to the corresponding amide by CYP3A4, ^{18}O-labeling experiments were conducted in which pinacidil was incubated with

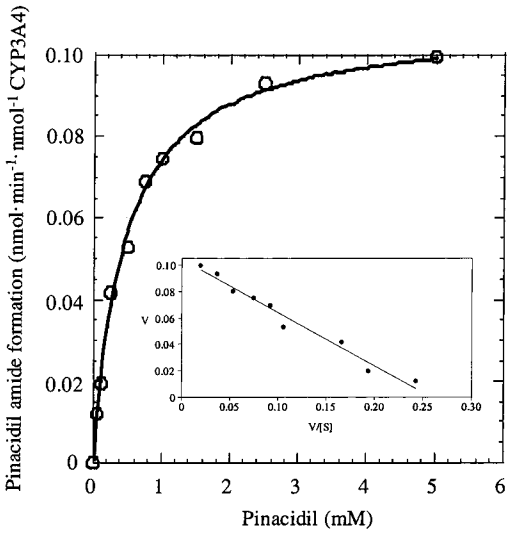

FIGURE 3: Effects of pinacidil concentrations on the rates of pinacidil amide formation catalyzed by CYP3A4. The Michaelis plot shows the saturation curve for the formation of pinacidil amide in the presence of CYP3A4 (0.25 μM). The insert is an Eadie−Hofstee plot of the data. Data points represent the mean of triplicate analyses.

CYP3A4 in the presence of either $H_2{}^{18}O-H_2O$ (1:1) or $^{18}O_2$. Incorporation of ^{18}O into pinacidil amide was determined by LC/MS/MS analysis and was based on the relative intensities of the MH^+ ions at *m*/*z* 264 (unlabeled amide) and 266 ([^{18}O]pinacidil amide). The experiments demonstrated that no ^{18}O was incorporated into pinacidil amide from the incubations of pinacidil with CYP3A4 in the presence of $H_2{}^{18}O-H_2O$ (1:1). However, the product formed under an atmosphere of $^{18}O_2$ incorporated one atom of ^{18}O at the level of 92 atom % excess. The MS/MS spectra resulting from CID of the MH^+ ions of the unlabeled and ^{18}O-labeled variants of pinacidil amide are shown in panels A and B of Figure 4, respectively, and reflect the incorporation of ^{18}O into the carbonyl oxygen. Thus, the ions at *m*/*z* 247 and 163 in the spectrum of the unlabeled metabolite, which derive from losses of the elements of NH_3 and NH_3 plus trimethylpropene, respectively, shift 2 units to higher masses in the spectrum of ^{18}O-labeled pinacidil amide. Conversely, the ions at *m*/*z* 221, 204, 120, and 95, which do not retain the carbonyl moiety, do not shift in the spectrum of the labeled metabolite.

H_2O_2-Supported Formation of Pinacidil Amide by Purified His-CYP3A4. In the presence of H_2O_2 and the absence of both NADPH−cytochrome P450 reductase and NADPH, it was shown by LC/MS/MS that pinacidil amide and monooxygenated metabolites were formed in incubations of pinacidil with purified His-CYP3A4. The formation rate of pinacidil amide is 0.001 nmol·min^{-1}·(nmol of CYP3A4)$^{-1}$. The formation of pinacidil amide in corresponding control experiments which lacked His-CYP3A4 was below the LC/MS/MS detection limit.

DISCUSSION

Nitriles are a class of cyano-containing compounds. The cyano group is polarized with a large bond moment (~3.5 D), largely as a result of the electronegativity of the nitrogen and the lone pair orbital centered on the nitrogen atom (*17*). In chemical synthesis, the choice of reagent for the controlled

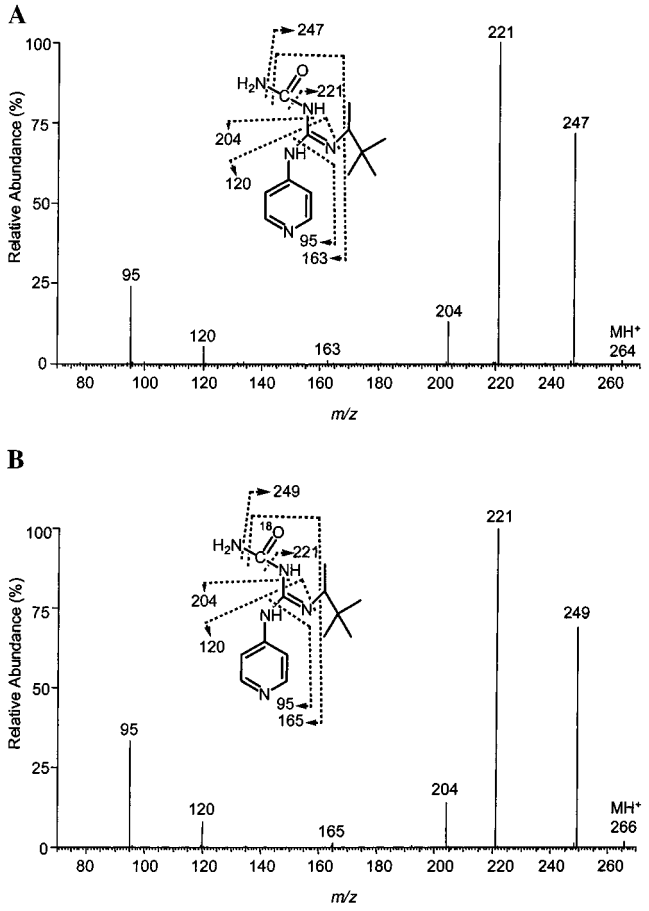

FIGURE 4: ESI-MS/MS spectrum of pinacidil amide obtained by CID of the MH^+ ion at *m/z* 264 (A) and the corresponding spectrum of the metabolite formed in incubations of pinacidil with CYP3A4 under an atmosphere of $^{18}O_2$ (B). The origin of characteristic fragment ions is as noted.

hydrolytic conversion of nitriles to the corresponding amides is aqueous NaOH (pH 9.3−9.9) with H_2O_2 as a catalyst, because the HOO^- ion, which is present at high concentration in basic solution, has a high nucleophilicity and readily attacks the carbon atom of the cyano group to facilitate the hydrolytic process (*18*, *19*). However, very little is known about the biotransformation of nitriles to the corresponding amides in biological systems. Our in vitro results showed that both CYP3A4 and CYP2D6 are capable of catalyzing the conversion of the cyano group of pinacidil to the amide in human liver microsomes. In view of the fact that CYP3A4 is the major CYP enzyme in human liver microsomes (*20*), it may be concluded that CYP3A4 is the primary CYP enzyme responsible for the conversion of the cyano group of pinacidil to the corresponding amide in these microsomes.

Two alternative mechanisms can be proposed to explain the conversion of the cyano functional group of pinacidil to the corresponding amide. The first involves formation of a nitrile oxide, a known class of synthetic organic compounds (*21*). However, it is energetically unfavorable to oxidize the −C≡N moiety directly to form the corresponding nitrile oxide using chemical oxidants (*21*). Furthermore, if nitrile oxide **1** were formed enzymatically as a result of the oxidation of pinacidil catalyzed by CYP3A4, the product **3** would incorporate one atom of ^{18}O from incubations conducted in $H_2{}^{18}O$ (Figure 5). No such labeling was observed, in contrast to the situation where pinacidil was

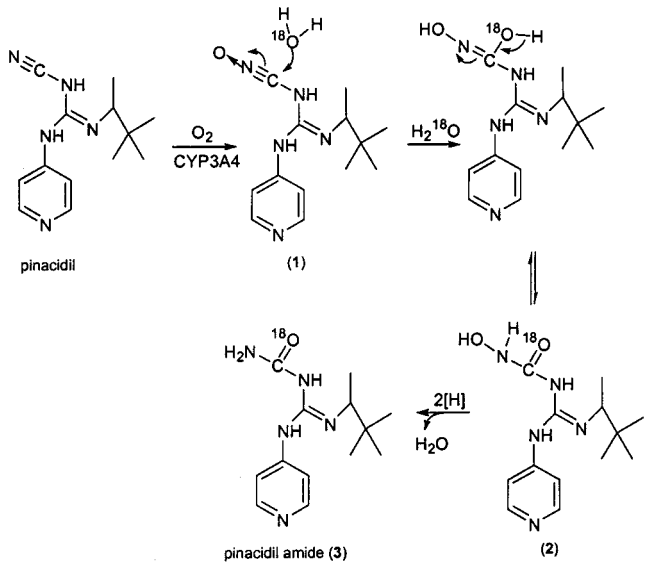

FIGURE 5: Hypothetical mechanism for the formation of pinacidil amide via the nitrile oxide pathway, which would lead to ^{18}O incorporations from water of the medium.

incubated under $^{18}O_2$, where essentially all of the pinacidil amide molecules incorporated a single atom of ^{18}O into the C═O bond of amide group (Figure 4B). The most likely explanation for such complete ^{18}O incorporation is the direct transfer of an ^{18}O atom from the enzyme to the carbon atom of the −C≡N triple bond to give the ^{18}O-labeled pinacidil amide **3**. Because the formation of pinacidil amide was known to be both CYP- and NADPH-dependent, it is very likely that sequential oxidation and reduction reactions are involved in the mechanism.

The catalytic cycle of CYP enzymes is known to involve multiple intermediates, one of which is the ferric peroxide species (Fe^{3+}−OOH). This ferric peroxide intermediate can decay to give the reactive ferryl-oxy species Enz−$(FeO)^{3+}$S or participate in nucleophilic addition to the substrate to form an Enz−Fe(III)−OO−S intermediate (*22*). The former pathway has been proposed for most CYP-mediated reactions (*1*), whereas the latter mechanism is relatively rare and has only been invoked in situations in which the ferric peroxide intermediate adds to aldehyde and keto groups. Examples of this nucleophilic addition mechanism include the CYP-mediated deformylation of aldehydes (*23*), the aromatization of the A ring of androst-4-ene-3,17-dione (*24*, *25*), and the 14α-demethylation of lanosterol (*26*).

In the case of pinacidil, the guanidine group is very basic, and at physiological pH it functions as an electron sink which further draws electron density from the carbon of the cyano group. In the CYP3A4/OR/NADPH/$^{18}O_2$ system, this electron-deficient carbon atom would be susceptible to nucleophilic attack by the deprotonated ferric peroxide intermediate (**4**) (Fe^{3+}−^{18}O−$^{18}O^-$) to form the Enz−Fe(III)−^{18}O−^{18}O−C(R)═NH intermediate (**5**), followed by cleavage of the ^{18}O−^{18}O bond to give ^{18}O-labeled pinacidil amide (**3**) and, presumably, the [^{18}O]ferryl-oxy enzyme species (**6**) (Figure 6). The substrate-free ferryl-oxy species (**6**) could then be reduced by NADPH to return the enzyme to its resting state. In light of the fact that formation of monooxygenated metabolites of pinacidil by CYP3A4 occurs to a greater extent than pinacidil amide formation (Figure 2), the partitioning between the decay of the ferric peroxide

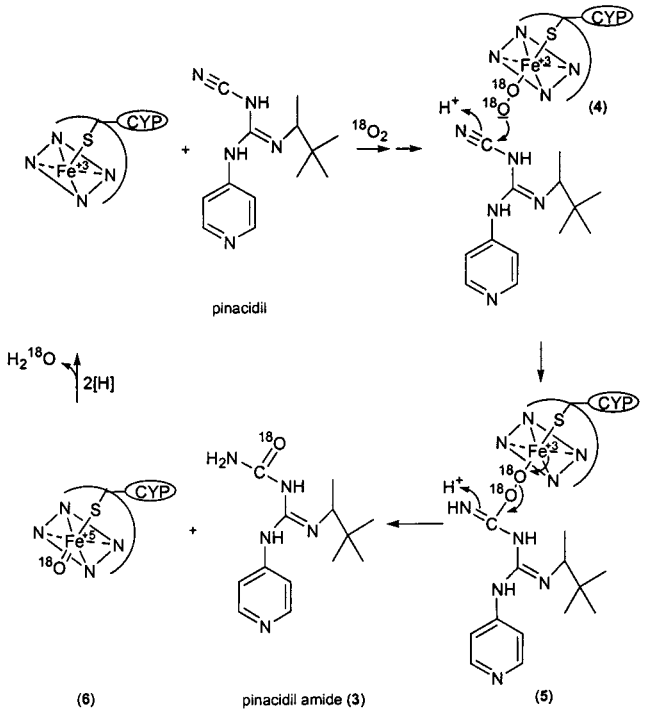

FIGURE 6: Proposed mechanism for the CYP3A4-mediated formation of pinacidil amide via the peroxide addition pathway under an atmosphere of $^{18}O_2$.

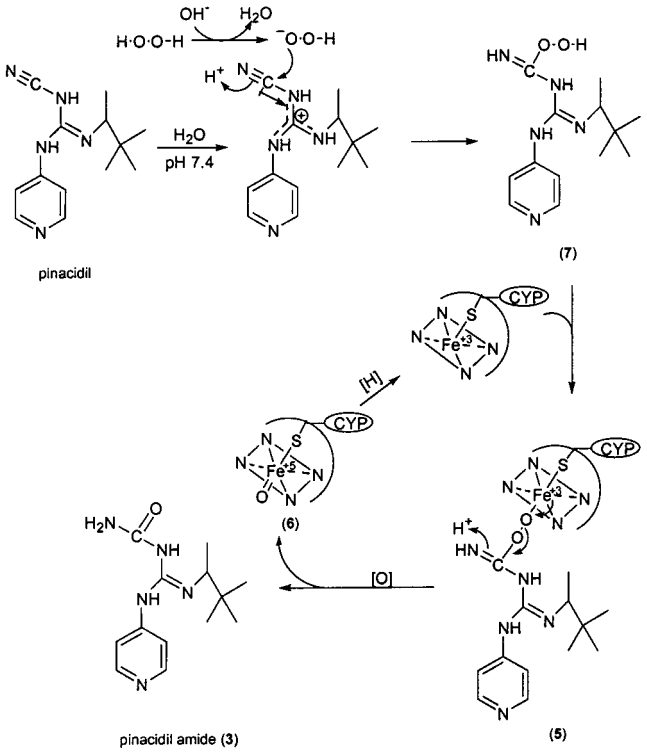

FIGURE 7: Proposed alternative mechanism for the formation of pinacidil amide in the presence of CYP3A4 and H_2O_2.

intermediate (**4**) to give the reactive ferryl-oxy species Enz−(FeO)$^{3+}$S and the addition of ferric peroxide (**4**) to substrate to form the Enz−Fe(III)−O−O−C(R)═NH intermediate (**5**) apparently favors the decay pathway. Compared to the disappearance of pinacidil at the rate of 0.383 nmol·min^{-1}·(nmol of CYP3A4)$^{-1}$, the formation of pinacidil amide at the rate of 0.012 nmol·min^{-1}·(nmol of CYP3A4)$^{-1}$ in the CYP3A4 incubations shows that it accounts for 3.1% of the total metabolism in the first 30 min period.

It is possible to propose an alternative mechanism in which the reactive ferryl-oxy species Enz−(FeO)$^{3+}$S generated from the decay pathway adds to the carbon atom of the cyano group to give Enz−Fe(V)−O−C(R)═NH, which subsequently collapses to the amide. However, the nitrile carbon is highly electron deficient not only due to the electronegativity of the nitrile nitrogen but also because of the strongly adjacent electron-withdrawing guanidine group. Transfer of the electrophilic ferryl oxygen to this carbon is therefore extremely unlikely, particularly as transfer to the terminal nitrile nitrogen would be greatly favored. Oxidation of the nitrogen does not occur, however, as it would result in incorporation of an oxygen from water into the amide (Figure 5), a result contrary to observation.

The proposed mechanism is supported by H_2O_2 shunt experiments in the presence of CYP3A4/H_2O_2 but absence of OR/NADPH in which pinacidil amide was shown to be formed. Because the pinacidil concentration was much higher than the level of CYP enzyme in these incubations, it is possible that pinacidil is attacked directly by a trace of the highly nucleophilic HOO^- species to form pinacidil peroxide (**7**) (Figure 7). Like other peroxides, pinacidil peroxide (**7**) could then react with the resting state of CYP3A4 to give the Enz−Fe(III)−O−O−C(R)═NH intermediate (**5**). Cleavage of the O−O bond of intermediate **5** then would follow the same course as shown in Figure 6. In the absence of NADPH, the substrate-free ferryl-oxy species (**6**) could oxidize other substrates or the protein itself. Because the concentration of the HOO^- species is very small at physiological pH, in contrast to the strongly basic conditions used for the H_2O_2-dependent hydrolysis of nitriles (*18*, *19*), the contribution to pinacidil amide formation from the addition of free HOO^- to the cyano group in the CYP3A4/H_2O_2 system is likely to be minor. This inference is reinforced by the fact that H_2O_2 does not support the formation of pinacidil amide at physiological pH in the absence of CYP3A4 (control experiment). All of these results point, as proposed, to the involvement of the shunt pathway in which H_2O_2 directly forms the protonated ferric peroxide intermediate and thus leads to the formation of both the monooxygenated metabolites and pinacidil amide. Not unexpectedly, the rate of the formation of pinacidil amide [0.001 nmol·min^{-1}·(nmol of CYP3A4)$^{-1}$] in the H_2O_2/His-CYP3A4 system is much slower than that in the CYP3A4/OR/NADPH system [0.012 nmol·min^{-1}·(nmol of CYP3A4)$^{-1}$]. These rate differences stem from the differences in the rate of formation of the deprotonated (Fe^{3+}−OO^-) species in the NADPH- versus H_2O_2-dependent systems and the fact that H_2O_2 causes degradation of the CYP3A4 protein.

Like other CYP enzymes, CYP3A4 exhibits substrate specificity with respect to the conversion of nitriles to amides. Two additional compounds [1-cyano-3-(3,4-xylyl)guanidine and tiotidine], which have structures similar to that of pinacidil, were incubated with CYP3A4. Under these conditions, only 1-cyano-3-(3,4-xylyl)guanidine was enzymatically transformed to the corresponding amide. This amide also was detected in incubations with human hepatocytes (unpublished results).

Nitric oxide synthase is a cytochrome P450-like hemoprotein that utilizes NADPH and O_2 to convert L-arginine

to nitric oxide and L-citrulline via the intermediate *N*-hydroxy-L-arginine (*28*). The mechanism of the second step in this transformation is thought to involve a nucleophilic addition of the NOS−Fe^{3+}−O−O^- species to the carbon atom of the C═N−$O^•$ group derived from the *N*-hydroxy-L-arginine by hydrogen abstraction to give a NOS−Fe^{3+}−O−O−C(NH_2)(R)−$NO^{•-}$ intermediate. The present evidence that a cytochrome P450 ferric peroxide intermediate adds to a −C═N π-bond appears to provide an example in a cytochrome P450 system of a reaction similar to that invoked in the catalytic mechanism of the nitric oxide synthases.

In conclusion, our results indicate that CYP3A4 can catalyze the biotransformation of nitriles to amides. The mechanism for pinacidil amide formation most likely involves nucleophilic attack by a deprotonated ferric peroxide derivative (Fe^{3+}−O−O^-) of CYP3A4 on the carbon atom of the −C≡N group to form an Enz−Fe(III)−O−O−C(R)═NH intermediate, followed by cleavage of the O−O bond to give pinacidil amide. The sequential oxidation and reduction reactions may occur at the iron center of the enzyme. Our studies provide an example of the CYP-mediated conversion of a nitrile to an amide and of a P450 reaction in which the Fe^{3+}−O−O^- intermediate adds nucleophilically to a −C≡N bond.

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