Identifying a Selective Substrate and Inhibitor Pair for the Evaluation of CYP2J2 Activity^S

Caroline A. Lee, J. P. Jones III, Jonathan Katayama, Rüdiger Kaspera, Ying Jiang, Sascha Freiwald, Evan Smith, Gregory S. Walker, and Rheem A. Totah

Department of Drug Metabolism, Pfizer Global Research, La Jolla, California (C.A.L., Y.J., S.F., E.S.); Department of Medicinal Chemistry, University of Washington, Seattle, Washington (J.P.J., J.K., R.K., R.A.T.); and Department of Drug Metabolism, Pfizer Global Research, Groton, Connecticut (G.S.W.)

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ABSTRACT:

CYP2J2, an arachidonic acid epoxygenase, is recognized for its role in the first-pass metabolism of astemizole and ebastine. To fully assess the role of CYP2J2 in drug metabolism, a selective substrate and potent specific chemical inhibitor are essential. In this study, we report amiodarone 4-hydroxylation as a specific CYP2J2-catalyzed reaction with no CYP3A4, or other drug-metabolizing enzyme, involvement. Amiodarone 4-hydroxylation enabled the determination of liver relative activity factor and intersystem extrapolation factor for CYP2J2. Amiodarone 4-hydroxylation correlated with astemizole O-demethylation but not with CYP2J2 protein content in a sample of human liver microsomes. To identify a specific CYP2J2 inhibitor, 138 drugs were screened using terfenadine and astemizole as probe substrates with recombinant

CYP2J2. Forty-two drugs inhibited CYP2J2 activity by $\geq 50\%$ at 30 μM , but inhibition was substrate-dependent. Of these, danazol was a potent inhibitor of both hydroxylation of terfenadine ($\text{IC}_{50} = 77 \text{ nM}$) and O-demethylation of astemizole ($K_i = 20 \text{ nM}$), and inhibition was mostly competitive. Danazol inhibited CYP2C9, CYP2C8, and CYP2D6 with IC_{50} values of 1.44, 1.95, and 2.74 μM , respectively. Amiodarone or astemizole were included in a seven-probe cocktail for cytochrome P450 (P450) drug-interaction screening potential, and astemizole demonstrated a better profile because it did not appreciably interact with other P450 probes. Thus, danazol, amiodarone, and astemizole will facilitate the ability to determine the metabolic role of CYP2J2 in hepatic and extrahepatic tissues.

Introduction

CYP2J2 is the only member of the human 2J subfamily, and, unlike other cytochrome P450 (P450) isozymes, it is predominantly expressed in extrahepatic tissues including the heart, skeletal muscle, placenta, small intestine, kidney, lung, pancreas, bladder, and brain (Zeldin et al., 1996, 1997; Enayetallah et al., 2004). In the liver and intestine, CYP2J2 constitutes 1 to 2% of total P450 content (Gaedigk et al., 2006; Paine et al., 2006). CYP2J2 is mostly known for its ability to convert arachidonic acid to regioselective epoxyeicosatrienoic acids, which play significant roles in maintaining the homeostasis of the kidney, heart, and lung by controlling crucial biological processes such as anti-inflammation, vasodilatation, relaxation of smooth mus-

cle, and angiogenesis (Kroetz and Zeldin, 2002; Spector et al., 2004). CYP2J2 is also highly expressed in tumor tissues and promotes tumor growth and proliferation (Jiang et al., 2005, 2009; Chen et al., 2011). Several drugs are metabolized by CYP2J2 including astemizole, ebastine, terfenadine, albendazole, amiodarone, and most recently vorapaxar (Matsumoto et al., 2003; Lee et al., 2010; Ghosal et al., 2011).

Most substrates identified for CYP2J2 are also metabolized by CYP3A4 and other isozymes (Lee et al., 2010); therefore, a specific reaction catalyzed by CYP2J2 is necessary to determine the contribution of CYP2J2 to overall P450-mediated drug metabolism. In our previous work, we identified amiodarone side chain hydroxylation as a CYP2J2-specific metabolic pathway based on P450 reaction phenotyping, which indicated that no other P450 appreciably contributed to the formation of this metabolite (Lee et al., 2010). A specific substrate/inhibitor pair for CYP2J2 may reveal a role for CYP2J2 in drug metabolism that may be underestimated, especially in extrahepatic tissues. CYP2J2-selective inhibition has been studied previously, and several compounds mostly related to the backbone structure of terfenadine have been identified (Lafite et al., 2006, 2007; Chen et al., 2009). However, selectivity of these agents against most

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ABBREVIATIONS: P450, cytochrome P450; RAF, relative activity factor; ISEF, intersystem extrapolation factor; HLM, human liver microsomes; HPLC, high-performance liquid chromatography; 1D, one dimensional; COSY, correlation spectroscopy; TOCSY, total correlation spectroscopy; HSQC, heteronuclear single quantum correlation; IS, internal standard; LC-MS/MS, liquid chromatography-tandem mass spectrometry; Cl_{int} , intrinsic clearance; DMSO, dimethyl sulfoxide; MRM, multiple reaction monitoring; CE, collision energies; DP, declustering potentials; NCE, new chemical entities; PF-05218881, (E)-3-(4-((2S,3S,4S,5R)-5-1-(3-chloro-2,6-difluorobenzyloxyimino)ethyl)-3,4-dihydroxytetrahydrofuran-2-yloxy)-3-hydroxyphenyl)-2-methyl-N(3aS,4R,5R,6S,7R,7aR)-4,6,7-trihydroxyhexahydrobenzo[d][1,3]dioxol-5-yl) acrylamide.

drug-metabolizing enzymes has not been determined. In addition, these compounds were all obtained through several steps of synthesis.

The aims of this study are as follows: 1) to fully characterize amiodarone hydroxylation as a CYP2J2 probe reaction, as well as the relative activity factor (RAF) and intersystem extrapolation factor (ISEF) for several individual preparations and one pooled human liver microsomal preparation; 2) to identify a readily available selective and potent CYP2J2 inhibitor by screening a panel of 138 marketed drugs; and 3) to refine a P450 cocktail assay in HLM that enables the evaluation of drug interactions incorporating potential involvement of CYP2J2.

Materials and Methods

General Chemicals and Reagents. All chemicals evaluated as inhibitors were purchased from Sigma-Aldrich (St. Louis, MO) and were used without further purification. Human CYP2J2 Supersomes (containing human CYP2J2, cytochrome P450 reductase and cytochrome *b*₅) and pooled human liver microsomes were purchased from BD Gentest (Woburn, MA). Terfenadine alcohol and terfenadine carboxylate were purchased from Ultrafine Chemical Co. (Manchester, England). High-performance liquid chromatography (HPLC)-grade ammonium acetate was purchased from Mallinckrodt Baker, Inc. (Phillipsburg, NJ). HPLC-grade acetonitrile and methanol were purchased from Honeywell Burdick & Jackson (Muskegon, MI).

Isolation of 3- and 4-Hydroxyamiodarone. Amiodarone was incubated at 37°C to a final concentration of 30 μ M in 100 mM potassium phosphate buffer (pH 7.4) containing 50 pmol/ml CYP2J2 Supersomes, 10 mM MgCl₂, and 1 mM NADPH. The total incubation volume was 60 ml, and total reaction time was 60 min. The incubation was quenched with 36 ml of acetonitrile and centrifuged at approximately 2000 relative centrifugal force for 10 min. The supernatant was removed and diluted to 600 ml with water containing 0.1% formic acid. The resulting solution was then centrifuged at approximately 40,000 relative centrifugal force for 30 min. Initial isolation of 4- and 3-hydroxyamiodarone was achieved using an Aqua C18 column (5 μ m, 10 $\times$ 250 mm; Phenomenex, Torrance, CA). The mobile phases consisted of 0.1% formic acid in water (mobile phase A) and acetonitrile (mobile phase B). The sample was introduced into the column via a loading pump (300 ml/run), and the metabolites were eluted with a linear gradient from 6% mobile phase B to 60% mobile phase B in 40 min at a flow rate of 4 ml/min. One-minute fractions were collected over the course of the run, and metabolite elution was monitored by UV detection (254 nm). Fractions containing 3- and 4-hydroxyamiodarone were combined and diluted with water containing 0.1% formic acid until the acetonitrile content was approximately 10%. Final isolation of the individual metabolites was achieved using a Luna C8 (2) column (5 μ m, 4.6 $\times$ 250 mm; Phenomenex). The mobile phases used were the same as those described above. The sample was introduced via a loading pump, and the metabolites were eluted using a linear gradient from 10% mobile phase B to 70% mobile phase B in 50 min at a flow rate of 1 ml/min. Metabolite elution was monitored as described above, and metabolites were collected manually.

Structural Characterization of 4- and 3-Hydroxyamiodarone by NMR. NMR spectra were recorded on a Bruker Avance 600 MHz system controlled by TOPSPIN (version 2.0), equipped with a 5-mm TCI CryoProbe (Bruker BioSpin Corporation, Billerica, MA). The 1D spectra were recorded using a sweep width of 12,000 Hz and a total recycle time of 7.2 s. The resulting time-averaged free induction decays were transformed using an exponential line broadening of 1.0 Hz to enhance signal to noise. Samples were dissolved in 0.15 ml of dimethyl sulfoxide-*d*₆ "100%" (Cambridge Isotope Laboratories, Andover, MA) and placed in 3-mm diameter tubes. All spectra were referenced using residual dimethyl sulfoxide-*d*₆ (¹H δ = 2.5 ppm and ¹³C δ = 39.5 relative to tetramethylsilane, δ = 0.00). Phasing, baseline correction, and integration were all performed manually. If needed, the BIAS and SLOPE functions for the integral calculations were adjusted manually. The final concentration of the isolated metabolites 4- and 3-hydroxyamiodarone were 0.31 and 0.22 mM, respectively, determined using the Siccio method (Walker et al., 2011). COSY, TOCSY, and multiplicity-edited HSQC data were recorded using the standard pulse sequences provided by Bruker. The 2D experiments were typically acquired using a 1K $\times$ 128 data matrix with 16 dummy scans. The data were zero-filled to a size of 1K $\times$ 1K. Unless

otherwise noted, for 2D experiments, a relaxation delay of 1.5 s was used between transients.

Formation of 4-Hydroxyamiodarone by Recombinant P450s. Assays were performed on a Biomek FX system (Beckman Coulter, Fullerton, CA). Amiodarone was incubated at a final concentration of 1 μ M with 11 different recombinant P450 isoforms (CYP1A2, -2A6, -2B6, -2C8, -2C9, -2C19, -2D6, -2E1, -2J2, -3A4, and -3A5) at final P450 concentration of 50 pmol/ml in 100 mM potassium phosphate buffer (pH 7.4) at 37°C with 3 mM MgCl₂. The reaction mixture was preincubated at 37°C before adding the NADPH-regenerating solution (10 mM NADP, 55 mM isocitric acid, 55 unit/ml isocitrate dehydrogenase). The final concentration of NADPH was 1 mM. A 50- μ l aliquot of the reaction mixture was removed at 0, 5, 10, 20, 30, and 45 min. Aliquots were quenched with 100 μ l of acetonitrile containing 500 ng/ml (*E*)-3-(4-((2S,3S,4S,5R)-5-1-(3-chloro-2,6-difluorobenzyloxyimino)ethyl)-3,4-dihydroxytetrahydrofuran-2-yloxy)-3-hydroxyphenyl)-2-methyl-N(3aS,4R,5R,6S,7R,7aR)-4,6,7-trihydroxyhexahydrobenzo[d][1,3]dioxol-5-yl) acrylamide (PF-05218881) [internal standard (IS)] and centrifuged at 2000 rpm for 10 min. Control incubations with each of the recombinant P450 isoforms were conducted without NADPH to monitor non-P450-mediated substrate disappearance. 4-Hydroxyamiodarone was quantified by liquid chromatography-tandem mass spectrometry (LC-MS/MS).

Relative Activity Factor and Intersystem Extrapolation Factor Determination. The CYP2J2 relative activity factor (RAF) was determined by monitoring the formation rate of 4-hydroxyamiodarone in HLM and recombinant CYP2J2 enzyme systems. The RAF value is the ratio of the activity of the probe substrate in HLM divided by the activity in recombinant P450, with HLM activity expressed as pmol $\cdot$ min⁻¹ $\cdot$ mg⁻¹ and recombinant P450 expressed as activity pmol $\cdot$ min⁻¹ $\cdot$ pmol CYP2J2⁻¹ such that the units are pmol CYP2J2/mg microsomal protein. The ISEF incorporates CYP2J2 content or abundance in the liver microsomal preparations and expressed as picomoles of CYP2J2 per milligram of microsomal protein. The ISEF value is determined by normalizing the RAF value to the CYP2J2 content in each HLM preparation, and therefore the ISEF value is unitless.

For recombinant CYP2J2, Cl_{int} was defined as the ratio of V_{max} and K_m determined by monitoring 4-hydroxyamiodarone formation rate under linear kinetic conditions. The kinetic parameters for CYP2J2 were determined under the following conditions: 0, 0.06, 0.12, 0.23, 0.46, 0.92, 1.9, 3.8, 7.5, 15, 30, and 60 μ M amiodarone, 40 pmol/ml recombinant CYP2J2, and 1 mM NADPH in 100 mM potassium phosphate buffer (pH 7.4) at 37°C. The reaction mixture was preincubated at 37°C for 5 min before adding the NADPH-regenerating solution (10 mM NADP, 55 mM isocitric acid, 55 unit/ml isocitrate dehydrogenase). Fifty microliters of the reaction mixture was removed after 20 min. The HLM Cl_{int} value was generated as the ratio of the formation rate of 4-hydroxyamiodarone divided by amiodarone concentration in the incubation. The HLM Cl_{int} value under linear conditions is similar to the ratio of V_{max} divided by K_m obtained by a complete Michaelis-Menten kinetic study. For the determination of HLM Cl_{int} value, individual prepared HLM or pooled HLM were incubated with amiodarone (at a concentration approximating its K_m value of 5 μ M), 0.4 mg/ml HLM and 1 mM NADPH in 100 mM potassium phosphate buffer (pH 7.4) at 37°C. A 50- μ l reaction mixture was removed after 20-min incubation time. 4-Hydroxyamiodarone formation was monitored by LC-MS/MS.

Determination of CYP2J2 Content in HLM. Mouse anti-human CYP2J2 antibody (Abnova, Walnut, CA) was used to detect and quantitate human CYP2J2 in HLM samples from the University of Washington, School of Pharmacy human tissue bank. Liver microsomal protein (50 μ g), and BD Gentest CYP2J2+OR+B₅ Supersomes as standards (0.1, 0.05, and 0.025 pmol/ μ l), were electrophoresed in NuPage Bis-Tris 12-well gels (gradient 8–12%) and transferred to polyvinylidene difluoride membranes. Blots were incubated with primary antibody for 4 h followed by secondary antibody (goat anti-mouse). Protein bands were visualized using an Odyssey infrared imager (Li-Cor Biosciences, Lincoln, NE), following the manufacturer's instructions, and quantified using a calibration curve generated from CYP2J2+OR+B₅ Supersomes.

Correlation Analysis of 4-Hydroxyamiodarone and Astemizole O-Demethylation Activity in Individually Prepared HLM and Pooled HLM. Amiodarone (5 μ M) or astemizole (0.3 μ M) (both at K_m) were incubated with various individual, prepared HLM and pooled HLM at a final concentration of 0.1 mg/ml (astemizole) or 1 mg/ml (amiodarone) in 100 mM potassium

TABLE 1

Ionization mode, *m/z* transitions, and retention times for 4- and 3-hydroxyamiodarone

<i>m/z</i> Ratios for 4- and 3-Hydroxyamiodarone					
Analyte	Q1 <i>m/z</i>	Q3 <i>m/z</i>	DP	CE	Retention Time (min)
4-Hydroxyamiodarone	662.4	100	60	40	12.7
3-Hydroxyamiodarone	662.4	100	60	40	13.2

phosphate buffer (pH 7.4) at 37°C. The reaction mixture was preincubated at 37°C before adding the NADPH-regenerating solution (10 mM NADP, 55 mM isocitric acid, and 55 unit/ml isocitrate dehydrogenase). Final concentration of NADPH was 1 mM. A 50- μ l aliquot of reaction mixture was removed at 5 or 20 min for astemizole or amiodarone, respectively. The aliquots were quenched with 100 μ l of acetonitrile containing 500 ng/ml of IS, PF-05218881. The amount of 4-hydroxyamiodarone or astemizole O-demethylated metabolites formed was monitored by LC-MS/MS.

Incubation Conditions for CYP2J2 Inhibition Screen. Recombinant CYP2J2 Supersomes (0.1 pmol/incubation), 0.2 μ M terfenadine (in methanol) or 1 pmol recombinant CYP2J2/incubation and 0.3 μ M astemizole (in ethanol) (both substrates at K_m), and potassium buffer (100 mM, pH 7.4, 200 μ l final volume) were incubated with each inhibitor [30 μ M final concentration in dimethyl sulfoxide (DMSO)] in a 96-well polypropylene plate (Nunc, Naperville, IL) and prewarmed at 37°C for 5 min. The reactions were initiated by addition of NADPH (1 mM final concentration in water). The final DMSO concentration in the incubations was 1% (v/v). Control incubations with DMSO and no inhibitor with or without the addition of methanol or ethanol to investigate solvent effects were compared with reactions without DMSO, methanol, or ethanol, and no significant differences in CYP2J2 activity were observed. After a 5-min reaction time, incubations were quenched with 200 μ l of ice-cold acetonitrile containing internal standard (clemizole for terfenadine incubations and norastemizole for astemizole incubations), immediately vortexed, and placed on ice. After cooling for 10 min, quenched samples were centrifuged at 14,000g for 5 min at room temperature. The supernatant was directly analyzed by LC-MS (terfenadine) or LC-MS/MS (astemizole).

Development of the Seven P450 Probe Substrate Cocktail for Drug Interactions Screening. The rate of metabolite formation of the cocktail probe substrates was monitored in the presence and absence of astemizole (0.3 μ M) or amiodarone (5 μ M) to evaluate whether the CYP2J2 probe would alter their metabolism. The final concentration of the P450 probe cocktail consisted of phenacetin (10 μ M, CYP1A2), paclitaxel (5 μ M, CYP2C8), diclofenac (5 μ M, CYP2C9), *S*-mephenytoin (40 μ M, CYP2C19), dextromethorphan (5 μ M, CYP2D6), and midazolam (2 μ M, CYP3A4) with either astemizole or amiodarone in 100 mM potassium phosphate buffer (pH 7.4) at 37°C. The 300- μ l reaction mixture was preincubated at 37°C for 15 min before adding the NADPH-regenerating solution (10 mM NADP, 55 mM isocitric acid, 55 unit/ml isocitrate dehydrogenase, and final concentration of NADPH was 1 mM). The reaction proceeded for an additional 8 min and was quenched with 600 μ l of acetonitrile containing 100 ng/ml IS, PF-05218881. The amount of metabolite formed from each probe substrate was monitored by LC-MS/MS.

Evaluation of Single Probe Versus Cocktail P450 Probe Substrate Assay. To confirm the robustness of the seven P450 probe substrate assay, a comparison of IC_{50} values was determined using the seven probe cocktail assay and the single probe substrate astemizole in pooled HLM. The seven P450 probe cocktail consisted of 10 μ M phenacetin, 5 μ M paclitaxel, 5 μ M

diclofenac, 40 μ M *S*-mephenytoin, 5 μ M dextromethorphan, 0.3 μ M astemizole, and 2 μ M midazolam. IC_{50} values were determined for danazol, pimozone, miconazole, or terfenadine using the seven P450 probe cocktail or astemizole (0.3 μ M) alone. The final inhibitor concentration range was 0.1, 0.3, 1, 3, 10, and 30 μ M. The 300- μ l reaction mixture containing inhibitor and seven P450 probe substrate or astemizole alone were preincubated at 37°C for 15 min before adding the NADPH-regenerating solution (10 mM NADP, 55 mM isocitric acid, 55 unit/ml isocitrate dehydrogenase, and final concentration of NADPH was 1 mM). The reaction proceeded for an additional 8 min before being quenched with 600 μ l of acetonitrile containing 100 ng/ml IS, PF-05218881. Metabolite formation of astemizole was monitored by LC-MS/MS.

Mechanism of Inhibition and K_i for Danazol Using Astemizole As Probe Substrate. Determination of K_i was performed with recombinant CYP2J2 (2 pmol/ml), diluted in 100 mM potassium phosphate buffer (pH 7.4), supplemented with astemizole (final concentration 0.15, 0.3, 0.6, 1.2, 2.4 μ M) and inhibitor danazol (0, 0.05, 0.1, 0.2, 0.4 μ M), and preincubated for 5 min at 37°C in a shaking water bath. Reactions were initiated by adding NADPH (1 mM final concentration) and allowed to proceed for 5 min. Incubations were quenched by adding an equal volume of ice-cold acetonitrile supplemented with internal standard (0.05 μ M terfenadine). Samples were vortexed then centrifuged for 10 min. Calibration standards were performed under assay conditions with heat-inactivated recombinant CYP2J2. Solvent concentrations were corrected for and are equal in each assay (0.01% DMSO, 0.4% ethanol, as lowest possible solvent concentrations due to low solubility of danazol).

For the determination of potential time and mechanism-based inhibition, recombinant CYP2J2 (0.5 pmol $\cdot$ ml⁻¹) was mixed with danazol (0.0003–10 μ M) and preincubated with and without 1 mM NADPH for 30 min. The reactions were initiated by the addition of 0.3 μ M astemizole. Quenching and analysis of incubations were similar to those described in the previous paragraph. To confirm time and mechanism-based inhibition, a dilution assay was also performed. Recombinant CYP2J2 (10 pmol/ml) in 100 mM potassium phosphate buffer (pH 7.4) was supplemented with 20 nM danazol only (time dependent), 20 nM danazol and 1 mM NADPH (mechanism-based), and with or without NADPH (controls). After each 0- and 30-min preincubation time, incubations were diluted 10-fold into an activity assay mixture containing 1 mM NADPH and 0.3 μ M astemizole (final concentration) in 200 μ l of 100 mM potassium phosphate buffer (pH 7.4). Incubation time for the activity assays was 5 min, and samples were processed as described in the paragraph above. Analysis of astemizole metabolites is described under *Analytical Methods*.

Analytical Methods. Quantification of 4- and 3-hydroxyamiodarone. A 10- μ l aliquot of quenched incubation sample was injected onto a Phenomenex Kromasil C4 (150 $\times$ 2 mm 3.5 μ ; Phenomenex) HPLC column with a CTC PAL autosampler (LEAP Technologies, Carboro, NC) and an integrated HPLC pumping system (Shimadzu Scientific Instruments, Columbia, MD). These compounds were then eluted and detected by an API 4000-triple quadrupole mass spectrometer (Applied Biosystems/MDS Sciex, Foster City, CA) fitted with a TurboIonSpray interface. Mobile phase A was 0.1% formic acid, mobile phase B was acetonitrile with 0.1% formic acid, and the flow rate was 0.2 ml/min. The starting condition for the HPLC gradient was 80:20 (A/B), and this condition was held for 0.3 min. From 0.3 to 9 min, the mobile phase composition changed linearly to 60:40 (A/B). This condition was held for 11 min. The gradient was returned in a linear fashion to 80:20 (A/B) from 11 min to 13.9 min and re-equilibrated until 15 min. Multiple

TABLE 2

m/z transitions and ionization mode for O-desmethyl astemizole and internal standard, PF-05218881

<i>m/z</i> Ratios for Desmethyl Astemizole and Internal Standard					
Analyte	Q1 <i>m/z</i>	Q3 <i>m/z</i>	DP	CE	CXP
Desmethyl astemizole	445.1	121.1	40	50	15
PF-05218881	687	320	60	30	15

CXP, collision cell exit potential.

TABLE 3

MRM transitions, collision energies, and declustering potentials for each P450 probe

Metabolite	P450	MRM	CE	DP
Acetaminophen	CYP1A2	<i>m/z</i> 152.0 > 110.0	20	61
6 α -Hydroxypaclitaxel	CYP2C8	<i>m/z</i> 870.5 > 525.4	25	80
4-OH-diclofenac	CYP2C9	<i>m/z</i> 312.3 > 231.1	30	60
4-OH- <i>S</i> -Mephenytoin	CYP2C19	<i>m/z</i> 235.2 > 150.1	25	71
Dextromethorphan	CYP2D6	<i>m/z</i> 258.1 > 199.1	40	80
Desmethyl astemizole	CYP2J2	<i>m/z</i> 445.1 > 121.1	50	40
1-OH-Midazolam	CYP3A4	<i>m/z</i> 342.3 > 203.1	40	60
Internal standard	PF-5218881	<i>m/z</i> 687 > 320	30	60

TABLE 4
Proton and ^{13}C NMR chemical shifts for amiodarone, M1, and M2

Amiodarone HCl			M1 (4-Hydroxy Amiodarone)		M2 (3-Hydroxy Amiodarone)	
	^1H Chemical shift in ppm (multiplicity, coupling constants, integration)	^{13}C Chemical shift in ppm	^1H Chemical shift in ppm (multiplicity, coupling constants, integration)	^{13}C Chemical shift in ppm	^1H Chemical shift in ppm (multiplicity, coupling constants, integration)	^{13}C Chemical shift in ppm
4	7.47 (d, $J = 7.8$, 1H)	120.9	7.42 (d, $J = 7.8$, 1H)	121.1	7.47 (d, $J = 7.4$, 1H)	120.3
5	7.29 (t, $J = 7.5$, 1H)	124.0	7.28 (t, $J = 7.6$, 1H)	124.4	7.29 (t, $J = 7.6$, 1H)	124.1
6	7.37 (t, $J = 7.5$, 1H)	124.9	7.36 (t, $J = 7.8$, 1H)	125.4	7.35 (t, $J = 7.4$, 1H)	124.4
7	7.67 (d, $J = 8.6$, 1H)	111.2	7.66 (d, $J = 8.2$, 1H)	111.5	7.67 (d, $J = 8.2$, 1H)	110.9
2'/6'	8.19 (s, 2H)	139.7	8.15 (s, 2H)	140.0	8.14 (s, 2H)	139.7
1 bubButyl	2.73 (t, $J = 7.3$, 2H)	27.6	2.75 (t, $J = 7.7$, 2H)	28.1	2.71/2.86 (cm, 2H)	24.5
2 Butyl	1.69 (cm, 2H)	29.4	1.73 (cm, 2H)	24.6	1.74 (cm, 2H)	36.3
3 Butyl	1.26 (cm, 2H)	22.0	1.39 (cm, 2H)	32.3	3.52 (bs, 1H)	65.5
4 Butyl	0.84 (t, $J = 7.4$, 3H)	13.2	3.36 (cm, 1H)	60.5	1.00 (d, $J = 6.2$, 3H)	23.6
OH	NA	NA	4.39 (t, $J = 5.2$, 1H)	NA	4.55 (s, 1H)	
1 DEAE	4.38 (t, $J = 4.9$, 2H)	67.2	4.04 (bs, 2H)	<i>a</i>	4.02 (t, $J = 6.7$, 2H)	71.6
2 DEAE	3.67 (q, $J = 4.6$, 2H)	49.9	2.99 (bs, 2H)	<i>a</i>	2.96 (t, $J = 6.7$, 2H)	51.6
4 DEAE	3.37 (cm, 4H)	47.1	2.64 (bs, 4H)	<i>a</i>	2.60 (q, $J = 7.4$, 4H)	47.5
5 DEAE	1.33 (t, $J = 7.2$, 6H)	8.6	1.02 (t, $J = 7.1$, 6H)	<i>a</i>	1.00 (t, $J = 7.1$, 6H)	12.4

d, doublet; t, triplet; q, quartet; s, singlet; cm, complex multiplet; bs, broad singlet; NA, not applicable; DEAE, diethyl amino ethanol.

^a No $^1\text{H}/^{13}\text{C}$ cross peak was observed.

reaction monitoring (MRM) was used to monitor the compounds. Table 1 lists the ionization mode, m/z transitions, and retention times for 4- and 3-hydroxyamiodarone.

Analysis of astemizole *O*-demethylated metabolite formation. A 20- μL aliquot of the quenched incubation sample containing *O*-desmethyl astemizole was injected onto a Synergi Polar-RP ($2 \times 30 \text{ mm } 4 \mu\text{m}$; Phenomenex) HPLC column with a CTC PAL autosampler (LEAP Technologies) and an integrated HPLC pumping system (Shimadzu Scientific Instruments). The metabolite was eluted and detected by an API 4000-triple quadrupole mass spectrometer (Applied Biosystems/MDS Sciex) fitted with a TurboIonSpray interface. Mobile phase A was 0.1% formic acid, mobile phase B was acetonitrile with 0.1% formic acid, the flow rate was 0.8 mL/min. The initial condition for the HPLC gradient was 99:1 (A/B). This condition was held for 0.3 min. From 0.3 to 1.2 min, the mobile phase composition changed linearly to 1:99 (A/B). This condition was held for 1.9 min. The gradient was returned in a linear fashion

to 99:1 (A/B) from 1.9 to 1.95 min and re-equilibrated until 2 min. MRM was used to monitor the compounds. Table 2 lists the m/z transitions and ionization mode for *O*-desmethyl astemizole and internal standard, PF-05218881. The retention times were 0.94 and 0.97 min for *O*-desmethyl astemizole and internal standard, respectively. The peak area ratio of the analyte to the internal standard was determined for each injection and used to quantify the amount of metabolite formed.

Astemizole inhibition screening assay. Metabolites and standards were measured by LC-MS/MS, performed on a Waters Quattro Premier XE Micromass system coupled to a Waters ACQUITY Sample Manager and Binary Solvent Manager (UPLC; Waters, Milford, MA), and detected by electrospray ionization (source temperature 350°C , capillary 3.5 kV, cone 45V, extractor 5V, cone gas flow $21 \cdot \text{h}^{-1}$, desolvation gas flow $800 \cdot \text{h}^{-1}$). Twenty-five microliters of sample was loaded onto an ACQUITY UPLC BEH Phenyl ($1.7 \mu\text{m}$, $2.1 \times 50 \text{ mm}$, column heat 50°C) column at a flow rate of 3.5 mL/min. Mobile phase A was ammonium formate (20 mM, pH 9.4) and mobile phase B was acetonitrile. The initial condition for the HPLC gradient was 90:10 (A/B). This was held for 0.5 min. From 0.5 to 1.5 min, the mobile phase composition changed linearly to 100% B and was maintained for 3.5 min. From 3.5 to 4.0 min, the gradient was returned to 90:10 (A/B). Mass ions were identified by fragmentation of 445.1 to 120.9 m/z (desmethyl astemizole: dwell 0.05 s, cone 50.0 V, collision 40.0 V) and 472.13 to 436.16 (terfenadine 3: 0.05 s, 35.0 V, 30.0 V).

Terfenadine hydroxylation inhibition screening assay. Analysis of terfenadine hydroxylation was performed on an HP 1100 LC/MSD system (Agilent Technologies, Santa Clara, CA) quadrupole mass spectrometer coupled to an HPLC system. Positive ions were generated from an electrospray ionization at 350°C . A 40- μL aliquot of quenched sample was loaded onto a reverse-phase HPLC system using a Zorbax Extend C-18 column ($5 \mu\text{m}$, $2.1 \times 50 \text{ mm}$; Agilent Technologies) at a flow rate of 300 $\mu\text{L}/\text{min}$. Chromatographic separation of terfenadine alcohol and potential terfenadine carboxylate was attained with a three-step linear gradient. Mobile phase A was 10 mM ammonium acetate, pH 5.5, and mobile phase B was methanol. The initial condition for the HPLC gradient was 50:50 (A/B) and increased linearly to 10:90 (A/B) to 2

FIG. 1. Time-dependent formation of 4-hydroxyamiodarone metabolite (M1) by recombinant CYP2J2 and pooled HLM. No other isozyme had any appreciable formation of 4-hydroxyamiodarone.

TABLE 5

Formation of CYP2J2-mediated metabolites of astemizole and amiodarone in various HLM preparations

HLM	CYP2J2 Content pmol/mg	Astemizole Desmethylation $\text{pmol}^{-1} \cdot \text{min} \cdot \text{pmol}^{-1}$ CYP2J2 (mean $\pm$ S.D.)	4-Hydroxyamiodarone $\text{pmol}^{-1} \cdot \text{min} \cdot \text{pmol}^{-1}$ CYP2J2 (mean $\pm$ S.D.)
130	0.047	1357 $\pm$ 77	82 $\pm$ 2
128	0.165	254 $\pm$ 18	18.8 $\pm$ 0.9
118	0.900	38 $\pm$ 3	7.2 $\pm$ 0.2
119	0.567	172 $\pm$ 12	12.7 $\pm$ 0.3
153	0.941	80 $\pm$ 34	7.9 $\pm$ 0.1
133	1.578	39 $\pm$ 2	2.8 $\pm$ 0.1
149	0.912	80.7 $\pm$ 0.6	4.5 $\pm$ 0.2
144	1.131	77 $\pm$ 1	3.2 $\pm$ 0.2
109	2.20	98 $\pm$ 1	0.9 $\pm$ 0.2
128	1.20	196 $\pm$ 7	2.1 $\pm$ 0.2
159	2.40	87 $\pm$ 17	0.9 $\pm$ 0.1
129	3.50	93 $\pm$ 3	1.6 $\pm$ 0.1
146	7.60	44 $\pm$ 6	0.5 $\pm$ 0.0
Pooled HLM	2.59	32 $\pm$ 1	3.1 $\pm$ 0.1

min, followed by a 2-min hold and then back to 50:50 (A/B) over 1 min. ChemStation Rev. A. 10.02 (Agilent Technologies) was used to set the selection of ion windows for single ion monitoring data acquisition. The mass ions monitored were m/z 488.0, 502.0, and 326.6 corresponding to terfenadine alcohol, terfenadine carboxylate, and clemizole (the internal standard), respectively. Dwell times were set at 889 ms per ion.

Six versus seven P450 probe cocktail assay. The reaction monitoring (MRM) LC-MS/MS analysis was conducted on an ABI 4000 Q TRAP Mass Spectrometer (Applied Biosystems, Foster City, CA) using a turbo ion spray source in positive ionization mode. MRM transitions, collision energies (CE), and declustering potentials (DP) are listed in Table 3.

Samples were separated using a Phenomenex Onyx Monolithic C18, 4.6 $\times$ 50 mm HPLC column with a CTC PAL autosampler (LEAP Technologies) and an integrated HPLC pumping system (Shimadzu Scientific Instruments). Mobile phase A was 0.1% formic acid in water and mobile phase B was acetonitrile with 0.1% formic acid. At the beginning of the injection, the primary gradient pump flow rates were 0.2 ml/min 99:1 (A/B), and the dilution pump flow rate was 2.8 ml/min of A (100%). After the analytes were loaded onto the column, the dilution pump was stopped (minimal flow was maintained at 0.01 ml/min flow to prevent back flow) and the primary gradient pumps were ramped to 3.0 ml/min to initiate the gradient. The primary gradient was changed to 90:10 (A/B) and held for 0.42 min. From 0.42 to 0.6 min, the mobile phase composition changed linearly to 75:25 (A/B). From 0.6 to 1.45 min, the mobile phase composition changed linearly to 35:65 (A/B). This condition was held to 1.57 min. The gradient was returned in a linear fashion to 90:10 (A/B) from 1.57 to 1.58 min and re-equilibrated for 1.9 min. The injection volume was 20 μ l. The metabolite concentrations were calculated using Analyst 1.4 software (Applied Biosystems, Carlsbad, CA).

Fig. 2. Correlation of astemizole O-demethylation and amiodarone 4-hydroxylation in a panel of individual HLM preparations.

Statistical Analysis. The general screen samples were conducted in duplicate and reported as the average, whereas IC_{50} and K_i experiments were performed in triplicate and reported as the average $\pm$ S.D. IC_{50} and K_i data analyses were performed by nonlinear regression using GraphPad Prism (version 5.02; GraphPad Software Inc., San Diego, CA).

Results

Structural Characterization of 4 and 3-Hydroxyamiodarone Metabolites. In a previous study that screened for CYP2J2 substrates, amiodarone was oxidized by CYP2J2 to a hydroxylated metabolite minimally formed by CYP3A4. Further analysis of the hydroxylated metabolite of amiodarone showed that this product was a mixture of two metabolites, 4- and 3-hydroxyamiodarone (Table 4). Comparison of the 1H spectrum of the M1 isolate with a similarly acquired spectrum of amiodarone revealed the absence of the terminal methyl of the butyl side chain and the presence of a new set of resonances at δ 3.36 and δ 4.39. COSY and TOCSY data of the M1 isolate indicated connectivity between the two new resonances and the other 1H resonances of the butyl side chain (see Supplemental Figs. 1–3). All other acquired NMR and mass spectral data are consistent with the structure of M1 as 4-hydroxyamiodarone analog of amiodarone.

The M2 isolate was identified as the 3-hydroxybutyl analog of amiodarone. Comparison of the 1H spectrum of the M2 isolate with a similarly acquired spectrum of amiodarone revealed the absence of the terminal methyl of the butyl side chain (triplet, δ 0.84, $J = 7.4$ Hz) and the presence of a new doublet (δ 1.00, $J = 6.2$ Hz) (Table 4). In addition, COSY and TOCSY data of the M2 isolate indicated connectivity between the new doublet and a broad singlet (not observed in amiodarone) at δ 3.52. Multiplicity-edited HSQC data contain a correlation between this broad singlet and a ^{13}C resonance at δ 65.5. The above data and all other acquired NMR data are consistent with the structure of M2 as the 3-hydroxybutyl analog of amiodarone (Supplemental Figs. 4–6).

4-Hydroxyamiodarone Is Specifically Formed by CYP2J2. P450 reaction phenotyping studies conducted with human liver microsomes (pooled HLM) and a panel of recombinant P450s (CYP1A2, -2B6, -2C8, -2C9, -2C19, -2D6, -2E1, -2J2, -3A4, and -3A5) revealed that the formation of 4-hydroxyamiodarone was CYP2J2-specific and was not formed by other drug-metabolizing enzymes, whereas 3-hydroxyamiodarone was formed by both CYP2J2 and CYP3A4 (data not shown). For CYP2J2, the formation of 4-hydroxyamiodarone was linear to 20 min as shown in Fig. 1 and metabolite formation followed Michaelis-Menten kinetics (Supplemental Fig. 7). Small levels of the 4- and 3-hydroxyamiodarone were detected in HLM (4-hydroxyamiodarone is shown in Fig. 1, but 3-hydroxyamiodarone data are not shown).

Examination of CYP2J2-Mediated Astemizole Demethylation and Amiodarone 4-Hydroxylation in Various HLM Preparations. CYP2J2 protein levels were quantified by Western blot analysis for 13

TABLE 6

Relative activity factor and intersystem extrapolation factor for CYP2J2 in HLM

HLM	CYP2J2 Content in HLM pmol/mg	RAF (CL_{im}) pmol/mg	CYP2J2 ISEF
130	0.047	0.0008	0.01760
165	0.002	0.0016	1.03472
128	0.165	0.0007	0.00401
118	0.900	0.0014	0.00154
119	0.567	0.0015	0.00271
153	0.941	0.0016	0.00169
133	1.578	0.0009	0.00060
149	0.912	0.0009	0.00096
144	1.131	0.0008	0.00068
Pooled HLM	2.590	0.0017	0.00066

TABLE 7

Percentage of inhibition of terfenadine hydroxylation (terfenadine) and astemizole O-demethylation (astemizole)

Values are average of duplicates.

Drug	% Activity Remaining Terfenadine (Astemizole)	Drug	% Activity Remaining Terfenadine (Astemizole)
Lansoprazole	0.7 (71.1)	Albendazole	29.3 (65.5)
Orphenadrine	1.2 (102.2)	Quercetin	29.8 (44.7)
Verapamil	1.3 (69.1)	Paroxetine HCl	31.6 (39.0)
Danazol	1.6 (8.0)	Mevinolin	37.7 (13.6)
Cisapride	3.2	Amiodarone	39.0 (18.7)
Miconazole	3.4 (10.1)	Methadone	43.0 (123.4)
Ketoconazole	5.8 (5.4)	Domperidone	43.5 (50.5)
Astemizole	8.3	Clomiphene	44.2 (108.8)
Nicardipine	8.9 (5.8)	Fluoxetine	44.3 (87.0)
Loratadine	11.4 (4.0)	Ranolazine	45.1 (204.1)
Tamoxifen	11.5 (9.1)	Nortriptyline	45.3 (70.1)
Clotrimazole	14.1 (5.6)	Methadone	45.7 (123)
Simvastatin	14.3 (5.0)	Omeprazole	46.1 (70.5)
Haloperidol	15.6 (14.7)	Fluvoxamine	48.7 (54.4)
Mefloquine	22.4 (44.6)	Cyclobenzaprine	48.9 (65.9)
Amodiaquine	22.5 (7.7)	Budesonide	50.8
Isradipine	23.7 (13.6)	Clozapine	51.3 (25.6)
Ivermectin	26.2 (20.9)	Quinapril	51.4 (64.1)
Pimozide	27.29 (6.6)	Thioridazine	51.7 (12.9)

individually prepared HLM and one pooled HLM samples. The CYP2J2 content (pmol CYP2J2/mg microsomal protein) was variable and ranged from 0.047 to 7.60 pmol/mg in the individual HLM samples (obtained from the School of Pharmacy human tissue bank). The pooled HLM sample contained 2.59 pmol/mg CYP2J2 (Table 5). The CYP2J2 protein content did not correlate with CYP2J2-catalytic activity assessed by either astemizole demethylation or 4-hydroxyamiodarone, as seen in Table 5. However, the catalytic activity measured for the two CYP2J2 probe substrates correlated, $r^2 = 0.97$ (Fig. 2). It is acknowledged that HLM 130 generates high CYP2J2 activity despite very low levels quantified by Western blot analysis, which greatly influenced the correlation analysis. Attempts were made to add additional liver samples to the correlation analysis, but no HLM samples were found to have activity between the cluster of HLM samples and HLM 130. It is duly noted that if one were to remove HLM 130, the r^2 value falls to 0.47, which indicates a lower level of

correlation; however, there is no scientific rationale to exclude HLM 130 from the correlation analysis.

RAF and ISEF for CYP2J2. The kinetic studies for recombinant CYP2J2 and HLM were conducted under linear conditions. Studies were conducted using recombinant CYP2J2, nine individually prepared HLM samples, and a single pooled HLM sample consisting of 60 livers. For recombinant CYP2J2, the Cl_{int} value was determined by taking the ratio of the V_{max} for 4-hydroxyamiodarone rate to its K_m value. For the HLM studies, the 4-hydroxyamiodarone rate was determined at an amiodarone substrate concentration approximating the K_m (5 μ M). For HLM, the Cl_{int} value was determined by taking the ratio of the 4-hydroxyamiodarone rate to substrate concentration. The CYP2J2 RAF values were determined as the ratio of the Cl_{int} in HLM to Cl_{int} in recombinant CYP2J2, with the units of pmol CYP2J2/mg HLM (Table 6). In the individually prepared HLM samples, the RAF based on Cl_{int} ranged from 0.0007 to 0.0017 and the RAF value determined using pooled HLM value was 0.0017. The ISEF values ranged from 0.0006 to 1.03 for the individually prepared HLM samples and 0.00066 for pooled HLM.

Screening 138 Drugs as CYP2J2 Inhibitors. From the 138 compounds screened for CYP2J2 inhibitors (Supplemental Table 1), 42 compounds were identified that inhibit terfenadine hydroxylation by 50% or more at 30 μ M, whereas eight compounds (danazol, ketoconazole, lansoprazole, loratadine, miconazole, nicardipine, orphenadrine, and verapamil) markedly reduced CYP2J2 activity by 90% or more (Table 7).

To identify a specific CYP2J2 inhibitor, the top 40 chemicals identified that inhibited terfenadine hydroxylation by 50% or more were also screened for inhibition of astemizole O-demethylation activity. Twenty-four compounds inhibited astemizole metabolism by 50% or more, whereas danazol, ketoconazole, loratadine, miconazole, and nicardipine inhibited terfenadine or astemizole metabolism by 90% or more (Table 7). To further refine the selection of a potent substrate-independent CYP2J2 inhibitor, an inhibition screen was conducted in pooled human liver microsomes with six selective probe substrates to test the inhibitory potential of the top 20 drugs identified as inhibitors of CYP2J2-mediated terfenadine and astemizole metabolism against CYP1A2, CYP2C8, CYP2C9, CYP2C19, CYP2D6, and CYP3A4 to determine respective IC_{50} values (Table 8). Five com-

TABLE 8
 IC_{50} values of potential CYP2J2 inhibitors against other P450s

Drug	IC_{50} (μ M)					
	CYP1A2	CYP2C8	CYP2C9	CYP2C19	CYP2D6	CYP3A4
Albendazole	>30	>30	>30	>30	>30	>30
Astemizole	>30	>30	>30	13.7	>30	10.6
Bepidil	>30	>30	>30	<0.1	>30	>30
Clotrimazole	1.25	0.803	0.136	7.41	0.883	<0.1
Danazol	>30	1.95	1.44	>30	2.74	>30
Haloperidol	>30	>30	>30	2.87	>30	8.20
Isradipine	28.3	5.00	3.66	>30	4.79	>30
Ivermectin	>30	>30	>30	>30	>30	>30
Ketoconazole	26	2.45	8.94	10.1	16.3	<0.1
Lansoprazole	6.46	5.75	19.2	13.4	2.90	>30
Loratadine	>30	2.95	>30	2.80	<0.1	>30
Mefloquine	>30	>30	>30	14.9	>30	0.667
Nicardipine	>30	1.56	0.378	3.30	1.59	>30
Orphenadrine	>30	>30	>30	24.2	>30	>30
Paroxetine	5.33	>30	>30	>30	0.96	24.1
Pimozide	>30	>30	>30	>30	>30	>30
Quercetin	1.55	1.39	10.2	10.5	18.8	2.11
Simvastatin	>30	3.70	>30	>30	>30	>30
Tamoxifen	>30	14.3	>30	>30	13.7	>30
Verapamil	>30	>30	>30	>30	>30	17.6

FIG. 3. Structure of danazol.

pounds (danazol, ketoconazole, loratadine, miconazole, and nicardipine) significantly reduced terfenadine or astemizole metabolism. However, only danazol (Fig. 3) seemed selective against CYP2J2 and modestly inhibited CYP2C8, CYP2D6, and CYP2C9 with IC_{50} values of 1.95, 2.74, and 1.44 μ M, respectively, and did not appreciably inhibit CYP3A4, CYP1A2, or CYP2C19 at concentrations as high as 30 μ M. The remaining compounds were either not potent inhibitors against both terfenadine and astemizole oxidation or inhibited at least one other cytochrome P450 in the low nanomolar range and were excluded from further consideration.

Seven Probe Substrate Cocktail. A seven probe cocktail that included CYP2J2 contribution was established containing phenacetin (CYP1A2), paclitaxel (CYP2C8), diclofenac (CYP2C9), *S*-mephenytoin (CYP2C19), dexamethorphan (CYP2D6), astemizole (CYP2J2), and midazolam (CYP3A4). Initial evaluation of the seven cocktail probes was conducted with amiodarone as the CYP2J2 probe substrate; however, addition of amiodarone resulted in substantial inhibition of other P450-mediated pathways. More specifically, amiodarone (5 μ M) inhibited CYP2C8, CYP2C9, CYP2C19, and CYP2D6, resulting in the decrease in metabolite formation from these isozymes by 33, 68, 29, and 37%, respectively (Table 9). When astemizole (0.3 μ M) was included as the seventh probe substrate, only marginal inhibition of CYP3A4 activity (20%) was observed with minimal effect on the other isoforms (Table 9). Four compounds (miconazole, pimozone, danazol, and terfenadine) were used to validate the seven probe cocktail in which CYP2J2-related IC_{50} values generated were similar to values measured in the single probe astemizole assay only (Table 10).

Recombinant CYP2J2 Inhibition Constants (IC_{50} and K_i) for Danazol. IC_{50} values were ascertained using both inhibition of terfenadine hydroxylation (0.077 ± 0.001 μ M) and astemizole O-demethylation (0.019 ± 0.006 μ M) (Fig. 4A) using recombinant CYP2J2 enzyme. A K_i of 20 nM for danazol was calculated based on astemizole O-demethylation activity (Fig. 4B), and the kinetics fit to a model of mixed inhibition with a high component of competitive inhibition ($\alpha = 18.3$). However, a precise characterization of the mechanism was severely hampered by 1) the assay's limit of quantitation (nanomolar range) and the distortion caused by substrate depletion, which was unavoidable at low substrate concentrations (astemizole-O-demethylation K_m value of 0.3 μ M); 2) a potential allosteric component of astemizole-O-demethylation; and 3) a possible residual solvent effect. A slight IC_{50} shift from 0.019 to 0.012 μ M using a 30-min preincubation in the presence of inhibitor and NADPH, but in absence of substrate, was found

(Fig. 4A) but could not be confirmed in dilution experiments commonly used for the determination of mechanism-based inhibition.

Discussion

In this study, we report 4-hydroxyamiodarone as a specific CYP2J2 metabolite, devoid of contributions by other drug-metabolizing isoforms. Moreover, 4-hydroxyamiodarone formation correlated with *O*-desmethyl astemizole, a previously reported metabolite of CYP2J2 (Matsumoto et al., 2002), in a panel of individually prepared HLM. CYP2J2 enzyme activity did not appear to correlate with protein content determined by Western blotting, as similarly observed by other investigators (Yamazaki et al., 2006). We also identified danazol as a selective inhibitor of CYP2J2, as assessed by inhibition of terfenadine hydroxylation ($IC_{50} = 77$ nM) and astemizole O-demethylation ($K_i = 20$ nM). Danazol is specific for CYP2J2 at low concentrations (~ 0.5 μ M), but higher concentrations can inhibit other isoforms such as CYP2C9, CYP2C8, and CYP2D6 with IC_{50} values of 1.44, 1.95, and 2.74 μ M, respectively.

We previously observed that amiodarone is hydroxylated on the butyl side chain predominantly by CYP2J2 (Lee et al., 2010). In this study, NMR analysis revealed that the hydroxylated metabolite is a mixture of two distinct products, 4- and 3-hydroxyamiodarone. These two metabolites have been recently identified in biliary excreta in human subjects after amiodarone dosing (Deng et al., 2011). Deng et al. (2011) identified 33 metabolites derived from amiodarone excreted in human bile by mass spectral fragmentation patterns or confirmation by comparison of chromatographic retention times and mass spectra with available reference standards. Our work confirms the formation of the 4- and 3-hydroxyamiodarone by CYP2J2 in HLM, and it is likely that the former metabolite can be made in extrahepatic tissues that may contribute to the overall in vivo formation.

Most substrates identified for CYP2J2 are also substrates for CYP3A4 or CYP2D6 (Lee et al., 2010). CYP2J2 catalyzes the formation of both 4- and 3-hydroxyamiodarone, whereas CYP3A4 and other isozymes were only capable of forming 3-hydroxyamiodarone. From reaction phenotyping data, 4-hydroxyamiodarone is a unique CYP2J2-mediated metabolite, providing a tool to characterize CYP2J2 inhibition potential in tissue microsomal preparations. Other probe substrates such as terfenadine and astemizole are good CYP2J2 probe substrates when studying recombinant CYP2J2. The formation of hydroxy-terfenadine (Rodrigues et al., 1995; Lafite et al., 2007) is predominantly formed by CYP3A4, whereas *O*-desmethyl astemizole is also formed by CYP2D6 (Matsumoto and Yamazoe, 2001). The identification of 4-hydroxyamiodarone as a CYP2J2-specific reaction will aid the assessment of CYP2J2 inhibitory potential of new chemical entities (NCE) in drug discovery and development, especially as we understand its role in extrahepatic tissues.

With the use of 4-hydroxyamiodarone formation, it is now possible to determine the RAF and ISEF values for this isoform to the overall P450-mediated metabolism in the liver. Because this isoform is largely found in extrahepatic tissues, it will be important to determine in the future a physiological based pharmacokinetic model that incorporates hepatic and extrahepatic tissue metabolism to depict overall

TABLE 9
Effect of amiodarone or astemizole on probe substrate activity

CYP2J2 Probe Substrate	% Inhibition of Metabolite Formation (Mean $\pm$ S.D.)					
	CYP1A2	CYP2C8	CYP2C9	CYP2C19	CYP2D6	CYP3A4
Amiodarone, 5 μ M	-0.6 ± 9.4	32.7 ± 1.9	68.3 ± 1.7	28.6 ± 20	36.5 ± 2.8	-18.4 ± 10
Astemizole, 0.3 μ M	-0.6 ± 4.3	1.1 ± 11.7	-2.7 ± 2.6	6.0 ± 8.2	8.5 ± 1.6	20.3 ± 3.0

TABLE 10

Comparison of IC_{50} values generated in single vs. multiple probe cocktail
Single probe substrate evaluated with astemizole O-demethylation.

Inhibitor	IC_{50} (μ M) Single Probe	IC_{50} (μ M) Seven Probe
Miconazole	0.58	0.64
Pimozide	>30	>30
Danazol	<0.1	<0.1
Terfenadine	1.13	1.83

contribution of various P450s. Among the various HLM preparations evaluated, the RAF value was rather consistent, varying only 2.6-fold. However, the value was quite low and reflected low overall CYP2J2 activity in the HLM. It is noteworthy that the ISEF value that accounts for potential variation in the amount of CYP2J2 in the HLM per milligram of protein is highly variable in the different HLM preparations ranging from 0.0006 to 1.03, a ~1700 fold variation among the individual preparations with pooled HLM ISEF value near the lower end of that range. The large variation in ISEF values is likely attributed to variability of P450 content or abundance in HLM because the recombinant P450 content is constant in this analysis. Moreover, the antibody binding to an epitope to CYP2J2 in the various HLM preparations cannot distinguish functional from nonfunctional protein. Taken together, these factors likely contribute to the large variation.

4-Hydroxyamiodarone correlated well with astemizole O-demethylation in a set of nine individually prepared HLM. It is noteworthy that correlation of either 4-hydroxyamiodarone or O-desmethyl astemizole with CYP2J2 protein content in the HLM was very low. The ability of the CYP2J2 antibody to recognize functional enzyme as well as apo-inactive enzyme will contribute to the lack of correlation. However, the high correlation between these two probe substrates suggests that the contribution of CYP2D6 to astemizole O-demethylation is relatively minor (Matsumoto and Yamazoe, 2001). CYP3A4 contributes mostly to the hydroxylation of astemizole and will not interfere with O-demethylation catalyzed by CYP2J2.

Danazol emerged as a specific inhibitor of CYP2J2 among the 138 drugs approved for clinical use in the United States that were screened. Inhibition screens were performed with terfenadine and astemizole because 4-hydroxyamiodarone was discovered after screens were conducted. Because CYP2J2 is the main cardiac isozyme, the list of drugs tested included several drug classes including those that modulate cardiac function or have known cardiac toxicity. When a similar list of drugs was screened to identify substrates of CYP2J2 (Lee et al., 2010), only eight substrates were identified. The list of potential inhibitors identified was much larger after 42 drugs met the initial criteria of >50% inhibition at

30 μ M. It is possible that some of the inhibitors are also substrates that have very low clearance and were not identified as CYP2J2 substrates. Danazol was also identified as a substrate, which supports the finding that it is mostly a competitive inhibitor of astemizole and terfenadine oxidation. This observation is also consistent with previous findings (Lee et al., 2010) in which the isoxazole ring is the site of metabolism, suggesting that this moiety is oriented toward the heme instead of the ethinyl moiety, which is a structural alert for potential mechanism-based inhibition. Inhibition of CYP2J2 in the cardiac tissue may partially be responsible for the cardiotoxicity observed by some of these agents. Work is currently underway to measure CYP2J2 inhibition in cardiac tissue and to determine whether this inhibition can lead to cardiac toxicity.

Based on structure activity studies using terfenadine as a backbone, Lafite et al. (2006, 2007) synthesized a series of compounds and tested their ability to inhibit CYP2J2 hydroxylation of ebastine. Two compounds were identified as selective potent inhibitors of CYP2J2, with compound 4 having a K_i of 160 nM. Chen et al. (2009) used the synthetic compounds designed by Lafite et al. (2006, 2007) to inhibit CYP2J2 in tumor cell lines. Whereas these compounds appear to be selective inhibitors of CYP2J2, a few caveats should be noted in that they were not tested against a large panel of P450s, especially CYP2D6, and they are not readily available.

To facilitate screening the large number of NCE synthesized in drug discovery programs as potential inhibitors of CYP2J2, evaluation of amiodarone and astemizole as a seventh probe substrate to include in the high-throughput P450 inhibition probe cocktail for drug interaction assessments was investigated. Whereas amiodarone and astemizole are ideal as single probe substrates to evaluate the potential of an NCE to inhibit recombinant CYP2J2, we found that only astemizole was suitable in a P450 cocktail assay setting because it had limited interactions with the other P450 isoforms. Unfortunately, amiodarone inhibited several P450s, namely CYP2C8, CYP2C9, CYP2C19, and CYP2D6, at 29% or greater, whereas astemizole only inhibited CYP3A4 to a minor extent. Moreover, the cocktail drug-drug interaction probes did not alter astemizole O-demethylation activity because similar IC_{50} values were generated for miconazole, pimozide, danazol, and terfenadine in the seven probe cocktail versus single probe assay evaluation.

In conclusion, 4-hydroxyamiodarone and danazol are specific CYP2J2 probe reaction and inhibitor, respectively. The utility of danazol as a specific CYP2J2 inhibitor will reveal the contribution of this isoform toward the overall P450-mediated clearance of an NCE. The presence of CYP2J2 activity in HLM will facilitate awareness of the potential extrahepatic contributions in total systemic clearance that may be underestimated using in vitro systems.

FIG. 4. A, IC_{50} determination for danazol inhibition of astemizole O-demethylation with and without a 30-min preincubation of the inhibitor with enzyme and NADPH. B, kinetic plots for velocity versus substrate concentration while varying danazol concentrations.

Furthermore, astemizole can be added to existing P450 probe cocktail assays to screen for CYP2J2 inhibition in drug discovery.

Authorship Contributions

Participated in research design: Lee, Jones, Kaspera, and Totah.

Conducted experiments: Jones, Katayama, Kaspera, Jiang, Freiwald, Smith, and Walker.

Contributed new reagents or analytic tools: Jones, Katayama, Kaspera, and Jiang.

Performed data analysis: Lee, Jones, Kaspera, Smith, Walker, and Totah.

Wrote or contributed to the writing of the manuscript: Lee, Jones, Kaspera, Smith, Walker, and Totah.

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Address correspondence to: Dr. Rheem A. Totah, Department of Medicinal Chemistry, Box 357610, University of Washington, Seattle, WA. E-mail: rtotah@uw.edu