Activity, Inhibition, and Induction of Cytochrome P450 2J2 in Adult Human Primary Cardiomyocytes[S]

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ABSTRACT

Cytochrome P450 2J2 plays a significant role in the epoxidation of arachidonic acid to signaling molecules important in cardiovascular events. CYP2J2 also contributes to drug metabolism and is responsible for the intestinal clearance of ebastine. However, the interaction between arachidonic acid metabolism and drug metabolism in cardiac tissue, the main expression site of CYP2J2, has not been examined. Here we investigate an adult-derived human primary cardiac cell line as a suitable model to study metabolic drug interactions (inhibition and induction) of CYP2J2 in cardiac tissue. The primary human cardiomyocyte cell line demonstrated similar mRNA-expression profiles of P450 enzymes to adult human ventricular tissue. CYP2J2 was the dominant isozyme with minor contributions from CYP2D6 and CYP2E1. Both terfenadine and astemizole oxidation were observed in this cell line, whereas midazolam was not metabolized suggesting lack of CYP3A activity. Compared with recombinant CYP2J2, terfenadine was hydroxylated in cardiomyocytes at a similar K_m value of 1.5 μM. The V_{max} of terfenadine hydroxylation in recombinant enzyme was found to be 29.4 pmol/pmol P450 per minute and in the cells 6.0 pmol/pmol P450 per minute. CYP2J2 activity in the cell line was inhibited by danazol, astemizole, and ketoconazole in submicromolar range, but also by xenobiotics known to cause cardiac adverse effects. Of the 14 compounds tested for CYP2J2 induction, only rosiglitazone increased mRNA expression, by 1.8-fold. This cell model can be a useful in vitro model to investigate the role of CYP2J2-mediated drug metabolism, arachidonic acid metabolism, and their association to drug induced cardiotoxicity.

Introduction

Cytochrome P450 2J2 has attracted particular attention for its ability to epoxidize arachidonic acid regioselectively to 5,6-, 8,9-, 11,12-, or 14,15-epoxyeicosatrienoic acids (EETs) (Roman, 2002). These EETs have many biological functions including, but not limited to, angiogenesis, regulation of vasodilation, inhibition of cytokine-induced endothelial cell adhesion-molecule expression, inhibition of vascular smooth muscle cell migration, protection of endothelial cells against hypoxia-reoxygenation injury, upregulation of endothelial nitric oxide biosynthesis, and protection of doxorubicin-induced cardiotoxicity (Larsen et al., 2007; Spector and Norris, 2007; Yang et al., 2009; Zhang et al., 2009; Campbell and Fleming, 2010; Pfister et al., 2010). All these events are involved in cardiac electrophysiology and protect the heart from ischemic-reperfusion injury (Spiecker and Liao, 2006). More specifically, the regioisomer 11,12-EET has been shown to be a potent activator of the ion channels sensitive to ATP, to directly decrease the membrane action potential in rat myocytes (Lu et al., 2001), and to enhance recovery of ventricular repolarization following ischemia reperfusion injury (Batchu et al., 2009). These investigations greatly increased interest in CYP2J2 with regard to its enzymology, localized expression, and the need for an in vitro model system suitable for studying the enzyme's importance in maintaining cardiomyocyte homeostasis.

CYP2J2 is predominantly expressed in extrahepatic tissues, particularly in the heart, but also in skeletal muscle, placenta, small intestine, kidney, lung, pancreas, bladder, and brain (Wu et al., 1997; Zeldin et al., 1997; Bieche et al., 2007). While a crystal structure has yet to be elucidated, molecular models suggest structural similarity between CYP2J2 and CYP3A4, explaining why the two enzymes share a number of substrates of diverse therapeutic areas, such as the antihistamine drugs terfenadine, astemizole, and ebastine (Matsumoto and Yamazoe, 2001; Hashizume et al., 2002; Matsumoto et al., 2002; Liu et al., 2006; Lafite et al., 2007), anticancer drug tamoxifen, and drugs such as thioridazine or cyclosporine (Lee et al., 2012). The combination of cardiac localization and involvement in the arachidonic acid metabolism makes CYP2J2 a particularly interesting target to mechanistically investigate drug-induced cardiotoxicity.

So far, no studies have demonstrated drug metabolism in the heart tissue. The inhibitory or inductive effect by such drugs on arachidonic acid metabolism could have profound downstream consequences by reducing EETs and their protective properties. However, a human heart model remains elusive and testing relies on animal-model, especially dog, cell systems or recombinant enzymes. Much of CYP2J2's activity has been assessed in such models as *Escherichia coli*-expressed or Baculovirus-infected insect cell–expressed enzyme (Supersomes) (Lafite et al., 2007), human liver microsomes (Lee et al., 2012), or in humanized animal models that overexpress the enzyme in cardiac tissue (Seubert et al., 2004; Deng et al., 2011).

In this study, we evaluate commercially available primary human cardiomyocytes for expression and activity of CYP2J2. We first cloned

ABBREVIATIONS: BHA, butylated hydroxyanisole; BHT, butylated hydroxytoluene; CE, collision energy; CPR, cytochrome P450 reductase; DMSO, dimethylsulfoxide; DP, declustering potential; EET, epoxyeicosatrienoic acid; hPSC, human pluripotent stem cells; hPSC-CMs, hPSC-derived cardiomyocytes; LC, liquid chromatography; MS/MS, tandem mass spectrometry; P450, cytochrome P450; PBS, phosphate-buffered saline; PXR, pregnane X receptor.

and expressed CYP2J2 and measured its activity. Second, we evaluated the expression of a range of important P450s in addition to CYP2J2 in human cardimyocytes by mRNA content compared with levels of P450 expression in human ventricular tissue. Third, we assessed the metabolic activity of CYP2J2 in the cardiomyocytes toward probe substrates and characterized the kinetic parameters compared with recombinantly expressed enzyme. Finally, we investigated the induction and inhibition of CYP2J2 in these cardiomyocytes by various compounds especially ones known to cause cardiotoxicity.

Materials and Methods

Chemicals and Cell Culture Materials. All chemicals including terfenadine and astemizole were purchased from Sigma-Aldrich (St. Louis, MO), unless otherwise stated, and used without further purification. Acetonitrile, methanol, water, ammonium formate, and formic acid were purchased from Fisher Scientific (Pittsburgh, PA). Adult-derived primary human cardiomyocytes, cell culture media (complete growth media and serum-free media), solutions, and cell culture materials (culture flasks and plates, precoated with proprietary matrix for cell adherence) were purchased from Celprogen Inc. (San Pedro, CA).

Cloning of the Expression Constructs. The CYP2J2 cDNA was a gift from Dr. Darryl Zeldin at the National Institute of Environmental and Health Sciences. An internal *Nde*I site in CYP2J2 was removed using the Quickchange II XL site-directed mutagenesis kit (Stratagene, La Jolla, CA) with primers 5′: GAAATTGTTTGTTTCT*CACATG*ATTGACAAACACAG, 3′:CTGTGTTTG-TCAAT*CATGTG*AGAAACAAACAATTTC (*Nde*I site in italics, change from wild-type underlined), one unit of *Pfx* polymerase, and cycling conditions of 95°C for 3 minutes followed by 18 cycles of 94°C for 30 seconds, 55°C for 45 seconds, 68°C for 10 minutes. The resulting construct (CYP2J2-*Nde*I) was excised and inserted into the pCWori expression vector (Guryev et al., 2001) used as a template to generate the pCW2J2 expression construct (Barnes et al., 1991). The constructs were generated by PCR amplification with the primers 5′: ACTCATATGGCTCTGTTATTAGCAGTTTTTCTCAAAAGACGGCGCC and the same reverse 3′ primer:ATTCAGGTCGACACCTGAGGAACAGCG-CAGAGGCGGTG, 1 unit of *Pfx* polymerase, and cycling conditions of 95°C for 3 minutes followed by 28 cycles of 95°C for 30 seconds, 55°C for 45 seconds, and 68°C for 2 minutes. These primers incorporated an *Nde*I site into the 5′ primer and a *Sal*I site into the 3′ primer and the pCWori plasmid contains a *Sal*I site followed by a 6xHis tag to facilitate subsequent purification. The N-terminus was consequently truncated (MLAAMGSLAAALWAVVHPRTL-LLGTVAFLLAADFLKRRRP to MARRRP). The resulting amplification products and the pCWori plasmid were digested with *Nde*I and *Sal*I, resolved on a 2% agarose gel, excised with a scalpel, and recovered with the Qiaquick gel extraction kit and ligated overnight with 1 IU of T4 DNA ligase.

Protein Expression. Protein expression was performed as previously described (Cheesman et al., 2003; Kaspera et al., 2011) and harvested cells were resuspended in storage buffer and stored in –80°C until purification.

Protein Purification. Frozen pellets were thawed on ice and resuspended in 100 mM potassium phosphate (pH 7.4) containing 20% glycerol and protease inhibitors. Purification was conducted following established procedures (Kaspera et al., 2011).

Measurement of P450 Concentration. CO-difference spectra were obtained to determine the concentration of purified CYP2J2 according to the method of (Omura and Sato 1964).

Determination of Kinetic Parameters K_m and V_{max}. Enzyme activity versus protein was determined for recombinant enzymes at varying protein concentrations from 0.02 to 1 pmol P450/ml (0.02, 0.05, 0.075, 0.1, 0.2, and 1 pmol P450/ml) at 0.1 μM terfenadine. To establish time linearity, time-course incubations of both Gentest 2J2 Supersome and reconstituted CYP2J2 were conducted for 0, 5, and 10 minutes. K_m and V_{max} determination were performed under linear conditions of time and protein concentration.

Recombinant CYP2J2 was reconstituted with reductase and lipid according to previously established protocols (Kaspera et al., 2011). Briefly, the mixture used was as follows: 1 pmol/ml recombinant CYP2J2 was mixed with 2 pmol/ml rat cytochrome P450 reductase (CPR), 1 pmol/ml cytochrome b$_5$, buffer containing 100 mM potassium phosphate (pH 7.4), and 50 μM DLPC on ice

for 40 minutes with intermittent mixing. Incubations were performed in a total volume of 200 μl buffer containing 100 mM potassium phosphate (pH 7.4), 1 pmol P450/ml reconstituted CYP2J2, and varying terfenadine concentrations (0, 0.05, 0.075, 0.1, 0.2, 0.5, 1, 2, 5, 10, and 20 μM in methanol). The final methanol concentration in the incubations was 1% and was previously determined to not affect enzyme activity. The reactions were initiated by addition of 1 mM NADPH following a 5-minute preincubation at 37°C (shaking at 70 strokes/min). Reactions were conducted for 5 minutes then quenched with 200 μl cold acetonitrile containing internal standard (0.1 μM midazolam), immediately vortexed, and placed on ice. After cooling for 10 minutes the samples were centrifuged at 14,000g for 5 minutes at room temperature. Supernatant was directly removed and analyzed by LC-MS.

Cardiomyocyte Cell Culture. Culturing of human cardiomyocytes was established following Celprogen's protocols. Cells were grown in an incubator set at 37°C with 5% CO$_2$ atmosphere. The batch obtained and used for all experiments in this study were of ventricular cardiac cells. All experiments were carried out with cells initiated from a cell stock frozen at passage four and cultured to passage six. Cells used for RNA work were detached by trypsin digestion, neutralized with media, harvested, and pelleted by centrifugation at 100g for 5 minutes. The pellet was then washed with phosphate-buffered saline (PBS), and stored in 30 μl of RNAlater solution (Life Technologies, Grand Island, NY) at –80°C.

Human Heart Tissue. Human heart transplantation residual tissue was obtained from the University of Washington Medical Center. Tissue from six individual donors ($n = 6$, 3 male, 3 female) undergoing transplant procedures were used in this study for comparison with the cardiac cell line. Only discarded residual tissues with no patient identifiers were used. Ventricular tissue obtained was immediately flash-frozen in liquid nitrogen and stored at –80°C until further processed. Upon thawing, the tissue was washed with phosphate-buffered saline and immediately processed.

P450 mRNA Detection. Cells used for RNA isolation were harvested from human cardiomyocytes when approximately 80% confluent. Total RNA was extracted from approximately 1 million cells using the MagMax-96 Total RNA Isolation Kit (Life Technologies, Carlsbad, CA) and from human heart tissue using Trizol Reagent and PureLink RNA Mini Kit (Life Technologies). Total RNA was then used to synthesize cDNA using Oligo dT$_{20}$ primers and the Superscript III First Strand Synthesis System (Life Technologies). Reverse-transcription polymerase chain reaction (RT-PCR) was then carried out using TaqMan (Life Technologies) FAM reporter primers for the various cytochrome P450s screened as well as the housekeeping gene *GusB*. Each biologic triplicate was performed in technical triplicates such that the values reported are an average of nine data points. Cycle threshold (C_T) values and the ΔC_T method followed by the $2^{\Delta CT}$ calculation were used to quantitate the amount of *CYP2J2* mRNA present in the cells relative to the *GusB* mRNA levels. In the case of the P450-enzyme screen, the mRNA levels were first determined in relation to the housekeeping gene using the ΔC_T method, and then the levels of each P450 mRNA were compared with the levels of *CYP2J2* mRNA levels using the $\Delta\Delta C_T$ calculation and relative P450-mRNA levels were reported using the $2^{-\Delta\Delta CT}$ calculation.

P450 Protein Content Determination. To determine protein content, approximately 1 million cells were pelleted and homogenized in potassium phosphate buffer (100 mM, 250 μl). The homogenate was then centrifuged for 10 minutes at 10,000 rpm. A 10.5-μl aliquot was subjected to trypsin digest using the Thermo Scientific Pierce In-Solution Tryptic Digestion and Guanidination Kit (Thermo Fisher, Pittsburgh, PA). The procedure for digestion was carried out according to manufacturer protocols. Briefly, the homogenate was added to a tube containing 50 mM stock NH$_4$HCO$_3$ (15 μl) and 100 mM stock dithiothreitol (1.5 μl). This solution was incubated at 95°C for 5 minutes and allowed to cool. Stock iodoacetamide (IAA; 100 mM, 3 μl) was subsequently added and the samples were incubated for 20 minutes at room temperature. The samples were then digested by adding 1 μl trypsin (100 ng/μl stock) and incubated for 1 hour at 37°C, followed by the addition of 1 μl trypsin and incubation of the samples for an additional 3 hours at 37°C. The reactions were quenched by the addition of 3.2 μl cold 100 mM phosphate buffer containing 1% formic acid. Additionally, 5 μl of internal standard (final concentration of 50 nM) was added.

The digested samples were then analyzed by quantitative ultra-performance liquid chromatography–tandem mass spectrometry (UPLC-MS/MS) using an Agilent 4000 mass spectrometer (Santa Clara, CA), connected to an Agilent LC

system. Ten microliters of the sample was injected on a Phenomenex (Torrance, CA) Aeris PEPTIDE XB-C18 column (1.7 μμ, 150 × 2.10 mm). The mobile phases consisted of aqueous phase A: 0.1% formic acid in H$_2$O, and organic phase B: 0.1% formic acid in acetonitrile. The samples were analyzed using the following gradient: mobile phase B: 0–3 minutes, 3%; 3–5 minutes, 3–10%; 5–8 minutes, 10–50%; 8–8.4 minutes, 50%; 8.4–8.5 minutes, 50–90%; 8.5–9.5 minutes, 90%; 9.5–10 minutes, 90–3%; 10–10.5 minutes, 3%. The column was re-equilibrated to initial conditions for 1 minute and the flow rate was 0.3 ml/min. The source temperature was 350°C, the capillary charge was 3500 V, and gas flow was 5 l/min.

The CYP2J2-specific peptide sequence monitored was VIGQGQQPSTAAR. Standards for mass spectrometry were custom ordered from and synthesized by Thermo Fisher (Rockford, IL). Similarly, the heavy-labeled peptide used as an internal standard was synthesized using a heavy ($^{13}C_6$, $^{15}N_4$) arginine residue at the C-terminal end of the fragment (+10 Da), also by Thermo Fisher. The transitions monitored were 656.85 > 602.33 (CYP2J2 fragment) and 661.9 > 612.1 (synthesized peptide internal standard). The protein content was determined using a standard curve containing the following concentrations of synthesized unlabeled peptide (nM): 0, 0.5, 1, 2.5, 5, 10, 25, 50, 100, 500. The internal standard concentration was the same as above (50 nM).

Kinetic Parameters of CYP2J2-Mediated Metabolism in Human Cardiomyocytes. Experiments to determine K_m and V_{max} of terfenadine and astemizole hydroxylation by the cells were carried out in triplicates. Kinetic parameters were measured under established linearity for cell density and time. Cells were plated in 96-well plates at an approximate density of 100,000 cells per well and allowed to adhere to the plate for 24 hours in 100 μl of complete media. The cells were then washed with phosphate-buffered saline (100 μl) and dosed with terfenadine or astemizole in serum-free media [100 μl, containing 0.1% dimethylsulfoxide (DMSO)] at varying concentrations (0, 0.02, 0.05, 0.1, 0.2, 0.5, 1, 2, 5, 10, 25, 50, and 100 μM). After 2 hours of incubation at 37°C, the reaction was quenched by the addition of acetonitrile (100 μl) containing 0.1 μM midazolam as internal standard. Vigorous pipetting was then used to facilitate cellular detachment from the plate and lysis. The samples were centrifuged (3500g, 10 minutes), and 150 μl was transferred to a new 96-well plate for spectrometric analysis.

To rule out potential involvement by CYP3A4 or CYP2C8, we also conducted activity experiments with probe substrates for CYP3A4 and CYP2C8. The incubations were carried out as outlined for K_m and V_{max} determination of CYP2J2 above but using midazolam (3 μM) or amodiaquine (2 μM) as probe substrates for CYP3A4 and CYP2C8, respectively, instead of terfenadine.

Metabolite Detection and Quantification. Metabolites and parent were quantified on a Sciex API4000 liquid chromatography–tandem mass spectrometry (LC-MS/MS; Applied Biosystems) connected to a Shimadzu HPLC System (LC-10AD, SCL-10A) equipped with a CTC PAL Autosampler (LEAP Technologies, Carrboro, NC). Ten microliters of supernatant was injected on an Agilent Zorbax XDB C8-column (2.1-μm, 5-cm) column. For terfenadine, the mobile phase consisted of aqueous phase A: 10 mM ammonium acetate (pH 5.5), and organic phase B: 10 mM ammonium acetate in methanol and analyzed using the following gradient: mobile phase B: 0 –1 minutes, 30%; 1–2 minutes, 30–70%; 2–4 minutes, 70–100%; 4–6.5 minutes, 100%; 6.5–6.6 minutes, 100–30%. The column was re-equilibrated at initial conditions for 1.4 minutes. The flow rate was 0.3 ml/min. MS/MS parameters: ion spray, 5,500 V; temperature, 450°C; collision gas, 6 l/min; ion gas, 15 l/min; curtain gas, 10 l/min. Compound detection: terfenadine (472.20 > 436.10; declustering potential (DP) 80, collision energy (CE) 37, hydroxyterfenadine (488.30 > 452.20, DP 90, CE 40), terfenadine acid (502.40 > 466.30, DP 100, CE 40), and midazolam (326.00 > 291.20, DP 50, CE 30). The dwell time for each ion was 50 millisecond. For astemizole, metabolites and standards were measured with identical instrumentation on an Agilent Zorbax SB C8-column (2.1 μm, 5 cm) using the following mobile phases: 0.1% v/v formic acid in water (A) and acetonitrile with 0.1% v/v formic acid (B), and gradient: 0–0.5 minutes, 20% B ; 0.5–1.5 minutes, increase to 100% B; hold until 3.5 minutes, decrease B to 20% within 0.1 minutes, and re-equilibrate for 1 minute. Mass transitions identified astemizole (459.20 > 135.10, DP 80, CE 50), desmethylastemizole (445.10 > 121.10, DP 40, CE 50), and midazolam (326.00 > 291.20, DP 50, CE 30).

Inhibition of CYP2J2 in Human Cardiomyocyte. Inhibition experiments were carried out in triplicates at 37°C. Controls included reactions without inhibitor, substrate, or cells. Two concentrations of inhibitors were used (10 μM and 1 μM, with a final solvent concentration of 0.1% DMSO). Cells were plated at an approximate density of 100,000 cells per well in a 96-well plate and allowed to adhere for 24 hours in complete media (100 μl). They were then washed with PBS to remove serum and incubated at 37°C for 2 hours in serum free media (100 μl) containing terfenadine (1.5 μM or 0.2 μM) and one of the following potential inhibitors: amiodarone, astemizole, cisapride, danazol, grepafloxacin, ketoconazole, lansoprazole, levomethadyl, pimozide, rofecoxib, and sertindole. Tacrolimus inhibition of terfenadine hydroxylation was also evaluated but only at a terfenadine concentration of 1.5 μM. An untreated control containing 0.1% DMSO was used to determine 100% activity. The reactions were then quenched with the addition of acetonitrile (100 μl) containing 0.1 μM midazolam as internal standard. Vigorous pipetting was then used to facilitate cellular detachment from the plate and cell lysis. The samples were centrifuged (3,500g, 10 minutes), and 150 μl was transferred to a new 96-well plate for analysis.

Induction of CYP2J2 mRNA in Human Cardiomyocytes. Cells that had been plated in 6-well plates and allowed to attach overnight were treated with potential inducers: phenytoin (100 μM), phenobarbital (100 μM), dexamethasone (100 μM), rifampin (10 μM), clotrimazole (100 μM), omeprazole (100 μM), rosiglitazone (100 μM), ritonavir (10 μM), β-naphthoflavone (100 μM), butylated hydroxyanisole (BHA, 100 μM), butylated hydroxytoluene (BHT; 100 μM), and carbamazepine (100 μM). Induction by 6β-estradiol and testosterone was also tested at different concentrations (0.01, 0.1, 1, 10, and 100 μM). The cells were kept for 48 hours in media containing the inducing agent. Media was changed at 24 hours to replenish inducers. After 48 hours, the cells were detached, pelleted, and mRNA content was analyzed as mentioned above. mRNA was extracted from approximately 1 million cells.

Induction of CYP2J2 Activity in Human Cardiomyocyte. Experiments were performed in triplicates. Cells were plated in 96-well plates at a density of approximately 100,000 cells/well. The cells were allowed to attach to the plate for 24 hours in complete media. The media was then aspirated and the cells were treated with serum-free media (100 μl) containing one of the following potential inducers: phenytoin (100 μM), phenobarbital (750 μM), dexamethasone (100 μM), rifampin (10 μM), clotrimazole (50 μM), omeprazole (100 μM), rosiglitazone(100 μM), ritonavir (10 μM), β-naphthoflavone (50 μM), BHA (100 μM), BHT (100 μM), and carbamazepine (100 μM). The cells were treated for 48 hours, after which the media was aspirated and the cells were washed with PBS (100 μl). Metabolic activity was measured by addition of serum-free media containing terfenadine (100 μl, 1.5 μM) and incubation at 37°C for two hours. The reaction was quenched by addition of acetonitrile (100 μl) containing 0.1 μM midazolam. The samples were analyzed as outlined under kinetic parameters of CYP2J2-mediated metabolism in human cardiomyocytes.

To further investigate the effect of ritonavir and rosiglitazone on protein stability and terfenadine levels in the cell, follow up studies were performed in which approximately 1 million cells were induced with 100 μM ritonavir, rosiglitazone, or BHT (as another control) for 48 hours, as described above, and compared with untreated cells. In one set of experiments at the end of the 48-hour induction period, the cells were washed with PBS, homogenized, and a trypsin digest was performed on the cells to determine if protein levels are affected by drug treatment. In another set of experiments, the induced cells were washed with PBS and treated with 1.5 μM terfenadine for 2 hours. After treating with terfenadine, the media was aspirated and the cells were washed with PBS, which was subsequently removed. The cells were then harvested by addition of 50% acetonitrile in water (500 μl) containing midazolam (100 nM). The cells were lysed using vigorous pipetting and then centrifuged at 3500 rpm (5 minutes, 4°C) to remove cell debris. A sample (200 μl) was moved to LC-MS vials and analyzed by mass spectrometry using the method outlined under kinetic parameters of CYP2J2-mediated metabolism in human cardiomyocytes.

Rosiglitazone Inhibition of CYP2J2 Activity. The ability of rosiglitazone to inhibit CYP2J2 biotransformation of terfenadine was determined by coincubating BD Gentest CYP2J2 Supersomes (1 pmol/ml; BD Biosciences, San Jose, CA), terfenadine (0.2 μM), and rosiglitazone (100 μM) in 100 mM potassium phosphate buffer (pH 7.4). The reaction mixture (90 μl) was preincubated for 5 minutes at 37°C, initiated with NADPH (1 mM final concentration), and quenched with cold acetonitrile (100 μl) containing midazolam (100 nM) after 5 minutes. Mass spectrometry analysis was carried out as previously described.

Data Analysis. Apparent Michaelis-Menten constants K_m and V_{max} were derived following nonlinear regression analysis of the kinetic data using
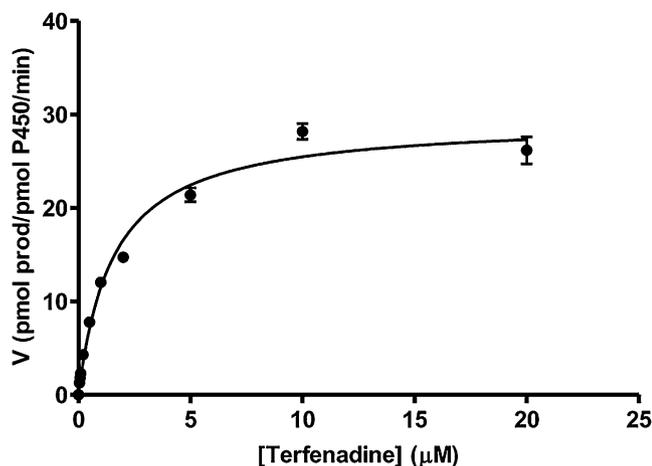
Fig. 1. Kinetic parameters of terfenadine hydroxylation using recombinant *E. coli*-expressed CYP2J2.

a Michaelis-Menten model (Prism 5 Windows version 5.02; GraphPad Software, Inc., La Jolla, CA). Kinetic data are reported as the mean $\pm$ S.D. of triplicates in cells and as the mean $\pm$ standard error of duplicates when using recombinant enzyme (computer generated).

Results

Expression and Kinetics of Recombinant *E. coli*-Expressed CYP2J2. SDS-PAGE analysis showed a band at 57 kDa consistent with full-length CYP2J2 protein, and a CO-difference spectrum showed active P450 and no inactive P420 present (data not shown). Expressed CYP2J2 protein was assayed for metabolic activity using terfenadine, which displayed Michaelis-Menten kinetics with a K_m of 1.55 μM (Fig. 1, Table 1). Enzyme activity was expressed as rate of alcohol metabolite formed, using the peak height as a quantitative comparison with internal standard.

Cytochrome P450 mRNA Screen. *CYP2J2* was the major isozyme expressed among the P450s that were screened in human cardiomyocytes (Fig. 2). *CYP2D6* and *CYP2E1* were also detected at levels approximately 20-fold below that of *CYP2J2*. In human heart tissue, *CYP2J2* had also the greatest expression level. Several other P450 isozymes complemented CYP2J2 expression in human heart tissue, including *CYP2C8, CYP2D6, CYP2E1, CYP2V1, CYP3A4, CYP4A11, CYP4B1,* and *CYP4F12,* but expression levels were at least 50-fold lower than that of *CYP2J2*.

CYP2J2 Protein Content Determination. Using mass spectrometry for detection, the average expression of CYP2J2 in cardiomyocytes is 2.96 pmol per 1 million cells.

Kinetic Parameters of Drug Metabolism in Human Cardiomyocytes. Drug metabolic activity was measured in the cells using

both terfenadine and astemizole as probe drugs. Both drugs were oxidized and exhibited Michaelis-Menten kinetics with a K_m of 1.51 μM (Fig. 3A, Table 1) for terfenadine hydroxylation and K_m of 5.22 μM for astemizole demethylation (Fig. 3B, Table 1). In contrast to astemizole, terfenadine was toxic to the cells at higher concentrations.

Inhibition of CYP2J2 in Human Cardiomyocytes. Inhibition was assessed at two concentrations of substrate [0.2 μM, Fig. 4A, and 1.5 μM (at K_m), Fig. 4B] and two concentrations of inhibitor (1 and 10 μM). Danazol and ketoconazole greatly inhibited the enzyme at both substrate concentrations. Danazol was equally potent at both concentrations of substrate, reducing activity about 95%, but ketoconazole was more potent at the lower substrate concentration. At 0.2 μM terfenadine (the K_m for terfenadine hydroxylation found using Supersomes), astemizole, and cisapride also inhibited CYP2J2 at both inhibitor concentrations. Pimozide reduced activity by >60% at the higher inhibitor concentration of 10 μM and by approximately 15% at an inhibitor concentration of 1 μM. Other drugs tested exhibited little to no inhibition. Levomethadyl and sertindole appear to activate the enzyme by up to 50%. At 1.5 μM terfenadine, inhibition of CYP2J2 activity was reduced, with many drugs exhibiting little (as much as 20%) to no inhibition (Fig. 4A). Astemizole, cisapride, and pimozide still inhibited enzyme activity, as much as 60% in the case of 1 μM astemizole, but the degree to which they inhibited was not as pronounced as it was at substrate concentration of 0.2 μM (Fig. 4B).

Hormone Effects on Gene Expression. *CYP2J2* induction by sex hormones β-estradiol and testosterone demonstrated that β-estradiol increased mRNA transcript levels in a concentration-dependent manner, while testosterone decreased transcription of *CYP2J2* (Fig. 5). However, changes in the levels of transcription were not statistically different from control untreated cells.

Induction of CYP2J2 in Human Cardiomyocytes. Fig. 6, A and B presents the mRNA and activity following induction using the following drugs and concentrations: phenytoin (100 μM), phenobarbital (100 μM expression, 750 μM activity), dexamethasone (100 μM), rifampin (10 μM), clotrimazole (100 μM expression, 50 μM activity), omeprazole (100 μM), rosiglitazone (100 μM), ritonavir (10 μM), β-naphthoflavone (100 μM expression, 50 μM activity), butylated hydroxyanisole (100 μM), butylated hydroxytoluene (100 μM), and carbamazepine (100 μM).

When examining *CYP2J2* mRNA expression, many of the compounds screened did not result in an increased gene expression (Fig. 6A). An increase in *CYP2J2* mRNA was observed when the cells were treated
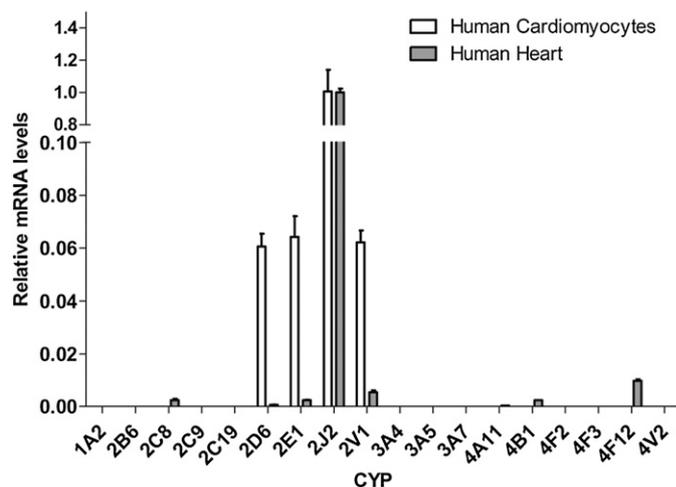
TABLE 1

Kinetic parameters of terfenadine and astemizole metabolism using recombinant enzyme or cardiomyocytes

	K_m	V_{max}
	μM	*pmol/pmol 2J2 per min*
Recombinant CYP2J2 terfenadine hydroxylation	1.6 ($\pm$0.2)	29.4 ($\pm$0.9)
Human cardiomyocyte terfenadine hydroxylation	1.5 ($\pm$0.2)	6.0 ($\pm$0.2)
Human cardiomyocyte astemizole demethylation	5.2 ($\pm$0.7)	3.2 ($\pm$0.1)

Fig. 2. Relative levels of mRNA expression in human cardiomyocytes and human ventricular heart tissue.
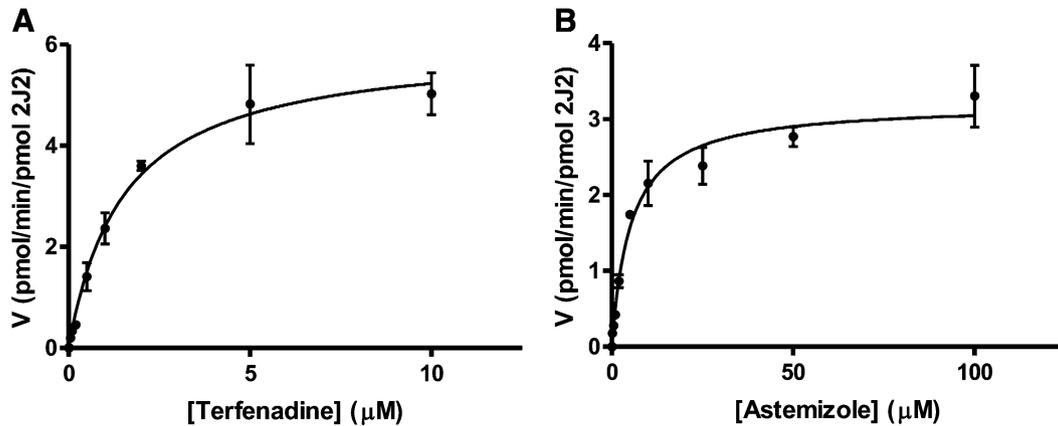
Fig. 3. Kinetic parameters of terfenadine hydroxylation (A) and astemizole demethylation (B) in human cardiomyocytes.

with rosiglitazone (>50% increase), BHA (50% increase), and BHT (40% increase). Slight decreases in mRNA content were observed in the cells when treated with dexamethasone, clotrimazole, and ritonavir.

The greatest increase in enzyme activity occurred when the cells were treated with carbamazepine (30% increase), though this was not significant. Ritonavir treatment showed >95% decrease in terfenadine hydroxylation by CYP2J2. Phenytoin, phenobarbital, rosiglitazone, omeprazole, and clotrimazole also reduced CYP2J2 activity (Fig. 6B). Other compounds did not appreciably affect the enzyme's ability to oxidize terfenadine.

Postinduction, there was no appreciable decrease in protein levels in cells treated with rosiglitazone, ritonavir, or BHT indicating that these agents do not affect protein stability. (Supplemental Fig. 1)

Intracellular levels of terfenadine postinduction were also measured. In cells treated with ritonavir and rosiglitazone, terfenadine levels were decreased by 50% compared with untreated cells but were unchanged relative to control when treated with BHT. (Supplemental Fig. 2)

Experiments to determine if rosiglitazone inhibited CYP2J2-mediated metabolism of terfenadine showed that rosiglitazone at 100 μM concentration does not inhibit CYP2J2 activity (data not shown).

Discussion

Here a primary cardiac cell line was examined for its potential use to screen for cardiac metabolism–related liabilities. These ventricular cells are derived from adult humans, which is important considering the interspecies differences in CYP2J activity previously reported (Ma et al., 2004; Yamasaki et al., 2004; Aiba et al., 2006; Elshenawy et al., 2013). Further, much of the drug-induced cardiotoxicity can be attributed to ventricular tissue. The P450 mRNA expression profile was similar to human cardiac ventricular tissue, with CYP2J2 by far the dominant isoform. The ability of the cells to metabolize CYP2J2 substrates astemizole and terfenadine was also established. Various compounds most notably danazol and ketoconazole readily inhibited CYP2J2 activity. However, CYP2J2 mRNA were mostly unchanged in the presence of potential inducers.

Others have shown the dominant presence of CYP2J2 in cardiac tissue, using immunoblotting or quantitative real-time PCR (Wu et al., 1996; Michaud et al., 2010). The expression of various P450 isozymes in the heart, including *CYP1A1, CYP2B6, CYP2C8, CYP2C19, CYP2J2,* and *CYP2E1,* are also reported (Wu et al., 1996; Thum and Borlak, 2000; Michaud et al., 2010). In the cardiac cell line, the expression of CYP2J2 agrees well with previously published data but the cellular expression levels of the CYP2C subfamily were below limits of detection. Delozier et al. (2007) detected CYP2C in cardiac tissue samples that were prepared from whole heart tissue. The cells investigated here are derived from ventricular tissue and do not contain endothelial cells. It is possible that the CYP2C expression in the heart tissue is localized to endothelial cells and not cardiomyocytes.
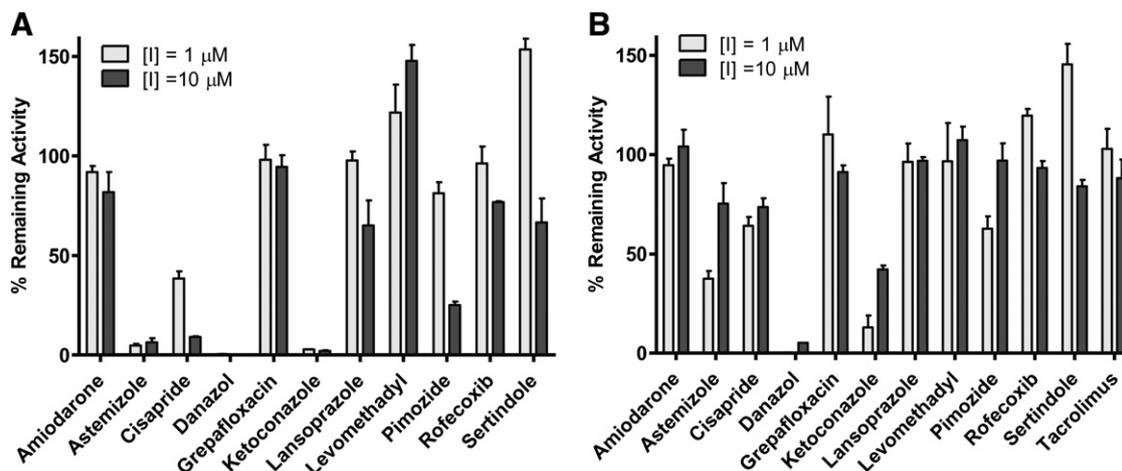

Fig. 4. Inhibition of terfenadine hydroxylation at 0.2 μM (A) and 1.5 μM (B) at 1-μM and 10-μM inhibitor concentrations after 2 hours of incubation in human cardiomyocytes.
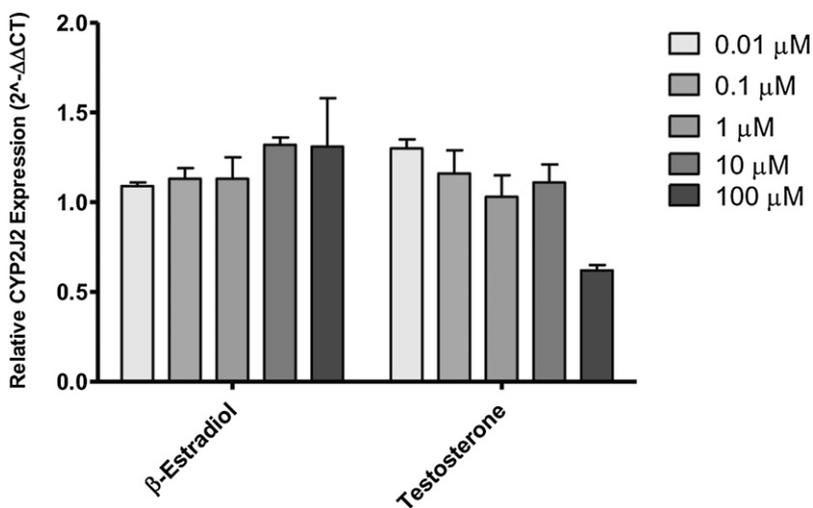
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Fig. 5. Induction of CYP2J2 mRNA expression with testosterone and β-estradiol at varying concentrations (values relative to untreated controls normalized to a value of 1.0).

K_m values for terfenadine hydroxylation were comparable in the cells and *E. coli*-expressed system but were 10-fold higher than Supersomes (1.5 μM versus 0.2 μM, respectively). The similarity of terfenadine hydroxylation seen in cells and *E. coli* models (with deviations at high substrate concentration due to inhibition or cell toxicity) is a promising indication that these cells present a well suited model of drug metabolism in the heart. Similar protein content of 0.2-0.3 pmol CYP2J2 were used for Km experiments carried out using the cardiomyocytes and E. coli expressed recombinant protein. It should be noted that the *E. coli*-expressed enzyme CYP2J2 has a truncation at the N-terminus and a 6xHis-tag at the C-terminus for purification purposes. It is unclear at this time whether these modifications alter the enzyme's activity to any significant degree. Another potential source of variability is the difference in the ratio between CYP2J2 and its redox partners cytochrome P450 reductase and cytochrome b5. Supersome systems by BD Gentest have variable ratios, while reconstituted systems maintain a 1:2:1 ratio of CYP/CPR/b5. Further, commercial Supersomes contain human CPR, while reconstituted systems use rat CPR. In addition, the role of specific and nonspecific binding of terfenadine to the cells in altering the K_m value cannot be determined at this time.

To test the inhibition of terfenadine hydroxylation in the heart, potential inhibitors with a documented history of cardiotoxicity were selected. Danazol was included because it is a specific inhibitor of CYP2J2 and causes congestive heart failure with prolonged use (Lee et al., 2012). Two inhibitor concentrations were used (1 and 10 μM) to resemble more closely plasma-level concentrations and accumulation due to inhibited metabolism or transport. Further, two concentrations of substrate (0.2 and 1.5 μM) were chosen to reflect the measured in vitro K_m values for terfenadine in the different in vitro systems. Using substrate concentrations at sub-K_m levels would reflect the competitive inhibition more clearly operating in the linear range of substrate turnover. As expected, danazol greatly inhibited CYP2J2 in this cell system, reinforcing CYP2J2's role in metabolism of terfenadine in the heart. The inhibition of CYP2J2 activity by drugs such as ketoconazole and ritonavir were also expected, particularly because these drugs are reported to inhibit CYP2J2 in Supersomes, and are also known to inhibit CYP3A4 (Lee et al., 2012). Interestingly, sertindole, tacrolimus, and levomethadyl at lower concentrations increased CYP2J2 activity, possibly due to allosterism or other cell distribution phenomena (such as transport) not accounted for in this study.
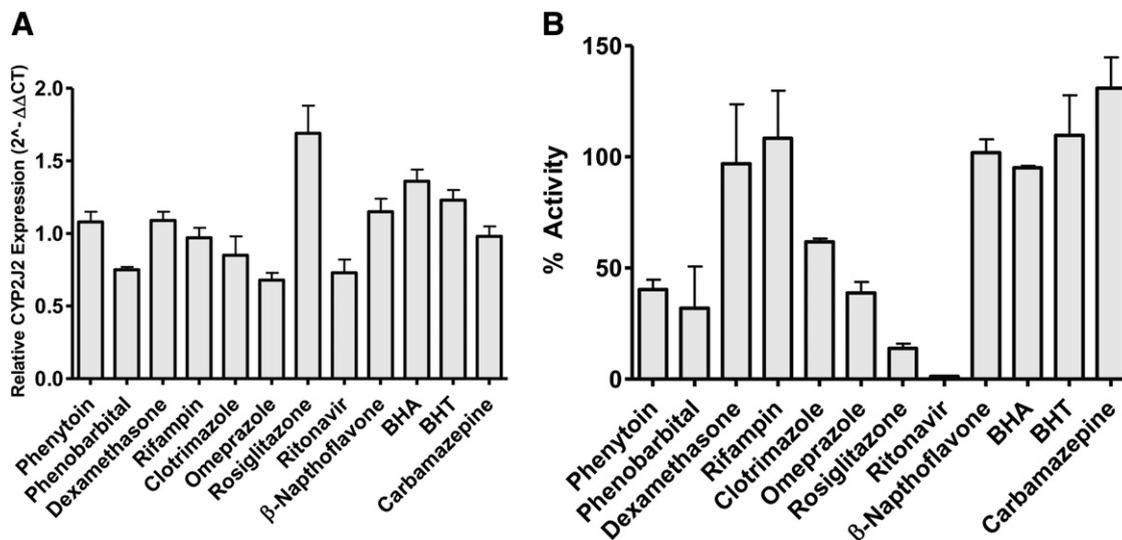

Fig. 6. CYP2J2 mRNA expression and activity following 48-hour induction with drug and then measuring (A) mRNA and (B) terfenadine hydroxylation [all values are relative to untreated controls containing 0.1% DMSO normalized to a value of 1.0 for (A) and 100% for (B)].

Induction of CYP2J2 was evaluated at both the transcriptional and protein activity levels. A 48-hour induction period was chosen after preliminary studies indicated that significant cell death occurred at 72 hours. Lee and Murray (2010) reported BHA as a CYP2J2 inducer in HepG2 cells. Further work by Ma et al. (2004) has shown that the mouse ortholog CYP2J5 is regulated by sex hormones in murine kidneys. The results of this study, however, show that in cardiomyocyte, neither BHA nor the sex hormone β-estradiol affect the transcription of the CYP2J2. Testosterone had a slight repressive effect at high concentration indicating possible gender differences in regulation. Incubation of the cells with terfenadine immediately following inducer treatment does not appear to result in increased protein activity, suggesting an unlikely change in protein levels.

It is possible that CYP2J2 is differentially regulated in various cell types and different organs. It is important to note that Lee and Murray (2010) induced their cells with BHA for 72 hours compared with the 48 hours of this study. Further, they replenished the BHA in their cell media frequently during their induction (at 6, 12, 18, 24, and 48 hours), whereas BHA was replenished at 24 hours in this study.

This inability to induce CYP2J2 in cardiomyocytes indicates an important endogenous function involving tightly regulated expression and activity to preserve or protect the cell. This is supported by the G-50T mutation, the only other notable CYP2J2-allele reported across ethnic groups. Carriers of this allele have decreased expression of the CYP2J2 gene and have been shown to have increased risk of adverse cardiac effects (Spiecker et al., 2004; Marciante et al., 2008; Zhang et al., 2008). A delicate balance of expression levels might be needed, and interference with physiologic pathways could have detrimental effects.

Other compounds tested for the ability to induce CYP2J2 transcription and CYP2J2 activity are classic P450 inducers, which bind to the pregnane X receptor (PXR) (Fahmi et al., 2012). Of note, rosiglitazone simultaneously induced transcription of mRNA but also inhibited terfenadine hydroxylation. Rosiglitazone is a known mild PXR inducer (Sinz et al., 2006); however, if rosiglitazone was operating through the PXR receptor, then rifampin should have induced mRNA as well. Rosiglitazone is potentially binding and inducing CYP2J2 through peroxisome proliferator-activated receptor (PPAR), which also induces mRNA of CYP2B and CYP4 enzymes (Rogue et al., 2010).

Also, while our goal was to find inducers of CYP2J2 transcription and CYP2J2 protein, it appears that some drugs reduced terfenadine hydroxylation, such as ritonavir and rosiglitazone. The decrease in terfenadine hydroxylation could potentially be due to the drug inhibiting the transporter responsible for uptake of terfenadine into the cell. Our data shows that the amount of terfenadine remaining in the cell was at least 50% lower than control samples (Supplemental Fig. 2). This indicates that terfenadine is perhaps unable to enter the cell following the induction treatment due to the inhibition of transporters by xenobiotics. Currently, not much is known about which drug transporters are expressed in these cardiomyocytes and further studies are needed. Protein degradation instigated by either ritonavir or rosiglitazone is another possible explanation. However, our studies indicate no significant decrease in the amount of CYP2J2 protein in these cells following drug treatment (Supplemental Fig. 1).

Cardiomyocytes derived from human pluripotent stem cells (hPSCs) are also being investigated for drug screening (Dick et al., 2010; Zeevi-Levin et al., 2012). Many of these studies, however, focus on the electrophysiological aspects of the cardiomyocyte, which are unfortunately absent in the cells presented in this study. Despite this, we have shown that these primary cells still maintain the ability to express drug-metabolizing enzymes, in agreement with published data in heart tissue. While the heart is not primarily involved in drug metabolism, the presence of these P450s, particularly CYP2J2, suggests the potential for drug-drug interactions in the heart. To our knowledge, there are no studies in hPSC-derived cardiomyocytes (hPSC-CMs) that characterize their expression of drug-metabolizing enzymes. Lastly, hPSC-CM currently have limitations such as large scale use, incomplete differentiation, and immaturity (Mordwinkin et al., 2013), making the primary cells investigated here a promising alternative.

In conclusion, this work provides an important step toward identifying a model that could investigate metabolism-related drug adverse effects in the heart during preclinical investigations. The cardiomyocyte cell line is of human-derived ventricular cells, but it is important to note that these primary lines exhibit potential drawbacks (e.g., heterogeneity of the donors, indefinite cultivation, donor age, donor drug use). Finding a model that is appropriate to all circumstances is difficult, but these primary human cardiomyocytes present a simpler applicable tool than in vivo studies and thus a promising avenue forward.

Authorship Contributions

Participated in research design: Evangelista, Kaspera, Mokadam.

Conducted experiments: Evangelista, Kaspera, Jones.

Contributed new reagents or analytic tools: Mokadam, Jones.

Performed data analysis: Evangelista, Kaspera, Jones, Totah.

Wrote or contributed to the writing of the manuscript: Evangelista, Kaspera, Mokadam, Totah.

References

Aiba I, Yamasaki T, Shinki T, Izumi S, Yamamoto K, Yamada S, Terato H, Ide H, and Ohyama Y (2006) Characterization of rat and human CYP2J enzymes as Vitamin D 25-hydroxylases. *Steroids* **71**:849–856.

Barnes HJ, Arlotto MP, and Waterman MR (1991) Expression and enzymatic activity of recombinant cytochrome P450 17 alpha-hydroxylase in Escherichia coli. *Proc Natl Acad Sci USA* **88**:5597–5601.

Batchu SN, Law E, Brocks DR, Falck JR, and Seubert JM (2009) Epoxyeicosatrienoic acid prevents postischemic electrocardiogram abnormalities in an isolated heart model. *J Mol Cell Cardiol* **46**:67–74.

Bièche I, Narjoz C, Asselah T, Vacher S, Marcellin P, Lidereau R, Beaune P, and de Waziers I (2007) Reverse transcriptase-PCR quantification of mRNA levels from cytochrome (CYP)1, CYP2 and CYP3 families in 22 different human tissues. *Pharmacogenet Genomics* **17**:731–742.

Campbell WB and Fleming I (2010) Epoxyeicosatrienoic acids and endothelium-dependent responses. *Pflugers Arch* **459**:881–895.

Cheesman MJ, Baer BR, Zheng YM, Gillam EM, and Rettie AE (2003) Rabbit CYP4B1 engineered for high-level expression in Escherichia coli: ligand stabilization and processing of the N-terminus and heme prosthetic group. *Arch Biochem Biophys* **416**:17–24.

Delozier TC, Kissling GE, Coulter SJ, Dai D, Foley JF, Bradbury JA, Murphy E, Steenbergen C, Zeldin DC, and Goldstein JA (2007) Detection of human CYP2C8, CYP2C9, and CYP2J2 in cardiovascular tissues. *Drug Metab Dispos* **35**:682–688.

Deng Y, Edin ML, Theken KN, Schuck RN, Flake GP, Kannon MA, DeGraff LM, Lih FB, Foley J, Bradbury JA, et al. (2011) Endothelial CYP epoxygenase overexpression and soluble epoxide hydrolase disruption attenuate acute vascular inflammatory responses in mice. *FASEB J* **25**:703–713.

Dick E, Rajamohan D, Ronksley J, and Denning C (2010) Evaluating the utility of cardiomyocytes from human pluripotent stem cells for drug screening. *Biochem Soc Trans* **38**:1037–1045.

Elshenawy OH, Anwar-Mohamed A, Abdelhamid G, and El-Kadi AO (2013) Murine atrial HL-1 cell line is a reliable model to study drug metabolizing enzymes in the heart. *Vascul Pharmacol* **58**:326–333.

Fahmi OA, Raucy JL, Ponce E, Hassanali S, and Lasker JM (2012) Utility of DPX2 cells for predicting CYP3A induction-mediated drug-drug interactions and associated structure-activity relationships. *Drug Metab Dispos* **40**:2204–2211.

Guryev OL, Gilep AA, Usanov SA, and Estabrook RW (2001) Interaction of apo-cytochrome b5 with cytochromes P4503A4 and P45017A: relevance of heme transfer reactions. *Biochemistry* **40**:5018–5031.

Hashizume T, Imaoka S, Mise M, Terauchi Y, Fujii T, Miyazaki H, Kamataki T, and Funae Y (2002) Involvement of CYP2J2 and CYP4F12 in the metabolism of ebastine in human intestinal microsomes. *J Pharmacol Exp Ther* **300**:298–304.

Kaspera R, Naraharisetti SB, Evangelista EA, Marciante KD, Psaty BM, and Totah RA (2011) Drug metabolism by CYP2C8.3 is determined by substrate dependent interactions with cytochrome P450 reductase and cytochrome b5. *Biochem Pharmacol* **82**:681–691.

Lafite P, Dijols S, Zeldin DC, Dansette PM, and Mansuy D (2007) Selective, competitive and mechanism-based inhibitors of human cytochrome P450 2J2. *Arch Biochem Biophys* **464**:155–168.

Larsen BT, Campbell WB, and Gutterman DD (2007) Beyond vasodilatation: non-vasomotor roles of epoxyeicosatrienoic acids in the cardiovascular system. *Trends Pharmacol Sci* **28**:32–38.

Lee AC and Murray M (2010) Up-regulation of human CYP2J2 in HepG2 cells by butylated hydroxyanisole is mediated by c-Jun and Nrf2. *Mol Pharmacol* **77**:987–994.

Lee CA, Jones JP, 3rd, Katayama J, Kaspera R, Jiang Y, Freiwald S, Smith E, Walker GS, and Totah RA (2012) Identifying a selective substrate and inhibitor pair for the evaluation of CYP2J2 activity. *Drug Metab Dispos* **40**:943–951.

Liu KH, Kim MG, Lee DJ, Yoon YJ, Kim MJ, Shon JH, Choi CS, Choi YK, Desta Z, and Shin JG (2006) Characterization of ebastine, hydroxyebastine, and carebastine metabolism by human liver microsomes and expressed cytochrome P450 enzymes: major roles for CYP2J2 and CYP3A. *Drug Metab Dispos* **34**:1793–1797.

Lu T, Hoshi T, Weintraub NL, Spector AA, and Lee HC (2001) Activation of ATP-sensitive K(+) channels by epoxyeicosatrienoic acids in rat cardiac ventricular myocytes. *J Physiol* **537**: 811–827.

Ma J, Graves J, Bradbury JA, Zhao Y, Swope DL, King L, Qu W, Clark J, Myers P, and Walker V, et al. (2004) Regulation of mouse renal CYP2J5 expression by sex hormones. *Mol Pharmacol* **65**:730–743.

Marciante KD, Totah RA, Heckbert SR, Smith NL, Lemaitre RN, Lumley T, Rice KM, Hindorff LA, Bis JC, and Hartman B, et al. (2008) Common variation in cytochrome P450 epoxygenase genes and the risk of incident nonfatal myocardial infarction and ischemic stroke. *Pharmacogenet Genomics* **18**:535–543.

Matsumoto S and Yamazoe Y (2001) Involvement of multiple human cytochromes P450 in the liver microsomal metabolism of astemizole and a comparison with terfenadine. *Br J Clin Pharmacol* **51**:133–142.

Matsumoto S, Hirama T, Matsubara T, Nagata K, and Yamazoe Y (2002) Involvement of CYP2J2 on the intestinal first-pass metabolism of antihistamine drug, astemizole. *Drug Metab Dispos* **30**:1240–1245.

Michaud V, Frappier M, Dumas MC, and Turgeon J (2010) Metabolic activity and mRNA levels of human cardiac CYP450s involved in drug metabolism. *PLoS ONE* **5**:e15666.

Mordwinkin NM, Burridge PW, and Wu JC (2013) A review of human pluripotent stem cell-derived cardiomyocytes for high-throughput drug discovery, cardiotoxicity screening, and publication standards. *J Cardiovasc Transl Res* **6**:22–30.

Omura T and Sato R (1964) The Carbon Monoxide-Binding Pigment of Liver Microsomes. I. Evidence for Its Hemoprotein Nature. *J Biol Chem* **239**:2370–2378.

Pfister SL, Gauthier KM, and Campbell WB (2010) Vascular pharmacology of epoxyeicosatrienoic acids. *Adv Pharmacol* **60**:27–59.

Rogue A, Spire C, Brun M, Claude N, and Guillouzo A (2010) Gene Expression Changes Induced by PPAR Gamma Agonists in Animal and Human Liver. *PPAR Res* **2010**:325183.

Roman RJ (2002) P-450 metabolites of arachidonic acid in the control of cardiovascular function. *Physiol Rev* **82**:131–185.

Seubert J, Yang B, Bradbury JA, Graves J, Degraff LM, Gabel S, Gooch R, Foley J, Newman J, and Mao L, et al. (2004) Enhanced postischemic functional recovery in CYP2J2 transgenic hearts involves mitochondrial ATP-sensitive K+ channels and p42/p44 MAPK pathway. *Circ Res* **95**:506–514.

Sinz M, Kim S, Zhu Z, Chen T, Anthony M, Dickinson K, and Rodrigues AD (2006) Evaluation of 170 xenobiotics as transactivators of human pregnane X receptor (hPXR) and correlation to known CYP3A4 drug interactions. *Curr Drug Metab* **7**:375–388.

Spector AA and Norris AW (2007) Action of epoxyeicosatrienoic acids on cellular function. *Am J Physiol Cell Physiol* **292**:C996–C1012.

Spiecker M and Liao J (2006) Cytochrome P450 epoxygenase CYP2J2 and the risk of coronary artery disease. *Trends Cardiovasc Med* **16**:204–208.

Spiecker M, Darius H, Hankeln T, Soufi M, Sattler AM, Schaefer JR, Node K, Börgel J, Mügge A, and Lindpaintner K, et al. (2004) Risk of coronary artery disease associated with polymorphism of the cytochrome P450 epoxygenase CYP2J2. *Circulation* **110**:2132–2136.

Thum T and Borlak J (2000) Cytochrome P450 mono-oxygenase gene expression and protein activity in cultures of adult cardiomyocytes of the rat. *Br J Pharmacol* **130**:1745–1752.

Wu S, Moomaw CR, Tomer KB, Falck JR, and Zeldin DC (1996) Molecular cloning and expression of CYP2J2, a human cytochrome P450 arachidonic acid epoxygenase highly expressed in heart. *J Biol Chem* **271**:3460–3468.

Wu S, Chen W, Murphy E, Gabel S, Tomer KB, Foley J, Steenbergen C, Falck JR, Moomaw CR, and Zeldin DC (1997) Molecular cloning, expression, and functional significance of a cytochrome P450 highly expressed in rat heart myocytes. *J Biol Chem* **272**:12551–12559.

Yamasaki T, Izumi S, Ide H, and Ohyama Y (2004) Identification of a novel rat microsomal vitamin D3 25-hydroxylase. *J Biol Chem* **279**:22848–22856.

Yang S, Wei S, Pozzi A, and Capdevila JH (2009) The arachidonic acid epoxygenase is a component of the signaling mechanisms responsible for VEGF-stimulated angiogenesis. *Arch Biochem Biophys* **489**:82–91.

Zeevi-Levin N, Itskovitz-Eldor J, and Binah O (2012) Cardiomyocytes derived from human pluripotent stem cells for drug screening. *Pharmacol Ther* **134**:180–188.

Zeldin DC, Foley J, Goldsworthy SM, Cook ME, Boyle JE, Ma J, Moomaw CR, Tomer KB, Steenbergen C, and Wu S (1997) CYP2J subfamily cytochrome P450s in the gastrointestinal tract: expression, localization, and potential functional significance. *Mol Pharmacol* **51**: 931–943.

Zhang L, Ding H, Yan J, Hui R, Wang W, Kissling GE, Zeldin DC, and Wang DW (2008) Genetic variation in cytochrome P450 2J2 and soluble epoxide hydrolase and risk of ischemic stroke in a Chinese population. *Pharmacogenet Genomics* **18**:45–51.

Zhang Y, El-Sikhry H, Chaudhary KR, Batchu SN, Shayeganpour A, Jukar TO, Bradbury JA, Graves JP, DeGraff LM, and Myers P, et al. (2009) Overexpression of CYP2J2 provides protection against doxorubicin-induced cardiotoxicity. *Am J Physiol Heart Circ Physiol* **297**: H37–H46.

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