Activity, inhibition and induction of cytochrome P450 2J2 in adult human primary cardiomyocytes

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Running Title: CYP2J2 activity, induction and inhibition in cardiomyocytes

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Abbreviations: CYP, cytochrome P450; hPSC, human pluripotent stem cells; hPSC-CMs, hPSC-derived cardiomyocytes; EET, epoxyeicosatrienoic acid; CPR, cytochrome P450 reductase
Abstract

Cytochrome P450 2J2 (CYP2J2) plays a significant role in the epoxidation of arachidonic acid to important signaling molecules 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 CYP-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 to 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/min and in the cells 6.0 pmol/pmol P450/min. CYP2J2 activity in the cell line was inhibited by danazol, astemizole and ketoconazole in sub-micromolar 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 (CYP2J2) has attracted particular attention for its ability to epoxidize arachidonic acid regio-selectively 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-re-oxygenation injury, up-regulation 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, directly decrease the membrane action potential in rat myocytes (Lu et al., 2001) and 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 to study 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 demonstrated drug metabolism in the heart tissue. The inhibitory or inductive effect by such drugs on arachidonic acid metabolism could have profound down-stream consequences by reducing EETs and their protective properties. However, a human heart model remains elusive and testing relies on animal models, especially dog, cell systems or recombinant enzymes. Much of CYP2J2’s activity has been assessed in such models as E.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 over express 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 and expressed CYP2J2 and measured its activity. Second, we evaluated the expression of a range of important CYPs in addition to CYP2J2 in human cardimyocytes by mRNA content compared to levels of CYP expression in human ventricular tissue. Third, we assessed the metabolic activity of CYP2J2 in the cardiomyocytes towards the probe substrates and characterized the kinetic
parameters compared to 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, USA), unless otherwise stated, and used without further purification.
Acetonitrile, methanol, water, ammonium formate and formic acid were purchased from
Fisher Scientific (Pittsburgh, PA, USA). Adult derived primary human cardiomyocytes,
cell culture media (complete growth media and serum-free media), solutions and cell
culture materials (culture flasks and plates, pre-coated 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 NIEHS. An internal NdeI site in
CYP2J2 was removed using the Quickchange II XL site directed mutagenesis kit
(Strategene, La Jolla, CA) with primers 5’:
GAAATTGTTTGTTTCTCA\textit{CAT}GATTGACAAACACAG,
3’:CTGTGTTTG\textit{G}TCAATCATG\textit{GT}GAGAAACAAACAAATTTTC (NdeI 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-\textit{NdeI}) 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’:ACTCATATGGCTCTGTTATTAGCAGTTTTTCTCAAAGACGCGCC and the same reverse 3’ primer:ATTCAGGTCGACACCTGAGGAACAGCGCAGGGCGGTG, 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 NdeI site into the 5’ primer and a SalI site into the 3’ primer and the pCWori plasmid contains a SalI site followed by a 6-histidine tag to facilitate subsequent purification and cloning. The N-terminus was consequently truncated (MLAAMGSLAAALWAVVHPRTLLLGTVALAADFLKRRP to MARRP). The resulting amplification products and the pCWori plasmid were digested with NdeI and SalI, resolved on a 2% agarose gel, excised with a scalpel and recovered with the Qiaquick gel extraction kit and ligated overnight with 1 Unit 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 (Omura and Sato, 1964).

Determination of Kinetic Parameters $K_m$ and $V_{max}$

Enzyme activity vs. protein was determined for recombinant enzymes at varying protein concentrations from 0.02 to 1 pmol P450 per 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 2pmol/mL rat cytochrome P450 reductase, 1pmol/mL Cytochrome b₅, buffer containing 100 mM potassium phosphate (pH 7.4), and 50 μM DLPC on ice for 40 min 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 1mM NADPH following a 5 minute preincubation at 37 °C (shaking at 70 strokes per minute). 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 14,000 x g 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% CO₂ 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 100xg for 5 mins. The pellet was then washed with PBS, and stored in 30 μL of RNA Later solution (Life Technologies, Carlsbad, CA) 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.
CYP mRNA Detection

Cells used for RNA isolation were harvested from human cardiomyocytes when approximately 80% confluent. Total RNA was extracted from approximately 1,000,000 cells using the MagMax 96 Total RNA Isolation kit (Invitrogen, Carlsbad, CA) and from human heart tissue using Trizol reagent and Purelink RNA Mini kit (Invitrogen, Carlsbad, CA). Total RNA was then used to synthesize cDNA using Oligo dT20 primers and the Superscript III First Strand Synthesis System (Invitrogen, Carlsbad, CA). RT-PCR was then carried out using TaqMan (Applied Biosystems, Carlsbad, CA) FAM reporter primers for the various cytochrome P450s screened as well as the housekeeping gene, GusB. Each biological 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 2ΔCT 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 CYP 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 CYP mRNA were compared to the levels of CYP2J2 mRNA levels using the ΔΔC_T calculation and relative CYP mRNA levels were reported using the 2-ΔΔCT calculation.

CYP protein content determination

To determine protein content, approximately one million cells were pelleted and homogenized in potassium phosphate buffer (100 mM, 250 μL). The homogenate was then centrifuged for 10 mins at 10000 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 NH₄HCO₃ (15 μL) and 100 mM stock DTT (1.5 μL). This solution was incubated at 95 °C for 5 minutes and allowed to cool. 100 mM stock iodoacetamide (IAA, 3 μL) was subsequently added and the samples were incubated for 20 mins at room temperature. The samples were then digested by adding 1 μL trypsin (100 ng/μL stock) and incubated for 1 hours 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 UPLC-MS/MS using an Agilent 4000 mass spectrometer (Santa Clara, CA), connected to an Agilent LC system. 10 μL of the sample was injected on a Phenomenex (Torrance, CA) Aeris PEPTIDE XB-C18 column (1.7 μ, 150 x 2.10 mm). The mobile phases consisted of aqueous phase A: 0.1 % formic acid in H₂O and organic phase B: 0.1 % formic acid in acetonitrile. The samples were analyzed using the following gradient: mobile phase B: 0-3 min, 3%; 3-5 min, 3-10%; 5-8 min, 10-50%; 8-8.4 min, 50%; 8.4-8.5 min, 50-90%; 8.5-9.5 min, 90%; 9.5-10 min, 90-3%; 10-10.5 min, 3%. The column was re-equilibrated to initial conditions for 1 min 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 2J2 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 (+10Da), also by Thermo-Fisher. The transitions monitored were 656.85>602.33 (2J2 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 % 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 (3500 x g, 10 min), and 150 μL was transferred to a new 96 well plate for spectrometric analysis.

In order 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 CYP2J2 above but using
midazolam (3 μM) or amodiaquine (2 μM) as probe substrates CYP3A4 and CYP2C8, respectively, instead of terfenadine.

**Metabolite Detection and Quantification**

Metabolites and parent were quantified on a Sciex API4000 LC/MS/MS (Applied Biosystems) connected to a Shimazu LC system (LC-10AD, SCL-10A) equipped with a CTC-PAL autosampler (LEAP Technologies, Carrboro, NC). 10 μL 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 min, 30% ; 1-2 min, 30 to 70% , 2 - 4 min 70 to 100% , 4-6.5 min 100% , 6.5-6.6 min 100-30%. The column was re-equilibrated at initial conditions for 1.4 min. 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 ms. 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 phase 0.1% v/v formic acid in water (A) and acetonitrile with 0.1% v/v formic (B), and gradient: 0-0.5 min, 20%B ; 0.5 to 1.5 min increase to 100% B, hold until 3.5 min, decrease B to 20% within 0.1 min and reequilibrate for 1 min. 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 1μM, 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 two 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 (3,500x
g, 10 min), 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), butylatedhydroxyanisole, butylatedhydroxytoluene (100 μM), and carbamazepine (100 μM). Induction by 6β-estradiol and testosterone was also tested at different concentration levels (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 1,000,000 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 twenty four 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), butylatedhydroxyanisole (BHA, 100 μM), butylatedhydroxytoluene (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 worked up as outlined under kinetic analysis.

To further investigate the effect of ritonavir and rosiglitazone on protein stability and terfenadine levels in the cell, follow up studies were performed where approximately one
million cells were induced with 100 μM ritonavir, rosiglitazone, or BHT (as another control) for 48 hours as described above and compared it 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 effected with 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 mins, 4 °C) to remove cell debris. 200 μL of sample was moved to LCMS vials and analyzed by mass spectrometry using the method outlined under kinetic analysis.

**Rosiglitazone inhibition of CYP2J2 activity**

The ability of rosiglitazone to inhibit CYP2J2 biotransformation of terfenadine was determined by co-incubating BD Gentest CYP2J2 supersomes (1 pmol/mL), 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 mins at 37 °C, initiated with NADPH (1 mM final concentration), and quenched with cold acetonitrile (100 μL) containing midazolam (100 nM) after 5 mins. Mass spectrometry analysis was carried out as previously described.
Data analysis

Apparent Michaelis–Menten constants $K_m$ and $V_{max}$ were derived after nonlinear regression analysis of the kinetic data using a Michaelis–Menten model (Prism 5 windows version 5.02, GraphPad Software, Inc., La Jolla, CA). Kinetic data is reported as the mean ± S.D. of triplicates in cells and as the mean ± 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 57kDa 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 (figure 1, table 1). Enzyme activity was expressed as rate of alcohol metabolite formed, using the peak height as a quantitative comparison to internal standard.

Cytochrome P450 mRNA Screen

*CYP2J2* was the major isozyme expressed among the CYPs that were screened in human cardiomyocytes (figure 2). *CYP2D6*, and *CYP2E1* were also detected at levels approximately twenty fold below that of *CYP2J2*. In human heart tissue, *CYP2J2* had also the greatest expression level. Several other CYP isozymes complemented CYP2J2 expression in human heart tissue, including *CYP2C8*, *CYP2D6*, *CYP2E1*, *CYP2V1*, *CYP3A4*, *CYP4A11*, *CYP4B1*, and *CYP4F12*, but expression levels were at least fifty fold lower than that of *CYP2J2*. 

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CYP2J2 Protein Content Determination

Using mass spectrometry, 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 (figure 3A, table 1) for terfenadine hydroxylation and $K_m$ of 5.22 μM for astemizole demethylation (figure 3B, table 1). In contrast to astemizole, terfinadine was toxic to the cells at at higher concentrations.

Inhibition of CYP2J2 in Human Cardiomyocytes

Inhibition was assessed at two concentrations of substrate (0.2 μM, figure 4A and 1.5 μM (at $K_m$), figure 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 (figure 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 (figure 4B).

**Hormone Effects on Gene Expression**

CYP2J2 induction by the 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 (figure 5). However, changes in the levels of transcription were not statistically different from control untreated cells.

**Induction of CYP2J2 in Human Cardiomyocytes**

Figure 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 (Figure 6A). An increase in CYP2J2 mRNA was observed when the cells were treated 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, omeprazol and clotrimazol also reduced CYP2J2 activity (Figure 6B). Other compounds did not appreciably affect the enzyme’s ability to oxidize terfenadine.

Post induction, 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 Figure 1)

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

Experiments to determine if rosiglitazone inhibited CYP2J2-mediated metabolism of terfenadine determined 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, many 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 CYP 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 sub-family were below limits of detection. DeLozier et al detected CYP2C in cardiac tissue samples, which were prepared from whole heart tissue (DeLozier et al., 2007). 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.
Km values for terfenadine hydroxylation were comparable in the cells and E. coli expressed system but were 10 fold higher than supersomes (1.5µM vs. 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 concentration of 0.2-0.3 pmol of CYP2J2 were used for Km experiments in the cardiomyocytes and E. coli expressed. It should be noted that the E. coli-expressed enzyme CYP2J2 has a truncation at the N-terminus and a 6x His-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 (CPR) 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 non-specific binding of terfenadine to the cells in altering the Km value cannot be determined at this time.

To test the inhibition of terfenadine hydroxylation in the heart, potential inhibitors with a documented history of cardio toxicity 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 concentrations closer to plasma levels and accumulation due to inhibited metabolism or transport. Further, two concentrations of substrate (0.2 and 1.5 µM) were chosen reflecting the measured in vitro Kₘ 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 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 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 not accounted for in this study (such as transport).

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. In 2010 Lee and Murray reported butylated hydroxyanisole (BHA) as a CYP2J2 inducer in HepG2 cells (Lee and Murray, 2010). Further work by Ma et al has shown that the mouse ortholog, CYP2J5, is regulated by sex hormones in murine kidneys (Ma et al., 2004). The results of this study, however, show that in cardiomyocyte, neither BHA nor the sex hormone $\beta$-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 induced their cells with BHA for 72 hours compared to the 48 hours of this study. Further, they replenished the BHA in their
cell media frequently during their induction (at 6h, 12h, 18h, 24h, and 48h) (Lee and Murray, 2010) whereas BHA was replenished at 24h in this study. This inability to induce CYP2J2 in cardiomyocytes indicates an important endogenous function with expression and activity tightly regulated in order 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 physiological pathways could have detrimental effects.

Other compounds tested for the ability to induce CYP2J2 transcription and CYP2J2 activity are classical CYP 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 PPAR (peroxisome proliferator-activated) receptor which also induces mRNA of CYP2B and CYP4 enzymes (Rogue et al., 2010).

Also, while our goal was to find potential 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 were at least 50% lower
than control samples (Supplemental Figure 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.

Another potential source of cardiomyocytes being investigated for drug screening are derived from human pluripotent stem cells (hPSCs) (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 CYPs, 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 have current limitations such as large scale use, incomplete differentiation and immaturity (Mordwinkin et al., 2013), making these primary cells a promising alternative.

In conclusion, this work provides an important step towards 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.
Author Contributions

Participated in research design: Evangelista, Kaspera, Mokadam

Conducted experiments: Evangelista, Kaspera, Jones

Contributed new reagents/Analytical Tools: Mokadam, Jones

Performed data analysis: Evangelista, Kaspera, Jones, Totah

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


Footnote

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Figure Legends

Figure 1: Kinetic parameters of terfenadine hydroxylation using recombinant E. coli expressed CYP2J2.

Figure 2: Relative levels of mRNA expression in human cardiomyocytes and human ventricular heart tissue.

Figure 3: Kinetic parameters of terfenadine hydroxylation (A) and astemizole demethylation (B) in human cardiomyocytes.

Figure 4: Inhibition of terfenadine hydroxylation at 0.2 μM (A) and 1.5 μM (B) at 1 μM and 10 μM inhibitor concentrations after two hours of incubation in human cardiomyocytes.

Figure 5: Induction of CYP2J2 mRNA expression with testosterone and beta-estradiol at varying concentrations (values relative to untreated controls normalized to a value of 1.0).

Figure 6: CYP2J2 mRNA expression and activity following 48h 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 panel (A) and 100% for panel (B)).
Table 1: Kinetic parameters of terfenadine and astemizole metabolism using recombinant enzyme or cardiomyocytes.

<table>
<thead>
<tr>
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<th>$K_m$ (μM)</th>
<th>$V_{max}$ pmol/pmol2J2/min</th>
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<tbody>
<tr>
<td><strong>Recombinant CYP2J2</strong></td>
<td>1.6 (±0.2)</td>
<td>29.4 (±0.9)</td>
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<tr>
<td>Terfenadine Hydroxylation</td>
<td></td>
<td></td>
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<tr>
<td><strong>Human Cardiomyocyte</strong></td>
<td>1.5 (±0.2)</td>
<td>6.0 (±0.2)</td>
</tr>
<tr>
<td>Terfenadine Hydroxylation</td>
<td></td>
<td></td>
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<tr>
<td><strong>Human Cardiomyocyte</strong></td>
<td>5.2 (±0.7)</td>
<td>3.2 (±0.1)</td>
</tr>
<tr>
<td>Astemizole Demethylation</td>
<td></td>
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Figure 1

![Graph showing the relationship between V (pmol prod/pmol P450/min) and [Terfenadine] (µM).]
Figure 2

- Relative mRNA levels

- CYP

- Human Cardiomyocytes
- Human Heart
Figure 5

[Bar chart showing relative CYP2J2 expression for βestradiol and testosterone at different concentrations (0.01 μM, 0.1 μM, 1 μM, 10 μM, 100 μM).]
Figure 6

A

Relative CYP2C2 Expression ($2^{\Delta\Delta CT}$)

B

% Activity

Phenytoin
Phenobarbital
Dexamethasone
Rifampin
Clotrimazole
Omeprazole
Rosiglitazone
Pitonavir
β-Naphthoflavone
BHA
BHT
Carbamazepine

Phenytoin
Phenobarbital
Dexamethasone
Rifampin
Clotrimazole
Omeprazole
Rosiglitazone
Pitonavir
β-Naphthoflavone
BHA
BHT
Carbamazepine