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ABSTRACT:

This study represents the first report on the characterization of luciferin-isopropyl acetal (LIPA) as a CYP3A4 substrate in human hepatocytes. LIPA metabolism by human hepatocytes was found to be linear with time up to 120 min and followed Michaelis-Menten kinetics, with apparent $K_M$ value of 15 $\mu$M and $V_max$ of 41 pmol/min/million hepatocytes for the hepatocytes used in the study. The non-specific cytochrome P450 (P450) inhibitor 1-aminobenzotriazole (ABT) and the CYP3A4-selective inhibitor ketoconazole (KTZ) caused concentration-dependent inhibition of LIPA metabolism, with more than 50% inhibition observed at the lowest concentrations evaluated of 7.8 $\mu$M (ABT) and 0.78 $\mu$M (KTZ), and near 100% inhibition observed at higher concentrations. Substantially lower inhibitory effects were observed for the non-CYP3A4 inhibitors diethyldithiocarbamate, furafylline, omeprazole, orphenadrine, sulfaphenazole, and quinidine. The commonly used organic solvents—acetonitrile, dimethyl sulfoxide (DMSO), and methanol—were found to inhibit LIPA metabolism, with approximately 50% inhibition at concentrations of 5, 1.25, and 5% (by volume), respectively. The comparatively higher inhibitory effects of DMSO relative to that for acetonitrile and methanol on LIPA metabolism were consistent with its known CYP3A4 inhibitory effects reported by others. LIPA metabolism in human hepatocytes was found to be induced by the treatment of human hepatocytes with the prototypical CYP3A4 inducers rifampin, carbamazepine, omeprazole, phenobarbital, and phenytoin but not by the CYP1A2 inducer 3-methylcholanthrene. Although the selectivity toward CYP3A4 needs to be definitively evaluated using cDNA-expressed P450 isoforms, the results suggest that LIPA is a suitable substrate to be used with human hepatocytes for the evaluation of CYP3A4 activities.

Cytochrome P450 (P450) is an important family of enzymes responsible for xenobiotic metabolism. Of the multiple isoforms, P450 isoform 3A4 (CYP3A4) is the most abundant of the isoforms in the human liver. CYP3A4 has been found to be responsible for the metabolism of a large variety of exogenous and endogenous substrates (Guengerich, 2006; Zhou, 2008). In drug development, there is a need to evaluate the inhibitory and inductive potential of drug candidates toward CYP3A4 to estimate their drug-drug interaction potential with the myriad drugs that are substrates of this important P450 isoform (Lin and Lu, 1998; Li, 2001).

Quantification of CYP3A4 activity of an enzyme system (e.g., liver microsomes, hepatocytes) is performed by incubation with a CYP3A4-selective substrate, followed by quantification of the metabolites formed. The most commonly used substrates are testosterone and midazolam, with $6\beta$-hydroxytestosterone (Chauret et al., 1998; Easterbrook et al., 2001) and 1’-hydroxymidazolam (Emoto and Iwasaki, 2007), respectively, as the CYP3A4-mediated metabolites (Guengerich, 2006). Quantification of these metabolites requires the use of high-performance liquid chromatography (HPLC), often facilitated by mass spectrometry (MS) (Yin et al., 2000).

In our laboratory, human hepatocytes are routinely used in the evaluation of drug metabolism, drug-drug interactions, and drug toxicity (Li, 2007). We recently embarked on research into higher throughput approaches for the evaluation of CYP3A4 inhibition and induction in intact human hepatocytes. We are interested in substrates that are metabolized specifically by CYP3A4 to form metabolites that are quantifiable by photometry using multiwell plate readers. Luciferin-isopropyl acetal (LIPA) is a novel substrate that is believed to be metabolized specifically by CYP3A4 to release luciferin, which can be quantified by luminescence through reaction with an ATP-luciferase reaction mixture (Fig. 1; J. Cali, personal communication). We report here the enzyme kinetics, the effects of organic solvents, and the effects of P450 inhibitors and inducers on LIPA metabolism by intact human hepatocytes. This study represents the first report on the characterization of LIPA metabolism in human hepatocytes.

Materials and Methods

Chemicals and Reagents. All the cell culture media and supplements, dimethyl sulfoxide (DMSO), and the P450 inhibitors and inducers were obtained from Sigma-Aldrich (St. Louis, MO). Acetonitrile and methanol were obtained from VWR (West Chester, PA). LIPA and luciferin standard were

ABBREVIATIONS: P450, cytochrome P450; HPLC, high-performance liquid chromatography; MS, mass spectrometry; LIPA, luciferin isopropyl acetal or 2-(4-(disisopropoxymethyl)-4,5-dihydrothiazol-2-yl)benzo[d]thiazol-6-olOMP; DMSO, dimethyl sulfoxide; KHB, Krebs-Henseleit buffer; ABT, 1-aminobenzotriazole.
gifts from Drs. Jim Cali and Mary Sobol of Promega (Madison, WI). The Luciferin Detection Reagent was obtained from Promega.

Human Hepatocytes. The human hepatocytes used in this study were isolated and subsequently cryopreserved in our laboratory from a nontransplantable human liver made available for use in research. Cryopreserved human hepatocytes (Hu4165) were isolated from a 57-year-old white woman (cause of death: anoxia) using our previously published methods for hepatocyte isolation and cryopreservation (Loretz et al., 1989; Li et al., 1992, 1995; Li, 2007, 2008). On thawing, the cells were 92% viable based on trypan blue exclusion, had a plating efficiency of 95%, and formed near-confluent monolayer cultures.

LIPA Metabolism. The cryopreserved human hepatocytes were thawed from cryopreservation using Cryopreserved Hepatocytes Recovery Medium (APSciences, Columbia, MD) and plated at 50,000 cells/well in 96-well collagen-coated plates (APSciences) in Cryopreserved Hepatocytes Plating Medium (APSciences) at a volume of 100 μl/well. The cells were cultured for 24 h in a cell culture incubator maintained at 37°C with a highly humidified atmosphere of 5% carbon dioxide and 95% air. The cells on the day after plating (24-h cultures) were used for the evaluation of LIPA metabolism. For the determination of K_m and V_max, the plating medium was removed and replaced with Krebs-Henseleit buffer (KHB) containing 3 mM salicylamide and various concentrations of LIPA. The cells were then incubated for 30, 60, and 120 min. Triplicate wells were used per LIPA concentration and time point. At the end of each incubation period, 50 μl of the incubated substrate solution from each well was removed and replaced into a white 96-well plate (BD Biosciences, San Jose, CA). After all the solutions were collected from the various time points, 50 μl of Luciferin Detection Reagent (Promega) was added to each well containing incubated substrate solution followed by quantification of luminescence using a Wallac Victor-3 plate reader (PerkinElmer Life and Analytical Sciences, Waltham, MA). Luminescence signals were converted to picomoles of luciferin based on a standard curve generated from 1.5 to 1500 pmol of standard luciferin (Promega). K_m and V_max Determination. K_m and V_max were determined based on Lineweaver-Burk analysis. The reciprocals of the initial rates of metabolism at various LIPA concentrations (derived from the slope of the time course) were plotted as the y-axis, and the reciprocals of the LIPA concentrations were plotted as the x-axis, with the x-intercept as the reciprocal of the K_m and the y-intercept as the reciprocal of the V_max.

Effects of P450 Inhibitors. The effects of various P450 inhibitors on LIPA metabolism by human hepatocytes were determined by incubating the hepatocytes at 37°C with 3 μM LIPA in KHB containing 3 mM salicylamide in the presence of various concentrations of P450 inhibitors.

Treatment with P450 Inducers. The cryopreserved human hepatocytes were thawed from cryopreservation using Cryopreserved Hepatocytes Recovery Medium (APSciences) and plated at 50,000 cells/well in 96-well collagen-coated plates (APSciences) in Cryopreserved Hepatocytes Plating Medium (APSciences) on the day of experimentation (day 1). After incubation overnight (day 2), the cells were overlaid with 0.2 mg/ml Matrigel (BD Biosciences) and returned to the cell culture incubator for another 24 h of culturing. On day 3, the cells were treated with the various P450 inducers for 3 days, after which the medium containing the inducers was removed and replaced with 100 μl of KHB containing 3 mM salicylamide and 3 μM LIPA. The plates were returned to the incubator for 120 min, after which a 50-μl aliquot was removed from each well and placed into wells of white 96-well plates for luciferin detection as described.

Data Analysis. Results for the inhibition or induction of LIPA metabolism are expressed as relative activity, which is calculated as a ratio of the activity in the presence of inhibitors or inducers to that of the solvent control using the following equation: Relative Activity (%) = [Activity (Treatment)/Activity (Solvent Control)] × 100.

Statistical Analysis. The statistical significance of inhibition or induction was determined by comparing treatment with solvent control based on Dunnett’s t test using JMP 6.0 statistical software (SAS Institute, Cary, NC), with p ≤ 0.05 as the level of significance.

Results

Kinetics of LIPA Metabolism. The time course for the metabolism of LIPA by human hepatocytes at LIPA concentrations ranging from 1.32 to 15 μM is shown in Fig. 2. The time course based on luminescence units was shown to illustrate the relatively high signal to noise ratio of the endpoint. The luminescence values of the LIPA media (at all LIPA concentrations from 1.32–15 μM) without incubation with hepatocytes were approximately 600 luminescence units, which is similar to that of a blank plate, indicating that LIPA has virtually no luminescence when treated with the detection reagent (data not shown). Linear or near-linear time-dependent increases in luciferin formation were observed at all the LIPA concentrations up to 120 min, except the lowest concentration of 1.32 μM, where a slightly slower rate of metabolism was observed at the longer time points of 90 and 120 min. Based on the amount of luciferin formed, the percentage of LIPA consumed at the various concentrations at the 120-min incubation period ranged from 9.7% at the lowest concentration of 1.32 μM and decreased with concentration to 6.5% at the highest concentration of 15 μM. Therefore, the linearity of the time course reflects the relatively unchanged substrate concentration during the incubation period.

Fig. 3. Lineweaver-Burk plot of LIPA metabolism in human hepatocytes. The y-axis, 1/V, is the reciprocal of the initial rate of metabolism (v) measured as picomoles of luciferin generated (representing picomoles of LIPA metabolized) per minute per million hepatocytes. The x-axis, 1/[S], is the reciprocal of the LIPA concentration in micromolars. The linear regression-derived equation and the coefficient of correlation (r^2) are shown. Each data point represents the average of triplicate observations.
The kinetics of LIPA metabolism was evaluated by Lineweaver-Burk analysis, plotting the reciprocal of initial velocity versus the reciprocal of substrate concentration as shown in Fig. 3. The plot was found to be linear (r² = 0.9965). The Kₘ and Vₘₐₓ values were calculated to be 15 μM and 41 pmol/min/million hepatocytes, respectively.

**Effects of P450 Inhibitors on LIPA Metabolism.** The LIPA metabolism in human hepatocytes was evaluated in the presence of various concentrations of model P450 inhibitors (Fig. 4). The non-specific, mechanism-based P450 inhibitor 1-aminobenzotriazole (ABT) and the CYP3A4 inhibitor ketoconazole were found to cause concentration-dependent inhibition of LIPA metabolism, with >50% inhibition at the lowest concentration evaluated of 7.8 μM ABT and 0.78 μM ketoconazole. Of the non-CYP3A4 selective inhibitors, omeprazole and quinidine showed the highest inhibitory effects. Statistically significant (p < 0.05) inhibition was observed for omeprazole at all the concentrations evaluated, with approximately 50% inhibition observed at the highest concentrations of 25 and 50 μM. Statistically significant inhibition was observed for quinidine at 12.5, 25, and 50 μM, with approximately 40 to 50% inhibition observed at these concentrations. Slight (less than 50%), but statistically significant (p < 0.05), decreases were observed for furafylline at 12.5 μM, with approximately 40 to 50% inhibition observed at these concentrations. Slight (less than 50%), but statistically significant (p < 0.05), decreases were observed for furafylline at 12.5 μM, diethyldithiocarbamate at all the concentrations evaluated, and for orphenadrine at 0.8, 25, and 50 μM. No statistically significant inhibition was observed for sulfaphenazole.

**Effects of Organic Solvents on LIPA Metabolism.** The effects of three commonly used organic solvents—acetonitrile, DMSO, and methanol—on LIPA metabolism in human hepatocytes are shown in Fig. 5. DMSO was found to have the highest inhibitory effects. Approximately 50% inhibition of LIPA metabolism was observed at approximately 1.25% v/v for DMSO (0.16 M), 5% v/v for acetonitrile (1.2 M), and 5% v/v for methanol (1.6 M).
Effects of P450 Inducers on LIPA Metabolism. Statistically significant and concentration-dependent induction of LIPA metabolism was observed for the model CYP3A inducers rifampin, carbamazepine, omeprazole, phenobarbital, and phenytoin (Fig. 6). Concentration-dependent induction of LIPA metabolism was observed. For rifampin, statistically significant induction was observed at all the concentrations evaluated from 0.78 to 50 \( \mu \text{M} \), with the highest level of 5398\% observed at 50 \( \mu \text{M} \). For carbamazepine, statistically significant induction was observed from 6.25 to 50 \( \mu \text{M} \), with the highest level of 8826\% for observed at 50 \( \mu \text{M} \). For phenobarbital, statistically significant induction was observed at 1.56 to 25 \( \mu \text{M} \), with the highest level of 3842\% observed at 12.5 \( \mu \text{M} \). For phenytoin, statistically significant induction was observed at 113 to 900 \( \mu \text{M} \), with the highest level of 8448\% observed at 12.5 \( \mu \text{M} \). Omeprazole showed the lowest level of induction, with statistically significant induction observed at only one concentration (25 \( \mu \text{M} \), 1257\%). No induction of LIPA metabolism was observed for the CYP1A inducer 3-methycholan-threne at the concentrations evaluated.

Discussion

We have recently optimized the procedures for the cryopreservation of human hepatocytes, yielding cells that, after thawing, retain high viability and the ability to be cultured as confluent, monolayer cells (Li, 2007, 2008). The activities of our laboratory are now focused on the development of practical experimental approaches to apply these “plateable” cryopreserved human hepatocytes in the understanding of human drug metabolism and drug-drug interactions.

The results of this report represent our research to develop higher throughput assays for the evaluation of CYP3A4 inhibition and induction assays using the plateable cryopreserved human hepatocytes. Quantification of CYP3A4 activities routinely requires the use of HPLC, preferably coupled with MS. The commonly used approach is the quantification of testosterone-6\( \beta \)-hydroxylation and midazolam 1-hydroxylation (Guengerich, 2006). Although the use of liquid chromatography/MS analysis is a routine practice and higher throughput automated approaches are available (e.g., Yin et al., 2000), it remains a bottleneck when a large number of samples are to be analyzed.

LIPA, if determined to be a CYP3A4-specific substrate, would greatly facilitate experimentation with this important P450 isoform. The metabolite of LIPA, luciferin, can be readily quantified by ATP-luciferin-based luminescence using a multiwell plate reader in a rapid, highly sensitive, and highly quantitative manner.

Our report here represents the first to report on the characterization of LIPA metabolism in human hepatocytes. We determined that human hepatocytes, as monolayer cultures, metabolized LIPA in a time- and concentration-dependent manner, following Michaelis-Menten kinetics. The quantification of luciferin by luminescence was found to have a high sensitivity, with a detection limit (based on the criteria of \( >3 \times \) background) of 6 pmol. This high sensitivity allows evaluation of LIPA metabolism in the 96-well plates, using 50,000 cells/well in 100 \( \mu \text{l} \) of total LIPA incubation medium, with quantification of luciferin in 50 \( \mu \text{l} \) of the medium, using LIPA concentration as low as 1.3 \( \mu \text{M} \). Linear time course for LIPA metabolism was observed at 3 \( \mu \text{M} \) and higher concentrations for up to 120 min. LIPA metabolism by the hepatocytes apparently followed Michaelis-Menten kinetics. With the specific lot of human hepatocytes used, the apparent \( K_m \) was found to be 15 \( \mu \text{M} \), and the \( V_{\text{max}} \) was 41 pmol/min/million hepatocytes. This observation of xenobiotic metabolism by intact hepatocytes as monolayer cultures following Michaelis-Menten kinetics has been previously reported by us using monolayer cultured hepatocytes with lidocaine as a substrate (Li et al., 1995) and others using hepatocytes in suspension (Griffin and Houston, 2004).

On showing that LIPA was readily metabolized by intact human hepatocytes, we focused our research on its specificity as a CYP3A4 substrate using P450 inhibitors. ABT, a nonspecific, mechanism-based P450 inhibitor, was found to be a potent inhibitor of LIPA metabolism, therefore suggesting P450-dependent metabolism. LIPA metabolism was inhibited in the presence of ketoconazole, a CYP3A4...
inhibitor, but not by the non-CYP3A4 inhibitors furafylline (CYP1A2-selective inhibitor), omeprazole (CYP2C19-selective inhibitor), orphenadrine (CYP2B6-selective inhibitor), sulfaphenazole (CYP2C9-selective inhibitor), quinidine (CYP2D6-selective inhibitor), and diethyldithiocarbamate (CYP2E1-selective inhibitor). That ABT and ketoconazole at the higher concentrations would cause virtually total inhibition of metabolism suggests that P450 metabolism, specifically of the CYP3A4 isoform, is entirely responsible for LIPA metabolism. The weak inhibitory effects of omeprazole and quinidine on LIPA metabolism are probably a result of them being CYP3A4 substrates rather than their non-CYP3A4 inhibitory effects. Omeprazole is known to be hydroxylated by CYP2C19 and sulfoxidized by CYP3A4 (Bertilsson et al., 1997). Quinidine 3-hydroxylation is known to be metabolized by CYP3A4 and CYP3A5 (Allqvist et al., 2007). The results with the inhibitors suggest that LIPA is a specific CYP3A4 substrate in human hepatocytes.

The results with model inducers on LIPA metabolism are also consistent with it being a CYP3A4 substrate. The known inducers of CYP3A4 in human hepatocytes—rifampin (Li et al., 1995; Roymans et al., 2004; Shou et al., 2008), carbamazepine (Shou et al., 2008), omeprazole (Masubuchi et al., 1998), phenobarbital (Shou et al., 2008), and phenytoin (Luo et al., 2002)—were found to cause concentration-dependent induction of LIPA metabolism. The observation of omeprazole as a less potent inducer of CYP3A4 than rifampin is consistent with historical data in our laboratory (unpublished data) and others (Roymans et al., 2004) using testosterone 6β-hydroxylation as the activity endpoint. As expected, the CYP1A2 inducer 3-methylcholanthrene did not induce LIPA metabolism. An interesting observation is the dynamic range of LIPA metabolism as an endpoint for CYP3A4 induction. The potent CYP3A4 inducers rifampin, carbamazepine, phenobarbital, and phenytoin increased LIPA metabolism to more than 30-fold of solvent control activities. The results suggest that LIPA metabolism can be used as a sensitive and time- and cost-effective endpoint for the quantification of CYP3A4 induction.

There are numerous reports on the inhibitory effects of organic solvents on P450-dependent metabolism (Chauret et al., 1998; Busby et al., 1999; Easterbrook et al., 2001; Chefson and Auclair, 2007). Our results on the effects of organic solvents on LIPA metabolism, that DMSO had a higher inhibitory potential than acetone or methanol, are consistent with previously published observations with CYP3A4 in cDNA-expressed human microsomes (Busby et al., 1999), human liver microsomes (Chauret et al., 1998; Chefson and Auclair, 2007), and our previous findings using testosterone as sub-
strate in human hepatocytes (Easterbrook et al., 2001). The results suggest that acetonitrile and methanol are preferred solvents to be used at concentrations up to 1% when LIPA is chosen as a substrate for the quantification of CYP3A4 activity. Because of its inhibitory effect, DMSO, if used, should be used at 0.1% or lower concentrations.

Although the selectivity toward CYP3A4 needs to be definitively evaluated using cDNA-expressed P450 isoforms, our results suggest LIPA is a substrate that can be used to effectively quantify CYP3A4 activity in human hepatocytes. The use of plateable cryopreserved human hepatocytes cultured in 96-well plates, with CYP3A activities quantified by LIPA metabolism, represents relatively high throughput approaches for the evaluation of CYP3A inhibition and induction.

References


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