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Brief communication

Simultaneous assessment of cytochrome P450 activity in cultured human hepatocytes for compound-mediated induction of CYP3A4, CYP2B6, and CYP1A2

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ABSTRACT

Introduction: The human nuclear receptors pregnane X receptor (PXR), constitutive androstane receptor (CAR), and aryl hydrocarbon receptor (*Ah*R) are known to regulate gene expression of the cytochrome P450 (CYP) enzymes, 3A4, 2B6, and 1A2, respectively. In conventional CYP induction studies, the activity of each CYP enzyme is assessed in a separate incubation with the appropriate marker substrate. The objective of this study was to assess, simultaneously, the induction of CYP3A4, CYP2B6, and CYP1A2 activity in cultured human hepatocytes treated with various prototypical ligands of PXR, CAR, and *Ah*R by utilizing an optimized substrate cocktail, as well as a rapid, sensitive liquid chromatography-mass spectrometry method. **Methods:** To evaluate the xenobiotic-mediated induction of hepatocellular gene expression, the prototypical inducers rifampicin (10 μM) and phenobarbital (1 mM) were used for CYP3A4, CITCO (1 μM) and artemisinin (50 μM) were used for CYP2B6, and 3-methylcholanthrene (1 μM) and omeprazole (50 μM) were utilized for induction of CYP1A2. Primary human hepatocytes were treated with each compound for 48 h, followed by a 30-min incubation of the hepatocyte culture along with the addition of three marker substrates for specific CYP activity: midazolam (CYP3A4; 5 μM), bupropion (CYP2B6; 50 μM), and phenacetin (CYP1A2; 100 μM). The assessment of CYP activity was performed with a rapid, sensitive liquid chromatography-tandem mass spectrometry method which simultaneously assessed activity of CYP3A4, CYP2B6, and CYP1A2 in a single 3-min method by examining the formation of the probe substrate metabolites, 1′-hydroxymidazolam, hydroxybupropion, and acetaminophen, respectively. **Results:** The average fold-induction of CYP3A4, CYP2B6, and CYP1A2 activity was comparable between the cocktail and the conventional assay. **Discussion:** The combination of three marker substrates in a single 30-min incubation, in addition to a rapid, sensitive LC-MS/MS method, resulted in an efficient and robust method for assessing cytochrome P450 induction as compared to the conventional methodology.

1. Introduction

Induction of human cytochrome P450s 3A4, 2B6, and 1A2 is important to consider in the selection of clinical candidate compounds in order to avoid the potential for adverse drug–drug interactions during clinical development (Hewitt et al., 2007). The human pregnane X receptor (PXR), constitutive androstane receptor (CAR), and aryl hydrocarbon receptor (*Ah*R) are known to regulate the gene expression of CYP3A4, CYP2B6, and CYP1A2 respectively (Faucette et al., 2006, 2007; Maglich et al., 2003). Emerging evidence that CAR plays an important role in regulating xenobiotic metabolism provides the rationale for assessment of this nuclear receptor (Faucette et al., 2004; Lamba et al., 2003). Conventionally, enzyme activity determinations are carried out as single probe substrate incubations and require separate LC-MS/MS methods for each metabolite (Roymans et al., 2005). Here, we have developed a unique probe substrate incubation cocktail along with a rapid and sensitive LC-MS/MS method to determine cytochrome P450 induction activities of 3A4, 2B6, and 1A2 in human hepatocytes that allow for simultaneous assessment of enzyme activity in a single 3-min method.

2. Materials

Reagents used included acetonitrile (HPLC grade, Burdick & Jackson, Madison, WI), methanol (HPLC grade, Burdick & Jackson),

Abbreviations: *Ah*R, aryl hydrocarbon receptor; CAR, constitutive androstane receptor; cDNA, complementary deoxyribonucleic acid; C_t, cycle threshold; CYP, cytochrome P450; CITCO, (6-(4-chlorophenyl)imidazo[2,1-*b*][1,3]thiazole-5-carbaldehyde-*O*-(3,4-dichlorobenzyl)oxime); DDI, (drug–drug interaction); DMSO, dimethyl sulfoxide; *goi*, gene of interest; HPLC, high performance liquid chromatography; LC-MS/MS, liquid chromatography-tandem mass spectrometry; mRNA, messenger ribonucleic acid; PXR, pregnane X receptor; qRT-PCR, real-time quantitative reverse transcription polymerase chain reaction.

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water (HPLC grade, JT Baker, Phillipsburg, NJ), dimethyl sulfoxide (DMSO), (EM Science, Darmstadt, Germany), InVitro*GRO* Hepatocyte Incubation Media (In Vitro Technologies, Baltimore, MD), Torpedo Antibiotic Mix (In Vitro Technologies), phosphate buffered saline (PBS) pH 7.4 (Sigma-Aldrich, Co., St. Louis, MO), Hanks Balanced Salt Solution (HBSS), pH 7.4 (Sigma-Aldrich), and 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid (Hepes) (Sigma-Aldrich). Rifampicin, phenobarbital, artemisinin, 3-methylcholanthrene, omeprazole, midazolam, bupropion, and phenacetin were purchased from Sigma-Aldrich. CITCO (6-(4-chlorophenyl)imidazo[2,1-*b*][1,3]thiazole-5-carbaldehyde-*O*-(3,4-dichlorobenzyl)oxime) was purchased from EMD Chemicals (Gibbstown, NJ). 1′-Hydroxymidazolam (Toronto Research Chemicals, North York, Ontario, Canada), hydroxybupropion (Toronto Research Chemicals), and acetaminophen (Sigma-Aldrich) were used as LC-MS standards for quantification of CYP3A4, CYP2B6, and CYP1A2 activity, respectively. Labetalol (Sigma-Aldrich) was used as an internal standard. Fresh human hepatocytes were purchased from CellzDirect (Pittsboro, NC). All other reagents and solvents were of the highest analytical grade supplied by Sigma-Aldrich.

3. Methods

3.1. Hepatocyte Incubations

Hepatocytes were obtained from CellzDirect in 24-well collagen-coated tissue culture plates with Matrigel® overlay. The shipping media was removed and replaced with InVitro*GRO* Hepatocyte Incubation Media supplemented with Torpedo Antibiotic Mix as directed and allowed to incubate at 37 °C, 95% humidity, and 5% CO_2 for approximately 2 h prior to initiating the experiment. Hepatocyte cultures from each of four donors were treated for two consecutive days (Roymans et al., 2005; Sinz, Wallace, & Sahi, 2008) with 10 μM rifampicin, 1 mM phenobarbital, 1 μM CITCO, 50 μM artemisinin, 1 μM 3-methylcholanthrene, or 50 μM omeprazole. The experimental control culture wells were treated with solvent alone (0.2% DMSO). The culture medium was exchanged every 24 h with a fresh supplemented culture medium containing vehicle or test articles over the two-day (48-h) experiment. All experimental conditions were carried out with cultured hepatocytes from four individual donor livers and performed in triplicate wells. All experiments were repeated in an additional set of culture plates to assess the effects of the inducers and vehicle control on hepatocyte viability.

3.2. Assessment of hepatocyte culture viability

After the final day of exposure, the media were aspirated from the hepatocyte cultures designated for evaluation of cell viability, rinsed with PBS (pH 7.4), and assessed with the ATPLite Luminescence ATP Detection Assay System (PerkinElmer, Boston, MA). Hepatocytes were lysed using 200 μL mammalian cell lysis solution and placed in an orbital shaker (IKA MTS 2/4 Digital Microtiter Shaker, VWR, West Chester, PA) for 5 min at 700 rpm. A 200-μL aliquot of the substrate solution was added to each well and placed in an orbital shaker for 5 min at 700 rpm. A 100-μL aliquot was transferred from each well of the culture plate into a white 96-well polypropylene plate (Costar, VWR, West Chester, PA). The plate was dark-adapted for 10 min and luminescence was measured using a Wallac Victor 1420 Multilabel HTS Counter (PerkinElmer).

3.3. Determination of CYP3A4, CYP2B6, and CYP1A2 activity in situ

After two days of treatment with the test articles, the media was aspirated from the hepatocyte cultures, rinsed with warm (37 °C) PBS (pH 7.4), and assessed for induction of CYP3A4, CYP2B6, and CYP1A2 activity by the addition of 10 mM Hepes-HBSS buffer supplemented either as a cocktail or as a single agent (conventionally) with 5 μM midazolam (CYP3A4), 50 μM bupropion (CYP2B6), and 100 μM phenacetin. The cells were incubated at 37 °C, 95% humidity, and 5% CO_2 for 30 min. After the 30-min incubation, a 100-μL aliquot of the cocktail buffer or buffer from the single substrate incubation was transferred from each well of their respective culture plate and into a clear 96-well polypropylene plate, followed by the addition of 100 μL 25% acetonitrile with the internal standard, 1 μM labetalol, for analysis by LC-MS/MS.

3.4. LC-MS/MS analysis of enzyme activity

Midazolam 1′-hydroxylase, bupropion hydroxylase, and phenacetin *O*-deethylase activities were quantitated using an LC-MS/MS system comprised of an HTS-PAL autosampler (Leap Technologies, Carrboro, NC), an HP1100 HPLC (Agilent, Palo Alto, CA), and an API4000 triple quadrupole mass spectrometer (PE Sciex, a division of Applied Biosystems, Foster City, CA). Chromatographic separation of the analytes and internal standard was achieved using a Discovery RP-Amide column (2.1 mm × 5 cm, 5.0 μm, Supelco, Sigma-Aldrich, St Louis, MO) in conjunction with gradient conditions using mobile phases A (0.1% acetic acid in HPLC-grade water) and B (0.1% acetic acid in 25:75 methanol: acetonitrile) and a flow rate of 0.6 mL/min. For the cocktail method, a total ion abundance of seven specific multiple reaction monitoring (MRM) transitions in electrospray positive ionization (ESI+) mode were monitored. Analyte responses were measured by MRM of transitions unique to each compound. The transitions of the protonated precursor ions to the selected product ions were *m/z* 326.0 to 291.0 for midazolam, *m/z* 342.1 to 324.4 for 1′-hydroxymidazolam, *m/z* 240.1 to 184.3 for bupropion, *m/z* 256.1 to 238.0 for hydroxybupropion, *m/z* 152.1 to 109.9 for both phenacetin and acetaminophen, and *m/z* 329.4 to 311.0 for labetalol, the internal standard. The total run time, including re-equilibration time, for a single injection was 3 min. The chromatographic separation of each analyte is shown in Fig. 1.

3.5. Real-time qRT-PCR analysis of enzyme mRNA

Total RNA was isolated from each culture using the RNeasy 96 kit for total RNA isolation (Qiagen, Valencia, CA). The RNA samples were DNase-treated and 12.5 μL of total RNA was reverse-transcribed using the High-Capacity cDNA Archive Kit (Applied Biosystems, Inc., Foster City, CA), which employs random hexamers, in a total reaction volume of 50 μL. Reverse-transcription of total RNA to cDNA was performed by a two-step thermocycle: 25 °C for 10 min and 37 °C for 120 min. The cDNA product was stored at −80 °C until analysis by quantitative real-time PCR (qRT-PCR). Applied Biosystems inventoried primers and FAM™ dye-labeled Taqman® MGB (minor groove binding) probes for CYP3A4 (assay ID: Hs00430021_m1), CYP2B6 (assay ID Hs00167937_g1), CYP1A2 (assay ID: Hs00167927_m1), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; assay ID: Hs99999905_m1) were purchased as 20x stocks and used as per the manufacturer's instructions (final concentration of 900 nM primers and 250 nM probe). Real-time qRT-PCR (7900HT Real-Time PCR System, Applied Biosystems, Foster City, CA) was performed on each sample for each of the four end points, CYP3A4, CYP2B6, CYP1A2, and GAPDH. Each 20 μL reaction contained one of the primer/probe sets, an aliquot of cDNA (2 μL), 2X-Taqman® Universal PCR Master Mix (10 μL), and RNase-free water q.s. to 20 μL. Real-time qRT-PCR conditions were as follows: 1 cycle at 50 °C, 2 min; 1 cycle at 95 °C, 10 min; 40 cycles at 60 °C, 1 min. Compound induced changes in gene expression were determined by the ΔΔCt method. The cycle threshold value (Ct) represents the PCR cycle at which the level of fluorescence during qRT-PCR for a specific gene of interest (*goi*) exceeds the baseline threshold. By this method, the Ct for the *goi* in response to each treatment condition (*i.e.,* compound or concentration) was normalized by the Ct for the housekeeping gene, GAPDH ($Ct_{goi} - Ct_{GAPDH} = \Delta Ct$). The ΔCt for each treatment condition was normalized by the ΔCt obtained with the vehicle control to obtain the ΔΔCt ($\Delta Ct_{compound} - \Delta Ct_{vehicle\ control} = \Delta\Delta Ct$).