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BIOTECHNOLOGY and BIOENGINEERING

Development of a Whole-Cell Screening System for Evaluation of the Human CYP1A2-Mediated Metabolism

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Received 22 March 2011; revision received 23 May 2011; accepted 20 June 2011

Published online 13 July 2011 in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/bit.23256

ABSTRACT: Cytochrome P450 1A2 (CYP1A2) is an important member of cytochrome P450 involved in drug metabolism. In this study, a cell line, Huh7-1A2-I-E, with high expression level of CYP1A2 is established based on Huh7 cells. To achieve this, we constructed a recombinant lentiviral vector, pLenti-1A2-I-E, containing a single promoter encoding CYP1A2 followed by an internal ribosome entry site (IRES) to permit the translation of enhanced green fluorescence protein (EGFP). Such a design has greatly facilitated the selection of stable cell lines because the translations of CYP1A2 and EGFP proteins would be based on a single bi-cistronic mRNA. The Huh7-1A2-I-E cells were evaluated as a cell-based model for identification of CYP1A2 inhibitors and for studies of cytotoxicity resulted from CYP-mediated drug metabolism. Treatment of Huh7-1A2-I-E cells and the Huh7-E control cells with aflatoxin B1 showed that cells with CYP1A2 expression are much more sensitive to aflatoxin B1 and the cellular toxicity of aflatoxin B1 in Huh7-1A2-I-E cells could be prevented by furafylline, a CYP1A2 inhibitor. A collection of approximately 200 drugs were screened using this system and results indicate that for most drugs the metabolism by CYP1A2 is unlikely to have made a major contribution to the in vitro cytotoxicity except for thimerosal and evoxine. Several previously unidentified CYP1A2 inhibitors such as evoxine and berberine were also identified in this study.

Biotechnol. Bioeng. 2011;108: 2932–2940.

KEYWORDS: cytochrome P450; drug metabolism; cytotoxicity

Abbreviations: CYPs, cytochrome P450; CYP1A2, cytochrome P450 1A2; IRES, internal ribosome entry site; EGFP, enhanced green fluorescence protein; CO, carbon monoxide; CEC, 3-cyano-7-ethoxycoumarin; CHC, 3-cyano-7-hydroxycoumarin; EDTA, ethylenediaminetetraacetic acid; PBS, phosphate buffered saline; HEPES, 4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid; DMSO, dimethylsulfoxide; ddH_2O, double-distilled water; MTS, 3-(4,5-di-methylthiazol-2-yl)-5-(3-carboxy-methoxy-phenyl)-2-(4-sulfophenyl)-2H-tetrazolium; PCR, polymerase chain reaction; PFU, plaque-forming unit; *S/N*, signal-to-noise ratio; *S/B*, signal-to-background ratio.

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Contract grant sponsor: National Health Research Institutes (NHRI) in Taiwan

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Introduction

Drug metabolism in human typically involves the chemical modifications of drugs and this process occurs mostly in liver where exist the majority of drug-metabolizing enzymes such as Cytochrome P450 (CYPs). CYPs catalyze most of the phase I drug metabolizing reactions such as oxidation and reduction (Lin and Lu, 1998; Tang et al., 2005).

CYPs were initially described by Klingenberg (1958) for their absorbance of light with wavelengths of approximately 450 nm upon formation of an adduct with carbon monoxide (CO) (Omura and Sato, 1962; Roos and Jakubowski, 2008). The substrates of CYPs include sterols, fatty acids, eicosanoids, vitamins, and xenobiotics such as drugs (Guengerich, 2008; Seliskar and Rozman, 2007). The drug metabolism process is essential for detoxification. However, the resulted CYP-mediated products may also be toxic or carcinogenic (Bai and Cederbaum, 2004; Ramaiah et al., 2001; Yan et al., 2008).

CYP1A2 is an important CYP subfamily that is important in metabolizing xenobiotics or medicine (Laika et al., 2010; Murray, 2006; Zhang et al., 2010). CYP1A2 is also well known for its role in the hepatoxicity of aflatoxin B1 (Gallagher et al., 1996). For research purpose, human

CYP1A2 can be derived from a variety of sources such as liver subcellular fractions, recombinant CYP1A2 enzymes, human hepatocytes, CYP1A2-expressed mammalian cell lines, among others. It is important to develop robust cell culture systems for the study of drug metabolism (Donato et al., 2008). In this study, we have developed a stable cell line, that is, Huh7-1A2-I-E, with constitutive expression of human CYP1A2 in Huh7 cells. We showed that high throughput screening for CYP1A2 inhibitors or for CYP1A2-associated cytotoxic agents could be facilely conducted using this system. The results and implications after screening of approximately 200 drugs is presented and discussed.

Materials and Methods

Chemicals and Reagents

3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxy-methoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) was purchased from Promega (Madison, WI). Screened drugs were obtained from MicroSource Discovery Systems, Inc. (Gaylordsville, CT). All other chemicals were commercially available products of analytical grade and purchased from Sigma–Aldrich (St. Louis, MO). Baculovirus supersome (recombinant CYP1A2 enzyme) was purchased from BD Biosciences (San Jose, CA).

Construction of Plasmids

Starting from the pLenti6/V5-D-TOPO plasmid (Invitrogen, Carlsbad, CA), two lentirvial vectors, pLenti-1A2-I-E and pLenti-E, were constructed (Fig. 1A and B). The names and sequences of the used primers were shown on Table I. Briefly, the CYP1A2 gene was amplified using polymerase chain reaction (PCR) from a human liver cDNA library kindly supplied by Dr. Shiu-Feng Huang (NHRI, Taiwan), and the PCR product was inserted into the *Sac*II site of

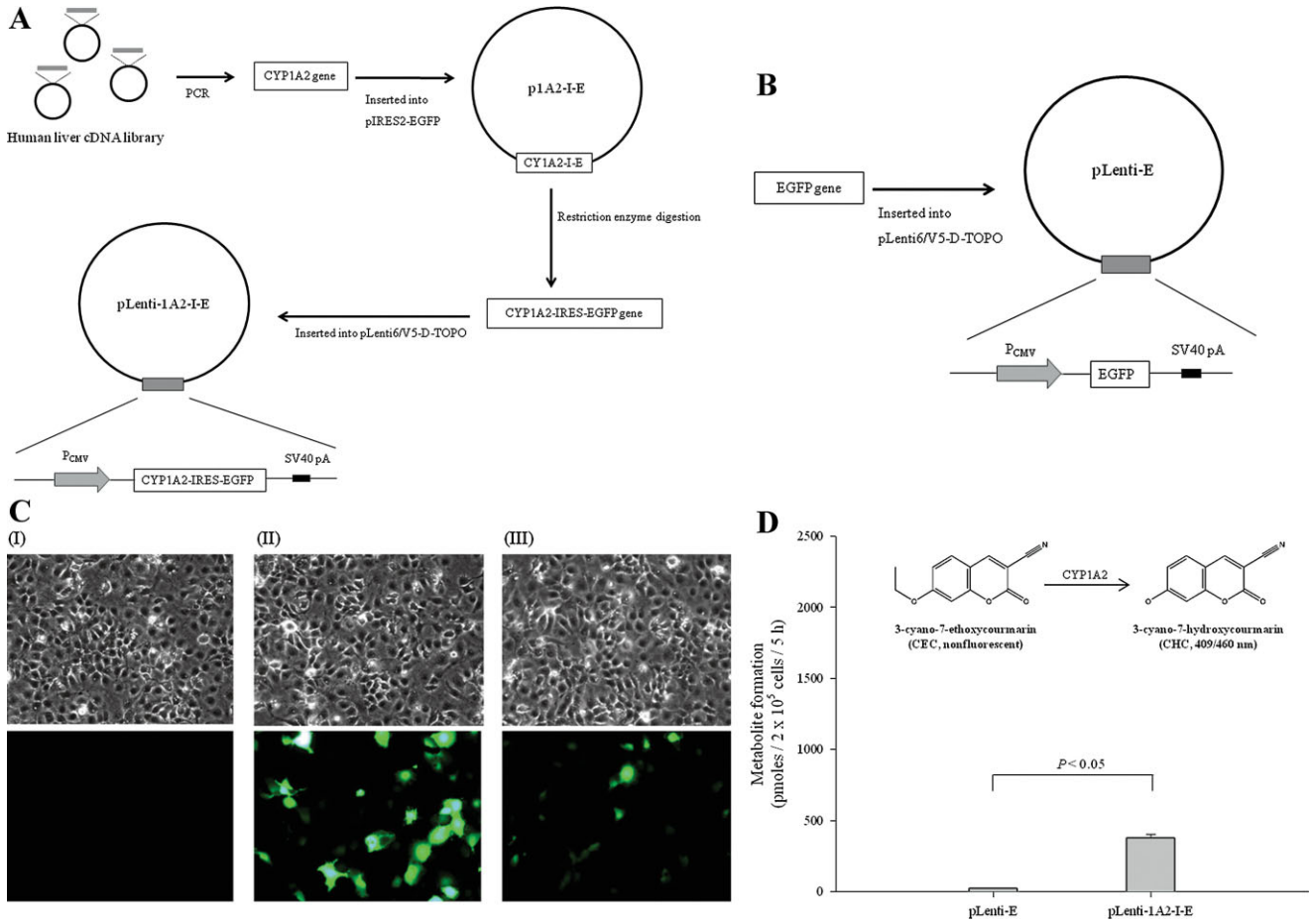

Figure 1. Construction of pLenti-1A2-I-E encoding functional human CYP1A2. **A**: The experimental procedure for the construction of the CYP1A2-expression vector, pLenti-1A2-I-E, is depicted. pLenti-1A2-I-E is a bi-cistronic vector where the expression of 1A2-I-E is under the control of the CMV promoter. (I: IRES: Internal Ribosome Entry Site; E: EGFP). **B**: The control vector, pLenti-E. **C**: Fluorescence microscopy of Huh7 cells (I); Huh7 cells transfected with pLenti-E (II) or pLenti-1A2-I-E (III), 48 h after transfection. **D**: Metabolic activity assays of Huh7 cells transfected with plasmids (pLenti-E and pLenti-1A2-I-E). 3-Cyano-7-ethoxycourmarin (CEC) was used as a CYP1A2 substrate and the fluorescent metabolite formation was measured by excitation (nm)/emission (nm): 409/460. The values were the averages of duplicate determinations. The statistical probability (*P*) is lower than 0.05.

Table I. The names and sequences of the primers.

Names	Sequences
CYP1A2	F: 5′-CACCATGGCATTGTCCCAGTCTG-3′
	R: 5′-CCGCGGTCAGTTGATGGAGAAGCGC-3′
CYP1A2-IRES-EGFP (1A2-I-E)	F: 5′-ACTAGTATGGCATTGTCCC-3′
	R: 5′-CTCGAGTTACTTGTACAGCTCG-3′
EGFP (E)	F: 5′-CACCATGGTGAGCAAGGGCGAGG-3′
	R: 5′-TTACTTGTACAGCTCGTCCATGCC-3′

F, forward primer; R, reverse primer.

pIRES2-EGFP plasmid (Clontech, Mountain View, CA) to generate a intermediate plasmid, p1A2-I-E. Subsequently, the fragment of CYP1A2-IRES-EGFP gene was directly subcloned into the *Spe*I–*Xho*I sites of pLenti6/V5-D-TOPO plasmid (Invitrogen). Finally, the CYP1A2 expression vector, pLenti-1A2-I-E, encoding the CYP1A2-IRES-EGFP fragment (CYP1A2 cDNA followed by IRES (I) element and EGFP (E) cDNA) was generated. Furthermore, EGFP gene was directly amplified using PCR, and inserted into the pLenti6/V5-D-TOPO plasmid to form the control plasmid, pLenti-E.

Cell Culture and Transfection

Huh7 cells were described previously (Pan et al., 2009). HEK293FT cells were from Invitrogen. Cells were transfected with various plasmids constructs using Lipofectamine 2000 reagent (Invitrogen). The process of transfection was performed according to the manufacturer's protocol.

Production of Recombinant Lentiviral Particles

The recombinant lentiviral plasmid and lentiviral package plasmids (pLP1, pLP2, and pLP/VSVG vectors) were co-transfected into the producer cell line, HEK293FT cells. Three days post-transfection, the viral particles were harvested from the culture medium. The production of recombinant lentiviral particles and the viral titer counts were performed according the manufacturer's protocol (Invitrogen). The viral particles (vLenti-1A2-I-E and vLenti-E) were stored in small aliquots at -80°C.

Stable Cell Line Selection

Huh7 cells were transduced with recombinant lentiviral particles (vLenti-1A2-I-E or vLenti-E) at an MOI of 0.5 for 24 h. At the end of transduction, the supernatants were removed and cells were maintained in basal medium plus 10 μg/mL blasticidin (Invitrogen) for the selection of blasticidin-resistant clones. After two passages, the survived cells were plated onto 96-well plates at a density of 30 cells per plate for the isolation of clonal lineages. Upon selection by blasticidin, 10 clonal cells harboring CYP1A2 bear desirable growth characteristics out of approximately 200 clonal cells that survived in 96-well plates. Subsequently, the clonal cells were examined for their CYP1A2 activity using 3-Cyano-7-ethoxycoumarin (CEC) as the substrate. As a result, clonal cells were isolated and two cell lines, Huh7-1A2-I-E and Huh7-E, were established. Subsequently, the stable cell lines were selected.

Fluorescence Microscopy and Metabolic Activity Assays

The EGFP could be observed using a fluorescence microscope. The CYP1A2 enzyme activity assays were performed by the direct incubation of intact cells with CYP1A2 substrates (CEC), as previously described (Donato et al., 2004). Briefly, Huh7-1A2-I-E cells were seeded at a density of 2×10^5 cells per well in 24-well plates or 5×10^3 cells per well in 96-well plates, and incubated at 37°C overnight. Subsequently, the culture medium was replaced with the CEC substrate incubation medium (30 μM CEC, 1 mM Na_2HPO_4, 137 mM NaCl, 5 mM KCl, 0.5 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM glucose, and 10 mM 4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid (HEPES), pH 7.4 in double-distilled water (ddH_2O)). After 5 h of incubation at 37°C, the assays were terminated by aspirating the incubation medium, and the fluorescent metabolite was quantified using a SpectraMax M2 microplate reader with excitation and emission wavelength maxima of 409 and 460 nm, respectively (Molecular Devices, Sunnyvale, CA). The fluorescent metabolite, CHC (3-cyano-7-hydroxycoumarin), was utilized to be a quantitative standard, and the measured fluorescent signals were proportional to the concentration of CHC. Results were expressed as pmoles of formed metabolite per 2×10^5 cells per 5 h.

Western Blotting

The procedure for Western blotting analysis is essential the same as described (Pan et al., 2009). Anti-CYP1A2 antibody was from Abcam (Cambridge, MA). Anti-β-actin antibody and the secondary antibody were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) or Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA).

Cell Viability Assays

The procedure for MTS assay is essential the same as described (Pan et al., 2009). MTS assay is based on the conversion of a tetrazolium salt into a colored aqueous-soluble formazan catalyzed by dehydrogenase enzymes in viable mitochondria. The amount of formazan is directly proportional to the number of living cells in the culture (Buttke et al., 1993; Malich et al., 1997). In this study, cells were seeded at a density of 5×10^3 cells per well in 96-well plates overnight. Then, compounds were added to the

complete culture medium to the test concentrations. After 72 h of treatment, MTS assay was performed and cell viability was measured using the follow equation: Cell viability (%) = [MTS value (compound treatment cells)/ MTS value (1% DMSO treatment cells)] × 100%.

Flowcytometry

Huh7, Huh7-E, and Huh7-1A2-I-E cells were harvested by 1× trypsin-ethylenediaminetetraacetic acid (EDTA) and suspended with 1× phosphate buffered saline (PBS) at 6×10^5 cells/mL. Samples were subjected to flow cytometry FACS Calibus (BD Biosciences) to detect green fluorescence (FL1-H) and 10,000 cells of each sample were collected. Data were analyzed by CellQuest Pro software (BD Biosciences) to obtain the mean fluorescence intensity of each sample. Experiments were performed in triplicate.

Calculation of *S/N*, *S/B*, and *Z′* Factor

The signal-to-noise ratio (*S/N*), signal-to-background ratio (*S/B*), and *Z′* factor values were calculated using the methods of Zhang et al. (1999). The statistical parameters were utilized to evaluate the performance of this developed assay for high-throughput screening. Before compound treatment, Huh7-1A2-I-E cells were seeded at a density of 5×10^3 cells per well in 96-well plates overnight. Cells were treated with 2.5 μM aflatoxin B1, as a background set, or 1% dimethylsulfoxide (DMSO), as a signal set, for 72 h, and cell viability was assessed by MTS assay.

Inhibition Assays of CYP1A2 Enzyme Activities

The assay was modified according to a previous report (Donato et al., 2004). CYP1A2 enzyme activity was assayed by incubating baculovirus supersome containing the recombinant CYP1A2 enzyme in 100 μL of 100 mM phosphate buffer, pH 7.4, with the NADPH-regenerating system (5 mM $MgCl_2$, 1 mM NADPH, 10 mM glucose 6-phosphate, and 0.3 U/mL glucose-6-phosphate dehydrogenase).

After the compounds were added into the enzyme mixture at 37°C for 1 h, CEC was then added to 30 μM. After 30 min of incubation at 37°C, the fluorescence due to the conversion of CEC to CHC, a fluorescent metabolite, was quantified using a SpectraMax M2 microplate reader.

Results

The Functional Expression of the Bi-Cistronic Constructs Was Evaluated by Transient Transfection in Huh7 Cells

To provide a robust cell model for evaluating the xenobiotic metabolism mediated by human CYP1A2, a lentiviral bi-cistronic vector, pLenti-1A2-I-E, was constructed (Fig. 1A). As a control, the pLenti-E plasmid was used (Fig. 1B). Huh7 cells were individually transfected with these two plasmids. As shown in Figure 1C, at 48 h post-transfection, the EGF expression in the pLenti-E transfected cells was evident under fluorescence microscopy. Some of the pLenti-1A2-I-E transfected cells also showed EGFP expression though it seemed that the expression level of EGFP was lower than that in the control. Presumably, this may be due to that the mRNA stability of the 1A2-IRES-EGFP is not as optimized as that of the EGFP.

The whole-cell CYP1A2 activity was measured. Results showed that the CYP1A2 activity in cells transfected with the bi-cistronic vector was approximately 17-fold higher than that in the pLenti-E transfected cells (Fig. 1D) indicating that the CYP1A2 gene encoded in the pLenti-1A2-I-E bi-cistronic vector is functional in Huh7 cells.

Establishment and Characterizations of Huh7-1A2-I-E and Huh7-E Cell Lines

Subsequently, recombinant lentiviral particles, vLenti-1A2-I-E and vLenti-E, were generated from the pLenti-1A2-I-E and the pLenti-E, respectively. The harvested lentiviral titers ranged between 2×10^5 and 5×10^5 PFU/mL (plaque-forming unit, PFU). Huh7 cells were then transduced with the lentiviral particles, and the cells harboring the genes of interest were selected with 10 μg/mL of blasticidin. Clonal cells were isolated and two cell lines, Huh7-1A2-I-E and Huh7-E, were established.

The GFP is a good marker for assessing transfection efficiency in the early stage. GFP was then used at later stage to ascertain that the expression cassette has remained intact in the clonal cells selected under the blasticidin selection pressure. Under fluorescence microscopy, GFP could be barely observed in Huh7-1A2-I-E cells (not shown). Stronger GFP in Huh7-E was observed (not shown). Results from flow cytometry analysis are consistent with the fluorescence microscopy observations (Fig. 2A). Thus, GFP under the IRES regulation was a successful strategy to provide a gene expression marker with limited protein expression capacity from cells.

Cells were analyzed for expression of CYP1A2 by Western blot using anti-CYP1A2 antibody. As a positive control, the recombinant human CYP1A2 produced from the baculovirus expression system appeared as a prominent band at a MW close to 55 kDa; similar to the expected size of CYP1A2 (Fig. 2B, lane 4). It is evident that CYP1A2 is overexpressed in Huh7-1A2-I-E cells (lane 3). The lower panel showed the levels of β-actin as a loading control. It is noteworthy that the mRNA of CYP1A2 could also be detected in Huh7-1A2-I-E but not in Huh7-E cells by reverse transcription-PCR (RT-PCR; not shown).

Huh7-1A2-I-E and Huh7-E cells were then examined for their whole-cell CYP1A2 activity by the addition of CEC into the medium of cultured cells. Results showed that the

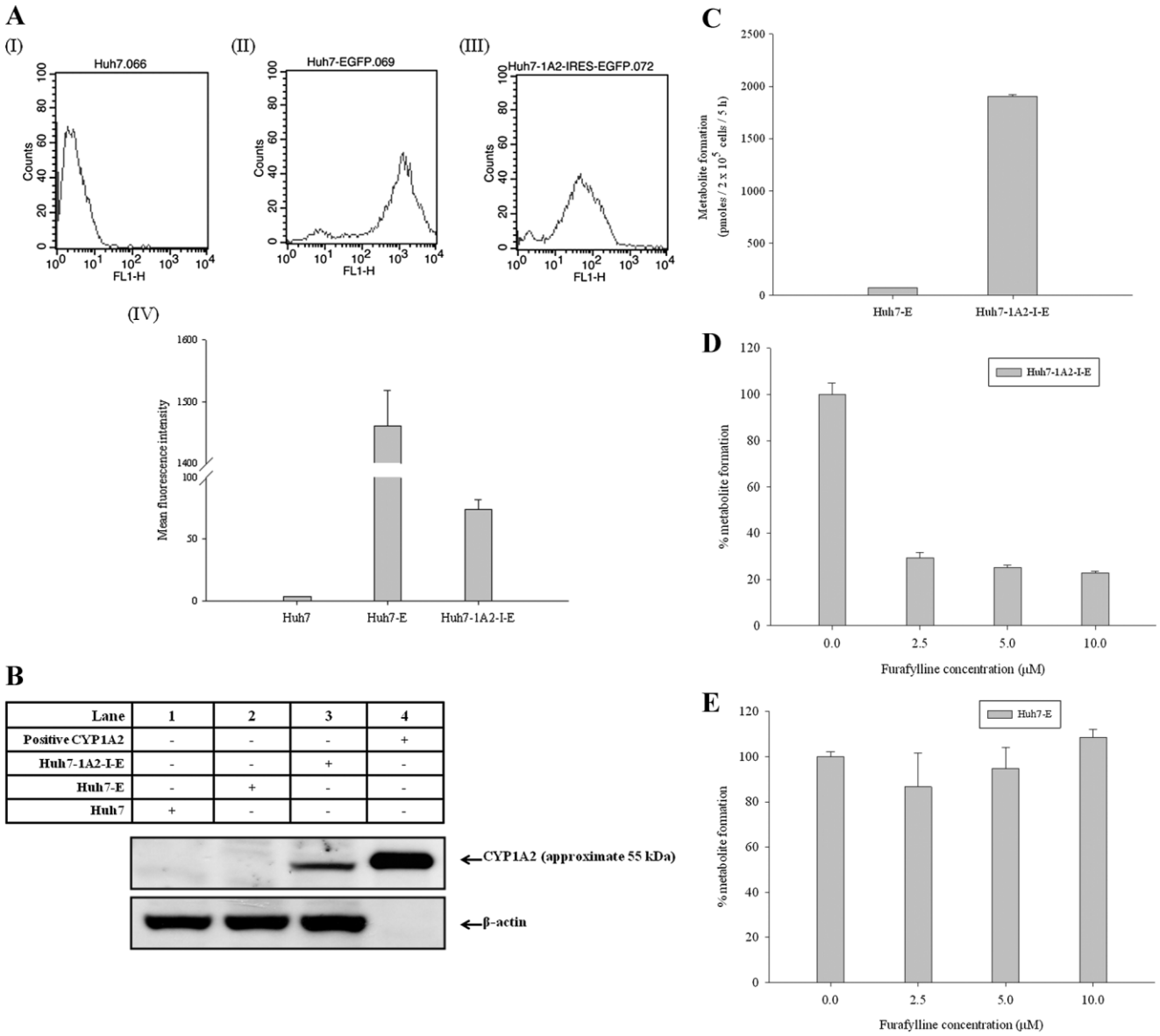

Figure 2. The characteristic comparison of stable Huh7 cell lines individually carried with human CYP1A2 and EGFP genes. **A**: The green fluorescence (FL1-H) of Huh7 (I), Huh7-E (II), and Huh7-1A2-IRES-E cells (III) detected by FACS Calibus. Averages of mean fluorescence intensity ($n=3$) were shown on IV. **B**: Western blotting. Samples were stained with CY1A2 antibody or β-actin antibody. From left to right: lane 1, Huh7 cell lysate; lane 2, Huh7-E cell lysate; lane3, Huh7-1A2-I-E cell lysate (lane 1, lane 2, and lane 3 were corresponding to 50 μg of protein/lane); lane 4, CYP1A2 positive standard (recombinant CYP1A2 from BD Biosciences, 1 μg of protein/lane). **C**: Metabolic activity assays of intact stable Huh7 cell lines by using fluorimetric probe, CEC. The values were the means of triplicate determinations. Inhibition effects of furafylline (known CYP1A2 inhibitor) on CYP1A2 enzyme activities in Huh7-1A2-I-E cell line (**D**) and the control Huh7-E cells (**E**). Cells were treated with indicated concentrations of furafylline for 72 h, and CYP1A2 activities were detected using CEC as the fluorimetric probe. The values were the means of triplicate determinations.

CYP1A2 activity in Huh7-1A2-I-E cells is much higher than that in Huh7-E cells (Fig. 2C). When cells were incubated with 30 μM of CEC for 5 h, approximately 2,000 pmol of the metabolite (CHC) was formed out of every 2×10^5 Huh7-1A2-I-E cells. However, the CHC formation was <100 pmol when Huh7-E cells were treated with CEC under the same conditions. Furthermore, close to 80% of the CYP1A2 activity can be inhibited by furafylline at 10 μM (Fig. 2D). Further, unlike in Huh7-1A2-I-E cells (Fig. 2D), the metabolite formation in Huh7-E cells was not affected by the CYP1A2 inhibitor furafylline up to 10 μM (Fig. 2E). Therefore, it is highly likely that the residual CYP1A2 activity measured in Huh7-E cells in Figure 2C is not due to the endogenous CYP1A2 in Huh7-E cells. These results indicate that CYP1A2 activity is present in the Huh7-1A2-I-E stable cell line. The cells have been propagated for more than 50 passages without loss of the enzymatic activity (not shown).

Evaluation of the CYP1A2-Mediated Toxicity in Huh7-1A2-I-E Cells

To investigate if the Huh7-1A2-I-E cells are suitable host cells for studying CYP1A2-mediated toxicity of drugs, Huh7-1A2-I-E and Huh7-E cell lines were treated with aflatoxin B1 that is known to produce toxic metabolites upon biotransformation by CYP1A2 (Mace et al., 1997; Manson et al., 1997). Cells were exposed to aflatoxin B1 at different concentrations for 72 h. For Huh7-E cells, approximately 80% of cell viability remained even at 10 μM of aflatoxin B1 (Fig. 3). In contrast, Huh7-1A2-I-E cells were much more sensitive to aflatoxin B1. The CC_{50} (drug concentration at which 50% of cells are viable compared to the control) of aflatoxin B1 in Huh7-1A2-I-E cells was approximately 0.3 μM. The sensitivity of Huh7-1A2-I-E cells to aflatoxin B1 disappeared when 5 μM of furafylline was applied to block the enzymatic activity of CYP1A2. Thus, it is highly likely that more toxic metabolites of aflatoxin B1 were formed in Huh7-1A2-I-E cells but not in the control Huh7-E cells. Therefore, the established cell lines may be important for identification of compounds or drugs that will generate toxic metabolites upon CYP1A2 transformation. In reverse, this system may also be valuable for studying CYP1A2-mediated detoxification of xenobiotics.

Robustness Analysis and Screening of Existing Drugs or Bioactive Compounds for Their Cytotoxic Effects in Huh7-1A2-I-E and Huh7-E Cell Lines

To assess whether Huh7-1A2-I-E cells can be adopted into a high throughput assay for cytotoxicity studies, assay parameters such as signal to noise ratio (*S*/*N*), signal to background ratio (*S*/*B*), and the *Z*′ factor were measured in the presence and absence of aflatoxin B1 as a reference drug. Huh7-1A2-I-E cells were treated with 10 μM of aflatoxin B1 for 72 h. The *S*/*N* ratio and *S*/*B* ratio were 12.8 and 5.7, respectively, and a *Z*′ value of 0.72 was obtained (Fig. 4A). Thus, this system is suitable for high throughput screenings.

To validate the utility of this cell-based CYP1A2 metabolism system, we screened a small collection of compounds consisting of ~200 drugs with known or potential anti-cancer and cytotoxic activity. The tested compounds are described in Supplementary Table 1. Huh7-1A2-I-E and Huh7-E cells were treated with the drugs at 10 μM of each compound in a 96-well format. After 72 h, cell viability was assessed with MTS assay and the results from the whole-cell cytotoxic screening are shown in Figure 4B.

The whole area of this viability plot can be generally divided into the following zones. Drugs, located at the straight line with equal distance to the *x*- and *y*-axis, showing equivalent cytotoxicity toward Huh7-1A2-I-E and Huh7-E cells constitute the majority of the tested drugs. Drugs that are more toxic to Huh7-E cells than to Huh7-1A2-I-E cells would be located at the upper left zone to indicate that these Huh7-E toxicants may be detoxified by CYP1A2 in Huh7-1A2-I-E cells. Whereas drugs that are more toxic for Huh7-1A2-I-E cells than for Huh7-E cells would fall in the lower right zone on the plot. Aflatoxin B1 indicated by the open triangle fell in this zone.

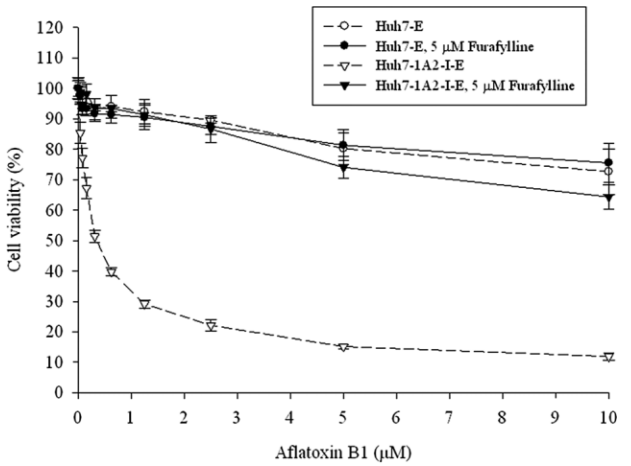

Figure 3. Cytotoxic effects of a CYP1A2-metabolized compound (Aflatoxin B1) in stable Huh7 cell lines. The cells were exposed to the indicated concentrations of aflatoxin B1 for 72 h, and cell viability was assessed by MTS assay. The values were the means of triplicate determinations. The symbols: Huh7-1A2-I-E cell line (-●-); Huh7-E cell line (-○-); Huh7-1A2-I-E cell line treated with 5 μM furafylline before aflatoxin B1 treatment for 1 h (-▼-).

Discovery and Characterizations of Previously Unknown Drugs Whose Bioactivity May Be Effected by CYP1A2

From the screened results, evoxine and thimerosal were further analyzed for their toxic effects in more details because they had a noticeable difference in cytotoxicity for Huh7-E and Huh7-1A2-I-E cells. As indicated by the * sign on Figure 4B, thimerosal at 10 μM killed almost all the Huh7-1A2-I-E cells while ~40% of Huh7-E cells remained viable. Also note that evoxine at 10 μM, indicated by the ** sign on Figure 4B, caused approximately 50% and 10% reduction in cell viability of Huh7-1A2-I-E and Huh7-E cells, respectively. These results indicate that more toxic metabolites may be formed in Huh7-1A2-I-E cells treated with thimerosal or evoxine.

Thimerosal and evoxine were examined by further dose-dependent cytotoxicity analyses. Thimerosal is a vaccine preservative and evoxine is a natural product existing in *Skimmia Reevesiana* (Epifano et al., 2007). The results showed that the CC_{50}s of thimerosal were close 3 and 1.5 μM in Huh7-E and Huh7-1A2-I-E cells, respectively. Evoxine at 10 μM caused >30% reduction in cell viability of Huh7-1A2-I-E cells but had no effect for the cell viability of Huh7-E cells (Fig. 4C and D). Results from this screening effort also illustrated that indeed this whole-cell screening system may be useful for systematically discovery of drugs with CYP1A2-mediated toxicity.

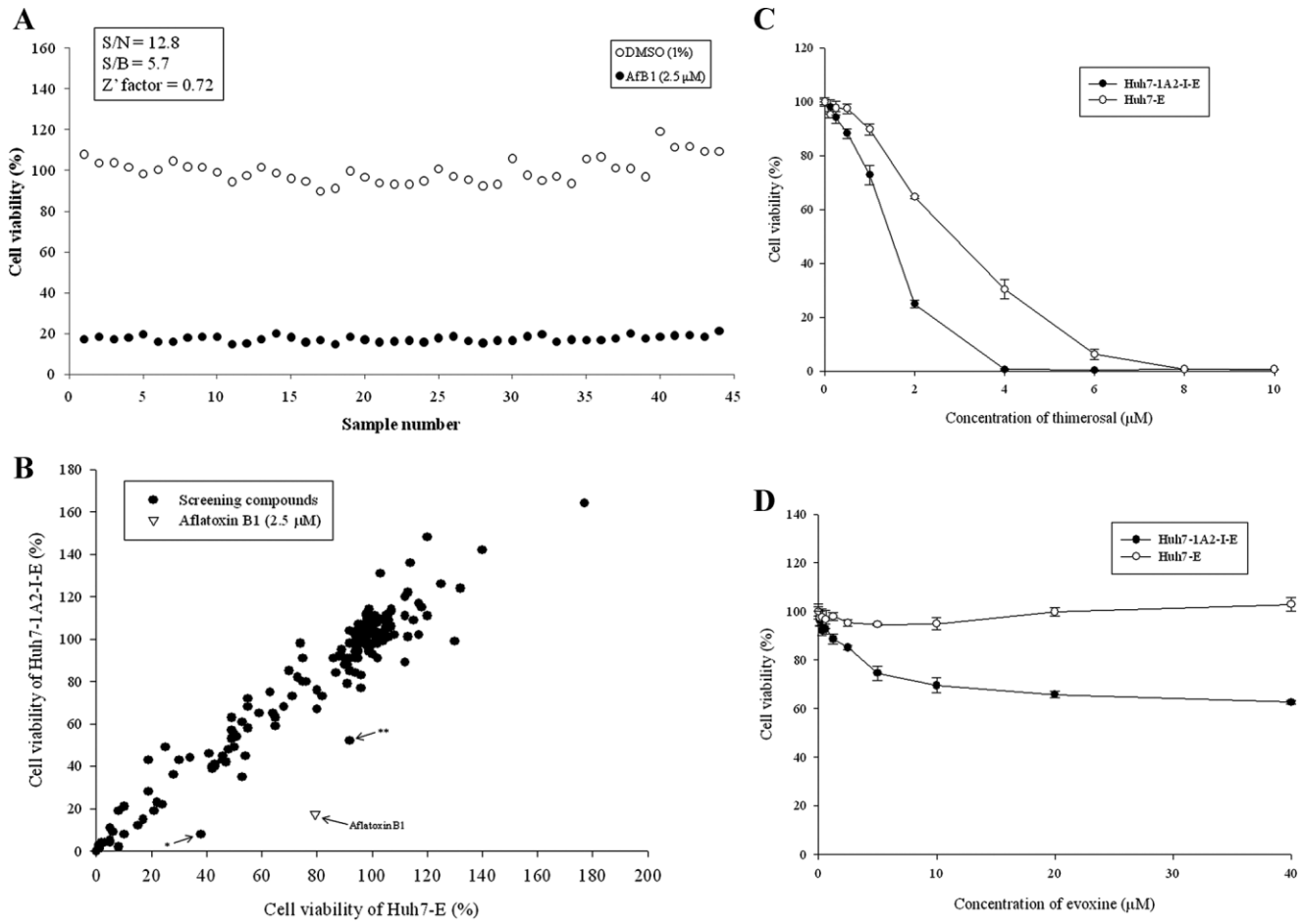

Figure 4. Evaluation of drugs for their CYP1A2-related cytotoxicity. **A**: Estimates of statistical parameters of the screening system. Huh7-1A2-I-E cells (5×10^3 cells per well in 96-well plates) were treated with 2.5 μM aflatoxin B1 (closed circles), as a background set, and 1% DMSO (open circles), as a signal set, for 72 h, and cell viability was assessed by MTS assay. The *S/N*, *S/B*, and *Z'* factor values were 12.8, 5.7, and 0.72, respectively. **B**: Cytotoxic effects of screened compounds in Huh7-E and Huh7-1A2-I-E cells. Cells were exposed to 10 μM compounds individually for 72 h, and cell viability was assessed by MTS assay. The values were the means of triplicate determinations. Highlighted compounds: Aflatoxin B1 (▽); Thimerosal (*); Evoxine (**). C and D: Dose-dependent effects of cell viability of thimerosal and evoxine, respectively. Cultured cells were exposed to compounds at different concentrations for 72 h, and cell viability was assessed by MTS assay. The values were the means of triplicate determinations. The symbols: Huh7-1A2-I-E cells (-●-); Huh7-E cells (-○-).

We also evaluated if the whole-cell CYP1A2 activity assay based on Huh7-1A2-I-E cells can be applicable for identification of potential enzyme inhibitors. Huh7-1A2-I-E cells were seeded in 96-well plates and then treated with test drugs at 10 μM. One hour after, the drug-containing medium was replaced with the CYP1A2 enzyme incubation medium containing CEC and incubated for another 5 h. The results are shown in Figure 5. In this whole-cell assay, 8 compounds showed >50% inhibition of CYP1A2 at concentrations not affecting cell viability. Among them, methoxsalen, miconazole, and thiabendazole are well known CYP1A2 inhibitors (Zhou et al., 2010). Surprisingly, it has never been previously reported that evoxine, berberine, benzo[a]pyrene, and 3-methylcholanthrene are potent CYP1A2 inhibitors. Results from the whole-cell assay were confirmed by enzyme-based assay using recombinant CYP1A2 produced by the baculovirus expression system. More detailed characterizations from the enzyme-based assay are shown in Supplementary Table 2. In summary, a whole-cell system was established in this study for identification of potential CYP1A2 inhibitors and for studying CYP1A2-mediated toxicity or detoxification in cells.

Discussion

In this study, we have succeeded in generating a novel cell line, Huh7-1A2-I-E, wherein high levels of CYP1A2 expression was achieved in Huh7 cells using the lentiviral gene transfer vector. Some hepatocyte-specific characteristics are maintained in Huh7 cells that were derived from a hepatocellular carcinoma (Nakabayashi et al., 1984). Like normal hepatocytes, Huh7 cells are able to secret some

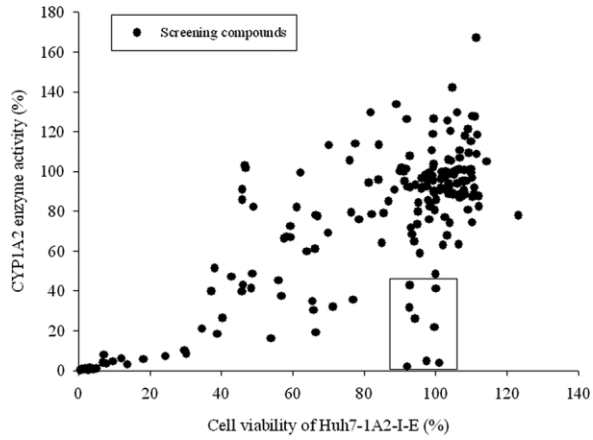

Figure 5. Screening of CYP1A2 inhibitors in the whole-cell assay. Huh7-1A2-I-E cells were seeded in 96-well plates and then treated with the drugs. The treatment concentration is 10 μM. One hour after, the drug-containing medium was replaced with the CYP1A2 enzyme incubation medium containing CEC as the substrate and incubated for another 5 h. CYP1A2 enzyme activity was assessed by the metabolic activity assay.

plasma proteins such as albumin, prealbumin, G6Pase, and FDPase, etc. (Nakabayashi et al., 1982, 1984; Seki et al., 1999). Further, the replication of hepatitis C virus RNAs is possible in this cell line indicating that Huh7 cells bear important hepatocellular properties (Blight et al., 2002). However, we found that several drug-metabolism CYP enzymes could not be detected in Huh7 cells. Therefore, we used the lentiviral protein expression technology to establish the whole-cell CYP1A2 system since the lentivirus-encoded genes can be efficiently integrated into the host genome (Buchschacher and Wong-Staal, 2000; Naldini, 1998).

Cell-based systems are important for studies of drug metabolism and many such in vitro models have been established and reviewed (Donato et al., 2008; Vermeir et al., 2005). To our knowledge, this study is the first report to describe the establishment of a cell-based assay with the heterologously expressed human CYP1A2 for high throughput screening of enzyme inhibitors or substrates that may be detoxificated or may produce more toxic metabolites upon conversion by CYP1A2. To validate that the system is indeed suitable for high throughput screening, we have tested that the cell-based enzymatic activity of CYP1A2 has been consistent up to more than 50 passages of the Huh7-1A2-I-E cells. Further, the high S/B ratios and low S/N ratios and a Z' factor of more than 0.5 indicated that the signal-to-noise scatter in the assay could be highly reproducible with little variation (Zhang et al., 1999). Thus, Huh7-1A2-I-E cells indeed represent a convenient cell-based recombinant system that may satisfy various needs for studying drug metabolism related to CYP1A2.

The reliability of the whole cell-based system was ascertained by the CYP1A2-mediated toxicity of aflatoxin B1 in Huh7-1A2-I-E cells. Aflatoxin was much more toxic to Huh7-1A2-I-E cells than to the control Huh7-E cells. Further, the toxicity of aflatoxin in Huh7-1A2-I-E cells disappeared when furafylline, a CYP1A2 inhibitor, was included. This result highly suggested that the conversion of aflatoxin B1 to a more toxic metabolite by CYP1A2 in Huh7-1A2-I-E cells (Gallagher et al., 1996; McDonagh et al., 1999). The results showed that the whole cell-based system has a potential for studying CYP1A2-metabolized compounds or drugs. Furthermore, a collection of ~200 drugs were tested for their CYP1A2-mediated toxicity using Huh7-1A2-I-E cells in 96-well plates. Results from this screening revealed that more toxic metabolites may be produced through the conversion of evoxine and thimerosal by CYP1A2. This observation indicates that, for most drugs examined in this study, the metabolism by CYP1A2 is unlikely to have made a major contribution to the in vitro cytotoxicity except for thimerosal and evoxine. Further investigations are needed to characterize the underlying mechanism.

In the screening for CYP1A2 inhibitors using this cell system, several known CYP1A2 inhibitors showed strong inhibition in the assay (Fig. 5). Interestingly, evoxine, berberine, benzo[a]pyrene, and 3-methylcholanthrene were also identified as probable CYP1A2 inhibitors in the cell-based system. Subsequent enzyme-based characterizations confirmed the observations. Evoxine, extracted from the stem bark of *Teclea gerrardii*, is an alkaloid with antiplasmodial activity (Waffo et al., 2007). Berberine is also an alkaloid with a multi-spectrum therapeutic activities (Kulkarni and Dhir, 2010; Vuddanda et al., 2010). Berberine was recently shown to be able to attenuates experimental autoimmune encephalomyelitis (EAE; Ma et al., 2010; Qin et al., 2010). Although there was few reported human adverse events regarding evoxine or berberine, results from this study warrant further studies to examine if the administration of evoxine or berberine through the intake of herb medicines may trigger potential drug–drug interactions involving CYP1A2.

In summary, this study describes a cell-based system stably expressing high level of CYP1A2. Theoretically, this system can be expanded to include other CYP or other genes, and the drug-metabolizing functions of hepatocytes may be restored in a cell line that can be facilely maintained.

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