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Evaluation of CYP3A4 inhibition and hepatotoxicity using DMSO-treated human hepatoma HuH-7 cells

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Abstract

A human hepatoma cell line (HuH-7) was evaluated as a metabolically competent cell model to investigate cytochrome P450 3A4 (CYP3A4) inhibition, induction, and hepatotoxicity. First, CYP3A4 gene expression and activity were determined in HuH-7 cells under three culture conditions: 1-week culture, 3-week culture, or 1% dimethyl sulfoxide (DMSO) treatment. HuH-7 cells treated with DMSO for 2 weeks after confluence expressed the highest CYP3A4 gene expression and activity compared to the other two culture conditions. Furthermore, CYP3A4 activity in DMSO-treated HuH-7 cells was compared to that in a human hepatoma cell line (HepG2/C3A) and human bipotent progenitor cell line (HepaRG), which yielded the following ranking: HepaRG > DMSO-treated HuH-7 >> HepG2/C3A cells. The effects of three known CYP3A4 inhibitors were evaluated using DMSO-treated HuH-7 cells. CYP3A4 enzyme inhibition in HuH-7 cells was further compared to human recombinant CYP3A4, indicating similar potency for reversible inhibitors (IC_{50} within 2.5 fold), but different potency for the irreversible inhibitor. Next, induction of CYP3A4 activity was compared between DMSO-treated HuH-7 and HepaRG cells using two known inducers. DMSO-treated HuH-7 cells yielded minimal CYP3A4 induction compared to that in the HepaRG cells after 48-h treatments. Finally, the cytotoxicity of five known hepatotoxicants was evaluated in DMSO-treated HuH-7 cells, HepG2/C3A, and HepaRG cells, and significant differences in cytotoxic sensitivity were observed. Overall, DMSO-treated HuH-7 cells are a valuable model for medium- or high-throughput screening of chemicals for CYP3A4 inhibition and hepatotoxicity.

Keywords

HuH-7 cells; DMSO; CYP3A4; Inhibition; Induction; Hepatotoxicity

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Conflicts of Interest

The authors declare no conflicts of interest.

Introduction

The liver contains essential drug-metabolizing enzymes and is the major organ for xenobiotic detoxification and/or bio-activation. Hepatotoxicity mediated by cytochrome P450, especially CYP3A4, along with drug-drug and drug-dietary supplement interactions are major concerns in drug development and consumer safety (Hisaka et al. 2010). Human primary hepatocytes are valuable for initial in vitro investigations of drug-drug interactions and hepatotoxicity prior to in vivo animal and human clinical studies. However, primary human hepatocytes are expensive, phenotypically unstable, functionally variable, and therefore less desirable for routine screening purposes (Liu et al. 2011). Therefore, robust, sensitive, and biologically relevant hepatoma cell lines are valuable as alternative models. Recently, various cell culture systems have been developed as in vitro models, such as human induced pluripotent stem cell-derived hepatocytes (Sjogren et al. 2014), hepatic cell lines transfected with drug-metabolizing enzyme transgenes, terminally differentiated human bipotent progenitor cell line HepaRG (Donato et al. 2013), and 3D cultured hepatoma cell lines (Malinen et al. 2014). Although these systems are useful, they often require complicated development procedures and extensive maintenance, which are not ideal for higher-throughput screening.

Human hepatoma HuH-7 is a continuous hepatocyte cell line derived in 1982 from a 57-year-old male with hepatocellular carcinoma (Nakabayashi et al. 1982). CYP3A4 is the most abundant cytochrome P450 enzyme in liver and intestine, and it is responsible for the metabolism of more than 50% of all marketed drugs (Liu et al. 2007). Non-confluent HuH-7 cells express CYP activity at a minimum level compared with primary human hepatocytes; for example, the average CYP3A4 activity over ten passages is around 1.5% of that in primary human hepatocytes (Lin et al. 2012). However, HuH-7 cells can exhibit higher drug-metabolizing enzyme activities when treated with DMSO (Choi et al. 2009) or when cultured over extended periods of time (Sivertsson et al. 2010). After a 2-week DMSO treatment, CYP3A4 activity in HuH-7 cells could reach nearly 9% of that in primary human hepatocytes (Choi et al. 2009). Additionally, after 5-week culture of confluent HuH-7 cells, microsomal CYP3A4 activity reached approximately 30% of the activity measured in human liver microsomes (Sivertsson et al. 2010). Although drug-metabolizing enzyme activities in HuH-7 cells remain lower than human primary cells, HuH-7 cells could be suitable for medium- or high-throughput screening due to its low cost, stability, and ease of use.

DMSO has been used historically to induce and maintain differentiation of primary cells and tumor cell lines. DMSO treatment induces HuH-7 cell differentiation and upregulates hepatocyte-specific genes, including albumin, hepatocyte nuclear factor 4- α (HNF4- α), and α -1-antitrysin (A1AT) (Sainz and Chisari 2006). Therefore, in this study, CYP3A4 gene expression and activity were first measured under different HuH-7 cell culture conditions. The optimized condition was then compared to CYP3A4 activity in HepG2/C3A cells, known to have low CYP3A4 activity, and HepaRG cells, which are known to have high CYP3A4 activity (Lübberstedt et al. 2011). The optimized DMSO-treated HuH-7 cell culture condition was then used to evaluate reversible and irreversible CYP3A4 inhibition and compared with human recombinant enzyme. Finally, CYP3A4 induction was evaluated

in DMSO-treated HuH-7 and HepaRG cells, and the cytotoxicity of model hepatotoxicants was compared among DMSO-treated HuH-7, HepG2/C3A, and HepaRG cells.

Methods and materials

Materials

Human hepatoma HuH-7 cells were purchased from Health Science Research Resources Bank, Japan Health Sciences Foundation (Osaka, Japan). HepG2/C3A cells were obtained from ATCC (Manassas, VA). HepaRG cells were purchased from Life Technologies (Grand Island, NY). Cell culture medium and medium supplements were obtained from Life Technologies. Fetal bovine serum was purchased from Atlanta Biologicals (Lawrenceville, GA). The following compounds (all 97% purity) were obtained from Sigma-Aldrich (St. Louis, MO): dimethyl sulfoxide (DMSO), ketoconazole, nifedipine, troleandomycin, rifampicin, dexamethasone, nordihydroguaiaretic acid (NDGA), nitrofurantoin, desipramine hydrochloride, troglitazone, nefazodone hydrochloride, bichinchonic acid, and copper sulfate. P450-Glo CYP3A4 assay (luciferin-IPA substrate), CellTiter-Fluor, and CellTiter-Glo cell viability assays were purchased from Promega (Madison, WI). Supersomes human CYP3A4 plus reductase and b5, NADPH regenerating system, and rat tail collagen type I were obtained from Corning (Corning, NY). RNeasy mini Kit and QIA shredder were purchased from QIAGEN (Valencia, CA). High-capacity cDNA reverse transcription kits, TaqMan gene expression assays (beta-actin, 18S and human CYP3A4), and NucRed Live 647 fluorescent dye were purchased from Life Technologies.

Cell culture

HuH-7 cells were cultured in a basal medium composed of Dulbecco's minimal essential medium (DMEM) with low glucose, pyruvate, Glutamax, MEM non-essential amino acids (1%), HEPES (10mM) and fetal bovine serum (10%). No antibiotics were added to the medium. HuH-7 cells were plated onto rat collagen type I surfaces (6×10^4 cells/cm²). HuH-7 cells were cultured under three conditions: (1) basal medium for 1 week, (2) basal medium for 3 weeks, and (3) basal medium for 1 week, followed by DMSO (1%) treatment for an additional 2 weeks. Before gene expression, enzyme, and hepatotoxicity studies, DMSO was removed from HuH-7 cell medium.

HepG2/C3A cells were cultured and plated (6×10^4 cells/cm²) in the same basal medium as HuH-7 cells, and cultured for 1 week to confluence before experiments.

HepaRG cells were thawed, seeded (3×10^5 cells/cm²), and maintained on tissue culture plates coated with rat tail collagen type I. CYP3A4 activity was determined in freshly plated HepaRG cells (4h after seeding). CYP3A4 induction and toxicity studies were conducted in HepaRG cells cultured in HepaRG Induction Working Medium (3 days before experiment) and Tox Working Medium (1 week before experiment), respectively, following the protocol provided by the manufacturer (Life Technologies).

qRT-PCR

Total RNA was isolated from HuH-7 cells using the RNeasy mini Kit and QIA shredder spin columns. mRNA reverse transcription was conducted using the High-Capacity cDNA Reverse Transcription Kit following the manufacture instructions. Real-time PCR was conducted on the 7500 Real-Time PCR system of Applied Biosystems by Life Technologies (Grand Island, NY) using TaqMan Gene Expression Assays primer/probe sets. Results were analyzed with the 7500 software v2.0.6 on the system using the relative quantification method. Beta-actin and 18S rRNA were both used as endogenous controls.

CYP3A4 luminescence assay

CYP3A4 enzyme activity was determined using P450-Glo CYP3A4 assay, which contained the substrate luciferin isopropyl acetal (luciferin-IPA) and luciferin detection reagent. Luciferin detection reagent was composed of a detergent to stop the reaction, along with Ultra-Glo luciferase, ATP, Mg^{2+} , and an esterase. Luminescence was measured at room temperature on a plate reader (BMG Labtech, Cary, NC). Assay conditions have been previously validated for temperature, time, and substrate concentration dependence (Promega technical bulletin TB325, 2015; Meisenheimer et al. 2011).

CYP3A4 inhibition in human recombinant enzymes

For reversible inhibition studies, the incubation mixture (50 μ L) consisted of the following: potassium phosphate buffer (0.1M, pH7.4), NADPH regenerating system, human recombinant CYP3A4 (2nM), luciferin-IPA (3 μ M; K_m value), and ketoconazole (0.01–1 μ M) or nifedipine (0.2–20 μ M). The reaction was initiated by adding human recombinant CYP3A4 and terminated after 10-min incubation at room temperature before measuring luminescence.

A time-dependent inhibition study was composed of two incubation steps: inhibitor pre-incubation followed by substrate incubation. The pre-incubation mixture (50 μ L) consisted of the following: potassium phosphate buffer (0.1M, pH7.4), NADPH regenerating system, human recombinant CYP3A4 (20nM, 10 $\times$), and troleandomycin (0.2–10 μ M; all 10 $\times$). The reaction was initiated by adding human recombinant CYP3A4 and pre-incubated for 0, 10, 20, 30, and 45min at room temperature. At each time point, 5 μ L of pre-incubation mixture was added to 45 μ L substrate incubation mixture with potassium phosphate buffer (0.1M), NADPH regenerating system, and luciferin-IPA (12 μ M, 4 $\times K_m$). The substrate mixture was further incubated for an additional 10min at room temperature before measuring luminescence.

CYP3A4 inhibition in DMSO-treated HuH-7 cells

For reversible inhibition studies, DMSO-treated HuH-7 cells were washed and incubated with ketoconazole (0.01–2 μ M) or nifedipine (0.2–50 μ M) simultaneously with CYP3A4 substrate luciferin-IPA (3 μ M, K_m value) at 37°C in serum-free medium for 30min. The reaction was terminated by transferring 50 μ L of culture medium from each well to a separate plate with wells containing 50 μ L of luciferin detection reagent at room temperature, followed by luminescence measurement. Cell viability was subsequently determined by the CellTiter-Fluor assay.

Time-dependent inhibition in DMSO-treated HuH-7 cells was performed in two steps: pre-incubation and substrate incubation. DMSO-treated HuH-7 cells were washed and pre-incubated with troleandomycin (2–50 μ M) for 0, 15, 30 and 60min at 37°C in serum-free medium. At different time points, cells were washed and incubated with CYP3A4 substrate luciferin-IPA (3 μ M, K_m value) for an additional 30min. The reaction was terminated by transferring 50 μ L of culture medium from each well to a separate plate with wells containing 50 μ L of luciferin detection reagent at room temperature, followed by luminescence measurement. Cell viability was subsequently determined by the CellTiter-Fluor assay.

CYP3A4 induction in DMSO-treated HuH-7 and HepaRG cells

After DMSO treatment, HuH-7 cells were incubated (37°C) with rifampicin (1–20 μ M) or dexamethasone (1–100 μ M) in serum-free medium for 48h, with treatments replenished after 24h. For HepaRG cells, HepaRG Induction Working Medium was used. Cells were then assayed for CYP3A4 activity (luciferin-IPA) and viability (CellTiter-Fluor) assays.

Hepatotoxicity assay

DMSO-treated HuH-7, HepG2/C3A and HepaRG cells were treated (24h, 37°C) with the following hepatotoxicants (1–100 μ M): nitrofurantoin, nordihydroguaiaretic acid (NDGA), troglitazone, nefazodone and desipramine. Compounds were dissolved in basal medium for DMSO-treated HuH-7 and HepG2/C3A cells, and HepaRG Tox Working Medium for HepaRG cells. Cell viability was determined by a luminescence assay (CellTiter-Glo).

Data analysis

GraphPad Prism (La Jolla, CA) was used to fit CYP3A4 inhibition data and calculate IC_{50} , k_{inact} , and K_I values. For competitive inhibition, IC_{50} was the half-maximal inhibitory concentration of ketoconazole or nifedipine determined using a non-linear regression Hill model. For time-dependent inhibition, the inactivation rate constant k_{obs} was obtained by plotting the natural logarithm of the remaining CYP3A4 activity (%) against pre-incubation time with troleandomycin. The kinetic parameters k_{inact} and K_I were obtained by fitting data to the following equation by non-linear regression,

$$k_{obs} = \frac{k_{inact}[I]}{K_I + [I]}$$

where $[I]$ is the inhibitor concentration.

GraphPad Prism (La Jolla, CA) was also used to fit cell viability data and calculate EC_{50} for hepatotoxicity studies. EC_{50} was the half-maximal cytotoxic concentration of hepatotoxicants determined using a non-linear regression Hill model.

Statistical analysis was conducted using SAS 9.2 (Cary, NC). Data were first analyzed to assure normality and homogeneity of variances. Significant differences were identified using a *t* test for data with only two groups or analysis of variance (ANOVA) followed by a least squares mean test for data with three or more groups.

Results

Cell morphology

Phase contrast photomicrographs of both dividing and confluent DMSO-treated HuH-7 cells are shown in Fig. 1a, b. The actively dividing cells (Fig. 1a) exhibited characteristic “cobblestone” morphology of proliferating epithelial cells. In contrast, cells treated with DMSO (Fig. 1b) displayed many of the characteristics of primary hepatocytes including dense packing, polygonal shape, large nuclei with prominent nucleoli, and numerous binucleated cells. Confocal images of DMSO-treated HuH-7 cells under bright field (Fig. 1c) and stained with a nuclei dye (Fig. 1d) are also presented. DMSO-treated HuH-7 cells displayed differentiated cell morphology resembling primary human hepatocytes.

CYP3A4 comparison in HuH-7, HepG2/C3A and HepaRG cells

First, CYP3A4 gene expression and activity were compared among 1-week cultured, 3-week cultured and 2-week DMSO-treated HuH-7 cells after confluence (Table 1). DMSO-treated HuH-7 cells expressed the highest levels of CYP3A4 activity and gene expression compared to the other two culture conditions. All of the following cell-based studies were conducted using DMSO-treated HuH-7 cells.

Next, CYP3A4 activity in DMSO-treated HuH-7 was further compared with that in HepG2/C3A and HepaRG cells. The highest CYP3A4 activity was measured in HepaRG cells (77.8 ± 9.3 pmol D-luciferin $\text{h}^{-1} \text{mg}^{-1}$ protein), followed by DMSO-treated HuH-7 cells (11.0 ± 1.8 pmol D-luciferin $\text{h}^{-1} \text{mg}^{-1}$ protein). Activity in HepG2/C3A cells was minimal and below the detection limit. Protein content in the cells was determined by the bicinchoninic acid assay (Smith et al. 1985).

Comparison of CYP3A4 inhibition between human recombinant enzyme and DMSO-treated HuH-7 cells

Inhibition of CYP3A4 by competitive inhibitors ketoconazole and nifedipine was evaluated. Ketoconazole (Fig. 2a) and nifedipine (Fig. 2b) displayed similar concentration-dependent inhibition of CYP3A4 activity in human recombinant enzyme and DMSO-treated HuH-7 cells. The IC_{50} values (Table 2) for these inhibitors agreed well with published data.

Time-dependent CYP3A4 inhibition by troleandomycin was compared in human recombinant enzyme (Fig. 3a) and DMSO-treated HuH-7 cells (Fig. 3b). In Fig. 3, the observed inactivation rate constants k_{obs} was plotted against troleandomycin concentrations, yielding a hyperbolic curve fit to the data points. Kinetic parameters K_I and k_{inact} for troleandomycin are summarized in Table 2. There was minimal cytotoxicity (<10%) observed for all compound concentrations.

Comparison of CYP3A4 induction between DMSO-treated HuH-7 and HepaRG cells

Induction of CYP3A4 activity (48h) by rifampicin and dexamethasone in DMSO-treated HuH-7 and HepaRG cells is presented in Table 3. For DMSO-treated HuH-7 cells, rifampicin (1 to 20 μM) concentration dependently induced CYP3A4 activity to a maximum of 1.7-fold ($p < 0.05$) compared with control cells without inducer. Similarly, dexamethasone

concentration dependently (1 to 100 μ M) induced CYP3A4 activity to a maximum of 2.7-fold ($p<0.05$) compared with control cells without inducer. For HepaRG cells, CYP3A4 activity was induced to a much higher level using both rifampicin (up to 21.5-fold, $p<0.05$) and dexamethasone (up to 6.7-fold, $p<0.05$). There was minimal loss of cell viability ($<10\%$) observed at all compound concentrations.

Cytotoxic effects of hepatotoxicants in DMSO-treated HuH-7, HepG2/C3A and HepaRG cells

Cytotoxicity after 24-h treatment with five hepatotoxicants was compared in DMSO-treated HuH-7, HepG2/C3A, and HepaRG cells. All five chemicals (1 to 100 μ M) exhibited concentration-dependent cytotoxicity. Under current experimental conditions, for the five hepatotoxicants, DMSO-treated HuH-7 cells were most sensitive (Fig. 4 and Table 4). In HepaRG and HepG2/C3A cells, hepatotoxicants nordihydroguaiaretic acid (NDGA), nitrofurantoin, and troglitazone caused minimal cytotoxicity ($<15\%$) up to 100 μ M and their EC_{50} values were not calculated.

Discussion

DMSO is a common solvent that has been shown to have various pharmacological, cellular, and molecular applications (Santos et al. 2003). One of the important applications of DMSO is to induce cell differentiation (Isom et al. 1985), which has been used in the development of the metabolically competent HepaRG cells. Although the mechanisms by which DMSO increases liver-specific functions in HepaRG cells are not fully understood, DMSO induces several genes corresponding to enzymes involved in xenobiotic metabolism as well as transporters (Guguen-Guillouzo and Guillouzo. 2010). Since DMSO treatment has successfully induced differentiation and drug-metabolizing enzyme activities in both HepaRG and HuH-7 cells, this approach may have the potential to be used with other hepatoma cells and stem cells.

Previous reports have shown that non-induced HuH-7 cells have low drug-metabolizing enzyme activities (Lin et al. 2012), but after DMSO treatment (Choi et al. 2009) or extended culture periods (Sivertsson et al. 2010), major cytochromes P450 enzymes (e.g. CYP1A2, CYP2C9, and CYP3A4) and glucuronyltransferases (e.g. UGT1A4 and UGT2B7) were induced. Our results (Table 1) showed DMSO (1%) treatment for 2 weeks after confluence induced CYP3A4 gene expression (~ 3 -fold) and enzyme activity (~ 12 -fold) compared to HuH-7 cells cultured for the same period without DMSO (i.e., 3-week culture). Therefore, DMSO treatment is an effective way to elevate CYP3A4 enzyme activity in HuH-7 cells. Although HepaRG cells had higher basal CYP3A4 activity, DMSO-treated HuH-7 cells were proven to be a suitable model for inhibition studies that was in good agreement with human recombinant enzymes and reported values (Table 2). These results for DMSO-treated HuH-7 cells, combined with the advantages of being cost-effective, ability to propagate in culture, and data reproducibility, support this model as effective for medium- and high-throughput screening.

Drug-metabolizing enzyme inhibition, especially time-dependent inhibition, is one of the leading causes for clinical drug-drug interactions and toxicity (McGinnity et al. 2006). In the

current study, human recombinant CYP3A4 reversible and irreversible inhibition kinetics were first investigated and compared to published data (Table 2). IC_{50} values for reversible inhibitors ketoconazole and nifedipine were consistent with a literature report using luciferin-IPA as a CYP3A4 substrate (Meisenheimer et al. 2011). K_I and k_{inact} values for the irreversible inhibitor troleanomycin determined using luciferin-IPA were in good agreement with values obtained using substrates midazolam (Atkinson et al. 2005) and testosterone (Chan and Delucchi 2000).

CYP3A4 inhibition in HuH-7 cells was then compared to data obtained using human recombinant CYP3A4. For ketoconazole and nifedipine, there was good agreement between IC_{50} values (<2.5-fold difference) generated in the DMSO-treated HuH-7 cells and human recombinant CYP3A4 enzyme. However, for troleanomycin, HuH-7 cells yielded a 2-fold lower k_{inact} and 70-fold higher K_I value compared to human recombinant CYP3A4 inhibition assay. A higher K_I value in HuH-7 cells could be attributable to slow diffusion into the cells or active removal from the cells by export pumps (Wang et al. 2013, Zhang et al. 2012). A comparison between HuH-7 cells and human recombinant CYP3A4 demonstrated a potential use for DMSO-treated HuH-7 cell line as a whole cell system for measuring CYP3A4 inhibition.

In addition to inhibition, drug-metabolizing enzyme induction could also cause clinical drug-drug interactions, such as loss of drug efficacy. In the 48-h induction studies (Table 3), DMSO-treated HuH-7 cells showed minimal induction by both rifampicin and dexamethasone compared to HepaRG cells. These results suggested that HuH-7 cells may not be an ideal model for CYP3A4 induction compared with other *in vitro* models such as primary human hepatocytes and HepaRG cells (Gerets et al. 2012; Templeton et al. 2011).

Hepatotoxicity is a major reason for drug withdrawal from the market after approval because liver toxicity is difficult to discover in pre-clinical and clinical trials (Kaplowitz 2005). A metabolically competent and cost-effective human cell culture system is valuable for early hepatotoxicity screening in drug development, especially for metabolism-associated hepatotoxicity. Here, DMSO-treated HuH-7 cells were compared with HepaRG and HepG2/C3A cells in detecting cytotoxicity (Fig. 4 and Table 4). Hepatotoxicity of the tested compounds has been reported, including the antibiotic nitrofurantoin (Hosomi et al. 2011), the anti-depressants nefazodone (Voican et al. 2014) and desipramine (Hosomi et al. 2011), the anti-diabetic and anti-inflammatory drug troglitazone (Gustafsson et al. 2014), and a bioactive compound nordihydroguaiaretic acid (NDGA) from a plant chaparral (Gordon et al. 1995; Lambert et al. 2002). Differences in cytotoxic sensitivity among the three cell lines were observed. These differences could be due to many reasons, such as differential generation of toxic metabolites or differential detoxification by various drug-metabolizing enzymes. Current results highlighted the importance of using multiple cell models when investigating hepatotoxicity in high-throughput screening.

Overall, HuH-7 cells treated with 1% DMSO after confluence differentiate into a robust and reproducible *in vitro* cell system with elevated CYP3A4 enzyme gene expression and activity, making it a useful model for CYP3A4 inhibition study. Furthermore, DMSO-treated HuH-7 cells were more sensitive to some hepatotoxicants than HepaRG or HepG2/C3A

cells. This observation suggests the potential application of DMSO-treated HuH-7 cells as a more predictive hepatotoxicity model than other commonly used liver cell lines. Future studies on the kinetics of other major drug-metabolizing enzymes, transporters, and liver-specific characteristics, such as bile canaliculi formation, will be valuable to further establish the HuH-7 cells as a useful model for medium- and high-throughput screening.

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Fig. 1. Phase contrast photomicrographs of (a) dividing (4 days) and (b) DMSO-treated (for 2 weeks post-confluence) HuH-7 cells with arrows pointing to binucleated cells. Confocal images of (c) DMSO-treated HuH-7 cells (for 2 weeks post-confluence) and (d) NucRed liver 647 nuclei dye stained DMSO-treated HuH-7 cells (for 2 weeks post-confluence). Bars represent 100 μ m.

Fig. 2. CYP3A4 competitive inhibition by (a) ketoconazole and (b) nifedipine in human recombinant enzyme and DMSO-treated HuH-7 cells normalized by cell viability (n = 3). Inhibition is expressed as mean% of control $\pm$ SD (n = 3)

Fig. 3. Plot of observed CYP3A4 inactivation rate constant (k_{obs}) and troleandomycin concentration in (a) human recombinant enzyme and (b) DMSO-treated HuH-7 cells normalized by cell viability ($n = 3$).

Fig. 4. Cell viability in DMSO-treated HuH-7, HepaRG, and HepG2/C3A cells after 24-h treatments with (a) nitrofurantoin, (b) nordihydroguaiaretic acid, (c) troglitazone, (d) nefazodone, (e) desipramine. Cell viability is expressed as mean% of control $\pm$ SD (n 3).

Table 1

CYP3A4 levels in HuH-7 cells under different culture conditions

	Basal medium one week	Basal medium three weeks	DMSO-treated ^a
Enzyme activity ^b	0.24±0.08	0.89±0.19	11.00±1.78
Gene expression ^c	1.00±0.63	9.74±2.67	27.83±4.50

Data expressed as mean±SD of 30 biological replicates for activity assays and three independent biological replicates for gene expression assays.

^a Basal medium for 1 week followed by DMSO (1%) treatment for an additional 2 weeks.

^b Enzyme activity is expressed as pmol D-luciferin h⁻¹mg⁻¹ protein. Protein content was determined by bicinchoninic acid assay (Smith et al. 1985).

^c CYP3A4 gene expression is expressed relative to the level in HuH-7 cells cultured for 1 week, which was defined as 1.00. Beta-actin and 18S were used as endogenous controls.

Table 2

CYP3A4 enzyme inhibition parameters

Inhibition type	Parameter	Human recombinant CYP3A4 enzyme		DMSO-treated Huh-7 cells
		Present study	Literature	
Ketoconazole	Reversible IC_{50} (μ M)	0.064	0.04 ^a	0.146
Nifedipine	Reversible IC_{50} (μ M)	2.45	3.24 ^a	5.19
Troleandomycin	Irreversible K_i (μ M)	0.11	0.18 ^b , 0.26 ^c	7.77
	k_{inact} (min^{-1})	0.061	0.12 ^c , 0.15 ^b	0.029

Data expressed as mean of at least two independent experiments with triplicates for each inhibitor concentration.

^aDetermined by luminescence assay using substrate luciferin-IPA (Meisenheimer et al. 2011).

^bDetermined by HPLC method using substrate testosterone (Chan and Delucchi 2000).

^cDetermined by LC/MS-MS method using substrate midazolam (Atkinson et al. 2005).

Table 3

Induction of CYP3A4 activity

	Concentration (μ M)	DMSO-treated HuH-7 cells	HepaRG cells
Rifampicin	1	1.3 $\pm$ 0.1	16.0 $\pm$ 1.0
	5	1.5 $\pm$ 0.2	21.7 $\pm$ 5.6
	20	1.7 $\pm$ 0.2	21.5 $\pm$ 3.2
Dexamethasone	1	1.6 $\pm$ 0.2	1.1 $\pm$ 0.2
	10	1.9 $\pm$ 0.3	2.3 $\pm$ 0.4
	100	2.7 $\pm$ 0.3	6.7 $\pm$ 0.6

CYP3A4 activity is expressed as fold change relative to control cells without inducers. Data is expressed as mean $\pm$ SD (n = 3).

Table 4Comparison of cytotoxicity EC_{50} (μ M) in the three cell lines

	DMSO-treated HuH-7	HepaRG	HepG2/C3A
Nitrofurantoin	78.8	N.D. ^a	N.D.
Nordihydroguaiaretic acid (NDGA)	63.6	N.D.	N.D.
Troglitazone	71.0	N.D.	N.D.
Nefazodone	32.9	55.3	43.2
Desipramine	67.3	72.9	95.6

Values were calculated based upon data in Fig. 4.

^aN.D. indicates “not determined” due to minimal cytotoxicity (<15%) up to 100 μ M.