The Impact of Various Solvents and Solvent Concentrations on *In Vitro* Enzyme Induction Assessment in Cultured Human Hepatocytes

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Introduction

Induction of cytochrome P450 (CYP) enzymes is one of the principal mechanisms of drug-drug interactions inasmuch as regulators recommend that new drug candidates be evaluated for their ability to induce CYP enzymes. The FDA (US), EMA (Europe) and PMDA (Japan) recently revised the guidelines for evaluating CYP induction (FDA, 2012). According to this new guidance document, a new chemical entity (NCE) should be evaluated at very high concentrations *in vitro* (*e.g.,* ((0.1*dose)/250 mL) or 50x the total $C_{max,ss}$) (EMA, 2013). Achieving these concentrations *in vitro* can be problematic if the test article is insoluble in preferred solvents (*e.g.,* 0.1% v/v DMSO) and/or cell culture medium. Consequently, test articles are evaluated for solubility in other solvents; however, potential cytotoxic effects and effects on CYP induction of these chemicals are often unknown.

In the present study, the effects of various solvents on CYP induction and cytotoxicity were evaluated in three preparations of sandwich-cultured cryopreserved human hepatocytes. These solvents include DMSO, ascorbic acid, acetic acid, acetonitrile, dimethylacetamide, ethyl acetate, and methanol. Cytotoxicity and enzyme induction potential was evaluated by *in situ* marker substrate incubations and mRNA expression.

Materials & Methods

1X TE Buffer (Ambion); gene expression assay, high capacity cDNA reverse transcription kit, TAQMan fast advanced master mix (Applied Biosystems); hydroxybupropion, 1′-hydroxymidazolam (Cerilliant); acetic acid, acetonitrile, ITS+, methanol, RNase-free Water (Fisher Scientific); penicillin-streptomycin (Invitrogen); buffer RLT, buffer RPE, DNase I, proteinase K, RNase inhibitor, RNeasy mini kit (Qiagen); acetaminophen-d_4, hydroxybupropion-d_6, 1′-hydroxy-midazolam-d_4 (proprietary information); acetaminophen, ascorbic acid, bupropion HCl, dexamethasone, dimethylacetamide, DMSO, ethanol, ethyl acetate, β-mercaptoethanol, midazolam, phenacetin, triton X-100 (Sigma-Aldrich); LDH cytotoxicity detection kit (Roche Diagnostics); Supplemented MCM (MCM+, XenoTech).

Hepatocyte cultures and LDH assay

Cryopreserved human hepatocytes were seeded and cultured at concentrations ranging from 0.28 – 0.32 x 10^6 cells/well in a collagen-Matrigel sandwich configuration in 48-well tissue culture plates in a humidified culture chamber (37 ± 1°C at 95% relative humidity and 95/5% air/CO_2) for 24 hours. Cultures were then treated once daily for 3 consecutive days with MCM+ medium containing one of four concentrations of DMSO, ascorbic acid (2 mg/mL), acetic acid, acetonitrile, dimethylacetamide, ethyl acetate or methanol (0.1, 0.5, 1.0 or 2.0% for each solvent). Media samples from like treatment groups were collected immediately prior to dosing and at cell harvest. Three wells from the DMSO treatment group were treated with a 1% Triton X-100 solution each day of treatment and incubated for 55 to 111 minutes. LDH activity for each sample was determined on a microtiter plate reader (λ = 490 nm) with a Synergy HT Multi-Detection Microplate Reader (Bio-Tek Instruments, Inc.) warmed to 37 ± 1°C and according to the LDH Detection Kit procedure (Roche Laboratories).

In situ CYP450 enzyme activity assays

Following collection of the spent media, approximately 24 hours after the last treatment, each well was rinsed two times with pre-warmed (37 ± 2°C) fresh culture media prior to incubation with probe substrates. Media was aspirated from the wells and reactions were started by addition of 200 µL pre-warmed media containing phenacetin probe substrate to each well. The culture multi-plates were placed in a humidified culture chamber (37 ± 1°C at 95% relative humidity and 95/5% air/CO_2) and incubations were carried out for 45 minutes, at which time an aliquot (150 µL) of the incubation mixture was removed and added to 150 µL of the stop reagent (acetonitrile) and internal standard. Following phenacetin incubations, the same wells were rinsed with pre-warmed MCM+ then were incubated with a cocktail of probe substrates, bupropion and midazolam, under the same conditions as the phenacetin incubations. The samples were centrifuged (*e.g.,* 2000 x *g* for 10 minutes at 2 to 8°C). An equal volume of supernatant fractions (100 µL from both substrate incubations) was pooled together and analyzed by LC-MS/MS for the formation (pmol/inc/min) of specific metabolites (see below).

Enzyme	Marker Substrate	Metabolite Monitored
CYP1A2	Phenacetin (100 µM)	Acetaminophen
CYP2B6	Bupropion (500 µM)	Hydroxybupropion
CYP3A4/5	Midazolam (30 µM)	1'-hydroxymidazolam

Analysis of mRNA expression

Following substrate incubations, hepatocytes were harvested with Buffer RLT. Total RNA was isolated from the cell lysates according to the Buffer RLT procedure (Qiagen) and purified using the RNeasy Mini Kit (Qiagen). RNA concentrations were determined by measuring absorbance at 260 and 280 nm on the NanoDrop (ThermoFisher Scientific). Single-stranded cDNA was prepared with the RT Master Mix using the AB 7900HT Fast Real Time PCR System (Applied Biosystems). For the qRT-PCR assay, to assess the mRNA expression of CYP1A2, CYP2B6 and CYP3A4, each PCR was performed in triplicate. Reactions were analyzed on an Applied Biosystems Real Time PCR sequence detection system (AB 7900HT). The relative quantity of the target cDNA compared with that of the endogenous control cDNA (GAPDH) was determined by the $\Delta\Delta C_t$ method (Applied Biosystems User Bulletin #2). Relative quantification measures change in mRNA expression in a test sample relative to that in a vehicle control sample (*e.g.,* 0.1% v/v DMSO).

Data processing

For cytotoxicity assessment (LDH release), data were processed with BIO-TEK KC4 Signature for Windows software (version 3.4 Rev 21). Data for all assays was graphed with the computer program Microsoft Excel 2003 or 2007 (Microsoft Corp.). For LDH release, percent LDH release was calculated based on the following equation:

$$\text{LDH release (\%)} = \frac{\text{ABCVM of Experimental Culture} - \text{ABCVM of Low Control}}{\text{ABCVM of High Control} - \text{ABCVM of Low Control}} \times 100$$

ABCVM = Average background – Corrected V_{max}
Low control is MCM+ only
High control (LDH positive control) is 1% Triton X-100 in MCM+

For enzyme activity, data were processed and graphed with the computer program Microsoft Excel 2003 or 2007 (Microsoft Corp.). Individual rates of reaction (expressed as pmol/inc/min) from like treatment groups were averaged, and fold change was calculated based on the following equation:

$$\text{Fold change} = \frac{\text{Enzymatic rate of each treatment group}}{\text{Enzymatic rate of the vehicle control}}$$

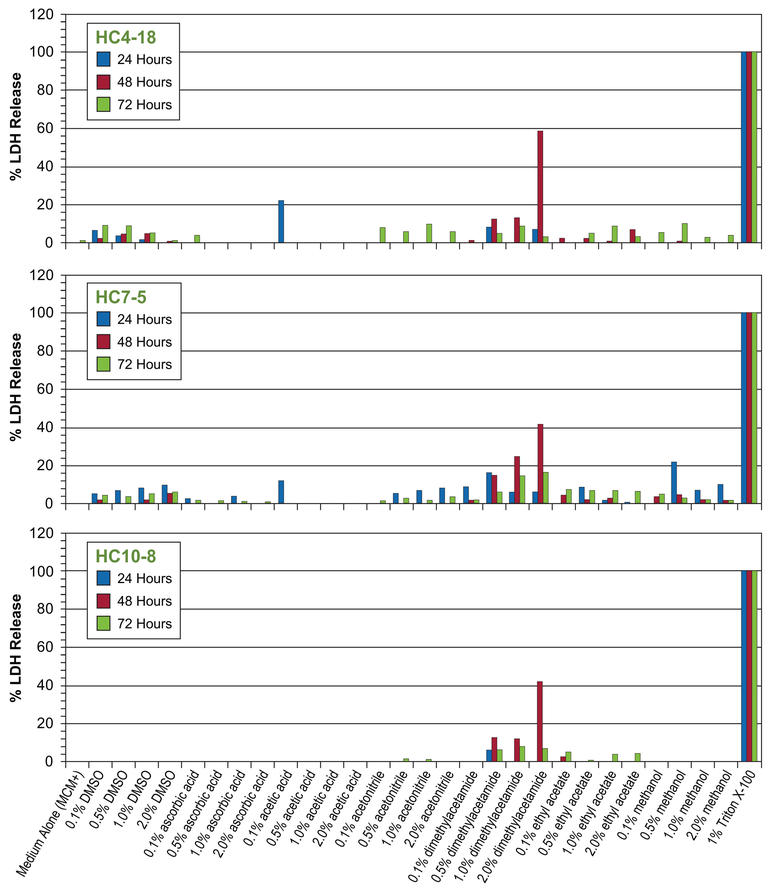
For mRNA by qRT-PCR, relative gene expression was analyzed by the Sequence Detection System (SDS) Software version 2.3 or 2.4, for Relative Quantification (Applied Biosystems) using the comparative C_t method ($\Delta\Delta C_t$). Calculations are as follows:

1. $\Delta C_t = C_t$ (target) − C_t (endogenous control)
2. $\Delta\Delta C_t = \Delta C_t$ (treated sample) − ΔC_t (untreated control)
3. Fold change in expression = $2^{-\Delta\Delta Ct}$

Results

Cytotoxicity was noted for several of the solvents tested (see Figure 1). Acetic acid caused up to a 22% increase in LDH release at 0.1% (the lowest concentration tested) following 24 hours of treatment, and complete cell death at all higher concentrations at 24 hours and all concentrations at 48 and 72 hours, and CYP activity and mRNA levels could not be determined. Hepatocytes treated with ethyl acetate or dimethylacetamide exhibited morphological changes (examined by light microscopy) including loss of membrane definition and increased cellular debris. Dimethylacetamide caused concentration-dependent increases in LDH release up to 58.5% following 48 hours of treatment, and complete cell death following 72 hours at 2% concentration. Treatment with up to 2% ethyl acetate caused concentration-dependent increases in LDH release up to 30.5% following 72 hours of treatment.

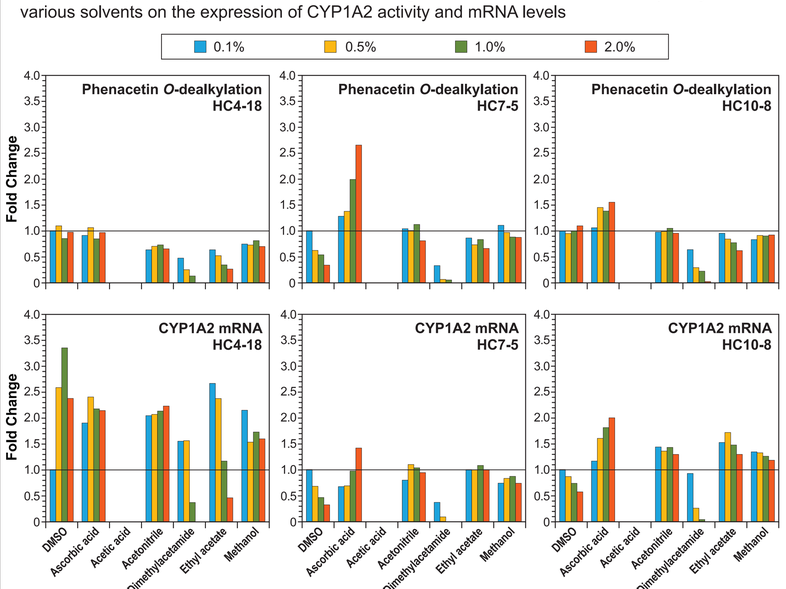
Figure 1. Cytotoxic evaluation: The effect of treating three lots of cultured human hepatocytes with various solvents on lactate dehydrogenase release



Negative Control: Medium Alone (MCM+) *Positive Control: 1% Triton X-100* *DMSO = Dimethyl sulfoxide*
Lack of a bar on the graph indicates that when compared to the negative control, there was not an increase in LDH release.

Induction results indicate that, when compared to 0.1% DMSO, multiple solvents caused a two-fold or greater change in at least one of the three cultures in CYP1A2, CYP2B6 or CYP3A4 mRNA levels, namely DMSO (up to 3.36-, 6.61- and 4.14-fold for CYP1A2, CYP2B6 and CYP3A4, respectively), ascorbic acid (up to 2.41-, 3.39- and 2.10-fold change for CYP1A2, CYP2B6 and CYP3A4, respectively), acetonitrile (up to 2.23- and 2.50-fold change for CYP1A2 and CYP2B6, respectively), and ethyl acetate (up to 2.67- and 2.41-fold change for CYP1A2 and CYP2B6, respectively). However, only DMSO caused concentration-dependent changes in CYP2B6 and CYP3A4 mRNA levels. In contrast, dimethylacetamide caused a greater than 50% decrease in CYP1A2, CYP2B6 and CYP3A4 mRNA levels (see Figure 2, Figure 3 and Figure 4).

Figure 2. CYP1A2 expression: The effect of treating three lots of cultured human hepatocytes with various solvents on the expression of CYP1A2 activity and mRNA levels



Unexpectedly, similar trends were not observed when CYP activity was evaluated. All concentrations of DMSO, ascorbic acid, acetonitrile and methanol had little or no effect (≤ 2-fold change) on CYP activity with a few exceptions (*i.e.,* CYP1A2: 2.0% ascorbic acid; CYP2B6: 0.5% DMSO; CYP3A4: 2.0% DMSO and 0.1% dimethylacetamide). However, dimethylacetamide and ethyl acetate caused a greater than 50% decrease in CYP1A2, CYP2B6 and CYP3A4 activity, which may be a result of the cytotoxic effects of these solvents (see Figure 2, Figure 3 and Figure 4).

Figure 3. CYP2B6 expression: The effect of treating three lots of cultured human hepatocytes with various solvents on the expression of CYP2B6 activity and mRNA levels

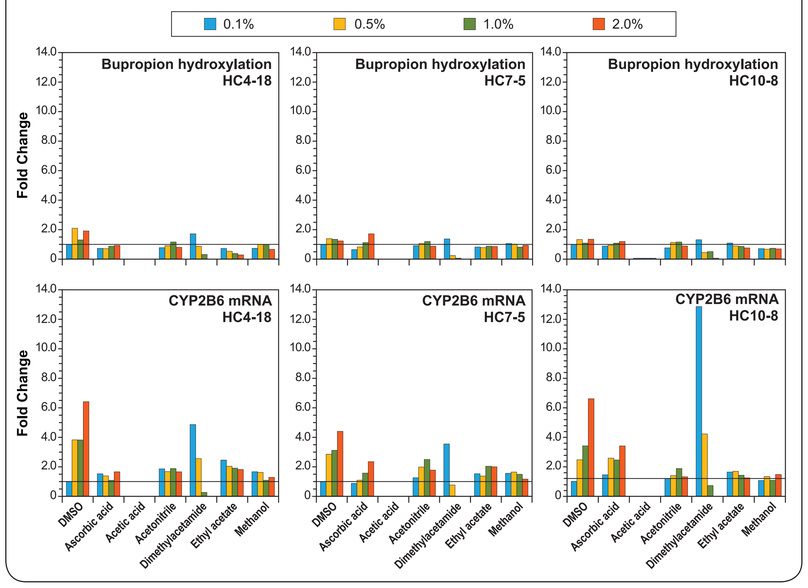
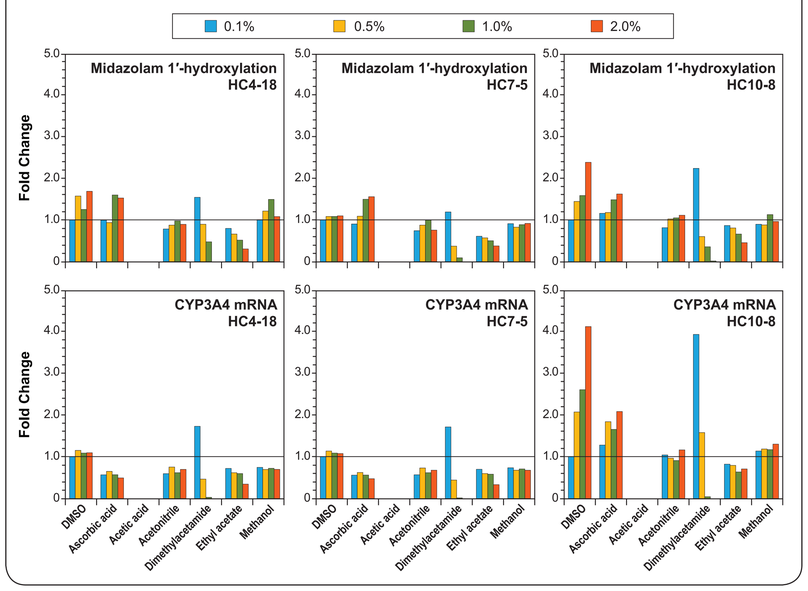


Figure 4. CYP3A4 expression: The effect of treating three lots of cultured human hepatocytes with various solvents on the expression of CYP3A4/5 activity and CYP3A4 mRNA levels



Conclusions

- Ascorbic acid (at 0.1% v/v), acetonitrile (up to 2% v/v), and methanol (up to 2% v/v) are acceptable substitutions to 0.1% DMSO as they caused little to no effect on cytotoxicity, CYP enzyme activity and CYP enzyme mRNA expression levels.
- Acetic acid, dimethylacetamide, DMSO (at ≥ 0.5% v/v) and ascorbic acid (at 0.2 to 2% v/v) were found to be unacceptable substitutions to 0.1% DMSO as they caused cytotoxic effects (morphological changes or increase LDH release) or a concentration dependent increase or decrease in CYP enzyme activity and CYP enzyme mRNA expression levels.
- Ascorbic acid (at ≥ 0.5% v/v) and ethyl acetate (up to 2% v/v) should be investigated further before accepting them as substitutions to 0.1% DMSO.
- The cytotoxic and enzyme induction or suppression effects of the solvent should be considered and predetermined when an alternate solvent is used to evaluate CYP induction in cultured hepatocytes.

References

1. U.S. Department of Health and Human Services, Food and Drug Administration (2012) Draft Guidance for Industry: Drug Interaction Studies—Study Design, Data Analysis, and Implications for Dosing and Labeling Recommendations. FDA (CDER), Rockville, MD.
2. [EMA] European Medicines Agency (2013) Guideline on the Investigation of Drug Interactions. European Medicines Agency, London. 60 p. EMA Guideline No.: CPMP/EWP/560/95/Rev.1. Corr.