The Use of Pooled Plated Cryopreserved Human Hepatocytes for the Determination of Metabolic Clearance, Cytochrome P450 Enzyme Induction and Uptake Transporter Studies

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Introduction

During early drug discovery and development, conventional test systems such as hepatic subcellular fractions or pooled suspended cryopreserved human hepatocytes are often used to assess the victim and perpetrator potential of new drug candidates. These test systems have limited stability (typically ≤ 4 hours) making it challenging to determine parameters such as intrinsic clearance (CL_{int}) and half-life ($t_{1/2}$) of low turnover drugs. Alternative test systems and techniques, such as micro-patterned hepatocytes co-cultured with fibroblasts and multi-day relay incubations of cryopreserved human hepatocytes, have been developed to prolong test system stability (Chan *et al.*, 2013; Di *et al.*, 2013), but can be cumbersome as well as time- and cost-prohibitive. In the case of CYP induction screening, the use of individual plated primary human hepatocytes can lead to inter-individual variability in the induction response or susceptibility to cytotoxicity. Hepatic uptake experiments are frequently conducted in pooled suspended hepatocytes and typically only with individual donor plated hepatocytes, resulting in similar experimental limitations associated with the induction assays.

Pooled plateable cryopreserved hepatocytes are a test system that may be able to address several limitations associated with the aforementioned test systems. As an alternative improved method, in the present study, we evaluated the utility of plated cryopreserved human hepatocytes pooled using a proprietary pooling process for metabolic clearance, CYP induction, and uptake transporter functionality experiments/assays.

Materials & Methods

Chemicals

Dextromethorphan, DMSO, diazepam, diclofenac, disopyramide, HEPES, midazolam, naloxone, omeprazole, phenacetin, phenobarbital, repaglinide, rifampin, *S*-warfarin, theophylline, timolol maleate, tolbutamide, Williams E, verapamil and zolmitriptan were all purchased from Sigma-Aldrich (St. Louis, MO). GlutaMax was purchased from Life Technologies (Grand Island, NY). The sources of all other reagents have been previously described (Paris *et al.*, 2009; Parkinson *et al.*, 2011).

Test system

Plateable human hepatocytes from non-transplantable livers (CryostaX™; n = 5) were isolated, cryopreserved and pooled using a proprietary single freeze process at XenoTech, LLC (Lenexa, KS).

Characterization of individual and pooled CYP, UGT and SULT enzyme activities

An assessment of enzyme activities was conducted in both individual and pooled hepatocyte preparations. Individual and pooled cryopreserved hepatocytes were cultured in 48-well plates (approximately 1.3 x 10^6 cells/mL) for 4 hours, followed by incubations of midazolam (CYP3A4/5; 30 µM), bupropion (CYP2B6; 500 µM), amodiaquine (CYP2C8; 20 µM), phenacetin (CYP1A2; 100 µM), tolbutamide (CYP2C9; 150 µM), *S*-mephenytoin (CYP2C19; 400 µM), dextromethorphan (CYP2D6; 75 µM) and 7-hydroxycoumarin (UGT, SULT; 500 µM) in Williams E + HEPES media. Incubations were performed for 30-45 min on a plate shaker (~150 rpm) in duplicate at 37°C with 95% humidity and 5% CO_2. Reactions were terminated by protein precipitation with one volume of acetonitrile containing internal standard, followed by centrifugation (10 min, 920 RCF) and LC/MS/MS analysis.

Determination of *in vitro* metabolic clearance

Pooled cryopreserved hepatocytes were cultured in 48-well plates for 4 hours, followed by incubations of 1 µM substrate for 0, 4, 8, 12, 24, 36 and 48 hours in Williams E + HEPES media. Incubations were performed on a plate shaker (~150 rpm) in duplicate at 37°C with 95% humidity and 5% CO_2. At the end of the incubation period, reactions were stopped by protein precipitation with the addition of one volume of acetonitrile containing internal standard (terfenadine). Samples were then centrifuged for 10 min at 920 RCF, followed by LC/MS/MS analysis. Data were processed with GraFit 7.0.2 (Erithacus Software Ltd., Surrey, UK) using single-exponentional fitting. CL_{int} was determined by the following equation: $CL_{int} = 0.693 \div t_{1/2}$ x 1800 g liver weight ÷ 70 kg body weight x incubation volume ÷ hepatocytes per well x 120 million hepatocytes per *g* liver (Chan *et al.*, 2013).

Assessment of *in vitro* CYP induction

Pooled cryopreserved hepatocytes were seeded and cultured in a collagen-Matrigel sandwich configuration in 48-well tissue culture plates in a culture chamber (37°C with 95% humidity and 5% CO_2) for 24 hours, followed by once daily treatment for 3 days with MCM+ medium containing either 0.1% DMSO, rifampin (0.1- 20 µM), omeprazole (50 µM) or phenobarbital (750 µM) in triplicate wells. Following treatment, enzyme activities of CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19 and CYP3A4/5 were assessed by a 2-step cocktail substrate *in situ* incubation (containing 100 µM phenacetin, 150 µM tolbutamide and 400 µM *S*-mephenytoin, followed by 500 µM bupropion, 20 µM amodiaquine and 30 µM midazolam). Following a double wash of the cells with MCM+, substrate incubations were conducted for 45 minutes for the first substrate cocktail, followed by two washes with MCM+ and a 30 min incubation of the second substrate cocktail. Incubations were stopped by removing an aliquot of the supernatant from the incubations and adding to an equal volume of acetonitrile containing internal standard, followed by centrifugation (10 min, 920 RCF) and LC/MS/MS analysis. After substrate incubations, cells were harvested for mRNA evaluation with Buffer RLT lysis to assess the mRNA expression of CYP1A2, CYP2B6, CYP2C8, CYP2C19, CYP2C9, and CYP3A4 by RT-qPCR. Total RNA was extracted from the cell lysates according to the Buffer RLT procedure (Invitrogen, Carlsbad, CA) and was purified using the RNeasy Mini Kit (Qiagen Inc., Valencia, CA). 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 control cDNA (GAPDH) was determined by the ΔΔCt method (Applied Biosystems User Bulletin #2). Relative quantification measures change in mRNA expression in a test sample relative to that in a control sample (*e.g.*, 0.1% v/v DMSO).

Assessment of uptake transporter functionality

Transporter functionality of the pooled cryopreserved hepatocytes) was assessed with 1 min incubations (with separate incubations of trace 0.1 µM radiolabeled or excess 100 µM cold compound) of estrone sulfate (OATP1B1), CCK-8 (OATP1B3), MPP+ (OCT1) or TCA (NTCP) in MCM+ media. Reactions were stopped by aspiration of the media followed by a 30 second rinse with 1x PBS containing 0.2% BSA. Samples were rinsed two additional times with 1x PBS followed by lysing of the cells with 0.1M NaOH and scintillation counting. Protein determinations were performed post incubation by isolation of microsomal fraction and measurement using the Pierce BCA assay (Pierce Chemical, Rockford, IL). Uptake ratios were determined based on the difference between uptake activities between trace substrate and excess substrate.

Analytical methods

Dextromethorphan, diazepam, diclofenac, disopyramide, midazolam, naloxone, phenacetin, repaglinide, theophylline, timolol, tolbutamide, verapamil, *S*-warfarin and zolmitriptan were quantified by liquid chromatography-tandem mass spectrometry (LC/MS/MS). All compounds were analyzed with a Waters Acquity (Milford, MA) or Shimadzu Nexera (Columbia, MD) LC system interfaced by electrospray ionization to an AB Sciex API4000 QTrap mass spectrometer (Foster City, CA) operated in multiple reaction monitoring (MRM) mode. Mobile phases were 0.2% formic acid in water and acetonitrile. Linear gradients were applied to a Waters Atlantis dC18, 5 µm (100 x 2.1 mm) column (Milford, MA), preceded by a direct connection Phenomenex Luna C8, 4 x 2 mm guard cartridge (Torrance, CA), for separation. Specific, selective MRM transitions were used for detection of each substrate and terfenadine was employed as a generic internal standard (IS). All other compounds were analyzed as previously described (Parkinson *et al.*, 2011).

Results

To compare the pooled plateable hepatocyte enzyme activities to the theoretical individual donor average enzyme activities, each individual donor lot of plateable hepatocytes and the pooled plateable hepatocytes were characterized for CYP, UGT and SULT activities as shown in **Table 1**. For all enzyme activities, the pooled plateable hepatocytes were within 2-fold of the theoretical average of individual donor activities. These results demonstrate that each donor is represented in the pool and one particular donor activity does not dominate substantially over the other donors.

The metabolic clearance data for 14 compounds with known, diverse clearance profiles (low, mid and high clearance) were evaluated in the pooled plated hepatocytes. As shown in **Figure 1** and **Table 2**, clearance values were determined for all compounds except theophylline. The rank order of *in vitro* CL_{int} was determined as follows: diclofenac > naloxone > midazolam > verapamil ≈ phenacetin ≈ repaglinide > dextromethorphan > diazepam > tolbutamide ≈ timolol > *S*-warfarin > disopyramide = zolmitriptan > theophylline. Results were consistent with their literature classification as high, medium or low clearance drugs. In comparison to literature values for other *in vitro* test systems, the pooled plated hepatocytes had comparable CL_{int} values for low, mid and high clearance drugs.

For CYP induction, prototypical inducers were tested with the pooled plated hepatocytes. As shown in **Figure 2** and **Table 3**, all CYPs were inducible with the greatest induction seen in CYP3A4 (rifampin 6.9-fold activity; 6.6-fold mRNA), CYP1A2 (omeprazole; 3.3-fold activity; 20-fold mRNA), and CYP2B6 (phenobarbital; 9.4-fold activity; 16-fold mRNA). Moderate induction of CYP2C8, CYP2C9 and CYP2C19 was also observed.

Lastly, as shown in **Table 4**, the uptake transporter activity of the pooled plateable hepatocytes was assessed using prototypical substrates for OATP1B1, 1B3, OCT1 and NTCP. Uptake ratios were >2, demonstrating functional uptake transporter activity, suitable for hepatic uptake studies.

Table 1. Individual donor hepatocyte and pooled plateable hepatocyte CYP, UGT and SULT activities

	Activity (pmol/min/10^6 cells)								
Hepatocyte Lot	1A2	2B6	2C8	2C9	2C19	2D6	3A4	SULT	UGT
Donor A	1.8	1.7	16	0.5	0.3	3.9	1.1	2.0	92
Donor B	3.1	1.7	102	1.3	2.9	5.7	9.0	4.2	159
Donor C	12	2.3	77	0.6	0.1	3.3	4.9	2.8	121
Donor D	5.5	13	390	3.5	2.1	18	16	19	600
Donor E	17	4.4	227	2.3	6.6	14	16	7.1	212
Individual Avg	8.0	4.7	162	1.6	2.4	8.9	9.4	7.0	237
Pooled	7.3	5.4	198	1.4	1.5	9.0	10.8	7.3	253

CYP: Cytochrome P450 UGT: UDP-glucuronosyltransferase SULT: Sulfotransferase

Figure 1. Metabolic stability of the low clearance drugs disopyramide, tolbutamide and *S*-warfarin in pooled plated hepatocytes

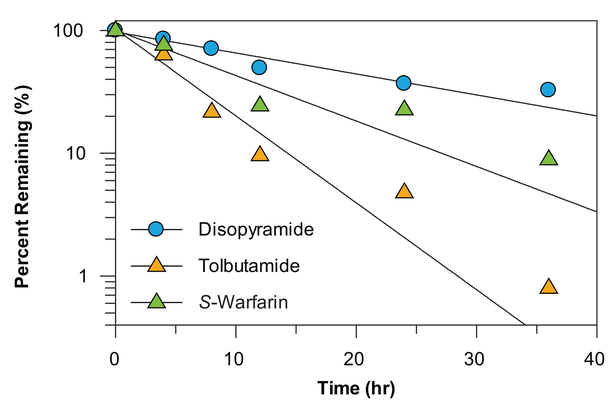


Table 2. CL_{int} values determined for 14 compounds with pooled plateable hepatocytes and comparison to literature values

Compound	*In vivo* CL_{int}	Elimination Rate Constant (k_{el})	Half Life (min)	CL_{int} (mL/min/kg)	CL_{int} (mL/min/kg) (Chan *et al.*, 2013)	CL_{int} (mL/min/kg) (Di *et al.*, 2012)
Dextromethorphan	Mid	0.0078	89	18.5	NA	NA
Diazepam	Low	0.0400	175	9.4	12.2	15.0
Diclofenac	High	0.0375	18	89.0	294.0	NA
Disopyramide	Low	0.0007	990	1.7	NA	4.8
Midazolam	High	0.0238	29	56.5	238.4	NA
Naloxone	High	0.0270	26	64.1	NA	NA
Phenacetin	High	0.0184	38	43.7	NA	NA
Repaglinide	High/Mid	0.0182	38	43.2	NA	NA
Theophylline	Low	ND	ND	ND	3.2	2.8
Timolol	Low	0.0019	365	4.5	NA	14.0
Tolbutamide	Low	0.0027	257	6.4	4.6	7.4
Verapamil	High	0.0189	37	44.9	NA	NA
S-Warfarin	Low	0.0014	495	3.3	3.6	4.2
Zolmitriptan	Low	0.0007	990	1.7	NA	3.5

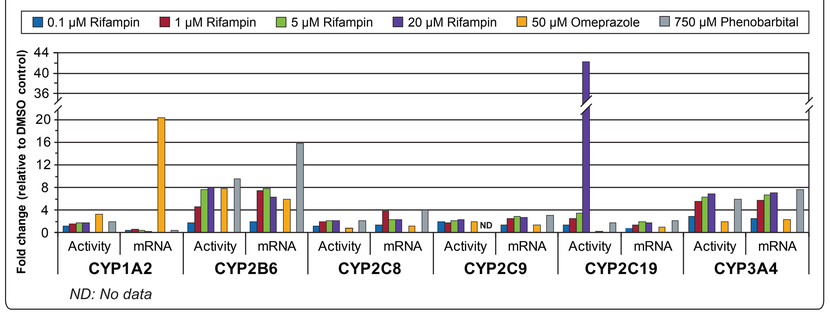
ND: No data NA: Not applicable

Table 3. Induction of CYP mRNA and enzymatic activities in pooled plateable hepatocytes by prototypical inducers

Inducer	CYP1A2		CYP2B6		CYP2C8		CYP2C9		CYP2C19		CYP3A4	
	Activity	mRNA	Activity	mRNA	Activity	mRNA	Activity	mRNA	Activity	mRNA	Activity	mRNA
20 µM Rifampin	1.8	0.3	8.0	6.4	2.2	2.3	2.3	2.7	42	1.8	6.9	7.1
50 µM Omeprazole	3.3	20	7.7	5.9	0.9	1.1	1.9	1.4	0.2	1.1	1.9	2.3
750 µM Phenobarbital	1.9	0.5	9.5	15.8	2.1	4.0	ND	3.0	1.7	2.1	6.0	7.7

= Prototypical inducer for given enzyme

Figure 2. Induction of CYP mRNA and enzymatic activity in pooled plateable hepatocytes by prototypical inducers



ND: No data

Table 4. Uptake transporter functionality assessment with pooled plateable human hepatocytes

Uptake Transporter	Marker Substrate	Rate (pmol/mg protein/min)	Uptake Ratio
OATP1B1	Estrone sulfate	1.21	4.5
OATP1B3	CCK-8	0.03	2.0
OCT1	MPP+	0.28	4.4
NTCP	TCA	0.31	3.3

Conclusions

- Enzyme activities of pooled plated hepatocytes are in good agreement with the values predicted based on the activities in cells from individual donors.
- Functional uptake transporter activity was demonstrated in pooled plated hepatocytes, establishing them as suitable for hepatic uptake studies.
- Pooled plated hepatocytes were as effective as other methods for evaluating turnover of low, mid and high clearance drugs.
- All inducible CYP enzymes evaluated were shown to be induced in the test system by prototypical inducers, demonstrating the utility of pooled plateable hepatocytes for CYP induction screening.
- Overall, pooled plateable hepatocytes represent a simple and cost effective test system for metabolic clearance, hepatic uptake and CYP induction screening.

References

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