**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 validity (typically ≤4 hours) making it challenging to determine parameters such as intrinsic clearance (CLint) and half-life (t1/2) of low turnover drugs. Alternative test systems, such as micro-patterned hepatocytes co-cultured with fibroblasts and multi-day release incubations of cryopreserved human hepatocytes, have been developed to provide a test system stability (Chan et al., 2010; D’Elia 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 sensitivity to cytoxocity. Hepatic uptake experiments are frequently conducted in pooled suspended hepatocytes with a key dependency on 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 pooled cryopreserved human hepatocytes pooled using a proprietary pooling process for metabolic clearance, CYP induction, and uptake transporter functionality experiments/assays.

**Materials & Methods**

**Chemicals**

Doxmethylorphan, DMSO, diazepam, diclofenac, disopyramide, HEPEs, midazolam, naloxone, omeprazole, phenacetin, phenobarbital, rifampin, S-warfarin, theophylline, timolol maleate, tolbutamide, Williams EL, verapamil and zolmitriptan were all purchased from Sigma-Aldrich (St. Louis, MO). GlutarMax 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 (Cryostat®X®, 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 10⁶ cells/well) 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), disopyramide (CYP2D6, 15 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% CO₂. Reactions were terminated by protein precipitation with one volume of acetonitrile containing internal standard, followed by centrifugation (10 min, 9200 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 Rifampin, 0.4, 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% CO₂. At the end of the incubation period, reactions were stopped by protein precipitation with the addition of one volume of acetonitrile containing internal standard (ternadine). Samples were then centrifuged for 10 min at 9200 RCF, followed by LC/MS/MS analysis. Data were processed with GraPhAT 7.0.2 (Erhus Software Ltd., Surrey, UK) using single-exponential fitting.

**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% CO₂) for 24 hours, followed by once daily treatment for 3 days with CM® 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, CYP2A6, CYP2B, CYP2C9, CYP2C19 and CYP3A4/45 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 buproprion, 200 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 an equal amount of acetonitrile containing internal standard, followed by centrifugation (10 min, 9200 RCF) and LC/MS/MS analysis. After substrate incubations, cells were harvested for RNA evaluation with Buffer RLT lysis to assess the mRNA expression of CYP1A2, CYP2A6, CYP2B, CYP2C9, CYP2C19, CYP2D6, 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). 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). The relative quantity of the target cDNA was compared with that of the control cDNA (GAPDH) which was determined by the 2⁻¹ΔCt method (Applied Biosystems User Bulletin #2). Relative quantitative changes in mRNA expression in a test sample relative to that in a control sample (e.g., 0.1% v/v DMSO).

**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 as activities shown in Table 1. For all enzyme activities, the pooled plateable hepatocytes were within 2-fold of the theoretical average of individual donor activities. The 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 plateable hepatocytes. As shown in Figure 1 and Table 2, clearance values were determined for all compounds except theophylline. The rank order of in vitro Clₘₐₓ was determined as follows: diclofenac > naloxone > midazolam > verapamil > phenacetin > rifampin > disopyramide > dextromethorphan > dexamethasone > tolbutamide > timolol > S-warfarin > disopyramide > zolmitriptan > theophylline. Results were consistent with their literate classification as high, medium or low clearance drugs. In comparison to literature values for other in vitro test systems, the pooled plateable hepatocytes had comparable Clₘₐₓ values for low, mid and high clearance drugs.**

**For CYP induction, prototypical inducers were tested with the pooled plateable 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), CYP2B (phenobarbital; 9.4-fold activity; 16-fold mRNA). Moderate induction of CYP2C8, CYP2C9 and CYP2C19 was also observed.**

**As shown in Figure 4 and Table 4, the uptake transporter activity of the pooled plateable hepatocytes was assessed using prototypical substrates for OATP1B1, OATP1B3 and NTCP. Uptake ratios were >2, demonstrating functional uptake transporter activity, suitable for hepatic uptake studies.**

**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 plateable hepatocytes, establishing them as suitable for hepatic uptake studies.
- Pooled plated hepatocytes were as effective as other methods for evaluating tumor 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**