Application of a low intrinsic clearance assay in preclinical drug discovery.

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Introduction

Progression of new chemical entities (NCEs) is a multi-parametric process involving a balance of *in vitro* and *in vivo* ADME, safety and potency properties. Early prediction of human pharmacokinetics (PK) is crucial to ensure efficient design and project progression and to give confidence in target engagement[1,2]. To accurately predict human PK and human efficacious dose, *in vitro* measurement of clearance (CL), is essential.

Low metabolic CL is often targeted to facilitate *in vivo* exposure and achieve appropriate half-life ($t_{1/2}$)[3]. Suspension primary human hepatocytes (PHHs) containing phase I and phase II metabolising enzymes have been successfully utilised in predictions of moderate and high CL compounds[4,5]. However, incubation times are limited (2-4 hours) due to declining levels of metabolising activity and an increase in cell mortality, hindering the limit of quantification[6].

The aims herein were to evaluate the application of a novel PHH media supplement, HepExtend™. HepExtend™ was compared to the widely used PHH maintenance medium (CM4000) and the necessity of an overlay for optimal performance was also evaluated.

Methods

Cells were incubated in a 24-well format for 5 days with one of the following culture medium:

1. CM4000
2. CM4000 + HepExtend™
3. CM4000 + Geltrex
4. CM4000 + HepExtend™ + Geltrex

Cells were incubated on day 1 and day 5 with 1 µM of compound in the culture medium. Samples were taken over 30 h to determine a CL_{int}. Depletion of parent compound was quantified using LC-MS/MS. Physiological scaling of *in vitro* CL_{int} was performed and the free fraction in the incubation was determined using the Kilford algorithm[7] to allow comparison to the *in vivo* human CL_{int}. A regression correction was subsequently applied.

Donor	Ethnicity	Gender	Age (y)	BMI (%)	Tobacco History	Alcohol History	Drug History	Medications
Hu1753	Caucasian	Female	43	22	Yes	Yes	None reported	Scopolamine patch (1.5 mg transdermal q 72 h prn)
Hu1824	Caucasian	Female	66	28	Yes	No	None reported	None reported
Hu8249	African American	Male	29	22	Yes	Yes	None reported	None reported

Table 1: Human donor demographics of the hepatocytes used in the study.

Results

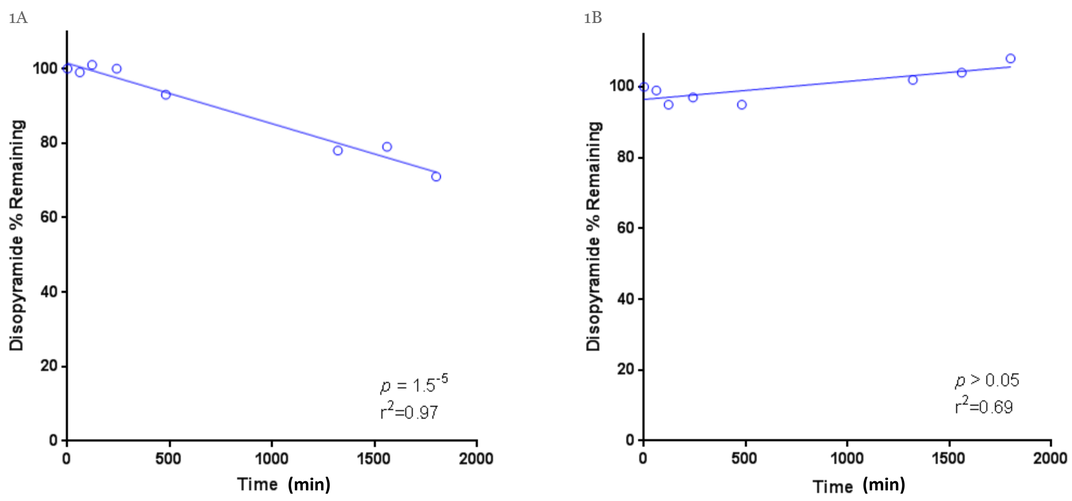

Figure 1. An example of a substrate depletion plot for disopyramide in Hu8249 treated with CM4000+Geltrex on day 1, with cells (A) and media alone (B).
Where, the CL_{int} for disopyramide with cells (A) was 0.19 µL/min/x10^6 cells, p = 1.5 x 10^{-5} and the CL_{int} for disopyramide with media only (B) was < 0.1 µL/min/x10^6 cells, p > 0.05.

- Robust and reproducible CL_{int} values of 0.1-0.2 µL/min/x10^6 cells were achieved for disopyramide; the stable control.
- Control incubations with no cells did not produce statistically significant CL_{int} values.

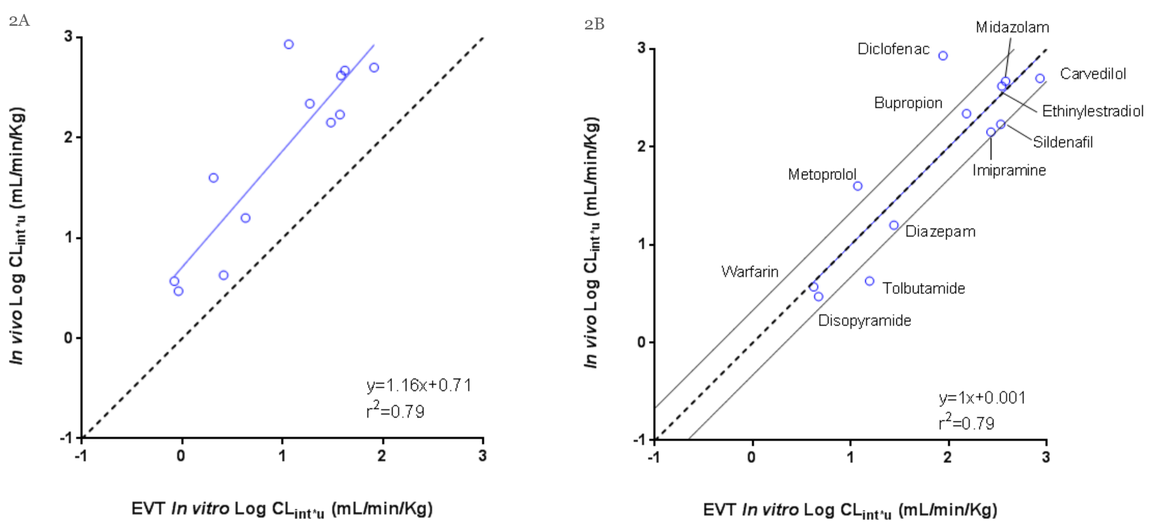

Figure 2: Correlation of scaled *in vitro* human intrinsic clearance with *in vivo* human intrinsic clearance for a set of 12 known drugs (Table 1) before a regression correction (A) and after a regression correction (B). A comparison of the *in vitro* and *in vivo* values was completed following the regression using Bland-Altman: bias 8x10^{-4}, 95% CI: -0.82-0.82.
Where, the solid lines represent a 2-fold offset.

Compound	Ion Class	Drug Metabolising Enzyme	Bonn *et al.*, 2016 PHH CL_{int} (µL/min/×10^6 cells)	Bonn *et al.*, 2016 Hµrel CL_{int} (µL/min/×10^6 cells)	Evotec CL_{int} (µL/min/×10^6 cells)
Bupropion	Base	CYP2B6, CYP1A2, CYP2A6, CYP3A4, CYP2E1	–	–	5.4
Carvedilol	Base	CYP2D6, CYP2C9	26.3	34.2	14.5
Diazepam	Neutral	CYP2C19, CYP3A4	0.8	1.3	0.7
Diclofenac	Acid	CYP2C9, UGT2B7	–	–	4.7
Disopyramide	Base	CYP3A4	0.2	0.4	0.1
Ethinylestradiol	Acid	UGT1A1, CYP3A4	–	–	3.3
Imipramine	Base	CYP1A2, CYP2C19, CYP2D6	8.6	1.7	8.5
Metoprolol	Base	CYP2D6, CYP3A4	2.2	0.8	0.9
Midazolam	Neutral	CYP3A4	–	–	5.1
Sildenafil	Base	CYP3A4, CYP2C9, CYP2C19	7.0	6.2	9.0
Tolbutamide	Acid	CYP2C9	–	–	0.8
Warfarin	Neutral	CYP2C9, CYP3A4	BLQ	0.7	0.3

Table 2: Compound information of those used in the regression correction.
Where, the CL_{int} data generated at Evotec is N=3 for Hu8249.

- The assay was robust and reproducible in providing statistically significant CL_{int} values at 0.1 µL/min/x10^6 cells over a 30 h period (p <0.05); lower than previously demonstrated.
- Human hepatic *in vivo* CL was predicted within 2-fold for 80% of compounds tested for three human donors, with an average fold error (AFE) of 2.2.
- This prediction accuracy was improved to 92% when an acceptance criteria of 4-fold was applied.
- The investigation demonstrated HepExtend™ was donor specific (N=3 human donors) in its abilities to retain enzyme activity.
- HepExtend™ was not detrimental for those donors where no advantage was observed.
- HepExtend™ and a Geltrex overlay were essential to maintain cell activity and viability out to 5 days.
- The morphology of the three human donors was further improved with the supplement.

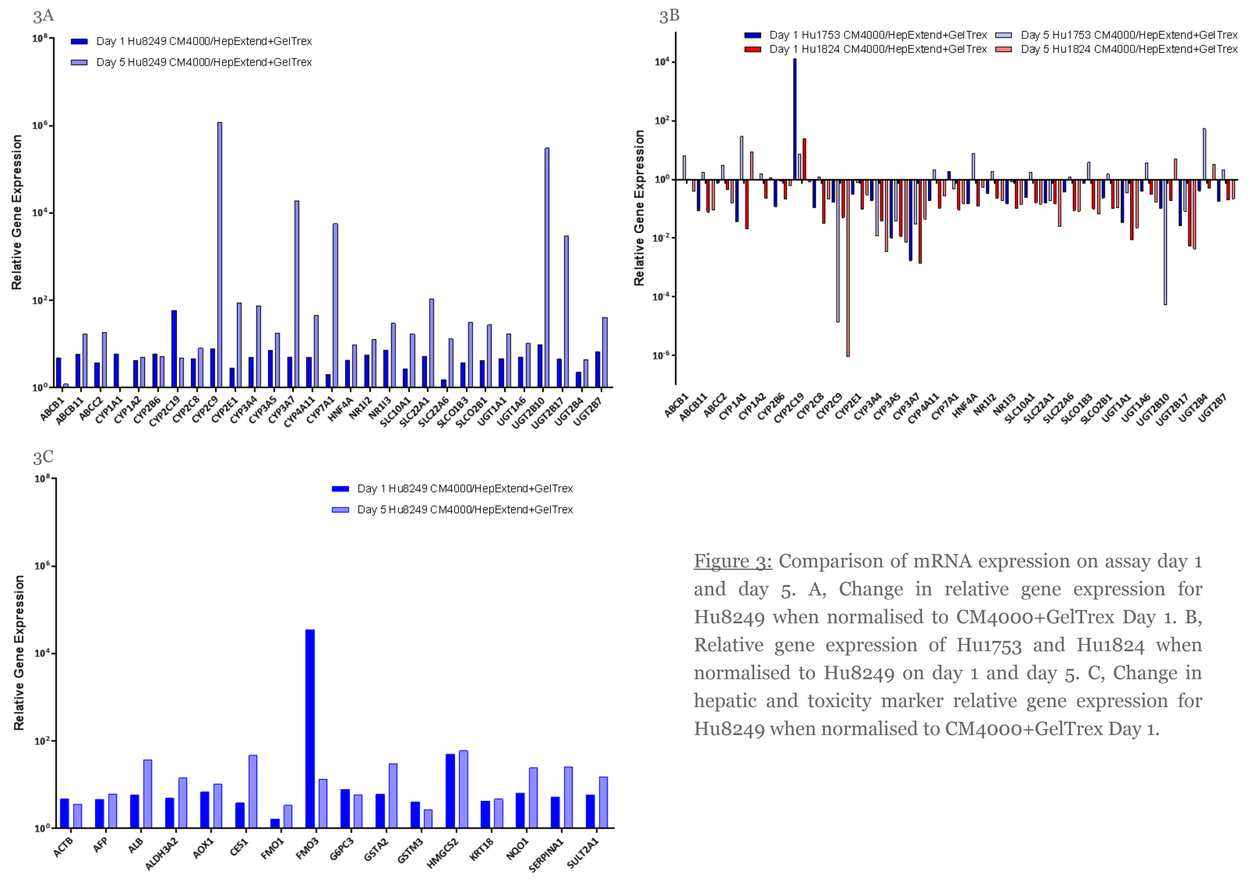

Figure 3: Comparison of mRNA expression on assay day 1 and day 5. A, Change in relative gene expression for Hu8249 when normalised to CM4000+GelTrex Day 1. B, Relative gene expression of Hu1753 and Hu1824 when normalised to Hu8249 on day 1 and day 5. C, Change in hepatic and toxicity marker relative gene expression for Hu8249 when normalised to CM4000+GelTrex Day 1.

- In comparison to CM4000±Geltrex, HepExtend™+Geltrex displayed a higher level of gene expression, particularly for the CYPs, nuclear receptors and UGTs.
- At day 5, the mRNA expression of the hepatic markers (e.g. β–Actin), toxicity markers (e.g. HMGCS2), transporters and UGTs were consistent with expression levels at day 1.

HTS Hepatocyte Assay	Low CL_{int} assay
Pooled hepatocytes (100 donors)	Individual donor
Regression correction	Regression correction
Suspension in 96-well plates	Plated in 24-well plates
No overlay	Matrix overlay (Geltrex)
Treatment immediately after thawing	Treatment 24 h post plating
6 time points over 2 h	8 time points over 30 h
Singlicate analysis	Duplicate analysis
LOQ 2.9 µL/min/x10^6 cells	LOQ 0.1 µL/min/x10^6 cells

Table 3: Differences between the high-throughput suspension assay and the low clearance plated assay.

Summary

The novel PHH medium, HepExtend™, with a matrix overlay offers significant improvement for determining CL_{int} values for compounds with low CL when compared to alternative approaches.
Robust and reproducible results obtained for CL_{int} down to 0.1 µL/min/×10^6 cells
The assay has been subsequently utilised for human PK predictions in preclinical drug discovery.

References
[1] Bonn *et al.*, 2016
[2] Grime *et al.*, 2013
[3] Di and Obach. 2015
[4] Riley *et al.*, 2005
[5] Grime and Riley. 2006
[7] Kilford *et al.*, 2008