Utilization and Optimization of Cryopreserved Human Hepatocytes as a Model to Assess CYP450 Inhibition

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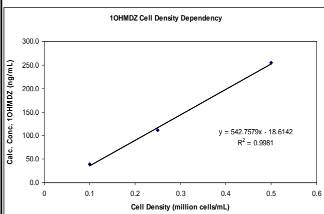
Introduction

Pharmacokinetic drug-drug interactions (DDIs) can occur when one drug alters the metabolism of a co-administered drug. To gain a better understanding of a drug's potential to cause pharmacokinetic-based DDIs via inhibition of drug metabolizing enzymes, a battery of *in vitro* screens can be employed to determine the extent of CYP inhibition. These assays typically use one or more of the following tools: recombinantly expressed CYPs (rCYPs) or human liver microsomes (HLM). Human primary hepatocytes, which provide an *in vitro* environment which more closely resembles that of the human liver by providing the full complement of Phase I and Phase II xenobiotic-metabolizing enzymes, have recently been suggested as a model to determine both reversible and time-dependent inhibition of CYPs. For this study we utilized cryopreserved human hepatocytes in suspension as a tool to assess reversible and time-dependent inhibition of CYP enzymes by isoform-specific inhibitors The inhibition potential of these compounds for the major drug metabolizing CYP enzymes (CYP1A2, phenacetin *O*-dealkylation; CYP2B6, bupropion hydroxylation; CYP2C8, paclitaxel 6α-hydroxylation; CYP2C9, tolbutamide hydroxylation; CYP2C19, (S)-mephenytoin 4'-hydroxylation; CYP2D6, dextromethorphan demethylation; CYP3A4, 6-hydroxylation; testosterone 6β-hydroxylation and midazolam 1'-hydroxylation) was assessed by measuring IC_{50} values for direct inhibition as well as K_I and k_{inact} values for time-dependent inhibition using known isoform-specific positive control inhibitors. Prior to determining the inhibition potential, the reactions conditions were optimized by assessing the linearity of time and cell density dependence and the K_m and V_{max} parameters for each of the isoforms were determined. These resulting kinetic parameters obtained are comparable to those determined for human liver microsomes and demonstrate that cryopreserved hepatocytes can be used for assessing both direct and time-dependent CYP inhibition potential by drug candidates.

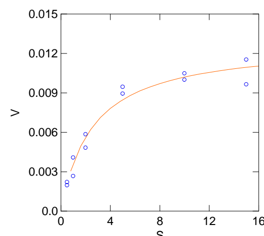
Figure 1 – Linearity assessment



Linearity of 1-hydroxymidazolam formation as a representative dataset for linearity assessment. Incubations for CYP3A4 (midazolam) were performed for 120 minutes at 37 °C

Linearity of metabolite formation from each of the probe substrates was assessed over a range of 10 to 120 minutes incubation and at cell concentrations of 0.1, 0.25 and 0.5 million cells per mL. Each probe substrate was incubated at 2 to 5 times the known K_m concentration in human liver microsomes. Incubations were started by the addition of hepatocytes and terminated by the addition of the appropriate stop solution containing internal standard. Metabolite formation was analysed using the HPLC-MS/MS methods from the CellzDirect validated human liver microsomal assays. Linear conditions were determined for each CYP isoform and all the HPLC-MS/MS methods were judged suitable for use.

Figure 2 – Kinetics assessment



Michaelis-Menten plot of 1-hydroxymidazolam formation as a representative dataset for the kinetics assessment. Incubations for CYP3A4 (midazolam) were performed for 120 minutes at 37 °C

Incubations for all isoforms assessed were performed under linear conditions for each probe substrate with hepatocyte concentrations of 0.1 – 0.5 million cells per mL and incubations times of 30 or 120 minutes. Variable concentrations of each probe substrate were used (Five (5) or six (6), depending on assay) and samples were terminated and analysed as described for the linearity assessment. Systat was used to perform the non-linear regression of the rates of metabolite formation at each substrate concentration. The kinetic parameters, K_m and V_{max}, were generated from the fit of the dataset.

Table 1 – Conditions used for incubations in cryopreserved human hepatocytes

Human CYP	Substrate	Substrate concentration (µM)	Cell concentration (million cells per mL)	Incubation time (minutes)
CYP1A2	Phenacetin	50	0.5	120
CYP2B6	Bupropion	60	0.1	120
CYP2C8	Paclitaxel	5	0.25	120
CYP2C9	Diclofenac	5	0.25	30
CYP2C19	(S)-mephenytoin	50	0.25	120
CYP2D6	Dextromethorphan	5	0.1	120
CYP3A4/5	Midazolam	5	0.1	120
CYP3A4/5	Testosterone	50	0.1	120

Incubations were performed under linear conditions at the the approximate Km concentration for each probe substrate. CellzDirect/Life Technologies cryopreserved human hepatocytes designated as Hu800, Hu8058, and Hu8066 were used in all incubations.

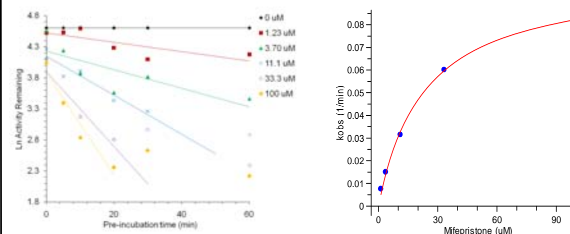
Table 2 – Comparison of kinetic parameters in cryopreserved human hepatocytes versus human liver microsomes

Human CYP	Substrate	K_m value in hepatocytes (µM)	K_m value in HLM (µM)
CYP1A2	Phenacetin	38	40
CYP2B6	Bupropion	11	56
CYP2C8	Paclitaxel	5.1	9.3
CYP2C9	Diclofenac	40	5.3
CYP2C19	(S)-mephenytoin	13	33
CYP2D6	Dextromethorphan	2.3	3.7
CYP3A4/5	Midazolam	2.6	2.8
CYP3A4/5	Testosterone	78	60

Table 3 – Percentage inhibition in cryopreserved hepatocytes when co-incubated with positive control inhibitors

Human CYP	Chemical inhibitor	Inhibitor concentration (µM)	% Inhibition
CYP1A2	Furafylline	5	42.2
CYP2B6	ThioTEPA	20	47.0
CYP2C8	Quercetin	10	11.9
CYP2C9	Sulfaphenazole	6.3	62.7
CYP2C19	Ticlopidine	1	73.2
CYP2D6	Quinidine	0.4	41.4
CYP3A4/5	Ketoconazole	0.1	58.4
CYP3A4/5	Ketoconazole	0.1	39.8

Figure 3 – Inhibition of CYP3A4/5 in cryopreserved hepatocytes by mifepristone



Inhibition parameters of K_I = 23.1 µM and k_{inact} = 0.101 min^{-1} were determined for the inhibition of CYP3A4/5 by mifepristone.

Table 4 – Percentage inhibition in cryopreserved hepatocytes when pre-incubated with positive control inhibitors

Human CYP	Chemical inhibitor	Inhibitor conc (µM)	% Inhibition
CYP1A2	Furafylline	1	70.7
CYP2B6	ThioTEPA	30	64.5
CYP2C8	Phenelzine	100	30.9
CYP2C9	Tienilic acid	3	41.7
CYP2C19	Ticlopidine	0.5	60.6
CYP2D6	MDMA	10	44.6
CYP3A4/5	Mifepristone	10	64.9
CYP3A4/5	Mifepristone	10	54.7

Results and Conclusions

- Hepatocytes can be a useful tool to determine both reversible and time-dependent inhibition of CYPs.
- The use of hepatocytes provides an *in vitro* environment which more closely resembles that of the human liver by providing the full complement of Phase I and Phase II xenobiotic-metabolizing enzymes.
- Assays for the identification of reversible and time-dependent inhibition of CYP 1A2, 2B6, 2C8, 2C9, 2C19, 2D6 and 3A4/5 were successfully developed and validated.
- The kinetic parameters determined for CYP 1A2, 2B6, 2C8, 2C9, 2C19, 2D6 and 3A4/5 in cryopreserved human hepatocytes were comparable to those determined for human liver microsomes.
- The utility of using hepatocytes was shown through the development and validation of a time-dependent assay for CYP3A4/5 using mifepristone as the positive control.