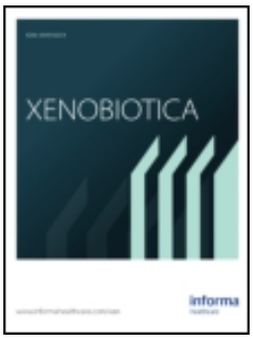

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RESEARCH ARTICLE

Comparison of intrinsic metabolic clearance in fresh and cryopreserved human hepatocytes

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Abstract

1. We compared the intrinsic clearance (CL_{int}) of a number of substrates in suspensions of fresh and cryopreserved human hepatocytes from seven donors.
2. CL_{int} values for a cocktail incubation of phenacetin, diclofenac, diazepam, bufuralol, midazolam, and hydroxycoumarin were 4.9 ± 3.4, 18 ± 7.2, 5.1 ± 4.9, 6.3 ± 3.3, 9.8 ± 5.8 and 22 ± 14 µl $min^{-1}/10^6$ cells, respectively, and they correlated well with corresponding CL_{int} values using cryopreserved hepatocytes from 25 different donors.
3. CL_{int} values of each cocktail substrate and 20 AstraZeneca new chemical entities were compared in fresh and cryopreserved hepatocytes from the same three donors. There was a statistically significant correlation between CL_{int} in fresh and cryopreserved hepatocytes for each of the three livers ($p < 0.002$) and the geometric mean of the ratio of fresh to cryopreserved CL_{int} values was 1.03.
4. In conclusion, the results add further support to the use of cryopreserved human hepatocytes as a screening model for the intrinsic clearance of new chemical entities.

Keywords: *Human hepatocytes; fresh; cryopreserved; intrinsic clearance; drug metabolism; drug development*

Introduction

The use of hepatocytes for *in vitro* studies of drug metabolism has increased in recent years. Hepatocytes have the advantage of cellular integrity (and thus a barrier between medium and intracellular enzymes), a full complement of enzyme systems, co-factors and membrane transport proteins (Brandon et al. 2003; Donato & Castell 2003; Hewitt et al. 2007). These *in vivo*-like properties add support to the use of hepatocytes to prediction *in vivo* hepatic clearance of xenobiotics (Houston 1994; McGinnity et al. 2004; Hallifax et al. 2008).

The limited availability of fresh human liver samples has driven the preference of cryopreserved hepatocytes as an alternative to freshly prepared hepatocytes. These cells are commercially available and commonly used for *in vitro* metabolic studies (Li et al. 1997, 1999). Several investigators have characterized cryopreserved rat and human hepatocytes with respect to metabolic capacities (Li et al. 1999; Naritomi et al. 2003; Floby et al. 2004; McGinnity et al. 2004) and showed cytochrome P450 (CYP) enzyme activities to be preserved after cryopreservation (Li et al. 1999; Naritomi et al. 2003; McGinnity et al. 2004). Phase 2 activities such as uridine diphosphate (UDP)-dependent glucuronosyl transferase (UGT) and sulfotransferases (SULTs) have also been shown to be retained in cryopreserved human hepatocytes, but the correlation with fresh hepatocytes was more scattered than for CYP enzymes (Li et al. 1999; McGinnity et al. 2004). Since this early comparison of drug-metabolizing enzymes, the methods of cryopreservation and thawing — the latter leading to the recovery of hepatocytes which could attach in culture — have been improved. The improvement

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in the ability to attach may also be mirrored in the metabolic function of these cells. Likewise, the presence of drug transporters, which play a role in the clearance of new chemical entities (NCEs), have been demonstrated to be preserved in cryopreserved rat hepatocytes (Houle et al. 2003). Despite these reports of favourable data using cryopreserved hepatocytes, there remains scepticism as to the routine use of these cells for screening of NCEs. Indeed, there is some evidence that cryopreserved human and rat hepatocytes have an impaired glutathione-conjugating capacity due to glutathione depletion in these cells (Sohlenius-Sternbeck & Schmidt 2005; Stevenson et al. 2007). With this in mind and considering the new cryopreservation and thawing techniques, we studied the use of cryopreserved human hepatocytes for the screening of stability of test compounds and compared them with data generated from fresh human hepatocytes.

In the present study, hepatocytes from seven donors were used to measure the intrinsic metabolic clearances (CL_{int} values) of six probe substrates for human drug-metabolizing enzymes. These substrates were phenacetin (CYP1A2), diclofenac (CYP2C9), diazepam (CYP2C19), bufuralol (CYP2D6), midazolam (CYP3A4/5), and 7-hydroxycoumarin (for glucuronidation and sulfation). The substrates were incubated as a cocktail and CL_{int} values were calculated by monitoring the depletion of the parent compounds. We have previously found this cocktail of probe substrates useful for the characterization of hepatocyte preparations since no interactions between the compounds occur either within the incubations or during analysis (Floby et al. 2004). We compared the mathematical mean CL_{int} values of each substrate using fresh hepatocytes with the corresponding values determined using single donor and pooled cryopreserved hepatocytes from 25 donors. In further studies, fresh and cryopreserved hepatocytes from the same donor were incubated with a total of 20 AstraZenenca NCEs.

Material and methods

Chemicals

Bufuralol was from Ultrafine Chemicals (Manchester, UK); diazepam, diclofenac, 7-hydroxycoumarin, midazolam, phenacetin, Krebs–Henseleit buffer, and Trypan blue were obtained from Sigma Chemical Co. (St Louis, MO, USA). AstraZeneca compounds were synthesized at AstraZeneca R&D Södertälje (Södertälje, Sweden). All other chemicals were of analytical grade and obtained from commercial suppliers.

Freshly isolated human hepatocytes

Fresh human hepatocytes from seven donors were supplied by CellzDirect, Inc. (see http://www.cellzdirect.com) and were isolated by a collagenase perfusion method (LeCluyse et al. 2005) (Durham, NC, USA). Hepatocyte viabilities were determined by Trypan blue exclusion and were greater than 75%. The donor demographics and hepatocyte metabolic characteristics (enzyme activities measured using K_m concentrations) are summarized in Table 1.

Cryopreserved hepatocytes from three donors

Hepatocytes from three of the donors (Hu 514, Hu 0587, and Hu 0832) were cryopreserved at CellzDirect and shipped to AstraZeneca in Södertälje, Sweden. Hepatocytes were thawed according to the supplier's instructions, subsequently incubated with the probe substrate cocktail and/or a total of 20 NCEs. The donor

Table 1. Characteristics of fresh human hepatocytes used in this study.

Donor	Sex of donor	Age of donor (years)	Hepatocyte viability (%)	ECOD activity (pmol $min^{-1}/10^6$ cells)[a]	7-OH coumarin sulphation activity (pmol $min^{-1}/10^6$ cells)	7-OH coumarin glucuronidation activity (pmol $min^{-1}/10^6$ cells)
Hu 8001 (Fresh)	Male	38	81	304	54	1000
Hu 0512 (Fresh)	Female	53	75	n.d.	n.d.	n.d.
Hu 0513 (Fresh)	Male	63	89	78	46	1041
Hu 0514 (Fresh)	Male	63	90	73	53	1324
(Cryo)			66	79	64	1583
Hu 0587 (Fresh)	Female	80	94	213	63	1918
(Cryo)			85	221	181	1437
Hu 8032 (Fresh)	Female	79	92	307	72	2936
(Cryo)			61	67	41	1528
Hu 0697 (Fresh)	Female	64	95	33	43	1596

Notes: [a]ECOD, 7-ethoxycoumarin *O*-deethylase. Enzyme activity measurements were performed at CellzDirect as follows: 0.5 million hepatocytes (1×10^6 cells ml^{-1}) were incubated for 30 min at 37°C with 100 μM of 7-ethoxycoumarin for ECOD activity or 100 μM of 7-hydroxycoumarin for sulfation and glucuronidation. Metabolites were quantified by high-performance liquid chromatography (HPLC) or liquid chromatography-tandem mass spectrometry (LC-MS/MS).
n.d., Not determined.

demographics and hepatocyte metabolic characteristics are summarized in Table 1.

Pools of cryopreserved hepatocytes

Cryopreserved hepatocytes, prepared according to Li et al. (1999), were from In Vitro Technologies, Inc. (Baltimore, MD, USA). Donor demographics and hepatocyte metabolic characteristics of the hepatocytes were available at http://www.invitrotech.com. Each vial was thawed according to the manufacturer's instructions. Hepatocyte viabilities (as determined by Trypan blue exclusion) were routinely approximately 80%. Incubation of the probe substrate cocktail with human hepatocytes was repeated five times with a mixed sex five-donor pool (that is, cryopreserved hepatocytes from individual donors had been thawed, pooled, and cryopreserved) and 31 times with cryopreserved hepatocytes from a total of 20 different individuals (ten males and ten females) where each experiment was performed with pools of hepatocytes from two to five individuals (that is, pooled after cryopreservation) in order to represent an average human donor pool.

Incubations of hepatocytes with the probe substrate cocktail and NCEs

The probe substrate stock solution consisted of 20 μM each of phenacetin, diclofenac, diazepam, bufuralol, midazolam, and 7-hydroxycoumarin in 1% dimethylsulphoxide (DMSO). The stock solution was stored in aliquots at –20°C until incubation. All incubations were performed in duplicates in 96-well deep microplates (Treff AG, Degersheim, Switzerland). The hepatocytes were diluted in Krebs–Henseleit buffer and 90 μl of the cell suspension was added to 10 μl of the cocktail stock solution to give a final hepatocyte density of 1×10^6 cells ml^{-1} and a final substrate concentration of 2 μM (final concentration of DMSO was 0.1%). Incubations were performed in duplicate at 37°C in an atmosphere of 5% CO_2/95% air, with gentle shaking, for 0, 15, 30, 45, 60 and 90 min. The reactions were stopped by the addition of 100 μl ice-cold acetonitrile. The plates were centrifuged (1900*g* for 5 min) and aliquots of supernatants were analysed by liquid chromatography-tandem mass spectrometry (LC-MS/MS).

Fresh and cryopreserved hepatocytes from Hu 0514, Hu 0587, and Hu 8032 were also incubated with a total of 20 AstraZeneca NCEs. The probe substrate cocktail was incubated with hepatocytes from all three donors, whereas each NCE was tested (as single incubations, that is, not as a cocktail) in hepatocytes from one donor only. The NCEs were dissolved in DMSO and diluted in Krebs–Henseleit buffer, pH 7.4, to give a concentration of 10 μM. The hepatocytes were incubated with each NCE at a concentration of 1 μM (final concentration of DMSO was 0.1%) in the same manner as that described for the cocktail.

LC-MS/MS analysis

LC-MS/MS analyses were performed with a Micromass Quattro Micro triple quadrupole (Micromass, Manchester, UK) coupled to two Shimadzu LC-10AD pumps working together as a binary pump (Shimadzu Corporation, Kyoto, Japan), a divert valve (Waters Corporation, Milford, USA) and a CTC HTS PAL autosampler (CTC Analytics, Zwingen, Switzerland) equipped with a 20 μl sample loop and a 100 μl syringe (SyrX G100, Hamilton Bonaduz AG, Bonaduz, Switzerland). The software MassLynx (which controls the LC system and the mass spectrometer), including QuanLynx (quantification) and QuanOptimize (MS/MS optimization), was used.

A HyPurity C-18, (3 μm, 2.1 × 30 mm, Thermo Electron Corporation, Waltham, USA) analytical column was used. Chromatography was performed using a generic gradient at a flow rate of 0.4 ml min^{-1}. The mobile phases consisted of A: 2:98 acetonitrile:0.1% acetic acid (v/v) and B: 80:20 acetonitrile:0.1% acetic acid (v/v). The gradient conditions were as follows: 0–0.2 min 0% B, 0.2–1.0 min 0–100% B, 1.0–2.5 min 100% B. The total time between injections was 3 min. The mobile phase was eluted to waste for the first 1.3 min, acquisition being performed between 1.3 and 2.5 min.

Electrospray ionization in both positive and negative mode (ESI+/ESI–) with multiple reaction monitoring (MRM) was used. The capillary voltage was set to 3 kV and the source block and desolvation temperatures were set at 150 and 350°C, respectively. The cone and desolvation gas flow rates were set to 20 and 910 $l \cdot h^{-1}$, respectively (high purity nitrogen). The analysers were set as follows: the low-mass and high-mass resolutions on both quadrupoles were set to 13.0 (approximately 0.9 amu Full Width at Half Maximum) with ion energies of 0.5 and 2.5 V. The collision entrance and exit lenses were –1 and 1 V, respectively. High-purity argon was used as a collision-induced dissociation (CID) gas. The CID gas was adjusted to give a collision cell pressure of approximately $4.5e^{-3}$ mBar.

MRM transitions were monitored as follows: diclofenac m/z 293.8→250.1, ESI–, cone voltage 19 V, collision energy 12 eV; 7-hydroxycoumarin m/z 160.9→133.1, ESI–, cone voltage 37 V, collision energy 19 eV; midazolam m/z 326.0→291.1, ESI+, cone voltage 46 V, collision energy 26 eV; diazepam m/z 285.1→154.1, ESI+, cone voltage 37 V, collision energy 26 eV; phenacetin m/z 180.1→110.1, ESI+, cone voltage 37 V, collision energy 19 eV; and bufarolol m/z 262.2→188.2, ESI+, cone voltage 19 V, collision energy 19 eV. The dwell time for each transition was 50 ms. QuanOptimize was used to obtain MRM transitions for all the NCEs.

Determination of intrinsic clearances

The concentration of each cocktail compound in the hepatocyte incubation was fitted to a first-order elimination equation:

$$C = C_0 \cdot e^{-k\triangle t}$$

where C is the measured concentration at any time; C_0 is the concentration at time zero; and k is the elimination constant. Intrinsic clearance was calculated as follows:

$$\mathrm{CL_{int}} = k \cdot V$$

where V is the volume of the hepatocyte suspension.

The calculations were performed by employing a Microsoft® Excel 2000-based standardized protocol and XLFit (version 2.1.2, Guildford, UK).

Data analysis

Data were analysed by linear regression analysis using Microsoft® Excel 2000. Means ± standard deviations (SDs) are given where appropriate.

The geometric mean for the comparison of $\mathrm{CL_{int}}$ values of compounds incubated in fresh and cryopreserved hepatocytes was calculates as follows:

$$\text{Geometric mean} = 10^{\left|\frac{1}{n}\log\Sigma\log\frac{\text{cryopreserved}}{\text{fresh}}\right|}$$

Results

Comparison of probe substrate CL_{int} in fresh and cryopreserved hepatocytes

There was a marked inter-individual variation within the seven donors tested in their capacity to metabolize the different probe substrates (Table 2). The average $\mathrm{CL_{int}}$ values for phenacetin, diclofenac, diazepam, bufuralol, midazolam, and hydroxycoumarin were 4.9 ± 3.4, 18 ± 7.2, 5.1 ± 4.9, 6.3 ± 3.3, 9.8 ± 5.8 and 22 ± 14 µl $\mathrm{min^{-1}}$ per 10^6 cells, respectively (Figure 1). Figure 1 also shows the comparison of $\mathrm{CL_{int}}$ values for each substrate with the corresponding average $\mathrm{CL_{int}}$ value obtained from incubating the same substrates in cryopreserved hepatocytes in a mixed sex five-donor pool (that is, cryopreserved hepatocytes from individual donors that had been thawed, pooled, and refrozen) and in cryopreserved hepatocytes from a total of 20 different donors. The mean $\mathrm{CL_{int}}$ values for the seven cocktail compounds obtained from the fresh human hepatocytes were in the same range as the corresponding cryopreserved values from the mixed sex five-donor pool and from the 20 individuals.

Comparison of CL_{int} of CYP substrates and NCEs with fresh and cryopreserved hepatocytes from the same donors

Fresh and cryopreserved hepatocytes from the same donor (Hu 0514, Hu 0587, and Hu 8032) were incubated with the CYP substrate cocktail and a total of 20 NCEs.

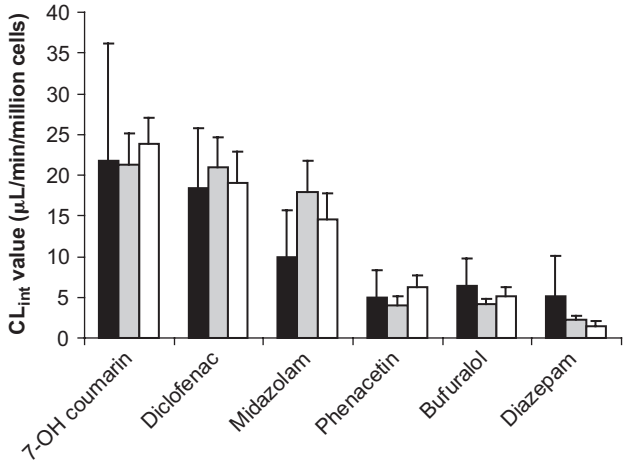

Figure 1. Mean intrinsic clearance for the cocktail compounds in suspensions of fresh human hepatocytes from seven donors (■) in a mixed sex five-donor pool (that is, cryopreserved hepatocytes from individual donors that had been thawed, pooled, and refrozen) (■), and in cryopreserved human hepatocytes from 20 different donors (□).

Table 2. $\mathrm{CL_{int}}$ values for cocktail substrates incubated with suspensions of fresh human hepatocytes from seven donors.

	$\mathrm{CL_{int}}$ (µl $\mathrm{min^{-1}}/10^6$ cells)						
	Donor						
Substrate	Hu 8001	Hu 512	Hu 513	Hu 514	Hu 0587	Hu 8032	Hu 0697
7-Hydroxycoumarin	42	28	16	31	n.d.	9.9	3.4
Diclofenac	10	18	17	22	29	24	9.1
Midazolam	2.9	18	12	16	5.2	10	4.8
Phenacetin	0.1	8.6	9.8	5.1	4.8	3.5	2.3
Bufuralol	12	8.3	4.6	8.8	2.7	3.9	4.1
Diazepam	0.6	11	8.7	11	0.4	1.4	2.8

Notes: Substrates were incubated as a cocktail (each at a final concentration of 2 µM) in hepatocytes from each donor. $\mathrm{CL_{int}}$ was measured from the depletion of parent compound over time.
n.d., Not determined.

The CYP cocktail was tested in hepatocytes from all three donors, whereas each NCE was tested in hepatocytes from one liver only. Figure 2 shows the correlation of CL_{int} values of compounds incubated with fresh and cryopreserved hepatocytes from the same donor. There was a statistically significant correlation between CL_{int} in fresh and cryopreserved hepatocytes for each of the three livers ($p = 2.4 \times 10^{-3}$, 3.3×10^{-5} and 3.4×10^{-4} for Hu 0514, Hu 0587, and Hu 8032, respectively). The highest correlation was achieved using hepatocytes incubated from donor Hu 0587 ($R^2 = 0.83$, compared with 0.75 and 0.64 for donors Hu 0514 and Hu 8032, respectively). The geometric mean of the ratio between CL_{int} in fresh and cryopreserved hepatocytes was 1.03.

Discussion

Hepatocytes are a valuable tool for *in vitro* drug metabolism research and more relevant to the *in vivo* environment because they contain a complete system of active metabolic enzymes with natural cellular localization of organelles and cofactors. Isolated hepatocytes are easy to handle and the cryopreservation procedure facilitates the use of hepatocytes at any time. Consequently, cryopreserved human hepatocytes are frequently used for the determination of the metabolic CL_{int} of new chemical entities (NCEs) in the pharmaceutical industry. A number of investigators have studied the drug-metabolizing capacity of cryopreserved rat and human hepatocytes (Li et al. 1999; Naritomi et al. 2003; Floby et al. 2004; McGinnity et al. 2004) and have shown that hepatocytes retain many cytochrome P450 (CYP) activities after cryopreservation (Li et al. 1999; Naritomi et al. 2003; McGinnity et al. 2004). Few have reported comparative enzyme activities in fresh and cryopreserved human hepatocytes from the same donor. In order for our laboratory to validate the use of cryopreserved human hepatocytes, we first had to show that the process of cryopreservation was not altering the metabolism of either prototypical substrates or, moreover, in-house AstraZeneca NCEs. To address this, the initial studies incorporated our cocktail method for the incubation of prototypical substrates for a number of CYPs, as well as UGTs and SULTs, to determine their $CL_{int.}$

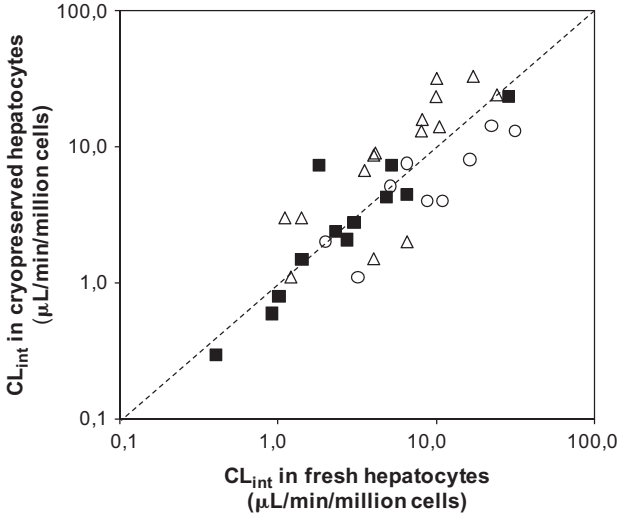

Figure 2. Comparison of CL_{int} values for 20 new chemical entities and prototypical cytochrome P450 (CYP) substrates in fresh and cryopreserved hepatocyte suspensions from donors Hu 0514 (○), Hu 0587 (■), and Hu 8032 (△).

As expected, there were notable inter-individual variations in the capacity of fresh human hepatocytes to metabolize different probe substrates. Likewise, this variation was also evident in the pools of cryopreserved hepatocytes. Differences in metabolism may be due, to a small extent, to different viabilities mainly due to a loss of cofactors through a leaky plasma membrane, rather than the drug-metabolizing enzymes themselves (Swales & Utesch 1998), but considering the high viability of these cells, the main reason for the differences is likely to be the well-known inter-individual variation in these enzymes. Moreover, the mean CL_{int} values obtained from cryopreserved human hepatocytes from 20 different donors were in the same range as those for obtained using fresh hepatocytes. Thus, these data support the use of cryopreserved human hepatocytes for CL_{int} determinations as an alternative to fresh hepatocytes, at least for the six CYP probe substrates tested. In addition, the CL_{int} values obtained from a mixed-sex five-donor pool were also in the same range as those values obtained using fresh hepatocytes. This demonstrates that the thawing and pooling of hepatocytes from individual donors and the subsequent refreezing of the obtained pool do not affect the metabolic capacities of the hepatocytes, at least not the activities tested with our cocktail. This also indicates that pre-pooled human hepatocytes from five donors can be used to represent an average population.

Fresh hepatocytes from three of the donors which were used for CL_{int} determinations were also cryopreserved and subsequently thawed and incubated with the substrate cocktail and with a total 20 AstraZeneca NCEs. The substrate cocktail was incubated with hepatocytes from all three donors, whereas each NCE was tested in hepatocytes from one liver only. Overall, these data support the use of cryopreserved human hepatocytes since CL_{int} values of NCEs were comparable in both fresh and cryopreserved hepatocytes from the same donor. The best correlation of CL_{int} values in fresh and cryopreserved hepatocytes was from donor Hu 0587. The correlation between CL_{int} values in fresh and cryopreserved hepatocytes from donors Hu 0514 and Hu 8032 were lower than donor Hu 0587, but this

was mainly due to one (Hu 0514) or two (Hu 8032) compounds for which the CL_{int} values were lower in cryopreserved hepatocytes compared with fresh cells. One explanation for the lower CL_{int} correlations for certain NCEs between fresh and cryopreserved hepatocytes may be differences in stability between different enzymes during cryopreservation. For example, glutathione conjugation capacity has been reported by some to be lower in cryopreserved hepatocytes than in fresh hepatocytes, due to glutathione depletion in these cells (Sohlenius-Sternbeck & Schmidt 2005; Stevenson et al. 2007). However, for Hu 8032, the two outliers are metabolized mainly by CYP3A4, CYP2D6 and CYP2C19, and the one outlier incubated with hepatocytes from donor Hu 0514 is metabolized mainly by CYP3A4/5 (unpublished data). However, considering the good correlation between fresh and cryopreserved hepatocyte clearance of the prototypical CYP substrates presented here and the known stability of these CYPs during cryopreservation, it is unlikely that the lower clearance in cryopreserved hepatocytes was due to selective loss of CYPs (Li et al. 1999). A more likely explanation for the observed outliers between fresh and cryopreserved hepatocytes is the donor-to-donor variation in their survival from the cryopreservation process. This is supported by the fact that hepatocytes from donor Hu 0587, which were the cells with the best correlation between fresh and cryopreserved CL_{int} values, also had the highest post-thaw viability, i.e. 85% compared with 66% and 61% for Hu 0514 and Hu 8032, respectively. We therefore recommend a minimum viability of 60% in order to avoid loss of enzyme activities. The importance of high viability and intact plasma membranes has also been noted by Li (2007).

Hepatocytes contain several drug transport proteins (Soars et al. 2007a, 2007b; Funk 2008) and it is possible that some of these proteins may be sensitive to cryopreservation, which may result in differences in CL_{int} between fresh and cryopreserved hepatocytes. It has, however, been demonstrated that certain drug transporter activities are preserved in cryopreserved hepatocytes (Houle et al. 2003; Li 2007). Further studies are necessary to reveal the full effect of cryopreservation on hepatic drug transport.

The use of cryopreserved human hepatocytes allows the testing of a compound in hepatocytes pooled from a large number of donors, potentially resembling an 'average' population. Experiments employing fresh hepatocytes are more costly and time consuming, since only hepatocytes from one donor can be tested at one time. By contrast, cryopreserved hepatocytes from a single donor or a selected pool of donors may be used on several occasions, allowing repeated studies. Clearly, fresh hepatocytes have the advantage of approximating the *in vivo* situation more closely than those which have been frozen. In our opinion, although the correlation between fresh and cryopreserved hepatocytes is not perfect, the determination of intrinsic metabolic clearances in fresh and cryopreserved hepatocytes is in many cases comparable. However, further comparative studies with fresh and cryopreserved hepatocytes are required in order fully to understand the applicability of cryopreserved hepatocytes for metabolic assays.

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