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Article *in* Drug Metabolism and Disposition: the Biological Fate of Chemicals · December 2004

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DRUG METABOLISM AND DISPOSITION
Vol. 32, No. 11
26/1178710
DMD 32:1247–1253, 2004
Printed in U.S.A.

EVALUATION OF FRESH AND CRYOPRESERVED HEPATOCYTES AS IN VITRO DRUG METABOLISM TOOLS FOR THE PREDICTION OF METABOLIC CLEARANCE

Dermot F. McGinnity, Matthew G. Soars, Richard A. Urbanowicz, and Robert J. Riley

Department of Physical & Metabolic Science, AstraZeneca R&D Charnwood, Leicestershire, United Kingdom

Received March 30, 2004; accepted July 30, 2004

ABSTRACT:

The intrinsic clearances (CL_{int}) of 50 neutral and basic marketed drugs were determined in fresh human hepatocytes and the data used to predict human in vivo hepatic metabolic clearance (CL_{met}). A statistically significant correlation between scaled CL_{met} and actual CL_{met} was observed ($r^2 = 0.48$, $p < 0.05$), and for 73% of the drugs studied, scaled clearances were within 2-fold of the actual clearance. These data have shown that CL_{int} data generated in human hepatocytes can be used to provide estimates of human hepatic CL_{met} for both phase I and phase II processes. In addition, the utility of commercial and in-house cryopreserved hepatocytes was assessed by comparing with data derived from fresh cells. A set of 14 drugs metabolized by the major human cytochromes P450 (P450s) (CYP1A2, 2C9, 2C19, 2D6, and 3A4) and uridine diphosphate glucuronosyltransferases (UGT1A1, 1A4, 1A9, and 2B7) have been used to characterize the activity of freshly isolated and cryopreserved human and dog hepatocytes. The cryopreserved human and dog cells retained on average 94% and 81%, respectively, of the CL_{int} determined in fresh cells. Cryopreserved hepatocytes retain their full activity for more than 1 year in liquid N_2 and are thus a flexible resource of hepatocytes for in vitro assays. In summary, this laboratory has successfully cryopreserved human and dog hepatocytes as assessed by the turnover of prototypic P450 and UGT substrates, and both fresh and cryopreserved human hepatocytes may be used for the prediction of human hepatic CL_{met}.

Most orally administered drugs are cleared primarily by the liver, and an accurate prediction of hepatic metabolism and an assessment of the potential first-pass clearance effect are paramount early in the drug discovery process. These processes are of importance both for the evaluation and optimization of potential candidate drugs and for the estimation of early human doses and exposures in the clinic. The ability to predict confidently human clearance values from human in vitro assays would therefore represent a major breakthrough in drug discovery.

Over the last 20 years there have been sporadic reports of the use of rat hepatic tissue in vitro to predict successfully rat CL in vivo (Houston, 1994a; Rane et al., 1977; Shibata et al., 2000). Various approaches to predicting human clearance have also been suggested, including allometric scaling, which has proved somewhat useful for renally excreted drugs (Mahmood, 1998), but less so for those cleared metabolically (Mahmood and Balian, 1999). Others have evaluated the use of animal and human hepatocytes together with animal in vivo data (Lave et al., 1997a, 1999). However, perhaps the most success for compounds that are eliminated primarily via hepatic metabolism has been achieved with in vitro human systems such as human liver microsomes (HLMs) (Hoener, 1994; Obach et al., 1997) and hepatocytes (Schneider et al., 1999; Zuegge et al., 2001; Lau et al., 2002; Bachmann et al., 2003).

Since most (~60%) marketed compounds are cleared metabolically by cytochrome P450 (P450) enzymes, the major activity in this area has focused on this family of enzymes (Bertz and Granneman, 1997). The now routine access to recombinant P450s and HLMs have made these in vitro tools highly valuable for determining the extent and route of the metabolism of new chemical entities and in screening for inhibitors of drug-metabolizing enzymes (Ayrton et al., 1998; Moody et al., 1999; McGinnity et al., 2000).

Although human hepatocytes are less readily available than either recombinant P450s or HLMs, they represent the most integrated in vitro experimental milieu containing the full complement of enzymes a compound is likely to encounter during first-pass hepatic metabolism. To evaluate human hepatocytes in predicting human in vivo metabolic clearance (CL_{met}), the intrinsic clearances (CL_{int}) of neutral and basic marketed drugs with a range of physicochemical properties and CL rates were determined. For the majority of the 50 drugs chosen, the primary clearance mechanism in human is metabolism via P450 and/or uridine diphosphate glucuronosyltransferase (UGT) catalysis. Substrates for all five major human hepatic P450 isoforms (CYP1A2, 2C9, 2C19, 2D6, and 3A4) and the major hepatic human UGT isoforms (UGT1A1, 1A4, 1A9, and 2B7) were represented.

Availability of good quality fresh liver tissue can limit human hepatocyte experiments in a drug discovery environment. The promise of hepatocytes becoming "off-the-shelf" reagents has been touted for several years via the advent of cryopreservation technology (Li, 1999; Lau et al., 2002), and indeed, commercial sources of cryopreserved human hepatocytes are available. Data from several laboratories have shown that P450 activities can be maintained in rat, dog, cynomolgus monkey, and human cryopreserved hepatocytes (Swales and Utesch,

Article, publication date, and citation information can be found at http://dmd.aspetjournals.org.
doi:10.1124/dmd.104.000026.

ABBREVIATIONS: CL, clearance; P450, cytochrome P450; UGT, uridine diphosphate glucuronosyltransferase; HLM, human liver microsome; CL_{int}, intrinsic clearance; CL_{met}, metabolic clearance; fu_{inc}, unbound fraction in incubation; fu_p, unbound fraction in plasma; BSA, bovine serum albumin; FBS, fetal bovine serum; COD, cause of death; HPLC, high-performance liquid chromatography.

1998; Silva et al., 1999; Steinberg et al., 1999; Hewitt et al., 2000, 2001).

The aims of this paper were, first, to evaluate the power of predicting in vivo CL_{met} of drugs via CL_{int} determined in human hepatocytes. Second, the usefulness of a commercial source of cryopreserved hepatocytes was assessed by comparing with data derived from fresh cells. This paper also describes this laboratory's efforts in cryopreserving hepatocytes from human and dog and compares CL_{int} estimates for a range of P450 and UGT substrates before and after cryopreservation.

Materials and Methods

Chemicals. The substrates acebutolol, (±)-atenolol, bepridil, bromocriptine, caffeine, carbamazepine, clozapine, cimetidine, codeine, cyclosporin A, desipramine, diazepam, diclofenac, diltiazem, dextromethorphan, diphenhydramine, doxepin, ethinylestradiol, famotidine, fluoxetine, gemfibrozil, imipramine, ketoprofen, lorazepam, (±)-metoprolol, midazolam, morphine, nadolol, naloxone, naproxen, nifedipine, nitrendipine, pindolol, pirenzipine, prazosin, propofol, (±)-propranolol, ranitidine, (−)-scopolamine, temazepam, triazolam, triprolidine, verapamil, β-nicotinamide adenine dinucleotide phosphate reduced form, Percoll (colloidal polyvinylpyrrolidone-coated silica), bovine serum albumin (BSA), and human serum albumin were purchased from Sigma-Aldrich (Gillingham, UK) and were of the highest grade available. Betaxolol and bisoprolol were purchased from Tocris Cookson Inc. (Bristol, UK). Carvedilol, cetirizine, chlorpheniramine, granisetron, isradipine, ondansetron, ritonavir, and zileuton were purchased from Sequoia Research Products Ltd. (Oxford, UK). Omeprazole was synthesized at AstraZeneca R&D Charnwood. Waymouth's MB 752/1 medium and fetal bovine serum (FBS) were purchased from Invitrogen (Paisley, UK).

Preparation of Dog Hepatocytes. Isolation of dog hepatocytes was performed essentially using a two-step in situ collagenase perfusion method. Briefly, the hepatic portal vein of an anesthetized male Beagle dog (weight range 12–18 kg) was cannulated just above the junction of the splenic and pyloric veins. Liver perfusion medium (Invitrogen) was perfused via the hepatic portal vein until the liver cleared to an even tan color. Liver digestion medium (Invitrogen) was then perfused until the liver displayed evidence of extensive dissociation. The liver was dissected from the dog and cells were gently teased out of the liver capsule into a beaker containing ice-cold hepatocyte suspension buffer [2.2 g of $NaHCO_3$, 2.34 g of Na HEPES, 2 g of BSA, 1 liter powder equivalent of Dulbecco's modified Eagle's medium (Sigma-Aldrich) diluted in 1 liter of water and adjusted to pH 7.4 with 1 M HCl]. The cell suspension was passed through a 250-μm mesh into a precooled tube and centrifuged at 40*g* for 5 min at 4°C. The supernatant was decanted, the cell pellet was resuspended in suspension buffer containing no BSA, and the centrifugation step was repeated. The resulting pellet of cells was resuspended in 200 ml of suspension buffer (without BSA), and an estimation of hepatocyte yield and viability was obtained using the trypan blue dye exclusion method.

Preparation of Human Hepatocytes. Human hepatocytes were prepared from an isolated lobe of human liver (obtained from local hospitals with ethical approval). The cold ischemia time was 10 min and the warm ischemia time was 5 min. Sections from both the left and right lobes were used. Perfusion was essentially the same as above except that an excised, rather than in situ, lobe of liver was perfused. Isolation of hepatocytes was also performed as above except that BSA was replaced with human serum albumin in the initial hepatocyte suspension buffer. The donor demographics for the five hepatocyte donors that were subsequently cryopreserved were as follows: four white men aged 59 to 71 years, via surgical resection, medical history not available; and one mixed race female aged 25, cause of death (COD) follicular nodular hyperplasia, nonsmoker, used salbutamol.

Cryopreservation of Dog and Human Hepatocytes. Before the cryopreservation of hepatocytes, cryopreservation medium A (65% Waymouth's, v/v; 20% FBS, v/v, 15% dimethyl sulfoxide, v/v) and medium B (60% Waymouth's, v/v, 20% FBS, v/v, 20% dimethyl sulfoxide, v/v) were prepared and stored at 4°C.

Freshly isolated hepatocytes were diluted to a cell concentration of approximately 8 million/ml using hepatocyte suspension buffer without added albumin, and dog hepatocytes only were incubated at 37°C for 30 min. Cells were centrifuged at 40*g* for 5 min at 4°C, the supernatant was removed by aspiration, and the pellet was resuspended initially in 2 ml of chilled Waymouth's medium followed by the addition of another 43 ml. The mixture was centrifuged at 40*g* for 5 min at 4°C, 15 ml of the resultant supernatant was removed, and the hepatocytes were resuspended in the remaining supernatant. Chilled cryopreservation medium A (15 ml) was added drop-wise to the cells in 5-ml aliquots, ensuring an even dispersal by inversion after the addition of each aliquot. The cells were centrifuged at 40*g* for 5 min, and 15 ml of the resultant supernatant was aspirated and replaced with 15 ml of chilled cryopreservation medium B, which was added drop-wise in 5-ml aliquots. The concentration and viability of the hepatocytes were determined using trypan blue exclusion, and the cells were resuspended at a concentration of 7 million cells/ml. Then, 1.5 ml of the resultant mixture was aliquoted into cryovials (giving 10 million cells per vial), which were transferred into Mr Frosty isopropanol containers (Nalgene; Nalge Nunc International, Rochester, NY) and stored at −80°C (freezing rate −1°C/min) for 12 h. Liquid nitrogen was used for the long-term storage of cryopreserved cells at −196°C.

Thawing of Cryopreserved Cells. Aliquots (20 ml) of hepatocyte suspension buffer (with no added albumin) were prewarmed to 37°C. Cryopreserved cells were removed from liquid N_2 and immediately immersed in a water bath that had been preheated to 37°C. The vials were shaken gently until the contents were completely free of ice crystals (approximately 90–120 s) and were then emptied into the prewarmed hepatocyte suspension buffer. The cells were centrifuged at 40*g* for 5 min at 19°C, the supernatant was removed by aspiration, and the resultant pellet was suspended in hepatocyte suspension buffer. The concentration and viability of the hepatocytes were determined using trypan blue exclusion, and the cells were resuspended at a concentration of 2 million cells/ml.

Commercial cryopreserved human hepatocytes were purchased from In Vitro Technologies, Inc. (Baltimore, MD). Six donors were purchased: DQ (lot 70), 57 years, white male (COD, cerebrovascular accident; used tobacco, alcohol, and tetrahydrocannabinol; medical history of hypertension); EB (lots 73 and 78), 50 years, white male [COD, head trauma; used tobacco, did not use alcohol or other substances; medical history of schizophrenia (antipsychotic medications]; EC (lot 75) 1.25 years, white male (COD, head trauma; did not use tobacco, alcohol, or other substances; medical history, eye surgery); DX (lot 76), 57 years, white male (COD, cerebrovascular accident; used tobacco, did not use alcohol or other substances; medical history of cardiac defibrillator implant); EA (lot 77), 58 years, white male (COD, intracranial hemorrhage; smoker, used alcohol, did not use other substances; medical history of depression, hypertension, and cardiac blockage). These cells were thawed using the same protocol.

Determination of CL_{int} Estimates using Fresh and Cryopreserved Hepatocytes. Drug stocks were prepared in dimethyl sulfoxide at 100-fold incubation concentration (100–300 μM). Of this ×100 stock, 10 μl were added to a vial containing 490 μl of hepatocyte suspension buffer. A 7-ml glass bijou vial containing 250 μl of hepatocytes at a concentration of 2 million cells/ml was preincubated for 5 min in a shaking (80 oscillations/min) water bath at 37°C along with the vial containing the drug/buffer mix. Reactions were started by adding 250 μl of drug/buffer mix to the 250 μl of hepatocytes, giving a final substrate concentration of between 1 and 3 μM, chosen to be below the K_m for most substrates but still demonstrating sufficient analytical sensitivity. To minimize the known P450-inhibitory effects of dimethyl sulfoxide, the final concentration in all incubations was 1% (v/v), which mimics the incubation conditions used in a typical drug discovery program to counter solubility issues. Then, 50-μl aliquots were removed at 5, 10, 20, 40, 60, and 90 min, ensuring adequate mixing, and samples were quenched in 100 μl of ice-cold methanol. Samples were subsequently frozen for 1 h at −20°C and then centrifuged at 3500 rpm for 15 min at 4°C. The supernatants were removed and transferred into HPLC vials and analyzed as described below. Assays were performed in triplicate where possible, depending on cell availability. Since data were collected over several years, all compounds were not incubated in the same livers.

Analytical Methods. Aliquots (20 μl) were analyzed by either HPLC-UV, HPLC-fluorescence, HPLC-mass spectroscopy, or HPLC-tandem mass spectroscopy for parent loss. All HPLC-UV or fluorescence was carried out using a Hewlett Packard 1100 Chemstation and a Hewlett Packard 1046A fluorescence detector (Hewlett Packard, Palo Alto, CA). HPLC-mass spectroscopy

was conducted using a Micromass ZMD single-quadrupole mass spectrometer with an Alliance HT Waters 2790 HPLC system (Waters, Watford, UK) for separation. Electrospray ionization was used for all mass spectrometry methods. Further mass spectrometry was conducted on a Micromass Quattro Ultima triple quadrupole by using an Alliance HT Waters 2790 HPLC system for separation. Analysis was by multiple reaction monitoring, and conditions were optimized for each compound. A symmetry shield RP8 3.9 × 50 mm cartridge (Waters) and a mobile phase of 0.025% (w/v) ammonium acetate and methanol were used for the chromatography of most analytes. The flow rate for all methods was 1.5 ml · min^{-1} and typically used a 5-min linear gradient from 20% to 80% methanol.

Data Analysis. CL_{int} estimates were determined using the rate of parent disappearance (at a single, low substrate concentration) described by Obach (1999). Dose/C_0 gives a term for the volume of the incubation (expressed in milliliters per 10^6 cells). The slope of the linear regression from log [substrate] versus time plot ($-k$) was determined and since elimination rate constant $k = 0.693/t_{1/2}$, an equation expressing CL_{int} in terms of $t_{1/2}$ of parent loss can be derived: CL_{int} = volume × $0.693/t_{1/2}$. The vast majority of the substrates demonstrated monoexponential parent loss, thus negating the usefulness of determining CL_{int} via area under the curve of the [S] versus time.

Hepatocyte CL_{int} (units, μl/min/10^6 cells) was scaled to in vivo CL_{int} (units, ml/min/kg) using the physiological parameters, human liver weight 22 g/kg body weight and hepatocellularity 120 × 10^6 cells/g of liver (Soars et al., 2002). Projection of human in vivo clearance was made using an adapted version of the nonrestrictive well stirred model (Houston, 1994b): $CL_{met} = (CL_{int} \times Q_h)/(CL_{int} + Q_h)$, where Q_h is hepatic blood flow (human Q_h = 20 ml/min/kg). An estimate of in vivo CL_{int} from observed CL_{met} can be made rearranging the well stirred model equation. No correction factor was made for any differential in vitro and in vivo binding, and the distribution of drug between the plasma and blood was assumed to be unity.

Results

Prediction of Human Metabolic CL of Neutrals and Bases using Human Hepatocytes. Table 1 shows the mean CL_{int} determined for each of the neutral, basic, or zwitterionic compounds using human hepatocytes, scaled CL_{met}, and measured CL_{met} determined from human studies. CL_{int} was scaled using the nonrestrictive well stirred model as described under *Materials and Methods*. No metabolism was detectable (defined as <1 μl/min/10^6 cells) using three or more different donors for five compounds: atenolol, pirenzipine, cetirizine, nadolol, and famotidine. For these compounds, scaled CL_{met} was consistent with the observed CL_{met} (<2.3 ml/min/kg for both). These data were excluded from Fig. 1A, which shows a correlation between scaled CL_{met} and observed CL_{met} ($r^2 = 0.48$, $p < 0.05$) for the remaining 45 compounds where turnover in human hepatocytes was determined. For 33 of the 45 drugs (73%) studied, scaled clearances were within 2-fold of the actual clearance. Figure 1B shows scaled versus observed in vivo CL_{int} where dashed lines signify 2-fold error between predicted and actual values. For 24 of 44 (55%) compounds, scaled CL_{int} was within 2-fold of the observed in vivo CL_{int}. For 6 of 44 (14%) compounds, CL_{int} was >2-fold under-predicted, whereas for 14 of 44 (32%), CL_{int} was >2-fold over-predicted. For this analysis CL_{int} was not corrected for unbound fraction in the incubation (fu_{inc}) or plasma (fu_p).

Commercial Cryopreserved Human Hepatocytes. The metabolic competence of commercially available cryopreserved hepatocytes was assessed. CL_{int} estimates were generated using the P450 substrates dextromethorphan, propranol, diltiazem, and verapamil, and one AstraZeneca proprietary compound known to undergo *O*-glucuronidation (AR-C1). The data from several individual cryopreserved donors were compared with the mean data using fresh hepatocytes from several donors. Under the same incubation conditions, the CL_{int} estimates generated from cryopreserved donor EC were compared with the mean CL_{int} generated from fresh human hepatocyte donors (a subset of donors reported in Table 1). Dextromethorphan (mean CL_{int} ± S.D. from fresh hepatocytes was 5.8 ± 4.8 μl/min/10^6 cells versus CL_{int} ± S.D. using cryopreserved hepatocytes of 4.6 ± 3.6 μl/min/10^6 cells), propranolol (7.8 ± 4.0 versus 5.6 ± 1.8), diltiazem (6.0 ± 2.7 versus 5.5 ± 2.2), verapamil (16.2 ± 6.0 versus 15.2 ± 4.7), and AR-C1 (30 ± 12 versus 32 ± 13).

Cryopreservation and Thawing of Human and Dog Hepatocytes. The cryopreservation and thawing of human and dog hepatocytes are detailed under *Materials and Methods*. Cryopreserving essentially entails slowly increasing the amount of dimethyl sulfoxide to cells through a series of centrifugations. The subsequent suspension is frozen gradually at a rate of −1°C/min and the cells are stored at −196°C. For use, cells are thawed rapidly, and the cryopreservant (dimethyl sulfoxide) is diluted in hepatocyte suspension buffer maintained at 37°C.

Experience suggests that the quality and viability of fresh hepatocytes are critical for successful cryopreservation. For both dog and human batches, the higher the viability of the fresh cells, the better the quality of the thawed cryopreserved cells. Hepatocyte recovery/viability was also sensitive to the speed of centrifugation during the cryopreservation procedure. Initial data suggested that whereas good recovery of cells was obtained using centrifugation at 50g, the cell viability decreased up to 10%. However, no such loss in cell viability was observed when centrifugation speeds of 40g or less were used.

Hepatocytes are classified as live cells by their ability to exclude trypan blue dye. In this laboratory, typical hepatocyte CL_{int} incubations use 1 million live cells/ml. Laboratories generally accept hepatocytes for experimentation when viability is >75%. Percoll has often been used for cell separation and to increase viability by separating live from dead cells (Lloyd et al., 2003). Table 2 shows the effect of a range of Percoll treatments on the percentage viability and recovery of cryopreserved hepatocytes from three individual human and dog donors. In general, the gain in cell viability was less with decreasing Percoll concentrations. A 25% Percoll treatment increased dog hepatocyte viability from 52 ± 1 to 78 ± 8% and human hepatocyte viability from 66 ± 2 to 88 ± 3%, and was only associated with a minor decrease in recovery. Percoll concentrations above 25% led to an unacceptable loss of cells in both species.

Characterization of Human and Dog Cryopreserved Hepatocytes. A set of 14 markers metabolized by the major human P450s (CYP1A2, 2C9, 2C19, 2D6, and 3A4) and UGTs (1A1, 1A4, 1A9, and 2B7) have been used to characterize the activity of freshly isolated and cryopreserved human and dog hepatocytes. The major enzymes responsible for human metabolism are as follows: caffeine, CYP1A2; phenytoin, CYP2C9; tolbutamide, CYP2C9; diazepam, CYP2C19 > 3A; dextromethorphan, CYP2D6 > 3A/2C19; metoprolol, CYP2D6; diltiazem, CYP3A; midazolam, CYP3A; verapamil, CYP3A; ketoprofen, UGT1A9/2B7; furosemide, UGT1A9/2B7; diclofenac, CYP2C9 > 3A/UGT2B7; propranolol, CYP2D6 > 1A2/2C19/UGT; imipramine, CYP2D6/1A2/2C19/3A/UGT1A4; ethinylestradiol, UGT1A1 > CYP3A; naproxen, UGT2B7 > CYP2C9; gemfibrozil, UGT1A9/2B7; and propofol, UGT1A9. For each of these substrates, CL_{int} generated in fresh hepatocytes was compared with data from the respective donor after cryopreservation.

Figure 2A shows the relationship ($r^2 = 0.99$, $p < 0.001$) between the metabolic activity of the marker compounds before and after cryopreservation of five human hepatocyte donors. Figure 2B shows the relationship ($r^2 = 0.95$, $p < 0.001$) between the metabolic activity of the marker compounds before and after cryopreservation of six dog hepatocyte donors. Cryopreserved human and dog cells retained on average 94% and 81%, respectively, of the CL_{int} determined in fresh cells. As well as the comparable rates of metabolism between fresh

TABLE 1

Prediction of metabolic clearance from human hepatocyte CL_{int}

Results are expressed as the mean CL_{int} ± standard deviation from n >3 donors. Results not expressing a S.D. are from <3 donors. The CL_{int} determinations were carried out as described under *Materials and Methods*. All substrates were not incubated in the same donor livers. Assays were performed in triplicate. Compounds are classified as neutrals (N), bases (B), or zwitterions (Z).

Compound	N/B/Z	Donors	Human Hepatic CL_{int}	Scaled CL_{int}	Scaled CL_{met}	Observed CL_{met} [a]	Observed in Vivo CL_{int}	Enzymes Responsible for Metabolism
			μl/min/10⁶ cells			*ml/min/kg*		
Bromocriptine	N	1	37	98	17	15	60	CYP3A4
Caffeine	N	1	3.3	8.7	6.1	2.1	2.3	CYP1A2
Carbamazepine	N	1	2.0	5.3	4.2	0.4	0.4	CYP3A4 > 2C8/9
Cimetidine	N	9	1.2 ± 0.4	3.2	2.7	3.2	3.8	P450
Cyclosporin A	N	8	3.5 ± 1.5	9.2	6.3	4.7	6.1	CYP3A4
Diazepam	N	1	0.3	0.8	0.8	0.4	0.4	CYP2C19 > 3A
Ethinylestradiol	N	5	7.0 ± 2.0	19	9.6	5.4	7.4	UGT1A1>CYP3A4
Famotidine	N	6	<1.0		<2.3	2.1	2.3	P450 (minor)
Isradipine	N	1	18	47	14	8	13	CYP3A4
Lorazepam	N	1	1.0	2.6	2.3	1	1.1	UGT
Midazolam	N	5	14 ± 8.0	37	13	6.6	9.9	CYP3A4
Nifedipine	N	5	5.6 ± 1.5	15	8.5	8.5	15	CYP3A4
Nitrendipine	N	7	7.4 ± 3.5	20	9.9	20		CYP3A4
Omeprazole	N	2	1.7	4.5	3.7	7.5	12	CYP2C19 > 3A4
Prazosin	N	7	2.3 ± 1.7	6.1	4.7	2.7	3.1	P450
Propofol	N	5	107 ± 26	283	19	11	24	UGT1A9 >> P450
Ritonavir	N	7	2.1 ± 3.0	5.5	4.3	1.2	1.3	CYP3A4 >> 2D6
Temazepam	N	2	2.0	5.3	4.2	1.3	1.4	UGT > P450
Triazolam	N	2	1.0	2.6	2.3	2.5	2.9	CYP3A4
Zileuton	N	16	2.1 ± 1.8	5.5	4.3	6.0	8.6	UGT
Acebutolol	B	4	1.8 ± 1.5	4.8	3.8	4.1	5.2	Acetylation
Atenolol	B	5	<1.0		<2.3	<1.0		Insignificant
Bepridil	B	1	2.0	5.3	4.2	5.3	7.2	CYP3A4
Betaxolol	B	6	2.5 ± 1.0	6.6	4.9	3.9	4.8	P450
Bisoprolol	B	8	1.6 ± 1.4	4.2	3.5	1.3	1.4	P450
Carvedilol	B	5	35 ± 11	93	16	8.7	15	P450/UGT
Chlorpheniramine	B	4	2.8 ± 1.3	7.4	5.4	1.3	1.4	P450 >> UGT
Clozapine	B	1	6.0	16	8.8	7.4	12	CYP1A2 >> UGT, CYP2D6, 3A4
Codeine	B	2	23	61	15	7.5	12	UGT > CYP2D6 > CYP3A4
Desipramine	B	1	3.0	7.9	5.7	12	30	CYP2D6 > UGT
Dextromethorphan	B	81	7.6 ± 8.1	20	10.0	6.0	8.6	CYP2D6 > 3A/2C19
Diltiazem	B	88	9.0 ± 5.0	24	11	12	30	CYP3A4
Diphenhydramine	B	1	6.0	16	8.8	9.6	19	P450 >> UGT
Doxepin	B	1	13	34	13	14	47	P450
Fluoxetine	B	1	1.0	2.6	2.3	7.8	13	CYP2D6
Granisetron	B	4	9.0 ± 8.7	24	11	11	24	CYP3A4
Imipramine	B	3	8.0 ± 2.5	21	10	17	113	CYP2D6/1A2/2C19/3A/ UGT1A4
Metoprolol	B	5	7.0 ± 2.9	19	9.6	13	37	CYP2D6
Morphine	B	2	24	63	15	18	180	UGT2B7
Nadolol	B	3	<1.0		<2.3	0.8	0.8	Insignificant
Naloxone	B	2	216	570	19	13	37	UGT2B7
Ondansetron	B	5	1.4 ± 0.5	3.7	3.1	5.9	8.4	CYP2D6, 1A2, 3A4
Pindolol	B	4	2.8 ± 1.0	7.4	5.4	4.2	5.3	P450 > UGT
Pirenzepine	B	4	<1.0		<2.3	<1.0		Insignificant
Propranolol	B	90	10 ± 5.0	26	11	16	80	CYP2D6 > 1A2/2C19/UGT
Ranitidine	B	5	1.0 ± 0.0	2.6	2.3	2.9	3.4	FMO3 / P450
Scopolamine	B	1	7.0	19	9.6	11	24	Esterase
Triprolidine	B	4	4.3 ± 3.3	11	7.2	8	13	P450
Verapamil	B	86	18 ± 12	46	14	15	60	CYP3A4
Cetirizine	Z	5	<1.0		<2.3	<1.0		P450 (minor)

[a] Human pharmacokinetic parameters (from i.v. studies) were sourced from several key compendiums including Goodman et al. (1996), Hardman et al. (2001), Dollery (1999), Taeschner and Vozeh (1998), and Bertz and Granneman (1997).

and cryopreserved hepatocytes, the routes of metabolism for substrates dextromethorphan and propranolol were assessed and found to be equivalent. Donor cells from dog and human stored in liquid N_2 for approximately 1 year have shown no significant decrease in viability or activity upon thawing, compared with the initial thaw, which was carried out within a week of cryopreservation.

Discussion

Predicting human pharmacokinetic parameters from preclinical data is one of the major challenges for drug metabolism scientists within the pharmaceutical industry. Increasingly accurate predictions of both human absorption and volume of distribution are being made for candidate drugs from a consideration of the physicochemical properties and pharmacokinetic parameters in preclinical species. Direct in vitro-in vivo scaling for compounds known to be cleared metabolically is a powerful tool for optimizing hepatic clearance, with the concomitant effect on lengthening half-life and increasing oral bioavailability. Whereas much of the supporting data has been generated in the rat (Houston, 1994b; Houston and Carlile, 1997), several recent publications have provided human data (Hoener, 1994; Iwatsubo et al., 1997; Lave et al., 1997b; Li, 1999; Obach, 1999; Lau et al., 2002; Shibata et al., 2002; Soars et al., 2002; Bachmann et al., 2003). These studies have shown that CL_{int} data can be used to provide estimates of human clearance for both phase I and phase II metabolic

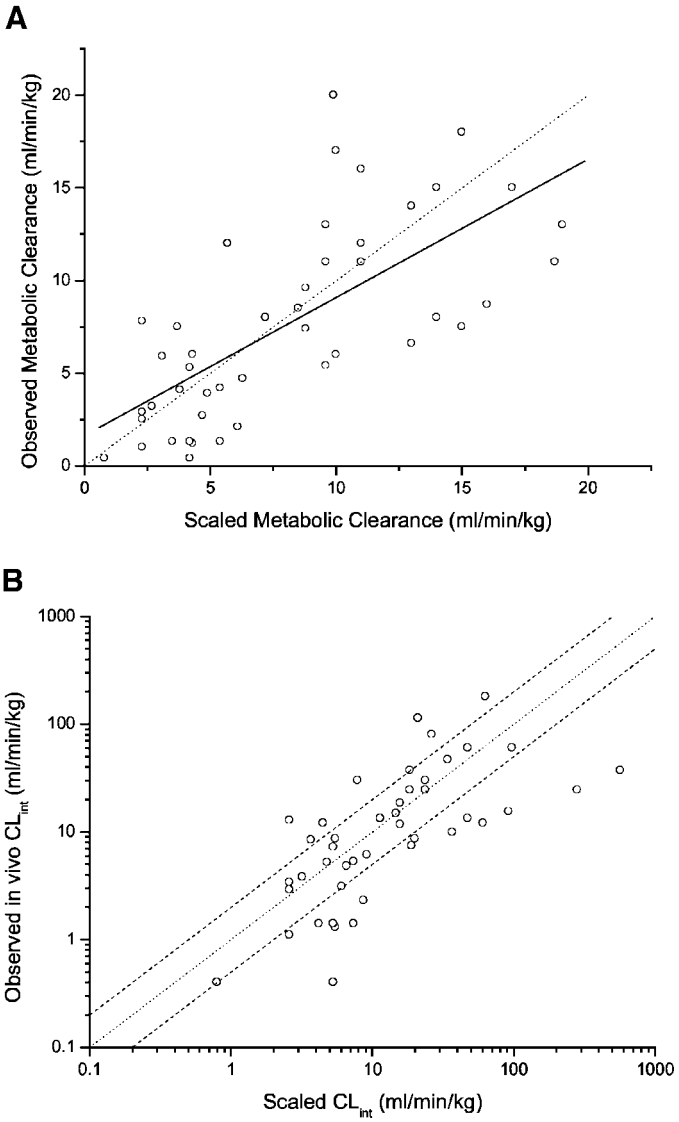

FIG. 1. A, prediction of CL_{met} using CL_{int} from human hepatocytes. The CL_{int} determinations were conducted as described under *Materials and Methods*. Assays were performed in triplicate where possible, depending on cell availability. The dotted line is unity. The solid line indicates linear regression of the data ($r^2 = 0.48$, $p < 0.05$). The data from this plot are tabulated in Table 1. B, accuracy of scaled CL_{int} versus observed in vivo CL_{int}. The dotted line indicates scaled CL_{int} = observed in vivo CL_{int}. The dashed lines signify 2-fold errors between the predicted and observed values. A total of 24 of 44 scaled Cl_{int} values are within 2-fold of the observed in vivo CL_{int}.

TABLE 2

Effect of Percoll on the viability and recovery of dog and human hepatocytes

Results are expressed as the mean CL_{int} ± standard deviation from three human and dog donors. The viability determinations were carried out as described under *Materials and Methods*.

Percoll Percentage	Human Hepatocytes		Dog Hepatocytes	
	Viability	Recovery	Viability	Recovery
	%		%	
0	66 ± 2	46 ± 10	52 ± 1	40 ± 11
10	64 ± 11	31 ± 10	53 ± 5	42 ± 18
15	72 ± 3	39 ± 14	58 ± 6	37 ± 12
20	74 ± 5	34 ± 9	67 ± 5	35 ± 3
25	88 ± 3	31 ± 8	78 ± 8	33 ± 8

processes. Moreover, a recent review of several methods (Zuegge et al., 2001) suggested that CL_{int} determined from human hepatocytes was the best predictor of human hepatic clearance when compared to

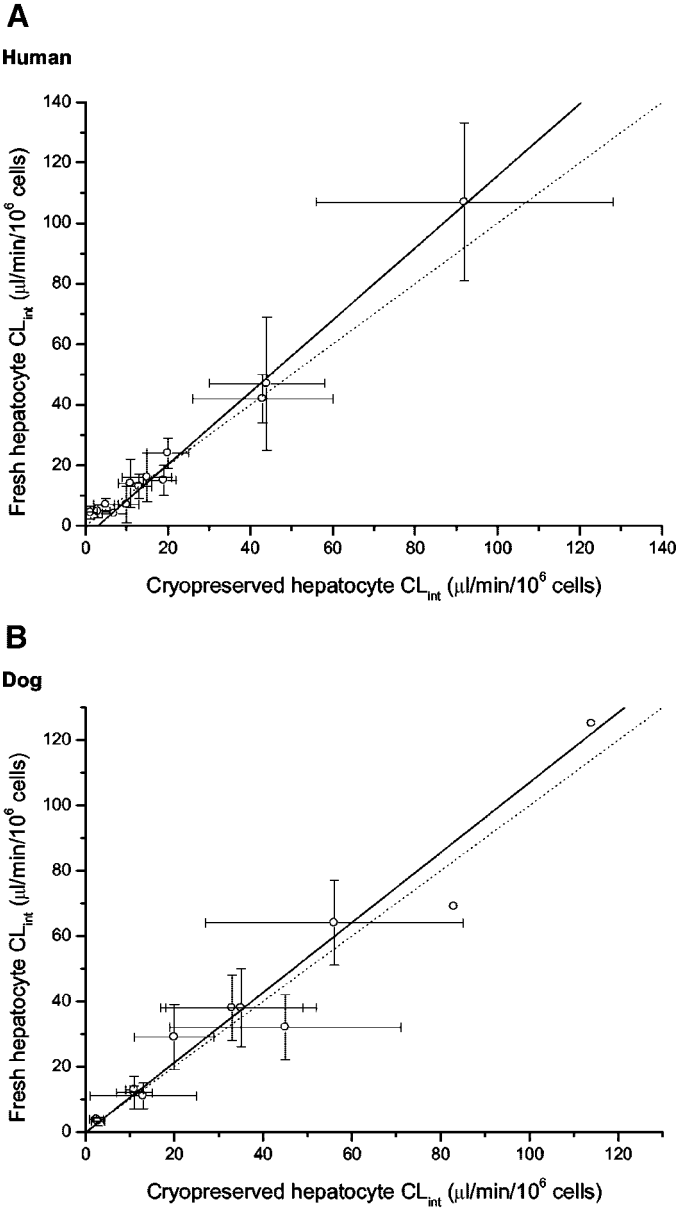

FIG. 2. A, CL_{int} of substrates determined in fresh and cryopreserved human hepatocytes. The data points represent the mean CL_{int} determinations and the error bars reflect the standard deviation from the mean. The CL_{int} determinations were carried out as described under *Materials and Methods*. Experiments were carried out in duplicate from six individual human donors. Results not expressing a S.D. are from two donors only. The dotted line is unity. The solid line indicates linear regression of the data ($r^2 = 0.99$, $p < 0.001$). The data from this plot are tabulated in Table 3. B, CL_{int} of substrates determined in fresh and cryopreserved dog hepatocytes. The data points represent the mean CL_{int} determinations and the error bars reflect the standard deviation from the mean. The CL_{int} determinations were carried out as described under *Materials and Methods*. Experiments were carried out in duplicate from six individual dog donors. Results not expressing a S.D. are from two donors only. The dotted line is unity. The solid line indicates linear regression of the data ($r^2 = 0.95$, $p < 0.001$). The data from this plot are tabulated in Table 3.

data from rat/dog hepatocytes or allometric scaling, and inclusion of in vivo data did not improve significantly the prediction accuracy.

Intrinsic clearance best describes the efficiency of metabolism and can be scaled to in vivo CL_{met} with knowledge of physiological parameters such as microsomal content, hepatocellularity, liver weight, and a model of hepatic extraction such as the well stirred model. Predictions of human in vivo metabolic clearance were achieved for compounds primarily cleared in humans by the P450s,

UGTs, or a combination of the two enzyme families. Evidently, caution should be exercised for compounds undergoing extensive conjugation, due to the potential for significant extrahepatic metabolism in the kidney and gut (Obach, 1999; Soars et al., 2002).

Clearly, in the context of discovering once daily oral therapies, an under-prediction of in vivo CL is of most concern since the higher than expected CL may preclude such a therapeutic dosing regimen. Encouragingly for the drugs studied, there was a good overall agreement between scaled clearance and human in vivo metabolic clearance using human hepatocytes (Table 1; Fig. 1). No metabolism was detected for several compounds (atenolol, pirenzipine, cetirizine, nadolol, and famotidine), and this agreed with the observation that in vivo metabolic CL is minimal for these compounds.

For scaling hepatic metabolic clearance, no fu_p correction was applied since previous studies have suggested this results in a systematic under-prediction of in vivo CL (Obach, 1997; 1999; Lau et al., 2002). To desensitize the analysis somewhat to the effects of "modeling" hepatic clearance, a direct comparison of scaled and observed in vivo CL_{int} was also conducted (Fig. 1B). For 24 of 44 compounds, prediction of in vivo CL_{int} was within 2-fold. Recent analysis of microsomal data (Houston and Galetin, 2003) has suggested that unbound in vivo CL_{int} may be somewhat higher than that projected from in vitro data. At present, all the fu_p and fu_{inc} data have not been generated for this data set. However, as a typical example, computing the unbound in vivo CL_{int} for verapamil ($fu_p = 0.1$) would yield an estimate of 467 ml/min/kg versus a scaled unbound CL_{int} of 92 ml/min/kg [assuming 50% binding in vitro (Austin et al., 2002)]. This preliminary analysis supports the notion that under the incubation conditions chosen (buffer, cell number, etc.), a similar "offset" may exist for human hepatocytes.

A clearer understanding of the relationships between physicochemical properties of compounds with differing in vitro milieu should assist in designing the appropriate in vitro experiments and the accuracy of in vivo CL_{met} predictions. Indeed, it has been shown that this binding in microsomes can be predicted purely from a knowledge of lipophilicity and ionization (Austin et al., 2002). For acids, because of a generally high affinity for the plasma protein albumin, incorporation of a fu_p correction in the well stirred model and incubating hepatocytes in the presence of serum to accommodate differential binding have been proposed as "remedies" (Shibata et al., 2000).

Variability of predicted CL_{met} from in vitro studies was a reflection of both interdonor differences and experimental error. Any interdonor differences in CL_{int} may reflect variability in observed human CL_{met}. Determining CL_{int} from human hepatocytes isolated from several individual donors should give an indication of the inherent pharmacokinetic variability in the human population. In addition, knowledge of the enzymology of metabolism may help predict variability in pharmacokinetics due to polymorphism (e.g., CYP2D6) or differences in enzyme activity. It is therefore important to assess the activity of each individual hepatocyte preparation, and a subset of the compounds (verapamil, diltiazem, dextromethorphan, propranolol, diclofenac, and ethinylestradiol) were identified as suitable markers to be incubated routinely for such analysis.

Data from this study support the use of CL_{int} derived from human hepatocytes as a reasonable predictor of human in vivo CL_{met}, and indeed, human hepatocytes are becoming an integral part of the drug discovery screening cascade. However, a lack of supply of good quality human tissue may limit their use in routine screens. Cryopreserved hepatocytes offer the advantage of being a continuously available screening tool. A scan of commercially available cryopreserved human hepatocytes from several donors showed a range of oxidation and glucuronidation rates compared with those of fresh hepatocytes. However, the activity of one particular donor (EC) matched closely that of the mean of several fresh human hepatocyte donors.

Efforts by this laboratory to establish an in-house cryopreservation and thawing technique for human and dog hepatocytes have proved successful. Experience suggests that the quality and viability of fresh hepatocytes are critical for successful cryopreservation. The viability of fresh hepatocytes to be used for cryopreservation should be >80%, respectively, or a Percoll step should be added to increase viability before initiating the cryopreservation technique. By using a more controlled freezing procedure than that described here, the recovery of viable cells may be improved (Hengstler et al., 2000; Houle et al., 2003). Cells used for metabolism incubations should typically be >75% viable, and the results shown in Table 2 indicate that a Percoll step can be used post-thaw to increase viability with an acceptable loss of material. Although dead cells are effectively ignored in metabolism experiments, they may contribute to the apparent CL_{int} either positively, via retained metabolic activity, or negatively, for example, by increasing nonspecific binding.

This study was designed only to assess the viability and metabolic capacity for substrates of cytochrome P450 and UGT enzyme families. There are reports of cryopreserved hepatocytes also retaining other enzymatic activities (Li et al., 1999; Hengstler et al., 2000; Hewitt et al., 2001; Fisher et al., 2002; Bachmann et al., 2003; Houle et al., 2003) transporter activity (Houle et al., 2003) and properties suitable for characterizing P450 induction (Silva et al., 1999). These applications remains to be assessed for our in-house batches. The activity of thawed cryopreserved human and dog hepatocytes was on average 94 and 81% of that of fresh hepatocytes for a range of P450 and UGT substrates (Table 3; Fig. 2). In addition, for two compounds, the routes of metabolism were also shown to be similar between fresh and cryopreserved hepatocytes. Since fresh human hepatocytes appear to be predictive for human CL_{met} for a range of P450 and UGT substrates, this work indicates that fresh and cryopreserved human hepatocytes can be used interchangeably for this application. In addition, the successful cryopreservation of dog hepatocytes has reduced this laboratory's requirement for freshly prepared cells. Consistent with findings from other laboratories (Li et al., 1999; Houle et al., 2003), the length of storage in liquid N_2 (currently up to 1 year) has

TABLE 3

CL_{int} estimates determined from fresh and cryopreserved dog and human hepatocytes

Results are expressed as the mean CL_{int} ± standard deviation from five human and six dog donors. Results not expressing a S.D. are from two donors only. The CL_{int} determinations were carried out as described under *Materials and Methods*. The CL_{int} estimates reported in Table 1 represent a different donor pool from the one reported in this table, which can result in different mean values for the same substrate.

Compounds	CL_{int}			
	Human hepatocytes		Dog hepatocytes	
	Fresh	Cryo	Fresh	Cryo
	μl/min/10⁶ cells		*μl/min/10⁶ cells*	
Dextromethorphan	16 ± 8	15 ± 6	64 ± 13	56 ± 29
Diazepam	<1	<1	>150	>150
Diclofenac	47 ± 22	44 ± 14	12 ± 5	11 ± 4
Diltiazem	13 ± 4	13 ± 3	29 ± 10	20 ± 9
Ethinylestradiol	7 ± 2	5 ± 3	69	83
Gemfibrozil	24 ± 5	20 ± 5	13 ± 1	11 ± 2
Imipramine	4	7 ± 3	32 ± 10	45 ± 26
Ketoprofen	4 ± 2	1 ± 1	4 ± 0.4	3 ± 2
Metoprolol	7 ± 6	10 ± 3	11 ± 4	13 ± 12
Midazolam	14 ± 8	11 ± 3	125	114
Naproxen	5 ± 2	3 ± 3	3 ± 1	3 ± 1
Propofol	107 ± 26	92 ± 36	>150	>150
Propranolol	15 ± 5	19 ± 3	38 ± 10	33 ± 16
Verapamil	42 ± 8	43 ± 17	38 ± 12	35 ± 17

had no deleterious effect on the post-thaw viability or activity for both dog and human hepatocytes.

In conclusion, this study has demonstrated the ability to predict human CL_{met} from human hepatocyte CL_{int} for a range of drugs. Successful in-house cryopreservation of hepatocytes has made this important tool readily available, more economical, flexible, and convenient. However, before any scaling exercise in humans, a priori knowledge of the likely clearance mechanism is paramount. In addition, accurate prediction of clearance in different preclinical species increases confidence that the approach would be successful in humans. Confidence may currently be higher for metabolic routes when knowledge of enzymology and the tissue distribution exists, such as for those compounds primarily cleared by the P450 and, increasingly, the UGT family of enzymes.

Acknowledgments. Many current and former AstraZeneca colleagues have contributed to this work. The contributions of Philip Gardiner, Iain Martin, Lee Reading, and Emma Roe warrant specific acknowledgment.

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Address correspondence to: Dr. Dermot McGinnity, Department of Physical & Metabolic Science, AstraZeneca R&D Charnwood, Bakewell Road, Loughborough, Leicestershire, LE11 5RH, UK. E-mail: dermot.f.mcginnity@astrazeneca.com