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Determination of Human Hepatocyte Intrinsic Clearance for Slowly Metabolized Compounds: Comparison of a Primary Hepatocyte/Stromal Cell Co-culture with Plated Primary Hepatocytes and HepaRG[S]

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ABSTRACT

A key requirement in drug discovery is to accurately define intrinsic clearance (CL_{int}) values of less than 1 μl/min/10^6 hepatocytes, which requires assays that allow for longer incubation time as a complement to suspended hepatocytes. This study assessed the effectiveness of plated HepaRG cells, plated primary human hepatocytes (PHHs), and the HμREL human hepatocyte/stromal cell co-cultures for determination of low CL_{int} values. The investigation demonstrated that the systems were capable of providing statistically significant CL_{int} estimations down to 0.2 μl/min/10^6 cells. The HμREL assay provided a higher level of reproducibility, with repeat significant CL_{int} values being defined in a minimum of triplicate consecutive assays for six of seven of the low CL_{int} compounds compared with four of seven for PHHs and two of seven for HepaRG. The assays were also compared with a suspension assay using drugs with higher CL_{int} values and diverse enzymology. The CL_{int} values from the PHH and HμREL assays were similar to those defined by a hepatocyte suspension assay, indicating that they can be used interchangeably alongside a standard assay. Finally, data from these two assays could also predict in vivo hepatic metabolic CL_{int} to within 3-fold for greater than 70% of the compounds tested, with average fold errors (AFE) of 1.6 and 2.3, respectively, whereas the HepaRG data were predictive to within 3-fold for only 50% of compounds (AFE 2.9). In summary, all systems have utility for low CL_{int} determination, but the HμREL co-culture appears slightly superior regarding overall assay performance.

Introduction

A crucial task for drug metabolism and pharmacokinetics (DMPK) scientists in drug discovery is to predict human pharmacokinetics (PK) and to design drug candidates with PK properties commensurate with effective and safe therapeutic treatment. In this context, clearance (CL) is a critical parameter. Particularly when distribution volume is low, CL is consequently required to be low for an effective half-life with a minimum C_{max}/C_{min} ratio.

Hepatic metabolism is responsible for the clearance of approximately 70% of marketed drugs (Wienkers and Heath, 2005). Human metabolic CL is commonly predicted using in vitro hepatocyte or hepatic microsomal intrinsic clearance (CL_{int}) data (Houston, 1994; Laine, 2008; Obach, 2011), with primary hepatocytes accepted as the most appropriate in vitro system (Soars et al., 2007; Di et al., 2013). Determination of CL_{int} can be performed using either metabolite formation or depletion of parent, but since knowledge of primary metabolites is seldom available in screening phases of drug discovery, the substrate depletion method is more or less exclusively used for this purpose (Obach and Reed-Hagen, 2002; Jones and Houston, 2004). Despite the improvement in clearance predictions based on analyses of large data sets (Riley et al., 2005; Grime and Riley, 2006; Obach, 2011; Sohlenius-Sternbeck et al., 2012), a remaining difficulty is the ability to accurately define CL_{int} for slowly metabolized compounds. To obtain a meaningful result, the metabolism of the compound over the incubation period must be significantly greater than the variability in procedures used to determine CL_{int}. Suspended primary hepatocytes suffer from rapid loss of drug-metabolizing enzyme activity (Smith et al., 2012), which limits the incubation time and makes this system inappropriate for CL_{int} determinations for low turnover compounds. For moderate to low volume of distribution compounds, CL_{int} may typically be less than 1 μl/min/10^6 cells (Grime et al., 2013). Such a CL_{int} relates to only a 2-fold change in drug concentration in the in vitro incubation over 12 hours in an assay format using one million cells/ml. The absence of a robust assay denies the DMPK scientist the ability to discriminate between compounds of interest in the drug discovery and of course signifies a lack of accuracy in predictions of in vivo hepatic metabolic CL.

Despite these difficulties, recent studies have demonstrated that, with care, progress can be made using hepatocyte suspension incubations. The Relay method in which supernatants from hepatocyte suspension incubations are repeatedly transferred to fresh cells, enables continual metabolism of parent compound by fresh metabolic enzymes for times exceeding 4 hours (Di et al., 2012, 2013). Nonetheless, the technical

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ABBREVIATIONS: AFE, average fold error; AZ01, 4-amino-N-[(1S)-1-cyano-2-[4-(4-cyanophenyl)phenyl]ethyl]tetrahydropyran-4-carboxamide; AZ02, N-[2-[(2,3-difluorophenyl)methylsulfanyl]-6-[[(1R,2R)-2,3-dihydroxy-1-methyl-propyl]amino]pyrimidin-4-yl]azetidine-1-sulfonamide; CYP, cytochrome P450; DMPK, drug metabolism and pharmacokinetics; DMSO, dimethyl sulfoxide; fu_b, fraction of drug unbound in blood; fu_{inc}, fraction of drug unbound in in vitro incubation; LC-MS/MS, liquid chromatography-tandem mass spectroscopy; PHH, plated primary human hepatocytes; PK, pharmacokinetics.

manipulation issues can make even this assay challenging, and since the method represents a mathematical approach to integrate several separate 4-hour incubations into one algorithm, there is a potential issue with error propagation and statistical significance of the defined CL_{int}. Beyond two-dimensional cultures of primary or immortalized hepatocytes, three-dimensional and co-cultures are becoming readily available (Godoy et al., 2013). Examples include HepatoPac (Chan et al., 2013), hepatocyte spheroids (Ohkura et al., 2014), and HμREL (Novik et al., 2010), all of which have been shown to maintain liver-specific functions for several days. A recent article has reviewed the available literature on different CL_{int} assays, with particular emphasis on slowly metabolized compounds and advantages or disadvantages with regard to prediction of human hepatic CL (Hutzler et al., 2015). The review highlighted the fact that although several assay options are emerging around the low CL_{int} issue, data supporting definitive evaluation are still sparse. Evident from the analysis compiled by Hutzler and colleagues is that there is requirement for greater knowledge on the functionality of multidonor pools of plated primary human hepatocytes (PHHs) and the need for comparisons of the same hepatocyte batch in different in vitro systems.

In this work, we appraised three different assay formats regarding the ability to determine CL_{int} values to 1 μl/min/10^6 hepatocytes and below and for accuracy of human in vivo CL prediction. The assay formats were chosen to facilitate longer incubation times as well as having a reasonable throughput appropriate for early drug discovery. The three assays evaluated were a five-donor pool of PHH plated on type I collagen, the same hepatocyte pool in the HμREL co-culture of primary hepatocytes/stromal cells, and finally plated HepaRG cells, a human hepatoma cell line that can be differentiated toward hepatocyte-like cells showing stable phenotype (Aninat et al., 2006; Kanebratt and Andersson, 2008). These three assays serve as potential tools for drug discovery to determine CL_{int} and to more accurately predict human hepatic CL for low turnover compounds.

Materials and Methods

Chemicals and Materials. Cryopreserved HepaRG cells (lot 2279582 and 2207183) plus additives for cell culture medium (HepaRG Thawing/Plating Medium, HepaRG Culture Medium and HepaRG Serum Free Induction Medium) were purchased from Millipore (Molsheim, France). For monoculture experiments, a five-donor pool of platable cryopreserved human hepatocytes (lot 1310168, 3 females and 2 males), thawing kit (K2000), resuspension medium (K2200), and culture medium (K2300) were purchased from XenoTech (Kansas City, KS). Co-culture of the five-donor pool (lot 1310168, XenoTech) and nonparenchymal stromal cells (stromal cell type and ratio of hepatocyte per stromal cell are proprietary information) in type I collagen coated 24-well plates were purchased from HμREL (North Brunswick, NJ). A ten-donor pool of cryopreserved human hepatocytes (lot IRK), used for the hepatocyte suspension assay, were obtained from Celsis In Vitro Technologies (Brussels, Belgium). Leibovitz's L-15 medium, without phenol red, with L-glutamine (Gibco 21083-027), was purchased from Invitrogen (Stockholm, Sweden). Trypan blue was purchased from Tebu-bio (Le Perray-en-Yvelines, Cedex, France).

S-warfarin, diazepam, disopyramide, metoprolol, theophylline, sildenafil, carvedilol, imipramine, ketanserin, ketoprofen and 5,5-diethyl-1,3-diphenyl-2-iminobarbituric acid (no. 39) were purchased from Sigma-Aldrich (St. Louis, MO). AZ01 (4-amino-N-[(1S)-1-cyano-2-[4-(4-cyanophenyl)phenyl]ethyl] tetrahydropyran-4-carboxamide), and AZ02 (N-[2-[(2,3-difluorophenyl) methylsulfanyl]-6-[[(1R,2R)-2,3-dihydroxy-1-methyl-propyl]amino]pyrimidin-4-yl]azetidine-1-sulfonamide) were obtained as dimethyl sulfoxide (DMSO) stock solutions from AstraZeneca compound management team (Gothenburg, Sweden).

Acetonitrile and methanol were purchased from Rathburn Chemicals Ltd, (Walkerburn, Scotland). All other chemicals were of analytical grade and highest quality available.

CL_{int} Validation Set. For validation of the different hepatocyte assay formats and their potential to determine CL_{int}, a set of slowly metabolized compounds was selected (Table 1). In addition, five compounds with intermediate to high CL_{int} values (5–40 μl/min/10^6 cells) were assayed to validate the novel systems through agreement of the recognized standard system of hepatocyte suspension incubations (Table 1). Chemical structures of the test set are available in supplemental information (Supplemental Fig. 1). All compounds were incubated at 1 μM.

Culture of HepaRG Cells. The cryopreserved HepaRG cells were thawed and washed according to protocol from the vendor. The HepaRG cells were thawed in prewarmed (37°C) HepaRG Thawing/Plating Medium, followed by centrifugation at room temperature. The supernatant was removed, and the cell pellet was resuspended in HepaRG Thawing/Plating Medium at room temperature. The amount of cells and the viability were determined by the trypan blue exclusion method. Aliquots of 100 μl of cell suspension (0.72 million cells/ml) were seeded into each well of flat-bottom 96-well plates, coated with type 1 collagen and allowed to attach for about 24 hours in incubator at 37°C in humidified atmosphere containing 95% air and 5% CO_2. After 24 hours, the cell morphology was checked and the medium was renewed with HepaRG Culture Medium at room temperature. After about 60 hours, the medium was renewed with HepaRG serum free induction medium, and 24 hours later, the cells were ready to be exposed to the test compounds. The test compounds were dissolved and diluted in 50% acetonitrile before addition to serum-free Induction Medium giving final concentrations of solvent in the incubations of less than 1% acetonitrile and 0.005% DMSO. The incubations were initiated by addition of 100 μl of test compound to each well using one well per time point. Plates were kept without shaking in the incubator during the experiments, and eight samples, evenly distributed over 24 hours, were withdrawn during incubation. At termination, 50 μl of medium from each well (one well per time point) was transferred to a 96 deep-well plate and quenched in 150 μl of ice-cold acetonitrile containing 0.8% formic acid and 25–200 nM verapamil and/or no. 39 as the bioanalytical internal standard. Samples were centrifuged at 4°C for 20 minutes at 3220*g*, after which clear aliquots of supernatant were diluted in equal amounts of water and kept at −20°C until quantification of parent using liquid chromatography mass spectroscopy (LC-MS/MS). Compounds were incubated in singlicates, and incubations were repeated three times.

Monoculture of Primary Human Hepatocytes. The five-donor pools of cryopreserved human hepatocytes (lot 1310168, XenoTech) were thawed and washed according to vendor protocol. Hepatocytes were thawed in DMEM and subsequently washed in isotonic Percoll gradient (material included in thawing kit K2000), after which the amount of cells and viability were determined by the trypan blue exclusion method. Viability exceeded 80% before seeding of 0.4 million hepatocytes in 300 μl of resuspension medium into each well of type I collagen-coated flat-bottom 24-well plates. Hepatocytes were left to attach for about 4 hours in incubator at 37°C in humidified atmosphere containing 95% air and 5% CO_2. Test compound was dissolved in DMSO, acetonitrile, and water and finally diluted to 1 μM in culture medium, giving final concentrations of solvent in incubations of less than 1% acetonitrile and 0.005% DMSO. Compound incubation was initiated by aspiration of resuspension medium, washed with 200 μl of blank culture medium, followed by the addition of 600 μl of culture medium per well containing 1 μM test compound. Plates were kept without shaking in the incubator during the experiments. Depending on the expected depletion rate, 8–18 samples of 10 μl medium, evenly distributed over 12 hours, were withdrawn from each well during incubation. Samples were quenched with 90 μl of ice-cold acetonitrile containing 0.8% formic acid and 25–200 nM verapamil and/or no. 39 as bioanalytical internal standard. Samples were centrifuged at 4°C for 20 minutes at 3220*g*, after which clear aliquots of supernatant were diluted in equal amount of water and kept at −20°C until quantification of parent using LC-MS/MS. Compounds were incubated in singlicates, and incubations where repeated three times.

HμREL-Co-cultured Primary Human Hepatocytes and Stromal Cells. Co-culture plates were carefully shipped in maintenance medium, at 37°C, from HμREL (North Brunswick, NJ). Amount of seeded viable hepatocytes was 0.188 million per well. The cells were co-cultured for 6 days before arrival at AstraZeneca R&D Gothenburg, Sweden, where the cells were left to acclimatize in HμREL PlatinumHeps maintenance medium overnight in incubator at 37°C in humidified atmosphere containing 95% air and 5% CO_2. Test compound was dissolved in DMSO, acetonitrile, and water and finally diluted to 1 μM in HμREL PlatinumHeps media. Final concentration of solvent in the incubations

TABLE 1

Compound properties, responsible drug metabolizing enzyme and observed proteinbinding and in vivo CL for the 12 test compounds

	Ion Class	MW	$LogD_{7.4}$	Main Responsible Enzyme	In Vivo CL_p	fu_b	fu_{inc}	Observed In Vivo CL_{int}
					ml/min/kg			*ml/min/kg*
Low CL_{int} compounds								
S-Warfarin	Neutral	308.3	0.2	2C9 > 3A4	0.06[a]	0.018[a]	0.953	0.06
Diazepam	Neutral	284.7	2.8	2C19 > 3A4	0.5[a]	0.036[a]	0.808	0.5
Disopyramide	Base	339..5	−0.2	3A4	0.9[b]	0.16[b]	0.827	0.9
Metoprolol	Base	267.4	−0.4	2D6	13[b]	0.88[b]	0.922	1.4
Theophylline	Neutral	180.2	−0.05	1A2	1.3[a]	0.53[a]	0.956	37.1
AZ01	Base	374.4	1.5	3A4	na			
AZ02	Acid	475.5	1.9	3A4	2.1	0.039	0.893	4.7
Intermediate to high CL_{int} compounds with diverse enzymology								
Ketoprofen	Acid	254.3	−0.3	UGT2B2	1.2[a]	0.17[a]	0.956	2.4
Sildenafil	Base	474.6	2.5	2C9/2C19/3A4	6.0[a]	0.094[a]	0.892	8.6
Carvedilol	Base	406.5	3.1	2D6 > 2C9	8.7[a]	0.030[a]	0.434	15.4
Imipramine	Base	280.4	2.3	2C9 > 2D6 > 3A4 > 1A2	13.5[a]	0.13[a]	0.127	41.5
Ketanserin	Base	395.4	3.0	AKR/CR	6.7[b]	0.094[b]	0.752	10.1

MW, molecular weight; na, not available.
[a]Hallifax et al., 2010.
[b]Obach et al., 2008.

was less than 1% acetonitrile and 0.005% DMSO. Compound incubation was initiated by careful aspiration of resuspension media, washed with 200 μl of blank culture media, followed by the addition of 600 μl of culture media containing 1 μM test compound per well. Plates were kept without shaking in the incubator during the experiments. Depending on the expected depletion rate, 12–22 samples of 10 μl of medium evenly distributed over 72 hours, were withdrawn from each well during incubation. Samples were quenched with 90 μl of ice-cold acetonitrile containing 0.8% formic acid and 25–200 nM verapamil and/or no. 39 as bioanalytical volume reference. Samples were centrifuged at 4°C for 20 minutes at 3220*g*, after which clear aliquots of supernatant were diluted in equal amount of water and kept in a freezer until quantification of parent using LC-MS/MS. Compounds were incubated in singlicates and incubations were repeated four times.

Primary Human Hepatocytes in Suspension. Data from suspended hepatocytes were from historical in-house data generated using a standard automated hepatocyte assay a minimum of three times. The 10-donor pool of cryopreserved human hepatocytes (lot IRK, Celsis) were thawed and washed in Leibovitz's L-15 medium. The cells were thawed in prewarmed (37°C) medium and subsequently washed in two steps in room temperature medium, after which the amount of cells and viability were determined by the trypan blue exclusion method. The cell-suspension was diluted to one million cells/ml (viability > 80%) and seeded into round-bottom 96-well plates (247.5 μl/well). The test compounds were dissolved in DMSO and further diluted in 50% acetonitrile, with a final concentration of 0.1% DMSO and 1% acetonitrile in the incubation. Incubation was performed in 37°C with agitation at a frequency of 13 Hz. The hepatocytes were preincubated for 15 minutes, and the reaction was initiated by the addition of one test compound/well, giving a final concentration of 1 μM and a final incubation volume of 250 μl/well. Ten samples evenly distributed over 120 minutes were withdrawn from each well during incubation. At termination, 15 μl of medium was quenched in 45 μl of ice-cold acetonitrile containing 0.8% formic acid and 100 nM no. 39 as a bioanalytical volume reference. The samples were centrifuged for 30 minutes at 4°C and 3220*g*. Clear aliquots of supernatant were diluted in equal amount of water and kept at 4°C until quantification of parent using LC-MS/MS. All incubations were performed using a Tecan Freedom Evo 200 robot with integrated plate centrifuge Rotanta 46 RSC (Hettich), plate shaker Variomag Teleshake 70 with TEC Control 485, Tecan Fredom Evo-2 200.

Bioanalysis. The concentration of test compound and bioanalytical internal standard in all samples was analyzed by high-performance liquid chromatography connected to a triple quadrupole tandem mass spectrometer using electrospray ionization and multiple reaction monitoring. A typical bioanalytical setup was an Acquity ultra-performance liquid chromatography system coupled to a Waters Xevo TQ MS mass spectrometer with an atmospheric pressure electrospray interface (Waters, Zellik, Belgium). Separation was performed on a CSH C18 column (1.7 mm × 2.1 mm × 30 mm, Waters). A gradient composed of eluent A (H_2O + 0.1% formic acid) and eluent B (acetonitrile + 0.1% formic acid) was used. For the first 0.3 minutes, isocratic conditions was applied with 96% eluent A; then a linear gradient from 96% to 5% was performed from 0.30 minutes to 0.70 minutes, followed by a plateau of 5% eluent A for 0.3 minutes before going back to 96% eluent A. Flow rate was 1 ml/min. Column temperature was set to 40°C and sample temperature to 10°C. Waters TargetLynx version 4.1 SCN 905 was used for LC-MS/MS system control and data analysis. Each compounds parameters were optimized automatically by MassLynx/IntelliStart, and a chromatographic test was performed before analysis. Mass transitions and additional information for each compound can be found in supplemental information (Supplemental Table 1).

Calculations. In vitro CL_{int} was calculated from parent compound loss data (eq. 1). For all three systems CL_{int} was calculated using the total amount of seeded cells.

$$CL_{int} = -slope\ of \ln(\%drug\ remaining)\ vs\ time\ plot \times \frac{ml\ incubation}{10^6\ cells}\mu l \cdot min^{-1} \cdot 10^6 cells^{-1} \quad (1)$$

To assign statistical relevance to the estimated CL_{int}, a two-sided *t* test was applied to check the validity of the overall slope, determined from substrate depletion data, and to determine whether it deviated significantly from zero. The degrees of freedom were considered (number of data-2) of the slope regression. Only when the confidence was over 95% was the CL_{int} reported. Bioanalytical internal standard was added as a part of the quality control of the assay.

From in vitro CL_{int}, the predicted in vivo CL_{int} was obtained by taking physiological human scaling factors, incubational binding, and drug binding in blood into account (eq. 2). Incubational binding (fu_{inc}) may potentially differ between systems, e.g. plated vs. hepatocytes in suspension. Despite its limitations, however, a simplified approach using predicted fu_{inc} was used in this study. The predictions of fu_{inc} were done based on $logD_{7.4}$ (acidic and neutral compounds) or logP (basic drugs) according to what was previously described by Kilford et al. (2008):

$$Predicted\ In\ vivo\ CL_{int} = \frac{CL_{int} \times \frac{120 \times 10^6 cells}{g\ liver} \times \frac{24g\ liver}{kg} \times fu_b}{fu_{inc}} ml \cdot min^{-1} \cdot kg^{-1} \quad (2)$$

A regression line correction method was used for the prediction of in vivo CL_{int} as described previously (Sohlenius-Sternbeck et al., 2012). The regression line coefficients were obtained from data on human hepatocyte incubations in suspension with slope and intercept at 0.7500 and 0.6532 respectively.

Observed human in vivo CL_{int} was calculated using well-stirred model (Brown et al., 2007) (eq. 3) using human CL data from relevant scientific literature:

$$Observed\ In\ vivo\ CL_{int} = \frac{CL_b}{\left(1 - \frac{CL_b}{Q_H}\right)} \quad (3)$$

where Q_H is hepatic blood flow (20 ml/min/kg) and CL_b is hepatic blood clearance.

For prediction of in vivo human hepatic intrinsic clearance, the accuracy of each prediction was assessed with the average fold error (AFE) of the observed/predicted in vivo CL_{int} (eq. 4):

$$AFE = 10^{\frac{\sum \log(predicted\ \ CLint/observed\ \ CLint)}{number\ \ of\ \ observations}} \quad (4)$$

Results

HepaRG Cells. The mean CL_{int} values and the variation between experiments for the low turnover compounds are presented in Fig. 1, and examples of substrate depletion plots are presented in Fig. 2. In HepaRG, the compounds were incubated up to 24 hours, and CL_{int} values as low as 0.2 μl/min/10^6 cells were obtained (s-warfarin and disopyramide). However, for only two of the seven low turnover compounds (AZ01 and AZ02), the CL_{int} was reproducible, and approved CL_{int} was obtained in three consecutive experiments. For theohylline, no statistically significant CL_{int} was determined in any experiment. For the comparison of compounds with higher CL_{int}, ketoprofen, carvedilol, imipramine, and ketanserin gave no or considerably lower CL_{int} values with HepaRG than with suspended hepatocyte incubations, whereas sildenafil was comparable between the assays (Table 2). The accuracy in predicting human in vivo CL_{int} from HepaRG data were as follows: from the eight compounds where CL_{int} could be determined, the AFE was 2.9 and four of these eight compounds, (diazepam, disopyramide, sildenafil and carvedilol) gave predictions within 3-fold of observed (Table 2).

Monoculture of Primary Human Hepatocytes. In the monoculture assay, compounds were incubated up to 12 hours and a statistically significant value as low as 0.2 μl/min/10^6 cells (disopyramide) could be defined. However, an approved CL_{int} could not be obtained repeatedly for this compound. For four of the seven low turnover compounds (diazepam, metoprolol, AZ01, and AZ02), CL_{int} values could be defined on replicate occasions, diazepam having the lowest CL_{int} of 0.8 μl/min/10^6 cells (Fig. 1). No CL_{int} values were obtained for s-warfarin and theophylline. Examples of substrate depletion plots are presented in Fig. 2. For comparison with the standard hepatocyte suspension assay of compounds with higher CL_{int}, PHH showed comparable values for four of five compounds tested (ketoprofen, sildenafil, carvedilol, and imipramine) but a lower turnover for ketanserin (Table 2). Where CL_{int} could be determined for compounds with clinical pharmacokinetic data, seven of nine (diazepam, disopyramide, metoprolol, sildenafil, carvedilol, imipramine, and ketanserin) had predicted human in vivo CL_{int} within 3-fold of the observed values (Table 2); the AFE in the prediction was 1.6.

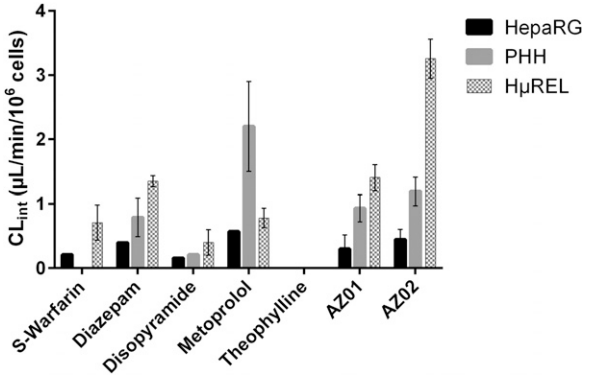

Fig. 1. Comparison of CL_{int} values for low turnover compounds obtained in HepaRG, PHH, and HμREL. Mean results from consecutive experiments with error bars represented by S.D. (n = 3 for HepaRG and PHH, and n = 4 for HμREL).

HμREL-Co-cultured Primary Human Hepatocytes and Stromal Cells. The HμREL co-culture assay was carried out for 72 hours and allowed several samples taken on each day of incubation (Fig. 2). In this assay, statistically significant CL_{int} values could be defined on replicate occasions for the compound with the lowest CL_{int}, disopyramide (four consecutive assays defined CL_{int} values of 0.3, 0.2, 0.6, and 0.5 with a mean of 0.4 μl/min/10^6 cells). The assay was able to provide CL_{int} values from a minimum of triplicate consecutive assays for six of seven low CL_{int} compounds assayed (s-warfarin, diazepam, disopyramide, metoprolol, AZ01, and AZ02) (Fig. 1). The only compound for which no CL_{int} was obtained was theophylline. When comparing suspended hepatocytes, this assay gave similar results for the higher turnover compounds, with the exception of imipramine (Table 2). Finally, the HμREL assay data were able to predict human in vivo CL_{int} to within 3-fold of the observed values for seven of 10 compounds (diazepam, disopyramide, AZ02, sildenafil, carvedilol, imipramine, and ketanserin) where CL_{int} could be determined (Table 2), with the AFE in the prediction being 2.3.

Discussion

An increasingly common problem for DMPK scientists is to define CL_{int} for slowly metabolized compounds using the substrate depletion method. Hepatocytes in suspension incubations suffer a rapid loss of drug-metabolizing enzyme activity (Smith et al., 2012), and such suspension assays are therefore inappropriate for consistent and accurate determination of CL_{int} values in the range of 1 μl/min/10^6 cells and lower unless the procedure is greatly modified as with the Relay method for example (Di et al., 2012, 2013). The objective of this investigation was to assess the capability of HepaRG and a multidonor primary hepatocyte pool plated as a monoculture and in the HμREL co-culture in robustly defining CL_{int} and accurately predicting human in vivo hepatic clearance for slowly metabolized compounds. Whereas the assay setups are more complex than the suspension format on which the Relay method is based, the sampling and sample treatment elements are uncomplicated and minimal. The results indicate that, despite the fact that it was not possible to determine a CL_{int} value for theophylline in any of the plated human hepatocytes assays, all three offer improved potential for CL_{int} determination of slowly metabolized compounds over a standard suspension assay format where incubation time is typically limited to 2–4 hours. All three assays allowed CL_{int} values in the range of 1 μl/min/10^6 and below to be obtained, and it was possible to define values as low as 0.2 μl/min/10^6 cells (disopyramide). However, only with the HμREL co-culture assay could CL_{int} values be defined on replicate occasions for disopyramide, the compound with the lowest CL_{int}. The three assays did allow for different times of incubation, limited by decline in enzyme activity. Smith et al. (2012) previously reported retained activity for cytochrome P450 3A4 (CYP3A4) and CYP1A2 in monocultures of primary human hepatocytes up to approximately 10 hours. This is in line with data from our laboratory on the five-donor hepatocyte pool in monoculture used in this study. Comparison of the same pool of primary human hepatocytes in HμREL co-cultures made it apparent that a log-linear compound concentration-time relationship was retained up to 72 hours. Taking the cell densities and the incubation times for these three systems (see *Materials and Methods*) into account reliable CL_{int}, limiting extrapolation of half-life to twice the incubation time, could

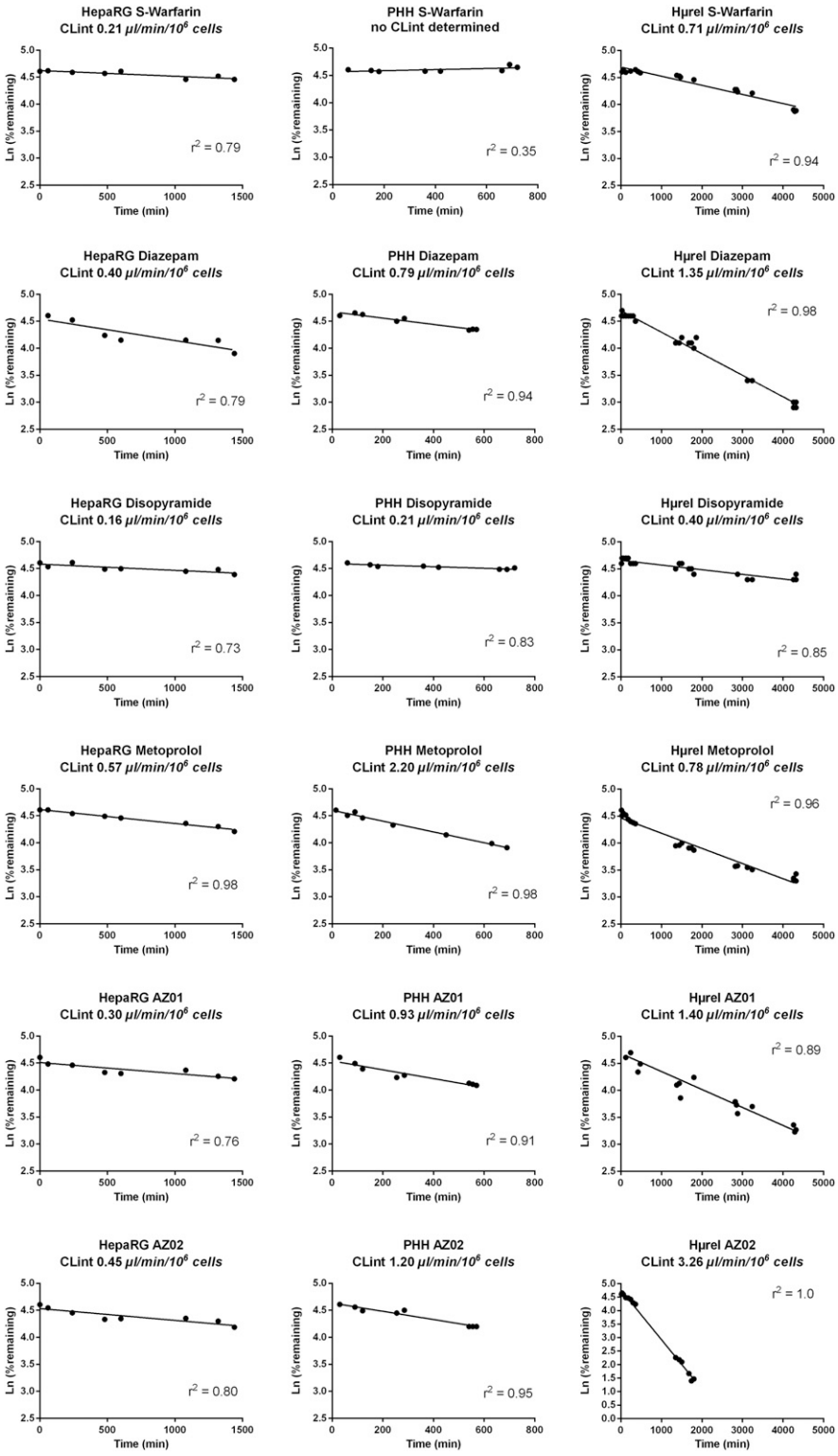

Fig. 2. Examples of substrate depletion plots using HepaRG, PHH, and HμREL.

theoretically be determined down to 0.3 μl/min/10^6 cells in HepaRG, 0.5 μl/min/10^6 cells in PHH, and 0.2 μl/min/10^6 cells in HμREL. Accordingly the longer incubation times of 24 and 72 hours afforded by the HepaRG and HμREL assays should contribute to increasing robustness and reproducibility of the data compared with PHH; indeed, the HμREL co-culture assay was able to provide CL_{int} data from a minimum of triplicate consecutive assays for 85% of the low CL_{int} compounds assayed. Besides a longer incubation time, the number of data points (i.e., replicates and or additional time points) is of importance when trying to assign statistically significant CL_{int} values in the low range, and this possibility also assisted in the HμREL assay (see example of substrate depletion plots in Fig. 2). The systems tested evidently have too short incubation time spans or too low enzymatic activity to show theophylline depletion. This finding is supported by

TABLE 2

In vitro clearance $(CL)_{int}$ (mean ± S.D., n = 3 for HepaRG and PHH, n = 4 for HμREL), predicted in vivo CL_{int} (fold difference between predicted and observed in parentheses) and observed in vivo CL_{int}

Compounds	In vitro CL_{int}				Predicted In Vivo CL_{int}			Observed In Vivo CL_{int}
	Suspended hepatocytes	HepaRG	PHH	HμREL	HepaRG	PHH	HμREL	
		μl/min/10^6 cells				*ml/min/kg*		*ml/min/kg*
S-warfarin	nv	0.21[b]	nv	0.71 ± 0.3	0.2 (3.3)	nv	0.4 (6.7)	0.06
Diazepam	nv	0.40[b]	0.79 ± 0.3	1.35 ± 0.09	0.5 (1.0)	0.8 (1.6)	1.2 (2.4)	0.5
Disopyramide	nv	0.16[a]	0.21[a]	0.40 ± 0.2	0.7 (−1.3)	0.9 (1.0)	1.5 (1.7)	0.9
Theophylline	nv	nv	nv	nv	nv	nv	nv	1.4
Metoprolol	nv	0.57[b]	2.2 ± 0.7	0.78 ± 0.15	6.3 (−5.9)	12.3 (−3.0)	8.0 (−4.6)	37.1
AZ01	nv	0.30 ± 0.2	0.93 ± 0.2	1.40 ± 0.2				nv
AZ02	nv	0.45 ± 0.2	1.2 ± 0.2	3.26 ± 0.3	0.5 (−9.4)	1.1 (-4.3)	2.3 (−2.0)	4.7
Ketoprofen	6.6 ± 0.3	nv	3.9[b]	4.3[a]	nv	7.5 (3.1)	8.1 (3.4)	2.4
Sildenafil	9.7 ± 0.9	5.3[a]	7.0 ± 2.0	6.2 ± 1.7	6.4 (−1.3)	7.9 (−1.1)	7.2 (−1.2)	8.6
Carvedilol	38.1 ± 7.0	8.6[a]	26.3 ± 3.3	34.2 ± 2.4	6.7 (−2.3)	15.6 (1.0)	19.0 (1.2)	15.4
Imipramine	4.7 ± 1.1	nv	8.6 ± 3.1	1.7 ± 0.06	nv	50.9 (1.2)	15.3 (−2.7)	41.5
Ketanserin	33.1 ± 3.6	0.65[a]	8.3 ± 5.8	14.1 ± 0.5	1.5 (−6.7)	10.2 (1.0)	15.3 (1.5)	10.1

nv, no value.
[a]n = 1.
[b]n = 2.

data from attempts to determine CL_{int} for theophylline using multidonor platable pools of hepatocytes in plated monoculture or co-culture, communicated at recent conferences (Kazmi et al., 2014) XenoTech) poster presented at ISSX/JSSX Conference 2014 in San Francisco, CA and Hieronymus et al. (2015) poster presented at AAPS annual meeting and exposition 2015 in Orlando, FL).

At AstraZeneca, human hepatocyte CL_{int} values for novel compounds are used in combination with a regression line equation, defined by an in vitro-in vivo correlation for drugs with known in vivo CL, to predict human hepatic clearance (Sohlenius-Sternbeck et al., 2012). To test the extent to which HepaRG, PHH and HμREL assays give CL_{int} values comparable with those defined in the standard AstraZeneca human hepatocyte suspension assay, a limited number of compounds with diverse enzymology from that standard assay were also incubated in the three plated hepatocyte assays. HepaRG gave lower CL_{int} values compared with the primary hepatocyte incubations, compounded by low CYP2D6 activity (Kanebratt and Andersson, 2008) accounting for poor CL_{int} definition for carvedilol and imipramine. Surprisingly, imipramine had a CL_{int} almost 3-fold lower in the HμREL assay than the standard assay, resulting in a 2.7-fold underprediction of the in vivo CL_{int} value. Since the HμREL assay gave similar CL_{int} to the standard assay for sildenafil and carvedilol, which cover the CYP2C9 and CYP2D6 enzymology primarily responsible for imipramine metabolism, this result cannot easily be rationalized. Evidently, aldoketoreductase activity, responsible for the metabolism of ketanserin (Akabane et al., 2012), may also be lower in the HepaRG cells since the CL_{int} was only 2% of that from the suspension hepatocyte incubation. Ketanserin also displayed a slower rate of metabolism in the PHH assay compared with the suspension and HμREL assays. The ketoprofen data indicate that UGT2B7 activity was preserved and comparable with hepatocytes in suspension, both when culturing the primary hepatocytes as monoculture and in HμREL co-culture. Although this was a limited compound set, major drug-metabolizing cytochrome enzymology was covered and the HμREL, and PHH assays in particular appears to offer promise as an assay that can be used seamlessly and interchangeably alongside a standard suspension hepatocyte assay.

To add value in a drug discovery setting, these assays most of all need to be capable of accurately predicting in vivo human hepatic clearance. For the compounds with available clinical PK data, the ability of each in vitro assay to accurately predict in vivo human hepatic CL_{int} was assessed as described previously (Sohlenius-Sternbeck et al., 2012). The HμREL and PHH assay data were able to predict in vivo human hepatic CL_{int} to within 3-fold of the observed values for seven of 10 (HμREL) and seven of nine (PHH) compounds where CL_{int} could be determined, with the AFE in prediction being 2.3 and 1.7, respectively. This good predictivity shows that, for this purpose, either of these two assay formats offers an alternative to the Relay method and the HepatoPac culture system, both of which have been shown to give good clearance predictions (Di et al., 2012; Chan et al., 2013). For the HepaRG assay, the predictions were less successful, with only four of the eight compounds for which a CL_{int} could be obtained having a predicted in vivo human hepatic CL_{int} within 3-fold of observed.

In summary, all three investigated systems allow for longer incubation times than a standard hepatocyte suspension format and demonstrated the ability to define CL_{int} values as low as 0.2 μl/min/10^6 hepatocytes. The HμREL co-culture assay was able to provide a higher level of reproducibility than the PHH and HepRG assays for low CL_{int} compounds. In addition, both the HμREL and PHH assays gave similar CL_{int} values to those defined by a standard hepatocyte suspension assay for a small set of drugs with higher CL_{int} values and diverse enzymology, and data from both assays could predict in vivo human hepatic CL_{int} to within 3-fold for 70% of the compounds tested. Therefore, whereas both PHH and the HμREL hepatocyte/stromal cell co-culture appear to be promising solutions to the problem of robustly defining CL_{int} for slowly metabolized compounds, the HμREL assay appears somewhat superior regarding overall assay performance, with the opportunity to run incubations up to 72 hours. Apart from accurate estimation of low CL_{int} values, the assay also provides the opportunity for metabolite identification in a human hepatic cell system for compounds that are stable in suspended hepatocyte incubations.

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Authorship Contributions

Participation in research design: Bonn, Svanberg, Janefeldt, Grime.
Conducted experiments: Svanberg, Janefeldt, Hultman.
Performed data analysis: Bonn, Svanberg, Grime.
Wrote and contributed to the writing of the manuscript: Bonn, Svanberg, Janefeldt, Hultman, Grime.

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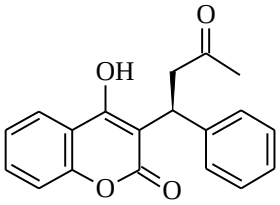
TITLE: Determination of Human Hepatocyte Intrinsic Clearance for Slowly Metabolized Compounds: Comparison of a Primary Hepatocyte/Stromal Cell Co-Culture with Plated Primary Hepatocytes and HepaRG

Britta Bonn, Petter Svanberg, Annika Janefeldt, Ia Hultman and Ken Grime



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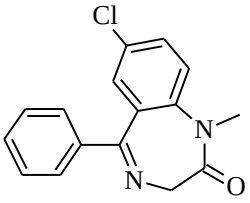

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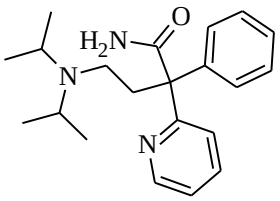

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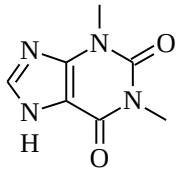

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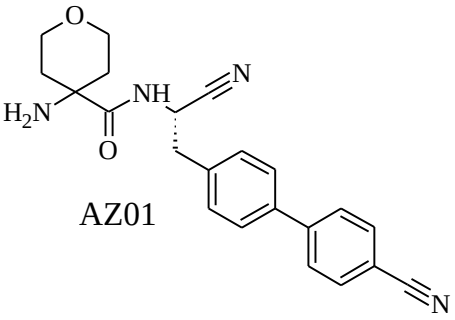

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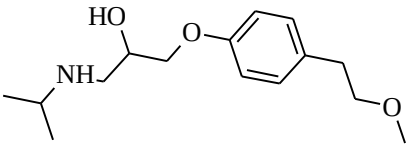

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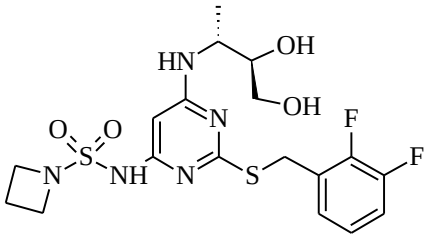

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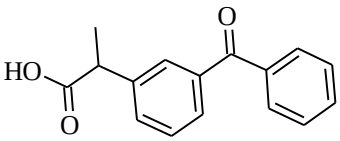

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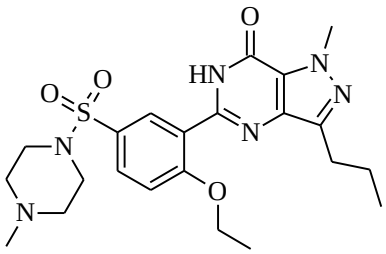

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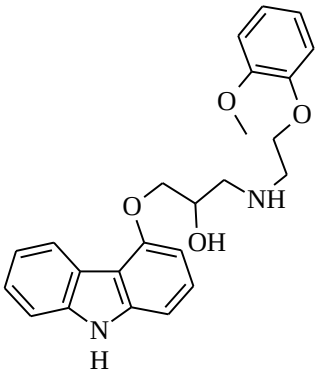
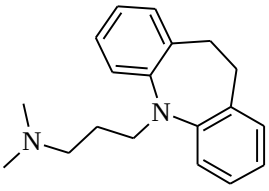
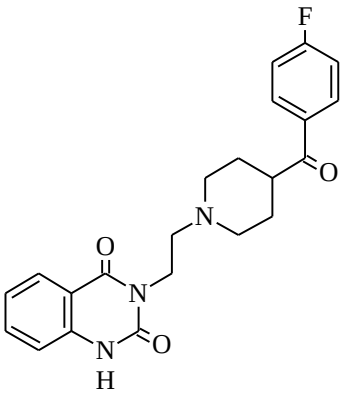


Carvedilol



Imipramine



Ketanserin

Supplemental Figure 1 *Chemical structures of CL_{int} validation test set.*

Supplemental Table 1 *Monoisotopic mass, ionization mode, mass transitions, cone voltage and collision voltage used for the LC-MSMS analysis of the 12 test compounds.*

Compound name	Monoisotopic mass	Charge	Parent (m/z)	Daughter (m/z)	Cone voltage	Collision voltage
S-warfarin	308.105	ES-	307.0994	160.8722	34	16
Diazepam	284.072	ES+	285.3267	153.9804	46	28
Disopyramide	339.231	ES+	340.6594	194.0255	46	46
Metoprolol	267.183	ES+	268.2363	116.0694	22	22
Theophylline	180.065	ES+	181.0444	123.9884	46	16
AZ01	374.174	ES-	373.0513	345.898	26	10
AZ02	475.116	ES-	474.0119	352.8891	34	22
Ketoprofen	254.094	ES+	255.0214	208.9927	12	10
Sildenafil	474.205	ES+	475.101	99.406	47	28
Carvedilol	406.189	ES+	407.1971	100.0039	35	28
Imipramine	280.194	ES+	481.1235	85.8675	47	28
Ketanserin	395.165	ES+	396.1723	145.9566	35	50