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A unified model for predicting human hepatic, metabolic clearance from *in vitro* intrinsic clearance data in hepatocytes and microsomes

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Running Title

Predicting human drug metabolic clearance

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Abbreviations

SF, scaling factor; Cl_{int}, intrinsic clearance; Cl_h, hepatic clearance; $Cl_{int,\ in\ vitro}$ *in vitro* intrinsic clearance; $Cl_{int,\ in\ vivo}$, *in vivo* intrinsic clearance; fu_p, plasma unbound fraction; R_B, blood-to-plasma concentration ratio; fu_{inc}, unbound fraction in incubations *in vitro*; Q_h, hepatic blood flow; $Cl_{h,}$, hepatic clearance; *afe*, average fold-error.

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Abstract

The aim of this study was to evaluate a unified method for predicting human *in vivo* intrinsic clearance ($Cl_{int,\ in\ vivo}$) and hepatic clearance (Cl_h) from *in vitro* data in hepatocytes and microsomes applying the unbound fraction in blood (fu_b) and *in vitro* incubations (fu_{inc}). Human $Cl_{int,\ in\ vivo}$ was projected using *in vitro* data together with biological scaling factors and compared with the unbound intrinsic clearance ($Cl_{int,\ ub,\ in\ vivo}$) estimated from clinical data using liver models with and without the various fu terms. For incubations conducted with FCS (N = 14), the observed $Cl_{int,\ in\ vivo}$ was modelled well assuming fu_{inc} and fu_b were equivalent. $Cl_{int,\ ub,\ in\ vivo}$ was predicted best using both fu_b and fu_{inc} for other hepatocyte data (N = 56; r^2 = 0.78, p = 3.3 x 10^{-19}, average fold error = 5.2). A similar model for $Cl_{int,\ ub,\ in\ vivo}$ was established for microsomal data (N = 37; r^2 = 0.77, p = 1.2 x 10^{-12}, average fold error = 6.1). Using the model for $Cl_{int,\ ub,\ in\ vivo}$ (including a further empirical scaling factor), the Cl_h in humans was also calculated according to the well-stirred liver model for the most extensive dataset. $Cl_{int,\ in\ vivo}$ and Cl_h were both predicted well using *in vitro* human data from several laboratories for acidic, basic and neutral drugs. The direct use of this model using *in vitro* human data only to predict the metabolic component of Cl_h is attractive, as it does not require extra information from pre-clinical studies in animals.

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Existing methods for the prediction of drug clearance in humans involve the use of *in vitro* human metabolic stability (intrinsic clearance, Cl_{int}) data (Iwatsubo et al., 1997), consideration of preclinical animal data (Boxenbaum, 1982) or a combination of these approaches (Lave et al., 1997; Naritomi et al., 2001). *In vitro* drug metabolism kinetic parameters can provide an estimate of *in vivo* Cl_{int} via "scaling" with established biological scaling factors (SFs) e.g. hepatocellularity for isolated hepatocytes, or a SF for microsomes based on incomplete microsomal recovery from human liver tissue using the cytochrome P450 (CYP) content in homogenate and microsomes (Houston, 1984). Cl_{int} may subsequently be used to provide an estimate of hepatic clearance ($Cl_{hepatic}$ or Cl_h) using several liver models (Houston, 1984; Ito and Houston, 2004).

To date the more extensive analyses of human clearance predictions have concentrated on CYP substrates and data has therefore been generated in human liver microsomes (Iwatsubo et al., 1997; Naritomi et al., 2001; Obach, 1999). In general, these studies have been less comprehensive in the range of approaches investigated with only occasional attention given to chemical class (Obach et al., 1997). Interestingly, these reports have also assessed the ability to predict human Cl_h rather than more fundamental parameter, Cl_{int} , as advocated initially (Houston, 1984; Ito and Houston, 2004). Some controversy also still exists over use of fu_{inc}, with some labs having suggested that fu_{inc} and fu_b may cancel, negating their inclusion in liver models (Obach et al., 1997). Recent reports have challenged this assumption (Obach, 1999; Austin et al, 2002) and perhaps suggest that consideration of Cl_h rather than $Cl_{int, in\ vivo}$ may de-sensitise such analyses, particularly to errors associated with higher enzyme activities (Ito and Houston, 2004).

The aim of this study was to investigate direct *in vitro-in vivo* scaling of human *in vitro* data generated in hepatocytes and microsomes for predicting human clearance *in vivo* applying recently described models for estimating fu_{inc} (Austin et al., 2002, Austin et al., 2005). In order to provide a more mechanistic insight, i*n vitro* human Cl_{int} data were

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compiled from recent in-house and published studies and associated *in vivo* Cl_{int} values were derived from published Cl_h data. Combining datasets permitted extension of models described in previous studies. In addition, the relative drug binding within blood and *in vitro* incubation matrices is considered further, with respect to their incorporation into liver models.

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Methods

Data collection

$Cl_{int,\ in\ vitro}$ data derived from hepatocyte (Lave et al., 1997; Lau et al., 2002; Shibata et al., 2002; Naritomi et al., 2003) or microsomal incubations (Naritomi et al., 2001; Obach, 1999; Carlile et al., 1999; Andersson et al., 2004) were generated in the authors' laboratory and collated from several published studies (Tables 1-3). Incubation conditions for data generated in the authors' laboratory have been detailed previously (Austin et al., 2002; McGinnity et al., 2004). Data from microsomal studies reflected a variety of methods including formal Michaelis-Menten kinetic analysis (Cl_{int} = Vmax/Km for specific metabolite(s) formation (Carlile et al., Andersson et al., 2004) and substrate depletion at low substrate concentrations (Obach, 1999; Naritomi et al., 2001), which was used for all hepatocyte data. Datasets were compiled with several key objectives in mind: to expand existing databases substantially; to provide some assessment of inter-lab variability; and to complement external datasets in terms of representation from different chemical classes covering a range of physico-chemical properties. Particular emphasis was put on hepatocyte data generated in this laboratory for acidic drugs, which were sparsely represented in external datasets. Data produced in the authors' laboratory represent the mean of at least 3 hepatocyte donors. Replicates and variability in other data can be found in the original reports.

The hepatocyte data were compiled from several sources: incubations conducted with more simple cell suspensions (Lau et al, 2002; Naritomi et al, 2003; authors' laboratory); and cells cultured in the presence of exogenous protein. Additional protein was either autologous (human) serum (Shibata et al, 2002) or 10% fetal calf serum (FCS) for incubations conducted with cultured cells for up to 72 h (Lave et al., 1997; Schneider et al., 1999). $Cl_{int,\ in\ vitro}$ was estimated using only an fu_p correction for incubations which

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included serum since binding to plasma proteins was deemed to be greater than any hepatocyte binding (Shibata et al., 2002; Austin et al., 2005). Since inter-species differences in plasma protein binding precluded such a simple correction for incubations conducted in the presence of FCS, a correction was made only for binding to cells and this dataset was treated separately. For the remaining datasets, the unbound fraction in the incubation (fu_{inc}) was applied either as reported by the authors (Naritomi et al., 2003) or predicted from a consideration of chemical class and either $logD_{7.4}$ or logP:

For microsomes: $\log(1\text{-}fu/fu) = 0.53\log P/D - 1.42$ (Austin et al., 2002)

For hepatocytes: $\log(1\text{-}fu/fu) = 0.40\log P/D - 1.38$ (Austin et al., 2005)

$Cl_{int,,in\ vivo,\ ub}$ was calculated from values of Cl_h, the unbound fraction in plasma (fu_p) and blood-to-plasma concentration ratio (R_B; fraction unbound in blood, $fu_b = fu_p/R_B$) reported for each drug according to the well-stirred or dispersion liver model (Shibata et al, 2002). Where values of R_B were not provided or readily available, this parameter was assumed to be unity for neutral and basic compounds and 0.55 for acids. The assumptions of the various liver models have been detailed previously (Ito and Houston, 2004).

Where the same drug had been studied by several laboratories (Tables 4 and 5), individual values for Cl_h and f_{ub} were used to estimate $Cl_{int,\ ub,\ in\ vivo}$ and $Cl_{int,\ in\ vitro}$ and fu_{inc} were used to project (scaled) $Cl_{int,\ ub,\ in\ vivo}$. The mean values for key parameters were then used for further modeling and statistical analyses. The sources used to derive key parameters are too extensive to be listed here but can be found in the original references cited.

Prediction of the in vivo intrinsic clearance

$Cl_{int,\ in\ vivo}$ was predicted using using SFs for hepatocytes and microsomes to convert the unit of the Cl_{int} from ul/min/10^6 cells or ul/min/mg protein to ml/min/kg using an estimate

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of human liver hepatocyte content. Hepatocyte content (or hepatocellularity) was routinely 120 x 10^6 cells/g liver. Some variability was evident in the microsomal protein concentration per gram of liver (45 – 50 mg protein/g liver), and the human liver weight values (1500 – 1800 g liver/70 kg) cited but this was not considered to impact significantly on the conclusions of this study.

Impact of plasma and in vitro binding

In *in vitro* studies, some drugs bind nonspecifically within the matrix, hence the kinetic parameters estimated need to be corrected to reflect the unbound drug. Obviously, if fu_b and fu_{inc} were equivalent, their terms in the equations for liver models would cancel out :

$$Cl_h = (Q_h \times fu_b \times (Cl_{int,}*/fu_{inc})) / (Q_h + fu_b \times (Cl_{int}*/fu_{inc}))$$

where $Cl_{int}* = (Cl_{int,\ in\ vitro} \times SF)$ and fu_{inc} is the unbound fraction in hepatocytes or microsomes, Q_h = liver blood flow (20 ml/min/kg).

The role of fu_{inc} and fu_b was investigated using several approaches. Firstly, the human $Cl_{int,\ in\ vivo}$ values were predicted assuming fu_{inc} to be unity and compared with $Cl_{int,\ in\ vivo}$ calculated from "deconvolution" of the well-stirred model, acknowledging the potential limitations of this model for highly extracted compounds (Ito and Houston, 2004):

$$Cl_{int,\ in\ vivo} = (Q_h \times Cl_h)/ (fu_b \times (Q_h – Cl_h))$$

$Cl_{int,\ in\ vivo}$ data was provided in several reports (Shibata et al, 2002; Naritomi et al, 2003). Relationships obtained using the parallel tube model to compute $Cl_{int,\ in\ vivo}$ were very similar to those reported (data not shown).

$Cl_{int,\ in\ vivo}$ values were also compared without consideration of both fu_{inc} and fu_b. Finally, $Cl_{int,\ in\ vivo}$ predictions using fu_{inc} were compared with estimates from clinical PK invoking fu_b ($Cl_{int,\ ub,\ in\ vivo}$).

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Prediction of hepatic (blood) clearance

Using the *in vivo* intrinsic clearance estimates outlined above, $Cl_{h,}$ was calculated according to the 'well-stirred' liver model as follows:

$$CL_{h,} = (Q_h \times (fu_b \times Cl_{int,\ in\ vivo})) / (Q_h + (fu_b \times CL_{int,\ in\ vivo}))$$

Accuracy of predictions

Quantitative linear regression analysis was not considered appropriate for the predicted Cl_h data since it is clearly not homoscedastic ie. the error in the y data is not even approximately constant across the full range of the data: the spread in the y data decreases with increasing log(predicted clearance). This behaviour is a consequence of the format of the well-stirred model, which gradually forces compounds with increasingly high intrinsic clearance towards the same value of predicted clearance, that of hepatic blood flow, Q_h.

Quantitative regression analyses were performed however for the log (predicted) and log (observed) values for $CL_{int,\ in\ vivo}$ to obtain the regression equation, correlation coefficient (r^2) and a summary of its statistics (standard deviation, SD, the F statistic and the p value). The average fold error (*afe*) of each prediction method was also calculated to provide a measure of bias with equal value to under- and over-predictions:

$$afe = 10^{\left|\frac{1}{N}\sum \log\frac{\text{Predicted}}{\text{Observed}}\right|}$$

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Results

Prediction of $Cl_{int,\ ub}$ and Cl_h from hepatocyte incubations

Figure 1 (A – C) shows plots of log(observed Cl_h) against log(predicted Cl_h) where hepatic clearance is predicted from the well-stirred model, including or excluding the various fu terms. For incubations conducted without FCS, the model with both fu_{inc} and fu_b corrections shows the best *qualitative* relationship between log(observed Cl_h) and log(predicted Cl_h). The model with an fu_b correction only (ie. ignoring fu_{inc}) appears to show the next best *qualitative* trend, with the uncorrected model showing a very scattered relationship with the acidic compounds becoming separated from the neutral and basic compounds.

Inter-laboratory variability in $Cl_{int,\ in\ vitro}$ was acceptable ($\leq$ 3-fold) for some compounds studied in hepatocytes under similar conditions (diclofenac, imipramine, naloxone, propranolol and tolbutamide) and microsomes (diazepam, ibuprofen and tolbutamide). Variability was more significant for other compounds, possibly due to differences in quality of liver samples, established inter-donor differences, incubations conditions eg. potential effects of co-incubating hepatocytes with serum. This highlights the challenges in compiling datasets from several sources.

A more appropriate transformation of the data for linear regression analysis is to consider $Cl_{int,\ ub}$ ie. log($Cl_{int,\ in\ vivo,\ ub}$) plotted against log(predicted $Cl_{int,\ ub}$) as shown in Figure 1D – F, which also provides a substantial dynamic range with which to study *in vitro-in vivo* comparisons at a detailed, mechanistic level (Ito and Houston, 2004). These plots show a fairly constant spread in the y data across the full range. Linear regression has been applied to the 3 different scaling models in Figures 1D to 1F, and the resulting statistical parameters are given in Table 6, along with the average fold error of each method. In

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terms of the statistics of linear regression, the model which includes both fu_{inc} and fu_b corrections provides the best fit to the data as shown by the significantly higher r^2 value and lower regression standard deviation (SD) compared with the other two models (Table 6). The F statistic and p-value of this model is also clearly superior to the other two models. The slope of the model including both correction terms (1.08) is close to unity, but the intercept (0.38) reveals a constant bias in the model leading to an approximately 5-fold under-prediction of $Cl_{int,, ub}$ across the range of compounds. Despite evidence of inter-laboratory variability for some data, this model appears largely independent of lab and chemical class. In terms of regression statistics, the model utilizing only an fu_b correction (ie. omitting fu_{inc}) has the next best performance, with the uncorrected model performing poorly.

Interestingly, sub-dividing this dataset into chemical class indicated that the predictions using the model without f_{ub} and fu_{inc} corrections were very poor for acidic drugs, in particular: predictions appeared better for basic and neutral compounds. Closer inspection of the ratio between fu_{inc} and fu_b for each chemical class provided further mechanistic insight (Figure 3). For the majority of the basic and neutral drugs studied, this ratio was between 1 and 10. However for some neutral (including the benzodiazepines diazepam, oxazepam and the lipophilic calcium channel blocker, felodipine) and lipophilic, basic compounds (for example, mibefradil), the ratio was somewhat larger. By contrast, for acidic drugs, this ratio was much higher on average reflecting the extensive binding to albumin for many of these compounds *in vivo*.

Prediction of $Cl_{int, ub}$, and Cl_b from hepatocyte incubations containing FCS

By contrast, for hepatocyte incubations conducted (up to 72 h) in the presence of FCS, Cl_b was described well under all conditions and $Cl_{int, ub, in\ vivo}$ was predicted well from $Cl_{int, in\ vitro}$ (Figure 2 and Table 6). However, models incorporating only fu_b or both fu_{inc} and

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fu_b, while statistically significant, yielded a large bias as evidenced by deviations in the gradient, intercept and the associated *afe* (42-86 fold; Table 6). The most robust model (in terms of bias in gradient, intercept and *afe*) for $Cl_{int,\ ub,\ in\ vivo}$ was derived from ignoring both fu_{inc} and fu_b.

Prediction of $Cl_{int,\ ub}$, from microsomal data

Applying this knowledge to the microsomal data again yielded a robust, highly significant relationship between the scaled $Cl_{int,\ ub,\ in\ vivo}$ estimate (using both fu_b and fu_{inc}) and that derived from clinical PK data ($r^2 = 0.77$, $p = 1.22 \times 10^{-12}$; Figure 4). The *afe* (6.1) for this dataset was very similar to the larger human hepatocyte dataset.

Further model development

For the most extensive hepatocyte dataset, since the fu_{inc} and fu_b corrected model was highly correlated but with a constant offset, the regression equation was applied to data in order to evaluate the most precise clearance prediction for test compounds:

$$Cl_{int,\ in\ vivo} = Cl_{int}^{*} \times fu_p/(R_B \times fu_{inc})$$

and ….

$$Cl_h = (Cl_{int,\ in\ vivo} \times Q_h)/(Cl_{int,\ in\ vivo} + Q_h)$$

Transformation of these predictions of $Cl_{int,\ in\ vivo}$ to projections of Cl_b finally give *afe* values of 1.9 for the fu_{inc} and fu_b corrected method, 2.0 for the fu_b corrected method and 15.1 for the uncorrected method. Therefore the model with both correction terms has the potential for making the most precise predictions of Cl_h for external test compounds.

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Discussion

Prediction of human pharmacokinetics remains an intense area of research. It is widely accepted that simple allometric methods are generally not suitable for predicting human clearance for compounds, which exhibit low to intermediate extraction *via* metabolism (Lave et al., 1997; Nagilla and Ward, 2004). *In vitro-in vivo* scaling of metabolic clearance has received much attention (Houston, 1984; Iwatsubo et al., 1997; Houston and Carlile, 1997; Naritomi et al., 2001) and also provides input for physiologically-based pharmacokinetic (PBPK) models (Theil et al., 2003). Early, extensive investigations of *in vitro-in vivo* scaling of hepatic metabolic clearance focused largely on the rat as a model species (Houston, 1984; Houston and Carlile, 1997). More recently, several labs have extended these analyses of human clearance predictions. These studies have tended to concentrate on a relatively small number of drugs metabolised by CYPs and data has therefore been generated in human liver microsomes (Iwatsubo et al., 1997; Obach et al., 1997). In general, these studies have been less comprehensive in the range of approaches investigated with only occasional attention given to chemical class (Obach, 1999). Interestingly, these reports have also assessed the ability to predict human $Cl_{hepatic}$ rather than more fundamental parameter, Cl_{int}, as advocated in the seminal work by Houston and colleagues (Houston, 1984; Houston and Carlile, 1997; Ito and Houston, 2004).

In order to assess various approaches to predicting human *in vivo* hepatic metabolic clearance a database has been collated from in-house and literature sources. Hepatocytes appear the most appropriate *in vitro* system to assess hepatic metabolic stability. Studies in the rat have advocated hepatocytes for more accurate prediction of rapid clearance (Houston, 1984; Houston and Carlile, 1997) and hepatocytes not only contain CYPs and the major phase 2 enzymes but also hepatobiliary transporters, which

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may modulate concentrations of substrate accessible to drug metabolising enzymes (Shitara et al, 2003; Liu and Pang, 2005). Advances in cryopreservation technology have recently enabled studies with human hepatocytes to become more routine (Li et al., 1999; McGinnity et al., 2004). The present analysis considered primarily *in vitro* and *in vivo* Cl_{int} estimates, the latter obtained from 'deconvoluting' Cl_h to generate a wide range of values to allow detailed, mechanistic comparisons. Predictions of the kinetic parameter Cl_h were also evaluated.

The role of plasma protein binding in clearance prediction has been the subject of some controversy. While the basic tenet of pharmacokinetics states that the unbound drug concentration in the plasma dictates tissue distribution, some reports using microsomes have suggested that *in vitro* Cl_{int} may provide a better estimate of *in vivo* clearance of *total* rather than *unbound* drug (Obach et al., 1997; Lin et al., 1999). Presumably, the assumption was that fu_b and fu_{inc} effectively nullified in the liver model calculation, negating the measurement of either process. However, measurements of *in vitro* binding has shown that drug binding within these two matrices is not equivalent and hence fu_{inc} should not be ignored, in principle (Obach, 1999; Austin et al., 2002; Austin et al., 2005). Most models were reasonable for neutral and basic drugs, particularly for Cl_h as reported previously (McGinnity et al., 2004; Riley and Kenna, 2004; Davis and Riley, 2004). However, examination of the hepatocyte data indicated the need to consider both fu_{inc} and fu_b for incubations not conducted in presence of exogenous protein to include all chemistries. Previous studies have shown that, as for microsomes, fu_{inc} for hepatocytes may be substantial for lipophilic neutral and basic compounds and can be predicted from consideration of charge at physiological pH and lipophilicity (Austin et al., 2005). Some neutral compounds (eg. the benzodiazepines diazepam and oxazepam) and lipophilic, bases eg. mibefradil (Figure 3) also showed a similar effect, as suggested previously (Riley and Kenna, 2004). By contrast, hepatocyte binding for most acidic drugs is low

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compared with their binding to distinct sites on albumin. Inclusion of both fu_{inc} and fu_b terms resulted in a unified model, the robustness of which was indicated by its ability to translate across both labs and chemical classes.

Interestingly, several reports on relatively small numbers of compounds have indicated that data generated from hepatocyte incubations containing exogenous protein (either FCS or serum) may yield more direct estimates of $Cl_{int,\ in\ vivo}$ and Cl_h (Lave et al, 1997; Shibata et al, 2003). However, the non-physiological composition of such assays and effects other than those attributable to protein binding (Blanchard et al, 2005) remain the subject of much controversy and may contribute to some of the variability depicted in Table 4. Furthermore, such methodology poses challenges in terms of determining low Cl_{int} values accurately and may necessitate long incubation periods (up to 72 h) with cultured cells, which may incur a (differential) loss of CYP activity.

Previous reports have debated the pros and cons of inclusion of fu_{inc} for data from HLM, hence this topic was not analysed in depth here. Inclusion of both fu_{inc} and fu_b yielded a highly significant correlation between predicted and observed $Cl_{int,,\ ub}$ with a bias or offset similar to that shown in Figure 1F, as suggested previously for both Cl_h and Cl_{int} (Iwatsubo et al., 1997; Obach, 1999; Naritomi et al., 2001).

A model incorporating *in vivo* and *in vitro* data from preclinical and clinical studies has the potential advantage of providing a drug-specific factor that would theoretically correct for any systematic difference between *in vitro* and *in vivo* parameters (Naritomi et al., 2001; Naritomi et al., 2003). However, further work using larger databases is required to validate this approach and evaluate whether various “factors” (which may be passive or active in origin) translate routinely across a range of species.

In summary, human *in vitro* Cl_{int} provided accurate predictions of $Cl_{int,\ ub}$ and Cl_h with more robust models resulting from incorporation of fu_{inc} for both hepatoyctes and microsomes. Using the standard biological SFs alone to scale *in vitro* Cl_{int} to provide *in vivo* CL_{int}

estimates resulted in a systematic under-prediction for data from both hepatocytes and microsomes – an observation consistent with other studies using *human* tissues. Interestingly, previous analyses of rat predictions from both *in vitro* systems did not show such a systematic bias (Ito and Houston, 2004). While a scientific rationale for this observation is lacking currently for these specific datasets, likely contributors include: active transport processes *in vivo* not reflected adequately *in vitro* (Liu and Pang, 2005, Shitara et al., 2005); incorrect assumptions within the liver models; inter-individual variability atypical kinetics eg. for CYP3A4 substrates (Houston and Galetin, 2004); quality of tissue used for hepatocyte preparations; some extra-hepatic metabolism *in vivo*. Future studies will aim to provide a systematic analysis and mechanistic interpretation, which could then be applied to modify existing scaling strategies further.

References

Andersson, TB, Bredberg, E, Ericsson, H and Sjoberg, H (2004). An evaluation of in vitro metabolism data for predicting the clearance and drug-drug interaction potential of CYP2C9 substrates. *Drug Metab. Dispos* **32**: 715-721.

Austin RP, Barton P, Cockroft SL, Wenlock MC, and Riley RJ (2002). The influence of nonspecific microsomal binding on apparent intrinsic clearance, and its prediction from physicochemical properties. *Drug Metab Dispos* **30**:1497-1503.

Austin RP, Barton P, Mohmed, S and Riley RJ (2005) The binding of drugs to hepatocytes and its relationship to physicochemical properties. *Drug Metab Dispos* **33**: 419-425.

Blanchard, N, Alexandre, E, Abadie, C, Lave, T, Heyd, B, Mantion, G, Jaeck, D, Richert, L and Coassolo (2005). Comparison of clearance predictions using primary cultures and suspensions of human hepatocytes. Xenobiotica **35**: 1-15.

Boxenbaum, H (1982). Interspecies scaling, allometry, physiological time, and the ground plan of pharmacokinetics. *J. Pharmacokin. Biopharm.* **10**: 201-227.

Carlile, DJ, Hakooz, N, Baylss, MK and Houston, JB (1999). Microsomal prediction of in vivo clearance of CYP2C9 substrates in humans. *Br. J clin. Pharmacol*. **47**: 625-635.

Davis, AM and Riley, RJ (2004). Predictive ADMET studies: the challenges and the opportunities. *Curr Opin in Chem Biol* **8**: 378–386.

Houston, BJ and Carlile, DJ (1997). Prediction of hepatic clearance from microsomes, hepatocytes and liver slices. *Drug Met. Rev*. **29**: 891-922.

Houston, JB (1984). Utility of *in vitro* drug metabolism data in predicting *in vivo* metabolic clearance. *Biochem. Pharmacol.* **47**:1469-1479.

Houston, JB and Galetin, A (2004). Progress towards prediction of human pharmacokinetic parameters from *in vitro* technologies. *Drug Met. Rev*. **35**: 393-415.

Ito, K and Houston, JB (2004). Comparison of the use of liver models for predicting drug clearance using in vitro kinetic data from hepatic microsomes and isolated hepatocytes. *Pharm Res.* **21**: 785- 792.

Iwatsubo, Hirota, N, Ooie, T, Suzuki, H, Shimada, N, Chiba, K, Ishizaki, T, Green, CE, Tyson, CA and Y. Sugiyama, Y (1997). Prediction of *in vivo* drug metabolism in the human liver from *in vitro* metabolism data. *Pharmacol. Ther.* **73**:147-171.

Lau YY, Sapidou E, Cui X, White RE, and Cheng KC (2002). Development of a novel in

vitro model to predict hepatic clearance using fresh, cryopreserved, and sandwich-cultured hepatocytes. *Drug Metab Dispos* **30**:1446-1454.

Lave T, Dupin S, Schmitt C, Valles B, Ubeaud G, Chou RC, Jaeck D, and Coassolo P (1997). The use of human hepatocytes to select compounds based on their expected hepatic extraction ratios in humans. *Pharm Res* **14**:152-155.

Lave, T, Dupin, S, Schmitt, C, Chou, RC, Jaeck, D and Coassolo, P (1997). Integration of *in vitro* data into allometric scaling to predict hepatic metabolic clearance in man: Application to 10 extensively metabolized drugs. *J. Pharm. Sci.* **86**:584-590.

Li AP, Lu C, Brent JA, Pham C, Fackett A, Ruegg CE, and Silber PM (1999) Cryopreserved human hepatocytes: Characterization of drug-metabolizing activities and applications in higher throughput screening assays for hepatotoxicity, metabolic stability, and drug-drug interaction potential. *Chem Biol Interact* **121**:17-35.

Lin, HJ, Chiba, M and Baillie, TA (1999). Is the role of the small intestine in first-pass metabolism overemphasized? *Pharmacol. Rev.* **51**:135-157.

Liu, L and Pang, KS (2005). The roles of transporters and enzymes in hepatic drug processing. *Drug Metab Dispos* **33**:1-9.

McGinnity DF, Soars MG, Urbanowicz RA and Robert J. Riley (2004). Evaluation of fresh and cryopreserved hepatocytes as *in vitro* drug metabolism tools for the prediction of metabolic clearance. *Drug Metab Dispos* **32**: 12547-1253.

Nagilla, R and Ward, KW (2004). A comprehensive analysis of the role of correction factors in the allometric predictivity of clearance form rat, dog and monkey to humans. *J. Pharm. Sci* **93**: 2522-2534.

Naritomi Y. Terashita S, Kagayama A, Sugiyama Y (2003). Utility of hepatocytes in predicting drug metabolism: comparison of hepatic intrinsic clearance in rats and humans in vivo and in vitro. *Drug Metab Dispos* **31**: 580-588.

Naritomi, Y, Terashita, S, Kimura, S, Suzuki, A, Kagayama, A and Sugiyama, Y (2001). Prediction of human hepatic clearance from *in vivo* animal experiments and *in vitro* metabolic studies with liver microsomes from animals and humans. *Drug Metab. Dispos.* **29**:1316-1324.

Obach, RS (1999). Prediction of human clearance of twenty-nine drugs from hepatic microsomal intrinsic clearance data: An examination of *in vitro* half-life approach and nonspecific binding to microsomes. *Drug Metab. Dispos.* **27**:1350–1359.

Obach, RS, Baxter, JG, Liston, TE, Silber, BM, Jones, BC, MacIntyre, F, Rance, DJ and

Wastall, P (1997). The prediction of human pharmacokinetic parameters from preclinical and *in vitro* metabolism data. *J. Pharmacol. Exp. Ther.* **283**:46-58.

Riley, RJ and Kenna, JG (2004). Cellular models for ADMET predictions and evaluation of drug-drug interactions. *Curr Opin Drug Discov Devel*. **7**: 86-99.

Schneider G, Coassolo P and Lave T (1999). Combining in vitro and in vivo pharmacokinetic data for prediction of hepatic drug clearance in humans by artificial neural networks and multivariate statistical techniques. *J Med Chem*. **42**: 5072-5076.

Shibata Y, Takahashi H, Chiba M, and Ishii Y (2002) Prediction of hepatic clearance and availability by cryopreserved human hepatocytes: An application of serum incubation method. *Drug Metab Dispos* **30**:892-896.

Shitara, Y, Li, AP, Kato, Y, Lu, C, Ito, K, Itoh, T and Sugiyama Y (2003). Function of uptake transporters for taurocholate and estradiol 17-b-D-glucuronide in cryopreserved hepatocytes. Drug Metab Pharmacokinetc **18**: 33-41.

Shitara Y, Sato H and Sugiyama Y (2005). Evaluation of drug-drug interaction in the hepatobiliary and renal transport of drugs. Annu Rev Pharmacol Toxicol **45**: 689-723.

Theil, F-P, Guentert, TW, Haddad, S and Poulin, P (2003). Utility of physiologically based pharmacokinetic models to drug development and rational drug discovery candidate selection. Tox Letts **138**: 29-49.

Footnotes

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Legends for Figures

Figure 1. Correlation between the observed and predicted human Cl_h (A-C) and $Cl_{int,\ ub}$ (D-F) for a dataset of 57 drugs from hepatocyte incubations without added FCS.

Panels A and D refer to data modeled with fu_b only; B and E assuming $fu_b = fu_{inc}$; and C and F incorporating both fu_b and fu_{inc} terms. Symbols depict different chemical classes (,acid; base; □ neutral). Dotted lines indicate regression analysis. The equation of best fit for F is given by $y = 1.08x + 0.38$ (SD = 0.38, $r^2 = 0.78$, F = 187.5, $p = 3.3 \times 10^{-19}$, *afe* = 5.2).

Figure 2. Correlation between the observed and predicted human Cl_h (A-C) and $Cl_{int,\ ub}$ (D-F) for a dataset of 14 drugs from hepatocyte incubations with added FCS.

Panels A and D refer to data modeled with fu_b only; B and E assuming $fu_b = fu_{inc}$; and C and F incorporating both fu_b and fu_{inc} terms. Dotted lines indicate regression analysis.

Figure 3. Distribution of fu_{inc}: fu_b ratio for drugs studied in hepatocytes classified by chemical class.

Figure 4. Correlation between the observed and predicted human Cl_h (A) and $Cl_{int,\ ub,\ \textit{in vivo}}$ (B) for a dataset of 37 drugs from microsomal incubations.

Symbols depict different chemical classes (acid; base; □ neutral). The equation of best fit (indicated by dotted line in B) is given by $y = 0.88x + 0.71$ (SD = 0.48, $r^2 = 0.77$, F = 115.8, $p = 1.2 \times 10^{-12}$, *afe* = 6.1).

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Table 1. Data for human hepatocyte incubations conducted in the absence of fetal calf serum (corrected for both fu_b and fu_{inc}).

Compound	Chemical class		$Cl_{int,\ ub,\ in\ vivo}$ (ml/min/kg)		Cl_h (ml/min/kg)	
		fu_b	Predicted	Observed	Predicted	Observed
Diclofenac[1]	A	0.0055	618.36	2083.46	2.92	7.33
Diflunisal[2]	A	0.0053	9.86	34.8	0.05	0.18
Etodolac[2]	A	0.02	81.2	82.84	1.28	1.31
Fenoprofen[2]	A	0.01	56.52	216.15	0.47	1.69
Furosemide[1]	A	0.029	5.95	22.85	0.14	0.59
Gemfibrozil[2]	A	0.005	325.82	773.37	1.43	3.09
Glipizide[2]	A	0.02	7.13	60.52	0.12	0.96
Ibuprofen[1]	A	0.0182	71.34	82.7	0.64	1.4
Indomethacin[2]	A	0.02	27.13	145.77	0.46	2.24
Irbesartan[2]	A	0.04	58.75	131.31	1.93	3.85
Ketoprofen[2]	A	0.02	22.44	103.95	0.52	2.22
Montelukast[2]	A	0.0009	96.27	1495.15	0.09	1.27
Oxaprozin[2]	A	0.0007	24.4	100.36	0.02	0.07
Tenoxicam[3]	A	0.0164	8.77	4.46	0.14	0.073
Tolbutamide[1]	A	0.04	6.91	8.99	0.24	0.3
Troglitazone[4]	A	0.0017	306.36	10000	0.51	9
Warfarin[3]	A	0.018	3.69	8.22	0.07	0.16
Buspirone[5]	B	0.05	613.8	1582	12.11	19.2[a]
Carvedilol[2]	B	0.03	281.58	521.97	5.87	8.7
Chlorpromazine[3]	B	0.03	230.33	502.92	5.14	8.6
Cimetidine[2]	B	0.9	3.35	4.23	2.62	3.2
Desipramine[3]	B	0.17	127.16	124.92	10.39	10.3
Diltiazem[1]	B	0.22	77.81	143.61	10.70	12.8
Granisetron[2]	B	0.7	29.72	35.14	10.17	11
Imipramine[1]	B	0.1	92.57	125.59	6.33	9.46
Lidocaine[5]	B	0.3	24.61	100.68	5.34	15.0
Metoprolol[1]	B	0.747	13.87	40.62	5.95	12.15
Naloxone[1]	B	0.56	150.28	924.35	16.16	19.5[a]
Pindolol[2]	B	0.9	9.28	5.91	5.89	4.2
Propranolol[1]	B	0.12	59.2	291.87	6.46	16.11
Quinidine	B	0.15	12.95	48.63	1.73	5.33
Ranitidine[2]	B	0.77	3	4.4	2.07	2.9
Timolol[5]	B	0.4	6.55	22.75	2.32	9.17
Tiprolidine	B	0.1	39.61	133.33	3.31	8
Verapamil[1]	B	0.115	278.92	388.33	11.65	14.66
Acetaminophen[4]	N	0.79	2.53	6.71	1.81	4
Antipyrine[1]	N	0.94	0.82	0.69	0.74	0.6
Caffeine[1]	N	0.685	2.89	2.25	2.07	1.4
Cyclosporin A[2]	N	0.04	13.46	155.27	0.52	4.7
Diazepam[1]	N	0.012	6.41	31.29	0.08	0.43
FK079[4]	N	0.0288	56.38	636	0.91	4.4
FK1052[4]	N	0.021	32.38	1570	0.64	12.2
FK480[4]	N	0.008	49.41	336	0.40	2.4
Lorazepam[3]	N	0.094	1.16	12.38	0.11	1.1
Methylprednisolone[3]	N	0.23	37.08	52.17	5.98	7.5
Midazolam[3]	N	0.04	40.08	246.27	1.48	6.6
Nifedipine[1]	N	0.05	32.6	253.7	1.46	7.8
Ondansetron[2]	N	0.68	5.23	12.4	3.00	5.9
Oxazepam[3]	N	0.03	8.23	38.8	0.24	1.1
Phenacetin[5]	N	0.594	76.01	212.5	13.90	19.2[a]
Prazosin[2]	N	0.07	6.16	42.23	0.45	2.7
Prednisolone[3]	N	0.26	35.54	59.22	6.32	8.7
Ritonavir[2]	N	0.0148	30.51	86.26	0.44	1.2
Sildenafil[3]	N	0.04	24.35	214.29	0.93	6
Theophylline[3]	N	0.4	1.67	1.68	0.65	0.65
Zidovudine[4]	N	0.8	9.87	42.1	5.66	12.4

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[1]Mean data (see Table 4); [2]This laboratory; [3]Lau et al., 2002; [4]Naritomi et al., 2003; [5]Shibata et al., 2004.

[a]Re-calculated from data provided in original reference, assuming oral bioavailability = hepatic availability = $1 - Cl_h/(R_B \times Q_h)$. $Cl_{int,\ ub,\ in\ vivo}$ was calculated from the dispersion model using Exel Goal seek tool.

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Table 2. Data for human hepatocyte incubations conducted in the presence of fetal calf serum corrected for both binding to hepatocytes (fu_{inc}) and fu_b.

Compound	Chemical Class		$Cl_{int,ub,\ in\ vivo}$ (ml/min/kg)		Cl_h (ml/min/kg)	
		fu_b	Predicted	Observed	Predicted	Observed
Antipyrine[1]	N	0.94	0.23	0.60	0.212	0.55
Bosentan[1]	A	0.02	0.67	42.1	0.0134	3.65
Caffeine[1]	N	0.83	0.37	1.40	0.303	1.05
Diazepam[1]	N	0.012	3.44	42.78	0.040	0.5
Diltiazem[1]	B	0.22	8.45	205.44	1.70	13.35
Felodipine[2]	N	0.004	72.08	6111.11	0.28	11
Lorazepam[1]	N	0.094	1.24	16.71	0.12	1.45
Mibefradil[2]	B	0.005	41.3	4888.9	0.20	11
Midazolam[1]	N	0.04	25.74	599.04	0.98	10.9
Oxazepam[1]	N	0.03	1.52	36.94	0.046	1.05
Propranolol[1]	B	0.1	21.67	388.75	1.96	13.2
Theophylline[1]	N	0.4	0.35	1.55	0.141	0.6
Tolcapone[1]	A	0.0018	6.41	1650.32	0.074	2.65
Warfarin[3]	A	0.018	0.34	6.02	0.006	0.12

[1]Mean data (see Table 4); [2]Schneider et al., 1999; [3]Lave et al., 1997

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Table 3. Data for human microsome incubations corrected for both fu_b and fu_{inc}.

Compound	Chemical class	HLM $Cl_{int, in\ vitro}$ (μl/min/mg)	$Cl_{int, ub, in\ vivo}$ (ml/min/kg)	
			Predicted	Observed
Alprazolam[1]	N	1.6	2.4	1.9
Amitryptyline[1]	B	14	94.3	516.0
Amobarbital[1]	A	0.94	1.2	1.4
Chlorpromazine[1]	B	25	229.6	381.3
Clozapine[1]	B	4.6	35.7	59.0
Desipramine[1]	B	17	81.8	160.0
Dexamethasone[1]	N	3	3.0	13.6
Diazepam[2]	N	3	11.8	28.0
Diclofenac[2]	A	378.02	183.8	1667.3
Diltiazem[3]	B	33.5	77.7	232.6
Diphenhydramine[1]	B	2.10	7.3	53.5
FK1052[4]	N	58.3	182.0	1525.0
FK480[4]	N	73.5	662.0	327.3
Fluvastatin[5]	A	47.06	75.4	1052.0
Hexobarbital[1]	A	2.3	2.9	8.3
Ibuprofen[2]	A	21.79	12.3	102.4
Imipramine[1]	B	19	106.6	330.0
Lorcainide[1]	B	50	97.1	924.0
Methohexital[1]	A	49	57.6	207.4
Methoxsalen[1]	N	40	43.0	1340.0
Metoprolol[3]	B	5	6.8	20.2
Midazolam[1]	N	160	183.7	163.2
Nicardipine[4]	N	1719	13460.0	1806.7
Nilvadipine[4]	N	1695	3867.0	8123.4
Omeprazole[4]	N	97.1	101.0	502.7
Phenacetin[3]	N	9	9.9	212.5
Phenytoin[6]	A	0.18	0.5	4.0
Prednisone[1]	N	2.7	13.6	21.5
Propafenone[1]	B	166	644.9	6650.0
Propranolol[3]	B	13	16.3	284.5
Quinidine[1]	B	3.4	10.7	22.1
Tenidap[1]	A	8.3	26.2	80.4
Tenoxicam[1]	A	1.7	2.2	2.2
Tolbutamide[2]	A	1.94	1.3	6.4
Triazolam[1]	N	19	24.6	38.1
Verapamil[2]	B	122	553.6	935.3
Zolpidem[4]	N	28.7	17.9	115.5

[1]Obach, 1999; [2]Mean data (see Table 5); [3]This laboratory; [4]Naritomi et al., 2001; [5]Andersson et al., 2004; [6]Carlile et al.,1999.

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Table 4. Human hepatocyte data concordance across different laboratories for dataset studied.

Compound	Chemical class	$LogD_{7.4}/P$	$fu_{,inc}$	$Cl_{int,\ in\ vitro}$ (µl/min/10^6 cells)					
				+ FCS		- FCS			
				Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 6[a]
Antipyrine	N	0.23	0.95	0.04	0.1	0.47			0.03
Bosentan	A	-0.37	0.97	0.22	0.2				
Caffeine	N	-0.07	0.96	0.13	0.1	1.5			0.2
Diazepam	N	2.89	0.63	0.69	0.7	1.4	1.32		3.85
Diclofenac	A	1.09	0.90					150	256.7
Diltiazem	B	2.8	0.65	1.62	1.9	4.7	23.35	9.0	
Furosemide	A	-0.61	0.98			0		2.2	
Ibuprofen	A	0.98	0.91			4.2		24.1	
Imipramine	B	4.44	0.29			8.2			8.92
Lorazepam	N	2.4	0.73	0.28	0.3	0.27			
Metoprolol	B	1.88	0.81					7.0	1.70
Midazolam	N	3.27	0.54	4.49	4.5	7			
Moferatone	N	8	0.02	1.83	2				
Naloxone	B	2.09	0.78	16.5	16.7	28			47.4
Oxazepam	N	2.24	0.75	0.37	0.37	2			
Propranolol	B	1.6	0.85	4.24	4.2	10		10.0	18.54
Theophylline	N	-0.14	0.97	0.11	0.1	0.52			
Tolbutamide	A	2.34	0.74			1.6		2.4	
Tolcapone	A	2.7	0.67	1.1	1.2				
Verapamil	B	3.79	0.42			16		48	60.0
Warfarin	A	0.75	0.92	0.1		1.1			

[a]Corrected for serum binding in assay

Lab 1 = Lave et al., 1997, Lab 2 = Schneider et al., 1999; Lab 3 = Lau et al., 2002; 4 = Naritomi et al., 2003;

Lab 5 = this laboratory; Lab 6 = Shibata et al., 2002

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Table 5. Human microsome data concordance across different laboratories for dataset studied.

	Cl_{int}*, ub, *in vitro* (ml/min/kg)					$Cl_{int, ub, in vivo}$ (ml/min/kg)				
Compound	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5	Lab 1	Lab 2	Lab 3	Lab 4	Lab 5
Diazepam		8.3		20.13	6.8		33.8		20.1	30
Diclofenac	38.5	190.9	415.4		90.6	623.1	1348.4	2464.7		2233
Diltiazem		19.9		137	74.2		136.4		287.9	253
Ibuprofen	9.2 (2- & 3-OH)	10.6	20.6		8.9	91	89.2	81.2		146
Tolbutamide	1.28	0.96	1.8		1.2	2.0	5.0	12.5		6.1
Verapamil		286.6			951.5		2926.0			388.5
Zolpidem		4.9		31			75.7		155.3	

Lab 1 = Carlile et al., 1999; Lab 2 = Obach, 1999; Lab 3 = Andersson et al., 2004; Lab 4 = Naritomi et al., 2001; Lab 5 = this laboratory

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Table 6. Regression analysis summary for various models applied to hepatocyte incubations conducted in the presence (+ FCS) and absence (- FCS) of fetal calf serum

	+ FCS			- FCS		
	- fu_{inc}	$fu_{inc} = fu_b$	fu_{inc} and fu_b	- fu_{inc}	$fu_{inc} = fu_b$	fu_{inc} and fu_b
m	1.44	1.04	1.40	1.09	0.64	1.08
c	1.34	0.18	1.13	0.57	-0.079	0.38
r^2	0.78	0.70	0.87	0.65	0.25	0.78
SD	0.61	0.50	0.47	0.57	0.68	0.38
F	39.9	25.9	73.8	99.6	18.2	187.5
p	5.7×10^{-5}	3.5×10^{-4}	3.3×10^{-6}	7.4×10^{-14}	8.2×10^{-5}	3.3×10^{-19}
afe	86.4	3.1	42.3	12.5	16.3	5.2

Figure 1

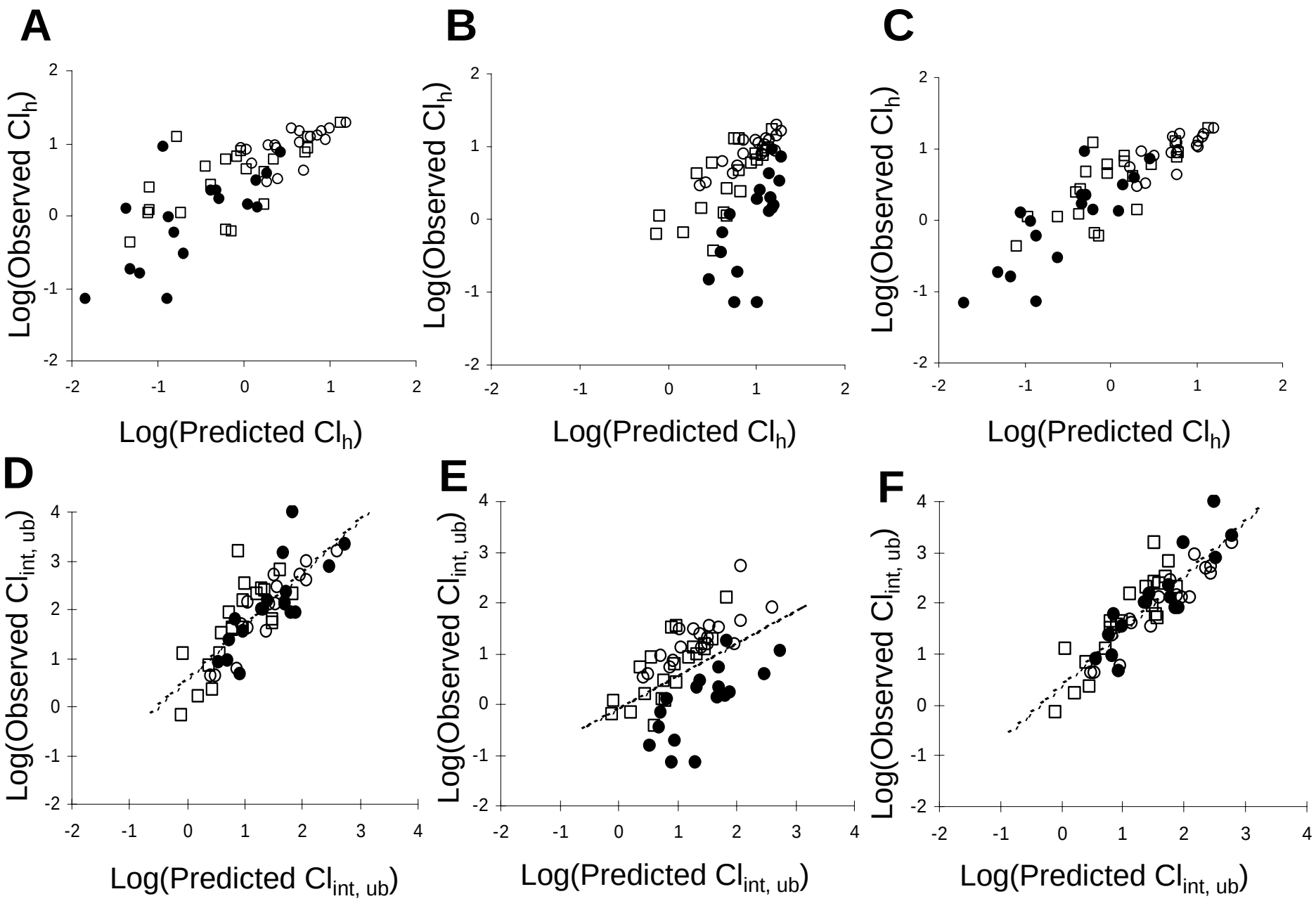

Figure 2

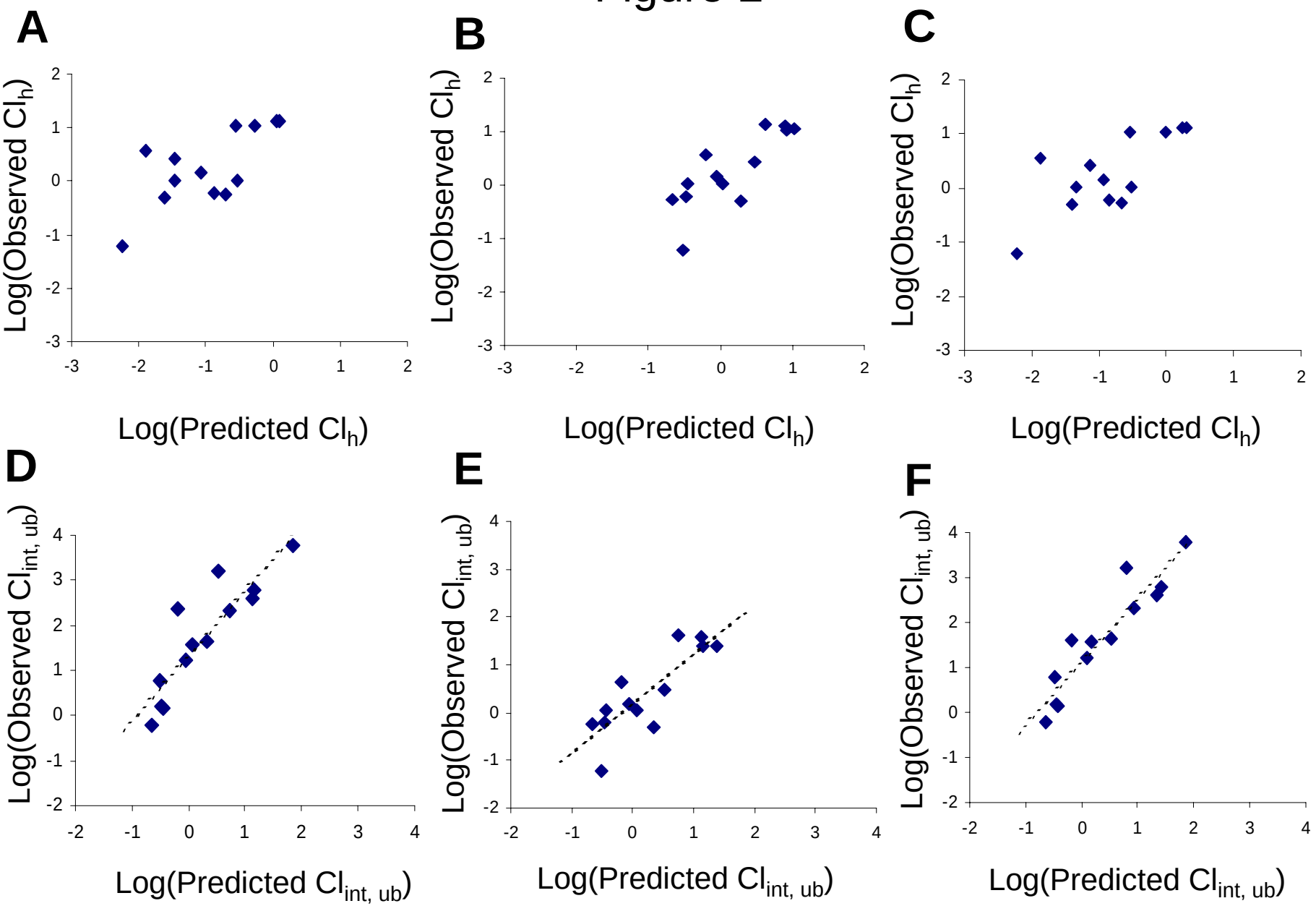

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Figure 3

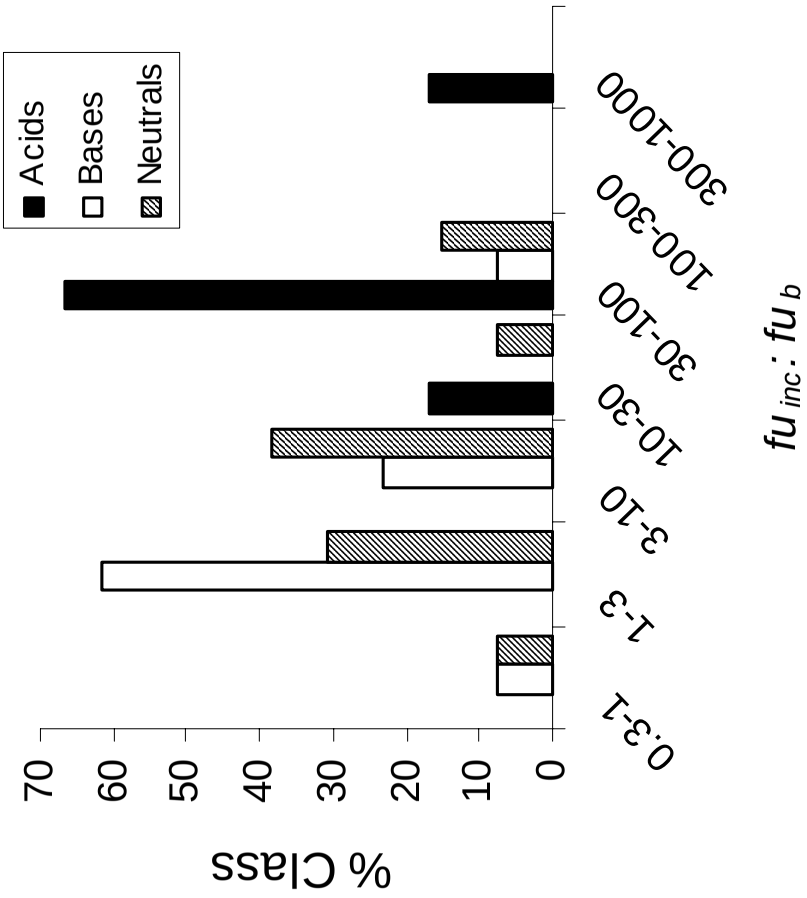

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Figure 4