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*Review Article*

*Theme: Towards Integrated ADME Prediction: Past, Present, and Future Directions*
*Guest Editors: Lawrence X. Yu, Steven C. Sutton, and Michael B. Bolger*

# Prediction of Hepatic Clearance in Human From *In Vitro* Data for Successful Drug Development

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***Abstract.*** The *in vivo* metabolic clearance in human has been successfully predicted by using *in vitro* data of metabolic stability in cryopreserved preparations of human hepatocytes. In the predictions by human hepatocytes, the systematic underpredictions of *in vivo* clearance have been commonly observed among different datasets. The regression-based scaling factor for the *in vitro*-to-*in vivo* extrapolation has mitigated discrepancy between *in vitro* prediction and *in vivo* observation. In addition to the elimination by metabolic degradation, the important roles of transporter-mediated hepatic uptake and canalicular excretion have been increasingly recognized as a rate-determining step in the hepatic clearance. It has been, therefore, proposed that the *in vitro* assessment should allow the evaluation of clearances for both transporter(s)-mediated uptake/excretion and metabolic degradation. This review first outlines the limited ability of subcellular fractions such as liver microsomes to predict hepatic clearance *in vivo*. It highlights the advantages of cryopreserved human hepatocytes as one of the versatile *in vitro* systems for the prediction of *in vivo* metabolic clearance in human at the early development stage. The following section discusses the mechanisms underlying the systematic underprediction of *in vivo* intrinsic clearance by hepatocytes. It leads to the proposal for the assessment of hepatic uptake clearance as one of the kinetically important determinants for accurate predictions of hepatic clearance in human. The judicious combination of advanced technologies and understandings for the drug disposition allows us to rationally optimize new chemical entities to the drug candidate with higher probability of success during the clinical development.

**KEY WORDS:** hepatic clearance; human hepatocytes; human PK prediction; OATP1B1.

## INTRODUCTION

It has become critically important to discover more bioavailable drug candidates in many therapeutic targets where the drug would be preferably given per oral to the patients. At the early discovery stage in the development of new drug, the *in vitro* metabolic stability is routinely examined in a high-throughput manner at the pharmaceutical companies. The rationale of this strategy is that the *in vitro* metabolic stability in the preparations from human should reasonably well predict *in vivo* clearance in human. In parallel with the *in vitro* assessment for the metabolic stability in preclinical species as well as human, the pharmacokinetics of new chemical entities is often examined in order to identify the major route(s) of elimination in animals from *in vitro*/*in vivo* correlation analysis. These data assure that any *in vitro* data using hepatic derived systems would be meaningful for the quantitative prediction in human. Predictions based on the metabolic clearance from *in vitro* samples have been able to account for the potential interspecies differences in the metabolism. However, much evidence such as drug–drug interactions through the inhibition of transporters (1–3) and interindividual variations of pharmacokinetics through the genetic polymorphism in the transporters (4,5) has also suggested that the transporter-mediated uptake and excretion processes, often or partly at least, become a rate-determining step as a determinant of hepatic clearance and pharmacokinetics in human for many drugs (4,6–8). Therefore, the accuracy of prediction for the hepatic clearance in human will be certainly improved by integrating transporter-mediated process(es) into the existing paradigm for the prediction, based on the understanding of rate-determining step in the hepatic clearance in human.

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## PREDICTION OF HEPATIC CLEARANCE: FROM LIVER MICROSOMES TO HEPATOCYTES

Based on the accumulated data for the quantitative prediction of hepatic clearance from *in vitro* studies with rat liver microsomes and isolated rat hepatocytes, the prediction strategy was proposed by Houston in 1994 (9) and 1997 (10).

Following those reports, many results were published and consistently indicated that the *in vivo* hepatic (or total body) clearance could be reasonably well predicted from the metabolic (intrinsic) clearance determined in the *in vitro* system on the basis of existing pharmacokinetic premises (9–26). Most studies in early years used rat liver microsomes and/or rat hepatocytes for the prediction of clearance due to the feasibility of *in vitro* experiments and limited availability of human samples (9–17,21,24). The prediction strategy established in rat has been extended to that for human as the *in vitro* samples (liver microsomes, liver slices, and freshly isolated hepatocytes) from human have become prevalent since early 1990s (18–20,22,23,25–27). The prediction of pharmacokinetics in human with reasonable level of confidence is one of the crucial components in the assessment of probability of success (POS) of drug candidate at the preclinical evaluation stage. Therefore, the *in vitro* studies with widely and routinely available human preparations certainly contributed to the reduction of attrition rate attributable to the inappropriate pharmacokinetics in human during the clinical development at the pharmaceutical companies (28,29).

It has been long recognized that the isolated rat hepatocytes generally provide better *in vitro* grounds for the quantitative/qualitative predictions of *in vivo* metabolism than liver slices and subcellular fractions (10). The advantages of using isolated rat hepatocytes over other *in vitro* preparations have been well documented in the context of quantitative predictions of *in vivo* metabolism. For example, the predictions by isolated rat hepatocytes for the inhibitory effects of metabolite(s) on the disposition of parent compounds (*i.e.*, product inhibition) were quantitatively successful for diazepam (12) and phenytoin (17) in rat: the overwhelming accumulation of inhibitory metabolite(s) in the liver microsomes underpredicted *in vivo* metabolism of parent drugs due to the lack of conjugation activities. *In vitro* preparations with activated liver microsomes also underpredicted *in vivo* UDP-glucuronosyltransferase activities in rat (30) and human (31,32), likely due to the insufficient activation of conjugative activity in liver microsomes to the extent relevant to *in vivo* (31) and/or to the potent competitive inhibitions by unsaturated long-chain fatty acids (oleic, linoleic, and arachidonic acids) released during microsomal incubations (33); in contrast, the metabolic clearance primarily catalyzed by glucuronidation in hepatocytes generally well predicted *in vivo* clearance in human (31,34,35). It has been consistently demonstrated that the accuracy of predictions for hepatic clearance by hepatocytes was superior to that by liver microsomes for a wide range of drugs in rat (10,21) and for the drugs predominantly metabolized by glucuronidation in human (31).

Despite the data suggesting that the preparation of freshly isolated hepatocytes is a superior *in vitro* tool for the prediction of metabolic clearance in human to that of subcellular fractions, the unpredictable availability of fresh liver from human precluded this *in vitro* system from routine applications. Cryopreserved preparation of human hepatocytes has instead become prevalent and versatile *in vitro* system alternative to freshly isolated human hepatocytes. Cryopreserved human hepatocytes have been reported to retain qualitatively and quantitatively comparable metabolic activities with those in hepatocytes from fresh liver (36–42). However, the comparisons between predicted and observed metabolic clearance in human in different datasets (27,35,41,43,44) have indicated that the *in vitro* metabolic clearance obtained from both human liver microsomes (43) and cryopreserved human hepatocytes (27,35,41,44,45) systematically underpredicted *in vivo* metabolic clearance by ~9 and 3~6-fold, respectively. Consequently, in order to mitigate the quantitative discrepancy between *in vitro* prediction and *in vivo* observation in human, the empirical and regression-based scaling factors specifically obtained from the *in vitro* preparation used for the prediction, instead of biological (anatomical) scaling factors such as microsomal content and hepatocellularity, were employed for the extrapolation of *in vitro* data to *in vivo* (27,35,43,45). Empirical (or regression-based) scaling factor represents the discrepancy (observed/predicted) between calculated metabolic (or intrinsic clearance) from *in vivo* hepatic clearance and those predicted from *in vitro* metabolic clearance in the cryopreserved human hepatocytes by using hepatocellularity or in human liver microsomes by using liver microsomal content as a biological scaling factor. For example, regression analysis indicated that the calculated metabolic clearance from *in vivo* was approximately threefold larger ($8.5\times10^9$ cells per kilogram body weight, solid line in Fig. 1) than those predicted from hepatocellularity ($3.1\times10^9$ cells per kilogram body weight, dotted line in Fig. 1) (27). Similar regression analyses for different datasets found that the empirical scaling factors necessary for accurate predictions were 5.2-fold (35) and 5.6-fold larger (45) than hepatocellularity ($2.5–3.1\times10^9$ cells per kilogram body weight) and ninefold larger than the biological scaling factor based on human liver microsomal content (856-mg microsomal protein per kilogram body weight) (43). In order to examine the potential discrepancy and adequate (regression-based) scaling factor for the prediction in human, the values of *in vitro* metabolic clearance were determined for seven standard drugs (naloxone, buspirone, verapamil, lidocaine, imipramine, metoprolol, and timolol) in the preparations of cryopreserved human hepatocytes from ten different donors (Fig. 1) (27). Consistent with the observations in other datasets (27,35,41,43–45), large interindividual variations were obtained in the *in vitro* metabolic clearance among different donors by a maximum of tenfold. Interestingly, the prediction for metabolic

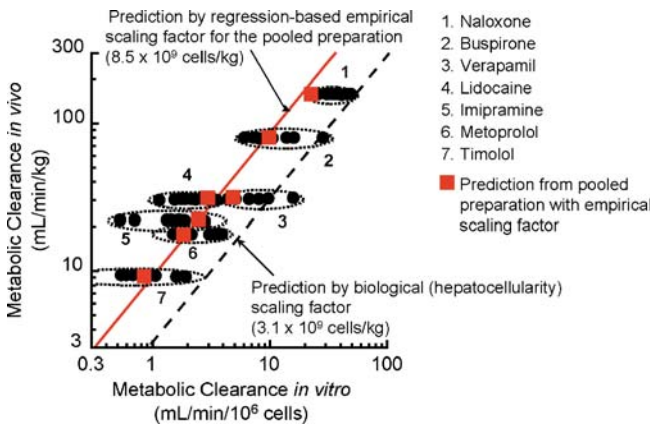


**Fig. 1.** Interindividual variation of metabolic clearance among cryopreserved human hepatocytes and an empirical scaling factor for the quantitative prediction. Data are from (27)

clearance in rat from rat liver microsomes and rat hepatocytes did not show any significant systematic bias (21), suggesting that the discrepancy can be only evident in human (27,35,41,43–45). Possible mechanisms for the systematic underprediction by biological scaling factors (41,46) are discussed in the following section. For human tissue preparation, there is an impact from extrinsic factors such as tissue handling and storage procedures on the preparation used for the prediction (43). Therefore, the empirical scaling factor needs to be determined in the human preparation used for the extrapolation of *in vitro* data to *in vivo*.

Negatively biased interindividual variations of metabolic clearance in the *in vitro* human preparations suggested that a careful pooling of liver preparations as well as an empirical evaluation of scaling factor would improve accuracy of prediction by minimizing potentially biased coverage of metabolizing enzyme activities in the individual preparation. Figure 2 shows the example of the pooling process for the *in vitro* preparations consisting of cryopreserved human hepatocytes from multiple donors. The calculated values of metabolic clearances from *in vivo* data (reported from clinical studies) for seven standard drugs (naloxone, buspirone, verapamil, lidocaine, imipramine, metoprolol, and timolol which are known to undergo the metabolic degradation catalyzed by different enzymes responsible for both phases I and II metabolism in human) were compared with those determined in the individual preparation of cryopreserved human hepatocytes from ten different donors (donor # a to j). As shown in the figure, metabolic activity for imipramine (standard compound *e*) in the preparations (donor # b and d) deviated from the regression lines between *in vivo* and *in vitro* metabolic clearance, suggesting that these two preparations had significantly lower activities for imipramine metabolism than those calculated by the empirical scaling factor (*i.e.*, slope of regression line, cells per kilogram). Therefore, the regression line with better correlation between *in vivo* and *in vitro* metabolic clearance represents a better coverage by empirical (regression-based) scaling factor for major metabolizing enzymes in human. In this study, the *in vitro* metabolic clearance determined in the pooled sample consisting of preparations from donors f and h provided predictions of *in vivo* metabolic clearance based on the best regression line for the *in vitro*-to-*in vivo* extrapolation for 12 drugs [*i.e.*, seven standard drugs (naloxone, buspirone, verapamil, lidocaine, imipramine, metoprolol, and timolol) five test drugs (propranolol, diclofenac, quinidine, phenacetin, and caffeine)].

Metabolic stability is routinely examined for new chemical entities at the discovery stage in conjunction with the early identification of metabolic "soft spot" which facilitates structure modification for the lead optimization. Because of the presence of all hepatic drug-metabolizing enzymes and cofactors at physiological levels, the intact hepatocytes are generally more relevant *in vitro* experimental system than liver microsomes even for the early screening of metabolic stability (40,47,48). In order to clarify the limited predication ability of the results from metabolic stability data with liver microsomes for the *in vivo* metabolic clearance, the values of metabolic stability in liver microsomes (as percent remaining)

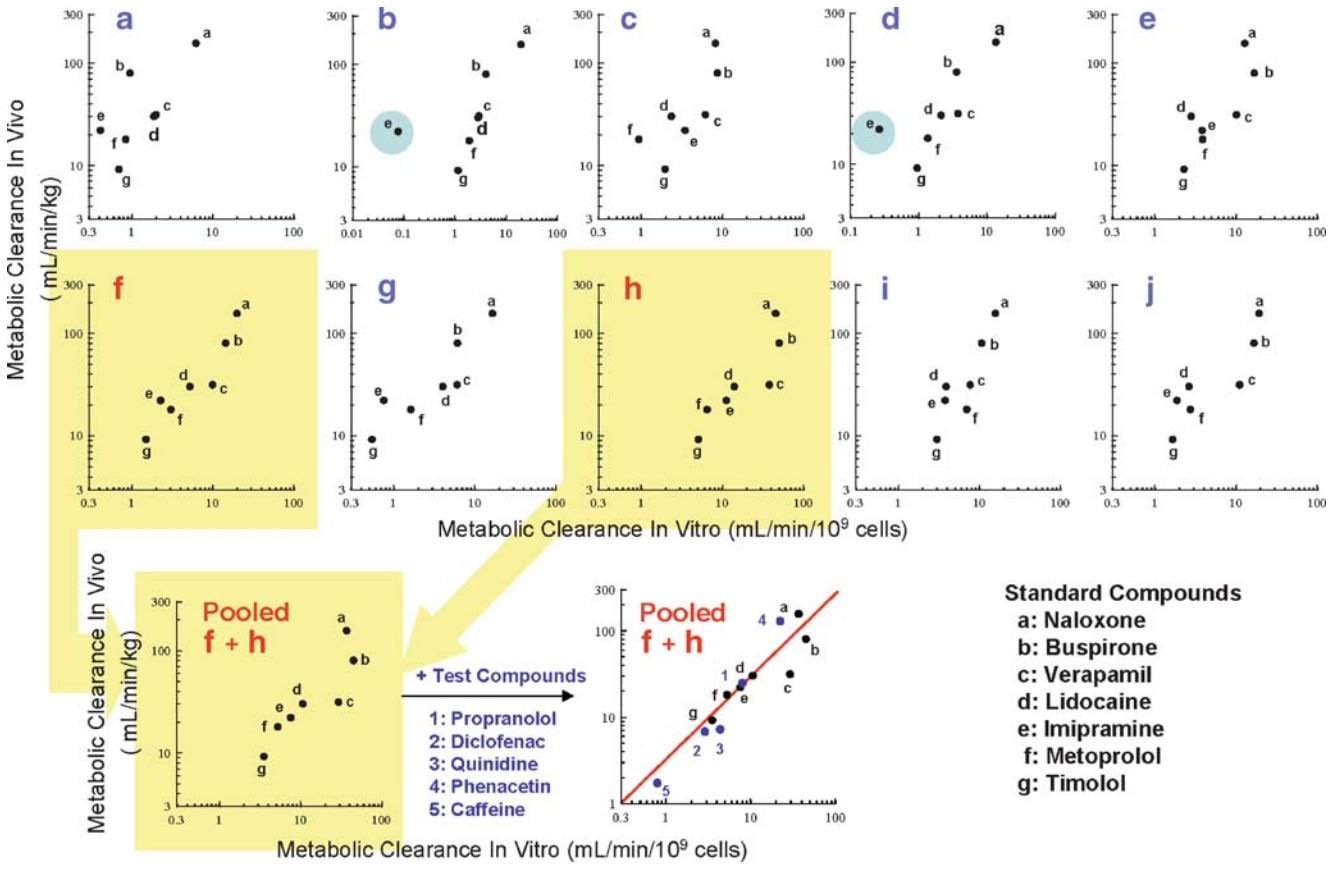


**Fig. 2.** Correlation of metabolic clearance between *in vitro* and *in vivo* among cryopreserved hepatocytes from different donors

are plotted against those in hepatocytes (as metabolic clearance) based on in-house dataset (Fig. 3). As clearly shown in these figures, the compounds having metabolic stability in liver microsomes >30% remaining could have a wide range of metabolic stability (metabolic clearance) in the corresponding hepatocytes. In contrast, the compounds having metabolic stability in liver microsomes <30% remaining were very unstable also in the hepatocytes with the metabolic clearance of >100 mL/min per kilogram. Dataset with rat liver microsomes and hepatocytes was much larger than that with human samples, while no species difference was observed in the cutoff (30%) for the relation between percent remaining in liver microsomes and metabolic clearance in hepatocytes. Therefore, the usefulness of the screening study with human liver microsomes would be limited to the higher-throughput screening of no promising compounds in terms of metabolic stability which needs to undergo structure modification based on the information on the metabolic "soft spot."

One of the goals at drug discovery stage is to identify the drug candidates which likely possess appropriate pharmacokinetics with lower hepatic clearance and higher oral bioavailability in human especially when the drug is favorably given to the patients by oral administration. Shibata *et al.* (27) reported that the *in vivo* hepatic clearance in human was successfully predicted with cryopreserved human hepatocytes suspended in 100% human serum for the model drugs which mainly undergo the elimination by the metabolism in human liver. The subsequent reports consistently indicated that the hepatocytes suspended in 100% serum have more accurately predicted *in vivo* metabolic clearance in rat (49) and human (47,50) than those in the absence of serum. The method has been applied to the prediction of pharmacokinetics (clearance, bioavailability, and terminal half-life) in human for drug candidates, and the accuracy of predictions was evaluated by the comparison of predicted values with the observed pharmacokinetics in first-in-human (FIH) studies (Table I and Fig. 4). The oral clearance ($CL_{oral}$) was calculated from area under the curve (AUC) and dose after oral administrations of drug candidates in the FIH studies as follows:

$$CL_{oral} = \frac{Dose}{AUC_{0-\infty}} \tag{1}$$

For the prediction of $CL_{oral}$ from *in vitro* data, the metabolic clearance determined in the cryopreserved human hepatocytes suspended in 100% human serum was extrapolated to that for *in vivo* ($CL_{met}$) with an aid of the regression-based empirical scaling factor for the preparation used as previously described. The hepatic clearance ($CL_H$) and hepatic availability ($F_H$) in human were calculated by the dispersion model (51) incorporating predicted metabolic clearance ($CL_{met}$), dispersion number ($D_N$, 0.17) (51), and hepatic blood flow in human ($Q_H$, 21 mL/min per kilogram) (52) as follows:

$$CL_H = Q_H \times R_B \times \left[1 - \left(\frac{4a}{(1-a)^2 \exp\left[\frac{a-1}{2D_N}\right] - (1-a)^2 \exp\left[-\frac{a+1}{2D_N}\right]}\right)\right] \tag{2}$$

$$F_H = 1 - \frac{CL_H}{Q_H \times R_B} \tag{3}$$

where $a = (1 + 4 \times R_N \times D_N)^{0.5}$, $R_N = CL_{met}/(Q_H \times R_B)$, and $R_B$ represents blood-to-plasma concentration ratio. By using the calculated $CL_H$ and the mean value of apparent volume of distribution ($Vd$) from preclinical species corrected by the species difference in the unbound fraction in plasma

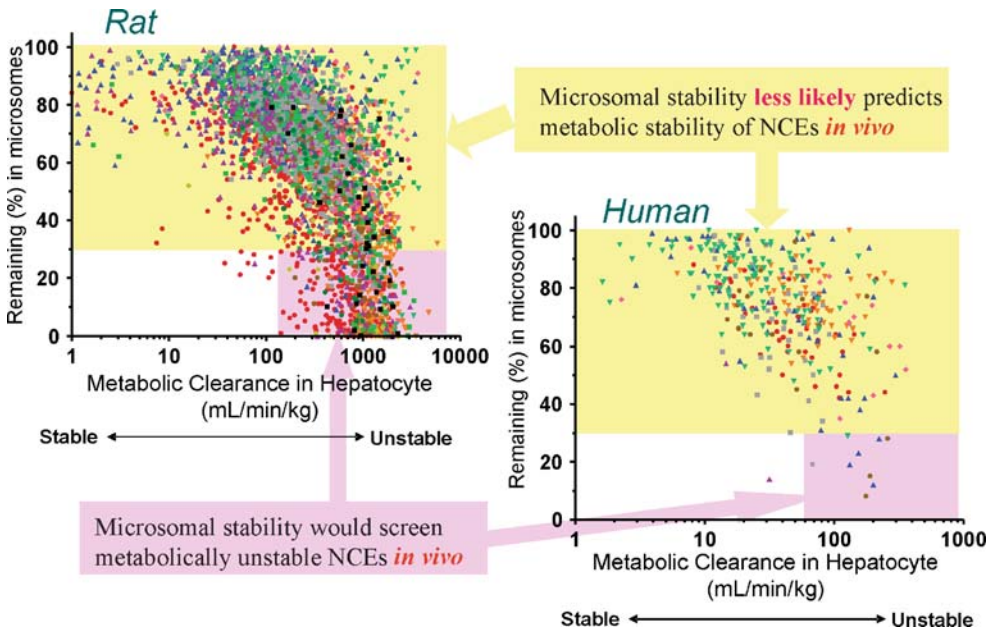


**Fig. 3.** Comparison between liver microsomes and hepatocytes for the metabolic stability screening at discovery stage

**Table I.** Prediction of Terminal Half-life and Oral Clearance in First-in-Human Studies (Data From In-house Database)

| | Observed in FIH study | | | Predicted | | |
|---|---|---|---|---|---|---|
| Compound # | Dose (mg) | $t_{1/2}$ (h) | $CL_{oral}$ [a] (mL/min/kg) | $t_{1/2}$ [b] (h) | $f_a$ [c] | $CL_{oral}$ [d] (mL/min per kilogram) |
| 1 | 0.5 | 5.1 | 0.36 | 2–5 | 0.59 | 1.3 |
| 2 | 1 | 11.4 | 5.5 | 4–5 | 0.66 | 6.1 |
| 3 | 0.4 | 9.4 | 1.2 | 6–11 | 0.80 | 0.54 |
| 4 | 5 | 7.9 | 2.4 | 2–3 | 0.84 | 7.9 |
| 5 | 12.5 | 13.1 | 10.9 | 4–10 | 0.82 | 9.3 |
| 6 | 2.5 | 14.1 | 33.4 | 14 | 0.68 | 35.8 |
| 7 | 0.5 | 3.5 | 26.5 | 3–6 | 0.69 | 22.5 |
| 8 | 5 | 40 | 1.0 | 20 | 0.76 | 3.6 |
| 9 | 10 | 19 | 2.1 | 9 | 0.79 | 9.2 |
| 10 | 350 | 8.9 | 12.8 | 9 | 0.50 | 24.6 |

[a] $CL_{oral} = \frac{Dose}{AUC_{0-\infty}}$

[b] $t_{1/2} = 0.693 \times \frac{Vd}{CL_H}$ where $Vd$ represents the observed $Vd$ in preclinical species (corrected by unbound fractions) and $CL_H$ represents the value predicted from the incubation of cryopreserved human hepatocytes suspended in 100% human serum

[c] Combined fractions of radioactivity (percent of dose) excreted into bile and urine after PO dosing of radiolabeled compound to bile duct cannulated rats

[d] $CL_{oral} = \frac{CL_H}{f_a \times F_H}$ where $CL_H$ and $F_H$ represent the values predicted from cryopreserved human hepatocytes suspended in 100% serum.

(18,23,53), the terminal half-life ($t_{1/2}$) in human was predicted as follows (18):

$$t_{1/2} = 0.693 \times \frac{Vd}{CL_H} \tag{4}$$

Based on the reports that the rat can serve as a reliable animal model to predict drug absorption in human based on the linear correlation in the percent of absorbed dose between rat and human (54,55), the absorption fraction ($f_a$) in human was predicted from the combined recovery of radioactivity from bile and urine after an oral administration of radiolabeled compound to the bile duct cannulated rat. Assuming that the intestinal metabolism was negligible for the compounds (*i.e.*, $F_G = 1$ in Eq. 5), the predicted $f_a$ was then used for the prediction of $CL_{oral}$ as follows (23):

$$CL_{oral} = \frac{CL_H}{f_a \times F_G \times F_H} \tag{5}$$

As shown in Fig. 4, the predicted terminal half-lives and $CL_{oral}$ reasonably well agreed with those observed in FIH studies for all ten drug candidates. These data suggested that the *in vitro* system consisting of cryopreserved human hepatocytes suspended in 100% serum can predict pharmacokinetics in human for the drug candidates which undergo hepatic metabolism as a predominant route of elimination. The prediction has facilitated prioritization/selection of clinical development candidates with POS assessment of human pharmacokinetics at the preclinical stage prior to the entry of clinical studies.

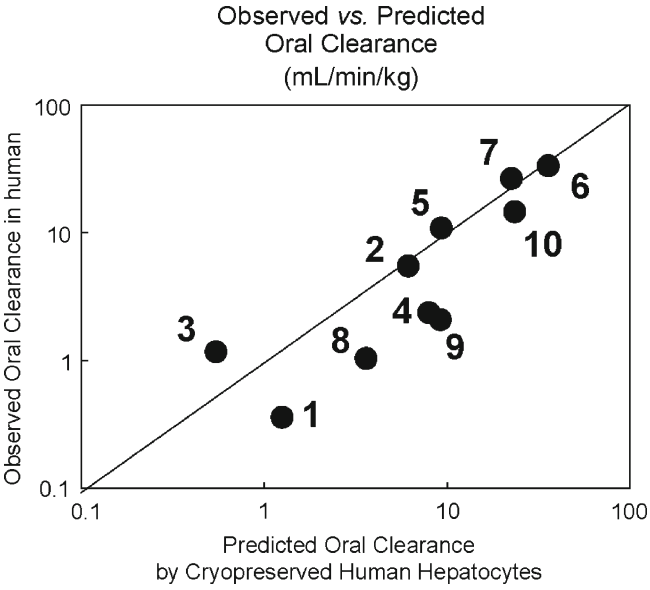


**Fig. 4.** Comparison of predicted and observed oral clearance in First-in-Human (FIH) studies. *Numbers* are corresponding to those compounds in Table I

## LIMITATION OF *IN VITRO* METABOLIC CLEARANCE TO PREDICT *IN VIVO* INTRINSIC CLEARANCE

Systematic underpredictions of *in vivo* metabolic clearance have been reported for human when the standard biological scaling factors were used to extrapolate the values of metabolic clearance determined in human liver microsomes and isolated (or cryopreserved) human hepatocytes to those *in vivo* as described in the previous section (22,23,26,27,35,41,43–46). Iwatsubo *et al.* (26) reported significant discrepancy between *in vivo* intrinsic clearance and *in vitro* metabolic clearance for 25 drugs in human (Fig. 5). As shown in the figure, for most outliers having >5-fold difference between *in vivo* and *in vitro*, the calculated values of *in vivo* intrinsic clearance from human were sevenfold to 80-fold higher than that predicted from the metabolic clearance determined in the *in vitro* studies. Several possible reasons for the discrepancy are discussed in the following sections.

The interindividual variation in the metabolic activities would likely be caused by the intrinsic factors such as genetic

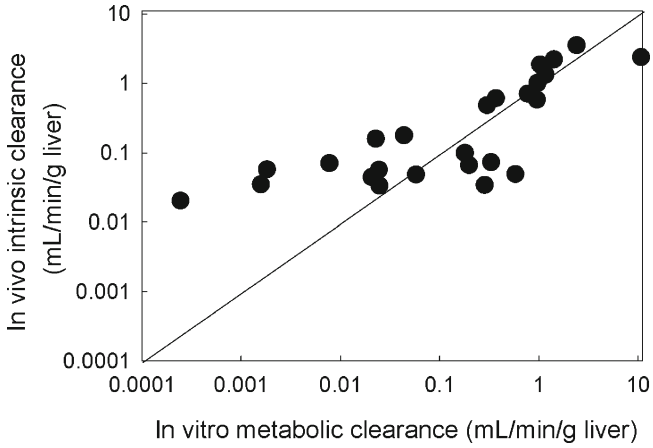


**Fig. 5.** Correlation between *in vivo* intrinsic clearance and *in vitro* metabolic clearance for 25 drugs in human. Data are taken from (26)

polymorphism, smoking, alcohol drinking, or drug administration. However, the intrinsic variability itself would result in a uniform variation, leading to both overpredictions and underpredictions of *in vivo* clearance by *in vitro* metabolic clearance. The values of *in vitro* metabolic clearance of midazolam determined in S-13 fractions prepared from biopsy samples were extrapolated to the hepatic clearance by using standard biological scaling factor (56,57). The predicted clearance was well correlated with *in vivo* clearance calculated from pharmacokinetic information on the same individuals, irrespective of a large interindividual difference in the CYP3A content between 1.6 and 27.3 pmol/mg of S-13 protein. These results suggested that the extrapolation of *in vitro* metabolic clearance determined in the *in vitro* samples prepared under well-controlled condition (*e.g.*, biopsy) well predicts *in vivo* metabolic clearance and interindividual variation in human by using biological scaling factor (56,57) as observed for rat studies (21). Therefore, the extrinsic factors such as preparation process and/or storage condition of liver samples from human are likely responsible for the potential loss of metabolic activity, resulting in the systematic underprediction by biological scaling factors for human samples (23,27,38,41).

The extrahepatic metabolism for the drug in human has been well documented (58–60), which could theoretically contribute to the discrepancy between *in vivo* intrinsic clearance and *in vitro* metabolic clearance (*in vivo*>*in vitro*). Metabolizing enzymes in the extrahepatic tissues include CYP isoforms (61), flavin-containing monooxygenases (62,63), carboxylesterase (64–67), and UDP-glucuronosyltransferases (68). Especially for the compounds undergoing CYP3A-mediated metabolism in the liver with negligible urinary excretion as a parent form, the intestinal first-pass metabolism (as a clearance in the intestine, $CL_G$, in Eq. 6) can significantly contribute to the total body clearance ($CL_{tot}$) as a hidden factor as follows (69–71):

$$CL_{tot} = CL_H + F_H \times CL_G \tag{6}$$

Therefore, the hepatic clearance (and *in vivo* metabolic clearance in the liver) calculated from total body clearance is likely overestimated by ignoring potential clearance from intestinal metabolism (Eq. 6). However, the contribution of intestinal first-pass metabolism has been reported to be quantitatively significant for CYP3A substrates only when the compound undergoes extensive first-pass metabolism in the liver with the hepatic metabolic clearance for unbound drug of >100 mL/min per kilogram (which corresponds to >4 mL/min per gram liver assuming that the liver and body weights in human are 1,700 g and 70 kg, respectively) (72,73). These data suggested that the overestimation would be only evident for the compounds extensively metabolized by both liver and intestine such as cyclosporine (74), tacrolimus (75), and midazolam (76,77). Generally, the quantitatively important contribution of extrahepatic metabolism to the total body clearance becomes discernible by the specialized surgical procedures applied to assess the AUC values after drug administration and blood sampling at a number of sites relative to the liver and the extrahepatic organs of interest, which is practical only in the experimental animals (30,70,78). Instead, the judicious combination of *in vitro* metabolic clearance from preclinical animals as well as human and *in vivo* total body clearance in preclinical animals has been successfully adopted for the prediction of hepatic clearance in human with an aid of empirical and drug-specific scaling factor for the *in vitro*-to-*in vivo* extrapolation (43,79,80). Drug-specific scaling factor represents a correction factor for any systematic difference between *in vitro* and *in vivo* parameters or systematic underprediction of *in vivo* clearance caused by indiscernible factor(s) including potential contribution of extrahepatic metabolism determined by the *in vitro*/*in vivo* correlation (or *IVIVC*) approach. Although the mechanism(s) of discrepancy would not be fully understood by the time when the new drug candidate enters the predevelopment stage, the *IVIVC* method has been helpful for the preliminary prediction of clearance in human.

Systematic underprediction of *in vivo* metabolic clearance can be also caused by the lack of appropriate correction of nonspecific bindings of compounds to the *in vitro* incubation matrices such as microsomal lipids and cellular components. It is an important tenet of pharmacokinetics that the only unbound compound, which is assumed to undergo the free exchange and reach rapid equilibrium between outside and inside cells, can be subject to the metabolism and excretion. Therefore, the unbound fractions in blood and/or in the *in vitro* incubation matrices have been often used to convert the values from enzyme kinetics into that for the unbound compound in order to quantitatively predict pharmacokinetics in experimental animals and human (19,35,43,81,82). The prediction ability of *in vitro* metabolic data obtained from human liver microsomes was examined for 29 drugs with wide ranges of structure and physicochemical property (19): predictions for the hepatic clearance ($CL_H$) were carried out with "well-stirred" model (83) either disregarding both unbound fractions in the blood ($f_B$) and in the microsomal reaction mixture ($f_m$; Eq. 7), incorporating only $f_B$ (Eq. 8) or incorporating both $f_B$ and $f_m$ (Eq. 9).

$$CL_H = \frac{Q_H \times CL_{u,met}}{Q_H + CL_{u,met}} \tag{7}$$

$$CL_H = \frac{Q_H \times f_B \times CL_{u,met}}{Q_H + f_B \times CL_{u,met}} \tag{8}$$

$$CL_H = \frac{Q_H \times f_B \times \frac{CL_{u,met}}{f_m}}{Q_H + f_B \times \frac{CL_{u,met}}{f_m}} \quad (9)$$

As shown in Fig. 6, the predictions based on the *in vitro* values reasonably well agreed with human hepatic clearance when both unbound fractions were taken into predictions (shown by open circles in Fig. 6). For these drugs, the systematic underprediction of hepatic clearance was observed if only unbound fraction in the blood was incorporated into the equation (shown by closed triangles in Fig. 6). On the other hand, the discrepancy (as expressed by fold error between predicted and observed hepatic clearance) became indiscernible among prediction models with or without taking unbound fractions into the prediction for the drugs with high hepatic clearance. As the hepatic clearance becomes closer to hepatic blood flow, the clearance is more independent of metabolic (intrinsic) clearance consisting of unbound fractions (in blood/microsomal incubation mixture) and intrinsic clearance for unbound form. Results indicated that the incorporation of both unbound fractions ($f_B$ and $f_m$) would provide best predictions of human hepatic clearance based on the *in vitro* microsomal data regardless of structure, physicochemical property, or hepatic clearance of drugs.

The hepatic clearance is described by different equations, depending on the mathematical model used for describing disposition of compounds in the liver, and the relation between hepatic clearance and intrinsic clearance ($CL_{int}$) depends on the models as shown by Fig. 7. Under the assumption that the drug (or chemical entity) is mixed infinitely well inside the liver, the "well-stirred" model is applicable to the hepatic clearance (83):

$$CL_H = \frac{Q_H \times CL_{int}}{Q_H + CL_{int}} \quad (10)$$

In the opposite extreme case in terms of flow dynamics of solutes in the sinusoidal space of the liver, the drug is mixed only in the infinitely small section along the flow path from input to output of the liver, the "parallel-tube" model is applicable (83):

$$CL_H = Q_H \times \left(1 - e^{-\frac{CL_{int}}{Q_H}}\right) \quad (11)$$

In addition to the aforementioned two extreme cases, based on the analysis on the distribution of hepatic residence time of solutes after the bolus injection into the liver, the "dispersion" model (Eq. 2) described in the previous section was introduced as the mathematical model more relevant to the observed flow dynamics in the liver than others (84):

$$CL_H = Q_H \times R_B \times \left[1 - \left(\frac{4a}{(1-a)^2 \exp\left[\frac{a-1}{2D_N}\right] - (1-a)^2 \exp\left[-\frac{a+1}{2D_N}\right]}\right)\right] \quad (12)$$

where $a = (1 + 4 \times R_N \times D_N)^{0.5}$ and $R_N = CL_{int}/Q_H$. $D_N$ is the dispersion number which determines the extent of dispersion of solutes in the liver: the "dispersion" model (Eq. 2) becomes both "well-stirred" (Eq. 10) and "parallel-tube" (Eq. 11) models when the $D_N$ approaches infinite (*i.e.*, infinitely mixed condition) and zero (*i.e.*, no mixing condition), respectively. The prediction of *in vivo* hepatic clearance from the same intrinsic clearance is not significantly different among models, while the calculation of *in vivo* intrinsic clearance from the observed hepatic clearance is more dependent on the models for the compounds with higher hepatic clearance, closer to the hepatic blood flow rate (Fig. 7a). For example, the values of *in vivo* intrinsic clearance for the hypothetical compound having hepatic clearance of 20 mL/min per kilogram are calculated to be 420, 93, and 64 mL/min per kilogram by "well-stirred," "dispersion," and "parallel-tube" models, respectively, when the hepatic blood flow rate ($Q_H$) is assumed to be 21 mL/min per kilogram (52). For the direct comparison of intrinsic (metabolic) clearance between *in vivo* and *in vitro*, the "*in vivo*" intrinsic clearance is often calculated from *in vivo* clearance by using "well-stirred" model because of the mathematical simplicity. The calculation can yield the "overestimated *in vivo*" intrinsic clearance, leading to the underprediction by the *in vitro* intrinsic (metabolic) clearance. On the other hand, the prediction of oral clearance (Fig. 7b), which directly relates hepatic clearance and availability with information on absorption fraction to AUC value after oral administration (Eqs. 1 and 5), highly depends on the model used for the prediction, especially for the compounds with relatively high intrinsic clearance (> 20 mL/min per kilogram). Comparisons of the accuracy in the predictions among different mathematical models have been reported (10,21,79). Results indicated that the three models predicted hepatic clearance with equal levels of accuracy (10,21), while the predictions of *in vivo* metabolic clearance from *in vitro* data were comparable between "parallel-tube" and "dispersion" models, and both more accurate with less biased and higher precision than "well-stirred" model (21) or the "dispersion" model provided more reliable prediction for the high-clearance drugs than the other models (20,85–87).

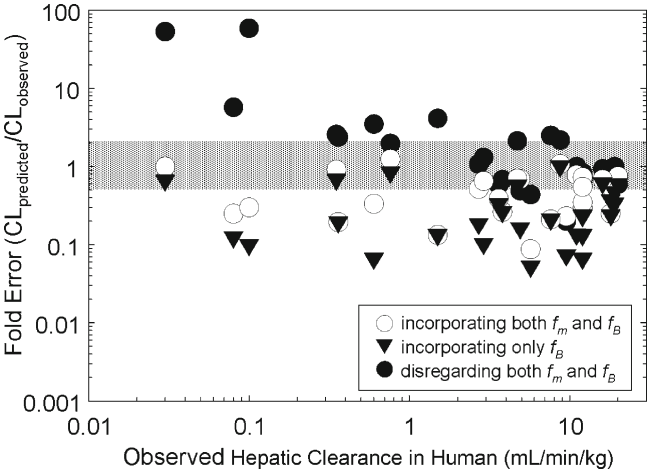


**Fig. 6.** Accuracy of prediction for the hepatic clearance in human from *in vitro* metabolic clearance with or without incorporating unbound fractions. Data in the *shaded area* are considered to be successful (0.5<fold error<2). Data are taken from (19)

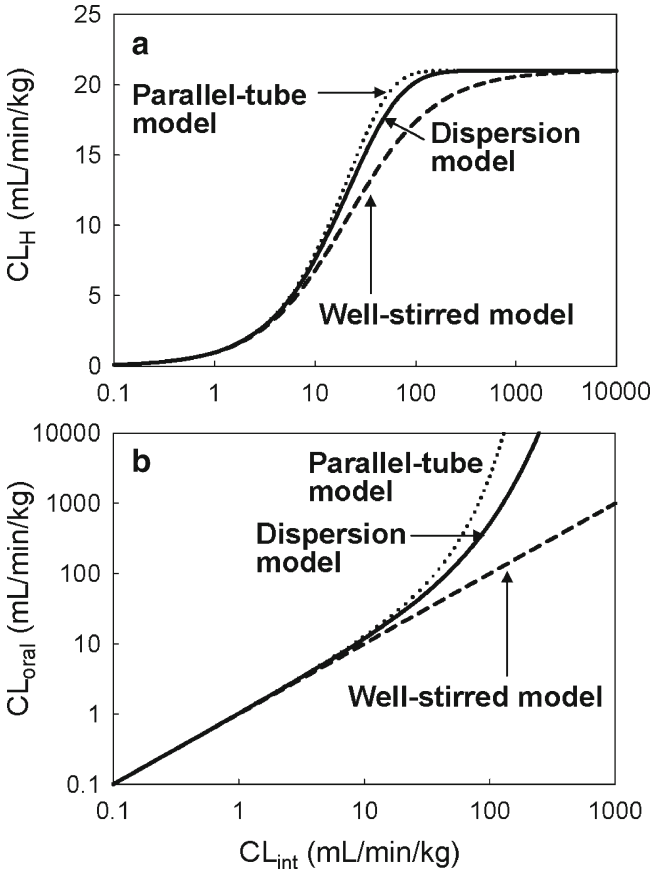


**Fig. 7.** Effects of intrinsic clearance ($CL_{int}$) on hepatic clearance ($CL_H$; **a**) and oral clearance ($CL_{oral}$; **b**) in various mathematical models. Simulations were carried out by "well-stirred" model (Eq. 10), "parallel-tube" model (Eq. 11) and "dispersion" model (Eq. 2). The values of $Q_H$, $f_B$, and $D_N$ are 21 (mL/min/kg), 1.0, and 0.17, respectively. The value of $CL_{oral}$ was calculated from $CL_H$ and $F_H$ (Eq. 3) based on the Eq. 5, assuming $f_a \times F_G = 1$

The expression of intrinsic clearance for the unbound compound involved in the overall elimination in the liver ($CL_{int,all}$) has been further expanded into the incorporation of distinct processes representing diffusions on the sinusoidal membrane and biliary excretion as well as metabolic degradation (88–90). The general (or expanded) expression of $CL_{int,all}$ (Eq. 13) consists of clearance for the diffusions through membrane in the direction of sinusoid → hepatocytes ($PS_{inf}$) and that of hepatocytes → sinusoid ($PS_{eff}$) as well as the clearance for metabolic degradation and/or biliary excretion as a parent form if any ($CL_{int}$). The equation has become more versatile and crucial as the roles of uptake and efflux transporters as well as diffusion barriers have been increasingly recognized as a rate-determining step in the elimination from the liver (4,6,7).

$$CL_{int,all} = \frac{PS_{inf} \times CL_{int}}{PS_{eff} + CL_{int}} \quad (13)$$

Equation 13 indicates that the rate-determining step in the overall elimination in the liver changes according to the relative magnitude of $PS_{eff}$ *versus* $CL_{int}$. In the case that the membrane permeability (or $PS_{eff}$) of the compound is much less than the metabolic clearance (or the clearance for the metabolism plus biliary excretion if any, *i.e.*, $PS_{eff} << CL_{int}$), $CL_{int,all}$ becomes equal to $PS_{inf}$ as follows:

$$CL_{int,all} = \frac{PS_{inf} \times CL_{int}}{PS_{eff} + CL_{int}} \rightarrow PS_{inf} \text{ when } PS_{eff} << CL_{int} \quad (14)$$

This indicates that the calculated *in vivo* intrinsic clearance from *in vivo* hepatic clearance does not necessarily represent the clearance for the metabolism and/or biliary excretion. The *in vivo* intrinsic clearance can be much larger than the predicted intrinsic clearance from *in vitro* (metabolism) studies if the $PS_{inf}$ is larger than $CL_{int}$. The value of $PS_{inf}$ is more contributing to the *in vivo* intrinsic clearance when transporter(s) is (are) more involved in the influx process especially for the less lipophilic compounds in Fig. 5. These compounds likely show larger discrepancies between *in vivo* (overall) intrinsic clearance and *in vitro* metabolic clearance (*i.e.*, *in vivo* intrinsic clearance > *in vitro* metabolic clearance). In contrast, for the highly membrane permeable compounds ($PS_{inf}$ and $PS_{eff} >> CL_{int}$), the contribution of transporter(s) to either $PS_{inf}$ or $PS_{eff}$ would be less significant, and the passive diffusion determines the clearance for both directions ($PS_{inf} \approx PS_{eff}$). Then, $CL_{int,all}$ becomes equal to $CL_{int}$ as follows:

$$CL_{int,all} = \frac{PS_{inf} \times CL_{int}}{PS_{eff} + CL_{int}} \rightarrow CL_{int} \text{ when } PS_{inf} \approx PS_{eff} >> CL_{int} \quad (15)$$

Therefore, under the condition in Eq. 15, the *in vivo* intrinsic clearance can represent the clearance by metabolism (plus biliary excretion as a parent form if any). One may need to be aware that the relative magnitude of $PS_{eff}$ *vs.* $CL_{int}$ can also change along with the escalation of dose, due to the decreased $CL_{int}$ by the saturation of metabolism (91,92).

Recent analysis on the relation between plasma protein binding and transporter-mediated hepatic uptake also provided possible explanations for the underprediction of hepatic clearance (93). The trend for the underprediction of *in vivo* hepatic clearance in human was more evident for the compounds which were highly bound to the plasma proteins (unbound fraction<5%) and substrates of hepatic uptake transporters (40). These observations suggested that the overestimation of the effect of plasma protein binding can lead to an inaccurate prediction of *in vivo* intrinsic clearance: the plasma protein binding would not restrict the access of compounds to the hepatocytes as much as anticipated from the assumption of rapid equilibrium between unbound and bound forms of compound in the process of high extraction by a transporter-mediated hepatic uptake (93,94).

## PREDICTION OF TARNSPORTER-MEDIATED HEPATIC UPTAKE CLEARANCE FROM *IN VITRO* DATA

Theoretically, the transporter-mediated uptake often or partly at least becomes a rate-determining step as a determinant of hepatic intrinsic clearance as described in the previous

section (4,7,8,95). Much evidence has been accumulated to support the notion that the transporter-mediated hepatic uptake has been playing an important role in the hepatic intrinsic clearance (and hepatic clearance) in human for many drugs (4,7). Such evidence includes the drug–drug interaction by the inhibition of transporters (1–3) and interindividual variation of pharmacokinetics by the genetic polymorphism of transporters (96–100). For example, cyclosporine A significantly increased systemic exposures of cerivastatin (1) and repaglinide (3) by inhibiting OATP1B1-mediated hepatic uptake (2). Genetic polymorphism of OATP1B1 (96–100) caused variation in the pharmacokinetics in human for many drugs such as fexofenadine (101), pitavastatin (102,103), pravastatin (104–110), repaglinide (111,112), rosuvastatin (113), temocapril (109), and valsartan (109). Although these data strongly suggest that the transporters are involved in the elimination process of drugs as much as the pharmacokinetics are affected by the perturbations of transporter activities, the quantitative prediction of *overall* intrinsic clearance ($CL_{int,all}$ in Eq. 13) by each individual clearance remains to be challenging (90).

For the quantitative extrapolation of human CYP-mediated metabolic activity in the recombinant CYP isoform to that in the liver microsomes, the utilization of relative activity factor (RAF) was proposed by Crespi (114). The RAF represents the ratio of the metabolic activity of the CYP-isoform-specific marker substrate in the human liver microsomes to that in the complementary-DNA-expressed system for the same isoform, and the value quantitatively facilitates bridging metabolic activities between both *in vitro* systems (115–118). Successful extrapolations of *in vitro* metabolic clearance obtained in the recombinant CYP isoforms to that *in vivo* have been well documented for the compounds with less significant contribution of hepatic uptake to overall clearance in human (115,119–121). This approach cannot only provide quantitative information on the relative contribution of CYP isoform(s) involved in the metabolism of the compound of interest but also the preliminary prediction of hepatic clearance attributable to the CYP-mediated metabolism in human at discovery stage (87,116,122,123). Similar methods have been applied to the quantitative assessment for relative contribution of particular transporter to the overall uptake of the compound of interest in hepatocytes by using RAFs with the reference ligands such as taurocholate (124) and estradiol-17β-D-glucuronide ($E_2$17βG) (125) to Ntcp and Oatp1a1, respectively. In these approaches, the values of RAF were calculated as the ratios of uptake clearance of test compound to that of reference ligand in the transporter expressing systems ($R_{exp}$) and hepatocytes ($R_{hep}$). The percentage of contribution was then calculated as follows (124,125):

$$\%\ contribution = \frac{R_{exp}}{R_{hep}} \times 100 \qquad (16)$$

The method has been further extended to the assessment of percentage contributions of OATP1B1- and OATP1B3-mediated hepatic uptakes of pitavastatin (126), $E_2$17βG (126), and fexofenadine (127) in human hepatocytes by applying reference ligands, estrone 3-sulfate and cholecystokinin octapeptide for OATP1B1 and OATP1B3, respectively, to the uptake clearance in the transporter-expressing HEK293 cells and cryopreserved human hepatocytes. The concept of RAF with reference ligand to the specific transporter has been successfully utilized to predict percentage contribution of particular transporter to overall hepatic uptake process observed in hepatocytes, and the method warranted potential application to the quantitative predictions for the hepatic clearance in human based on the clearance determined in the specific transporter-expressing cells.

Cryopreserved human hepatocytes have been used for the assessment of hepatic clearance as they have demonstrated to retain the activities of OATP transporters which were reasonably comparable with those in freshly isolated hepatocytes from human (128–130). A typical experimental protocol for the determination of initial uptake rate includes a rapid separation of cellular component from the incubation medium by the centrifugation of cell suspension layered over silicone oil, followed by the determination of compound amount in the cell lysate after the digestion with potassium hydroxide or trichloroacetic acid (131,132). The designated time points for the sampling need to be short enough to calculate the initial slope for the linear uptake into the cell along the incubation time, when the uptake process is assumed to be predominant in the accumulation of compounds in the cell. Also, the radiolabeled compound is often used in order to avoid the potential underestimation of the transported amount in the cell caused by the metabolic degradation. Therefore, most of published studies have examined the hepatic uptake of radiolabeled compounds into the cells through the silicon oil layer by the rapid centrifugation (126,128,133,134), while the method is not practical at the early discovery stage when the radiolabeled compound is not routinely available. Recently, the "media loss" assay in the isolated rat hepatocytes, which determines the loss of parent compound from the incubation medium, has been proposed to quantitatively predict the impact of hepatic uptake on the intrinsic clearance *in vivo* for the unbound compound in rat (90,135). The values of (unbound) intrinsic clearance for 36 compounds, which were not significantly eliminated into bile or urine as parent forms, were calculated from (1) the initial disappearance rate from whole suspension (conventional method), (2) the AUC of compound concentrations in the incubation medium, and (3) the initial disappearance rate from the incubation medium (Fig. 8). Predicted intrinsic clearance was compared with the observed *in vivo* values, and the comparison indicated that the conventional prediction (based on the disappearance from whole-cell suspension) yielded the poorest projection ($r^2$=0.25 and average ratio=57) among three approaches, while the prediction from the initial disappearance rate from medium provided the best projection ($r^2$=0.72 and average ratio=3) (135). Large differences (as the fraction of drug in the incubation medium<0.65) were observed for the majority of compounds between concentrations in the whole-cell suspension (conventional assay) and in the incubation medium ("media loss" assay), suggesting that the incorporation of hepatic uptake clearance is crucial for the accurate prediction of *in vivo* intrinsic clearance for most of the compounds tested in the study. The values of *in vivo* intrinsic clearance for the marketed drug transporter substrates (monelukast, bosentan, atorvastatin, and pravastatin) were also predicted well by the *in vitro* intrinsic clearance based on the initial disappearance rate in the "media loss" assay with freshly isolated human hepatocytes (135) (Table II). Interestingly, the

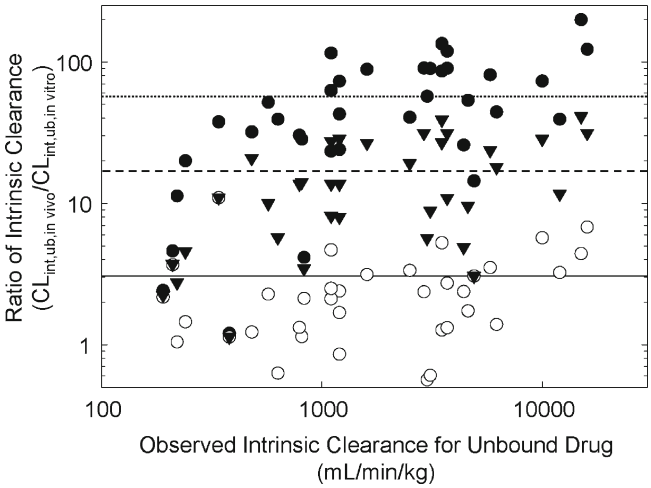


**Fig. 8.** Ratio of intrinsic clearance for unbound drugs calculated from plasma clearance in rat ($CL_{int,\ ub,\ \textit{in vivo}}$) to that determined in the incubation with isolated rat hepatocytes ($CL_{int,\ ub,\ \textit{in vitro}}$). Calculations of *in vitro* intrinsic clearance ($CL_{int,\ ub,\ \textit{in vitro}}$) were based on (1) the initial disappearance rate in whole suspension [conventional method] (*filled circles*, with average ratio=57, *dotted line*), (2) the AUC in the medium (*filled triangles*, with average ratio=16) and (3) the initial disappearance rate in the media (*empty circles*, with average ratio=3, *solid line*) in the isolated rat hepatocytes. Data are taken from (135)

prediction by the "media loss" assay based on the AUC resulted in more significant underprediction (average fold error=16) than that based on the initial disappearance rate (average fold error=3). Theoretically, the intrinsic clearance calculated from "media loss" represents the value for overall elimination consisting of diffusions on the sinusoidal membrane and biliary excretion as well as metabolic degradation (Eq. 13) only when the calculation is based on the AUC from the infinite integration of medium concentration. Therefore, the deviation of calculated intrinsic clearance based on the AUC from observed *in vivo* intrinsic clearance (Fig. 8) suggests potential degradation of cellular activities for transporters/enzymes during incubation for a long time period for the accurate assessment of AUC values (136,137). It was also suggested that the "media loss" assay from the initial disappearance rate may effectively eliminate the terminal phases of polyexponential profiles, the component of which can be affected by the artifacts inherent in the *in vitro* systems: the *in vitro* hepatocytes system may have a greater efflux (hepatocytes → incubation medium) clearance by passive diffusion through the larger surface area than the cells only facing to the perisinusoidal space of Disse in the intact liver (138). The concept of "media loss" assay for the accurate assessment of hepatic uptake clearance has been extended to the two-compartment model which enabled accurate predictions of the clearances for hepatic uptake, passive diffusion, and metabolism for atorvastatin, cerivastatin, and indomethacin by simultaneous fittings to the changes of drug concentrations in both incubation medium and cells along the incubation time in rat hepatocytes (139). The hepatic clearances for these drugs were predicted from the obtained parameters, and the predicted values from "media loss" assay well agreed with the observed hepatic clearance, in contrast to that from conventional assay (based on the disappearance from whole-cell suspension). This approach allows the predictions for the contributions of hepatic uptake to both overall hepatic clearance (Eq. 13) and unbound drug concentrations in the hepatocytes. Compartment-model-based analysis has been further extended to the mechanistic assessment of Michelis–Menten parameters ($K_m$ and $V_{max}$) responsible for the transporter-mediated hepatic uptake, bidirectional passive diffusion, and nonspecific binding which directly reflect those obtained from *in vitro* experiments (140). These mechanistic approaches can provide an integrated tool not only for the accurate predictions of pharmacokinetics but also for the predictions of potential drug–drug interactions by transporter(s) and/or CYP(s) and of efficacy by liver-targeted drug candidates.

Future prediction of hepatic and oral clearance in human judiciously incorporates both information on metabolic clearance and transporter-mediated membrane permeability of drug candidates into the prediction paradigm as described in Fig. 9. Potential contribution of transporter-mediated clearance to overall hepatic clearance would be identified by the evaluation of difference in the concentrations between whole cells and medium in the incubation with cryopreserved human hepatocytes (90,135). Following the identification of positive contribution, the compartment-model based analysis for the time-course of drug concentrations in the hepatocytes (139,140) provides parameters for each transporter-mediated kinetic process in hepatic clearance (Eq. 13). The integrated approach to the *in vitro*-to-*in vivo* extrapolation of hepatic clearance by combining *in vitro* metabolic and transporter-mediated clearance would allow the predictions of hepatic and oral clearance for the drug candidates undergoing kinetically significant transporter-mediated clearance process (es) in human.

**Table II.** Prediction of *In Vivo* Intrinsic Clearance for Unbound Drugs Using Human Hepatocytes Based on the Initial Disappearance Rate in "Media Loss" Assay (Data From (135))

| | | *In vivo* intrinsic clearance for unbound drug (mL/min per kilogram) | |
|---|---|---|---|
| Compound | Mean $CL_{int}$ (μL/min/$10^6$ cells) | Predicted | Observed |
| Montelukast | 360±250 | 1,100 | 2,700 |
| Prazosin | 12±1.2 | 36 | 50 |
| Pravastatin | 2.1±1.4 | 6.6 | 23 |
| Atorvastatin | 100±19 | 320 | 910 |
| Bosentan | 38±6.5 | 120 | 340 |

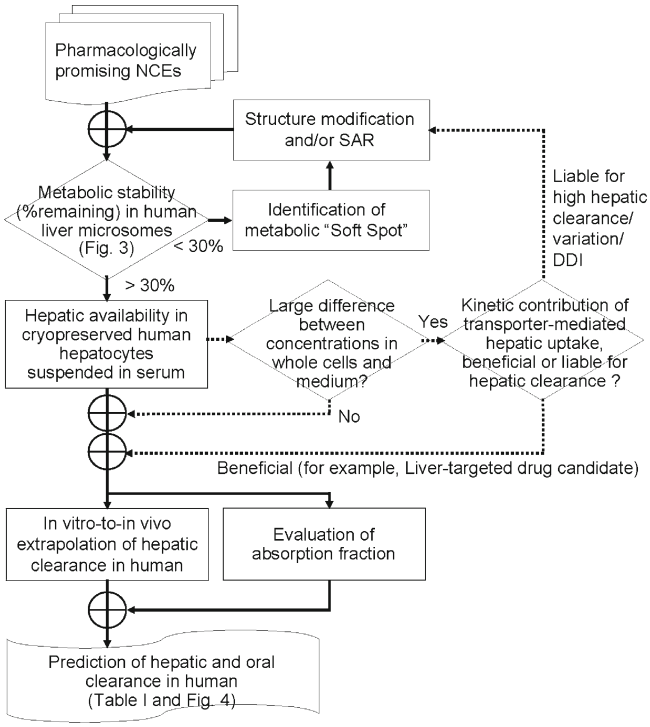


**Fig. 9.** Lead optimization for the successful development candidate by the predictions of hepatic and oral clearance in human. Future workflow for the incorporation of transporter-mediated hepatic uptake into the optimization strategy is shown by *dotted lines*

## CONCLUSION

With recent advance in technology and continued accumulation of our knowledge for drug transporters (4,7), the identification of transporter(s) responsible for the pharmacokinetics of drug candidates has greatly facilitated both discovery and development of new drug candidates with higher POS from drug metabolism and pharmacokinetics point of view. For example, the transporter(s) involved in the hepatobiliary transport in human can be directly identified (141–143) by using the panels of double transfectant (or quadruple transfectant) such as OATP1B1/MRP2 (142,144), OATP1B1/MDR1 (142), OATP1B1/BCRP (142), OATP1B3/MRP2 (145,146), OATP1B1 OATP1B3 OATP2B1/MRP2 (147) and OCT1/MDR1 (148), which consist of the transporters for sinusoidal uptake/canalicular efflux on the basal/apical membranes in the polarized cell lines. Like the identification of CYP isoform(s) involved in the metabolism of the drug candidate, the information on the identified transporter(s) would be very useful to evaluate the liability of potential drug–drug interaction/genetic polymorphism and to assess the POS for liver-targeted candidates. However, unlike the metabolism study with recombinant CYP isoforms, the quantitative predictions of *in vivo* clearances from the transcellular transport data in the transporter(s)-transfected cell lines remain to be explored (95,149).

Cryopreserved human hepatocytes have been widely and routinely utilized as the most versatile *in vitro* system which maintains both metabolizing and transporting activities reasonably comparable with those in the freshly isolated human hepatocytes (27,36,40,42,44,45,47,48). Despite the fact that the values and advantages of hepatocytes for the prediction of hepatic clearance in human have been demonstrated as described herein, the limitation of capability for the quantitative prediction, especially characterized by a systematic underprediction of scaled-up hepatic clearance with a biological scaling factor (hepatocellularity), has emerged from different datasets as a significant concern. In order to mitigate the discrepancy between *in vitro* predication and *in vivo* observation, the empirical and regression-based scaling factors have been inevitably employed for the accurate predictions by human hepatocytes (27,34,35,43,45,79,135). The understanding of potential mechanism(s) underlying the systematic underprediction will improve our confidence of prediction by rationalizing the empirical strategy for the *in vitro*-to-*in vivo* extrapolation of data from human hepatocytes (40,90).

The hepatic clearance has been recognized as a hybrid parameter consisting of distinct clearance processes such as passive diffusion, hepatic (sinusoidal) uptake, efflux from cell to sinusoid, metabolism, biliary excretion, and (canalicular) transporter-mediated efflux into the bile. Although the equation for (overall) intrinsic clearance in the hepatic elimination (Eq. 13) can be simplified under certain circumstances (Eqs. 14 and 15), it is generally difficult to identify the rate-determining step in the hepatic disposition, especially when the information on relative magnitude of clearance representing each process is limited for the drug candidate of interest at discovery stage. The assay of both concentrations in the incubation medium and whole suspension of hepatocytes along with the incubation time can serve as one of the simple methods to identify the compounds having a significant contribution of hepatic uptake to the *in vitro* intrinsic clearance (135). The positive data from this assay will provide strong rationale for further studies on the identification of transporter(s) and the quantitative assessment of hepatic uptake process as a potential rate-determining step in hepatic clearance (139,140).

Future prediction of pharmacokinetics in human will increasingly rely on the systematic and integrated approaches to the hepatic and total-body clearance. Successful prediction paradigm will certainly need to incorporate the *in vitro* piece of quantitative information on physicochemical properties, kinetics of transporter-mediated (sinusoidal and canalicular) transports, and metabolic degradation in the liver as well as qualitative information on the identified transporter(s) and enzyme(s) responsible for the pharmacokinetics in human. Figure 9 shows lead optimization process for the successful development candidate by the predictions of hepatic and oral clearance in human described in this article. Future workflow is also extended by incorporating the evaluations of transporter-mediated hepatic clearance into the optimization strategy as shown by dotted lines. For metabolically promising NCEs, kinetically significant contribution of transporter-mediated clearance process(es) to overall hepatic clearance (Eq. 13) would be subsequently identified by *in vitro* experiments using cryopreserved human hepatocytes. The information on transporter(s) allows the assessment whether the transporter-mediated clearance would likely be liable for high hepatic clearance/interindividual variation/DDI and the con-

tribution should be mitigated by structure modification; or the transporter-mediated clearance would likely be beneficial (for example, for liver-targeted drug candidates) and the discovery should proceed to further evaluations for the development. A judicious combination of quantitative and qualitative data from *in vitro* studies can allow the predictions for the potential effects of transporter-mediated and/or CYP-mediated drug–drug interactions and changes of pathophysiological conditions on the unbound drug concentration in the liver and target organ. It also allows a rational optimization of the pharmacokinetic properties of drug candidates, leading to the successful development of ideal drugs with fewer interindividual differences, fewer drug interactions, and more selective delivery to the target organ (7).

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