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Beyond the Michaelis-Menten: Accurate Prediction of *In Vivo* Hepatic Clearance for Drugs With Low K_M

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Clearance (CL) is the major pharmacokinetic parameter for evaluating systemic exposure of drugs in the body and, thus, for developing new drugs. To predict *in vivo* CL, the ratio between the maximal rate of metabolism and Michaelis-Menten constant (V_{max}/K_M estimated from *in vitro* metabolism study has been widely used. This canonical approach is based on the Michaelis-Menten equation, which is valid only when the K_M value of a drug is much higher than the hepatic concentration of the enzymes, especially cytochrome P450, involved in its metabolism. Here, we find that such a condition does not hold for many drugs with low K_M, and, thus, the canonical approach leads to considerable error. Importantly, we propose an alternative approach, which incorporates the saturation of drug metabolism when concentration of the enzymes is not sufficiently lower than K_M. This new approach dramatically improves the accuracy of prediction for *in vivo* CL of high-affinity drugs with low K_M. This indicates that the proposed approach in this study, rather than the canonical approach, should be used to predict *in vivo* hepatic CL for high-affinity drugs, such as midazolam and propafenone.

Study Highlights

WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?
☑ For over half a century, *in vivo* intrinsic drug clearance (CL) has been predicted by simply scaling the *in vitro* intrinsic drug CL, that is, the ratio between maximal rate of metabolism and Michaelis-Menten constant (V_{max}/K_M).
WHAT QUESTION DID THIS STUDY ADDRESS?
☑ The canonical approach works only when hepatic enzyme concentration is sufficiently lower than the K_M of the drug. We find that such a condition does not hold for drugs with low K_M, and, thus, the canonical approach leads to considerable error in predicting *in vivo* CL.

WHAT DOES THIS STUDY ADD TO OUR KNOWLEDGE?
☑ We propose a new approach, which incorporates the saturation of the metabolism rate at high enzyme concentrations. This accurately predicts the intrinsic *in vivo* CL of high affinity drugs from V_{max} and K_M estimated with *in vitro* experiments.
HOW MIGHT THIS CHANGE CLINICAL PHARMACOLOGY OR TRANSLATIONAL SCIENCE?
☑ The approach proposed in this work improves the accuracy and precision of human hepatic CL prediction using *in vitro* experiments, which is critical for drug development.

Drug clearance (CL), which has been studied in > 65,000 publications,[1] is the primary pharmacokinetic (PK) parameter for predicting human systemic drug disposition.[2] To predict *in vivo* hepatic CL (CL_h) from *in vitro* metabolism studies, well-stirred, parallel tube, or dispersion liver models have been widely used, which commonly require the estimation of intrinsic CL in the liver.[3] The intrinsic CL_h of a drug has been predicted by scaling the ratio between the maximal rate of metabolism and Michaelis-Menten constant (i.e., V_{max}/K_M) estimated from *in vitro* study using human liver microsomes (HLMs) or hepatocytes.[4] This canonical approach assumes that *in vivo* drug metabolism is accurately captured by the Michaelis-Menten (MM) equation, similar to *in vitro* drug metabolism.[4,5]

The MM equation becomes inaccurate when a significant fraction of the drug binds to the enzyme.[6–8] To avoid this, the K_M value of the drug needs to be much larger than the hepatic concentration of the enzymes, especially cytochrome P450 (CYP), involved in its metabolism.[6–8] To the best our knowledge, the validity of this condition has not been systematically investigated. In this study, we find that the condition does not hold for many high-affinity drugs with low K_M, including coumarin (CYP2A6), paclitaxel (CYP2C8), propafenone (CYP2D6), and midazolam (CYP3A4). Thus, under these circumstances, estimating their intrinsic CL_h using the canonical approach based on the MM equation results in considerable error. To resolve this problem, we propose an alternative to the canonical

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approach based on an enzyme kinetic equation, which is accurate over a much wider range of the ratio of K_M to enzyme concentration than the MM equation.[7,8] This new approach accurately predicts intrinsic CL_h for drugs regardless of the ratio of their K_M values to enzyme concentrations, which dramatically improves the accuracy and precision of in vitro-in vivo extrapolation (IVIVE) for the CL_h.

METHODS
Data collection
Drugs in **Table 1** were selected and their K_M, V_{max}, and in vivo CL_h in **Table 2** were obtained using the following criteria:

1. *Selection of drug*: We selected drugs whose metabolism by a specific major CYP isoform in the liver is the main excretion route for the selected drugs (**Table 1**). Thus, metabolism by other CYP enzymes and CL in other organs have small contributions to the drug CL. Furthermore, we selected drugs whose K_M is less than $10 \cdot E_T$, where E_T is the hepatic concentration of the major CYP isoform involved in drug metabolism. ET is estimated under the assumption that CYPs are evenly distributed in the liver is used (**Table 1**). If the estimated E_T increases considering the subcellular localization of CYPs in the liver (**Supplementary Table S1**), K_M/E_T would be less than 10 for a larger number of drugs than those in **Table 1**.
2. K_M *and* V_{max}: To estimate the in vitro CL by the major CYP, in vitro K_M and V_{max} values of the majority of the drugs (**Table 2**) were obtained from previous studies where the values were estimated with product formation by HLM. However, as the values from the product formation study were not available for saquinavir, docetaxel, and valspodar, their K_M and V_{max} values were obtained from substrate depletion studies. In these in vitro experiment, $E_T \ll K_M$ so that using the MM leads to accurate estimation of K_M and V_{max}.[7]
3. *Estimation of in vivo* CL_h: In vivo CL_h in **Table 2** was estimated from total in vivo CL of drugs administered by an i.v. route, except for propafenone and indinavir. As their i.v. route has not been measured, in vivo CL of drugs administrated by an oral route was used. When extrahepatic CL is known, we excluded it from total in vivo CL to estimate in vivo CL_h:

   - For paclitaxel, cyclosporine, midazolam, cabazitaxel, indinavir, and felodipine, we subtracted the estimated renal and/or bile elimination rate of their unchanged forms from their total in vivo CL.
   - For docetaxel, the fraction of its hydroxylated metabolite in the bile, the major elimination pathway, is multiplied to total in vivo CL.
   - For saquinavir, the fraction of its hydroxylated metabolite in systemic circulation is multiplied to total in vivo CL.

As the extrahepatic CL of valspodar is unknown, total in vivo CL was used as in vivo CL_h. Furthermore, as coumarin and propafenone have multiple metabolism pathways, we used the CL by a specific CYP as in vivo CL_h: for propafenone, CL from the parent to metabolite (5-OH propafenone) by CYP2D6 and for coumarin, CL from the parent to metabolite (7-OH coumarin) by CYP2A6.

Estimation of CYP concentration in a whole human liver
The CYP concentration in a whole human liver ($CYP_{conc.}^{liver}$; **Table 1**) is estimated using the following equations under the assumption that CYP is evenly distributed in the liver:

$$CYP_{conc.}^{liver} (\mu M) = \frac{CYP\,amount}{liver\,volume}$$
$$= \frac{CYP\,abundance \times MPPGL \times liver\,weight}{liver\,volume}$$

CYP abundance (pmol/mg) is the amount of CYP enzyme per mg microsomal protein, which is obtained from published data.[9–11] Thirty-two and 52.5 were used as the lowest and the highest microsomal protein per gram of liver (MPPGL).[12,13] Liver weight and density are 1,800 g and 1.05 g/mL, respectively,[14] which led to the estimation of the liver volume as $1,800/1.05 \sim 1,714$ mL.

Simulation and calculation
All the simulation was done using MATHEMATICA 11.0 (Wolfram Research, Champaign, IL). See **Supplementary Excel File** for the detailed calculation of both canonical and new IVIVE approaches.

Data availability
The datasets generated during and/or analyzed during the current study are available in the **Supplementary Excel File**.

RESULTS
Detailed derivation of canonical IVIVE for CL_{int}
The MM equation has been used for over a century to describe the enzyme reaction between a single enzyme (e.g., CYP isoform) and a single substrate (e.g., free drug)[15]:

$$S + E \underset{k_b}{\overset{k_f}{\longleftrightarrow}} C \overset{k_{cat}}{\longrightarrow} P + E \qquad (1)$$
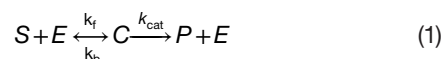
In this system, the free enzyme (E) reversibly binds with the substrate (S) to form the complex (C), and the complex irreversibly dissociates into the product (P) of metabolism and the free enzyme, where the total enzyme concentration, $E_T \equiv C + E$ (μM), is conserved. k_f ($\mu M^{-1} \cdot min^{-1}$) and k_b (min^{-1}) are the forward and reverse rates of substrate binding to the enzyme, and k_{cat} (min^{-1}) is the catalytic constant, which is the maximal product formation rate per enzyme. The enzyme kinetics (Eq. 1) can be described with a simplified MM model under a suitable condition[6] (see **Supplementary Note** for details):

$$\frac{dP}{dt} = \frac{k_{cat} E_T S}{S + K_M}, \qquad (2)$$

where $K_M = \frac{k_b + k_{cat}}{k_f}(\mu M)$ is the Michaelis-Menten constant. When the initial substrate concentration (S_T) is substituted into the

above equation, the initial velocity of the metabolite formation (v) can be obtained, which is known as the MM equation:

$$v = \frac{k_{cat}E_T S_T}{S_T + K_M}. \tag{3}$$

By fitting the MM equation to the velocity data obtained from *in vitro* metabolism experiments, the values of k_{cat} and K_M can be estimated.[16]

In enzyme kinetic field, V_{max} usually indicates the maximal rate of metabolic formation (i.e., $k_{cat}E_T$ µM·min^{-1}). On the other hand, in the drug metabolism field, V_{max} normalized with pmol CYP or mg protein of the microsomes is commonly used.[17] That is, the V_{max} value is reported per pmol CYP (i.e., pmol·min^{-1}·pmol^{-1} CYP) when data are obtained using supersomes prepared from recombinant systems expressing human CYP. When HLM are used, it is reported per mg protein of the microsomes (i.e., pmol·min^{-1}·mg^{-1}). To convert the rate per mg protein of the microsomes to rate per pmol CYP, the CYP abundance in the HLM sample (pmol/mg) is required. However, as few studies of *in vitro* enzyme kinetics with HLM measure the CYP abundance in the sample, we use mean population abundance values for the conversion as has frequently been done in other studies.[17] Through the conversion, we use the unit of pmol·min^{-1}·pmol^{-1} CYP for V_{max} throughout the study. This allows us to replace k_{cat} with the normalized V_{max} in Eq. 2 and, thus, to follow the conventional notation of IVIVE as described below. Note that the estimation results obtained throughout this work do not change when the pmol·min^{-1}·mg^{-1} is chosen for the unit of V_{max} (see **Supplementary Excel File**).

As therapeutic drug concentrations rarely reach their K_M (i.e., $S \ll K_M$ and $S + K_M \approx K_M$),[18] Eq. 2 can be further simplified:

$$\frac{dP}{dt} = \frac{V_{max}E_T S}{S + K_M} \approx \frac{V_{max}E_T S}{K_M} = CL_{int}^{vitro} E_T S, \tag{4}$$

which is referred to as the *canonical* model in this study. The $CL_{int}^{vitro} = \frac{V_{max}}{K_M}$ (mL·min^{-1}·pmol^{-1} CYP) is the intrinsic clearance per enzyme estimated with the *in vitro* metabolism experiment. This model predicts that the drug is metabolized exponentially with the rate of $CL_{int}^{vitro}E_T$ (min^{-1}) for varying E_T (µM). This allows for the estimation of the intrinsic hepatic clearance with a simple scaling of E_T. Specifically, by multiplying CL_{int}^{vitro} by the amount of hepatic CYP isoform ($\tilde{E}_T$ (pmol)), the intrinsic hepatic clearance is predicted:

$$CL_{int}^{liver} = CL_{int}^{vitro} \tilde{E}_T \,(\text{mL/min}), \tag{5}$$

where $\tilde{E}_T$ can be estimated by multiplying the abundance of CYP (pmol/mg) by mg MPPGL and liver weight.[19] This is the *canonical* approach to extrapolate CL_{int}^{liver} from CL_{int}^{vitro} estimated with *in vitro* metabolic data. Note that the canonical approach (Eq. 5) assumes that *in vivo* drug metabolism can be described with the canonical model (Eq. 4) based on the MM model (Eq. 2), similar to *in vitro* metabolism.

The limitation of the canonical approach

In fact, the MM model (Eq. 2) is accurate under a limited condition when enzyme concentration is sufficiently low (i.e., $E_T \ll K_M + S$) that an insignificant fraction of substrate is bound to the enzyme and, thus, the metabolism rate is proportional to the concentration of enzyme.[7,20] Because therapeutic drug concentrations (S) are typically much lower than K_M (i.e., $S \ll K_M$),[18] a much lower enzyme concentration than K_M (i.e., $E_T \ll K_M$) is required to use the canonical model (Eq. 4). This is why *in vitro* experiments have been performed using low concentrations of enzyme (~nM).[21]

Does $E_T \ll K_M$ hold in the liver? To investigate this question, we first estimated hepatic concentrations of various CYP isoforms (E_T) (see Methods for details). To derive the concentration from the amount of hepatic CYP ($\tilde{E}_T$) we need to estimate the volume of CYP distribution (V). If CYP is evenly distributed in the whole liver, the volume of CYP distribution would be the liver volume, 1,714 mL.[14] For instance, the amount of CYP2D6 in the whole liver (i.e., abundance of CYP2D6 in HLM·MPPGL·liver weight) is ~0.54–1.63 µmol (**Table 1**). By dividing this with 1,714 mL, we can estimate that the hepatic concentration of CYP2D6 would be ~ 0.31–0.95 µM (**Table 1**).

Surprisingly, $E_T \ll K_M$ does not hold for many drugs with low K_M (**Table 1**). For instance, the K_M of propafenone (~ 0.12 µM) is even lower than the hepatic concentration of its major metabolizing enzyme, CYP2D6 (~ 0.31–0.95 µM).[22] As the validity condition of the MM model ($E_T \ll K_M$) does not hold, we hypothesized that the canonical approach based on the MM model (Eq. 5) leads to an inaccurate prediction for CL_{int}^{liver} of the propafenone. To investigate this, we compared the kinetics of propafenone metabolism simulated using the canonical model (Eq. 4) and the *full* model (Eq. 8 in **Supplementary Note**). As the full model describes the enzyme kinetics (Eq. 1) based on mass action kinetics without the model simplification to derive the MM model (Eq. 2), it accurately captures the drug metabolism regardless of enzyme concentration. First, we considered the concentration of CYP2D6 (0.0017 µM), which is used in *in vitro* metabolism experiments for propafenone.[22] In this case, as the concentration of CYP2D6 is 100-fold lower than the K_M of the propafenone, the canonical model accurately captures the drug metabolism simulated by the full model (**Figure 1a**). On the other hand, when the concentration of CYP2D6 is increased to its *in vivo* level (~ 0.95 µM; **Table 1**), the canonical model overestimates the rate of drug metabolism by approximately seven-fold higher than the full model (**Figure 1a** inset). This indicates that indeed the canonical approach to extrapolating CL_{int}^{liver} from CL_{int}^{vitro} (Eq. 5) is inaccurate when the K_M of the drug is not much higher than the hepatic concentration of its metabolizing CYP, and, thus, a significant fraction of the drug is bound to the CYP.

The new approach to extrapolate CL_{int}^{liver} from CL_{int}^{vitro}

With the simple modification of the canonical model (Eq. 4), we can derive an alternative model that accurately describes the hepatic drug metabolism even when the K_M of the drug is not sufficiently higher than the hepatic CYP concentration[7,8,23,24] (see **Supplementary Note** for details):

$$\frac{dP}{dt} \approx \frac{V_{max}E_T \overline{S}}{K_M + E_T} = CL_{int}^{vitro} E_T \frac{K_M}{K_M + E_T} \overline{S}, \tag{6}$$

where $\bar{S} = S + C$ is the total substrate concentration regardless of its form. This model is referred to as the *new* model in this study, which leads to a *new* prediction formula for the intrinsic hepatic clearance:

$$CL_{int}^{liver} = CL_{int}^{vitro} \tilde{E}_T \frac{K_M}{K_M + E_T} \, (mL/min), \quad (7)$$

where CL_{int}^{vitro} (mL·min^{-1}·pmol^{-1} CYP) is the intrinsic clearance per enzyme and $\tilde{E}_T$ (pmol) is the amount of hepatic CYP isoform. Next, we investigated whether the new model (Eq. 6) and, thus, the new formula (Eq. 7) are accurate using the example of propafenone. Indeed, the new model accurately simulates the metabolism of propafenone under both low CYP2D6 (0.0017 µM; **Figure 1b**) and high CYP2D6 (0.95 µM) conditions (**Figure 1b** inset). This indicates that the new formula, which is obtained by simply multiplying $\frac{K_M}{K_M + E_T}$ with the canonical formula (Eq. 5), can provide accurate prediction of the intrinsic hepatic clearance rate even when $E_T \ll K_M$ does not hold, unlike the canonical formula. Note that as we assume that therapeutic drug concentrations are much lower than K_M, the new approach also predicts that the drug is metabolized exponentially, similar to the canonical approach (**Figure 1b**). That is, the qualitative dynamics of hepatic drug metabolism predicted with the canonical and new approach are the same. However, their quantitative prediction of the metabolism rates differ by $\frac{K_M}{K_M + E_T}$.

When $E_T \ll K_M$ and, thus, $\frac{K_M}{K_M + E_T} \approx 1$, the new formula (Eq. 7) is nearly the same as the canonical one (Eq. 5). On the other hand, as E_T increases, the new formula predicts that CL_{int}^{liver} becomes saturated, which differs from the unrealistic prediction of the canonical formula (Eq. 5): the unlimited increase of CL_{int}^{liver} with the increase of E_T (**Figure 2a**). The difference between the canonical and new formulas becomes notable when E_T/K_M becomes greater than 0.1 (**Figure 2a**). Thus, unless the K_M of a drug is 10-fold higher than its major metabolizing CYP concentration in the liver, the CL_{int}^{liver} predicted using the canonical approach should be reduced by multiplying $\frac{K_M}{K_M + E_T}$. We compared the CL_{int}^{liver} predicted using the canonical approach and the new approach for 11 drugs whose K_M is not 10-fold higher than their major metabolic CYP concentration in the liver (**Figure 2b** and **Table 1**; see Method for details of drug selection). For these drugs, the canonical approach considerably overestimates CL_{int}^{liver} compared with the new approach.

The new approach more accurately predicts CL_h

For drugs whose K_M is not 10-fold higher than E_T, the canonical approach predicts larger CL_{int}^{liver} than the new approach (**Figure 2b**). Thus, when the predicted CL_{int}^{liver} is substituted into the dispersion model (see **Supplementary Note** for details), a larger CL_h is predicted with the canonical approach than with the new approach (**Table 2**). Importantly, the predicted CL_h with the canonical approach is much larger compared to experimentally measured CL_h. Such overestimation is considerably reduced with the new approach (**Figure 3a,b**). Thus, the new approach improves the accuracy and precision of the prediction for CL_h compared with the canonical approach (**Tables 2** and **3**). Such considerable improvement using the new approach is also observed when other CL_h

Table 1 Estimated CL_{int}^{liver} using the canonical and new approaches

Drugs[a]	K_M (µM)[b]	V_{max} (pmol·min^{-1}·pmol CYP^{-1})[c,d]	Major CYP	CYP abundance in HLM (pmol/mg)	$\tilde{E}_T$ (µmol)[c,d]	E_T (µM)[e]	CL_{int}^{liver} (mL/min) Canonical[f]	CL_{int}^{liver} (mL/min) New[g]
Coumarin	0.75	39.2	2A6	30.7 ~ 56.2[9,22]	1.77 ~ 5.31	1.03 ~ 3.10	92,457 ~ 277,683	38,924 ~ 54,122
Paclitaxel	5.5	4.64	2C8	26.9 ~ 43.0[9,22]	1.55 ~ 4.06	0.90 ~ 2.37	1,308 ~ 3,429	1,123 ~ 2,396
Propafenone	0.12	4.83	2D6	9.34 ~ 17.2[11,22]	0.54 ~ 1.63	0.31 ~ 0.95	21,626 ~ 65,339	5,982 ~ 7,340
Midazolam	1.6	24.4	3A4	32.6 ~ 60.4[11,22]	1.88 ~ 5.71	1.10 ~ 3.33	28,630 ~ 87,025	16,995 ~ 28,246
Indinavir	2.31	9.28					7,535 ~ 22,905	5,112 ~ 9,382
Cyclosporine	5	4.44					1,666 ~ 5,063	1,366 ~ 3,039
Saquinavir	0.61	40.4					124,247 ~ 377,671	44,443 ~ 58,479
Cabazitaxel	2.1	9.5					8,499 ~ 25,835	5,586 ~ 9,992
Docetaxel	1.1	0.20					348 ~ 1,059	175 ~ 263
Valspodar	1.3	2.49					3,588 ~ 10,906	1,947 ~ 3,062
Felodipine	2.81	36.2					24,156 ~ 73,427	17,381 ~ 33,606

CL_{int}^{liver}, intrinsic clearance of the liver; HLM, human liver microsome; K_M, Michaelis-Menten constant; V_{max}, maximal rate of metabolism.
[a]See Methods for the criteria for the drug selections. [b]See **Supplementary Excel File** for references of K_M and V_{max} values.
[c]Obtained by dividing the measured rate per mg of the microsomes by the rate per pmol CYP with mean population abundance of CYP (pmol/mg): CYP2A6 (45.37), CYP2C8 (32.30), CYP2D6 (13.26), and CYP3A4 (45.07).[9–11,22] [d]The hepatic CYP amount is estimated by multiplying CYP abundance in HLM (pmol/mg) by MPPGL and liver weight (see Methods for details).
[e]The hepatic CYP concentration is estimated based on the assumption that CYPs are evenly distributed (see Methods for details).
[f]Canonical $CL_{int}^{liver} = \frac{V_{max}}{K_M} \cdot \tilde{E}_T$. [g]New $CL_{int}^{liver} = \frac{V_{max}}{K_M} \cdot \tilde{E}_T \cdot \frac{K_M}{K_M + E_T} = $ Canonical $CL_{int}^{liver} \cdot \frac{K_M}{K_M + E_T}$. See **Supplementary Excel File** for detailed calculation.

models, such as the parallel tube model and the well-stirred model are used (**Tables 2 and 3**).

DISCUSSION

For drugs with low K_M values (**Table 1**), prediction of *in vivo* drug clearance from *in vitro* data using the canonical approach has been unsatisfactory.[12,21,25] Although various causes of the problem, such as the choice of liver model,[3,26] have been proposed, here, we propose that it could be mainly due to the fact that the PK behavior of drugs with low K_M in the liver cannot be captured by the conventional MM equation. The detailed analysis of enzyme kinetics shows that when the K_M of drugs is low and, thus, not 10-fold higher than the hepatic concentration of their major metabolic CYP (E_T), a significant fraction of the drug ($\sim \frac{E_T}{K_M + E_T}$) is bound to CYP. In this case, the fundamental scaling assumption of the canonical approach (i.e., CL_{int}^{liver} doubles as the amount of hepatic enzyme doubles) does not hold, which causes considerable error in predicting CL_{int}^{liver} with the canonical approach (**Figure 1a**). Thus, we propose an alternative approach (Eq. 7) considering the fact that the saturation of metabolism rate occurs when the enzyme concentration is not sufficiently lower than K_M (**Figure 2a**). The new approach accurately predicts the CL_{int}^{liver} of 11 drugs with low K_M (**Figure 1b** and **2b**), which leads to a considerable improvement in predicting their CL_h compared with the canonical approach (**Figure 3** and **Table 2**). Such improvement in prediction of CL_h with the new approach proposes the validity check for the canonical approach in the current IVIVE process (**Figure 4**).

The V_{max} and K_M values, needed to compare the canonical and new approaches (Eq. 7), have been reported for a few drugs with low K_M because drug candidates with low K_M might be excluded from drug development due to potential problems of low bioavailability and drug-drug interactions. Furthermore, we selected drugs that are mainly metabolized by a single CYP enzyme and do not exhibit inhibitory and inducible activities for CYP (see Methods for details). Thus, small number of drugs are used to investigate the accuracy and precision of canonical and new IVIVE (**Figure 3**) compared with recent IVIVE studies.[5,27,28] Thus, further studies are needed with a large number of drugs to fully confirm the advantage of using the new approach over the canonical approach. This might be achieved by considering a large number of drugs metabolized by multiple CYP enzymes. For drugs metabolized by multiple CYPs, the canonical approach estimates the contribution of each CYP to net metabolism with the V_{max}/K_M and then combines them to estimate global CL_{int}^{liver}.[17] Similarly, our new approach can be extended for drugs with multiple metabolic pathways by simply combining the estimated contribution of each CYP to metabolism with the new approach. It would be interesting in future work to investigate how this extended approach improves the estimation of drug CL.

The values of CL_{int}^{liver} and CL_h estimated with the canonical approach in this study (**Table 2**) do not match with those estimated in all previous studies, which have reported values with a large range. For instance, the range of CL_{int}^{liver} for midazolam and propafenone reported by previous studies

Table 2 Predicted CL_h using the canonical and new approaches and measured *in vivo* CL_h

Drugs	Major CYP	$f_{u\text{-}blood}$[a]	$f_{u\text{-}mic}$[a]	Dispersion model Canonical	Dispersion model New	Well-stirred model Canonical	Well-stirred model New	Parallel tube model Canonical	Parallel tube model New	*In vivo* CL_h (mL/min)[a,c]
				Predicted CL_h (mL/min)						
Coumarin	2A6	0.02	0.99[b]	971 ~ 1,357	547 ~ 718	814 ~ 1,151	508 ~ 622	1,048 ~ 1,418	606 ~ 765	625
Paclitaxel	2C8	0.12	0.66	215 ~ 484	187 ~ 364	204 ~ 436	179 ~ 335	219 ~ 507	190 ~ 376	141
Propafenone	2D6	0.06	0.26	1,329 ~ 1,444	822 ~ 919	1,119 ~ 1,320	701 ~ 775	1,400 ~ 1,449	881 ~ 990	400
Midazolam	3A4	0.07	0.54	1,260 ~ 1,436	1,060 ~ 1,256	1,050 ~ 1,288	884 ~ 1,046	1,345 ~ 1,449	1,146 ~ 1,341	429
Indinavir		0.40	0.97	1,190 ~ 1,425	1,028 ~ 1,264	988 ~ 1,256	859 ~ 1,054	1,279 ~ 1,447	1,111 ~ 1,349	624
Cyclosporine		0.07	0.37	281 ~ 669	236 ~ 462	263 ~ 584	223 ~ 418	288 ~ 711	241 ~ 482	209
Saquinavir		0.02	0.58	1,292 ~ 1,440	876 ~ 1,005	1,081 ~ 1,303	742 ~ 841	1,372 ~ 1,449	942 ~ 1,085	452
Cabazitaxel		0.1	0.42[b]	1,051 ~ 1,389	855 ~ 1,120	876 ~ 1,193	726 ~ 931	1,135 ~ 1,435	919 ~ 1,209	790
Docetaxel		0.07	0.01[b]	1,286 ~ 1,440	1,024 ~ 1,194	1,075 ~ 1,300	855 ~ 991	1,367 ~ 1,449	1,106 ~ 1,283	504
Valspodar		0.02	0.07[b]	721 ~ 1,215	467 ~ 650	624 ~ 1,010	422 ~ 568	769 ~ 1,304	488 ~ 689	182
Felodipine		0.01	0.08	1,179 ~ 1,423	1,042 ~ 1,289	979 ~ 1,251	869 ~ 1,077	1,269 ~ 1,446	1,125 ~ 1,369	829

CL_h, hepatic clearance; $f_{u\text{-}blood}$, drugs unbound fraction in blood; $f_{u\text{-}mic}$, drugs unbound fraction in microsome.
[a]See **Supplementary Excel File** for references of $f_{u\text{-}blood}$, $f_{u\text{-}mic}$, and *in vivo* CL_h. [b]$f_{u\text{-}mic}$ of coumarin, cabazitaxel, docetaxel, and valspodar were estimated using this equation, $\frac{1}{C \times 10^{0.56 \log P/D - 1.41} + 1}$, as they have not been measured.[17] Microsomal protein concentrations (C) for coumarin, cabazitaxel, docetaxel, and valspodar were 0.03, 0.5, 1, and 1 mg/mL, respectively. Log $P = 1.39$ for coumarin and Log $D_{7.4} = 3.3$ for cabazitaxel were calculated using ACD/Labs Percepta Platform—PhysChem Module. Log $D_{7.4} = 6.5$ and 4.5 for docetaxel and valspodar were referenced by Bu, 2006. [c]See Methods and **Supplementary Excel File** for detailed derivation of *in vivo* CL_h.
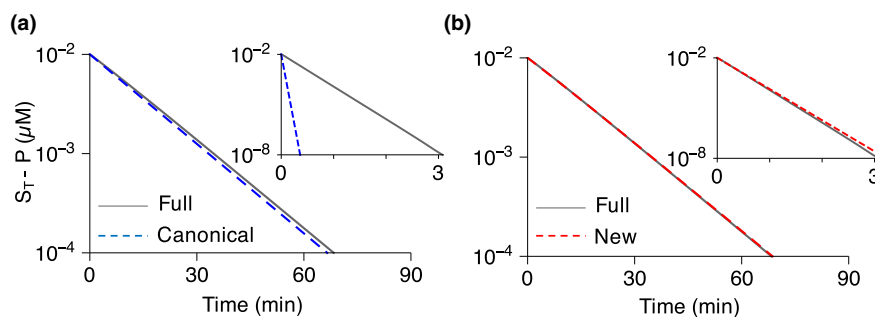
Figure 1 Simulated propafenone metabolism under *in vitro* (CYP2D6 = 0.0017 μM) and *in vivo* (CYP2D6 = 0.95 μM) conditions. (**a**) The canonical model (Eq. 4) accurately simulates propafenone metabolism under the *in vitro* condition, but not the *in vivo* condition (inset). Here, S_T represents the initial drug concentration. For the simulations, $V_{max} = 4.83$ pmol·min^{-1}·pmol^{-1} CYP and $K_M = 0.12$ μM are used based on the experimental measurement (**Table 1** and **S1**). $E_T = 0.0017$ μM[22] and $E_T = 0.95$ μM are used for *in vitro* and *in vivo* simulations, respectively (**Table 1**). $k_f = 100$ μM/ min and $k_b = 7.17$ min^{-1} are used for the full model (Eq. 8 in **Supplementary Note**) simulation so that $K_M = 0.12$ μM. $S(0) = 0.01$ μM, $C(0) = 0$ and $P(0) = 0$. (**b**) The new model (Eq. 6) accurately simulates the drug metabolism under both *in vitro* and *in vivo* conditions (inset). K_M, Michaelis-Menten constant; V_{max}, maximal rate of metabolism.

are 8.8 ~ 200 mL/min/kg [25,28–32] and 6.5 ~ 644.9 mL/min/kg,[28,30,31] respectively. Thus, our estimations are consistent with those in some previous works (midazolam[25,30–32] and propafenone[31]), but not the others (midazolam[28,29] and propafenone[28,30]). Such differences were mainly attributed to different experimental systems used in previous studies. For instance, they used V_{max} and K_M obtained with hepatocytes,[28,29] whereas we used ones estimated with HLM. Furthermore, they used the estimated values of f_{u-mic} and $f_{u-blood}$,[28,30] whereas we used experimentally measured ones.

As the CYP amount increases, both the canonical approach and the new approach predict the increase of CL_{int}^{liver} and, thus, CL_h, but with different rates: the new approach predicts a lower increase than the canonical approach when $E_T/K_M > 0.1$ (**Figure 2a**). For instance, if the amount of CYP3A4 in the liver ($\tilde{E}_T$) is increased from 1.8 μmol by 10-fold, the canonical approach predicts that the CL_h of cyclosporine increases by ~ 325%. On the other hand, our new approach predicts the increase by only ~65% because E_T in the denominator of Eq. 7 also increses and, thus, saturation of metabolism occurs. Consistent with the prediction of the new approach, when CYP3A4 is increased by 600 mg rifampin for 11 days, the CL of cyclosporine administered by intravenous route was increased by only ~ 40%,[33] although rifampin increases CYP3A4 activity in primary human hepatocytes by 10-fold.[34] Unlike CL_h, as the level of CYP3A4 is much lower in the gut than in the liver,[11] the new approach predicts that the induction of intestinal CYP3A4 greatly increases the CL of cyclosporine in the gut (i.e., the range of $E_T/K_M < 0.1$ in **Figure 2a**). Indeed, when CYP3A4 is induced by rifampin, the CL of cyclosporine administrated by the oral route, which occurs considerably in the gut, was increased by 270%.[33] These results indicate that when CYP is induced the saturation of metabolism rate seems to occur for drugs with low K_M in the liver. However, note that p-glycoprotein-mediated cellular efflux of cyclosporine can be increased by rifampin,[35] leading to decrease in cyclosporine bioavailability through elevation of efflux in the gut. In addition, hepatic induction of p-glycoprotein by rifampin may increase hepatic CL of cyclosporine via bile, although hepatic metabolism plays a major role in CL of cyclosporine.

The canonical and new approaches make similar prediction of *in vivo* CL_h for drugs with high K_M (**Figure 3a**). On the other hand, the new approach decreases the prediction of *in vivo* CL_h compared with the canonical approach when $K_M/E_T < 10$. This seems to be paradoxical as the canonical approach frequently leads to underestimation of *in vivo* CL for many drugs.[20,28,31] However, interestingly, compared with drugs with high K_M, such an underestimation less frequently occurs for drugs with low K_M, including ones we have discussed (e.g., midazolam and saquinavir),[27,30–32,36] which is consistent with our findings (**Figure 3**). That is, the level of the underestimation of canonical IVIVE seems to vary depending on the K_M of the drug, which could be due to the inaccurate prediction of the canonical approach for drugs with low K_M. Thus, using the new approach, which can provide a consistent prediction regardless of K_M, might be helpful in finding a way to resolve the underestimation problem (e.g., universal scaling regardless of K_M).[37] Furthermore, as enzyme concentration is typically low (~nM) in the *in vitro* experiment[9,11,22] and, thus, $E_T \ll K_M + S$ holds, using the MM equation to estimate K_M is valid. However, if such condition does not hold, K_M can be dramatically overestimated,[7] which can lead to the underprediction of *in vivo* CL_h. Such overestimation of K_M can be resolved by using the Eq. 9 in the Supplementary Note (ref. [7]), which might help to resolve underprediction problem of IVIVE.

If the estimated concentration of hepatic CYPs is too high (**Table 1** and **Figure 4**), they may not be fully inhibited by CYP inhibitors as CYP inhibitors do not reach sufficient concentrations *in vivo* to inhibit target CYPs.[38] For instance, the K_i value of voriconazole, a CYP3A4 inhibitors, is 0.66 μM,[39] and hepatic concentration of its free form is 4.96 μM with a typical dose (i.e., 200 mg).[40–42] Our estimation of hepatic CYP3A4 concentration is 1.10 ~ 3.33 μM (**Table 1**). When these values are substituted into the formula of K_i (i.e., the ratio between the concentration of complex and reactants), ~ 78–86% of CYP3A4 is expected to form a complex with voriconazole (**Table S4**). However, due to the short half-life of voriconazole (~ 5 hours), it is questionable to use the static equation of K_i. When the equation is applied to another CYP3A4 inhibitor, fluconazole, which has a long
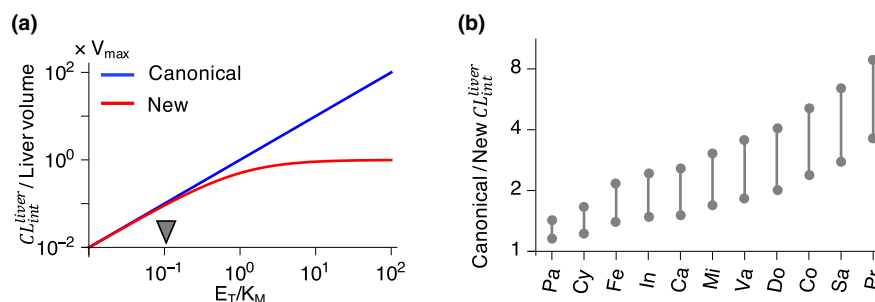
Figure 2 CL_{int}^{liver} predicted with the canonical approach and the new approach. (**a**) The canonical approach (Eq. 5) predicts an unlimited increase of CL_{int}^{liver} as enzyme concentration increases. On the other hand, the new approach (Eq. 7) predicts the saturation of CL_{int}^{liver}. Unless $E_T/K_M < 0.1$, which is highlighted by the arrow, the two approaches lead to different predictions for CL_{int}^{liver}. (**b**) The canonical approach predicts considerably larger CL_{int}^{liver} than the new approach for drugs whose K_M is not 10-fold higher than their major metabolizing CYP concentration in the liver (**Table 1**): Pa, paclitaxel; Cy, cyclosporine; Fe, felodipine; In, indinavir; Ca, cabazitaxel; Mi, midazolam; Va, valspodar; Do, docetaxel; Co, coumarin; Sa, saquinavir; Pr, propafenone. See **Table 1** for the detailed calculation of CL_{int}^{liver}. See Methods for the detailed description of drug selection. CL_{int}^{liver}, intrinsic clearance of the liver; V_{max}, maximal rate of metabolism; K_M, Michaelis-Menten constant.; E_T, total enzyme concetnration.
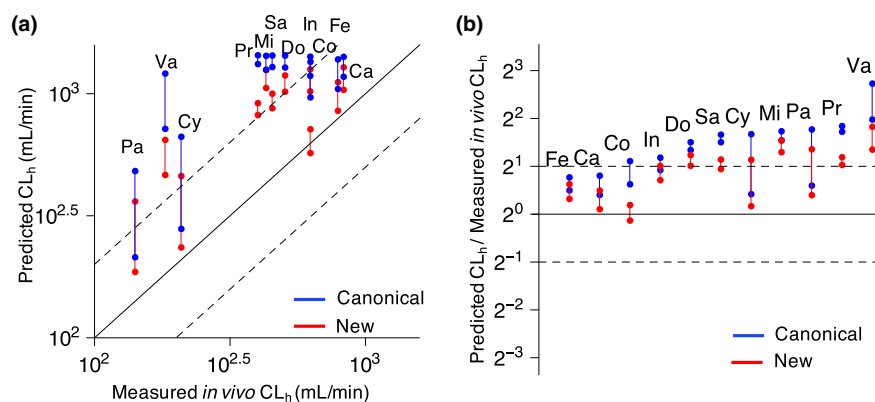

Figure 3 The new approach provides more accurate prediction of hepatic clearance (CL_h) than the canonical approach. (**a**) Relationships between measured *in vivo* CL_h and predicted CL_h (**Table 2**). Here, the dispersion model is used to predict CL_h based on intrinsic clearance of the liver (CL_{int}^{liver}) estimated by either the canonical or the new approach (see Methods and **Table 2** for details). The solid and dashed lines represent the line of identity and twofold error, respectively. (**b**) Precision error of CL_h predicted with the canonical and new approaches. Pa, paclitaxel; Cy, cyclosporine; Fe, felodipine; In, indinavir; Ca, cabazitaxel; Mi, midazolam; Va, valspodar; Do, docetaxel; Co, coumarin; Sa, saquinavir; Pr, propafenone.

half-life (~ 22 hours),[43] ~ 93–94% of CYP3A4 is expected to form a complex with fluconazole (**Table S4**). On the other hand, for itraconazole and ketoconazole having high protein-binding affinity, extremely low fraction of CYP3A4 (i.e., 2% and 1%, respectively) is expected to form a complex (**Table S4**), which raises the question of our estimation for CYP3A4 concentration. In fact, their free form concentration is much less than 10-fold of K_i. Thus, the static equation of K_i predicts, even when CYP3A4 concentration is extremely low, high fraction of CYP3A4 cannot be in complex with itraconazole and ketoconazole in contrast to their strong CYP3A4 inhibition. This paradox of itraconazole has been resolved by a recent study, which identifies that even metabolites of itraconazole inhibit the CYP3A4 and raises the question of using the concentration of free form of itraconazole to predict its strong inhibitory effect.[44] Taken together, the estimation with CYP3A4 inhibitors having weak protein binding affinity (fluconazole and voriconazole), but not with those having strong protein binding affinity (itraconazole and ketoconazole), indirectly support that the estimated CYP

concentration (**Table 1**) is in a reasonable range. However, it is difficult to make rigorous conclusion with the static equation of K_i, and more detailed analysis is needed considering the properties of CYP inhibitors, such as selectivity, inhibitory mode, nonspecific binding, and PK behavior.

In this study, to test the validity condition ($K_M/E_T > 10$), we used the concentration of CYPs (E_T) estimated under the assumption that CYPs are evenly distributed in hepatocytes (**Table 1**). However, the actual concentration of CYP isoforms can be higher because CYPs are primarily localized in the membrane of the endoplasmic reticulum, which is about 20% of the total cell volume. If we adopt the increased concentration of CYP isoforms, the prediction of the CL_h with the new approach changes considerably (**Supplementary Figure S1** and **Tables S1 and S2**). Furthermore, a larger number of drugs than those presented in this study (**Table 1**) fail to satisfy the validity condition of the canonical approach ($K_M/E_T > 10$), and, thus, necessitate the new approach presented in this study (**Figure 4**). However, restricting the compartment to the membrane of the endoplasmic reticulum

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Table 3 Accuracy and precision of predicted CL_h using the canonical and new approaches

	Dispersion model		Well-stirred model		Parallel tube model	
	Canonical	New	Canonical	New	Canonical	New
AFE	2.46	1.83	2.14	1.58	2.57	1.94
AAFE	2.46	1.84	2.14	1.62	2.57	1.95
RMSE (mL/min)	709	433	560	305	753	494
R-RMSE	2.06	1.15	1.67	0.88	2.20	1.28

AAFE, absolute average fold error; AFE, average fold error; CL_h, hepatic clearance; RMSE, root mean squared error; R-RMSE, relative-root mean squared error.
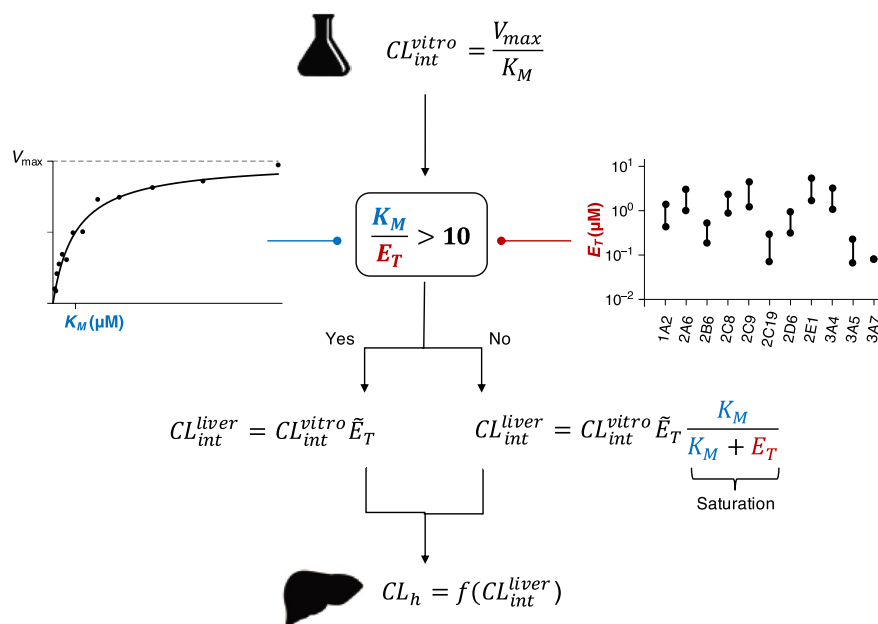
Figure 4 Incorporation of the validity check for the canonical approach into *in vitro-in vivo* extrapolation for clearance (CL). If K_M of the drug is not 10-fold higher than the hepatic concentration of its major CYP (E_T), the canonical approach cannot capture the saturation of metabolism caused by the binding of a significant fraction of the substrate to the enzyme. Thus, to extrapolate CL_{int}^{liver} from CL_{int}^{vitro}, the new approach should be used, which incorporates the saturation of metabolism. See **Supplementary Table S3** and Methods for the detailed estimation procedure for the hepatic E_T. CL_{int}^{liver}, intrinsic clearance of the liver; CL_{int}^{vitro} *in vitro* intrinsic clearance of the liver; K_M, Michaelis-Menten constant; V_{max}, maximal rate of metabolism.

can disrupt the correlation between *in vivo* and *in vitro* systems. For instance, it is questionable to use the same value of K_M for both the *in vitro* system based on a homogenous distribution of the substrate and the *in vivo* system based on an inhomogeneous cellular localization of the substrate. In particular, if the volumes occupied by substrate and enzyme are different, then even when enzyme concentration is higher than the substrate concentration, the amount of substrate can be in excess of the amount of enzyme. In this case, unlike our analysis based on concentration, the amount of substrate bound to enzyme is negligible and the canonical approach can be still valid. Thus, further study is needed to estimate the drug CL considering the subcellular localization of CYP isoforms in the liver.

When a drug-enzyme interaction occurs at multiple sites cooperatively, the relationship between substrate concentration and the velocity of metabolism becomes sigmoidal rather than hyperbolic.[18,45] In this case, instead of the MM model (Eq. 2), the model based on the Hill equation has been used to investigate CL.[18] On the other hand, when substrate inhibition is observed, a modified MM model incorporating the inhibition effect of the substrate has been used.[18] However, similar to the MM model, all of these current models are valid only when the enzyme concentration is sufficiently low, relative to the K_M value,[46] which does not always hold in the liver, as shown in this study (**Tables 1 and S1**). Thus, alternative models, which consider the saturation of metabolism at high enzyme concentrations, need to be developed as done in this study.

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