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**When does the rate-determining step in the hepatic clearance of a drug switch from sinusoidal uptake to all hepatobiliary clearances? Implications for predicting drug-drug interactions**

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**d) Nonstandard abbreviations:** AUCR = area under the curve ratio;  $CL_{\text{bile}}$  = biliary (canalicular) efflux clearance;  $CL_{\text{int}}$  = intrinsic clearance;  $CL_{\text{met}}$  = metabolic clearance;  $CL_{\text{in}}^{\text{s}}$  = sinusoidal influx clearance;  $CL_{\text{ef}}^{\text{s}}$  = sinusoidal efflux clearance; DDI = drug – drug interaction; ECM = extended clearance model; IVIVE = *in vitro* to *in vivo* extrapolation; HPGL =  $10^6$  hepatocytes per gram

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liver; NDA = new drug application; NME = new molecular entity; OATP = organic anion transporting polypeptide;  $PI_{\text{met+bile}}$  = percent inhibition of  $CL_{\text{met+bile}}$  necessary for  $RDS_{\text{uptake}}$  to switch to  $RDS_{\text{all}}$ ; PBPK = physiologically based pharmacokinetics; MPPGL = microsomal protein per gram liver; SCRH = sandwich cultured rat hepatocytes; SCHH = sandwich cultured human hepatocytes; RDS = rate-determining step

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## ABSTRACT

For dual transporter/enzyme substrate drugs, the extended clearance model (ECM) can be used to predict the rate-determining step(s) (RDS) of a drug and hence predict its drug-drug interaction (DDI) liabilities (i.e. transport, metabolism, or both). If the RDS of the hepatic clearance of the drug is sinusoidal uptake clearance ( $CL_{in}^s$ ), even if the drug is mainly eliminated by hepatic metabolism, its DDI liability (as viewed from changes to systemic drug concentrations) is expected to be inhibition or induction of uptake transporters but not hepatic enzymes. However, this is true only if the condition required to maintain  $CL_{in}^s$  as the RDS is maintained. Here, we illustrate through theoretical simulations that the RDS condition may be violated in the presence of a DDI. That is, the RDS of a drug can switch from  $CL_{in}^s$  to all hepatobiliary clearances (i.e. metabolic/biliary clearance [ $CL_{met+bile}$ ] and  $CL_{in}^s$ ) leading to unexpected systemic DDI's, such as metabolic DDI's when only transporter DDI's are anticipated. As expected, these analyses revealed that the RDS switch depends on the ratio of  $CL_{met+bile}$  to sinusoidal efflux clearance ( $CL_{ef}^s$ ). Additional analyses revealed that for intravenously administered drugs, the RDS switch also depends on the magnitude of  $CL_{in}^s$ . We analyzed published *in vitro* quantified hepatobiliary clearances and observed that most drugs have  $CL_{met+bile}/CL_{ef}^s$  ratio < 4, and hence in practice, the magnitude of  $CL_{in}^s$  must be considered when establishing the RDS. These analyses provide insights, previously not appreciated, and a theoretical framework to predict DDI liabilities for drugs that are dual transporter/enzyme substrates.

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## INTRODUCTION

Identifying liabilities with respect to drug – drug interactions (DDI) is important in drug development. In 2015, 25 out of the 33 new drug applications (NDA's) contained *in vitro* transporter data and out of 20 clinical trials using the new molecular entities (NME's) as victim drugs, only 9 resulted in a significant area under the curve (AUC) change (Yu et al., 2017). These data acknowledge that drug transporters are important in determining drug disposition (Giacomini et al., 2010; Hillgren et al., 2013; Patel et al., 2016).

As shown by the hepatic extended clearance model (ECM), when a drug is both transported into and metabolized/biliary excreted by the liver, the rate-determining step (RDS) in the systemic clearance of the drug can be its hepatic uptake clearance, metabolic clearance, biliary (canalicular efflux) clearance, or all hepatobiliary clearances (Miyachi et al., 1987; Sirianni and Pang, 1997; Shitara et al., 2006; Kushara and Sugiyama, 2009; Li et al., 2014; Patilea-Vrana and Unadkat, 2016). The RDS of a drug can be identified using models such as the Extended Clearance Concept Classification System (ECCCS) and the Extended Clearance Classification System (ECCS) that use the drug's *in vitro* quantified hepatobiliary clearance values or the drug's physicochemical properties, respectively (Camenisch and Umehara, 2012; Varma et al., 2015). Using such models is advantageous since the RDS of a drug helps identify where the DDI liabilities lie. Of note, unless indicated otherwise, all subsequent reference to DDI should be interpreted as those DDI that can be observed from measurement of the systemic concentrations of the victim drug. For example, if the RDS of a drug is its hepatic uptake clearance ( $RDS_{\text{uptake}}$ ), then the focus of the DDI studies should be transporter-based (e.g. hepatic organic anion-transporting polypeptide (OATP) – mediated uptake of atorvastatin, (Maeda et al., 2011)) or if the RDS is both hepatic uptake and metabolic/biliary clearance ( $RDS_{\text{all}}$ ), the focus of DDI studies should be all hepatobiliary pathways (e.g. OATP and CYP-mediated clearance of cerivastatin, (Mück et al., 1999; Backman et al., 2002)).

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Here, we asked if knowledge of the RDS of a drug is enough to predict DDI liabilities for drugs that are dual transporter/enzyme substrates? If it is not, the focus of DDI studies will be misdirected and will result either in a negative or unexpected DDI and therefore toxicity. Under the worst-case scenario, the latter will lead to discontinuation of drug development. The end result is that both outcomes will increase drug development cost (Paul et al., 2010). For these reasons, it is important to ask: can the RDS switch from hepatic uptake clearance to all hepatobiliary clearance pathways, thus resulting in unexpected systemic DDIs? Using the ECM theory and simulations, we aimed to: i) provide a theoretical framework of when the  $RDS_{\text{uptake}}$  switches to  $RDS_{\text{all}}$  in the presence of a DDI, and ii) apply the RDS framework to predict DDI liabilities through theoretical and practical examples. The resulting analyses and simulations provide novel insights, hitherto not appreciated, into factors that determine when a victim drug experiences the  $RDS_{\text{uptake}}$  switch to  $RDS_{\text{all}}$  and elucidate important considerations when predicting DDI liabilities for drugs that are substrates of both hepatic transporters and enzymes.

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## MATERIALS AND METHODS

### Theoretical background

The ECM describes complex hepatobiliary clearance in terms of transport at the sinusoidal membrane via sinusoidal influx ( $CL_{in}^s$ ) and efflux ( $CL_{ef}^s$ ), transport at the canalicular membrane via biliary efflux ( $CL_{bile}$ ), metabolism ( $CL_{met}$ ), hepatic blood flow ( $Q_h$ ), and fraction unbound in blood ( $f_{ub}$ ) (Eq. 1).  $CL_{in}^s$  and  $CL_{ef}^s$  terms incorporate both transport-mediated plus passive diffusion clearance while  $CL_{bile}$  describes active transport only. The interrelationships between the hepatobiliary clearances defined by the ECM create the RDS in the hepatic clearance of a drug. As described by us and others (Miyachi et al., 1987; Sirianni and Pang, 1997; Shitara et al., 2006; Patilea-Vrana and Unadkat, 2016), these can be: i)  $RDS_{met+bile}$  when the metabolic and biliary efflux clearances of the drug are much less than sinusoidal efflux clearance ( $CL_{met+bile} \ll CL_{ef}^s$ ) and the drug is highly permeable (passive diffusion  $\gg$  active transport,  $CL_{in}^s \approx CL_{ef}^s$ ) and can thus rapidly distribute across the sinusoidal membrane, ii)  $RDS_{uptake}$  when the metabolic plus biliary efflux clearances are much greater than the sinusoidal efflux clearance ( $CL_{met+bile} \gg CL_{ef}^s$ ), or iii)  $RDS_{all}$  when a drug has both active transport and metabolism but the two extreme scenario from above do not apply ( $CL_{in}^s \neq CL_{ef}^s$ ).

$$CL_h = \frac{Q_h f_{ub} CL_{in}^s (CL_{met} + CL_{bile})}{Q_h (CL_{ef}^s + CL_{met} + CL_{bile}) + f_{ub} CL_{in}^s (CL_{met} + CL_{bile})} \quad (1)$$

Identifying the RDS of a drug can be used to predict the liability of transporter versus metabolic DDI's (see Patilea-Vrana and Unadkat, 2016 for simulations of systemic and hepatic AUC when hepatobiliary clearances are inhibited). For example, while a victim drug has  $RDS_{uptake}$ , inhibition of  $CL_{met+bile}$  will not result in a significant increase in the systemic AUC even though such DDI could result in significant drug accumulation in the liver and hence potentially enhanced hepatic efficacy or toxicity of the drug. That is, from the point of view of a systemic (e.g. victim plasma concentrations) measurements, inhibition of  $CL_{met+bile}$  will be incorrectly

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interpreted as negative because there will be no change in systemic concentrations of the drug. On the other hand, inhibition of  $CL_{in}^s$  will result in an increase in the drug's systemic AUC (and therefore potentially non-hepatic efficacy and toxicity of the drug) but will result in no changes in the hepatic AUC provided the liver is the primary eliminating organ (see Patilea-Vrana and Unadkat, 2016 for examples). However, less appreciated is the fact that in the presence of metabolic/biliary efflux DDI, the RDS of a drug can switch from  $RDS_{uptake}$  to  $RDS_{all}$  and hence switch the DDI liability from uptake transporters to both metabolic/biliary and uptake pathways. Consequently, the drug's systemic AUC will significantly change due to metabolic and biliary efflux DDI's even though uptake was the RDS of the drug in the absence of a DDI. This would lead to unexpected DDIs as viewed from the systemic concentrations of the victim drug. Therefore, through MATLAB simulations (R2016a; MathWorks, Natick, MA), we illustrated when the  $RDS_{uptake}$  to  $RDS_{all}$  switch occurs for a victim drug in the presence of a DDI. We then applied our proposed RDS framework to published *in vitro* hepatobiliary clearances to determine if *in vivo* observed DDI liabilities can be correctly predicted. While the insights illustrated can be derived from analytical solutions of the ECM equation (Eq. 1), for clarity, we chose to use simulations to illustrate the principles of these DDI liabilities within the RDS framework.

### Simulation assumptions

The hepatic ECM was simulated using the governing differential equations as previously described (Endres et al., 2009; Patilea-Vrana and Unadkat, 2016) and for simplicity, the following assumptions about the victim drug were made: i) it was administered intravenously (IV); ii) fraction unbound ( $f_u$ ) in blood and tissue (liver) was set to 1; iii) liver was the only eliminating organ; iv)  $Q_h$  was set to 1 L/min. All references to systemic AUC are derived from drug concentrations in blood. Our conclusions regarding the RDS switch are generalizable to when victim drugs are administered orally but our conclusions of the RDS dependence on  $CL_{in}^s$  only apply for IV administered drugs (see text below). Furthermore, for oral drug administration,



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our findings apply only to changes to the hepatic clearance/bioavailability of the victim drug and do not address the intestinal availability of the victim drug. If there is significant non-hepatic clearance, our conclusions will stand except that the magnitude of the change observed in the systemic and/or hepatic AUC of the drug will differ (Patilea-Vrana and Unadkat, 2016).

### Identifying when the $RDS_{\text{uptake}}$ switches to $RDS_{\text{all}}$ and factors that influence this switch

First, we determined when the RDS of a drug switches from uptake clearance to all hepatobiliary clearance pathways. This requires violating the condition  $CL_{\text{met+bile}} \gg CL^{\text{S}}_{\text{ef}}$ , the condition necessary for uptake clearance to be the RDS in the hepatic clearance of drug. To illustrate this effect, for three theoretical victim drugs where  $CL_{\text{met+bile}} \gg CL^{\text{S}}_{\text{ef}}$ , ( $CL_{\text{met+bile}} = 1, 10, 100$  L/min,  $CL^{\text{S}}_{\text{ef}} = 0.1$  L/min, and  $CL^{\text{S}}_{\text{in}} = 1 \times Q_h$ ), the systemic AUC ratio (AUCR) of the victim drug in the absence and presence of 10-99% inhibition of  $CL_{\text{met+bile}}$  was simulated. Following the FDA guidelines, an AUCR of 1.25 was considered to be significant.

To illustrate that the  $CL_{\text{met+bile}}/CL^{\text{S}}_{\text{ef}}$  ratio and not the absolute magnitude of  $CL_{\text{met+bile}}$  and/or  $CL^{\text{S}}_{\text{ef}}$  determines when the  $RDS_{\text{uptake}}$  switches to  $RDS_{\text{all}}$  we conducted the following simulations: the systemic AUC of the drug was simulated for  $CL^{\text{S}}_{\text{ef}}$  values ranging from 0.1 to 10 L/min (representing 0.1x to 10x $Q_h$ ) with  $CL_{\text{met+bile}}$  set to 1-20 fold the value of the corresponding  $CL^{\text{S}}_{\text{ef}}$  value. The simulated systemic AUC's when  $CL_{\text{met+bile}}/CL^{\text{S}}_{\text{ef}}$  ratio was held constant were compared to the simulated systemic AUC's when  $CL_{\text{met+bile}}/CL^{\text{S}}_{\text{ef}}$  ratio varied.

Next, we defined the tipping point as the  $CL_{\text{met+bile}}/CL^{\text{S}}_{\text{ef}}$  ratio at which  $RDS_{\text{uptake}}$  switches to  $RDS_{\text{all}}$ . Following the same strategy as above, we simulated the AUCR for various  $CL_{\text{met+bile}}/CL^{\text{S}}_{\text{ef}}$  ratios for victim drugs that originally had  $RDS_{\text{uptake}}$  to illustrate the  $CL_{\text{met+bile}}/CL^{\text{S}}_{\text{ef}}$  ratio at which  $AUCR = 1.25$ , thus signifying that  $RDS_{\text{uptake}}$  switched to  $RDS_{\text{all}}$ . The systemic AUC where the RDS is uptake was simulated such as  $CL_{\text{met+bile}}/CL^{\text{S}}_{\text{ef}}$  ratio = 1000 ( $AUC_{\text{ratio = control}}$ ,  $CL_{\text{met+bile}} = 100$  L/min,  $CL^{\text{S}}_{\text{ef}} = 0.1$  L/min). Then, systemic AUC was simulated for  $CL_{\text{met+bile}}/CL^{\text{S}}_{\text{ef}}$

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“test” ratios ranging from 0.1 – 10 ( $CL_{\text{met+bile}} = 0.01 - 1 \text{ L/min}$ ,  $CL_{\text{ef}}^s = 0.1 \text{ L/min}$ ) and the resulting AUC ( $AUC_{\text{ratio = test}}$ ) was normalized to the control simulation ( $AUCR = AUC_{\text{ratio = test}}/AUC_{\text{ratio = control}}$ ). The decrease in  $CL_{\text{met+bile}}/CL_{\text{ef}}^s$  ratio is akin to inhibition of  $CL_{\text{met+bile}}$  since  $CL_{\text{ef}}^s$  is held constant. The  $CL_{\text{met+bile}}/CL_{\text{ef}}^s$  ratio which resulted in a significant change to the systemic AUC ( $AUCR = 1.25$ ) compared to control was identified as the tipping point.

To illustrate that the magnitude of  $CL_{\text{in}}^s$  contributes to the tipping point, we simulated the tipping point for  $CL_{\text{in}}^s$  values ranging from  $0.01xQ_h - 4xQ_h$  (henceforth, for simplicity,  $CL_{\text{in}}^s$  notation will be used instead of  $f_u CL_{\text{in}}^s$  since  $f_u = 1$ ). The tipping point can be explicitly derived from the ECM (Eq. 1) by defining the RDS switch for any chosen AUCR as  $AUCR = RDS_{\text{uptake}}/RDS_{\text{all}}$  and solving for the  $CL_{\text{met+bile}}/CL_{\text{ef}}^s$  ratio (Eq. 2). This relationship (Eq. 2 with  $AUCR = 1.25$ ) was used later to identify DDI liabilities when considering  $CL_{\text{in}}^s$  magnitude and  $CL_{\text{met+bile}}/CL_{\text{ef}}^s$  ratio of a drug.

$$\text{Tipping point} = \frac{1}{(AUCR - 1) \left(1 + \frac{CL_{\text{in}}^s}{Q_h}\right)} \quad (2)$$

Quantifying when a drug with  $RDS_{\text{uptake}}$  will switch to  $RDS_{\text{all}}$  due to metabolic/biliary efflux DDI's

We defined  $PI_{\text{met+bile}}$  as the percent inhibition of  $CL_{\text{met+bile}}$  required for  $RDS_{\text{uptake}}$  to switch to  $RDS_{\text{all}}$ . This quantifies when a significant DDI ( $AUCR \geq 1.25$ ) will occur due to inhibition of  $CL_{\text{met+bile}}$  even when uptake was the RDS in the absence of DDI. For  $CL_{\text{met+bile}}/CL_{\text{ef}}^s$  ratios ranging from 1-100,  $CL_{\text{met+bile}}$  was inhibited 10-99%. Simulations were conducted for  $CL_{\text{in}}^s$  values = 0.25x, 1x, 4x $Q_h$ .  $CL_{\text{in}}^s$  values were chosen to represent ER = 0.2, 0.5, and 0.8 (low, mid, and high extraction ratio (ER), respectively) and were back calculated from Eq. 3-4. The percent inhibition of  $CL_{\text{met+bile}}$  at which the  $CL_{\text{met+bile}}/CL_{\text{ef}}^s$  ratio reaches the tipping point (i.e.  $PI_{\text{met+bile}}$ ) and thus causes the  $RDS_{\text{uptake}}$  to switch to  $RDS_{\text{all}}$  was calculated as shown in Eq. 5.

$$CL_h = Q_h * ER \quad (3)$$

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$$CL_h = \frac{Q_h f_{ub} CL_{in}^s}{Q_h + f_{ub} CL_{in}^s} \quad (4)$$

$$PI_{met+bile} (\%) = \frac{CL_{met+bile}/CL_{ef}^s - \text{tipping point}}{CL_{met+bile}/CL_{ef}^s} \times 100 \quad (5)$$

### Applying the RDS framework to *in vitro* and *in vivo* examples

Published data sets where all hepatobiliary clearance pathways ( $CL_{in}^s$ ,  $CL_{ef}^s$ ,  $CL_{bile}$ ,  $CL_{met}$ ) were quantified *in vitro* were collected. The *in vivo* hepatobiliary clearances must be used to identify the RDS of a drug. As such, the provided *in vitro* to *in vivo* extrapolated (IVIVE) clearances were utilized; otherwise, *in vitro* hepatobiliary clearance values were scaled to *in vivo* using IVIVE scaling factors (i.e. MPPGL, HPGL, liver weight) as provided by the authors. For all drugs,  $f_{ub} CL_{in}^s / Q_h$  was used to calculate the tipping point using Eq. 2 (see Results section below). RDS was labeled as  $RDS_{uptake}$  and  $RDS_{all}$  if the  $CL_{met+bile} / CL_{ef}^s$  ratio was above and below the tipping point, respectively. For drugs with  $RDS_{uptake}$ , the  $PI_{met+bile}$  was calculated using Eq. 5. Finally, for selected drugs, the predicted DDI liabilities using the RDS and  $PI_{met+bile}$  were compared to the observed *in vivo* data. To ensure that only the systemic clearance, and not bioavailability of the victim drug was affected, clinical DDI studies were included if the victim was a dual transporter/enzyme substrate and co-administered with a selective enzyme inhibitor administered IV. It should be noted that the availability of such studies was limited.

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## RESULTS

### Identifying the tipping point (i.e. when $RDS_{\text{uptake}}$ switches to $RDS_{\text{all}}$ ) and factors that influence this switch

As described under theoretical background,  $RDS_{\text{uptake}}$  occurs when  $CL_{\text{met+bile}} \gg CL_{\text{ef}}^{\text{s}}$ , and as such, inhibition of  $CL_{\text{met+bile}}$  will not manifest in the systemic AUC of a victim drug. However, when the above condition is violated due to extensive inhibition of  $CL_{\text{met+bile}}$ , there will be a significant increase in the systemic AUC of the victim drug when  $CL_{\text{met+bile}}$  is inhibited further. In other words, when  $CL_{\text{met+bile}}$  is no longer  $\gg CL_{\text{ef}}^{\text{s}}$ , then  $RDS_{\text{uptake}}$  switches to  $RDS_{\text{all}}$ . In Fig.1A, 84%, 98%, 99.8% inhibition of  $CL_{\text{met+bile}}$  led to a clinically significant increase in the systemic AUC of the three theoretical victim drugs shown ( $AUCR \geq 1.25$ ). Even though the victim drugs had different pre-inhibition  $CL_{\text{met+bile}}$  values (1, 10, 100 L/min), the post-inhibition  $CL_{\text{met+bile}}$  values were all the same (0.2 L/min). Since  $CL_{\text{ef}}^{\text{s}}$  was kept constant (0.1 L/min), an  $AUCR$  of 1.25 was observed when  $CL_{\text{met+bile}}/CL_{\text{ef}}^{\text{s}} = 2$  for all three victim drugs. This simulation illustrates that the  $RDS_{\text{uptake}}$  switch to  $RDS_{\text{all}}$  depends on the  $CL_{\text{met+bile}}/CL_{\text{ef}}^{\text{s}}$  ratio and not the extent of  $CL_{\text{met+bile}}$  inhibition.

To further emphasize the dependence on the  $CL_{\text{met+bile}}/CL_{\text{ef}}^{\text{s}}$  ratio, we simulated the systemic AUC of the victim drug (in the absence of DDI) for different  $CL_{\text{met+bile}}$  and  $CL_{\text{ef}}^{\text{s}}$  values while holding  $CL_{\text{in}}^{\text{s}}$  constant. The systemic AUC remained unchanged when the  $CL_{\text{met+bile}}/CL_{\text{ef}}^{\text{s}}$  ratio remained fixed even though the  $CL_{\text{met+bile}}$  and  $CL_{\text{ef}}^{\text{s}}$  values varied, demonstrating that the RDS in the hepatic clearance of a drug is dependent on the  $CL_{\text{met+bile}}/CL_{\text{ef}}^{\text{s}}$  ratio and not on the absolute value of these clearances (Fig. 1B). This was true for both when  $CL_{\text{met+bile}}$  was higher and lower than  $CL_{\text{ef}}^{\text{s}}$  (also see Supplementary Fig. 1). Since the systemic AUC decreased as the  $CL_{\text{met+bile}}/CL_{\text{ef}}^{\text{s}}$  ratio increased, only the  $CL_{\text{met+bile}}/CL_{\text{ef}}^{\text{s}}$  ratio needs to be considered when determining when the  $RDS_{\text{uptake}}$  switches to  $RDS_{\text{all}}$  for a victim drug.

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Next, we identified the tipping point, defined here as the  $CL_{\text{met+bile}}/CL^{\text{s}}_{\text{ef}}$  ratio when  $RDS_{\text{uptake}}$  switches to  $RDS_{\text{all}}$ . The  $RDS_{\text{uptake}}$  switch to  $RDS_{\text{all}}$  signifies when DDI's due to inhibition of  $CL_{\text{met+bile}}$  start to become significant for a victim drug that has  $RDS_{\text{uptake}}$ . As demonstrated above, the  $RDS_{\text{uptake}}$  switch to  $RDS_{\text{all}}$  depends on the  $CL_{\text{met+bile}}/CL^{\text{s}}_{\text{ef}}$  ratio. As such, we identified the tipping point as the  $CL_{\text{met+bile}}/CL^{\text{s}}_{\text{ef}}$  ratio at which the systemic AUC increases significantly (AUCR = 1.25) due to decrease in the  $CL_{\text{met+bile}}/CL^{\text{s}}_{\text{ef}}$  ratio for a victim drug that has  $RDS_{\text{uptake}}$  (Fig. 1C). As demonstrated in Fig. 1C, the tipping point for a low, mid, and high ER drug was 3.2, 2, and 0.8, respectively.

Since the tipping point varied for a low, mid, and high ER, the magnitude of  $CL^{\text{s}}_{\text{in}}$  is also an important factor in determining when the  $RDS_{\text{uptake}}$  switches to  $RDS_{\text{all}}$  (Fig. 1C). Extending the simulations to identify the tipping point across a range of  $CL^{\text{s}}_{\text{in}}$  values, we established a theoretical (Eq. 2) and practical (Fig 2.) relationship between  $CL^{\text{s}}_{\text{in}}/Q_h$  and the tipping point. The tipping point decreases as  $CL^{\text{s}}_{\text{in}}$  increases. In other words, as a drug's  $CL^{\text{s}}_{\text{in}}$  (and therefore its ER) increases, the drug is more likely to have  $RDS_{\text{uptake}}$  and a larger  $PI_{\text{met+bile}}$ , therefore making the drug more resistant to switching its RDS. In addition, as the influx across the sinusoidal membrane becomes large, hepatic clearance becomes limited by blood flow and therefore less likely to result in a change in AUCR when either  $CL^{\text{s}}_{\text{in}}$  (or for that matter  $CL_{\text{met+bile}}$ ) is inhibited. On the other hand, when  $CL^{\text{s}}_{\text{in}}$  (or ER) is small and the hepatic clearance becomes proportional to  $CL^{\text{s}}_{\text{in}}$ , the victim drug becomes more susceptible to a change in RDS. This demonstrates that low ER drugs are more susceptible to  $RDS_{\text{uptake}}$  switching to  $RDS_{\text{all}}$  whereas high ER drugs are more resistant to the RDS switch.

It should be noted that the relationship between  $CL^{\text{s}}_{\text{in}}/Q_h$  and the tipping point (Eq. 2 and Fig. 2) depends on the chosen AUCR cutoff. Here, an AUCR of 1.25 was chosen based off FDA guidelines of what constitutes a positive DDI. If a higher AUCR cutoff were to be selected (Supplementary Fig. 2), this would lead to estimation of lower tipping points, thus making it more

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likely that drugs are labeled with  $RDS_{\text{uptake}}$ . Labeling a drug with  $RDS_{\text{uptake}}$  when in fact it has  $RDS_{\text{all}}$  can lead to underpredictions of DDI liabilities due to metabolic enzymes and biliary transporters.

By understanding the relationship between  $CL^{\text{S}}_{\text{in}}$  and the tipping point, the RDS can be identified for any combination of a drug's hepatobiliary clearance values (Fig. 2). For example, a high ER drug with a  $CL_{\text{met+bile}}/CL^{\text{S}}_{\text{ef}}$  ratio of 3 will have  $RDS_{\text{uptake}}$  but a low ER drug with the same  $CL_{\text{met+bile}}/CL^{\text{S}}_{\text{ef}}$  ratio will have  $RDS_{\text{all}}$ . Furthermore, a drug will always have  $RDS_{\text{uptake}}$  if the  $CL_{\text{met+bile}}/CL^{\text{S}}_{\text{ef}}$  ratio is greater than 4, irrespective of the magnitude of  $CL^{\text{S}}_{\text{in}}$ . It should be noted that for orally administered drugs, the tipping point will no longer depend on the magnitude of  $CL^{\text{S}}_{\text{in}}$ , and therefore will always be 4, because blood flow limitations from systemic clearance are cancelled out by blood flow limitations of hepatic bioavailability.

#### Quantifying the $PI_{\text{met+bile}}$ for drugs with $RDS_{\text{uptake}}$

Identifying the RDS of a drug as well as when the  $RDS_{\text{uptake}}$  to  $RDS_{\text{all}}$  switch will happen identifies the drug's DDI liabilities. We quantified the  $PI_{\text{met+bile}}$ , defined here as the percent inhibition of  $CL_{\text{met+bile}}$  needed to cause the  $RDS_{\text{uptake}}$  switch to  $RDS_{\text{all}}$ , to understand when inhibition of  $CL_{\text{met+bile}}$  starts to become a DDI liability for victim drugs that have  $RDS_{\text{uptake}}$ . As the  $CL_{\text{met+bile}}/CL^{\text{S}}_{\text{ef}}$  ratio of the victim drug (prior to inhibition) increases, the  $PI_{\text{met+bile}}$  increases (Fig. 3A). This is because as  $CL_{\text{met+bile}}$  becomes  $\gg$  than  $CL^{\text{S}}_{\text{ef}}$ , the victim drug become resistant to the  $RDS_{\text{uptake}}$  switch to  $RDS_{\text{all}}$ . High ER drugs have a higher  $PI_{\text{met+bile}}$  than low ER drugs, demonstrating again that high ER drugs are resistant to the RDS switch while low ER drugs are sensitive (Fig. 3A). Fig. 3B illustrates that while a low, mid, and high ER victim drug with  $CL_{\text{met+bile}}/CL^{\text{S}}_{\text{ef}}$  ratio of 6 have  $RDS_{\text{uptake}}$  (prior to inhibition), inhibition of  $CL_{\text{met+bile}}$  greater than 46%, 66%, and 87%, respectively, will cause the  $RDS_{\text{uptake}}$  to switch to  $RDS_{\text{all}}$ . This translates to observing a positive DDI due to inhibition of  $CL_{\text{met+bile}}$  for a victim drug that has been identified to

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have  $RDS_{\text{uptake}}$  (prior to inhibition). Without knowledge of the  $PI_{\text{met+bile}}$ , such a DDI may not be expected.

The purpose and conclusions of the simulations that have been used to establish the RDS framework up to this point are summarized in Fig.4. As discussed, identifying the drug's RDS is not enough to correctly predict the drug's DDI liabilities. The tipping point concept is an important consideration when identifying DDI's for victim drugs that are dual substrates of enzymes and transporters.

The flowchart in Fig. 5 can be used as a guide to identify the DDI liabilities for dual transporter/enzyme substrates. All drugs with  $CL_{\text{met+bile}}/CL_{\text{ef}}^{\text{s}}$  ratio  $> 4$  will have  $RDS_{\text{uptake}}$  whereas drugs with  $CL_{\text{met+bile}}/CL_{\text{ef}}^{\text{s}}$  ratio  $< 4$  will have  $RDS_{\text{uptake}}$  as long as this ratio is greater than the tipping point. Drugs with  $CL_{\text{met+bile}}/CL_{\text{ef}}^{\text{s}}$  ratio less than the tipping point will have  $RDS_{\text{all}}$ . If the drug has  $RDS_{\text{uptake}}$ , then uptake transporters will become a DDI liability, whereas if the drug has  $RDS_{\text{all}}$ , then transporters and enzymes will be a DDI liability. However, even for drugs that have  $RDS_{\text{uptake}}$ ,  $CL_{\text{met+bile}}$  can become a DDI liability if inhibition of  $CL_{\text{met+bile}}$  is greater than the predicted  $PI_{\text{met+bile}}$  and thus causes the  $RDS_{\text{uptake}}$  switch to  $RDS_{\text{all}}$ . The flowchart identifies the  $CL_{\text{met+bile}}/CL_{\text{ef}}^{\text{s}}$  ratios at which 25%, 50%, 75%, and 95% expected inhibition of  $CL_{\text{met+bile}}$  is going to result in the RDS switch. This information can be used to assess when  $CL_{\text{met+bile}}$  starts to become a DDI liability for drugs with  $RDS_{\text{uptake}}$ . It also helps answer the question of how much larger does  $CL_{\text{met+bile}}$  need to be compared to  $CL_{\text{ef}}^{\text{s}}$  in order for sinusoidal uptake clearance to become and maintain as the RDS in the hepatic clearance of any drug. Such information may be used during drug development to select drug candidates if a certain RDS is desired.

#### Applying the RDS framework to *in vitro* and *in vivo* examples

To provide context to the theoretical framework presented, examples from literature, where available, were utilized. For drugs with *in vitro* quantified hepatobiliary clearances that

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were extrapolated to *in vivo* via IVIVE, the tipping point and  $PI_{\text{met+bile}}$  were calculated using Eq. 2 and 5, and a subset of the analyzed data set, which includes primarily statin drugs, is shown in Fig. 6 (also see Supplementary Table 1) (Camenisch and Umehara, 2012; Jones et al., 2012; Varma et al., 2014; Kunze et al., 2015; Riede et al., 2017). If no empirical scaling factors (such as for active uptake clearance to match observed *in vivo* clearance) are included in the IVIVE process, then almost all drugs have  $RDS_{\text{all}}$ , except for valsartan and pravastatin (Fig. 6A). This is because most  $CL_{\text{met+bile}}/CL^{\text{s}}_{\text{ef}}$  ratios  $< 4$  and since the IVIVE  $CL^{\text{s}}_{\text{in}}$  magnitudes were small, most  $CL_{\text{met+bile}}/CL^{\text{s}}_{\text{ef}}$  ratios were less than the tipping point. Because many statins have been identified to have  $RDS_{\text{uptake}}$ , the trend in Fig. 6A suggests that  $CL^{\text{s}}_{\text{in}}$  was underestimated *in vitro*. When hepatobiliary clearances were adjusted by empirical scaling factors (Varma et al., 2014) or parameters were fitted from *in vivo* IV concentration-time profiles using a PBPK model (Jones et al., 2012), the distribution of drugs is altered such as low drugs ( $ER < 0.2$ ) tended to have  $RDS_{\text{all}}$  whereas mid and high ER drugs ( $ER > 0.2$ ) were more likely to have  $RDS_{\text{uptake}}$  (Fig. 6B). This analysis of the published *in vitro* hepatobiliary clearances provides insight that drugs with  $RDS_{\text{uptake}}$  exist within the moderate RDS framework space, meaning that in general their  $CL_{\text{met+bile}}/CL^{\text{s}}_{\text{ef}}$  ratio is  $< 4$  and are quite susceptible to the RDS switch (Supplementary Table 1). It further elucidates that current *in vitro* quantification techniques may underestimate  $CL^{\text{s}}_{\text{in}}$  which can lead to erroneous labeling of the RDS and thus incorrect DDI liability predictions (Fig 7, Supplementary Fig. 3).

To further illustrate the applicability of the RDS framework, predicted DDI liabilities using the RDS framework were compared to *in vivo* DDI examples. As indicated in Table 1, when empirical scaling factors are utilized during the IVIVE process or hepatobiliary clearances were estimated from *in vivo* via PBPK, atorvastatin and repaglinide have  $RDS_{\text{uptake}}$  and  $PI_{\text{met+bile}}$  of 10-51% and 15-40%, respectively, while bosentan has  $RDS_{\text{all}}$ . For atorvastatin and repaglinide, the *in vitro* data predicted that uptake transporters (OATPs) are the primary DDI liability with the



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drugs' major metabolic enzymes (CYP3A and CYP2C8, respectively) becoming a potential liability only if the *in vivo* hepatic metabolic inhibition is greater than the  $PI_{\text{met+bile}}$ . For bosentan, the *in vitro* data predicted that both OATPs and CYP3A4 are a potential DDI liability. Clinically, for atorvastatin, co-administration of rifampin (an OATP inhibitor) lead to AUCR of 12 whereas 33% inhibition of CYP3A4 due to IV itraconazole (as measured using CYP3A4 probe midazolam) did not change atorvastatin systemic AUC even though inhibition of atorvastatin metabolism was observed via a decrease in the 2-hydroxyatorvastatin concentrations (Maeda et al., 2011). In a similarly conducted experiment, co-administration of rifampin resulted in AUCR of 3.2 and 1.9 for bosentan or repaglinide, respectively, whereas 73% inhibition of CYP3A4 due to IV itraconazole did not significantly change the systemic AUC of these drugs (Yoshikado et al., 2017). Furthermore, repaglinide co-administered with PO rifampin and trimethoprim (CYP2C8 selective inhibitor) resulted in AUCR was 2.6 and 1.8, respectively (Kim et al., 2016). The *in vivo* DDI liability for OATPs was well predicted for all three victim drugs. The *in vivo* DDI liability for CYP3A4 was well predicted for atorvastatin. Since a probe was not used to assess the degree of CYP2C8 inhibition, it is difficult to interpret if the significant DDI when repaglinide was co-administered with trimethoprim was because  $RDS_{\text{uptake}}$  switched to  $RDS_{\text{all}}$ , or because repaglinide truly has  $RDS_{\text{all}}$ . The *in vitro* metrics as well as a whole-body PBPK DDI model suggests that repaglinide has  $RDS_{\text{uptake}}$  (Varma et al., 2013) and thus the repaglinide-trimethoprim DDI is likely due to the RDS switch. Lastly, since bosentan was predicted to have  $RDS_{\text{all}}$ , a DDI was expected due to CYP3A4 inhibition but none was observed. It should be noted that the metabolic DDI liability prediction is assuming one main drug metabolizing enzyme and no significant biliary efflux (e.g.  $CL_{\text{met+bile}} = CL_{\text{CYP3A4}}$  for atorvastatin and bosentan). This assumption predicts the highest DDI risk due to inhibition of  $CL_{\text{met+bile}}$  and has a higher chance of predicting false positive DDI results.

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In the published *in vitro* datasets, discrepancies in the *in vitro* quantified values, particularly for  $CL_{in}^s$ , can be observed (Table 1 and Supplementary Table 1). For example, in one report the authors used empirical scaling factors for active sinusoidal uptake clearance in order to match hepatic clearance with clinical observed data that ranged from 1.1 – 101.8 with geometric mean of 10.6 (Varma et al., 2014). However, the scaling factor used severely impacted the labeling of the RDS (e.g. fluvastatin, glyburide, and pravastatin) or impacted the predicted  $PI_{met+bile}$  of drugs (e.g. atorvastatin, rosuvastatin, fluvastatin, and repaglinide) (Supplementary Table 1). Assumptions regarding  $CL_{ef}^s$  also caused discrepancies. In all reports,  $CL_{ef}^s$  was assumed to be equal to passive diffusion across the sinusoidal membrane, except in one report where  $CL_{ef}^s$  was back-calculated from total SCHH  $CL_{int}$  (Camenisch and Umehara, 2012). The assumptions surrounding  $CL_{ef}^s$  impacted the  $CL_{met+bile}/CL_{ef}^s$  ratio which either changed how the RDS was labeled or the magnitude of the  $PI_{met+bile}$  (e.g. aliskerin, ciprofloxacin, and digoxin) (Supplementary Table 1). All in all, mispredictions of any of the hepatobiliary clearances impact the RDS labeling, magnitude of the  $PI_{met+bile}$ , and DDI liability predictions.

Errors from *in vitro* quantification of hepatobiliary clearances can propagate when establishing the RDS and the predicted DDI liabilities. Underprediction of both  $CL_{in}^s$  and  $CL_{met+bile}$  may erroneously label a drug with  $RDS_{all}$  when it is truly  $RDS_{uptake}$  (Fig. 7).  $CL_{met+bile}$  is the more sensitive parameter when determining the RDS because underpredictions of  $CL_{in}^s$  may mislabel the RDS only for drugs with  $CL_{met+bile}/CL_{ef}^s$  ratio < 4 (Supplementary Fig. 4). For such drugs, even moderate (e.g. 2-5 fold) underpredictions of either clearance pathway will lead to RDS mislabeling (Supplementary Fig. 4). Furthermore, underpredictions of both  $CL_{in}^s$  and  $CL_{met+bile}$  leads to underprediction of  $PI_{met+bile}$ , resulting in predicting a larger DDI liability due to  $CL_{met+bile}$  inhibition for a drug with  $RDS_{uptake}$  (Fig. 7; Supplementary Fig. 4). While underpredictions of hepatobiliary clearances will result in conservative DDI decisions, they also increase the chances of negative DDI studies.

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## DISCUSSION

We built a theoretical RDS framework and identified important considerations when predicting DDI liabilities for dual transporter/enzymes substrate drugs. First, inhibition of  $CL_{\text{met+bile}}$  can cause the RDS of a victim drug to switch from  $RDS_{\text{uptake}}$  to  $RDS_{\text{all}}$  and hence result in an unexpected systemic DDI. Two metrics have been developed to identify when the RDS switch occurs: the tipping point, defined as the  $CL_{\text{met+bile}}/CL^{\text{s}}_{\text{ef}}$  ratio at which  $RDS_{\text{uptake}}$  will switch to  $RDS_{\text{all}}$  and the  $PI_{\text{met+bile}}$ , defined as the percent inhibition of  $CL_{\text{met+bile}}$  at which a significant AUC change ( $AUCR > 1.25$ ) for a drug with  $RDS_{\text{uptake}}$  will start to be observed. The tipping point depends on the drug's  $CL_{\text{met+bile}}/CL^{\text{s}}_{\text{ef}}$  ratio and on the magnitude of  $CL^{\text{s}}_{\text{in}}$ . The former but not latter condition is relevant when victim drugs are administered orally. Second, we showed that the  $CL_{\text{met+bile}}/CL^{\text{s}}_{\text{ef}}$  ratio must be  $> 4$  in order for any drug to have  $RDS_{\text{uptake}}$ . Third, we applied the RDS framework to *in vitro* quantified hepatobiliary clearances and observed that most drugs have  $CL_{\text{met+bile}}/CL^{\text{s}}_{\text{ef}}$  ratio  $< 4$ , and hence in practice, the magnitude of  $CL^{\text{s}}_{\text{in}}$  must be considered when establishing the RDS.

Our theoretical analysis demonstrates that the  $CL_{\text{met+bile}}/CL^{\text{s}}_{\text{ef}}$  ratio and not the absolute magnitudes of the clearances determines the RDS in the hepatic clearance of a drug. Previous publications allude to this relationship. The authors of the ECCCS observed through experimental data that when  $CL_{\text{met+bile}}$  is  $2 \times CL^{\text{s}}_{\text{ef}}$ , drugs that have  $RDS_{\text{uptake}}$  can be separated from those that do not (Riede et al., 2016). Furthermore,  $\beta$  value ( $\beta = CL_{\text{met+bile}} / (CL_{\text{met+bile}} + CL^{\text{s}}_{\text{ef}})$ ) introduced by Yoshikado et al., can be used to differentiate the RDS, such as when  $\beta$  approaches unity (i.e.  $CL_{\text{met+bile}} \gg CL^{\text{s}}_{\text{ef}}$ ), a drug has  $RDS_{\text{uptake}}$  (Yoshikado et al., 2016). Our analyses corroborate and expand upon these results to provide a quantitative definition of the demarcation point between  $RDS_{\text{uptake}}$  and  $RDS_{\text{all}}$ , i.e. the tipping point, and illustrate that the magnitude of  $CL^{\text{s}}_{\text{in}}$  in addition to the  $CL_{\text{met+bile}}/CL^{\text{s}}_{\text{ef}}$  ratio is an important factor in determining the

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RDS of a drug. That is, as a drug's  $CL_{in}^s$  value increases, the drug is more likely to have  $RDS_{uptake}$  and to become resistant to the  $RDS_{uptake}$  switch to  $RDS_{all}$ .

We found good agreement for atorvastatin *in vivo* predicted DDI liabilities (Table 1). For bosentan, the overprediction of expected DDI due to inhibition of  $CL_{met+bile}$  may be due to errors in the quantification of the hepatobiliary clearances. Indeed, a study in cynomolgus monkey where bosentan plasma and liver drug concentrations were quantified found that the *in vitro* scaled  $CL_{in}^s$  and  $CL_{met}$  were 28 and 13-fold underpredicted while  $CL_{ef}^s$  (assumed equal to passive diffusion) was overpredicted by 2-fold when compared to the *in vivo* fitted values (Morse et al., 2017). Combining the *in vitro* metrics that identify  $RDS_{uptake}$  for repaglinide with *in vivo* repaglinide DDI's, it appears CYP2C8 but not CYP3A4 inhibition may lead to  $RDS_{uptake}$  switch to  $RDS_{all}$ . Indeed, inhibition of repaglinide with gemfibrozil (CYP2C8 and OATP1B1 inhibitor) led to an 8-fold increase in systemic AUC while co-administration of itraconazole or cyclosporine (OATP1B1 and CYP3A4 inhibitor) led to much more modest 1.4 and 2.4-fold increase in systemic AUC (Niemi et al., 2003; Kajosaari et al., 2005).

The DDI liabilities discussed so far are relevant for systemic drug exposure but not necessarily for hepatic drug exposure and thus efficacy/toxicity if the site of action is in the liver. For example, the LDL cholesterol lowering effect mediated by atorvastatin does not change for subjects with OATP1B1 polymorphism c.521T>C even though there is a significant increase in atorvastatin systemic AUC (Maeda, 2015). This is because if the liver is the main eliminating organ, changes to sinusoidal uptake alters the hepatic concentration-time profile but not the hepatic AUC. However, systemic increase of atorvastatin may lead to off-target toxicity, such as muscle myopathy. We refer the readers to our previous publication (Patilea-Vrana and Unadkat, 2016), where simulations demonstrate the impact of inhibition of uptake or metabolism on both systemic and hepatic AUC when the liver is and is not the main eliminating organ.

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The contrast between *in vitro* quantified  $CL_{in}^s$  with and without empirical scaling factors in Fig. 6 demonstrates that IVIVE of accurate transporter mediated clearance remains challenging (Chu et al., 2013; Feng et al., 2014). The system used for *in vitro* quantification may be crucial, since  $CL_{in}^s$  for statins quantified in SCHH appeared to be lower in magnitude than when quantified in suspended hepatocytes (Table 1 and Supplementary Table 1). This may be mediated by significant intracellular localization of plasma membrane transporters (Kumar et al., 2017), or high interindividual variability when using individual donors (Vildhede et al., 2014). These reasons may also cause underpredictions of  $CL_{in}^s$  or  $CL_{bile}$ . For transporter IVIVE, we have previously recommended using a bottom-up proteomic approach and adjusting for *in vitro* activity via *in vitro* to *in vivo* transporter expression based scaling factors (Prasad and Unadkat, 2014). We have recently demonstrated the successful prediction of hepatobiliary clearance of rosuvastatin in rat using the aforementioned approach (Ishida et al., 2017).

Special emphasis needs to be put on quantifying  $CL_{ef}^s$  along with  $CL_{met+bile}$  since the  $CL_{met+bile}/CL_{ef}^s$  ratio is one of the anchor points when establishing the RDS. Because  $CL_{ef}^s$  is a difficult parameter to quantify *in vitro*, it is typically assumed to be equal to passive diffusion across the sinusoidal membrane. However, there are examples of active sinusoidal efflux transport, such as MRP3 efflux of rosuvastatin (Pfeifer et al., 2013). Active sinusoidal efflux would increase the magnitude of  $CL_{ef}^s$  and decrease the  $CL_{met+bile}/CL_{ef}^s$  ratio, making a drug more likely to have  $RDS_{all}$ . One approach to measuring  $CL_{ef}^s$  is to use an integrative temporal modeling approach in SCRH (Pfeifer et al., 2013; Ishida et al., 2017).

Errors in the quantification of  $CL_{in}^s$  and/or the  $CL_{met+bile}/CL_{ef}^s$  ratio can impact DDI liability predictions. For example, patients with OATP1B1 polymorphism c.521T>C have a ~2-fold higher atorvastatin AUC compared to the wild type allele (Maeda, 2015). Because of the lower  $CL_{in}^s$  and therefore higher susceptibility to the  $RDS_{uptake}$  to  $RDS_{all}$  switch, patients with OATP1B1 polymorphism may experience a DDI due to inhibition of CYP3A whereas patients with the wild-

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type allele may not. The same trend would be true for patients with polymorphic enzymes that result in lower  $CL_{\text{met+bile}}$  and thus lower  $CL_{\text{met+bile}}/CL_{\text{ef}}^{\text{s}}$  ratios. Polypharmacy use can also impact DDI liability predictions. For example, highly active antiretroviral therapy (HAART) typically includes potent CYP3A4 and moderate OATP inhibitor ritonavir among other drugs, which can impact the  $CL_{\text{met+bile}}/CL_{\text{ef}}^{\text{s}}$  ratio more severely than if only one drug is administered. Indeed, the systemic AUC of atorvastatin increased 3.9 and 9.4-fold when co-administered with saquinavir/ritonavir and tipranavir/ritonavir, respectively (Fichtenbaum et al., 2002; Pham et al., 2009). Lastly, saturation of enzymes, leading to a lower  $CL_{\text{met}}$  with increased dose, may lower the  $CL_{\text{met+bile}}/CL_{\text{ef}}^{\text{s}}$  ratio and cause DDI's due to the  $RDS_{\text{uptake}}$  switch to  $RDS_{\text{all}}$ .

If a victim drug has  $RDS_{\text{all}}$  but it has been mislabeled as  $RDS_{\text{uptake}}$ , then the DDI liability due to inhibition of both transporter and metabolic activity could be underestimated. Considering potential DDI risks, it would be most conservative to assume a drug has  $RDS_{\text{all}}$ ; however, making such an assumption would lead to an increase in negative DDI studies, particularly when conducting metabolic/biliary efflux DDI studies if the drug has  $RDS_{\text{uptake}}$ . An analysis of the DDI's performed for a cohort of NME's in 2013 showed a modest return on investment because 57% (n=141) of all *in vivo* DDI's were negative (Lesko and Lagishetty, 2016). Given the high prevalence of negative DDI's, it may be more appropriate to make mechanistic-based rather than conservative decisions with regard to DDI liabilities.

The RDS framework presented here should be used as a guide when identifying the DDI liabilities whereas PBPK models should be used to predict the direction and magnitude of complex transporter-enzyme DDI's. Several examples of such models (e.g. repaglinide, simvastatin, rosuvastatin) exist that predict complex interactions due to chemical inhibition or genetic polymorphism (Varma et al., 2013; Rose et al., 2014; Tsamandouras et al., 2015). Even with PBPK models, there are limitations. For example, when a drug has  $RDS_{\text{uptake}}$ , the  $CL_{\text{met+bile}}$  is unidentifiable from plasma concentrations data since only  $CL_{\text{in}}^{\text{s}}$  plays a significant role in

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determining hepatic clearance. Focusing on capturing the correct  $CL_{\text{met+bile}}$  magnitude and not the  $CL_{\text{met+bile}}/CL_{\text{ef}}^{\text{s}}$  ratio can be misleading and will impact PBPK predictions. For instance, in an atorvastatin PBPK model, when cyclosporine CYP3A4  $K_i$  was modulated 100-fold, a maximum 1.6-fold AUCR was achieved (Duan et al., 2017). While the tendency is to run sensitivity analysis on the active components (transport and metabolism), a sensitivity analysis on  $CL_{\text{ef}}^{\text{s}}$  value (in the model it was assumed to be equal to passive diffusion) should also be run as, for the specific example provided, it would likely have revealed a larger impact of cyclosporine on atorvastatin systemic AUC. Such an analysis may be helpful in consolidating *in vitro*  $K_i$  data with observed *in vivo* DDI data.

In summary, we introduced a theoretical RDS framework to better predict DDI liabilities for drugs that are dual transporter/enzyme substrates. We provide useful insights, such as: i) the  $RDS_{\text{uptake}}$  switch to  $RDS_{\text{all}}$  depends on the ratio of  $CL_{\text{met+bile}}/CL_{\text{ef}}^{\text{s}}$  and the magnitude of  $CL_{\text{in}}^{\text{s}}$ , ii)  $CL_{\text{met+bile}}/CL_{\text{ef}}^{\text{s}}$  ratio > 4 ensures  $RDS_{\text{uptake}}$  independent of  $CL_{\text{in}}^{\text{s}}$  magnitude or administration route iii) existing drugs exist within a moderate space within the RDS framework and are susceptible to the  $RDS_{\text{uptake}}$  switch to  $RDS_{\text{all}}$ . While the above insights were obtained from the hepatic ECM, they can be equally applied to other organs such as the kidneys where vectorial (basal to apical) transport of drugs is possible.

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## **AUTHORSHIP CONTRIBUTIONS**

*Participated in research design:* Patilea-Vrana and Unadkat

*Conducted experiments:* Patilea-Vrana

*Contributed new reagents or analytical tool:* Patilea-Vrana

*Performed data analysis:* Patilea-Vrana

*Wrote or contributed to the writing of the manuscript:* Patilea-Vrana and Unadkat

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## FOOTNOTES

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## FIGURE LEGENDS

### Figure 1. Identifying when $RDS_{\text{uptake}}$ switches to $RDS_{\text{all}}$ , i.e. the tipping point. **A)**

Extensive inhibition of  $CL_{\text{met+bile}}$  can lead to a significant increase in the systemic AUC for three theoretical victim drugs that have  $RDS_{\text{uptake}}$  (i.e.  $CL_{\text{met+bile}} \gg CL_{\text{ef}}^{\text{s}}$ ) in the absence of DDI. When inhibition of  $CL_{\text{met+bile}}$  eventually violates the condition required for  $RDS_{\text{uptake}}$ , the  $RDS_{\text{uptake}}$  switches to  $RDS_{\text{all}}$ . An AUCR  $\geq 1.25$  was observed when  $CL_{\text{met+bile}}$  was inhibited  $\geq 84\%$ ,  $\geq 98\%$ , and  $\geq 99.8\%$  for  $CL_{\text{met+bile}} = 1, 10, 100$  L/hr, respectively. However, for all three victim drugs, the  $CL_{\text{met+bile}}$  value after such inhibition was similar (0.2 L/min) as was the  $CL_{\text{met+bile}}/CL_{\text{ef}}^{\text{s}}$  ratio (= 2).

Simulations were performed as follows:  $CL_{\text{in}}^{\text{s}} = 1 \times Q_{\text{h}}$ ,  $CL_{\text{met+bile}} = 1, 10, 100$  L/hr,  $CL_{\text{ef}}^{\text{s}} = 0.1$

L/min. **B)** The systemic AUC (in the absence of any DDI) of a theoretical drug remains unchanged when the  $CL_{\text{met+bile}}/CL_{\text{ef}}^{\text{s}}$  ratio remains fixed (blue bars) but not when the  $CL_{\text{met+bile}}/CL_{\text{ef}}^{\text{s}}$  ratio is varied (yellow bars) even though the absolute value of  $CL_{\text{met+bile}}$  and  $CL_{\text{ef}}^{\text{s}}$  is varied in both scenarios. This trend was observed irrespective of the value of  $CL_{\text{in}}^{\text{s}}$

(Supplementary Fig. 1). Furthermore, this trend is true for when  $CL_{\text{met+bile}} > CL_{\text{ef}}^{\text{s}}$  or  $CL_{\text{met+bile}} < CL_{\text{ef}}^{\text{s}}$  (also refer to Supplementary Fig. 1). Thus, the  $CL_{\text{met+bile}}/CL_{\text{ef}}^{\text{s}}$  ratio, irrespective of the magnitude of the absolute values of these clearances, is important for establishing the RDS and henceforth when the RDS switches from uptake to all hepatobiliary clearances. Simulations

were performed as follows:  $CL_{\text{in}}^{\text{s}} = 0.25 \times Q_{\text{h}}$  and the other input clearance values for scenarios

A-E are shown in the table provided. **C)** Since the RDS depends on the  $CL_{\text{met+bile}}/CL_{\text{ef}}^{\text{s}}$  ratio, we define the tipping point as the  $CL_{\text{met+bile}}/CL_{\text{ef}}^{\text{s}}$  ratio at which  $RDS_{\text{uptake}}$  switches to  $RDS_{\text{all}}$ . Similar to panel A, the  $RDS_{\text{uptake}}$  switch to  $RDS_{\text{all}}$  is represented by an AUCR of 1.25 and the decrease in  $CL_{\text{met+bile}}/CL_{\text{ef}}^{\text{s}}$  ratio is akin to inhibition of  $CL_{\text{met+bile}}$  when  $CL_{\text{ef}}^{\text{s}}$  is kept constant. As shown by the gray arrows, the tipping point for a low, mid, and high ER drug is 3.2, 2, and 0.8, respectively.

For example, if the  $CL_{\text{met+bile}}/CL_{\text{ef}}^{\text{s}}$  ratio for the low ER drug is above the tipping point (i.e.  $CL_{\text{met+bile}}/CL_{\text{ef}}^{\text{s}} > 3.2$ ), then the drug will have  $RDS_{\text{uptake}}$  and therefore DDI's due to

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$CL_{in}^s$  but not  $CL_{met+bile}$  should be expected. However, inhibition of  $CL_{met+bile}$  that makes the  $CL_{met+bile}/CL_{ef}^s$  ratio lower than the tipping point (i.e.  $CL_{met+bile}/CL_{ef}^s < 3.2$ ) will lead to a significant increase in the systemic AUC. Crossing the tipping point is indicative of the  $RDS_{uptake}$  switch to  $RDS_{all}$ . Simulations were performed as follows: systemic AUC were simulated for  $CL_{in}^s = 0.25x, 1x, 4xQ_h$  (representing low, mid, and high ER, respectively) and  $CL_{met+bile}/CL_{ef}^s$  ratios from 1-10, then normalized to a control simulation where the  $CL_{met+bile}/CL_{ef}^s$  ratio was set to 1000 (i.e.  $RDS_{uptake}$ ).

**Figure 2. The RDS framework helps identify DDI liabilities.** The  $CL_{met+bile}/CL_{ef}^s$  ratio and  $CL_{in}^s$  magnitude of a drug determines the RDS of the drug and when  $RDS_{uptake}$  switches to  $RDS_{all}$ . Combinations of hepatobiliary clearances found in the shaded area have  $RDS_{all}$  while those in the non-shaded area have  $RDS_{uptake}$ . Any alterations in hepatobiliary clearances that causes a drug to switch from the non-shaded to the shaded area will cross the tipping point (dashed line – Eq. 2) and therefore switch the RDS from uptake to all hepatobiliary clearances. The consequence of this switch is that DDI's due to inhibition of  $CL_{met+bile}$  will now manifest in the systemic AUC of a victim drug that originally had  $RDS_{uptake}$ . Consistent with Fig. 1C, the tipping point decreases as the magnitude of  $CL_{in}^s$  (and therefore the drug's ER) increase. This suggests that the greater the ER of the drug, the more likely it will have  $RDS_{uptake}$  and it will be more resistant to switch to  $RDS_{all}$ . Furthermore, when  $CL_{met+bile}/CL_{ef}^s > 4$ , the RDS will always be uptake clearance irrespective of the value of  $CL_{in}^s/Q_h$ . However, when  $CL_{met+bile}/CL_{ef}^s < 4$ , the RDS can be either uptake or all hepatobiliary pathways depending on the magnitude of  $CL_{in}^s$ . It should be noted that if a drug is administered orally, the tipping point will always be 4 because the blood flow limitations are no longer relevant. Simulations were performed as follows: the tipping point was simulated for  $CL_{in}^s$  values ( $0.01xQ_h - 4xQ_h$ ) using Eq. 2.

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**Figure 3. Identifying when drugs with  $RDS_{\text{uptake}}$  will start to experience a DDI due to inhibition of  $CL_{\text{met+bile}}$ .** **A)** The  $PI_{\text{met+bile}}$ , defined as the % inhibition of  $CL_{\text{met+bile}}$  required for the  $RDS_{\text{uptake}}$  switch to  $RDS_{\text{all}}$ , depends on the  $CL_{\text{met+bile}}/CL^{\text{S}}_{\text{ef}}$  ratio (prior to inhibition) and the magnitude of  $CL^{\text{S}}_{\text{in}}$  (represented as low, mid, and high ER drugs). The  $PI_{\text{met+bile}}$  identifies when a positive DDI due to inhibition of  $CL_{\text{met+bile}}$  for a drug with  $RDS_{\text{uptake}}$  would be expected. Lower  $CL_{\text{met+bile}}/CL^{\text{S}}_{\text{ef}}$  ratios as well as low ER drugs are the most susceptible for the  $RDS_{\text{uptake}}$  switch to  $RDS_{\text{all}}$  due to  $CL_{\text{met+bile}}$  inhibition. **B)** In order for  $RDS_{\text{uptake}}$  to switch to  $RDS_{\text{all}}$  for a theoretical victim drug with  $CL_{\text{met+bile}}/CL^{\text{S}}_{\text{ef}}$  ratio of 6,  $CL_{\text{met+bile}}$  must be inhibited by >46%, >66%, or >87% if the drug is low, mid, and high ER, respectively. Visually, the  $RDS_{\text{uptake}}$  switch to  $RDS_{\text{all}}$  happens when the theoretical victim drug crosses the dashed line (the tipping point) from the unshaded area ( $RDS_{\text{uptake}}$ ) to the shaded area ( $RDS_{\text{all}}$ ). Additional examples of  $PI_{\text{met+bile}}$  are given in the table provided. Simulations were performed as follows:  $PI_{\text{met+bile}}$  was calculated using Eq. 5 for  $CL_{\text{met+bile}}/CL^{\text{S}}_{\text{ef}}$  ratios ranging 1-40 and for  $CL^{\text{S}}_{\text{in}} = 0.25x, 1x, 4xQ_h$  (representing low, mid, and high ER, respectively).

**Figure 4. Summary of the purpose and conclusions for the simulations used to establish the RDS framework.**

**Figure 5. Applying the RDS framework to identify DDI liabilities for dual transporter/enzyme substrate drugs.** If  $CL_{\text{met+bile}}/CL^{\text{S}}_{\text{ef}} > 4$ , then the drug will have  $RDS_{\text{uptake}}$ , irrespective of the magnitude of  $CL^{\text{S}}_{\text{in}}$ . For drugs with  $RDS_{\text{uptake}}$ , DDI's due to inhibition of  $CL_{\text{met+bile}}$  can become significant depending on the drug's  $CL_{\text{met+bile}}/CL^{\text{S}}_{\text{ef}}$  ratio and the expected inhibition of  $CL_{\text{met+bile}}$ . For example, 50% inhibition of  $CL_{\text{met+bile}}$  may result in a significant DDI for a drug with  $RDS_{\text{uptake}}$  and  $CL_{\text{met+bile}}/CL^{\text{S}}_{\text{ef}}$  ratio < 8 but no DDI will be observed if the drug has  $CL_{\text{met+bile}}/CL^{\text{S}}_{\text{ef}}$  ratio > 8. The DDI liability due to inhibition of  $CL_{\text{met+bile}}$  increases as the  $CL_{\text{met+bile}}/CL^{\text{S}}_{\text{ef}}$  ratio decrease and the expected  $CL_{\text{met+bile}}$  inhibition increases.

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**Figure 6. The distribution of drugs within the RDS framework using hepatobiliary clearance quantified *in vitro* and extrapolated to *in vivo*.** Published *in vitro* hepatobiliary clearance values, when extrapolated to *in vivo* via IVIVE, can identify the RDS based on  $f_u \text{CL}^{\text{S}_{\text{in}}}/Q_h$  and  $\text{CL}_{\text{met+bile}}/\text{CL}^{\text{S}_{\text{ef}}}$  ratio (Eq. 2). **A)** When no empirical scaling factors, such as to scale up active transport, are applied during the IVIVE process, all drugs except for valsartan and pravastatin have  $\text{RDS}_{\text{all}}$ . Most drugs had  $\text{CL}_{\text{met+bile}}/\text{CL}^{\text{S}_{\text{ef}}}$  ratio  $< 4$ , indicating drugs primarily exist within the moderate RDS framework space. Furthermore, most drugs have  $f_u \text{CL}^{\text{S}_{\text{in}}}/Q_h < 0.4$ , indicating severe underprediction of  $\text{CL}^{\text{S}_{\text{in}}}$ . **B)** When empirical scaling factors are used or hepatobiliary clearances are estimates from *in vivo* data using PBPK modeling, the RDS of the drugs is altered severely. Now,  $\text{RDS}_{\text{uptake}}$  occurs more often for mid and high ER drugs with  $\text{RDS}_{\text{all}}$  primarily for low ER drugs (ER was calculated from *in vivo* hepatic clearance and blood flow). Furthermore, since all drugs have  $\text{CL}_{\text{met+bile}}/\text{CL}^{\text{S}_{\text{ef}}}$  ratio  $< 4$ , information about both the magnitude of  $f_u \text{CL}^{\text{S}_{\text{in}}}$  and the  $\text{CL}_{\text{met+bile}}/\text{CL}^{\text{S}_{\text{ef}}}$  ratio is necessary to correctly predict DDI liabilities. The dashed line represents the tipping point (Eq. 2). The data shown are from Jones et al., 2012 and Varma et al., 2014, and represent a subset of the complete data set presented in Supplementary Table 1.

**Figure 7. The impact of underpredictions of hepatobiliary clearance on DDI liability predictions.** A representative 3-fold underprediction of either **A)**  $\text{CL}^{\text{S}_{\text{in}}}$  or **B)**  $\text{CL}_{\text{met+bile}}$  can lead to erroneous labeling of the RDS for low, mid and high ER drugs (shown by the filled circles crossing from the non-shaded to shaded area, i.e.  $\text{RDS}_{\text{uptake}}$  switches to  $\text{RDS}_{\text{all}}$ ). Mislabeling the RDS impacts the expected DDI risk due to transporters versus enzymes. Furthermore, underpredictions of either  $\text{CL}^{\text{S}_{\text{in}}}$  or  $\text{CL}_{\text{met+bile}}$  leads to identifying both transporters and enzymes as DDI liabilities when truly only uptake transporters are the true DDI liability. Please refer to Supplementary Fig. 4 for more detailed simulations.

**Table 1. Comparison of predicted DDI liabilities from *in vitro* data to *in vivo* clinical studies**

Hepatobiliary clearances, following IVIVE, can be used to identify the RDS of a drug, such as if the  $CL_{met+bile} / CL_{ef}^{s_{ef}}$  ratio is > or < than the tipping point (Eq. 2), then the drug will have  $RDS_{uptake}$  or  $RDS_{all}$ , respectively. If a drug has  $RDS_{uptake}$ , then the  $PI_{met+bile}$  can be quantified (Eq. 5) in order to predict when a significant DDI should be expected due to inhibition of metabolic/biliary efflux clearance. An expanded analysis is shown in Supplementary Table 1.

Drug	Hepatobiliary clearance (ml/min/kg)				$f_u CL_{in}^{s_{in}} / Q_h^f$	$CL_{met+bile} / CL_{ef}^{s_{ef}}$	Tipping point	$PI_{met+bile}$	RDS <i>in vitro</i>	RDS <i>in vivo</i>	REF
	$CL_{in}^{s_{in}}$	$CL_{ef}^{s_{ef}}$	$CL_{met}$	$CL_{bile}$							
Atorvastatin	61 <sup>a</sup>	24.9	58.3	4.3	0.12	2.52	3.59	--	all	uptake <sup>g</sup>	Varma et al., 2014 Kamenisch and Umehara, 2012 Kunze et al., 2015 Maeda et al., 2011
	1194 <sup>b</sup>	24.9	58.3	4.3	2.27	2.52	1.22	>51%	uptake		
	405 <sup>c</sup>	24.9	58.3	4.3	0.77	2.52	2.26	>10%	uptake		
	198 <sup>a</sup>	359	64.6	11.8	0.80	0.21	2.22	--	all		
	198 <sup>a</sup>	57.7	64.6	11.8	0.80	1.32	2.22	--	all		
Bosentan	132 <sup>a</sup>	28.9	19.5	5.8	0.36	0.87	2.95	--	all	uptake <sup>g</sup>	Varma et al., 2014 Jones et al., 2012 Yoshikado et al., 2017
	142 <sup>b</sup>	28.9	19.5	5.8	0.38	0.87	2.90	--	all		
	1117 <sup>c</sup>	28.9	19.5	5.8	3.02	0.87	0.99	--	all		
	35 <sup>a</sup>	12.1	--	39 <sup>e</sup>	0.02	3.24	3.93	--	all		
	2035 <sup>d</sup>	14	--	5.0 <sup>e</sup>	1.09	0.36	1.91	--	all		
Repaglinide	166 <sup>a</sup>	63.6	128	0.3	0.19	2.01	3.35	--	all	uptake <sup>g</sup> all <sup>h</sup>	Varma et al., 2014 Jones et al., 2012 Yoshikado et al., 2017
	1983 <sup>b</sup>	63.6	128	0.3	2.32	2.01	1.21	>40%	uptake		
	1151 <sup>c</sup>	63.6	128	0.3	1.35	2.01	1.71	>15%	uptake		
	299 <sup>a</sup>	223	125	0.0	0.22	0.56	3.27	--	all		
	3671 <sup>d</sup>	352	125	0.0	2.73	0.35	1.07	--	all		

<sup>a</sup> – *in vitro* quantified + IVIVE

<sup>b</sup> – *in vitro* quantified + IVIVE + empirical scaling factor for active uptake transport (individual scaling factor)

<sup>c</sup> – *in vitro* quantified + IVIVE + empirical scaling factor for active uptake transport (geometric mean scaling factor)

<sup>d</sup> – fitted parameters from *in vivo* using PBPK model

<sup>e</sup> – composite  $CL_{met+bile}$

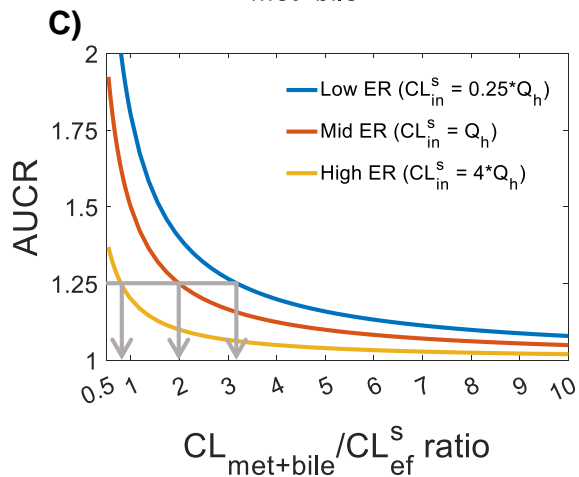
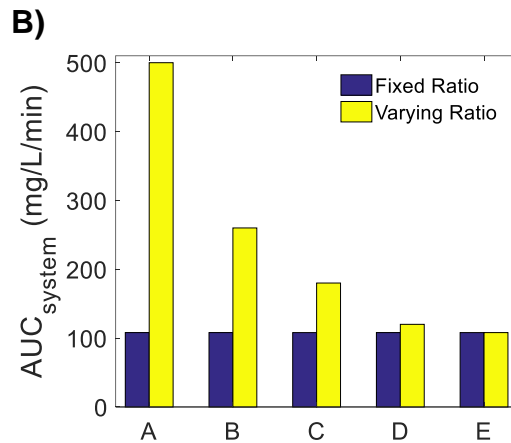
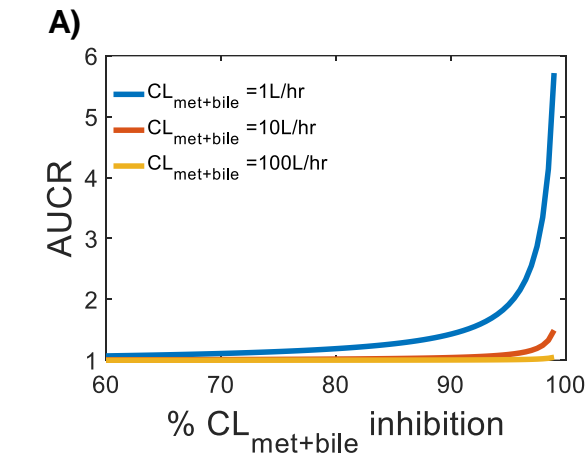
<sup>f</sup> –  $f_u$  and  $Q_h$  values as noted in each reference were used for analysis – note that  $f_u$  may vary for the same drug across different references

<sup>g</sup> –  $RDS_{uptake}$  was established *in vivo* for atorvastatin and bosentan, since there was no significant AUC change to victim drugs when co-administered with IV itraconazole (CYP3A inhibitor) which resulted in 33% and 73% CYP3A inhibition, respectively (Maeda et al., 2011; Yoshikado et al., 2017). Midazolam, a CYP3A

probe, was used to assess magnitude of CYP3A inhibition.  $RDS_{\text{uptake}}$  was established for repaglinide via whole-body PBPK modeling of complex transporter- and enzyme-mediated DDI's (Varma et al., 2013).

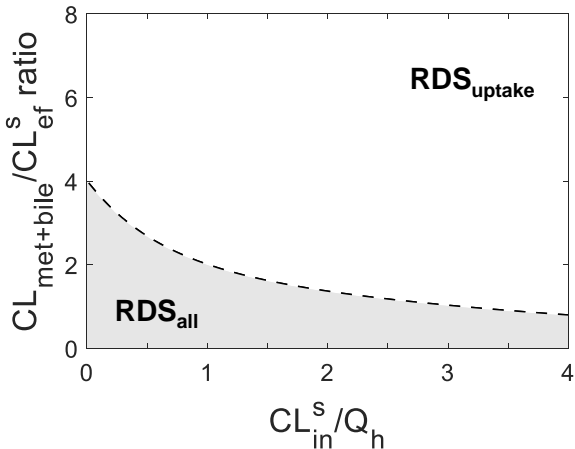
<sup>h</sup> –  $RDS_{\text{all}}$  was established *in vivo* for repaglinide since even though there was no change to systemic AUC by IV itraconazole, because CYP2C8 is the major hepatic drug metabolizing enzyme (Yoshikado et al., 2017). In a different study, PO trimethoprim, a selective CYP2C8 inhibitor, increased repaglinide AUC by 1.8-fold (Kim et al., 2016).

**Figure 1**



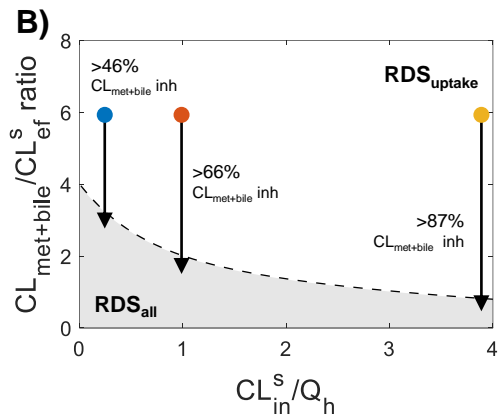
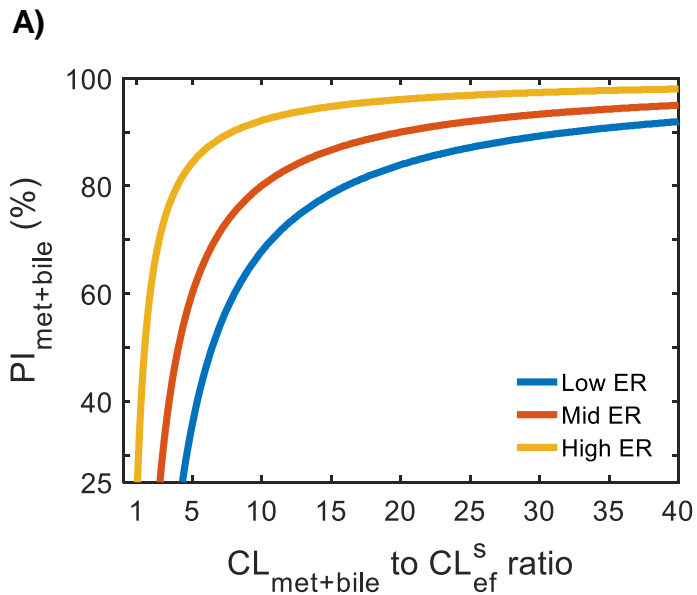
	A	B	C	D	E	
Fixed Ratio	$CL_{met+bile}$ (L/min)	1	4	10	40	100
	$CL_{ef}^s$ (L/min)	0.1	0.4	1	4	10
	$CL_{met+bile} / CL_{ef}^s$	10	10	10	10	10
Varying Ratio	$CL_{met+bile}$ (L/min)	0.2	0.4	1	4	10
	$CL_{ef}^s$ (L/min)	1	1	1	1	1
	$CL_{met+bile} / CL_{ef}^s$	0.2	0.4	1	4	10

**Figure 2**





**Figure 3**



$CL_{\text{met+bile}}/CL_{\text{ef}}^{\text{s}}$	$PI_{\text{met+bile}} (\%)$		
	Low ER	Mid ER	High ER
4 – fold	$>19\%$	$>50\%$	$>80\%$
8 – fold	$>60\%$	$>75\%$	$>87\%$
16 – fold	$>80\%$	$>90\%$	$>95\%$

**Figure 4**

How to predict DDI liabilities for dual enzyme/transporter substrates?

What factors determine the switch from  $RDS_{\text{uptake}}$  to  $RDS_{\text{all}}$ ?

**Fig. 1A**

The magnitude of  $CL_{\text{met+bile}}$  inhibition that leads to a DDI for a drug with  $RDS_{\text{uptake}}$  depends on the starting  $CL_{\text{met+bile}}$  value.

**Fig. 1B**

The  $RDS_{\text{uptake}}$  switch to  $RDS_{\text{all}}$  depends on the  $CL_{\text{met+bile}}/CL_{\text{ef}}^{\text{s}}$  ratio and not the absolute  $CL$  magnitude.

**Fig. 1C**

The  $CL_{\text{met+bile}}/CL_{\text{ef}}^{\text{s}}$  ratio that results in an  $AUCR = 1.25$  signifies when  $RDS_{\text{uptake}}$  will switch to  $RDS_{\text{all}}$ . This ratio is defined as the tipping point.

How to use the RDS framework to determine DDI liabilities?

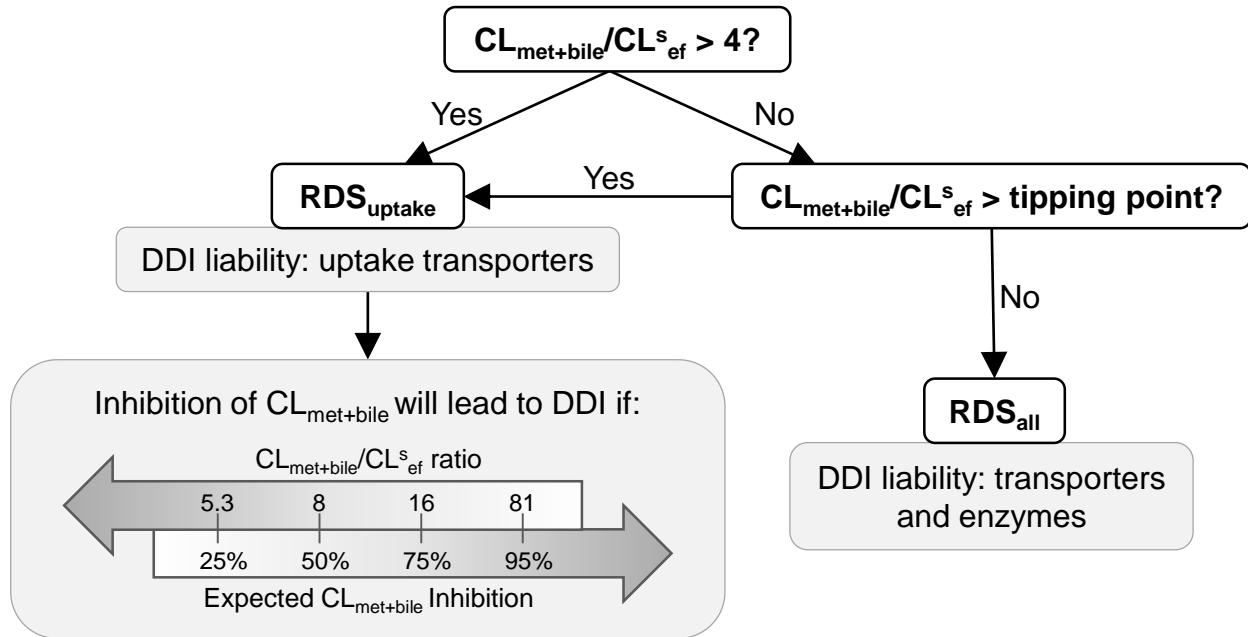
**Fig. 2**

1. Identify the drug's RDS in the absence of DDI.
  - The  $CL_{\text{met+bile}}/CL_{\text{ef}}^{\text{s}}$  ratio and  $CL_{\text{in}}^{\text{s}}/Q_{\text{h}}$  in relation to the tipping point defines the RDS of the drug.

**Fig. 3**

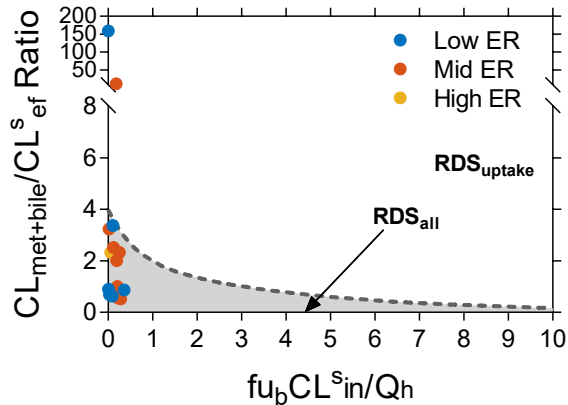
2. Identify when  $RDS_{\text{uptake}}$  will switch to  $RDS_{\text{all}}$  in the presence of DDI.
  - Inhibition of  $CL_{\text{met+bile}}$  that results in the  $CL_{\text{met+bile}}/CL_{\text{ef}}^{\text{s}}$  ratio to be less than the drug's tipping point, is defined as  $PI_{\text{met+bile}}$ .
  - $PI_{\text{met+bile}}$  identifies when inhibition of  $CL_{\text{met+bile}}$  starts to become a DDI liability for victim drugs that have  $RDS_{\text{uptake}}$ .

Figure 5



# Figure 6

## A)



## B)

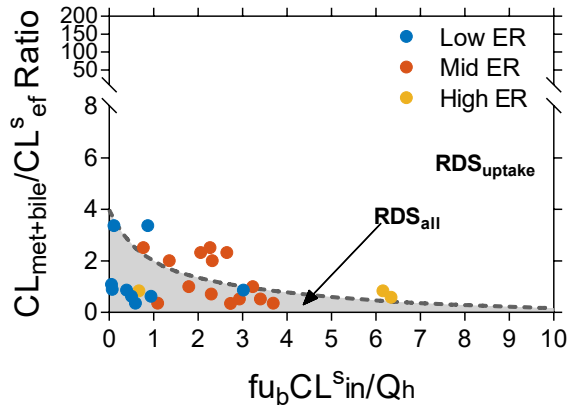


Figure 7

