Citation: Clin Transl Sci (2020) 13, 47–52; doi:10.1111/cts.12691

BRIEF REPORT

Variability in *In Vitro* OATP1B1/1B3 Inhibition Data: Impact of Incubation Conditions on Variability and Subsequent Drug Interaction Predictions

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As the research into the organic anion transporting polypeptides (OATPs) continues to grow, it is important to ensure that the data generated are accurate and reproducible. In the *in vitro* evaluation of OATP1B1/1B3 inhibition, there are many variables that can contribute to variability in the resulting inhibition constants, which can then, in turn, contribute to variable results when clinical predictions (*R*-values) are performed. Currently, the only experimental condition recommended by the US Food and Drug Administration (FDA) is the inclusion of a pre-incubation period.[1] To identify other potential sources of variability, a descriptive analysis of available *in vitro* inhibition data was completed. For each of the 21 substrate/inhibitor pairs evaluated, cell type and pre-incubation were found to have the greatest effect on half-maximal inhibitory concentration (IC_{50}) variability. Indeed, when only HEK293 cells and co-incubation conditions were included, the observed variability for the entire data set (highest IC_{50}/lowest) was reduced from 12.4 to 5.2. The choice of probe substrate used in the study also had a significant effect on inhibitor constant variability. Interestingly, despite the broad range of inhibitory constants identified, these two factors showed little effect on the calculated *R*-values relative to the FDA evaluation cutoff of 1.1 triggering a clinical evaluation for the inhibitors evaluated. However, because of the small data set available, further research is needed to confirm these preliminary results and define best practice for the study of OATPs.

Study Highlights

WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?

✔ Significant variability in *in vitro* parameters for organic anion transporting polypeptides (OATPs)1B1/1B3 inhibition exists in the literature, due in part to differences in experimental conditions; however, the current regulatory guidelines are limited with regard to experimental setup for the *in vitro* assessment of OATP1B1/1B3 inhibition.

WHAT QUESTION DID THIS STUDY ADDRESS?

✔ For the assessment of *in vitro* OATP1B1/1B3 inhibition, what are the primary sources of variability and how does this variability translate to *in vivo* predictions?

WHAT DOES THIS STUDY ADD TO OUR KNOWLEDGE?

✔ This study identified two main sources of variability in the determination of inhibitory constants for OATP1B1/1B3—cell system and pre-incubation with the inhibitor. These conditions, however, do not seem to show a strong effect on subsequent clinical prediction (*R*-value).

HOW MIGHT THIS CHANGE CLINICAL PHARMACOLOGY OR TRANSLATIONAL SCIENCE?

✔ This work suggests that performing experiments with clinically relevant substrates and/or multiple substrates could serve to improve *in vitro* to *in vivo* predictions through a reduction in observed *in vitro* variability.

Organic anion transporting polypeptides (OATPs) 1B1 and 1B3 are the major hepatic uptake transporters involved in the distribution and disposition of many drugs. As such, the US Food and Drug Administration (FDA) recommends that all new drug entities are tested as inhibitors of both OATP1B1 and OATP1B3 *in vitro* in order to predict the risk of *in vivo* drug interactions. The 2017 FDA guidance on clinical drug interaction studies recommends the determination of the inhibitory potency (half-maximal inhibitory concentration (IC_{50}) or inhibition constant (K_i)) of the compound in cells overexpressing the relevant transporter, with a pre-incubation period of at least 30 minutes with the inhibitor.[1] As there are many other experimental variables used, such as cell type, culture conditions, and probe substrate, a thorough descriptive analysis of the literature data was completed to determine which factors may contribute to the observed interlaboratory variability in inhibition potency, which may subsequently affect drug interaction predictions based on those values.

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Received: June 25, 2019; accepted: August 8, 2019. doi:10.1111/cts.12691

METHODS

Literature data were retrieved from the University of Washington Drug Interaction Database (www.druginteractioninfo.org). All studies showing *in vitro* inhibition of OATP1B1 and/or OATP1B3 were collected, and those studies providing K_i and/or IC_{50} data were retained. Experiments with K_i values were analyzed separately from IC_{50} values for consistency of data. Data were collated by inhibitor/substrate pair (ISP) and, to allow for adequate data comparison, those with a minimum of three experimental results were analyzed by calculating variability ratios (VRs—highest value for a given data set relative to lowest value). VRs were calculated for all data as well as after considering key experimental factors to evaluate sources of variability. To determine the effect of *in vitro* variability on drug-drug interaction (DDI) predictions, *R*-values were calculated and the variability (range as well as fold-change) were determined for the full data sets as well as for individual experimental conditions. Due to the small data set available for evaluation, only a descriptive analysis was able to be completed.

RESULTS

A total of 128 studies from 44 publications published between 2001 and 2018 were examined in the final data set. For OATP1B1, 21 ISPs were identified with ≥ 3 IC_{50} values available and seven ISPs were identified with ≥ 3 K_i values, with five pairs having both IC_{50} and K_i data (**Table S1**). For OATP1B3, only two ISPs were identified with ≥ 3 IC_{50} values, and none of the ISPs had K_i data reaching the required number of studies (**Table S1**). For both transporters, the most commonly used substrate was estradiol-17-β-glucuronide ($E_2 17\beta G$; 62% of all studies), whereas the top three inhibitors used were rifampin (27%), cyclosporine (25%), and gemfibrozil (18%), and the most commonly used cell type was HEK293 cells (79%).

IC_{50} variability

The largest IC_{50} VRs calculated for OATP1B1 were cyclosporine/$E_2 17\beta G$ (86.4; n = 11), and rifampin/bromosulfophthalein (BSP; 43.6; n = 3), whereas the highest for OATP1B3 was rifampin/$E_2 17\beta G$ (58.2; n = 7). Lower variability was observed in OATP1B1 K_i values, where the highest VR was for gemfibrozil/$E_2 17\beta G$ (7.2; n = 3). Two experimental factors were found to contribute the most to inhibition constant variability—cell type and pre-incubation vs. co-incubation with the inhibitor. For OATP1B1, IC_{50} values, the mean VR for all 21 substrate/inhibitor pairs was 11.7, reduced to 8.5 and 7.3 when only HEK293 cells and co-incubation were considered, respectively, and further reduced to 4.2 when both factors were considered together (**Figure 1a**). Regarding K_i data, none of the experiments that were analyzed involved pre-incubation, therefore, only the effect of cell type could be analyzed, and the average VR was reduced from 3.8 to 2.0 when only studies conducted in HEK293 cells were considered (**Figure 1b**). For OATP1B3, only two ISPs were analyzed, and a similar decrease in variability was observed when only experiments with co-incubation were considered relative to the complete data set (**Figure 1c**). Because a majority of the experiments identified for OATP1B3 were conducted in HEK293 cells, the contribution of cell type to the variability could not be evaluated.

In order to determine the contribution of the substrate used to inhibitory constant variability, inhibitors that were tested with the largest array of substrates were analyzed. Of the OATP1B1 inhibitors evaluated, cyclosporine and rifampin were studied with the largest number of substrates, and, interestingly, the highest variability in IC_{50} values was observed with nonclinically relevant substrates, namely $E_2 17\beta G$, estrone-3-sulfate (E3S), and BSP. When cyclosporine was used as the inhibitor, the VR for atorvastatin, $E_2 17\beta G$, and pitavastatin were 3.4, 86.3, and 12.7 for all data and 3.4, 12.6, and 3.0 when only HEK293 cells and co-incubation was considered, respectively. For rifampin, the VR for atorvastatin, BSP, $E_2 17\beta G$, and E3S were 3.9, 43.6, 15.8, and 11.9 for all data and 3.9, 4.3, 6.9, and 7.9 when only HEK293 cells and co-incubation was considered, respectively. These data indicate that the substrate used contributes substantially to the observed variability.

R-value variability

To determine the effect of the observed variability on clinical predictions, *R*-values, the predicted area under the concentration-time curve ratio (AUCR) of a substrate in the presence and absence of the inhibitor as described in the 2017 FDA *in vitro* guidance,[1] were calculated for each constant and the range and fold-change was determined for each ISP, as well as each inhibitor overall. Despite marked changes in VR when incubation conditions were accounted for, the resulting *R*-values did not show a substantial shift with respect to the FDA cutoff value for prompting a clinical evaluation ($R \geq 1.1$; **Figure S1**). For the recommended index inhibitors cyclosporine and rifampin, all calculated *R*-values were ≥ 1.1 regardless of the *in vitro* conditions. For cyclosporine, the fold-change ranged from 2.3 with atorvastatin to 51.1 with $E_2 17\beta G$, whereas the same substrates showed a 3.1-fold and 12.8-fold change, respectively, with rifampin (**Table 1**). This variability, both within the pairs and for the inhibitors overall, was decreased when the two primary sources of variability were accounted for, resulting in *R*-values ranging from 2.3–8.6 for cyclosporine and 2.8–5.7 for rifampin. In contrast, for gemfibrozil, a known *in vivo* inhibitor, only 5 of 14 (36%) of the *R*-values met the FDA cutoff, however, this is likely due to the major circulating metabolite, gemfibrozil-1-O-β-glucuronide, which is also an OATP inhibitor and, therefore, contributes to *in vivo* inhibition, which was not considered in this calculation.[2]

Similarly, for lopinavir, rifamycin, saquinavir, and troglitazone, all *R*-values calculated were greater than the cutoff value, regardless of the *in vitro* conditions, whereas in contrast, ritonavir and verapamil had *R*-values on both sides of the FDA cutoff, even using the most uniform data set (HEK293 and co-incubation). Interestingly, very few of these drugs had clinical data with a sensitive OATP1B1/1B3 substrate available, with no supporting clinical data identified for rifamycin or troglitazone. Ritonavir did not show significant inhibition of OATP1B1/1B3 (maximum observed AUCR of 1.31-fold with pravastatin[3]) when

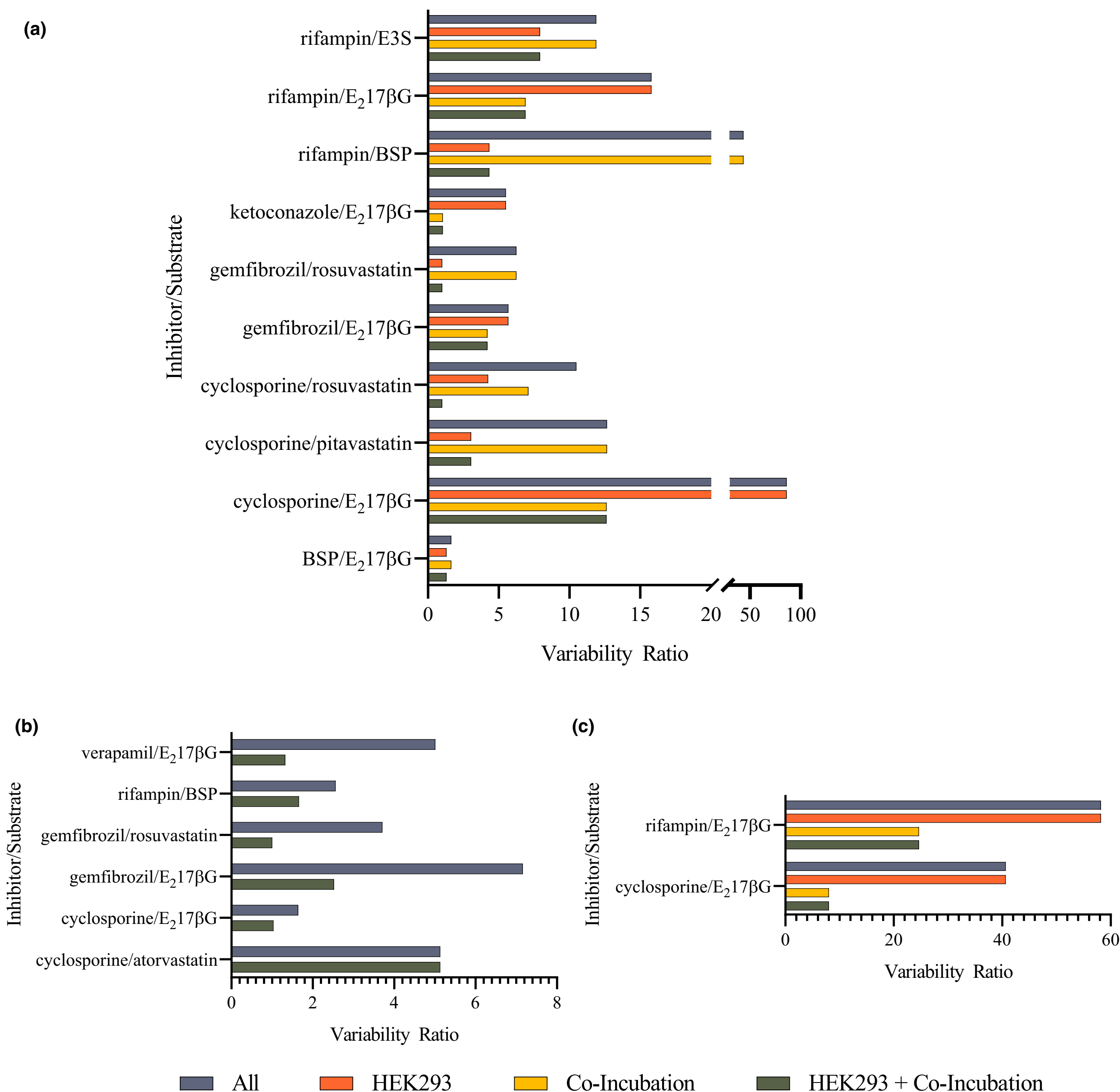

Figure 1 Effect of experimental conditions on variability ratio (VR). Only those inhibitor/substrate pairs where the VR changed are shown for clarity. (**a**) Organic anion transporting polypeptide (OATP)1B1 half-maximal inhibitory concentration (IC_{50}) VR, (**b**) OATP1B1 inhibition constant VR, (**c**) OATP1B3 IC_{50} VR. Blue bars are all collected data, orange bars are experiments performed in HEK293 cells only, yellow bars are only co-incubation with inhibitors, and green bars and HEK293/co-incubation only. BSP, bromosulfophthalein; $E_2$17Bg, estradiol-17-β-glucuronide; E3S, estrone-3-sulphate.

administered alone; however, when ritonavir was administered as part of a combination therapy with lopinavir or saquinavir, significant clinical inhibition was observed (maximum observed AUCR of 2.08 for lopinavir + ritonavir/rosuvastatin[4] and 3.93 saquinavir + ritonavir/atorvastatin[5]). Only one study with a sensitive OATP1B1/1B3 substrate was identified for verapamil, conducted with pravastatin. The observed change in exposure was minimal (1.32-fold) and could be attributable, at least in part, to inhibition of P-glycoprotein (P-gp).[6] In the case of ketoconazole, by narrowing the data set to the most uniform (HEK293 cells and co-incubation only) all of the resulting *R*-values were below the FDA cutoff, although the sample size was reduced to $n = 2$, which is supportive of ketoconazole not being an OATP1B inhibitor *in vivo*.[7]

DISCUSSION

Interlaboratory variability involving inhibition of transporters, specifically P-gp, has been discussed and addressed

Table 1 ***R*-value ranges calculated for OATP1B1/1B3 inhibition data using IC_{50} values**

Inhibitor	Substrate	All data			HEK293 only			Co-incubation only			HEK293 + Co-incubation			*In vivo* inhibitor?[a]
		Range	Fold-change	*N*	Range	Fold-change	*N*	Range	Fold-change	*N*	Range	Fold-change	*N*	
Cyclosporine	Atorvastatin	2.24–5.24	2.3	3		NC[b]			NC			NC		Y
	$E_2$17βG	2.42–123.78	51.1	11	2.42–123.78	51.1	10	2.42–20.77	8.6	9	2.42–20.77	8.6	8	
	$E_2$17βG (1B3)	2.79–73.9	26.4	4		NC		2.79–15.4	5.5	3		NC		
	Pitavastatin	1.80–11.14	6.2	4	4.33–11.14	2.6	3		NC		4.33–11.14	2.6	3	
	Rosuvastatin	2.06–12.11	5.9	4	3.62–12.11	3.3	2	2.06–8.53	4.1	3	3.62	–[c]	1	
Gemfibrozil	Atorvastatin	**1.02**[d]**–1.08**[d]	1.1	4		NC			NC			NC		Y
	$E_2$17βG	**1.06**[d]–1.34	1.3	7		NC		**1.06**[d]–1.25	1.2	6		NC		
	Rosuvastatin	**1.10**[d]–1.64	1.5	3	1.13	–	1		NC		1.13	–	1	
Ketoconazole	$E_2$17βG	**1.07**[d]–1.36	1.3	3		NC		**1.07**[d]**–1.07**[d]	1.0	2		NC		N
Lopinavir	Atorvastatin	1.60–1.81	1.1	3		NC			NC			NC		Y
Rifampin	Atorvastatin	3.97–12.42	3.1	3	3.97–12.42	3.1	2		NC		3.97–12.42	3.1	2	Y
	BSP	1.12–6.4	5.7	3	2.25–6.4	2.8	2		NC		2.25–6.40	2.8	2	
	$E_2$17βG	4.92–62.85	12.8	11	4.92–62.85	12.8	9	4.92–27.99	5.7	10	4.92–27.99	5.7	8	
	$E_2$17βG (1B3)	3.32–135.95	41.0	7	3.32–135.95	41.0	6	3.32–58.1	17.5	6	3.32–58.1	17.5	5	
	E3S	2.42–17.87	7.4	6	3.13–17.87	5.7	4		NC		3.13–17.87	5.7	4	
	Pitavastatin	7.75–27.51	3.6	4	7.75–27.51	3.6	2		NC		7.75–27.51	3.6	2	
Rifamycin	$E_2$17βG	23.46–144.75	6.2	5		NC			NC			NC		Y
Ritonavir	$E_2$17βG	**1.09**[d]–1.28	1.2	6	**1.09**[d]–1.28	1.2	5	**1.09**[d]–1.28	1.2	5	**1.09**[d]–1.28	1.2	4	Y/N[e]
	Pitavastatin	**1.08**[d]–1.22	1.1	3	1.11–1.22	1.1	2		NC		1.11–1.22	1.1	2	
Saquinavir	$E_2$17βG	2.95–8.61	2.9	3	2.95–8.61	2.9	2		NC		2.95–8.61	2.9	2	Y
Troglitazone	$E_2$17βG	1.24–2.89	2.3	3	1.24–2.89	2.3	2		NC		1.24–2.89	2.3	2	ND
Verapamil	$E_2$17βG	**1.03**[d]–1.12	1.1	3		NC			NC			NC		ND

BSP, bromosulfophthalein; $E_2$17Bg, estradiol-17-β-glucuronide; E3S, estrone-3-sulphate; IC_{50}, half-maximal inhibitory concentration; NC, no change; ND, not determined; OATP, organic anion transporting polypeptide.
All values are for inhibition of OATP1B1 unless otherwise specified.
[a]Supporting clinical data are presented in **Tables S2** and **S3**.
[b]No change from full data set (all data).
[c]Fold-change was not calculated when $N = 1$ and is indicated with a dash (–).
[d]Values in **bold** indicate $R < 1.1$.
[e]When used as monotherapy, no clinical inhibition was observed. However, significant inhibition has been observed for ritonavir-containing treatments.

in previous years, however, a similar analysis has not yet been performed for OATPs.[8,9] With the importance of OATP1B1/1B3 in drug disposition becoming increasingly apparent, addressing this variability, and the subsequent effect on *in vivo* predictions, is prudent. The descriptive analysis performed herein evaluated a broad data set, identifying two main areas of experimental design that contributed to this variability—cell system and pre-incubation vs. co-incubation with the inhibitor. By accounting for these factors, the variability in the overall data set dropped substantially. In addition, the choice of substrate influenced inhibitory constant variability.

The latest revision to the FDA guidance on *in vitro* assessment of DDIs requires that studies should be completed with a 30-minute pre-incubation with inhibitor before the addition of substrate. The pre-incubation data analyzed within the overall data set tended toward lower IC_{50} values than co-incubation, which represents more of a worse-case scenario for *in vivo* predictions. This has been confirmed by Tátrai *et al*.,[10] showing that pre-incubation resulted in a decrease in the IC_{50} for many inhibitors, increasing the likelihood of drugs being flagged for follow-up from *in vitro* DDI risk assessments. It is likely that this experimental design will be reflected in the literature in coming years as this approach is implemented. Similarly, it seems that although there is no current recommendation for cell system, there is a trend toward a singular preferred cell system for the determination of inhibition constants, HEK293 cells. In the overall analyzed data set (2001–2018), HEK293 cells were used in 68% of assays, whereas they were used in ~ 80% of experiments performed in the last 5 years (**Figure S2**). Aside from cell type and pre-incubation with inhibitor, another experimental condition that seemed to contribute to variability was the choice of probe substrate. Although there is known substrate dependence for inhibition of OATP transporters, that alone does not explain the variability observed within a single ISP.[11] In general, there were two classes of compounds used, *in vitro* probes ($E_2 17\beta G$, E3S, and BSP), and statins (atorvastatin, rosuvastatin, pravastatin, and pitavastatin). When the *in vitro* probes were used, the variability was higher than when statins were used. Many experimental factors could contribute to this, including substrate permeability, dynamic range of uptake for an individual substrate within the cell line, and analytical detection method of substrate, as the *in vitro* probes tend to be radiolabeled, whereas the statins require mass spectrometry for analysis.[12] These factors lend credence to the *in vitro* use of more clinically relevant substrates, such as statins, which may lead to improved *in vitro* to *in vivo* predictions based on not just inhibitor potency, but also reproducibility through the reduced variability for a substrate/inhibitor pair.

Although the most uniform experimental system seems to be HEK293 cells and accounting for pre-incubation, this analysis does not identify an experimental procedure or substrate that outperforms the others. For strong inhibitors, the observed *in vitro* variability does not seem to have an effect on clinical predictions relative to the FDA cutoff. Even when these two factors were accounted for, moderate and weak inhibitors showed values above and below 1.1, which was, for those with clinical data available, reflected in the low level of *in vivo* inhibition observed. However, the lack of specific OATP1B1/1B3 substrates or inhibitors confounds the direct translation of *in vitro* findings to *in vivo* effects and should be taken into account when considering clinical interactions.

It is important to note, however, that this descriptive analysis was limited by the availability of literature data regarding *in vitro* OATP1B1/1B3 inhibition. As many compounds are tested as inhibitors only during the drug development stage, requiring at least three experimental values significantly decreased the number of inhibitors that could be evaluated. Due to this criterion, it is possible that the conclusions reached here may underestimate the variability in OATP1B1/1B3 inhibitory constants, and subsequently *R*-values, as the drugs with the most studies available for analysis are marker inhibitors, as recommended by the FDA and, therefore, are likely to show more consistent results.[1] It should also be noted that although high variability was observed in the IC_{50} determination for OATP1B1 (~ 12-fold for the data set overall, reduced to 5-fold when controlling for incubation conditions), this is much lower than what has been observed for P-gp interactions with a range of IC_{50} values of over 700-fold being seen for a single ISP.[8] The current analysis did, however, identify similar parameters that contribute to *in vitro* inhibitor constant variability found in the prospective P-gp study. In both studies, controlling for cell system and substrate/inhibitor were found to reduce the observed variability in IC_{50} and K_i values and decreased the interlaboratory variability. However, it was found that variability also existed between experiments within the same laboratory, the effect of which cannot be tested in the current literature analysis for OATP1B1/1B3.[8] Despite the lower variability observed for OATP1B1/1B3 IC_{50} values, it remains prudent to further evaluate the potential sources of interlaboratory variability so as to ensure accurate and reproducible data are being generated.

In summary, accounting for cell type and inclusion of a pre-incubation period significantly reduced the observed variability in IC_{50} values for the data set overall as well as for specific ISP. Additionally, the choice of substrate also contributed to the variability with clinical substrates, such as statins, showing lower variability overall compared with *in vitro* probe substrates. The preclinical variability did not seem to affect *in vitro* to *in vivo* predictions for the inhibitors evaluated, as almost all calculated *R*-values were above the FDA cutoff value. As more data become available, evaluating the relationship between the extent of variability and inhibitory potency and its impact on clinical predictions would be valuable to confirm these preliminary results.

Supporting Information. Supplementary information accompanies this paper on the *Clinical and Translational Science* website (www.cts-journal.com).

Supplemental Data.
Supplemental Methods—Equations.

Funding. No funding was received for this work.

Conflict of Interest. The authors declared no competing interests for this work.

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