Validation of a total IC\(_{50}\) method which enables \textit{in vitro} assessment of transporter inhibition under semi-physiological conditions

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Abstract

1. Accurate predictions of clinical transporter-mediated drug–drug interactions (DDI) from \textit{in vitro} data can be challenging when compounds have poor solubility and/or high nonspecific binding. Additionally, current DDI predictions for compounds with high plasma–protein binding assume that the unbound fraction in plasma is 0.01, if the experimental value is less than 0.01 or cannot be determined. This approach may result in an overestimation of DDI risk. To overcome these challenges, it may be beneficial to conduct inhibition studies under physiologically relevant conditions.

2. Here, IC\(_{50}\) values, determined in the presence of 4% bovine serum albumin approximating human plasma albumin concentrations, were successfully used to predict DDI for uptake transporters, OATP1B1/1B3, OCT1/2, OAT1/3 and MATE1/2K.

3. The IC\(_{50}\) values of reference inhibitors with 4% bovine serum albumin, considered total IC\(_{50}\), were comparable to the predicted values based on nominal IC\(_{50}\) values determined under protein-free conditions and unbound fraction in plasma. Calculation of R-total and C\(_{\text{max}}\)/IC\(_{50,\text{total}}\) values using total plasma exposure and total IC\(_{50}\) values explained the clinical DDI or absence of it for these inhibitors.

4. These results suggest that IC\(_{50}\) determinations in the presence of 4% albumin can be used, in the context of clinical total exposure, to predict DDI involving uptake transporters.

Keywords

Albumin, DDI, drug–drug interactions, transporters, total IC\(_{50}\) values

Introduction

Drug transporters are increasingly recognized as important vehicles to govern drug absorption, distribution, and excretion. In the last decade, following extensive review of nonclinical and clinical data, including pharmacogenomics, pharmacokinetics, and transporter-mediated drug–drug interactions (DDIs), multiple transporters of clinical relevance have been identified. These transporters include P-glycoprotein, breast cancer resistance protein, organic anion transporting polypeptide (OATP) 1B1, OATP1B3, organic cation transporter (OCT) 1, OCT2, organic anion transporter (OAT) 1, OAT3, multidrug and toxin extrusion (MATE) 1 and MATE2K (Giacomini et al., 2010; Tweedie et al., 2013). To assess and mitigate the potential risk of transporter-mediated DDIs, Drug–Drug Interaction guidance documents from regulatory agencies (US Food and Drug Administration (FDA), European Medicines Agency (EMA) and Pharmaceuticals and Medical Devices Agency (PMDA)) have been updated to include broader characterization of investigational drugs as transporter substrates or inhibitors during drug development (EMA, 2012; FDA, 2012; PMDA, 2014).

Assessment of inhibition potential is recommended for all investigational drugs because such interactions may alter the pharmacokinetics and potentially pharmacodynamics of co-administered transporter substrates. Multiple \textit{in vitro} tools are available to determine the transporter inhibition including cell- and vesicle-based models overexpressing the transporter of interest (Brouwer et al., 2013). Generally, these inhibition studies (e.g. IC\(_{50}\) determination) are conducted in a protein-free environment so that the result represents the unbound IC\(_{50}\) value (IC\(_{50,\text{unbound}}\)) of test compound under the assumption that there is no significant loss of compound during assay incubation. IC\(_{50,\text{unbound}}\) can be used along with clinical unbound exposure to predict the likelihood of clinical inhibition (Giacomini et al., 2010). However, when an investigational compound has poor aqueous solubility, high plasma–protein and/or high nonspecific binding, there can be uncertainty in the actual effective concentration being studied \textit{in vitro}. For compounds with poor aqueous solubility or high nonspecific binding, \textit{in vitro} IC\(_{50}\) values are considered nominal and may not necessarily represent the intrinsic unbound IC\(_{50}\) values. It is quite possible that the IC\(_{50}\) determinations under the standard protein-free condition may overestimate the intrinsic unbound IC\(_{50}\) values, potentially...
leading to an underestimation of the DDI risk. Additionally, current DDI predictions for compounds with high plasma-protein binding assume the unbound fraction in plasma \( f_{u,p} \) is 0.01 if the experimentally derived value is less than 0.01 or if it cannot be determined (EMA, 2012; FDA, 2012). If the actual \( f_{u,p} \) is less than 0.01, defaulting to this assumption may result in an overestimation of the DDI risk, yield a false positive prediction and lead to an unnecessary clinical DDI study.

An apparent disconnect in the in vitro–in vivo correlation of transporter inhibition for a highly protein-bound compound has already been reported. Dolutegravir (>99% bound to plasma proteins (Bollen et al., 2015)) is an in vitro inhibitor of both OCT2 and MATE1 with apparent \( IC_{50} \) values of 1.93 and 6.34 \( \mu \)M, respectively (GlaxoSmithKline, Reese et al., 2013). Following a 50 mg QD dose, the plasma maximum concentration of dolutegravir is 8 \( \mu \)M (Song et al., 2010). When the \( f_{u,p} \) is assumed as 0.01, \( C_{u,\text{max}}/IC_{50} \) values for OCT2 and MATE1 are 0.041 and 0.013, respectively, suggesting low likelihood of clinical interactions with substrates for these transporters such as metformin. However, plasma exposures of metformin were significantly increased (1.8–2.5 fold) when co-administered with dolutegravir at 50 mg QD or BID (Zong et al., 2014). This indicates the overestimation of \( IC_{50} \) value and accordingly underestimation of DDI risk when the transporter inhibition of dolutegravir is assessed in the standard protein-free condition.

The possible limitations in the currently available tools as well as an apparent in vitro–in vivo disconnect observed clinically warrant the development of a new experimental method, which enables in vitro assessment of transporter inhibition under more physiologically relevant conditions. One approach to refine DDI predictions, for challenging compounds, is to run in vitro \( IC_{50} \) studies in the presence of 4% w/v albumin, which approximates human plasma albumin concentrations (Davies & Morris, 1993). \( IC_{50} \) values determined under this condition can be regarded as ‘total’ \( IC_{50} \) values. Theoretically, these values can be compared with total, but not unbound, exposure of test compounds in DDI predictions assuming all plasma–protein binding is ascribed to albumin. The present study was aimed to validate this alternate approach in the DDI prediction for uptake transporters.

**Materials and methods**

**Materials**

Human embryonic kidney (HEK) cell lines stably expressing OATP1B1 and OATP1B3 (HEK-OATP1B1 and HEK-OATP1B3), and the corresponding mock-transfected cell line (HEK-WT) were obtained from Prof. Dietrich Keppler (German Cancer Research Center (DKFZ), Heidelberg, Germany). OATP1B3 used in this study has a mutation at amino acid 112 (serine to alanine), which has been shown to exhibit the same substrate specificity as the native form of OATP1B3 (Letschert et al., 2004; Smith et al., 2007). The HEK-OATP1B3 (S112A) cells were used because they exhibited 2.5-fold higher transport activity \( (V_{\text{max}}) \) due to higher expression density, compared with the HEK-OATP1B3 cells. HEK cell lines stably expressing OCT1, OCT2, OAT1, OAT3, MATE1, and MATE2K (HEK-OCT1, HEK-OCT2, HEK-OAT1, HEK-OAT3, HEK-MATE1, and HEK-MATE2K) and the corresponding mock-transfected cell line (HEK-EV) were obtained from Prof. Kathleen M. Giacomini (Department of Biopharmaceutical Sciences, School of Pharmacy, University of California San Francisco). Estradiol 17-\( \beta \)-d-glucuronide (E\(_2\)17\( \beta \)G) and pitavastatin, para-aminohippuric acid (PAH), estrone sulfate (ES) and 1-methyl-4-phenylpyridinium (MPP+) were used as probe substrates for OATP1B1/OATP1B3, OAT1, OAT3 and OCT1/OCT2/MATE1/MATE2K, respectively. \[ ^{\text{3}}\text{H}\]E\(_2\)17\( \beta \)G (34–42 Ci/mol), \[ ^{\text{3}}\text{H}\]PAH (3–5 Ci/mmol), \[ ^{\text{3}}\text{H}\]ES (44–46 Ci/mmol) and \[ ^{\text{3}}\text{H}\]MPP+ (82–84 Ci/mmol) were purchased from PerkinElmer (Waltham, MA), and \[ ^{\text{3}}\text{H}\]pitavastatin (5 Ci/mmol) was from American Radiolabeled Chemicals (St. Louis, MO). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless stated otherwise.

**Uptake experiments**

Cells were cultured as described previously (Chiou et al., 2014; Kikuchi et al., 2013). For transport studies, the cells were seeded in 24-well poly-d-lysine-coated plates and cultured for 2–3 days. Cells were washed three times with incubation buffer (Hanks Balanced Salt Solution (HBSS), supplemented with 10 mM HEPES, pH 7.4) at 37°C. For MATE1 and MATE2K studies only, the cells were instead pre-incubated with an acidification buffer (HBSS containing 10 mM HEPES and 30 mM NH\(_4\)Cl, pH 7.4) at 37°C for 20 minutes. For the inhibition studies in the absence of proteins, uptake was initiated by adding incubation buffer containing radiolabeled probe substrate, 2 \( \mu \)M \[ ^{\text{3}}\text{H}\]E\(_2\)17\( \beta \)G (0.5 \( \mu \)Ci/mL) or 0.2 \( \mu \)M \[ ^{\text{3}}\text{H}\]pitavastatin (0.1 \( \mu \)Ci/mL) for OATP1B1, 2 \( \mu \)M \[ ^{\text{3}}\text{H}\]E\(_2\)17\( \beta \)G (0.5 \( \mu \)Ci/mL) for OATP1B3, 1 \( \mu \)M \[ ^{\text{3}}\text{H}\]PAH (0.2 \( \mu \)Ci/mL) for OAT1, 0.1 \( \mu \)M \[ ^{\text{3}}\text{H}\]ES (0.5 \( \mu \)Ci/mL) for OAT3 and 1 \( \mu \)M \[ ^{\text{3}}\text{H}\]MPP+ (0.2 \( \mu \)Ci/mL) for OCT1, OCT2, MATE1 and MATE2K, in the presence or absence of multiple concentrations of test compounds. Those are rifampicin, rifamycin SV, indinavir, saquinavir, and troglitazone for OATP1B1, rifampicin for OATP1B3, pyrimethamine for OCT1, OCT2, MATE1, and MATE2K, and probenecid for OAT1 and OAT3. For the inhibition studies in 100% human plasma or in the presence of 4% w/v bovine serum albumin (BSA), different concentrations of probe substrates were used for OATP1B1, OATP1B3 and OAT3; 5 \( \mu \)M \[ ^{\text{3}}\text{H}\]E\(_2\)17\( \beta \)G (1.25 \( \mu \)Ci/mL) or 1 \( \mu \)M \[ ^{\text{3}}\text{H}\]pitavastatin (0.5 \( \mu \)Ci/mL) for OATP1B1, 5 \( \mu \)M \[ ^{\text{3}}\text{H}\]E\(_2\)17\( \beta \)G (1.25 \( \mu \)Ci/mL) for OATP1B3, and 6 \( \mu \)M \[ ^{\text{3}}\text{H}\]ES (3 \( \mu \)Ci/mL) for OAT3. The concentrations of the other probe substrates were the same as those used in the inhibition studies in the absence of proteins. Uptake was terminated after a fixed incubation time by aspirating the incubation buffer and washing the cells three times with ice-cold HBSS. The incubation time was 5 minutes (E\(_2\)17\( \beta \)G) or 2 minutes (pitavastatin) for OATP1B1 and OATP1B3, 2 minutes for OCT1, and 1 minute for OCT2, OAT1, OAT3, MATE1 and MATE2K. The uptake of probe substrates for hepatic transporters (OATP1B1, OATP1B3 and OCT1) (Supplemental Figure 1) and renal transporters (OCT2, OAT1, OAT3, MATE1 and MATE2K) (Kikuchi et al., 2013) were shown to be linear within these incubation
times. Cells were then lysed in 200 μL of phosphate buffered saline containing 0.5% Triton X-100 with shaking for one hour at room temperature. Aliquots (150 μL) of cell lysate and 20 μL of incubation buffers were transferred to a 24-well micro-β counting plate containing 0.5 mL of scintillation cocktail. Radioactivity associated with the cells and incubation buffers was determined by Micro-β counter (PerkinElmer, Waltham, MA). The cell lysate protein concentrations were determined by the Pierce BCA Protein Assay (Thermo Scientific, Waltham, MA) according to the manufacturer’s instructions. Uptake in each well was normalized by the corresponding protein concentrations and expressed as picomole per milligram protein. Transporter-specific uptake was obtained by subtracting the uptake into mock-transfected cells from that into transporter-expressing cells. IC50 values were determined assuming competitive inhibition using the following equation:

\[
Y = \text{Bottom} + \frac{100 - \text{Bottom}}{1 + 10^{X - \log IC50}}
\]

where \(X\) is log concentration of inhibitor, \(Y\) is % uptake of control, and \(\text{Bottom}\) is the lowest values of a fitted curve in GraphPad Prism (ver. 6.04) (GraphPad Software, La Jolla, CA). Mean and standard deviation (SD) of IC50 values were calculated from three or four independent experiments each run in duplicate.

**Results**

**Uptake of probe substrates in the presence of 100% human plasma or 4% BSA**

In order to determine and compare IC50 values of test compounds under the same unbound concentration of probe substrates, the total concentration of \(E_217\gamma\) (OATP1B1/OATP1B3) and ES (OAT3) was increased from 2 to 5 μM and from 0.1 to 6 μM, respectively. Probe substrate concentrations were determined by considering their plasma unbound fractions, 0.39 and 0.016, respectively (Giorgi & Crosignani, 1969; Rosenthal et al., 1972), and assuming similar binding in 4% BSA. No adjustment for probe substrate concentrations was made for PAH (OAT1) and MPP+ (OCT1, OCT2, MATE1 and MATE2K) because the protein binding of these compounds was expected to be minimal. The fold difference values of the uptake of probe substrates between transporter-expressing cells and mock-transfected cells in the absence of inhibitors are summarized in Supplemental Table 1; these are at least 3.7-fold greater than that in mock-transfected cells, giving sufficient window for the subsequent IC50 studies.

**Comparison of OATP1B1 total IC50 values between 4% BSA and 100% human plasma**

Total IC50 values of reference inhibitors for OATP1B1 (rifampicin, rifamycin SV, indinavir and saquinavir) were determined in the presence of 4% BSA or 100% human plasma (Table 1). The observed total IC50 values were compared (within three-fold) whether using 4% BSA or 100% human plasma (Figure 1). Representative IC50 curves are shown in Figure 2 and Supplemental Figure 2. Due to the greater uptake window in the presence of 4% BSA than human plasma (Supplemental Table 1), 4% BSA was used in the subsequent total IC50 studies for the other transporters.

**IC50 determination of reference inhibitors in the absence and presence of 4% BSA**

IC50 values of reference inhibitors for OATP1B1, OATP1B3, OCT1, OCT2, OAT1, OAT3, MATE1, and MATE2K were determined in the absence and presence of 4% BSA (Figure 2, Supplemental Figure 3, and Table 1). The IC50 values determined in the presence of 4% BSA were higher than those determined in the absence of proteins. The unbound IC50 values were extrapolated to total IC50 values using the following equation (Table 1):

\[
\text{IC50, total, predicted} = \frac{\text{IC50, unbound}}{f_{u,p}}
\]

### Table 1. Unbound and total IC50 values of reference inhibitors.

| Transporter | Compound     | IC50,unbound (μM) | \(f_{u,p}\) | Predicted IC50,total (μM) | 4% BSA | 100% human plasma |
|-------------|--------------|-------------------|-------------|--------------------------|--------|--------------------|
| OATP1B1*   | Rifampicin   | 0.59 ± 0.25       | 0.20        | 3.0 ± 1.3                | 4.9 ± 1.6 | 2.4 ± 0.2          |
|            | Rifampicin SV| 0.050 ± 0.004     | 0.20        | 0.25 ± 0.02              | 0.80 ± 0.26 | 1.2 ± 0.6          |
|            | Indinavir    | 8.3 ± 2.3         | 0.40        | 21 ± 6                   | 13 ± 6   | 11 ± 2             |
|            | Saquinavir   | 0.41 ± 0.16       | 0.020       | 21 ± 8                   | 18 ± 11  | 22 ± 10            |
| OATP1B1 (pitavastatin) | Rifampicin | 0.56             | 0.20        | 2.8                      | 4.1     | –                  |
| OATP1B3*   | Rifampicin   | 0.22 ± 0.12       | 0.20        | 1.1 ± 0.6                | 1.4 ± 0.4 | –                  |
| OCT1       | Pyrimethamine| 1.8 ± 0.5         | 0.13        | 14 ± 4                   | 7.6 ± 3.2 | –                  |
| OCT2       | Pyrimethamine| 24 ± 14           | 0.13        | 186 ± 105                | 160 ± 52 | –                  |
| OAT1       | Probenecid   | 20 ± 4            | 0.11        | 179 ± 34                 | 160 ± 65 | –                  |
| OAT3       | Probenecid   | 4.1 ± 1.0         | 0.11        | 38 ± 9                   | 24 ± 12  | –                  |
| MATE1      | Pyrimethamine| 0.13 ± 0.02       | 0.13        | 1.0 ± 0.2                | 0.92 ± 0.74 | –              |
| MATE2K     | Pyrimethamine| 0.19 ± 0.06       | 0.13        | 1.4 ± 0.4                | 0.75 ± 0.34 | –              |

The unbound IC50 values were extrapolated to predicted total IC50 values as described in the text. The observed IC50 values represent the experimentally determined values in the presence of 4% BSA or in 100% human plasma. Two different probe substrates shown in parentheses were used in the IC50 determination for OATP1B1.

*Unbound IC50 values for OATP1B1 (E217G) and OATP1B3 were cited from our previous study (Chiou et al., 2014).

*Mean ± SD values of three or four independent experiments except rifampicin IC50 values on the uptake of pitavastatin by OATP1B1 which are the mean of two independent experiments.

\(f_{u,p}\) values are from either product labels (rifampicin, indinavir, saquinavir, and pyrimethamine), Campbell et al., 2004 (rifamycin SV), or Laurence L. Brunton, et al. (Eds), Goodman & Gilman’s The Pharmacological Basis of Therapeutics, 12e (probenecid).
Predicted total IC₅₀ values (IC₅₀,total,predicted) were compared with the observed total IC₅₀ values (IC₅₀,total) (Figure 3). The observed total IC₅₀ values of reference inhibitors in 4% BSA were within three-fold of the predicted values based on their unbound IC₅₀ and fₚ,u values.

**OATP1B1 IC₅₀ value of rifampicin using a clinically relevant probe substrate pitavastatin**

Additional IC₅₀ studies were conducted in order to validate the translatability of the total IC₅₀ method in predicting a clinical DDI. The IC₅₀ values of rifampicin, a clinically relevant inhibitor, on the OATP1B1-mediated uptake of pitavastatin were 0.56 μM and 4.1 μM in the absence and presence of 4% BSA, respectively (Figure 4 and Table 1).

**DDI prediction using total IC₅₀ values**

Static DDI predictions for reference inhibitors on hepatic transporters (OATP1B1, OATP1B3 and OCT1) and renal transporters (OCT2, OAT1, OAT3, MATE1 and MATE2K) were conducted using either unbound or total IC₅₀ values. For hepatic uptake transporters, R-free and R-total were calculated using the following equations:

\[
R_{\text{free}} = \frac{1}{1 + \frac{f_{u,p}}{C_{I_{\text{in}},\text{max}}/IC_{50,\text{unbound}}}}
\]

\[
R_{\text{total}} = \frac{1}{1 + \frac{I_{\text{in},\text{max}}}{IC_{50,\text{total}}}}
\]
where $I_{in,max}$ is the estimated maximum inhibitor concentration at the inlet to the liver and is equal to: $C_{max} + (k_a \times \text{Dose} \times F_uF_g)/Q_h$. $C_{max}$ is the maximum systemic plasma concentration of inhibitor; Dose is the inhibitor dose; $F_uF_g$ is the fraction of the dose of inhibitor which is absorbed; $k_a$ is the absorption rate constant of the inhibitor, and $Q_h$ is the estimated hepatic blood flow (1500 mL/min) (FDA, 2012). For renal transporters, unbound $C_{max} (C_{u,max})/IC_{50,unbound}$ and $C_{max}/IC_{50,total}$ values were calculated. A summary of the predicted inhibition of hepatic transporters and renal transporters by reference inhibitors (tested under both assay conditions) is listed in Tables 2 and 3, respectively. For hepatic uptake transporters, $R$-total values, which were derived from total plasma exposure and total $IC_{50}$ values, were comparable to the $R$-free values which are calculated using unbound exposure and unbound $IC_{50}$ values (Table 2). Likewise, $C_{u,max}/IC_{50,unbound}$ and $C_{max}/IC_{50,total}$ values were comparable for renal transporters (Table 3).

**IC$_{50}$ determination of troglitazone in the absence or presence of 4% BSA**

Troglitazone is extensively bound in human plasma: $f_{u,p}$ equals 0.0011 (Shibukawa et al., 1995). The $IC_{50}$ value of troglitazone for OATP1B1 was determined in the absence and presence of 4% BSA (Figure 5). Troglitazone inhibited OATP1B1 in the absence and presence of 4% BSA with $IC_{50}$ values of 0.32 ± 0.20 μM and 40 ± 16 μM (mean ± SD), respectively.

**Discussion**

The *in vitro* characterization of transporter interactions can be challenged by poor aqueous solubility and/or high nonspecific binding of a compound because there is an uncertainty in the effective concentration of test compound during the assay incubation, i.e. it can be lower than the nominal concentrations due to a potential loss of compounds by precipitation or nonspecific binding in the assay system (e.g. plastic wells). There are multiple approaches to improve transporter DDI characterization for these classes of compounds. For example, an $IC_{50}$ value may be estimated based on experimentally determined concentrations of solubilized compound in the assay, rather than nominal concentrations. Another approach may be to conduct an $IC_{50}$ study in the presence of proteins (e.g. albumin) or plasma and normalize the resulting $IC_{50}$ value (i.e. total $IC_{50}$ value) by the *in vitro* unbound fractions which are determined in a separate experiment. These approaches would be considered ideal because they may provide the intrinsic unbound $IC_{50}$ value even for a compound with poor aqueous solubility and/or high nonspecific binding. However, an accurate determination of aqueous solubility and (plasma) protein binding, which are required to provide a reliable DDI prediction in the current methodology, can be challenging for such compounds (Riccardi et al., 2015).

In the present study, a new *in vitro* method which enables the *in vitro* assessment of transporter inhibition under semi-physiological conditions was developed and validated using reference inhibitors for uptake transporters. $IC_{50}$ values determined for rifampicin, rifamycin SV, indinavir and saquinavir against OATP1B1 in 4% BSA or 100% human plasma were comparable (Figure 1), suggesting that 4% BSA can be used as a surrogate for plasma. The $IC_{50}$ values of reference inhibitors for hepatic and renal transporters in the presence of 4% BSA were comparable to the predicted values based on their nominal $IC_{50}$ values determined under protein-free conditions (i.e. unbound $IC_{50}$) corrected for unbound fraction in plasma (Table 1, Figure 3). Accordingly, the $R$-total and $C_{max}/IC_{50,total}$ values, which are considered DDI predictions using total plasma exposure of test compounds...
in duplicate. The IC\textsubscript{50} values are 0.32 ± 0.20 instead of unbound exposure, were comparable to the R-free and R-total IC\textsubscript{50} values.

Table 2. Static DDI prediction for hepatic transporters using unbound or total IC\textsubscript{50} values.

| Transporter | Compound | Dose (mg)\textsuperscript{a} | C\textsubscript{max} (µM)\textsuperscript{b} | u,max/IC50,unbound values, respectively (Tables 2 and 3).

In addition, transient benign unconjugated hyperbilirubinemia has been observed clinically with these compounds, which has been ascribed to the inhibition of OATP1B1 and/or OATP1B3 clinically. The draft DDI guidance from FDA recommends that compounds should be investigated clinically as potential OATP1B1 or OATP1B3 inhibitors if the R-free value is greater than 1.25 (FDA, 2012). Indeed, R-free values calculated in the present study successfully differentiated the clinical inhibition potential of inhibitors of OATP1B1 and/or OATP1B3 for rifampicin, rifamycin SV, indinavir and saquinavir using the cutoff value of 1.25 (Table 2). Importantly, R-total values can also explain a clinical inhibition or its absence for these inhibitors by applying the same cutoff value. R-total values were greater than 1.25 for rifampicin, rifamycin SV and indinavir, while it was borderline (1.3) for saquinavir (similar to the R-free value of 1.2) (Table 2). Pyrimethamine is predicted to be a clinical inhibitor of OCT1 (R-free and R-total values greater than 1.25), however, this has not been examined clinically. For DDI predictions on renal transporters, the International Transporter Consortium recommends the cutoff value of 0.1 for C\textsubscript{u,max}/IC\textsubscript{50,unbound} (Tweedie et al., 2013). In the present study, both C\textsubscript{u,max}/IC\textsubscript{50,unbound} and C\textsubscript{max}/IC\textsubscript{50,total} values were greater than 0.1 for probenecid on OAT1 and OAT3 and pyrimethamine on MATE1 and MATE2K (Table 3). This is consistent with known clinical DDIs between probenecid and OAT1 or OAT3 substrates such as famotidine, and between pyrimethamine and MATE1 or MATE2K substrate (metformin) (Morrissey et al., 2013). The translatability of total IC\textsubscript{50} method in predicting a clinical DDI was further explored using a clinically relevant combination of victim and perpetrator drugs. Rifampicin inhibited the OATP1B1-mediated uptake of pitavastatin in the presence of 4% BSA (Figure 4). The resulting R-total value (4.2) was greater than the cutoff value (1.25), consistent with the clinical DDI observed between these two compounds (Table 3).
IC50 values combined with total plasma exposure could explain the clinical transporter interactions for these reference inhibitors. These results suggest that IC50 determinations in the presence of 4% albumin can be used in the prediction of transporter DDI by using clinical total plasma exposure in the calculation of R-total and Cmax/IC50,total values.

The in vitro method developed in the present study, the ‘‘total IC50 method’’, may offer an advantage to the conventional protein-free method in the transporter characterization for compounds with poor solubility and/or high plasma protein- and nonspecific binding because: (1) the presence of albumin is expected to minimize nonspecific binding and uncertainty in the effective concentration in the test system, and (2) an accurate fu,p value is no longer required in DDI predictions as in vitro IC50 values are determined in the presence of a physiological albumin concentration and are directly compared to total plasma concentrations. The total IC50 method was applied for a highly-bound compound, troglitazone, of which fu,p equals 0.0011 (Shibukawa et al., 1995) (Figure 5). Troglitazone was confirmed as an OATP1B1 inhibitor with a nominal IC50 value of 0.32 μM in the conventional protein-free assay condition. This value is 8-fold lower than the previously reported value (2.5 μM) (van de Steeg et al., 2013) even though the studies were conducted in almost identical experimental conditions; inhibition of [3H]E217 uptake in HEK cells stably expressing OATP1B1 was examined. This may imply an uncertainty in the nominal IC50 values potentially due to the nonspecific adsorption of troglitazone to assay apparatus, which could vary across different laboratories. Albumin plays a major role in the protein binding of troglitazone in plasma (Shibukawa et al., 1995). With the experimentally determined fu,p of 0.0011, the total IC50 value of troglitazone for OATP1B1 was expected to be 291 μM. However, in contrast to the reference inhibitors used to validate the method, the observed total IC50 value of troglitazone (40 μM) was significantly lower than the predicted value. This indicates a significant loss of compound in the absence of added protein, resulting in an overestimation of intrinsic unbound IC50 value. The total IC50 method may improve the DDI characterization for compounds with poor solubility and/or high plasma protein- and nonspecific binding like troglitazone.

In conclusion, the present study describes a new in vitro method, namely the total IC50 method, which enables the in vitro assessment of transporter inhibition under semi-physiological conditions. IC50 determinations in the presence of 4% bovine serum albumin can be used, in context of clinical total exposure, as an alternate to traditional protein-free methods to predict clinical DDI involving uptake transporters.

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References
Bollen P, Reiss P, Schapiro J, Burger D. (2015). Clinical pharmacokinetics and pharmacodynamics of dolutegravir used as a single tablet regimen for the treatment of HIV-1 infection. Expert Opin Drug Saf 14:1457–72.
Brouwer KL, Keppler D, International Transporter Consortium, et al. (2013). In vitro methods to support transporter evaluation in drug discovery and development. Clin Pharmacol Ther 94:95–112.
Campbell SD, De Morais SM, Xu JJ. (2004). Inhibition of human organic anion transporting polypeptide OATP 1B1 as a mechanism of drug-induced hyperbilirubinemia. Chem Biol Interact 150:179–87.
Chiou WJ, De Morais SM, Kikuchi R, et al. (2014). In vitro OATP1B1 and OATP1B3 inhibition is associated with observations of benign clinical unconjugated hyperbilirubinemia. Xenobiotica 44:276–82.
Davies B, Morris T. (1993). Physiological parameters in laboratory animals and humans. Pharm Res 10:1093–5.
EMA. (2012). Guideline on the investigation of drug interactions. Available from: http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2012/07/WC500129606.pdf.
FDA. (2012). Guidance for industry. Drug interaction studies – study design, data analysis, implications for dosing, and labeling recommendations: draft guidance. Available from: http://www.fda.gov/downloads/Drugs/GuidanceComplianceReulatoryInformation/Guidances/UCM292362.pdf.
Fichtenbaum CJ, Gerber JG, Rosenkranz SL, NIAID AIDS Clinical Trials Group, et al. (2002). Pharmacokinetic interactions between protease inhibitors and stavitis in HIV seronegative volunteers: ACTG study A5047. AIDS 16:569–77.
Giacomini KM, Huang SM, Tweedie DJ, et al. (2010). Membrane transporters in drug development. Nat Rev Drug Discov 9:215–36.
Giorgi EP, Croisignani PG. (1989). Competitive binding of free and conjugated oestrogens to plasma proteins. J Endocrinol 44:219–30.
Glaxosmithkline, Tivicay (dolutegravir) prescribing information. Available from: https://www.gsksource.com/pharma/content/dam/GlaxoSmithKlineUS/en/Prescribing_Information/Tivicay/pdf/TIVICAY-PI-PIL.PDF [last accessed 7 July 2015].
Jacobson JM, Davidian M, Rainey PM, et al. (1996). Pyrimethamine pharmacokinetics in human immunodeficiency virus-positive patients seropositive for toxoplasma gondii. Antimicrob Agents Chemother 40:1360–5.
Kikuchi R, Lao Y, Bow DA, et al. (2013). Prediction of clinical drug-drug interactions of veliparib (ABT-888) with human renal transporters (OAT1, OAT3, OCT2, MATE1, and MATE2K). J Pharm Sci 102:4426–32.
Letschert K, Keppler D, Konig J. (2004). Mutations in the SLCO1B3 gene affecting the substrate specificity of the hepatocellular uptake transporter OATP1B3 (OATP8). Pharmacogenetics 14:441–52.
Morrissey KM, Stocker SL, Wittwer MB, et al. (2013). Renal transporters in drug development. Annu Rev Pharmacol Toxicol 53:503–29.
PMDA. (2014). Drug Interaction Guideline for Drug Development and Provision of Appropriate Information (draft). In Japanese. Available from: http://www.mhlw.go.jp/mhlw/20131488.pdf.
Prueksaritanont T, Chu X, Evers R, et al. (2014). Pitavastatin is a more sensitive and selective organic anion-transporting polypeptide 1B clinical probe than rosuvastatin. Br J Clin Pharmacol 78:587–98.
Reese MJ, Savina PM, Generaux GT, et al. (2013). In vitro investigations into the roles of drug transporters and metabolizing enzymes in the
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