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In Vitro Evidence of OATP1B1 Induced Drug-Serum Protein Binding Shift and Its Implications on Predicting Drug Clearance and Drug-Drug Interactions

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ABSTRACT

BACKGROUND: We recently proposed a Transporter-Induced Protein Binding Shift (TIPBS) hypothesis to describe the effects of serum proteins on transporter-mediated drug transport. This work provides *in vitro* evidence of drug- and transporter-dependent TIPBS effects that may substantiate the hypothesis and potentially improve prediction of clearance and DDI for certain high-protein bound drugs.

METHODS: OATP1B1 mediated transport of given major statins and Rifampicin IC_{50}s were measured in protein-free HBSS and human serum, using CHO cells stably expressing the transporter. Serum unbound fraction (fu) of each compound, measured with rapid equilibrium dialysis, was used to calculate substrate transport or inhibitor IC_{50}s in serum from the constants measured in HBSS. The predicted values were contrasted to that measured from assays conducted in human serum.

RESULTS: The fu adjustment method generally under-estimated substrate uptake and inhibitor potency in serum. For examples, actual OATP1B1 mediated uptake of 5μM atorvastatin in serum (fu=3.95%) is 2.8x higher than that of HBSS with the same unbound drug; depending on the substrate used, Rifampicin (fu=11%) OATP1B1 IC_{50} values in serum was 2.5x-4.5x lower than the predicted ones. Our data on various substrates and inhibitors with different fu indicated that the extent of underestimation were drug AND transporter dependent, possibly due to difference in drug binding affinities to serum proteins and transporters as predicted by our TIPBS models.

CONCLUSION: Our work suggests that transporter-mediated drug uptake and its inhibition in serum may be under-predicted by simply applying unbound drug fraction to correct *in vitro* assay results performed in protein-free buffers. This raises the question on whether/when is appropriate to use the conventional fu adjustment method for predicting in vivo drug clearance and DDIs without using empirical drug-dependent scaling factors.

INTRODUCTION

Transporters play a crucial role in drug clearance and drug-drug interactions (DDI). Currently *in vitro* cell-based assays are conducted to predict potential clinical DDI. Typically, in these assays transporter kinetic parameters (Vmax, Km, and Ki/IC_{50}) are measured in protein free buffers (e.g. HBSS without albumin). However, the presence of proteins in serum may affect significantly the free concentration of drugs. Thus conventional "equilibrium model" is applied by applying drug unbound fraction at equilibrium (fu), in predicting *in vivo* drug clearance and DDIs. This approach assumes that drugs are in binding equilibrium with serum proteins and only the free fraction (fu) is transported or has inhibitor effect.

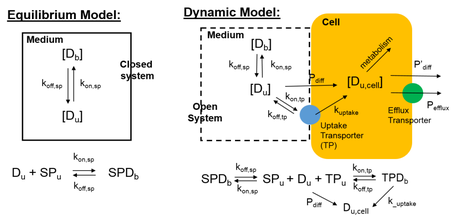

Figure 1. Schematic illustration of the conventional rapid equilibrium model (left) whereas $[D_u]=[D_{total}]$ x f_u, $f_u=\frac{1}{1+[A]/K_d}$, and our dynamic TIPBS model (right) in which $[D_u]$ at cell surface may not be determined by the f_u measured under equilibrium conditions

We have previously shown for certain highly protein bound transporter substrates and inhibitors, the apparent transport rate or inhibition potency measured in 100% human serum were significantly higher than those calculated based on equilibrium fu and the intrinsic transporter IC_{50}s assessed in HBSS [1]. We further hypothesized that such discrepancies may arise from transporter induced protein binding shift (TIPBS) for compounds which exhibit higher binding affinities toward transporters than serum binding proteins; thus the transporters may pull compounds away from binding proteins then clear them out, creating a "sink" condition under which binding equilibrium may not be reached (Open system) [2]. Here we aim to further interrogate experimentally TIPBS effects on OATP1B1 mediated transport of highly protein bound statins and its inhibition by Rifampicin. The extent of shift in active uptake is correlated with estimated compound binding affinities toward the transporter and serum albumin.

MATERIALS AND METHODS

CELL CULTURE: CHO cells stably expressing human OATP1B1 transporters were maintained in Dulbecco's modified Eagle's medium (DMEM) at 37 °C in a humidified atmosphere with 5% CO_2. Approximately 48 hr before assay, cells were seeded on Corning BioCoat™ 96-well flat bottom plates. CHO parental cells were also used as a control.

TRANSPORT ASSAY: Briefly, cells were washed and pre-incubated with HBSS for 15 min at 37°C. Radioisotope-labelled statins (atorvastatin, pitavastatin, lovastatin, simvastatin and fluvastatin) in HBSS or human serum were incubated with cells for 2 min based on the previously determined linear range for each statin. The intracellular contents of statins were extracted by adding 50% acetonitrile in water and quantified by radiometry. To determine the intrinsic Km and Vmax values for each statin as a substrate of OATP1B1, concentrations of up to 100 μM in HBSS were tested for each statin as a free monomer. To evaluate the IC_{50} of Rifampicin against the transport of each statin, a series concentrations of Rifampicin (0.1-100 μM) was mixed with a fixed concentration of each statin tested at 100nM in HBSS and 10μM in serum (except for Atorvastatin tested at 5μM). The mixture was in either HBSS buffer or human serum. Actual concentrations of inhibitor Rifampicin was quantified by LC-MS/MS and used in calculation of IC_{50} values by non-linear regression using GraphPad Prism.

DETERMINATION OF DRUG SERUM UNBOUND FRACTION F_U USING RAPID EQUILIBRIUM DIALYSIS: Rapid Equilibrium Dialysis (RED) kit (Thermo Scientific, Rockford, IL) was used to estimate the extent of statins and Rifampicin binds to serum proteins. A series concentrations of drugs were prepared in 100% human serum and 200 μL was loaded the sample chamber. 350 μL PBS was added to the buffer chamber. The device was sealed and incubated at 37°C on an orbital shaker at approximately 250 rpm for 4 hr. 50 μL samples was taken each chamber and measured by either scintillation counter or LC-MS/MS. The fraction unbound was calculated by dividing the concentration in the buffer chamber by the concentration in corresponding serum sample chamber.

F_U ADJUSTMENT METHOD FOR PREDICTING SUBSTRATE TRANSPORT AND INHIBITOR POTENCY IN SERUM: To distinguish transport and inhibition constants in HBSS and serum, we hereby denote constants measured in protein-free HBSS as *intrinsic* as they reflect the "true" kinetic properties without influence from protein binding. The f_u adjustment method as defined by the following equations are used to predict substrate transport and *apparent* inhibitor IC_{50} in serum.

Substrate: $V_{serum}=\frac{V_{max}}{K_m} \times C_{total} \times fu$, assuming $C_{total} \times fu << K_m$ Inhibitor: $IC_{50,\,serum}=IC_{50}/f_u$

F_{TIPBS} AND EXTENT OF TIPBS EFFECT: We define F_{TIPBS} as the ratio of measured and predicted transport rate or inhibition potency in serum. Larger F_{TIPBS} value suggests more profound shift in serum protein binding.

Substrate: $F_{TIPBS} = V_{serum,\,measured} / V_{serum,\,predicted}$ Inhibitor: $F_{TIPBS} = IC_{50,\,serum,\,predicted} / IC_{50,\,serum,\,measured}$

ESTIMATION OF COMPOUND DISSOCIATION CONSTANT K_d FOR TRANSPORTER AND ALBUMIN: For simplicity of calculation, it is assumed that albumin is the dominant serum binding protein and serum albumin concentration is 660μM. Albumin K_d is calculated from measured equilibrium f_u. Transporter K_d is approximated by K_m assuming $k_{off,tp} >> k_{uptake}$ (Fig. 1).

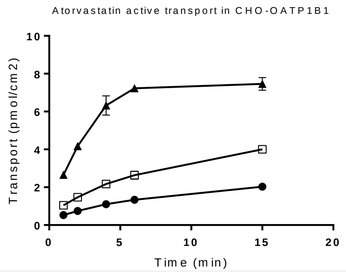

Figure 2. Time-dependent active transport of 100nM Atorvastatin in HBSS (solid circle) and 5μM Atorvastatin in 100% human serum (measured f_u 3.95%) mediated by OATP1B1. Measured Atorvastatin active uptake (solid triangle) in serum is significantly higher than that of predicted (open square).

RESULTS, DISCUSSION AND CONCLUSIONS

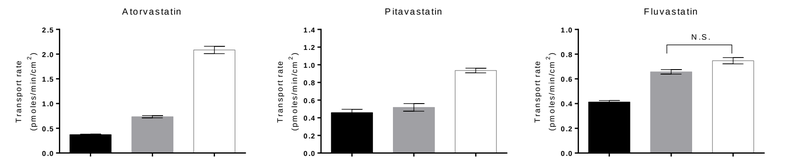

Figure 3. Representative measured active transport rate of 100nM statins in HBSS (black), predicted (grey) and actual (open) uptake rate of 5μM Atorvastatin, 10μM Pitavastatin and Fluvastatin, in 100% human serum

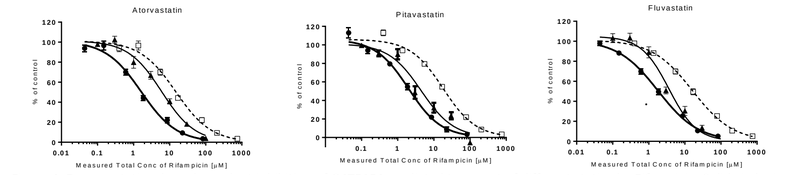

Figure 4. Representative dose-dependent inhibition of OATP1B1 mediated transport of different statins by Rifampicin. Solid circle: in HBSS, open square: predicted in serum, solid triangle: measured in serum

OATP1B1 Substrate	Meas'd f_u	Est. AB K_d (μM)	Meas'd OATP1B1 K_m (μM)	Affinity ratio (K_d/K_m)	Calc'd F_{TIPBS}
Atorvastatin	3.95%	27.1	1.9	14.3	2.84
Pitavastatin	1.13%	7.5	1.7	4.5	1.81
Simvastatin	3.17%	21.6	6.0	3.6	1.60
Lovastatin	5.92%	41.5	9.8	4.3	1.19 (ns)
Fluvastatin	1.59%	10.7	10.6	1.0	1.14 (ns)
E17βG	2.62%	17.8	3.0	5.9	2.2

Table 1. Summary of protein binding, active transport constants and TIPBS effect of various statins and reference OATP1B1 substrate E17βG (ns: no statistical difference)

OATP1B1 Substrate	Meas'd HBSS IC_{50} (μM)	Meas'd HS IC_{50} (μM)	Pred'd HS IC_{50} (μM)	Calc'd F_{TIPBS}
Atorvastatin	1.6	6.0	14.8	2.5
Pitavastatin	2.0	4.4	18.0	4.1
Simvastatin	3.3	7.6	30.0	3.9
Lovastatin	1.5	5.3	13.6	2.6
Fluvastatin	1.8	3.6	16.3	4.5
E17βG	0.6	1.7	5.5	3.2

Table 2. Summary of measured and predicted Rifampicin IC_{50}s of different substrates in HBSS and human serum (measured Rifampicin f_u 11%)

The presented *in vitro* data substantiate our previous experimental findings and TIPBS hypothesis. We demonstrated that both OATP1B1 mediated transport of highly protein bound substrates in human serum, and their inhibition by Rifampicin, can be under-estimated using the conventional f_u adjustment approach. The under-estimation can attribute to the reported compound-dependent scaling factors required in predicting *in vivo* hepatic clearance of these drugs and DDIs.

Moreover, except for Lovastatin, there is a positive correlation between the albumin/OATP1B1 binding affinity difference and the extent of TIPBS effect (Table 1), supporting our previous hypothesis, based on mathematical simulations using a dynamic binding model [2], that high protein binding itself may not be sufficient to indicate whether a compound is subject to binding shift due to its interactions with transporters.

Our current and previous experimental studies and theoretical modeling collectively suggest that high affinity binding to a transporter by its substrate or inhibitor may displace the compound's binding to serum binding proteins, hence, the conventional approach of using equilibrium unbound fraction f_u may not be valid in predicting *in vivo* clearance and DDI for such compound. We therefore call for more research on this subject in order to better understand and predict drug transport in complex, protein-rich biological matrixes.

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