

# Is the Protein-Mediated Uptake of Drugs by Organic Anion Transporting Polypeptides a Real Phenomenon or an Artifact?<sup>§</sup>

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## ABSTRACT

Plasma proteins or human serum albumin (HSA) have been reported to increase the *in vitro* intrinsic uptake clearance ( $CL_{int,uptake}$ ) of drugs by hepatocytes or organic anion transporting polypeptide (OATP)-transfected cell lines. This so-called protein-mediated uptake effect (PMUE) is thought to be due to an interaction between the drug-protein complex and the cell membrane causing an increase in the unbound drug concentration at the cell surface, resulting in an increase in the apparent  $CL_{int,uptake}$  of the drug. To determine if the PMUE on OATP-mediated drug uptake is an artifact or a real phenomenon, we determined the effect of 1%, 2%, and 5% HSA on OATP1B1-mediated [human embryonic kidney (HEK)293 transfected cells] and passive  $CL_{int,uptake}$  (mock HEK293 cells) on a cocktail of five statins. In addition, we determined the non-specific binding (NSB) of the statin-HSA complex to the cells/labware. The increase in uptake of atorvastatin, fluvastatin, and rosuvastatin in the presence of HSA was completely explained by the extent of NSB of the statin-HSA complex, indicating that the PMUE for these statins is an artifact. In contrast, this was not the case for OATP1B1-mediated uptake of

pitavastatin and passive uptake of cerivastatin, suggesting that the PMUE is a real phenomenon for these drugs. Additionally, the PMUE on OATP1B1-mediated uptake of pitavastatin was confirmed by a decrease in its unbound  $IC_{50}$  in the presence of 5% HSA versus Hank's balanced salt solution buffer (HBSS). These data question the utility of routinely including plasma proteins or HSA in uptake experiments and the previous findings on PMUE on OATP-mediated drug uptake.

## SIGNIFICANCE STATEMENT

Here we report, for the first time, that the protein-mediated uptake effect (PMUE) on organic anion transporting polypeptide (OATP)-transported drugs could be an artifact of the non-specific binding (NSB) of the drug-albumin complex to cells/labware. Future experiments on PMUE must take into consideration such NSB. In addition, mechanisms other than PMUE need to be explored to explain the underprediction of *in vivo* OATP-mediated hepatic drug clearance from *in vitro* uptake studies.

## Introduction

Successful *in vitro* to *in vivo* extrapolation (IVIVE) of transporter-mediated hepatic drug clearance ( $CL_h$ ) is important in drug development. Underprediction of *in vivo*  $CL_h$  of highly protein-bound organic anion transporting polypeptide (OATP)-substrate drugs is widely reported when using IVIVE approaches (Soars et al., 2007; Jones et al., 2012; Bowman and Benet, 2016; Wood et al., 2017; Miyauchi et al., 2018). The mechanistic basis of this discrepancy is not clear. Numerous investigators, including us, have suggested that the absence of plasma proteins in the *in vitro* uptake studies is a contributor to this *in vitro* to *in vivo* discrepancy in  $CL_h$  (Miyauchi et al., 2018, 2022; Bowman

et al., 2019, 2020, 2021; Kim et al., 2019; Liang et al., 2020; Bi et al., 2021; Francis et al., 2021; Kumar et al., 2021). These investigations have demonstrated that inclusion of plasma or plasma proteins in the *in vitro* uptake studies increases the apparent intrinsic uptake clearance ( $CL_{int,uptake}$ ) of OATP-substrate drugs by hepatocytes and OATP-expressing cells. This phenomenon is called the protein-mediated uptake effect (PMUE) (i.e., plasma proteins present *in vivo* increase the  $CL_h$  of OATP substrate drugs beyond that quantified by *in vitro* uptake studies conducted in protein-free buffer). Indeed, studies have shown that IVIVE of  $CL_h$  of OATP substrate drugs is improved by including plasma/plasma proteins in the *in vitro* uptake studies (Mao et al., 2018; Miyauchi et al., 2018; Poulin and Haddad, 2018; Kim et al., 2019; Li et al., 2020b; Liang et al., 2020; Bi et al., 2021; Kumar et al., 2021).

Several potential mechanisms for the PMUE have been proposed (Bowman and Benet, 2018; Bteich et al., 2019; Francis et al., 2021; Miyauchi et al., 2022). Of these, the most accepted is the protein-lipid interaction (PLI) mechanism. This mechanism hypothesizes that an *in vivo* interaction between the drug-protein complex and the lipid membrane of the cell results in enhanced dissociation of the drug-protein complex and therefore increased local unbound drug

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**ABBREVIATIONS:** ATV, atorvastatin; BCA, bichinchoninic acid assay;  $CL_h$ , hepatic clearance;  $CL_{int,uptake}$ , intrinsic uptake clearance; CRV, cerivastatin; FLV, fluvastatin;  $f_u$ , fraction unbound;  $f_{u,p}$ , fraction unbound in plasma; HBSS, Hank's balanced salt solution; HEK, human embryonic kidney; HSA, human serum albumin;  $IC_{50,u}$ , unbound  $IC_{50}$ ; IVIVE, *in vitro* to *in vivo* extrapolation;  $K_m$ , substrate concentration at half of  $V_{max}$ ; LC-MS/MS, liquid chromatography-tandem mass spectrometry; NSB, nonspecific binding; OATP, organic anion transporting polypeptide; PLI, protein-lipid interaction; PMUE, protein-mediated uptake effect; PTV, pitavastatin; RIF, rifampicin; RSV, rosuvastatin.

concentration at the cell surface. Consequently, in the presence of plasma proteins, the *in vivo* apparent  $CL_{int,uptake}$  of the drug is greater than that estimated from *in vitro* uptake studies conducted in the absence of proteins.

If the PLI hypothesis is correct, the following should be observed in the *in vitro* OATP uptake studies in the presence versus absence of plasma proteins: 1) the OATP-mediated and passive  $CL_{int,uptake}$  of the drug should increase to the same extent; 2) the slope (i.e., the  $CL_{int,uptake}$ ), but not the intercept, of the unbound concentration-normalized uptake versus time curve should increase. However, all publications supporting the PLI mechanism report an increase in both slope and intercept of the uptake curve in the presence of plasma or plasma proteins [e.g., human serum albumin (HSA)] (Nunes et al., 1988; Miyachi et al., 2018; Li et al., 2020b; Liang et al., 2020; Bi et al., 2021). An increase in the intercept is usually interpreted as non-specific binding (NSB) of the drug to the cells/labware. Therefore, we hypothesized that the PLI mechanism, and therefore the PMUE on OATP-mediated drug uptake, is an artifact caused by NSB and not a real phenomenon.

The PMUE may be an artifact because of the way in which *in vitro* uptake clearance of a drug is routinely determined. Ideally, drug uptake should measure the intracellular concentration of the drug when the uptake experiment is terminated by washing the cells with cold drug-free buffer. However, in practice, it is analytically impossible to distinguish between intracellular drug and that bound to the cell surface/labware. If the drug uptake study is conducted in the presence of plasma proteins, the amount of drug bound to the proteins in the media will be large relative to the unbound drug concentration in the media and that taken up by the cells. Consequently, any remaining drug-protein complex bound to the cells/labware (i.e., residual drug-protein complex) will be erroneously interpreted as drug taken into the cells. Such erroneous interpretation will result in an increase in intercept and, if the NSB is time dependent, an apparent increase in slope (i.e.,  $CL_{int,uptake}$ ) of drug uptake profile.

To test our hypothesis that the PMUE is due to NSB, we determined the total, active (OATP1B1-mediated), and passive  $CL_{int,uptake}$  of a cocktail of five OATP1B1-transported statins, namely atorvastatin (ATV), cerivastatin (CRV), fluvastatin (FLV), pitavastatin (PTV), and rosuvastatin (RSV) in the absence (protein-free buffer) and presence of 1%, 2%, or 5% HSA. These statins were chosen as they have varying degrees of binding to albumin and their uptake by OATP1B1 has been shown to demonstrate PMUE (Bowman et al., 2019, 2020; Kim et al., 2019; Liang et al., 2020; Bi et al., 2021). Albumin, and not plasma, was chosen to simplify the number of plasma proteins included in the incubations. Total  $CL_{int,uptake}$  of the statins was determined by using OATP1B1-transfected human embryonic kidney (HEK)293 cells, whereas passive  $CL_{int,uptake}$  was determined using mock or OATP1B1-transfected HEK293 cells incubated with high unbound concentration of rifampicin (500  $\mu$ M) (henceforth called OATP1B1\_RIF cells). The NSB of HSA was determined by quantifying the residual albumin by quantitative targeted proteomics. Finally, to confirm or refute that the PMUE is an artifact, we determined a parameter that should be independent of NSB of the statin-HSA complex, namely the unbound inhibitory capacity of a statin [unbound inhibitor concentration that results in 50% inhibition ( $IC_{50,u}$ )] toward OATP1B1-mediated transport of another statin.

## Materials and Methods

### Chemicals and Reagents

The dithiothreitol (DTT), iodoacetamide (IAA), mass spectrometry grade trypsin, total protein quantification bicinchoninic acid assay (BCA) kit, and Hank's balanced salt solution (HBSS) with calcium and magnesium were obtained from Thermo Scientific (Rockford, IL). Dulbecco's modified Eagle's medium

(DMEM), fetal bovine serum (FBS), nonessential amino acids, geneticin, blasticidin S HCl, penicillin, and streptomycin solution were obtained from Thermo Fisher Scientific (Waltham, MA). Atorvastatin (ATV), cerivastatin (CRV), fluvastatin (FLV), pitavastatin (PTV), rosuvastatin (RSV), diclofenac sodium salt (DCL), rifampicin (RIF), fatty acid-free human serum albumin (HSA), and formic acid were purchased from Sigma-Aldrich (St. Louis, MO). Bovine serum albumin (BSA) and HEPES buffer were purchased from MP Biomedicals (Solon, OH). Synthetic signature peptides for HSA were obtained from New England Peptides (Boston, MA). High-performance liquid chromatography (HPLC)-grade acetonitrile and sodium dodecyl sulfate (SDS) were purchased from Fischer Scientific (Fair Lawn, NJ). All reagents were analytical grade. The Calbiochem ProteoExtract Native Membrane Extraction Kit and the Centrifree Ultrafiltration Device were purchased from EMD Millipore Corporation (Billerica, MA). Poly-D-lysine-coated 24-well plates were purchased from Corning (Kennebunk, ME). OATP1B1-expressing and mock human embryonic kidney (HEK)293 cells were generously provided by Dr. Yurong Lai of Gilead Sciences Inc. (Foster City, CA).

### Uptake of Statins by OATP1B1-Expressing or mock HEK293 Cells in the Absence or Presence of 1%, 2%, and 5% HSA

OATP1B1-expressing or mock HEK293 cells were seeded in 24-well poly-D-lysine-coated plates at a density of  $3 \times 10^5$  cells per well with 1 ml of high-glucose Dulbecco's modified Eagle's medium (DMEM) (containing 10% FBS, 100 U/ml penicillin and streptomycin, 25 mM HEPES, and 0.1 mM MEM non-essential amino acid solution) for 48 hours at 37°C, 90% relative humidity, and 5% CO<sub>2</sub>. OATP1B1-expressing HEK293 cells were supplemented with 600  $\mu$ g/ml geneticin and 10  $\mu$ g/ml blasticidin.

For the uptake assays, OATP1B1-expressing or mock HEK293 cells were rinsed twice with 1 ml of warm HBSS buffer (37°C, pH 7.4). Then, the cells were preincubated with HBSS buffer or HSA solution (1%, 2%, or 5% HSA w/v in HBSS buffer) for 10 minutes. After aspiration of these solutions from the wells, statin uptake was initiated by adding 0.5 ml of a cocktail containing unbound concentrations of approximately 0.2  $\mu$ M ATV, 0.1  $\mu$ M CRV, 0.2  $\mu$ M FLV, 0.2  $\mu$ M PTV, and 1  $\mu$ M RSV. The above uptake studies with OATP1B1-expressing cells were repeated in the presence of RIF (unbound concentration of 500  $\mu$ M). Statin uptake was terminated at designed timepoints (5, 30, 60, and 120 seconds, all within the linear range) by aspirating the drug solution and washing the cells three times with ice-cold HBSS buffer. Then, the cells were lysed using the quench solution (80% acetonitrile containing 1 nM diclofenac sodium salt as an internal standard). After centrifuging the lysate at 18,000 g for 20 minutes, 10  $\mu$ l of the supernatant was injected onto the liquid chromatography-tandem mass spectrometry (LC-MS/MS) systems to quantify the cell-lysate statin concentration. For every experiment, extra wells were included for total protein quantification by the BCA assay following cell lyses by 1 ml 2% SDS. Three to five independent experiments were conducted, each in triplicate. Of note, the final dimethyl sulfoxide (DMSO) concentration (used to make the statin stock solution) was maintained at 1% (v/v) in each uptake experiment. After the uptake study, the total unbound (and bound) statin concentration in the uptake media (HBSS or HSA) in every uptake study was estimated using ultrafiltration as described below. The unbound RIF concentration was estimated based on its published binding to albumin (Boman and Ringberger, 1974).

### Inhibitory Effect of ATV, PTV, or FLV on OATP1B1-Mediated Uptake of RSV in the Absence or Presence of HSA

The uptake of rosuvastatin (nominal unbound concentration of 1  $\mu$ M) by the OATP1B1-expressing cells was determined (over 1 minute) in the presence of the inhibitors ATV, PTV, or FLV. The range of nominal unbound concentration of ATV was 0–200  $\mu$ M (in HBSS or 2% HSA solution); of PTV was 0–100  $\mu$ M in HBSS and 0–25  $\mu$ M in 5% HSA solution; and of FLV was 0–100  $\mu$ M in HBSS buffer, 0–2  $\mu$ M in 2% HSA solution, and 0–1  $\mu$ M in 5% HSA solution (different nominal unbound concentrations and the percentage of HSA used was dictated by the solubility of the stock solutions). The total, unbound (and bound) substrate and inhibitor concentrations were estimated using ultrafiltration as described below.

### Quantification of the Statin Unbound Fraction in 1%, 2%, and 5% HSA Solution

The unbound fraction of the statins in the uptake solution was determined using the Centrifree Ultrafiltration Device according to the manufacturer's specifications. The fraction unbound of the statins was corrected for NSB of the

statins to the ultrafiltration device determined by filtering 500  $\mu\text{l}$  of the statin HBSS solution used in the above uptake experiments. Briefly, the samples were centrifuged at 37°C for 2 minutes at 1,200  $g$  to keep the filtrate volume <15% of the initial volume. After centrifugation, 10  $\mu\text{l}$  of the statin solution/filtrate was diluted up to 1 ml or 10 ml by the quench solution described above. Then, after centrifuge at 18,000  $g$  for 20 minutes, 10  $\mu\text{l}$  of the supernatant was injected onto the LC-MS/MS systems.

#### Quantification of Statins by LC-MS/MS

All of the above samples were analyzed on the AB SCIEX Triple Quad 6500 (Framingham, MA) coupled with the Waters ACQUITY UPLC System (Hertfordshire, UK). Ten microliters of the sample was injected onto an ultra-performance liquid chromatography (UPLC) column (ACQUITY UPLC BEH C18 Column, 1.7  $\mu\text{m}$ , 2.1 mm  $\times$  50 mm; Waters). The LC-MS/MS conditions are summarized in Supplemental Table 1.

#### Quantification of Residual Albumin in Cell Lysates Using Quantitative Target Proteomics

The residual albumin in the cell lysates (i.e., the NSB of albumin-drug complex) was measured in the absence and presence of HSA (1%, 2%, and 5%) using either the relative quantification approach (when the unlabeled HSA surrogate peptide was not immediately available) or the absolute quantification approach (when the unlabeled HSA surrogate peptide was available).

**Relative Quantification of Residual Albumin in the Cell Lysates in the Absence or Presence of 1%, 2%, and 5% HSA.** After termination of uptake in the above uptake studies (excluding the  $\text{IC}_{50,u}$  studies), the cells were lysed for 1 hour at 4°C (200  $\mu\text{l}$ /well) with equal mixture of 2% SDS and EBII buffer from the Calbiochem ProteoExtract Native Membrane Extraction Kit. Following reduction, alkylation, and digestion by trypsin as previously described (Storelli et al., 2021), 10  $\mu\text{l}$  of a mixture of the stable-labeled surrogate peptides (Supplemental Table 1), prepared in 80% acetonitrile plus 0.2% formic acid and 5  $\mu\text{l}$  of 80% acetonitrile plus 0.2% formic acid, was added to 40  $\mu\text{l}$  of trypsin digest (in 50 mM ammonium bicarbonate buffer). After centrifugation (5000  $g$ , 4°C, 5 minutes), 5  $\mu\text{l}$  of supernatant was injected onto the LC-MS/MS system (described above) and analyzed using the settings and procedure described in Supplemental Table 1. Any measured HSA under the HBSS condition (in OATP1B1-expressing HEK293 cells) was assumed to be endogenous and identical to that in mock HEK293 cells and unaffected by the addition of the statins.

**Absolute Quantification of Residual Albumin in the Cell Lysates in the Presence of 5% HSA.** To estimate the amount of statin-HSA complex nonspecifically bound to the cells, absolute quantification of HSA in the cell lysates is required at all timepoints of the uptake studies. For these experiments, the preincubation step was eliminated from the uptake studies primarily to replicate the NSB of the statin-HSA complex during the uptake phase of the experiments. In addition, the preincubated albumin will not carry any drug into the cells, and any remaining albumin from the preincubation mixture (after aspiration) will be negligible relative to that added when the uptake study is conducted in the presence of HSA. The absolute amount of albumin in the cell lysates was quantified as described above except that the calibrators (14.2–455 nM of the unlabeled albumin surrogate peptide) and quality control samples (28.7, 56.8, and 114 nM of the unlabeled albumin surrogate peptide) were included in the LC-MS/MS analyses. These were prepared by spiking 5  $\mu\text{l}$  of the unlabeled peptide standard and 10  $\mu\text{l}$  of the labeled peptide (both in 80% acetonitrile and 0.2% formic acid solution) to 40  $\mu\text{l}$  of 50 mM ammonium bicarbonate buffer.

#### Data and Statistical Analyses

**Determination of OATP1B1-Mediated and Passive Apparent  $\text{CL}_{\text{int,uptake}}$  of Statins.** The initial uptake rate was estimated from the slope of the drug uptake versus time profile (passive: in mock cells or OATP1B1\_RIF cells; total: in OATP1B1-expressing cells) using simple linear regression in GraphPad Prism version 9 (GraphPad Software, La Jolla, CA). The apparent  $\text{CL}_{\text{int,uptake}}$  was calculated as the ratio of the

initial uptake rate and the measured unbound concentration of the drug in HBSS or HSA-containing buffer. To allow comparison across the statins, the uptake data presented in the figures were normalized to a nominal 1  $\mu\text{M}$  unbound concentration of each statin. The OATP1B1-mediated apparent  $\text{CL}_{\text{int,uptake}}$  was calculated by subtracting the apparent passive  $\text{CL}_{\text{int,uptake}}$  (in mock or OATP1B1\_RIF cells) from the apparent total  $\text{CL}_{\text{int,uptake}}$  (in OATP1B1-expressing cells).

**Estimation of the  $\text{IC}_{50,u}$  or the Degree of Inhibition of OATP1B1-Mediated RSV Uptake by ATV, FLV, or PTV.** First, the total percentage of RSV uptake in the presence of the inhibitor (expressed relative to the uptake in the absence of the inhibitor) was corrected for the percentage of passive uptake of RSV to derive the percentage of OATP1B1-mediated RSV uptake. The percentage of passive uptake of RSV was assumed to equal the percentage of RSV uptake at maximum inhibitor concentration and was comparable to the passive uptake obtained in mock or OATP1B1\_RIF cells. Then, the  $\text{IC}_{50,u}$  value of ATV or PTV was estimated by fitting an inhibition model to the percentage of OATP1B1-mediated RSV uptake as a function of the unbound inhibitor concentrations using GraphPad Prism version 9:

$$\% \text{ OATP1B1 - mediated RSV uptake} =$$

$$100\% / [1 + (\text{IC}_{50,u} / \text{unbound inhibitor concentration})^{\text{Hill Slope}}] \quad (1)$$

**Estimation of the Residual Statin-HSA Complex Amount in the Cell Lysates in the Presence of 5% HSA.** The bound statin (i.e., statin-HSA complex) amount per well was the difference between the total statin amount and the unbound statin amount per well calculated as the total or unbound statin concentration per well times 500  $\mu\text{l}$  uptake media per well.

Assuming that the ratio of the residual HSA and the total HSA (in 500  $\mu\text{l}$ ) equals the ratio of the residual bound statin and the total bound statin (in 500  $\mu\text{l}$ ), the amount of residual statin-HSA complex at different uptake times was estimated as follows:

$$\begin{aligned} \text{Residual bound statin (pmol/well)} &= \text{Total bound statin (pmol/well)} \\ &\times \frac{\text{Residual HSA (pmol/well)}}{\text{Total HSA (pmol/well)}} \end{aligned} \quad (2)$$

where the total HSA per well [5% (w/v) HSA in 500  $\mu\text{l}$ ] was 0.36  $\mu\text{mol}$ , based on HSA molecular weight of 69,367  $\text{g/mol}$  (<https://www.uniprot.org/uniprot/P02768>), and the residual HSA was quantified by proteomics as described above.

#### Calculation of Increased Apparent Uptake in the Presence of 5% HSA versus HBSS

To take into consideration small variation in the measured unbound statin concentration and the total protein content in HSA versus HBSS uptake studies, statin uptake at each timepoint in these studies was corrected for these variables before estimating the increased apparent uptake in the presence of 5% HSA versus HBSS:

Normalized statin uptake in HBSS = Apparent statin uptake in HBSS

$$\begin{aligned} (\text{pmol/well}) \times \frac{\text{Unbound statin concentration in 5\% HSA } (\mu\text{M})}{\text{Unbound statin concentration in HBSS } (\mu\text{M})} \\ \times \frac{\text{Total protein amount in 5\% HSA (mg/well)}}{\text{Total protein amount in HBSS (mg/well)}} \end{aligned} \quad (3)$$

where the unbound statin concentration was measured using ultrafiltration and the total protein amount was determined by the BCA assay. Then, the increase in statin uptake in the presence of HSA versus HBSS at each timepoint was estimated as follows:

Increased apparent uptake in the presence of 5% HSA vs. HBSS

$$= \text{Apparent statin uptake in 5\% HSA} \\ - \text{normalized statin uptake in HBSS} \quad (4)$$

**Statistical Analysis.** Estimates of the NSB of statin-HSA complex to the cells and the measured increase in statin uptake in the presence of 5% HSA (Fig. 4), as well as the  $IC_{50,u}$  of the statins in absence and presence of HSA (Fig. 5), were statistically compared by the unpaired (Fig. 4) or the paired (Fig. 5) Student's *t* test using GraphPad Prism version 9.

## Results

**Unbound Fraction in 1%, 2%, and 5% HSA.** The extent of protein binding of the five statins followed the order FLV > PTV > CRV > ATV > RSV (Table 1; corrected for the NSB of statins to the ultrafiltration device 0.03 to 0.44). Three statins (CRV, FLV, and PTV) were highly protein-bound drugs (>0.97), with fraction unbound in 5% HSA ( $f_{u,5\%HSA}$ ) ranging from 0.003 to 0.023. No difference in  $f_{u,5\%HSA}$  values was noted for each drug when determined singly (data not shown) versus as a cocktail. The  $f_{u,5\%HSA}$  values (physiologically relevant, Table 1) of the statins were in agreement with their reported fraction unbound values in plasma ( $f_{u,p}$ ) except for the highly protein-bound statin FLV ( $f_{u,5\%HSA}$  0.003 vs.  $f_{u,p}$  0.009) (Riccardi et al., 2019).

**The Presence of HSA Increased the Apparent In Vitro Uptake of Statins into Both OATP1B1-Expressing and mock HEK293 Cells.** In the presence of HSA (with the unbound statin concentration kept approximately the same), slope of the uptake curves (Fig. 1) increased with the increase in HSA concentration, suggesting a PMUE on the statins. However, the ratio (HSA/HBSS) of the  $CL_{int,uptake}$  (a reflection of the slope), also interpreted as the PMUE, was much smaller for OATP1B1-expressing cells (1.0 to 4.4 for 5% HSA) versus mock or OATP1B1\_RIF cells, especially for ATV and RSV (<2) (Figs. 1 and 2; Supplemental Fig. 1). In contrast, the ratio of the slope (HSA/HBSS) of the uptake curve for the mock cells (passive uptake) was 3 to 16, with the largest ratios following the order FLV (Fig. 1C.i) > PTV (Fig. 1D.i) > RSV (Fig. 1E.i). The same trend was observed for OATP1B1\_RIF cells (Supplemental Fig. 1). Indeed, this apparent PMUE on statin uptake was confirmed when the apparent total OATP1B1-mediated and passive in vitro  $CL_{int,uptake}$  of the statins was estimated (Supplemental Table 1). The ratio (HSA/HBSS) of the apparent passive  $CL_{int,uptake}$  (i.e., in mock cells) was greater than that of the apparent OATP1B1-mediated  $CL_{int,uptake}$  (Fig. 2).

Interestingly, for OATP1B1-expressing, mock, and OATP1B1\_RIF cells, not only the slopes but also the y-intercepts of uptake curves were considerably increased in the presence of HSA (Fig. 1; Supplemental Fig. 1). The y-intercept under the 2% and 5% HSA conditions was approximately 2- and 5-fold of that in 1% HSA condition, respectively (Fig. 3, A–E).

**The Amount of HSA Nonspecifically Bound to the Cells/Labware Increased in the Presence of HSA.** Using quantitative targeted proteomics, we confirmed the presence of residual HSA in the cell lysate after the cells had been washed three times with the ice-cold wash buffer (Fig. 3F). As was the case for the y-intercept (Fig. 3, A–E), the amount of HSA nonspecifically bound to the cells/labware at 1 minute was directly proportional (1:1) to the HSA concentration used to conduct the uptake experiment (Fig. 3F).

**The Amount of Statin-HSA Complex Nonspecifically Bound to the Cells (Except for Pitavastatin/OATP1B1 Cells or Cerivastatin/mock Cells) Completely Explained the Increase in the Apparent Uptake in the Presence of 5% HSA.** The NSB of statin-HSA complex completely explained the increase in apparent uptake of ATV, FLV, and RSV by OATP1B1-expressing, mock, and OATP1B1\_RIF HEK293 cells in the presence of HSA (Fig. 4, A, C, and E; Supplemental Fig. 2, A, C, and E). Surprisingly, the amount of PTV-HSA and CRV-HSA complex nonspecifically bound to the OATP1B1-expressing cells/labware explained only 35% and 27% of the increase in total uptake in the presence of 5% HSA (vs. HBSS buffer) (Fig. 4, B.i,ii and D.i), implying that the PMUE on total (passive + active) uptake of PTV and CRV is a real phenomenon. Moreover, in the presence of 5% HSA, the increase in passive uptake of PTV, but not that of CRV, was completely explained by NSB (Fig. 4, B.ii and D.ii; Supplemental Fig. 2, D and B), implying the PMUE on only the OATP1B1-mediated PTV uptake and on passive uptake of CRV.

**PMUE on OATP1B1-Mediated Pitavastatin Uptake Was Confirmed by Its Lower Unbound OATP1B1  $IC_{50}$  in the Presence versus Absence of 5% HSA.** The unbound  $IC_{50}$  ( $IC_{50,u}$ ) of PTV was decreased by more than 80% in the presence ( $P = 0.03$ ) of 5% HSA versus HBSS buffer (Fig. 5A). Conversely, no difference in the  $IC_{50,u}$  of ATV (negative control) was observed between HBSS and 2% HSA conditions ( $P = 0.3$ ) (Fig. 5B; due to solubility issues, these studies could not be conducted with 5% HSA). Although the  $IC_{50,u}$  of FLV could not be determined in the presence of HSA due to the same solubility issue, we did not observe greater inhibitory effect of FLV on RSV uptake in the presence versus absence of HSA (Fig. 5C).

## Discussion

To our knowledge, this is the first study to show that, except for PTV, the previously reported in vitro PMUE on OATP1B1-mediated uptake of statins is likely an artifact caused by NSB of the statin-HSA complex to cells/labware. Such an artifact is not surprising. We were able to detect this artifact because we used a rigorous experimental strategy that included appropriate controls and measures not employed by others on the same subject. First, we estimated the PMUE on not only the total uptake of the statins but also on OATP1B1-mediated and passive uptake of the statins. Second, we expressed ALL of our data with respect to the media unbound statin concentration. Except for the  $IC_{50,u}$  studies, these concentrations were maintained below the reported

TABLE 1

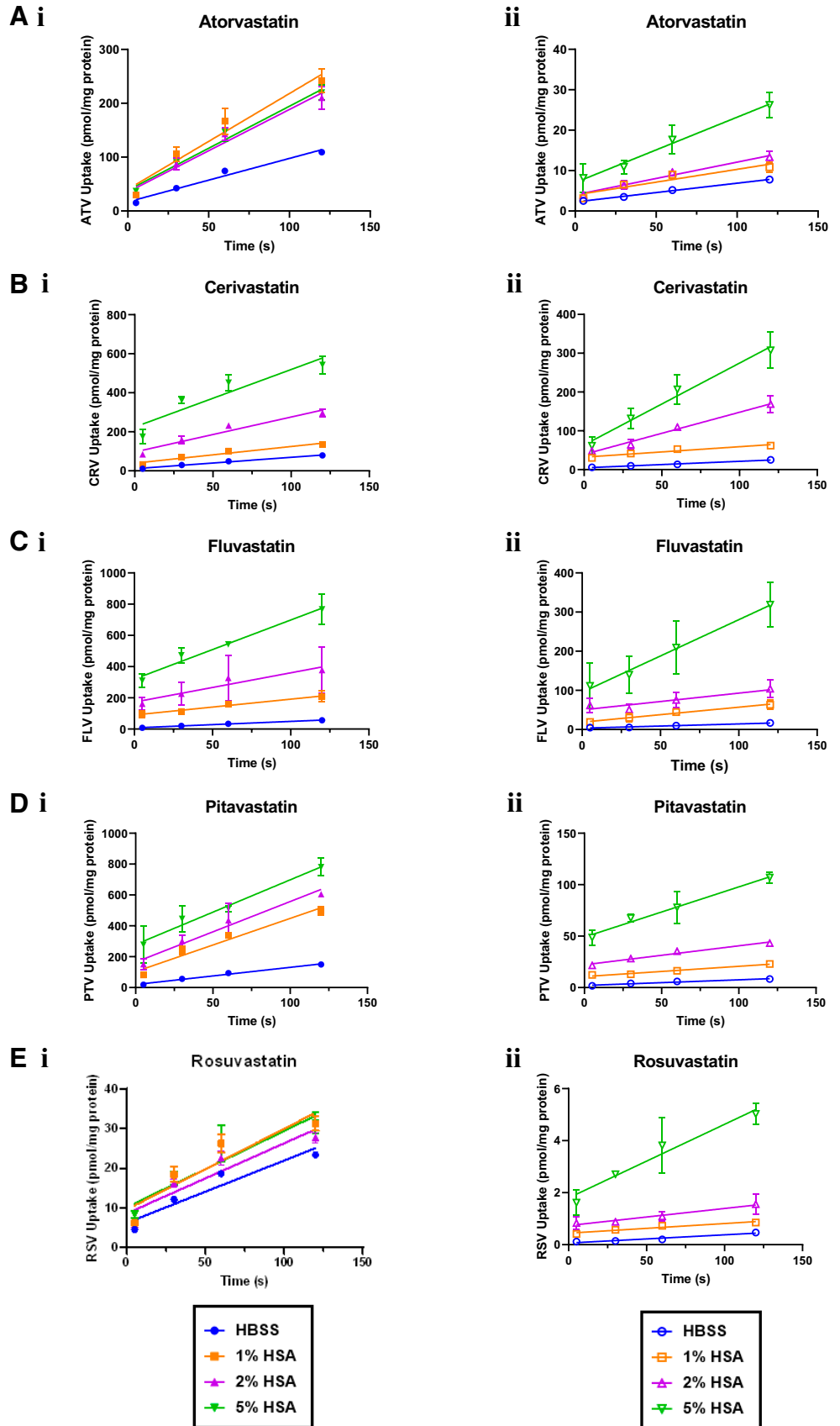
Fraction unbound ( $f_u$ ) of the statins in buffer containing 1%–5% HSA compared with that reported in human plasma. Data are mean  $\pm$  S.D. of 10–12 uptake experiments and corrected for NSB of the statin to the ultrafiltration device.

Compound	$F_{u,1\% \text{ HSA}}$	$F_{u,2\% \text{ HSA}}$	$F_{u,5\% \text{ HSA}}$	Reported $F_{u,p}^a$
Atorvastatin	0.202 $\pm$ 0.041	0.102 $\pm$ 0.038	0.056 $\pm$ 0.014	0.048
Cerivastatin	0.058 $\pm$ 0.008	0.035 $\pm$ 0.007	0.023 $\pm$ 0.004	0.018
Fluvastatin	0.012 $\pm$ 0.002	0.006 $\pm$ 0.001	0.003 $\pm$ 0.0007	0.00922
Pitavastatin	0.032 $\pm$ 0.006	0.018 $\pm$ 0.002	0.008 $\pm$ 0.001	0.0080
Rosuvastatin	0.41 $\pm$ 0.04	0.27 $\pm$ 0.03	0.13 $\pm$ 0.01	0.16

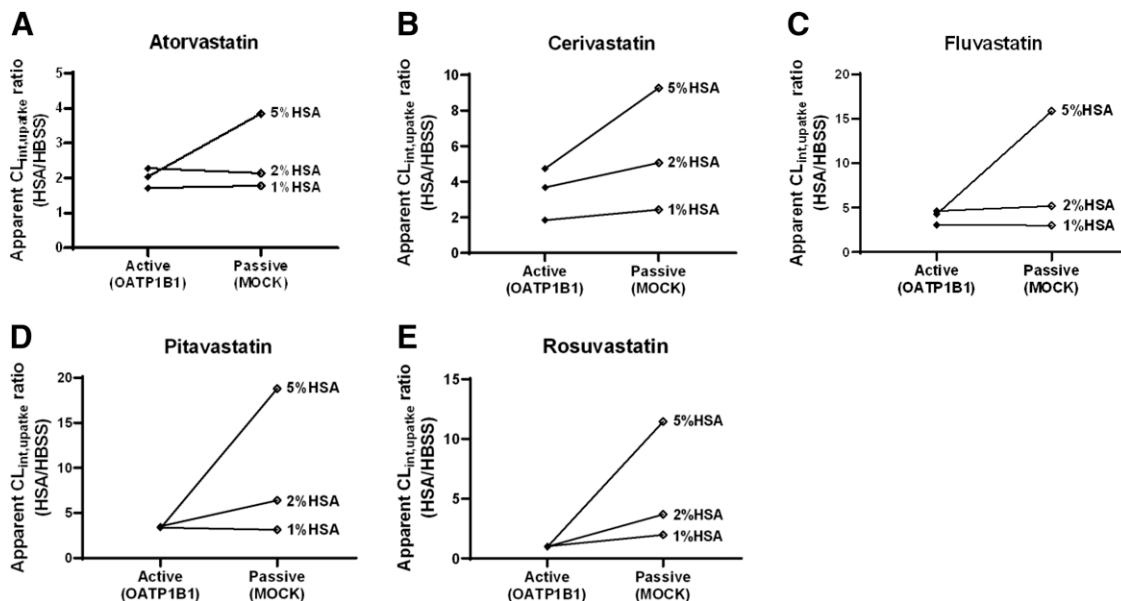
<sup>a</sup>Fraction unbound in human plasma ( $f_{u,p}$ ), determined using equilibrium dialysis, was obtained from Riccardi et al. (2019).

OATP1B1-expressing HEK293 cells

MOCK HEK293 cells



**Fig. 1.** The statin uptake-time profiles for OATP1B1-expressing (left panel) and mock HEK293 cells (right panel). The increase in the slope of the uptake curves in the presence of HSA vs. HBSS suggests a PMUE on the  $CL_{int,uptake}$  of the statins. The increased intercept in the presence of HSA indicates NSB of statin-HSA complex to the cells/labware. Data shown are mean  $\pm$  S.D. of statin uptake (normalized to  $1 \mu M$  unbound concentration) and represent three to five independent experiments, each conducted in triplicate at each timepoint.



**Fig. 2.** The ratio of the apparent active and passive  $CL_{int,uptake}$  of the statins in the presence of HSA vs. HBSS. These data indicate that the PMUE on  $CL_{int,uptake}$  of the statins was greater on passive vs. OATP1B1-mediated uptake. Data shown are mean of three to five independent experiments.

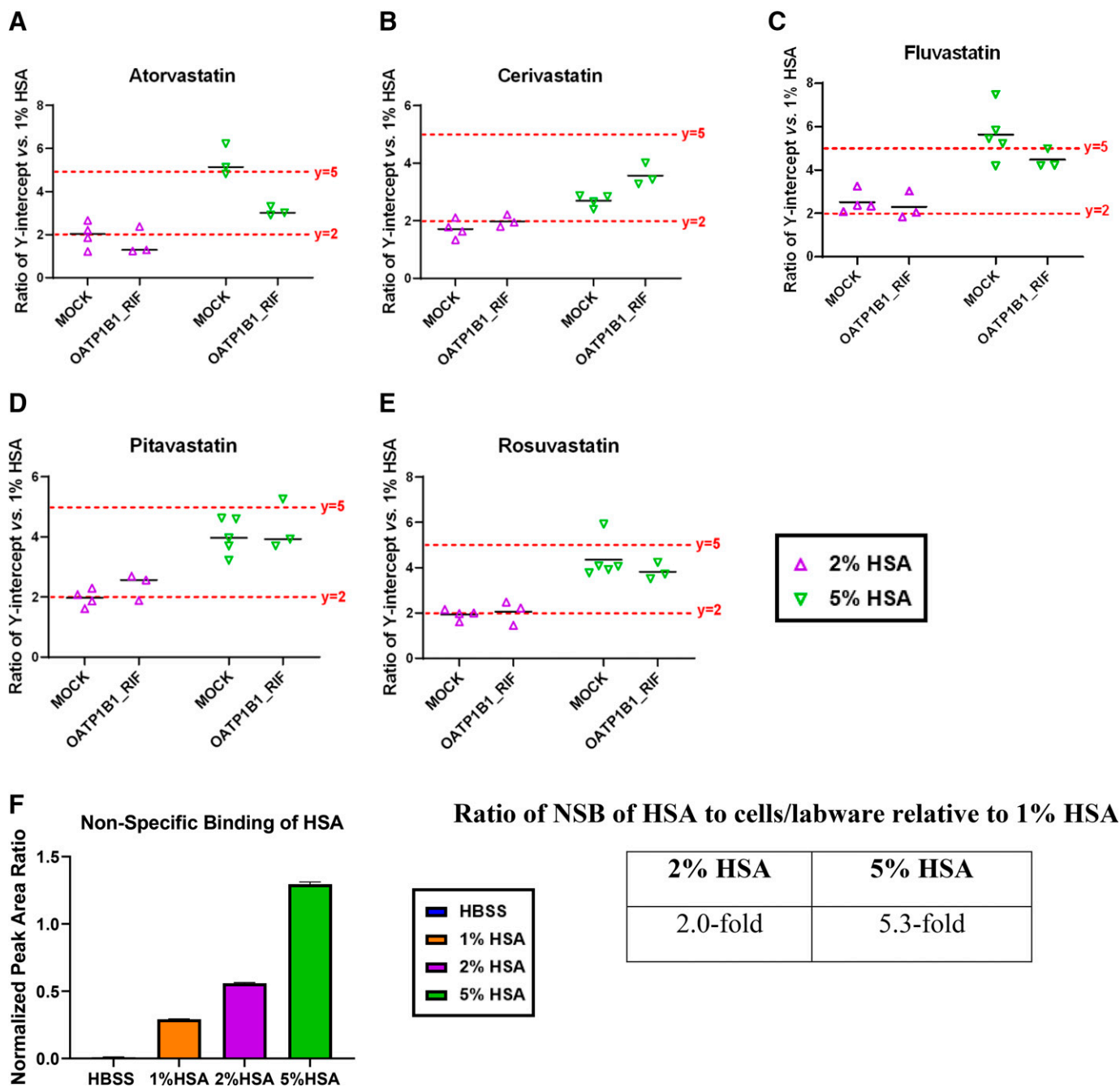
concentration at half of  $V_{max}$  ( $K_m$ ) for their OATP1B1-mediated transport (Lau et al., 2006; Van De Steeg et al., 2013; Izumi et al., 2015; Mitra et al., 2018). Third, by targeted quantitative proteomics we quantified the NSB of HSA (after the cells were washed three times) at each uptake timepoint for each HSA concentration used. This allowed us to estimate the confounding contribution of NSB of the statin-HSA complex (at 5% HSA) to the “apparent” PMUE on the OATP1B1-mediated and passive uptake of statins. Finally, to confirm or refute that the PMUE observed for the statins was an artifact, we determined the inhibitory capacity ( $IC_{50,u}$ ) of selective statins (ATV, FLV, and PTV) on OATP1B1-mediated RSV uptake in the absence and presence of HSA. If the PMUE is an artifact caused by NSB, the  $IC_{50,u}$  of these statins should be invariant in the absence or presence of HSA. This is because the  $IC_{50,u}$  of a drug (if the substrate concentration is  $<K_m$ ) is determined ONLY by the local unbound drug concentration or the interaction of the drug and the protein (in this case OATP1B1) and not dependent on passive diffusion or NSB of the drug. If the PMUE is a real phenomenon, the  $IC_{50,u}$  of the drug in the presence of plasma proteins should be significantly lower than that determined in the absence of plasma proteins.

Our finding of an increase in the slope (apparent  $CL_{int,uptake}$ ) of the time course of statin uptake by the OATP1B1-expressing cells in the presence of HSA versus HBSS (Fig. 1) is consistent with PMUE on OATP1B1-mediated apparent uptake of the statins reported by others (Kim et al., 2019; Li et al., 2020b; Liang et al., 2020; Bi et al., 2021). To gain insight into the above observations, we deconvoluted the apparent PMUE on the OATP1B1-mediated uptake versus that on passive uptake observed in the mock and OATP1B1\_RIF cells (Fig. 2). Surprisingly, we found that the PMUE was much greater on passive (in mock or OATP1B1\_RIF cells) compared with OATP1B1-mediated uptake of the statins (Fig. 2). In addition, the PMUE on passive uptake increased as the HSA concentration increased, whereas this change was much more modest (as expected) for the active uptake of the statin (Fig. 2). These data are NOT consistent with the PMUE caused by the PLI mechanism since this mechanism would result in the magnitude of the PMUE that would be identical for both the apparent OATP1B1-mediated and passive uptake of the statins. In contrast, our observations are consistent with the NSB

hypothesis provided that the NSB is time dependent (i.e., increases during the duration of the uptake experiments).

To confirm the NSB hypothesis, we quantified the amount of HSA remaining in the uptake experiments (i.e., NSB of HSA) at 1 minute. Not surprisingly, the amount of HSA remaining in the cell lysate was proportional to the HSA concentration used in the incubation media (Fig. 3F). Moreover, over the duration of the uptake experiments, the amount of residual HSA increased with time (Fig. 4). Collectively, both of these observations indicate that the so-called PMUE is likely an artifact of how uptake experiments are conducted. Indeed, as expected, NSB of the statin-HSA complex (5% HSA) to the cells/labware explained all of the increase in ATV, FLV, and RSV uptake by OATP1B1-expressing cells at each timepoint of the uptake study (Fig. 4). Surprisingly, this was not the case for PTV or CRV (Fig. 4, B.i and D.i). Since the uptake by the OATP1B1-expressing cells is a combination of active and passive uptake, we asked whether these observations were caused by a PMUE (or lack thereof) on the passive or active uptake of the statins or both. We found that the apparent PMUE on the uptake (both passive and active) of ATV, FLV, and RSV by OATP1B1-expressing cells was an artifact caused by NSB of the statin-HSA complex to the cells/labware (Fig. 4, A, C, and E). However, this was not the case for OATP1B1-mediated uptake of PTV (Fig. 4D.i) or the total uptake of CRV (Fig. 4B.i,ii). Interestingly, the ratio of CRV-HSA NSB to CRV uptake increase was almost identical in OATP1B1-expressing cells (27%, Fig. 4B.i) versus mock cells (28%, Fig. 4B.ii), suggesting that the total CRV uptake increase in the presence of 5% HSA is predominantly due to an increase in passive uptake of CRV.

To confirm that the PMUE on OATP1B1-mediated PTV uptake is a real phenomenon, we determined the unbound inhibitory capacity ( $IC_{50,u}$ ) of PTV toward OATP1B1 transport of RSV (Fig. 5A). As a negative control, we also determined the  $IC_{50,u}$  of ATV and FLV because their apparent PMUE could be completely explained by NSB (Fig. 5, B and C). The  $IC_{50,u}$  of PTV decreased in the presence of HSA versus HBSS but not for ATV (Fig. 5, A and B). Due to the higher fraction of ATV bound to HSA and limitations caused by poor solubility of ATV, the  $IC_{50,u}$  for ATV could be determined only at 2% HSA. For the same reasons, FLV  $IC_{50,u}$  could not be determined. Nevertheless,



**Fig. 3.** The ratio of Y-intercept of the uptake curve for 2% or 5% HSA conditions (vs. 1% HSA) for ATV (A), CRV (B), FLV (C), PTV (D), and RSV (E) uptake by mock or OATP1B1\_RIF HEK293 cells. In general, the intercept increased in proportion to the percentage of HSA used (A–E), indicating NSB of the statin-HSA complex to the cells/labware. This was subsequently confirmed by quantifying the relative amount of HSA remaining in the cell lysate (2% HSA or 5% HSA vs. 1% HSA) as quantified at 1 minute by the peak area ratio (PAR) of the HSA and sodium-potassium adenosine triphosphatase (Na<sup>+</sup>K<sup>+</sup>ATPase) peptide (membrane marker) using targeted proteomics (F). Data shown in (F) are mean ± S.D. and represent three independent experiments, each conducted in triplicate.

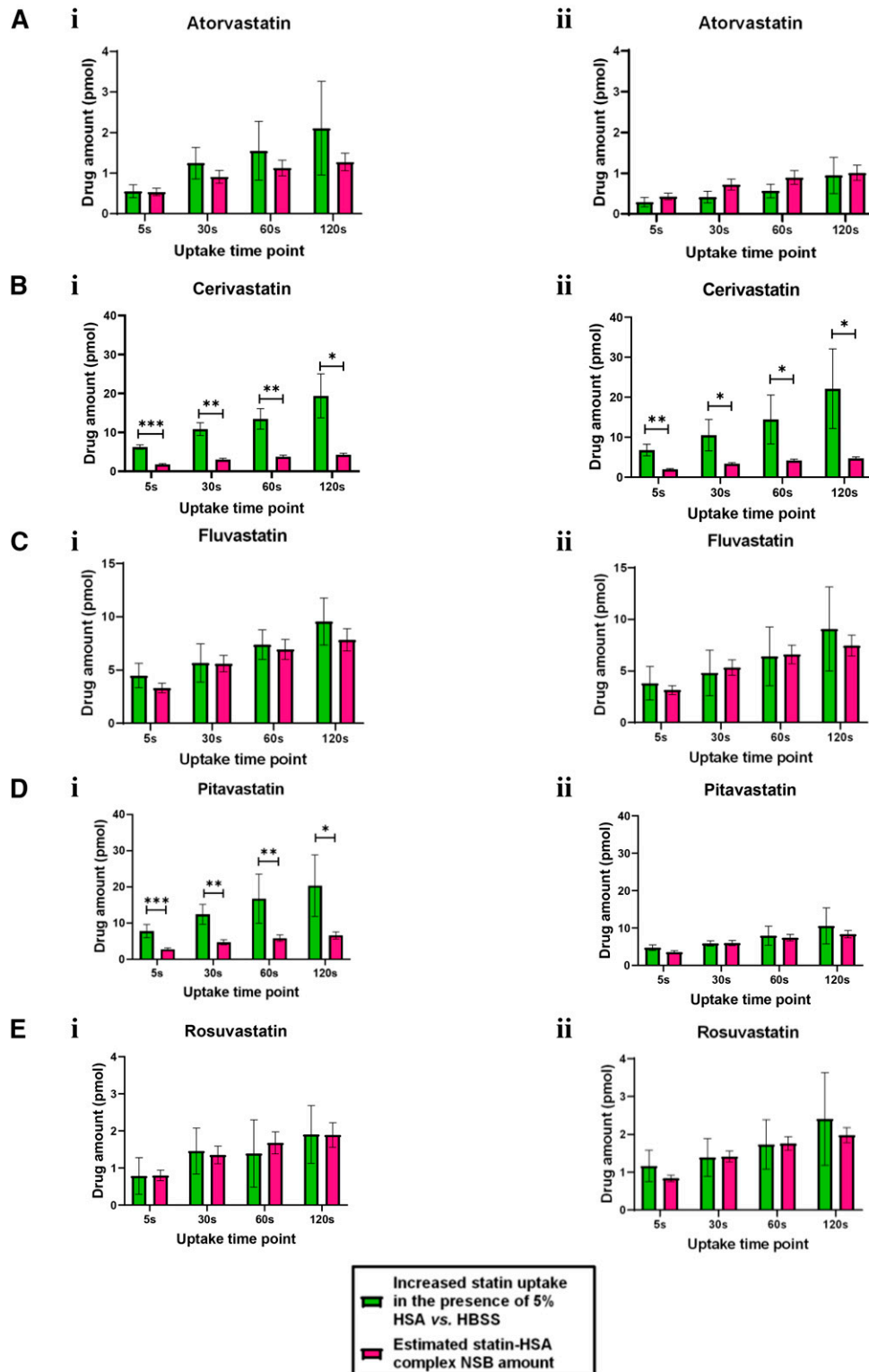
there was no clear difference between the ability of FLV to inhibit RSV uptake by OATP1B1-expressing cells in the absence or presence of HSA (Fig. 5C). Collectively, these results show that the presence of HSA results in a real PMUE on PTV, but not on ATV or FLV, uptake by OATP1B1. These results align well with the conclusions drawn from our proteomics data.

The above observations raise some intriguing questions. Why is the NSB of the statin-HSA complex time dependent and not instantaneous? Is this because there is time-dependent endocytosis of the statin-HSA complex by the HEK293 cells? HEK293 cells do express the albumin

receptor for endocytosis (Choi et al., 1999; Urae et al., 2020). Therefore, it is possible that the change in slope of the uptake curve of ATV, FLV, and RSV in the presence of HSA was due, at least in part, to endocytosis of statin-HSA complex. However, this mechanism should explain the PMUE for ALL of the statins. It does not. Also, it cannot account for the decrease in OATP1B1 IC<sub>50,u</sub> of PTV in the presence of HSA, and it is unable to explain why the proteomics data do not completely explain the apparent PMUE on passive uptake of CRV. Thus, other mechanisms must be invoked to explain our intriguing observations.

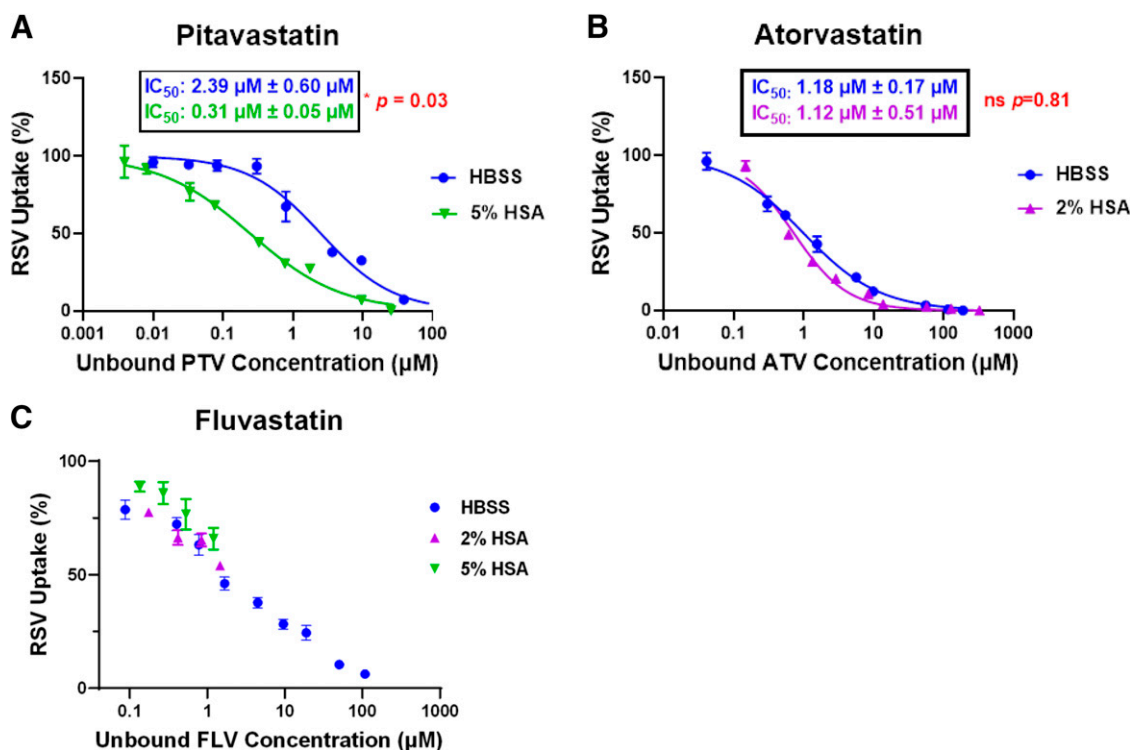
## OATP1B1-expressing HEK293 cells

## MOCK HEK293 cells



**Fig. 4.** Comparison of the amount of statin nonspecifically bound to the cells (as statin-I complex; pink bars) and the increase in statin taken up by OATP1B1-overexpressing (A.i–E.i) or mock (A.ii–E.ii) HEK293 cells in the presence of 5% HSA vs. HBSS (green bars). The increase in the uptake of ATV, FLV, and RSV in the presence of 5% HSA (vs. HBSS) can be completely explained by the NSB of the statin-HSA complex to the OATP1B1-expressing or mock HEK293 cells. However, this was not the case for PTV (OATP1B1-expressing cells) or CRV (both cells). The uptake data shown have been corrected for small differences in unbound concentration and total protein content between each experiment. Data shown are mean  $\pm$  S.D. of three independent experiments, each conducted in triplicate. Statistical comparison between the increase in statin uptake in the presence of 5% HSA and NSB was performed using Student's *t* test (\* $P$  < 0.05; \*\* $P$  < 0.01; \*\*\* $P$  < 0.001).





**Fig. 5.** Unbound OATP1B1 IC<sub>50</sub> (IC<sub>50,u</sub>) of PTV (A) or ATV (B) and inhibition of OATP1B1 by various concentrations of FLV (C), using RSV as a substrate. The IC<sub>50,u</sub> of PTV decreased in the presence of HSA, whereas that of ATV did not. In addition, OATP1B1 inhibition by FLV in the presence of HSA was not different from that in HBSS. These data suggest that the PMUE on OATP1B1-mediated PTV uptake appears to be a real phenomenon. Percentage of RSV uptake was the OATP1B1-mediated uptake (i.e., the total % RSV uptake corrected for the % passive uptake of RSV) (see *Materials and Methods*). The data shown are mean ± S.D. and represent three independent experiments, each conducted in triplicate. Solid line is the model fit to the data. IC<sub>50,u</sub> data shown are mean ± S.D. of three independent experiments, each conducted in triplicate. \*IC<sub>50,u</sub> in the presence of HSA vs. HBSS (A and B) was statistically compared using the paired Student's *t* test.

“Transporter-induced protein-binding shift (TIPBS)” has been proposed as another possible mechanism for the PMUE on OATP1B1/OATP1B3-mediated uptake of drugs (Baik and Huang, 2015; Bowman et al., 2019, 2020). According to this mechanism, the PMUE should be observed for only high extraction drugs. However, this mechanism cannot explain the PMUE on passive uptake of CRV and the minimal PMUE on active uptake of RSV, a high extraction ratio drug (Fig. 4) (Billington et al., 2019). In addition, PTV, for which PMUE is observed, is a low extraction drug (NDA-022363, 2009). Another possible mechanism is channeling (i.e., protein-protein interaction) (Budhu and Noy, 2002; Nelson et al., 2016). This mechanism should decrease the  $K_m$  of the statins, whereas  $V_{max}$  should remain unchanged. This cannot explain the CRV data but is consistent with the decrease in the IC<sub>50,u</sub> of PTV but inconsistent with the decrease in  $V_{max}$  of OATP1B1-mediated PTV uptake in the presence of plasma reported by Bowman et al. (2020). Finally, given that OATPs are known to be allosteric (Kindla et al., 2011), does HSA, PTV-HSA complex, or another constituent of HSA/plasma bind to the OATP1B1 transporter, causing a conformational change of OATP1B1 resulting in a reduction in the IC<sub>50,u</sub> of PTV? Preliminary studies in our laboratory, using human plasma filtrate, did not produce any PMUE on OATP1B1-mediated uptake of statins including PTV or CRV (data not shown), suggesting that this is not a viable hypothesis.

A key question is whether our findings apply to other statins (e.g., pravastatin) or other OATP1B1 substrate drugs when OATP1B1 cells or human hepatocytes are used to determine drug uptake. Published data using hepatocytes show an increase in the intercept of uptake curves, a PMUE on both active and passive uptake of drugs with the effect on passive uptake being greater than the active uptake of the drugs

(Miyachi et al., 2018; Bowman et al., 2019, 2020; Li et al., 2020b; Liang et al., 2020; Bi et al., 2021). Overall, these observations imply that the NSB of drug-protein complex may occur when using hepatocytes. There is no clear evidence that hepatocytes demonstrate endocytosis of albumin. Thus, whether the PMUE in hepatocytes is affected by endocytosis or NSB needs further investigation.

Our studies do have some limitations. First, to quantify the amount of statin-HSA complex to the cells/labware, we assumed that the binding of the statin to albumin at the cell surface was the same as that measured in vitro using ultrafiltration. If the latter underestimated unbound drug concentration at the cell surface, it may explain the PMUE observed on CRV (Fig. 4B; Supplemental Fig. 2B). If this explanation is correct, it is puzzling that this PMUE was not of equal magnitude on the active versus passive uptake of CRV (Supplemental Table 2). Moreover, this explanation cannot explain our PTV data, as the passive uptake of PTV was completely explained by NSB. Second, whether our observations will translate to plasma or other transporters is unknown. There could be protein constituents in plasma other than albumin that may result in a true PMUE on OATP1B1 drug uptake (but see above for our preliminary data).

Although many questions remain, here we aim to raise concerns about interpretation of the so-called PMUE on OATP1B1 drug uptake reported by others. Notably, in the last four years, more than 20 reports (too numerous to cite) have been published that the PMUE on OATP1B1 drug uptake is a real phenomenon. However, none of them have considered the possibility that this could be an artifact of NSB of the drug-protein complex to the cells/labware. If the PMUE for OATP1B1 drug substrates is an artifact, it is not necessary to include albumin or plasma in OATP1B1-mediated uptake experiments because

such inclusion unnecessarily complicates uptake studies and considerably raises their cost. In addition, including the PMUE in IVIVE does not appear to result in successful predictions of transporter-based  $CL_{th}$  (Kim et al., 2019; Li et al., 2020a,b; Bi et al., 2021). Therefore, future focus should be delineating the underlying mechanisms causing the underprediction of OATP-mediated  $CL_{th}$ .

### Acknowledgments

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### Authorship Contributions

*Participated in research design:* Yin, Storelli, Unadkat.

*Conducted experiments:* Yin.

*Performed data analysis:* Yin, Storelli.

*Wrote or contributed to the writing of the manuscript:* Yin, Storelli, Unadkat.

### References

- Baik J and Huang Y (2015) Transporter-induced protein binding shift (TIPBS): hypothesis and modeling, in ISSX Online Abstracts from 20th North American ISSX Meeting; 2015 Oct 18–22; Orlando, FL. Suppl 10 (No. 2):P272, International Society for the Study of Xenobiotics, Washington, DC.
- Bi YA, Ryu S, Tess DA, Rodrigues AD, and Varma MVS (2021) Effect of human plasma on hepatic uptake of organic anion-transporting polypeptide 1B substrates: studies using transfected cells and primary human hepatocytes. *Drug Metab Dispos* **49**:72–83.
- Billington S, Shoner S, Lee S, Clark-Snustad K, Pennington M, Lewis D, Muzi M, Rene S, Lee J, Nguyen TB, et al. (2019) Positron emission tomography imaging of [<sup>11</sup>C]rosuvastatin hepatic concentrations and hepatobiliary transport in humans in the absence and presence of cyclosporin A. *Clin Pharmacol Ther* **106**:1056–1066.
- Boman G, and Ringberger VA (1974) Binding of rifampin by human plasma proteins. *Eur J Clin Pharmacol* **7**:369–373.
- Bowman CM and Benet LZ (2016) Hepatic clearance predictions from in vitro-in vivo extrapolation and the biopharmaceutics drug disposition classification system. *Drug Metab Dispos* **44**:1731–1735.
- Bowman CM and Benet LZ (2018) An examination of protein binding and protein-facilitated uptake relating to in vitro-in vivo extrapolation. *Eur J Pharm Sci* **123**:502–514.
- Bowman CM, Chen B, Cheong J, Liu L, Chen Y, and Mao J (2021) Improving the translation of organic anion transporting polypeptide substrates using HEK293 cell data in the presence and absence of human plasma via physiologically based pharmacokinetic modeling. *Drug Metab Dispos* **49**:530–539.
- Bowman CM, Chen E, Chen L, Chen YC, Liang X, Wright M, Chen Y, and Mao J (2020) Changes in organic anion transporting polypeptide uptake in HEK293 overexpressing cells in the presence and absence of human plasma. *Drug Metab Dispos* **48**:18–24.
- Bowman CM, Okochi H, and Benet LZ (2019) The presence of a transporter-induced protein binding shift: a new explanation for protein-facilitated uptake and improvement for in vitro-in vivo extrapolation. *Drug Metab Dispos* **47**:358–363.
- Bteich M, Poulin P, and Haddad S (2019) The potential protein-mediated hepatic uptake: discussion on the molecular interactions between albumin and the hepatocyte cell surface and their implications for the in vitro-to-in vivo extrapolations of hepatic clearance of drugs. *Expert Opin Drug Metab Toxicol* **15**:633–658.
- Budhu AS and Noy N (2002) Direct channeling of retinoic acid between cellular retinoic acid-binding protein II and retinoic acid receptor sensitizes mammary carcinoma cells to retinoic acid-induced growth arrest. *Mol Cell Biol* **22**:2632–2641.
- Choi JS, Kim KR, Ahn DW, and Park YS (1999) Cadmium inhibits albumin endocytosis in opossum kidney epithelial cells. *Toxicol Appl Pharmacol* **161**:146–152.
- Francis LJ, Houston JB, and Hallifax D (2021) Impact of plasma protein binding in drug clearance prediction: a database analysis of published studies and implications for in vitro-in vivo extrapolation. *Drug Metab Dispos* **49**:188–201.
- Izumi S, Nozaki Y, Maeda K, Komori T, Takenaka O, Kusuhara H, and Sugiyama Y (2015) Investigation of the impact of substrate selection on in vitro organic anion transporting polypeptide 1B1 inhibition profiles for the prediction of drug-drug interactions. *Drug Metab Dispos* **43**:235–247.
- Jones HM, Barton HA, Lai Y, Bi YA, Kimoto E, Kempshall S, Tate SC, El-Kattan A, Houston JB, Galetin A, et al. (2012) Mechanistic pharmacokinetic modeling for the prediction of transporter-mediated disposition in humans from sandwich culture human hepatocyte data. *Drug Metab Dispos* **40**:1007–1017.
- Kim SJ, Lee KR, Miyachi S, and Sugiyama Y (2019) Extrapolation of in vivo hepatic clearance from in vitro uptake clearance by suspended human hepatocytes for anionic drugs with high binding to human albumin: improvement of in vitro-to-in vivo extrapolation by considering the “albumin-mediated” hepatic uptake mechanism on the basis of the “facilitated-dissociation model”. *Drug Metab Dispos* **47**:94–103.
- Kindla J, Müller F, Mieth M, Fromm MF, and König J (2011) Influence of non-steroidal anti-inflammatory drugs on organic anion transporting polypeptide (OATP) 1B1- and OATP1B3-mediated drug transport. *Drug Metab Dispos* **39**:1047–1053.
- Kumar V, Yin M, Ishida K, Salphati L, Hop CECA, Rowbottom C, Xiao G, Lai Y, Mathias A, Chu X, et al. (2021) Prediction of transporter-mediated rosuvastatin hepatic uptake clearance and drug interaction in humans using proteomics-informed REF approach. *Drug Metab Dispos* **49**:159–168.
- Lau YY, Okochi H, Huang Y, and Benet LZ (2006) Response to comments on “multiple transporters affect the disposition of atorvastatin and its two active hydroxy metabolites: application of in vitro and ex situ systems”. *J Pharmacol Exp Ther* **316**:1387.
- Li N, Badrinarayanan A, Ishida K, Li X, Roberts J, Wang S, Hayashi M, and Gupta A (2020a) Albumin-mediated uptake improves human clearance prediction for hepatic uptake transporter substrates aiding a mechanistic in vitro-in vivo extrapolation (IVIVE) strategy in discovery research. *AAPS J* **23**:1.
- Li N, Badrinarayanan A, Li X, Roberts J, Hayashi M, Virk M, and Gupta A (2020b) Comparison of in vitro to in vivo extrapolation approaches for predicting transporter-mediated hepatic uptake clearance using suspended rat hepatocytes. *Drug Metab Dispos* **48**:861–872.
- Liang X, Park Y, DeForest N, Hao J, Zhao X, Niu C, Wang K, Smith B, and Lai Y (2020) In vitro hepatic uptake in human and monkey hepatocytes in the presence and absence of serum protein and its in vitro to in vivo extrapolation. *Drug Metab Dispos* **48**:1283–1292.
- Mao J, Doshi U, Wright M, Hop CECA, Li AP, and Chen Y (2018) Prediction of the pharmacokinetics of pravastatin as an OATP substrate using plateable human hepatocytes with human plasma data and PBPK modeling. *CPT Pharmacometrics Syst Pharmacol* **7**:251–258.
- Mitra P, Weinheimer S, Michalewicz M, and Taub ME (2018) Prediction and quantification of hepatic transporter-mediated uptake of pitavastatin utilizing a combination of the relative activity factor approach and mechanistic modeling. *Drug Metab Dispos* **46**:953–963.
- Miyachi S, Kim SJ, Lee W, and Sugiyama Y (2022) Consideration of albumin-mediated hepatic uptake for highly protein-bound anionic drugs: bridging the gap of hepatic uptake clearance between in vitro and in vivo. *Pharmacol Ther* **229**:107938.
- Miyachi S, Masuda M, Kim S-J, Tanaka Y, Lee K-R, Iwakado S, Nemoto M, Sasaki S, Shimono K, Tanaka Y, et al. (2018) The phenomenon of albumin-mediated hepatic uptake of organic anion transport polypeptide substrates: prediction of the in vivo uptake clearance from the in vitro uptake by isolated hepatocytes using a facilitated-dissociation model. *Drug Metab Dispos* **46**:259–267.
- Nelson CH, Peng C-C, Lutz JD, Yeung CK, Zelter A, and Isoherranen N (2016) Direct protein-protein interactions and substrate channeling between cellular retinoic acid binding proteins and CYP26B1. *FEBS Lett* **590**:2527–2535.
- Nunes R, Kiang C-L, Sorrentino D, and Berk PD (1988) ‘Albumin-receptor’ uptake kinetics do not require an intact lobular architecture and are not specific for albumin. *J Hepatol* **7**:293–304.
- Poulin P and Haddad S (2018) Extrapolation of the hepatic clearance of drugs in the absence of albumin in vitro to that in the presence of albumin in vivo: comparative assessment of 2 extrapolation models based on the albumin-mediated hepatic uptake theory and limitations and mechanistic insights. *J Pharm Sci* **107**:1791–1797.
- Riccardi KA, Tess DA, Lin J, Patel R, Ryu S, Atkinson K, Di L, and Li R (2019) A novel unified approach to predict human hepatic clearance for both enzyme- and transporter-mediated mechanisms using suspended human hepatocytes. *Drug Metab Dispos* **47**:484–492.
- Soars MG, McGinnity DF, Grime K, and Riley RJ (2007) The pivotal role of hepatocytes in drug discovery. *Chem Biol Interact* **168**:2–15.
- Storelli F, Anoshchenko O, and Unadkat JD (2021) Successful prediction of human steady-state unbound brain-to-plasma concentration ratio of P-gp substrates using the proteomics-informed relative expression factor approach. *Clin Pharmacol Ther* **110**:432–442.
- Urae S, Harita Y, Udagawa T, Ode KL, Nagahama M, Kajiho Y, Kanda S, Saito A, Ueda HR, Nangaku M, et al. (2020) A cellular model of albumin endocytosis uncovers a link between membrane and nuclear proteins. *J Cell Sci* **133**:242859.
- van de Steeg E, Greupink R, Schreurs M, Nooijen IHG, Verhoeckx KC, Hanemaaijer R, Ripken D, Monshouwer M, Vlaming MLH, DeGroot J, et al. (2013) Drug-drug interactions between rosuvastatin and oral antidiabetic drugs occurring at the level of OATP1B1. *Drug Metab Dispos* **41**:592–601.
- Wood FL, Houston JB, and Hallifax D (2017) Clearance prediction methodology needs fundamental improvement: trends common to rat and human hepatocytes/microsomes and implications for experimental methodology. *Drug Metab Dispos* **45**:1178–1188.

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**SUPPLEMENTARY INFORMATION**

**Is the Protein-Mediated Uptake Of Drugs By OATPs A Real Phenomenon Or An Artifact?**

Mengyue Yin, Flavia Storelli and Jashvant D. Unadkat

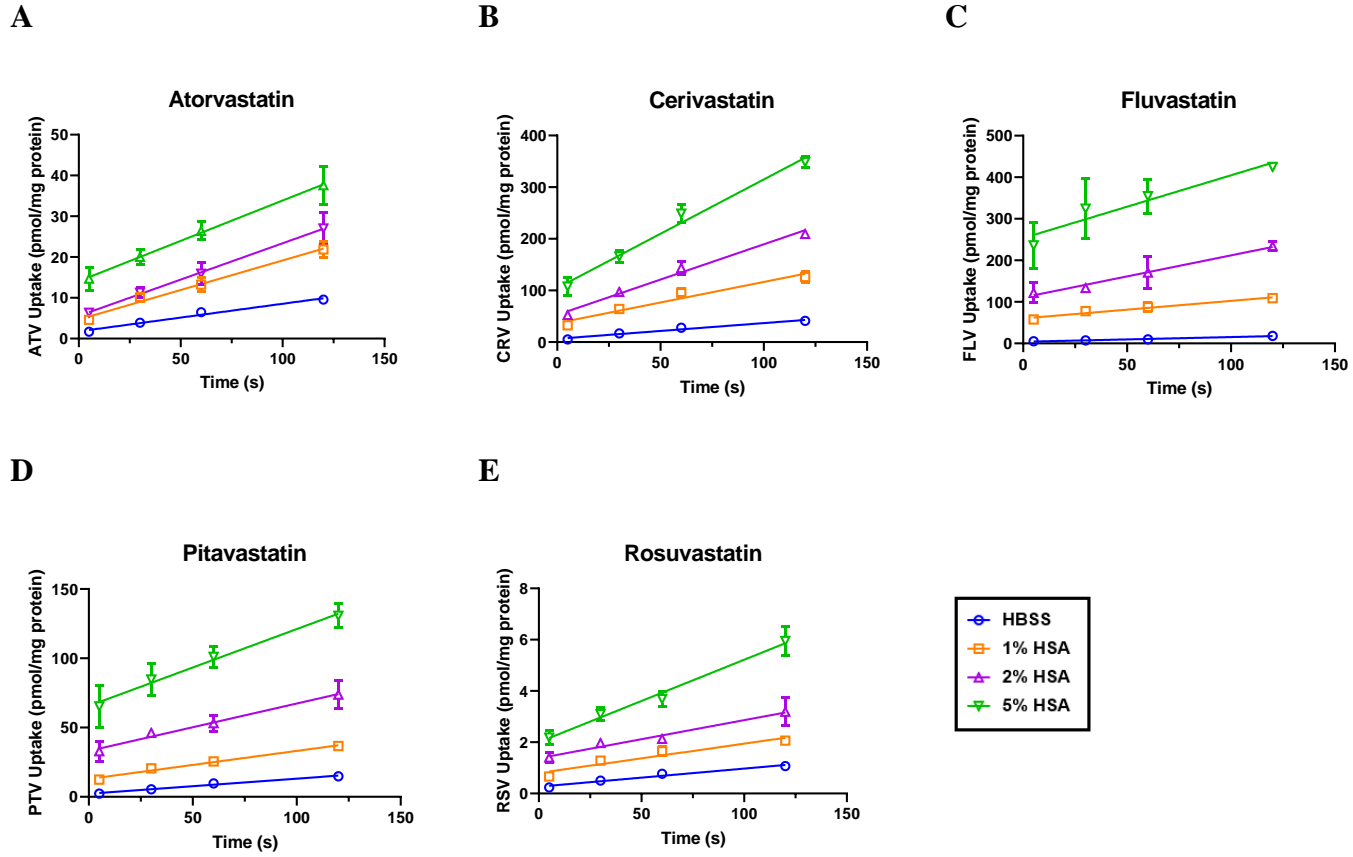
Department of Pharmaceutics, University of Washington, Seattle, Washington (MY, FS and JDU)

**Supplementary Figures**

**Supplementary Fig. 1. The statin uptake-time profiles for OATP1B1\_RIF cells.** The increase in the slope of the uptake curves in the presence of HSA *vs.* HBSS suggests a PMUE on the passive uptake of the statins. The increased intercept in the presence of HSA suggests NSB of statin-HSA complex to the cells/labware. Data shown are mean  $\pm$  standard deviation (SD) of statin uptake (normalized to 1  $\mu$ M unbound concentration) and are representative of three independent experiments, each conducted in triplicate at each time point.

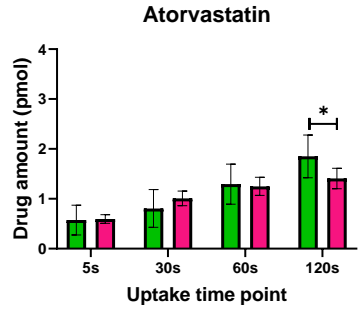
**Supplementary Fig. 2. Comparison of the amount of statin non-specifically bound to the cells (as statin-HSA complex; pink bars) and the increase in statin taken up by OATP1B1\_RIF cells in the presence of 5% HSA *vs.* HBSS (green bars).** The increase in the uptake of ATV, FLV, PTV and RSV in the presence of 5% HSA (*vs.* HBSS) can be completely explained by the NSB of the stain-HSA complex to the OATP1B1\_RIF cells. However, this was not the case for CRV. The uptake data shown have been corrected for small differences in unbound concentration and total protein content between each experiment. Data shown are mean  $\pm$  SD of three independent experiments, each conducted in triplicate. Statistical comparison between the increase in statin uptake in the presence of 5% HSA and NSB was performed using the Student's t test (\*  $p < 0.05$ ; \*\*  $p < 0.01$ ).

Supplementary Fig. 1.

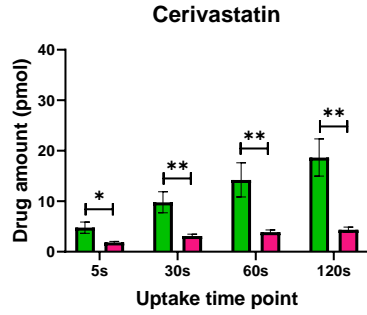


Supplementary Fig. 2.

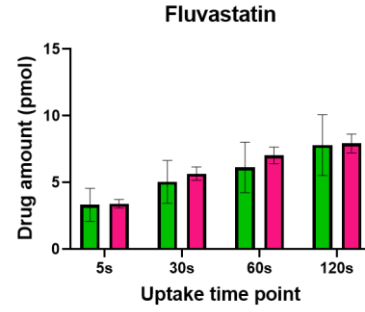
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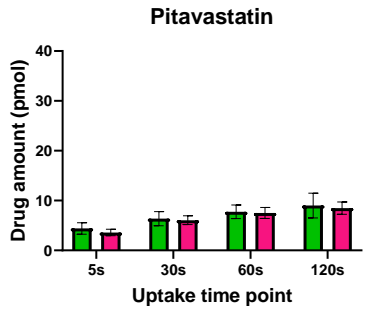
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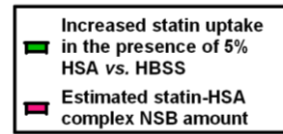
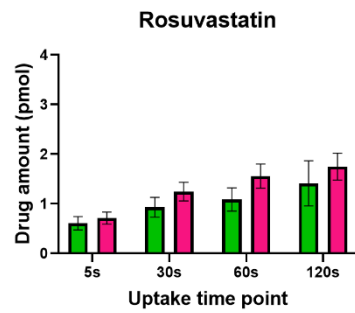
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D



E



**Supplementary Tables****Supplementary Table. 1 LC-MS/MS conditions****LC method for quantification of the 5 statins**

Time	Flow Rate	%A	%B
Initial	0.5	90	10
0.5	0.5	90	10
0.7	0.5	5	95
2.2	0.5	5	95
2.3	0.5	90	10
3	0.5	90	10

A: 0.1% formic acid in water; B: 0.1% formic acid in acetonitrile

**MS conditions for quantification of the 5 statins**

Compound ID	ESI Mode	Q1 Mass (Da)	Q3 Mass (Da)	DP (volts)	CE (volts)	CXP (volts)
Atorvastatin	Positive	559	250.1	60	60	10
Cerivastatin	Positive	460.3	356.3	100	40	10
Fluvastatin	Positive	412	224.3	60	41	15
Pitavastatin	Positive	422.2	290.2	90	35	17
Rosuvastatin	Positive	482.3	258.2	80	30	10
Diclofenac (IS)	Positive	295.82	213.9	40	45	9

ESI, electrospray ionization; DP, declustering potential, CE, collision energy; CXP, collision cell exit potential.

**LC method for quantification of HSA and Na<sup>+</sup>K<sup>+</sup>ATPase**

Time	Flow Rate	%A	%B
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Initial	0.3	97.0	3.0
3.00	0.3	97.0	3.0
10.00	0.3	87.0	13.0
20.00	0.3	75.0	25.0
22.00	0.3	66.7	33.3
22.90	0.3	50.0	50.0
23.00	0.3	20.0	80.0
24.10	0.3	20.0	80.0
24.20	0.3	97.0	3.0
28.00	0.3	97.0	3.0

A: 0.1% formic acid in water; B: 0.1% formic acid in acetonitrile

#### MS conditions for quantification of HSA and Na<sup>+</sup>K<sup>+</sup>ATPase

Compound ID	Peptide Sequence	ESI Mode	Q1 Mass (Da)	Q3 Mass (Da)	DP (volts)	CE (volts)	CXP (volts)
HSA F1	VFDEFKPLVEEPQNLIK (unlabeled)	Positive	682.65	712.65	170	45	12
HSA F2		Positive	682.65	970.75	170	45	12
HSA IS F1	VFDEFKPLVEEPQNLIK (labeled)	Positive	685.35	712.65	170	45	12
HSA IS F2		Positive	685.35	978.75	170	45	12
Na <sup>+</sup> K <sup>+</sup> ATPase F1	AAVPDAVGK (labeled)	Positive	414.235	685.388	61.3	18	12
Na <sup>+</sup> K <sup>+</sup> ATPase F2		Positive	414.235	586.320	61.3	18	12
Na <sup>+</sup> K <sup>+</sup> ATPase IS F1		Positive	418.242	693.402	61.3	18	12
Na <sup>+</sup> K <sup>+</sup> ATPase IS F2		Positive	418.242	594.334	61.3	18	12



ESI, electrospray ionization; DP, declustering potential, CE, collision energy; CXP, collision cell exit potential; F1/2, fragment 1/2. **Bolded letters** indicate the labeled amino acids.

**Supplementary Table. 2 In vitro apparent hepatic uptake intrinsic clearance of the 5 statins into OATP1B1-expressing or MOCK cells for the HBSS and HSA (1%, 2% and 5%) conditions**

Drug	HBSS				1% HSA				2% HSA				5% HSA							
	CL <sub>int,tota</sub> <sup>#</sup> ( $\mu$ /min/ mg protein )	CL <sub>int,passive</sub> <sup>%</sup> ( $\mu$ /min/mg protein)		CL <sub>int,OATP1B1</sub> <sup>*</sup> ( $\mu$ /min/mg protein)		CL <sub>int,tota</sub> <sup>#</sup> ( $\mu$ /min/ mg protein )	CL <sub>int,passive</sub> <sup>%</sup> ( $\mu$ /min/mg protein)		CL <sub>int,OATP1B1</sub> <sup>*</sup> ( $\mu$ /min/mg protein)		CL <sub>int,tota</sub> <sup>#</sup> ( $\mu$ /min/ mg protein )	CL <sub>int,passive</sub> <sup>%</sup> ( $\mu$ /min/mg protein)		CL <sub>int,OATP1B1</sub> <sup>*</sup> ( $\mu$ /min/mg protein)						
		Based on passive uptake in		Based on passive uptake in			Based on passive uptake in		Based on passive uptake in			Based on passive uptake in		Based on passive uptake in						
		MO CK	OATP1B1 _RIF	MO CK	OATP1B1 _RIF		MO CK	OATP1B1 _RIF	MO CK	OATP1B1 _RIF		MO CK	OATP1B1 _RIF	MO CK	OATP1B1 _RIF	MO CK	OATP1B1 _RIF			
Atorvast atin	43.2 (35.8)	2.1 (24.2 )	3.7 (24.2)	41.1	39.5	76.7 (9.5)	3.6 (17.2 )	6.6 (29.1)	73.1	70.1	101.5 (26.9)	4.3 (17.3 )	9.6 (20.5)	97.2	91.9	94.9 (30. 2)	7.8 (30.4 )	13.3 (39.5)	87.1	81.6
Cerivast atin	39.2 (18.3)	15.0 (22.1 )	17.5 (22.8)	24.2	21.7	84.5 (29.5)	40.1 (41.9 )	58.3 (23.4)	44.4	26.1	161.5 (26.4)	72.5 (10.5 )	100.1 (24.2)	88.9	61.4	199. 2 (34. 2)	121. 8 (9.4)	159.8 (28.9)	77.4	39.4
Fluvastat in	32.1 (20.6)	8.6 (27.7 )	7.5 (30.1)	23.5	24.6	95.1 (21.2)	23.3 (6.9)	23.2 (18.4)	71.7	71.9	151.8 (16.5)	43.2 (31.4 )	43.8 (33.6)	108. 6	108.0	224. 4 (12. 9)	123. 5 (7.5)	116.1 (47.2)	100.9	108.3
Pitavasta tin	79.2 (28.9)	3.7 (41.6 )	7.4 (23.0)	75.5	71.9	264.7 (19.9)	9.7 (33.2 )	11.4 (7.3)	255. 0	253.3	285.2 (17.4)	18.9 (42.9 )	26.2 (31.4)	266. 4	259.1	294. 7 (24. 2)	45.0 (29.6 )	39.1 (11.1)	249.7	255.7
Rosuvast atin	15.3 (40.0)	0.15 (33.4 )	0.48 (15.4)	15.1	14.8	15.7 (42.9)	0.30 (40.2 )	0.58 (2.8)	15.4	15.1	15.3 (16.2)	0.57 (33.1 )	0.94 (38.1)	14.8	14.4	16.3 (27. 0)	1.79 (25.9 )	1.62 (31.4)	14.5	14.7

# CL<sub>int,tota</sub> - Apparent total uptake clearance into OATP1B1-expressing HEK293 cells. % CL<sub>int,passive</sub> - Apparent passive uptake clearance into either MOCK or OATP1B1\_RIF HEK293 cells. \*CL<sub>int,OATP1B1</sub> = mean CL<sub>int,tota</sub> - mean CL<sub>int,passive</sub> where the CL<sub>int,passive</sub> measured in the MOCK cells or the OATP1B1\_RIF cells was used. Data shown are mean (CV%) values of three to five independent experiments.