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Title Page

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

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Running Title Page

Running Title: Is the Protein-Mediated Uptake Effect an Artifact?

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ABBREVIATIONS: ATV, atorvastatin; BCA, bicinchoninic acid assay; CL, clearance; CL_h, hepatic clearance; $CL_{int,uptake}$, intrinsic uptake clearance; CRV: cerivastatin; DCL, diclofenac sodium salt; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; FBS, fetal bovine serum; FLV, fluvastatin; HBSS, Hank's balanced salt solution; HEK, human embryonic kidney; HPLC, high-performance liquid chromatography; HSA, human serum albumin; IAA, iodoacetamide; IC_{50}, inhibitor concentration that produces 50% inhibition of uptake; IVIVE, *in vitro* to *in vivo* extrapolation; LC-MS/MS, liquid chromatography tandem mass spectrometry; NSB, non-specific binding; OATP, organic anion

transporting polypeptide; PLI, protein-lipid interaction; PMUE, protein-mediated uptake effect; PTV, pitavastatin; RIF, rifampicin; RSV, rosuvastatin; SDS, sodium dodecyl sulfate.

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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 (HEK293 transfected cells) and passive $CL_{int,uptake}$ (MOCK HEK293 cells) of 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 *vs.* HBSS buffer. 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 OATP-transported drugs could be an artifact of the 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 CL from *in vitro* uptake studies.

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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, 2021; 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 $CL_{int,uptake}$ of OATP substrate drugs by hepatocytes and OATP-expressing cells. This phenomenon is called the protein-mediated uptake effect (PMUE). That is, 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.*, 2020; 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.*, 2021). 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 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.

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If the PLI hypothesis is correct, the following should be observed in the *in vitro* OATP uptake studies in the presence *vs.* 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 *vs.* 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; Miyauchi *et al.*, 2018; Li *et al.*, 2020; 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 CL 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

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plasma proteins included in the incubations. Total $CL_{int.uptake}$ of the statins was determined by using OATP1B1-transfected HEK293 cells while passive $CL_{int.uptake}$ was determined using MOCK or OATP1B1-transfected HEK293 cells incubated with high unbound concentration of rifampicin (500 μM) (henceforth called OATP1B1_RIF cells). The NSB of HSA was determining 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 ($IC_{50,u,}$ unbound inhibitor concentration that results in 50% inhibition) towards OATP1B1-mediated transport of another statin.

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Materials and Methods

Chemicals and Reagents

The dithiothreitol (DTT), iodoacetamide (IAA), mass spectrometry grade trypsin, total protein quantification bicinchoninic acid assay (BCA) kit, Hank's balanced salt solution with calcium and magnesium (HBSS) 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), human serum albumin (HSA, fatty acid free) and formic acid was 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). Formic acid was purchased from Sigma-Aldrich (St. Louis, MO). All reagents were analytical grade. The Calbiochem ProteoExtract Native Membrane Extraction kit and the Centrifree® Ultrafiltration Device was 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 3×10^5 cells per well with 1 ml of high glucose DMEM medium (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℃,

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90% relative humidity, and 5% CO_2. OATP1B1-expressing HEK293 cells were supplemented with 600 µg/mL geneticin and 10 µg/mL blasticidin.

For the uptake assays, OATP1B1-expressing or MOCK HEK293 cells were rinsed twice with 1 mL of warm HBSS buffer (37℃, pH 7.4). Then, the cells were pre-incubated 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 approximately unbound 0.2 µM ATV, 0.1 µM CRV, 0.2 µM FLV, 0.2 µM PTV and 1 µM RSV. The above uptake studies with OATP1B1-expressing cells were repeated in the presence of RIF (unbound concentration 500 µM). Statin uptake was terminated at designed time points (5s, 30s, 60s and 120s, 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 µL of the supernatant was injected onto the liquid chromatography-tandem mass spectrometer (LC-MS/MS) 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 Atorvastatin (ATV), Pitavastatin (PTV) or Fluvastatin (FLV) on OATP1B1-Mediated Uptake of RSV in the Absence or Presence of HSA

The uptake of rosuvastatin (nominal unbound concentration 1 µM) by the OATP1B1-expressing cells was determined (over 1 min) in the presence of the inhibitors ATV, PTV or FLV. The range of nominal unbound concentrations of ATV was 0-200 µM (in HBSS or 2% HSA solution), for PTV was 0-100 µM in HBSS and 0-

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25 µ M in 5% HSA solution, and for FLV was 0-100 µM in HBSS buffer, 0-2 µM in 2% HSA solution and 0-1 µM in 5% HSA solution (different nominal unbound concentrations and the percent 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 as per 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 µL of the statin HBSS solution used in the above uptake experiments. Briefly, the samples were centrifuged at 37℃ for 2 minutes at 1,200 g to keep the filtrate volume <15% of the initial volume. After centrifugation, 10 µL 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 µL of the supernatant was injected onto the LC-MS/MS systems.

Quantification of Statins by Liquid Chromatography–Tandem Mass Spectroscopy (LC-MS/MS)

All the above samples were analyzed on AB Sciex Triple Quad 6500 (SCIEX, Farmingham, MA) coupled with Waters Acquity UPLC system (Waters, Hertfordshire, UK). Ten microliters of the sample were injected onto a UPLC column (ACQUITY UPLC® BEH C18 column, 1.7 µm, 2.1 mm x 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).

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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 $IC_{50,u}$ studies), the cells were lysed for 1 hour at 4°C with (200µL/well) 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 µL of a mixture of the stable-labeled surrogate peptides (Supplemental Table 1), prepared in 80% acetonitrile plus 0.2% formic acid and 5 ul of 80% acetonitrile plus 0.2% formic acid was added to 40 µL of trypsin digest (in 50 mM ammonium bicarbonate buffer). After centrifugation (5000g, 4°C, 5min), 5 µL 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 non-specifically bound to the cells, absolute quantification of HSA in the cell lysates is required at all time points of the uptake studies. For these experiments, the pre-incubation 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 pre-incubated albumin will not carry any drug into the cells and any remaining albumin from the pre-incubation 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, 114 nM of the unlabeled albumin surrogate peptide) were included in the LC-MS/MS analyses. These were prepared by spiking 5 µl of the unlabeled peptide standard and 10 µL of the labeled peptide (both in 80% acetonitrile and 0.2% formic acid solution) to 40 µL of 50 mM ammonium bicarbonate buffer.

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Data and Statistical Analyses

Determination of OATP1B1-Mediated and Passive Apparent Intrinsic Uptake Clearance ($CL_{int,uptake}$) of statins

The initial uptake rate was estimated from the slope of the drug uptake *vs.* 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 $CL_{int,uptake}$ 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 μM unbound concentration of each statin. The OATP1B1-mediated apparent $CL_{int,uptake}$ was calculated by subtracting the apparent passive $CL_{int,uptake}$ (in MOCK or OATP1B1_RIF cells) from the apparent total $CL_{int,uptake}$ (in OATP1B1-expressing cells).

Estimation of the $IC_{50,u}$ or the Degree of Inhibition of OATP1B1-Mediated RSV Uptake by ATV, FLV or PTV

First, the total % RSV uptake in the presence of the inhibitor (expressed relative to the uptake in the absence of the inhibitor) was corrected for the % passive uptake of RSV to derive the % OATP1B1-mediated RSV uptake. The % passive uptake of RSV was assumed to equal % RSV uptake at maximum inhibitor concentration and was comparable to the passive uptake obtained in MOCK or OATP1B1_RIF cells. Then, the $IC_{50,u}$ value of ATV or PTV was estimated by fitting an inhibition model to the % OATP1B1-mediated RSV uptake as a function of the unbound inhibitor concentrations using GraphPad Prism version 9 (GraphPad Software, La Jolla, CA):

$$\%\ \text{OATP1B1-mediated RSV uptake} = 100\% / [1+(IC_{50}/\text{unbound inhibitor concentration})^{\text{HillSlope}}] \quad \text{Eq. 1}$$

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 µL uptake media per well.

Assuming the ratio of the residual HSA and the total HSA (in 500 µL) equals the ratio of the residual bound statin and the total bound statin (in 500 µL), the amount of residual statin-HSA complex at different uptake times was estimated as follow:

$$\text{Residual bound statin (pmol/well)} = \text{Total bound statin (pmol/well)} \times \frac{\text{Residual HSA (pmol/well)}}{\text{Total HSA (pmol/well)}} \quad \text{Eq. 2}$$

Where the total HSA per well [5% (w/v) HSA in 500 µL] was 0.36 µmol, based on HSA molecular weight of 69367 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 vs. HBSS

To take into consideration small variation in the measured unbound statin concentration and the total protein content in HSA *vs.* HBSS uptake studies, statin uptake at each time point in these studies was corrected (Eq. 3) for these variables before estimating the increased apparent uptake in the presence of 5% HSA *vs.* HBSS.

$$\text{Normalized statin uptake in HBSS} = \text{Apparent statin uptake in HBSS (pmol/well)} \times \frac{\text{Unbound statin concentration in 5\%HSA (µM)}}{\text{Unbound statin concentration in HBSS (µM)}} \times \frac{\text{Total protein amount in 5\% HSA (mg/well)}}{\text{Total protein amount in HBSS (mg/well)}} \quad \text{Eq. 3}$$

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 *vs.* HBSS at each time point was estimated as follows:

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$$\text{Increased apparent uptake in the presence of 5\% HSA } vs. \text{ HBSS} = \text{Apparent statin uptake in 5\% HSA} - \text{normalized statin uptake in HBSS} \quad \text{Eq. 4}$$

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 (GraphPad Software, La Jolla, CA).

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Result

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 ($fu_{5\%HSA}$) ranging between 0.003 to 0.023. No difference in $fu_{5\%HSA}$ values was noted for each drug when determined singly (data not shown) *vs.* as a cocktail. The $fu_{5\%HSA}$ values (physiologically relevant, Table 1) of the statins were in agreement with their reported fraction unbound values in plasma (fu_p) except for the highly protein-bound statin FLV ($fu_{5\%HSA}$ 0.003 *vs.* fu_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) *vs.* MOCK or OATP1B1_RIF cells, especially for ATV and RSV (< 2) (Fig. 1 & 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. 1Ci) > PTV (Fig. 1Di) > RSV (Fig. 1Ei). 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 cells, 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 and 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. 3A-E).

The Amount of HSA Non-Specifically 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 thrice with the ice-cold wash buffer (Fig. 3F). As was the case for the y-intercept (Fig. 3A-E), the amount of HSA non-specifically 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 Non-Specifically 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. 4A, 4C, 4E; Supplemental Fig. 2A, 2C, 2E). Surprisingly, the amount of PTV-HSA and CRV-HSA complex non-specifically 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. 4Di, 4Bi, 4Bii), 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. 4Dii, 4Bii; Supplemental Fig. 2D and 2B), 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 *vs.* Absence of 5% HSA

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The unbound IC_{50} ($IC_{50,u}$) of PTV was decreased by more than 80% in the presence (p=0.03) of 5% HSA *vs.* 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). While 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 *vs.* absence of HSA (Fig. 5C).

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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 our data with respect to the media unbound statin concentration. Except for the $IC_{50,u}$ studies, these concentrations were maintained below the reported 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, we quantified by targeted quantitative proteomics, the NSB of HSA (after the cells were washed thrice) at each uptake time point 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 *vs.* 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.*, 2020; 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 *vs.* 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 while 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 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 1min. Not surprisingly, the amount of HSA remining 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 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 the increase in ATV, FLV, and RSV uptake by OATP1B1-expressing cells at each time point of the uptake study (Fig. 4). Surprisingly, this was not the case for PTV or CRV (Fig. 4Bi and 4Di). 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.4A, 4C and 4E). However, this was not the case for OATP1B1-mediated uptake of PTV (Fig. 4Di) or the total uptake of CRV (Fig. 4Bi,

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4Bii). Interestingly, the ratio of CRV-HSA NSB to CRV uptake increase was almost identical in OATP1B1-expressing cells (27%, Fig. 4Bi) *vs.* MOCK cells (28%, Fig. 4Bii), suggesting 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 towards 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. 5B-C). The $IC_{50,u}$ of PTV decreased in the presence of HSA *vs.* HBSS but not for ATV (Fig. 5A-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, 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 the statins. It does not. Also, it cannot account for the decrease in OATP1B1 $IC_{50,u}$ 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.

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"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 while V_{max} should remain unchanged. This cannot explain the CRV data, but is consistent with the decrease in the $IC_{50,u}$ of PTV but inconsistent with the decrease in V_{max} of OATP1B1-mediated PTV uptake in the persence of plasma reported by Bowman *et al*. (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 $IC_{50,u}$ 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 (Miyauchi *et al.*, 2018; Bowman *et al.*, 2019, 2020; Li *et al.*, 2020; Liang *et al.*, 2020; Bi *et al.*, 2021). Overall, these observations imply the NSB of drug-protein complex may occur when using hepatocytes. There is no clear evidence that

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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 *vs.* 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).

While 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. But, 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_h (Kim *et al.*, 2019; Li *et al.*, 2020, 2021; Bi *et al.*, 2021). Therefore, future focus should be delineating the underlying mechanisms causing the underprediction of OATP-mediated CL_h.

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Authorship Contributions

Participated in research design: Yin, Storelli and Unadkat.

Conducted experiments: Yin.

Performed data analysis: Yin and Storelli.

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

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Footnotes

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Figure Legends

Figure 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 ± standard deviation (SD) of statin uptake (normalized to 1 μM unbound concentration) and are representative of three to five independent experiments, each conducted in triplicate at each time point.

Figure 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 3-5 independent experiments.

Figure 3. The ratio of Y-intercept of the uptake curve for the 2% or 5% HSA conditions (*vs.* 1% HSA), for ATV (A), CRV (B), FLV (C), PTV (D), and RSV (E) uptake by the MOCK or OATP1B1_RIF HEK293 cells. In general, the intercept increased in proportion to the % 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 Na^+K^+ATPase peptide (membrane marker) using targeted proteomics (F). Data shown in F are mean ± SD and are representative of three independent experiments, each conducted in triplicate.

Figure 4. 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-overexpressing (Ai-Ei) or MOCK (Aii-Eii) 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 stain-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 ± 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; *** p<0.001).

Figure 5. Unbound OATP1B1 IC_{50} ($IC_{50,u}$) of PTV (A) or ATV (B) and inhibition of OATP1B1 by various concentrations of FLV (C), using RSV as a substrate. The $IC_{50,u}$ of PTV decreased in the presence of HSA while 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. % RSV uptake was the OAPT1B1-mediated uptake, i.e. the total % RSV uptake corrected for the % passive uptake of RSV (see methods). The data shown are mean ±SD and representative of three independent experiments, each conducted in triplicate. Solid line is the model fit to the data. $IC_{50,u}$ data shown are mean ±SD of three independent experiments, each conducted in triplicate. *$IC_{50,u}$ in the presence of HSA *vs.* HBSS (A, B) was statistically compared using the paired Student's t test.

Tables

Table 1: Fraction unbound (fu) of the statins in buffer containing 1%-5% human serum albumin (HSA) compared with that reported in human plasma

Compound	$fu_{1\% HSA}$	$fu_{2\% HSA}$	$fu_{5\% HSA}$	Reported fu_p [#]
Atorvastatin	0.202 ± 0.041	0.102 ± 0.038	0.056 ± 0.014	0.048
Cerivastatin	0.058 ± 0.008	0.035 ± 0.007	0.023 ± 0.004	0.018
Fluvastatin	0.012 ± 0.002	0.006 ± 0.001	0.003 ± 0.0007	0.00922
Pitavastatin	0.032 ± 0.006	0.018 ± 0.002	0.008 ± 0.001	0.0080
Rosuvastatin	0.41 ± 0.04	0.27 ± 0.03	0.13 ± 0.01	0.16

Data are mean ± SD of 10~12 uptake experiments and were corrected for NSB of the statin to the ultrafiltration device.

[#] Fraction unbound in human plasma (fu_p), determined using equilibrium dialysis, was obtained from (Riccardi *et al.*, 2019).

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Figure 1.

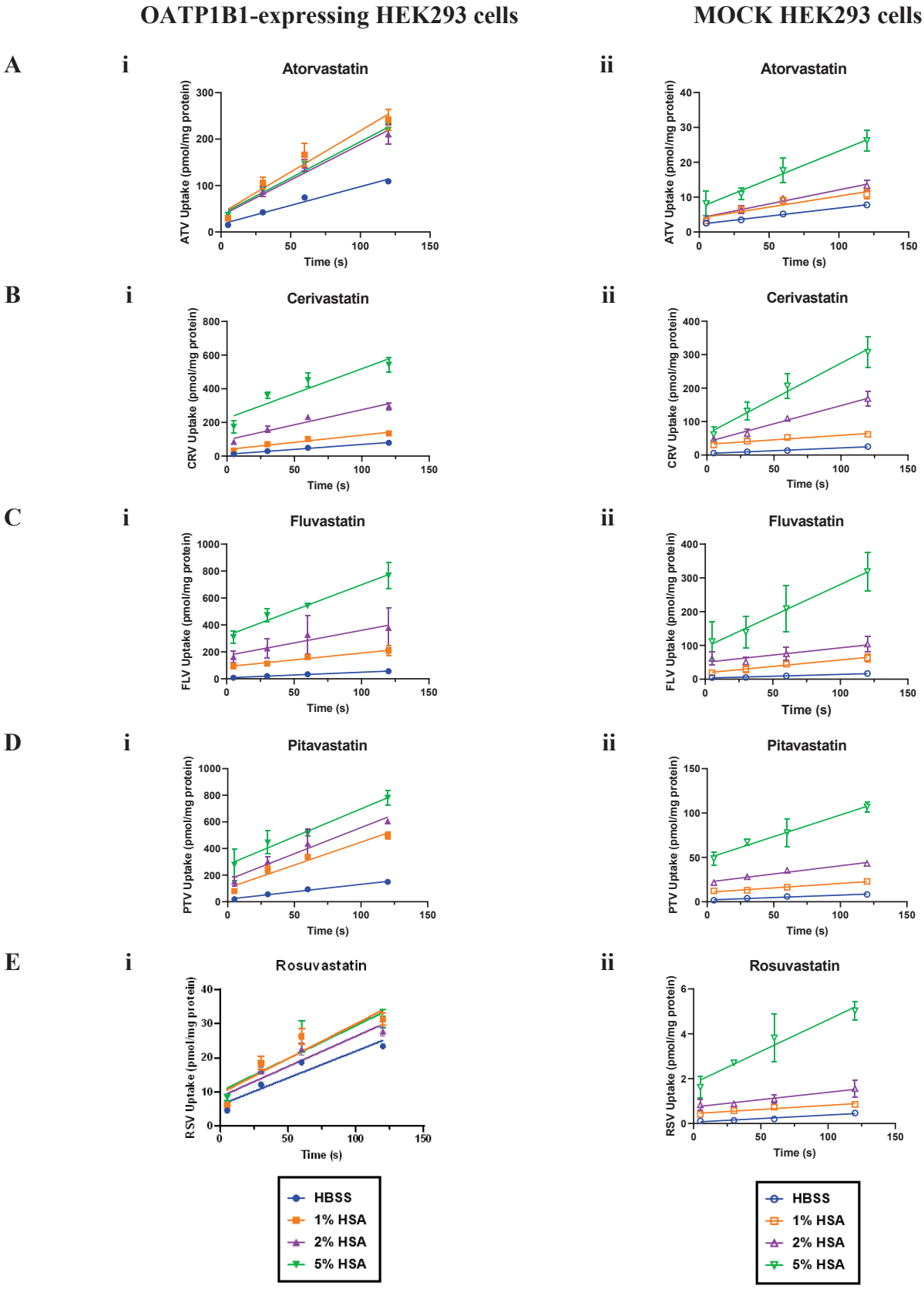

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Figure 2.

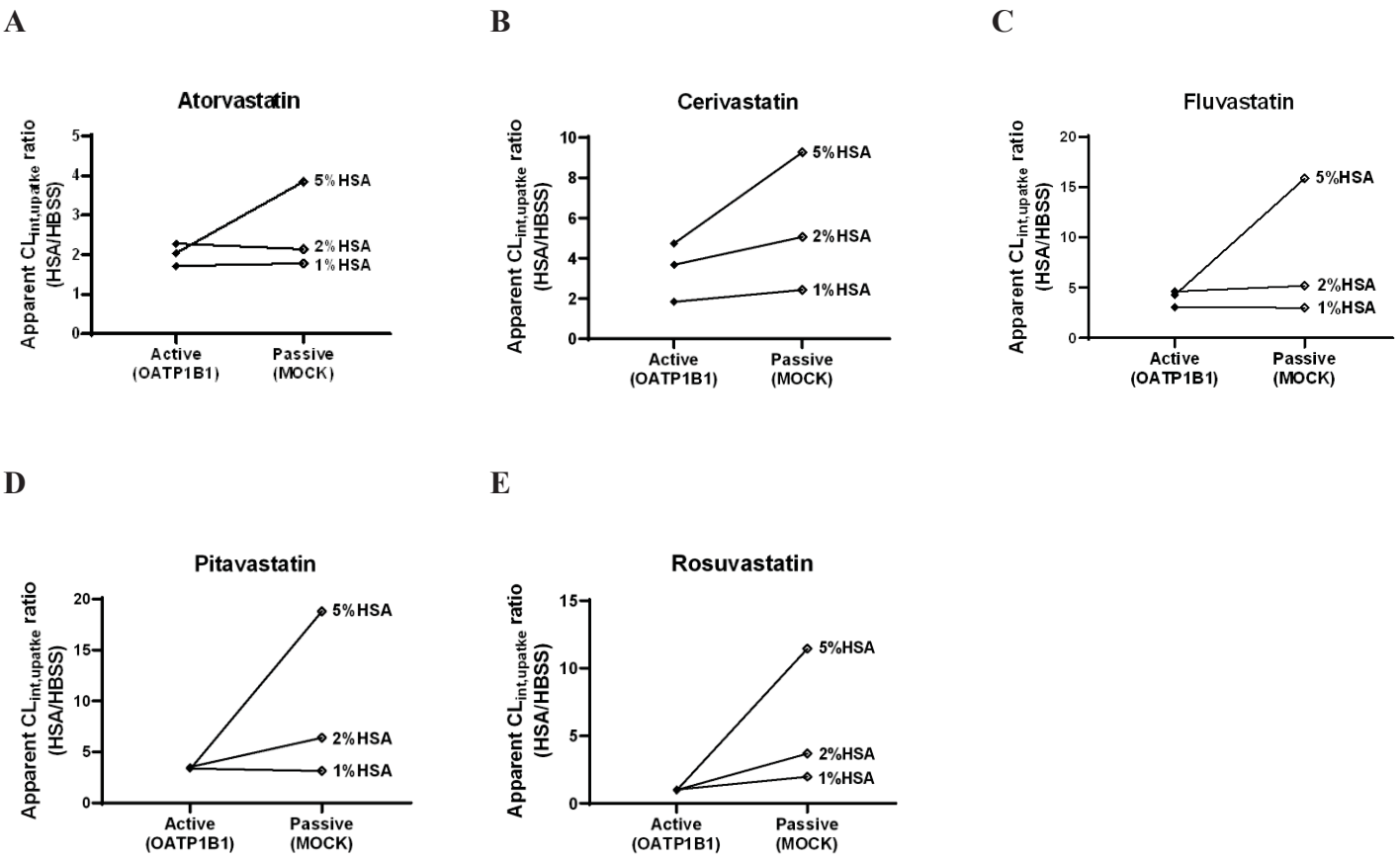

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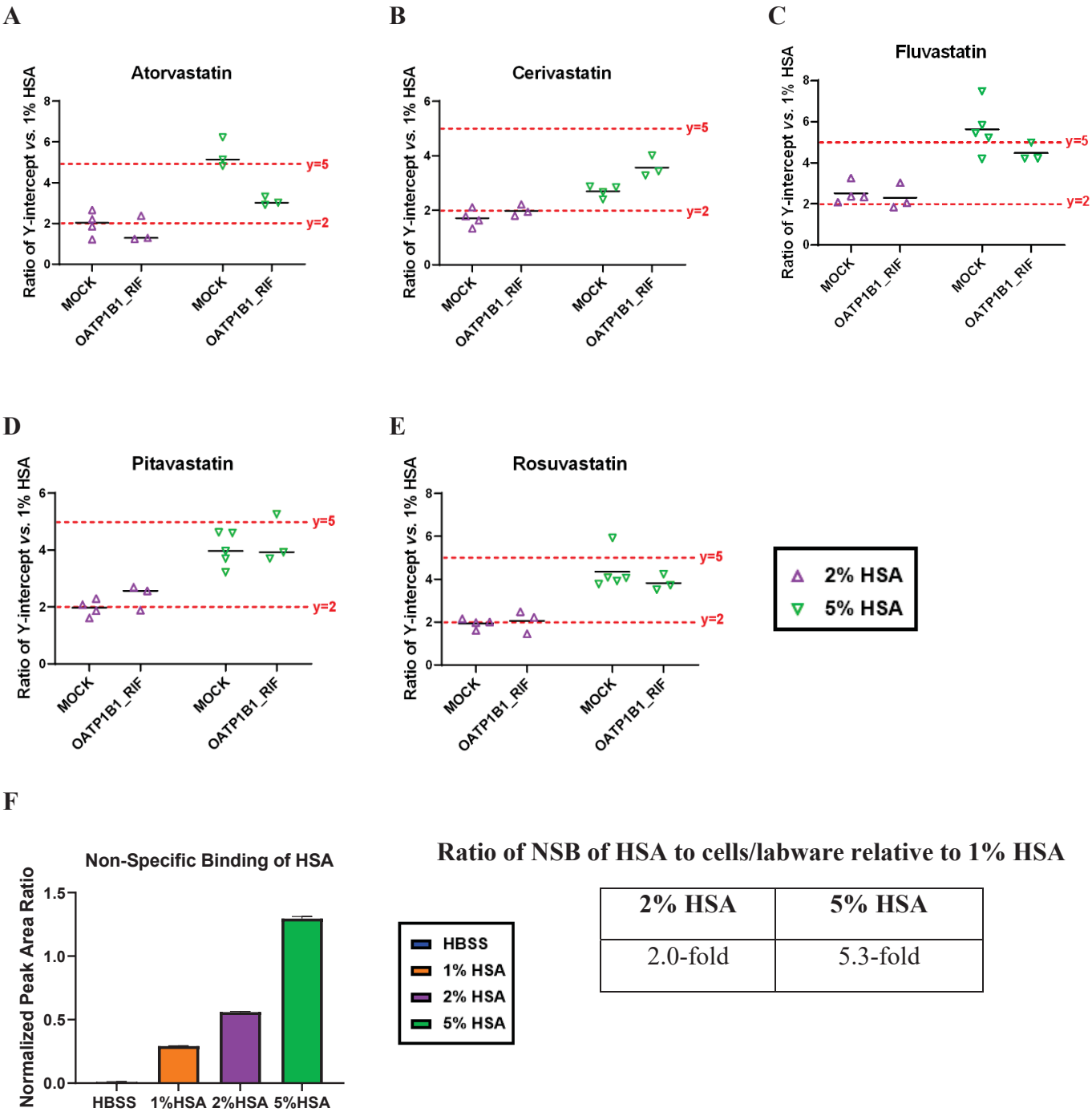
Figure 3.



Ratio of NSB of HSA to cells/labware relative to 1% HSA

2% HSA	5% HSA
2.0-fold	5.3-fold

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Figure 4.

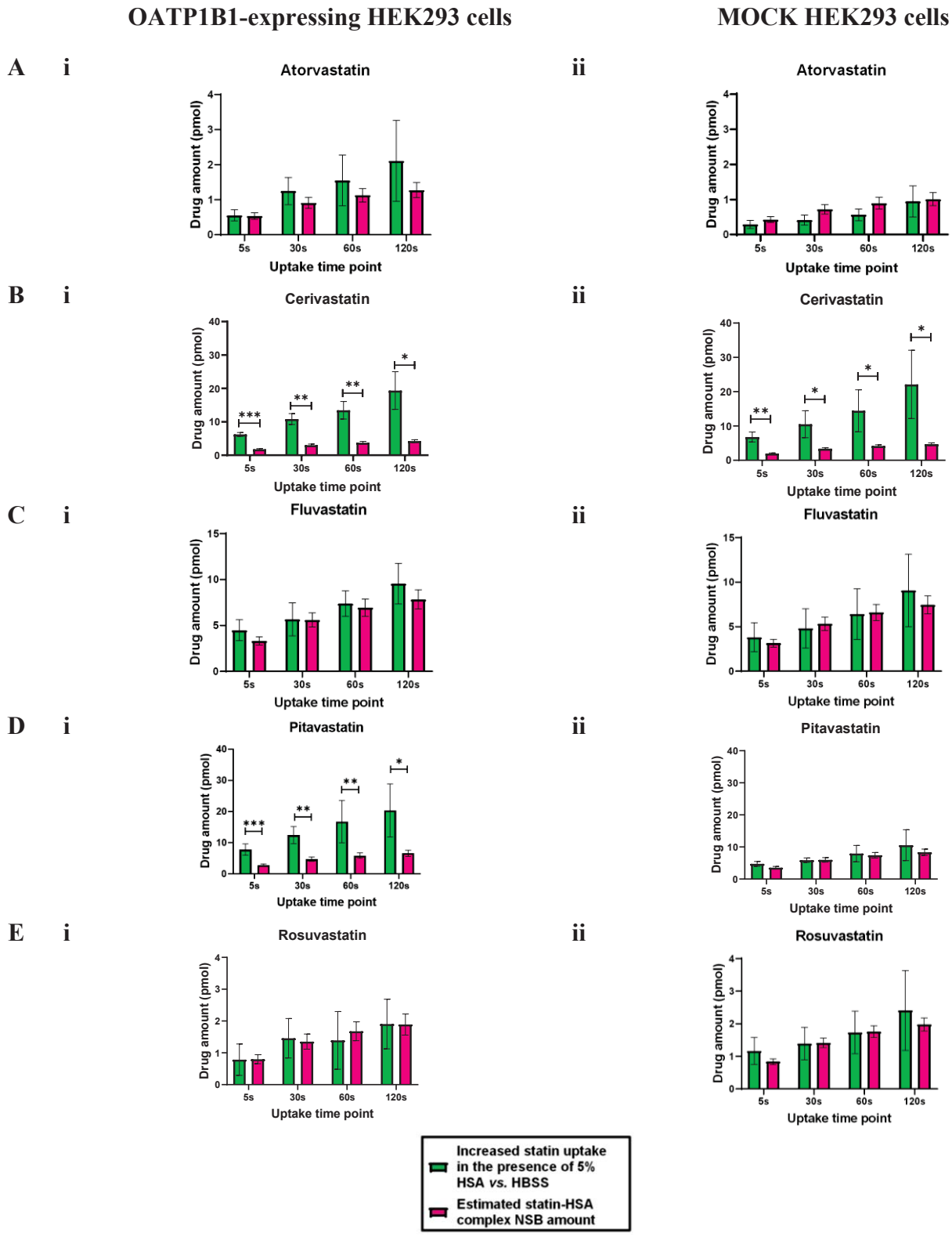

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Figure 5.