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Investigating the Impact of Albumin on the Liver Uptake of Pitavastatin and Warfarin in Nagase Analbuminemic Rats

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Non-standard abbreviations:

Area under the curve from 0-8 hr, $AUC_{0\text{-}8hr}$; below the limit of quantitation, BLQ; fraction unbound, fu; in vitro to in vivo correlation, IVIVC; intrinsic clearance, CL_{int}; Nagase analbuminemic rat, NAR; organic anion-transporting polypeptides, Oatps; free tissue-to-free plasma partitioning, Kp,u,u; unbound intrinsic clearance, $CL_{int,u}$; unbound tissue-to-plasma partition coefficient, $K_{p,u,u}$; wild-type, WT

Abstract

Albumin has been suggested to enhance the hepatic uptake of organic anion-transporting polypeptides (Oatps) substrates in various in vitro and liver perfusion models. However, it is not known if the interplay between albumin and Oatps is an experimental artifact, or whether this interaction occurs in vivo. The objective of this work was to investigate the hepatic uptake of warfarin and pitavastatin, which are both extensively bound to albumin, but only pitavastatin being an Oatp substrate. Experiments were conducted in Nagase analbuminemic rats (NAR) which exhibit reduced albumin levels compared with F344 (WT). The fraction unbound (f_u) was 140- and 10-fold greater in NAR plasma for warfarin and pitavastatin, respectively, whereas no meaningful differences were observed with tissue binding. In vitro, pitavastatin uptake into hepatocytes reconstituted in WT plasma was 17- and 3-fold greater than when reconstituted in buffer or NAR plasma, respectively. In vivo, free tissue-to-free plasma ratios ($K_{p,u,u}$) from brain and liver in intact WT and NAR were not significantly different for warfarin. Contrarily, liver $K_{p,u,u}$ of pitavastatin was 6-fold higher in WT animals which corresponded to 2.3-fold reduction in free plasma and 2.6-fold increase in free liver exposure. These results suggest that the enhanced hepatic uptake by albumin is not necessarily an experimental artifact but is also a relevant phenomenon in vivo. This work raises the possibility that additional plasma proteins may have similar impact on the function of other drug transporters, and that modulating plasma protein binding may exhibit meaningful clinical relevance in the disposition of drugs.

Significance Statement:

While the interplay between albumin and Oatps has been reported in hepatocytes and in liver perfusion studies, in vivo relevance of this interaction has not yet been elucidated. Utilizing NAR and its corresponding WT animal, the work presented here demonstrates that albumin may indeed enhance the hepatic uptake of pitavastatin in intact animals. In vivo demonstration of this interplay not only provides further justification for continued investigation into this particular mechanism, but also raises the possibility that other plasma proteins may affect additional drug transporters, and that modulating plasma protein binding may exhibit meaningful clinical relevance in the disposition of drugs.

Introduction

The fraction unbound in plasma (fu) is one of the key variables that is measured during drug discovery. There are several ways that fu values may be integrated into the characterization of compounds. For example, fu can be used to scale in vitro intrinsic clearance (CL_{int}) to in vivo clearance (CL) (Obach, 1999; Jones et al., 2017). In addition, fu can be used to bridge in vitro biochemical potency to in vivo efficacious concentrations. Furthermore, species differences in fu can be incorporated to adjust preclinical efficacious concentration to determine a clinical efficacious target, or to relate certain preclinical safety exposure to clinical exposure.

Molecules can bind to a variety of elements in plasma, with albumin being the most abundant plasma protein. Albumin is a water soluble anionic protein synthesized in the liver and is excreted out into the plasma in non-glycosylated form. In addition to serving as a carrier protein by binding to endogenous and exogenous molecules, physiological role of albumin has been reported such as regulation of colloid osmotic pressure (Bjorneboe, 1945), sequestration of toxic molecules such as bilirubin (Schmid et al., 1965; Brodersen, 1979), and its function as an antioxidant (Brown et al., 1989; Deigner et al., 1992).

The Nagase analbuminemic rat (NAR) model has been described to be deficient in their levels of albumin (Nagase et al., 1979). When compared with their corresponding wild-type (WT) strain Fischer 344 (F344), serum albumin levels in the NAR model is less than 5-10% of WT as determined with Western Blot (personal communication with Dr. Sanjeev Gupta from Albert Einstein College of Medicine), and was less than 2000-fold of WT when quantifying rat albumin with ELISA (Supplemental Figure 3). Instead, their cholesterol and triglyceride concentrations were elevated, and as a result, NAR animals develop hyperlipidemia. Similarly in tissues such as the liver and kidneys, albumin was not detected (Sugiyama et al., 1980). Therefore, NAR is an

intriguing model to investigate the role of albumin in various physiological and pharmacological processes. For instance, studies with mycophenolic acid showed that while the total exposure was 29-fold higher in the WT animals compared with NAR, the free exposures were comparable. Accordingly, the inhibition of inosine-5′-monophosphate dehydrogenase by mycophenolic acid was not significantly changed between the WT and NAR animals, underscoring that target engagement is driven by the free drug (Yoshimura et al., 2015).

The free drug hypothesis assumes that only free drug is able to interact with the pharmacological target (Mendel, 1989), and serves as the rationale for incorporating fu in profiling the properties of drugs. Plasma protein binding is used to determine free drug concentrations. CL_{int} is a measure of the ability for a particular system to eliminate compounds without limitations of blood flow nor binding to cells or proteins in blood. However, several studies have implied that albumin may alter the disposition of compounds, particularly for compounds that are substrates for uptake transporters such as organic anion-transporting polypeptides (Oatps). For example, in the bile-ducted cannulated model, biliary secretion of bilirubin was markedly higher in WT compared with NAR animals following administration of high concentration of bilirubin (Inoue et al., 1985). In addition, in the rat liver perfusion model, the hepatic uptake of fatty acids and bile acids such as oleic acid and taurocholic acid was higher in the presence of albumin (Forker and Luxon, 1981; Weisiger et al., 1981). In vitro, when compared with incubations conducted in buffer, statins that are highly bound to plasma proteins such as atorvastatin and pitavastatin demonstrated greater uptake into hepatocytes in the presence of plasma or albumin (Miyauchi et al., 2018; Bowman et al., 2019; Kim et al., 2019). Many reports regarding the potential effect of albumin on hepatic uptake have been studied in surgically modified animal models or with in vitro systems. The objective of the current work was to evaluate the impact of albumin on the

hepatic uptake of warfarin and pitavastatin in intact WT and NAR animals. Warfarin and pitavastatin were chosen because while both drugs are extensively bound to plasma proteins, particularly to albumin, pitavastatin is highly dependent on Oatps for elimination from the body.

Materials and Methods

Materials.

Warfarin sodium was purchased from Sigma-Aldrich (St. Louis, MO) and pitavastatin calcium was purchased from Cayman Chemical (Ann Arbor, MI). Propranolol was purchased from Sigma-Aldrich (St. Louis, MO). All other chemicals and reagents were of analytical grade and were purchased from Sigma-Aldrich (St. Louis, MO), unless specified otherwise.

Plasma protein binding and tissue binding.

Plasma, brain and liver harvested from F344 (WT) and NAR animals were used to determine binding of warfarin and pitavastatin. Experiments were conducted using a Single-use RED plate with inserts (Thermo Fisher Scientific Inc., Rockford, IL, USA) with the following set-up as described previously (Chen et al., 2019), which enables robust and reliable determination of fu for highly bound compounds. The positive control utilized in the current work was tolbutamide (internal historic coefficient of variation of 13%). In brief, 300uL of plasma or tissue homogenate spiked with drug on the donor chamber, and 500uL phosphate buffered saline (PBS) containing 133 mM potassium phosphate and 150 mM NaCl on the receiver chamber. Plasma protein binding and tissue binding experiments were performed at a final concentration of 5 μM and 1 uM, respectively, in a shaking incubator at 37ºC with 5% CO_2 at 450 rpm (VWR SymphonyTM). Experiments were conducted in triplicate using 100% plasma or 20% tissue homogenate in PBS buffer, and binding values were then extrapolated to 100% using the following equation:

$$\text{Fraction unbound in tissue } (f_{u,tissue}) = \frac{\frac{1}{Dilution\ Factor}}{\left(\frac{1}{fu,tissue'}-1\right)+\frac{1}{Dilution\ Factor}}$$

where fu,tissue' is fraction unbound determined with 20% tissue homogenate, and fu,tissue is fraction unbound when extrapolated to 100%. Following 6-hr incubation, aliquot was sampled from the RED device and equalized with equal amount of either plasma/tissue homogenate or PBS buffer (buffer was added to donor side and plasma/tissue homogenate was added to receiver side). Samples were then quenched in 1:3 (sample: acetonitrile) ratio with ice cold HPLC grade acetonitrile (EMD Millipore, Billerica, MA) with propranolol as the internal standard. The resulting mixture was shaken for 15 min at 500 rpm (Thermo Scientific Compact Digital MicroPlate Shaker), and the supernatant was collected by centrifuging for 15 min at 3750 rpm (Beckman Coulter Allegra X 12R). Subsequently, the supernatant was diluted further 1:1 with HPLC grade water (J.T. Baker, Center Valley, PA) for LC-MS/MS analysis. fu was calculated by dividing the peak area from the receiver chamber by the peak area from the donor chamber.

Hepatocyte uptake.

Pooled male F344 rat hepatocytes (Lot: OAE; BioIVT, Westbury, NY), at a density of 2 million cells/mL, were reconstituted in protein-free Krebs-Henseleit buffer (pH 7.4), or in plasma collected from WT and NAR animals. Rat hepatocytes in the various suspensions were pre-incubated at 37^{o}C for 10 min. To initiate the reaction, equal volume of pitavastatin solution (2 µM) prepared in buffer, NAR plasma or WT plasma was added to the hepatocyte suspension. The final incubation volume was 500 µL constituting of 1 million rat hepatocytes/mL and a nominal drug concentration of 1 µM. At 20, 45, 90 and 120 sec, 100 µL aliquots were sampled and centrifuged at 12000 rpm for 10 sec. Supernatant and hepatocyte pellets were quenched in acetonitrile containing propranolol as an internal standard. Samples were analyzed by LC-MS/MS.

In vivo free tissue-to-free plasma ratio ($K_{p,u,u}$).

All in vivo studies performed were approved by the Institutional Animal Care and Use Committee at Genentech, Inc. (South San Francisco, CA). Free liver-to-plasma ratio ($K_{p,u,u}$) was determined as described previously with minor modifications (Chang et al., 2019). Briefly, twelve male rats of each strain of F344 (background strain of NAR; Charles River Laboratories, Wilmington, MA) or NAR (Albert Einstein College of Medicine of Yeshiva University, Bronx, NY) were used for each compound to determine tissue and plasma concentrations, and tissue-to-plasma ratios. F344 rats weighed between 197 to 235g, and NAR weighed between 197 to 261g. Warfarin prepared in 10% DMSO/50% polyethylene glycol 400 (PEG 400)/40% PBS was administered as a single intravenous bolus dose of 1 mg/kg with dose volume of 2mL/kg. Pitavastatin prepared in 10% DMSO/50% PEG 400/40% water was administered as a single intravenous bolus dose of 0.5 mg/kg with a dose volume of 2 mL/kg. Animals were not fasted before dosing. Three animals/time point were euthanized by exsanguination under isoflurane anesthesia at 0.25, 0.5, 2, and 8 hr, post-dose to collect blood, brain and liver. Blood was centrifuged at 1500 to 2000g at 2 to 8°C to harvest plasma within 1 hr of collection. Tissue samples were diluted 1:4 weight (g) by volume (mL) with water and homogenized using the BeadBeater (BioSpec Products; Bartlesville, OK).

LC-MS/MS analysis.

Warfarin and pitavastatin concentrations were quantitated in blood, brain and liver with an LC-MS/MS, which includes a Shimadzu CBM-20A controller with SIL-30AD pumps, a Shimadzu Nexera X2 autosampler with a CTO-20AC column oven (Shimadzu, Columbia MD), and a Sciex QTrap 6500+ mass (Framingham, MA). Chromatographic separation for all samples was achieved with a Phenomenex kinetex XB-C18 column (30 x 2 mm, 100Å, 2.6um particle size) at a flow rate of 1 mL/min with gradient elution using mobile phases of water with 0.1% formic

acid (A) and acetonitrile with 0.1% formic acid (B). HPLC gradient was 3% B for the first 0.1 minute and was ramped up to 95% B from 0.10 to 0.35 minute. The gradient was maintained at 95% B for 0.50 minutes, before returning to initial composition of 3% B within 0.65 minutes. The total runtime was 2 minutes. The concentrations of warfarin and pitavastatin were determined using multiple reaction monitoring (MRM) in the positive ion mode. The following MRM transitions were monitored: m/z 422.1->290.1 for pitavastatin, m/z 309.1->163.2 for warfarin, and m/z 260.1->116.1 for propranolol which was the internal standard. The lower limit of quantitation for warfarin was 0.00989 µM, 0.00331 nmol/g and 0.0296 nmol/g in plasma, brain and liver, respectively; pitavastatin was 0.00242 µM, 0.0121 nmol/g and 0.0121 nmol/g, respectively.

Data analysis.

Mean concentrations measured in plasma and tissue homogenates were used to construct semi logarithmic plasma concentration-time curve. The significance of difference was determined with unpaired t-test using GraphPad Prism 7 (La Jolla, CA). Mean (+ SD) was reported and $P < 0.05$ was considered statistically significant. Free plasma and free tissue concentrations from individual animals were used to construct a composite area under the free concentration-time curve from 0-8 hr ($AUC_{0\text{-}8hr}$). $AUC_{0\text{-}8hr}$ was determined by non-compartmental methods using Phoenix™ WinNonlin®, version 6.4 (Pharsight Corporation, Mountain View, CA). Because the $AUC_{0\text{-}8hr}$ was calculated with a composite profile, standard deviation of the $AUC_{0\text{-}8hr}$ values could not be generated. Free tissue-to-free plasma partitioning ratio ($K_{p,u,u}$) was determined by dividing the free tissue $AUC_{0\text{-}8hr}$ by free plasma $AUC_{0\text{-}8hr}$, or by dividing the free tissue concentration by free plasma concentration.

CL_{int} was determined from the concentration of pitavastatin measured in the hepatocyte uptake assay by calculating the percent of pitavastatin depleted in the incubation media between 0 and 120 sec. Unbound CL_{int} was calculated by dividing CL_{int} by fu determined from NAR and WT plasma. fu for buffer was assumed to be 1.0. Mean (+ SD) was reported and $P < 0.0005$ calculated from one-way ANOVA followed by Tukey's post hoc test using GraphPad Prism 7 was considered statistically significant.

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Results

Binding of warfarin and pitavastatin in plasma, and in brain and liver homogenates

Binding of warfarin and pitavastatin to plasma as well as to homogenates prepared from brain and liver harvested from WT and NAR animals was determined. Table 1 summarizes that in the WT animals, warfarin and pitavastatin was highly bound to plasma, with fraction unbound in plasma ($f_{u,plasma}$) at 0.0031 and 0.0079, respectively. In NAR animals, $f_{u,plasma}$ for warfarin increased 140-fold to yield 0.43, whereas $f_{u,plasma}$ for pitavastatin increased 10-fold to yield 0.078. In brain homogenates prepared from WT animals, fraction unbound ($f_{u,brain}$) was 0.24 and 0.022 for warfarin and pitavastatin, respectively. Unlike with plasma, $f_{u,brain}$ of warfarin and pitavastatin was not markedly different between WT and NAR animals. Fraction unbound measured in liver homogenates ($f_{u,liver}$) prepared from WT animals for warfarin and pitavastatin was 0.078 and 0.023, respectively, and was not meaningfully different from $f_{u,brain}$. In addition, as observed with $f_{u,brain}$, values for $f_{u,liver}$ between WT and NAR animals were comparable.

In vitro uptake of pitavastatin into hepatocytes suspended in buffer, and in plasma from WT and NAR animals

Uptake experiments were conducted in hepatocytes reconstituted in buffer, or in plasma from WT and NAR animals. Because initial experiments indicated that total uptake of warfarin into hepatocytes was rapid and reached saturation prior to the first technical feasible timepoint of 15 sec, no further experiments were conducted due to the inability to accurately measure CL_{int}. This inability to measure earlier timepoints is a limitation of the current in vitro assay. Figure 1 shows the unbound CL_{int} of pitavastatin uptake into hepatocytes reconstituted in buffer was 160 $\pm$ 40 µL/min/million cells and was not statistically significant from hepatocytes reconstituted in NAR plasma at 510 $\pm$ 130 µL/min/million cells. However, pitavastatin uptake into hepatocytes reconstituted in WT plasma was greater than when the hepatocytes were re-suspended in buffer

or NAR plasma at 2800 ± 1100 µL/min/million cells, indicating that albumin was enhancing pitavastatin uptake into hepatocytes. The 17-fold increase in CL_{int} between WT and buffer for pitavastatin is comparable to what has been shown with plasma and albumin (Miyauchi et al., 2018; Bowman et al., 2019).

Brain and liver partitioning of warfarin and pitavastatin in intact WT and NAR animals

Following intravenous administration of warfarin and pitavastatin to WT and NAR animals, the plasma concentration and their distribution into brain and liver were measured at multiple timepoints between 0.25 to 8 hr. Total concentrations were converted to free concentrations by incorporating appropriate fu accordingly to the corresponding biological matrix. Figure 2 describes the free concentration time-profile of warfarin in WT and NAR animals, and the total concentration time-profiles are shown in Supplemental Figure 1. In both strains, the free concentration-time profiles were similar between plasma, brain and liver. In WT, Figure 2A indicates that the elimination of warfarin was slow since the free concentration remained relatively unchanged up to 8 hr. In contrast, Figure 2B shows that the free concentration of warfarin in plasma, brain and liver declined over time with half-life between 2-4 hr. The free concentration time-profiles of pitavastatin in WT and NAR animals are shown in Figure 3, and the total concentration time-profiles are shown in Supplemental Figure 2. Similar to warfarin, the shape of the free plasma and free liver concentration-time profiles of pitavastatin were comparable in both strains. Brain levels of pitavastatin were not reported because the concentrations were below the limit of quantitation (BLQ). In both WT and NAR animals, pitavastatin was quickly eliminated from the plasma and liver with half-life between 1-2 hr.

Warfarin and pitavastatin concentrations were measured in plasma, brain and liver, from individual animals at multiple timepoints. Plots of the free tissue-to-free plasma ratio ($K_{p,u,u}$) at

each timepoint are shown in Figure 4. Brain and liver $K_{p,u,u}$ for warfarin and pitavastatin in WT and NAR animals remained relatively constant over the various timepoints, which is a reflection of the free concentration-time profile being similar between plasma and tissues. Warfarin $K_{p,u,u}$ of the brain and liver from WT animals was approximately 0.8-1 and 7-10, respectively. In NAR, $K_{p,u,u}$ of the brain and liver was approximately 0.4-1 and 4-10, respectively, and therefore, no significant differences were observed between WT and NAR for brain and liver $K_{p,u,u}$ of warfarin. However, for pitavastatin, liver $K_{p,u,u}$ in WT was approximately 75-130 which was significantly higher than what was determined in the NAR at approximately 6-18. Concentrations at the various timepoints were used to construct a composite concentration-time profile to calculate the area under the curve from 0 to 8 hr ($AUC_{0\text{-}8hr}$). As summarized in Table 2, the free $AUC_{0\text{-}8hr}$ for warfarin in plasma, brain and liver from WT animals was 0.82 µM*hr, 0.73 nmol/g*hr and 6.9 nmol/g*hr, respectively. Exposure of warfarin in NAR animals was less than 2-fold of the WT animals and was not meaningfully different. Free $AUC_{0\text{-}8hr}$ for pitavastatin in plasma and liver from WT animals was 0.0088 µM*hr and 0.93 nmol/g*hr, respectively. In NAR animals, free plasma $AUC_{0\text{-}8hr}$ was 2.3-fold higher to yield 0.020 µM*hr, whereas free liver $AUC_{0\text{-}8hr}$ was 2.6-fold lower to yield 0.36 nmol/g*hr. Free $AUC_{0\text{-}8hr}$ values were utilized to calculate free tissue-to-free plasma ratio ($K_{p,u,u}$). Warfarin $K_{p,u,u}$ in WT animals was 0.89 and 8.4 in brain and liver, respectively, whereas $K_{p,u,u}$ was approximately 2-fold lower in the NAR animals at 0.43 and 0.44 in brain and liver, respectively. Pitavastatin $K_{p,u,u}$ in WT liver 110. However, $K_{p,u,u}$ decreased 6-fold in NAR liver to 18.

Discussion

Plasma protein binding is measured to determine free drug levels that is available to engage pharmacological target(s), which is based on the premise of the free drug hypothesis. CL_{int} is the inherent ability of a particular system to eliminate compounds without being limited by blood flow or binding to cells or proteins in blood. However, various studies have insinuated that albumin, the most abundant plasma protein, may modulate CL_{int}. For example, there have been several reports of albumin augmenting CL_{int} of drug metabolizing enzymes such as CYP2C9 (Tang et al., 2002), CYP2C8 (Wattanachai et al., 2011), CYP1A2 (Wattanachai et al., 2012) and UGTs (Rowland et al., 2007; Kilford et al., 2009). Further investigations have indicated that long-chain unsaturated fatty acids released from the in vitro membrane during the incubation was attenuating metabolic activity, and that albumin was sequestering these inhibitory fatty acids to boost metabolism. This interaction between albumin and fatty acids to affect CL_{int} is an in vitro artifact that is not relevant in vivo. As such, the practice of supplementing albumin to in vitro metabolism incubations has been employed to improve the in vitro and in vivo correlation (IVIVC) of clearance. In addition to drug metabolizing enzymes, there has been growing evidence to suggest that albumin may facilitate the in vitro uptake into hepatocytes, mediated by Oatps. The objective of this work was to investigate whether the increased uptake into hepatocyte in the presence of albumin is also an experimental artifact, or whether this phenomenon occurs in vivo. Warfarin and pitavastatin were chosen as probe substrates because while both compounds are extensively bound to albumin, pitavastatin is a strong substrate for Oatps. Hepatic uptake was monitored in NAR and in F344, its corresponding WT animals.

To evaluate whether NAR animals would be an appropriate model to investigate the effect of albumin on hepatic uptake, binding of warfarin and pitavastatin were compared between NAR

and WT animals. Table 1 shows that warfarin and pitavastatin are highly bound to WT plasma, but the extent of binding in NAR plasma was reduced by 140- and 10-fold, respectively, which corroborates the lower expression of albumin in NAR animals. In contrast, there was no meaningful difference in their binding to tissues which is consistent with studies showing that unlike binding to plasma, binding to tissues is species independent (Summerfield et al., 2008; Liu et al., 2018; Riccardi et al., 2018). The likely reason for the disparity in binding pattern between plasma and tissues is that binding to tissues is driven by non-specific binding, whereas binding to albumin is mediated by specific binding sites (Sudlow et al., 1975). Consequently, the distinct modalities of binding suggest that binding to tissues is not necessarily equivalent to binding to plasma proteins and is dependent on the properties of the compound. Indeed, in the current work with warfarin and pitavastatin, the ratio of $f_{u,plasma}$ to $f_{u,liver}$ in WT animals was 7.5-fold higher for pitavastatin than for warfarin at 0.3 and 0.04, respectively. However, in the absence of albumin in the NAR animals, $f_{u,plasma}$ to $f_{u,liver}$ ratio of pitavastatin and warfarin converged at 4.3 and 4.4, respectively. Similarly in the brain, the ratio of $f_{u,plasma}$ to $f_{u,brain}$ in WT animals was 40-fold higher for pitavastatin than for warfarin, but became more comparable in the NAR animals. This analysis also imply that rather than total ratios, $K_{p,u,u}$ is the more appropriate parameter to estimate free tissue availability as suggested previously (Yabe et al., 2011; Yoshikado et al., 2017).

In addition, the unbound CL_{int} of pitavastatin uptake into hepatocytes shown in Figure 1 was significantly greater in the presence of WT plasma than in buffer or NAR plasma. Thus, the differences in binding between WT and NAR plasma, and increased uptake of pitavastatin in hepatocytes suspended in WT plasma ascertained the utility of the NAR model to evaluate the potential impact of albumin on hepatic uptake. Figure 4A and 4B show that $K_{p,u,u}$ of warfarin for

brain and the liver was not significantly altered between WT and NAR animals. Nevertheless, it is worth noting that while not statistically significant, warfarin uptake was approximately 2-fold greater in WT. Recently, it has been reported that warfarin is a substrate for human OAT2 (Bi et al., 2018), and accordingly, hepatic $K_{p,u,u}$ was greater than unity in the current study. It is not known if the unexceptional impact of albumin on warfarin hepatic uptake is because the interplay between albumin and OAT2 is inconsequential or because OAT2 is not the rate-limiting step in warfarin elimination due to the rapid uptake of warfarin as observed in the in vitro experiment. Further investigations are needed to understand the role of OAT2 on the overall disposition of warfarin.

Albumin significantly increased the hepatic uptake of pitavastatin across all timepoints. When considering the composite $AUC_{0\text{-}8hr}$, $K_{p,u,u}$ was approximately 6-fold higher in WT than in NAR animals, as more free pitavastatin in the plasma was accompanied by less free pitavastatin in the liver of NAR animals by 2.3- and 2.6-fold, respectively. Besides albumin levels, there are several dissimilarities between WT and NAR animals. For example, reduced albumin is partly compensated by higher cholesterol and triglyceride concentrations which lead to hyperlipidemia in NAR animals (Nagase et al., 1979). It has also been shown that Cyp1a protein expression is greater in NAR animals (Kim et al., 2003), which may partly explain why the half-life of warfarin, whose metabolism is partially contingent on Cyp1a, is longer in the WT animals. No changes in expression of efflux transporters such as P-glycoprotein and multidrug resistance-associated protein 2 have been reported (Abe et al., 2008), although additional work is needed to examine the expression of Oatps in these models. Furthermore, while no striking differences were noted with physiological parameters such as hematocrit and mean arterial/venous blood pressure (Renkin et al., 1993), the NAR animals were associated with increases in liver weight

by 10% (Kim et al., 2003) and plasma volume by 22% (Renkin et al., 1993). These differences suggest that the central volume is dissimilar between the two models and may partly explain the significantly higher concentration observed with warfarin in WT at the first sampling timepoint. However, despite these differences, the overall effect on warfarin $K_{p,u,u}$ was inconsequential. Taken together, these data provide further evidence to strengthen the claim that albumin may enhance the hepatic uptake of pitavastatin (Miyauchi et al., 2018; Bowman et al., 2019; Kim et al., 2019); and importantly, this is the first in vivo evidence demonstrating the impact of albumin on the hepatic uptake of pitavastatin in intact animals.

Interplay between albumin and hepatic uptake has been previously described in liver perfusion models such as with taurocholate (Forker and Luxon, 1981). However, while liver perfusion is a powerful method to study liver disposition, albumin effect has been typically evaluated by adding exogenous albumin which may deviate the system from physiological conditions and obfuscates the experiment. For example, albumin effect with warfarin was only evident when exogenous albumin was added to the system, as the uptake $CL_{int,u}$ of warfarin increased by 9- and 11-fold in the presence of 1.6 g/dL of bovine serum albumin (Tsao et al., 1986). Meanwhile, one benefit of the current experimental design is that the role of albumin was evaluated in an in vivo system where the physiological system was intact and without perturbation to the system. Another merit of this work with intact animals is that unlike with drug metabolizing enzymes, it shows that the albumin effect is not an in vitro artifact. Therefore, modification to supplement albumin is not to overcome challenges of the in vitro system to enhance CL_{int} to improve IVIVC, but to optimize the in vitro system to better mirror in vivo conditions. Moreover, if the albumin effect is indeed a relevant in vivo phenomenon, these data raise the possibility that altering plasma protein binding may have meaningful clinical relevance to alter drug disposition for

drugs such as pitavastatin. For example, albumin levels have been shown to be lower in patients with severe liver disease or renal impairment. As observed in the current study, pitavastatin exposures were higher in patients with Child-Pugh grade B cirrhosis (Hui et al., 2005) compared with healthy volunteers. Pitavastatin exposures were also higher in severe renal impairment patients (Morgan et al., 2012), but since uremic toxins have been shown to inhibit OATPs (Sato et al., 2014), additional studies are needed to distinguish whether the changes are due to an "albumin-effect" or to direct inhibition of OATPs.

In summary, this work in WT and NAR models with distinct albumin expression was employed to illustrate the interplay between albumin and hepatic uptake of pitavastatin. Hepatic uptake of pitavastatin was reduced in NAR animals which exhibit lower expression of albumin, indicating that albumin effect on hepatic uptake was not an experimental artifact, but a pertinent mechanistic process in vivo. This finding provides further rationale to continue studying the potential mechanism of the interplay between albumin and Oatps. There are already several hypotheses that have been proposed to describe the interplay between albumin and Oatps. While this work is not able to provide additional insight into the various proposals, data showing that both albumin and glutathione are able to augment the hepatic uptake of bilirubin (Inoue et al., 1985), suggests that the observed interaction may not be restricted to albumin and Oatps. It is conceivable that there are other plasma proteins such as alpha-acid glycoprotein that can enhance the activity of additional transporters facing the blood compartment. In addition, the impact of albumin facilitated uptake of Oatp substrates may be less pronounced for compounds that are not as extensively bound to plasma protein as exemplified with compounds such as with pravastatin (Bowman et al., 2019). As such, experiments are ongoing in this laboratory to investigate some of these potential interactions with expanded set of compounds across various compartments

including the blood-brain-barrier and the kidneys, and to study whether modulation of plasma protein binding can in fact exhibit meaningful clinical relevance in the disposition of drugs.

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Authorship contribution

Participated in research design: Chang, Chen, Cheong, Pang

Conducted experiments: Chen, Cheong, Jones, Pang

Performed data analysis: Chang, Chen, Cheong, Jones, Pang

Wrote or contributed to the writing of the manuscript: Chang, Chen, Cheong, Jones, Pang

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Figure Legends

Figure 1. CL_{int} of pitavastatin uptake into F344 rat hepatocytes suspended in buffer, WT plasma or NAR plasma (N=4). Yellow bar represents hepatocytes reconstituted in buffer, purple bar represents hepatocytes reconstituted in WT plasma and orange bar represents hepatocytes reconstituted in NAR plasma. Data are presented as mean $\pm$ SD. Star "*" represents statistical difference of $p < 0.0005$ based on one-way ANOVA, and "n.s." is not significant.

Figure 2. Free concentration-time profile of warfarin following 1 mg/kg IV administration in A) WT and B) NAR animals (N=3). Circle with solid green line represents plasma, square with solid maroon line represents brain and triangle with broken blue line represents liver. Data are presented as mean $\pm$ SD.

Figure 3. Free concentration-time profile of pitvastatin following 1 mg/kg IV administration in A) WT and B) NAR animals (N=3). Circle with solid green line represents plasma and triangle with broken blue line represents liver. Data are presented as mean $\pm$ SD.

Figure 4. Log of free tissue-to-free plasma ratio ($K_{p,u,u}$) at 0.25, 0.5, 2 and 8 hr for A) warfarin in brain, B) warfarin in liver, and C) pitavastatin in liver. Pitavastatin brain $K_{p,u,u}$ is not available since the concentrations were BLQ. Orangebars represent WT and purple bars represent NAR animals. Data are presented as mean $\pm$ SD. Star "*" represents statistical difference of $p < 0.05$ based on unpaired t-test.

Table 1: Summary of fraction unbound (fu) values of warfarin and pitavastatin in plasma, brain and liver of F344 (WT) and NAR animals.

	$f_{u,plasma}$		$f_{u,brain}$		$f_{u,liver}$	
	NAR	F344	NAR	F344	NAR	F344
Warfarin	0.43	0.0031	0.33	0.24	0.097	0.078
Pitavastatin	0.078	0.0079	0.022	0.022	0.018	0.023

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Table 2: Summary of total $AUC_{0\text{-}8hr}$, free $AUC_{0\text{-}8hr}$ and $K_{p,u,u}$ of warfarin and pitavastatin in plasma, brain and liver in F344 (WT) and NAR animals (N=3). The $AUC_{0\text{-}8hr}$ was a composite constructed from 3 independent measurements. Units for brain and liver $AUC_{0\text{-}8hr}$ is nmol/g*hr; units for plasma $AUC_{0\text{-}8hr}$ is µM*hr. BLQ=below the limit of quantitation; NA=not applicable.

		WT			NAR		
		Total $AUC_{0\text{-}8hr}$	Free $AUC_{0\text{-}8hr}$	$K_{p,u,u}$	Total $AUC_{0\text{-}8hr}$	Free $AUC_{0\text{-}8hr}$	$K_{p,u,u}$
Warfarin	Brain	3.1	0.73	0.89	1.7	0.57	0.43
	Liver	89	6.9	8.4	60	5.9	4.4
	Plasma	260	0.82	-	3.1	1.3	-
Pitavastatin	Brain	BLQ	BLQ	NA	BLQ	BLQ	NA
	Liver	40	0.93	110	20	0.36	18
	Plasma	1.1	0.0088	-	0.26	0.020	-

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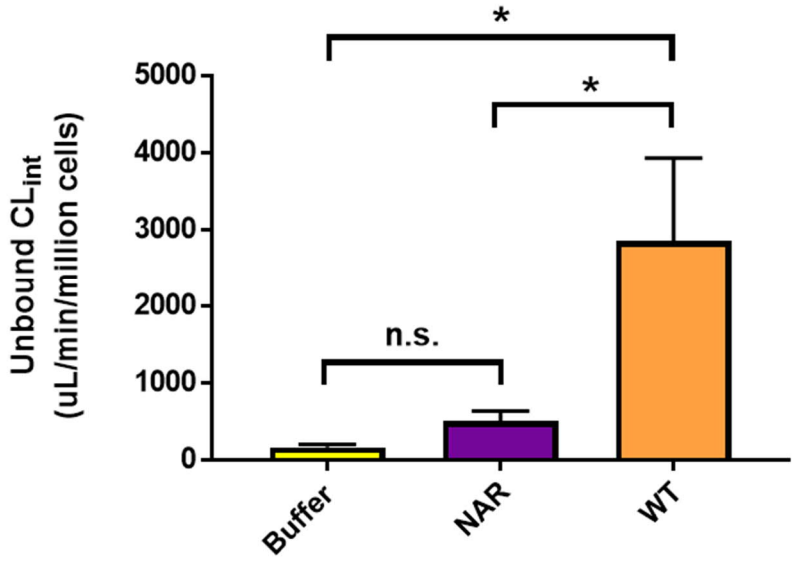

Figure 1

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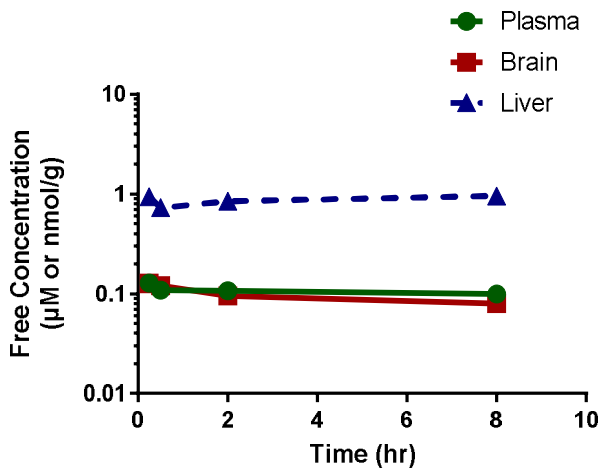

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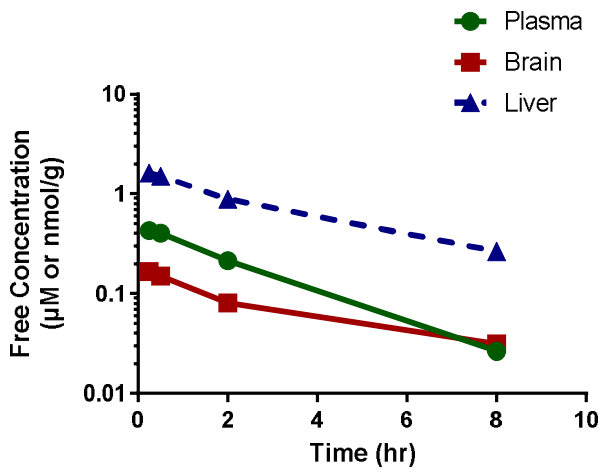

Figure 2

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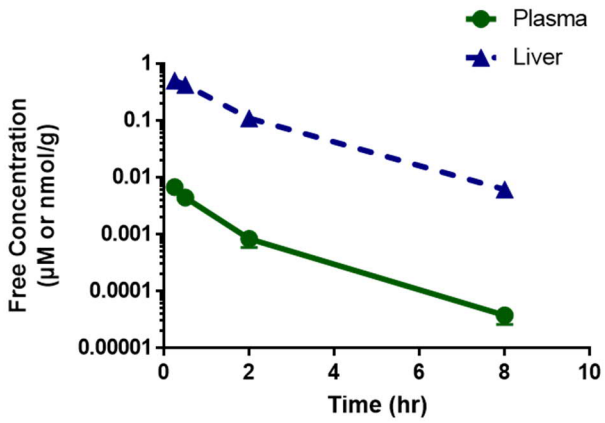

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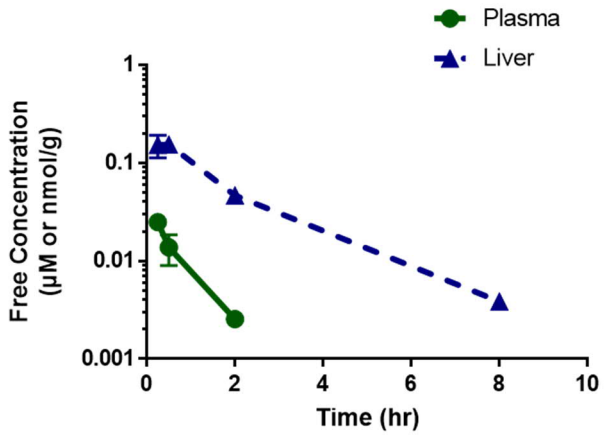

Figure 3

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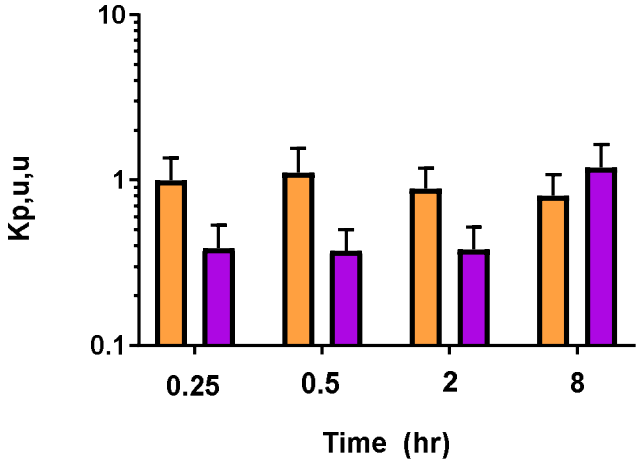

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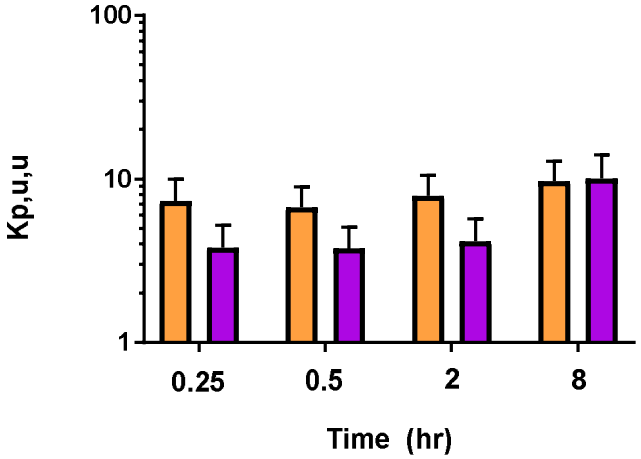

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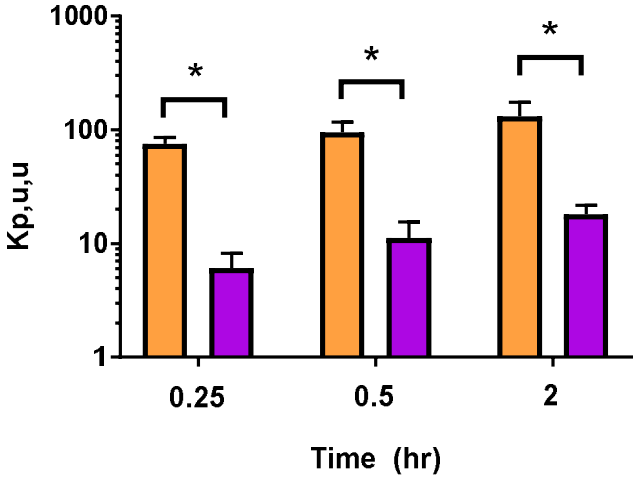

Figure 4