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P-Glycoprotein-Based Loperamide–Cyclosporine Drug Interaction at the Rat Blood–Brain Barrier: Prediction from *In Vitro* Studies and Extrapolation to Humans

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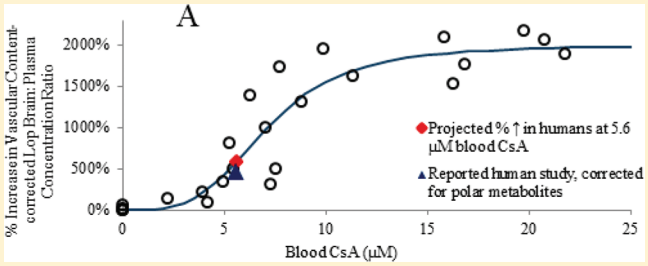
ABSTRACT: We have shown that the rat can quantitatively predict the verapamil–cyclopsorine A (CsA) drug–drug interaction (DDI) at the human blood–brain barrier (BBB). In addition, the potency (EC_{50}) of CsA to inhibit rat BBB P-gp can be predicted from *in vitro* studies in MDRI-transfected cells. To assess if these excellent agreements extend to other substrates, we determined the magnitude of P-gp-based DDI at the rat BBB between loperamide (Lop) or its metabolite, *N*-desmethyl Lop (dLop), and escalating CsA blood concentrations. The percent increase in the brain:blood Lop concentration ratio was described by the Hill equation, E_{max} = 2000%, EC_{50} = 7.1 μM and γ=3.7. The potency (EC_{50}) of CsA to inhibit P-gp at the rat BBB was independent of the substrate used (verapamil, Lop, or dLop). Like the verapamil–CsA DDI, the potency (EC_{50}) of CsA to inhibit rat BBB P-gp could be predicted from studies in MDRI-transfected cells. When ^{11}C-Lop was coadministered with a 10 mg/kg iv infusion of CsA[1]yielding ~5.6 uM CsA blood concentration to healthy volunteers, the brain distribution of ^{11}C-radioactivity was increased by 110%.[1] When corrected for diffusible Lop metabolite(s), this translates into an increase in ^{11}C-Lop brain distribution of 457%. Based on our rat data, we estimated a similar value at 5.6 μM blood CsA concentration, 588% increase in Lop brain distribution. These data support our conclusion that the rat is a promising model to predict P-gp based DDI at the human BBB.



KEYWORDS: *P-glycoprotein, blood–brain barrier, cyclosporin A, loperamide, N-desmethyl loperamide, drug–drug interaction, in vitro to in vivo correlation*

INTRODUCTION

P-glycoprotein (P-gp), an ATP-dependent efflux transporter, is recognized as an important component of the blood–brain barrier (BBB).[2] Localized at the luminal membrane of brain endothelial cells,[3] P-gp protects the brain by restricting the entry of harmful compounds and drugs.[2] The importance of P-gp at the BBB has been demonstrated in numerous small animal studies, mostly in mice and rats. In rodents, when the genes encoding P-gp (*mdr1a/b*) are ablated or P-gp is chemically inhibited, the brain distribution of numerous chemically diverse drugs is increased from 800 to 3600%.[3−7] While these rodent studies raise concerns for clinically significant drug–drug interactions (DDI) at the human BBB, these concerns are only relevant if the rodent is truly predictive of DDI at the human BBB. Until recently, it was not possible to test this hypothesis due to our inability to quantitatively and noninvasively measure drug distribution across the human BBB. Using noninvasive, quantitative, positron-emission tomography (PET) to assess P-gp-based DDI at the human BBB, we reported excellent quantitative correlation between the rat and the human for the verapamil–cyclosporin A (CsA) DDI.[8,9] At identical CsA blood concentration (~3 μM), the increase in brain:plasma concentration ratio of labeled verapamil in the human and the rat was 79 and 75% respectively. In addition, the potency of CsA to inhibit rat BBB P-gp was virtually identical to its potency to inhibit human P-gp in MDRI-transfected cells. Despite these excellent correlations, the study examined only one P-gp substrate–inhibitor pair. Therefore, studies in the rat and the human, with other P-gp substrates and inhibitors, are needed to determine if DDI at the rat BBB are predictive of those at the human BBB. This is particularly important for P-gp because it demonstrates allostery with multiple binding sites and therefore has the potential for substrate-dependent DDI.[10−15]

To assess if the above excellent agreements extend to other substrates, we determined the magnitude of P-gp-based DDI at the rat BBB between loperamide (Lop) or its metabolite, *N*-desmethyl Lop (dLop), and escalating CsA blood concentrations. In addition, we determined if the potency of CsA to inhibit P-gp at the rat BBB correlates with that obtained in MDR1-transfected cells. Using a similar study design as that of our ^{11}C-verapamil–CsA PET study, Passchier et al. showed that ^{11}C-Lop CNS distribution increased 110% when coadministered with a 10 mg/kg iv infusion of CsA.[1] Therefore,

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this study provides us an opportunity to determine whether the rat is predictive of this DDI at the human BBB.

METHODS

Materials. Loperamide (Lop) was purchased from Sigma-Aldrich (St. Louis, MO). *N*-Desmethyl loperamide (dLop) was a kind gift from GlaxoSmithKline. Cyclosporin A (Sandimune, 50 mg/mL) was purchased from Abbott Laboratories (Chicago, IL). Lop was formulated as 5 mg/mL in 650 mg of Cremophore EL and 32.9% ethanol by volume. All other reagents were of the highest grade available from commercial sources.

Animals. Male Sprague–Dawley rats (8–10 weeks, ~300 g) were purchased from Taconic Farms (Hudson, NY) and housed in a temperature- and humidity-controlled room with a 12 h light/dark cycle with free access to food and water. The experimental protocol was approved by the Institutional Animal Care and Use Committee of the University of Washington. All experimental procedures were conducted according to the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council, Washington, DC, 1996).

Experimental Protocol. Under isoflurane anesthesia (5% induction, 1–1.5% maintenance at 1.0 L/min), each animal was cannulated in both the left or right femoral artery and vein. Anesthesia was maintained throughout the experiment. The anesthesia plane and the condition of the animal were evaluated by a routine tail/toe pinching test, respiration rate and the palpebral reflex test. CsA (or saline) (0, 1.3, 2.3, 3.5, 5.0, 7.0, or 10.0 mg/kg in 0.1 mL) and Lop (3 mg/kg in 0.1 mL) were administered as an iv bolus, followed by a constant rate iv infusion of CsA plus Lop (0, 1.6, 3.0, 4.5, 6.5, 9.0, or 13.0 mg/kg of CsA plus 0.3 mg Lop/kg/h at the rate of 0.5 mL/h) via the femoral vein to achieve pseudo-steady-state blood CsA concentrations of 0, 2.7 (3.3), 4.2 (5.0), 6.0 (7.2), 8.3 (10), 12.0 (14.4), and 15.0 (18.0) μM (μg/mL) respectively. CsA blood samples (~0.5 mL) were collected in heparinized tubes via the femoral artery at 0 (pre-CsA), 30, 45, and 60 min. Hematocrit in each blood sample (~50 μL) was determined immediately following each blood draw. Immediately following the 60 min blood draw, the animal was sacrificed by decapitation and the brain harvested. This time point was chosen based on reported Lop pharmacokinetic studies in rats[16,17] along with our pilot study in rats. Collectively, the literature and our pilot study results indicate that this duration was adequate for Lop and its metabolite, dLop, to reach pseudoequilibrium between the brain and plasma, and exhibit linear pharmacokinetics at the Lop and dLop plasma concentrations studied.[16−18] Blood CsA concentrations were determined within 24 h by LC/MS (Department of Laboratory Medicine at the University of Washington Medical Center), while the brain and plasma samples were stored at −20 °C until analysis for Lop/dLop concentration by LC/MS.

Plasma and Brain Tissue Analysis. Brain samples (0.4–0.7 g) were homogenized with PBS (100 μL of PBS per 100 mg of brain). The plasma and brain homogenate (100 μL) samples were precipitated with 1:1 acetonitrile containing 500 ng/mL of saquinavir (internal standard), followed by centrifugation at 20800*g*. The supernatant was then injected (5–35 μL) onto an Agilent XDB-C18 reverse-phase column (2.1 × 50 mm, 5 μm, with an Agilent XDB-C18 guard column, 2.1 × 12.5 mm, 5 μm; Agilent Technologies, Santa Clara, CA) eluted at 0.25 mL/min with a gradient mobile phase consisting of mobile phase A, 0.1% acetic acid in water, and mobile phase B, 1:1 methanol:acetonitrile. The gradient was adjusted linearly as follows: 65% A/35% B for the first 1 min, 5% A/95% B from 1 to 4 min, maintained at 5% A/95% B from 4 to 6.4 min, 65% A/35% B from 6.4 to 6.5 min, and maintained at this composition from 6.5 to 7.5 min. The mass spectrometer was operated in atmospheric pressure ionization electrospray (API-ES) mode (spray chamber: gas temp 350 °C, Vcap (+) 3500 V). The standard curve contained 0.31 to 625 ng/mL Lop and dLop in plasma. Quality control samples of 1.2, 20, and 313 ng/mL Lop and dLop were prepared in both plasma and brain homogenate. The limit of detection for Lop and dLop in tissue and plasma samples was 0.6 ng/g or 0.6 ng/mL respectively.

Data Analysis. The brain:plasma ratio of Lop and dLop was adjusted for vascular contamination. We previously found that the brain vascular volume in the rat is 26.3 ± 10 μL/g.[8] Using this value, we estimated the vascular content of Lop/dLop in each brain sample. Next, the brain Lop/dLop concentration for each animal was corrected for the corresponding contamination from the vascular Lop/dLop content. Using nonlinear regression (WinNonlin; Pharsight Corporation), the Hill equation was fitted (using uniform weighting) to the percent increase in the brain:plasma ratio of Lop or dLop as a function of blood CsA concentration. Unless otherwise stated, data are presented as mean ± SD. Analysis of variance, followed by Student's *t* test, was used to determine the statistical significance of the difference ($p < 0.05$) between experimental groups.

RESULTS

Except at the higher CsA infusion rates, the targeted pseudo-steady-state blood CsA concentrations were achieved before the experimental end point (60 min) (Figure 1). The blood CsA

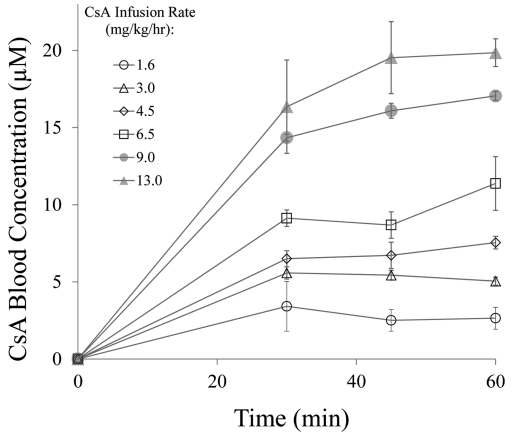

Figure 1. Except at the higher CsA infusion rates, the expected pseudo-steady-state blood CsA concentrations (mean ± SD of at least $n = 3$) were achieved by 30 min after an iv loading dose of CsA followed by an iv infusion of CsA.

concentrations achieved at the higher targeted CsA groups (11.4 ± 1.7, 17.1 ± 0.3 and 19.9 ± 0.9 μM) differed from the expected values (8.3, 12.0, and 15.0 μM, respectively), most likely due to nonlinearity in the pharmacokinetics of CsA.

Targeted pseudo-steady-state plasma Lop and dLop concentrations were also achieved and maintained for the duration of the experiment (Figure 2). The presence of CsA did not significantly affect the pseudo-steady-state plasma Lop

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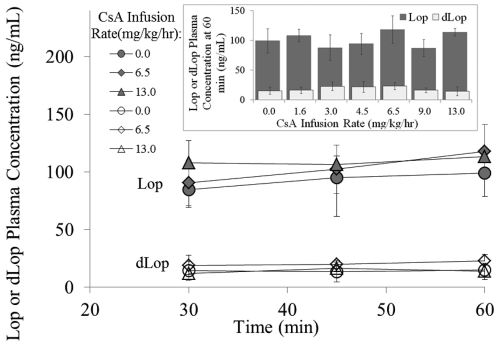

Figure 2. Target Lop (shaded circle, diamond and triangle) and dLop (open circle, diamond and triangle) pseudo-steady-state plasma concentrations (mean ± SD of at least $n = 3$) were achieved by 30 min and maintained thereafter. For clarity, only three CsA-infusion-rate groups are shown. Inset: Lop (shaded bar) and dLop (open bar) steady-state plasma concentrations (mean ± SD at 60 min) were not significantly affected by the presence of CsA (Student's t test).

or dLop concentrations (Student's t test) at the experimental end point (60 min) (Figure 2 inset).

In the absence of CsA, the brain:plasma concentrations for Lop and dLop were 0.10 ± 0.02 ($n = 5$) and 0.14 ± 0.04 ($n = 5$), respectively. When corrected for vascular content, the brain:plasma concentrations for both Lop and dLop changed modestly to 0.07 ± 0.02 ($n = 5$) and 0.11 ± 0.04 ($n = 5$), respectively. With increasing CsA blood concentration, the percent corrected Lop and dLop brain:plasma concentration ratio increased in a sigmoidal fashion (Figure 3A and 3B). Fitting the Hill equation to the data yielded the following estimates (% CV of the estimate) for Lop and dLop respectively: E_{max} = 2000% (8.5%), EC_{50} = 7.1 μM (8.6%), γ = 3.7 (28.9%) and E_{max} = 2200% (1.3%), EC_{50} = 6.9 μM (0.4%), γ = 4.8 (1.2%) (Figure 3).

DISCUSSION

Similar to the verapamil–CsA study,[8] vascular content correction had little effect on the magnitude of brain distribution of Lop or dLop in the absence of CsA. In addition, the maximum increase in the brain distribution of Lop (2000%) or dLop (2200%) produced by CsA was comparable to that of the CsA–verapamil interaction (2630%).[8]

In order to compare our rat data to the human PET data of Passchier et al.,[1] the blood CsA concentrations achieved in the human study should be available. Unfortunately they are not. Therefore, we estimated these concentrations based on our human ^{11}C-verapamil–CsA DDI study. Since the iv infusion rate of CsA in the Passchier study was double that in our study, based on linear pharmacokinetics, we predicted that the average blood CsA concentration achieved in their study was 5.6 μM. At 5.6 μM blood CsA concentration, our model predicted a 588% increase in the brain distribution of Lop (Figure 3A, shaded diamond). This estimated percent increase is much higher than the 110% observed in the CsA–Lop PET human study.[1] However, this discrepancy between our rat study and the reported human study is likely due to significant metabolism of Lop in humans. In our rat study, we measured the unchanged Lop concentrations in the brain and plasma, while in the human PET study, imaging is unable to distinguish between labeled Lop and its labeled metabolites. Thus, we estimated the brain concentration of the unchanged Lop in the PET study based on the following data. First, Passchier et al.

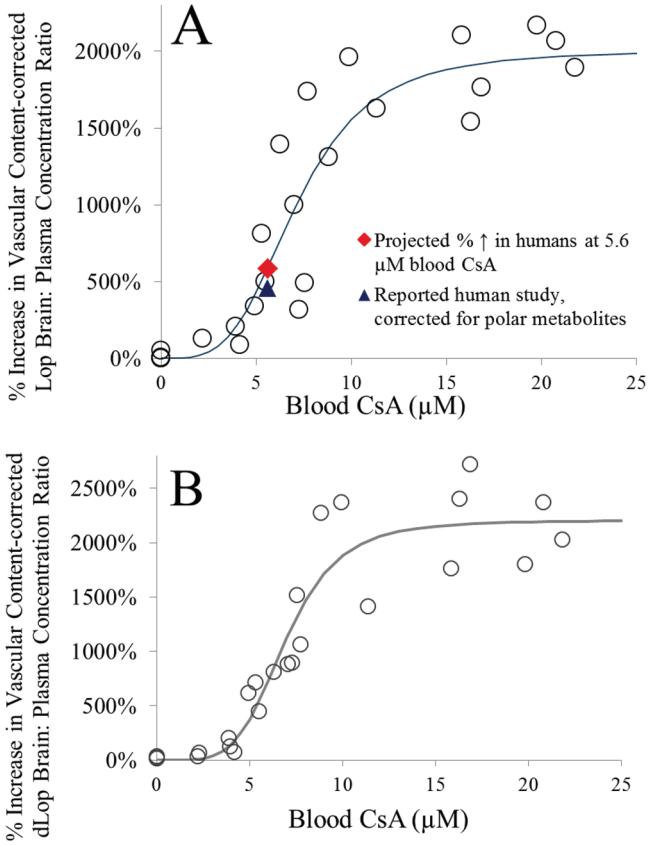

Figure 3. With increasing CsA blood concentrations, the percent increase in the vascular-content corrected brain:plasma Lop (A) or dLop (B) concentration ratio, expressed relative to that in the control group (absence of CsA), increased as the blood CsA concentration increased. (A) Fitting the Hill equation to the Lop data yielded the following estimates (% CV of the estimate): E_{max} = 2000% (8.5%), EC_{50} = 7.1 μM (8.6%), and γ = 3.7 (28.9%). For the reported PET study by Passchier et al. (estimated blood CsA concentration of 5.6 μM), the rat model predicted a 588% increase in the Lop brain:plasma concentration ratio (filled diamond) versus the observed 110% increase in total brain:plasma radioactivity. However, on correcting the latter for the labeled diffusible metabolites, the predicted increase in Lop brain:plasma concentration ratio (457%; filled triangle) was comparable with that predicted by the rat model. (B) Fitting the Hill equation to the dLop data yielded the following estimates (% CV of the estimate): E_{max} = 2200% (1.3%), EC_{50} = 6.9 μM (0.4%), and γ = 4.8 (1.2%). The consistency in CsA EC_{50} values between Lop, dLop and verapamil (EC_{50} 7.9 μM) indicates that CsA inhibits P-gp in a substrate-independent manner.

reported that 76% of the radioactivity in the plasma was due to ^{11}C-Lop and 24% was due to its metabolites (presumably polar metabolites).[1] Second, the brain:plasma concentration ratio of the Lop polar metabolites in the mouse is ~0.54.[16,19–21] Assuming that the same is true in humans and that, in the absence of P-gp inhibition, the human brain:plasma ratio of ^{11}C-Lop is similar to that in mice, one can correct the observed 110% increase for the presence of polar labeled metabolites in the human brain which would also be imaged in the PET study. When such a correction is made, the corrected but estimated increase in the ^{11}C-Lop brain:plasma ratio at 5.6 μM CsA blood concentrations is 457%, a number similar to the value predicted by our rat data (588%, Figure 3A). Several assumptions were made in arriving at this metabolite-corrected estimate. A rigorous ^{11}C-Lop PET study needs to be conducted in humans

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to test some of these assumptions. If our predictions are correct, then CNS toxicity of Lop may occur when P-gp at the human BBB is significantly inhibited. In a recent review, Vandenbossche and co-workers examined 595 postmarket Lop case reports and 10 clinical Lop drug interaction studies[22] but found no evidence of clinically significant drug interactions with Lop. However, these studies may not have achieved unbound plasma concentration of the P-gp inhibitor/perpetrator that significantly inhibits P-gp at the human BBB.

CsA was equipotent in inhibiting the *in vivo* efflux of verapamil, Lop or dLop across the rat BBB (EC_{50} [mean ± SD of the estimate] 7.9 ± 0.6, 7.1 ± 0.6 and 6.9 ± 0.4 μM and γ 3.7 ± 0.7, 3.7 ± 1.1, and 4.8 ± 0.6 respectively).[8] Although the CsA EC_{50} and γ appear to be independent of the substrate used, we have recently reported substrate dependence in a number of model P-gp inhibitors such as quinine and itraconazole[23] in an *in vitro* P-gp drug interaction study. In that study, depending on the probe substrate used (verapamil-bodipy or prazosin-bodipy), the potency (EC_{50}) of quindine, itraconazole and verapamil to inhibit P-gp differed by 20-, 10- or 2-fold, respectively.[23] Given these data from our laboratory and the reported variable sensitivity of dLop to P-gp modulators *in vivo*,[24] additional animal studies using Lop as the substrate and quinidine or itraconazole as the inhibitor seems to be the next logical step to further investigate the substrate dependency of P-gp inhibition *in vivo*. A value of γ (Hill coefficient) >1 is suggestive of possible P-gp allosterism. This is especially plausible for P-gp given its large drug binding pocket and multiple drug binding sites.[10,23,24] However, other factors may play a role in *in vivo* studies in giving rise to a γ of >1.

The comparable CsA E_{max} between the Lop−CsA, dLop−CsA, and verapamil−CsA studies suggest that the role of P-gp and other processes (e.g., passive diffusion) in the brain distribution of these three drug substrates is similar. However, if the drug substrate has a higher affinity for P-gp (i.e., lower brain distribution in the absence of P-gp activity) and/or if P-gp plays a larger role in preventing the brain distribution of that drug (vs other processes such as diffusion), then the impact of inhibiting P-gp by CsA may be much greater on its brain distribution than that for Lop, dLop or verapamil. Based on a survey of the literature, P-gp appears to play a larger role in excluding nelfinavir from the brain. Nelfinavir has one of the largest increases in the brain:blood concentration ratio in *mdr1a/b(−/−)* vs wild-type mice (3400% increase, versus 900% for verapamil).[5,7] Thus nelfinavir seems a logical P-gp substrate for a future study on the maximum magnitude of change in the brain distribution of a P-gp drug in the presence of an inhibitor. Studying the CsA−nelfinavir interaction at the BBB will also provide another opportunity to determine if such an interaction is substrate dependent (i.e., CsA EC_{50}).

For this study, our *in vivo* unbound CsA EC_{50} for Lop (calculated to be 0.50 ± 0.04 μM) was remarkably consistent with the *in vitro* unbound EC_{50} (0.78 ± 0.04 μM) reported by others[25] (unbound EC_{50} value was computed using the reported CsA fraction unbound of 7% in the rat (Bernareggi and Rowland, 1991)). This is consistent with the finding in our prior *in vitro−in vivo* correlation study using verapamil−CsA as the P-gp substrate pair (CsA *in vitro* EC_{50} of 0.6 ± 0.3 μM vs *in vivo* unbound EC_{50} of 0.47 ± 0.004 μM at the rat BBB[26]). The present data provide further support to the notion that inhibition of P-gp at the rat BBB is consistent with that observed with human P-gp expressed in LLCPK1-MDR1 cell line.

In summary, the above CsA−Lop interaction provides support to our conclusion based on our previous CsA−verapamil data that the rat is a promising model to predict P-gp based drug interactions at the human BBB. In addition, the potency (EC_{50}) of CsA to inhibit P-gp appears to be substrate independent when Lop, dLop or verapamil is used as the substrate. Furthermore, there is a remarkable *in vitro* (LLCPK1-MDR1 cells) to *in vivo* correlation of the potency of CsA to inhibit P-gp. However, given the dearth of human data available, additional human studies with other substrate−inhibitor pairs are needed to further validate whether the rat, together with *in vitro* studies in LLCPK1 cells, can be used to predict the magnitude of P-gp drug interactions at the human BBB.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

P-gp, P-glycoprotein; BBB, blood−brain barrier; CsA, cyclosporin A; Lop, loperamide; dLop, *N*-desmethyl loperamide; PET, positron emission tomography; DDI, drug−drug interaction

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