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In Vitro to *In Vivo* Prediction of P-glycoprotein Based Drug Interactions at the Human and Rodent Blood-Brain Barrier

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Running Title Page

Running Title: Predicting P-gp based interactions at the human BBB

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Abbreviations: P-gp, P-glycoprotein; BBB, blood-brain-barrier; CsA, cyclosporine A; PET, positron emission tomography.

ABSTRACT

In vitro inhibition of P-glycoprotein (P-gp) expressed in cells is routinely used to predict the potential of *in vivo* P-gp drug interactions at the human blood-brain barrier (BBB). The accuracy of such predictions has not been confirmed because methods to quantify *in vivo* P-gp drug interactions at the human BBB have not been available. With the development of a non-invasive Positron Emission Topography (PET) imaging method by our laboratory to determine P-gp based drug interactions at the human BBB, an *in vitro–in vivo* comparison is now possible. Therefore, we developed a high throughput cell-based assay to determine the potential of putative P-gp inhibitors (including cyclosporine A; CsA) to inhibit (EC_{50}) the efflux of verapamil-bodipy, a model P-gp substrate. LLCPK1-MDR1 cells, expressing recombinant human P-gp, or control cells lacking P-gp (LLCPK1) were used in our assay. Using this assay, quinine, quinidine, CsA and amprenavir were predicted to be the most potent P-gp inhibitors *in vivo*, at their respective therapeutic maximal unbound plasma concentrations. The *in vitro* EC_{50} of CsA (0.6 μM) for P-gp inhibition was virtually the same as our previously determined *in vivo* unbound EC_{50} at the rat BBB (0.5 μM). Moreover, at 2.8 μM CsA (total blood concentration), our *in vitro* data predicted an increase of 129% in [^{11}C]-verapamil distribution into the human brain, a value similar to that observed by us (79 %) using PET. These data suggest that our high throughput cell assay has the potential to accurately predict P-gp drug interactions at the human BBB.

INTRODUCTION

The *in vivo* importance of P-glycoprotein (P-gp) at the BBB has been well-demonstrated by studies in mdr1a/b (-/-) mice. For example, compared to the wild type mouse, in the mdr1a/b (-/-) mice, the brain:plasma concentration ratio (or the brain uptake) of the anti-HIV protease inhibitors is increased 7- to 36-fold, anti-cancer taxanes, paclitaxel or docetaxel are increased 6- to 28-fold, while that of verapamil is increased 8.5-fold (Endres et al., 2006). Similar data have been obtained in mice and rats where P-gp has been chemically ablated with selective inhibitors of P-gp such as PSC 833 (Valspodar), GF120918 (Elacridar), and LY335979 (Zosuquidar) (Lin and Yamazaki, 2003; Endres et al., 2006). For example, the brain:plasma ratio of verapamil is increased 24.1-fold when the rat is pretreated with cyclosporine A (CsA) (Hendrikse and Vaalburg, 2002). Based on these data and others, it has been widely postulated that P-gp plays a vital role in limiting drug distribution at the human BBB and that P-gp based drug interactions will result in profound increase in brain concentrations of the affected drugs and therefore their CNS efficacy or toxicity.

Although rodent studies make a compelling case for the importance of P-gp at the BBB in the CNS distribution of drugs, their ability to predict the magnitude of P-gp based drug interactions at the human BBB has not been investigated. Due to safety and ethical reasons, it has not been possible to measure *in vivo* human BBB P-gp activity. With the development by our laboratory of a non-invasive, **P**ositron **E**mission **T**opography (PET) imaging method to measure P-gp-based drug interactions at the human BBB, a quantitative comparison of drug interactions at the brain P-gp barrier is now possible (Sasongko et al., 2005; Hsiao et al., 2006).

In our PET imaging study, the brain distribution of [^{11}C]-verapamil, a well established P-gp substrate, was quantitatively monitored in healthy subjects, in the presence and absence of the P-gp inhibitor, CsA. The results showed that P-gp at the human BBB limits the entry of [^{11}C]-verapamil into the brain. In the absence of CsA, [^{11}C]-radioactivity AUC_{brain}:AUC_{blood} ratio was 0.55 $\pm$ 0.03,

and increased to 1.02 $\pm$ 0.05 in the presence of CsA. CsA almost doubled the entry of [^{11}C]-radioactivity into the brain by inhibiting P-gp (Sasongko et al., 2005). Consistent with this result, in rats and at the same blood concentrations of CsA as those achieved in humans (~3 μM), P-gp inhibition at the rat BBB was modest with excellent quantitative correlation with that obtained in humans (75% vs. 79% increase in total [^{3}H]-radioactivity distribution into the brain for rats and humans, respectively (Hsiao et al., 2006)).

While the above correlation is excellent, it is a correlation of *in vivo* rodent data with *in vivo* human data. Therefore, we asked whether such interactions could be predicted from *in vitro* cell based assay, which is high-throughput, simple, and cost-effective. In this communication, we report the inhibition of P-gp by CsA and several other inhibitors, in stable LLCPK1 cells expressing recombinant MDR1 gene (LLCPK1-MDR1) (Woodahl et al., 2004), using verapamil-bodipy as a substrate. Then, we determined if the EC_{50} of P-gp inhibition by CsA in this cell system was predictive of the EC_{50} previously observed by us at the rat BBB (Hsiao et al.). In addition, we asked if the *in vitro* LLCPK1-MDR1 cells could quantitatively predict the inhibition of P-gp observed by us *in vivo* at the human BBB using PET and [^{11}C]-verapamil (Sasongko et al).

MATERIALS AND METHODS

Materials. Bodipy® FL verapamil, hydrochloride, culture media, fetal calf serum, medium supplements, antibiotics were purchased from Invitrogen (Carlsbad, CA). All other reagents were of the highest grade available from commercial sources.

Cell Culture. Stable LLCPK1 cells expressing recombinant MDR1 (LLCPK1-MDR1) or control cells (no detectable P-gp expression) were grown in complete media consisting of RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum and 1% (v/v) antibiotic-antimycotic and grown at 37°C in the presence of 5% CO_2. The characteristics of these cells, including P-gp expression in the LLCPK1-MDR1 and the control cells, has been described elsewhere (Woodahl et al., 2004).

Verapamil-bodipy Accumulation Assay. Cells with passage number 12 to 40 were plated at a density of 1×10^5 cells/well (100 μL per well) on a 96-well plate (Corning) and grown overnight. The cells were washed with PBS followed by incubation for 1 h at 37ºC in serum free media in the presence of 5% CO_2 containing 0.3μM verapamil-bodipy, 0.2% dimethyl sulfoxide (DMSO) and varying concentration of P-gp inhibitors (10 concentrations of each inhibitor with each concentration conducted in quadruplicate). After washing and replacing with PBS, the verapamil-bodipy content of cells were measured by a fluorescence plate reader (ex/em: 485/535 nm).

Data Analysis. Using nonlinear regression (WinNonlin®; Pharsight Corporation), the Hill equation was fit to the fold-increase in intracellular fluorescence (relative to that observed in the absence of the inhibitor) as a function of increasing inhibitor concentration. The mean EC_{50} of each inhibitor was determined from at least 3 independent experiments. Unless otherwise stated, data are presented as mean ± S.D.

Comparison with previously published *in vivo* rat and human PET data. We have previously published studies (Sasongko et al., 2005; Hsiao et al., 2006) where we determined the

inhibition of P-gp mediated radiolabeled-verapamil efflux across the BBB by CsA. In the rat study, we determined the EC_{50} of CsA P-gp inhibition at increasing pseudo steady-state CsA blood concentrations, while in the human study; we determined P-gp inhibition at the BBB at a single steady-state blood CsA concentration of 2.8 μM.

In the rat study, anesthetized male Sprague Dawley rats were administered i.v. infusion of CsA to achieve increasing pseudo steady-state blood concentrations until maximal P-gp inhibition was achieved. An i.v. bolus dose of [^{3}H]-verapamil (~14 μCi) was administered when pseudo steady-state blood CsA concentration was achieved (n = 5 or greater per each concentration group). The animals were sacrificed 20 minutes post [^{3}H]-verapamil dose administration to determine blood, plasma and brain [^{3}H]-radioactivity by scintillation counting. Details of the sampling scheme, specimen processing techniques, and data analysis procedures are described thoroughly in our previously published study (Hsiao et al., 2006).

For the human study, similar experimental conditions were employed as the rat study, except that the distribution of [^{11}C]-radioactivity in the brain was measured at a single pseudo steady-state blood CsA concentration of 2.8 μM using PET. [^{11}C]-verapamil (~ 0.2 mCi/kg) was administered to healthy volunteers (n = 12, 6 females and 6 males) as an i.v. infusion over ~ 1 min before and after at least 1 h infusion of CsA (2.5 mg/kg/hr). Arterial blood samples and brain PET images were obtained at frequent intervals over 45 min. The brain uptake of ^{11}C-radioactivity (brain:blood at 45 min) was determined in the presence and absence of CsA. For additional details on the methods, the reader is referred to Sasongko et al. (Sasongko et al., 2005).

RESULTS AND DISCUSSION

We had previously determined that a seeding density of 1×10^5 cells/100 μL media/well with 24 hr incubation in the Corning Costar plates was necessary to form a confluent monolayer. The monolayer confluency was critical in minimizing well-to-well variability since the fluorescence plate reader only measure a small region inside each well. Based on a prior optimization study carried out over a period of 4 hours with 15 minutes measuring time intervals, one hour incubation time was necessary for the verapamil-bodipy and the inhibitors to achieve maximal inhibition of efflux. Similarly, based on a study using concentrations ranged from 0.1 to 5 μM, the optimal verapamil-bodipy incubation concentration was determined to be 0.3 μM to allow the range of intracellular fluorescence accumulations to fall within the linear range of the fluorescence plate reader (0 to 1.2×10^6 fluorescence relative units).

The EC_{50} of each inhibitor was determined based on 3 or more independent experiments (Table 1). The Hill equation was fit to the fold-increase in intracellular fluorescence as a function of increasing inhibitor concentration to estimate the E_{max}, the EC_{50} and the Hill coefficient (γ). The observed EC_{50} values were specific to P-gp as no or minimal change in intracellular fluorescence was observed in the control cells. Although the EC_{50} of many of these drugs have been previously determined, the reported values (Table 1) have a huge variability. This makes it impossible to utilize these values for *in vitro* to *in vivo* prediction of drug interactions. Hence, we determined the EC_{50} of these drugs in our own laboratory using a single methodology and a single transfected cell line.

The *in vivo* potency of an inhibitor will be determined by the ratio of the maximal therapeutic plasma concentration of the drug and EC_{50}. Therefore, this ratio was calculated to provide a rank order of "potency" of the drugs to produce P-gp based drug interaction at the human BBB. This ratio can be computed for either the total or unbound therapeutic plasma concentration

of the inhibitor. Since it is not clear which should be used (Endres et al., 2006), we computed both these ratios. When the total plasma concentrations were used, the drugs predicted to be potent inhibitors of P-gp at the human BBB ranked as follows: tipranavir>quinine>quinidine>lopinavir>ketoconazole=itraconazole>amprenavir (Table 1). When the unbound therapeutic plasma concentrations were used, the rank order changed and became quinine>quinidine>amprenavir>CsA>itraconazole>lopinavir. In addition, the potential for these drugs to inhibit P-gp *in vivo* was considerably reduced. Nevertheless, quinine and quinidine were predicted to be potent inhibitors of P-gp activity *in vivo*.

The *in vitro* EC_{50} of CsA (0.6 ± 0.3 μM) (obtained using protein free media) was remarkably consistent with the *in vivo* unbound EC_{50} of 0.47 ± 0.004 μM at the rat BBB (Table 1 and Fig. 1, the unbound EC_{50} value was computed using the reported CsA fraction unbound of 6% in the rat (Bernareggi and Rowland, 1991; Hsiao et al., 2006)).

In the presence of complete inhibition of P-gp, the maximal increase in verapamil accumulations in the LLCPK1-MDR1 cells is much less (~150% increase, Fig. 1) than the maximal increase in the brain distribution of ^{3}H-verapamil observed in the rat (Endres et al., 2006; Hsiao et al., 2006). This difference is not surprising, as the *in vitro* system does not incorporate binding of verapamil to brain tissue. Therefore, the ratio of the E_{max} of the two systems can be used as a scaling factor to conduct *in vitro* to *in vivo* predictions of verapami-CsA interaction at the human BBB. Such a scaling factor assumes that the E_{max} at the human BBB is similar to that observed at the rat BBB.

We then asked if this *in vitro* data as well as the *in vitro* EC_{50} of CsA, adjusted by the scaling factor, would have predicted the magnitude of *in vivo* P-gp inhibition obtained in humans using PET. In our human PET study (Sasongko et al., 2005), a steady-state CsA blood concentration of 2.8 μM (0.2 μM unbound) resulted in 79% increase in the distribution of [^{11}C]-

radioactivity into the brain. At the same unbound CsA blood concentration, our *in vitro* studies predicted a similar increase of 129% in the human brain:blood radioactivity (Fig. 2).

The above data have demonstrated the utility of our high throughput *in vitro* assay to predict verapamil-CsA interaction at the rat and human BBB. We recognize that these excellent correlations are all based on a single P-gp substrate-inhibitor combination, verapamil-CsA. Similar studies with additional P-gp inhibitors are needed to test if these excellent *in vitro-in vivo* correlations can be extended to other inhibitors (i.e. generalized). For these reason we have determined the potency (EC_{50}) of other drugs to inhibit P-gp at the human BBB (Table 1). Based on these data, the drugs that should be tested in humans are quinine and quinidine. Indeed, quinidine has been shown to increase the CNS effects of loperamide in humans (Sadeque et al., 2000). Such validation with other inhibitors is particularly important for P-gp because P-gp has multiple substrate/inhibitor binding sites (Martin et al., 2000). Thus, it is possible that the [^{11}C]-verapamil-CsA inhibition in our human study might have been more profound if another inhibitor had been used.

In conclusion, this is the first *in vitro* study to quantitatively predict the *in vivo* inhibition of P-gp transport activity at the human BBB. The remarkable agreement between the *in vitro* and the *in vivo* data suggests that our *in vitro* cell culture method has the potential to be an excellent model to predict the *in vivo* inhibition of P-gp at the human BBB. The utility of this high throughput *in vitro* assay, in conjunction with the rat, to predict P-gp based drug interactions at the human BBB appears to be promising. As discussed above, additional *in vitro* and *in vivo* human studies with other inhibitors, such as quinine or quinidine, are needed to further validate this excellent *in vitro* to *in vivo* prediction.

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FIGURE LEGENDS

Fig 1. P-gp inhibition in the LLCPK1-MDR1 cell assay (open circles; mean ± SE and dashed line is the model prediction) is quantitatively predictive of the magnitude of inhibition of P-gp at the human (open triangle) and the rat BBB (gray circles, solid line is the model prediction). Note the x-axis is unbound CsA concentration.

Fig 2. At identical pseudo steady-state CsA blood concentrations of 0 and ~3 μM, the brain: blood ratio of total [^{3}H]-radioactivity in the rat (gray bars, n=15) is virtually identical to that previously obtained by us in humans (open bars; n=12) using PET. At the same unbound CsA blood concentration, our *in vitro* studies predicted an increase in brain:blood radioactivity that is similar to that observed in humans. The numerical values of the percent increase in brain: blood total [^{3}H]-radioactivity (relative to the absence of CsA) produced by pseudo steady-state CsA blood concentrations of ~3 μM are listed above the each bar. The value of n represents the number of subjects/experiments per CsA blood concentration.

TABLES

Table 1. The *in vitro* EC_{50} values of the P-gp inhibitors compared to those reported previously (Ekins et al., 2002; Rautio et al., 2006). The ratios, therapeutic C_{max}/EC_{50} and C_{maxu}/EC_{50}, provide a rank order of the "potency" of the drug to produce drug interactions at the human BBB. Fraction unbound values are based on reported human data and drug package inserts. Based on the FDA guidance of 0.1 or greater (*) therapeutic C_{max}/EC_{50} as the screening criteria, all the drugs listed below, except indinavir, would be classified as potential *in vivo* inhibitors of P-gp. Experimental values are mean±SD of at least three independent experiments.

		EC_{50} (μM) mean±SD	Previous Reported EC_{50} (μM)	E_{max} mean±SD	γ (gamma) mean±SD	Therapeutic C_{max} (μM)	C_{maxu} (μM)	Therapeutic C_{max}/EC_{50}	C_{maxu}/EC_{50}
Cyclosporine A		0.6 ± 0.3	0. 46 ~ 6.2	2.5 ± 0.4	2.0 ± 0.7	1.11	0.078	1.78	0.12
Quinidine		0.9 ± 0.2	2 ~ 55	1.6 ± 0.3	0.8 ± 0.4	8.94	1.16	9.57	1.24
Quinine		1.2 ± 0.3	NA	1.7 ± 0.04	1.1 ± 0.3	18.8	2.44	15.2	1.97
Verapamil		3.8 ± 1.3	1.5~ 61	4.3 ± 0.8	0.8 ± 0.2	0.66	0.07	0.17	0.02
Ketoconanzole		2.8 ± 1.2	0.7 ~ 29	2.9 ± 0.4	0.8 ± 0.1	8.47	0.085	2.98	0.03
Itraconazole		0.3 ± 0.2	0.5	2.0 ± 0.1	1.0 ± 0.2	0.92	0.033	2.98	0.11
HIV inhibitors	Amprenavir	5.8 ± 0.9	> 100	4.2 ± 0.7	1.1 ± 0.2	15.15	1.52	2.61	0.26
	Indinavir	ND	> 50	ND	ND	9.8	3.82	ND	ND
	Nelfinavir	18.1 ± 8.6	1.4 ~ 44	5.6 ± 2.0	1.0 ± 0.2	5.64	0.11	0.31	0.01

	Ritonavir	12.1 ± 5.0	3.8	4.3 ± 1.2	1.6 ± 0.4	15.26	0.31	1.26	0.03
	Saquinavir	15.9 ± 4.1	6.5	4.4 ± 1.2	1.2 ± 0.3	5.64	0.11	0.35	0.01
	Atazanavir	13.2 ± 4.8	NA	1.9 ± 0.1	1.5 ± 0.3	7.66	1.07	0.58	0.08
	Lopinavir	3.1 ± 0.8	NA	1.8 ± 0.1	0.8 ± 0.1	15.59	0.31	5.03	0.10
	Tipranavir	2.9 ± 0.9	NA	1.5 ± 0.3	1.0 ± 0.4	94.8	0.09	32.39	0.03
Erythromycin		44.6 ± 18	38	3.5 ± 0.6	1.5 ± 0.5	4.77	0.76	0.11	0.02
Clarithromycin		26.4 ± 10	NA	3.8 ± 1.2	1.1 ± 0.2	3.21	1.86	0.12	0.07

EC_{50} – concentration of drug that produces half-maximal (Emax) inhibition of P-gp
γ (gamma) – the Hill coefficient
C_{max} – maximal therapeutic plasma (or blood) concentrations
C_{maxu} – unbound Cmax
ND – could not be determined
NA – not available

(*) US FDA Guidance for Industry, "Drug Interaction Studies—Study Design, Data Analysis, and Implications for Dosing and Labeling, Draft issued 9/11/06 (http://www.fda.gov/cdr/guidance/6695dft.pdf).

Figure 1.

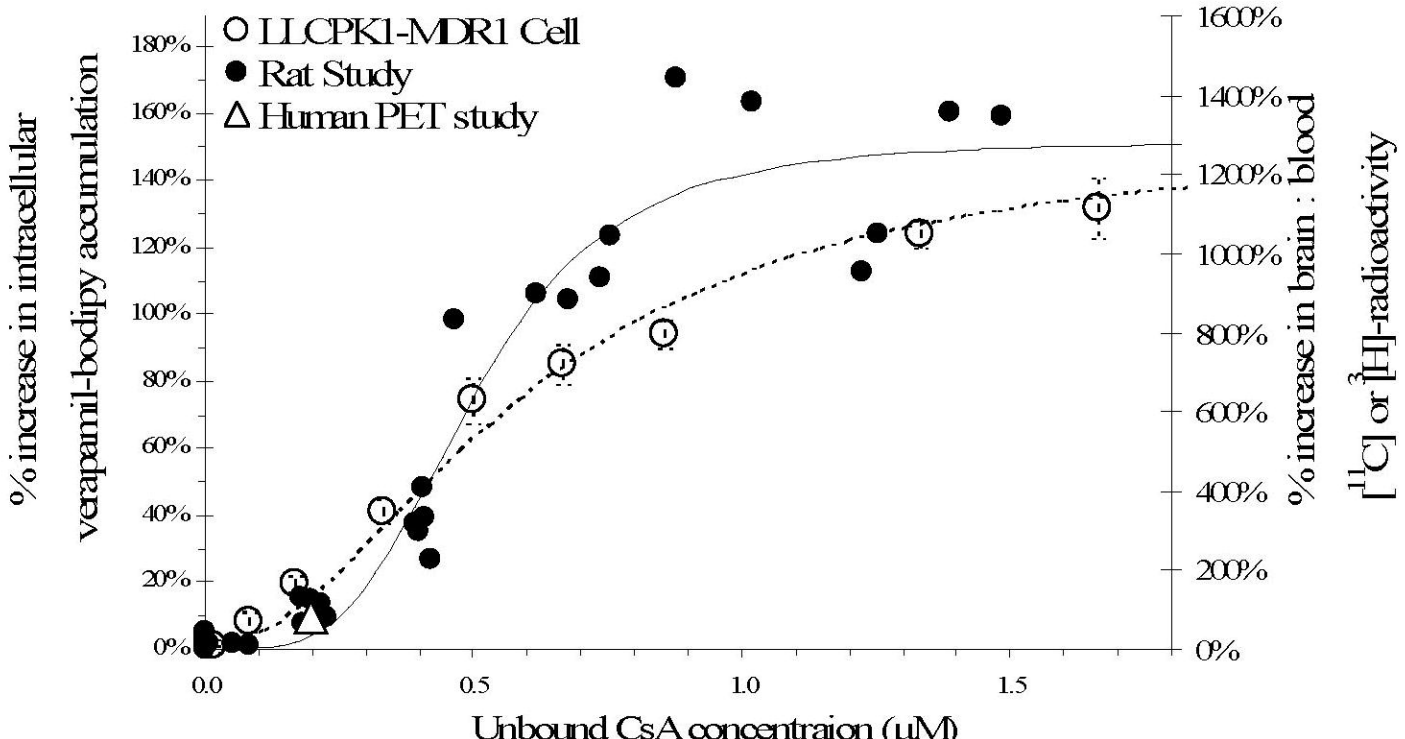
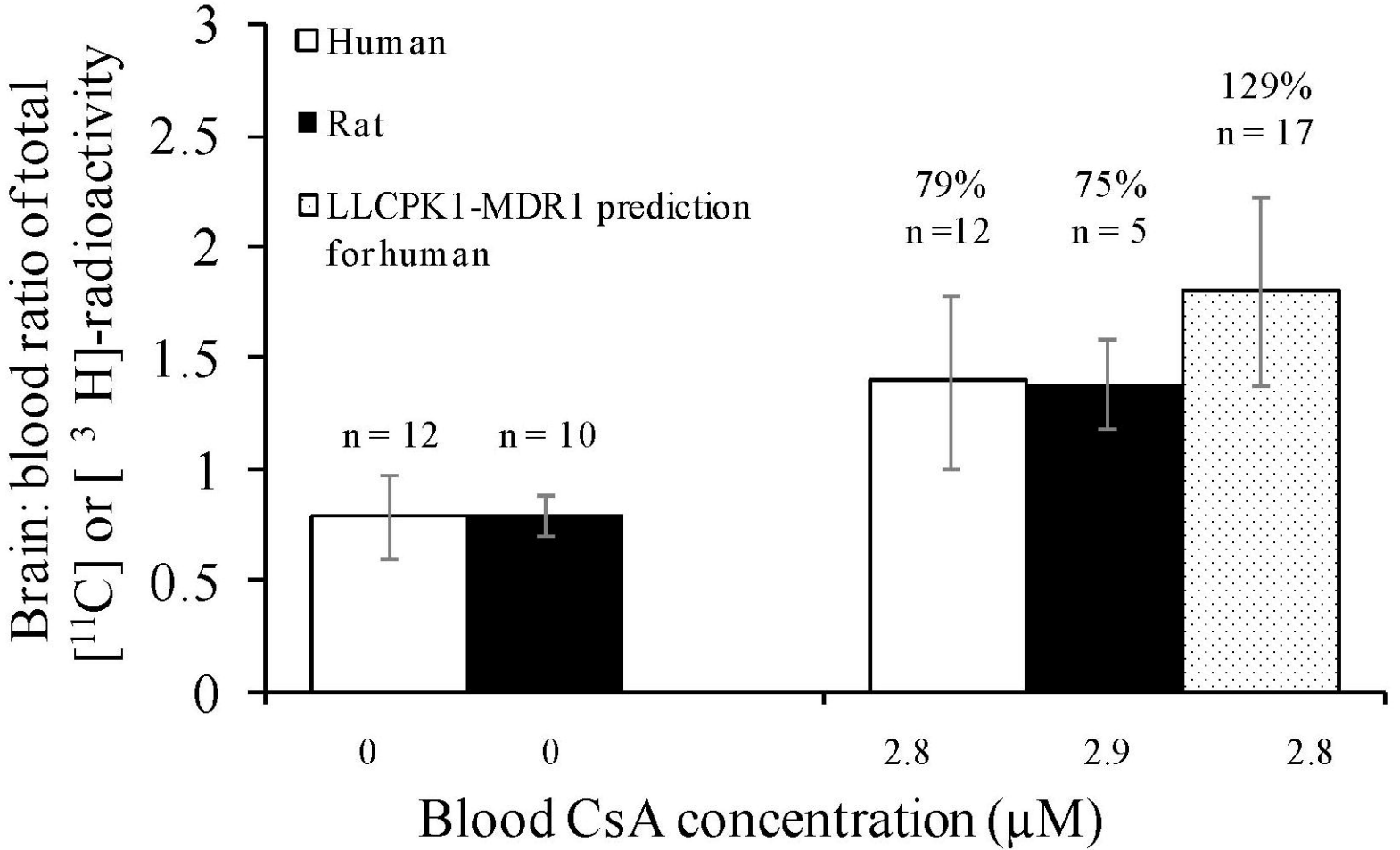

LLCPK1-MDR1 Cell
Rat Study
Human PET study
% increase in intracellular verapamil-bodipy accumulation
% increase in brain : blood [11C] or [3H]-radioactivity
Unbound CsA concentraion (μM)
0%
20%
40%
60%
80%
100%
120%
140%
160%
180%
200%
400%
600%
800%
1000%
1200%
1400%
1600%
0.0
0.5
1.0
1.5

Figure 2.


Human
Rat
LLCPK1-MDR1 prediction for human
Brain: blood ratio of total [11C] or [3H]-radioactivity
3
2.5
2
1.5
1
0.5
0
n = 12
n = 10
79%
n =12
75%
n = 5
129%
n = 17
0
0
2.8
2.9
2.8
Blood CsA concentration (μM)