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Effect of P-glycoprotein-mediated efflux on cerebrospinal fluid concentrations in rhesus monkeys

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ABSTRACT

Brain penetration of drugs which are subject to P-glycoprotein (Pgp)-mediated efflux is attenuated, as manifested by the fact that the cerebrospinal fluid concentration (C_{CSF}), a good surrogate of the unbound brain concentration (C_{ub}), is lower than the unbound plasma concentration (C_{up}) for Pgp substrates. In rodents, the attenuation magnitude of brain penetration by Pgp-mediated efflux has been estimated by correlating the ratio of CSF to plasma exposures (C_{CSF}/C_p) with the unbound fraction in plasma (f_u) upon the incorporation of the in vivo or in vitro Pgp-mediated efflux ratios (ERs). In the present work, we investigated the impact of Pgp-mediated efflux on C_{CSF} in monkeys. Following intravenous administration to cisterna magna ported rhesus monkeys, the CSF and plasma concentrations were determined for 25 compounds from three discovery programs. We also evaluated their f_u in rhesus plasma and ER in human and African green monkey MDR-transfected LLC-PK1 cells. These compounds varied significantly in the f_u (0.025–0.73), and 24 out of 25 are considered Pgp substrates based on their appreciable directional transport (ER > 2). The C_{CSF}/C_p was significantly lower than the corresponding f_u ($\geq$3-fold) for 16 compounds regardless of a significant correlation ($R^2 = 0.59$, $p = 4 \times 10^{-5}$) when the C_{CSF}/C_p was plotted against the f_u. When the f_u was normalized to the ER (f_u/ER) the correlation was improved ($R^2 = 0.75$, $p = 8 \times 10^{-8}$). More importantly, only one compound showed the C_{CSF}/C_p that exceeded 3-fold of the normalized f_u. The results suggest that the impact of Pgp-mediated efflux in monkeys, similar to the case in rodents, is reasonably reflected by the gradient between the free concentrations in plasma and in CSF. Therefore, f_u and Pgp ER may serve as useful measurements in estimating in vivo C_{CSF}/C_p ratios in monkeys, and potentially in humans.

1. Introduction

According to the free drug hypothesis, only the unbound or free drug is the species available for interaction with drug targets within the body. For drugs intended to act in the central nervous system (CNS), the unbound drug in interstitial spaces in the brain (C_{ub}) is considered to be in direct contact or in equilibrium with the target. Therefore, the knowledge of C_{ub} is critical for proving the mechanism of action, validating the animal model, and defining the pharmacokinetic/pharmacodynamic relationship in drug discovery and development. However, it has been a challenge to directly measure C_{ub} due to the anatomical confinement of brain tissue by the skull and the functional separation of brain fluid by the blood–brain barrier (BBB) and the blood–CSF barrier (BCSFB). While microdialysis, an invasive method, has been used to measure C_{ub} for endogenous or extrogenous water soluble substances, its application is limited in dealing with lipophilic compounds because of the high non-specific binding and low recovery [1,2]. In addition, the strenuousness in setting up microdialysis does not agree with the spirit of high-throughput in current drug discovery scenario. As a result, surrogates of C_{ub} have been vigorously sought and validated. Numerous cases have been reported which demonstrated the reasonable representation of C_{ub} by the surrogates.

Abbreviations: Pgp, P-glycoprotein; MDR1, multidrug resistance protein; CNS, central nervous system; CSF, cerebral spinal fluid; ISF, interstitial fluid; BBB, blood–brain barrier; BCSFB, blood–CSF barrier; C_{CSF}, compound concentration in CSF; C_{up}, unbound compound concentration in plasma; C_{ub}, unbound compound concentration in brain tissues; C_p, total compound concentration in plasma; C_b, total compound concentration in brain; ER, efflux ratio; f_u, unbound fraction in plasma; AGM, African green monkey; CMP, cisterna magna ported.

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One surrogate of C_{ub} is unbound plasma concentration (C_{up}). For highly permeable compounds with passive diffusion as the primary mechanism for their entry into the brain, their C_{ub} would be equivalent to their C_{up} at steady state or at equilibrium. For instance, codeine and enadoline rapidly reached a distributional equilibrium with equal unbound concentrations in blood and brain [3,4]. It is known that most marketed CNS drugs exhibited similar unbound plasma and brain exposures [5,6]. However, in the presence of active transport, the C_{ub} may be deviated from the C_{up} [7,8]. Another surrogate is the cerebrospinal fluid concentration (C_{CSF}). CSF in ventricular and subarachnoid spaces communicates with brain extracellular fluid, or brain interstitial fluid (ISF) through fenestrated ependyma and pia mater. Approximately 40% of CSF originates from ISF. Therefore, CSF content is significantly influenced by the composition of solutes in brain ISF [7]. In this sense, C_{CSF} is assumed to be a better surrogate than C_{up} for C_{ub}. In general, the equivalence C_{CSF} and C_{ub} appears to hold true for passive diffusion and transport-mediated efflux, while C_{CSF} is lower than the C_{up} in the presence of the efflux [7,9]. It has been reported that in rats and mice the C_{CSF} quantitatively reflects the equilibrium shift of unbound drugs across BBB by Pgp because incorporation of the in vitro efflux ratio (ER: the permeability difference between basolateral to apical and apical to basolateral transport) resulted in strong correlation between the plasma unbound fraction (f_u) and the CSF/plasma exposure ratio (C_{CSF}/C_p) with the regression slope close to unity [10,11], suggesting the C_{CSF}/C_p ratio is almost identical to the normalized f_u (f_u/ER). These findings have reinforced the approach to utilizing C_{up} and in vitro Pgp efflux data to estimate in vivo brain exposure at the drug discovery stage.

However, uncertainty remains whether the aforementioned findings from rodents would be expended to humans. It is generally thought that differences among species in many physiological and biochemical functions quite often make it difficult to extrapolate to humans from rodent data. Due to ethical considerations, it is difficult to generate sufficient data in this regard from clinical studies. Therefore, information from species which are genetically closer to humans, such as nonhuman primates, would help bridge the gap. Nonhuman primates are commonly used throughout the pharmaceutical industry as preclinical species. They have successfully mimicked drug interactions verified in humans [12–15]. In addition, nonhuman primates have been widely used as animal models for the discovery and development of drugs targeting a variety of diseases. Neurological diseases are one of the areas where rhesus monkeys are of unique value in preclinical and clinical studies [16–18]. With noninvasive imaging technologies, such as positron emission tomography, it has been demonstrated that some Pgp substrates are excluded from rhesus brain regions and treatment of Pgp inhibitors have increased their brain uptake [19–21]. However, it is unknown if these findings can be captured by any changes in the corresponding CSF concentrations. While the surrogate validity of the CSF concentration holds true across species for drugs with passive diffusion as the primary mechanism of brain entry, the evidence supporting this notion for Pgp substrates in monkeys is lacking. In this communication, we disclose our efforts in evaluating the effect of Pgp-mediated efflux on the C_{CSF} in rhesus monkeys for 25 compounds. The results suggest that the impact of Pgp-mediated efflux in monkeys is reasonably reflected by the gradient between the free concentrations in plasma and in CSF.

2. Materials and methods

2.1. Chemicals

Twenty five compounds of interest were synthesized in the Department of Medicinal Chemistry, Merck Research Laboratories. Frozen rhesus monkey plasma was produced in house. Artificial CSF was purchased from Medical Analysis System, Inc. (Canarillo, CA). Isotonic buffer was prepared from 0.067 M sodium phosphate and 0.05 M sodium chloride (pH 7.4, Sigma, St. Louis, MO).

2.2. Transport studies

Human MDR1 transfectants and their parental cell line LLC-PK1 (porcine renal epithelial cells) were kindly provided by Dr. Alfred H. Schinkel (The Netherlands Cancer Institute, Amsterdam, The Netherlands) and used under a license agreement. African green monkey (AGM) MDR1 (GenBank accession no. AY924201) has been cloned and transfected in LLC-PK1 cells in house. Details of cell culture have been described elsewhere [22,23].

Directional transport studies to determine Pgp ER were performed according to published methods [22,23] using Multi-screen Caco-2 96-well filter plates (Millipore Corporation, Billerica, MA). Passive membrane permeability values were determined as the average of the permeability coefficients for apical to basolateral (A-B) and basolateral to apical (B-A) transport in the parental LLC-PK1 cells, whereas the Pgp ER is measured as the ratio of B-A/A-B permeation. Transport studies in the parental and MDR1-transfected cells were conducted for 3 h with the compound concentration of 1 or 5 μM. Upon completion of the study, aliquots (50 μL) were taken from the opposite compartment into a 96-well plate. An equal volume of acetonitrile containing the internal standard was added to each well prior to the analysis by LC–MS/MS.

2.3. Plasma protein binding

Plasma unbound fractions (f_u) were determined in 96-well equilibrium dialyzer (5000 Da molecular weight cut off membrane, Harvard Apparatus, Holliston, MA). Compounds of interest in the stock solutions (1 mM in 50% aqueous acetonitrile) were added to rhesus plasma to achieve a final concentration of 2 μM. Aliquots of the plasma (200 μL) were loaded into the donor side, and the same volume of the buffer into the receiver side of the dialyzer. The dialysis was performed at 37 °C for 18 h with rotation. Aliquots of 50 μL samples from both donor and receiver sides of the membrane were taken for LC–MS/MS analysis. The extent of non-specific binding to the well of the dialyzer was evaluated by the recovery of the compound with the plasma being replaced with the buffer at the donor site. The f_u was determined by the ratio of compound concentration in the receiver buffer to the concentration in the donor plasma after dialysis.

2.4. Cisterna magna ported (CMP) rhesus monkey study

Rhesus monkeys chronically implanted with catheters in the cisterna magna allowed repeated sampling of CSF and plasma in conscious animals [24]. There was one- or two-week washing period between each study. All animal procedures were done in accordance with guidelines from the Institutional Animal Care and Use Committee at Merck.

All compounds were administered via intravenous bolus at 2 mg/kg doses. Compounds were dissolved in the vehicle of 10% ethanol/50% PEG400/40% saline. Plasma samples were collected at 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 5 and 6 h, and CSF were collected at 1, 2, 3, 4, 5 and 6 h post-dose. Samples were stored at −70 °C until analysis.

2.5. Sample analysis

Protein precipitation with acetonitrile was applied to extract compounds in rhesus plasma and CSF and samples from in vitro studies. In general, acetonitrile containing the internal standard was added to samples (2:1 in volume) followed by vigorous

vortexing for 2 min. An aliquot of acetonitrile supernatants was dried under a nitrogen stream and reconstituted with the mobile phase prior to LC–MS/MS analysis. Each type of sample was quantitated against the calibration curves prepared with the corresponding matrix.

The separation of test compounds and internal standard was accomplished on a Betasil C18 column (2.1 mm × 50 mm, 4 μm, 80 Å, Phenomenex, Torrance, CA) using PE 200 binary pumps (PerkinElmer Life And Analytical Sciences, Inc., Wellesley, MA). Solvent A consisted of 0.02% aqueous acetic acid, pH adjusted to 4.5 with NH_4OH, and acetonitrile (90:10), and solvent B consisted of acetonitrile and water (90:10). The mobile phase was delivered at a flow rate of 0.5 mL/min with a linear increase of solvent B from 10% to 85% over a period of 2 min. This value was held for 0.5 min before returning to 10% over 0.1 min. Equilibration was allowed for an additional 0.9 min, giving a total chromatographic run time of 3.5 min. A tandem mass experiment was performed on a Sciex (Concord, Ontario, Canada) Model API 3000 or 4000 triple quadrupole mass spectrometer interfaced to the column eluant via a Sciex turbospray probe operating at 350 °C. Operating conditions and ions for selected reaction monitoring were optimized using the Auto-tune with infusion of the test compounds and internal standards. The lower limits of quantitation in general were 1 ng/mL.

2.6. Data analysis

The area under the curve from time 0 to the last point was calculated using the trapezoidal rule. The regression analyses were conducted using the least square model built in Excel software (Microsoft 2003).

3. Results

3.1. Physicochemical properties

Compounds cited in this communication came from three discovery programs with central target. As summarized in Table 1, the molecular weight of these compounds ranges from 381 to 550 Da and calculated log *P* from 2.5 to 7.3, suggesting a category of moderate molecular weight and lipophilic compounds.

Table 1
Calculated properties of compounds tested in the present study.

Compound ID	Mol. wt.	cLog *P*	Number of HBD	Number of HBA	pKa	PSA
1	426.58	5.53	1	2	8.99	49.22
2	444.57	5.04	1	2	8.99	48.09
3	491.45	5.33	1	2	9.08	47.95
4	491.45	5.33	1	2	9.08	47.52
5	447.00	5.15	1	2	9.05	47.77
6	462.56	5.39	1	2	8.94	48.56
7	462.56	5.39	1	2	8.94	48.07
8	464.99	4.89	1	2	9.01	48.34
9	464.99	4.89	1	2	9.01	47.80
10	451.59	4.80	1	3	8.96	73.70
11	449.02	5.64	1	2	8.94	44.73
12	449.02	5.64	1	2	8.94	44.77
13	431.03	5.90	1	2	8.99	45.10
14	431.03	5.90	1	2	8.99	44.96
15	434.99	5.08	1	2	9.01	45.62
16	475.48	6.08	1	2	9.02	47.76
17	477.07	6.55	1	2	9.13	47.31
18	495.06	6.29	1	2	9.09	48.85
19	495.06	6.29	1	2	9.09	47.97
20	549.56	7.34	1	2	9.03	47.37
21	489.64	5.12	1	3	9.00	55.80
22	520.61	4.32	0	5	8.26	78.10
23	381.34	2.51	0	4	5.91	80.84
24	458.06	5.74	1	2	8.99	48.23
25	430.43	2.57	1	5	13.84	80.23

cLog *P*, calculated log *P*; HBD, hydrogen-bond donor; HBA, hydrogen-bond acceptor; PSA, polar surface area.

3.2. Bidirectional transport across human and monkey MDR1-transfected LLC-PK1 cells

The marker Pgp substrate verapamil exhibited an ER of 4–5 in both human and AGM MDR1-transfected cells. The value was close to 1.0 with a permeability of 24×10^{-6} cm/s in the parental cells. In line with their moderate molecular weight and lipophilicity, all compounds showed good passive permeability in the parent LLC-PK1 cells (P_{app} values $>20 \times 10^{-6}$ cm/s, Table 2). However, 24 out of 25 test compounds showed directional transport with the ER ≥ 2 (2.5–25) determined in AGM MDR1-transfected cells, indicating that the majority of the test compounds are subject to Pgp-mediated efflux to different degrees in African green monkeys.

As rhesus MDR1-transfected cells have not been established in-house, the validity of AGM MDR1 to represent rhesus MDR1 has been evaluated via a pilot work which compared the ER values for two Pgp substrates (verapamil and a proprietary compound). The ER values were 4 versus 5 for verapamil and 13 versus 10 for the proprietary compounds in cynomolgus (a species whose MDR1 amino acid sequence showed >99% similarity to rhesus MDR1) and AGM MDR1-transfected LLC-PK1 cells, respectively (in-house data). To substantiate this finding, the present study compared the ER values determined in human and AGM MDR1-transfected cells for these 25 compounds. It was found that the two sets of data correlated reasonably well ($R^2 = 0.64$, $p = 2 \times 10^{-5}$) with the regression slope of 1.03 when the ERs were below 20 (Fig. 1). However, deviation was observed when the ER became very high. It is conceivable that a compound highly susceptible to Pgp efflux would show a very low concentration in the receiver solution upon A-to-B transport. Therefore, a slight experimental variation could result in considerable difference in the ER value. Therefore, compound **21** was not included in the correlation because it showed an ER of 52 for human Pgp.

3.3. Plasma protein binding

Reversible binding of the compounds to rhesus plasma proteins was determined using an equilibrium dialysis method. The non-specific binding to the dialyzer was minor because the recovery was ≥70% in the buffer controls where comparable concentration was obtained in both the donor and receiver sides after the dialysis. The compounds of interest showed a wide range of unbound fraction (f_u) from 0.025 to 0.73, a greater than 20-fold difference (Table 2).

3.4. CSF and plasma concentrations in CMP rhesus monkeys and comparison of CSF/plasma ratios to the unbound fractions with correction to Pgp ER

The CSF and plasma were sampled up to 6 h following intravenous administration to the CMP rhesus monkeys. Most compounds appeared to reach equilibrium between CSF and plasma compartments by 2 h post-dose as the C_{CSF}/C_p ratios were quite constant from then (data not shown). As compounds varied in the half-life, some compounds showed a CSF level that was below the limit of quantitation prior to 6 h. Therefore, the area under the compound concentration curve versus time (AUC) was calculated up to the last data point (Table 2).

To estimate the impact of Pgp-mediated efflux on the C_{CSF}, the CSF/plasma AUC ratios (denoted as C_{CSF}/C_p) were plotted against the f_u values. As delineated by Fig. 2A, there appeared to be a significant correlation between these two parameters ($R^2 = 0.59$, $p = 4 \times 10^{-5}$), indicating the free concentration as one of the

Table 2
In vivo and in vitro results for 30 compounds from four discovery programs.

Compound ID	In vivo				In vitro					f_u/f_{ER}	$f_u/(C_{CSF}/C_p)$	$(C_{CSF}/C_p)/(f_u/ER)$
	Duration (h)	AUC_{0-t} (μM h)		C_{CSF}/C_p ratio	Rhesus f_u	$P_{app} \times 10^{-6}$ (cm/s)	ER (B-A/A-B)					
		Plasma	CSF				Control	MDR1 (AGM)	MDR1 (human)			
1	0–6	1.702	0.289	0.170	0.501	35	0.9	4.4	2.6	0.115	3.0	1.5
2	0–6	1.458	0.223	0.153	0.586	35	1.1	8.1	7.1	0.072	3.8	2.1
3	0–3	1.250	0.114	0.091	0.526	22	0.9	16.5	15	0.032	5.8	2.9
4	0–6	2.083	0.269	0.129	0.464	27	1.4	10.9	14	0.042	3.6	3.0
5	0–6	1.397	0.179	0.128	0.729	21	0.8	12.9	11	0.056	5.7	2.3
6	0–6	1.916	0.338	0.177	0.522	36	1.1	5.1	4.6	0.102	3.0	1.7
7	0–6	2.495	0.484	0.194	0.558	40	1.1	4.1	5	0.135	2.9	1.4
8	0–6	1.627	0.151	0.093	0.557	29	1.1	8.2	13	0.068	6.0	1.4
9	0–6	1.780	0.163	0.091	0.458	30	1.3	7.2	12	0.063	5.0	1.4
10	0–6	1.742	0.287	0.165	0.661	34	1.2	10	11	0.065	4.0	2.5
11	0–4	0.807	0.104	0.122	0.264	38	1.2	3.5	7.5	0.076	2.2	1.6
12	0–6	1.534	0.165	0.114	0.254	34	1.1	5.1	5	0.050	2.4	2.1
13	0–6	1.884	0.114	0.061	0.260	36	1.0	6.4	11	0.040	4.3	1.5
14	0–6	2.519	0.232	0.092	0.318	33	0.9	7.4	7.4	0.043	3.5	2.1
15	0–6	1.441	0.265	0.184	0.483	36	1.1	6.3	11	0.077	2.6	2.4
16	0–2	1.737	0.039	0.022	0.198	33	1.1	7.7	12	0.026	8.8	0.9
17	0–6	2.577	0.101	0.039	0.167	29	1.2	10.2	15	0.016	4.1	2.5
18	0–6	0.966	0.079	0.082	0.133	36	1.3	3.9	3.5	0.034	1.6	2.4
19	0–6	2.999	0.184	0.061	0.126	30	1.1	4.3	3.5	0.029	2.1	2.1
20	0–3	1.849	0.016	0.009	0.025	24	1.0	4.7	2.3	0.005	2.9	1.6
21	0–6	13.521	0.147	0.011	0.280	23	1.7	25	52	0.011	26	1.0
22	0–6	2.330	0.146	0.063	0.085	33	1.0	1.5	1.2	0.056	1.4	1.1
23	0–6	28.798	0.634	0.021	0.123	35	0.7	4.4	1.5	0.028	5.6	0.8
24	0–8	10.666	1.281	0.120	0.490	29	0.5	9.7	6.8	0.051	4.1	2.4
25	0–6	3.141	0.201	0.064	0.120	35	0.7	2.5	1.9	0.048	1.9	1.3

determinants of the CSF concentration. However, the C_{CSF}/C_p ratios were remarkably lower than the f_u for 16 of 25 compounds (by >3-fold lower), as suggested by the regression slope of 0.21. This difference may reflect the concentration gradient between CSF and unbound compound in plasma which was maintained by the Pgp pump. When the f_u was normalized to the ER (f_u/ER), the correlation of the C_{CSF}/C_p ratios with the normalized f_u was improved ($R^2 = 0.75$, $p = 8 \times 10^{-8}$), as illustrated by Fig. 2B. More importantly, the C_{CSF}/C_p ratios of the majority of the compounds (24 out of 25) fell within the range of less than 3-fold of the f_u/ER (Table 2). Of note is the regression slope of 1.5 in Fig. 2B. As shown in Table 2, the difference between the C_{CSF}/C_p ratio and the f_u/ER ranged from 1.5 to 2.5 for the majority of the compounds, suggesting a slight overcorrection of f_u by the ER.

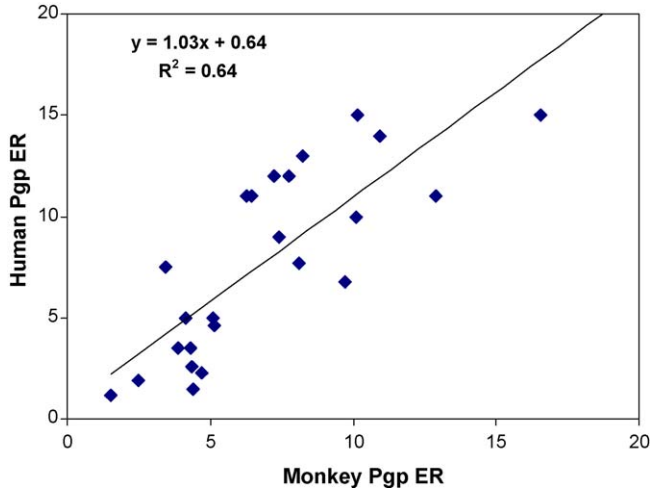

Fig. 1. Correlation of human Pgp ER with AGM Pgp ER. The regression analysis shows a statistically significant correlation of these two parameters ($R^2 = 0.64$ and $p = 2 \times 10^{-5}$).

4. Discussion

The C_{CSF}/C_p ratio of the majority of the test compounds in our study was significantly lower than their f_u (>3-fold lower) in rhesus monkeys (Table 2), indicating a gradient between C_{CSF} and C_{up} that was maintained by efflux transporters. The most likely one involved is Pgp since it is in general the most prevalent efflux transporter for lipophilic compounds across species. Ideally, the impact of the transporter would be best characterized by rhesus MDR1-transfected cells. However, AGM Pgp may reasonably represent rhesus Pgp for substrate selectivity based on several lines of evidence. First, rhesus, cynomolgus and African green monkeys all belong to Cercopithecidae family. Their Pgp proteins share very high homology in amino acid sequence [25]. The identity is 99.6% between rhesus and cynomolgus monkeys and 98.1% between AGM and rhesus/cynomolgus monkeys. The homology is also high between these monkeys and humans (~96%). Second, highly similar ER values were obtained for some compounds evaluated with cynomolgus and AGM MDR1-transfected cell. Third, there was a good correlation between the ER values determined with human and AGM MDR1-transfected cells (Fig. 1) for 24 compounds in the current study. In addition, a good correlation between human and cynomolgus Pgp ER values was observed for eight proprietary compounds ($R^2 = 0.86$, in-house data). It appears that the similar substrate selectivity is consistent with the high homology in Pgp amino acid sequence across these species. Further supporting evidence is the finding that compounds with human Pgp ER > 3 shows a low C_b/C_p ratio (<0.2) in African green monkeys, while the ratio falls between 0.4 and 1.5 for compounds not subject to efflux by human Pgp (ER < 2) [31]. Although the C_b/C_p ratio may be influenced by the level of non-specific brain tissue binding, given the similar physicochemical properties of those compounds derived from the same structure template, we consider the data convincingly demonstrate the similarity between human and AGM Pgp. Therefore, it would be difficult to argue against the similarity between AGM Pgp and

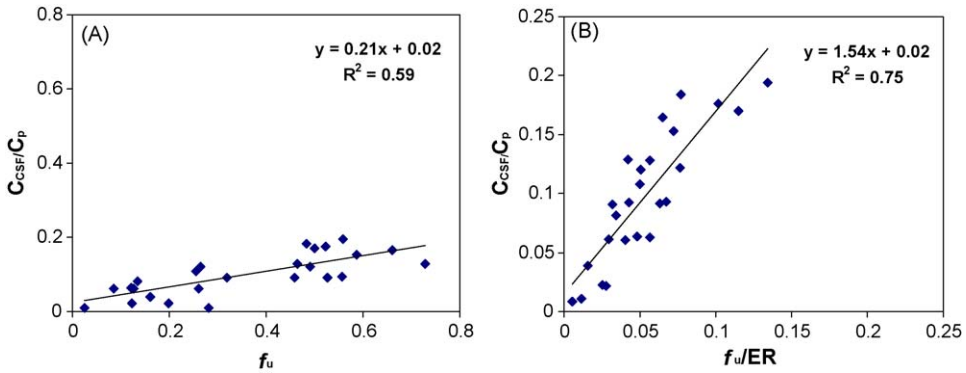

Fig. 2. Correlation of rhesus C_{CSF}/C_p ratio with unbound fraction (f_u) in rhesus plasma before (A) and after (B) correction with monkey Pgp ER. The regression analysis shows a statistically significant correlation of C_{CSF}/C_p ratio and f_u before or after ER correction (A: $R^2 = 0.59$ and $p = 4 \times 10^{-5}$; B: $R^2 = 0.75$ and $p = 8 \times 10^{-8}$).

rhesus Pgp relative to human Pgp. In this sense, AGM Pgp should in general be a good surrogate for rhesus Pgp. Therefore, the majority of compounds in this study may be categorized as monkey Pgp substrates (ER > 2).

In agreement, when rhesus f_u was corrected by the in vitro ER determined with AGM MDR1-transfected cells, the difference between C_{CSF}/C_p ratio and the corrected f_u (f_u/ER) became less significant. Meanwhile, the correlation of these two parameters was improved from 0.59 to 0.75. The results suggest that the impact of Pgp-mediated efflux in rhesus monkeys is reasonably reflected by the gradient between C_{CSF} and C_{up}. While the involvement of other efflux transporters cannot be excluded, the significantly reduced difference between C_{CSF}/C_p ratio and the corrected f_u along with the improved correlation between these two measurements upon Pgp efflux incorporation indicate that the contribution of other transporters, if any, may be negligible.

This findings in our work are analogous to what was reported in rats and mice [10,11], but there are some differences in comparison with those rodent studies. The major difference resides on the slope of regression curve for C_{CSF}/C_p and f_u/ER. Ohe et al [10] and He et al. [11] reported a slope close to unity in rats and mice, respectively, suggesting an almost 1:1 relation between in vivo and in vitro Pgp activity in their cases. In our study, the slope was 1.5, indicating on average a 50% overestimation of in vivo Pgp impact by in vitro Pgp activity. This in vivo and in vitro disparity may be associated with the difference in Pgp expression levels. A recent comparative analysis revealed that Pgp protein level based on protein content in microvessels from rat brain is higher than from human brain [26]. However, it is unknown what level of Pgp expression is in the BBB of rhesus relative to rodents. This information would facilitate setting up an in vitro system which is more comparable to the in vivo situation if a more definitive in vivo–in vitro correlation is intended. It is clear that the comparable level of in vitro Pgp expression across species may not be quantitatively predictive if the in vivo expression level varies.

Alternatively, this overestimation of in vivo Pgp impact could be related to the deviation of C_{CSF} from C_{ub} of Pgp substrates. It has been recognized that once active transport is involved, C_{CSF} may be divergent from C_{ub} even though it still appears to be more accurate than C_{up} to represent C_{ub} [9]. It has been noticed that C_{CSF} is less sensitive than C_b to the modulation of Pgp activity. For instance, the C_b of three Pgp substrates loperamide, quinidine and verapamil in Pgp mdr1a/1b knockout mice is 9-, 36- and 16-fold higher than in wild type mice, while the difference is 6-, 10- and 8-fold for C_{CSF} [5]. With the treatment of a Pgp inhibitor, the C_b of a Pgp substrate in rats increased by >10-fold, while C_{CSF} by ~5-fold (in-house data). Clearly, the C_{CSF} does tract the change of C_b which should directly respond to the Pgp activity at BBB, but the magnitude for C_{CSF} change in general appears lower than for C_b, which suggests a gradient of the unbound compound in the CSF and brain tissues. Therefore, it is possible that the Pgp impact manifested by C_{CSF} is overestimated using the Pgp ER even though Pgp expression levels in vivo and in vitro are normalized. However, more cases are needed to better understand the different regression slopes observed in the present and those rodent studies.

Another observation of note is the statistically significant correlation between C_{CSF}/C_p and f_u ($R^2 = 0.59$) regardless of the regression slope of 0.21 (Fig. 2A). This result indicates that the level of C_{up} is another factor influencing the level of C_{CSF}. In other words, passive diffusion across BBB or BCSFB can offset the impact of efflux on CSF composition to a certain degree. Given the origin of CSF, the anatomical and structural difference between BBB and BCSFB, cerebral flow dynamics and CSF sink action [7], it is reasonable to speculate a higher permeability at BCSFB than in BBB. For a compound highly susceptible to Pgp efflux, its C_{ISF} (C_{ub}) will be enormously reduced by the transporter located on the BBB, leading to a C_{ISF} (C_{ub}) much lower than its C_{up}. In contrast, Pgp does not appear to play a significant role in limiting the entry into ventricular CSF. Although subapical expression of Pgp on choroid plexus epithelial cells has been reported [27], its expression level was found to be only <1% of that in BBB in rats [26]. Besides, the transport by Pgp on choroid plexus, if any, is thought to be towards CSF [27,28]. More importantly, the capillaries in choroid plexus are fenestrated and the epithelial layer is more permeable than brain endothelial cells [29]. Therefore, the concentration of a Pgp substrate in the CSF secreted from choroid plexus is likely close to its C_{up}. Once this portion of CSF is mixed later with the ISF entering ventricles from cerebral regions, the substrate concentration in CSF will be diluted. This may in part explain why C_{CSF} could be higher than C_{ub} but still reflects the Pgp impact on BBB. However, equilibrium between CSF and ISF is rarely achieved because the cerebral fluid dynamics and CSF "sink action" result in more favored flow from brain to CSF than the opposite direction. As a result, a concentration gradient may be generated between CSF and ISF for a Pgp substrate. The magnitude of the gradient depends on the BBB permeability, ISF bulk flow, CSF turnover and the distance from ISF at a particular cerebral site to CSF [7]. The work of Venkatakrishnan et al. [8] showed a 7-fold difference between C_{CSF} and C_{ISF} for a strong Pgp substrate in rats. Recently Watson et al. [30] demonstrated a better prediction of D_2 receptor occupancy with C_{ub} than with C_{CSF} for six marketed antipsychotics whose entry into brain tissue is influenced by factors other than simple passive diffusion. In our on-going endeavor, some compounds highly subject to Pgp-mediated efflux will be evaluated for their receptor occupancy in rhesus. Coupled with in vitro potency (K_i) and C_{CSF}, the occupancy data should shed more light on how quantitatively the C_{CSF} deviates from the C_{ub} for Pgp substrates in monkeys. It would be also important to extend the findings from

rodents to monkeys that C_{ub} derived from unbound fraction in brain homogenate and total C_b provides a better prediction of the occupancy of receptors located inside brain [30].

In conclusion, we have shown that incorporation of in vitro Pgp ER has improved the correlation between C_{CSF}/C_p ratio and f_u in rhesus monkeys. This finding indicates that C_{CSF} reasonably reflects the impact of Pgp-mediated efflux for this set of compounds. Analogous to the findings in rodents, f_u and Pgp ER may serve as useful measurements in estimating in vivo C_{CSF}/C_p ratios in monkeys. In light of the genetic closeness as well as functional and biochemical resemblance between nonhuman primates and humans, similar findings can be expected in humans.

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