A Practical Perspective on the Evaluation of Small Molecule CNS Penetration in Drug Discovery

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Abstract: The separation of the brain from blood by the blood-brain barrier and the blood-cerebrospinal fluid (CSF) barrier poses unique challenges for the discovery and development of drugs targeting the central nervous system (CNS). This review will describe the role of transporters in CNS penetration and examine the relationship between unbound brain (C_u-brain) and unbound plasma (C_u-plasma) or CSF (C_u-CSF) concentration. Published data demonstrate that the relationship between C_u-brain and C_u-plasma or C_u-CSF can be affected by transporter status and passive permeability of a drug and C_u-CSF may not be a reliable surrogate for CNS penetration. Indeed, C_u-CSF usually over-estimates C_u-brain for efflux substrates and it provides no additional value over C_u-plasma as the surrogate of C_u-brain for highly permeable non-efflux substrates. A strategy described here for the evaluation of CNS penetration is to use in vitro permeability, P-glycoprotein (Pgp) and breast cancer resistance protein efflux assays and C_u-brain/C_u-plasma in preclinical species. C_u-plasma should be used as the surrogate of C_u-brain for highly permeable non-efflux substrates with no evidence of impaired distribution into the brain. When drug penetration into the brain is impaired, we recommend using (total brain concentration * unbound fraction in brain) as C_u-brain in preclinical species or C_u-plasma/ in vitro Pgp efflux ratio if Pgp is the major limiting mechanism for brain penetration.

Keywords: Blood-brain barrier, blood-CSF barrier, BCRP, CNS penetration, CSF, P-glycoprotein, unbound brain concentration.

1. INTRODUCTION

Brain cells, unlike cells in the other organs are separated from blood by two barriers: the blood-brain barrier (BBB) and the blood-cerebrospinal fluid barrier (BCSFB) [1, 2]. The barriers are essential elements in the regulation of the brain microenvironment necessary for optimal function, but can limit the drugs targeting the central nervous system (CNS) from accessing the brain in concentrations sufficient for maximal efficacy. Moreover, the barriers make it difficult to estimate free drug concentration in the brain (C_u-brain) and translate preclinical findings to humans during the process of drug discovery and development.

Fig. (1) shows the schematic model of drug distribution among the blood, brain and CSF compartments. The BBB has a large surface area (~15-25 m² in humans) [3]. It is formed by capillary endothelial cells connected with tight junctions and surrounded by basal lamina and astrocytic perivascular endfeet. The BBB is the major barrier for CNS penetration [1, 2, 4]. The ability of a small molecule to cross the BBB depends on its physicochemical properties and whether it is a substrate for transporters at the BBB. A drug must be highly permeable to efficiently cross the BBB as paracellular transport across the BBB is minimal under normal physiological conditions. Rarely, poorly permeable compounds are transported across the BBB by uptake transporters [5-7], but the physicochemical properties necessary for an uptake transporter restrict the chemical space available for designing potent CNS-active drugs and limit exploiting uptake transporters as a drug-design strategy. More frequently, brain penetration of many small molecules is limited by efflux transporters such as P-glycoprotein (Pgp) and breast cancer resistant protein (BCRP) [8-10]. A strategy of designing drug candidates with reduced efflux while maintaining sufficient passive permeability has been widely employed in drug discovery and has shown some success [11, 12].

The BCSFB is different from the BBB in several aspects. Firstly, the BCSFB is formed by the choroid plexus epithelia connected with tight junctions, whereas the capillaries of the choroid plexus are fenestrated allowing the free-movement of small molecules [13]. Secondly, evidence that some poorly permeable compounds such as atenolol can distribute into CSF [14] suggests that the tight junctions of the choroid plexus epithelia are not as tight as those in the endothelia of the BBB. In addition, transporters expressed at the BCSFB
are qualitatively and quantitatively different than those at the BBB and actively modulate molecule movement into and out of the CSF [13].

Some evidence suggests that CSF is mainly produced by the choroid plexus and approximately one-third originates from brain extracellular fluid (ECF) [2, 13]. CSF formed at the choroid plexus flows through the ventricles into the subarachnoid space and is reabsorbed into venous blood via arachnoid villi [2]. It is generally accepted that brain ECF flows towards the CSF and the movement from the CSF into the brain is minimal under normal physiological conditions. This is supported by the observation that $^{14}$C-sucrose injected into ventricular CSF only moved from the CSF compartment to the adjacent brain tissue by simple diffusion [15]. There is, however, evidence of para-arterial influx of subarachnoid CSF into the brain interstitium by what has been termed as glymphatic flux. Iliff et al. [16] reported that CSF entered the parenchyma along para-vascular spaces and brain ECF was cleared along para-venous drainage pathways based on in vivo imaging of small fluorescent markers administered via the cisterna magna in the anesthetized mice. The relevance of this CSF influx in conscious animals and indeed its quantitative importance to the impact on drug distribution into the brain is unknown.

This review will describe the role of transporters in CNS penetration; examine the relationship between $C_{u\text{-brain}}$, CSF concentration ($C_{CSF}$) and unbound plasma concentration ($C_{u\text{-plasma}}$) in preclinical species; and discuss an evaluation strategy for CNS penetration in drug discovery.

2. ROLE OF TRANSPORTERS IN BRAIN PENETRATION

Various uptake and efflux transporters are expressed in many tissues including intestine, liver, kidney and brain and play an important role in drug disposition [17]. Table 1 lists some of the transporters expressed at the BBB including glucose transporter 1, monocarboxylate transporter 1 (MCT1), Pgp, BCRP and multidrug resistance protein 4 (MRP4). Protein levels of organic anion transporting polypeptides (OATPs) in human brain microvessels were below

![Fig. (1). Schematic model of drug distribution among blood, brain and CSF compartments. ICF, intracellular fluid; ECF, extracellular fluid.](image)

<table>
<thead>
<tr>
<th>Table 1. Protein expression, measured by LC-MS/MS, of selected transporters in brain microvessels across species.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Transporters</strong></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>OAT3/Oat3</td>
</tr>
<tr>
<td>OATPA, OATP2B1/Oatp-2</td>
</tr>
<tr>
<td>OATP-F/Oatp14</td>
</tr>
<tr>
<td>MCT1/Mct1</td>
</tr>
<tr>
<td>GLUT1/Glut1</td>
</tr>
<tr>
<td>MDR1/Mdr1a</td>
</tr>
<tr>
<td>BCRP/Bcrp</td>
</tr>
<tr>
<td>MRP4/Mrp4</td>
</tr>
</tbody>
</table>

Data compiled from [19-22]. NA, not available.
the limits of quantitation (<0.695, <0.337 and <0.208 fmol/µg protein for OATP-A, OATP-B and OATP-F, respectively) by the method of liquid chromatography tandem mass spectrometry (LC-MS/MS) [18], but OATP2B1 was readily detected in human capillary endothelia by immunohistochemistry [19-22]. In terms of protein expression at the BBB measured by LC-MS/MS, primates (humans and monkeys) have a lower overall expression of Pgp, Mrp4, organic anion transporter (Oat), Mct1 and Oatp than rodents. Conversely, Bcrp expression at the BBB is 2 to 3-fold higher in the primates than in rodents. Glucose transporter 1(GLUT1) expression was similar across species (Table 1).

Much of what is known about functional importance of transporters in CNS penetration has been deduced from the brain exposure data in knockout (KO) mice and rats, where one or more transporter genes have been deleted (Table 2). Overlapping substrate specificity, the array of transporters present and potential changes in the expression of other transporters should all be considered when interpreting the results from KO animals. Despite these complexities, the KO animals offer tremendous insight into the roles of transporters in CNS penetration. Therefore, in this review, we focus on those transporters for which KO animals are available.

### Table 2. Transporter expression, brain and CSF exposure in transporter deficient animals.

<table>
<thead>
<tr>
<th>Transporter (Gene Symbol)</th>
<th>Expression*</th>
<th>Animals</th>
<th>Exposure in KO or Mutant Animals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pgp (MDR1, ABCA1)</td>
<td>Rat and human: BBB&gt;&gt;BC SF B</td>
<td>Pgp deficient mice</td>
<td>≥10-fold † for amprenavir, dihydroxydramine, eleriptan, indinavir, ivermectin, cyclosporine, loperamide, nelfinavir, quinidine, vinblastine, etc.</td>
</tr>
<tr>
<td></td>
<td>Mdr1a(-/-) rats</td>
<td>at least 10-fold † for loperamide, digoxin, amprenavir, quinidine and 9-OH risperidone</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mdr1b deficient collie dogs</td>
<td>hypersensitive to ivermectin and loperamide; &quot;mTc-sestamibi distribution †</td>
<td></td>
</tr>
<tr>
<td>BCRP (ABCG2)</td>
<td>Rat and human: BBB&gt;&gt;BC SF B</td>
<td>Abcg2(-/-) mice</td>
<td>≤2-fold † for lapatinib, imatinib, prazosin, flavopiridol, sunitinib, gefitinib, vemurafenib, erlotinib; 3 to 6-fold † for dantrolene; 6 to 9-fold † for dazidein and genestein.</td>
</tr>
<tr>
<td></td>
<td>Abcg2(-/-) rats</td>
<td>2 to 3-fold † for dantrolene; ↔ for compound X</td>
<td></td>
</tr>
<tr>
<td>Pgp (MDR1, ABCA1) + BCRP (ABCG2)</td>
<td>Rat and human: BBB&gt;&gt;BC SF B</td>
<td>Mdr1a(−/-)/Abcg2(-/-) mice</td>
<td>≥30-fold † for lapatinib, imatinib, sunitinib, gefitinib and vemurafenib; 5 to 7-fold † for flavopiridol and prazosin.</td>
</tr>
<tr>
<td></td>
<td>Mdr1a(−/-)/Abcg2(-/-) rats</td>
<td>40-fold † for imatinib</td>
<td></td>
</tr>
<tr>
<td>MRP1 (ABCC1)</td>
<td>Rat and human: BCSFB&gt;&gt; BBB</td>
<td>Abcc1(-/-) mice</td>
<td>↓ for 6-bromo-7-[11C]methylpurine glutathione conjugate and 6-bromo-7-(2-[18F]fluoroethyl) purine glutathione conjugate (brain efflux); ↔ for etoposide and vincristine.</td>
</tr>
<tr>
<td></td>
<td>Mdr1a(1b(-/-)/Abcc1(-/-) mice</td>
<td>↔ for etoposide compared to Mdr1a(1b(-/-)).</td>
<td></td>
</tr>
<tr>
<td>MRP4 (ABCC4)</td>
<td>Rodents: BBB &amp; BCSFB</td>
<td>Abcc4(−/-) mice</td>
<td>3-fold † at 6 h following an IV bolus dose of topotecan; 4 to 6-fold † for RO 64-0802; ↔ for adefovir and tenofovir.</td>
</tr>
<tr>
<td></td>
<td>Abcc4(−/-) mice</td>
<td>10-fold † for topotecan (ventricular Cso).</td>
<td></td>
</tr>
<tr>
<td>PEPT2 (SLC15A2)</td>
<td>Rat: BCSFB</td>
<td>SLC15a2(-/-) mice</td>
<td>2-fold † for glycosylsarcosine and cefadroxil; ↔ for 5-aminovaleric acid; ↓ 50% for carnosine</td>
</tr>
<tr>
<td></td>
<td>SLC22a8(-/-) mice</td>
<td>3 to 9-fold † for glycosylsarcosine, cefadroxil, carnosine and 5-aminovaleric acid.</td>
<td></td>
</tr>
<tr>
<td>OAT3 (SLC22A8)</td>
<td>Mouse: BCSFB</td>
<td>SLC22a8(-/-) mice</td>
<td>↔ for Ro 64-0802 after 24 h subcutaneous infusion; † for DHEAS, estrone-3-sulfate, RO 64-0802 and metabolite of N,N'-1,2-ethylenediybis-L-cysteine diethyl ester (brain efflux).</td>
</tr>
</tbody>
</table>

* [53, 57, 60-64]. †, increase; ↓, decreased; ↔, no significant change.
Pgp and BCRP, members of the ATP-dependent transporter family, are expressed in the luminal membrane of brain capillary endothelia [65]. They can actively efflux a wide variety of compounds out of cells and limit the distribution of drugs into the brain. Pgp and BCRP have distinct, but overlapping, substrate specificity. In general, Pgp substrates are hydrophobic and neutral or positively charged at physiological pH; examples include HIV protease inhibitors, calcium channel blockers and anticancer drugs of the vinca alkaloid, anthracycline and taxane classes [66]. In contrast, many organic anions such as statins, sulfasalazine, glutathione and sulfate conjugates are good BCRP substrates [9]. However, a number of drugs such as imatinib, prazosin and lapatinib were found to be transported by both Pgp and BCRP [41, 42].

2.1. Pgp Plays a Critical Role in CNS Penetration

The impact of Pgp, the most important gatekeeper at BBB, on CNS penetration depends on efficiency of Pgp-mediated efflux, passive permeability and whether compounds are substrates of other transporters. While brain exposure in Pgp deficient mice increased profoundly for many Pgp substrates including ivermectin, digoxin, cyclosporine, loperamide, quinidine, amphenavir, indinavir and nelfinavir, the difference in brain exposure between wild-type (WT) and Pgp KO [Mdr1a(-/-) or Mdr1a/1b(-/-)] mice was small (< 3-fold) for some Pgp substrates such as fexofenadine, sumatriptan and flavoridol [24, 25, 28, 34, 41, 42, 67]. The small change in brain exposure of these Pgp substrates in the Pgp KO mice could be due to limited passive permeability or contribution of other efflux transporters. For example, passive permeability of fexofenadine and sumatriptan is low [67, 68], and flavoridol has been identified as a BCRP substrate [42].

Similar to mice, brain exposure of Pgp substrates including risperidone, digoxin, loperamide, imatinib and amphenavir increased significantly in Pgp KO [Mdr1a(-/-)] rats. The magnitude of the increase was comparable to that in the Pgp KO mice [35-37]. Certain dogs of the collie lineage displayed hypersensitivity to ivermectin [69, 70]. These dogs showed neurotoxic signs at doses as low as 0.1 mg/kg, whereas doses up to 2 mg/kg were well tolerated in normal dogs. The ivermectin-sensitive dogs were later found lacking Bcrp [73]. However, a number of drugs such as imatinib, prazosin and lapatinib were found to be transported by both Pgp and BCRP [41, 42].

2.2. BCRP (ABCG2) is Another Important Efflux Transporter at the BBB

The absolute amount of Bcrp protein, measured by LC-MS/MS, was approximately 4-fold lower than Pgp in rodents but higher than Pgp in monkey and human (Table 1). As Bcrp is a half transporter and is believed to function as a homodimer [74], the amount of Bcrp as functioning units at the BBB is approximately 8-fold lower than Pgp in rodents, but comparable to Pgp in monkey and human. Consistent with the expression level of Pgp and Bcrp at the BBB, Pgp and BCRP dual substrates generally showed higher brain exposure in Mdr1a/1b(-/-) mice compared to Bcrp KO [Abcg2(-/-)] mice [41, 42, 46, 47].

In contrast to the large differences in brain exposure of many Pgp substrates between WT and Pgp deficient mice, brain exposure of many BCRP substrates in Abcg2(-/-) mice was only modestly affected (≤3-fold) (Table 2). Although this small effect of Abcg2 deletion on brain exposure may be partly due to the lower expression of Bcrp than Pgp at the BBB, it is likely that the absence of Bcrp is compensated for by the presence of other efflux transporters. This is supported by the findings that many BCRP substrates are also transported by other efflux transporters such as Pgp and MRP [42, 75-77]. Brain exposure was ≥10-fold higher in the Pgp and Bcrp double KO [Mdr1a/1b(-/-)/Abcg2(-/-)] mice compared to the Mdr1a/1b(-/-) mice for dual substrates of Pgp and BCRP including lapatinib, imatinib, sunitinib and vemurafenib [41, 42, 46, 47]. These data support the importance of BCRP at the BBB, especially in humans considering higher BCRP expression than Pgp in humans.

2.3. Potential Role of Additional Transporters Contributing to Brain Exposure

In addition to Pgp and BCRP, other transporters such as MRPs and OATPs are also expressed at the BBB [10, 61]. However, information on their roles in brain penetration is limited. Zhou et al. [42] reported that brain exposure of PF-40728, a Bcrp substrate with high passive permeability, remained very low (total brain to plasma concentration ratio, Cbrain/Cplasma = 0.03) in the Mdr1a/1b(-/-)/Abcg2(-/-) mice. In addition to Pgp and BCRP, the compound may be transported by other efflux transporters at the BBB, such as MRP4.

MRP4 is expressed at the luminal membrane of brain capillary endothelial cells and the basolateral membrane of choroid plexus epithelia [54]. The location of MRP4 suggests that it may limit brain penetration and transport compounds from the CSF to the blood. Brain distribution of topotecan, RO 64-0802, adefovir and tenofovir have been assessed in MRP4 KO [Abcc4(-/-)] mice [54-56]. Following IV infusion, Cbrain/Cplasma was 0.07 ± 0.02, and 0.09 ± 0.04 for adefovir, 0.015 ± 0.003 and 0.019 ± 0.011 for tenofovir in the WT and Abcc4(-/-) mice, respectively. Although the absence of MRP4 in mice led to an increase in brain exposure of RO 64-0802, Cbrain/Cplasma remained low (~0.04) in the Abcc4(-/-) mice following subcutaneous infusion for 24 hrs. However, these drugs and other known MRP4 substrates, including azidothymidine, cAMP, cGMP, methotrexate, prostaglandins, estradiol-17β-glucuronide, topotecan, olmesartan, and β-lactam antibiotics [54, 78-81], are not useful tool compounds to assess at the BBB. These MRP4 substrates have limited passive permeability and/or may be transported by other transporters. For example, topotecan has been identified as a substrate for Pgp and BCRP [82], and adefovir showed low passive permeability [83]. A suitable tool compound for assessing the role of MRP4 at the BBB would require an MRP4-specific substrate with high passive permeability and as yet that has not been reported.
2.4. Species Differences in Pgp and BCRP at the BBB

The literature supports no fundamental differences in Pgp-, and BCRP-mediated efflux at the BBB between humans and rodents. Generally, the demonstration that Pgp or Bcrp plays a role in brain exposure of a drug in rodents can be extrapolated to humans. However, precise quantitative extrapolation may be challenging given that species differences in sequence and expression level exist. In addition, species differences may be magnified or mitigated by the array of transporters present at the BBB for those compounds that are substrates of multiple transporters.

Pgp has a high sequence homology across multiple species; amino acid homology to human is 87, 85, 87 and 93% for mouse, rat, dog and rhesus monkey, respectively [84]. Feng et al. [85] found few differences in substrate susceptibility between human and mouse Pgp among the 3300 compounds evaluated. Aman and colleagues [86] reported that more than 8000 compounds are structurally diverse in pig kidney epithelial cells transfected with human MDR1 gene (MDR1-LLC-PK1) or rat Mdr1a gene (Mdr1a-LLC-PK1). Most compounds showed comparable efflux ratio (ER) between these two cell lines. Only 3.4% of compounds had an ER outside of the 3-fold boundary (Fig. 2). Although species differences in substrate recognition may exist for some compounds [32, 86], efflux is comparable between human and rodent Pgp for most compounds. Screening compounds for human Pgp only (not both human and rodent Pgp) should be adequate for CNS drug discovery in most cases.

The ABCG2 gene is highly conserved [9], though data on species differences in BCRP substrate recognition are limited. Fluorescent BCRP substrates including mitoxantrone, Hoechst 3342, BODIPY-prazosin, daunorubicin and rhodamine123 were similarly transported by human and mouse BCRP using flow cytometry [87]. We measured bidirectional transport across MDCK cells expressing rat, dog, monkey and human BCRP for a set of compounds including pitavastatin, fluvasatin, gleevec, prazosin and proprietary compounds. No substantial differences in efflux ratios across these cell lines were observed for the compounds evaluated, suggesting that species differences in BCRP substrate recognition are uncommon.

Protein expression of Pgp and BCRP at the BBB varies 2 to 3-fold across species (Table 1). It is still debatable whether this modest species difference in Pgp and BCRP expression would result in significant species differences in brain exposure of Pgp and BCRP substrates. It has been reported that estimated in vivo unbound brain to unbound plasma concentration ratio (Cbrain/Cplasma) of risperidone and 9-OH risperidone was 4 to 7-fold higher in dogs and monkeys than in rats following subcutaneous administration of risperidone [88]. Syvänen et al. [84] reported difference in Cbrain/Cplasma of \([^{11}C]\)-verapamil, \([^{11}C]\)-GR205171 and \([^{18}F]\)-altanserin among rats, monkeys and humans. For example, Cbrain/Cplasma of \([^{11}C]\)-GR205171 was almost 3- and 9-fold higher in monkeys and humans, respectively, compared to rats. However, the difference was not abolished after co-administration of Pgp inhibitor cyclosporine, suggesting that the difference in Pgp activity alone does not explain the observed species differences in Cbrain/Cplasma of these compounds. Uchida et al. [89, 90] measured Cbrain/Cplasma of five Pgp substrates indinavir, quindine, loperamide, paclitaxel and verapamil and one non-Pgp substrate diazepam in both WT mice and monkeys following intravenous (IV) infusion. While Cbrain/Cplasma of indinavir, quindine, paclitaxel and diazepam was similar (<3-fold difference) between mouse and monkey, Cbrain/Cplasma of loperamide and verapamil was 5 to 11-fold higher in mouse than in monkey (Table 3). However, the differences in Cbrain/Cplasma of loperamide and verapamil between monkey and mouse was mainly due to species differences in plasma protein binding. Estimated in vivo unbound to plasma concentration ratio (C_u-brain/C_u-plasma) of the six compounds evaluated was comparable (<3-fold difference) between monkeys and WT mice (Table 3).

Following an IV bolus administration of nelfinavir to monkeys, Cbrain/Cplasma (34 to 75 min post dose) increased dramatically (from 0.044 to 6.4) by co-administration of zosuquidar (3 mg/kg, IV administration), a Pgp inhibitor [73]. The magnitude of the increase was comparable to what was observed in the Pgp deficient mice; Cbrain/Cplasma of \([^{14}C]\)-nelfinavir was 0.08 and 2.3 in the WT and Pgp deficient mice, respectively (2hr after an IV bolus dose of 5 mg/kg) [91]. In recent years, Positron Emission Tomography (PET) with the use of carbon 11-labeled Pgp substrates such as verapamil and N-desmethyl loperamide has been conducted to quantify Pgp activity at the human BBB [92, 93]. N-desmethyl loperamide showed high passive permeability and had an in vitro ER of 9 at 5 µM in MDR1-LLC-PK1 cells under our experimental conditions (data not shown). Following IV administration of \([^{11}C]\)-N-desmethyl loperamide to healthy human subjects, brain distribution was the lowest among the tissues examined; distribution of radioactivity into the pituitary, which is not protected by the BBB, was about 50-fold higher than that into the brain cortex [93]. Furthermore, co-administration with a Pgp inhibitor (tariquidar at 6 mg/kg) increased brain distribution of \([^{11}C]\)-N-desmethyl-loperamide 4-fold [94]. These data support a similar role of Pgp at the BBB between human and rodents and the modest species differences in Pgp expression should
have a small impact on brain penetration for most Pgp substrates.

Collectively, the current literature supports the use of brain exposure data from preclinical species in clinical translation for most Pgp substrates because 1) there are no species differences in Pgp substrate recognition for the majority of compounds; 2) modest species differences in Pgp expression should have a small impact on brain penetration for most Pgp substrates; 3) available in vivo data, albeit sparse for humans, support a similar role of Pgp at the BBB between humans and preclinical species.

3. FACTORS THAT MAY IMPACT CSF EXPOSURE

Drug concentration in CSF (C_{CSF}) has been widely measured in both preclinical species and humans due to its contact with brain tissue and the feasibility of sampling in the clinic (compared to any brain measurement) [1, 2, 95, 96]. C_{CSF} has been suggested as a useful surrogate for brain exposure [1, 95, 96]. However, the value of using C_{CSF} as the surrogate of unbound brain concentration (C_{u-brain}) is questionable in the majority of situations as C_{CSF} may be affected by a number of factors, including sampling site, sampling time and transporters at the BBB and the BCSFB. Furthermore, although not discussed here, the manner and validity of the method used to collect CSF and bioanalytical complexities such as the impact of non-specific binding (not associated with plasma) could affect the accuracy of drug quantitation from the CSF matrix [97, 98].

3.1. Collection Site

The CSF compartment is not homogenous. C_{CSF} may vary depending on the location from which CSF samples are collected. While CSF is usually taken from the cisternal magna in preclinical species, it is obtained from the lumbar region in humans, which is far away from the brain. Experiments with lamivudine provide an example for the impact of collection site on C_{CSF} [99]. Following an IV bolus administration of lamivudine to rhesus monkeys, concentration-time profiles of lamivudine in CSF differed significantly between the lumbar and the ventricular sites. Area under curve (AUC) of lamivudine was significantly higher in the lumbar CSF than in the ventricular CSF (CSF to plasma AUC ratio, AUC_{CSF}/AUC_{plasma} = 0.41 vs. 0.079).

3.2. Collection Time

With the exception of an indwelling catheter, CSF samples are usually only collected at limited time points. However, rapid equilibrium between CSF and brain ECF or blood cannot be assumed due to factors such as bulk flow of fluid movement, poor permeability and transporters, etc. For highly permeable non-efflux substrates such as benzodiazepines, rapid equilibrium between blood and CSF can be reached. For example, following an IV bolus dose of benzodiazepines (diazepam, desmethyldiazepam, midazolam, lorazepam, alprazolam, triazolam, flunitrazepam and clobazam) to cats, all the eight benzodiazepines rapidly entered CSF (collected from the cisternal magna) with peak concentrations usually attained within 15 min post dose. After distribution equilibrium was reached, the disappearance of benzodiazepines was parallel between plasma and CSF [100]. For efflux substrates or compounds with poor passive permeability, however, sampling time alone can be a significant determinant of C_{CSF}/C_{u-plasma} as concentration-time profiles of plasma and CSF may diverge. For example, ceftazidime entered into CSF slowly with peak concentrations observed at 7 hr while serum concentrations declined bi-exponentially following a 30 min IV infusion to patients who had undergone external ventriculostomy [101]. In another example, Haas et al. [102] characterized indinavir pharmacokinetics with intensive lumbar CSF sampling in the clinic. C_{CSF}/C_{u-plasma} of indinavir, which is a Pgp substrate [68], was 0.05 from 0.5 to 1.5hr and 1.47 at 8hr.

3.3. Transporters

The expression of transporters at choroid plexus epithelial cells is qualitatively and quantitatively different than the BBB (Fig. 1) [10, 57]. While peptide transporter 2 (PEPT2), organic anion transporter 3 (OAT3) and MRP4 are highly expressed at the BCSFB, Pgp and BCRP appeared to be expressed in the apical membrane of choroid plexus epithelia at a much lower level than at cerebral microvessels [57, 64, 103, 104]. Shen et al. [105] performed intracerebral microdialysis studies to understand the role of Pgp and Bcrp at the choroid plexus. Following a single IV administration,

### Table 3. Estimated in vivo C_{u-brain}/C_{u-plasma} in monkeys and WT mice following IV infusion.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>C_{u-brain}/C_{u-plasma}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Monkey</td>
</tr>
<tr>
<td>Indinavir</td>
<td>0.061</td>
</tr>
<tr>
<td>Quinidine</td>
<td>0.074</td>
</tr>
<tr>
<td>Loperamide</td>
<td>0.040</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>0.113</td>
</tr>
<tr>
<td>Verapamil</td>
<td>0.154</td>
</tr>
<tr>
<td>Diazepam</td>
<td>1.050</td>
</tr>
</tbody>
</table>

Data compiled from [90, 91].
ventricular CSF to unbound plasma AUC ratio (AUCCSF/AUCu-plasma) of topotecan lactone decreased from 3.07 ± 0.09 in WT mice to 0.86 ± 0.05 in Mdr1a/b(-/-)/Abcg2(-/-) mice, whereas unbound brain to unbound plasma exposure ratio (AUC u-brain/AUCu-plasma) increased from 0.36 ± 0.06 in the WT mice to 0.88 ± 0.07 in the KO animals. Tsuchiya et al. [106] recently reported that CSF of raltegravir in humans was associated with ABCG2 421C>A polymorphism. The mean CSF of raltegravir was significantly lower in patients with ABCG2 421AA genotype (<10 ng/mL) compared to those with CC genotype (103.6 ng/mL) with the heterozygotes possessing an intermediate phenotype (25 ng/mL). These data suggest that Pgp and BCRP are expressed in the apical membrane of epithelial cells at the choroid plexus and may help move drugs across choroid plexus epithelia into the CSF compartment.

In contrast to the study reported by Shen et al. [105], concentrations of Pgp and/or BCRP substrates in CSF samples collected from the cisterna magna generally increased in Pgp and/or Bcrp KO animals (Fig. 3). Following oral administration of 20 compounds that possess similar physical chemical properties, CSF to unbound plasma concentration ratio (C CSF/C u-plasma) in rats correlated well with in vitro Pgp efflux ratio (ER) obtained from mouse Mdr1a-LLC-PK1 cells [107]. Similar results were also observed in monkeys [108]. A potential explanation for the difference in these results compared to the study reported by Shen et al. may be the site of CSF sampling. BBB may have more influence on C CSF in samples collected from the cisterna magna than from the ventricular as CSF flows past brain tissue before it reaches the cisterna magna.

While transporters at the BBB such as Pgp and BCRP have a more profound impact on C brain than C CSF (Fig. 3), transporters that are predominantly expressed at the BCSFB (choroid plexus) such as Mrp1 and Pep2 mainly affect C CSF as supported by studies in KO mice. For example, C CSF of etoposide increased approximately 10-fold in mice lacking both Mrp1 and Pgp compared to the mice lacking Pgp only. In contrast, C brain of etoposide was not affected by the absence of Mrp1 [53]. After intracerebroventricular administration of glycyrsarcosine and cefadroxil, the CSF clearance was approximately 4-times faster in KO mice than in Pep2 null mice, indicating that Pep2 at the choroid plexus removes compounds from CSF. Following IV administration, CSF distribution of four Pep2 substrates (glycylsarcosine, cefadroxil, carnosine and 5-aminolevulinic acid) increased 3 to 9-fold in the Pep2 KO mice, while the effects of Pep2 deletion on brain distribution were more modest or even the reverse to that found in the CSF [57].

4. RELATIONSHIP BETWEEN C u-brain, C u-plasma AND C CSF IN RODENTS

4.1. Highly Permeable Non-efflux Substances

Rapid equilibrium between blood and brain can be reached for highly permeable non-efflux substrates since brain is a highly perfused organ and this type of molecules...
can move across BBB quickly. Doran et al. [34] determined AUC of 34 drugs in plasma (AUC\textsubscript{plasma}), brain (AUC\textsubscript{brain}) and CSF (AUC\textsubscript{CSF}) in WT and Mdr1a/1b\textsuperscript{-/-} mice following subcutaneous administration. The difference in brain exposure between the WT and Mdr1a/1b\textsuperscript{-/-} mice was minimal to modest (<3-fold) for most of them, except metoclopramide, risperidone and 9-OH-risperidone. Brain exposure of metoclopramide, risperidone and 9-OH-risperidone increased 6 to 17-fold in the Mdr1a/1b\textsuperscript{-/-} mice. AUC\textsubscript{brain} and AUC\textsubscript{plasma} were converted to unbound AUC in the brain (AUC\textsubscript{u-brain}) and plasma (AUC\textsubscript{u-plasma}) using unbound fraction in plasma (f\textsubscript{u-plasma}) and unbound fraction in brain (f\textsubscript{u-brain}) taken from Maurer et al. [109] for a comparison between AUC\textsubscript{u-brain} and AUC\textsubscript{u-plasma} or AUC\textsubscript{CSF} in the WT mice. The Pgp substrates metoclopramide, risperidone, 9-OH-risperidone and the poorly permeable compound sulpiride, which showed average apparent permeability value (Papp) of <1 × 10\textsuperscript{-6} cm/s in MDCK cells, were excluded for the comparison. As shown in Fig. (4), AUC\textsubscript{u-brain} correlated well with AUC\textsubscript{u-plasma} or AUC\textsubscript{CSF} for these highly permeable non-efflux substrates. AUC\textsubscript{u-brain}/AUC\textsubscript{u-plasma} and AUC\textsubscript{u-brain}/AUC\textsubscript{CSF} was 0.95 ± 0.70 and 1.41 ± 1.64, respectively. Following IV infusion of 11 highly permeable non-efflux substrates (antipyrine, buspirone, caffeine, carbamazepine, citalopram, diazepam, midazolam, phenytoin, sertraline, thiopeptal and zolpidem) to rats, a similar relationship among Cu-brain, Cu-plasma and Cu-CSF was observed [44]. The fold difference between Cu-brain and Cu-plasma or Cu-CSF was <3-fold for all the 11 compounds. These data indicate that Cu-brain is generally similar to Cu-plasma or Cu-CSF for highly permeable non-efflux substrates.

### 4.2. Efflux Substrates or Compounds with Poor Permeability

As discussed in section 2, transporters at the BBB play important roles in brain exposure; Cu-brain is likely lower than Cu-plasma for efflux substrates or compounds with poor permeability. For example, Cu-plasma was >20-fold higher than Cu-brain for loperamide, amphenavir, quinidine, benzylpenicillin, cimetidine and sulpiride following IV infusion to rats [36, 37, 44]. For efflux substrates or compounds with poor permeability, Cu-CSF in general is lower than Cu-plasma as well [36, 37, 44, 107, 108].

Though Cu-CSF of Pgp and/or BCRP substrates increased in the Pgp and/or Bcrp KO animals for most compounds tested, the magnitude of the increase differed from Cu\textsubscript{brain} as shown in Fig. (3). Cu\textsubscript{CSF}, if used as the surrogate of brain exposure, likely under-estimates the impact of Pgp and Bcrp on brain distribution. For example, Cu\textsubscript{CSF} of amphenavir only increased 1.2-fold, while brain exposure increased 12-fold, in the Mdr1a\textsuperscript{-/-} rats [36]. Following IV infusion to rats, Cu\textsubscript{CSF} was >Cu\textsubscript{brain} (up to 4-fold) for the efflux substrates or compounds with poor passive permeability evaluated including verapamil, loperamide, quinidine, daidzein, genistein, cimetidine and sulpiride, etc [44]. Furthermore, the concentration gradient between Cu\textsubscript{CSF} and Cu\textsubscript{brain} was abolished in the Abcg2\textsuperscript{-/-} mice for daidzein and genistein, and in the Mdr1a/1b\textsuperscript{-/-} mice for verapamil and quinidine. These results indicate efflux is a major mechanism for the observed higher Cu\textsubscript{CSF} than Cu\textsubscript{brain}. While efflux substrates tend to have Cu\textsubscript{CSF}/Cu\textsubscript{brain} of >1, Cu\textsubscript{CSF}/Cu\textsubscript{brain} of <1 has been reported for some compounds. One rare example is morphine-6-glucuronide. Cu\textsubscript{brain} measured by transcortical microdialysis was 19-fold higher than Cu\textsubscript{CSF} in samples collected by cisternal puncture from separate animals following subcutaneous administration of morphine-6-glucuronide [110]. Brain uptake of morphine-6-glucuronide was significantly inhibited by digoxin and D-glucose in an in situ mouse brain perfusion study, suggesting that morphine-6-glucuronide is taken into mouse brain by GLUT1 and a digoxin sensitive transporter, which could be an Oatp [7].

Conclusively, Cu\textsubscript{brain} is close to Cu\textsubscript{plasma} or Cu\textsubscript{CSF} for highly permeable non-efflux substrates as rapid distribution equilibrium can be reached among the blood, the brain and the CSF compartment for these types of molecules. However, Cu\textsubscript{brain} can be significantly different from Cu\textsubscript{plasma} or Cu\textsubscript{CSF} for efflux substrates or compounds with poor passive permeability.

### 5. Commonly Used Approaches to Estimate Cu\textsubscript{brain} in Preclinical Species

For drugs targeting CNS, accurate estimation of Cu\textsubscript{brain} is valuable for understanding the in vitro-in vivo relationship of pharmacological effect and extrapolating preclinical findings to humans. Cu\textsubscript{brain}/Cu\textsubscript{plasma} can serve as a relative indicator for...

![Fig. (4).](image-url) Relationship between AUC\textsubscript{u-brain} and AUC\textsubscript{u-plasma} or AUC\textsubscript{CSF} in wild-type mice for highly permeable non-efflux substrates. Data derived from [33] using unbound fractions taken from [110]. The dashed lines are the 3-fold boundaries. (A higher resolution / colour version of this figure is available in the electronic copy of the article).
brain distribution, for example, comparison between WT and KO animals with the same drug. However, C_{brain}/C_{plasma} is ultimately determined by brain tissue binding, plasma protein binding and BBB transport. C_{brain}/C_{plasma} should not be a parameter for optimization, whereas C_{u-brain}/C_{plasma} is a critical parameter for assessing distribution equilibrium between the blood and the brain compartment. However, direct measurement of C_{u-brain} by microdialysis is generally not applicable in drug discovery due to its limitations including technical complexity, low throughput and technical issues related to low recovery for hydrophobic compounds [111]. We are therefore limited to indirect approaches or surrogates to estimate C_{u-brain} in drug discovery. Three commonly used methods are the use of C_{u-plasma} or C_{CSF} as a surrogate or experimental determination of f_{u-brain}*C_{brain}.

5.1. C_{CSF} as the Surrogate of C_{u-brain}

As discussed in sections 3 and 4, C_{CSF} is not a good surrogate of C_{u-brain} for transporters substrates or compounds with poor permeability. For some instances, we may even draw wrong conclusions about CNS penetration based on C_{CSF}. Here are a few examples: concentrations of 0.5 mTc-teasestami, a Pgp substrate, in CSF samples collected from the cisterna magna, was unchanged in dogs with Mdr1 mutation while brain levels were elevated [38]; co-administration of Pgp inhibitor zosuquidar to non-human primates led to a dramatic increase in brain distribution of nelfinavir, but had no effect on lumbar C_{CSF} [73]; zidovudine is well distributed into human CSF with C_{CSF}/C_{plasma}=1.35 at 4 hr post an oral dose [112], but its brain penetration was significantly limited in rodents [96, 113]. While C_{CSF} is similar to C_{u-brain} for highly permeable non-efflux substrates, in these situations obtaining C_{CSF} is an additional burden but offers no added value over C_{u-plasma} since C_{u-plasma} is an equally good surrogate of C_{u-brain} for these types of molecules.

5.2. C_{u-brain} Determined from f_{u-brain}*C_{brain}

C_{u-brain} in preclinical species can be calculated from the product of measured C_{brain} and f_{u-brain} (determined in vitro) with the assumption that there are no significant regional differences in C_{u-brain}. This assumption probably is valid for most compounds, though may not always hold. In human PET scan studies, brain distribution of N-desmethylloperamide or verapamil was low across regions. With Pgp inhibition, however, brain distribution did not appear to be homogenous [94, 114], which could be due to regional differences in brain components such as gray vs. white matter and blood flow, etc. Doran et al. [88] measured regional brain concentrations of PF-4778574, CE-157119, risperidone and 9-OH risperidone in dogs and monkeys. Brain concentrations of PF-4778574 and CE-157119 in both dogs and monkeys were similar among different regions, whereas small variations (within 2 to 3-fold) were observed for risperidone and 9-OH risperidone among the 8-regions examined with the cerebellum the lowest. This small variation could be due to specific binding or experimental variations. The commonly used in vitro methods for f_{u-brain} determination are the equilibrium dialysis or ultracentrifugation of brain homogenate and the brain slice uptake method. Though the brain slice uptake method may be more physiologically based than the brain homogenate method with respect to the integrity of cell membranes and pH gradients of the intracellular compartments [115], it is technically more demanding and has lower throughput. The more prevalent method in drug discovery is the method of brain homogenate, which adopts the same methodology used for the measurement of f_{u-plasma}. Obviously, the disruption of cells and organelles leads to the loss of pH gradient and may expose binding sites that otherwise would not be available for poorly permeable compounds. A pH gradient could also have significant effects on in vivo blood-to-brain partition for strong basic molecules and correction for pH effect may be needed as suggested by Fridén et al. [116]. Even though the brain homogenate method has these obvious limitations, good correlation was observed between C_{u-plasma} and C_{u-brain} from f_{u-brain}*C_{brain} for highly permeable non-efflux substrates (Fig. 4), suggesting that f_{u-brain} with the brain homogenate method is reflective of in vivo brain tissue binding for majority of highly permeable compounds. The in vitro determined f_{u-brain} from one species usually can be used to convert measured C_{brain} to C_{u-brain} across species [117].

5.3. C_{u-plasma} as the Surrogate of C_{u-brain}

Given the more intimate temporal relationship between the blood and the brain, C_{u-plasma} is a suitable and better surrogate than C_{CSF} for C_{u-brain} when compounds are highly permeable non-efflux substrates and show no evidence of brain distribution impairment. Of course, C_{u-plasma} differs from C_{u-brain} for efflux substrates or poorly permeable compounds and C_{u-brain} determination requires considerable additional resource to measure C_{brain} and f_{u-brain}. Considering that in vitro Pgp are generally predictive of in vivo brain penetration [118], using the ratio of C_{u-plasma} over in vitro Pgp ER (C_{u-plasma}/Pgp ER) as a surrogate can substantially reduce experimental efforts since this value is derived from experimental data already routinely available in drug discovery and development.

6. IN VITRO PERMEABILITY, Pgp AND BCRP DATA OF MARKETED CNS DRUGS

Various in vitro models have been explored to assess BBB penetration including parallel artificial membrane permeability assay, primary cultures, immortalized cell lines of cerebral origin and cell lines of non-cerebral origin. The use of primary cultures such as bovine brain endothelial cells co-cultured with astrocytes has diminished in the industry due to the limitations including labor intensiveness, low expression of Pgp and insufficient tight junctions. MDCK or LLC-PK1 cells stably expressing Pgp have become prevalently used as in vitro models for assessing CNS penetration.

The belief that ideal CNS drugs should have high passive permeability and low Pgp ER has been mainly based on in vitro permeability, efflux and in vivo brain exposure data of marketed CNS drugs tested so far [34, 119]. As in vitro data could be significantly affected by cell lines and how the assays are conducted [120], the in vitro criteria for ideal CNS compounds may vary from lab to lab. Mahar Doan et al. [119] evaluated 48 CNS drugs in MDRI-MDCK and concluded that compounds targeting CNS should ideally have an in vitro passive permeability of >15 (10^-6 cm/s) and ER of
<2.5 in MDR1-MDCK cells. However, Feng et al. [85] reported much lower apparent permeability (Papp) values for some CNS compounds. Under the experimental conditions these authors used to evaluate 32 marketed CNS drugs, the ER in MDR1-MDCK cells was ≤3.6 and Papp values in the apical to basolateral direction across MDCK cells ranged from 0.9 (10^{-6} cm/s) for sulpiride to 30.6 (10^{-6} cm/s) for caffeine. Chlorpromazine, fluoxetine, morphine, paroxetine and sertraline had measured Papp values of 4.1, 6.3, 1.8, 6.1 and 1.9 (10^{-6} cm/s), respectively.

Twenty-six marketed CNS drugs were evaluated in our in vitro permeability and efflux assays (Table 4). Consistent with the literature data, all the compounds tested had ER of ≤3 in the MDR1-MDCK cells. Similar results were observed in the BCRP-MDCK cells. A number of compounds including sertraline, chlorpromazine, fluoxetine, fluvoxamine, clozapine and haloperidone showed low recovery (<50%) and some of them had low measured Papp values. This highlights the importance of considering recovery data for the interpretation of permeability data. For compounds with low recovery, Papp values are likely underestimated, although their true values are uncertain. All the compounds with recovery of >50% had Papp values of >10 (10^{-6} cm/s), except sulpiride. Sulpiride had low Papp values of <1 (10^{-6} cm/s) with good recovery, which is consistent with its limited brain distribution. Its C_{u-brain}/C_{u-plasma} was 0.02 in rats following IV

Table 4. In vitro permeability and efflux data of marketed CNS drugs in our assays.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Permeability (10^{-6} cm/s)</th>
<th>ER in MDR1-MDCK</th>
<th>ER in ABCG2-MDCK</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulpiride</td>
<td>1.0</td>
<td>&gt;1.7</td>
<td>NC</td>
<td>93</td>
</tr>
<tr>
<td>Sertraline</td>
<td>1.2</td>
<td>3</td>
<td>3.1</td>
<td>26</td>
</tr>
<tr>
<td>Chlorpromazine</td>
<td>1.5</td>
<td>NC</td>
<td>NC</td>
<td>25</td>
</tr>
<tr>
<td>Fluoxetine</td>
<td>2.6</td>
<td>NC</td>
<td>1.8</td>
<td>36</td>
</tr>
<tr>
<td>Fluvoxamine</td>
<td>6.5</td>
<td>0.8</td>
<td>0.8</td>
<td>31</td>
</tr>
<tr>
<td>Clozapine</td>
<td>8.0</td>
<td>0.5</td>
<td>0.6</td>
<td>40</td>
</tr>
<tr>
<td>Haloperidol</td>
<td>12.1</td>
<td>1.2</td>
<td>1.0</td>
<td>46</td>
</tr>
<tr>
<td>Propoxyphene</td>
<td>12.1</td>
<td>0.8</td>
<td>1.6</td>
<td>60</td>
</tr>
<tr>
<td>Hydroxyzine</td>
<td>14.1</td>
<td>0.5</td>
<td>1.6</td>
<td>51</td>
</tr>
<tr>
<td>Citalopram</td>
<td>20.3</td>
<td>1.9</td>
<td>0.9</td>
<td>58</td>
</tr>
<tr>
<td>Paroxetine</td>
<td>23.6</td>
<td>1.1</td>
<td>1.0</td>
<td>68</td>
</tr>
<tr>
<td>Risperidone</td>
<td>28.5</td>
<td>1.9</td>
<td>1.5</td>
<td>76</td>
</tr>
<tr>
<td>Caffeine</td>
<td>29.4</td>
<td>1.5</td>
<td>1.3</td>
<td>102</td>
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<tr>
<td>Venlafaxine</td>
<td>30.3</td>
<td>1.3</td>
<td>0.8</td>
<td>77</td>
</tr>
<tr>
<td>Zolpidem</td>
<td>30.6</td>
<td>1.1</td>
<td>0.9</td>
<td>79</td>
</tr>
<tr>
<td>Midazolam</td>
<td>33.0</td>
<td>0.6</td>
<td>0.6</td>
<td>77</td>
</tr>
<tr>
<td>Metoclopramide</td>
<td>33.8</td>
<td>1.2</td>
<td>1.2</td>
<td>89</td>
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<tr>
<td>Diazepam</td>
<td>35.5</td>
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<td>0.5</td>
<td>81</td>
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<tr>
<td>Trazodone</td>
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</tr>
<tr>
<td>Buspirone</td>
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<td>1.4</td>
<td>85</td>
</tr>
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<td>Selegiline</td>
<td>39.3</td>
<td>0.7</td>
<td>1.5</td>
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<td>Hydrocodone</td>
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<td>1.3</td>
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<td>87</td>
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<td>Lamotrigine</td>
<td>41.6</td>
<td>1.1</td>
<td>0.9</td>
<td>94</td>
</tr>
<tr>
<td>Carbamazepine</td>
<td>68.9</td>
<td>1.3</td>
<td>1.1</td>
<td>107</td>
</tr>
</tbody>
</table>

VC-MDCK, MDR1-MDCK and ABCG2-MDCK were MDCK cells transfected with vector control, human MDR1 and ABCG2, respectively. Transport studies were conducted at 6 to 7-days post seeding at 5 μM with a 2-h incubation. 'average apparent permeability values of both directions in VC-MDCK; average recovery in VC-MDCK. NC, not calculated due to apparent permeability values of <1 (10^{-6} cm/s) in both directions.
Fig. (5). Evaluation Strategy for CNS Penetration. * the criteria should be determined by assay calibration in each lab; the cutoff of Papp values was for compounds incubated in buffer without protein and with recovery ≥50%; consider permeability acceptable if recovery is <50% in control cells, although exceptions may exist. **Poorly permeable compounds may get into brain through uptake transporters on rare occasions. PD, pharmacodynamic. (A higher resolution / colour version of this figure is available in the electronic copy of the article).

7. EVALUATION STRATEGY FOR CNS PENETRATION IN DRUG DISCOVERY

Obviously, Pgp and BCRP substrates should be avoided, whenever possible, for CNS targets. Ideal CNS compounds should have high passive permeability and low Pgp and BCRP ER, though the cutoff criteria may depend on the cell lines used, how the assays are performed. Quite often, however, it is challenging to balance Pgp and BCRP status with other properties such as potency, systemic exposure and safety profiles for some targets. In addition, other unknown mechanisms may limit brain penetration. When we have to select Pgp and BCRP substrates for development, which presents uncertainty in the estimation of C_{u-brain}, similar impact of Pgp and BCRP on brain penetration between humans and rodents is assumed.

We recommend using C_{u-plasma} as the surrogate for C_{u-brain} across species when compounds are non-efflux substrates with high passive permeability and have no evidence of impaired penetration into brain. Compounds with high passive permeability and low ER in Pgp and BCRP expressing cells do not always show good BBB penetration due to potential involvement of other transporters in brain penetration and efflux saturation under in vitro assay conditions. In general, Pgp is more susceptible to saturation in vitro than in vivo because unbound plasma concentrations is often lower than the concentrations used for in vitro assays and Pgp expression may be lower in the cell lines used in vitro assays than in vivo at the BBB [91]. Therefore, it is important to assess in vivo brain penetration (C_{u-brain}/C_{u-plasma}) for spot checking within a chemical scaffold to be confident that C_{u-plasma} is a valid surrogate for C_{u-brain} during drug discovery. When drug penetration into brain is impaired, we recommend using f_{u-brain} \times C_{u-brain} as C_{u-brain} in preclinical species or C_{u-plasma}/C_{u-brain} in vitro Pgp ER if Pgp is the major limiting mechanism for brain penetration.

Fig. (5) shows the framework for the evaluation of CNS penetration in drug discovery based on in house and literature data and in the consideration of judicious use of animal and fiscal resources. The framework can be summarized as follows.

1) In vitro permeability, Pgp and BCRP assays are the primary screening tools for understanding and optimizing for brain penetration. As noted, it is important to establish selection criteria for specific assays since results will be impacted by assay conditions. Based on data from our experiments (exemplified in Table 4), the criteria for compound selection are Pgp and BCRP ER of <3 and average Papp value from control cells of >10 (10^{-6} cm/s), and permeability is considered acceptable for compounds with recovery ≤50%. We chose the criteria for compound selection that are relatively loose in the consideration of not throwing out potentially therapeutically useful compounds due to experimental variations...
at the risk of allowing further characterization of what may turn out to be poor drug candidates.

2) Experimental determination of Cu-brain from fu-brain*Cbrain is important for checking the validity of using Cu-plasma or Cu-plasma/in vitro Pgp ER as a surrogate for Cu-brain in the screening strategy for a chemical scaffold.

3) If fu-brain*Cbrain/Cu-plasma is ~1 in rodents, Cu-plasma should be used as the surrogate for Cu-brain for all species including humans. If fu-brain*Cbrain/Cu-plasma is not consistent with in vitro permeability and efflux data, further investigation may be conducted.

4) For Pgp and BCRP substrates, similar impact of Pgp and Bcrp on brain penetration is assumed between humans and rodents. Since the difference in P-gp and BCRP protein expression levels at the BBB are 2- to 3-fold between humans and rodents (Table 1), the differences in Cu-brain/Cu-plasma of Pgp and BCRP substrates between humans and rodents could be typically less than 3-fold. Therefore, the measurement of Cu-brain/Cu-plasma in rodents during drug development would be useful in understanding brain penetration in humans.

CONCLUSION

In summary, transporters have differential effects on brain and CSF exposure. Though CSF is in direct contact with the brain, CCSF is not a reliable surrogate for Cu-brain. The general guidance for optimizing CNS penetration is to minimize efflux, while maintaining good permeability, and use Cu-plasma as the surrogate for Cu-brain for highly permeable non-efflux substrates. Experimental determination of Cu-brain from fu-brain*Cbrain can be used to check the validity of using Cu-plasma or Cu-plasma/in vitro Pgp ER as a surrogate for Cu-brain for a chemical scaffold.

LIST OF ABBREVIATIONS

AUC = Area Under Curve
AUC_{CSF} = AUC in CSF
AUC_{u-brain} = Unbound AUC in the Brain
AUC_{u-plasma} = Unbound AUC in Plasma
BBB = Blood-brain Barrier
BCRP = Breast Cancer Resistance Protein
BCRP-MDCK = Madin-Darby Canine Kidney Cells Expressing BCRP
BCSFB = Blood-cerebrospinal Fluid Barrier
C_{brain} = Total Brain Concentration
C_{CSF} = Drug Concentration in CSF
CNS = Central Nervous System
C_{plasma} = Total Drug Concentration in Plasma
CSF = Cerebrospinal Fluid
C_{u-brain} = Unbound Brain Concentration
C_{u-plasma} = Unbound Plasma Concentration
ECF = Brain Extracellular Fluid
ER = Efflux Ratio
fu-brain = Unbound Fraction in Brain
fu-plasma = Unbound Fraction in Plasma
GLUT1 = Glucose Transporter 1
IV = Intravenous
KO = Knockout
LC-MS/MS = Liquid Chromatography Tandem Mass Spectrometry
MCT1 = Monocarboxylate Transporter 1
MDCK = Madin-Darby Canine Kidney Cell
Mdr1a-LLC-PK1 = pig Kidney Epithelial Cells Transfected with rat Mdr1a Gene
MDR1-LLC-PK1 = Pig Kidney Epithelial Cells Transfected with the human MDR1 Gene
MDR1-MDCK = Mardin-Darby Canine Kidney Cells Transfected with Human MDR1 Gene
MRP4 = Multiple Drug Resistance Protein 4
OAT = Organic Anion Transporter
OATP = Organic Anion Transporting Poly-peptide
Papp = Apparent Permeability Value
PEPT2 = Peptide Transporter 2
Pgp = P-glycoprotein

CONSENT FOR PUBLICATION

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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