Central Nervous System Penetration for Small Molecule Therapeutic Agents Does Not Increase in Multiple Sclerosis- and Alzheimer’s Disease-Related Animal Models Despite Reported Blood-Brain Barrier Disruption

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ABSTRACT:
Therapy for central nervous system (CNS) diseases requires drugs that can cross the blood-brain barrier (BBB). BBB disruption has been reported in patients with multiple sclerosis (MS) and Alzheimer’s disease (AD) and the related animal models as evidenced by increased infiltration of inflammatory cells or increased staining of Igs in the central nervous system. Although CNS penetration of therapeutic agents under pathological conditions has rarely been investigated, it is commonly assumed that BBB disruption may lead to enhanced CNS penetration and also provide a “window of opportunity” through which drugs that do not normally cross BBB are able to do so. In this article, we have compared brain penetration of eight small molecules in naive animals and experimental autoimmune encephalomyelitis (EAE) mice, streptozotocin-induced mice, and TASTPM transgenic mice. The tool compounds are lipophilic transcellular drugs [GlaxoSmithKline (GSK)-A, GSK-B, GSK-C, and naproxen], lipophilic P-glycoprotein (P-gp) substrates (amprenavir and lopinavir), and hydrophilic paracellular compounds (sodium fluorescein and atenolol). Our data showed that rate and extent of CNS penetration for lipophilic transcellular drugs and P-gp substrates are similar in naive and all tested animal models. The brain penetration for paracellular drugs in EAE mice is transiently increased but similar to that in naive mice at steady state. Our data suggest that, despite reported BBB disruption, CNS penetration for small molecule therapeutic agents does not increase in MS- and AD-related animal models.

All organisms with a well developed CNS have a BBB (Abbott, 2005). The BBB is composed of a single layer of endothelial cells connected by tight junctions. Brain microvascular endothelial cells lack fenestrations, have few pinocytotic vesicles, and express a variety of metabolic enzymes and membrane efflux transporters, such as P-gp (Kusuhara and Sugiyama, 2001; Dauchy et al., 2008). The close association of pericytes and astrocytic foot processes with abluminal endothelial membrane presents an additional barrier to translocation. The BBB represents physical and enzymatic barriers that restrict and regulate the penetration of compounds into the brain. It is estimated that 98% of all small molecules do not cross the BBB. All large molecules, such as monoclonal antibodies (mAbs), recombinant proteins, or antisense agents, do not cross the BBB (Partridge, 2005). The BBB is essential to protect the CNS from potentially harmful agents in the peripheral circulation; however, it also prevents potential therapeutic agents from reaching the site of action and hence presents great challenges to CNS drug discovery and development.

Animal models are important tools to study pathology and test potential treatments for CNS diseases. A transgenic mouse bearing mutant transgenes linked to familial forms of AD for the amyloid precursor protein and presenilin-1 (TASTPM) showed Aβ plaque deposition and age-related histological changes in associated brain pathology (Howlett et al., 2008). TASTPM mice, therefore, may provide a means for assessment of novel therapeutic agents directed to modifying or halting progression of AD. Besides Aβ plaques, the other hallmark pathological characteristic of AD is the presence of neurofibrillary tangles containing hyperphosphorylated tau, a microtubule-associated protein. Administration of streptozotocin (STZ) depletes insulin and the STZ-induced mouse is a commonly used model for diabetic drug research. STZ was also shown to rapidly and largely increase tau phosphorylation in mouse brain (Clodfelder-Miller et al., 2006). This animal model has been adapted for AD drug discovery. It was reported that BBB permeability of insulin, a polysaccharide paracellular marker, increased at the mediobasal hypothalamus (p < 0.01), mediodorsal hypothalamus (p < 0.05), and periaqueductal gray (p < 0.01) in STZ-induced rats (Lorenzi et al., 1986). BBB permeability in TASTPM mice compared with that in naive mice has not been studied. Multiple sclerosis is a chronic inflammatory demyelinating disease of the CNS, characterized by the presence of sclerotic

ABBREVIATIONS: CNS, central nervous system; BBB, blood-brain barrier; P-gp, P-glycoprotein; mAb, monoclonal antibody; AD, Alzheimer’s disease; STZ, streptozotocin; EAE, experimental autoimmune encephalomyelitis; MS, multiple sclerosis; PK, pharmacokinetics; NaF, sodium fluorescein; GSK, GlaxoSmithKline; PSA, polar surface area; Br/Bl, brain/blood ratio; PTX, pertussis toxin; PBS, phosphate-buffered saline.
lesions or plaques scattered throughout the brain. EAE is a relevant animal model of MS characterized by perivascular CD4+ T cell and mononuclear cell inflammation and subsequent primary demyelination of axonal tracks in the CNS, leading to progressive hindlimb paralysis (Miller and Karpus, 2007). The integrity of the BBB was reported to be weakened in patients with MS (Stone et al., 1995) and in EAE animals (Tonra, 2002).

It is hypothesized but generally accepted that a compromised BBB may provide a “window of opportunity” through which drugs that do not normally cross BBB are able to do so. This finding is particularly important for therapeutic agents targeting the CNS because drug exposure at the target site of action may be altered in disease conditions and the pharmacokinetics (PK) and CNS penetration are investigated in naive animals only. As of now, a compromised BBB reported in patients with MS and AD and the related animal models was evidenced by increased infiltration of inflammatory cells (Couraud, 1998; Förster et al., 2007) or increased staining of Igs (Bowman et al., 2007) in the central nervous system. Little is known about CNS penetration of therapeutic agents under the pathological conditions. We have compared brain penetration of eight small molecules in naive mice and the related animal models for MS and AD. The compounds were chosen to cover the three BBB permeation mechanisms (passive diffusion, active efflux transport, and paracellular pathway) that are most relevant to small molecules (Abbott et al., 2007).

### Materials and Methods

#### Compounds Selection.

The molecular properties and BBB permeation mechanisms of tool compounds are listed in Table 1. Atenolol and NaF, characterized by hydrophilic, polar, and nonsubstrates for carrier or active transport systems, cross the BBB by a paracellular mechanism via the tight junction (Nakagawa et al., 2009). GSK-A, GSK-B, GSK-C, and naproxen, nonpolar and lipid-soluble molecules, passively diffuse through the cell membranes. There is a general correlation between the rate at which a lipophilic drug enters the CNS brain penetration and a compound’s lipid solubility, usually determined as the logD octanol/buffer partition coefficient at pH 7.4 (Clark, 2003). Other factors that restrict the entry of compounds into the CNS are a high polar surface area (PSA) (>80 Å), a tendency to form more hydrogen bonds (Gleeson, 2008), and a molecular mass in excess of 450 Da (Abbott et al., 2010). GSK-A [brain/blood ratio (Br/Bl) = 0.18; GSK internal results] has high PSA (103 Å) and a high number of hydrogen bonds (9). GSK-B (Br/Bl = 1.1; GSK internal results) has clogD (pH 7.4) at 3.2, 5 hydrogen bonds, and PSA at 53 Å. GSK-C (Br/Bl = 0.08; GSK internal results) has very high molecular weight (720), a high number of hydrogen bonds (8), and high PSA (76 Å). Naproxen (Br/Bl = 0.02) (Summerfield et al., 2006) has a low computed octanol/water partition coefficient (clogD) (pH7.4) at 0.3 despite reasonable molecular weight (230), hydrogen bonds (4), and PSA (47 Å). Active efflux transporters, such as P-gp, intercept and remove some lipid-soluble amphiphilic drugs such as ampranavir and loperamide, resulting in low brain penetration. The remaining BBB permeation mechanisms were not investigated because they are either for large molecules or small endogenous molecules. Carrier-mediated influx transporters mediate uptake of essential polar molecules, such as glucose and amino acids. Receptor-mediated transcytosis can transport large molecules, such as peptides and proteins, whereas adsorptive mediated transcytosis may be induced nonspecifically by cationic macromolecules such as albumin.

<table>
<thead>
<tr>
<th>Permeation Mechanism</th>
<th>Compound</th>
<th>Mol. Wt.</th>
<th>cLogD at pH 7.4</th>
<th>Hydrogen Bond Donor</th>
<th>Hydrogen Bond Acceptor</th>
<th>PSA (Å)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Paracellular pathway via tight junction</td>
<td>NaF</td>
<td>376</td>
<td>1.9</td>
<td>0</td>
<td>5</td>
<td>89</td>
</tr>
<tr>
<td></td>
<td>Atenolol</td>
<td>266</td>
<td>−1.7</td>
<td>3</td>
<td>5</td>
<td>85</td>
</tr>
<tr>
<td>Transcellular pathway via passive diffusion</td>
<td>GSK-A</td>
<td>426</td>
<td>1.7</td>
<td>1</td>
<td>8</td>
<td>103</td>
</tr>
<tr>
<td></td>
<td>GSK-B*</td>
<td>516</td>
<td>3.2</td>
<td>1</td>
<td>4</td>
<td>53</td>
</tr>
<tr>
<td></td>
<td>GSK-C</td>
<td>720</td>
<td>1.6</td>
<td>0</td>
<td>8</td>
<td>76</td>
</tr>
<tr>
<td></td>
<td>Naproxen</td>
<td>230</td>
<td>0.3</td>
<td>1</td>
<td>3</td>
<td>47</td>
</tr>
<tr>
<td>Active efflux transport P-gp substrates</td>
<td>Amprenavir</td>
<td>505</td>
<td>2.7</td>
<td>3</td>
<td>9</td>
<td>131</td>
</tr>
<tr>
<td></td>
<td>Loperamide</td>
<td>454</td>
<td>3.3</td>
<td>1</td>
<td>4</td>
<td>44</td>
</tr>
</tbody>
</table>

*The structure of GSK-B was shown as example 10h in a recent publication (Hall et al., 2010).
plasma, or brain homogenate were immediately frozen on dry ice and subsequently were stored at approximately −70°C until analysis.

**Brain Tissue Binding.** A 96-well equilibrium dialysis apparatus (HT Dialysis LLC, Gales Ferry, CT) was used to determine the free fraction in the brain from naive or EAE mice for naproxen, loperamide, GSK-A, and GSK-B. Membranes (3-kDa cutoff) were conditioned in deionized water for 40 min, followed by 80:20 deionized water-ethanol for 20 min, and then were rinsed with deionized water before use. The brain tissue was homogenized with 2× PBS in an ice bath. The diluted brain homogenate was spiked with 10 μM test compound, and 150-μl aliquots were loaded into the 96-well equilibrium dialysis plate. Dialysis against PBS was performed for 4 h in a 37°C air bath incubator (Stuart Scientific, Watford, UK) at 130 rpm. At the end of the incubation period, matrix in brain homogenate or PBS was balanced with corresponding opposite fluid before analysis. The unbound fraction was determined as the ratio of the peak area in buffer to that in brain, with correction for the dilution factor (Kalvass and Maurer, 2002).

**Analysis of Drugs in Mouse Blood and Brain.** Plasma and brain EAE samples from NaF experiments were analyzed using a fluorometer (SpectraMax M5; Molecular Devices, Sunnyvale, CA) with excitation at 485 nm and emission at 530 nm. Quantification of other compounds in blood and brain samples was analyzed by Waters ACQUITY ultraperformance liquid chromatography coupled with an API 5000 or 4000 Qtrap tandem mass spectrometry system (Applied Biosystems, Foster City, CA). Samples were processed by deproteinization using acetonitrile containing the appropriate internal standard. Settings for the mass spectrometers were optimized to achieve the highest signal for each analyte. The mass transitions used for quantification were m/z 506 to 245 for ampicillin, 477 to 266 for loperamide, 267 to 190 for atenolol, and 721 to 391 for GSK-C in positive mode and m/z 425 to 353 for GSK-A, 515 to 210 for GSK-B, 229 to 169 for naproxen, and 333 to 287 for NaF (TASTPM experiment) in negative mode.

**Statistical Analysis.** Data are presented as the mean ± S.D. A Student’s two-tailed unpaired t test was used to determine statistical significance. The significance level was p < 0.05.

**Results**

**Brain Penetration of Paracellular Compounds in Naive and EAE Mice.** A compromised BBB in EAE mice has been shown by liquid chromatography-tandem mass spectrometry system. Because NaF was quantified by the fluorescent detection method with NaF. The high dose (500 mg/kg) was used for the NaF experiment because NaF was quantified by the fluorescent detection method with low sensitivity, compared with a much lower dose for atenolol (5 mg/kg), which was quantified by a state-of-art ultra-performance liquid chromatography-tandem mass spectrometry system. Because absolute quantities in the CNS in the absence of measuring coincent changes in the peripheral compartment can be misleading, we considered changes to Br/Bl, a more relevant way to compare brain penetration and reduce intersubject variability. The mean Br/Bl for NaF was 0.040 ± 0.014 and 0.129 ± 0.002 for naive and EAE mice, respectively. The mean Br/Bl for atenolol was 0.005 ± 0.002 and 0.014 ± 0.002 for naive and EAE mice, respectively. The Br/Bl difference for both compounds was statistically significant (p < 0.01), whereas blood concentrations for both compounds were similar between naive and EAE mice. These data showed that brain penetration of paracellular compounds was elevated in EAE mice compared with naive mice at 10 min after intravenous bolus administration. However, in a following experiment at putative steady state after intravenous infusion for 2.5 h (>5 blood half-lives), brain penetration of atenolol was similar in EAE (Br/Bl = 0.014 ± 0.003) and naive mice (Br/Bl = 0.015 ± 0.001). To confirm these results, naive mice were given a loading dose by intravenous bolus administration immediately followed by intravenous infusion. The dosing regimen was designed to obtain quantifiable blood and brain concentrations at 10 min and 2.5 h. Br/Bl of atenolol in naive mice was 0.005 ± 0.001 at 10 min and 0.010 ± 0.002 (p < 0.01) at 2.5 h (Table 2). These results showed that the brain penetration rate, but not the extent of paracellular compounds increased in EAE mice compared with that in naive mice. Ideally brain penetration should be determined at steady state. However, EAE mice were too weak for vein cannulation surgery and intravenous infusion for an extended period of time. All subsequent experiments were conducted with two to three time points after single intravenous or oral dose administration to evaluate both the rate and extent of brain penetration.

**Brain Penetration of Transcellular Drugs in Naive and EAE Mice.** To include transcellular pathways in our investigations, BBB permeability studies of GSK-A and GSK-B, two lipophilic nonpolar compounds, were performed. GSK-A and GSK-B are compounds in drug discovery for MS and AD, respectively. The two compounds were chosen because of their low-to-intermediate (GSK-A Br/Bl = 0.18) and high brain penetration (GSK-B Br/Bl = 1.1), respectively. Compounds were given by oral gavage, and sufficient drug concentrations in brain and blood were achieved at 2 mg/kg dosing. Brain penetration of GSK-A and GSK-B was investigated at various EAE disease stages: after myelin oligodendrocyte glycoprotein and the first PTX administration (day 1), after the second PTX administration (day 3), before onset (day 7), onset (day 13), peak (day 20), and later stage (day 27). The brain penetration of GSK-A and GSK-B at all three time points (2, 4, and 8 h after dosing) was not significantly different between EAE and naive mice (Fig. 1, A and C). In addition, Br/Bl of GSK-A was similar in EAE and naive mice at 10 min after intravenous bolus administration at the peak stage (Fig. 1B). These data suggest that not only extent but also rate of brain penetration for transcellular compounds does not increase under disease conditions.

GSK-A and GSK-B are very lipophilic and highly protein-bound (blood and brain protein binding >99.5%). To investigate whether

**TABLE 2**

**Brain Penetration (Br/Bl) of paracellular markers in EAE and naive mice after intravenous administration**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Dosing Regimen</th>
<th>Sample Time</th>
<th>Naive</th>
<th>EAE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atenolol</td>
<td>Intravenous bolus (5 mg/kg, n = 5)</td>
<td>10 min</td>
<td>0.005 ± 0.002</td>
<td>0.014 ± 0.002*</td>
</tr>
<tr>
<td></td>
<td>Intraavenous infusion (10 mg/h/kg)*</td>
<td>2.5 h</td>
<td>0.014 ± 0.003</td>
<td>0.015 ± 0.001</td>
</tr>
<tr>
<td>Atenolol</td>
<td>Intravenous bolus (6 mg/kg)-intravenous infusion (10 mg/h/kg, n = 3)</td>
<td>10 min</td>
<td>0.005 ± 0.001</td>
<td>Not carried out</td>
</tr>
<tr>
<td>NaF</td>
<td>Intravenous bolus (500 mg/kg, n = 3)</td>
<td>10 min</td>
<td>0.040 ± 0.014</td>
<td>0.129 ± 0.002*</td>
</tr>
</tbody>
</table>

* p < 0.01.

* Br/Bl in naive and EAE mice was derived from four and two animals, respectively.
BBB permeability of a less lipophilic and lower protein-bound drug is altered in animal models, naproxen was given by intravenous administration at 5 mg/kg to naive and EAE mice. Naproxen, a nonsteroidal anti-inflammatory drug, is a non-P-gp compound with Br/Bl of only 0.02, and the protein binding of naproxen in brain homogenates and blood was 45.8 and 98.2%, respectively (Summerfield et al., 2006). Brain penetration of naproxen was not significantly different between EAE and naive mice (Fig. 1D). The mean Br/Bl of naproxen at 30 min after the intravenous dose was 0.012 ± 0.003 and 0.014 ± 0.005 in naive and EAE mice, respectively. The mean Br/Bl of naproxen at 1 h after the intravenous dose was 0.010 ± 0.003 and 0.011 ± 0.001 in naive and EAE mice, respectively.

Amprenavir and loperamide, two prototypical P-gp substrates, were evaluated for BBB permeability to span the spectrum to transporter functions in disease animal models. P-gp is the best characterized efflux transporter, expressed not only at luminal side of BBB but also at the apical side of endothelial cells in absorption and elimination organs such as gastrointestinal tract, liver, and kidney. Strong P-gp substrates often show low oral bioavailability and poor brain penetration. As a result, amprenavir and loperamide were given by intravenous bolus administration instead of oral gavage administration. A preliminary experiment was also performed in naive mice to determine the appropriate time point at which brain concentration can be quantifiable. Ten and 30 min were subsequently chosen to study brain penetration for amprenavir and loperamide, respectively. Our data showed that brain penetration of amprenavir and loperamide did not increase in EAE mice at the peak stage compared with that in naive mice (Fig. 2). The mean Br/Bl for amprenavir was 0.019 ± 0.006 and 0.013 ± 0.003 in naive and EAE mice, respectively. The mean Br/Bl for loperamide was 0.114 ± 0.033 and 0.112 ± 0.060 in naive and EAE mice, respectively.

Brain Penetration of Small Molecules in TASTPM Transgenic Mice and STZ-Induced Mice. To address whether unchanged brain penetration for lipophilic compounds was unique to EAE mice, BBB permeability of GSK-C, an exploratory compound for the treatment of AD, was investigated in the STZ-induced mouse model. The route of administration, dose, and time point were in line with the efficacy study conducted in these animals. The results are shown in Fig. 3. The mean Br/Bl for GSK-C at 6 h after the dose was similar in naive (0.085 ± 0.008) and STZ mice (0.091 ± 0.042), respectively. GSK-A and GSK-B, the two lipophilic compounds investigated in EAE mice, were administered to TASTPM mice by oral gavage. Early Aβ deposition was found at 3 months of age, and all mice exhibited Aβ deposition at 4 months and showed gender differences (more severe in females than in males). Four-month-old mice were used for the BBB permeability study. GSK-A and GSK-B were administered to male and female mice, respectively. Brain penetration for GSK-A and GSK-B at all three time points (2, 4, and 8 h postdose) was not significantly different between TASTPM and naive mice. BBB permeability to the paracellular compound in TASTPM and naive mice was investigated by intravenous administration of NaF only. In contrast with EAE mice, brain penetration of NaF in TASTPM mice was not enhanced compared with that in naive mice.

Brain Tissue Binding in EAE and Naive Mice. The central tenet in PK is that the unbound or free drug concentrations are responsible for drug action. To address whether free drug concentrations are altered in animal disease models, brain tissue binding was determined for naproxen, loperamide, GSK-A, and GSK-B. Free fraction was

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**Fig. 1.** Brain penetration of lipophilic nonpolar compounds GSK-A (A and B), GSK-B (C), and naproxen (D) in EAE at various stages of disease progression and in naive mice. The x-axis represents blood and brain sampling time after dosing.

**Fig. 2.** Brain penetration of lipophilic P-gp substrates at EAE peak stage and in naive mice. A single intravenous bolus 5 mg/kg dose of amprenavir or loperamide was administered to each mouse. Blood and brain samples were collected at 10 and 30 min for amprenavir and loperamide, respectively.
similar in brain homogenates obtained from naive and EAE mice (Table 3).

**Blood PK of the Tool Compounds in Naive and Animal Models.**

To address whether the disease state might have affected the blood PK in each animal model, the blood concentrations for various drugs used in previous experiments (Figs. 1, 2, and 3; Table 2) were tabulated and shown in Fig. 4. The blood concentrations in EAE mice, TASTPM transgenic mice, and STZ-induced mice were not significantly different from that in naive mice after intravenous bolus and oral administration of atenolol, NaF, naproxen, loperamide, GSK-A, GSK-B, and GSK-C. Among all groups compared, only the blood concentration of amprenavir was significantly greater in EAE mice than in naive mice, possibly because of dosing variability.

**Discussion**

There is growing evidence that the BBB is compromised in several neurological diseases including AD and MS. Increased leukocyte migration in MS has been reported to lead to reorganization of the actin cytoskeleton and breakdown of the tight junctions proteins occluding and ZO-1 (Couraud, 1998; Förster et al., 2007). From EAE, an animal model for MS, the inflammatory cytokines tumor necrosis factor-α and interleukin-1 have been delineated to be the key mediators inducing alterations in BBB permeability (Körner and Sedgwick, 1996). Bowman et al. (2007) have reported BBB impairment in a subgroup of patients with AD whose peripheral IgG has greater access to the CNS, BBB microvascular changes such as increased number of fragmented vessels with fewer intact branches, atrophic string vessels, and increased irregularity of capillary surfaces have been described in AD (Zlokovic, 2008). Because BBB permeability of leukocytes and large molecules such as peripheral IgG is enhanced, it is reasonable to hypothesize that therapeutic agents, especially small molecules (mol. wt. <1000), should have greater brain penetration in patients with MS and AD and their corresponding animal disease models. Compounds with good potency and developability but with poor brain penetration are usually discarded during neurodegenerative drug discovery. The potential increased brain penetration of therapeutic agents in diseases could be very valuable for CNS drug discovery because usually discarded compounds could have been saved for progression.

In this article, we have compared brain penetration of eight small molecules in AD and MS disease models and naive control animals. There are two fundamental components of assessing brain penetration. One is the rate of brain uptake at the initial state, and the other is the extent of brain exposure at steady state (Di et al., 2008). Although CNS indications such as hypnosis, epilepsy, and stroke require rapid onset, the extent of brain penetration is much more important than the rate for chronic diseases such as AD and MS. The brain penetration of atenolol and NaF, two well characterized paracellular markers, were significantly elevated in EAE mice compared with naive mice at 10 min after intravenous bolus administration. The elevated Br/Bl was not ascribed to altered blood PK, as the blood concentration was similar in naive and EAE mice. However, brain penetration of atenolol was similar in EAE and naive mice at a putative steady state after intravenous infusion for 2.5 h. These results showed that the brain penetration rate but not the extent of paracellular compounds increased in EAE mice compared with that in naive mice. Paracellular markers have been commonly used to evaluate BBB integrity in vivo. For example, Fabis et al. (2008) reported increased BBB permeability of NaF in EAE 10 min after intraperitoneal administration. BBB opening data obtained from paracellular markers should be interpreted with caution because the extent of BBB permeability to NaF may not increase. Brain penetration of lipophilic nonpolar compounds has been investigated in EAE, STZ-induced, and TASTPM transgenic mice. Our data showed that BBB permeability and blood PK to transcellular compounds did not alter in all animal disease models. There are various reports about the temporal features of increases in BBB permeability in EAE (Muller et al., 2005), either before the onset of clinical signs (Koh et al., 1993), at the time of onset of clinical signs, before the onset of inflammation (Floris et al., 2004), at the initial stage of cellular infiltration (Koh et al., 1993), or after cellular infiltration (Rausch et al., 2003). Irrespective of disease progression, BBB permeability (Br/Bl) to small molecule therapeutic agents re-

**TABLE 3**

<table>
<thead>
<tr>
<th>Name</th>
<th>% Unbound Fraction in Brain Homogenate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Naive</td>
</tr>
<tr>
<td>Naproxen</td>
<td>49.7 ± 6.6</td>
</tr>
<tr>
<td>Loperamide</td>
<td>0.65 ± 0.02</td>
</tr>
<tr>
<td>GSK-A</td>
<td>0.0053 ± 0.0012</td>
</tr>
<tr>
<td>GSK-B</td>
<td>0.22 ± 0.03</td>
</tr>
</tbody>
</table>

**FIG. 3.** Brain penetration of lipophilic GSK-A (A), GSK-B (B), and paracellular NaF (C) in naive and TASTPM mice (4-month old) and GSK-C (D) in naive and STZ-induced mice. The x-axis represents blood and brain sampling time after dosing.
mained unchanged at all EAE stages. Increased BBB permeability of inflammatory cells does not translate into increased permeability to much smaller molecules (mol. wt. <1000). Our results do not exclude the possibility that BBB permeability of small therapeutic molecules may increase in certain brain regions or local changes adjacent to brain microvessels. The increased brain penetration at the “injured” region may be diluted by a large volume of “normal” tissues. Further studies will be designed to investigate brain penetration of therapeutic agents in a disease-related brain region, such as the hippocampus for AD, by using positron emission tomography.

P-gp is a BBB efflux transporter that limits drug accumulation in the brain. Kooij et al. (2009) found that P-gp expression by immunohistochemical analysis decreased in human MS lesions and in Lewis rat EAE lesions A reduction in BBB P-gp expression and function could allow for improved delivery of therapeutic agents to a site of action for CNS diseases. However, our data showed that brain penetration of amprenavir and loperamide, two prototypical P-gp substrates, did not increase in EAE mice at the peak stage compared with that in naive mice 10 and 30 min after intravenous bolus administration, respectively. Our data are in line with expert opinion (Roberts and Goralski, 2008). In the acute phase of a CNS inflammatory reaction, there is an initial loss of P-gp expression and/or activity, thus leading to increased accumulation of P-gp substrates in the brain. As the acute inflammatory episode resolves or in a chronic neuroinflammatory reaction, expression of P-gp in the BBB is recovered to baseline or even enhanced. It is important to note that the direction and degree of change in P-gp activity depends on the in vivo or in vitro model used, the inflammatory mediator used, the time points chosen for observation, and the P-gp substrates analyzed (Roberts and Goralski, 2008). The time points in our study were chosen on the basis of a preliminary experiment performed in naive mice when brain concentrations can be quantifiable. Ideally brain penetration should be determined at steady state. However, EAE mice were too weak for vein cannulation surgery and intravenous infusion for an extended period of time. The influence of inflammation on the accumulation of drugs within the CNS may also depend on alterations in the activity of other efflux and uptake transporters. It is essential for future studies to address the impact of inflammation on the function of these other, at present least characterized, BBB drug transporters, such as breast cancer resistance protein.

For CNS drugs, the unbound brain concentration is the most relevant drug concentration for in vivo CNS activity (Liu and Chen, 2005). Br/Bl describes the ratio between brain and blood total concentrations. Br/Bl alone has limited value in drug discovery and should be combined with free drug fractions in the brain. Unbound brain concentrations can be directly measured by using microdialysis, but this method is resource-demanding and not applicable to highly lipophilic compounds because of poor recovery due to high nonspecific binding (Davies, 1999). Unfortunately, most compounds in CNS drug discovery, including the tool compounds used in this article, are very lipophilic. Use of brain homogenate is an alternative method to estimate free drug concentrations based on comparison of brain slice and brain homogenate methods with cerebrospinal fluid concentrations (Liu et al., 2006) and brain homogenate measurements compared with cerebrospinal fluid concentrations and interstitial fluid concentrations determined by microdialysis (Liu et al., 2009). Our results showed that the free fraction was similar in brain homogenates obtained from naive and EAE mice. Neither the total nor free concentration is determined by microdialysis, but this method is resource-demanding and not applicable to highly lipophilic compounds because of poor recovery due to high nonspecific binding (Davies, 1999). Unfortunately, most compounds in CNS drug discovery, including the tool compounds used in this article, are very lipophilic. Use of brain homogenate is an alternative method to estimate free drug concentrations based on comparison of brain slice and brain homogenate methods with cerebrospinal fluid concentrations (Liu et al., 2006) and brain homogenate measurements compared with cerebrospinal fluid concentrations and interstitial fluid concentrations determined by microdialysis (Liu et al., 2009). Our results showed that the free fraction was similar in brain homogenates obtained from naive and EAE mice. Neither the total nor free concentration is altered in diseased animals compared with naive mice. It is unrealistic to expect enhanced BBB permeability of poor brain-pene-
trable compounds under chronic pathological conditions.

In contrast to small molecule drugs, mAbs can interact with precisely defined targets and are thought to constitute a particularly promising class of bioactive molecules. However, their large size (~150 kDa for IgG) makes mAbs brain-impermeable, which is the biggest hurdle to development of therapeutic antibodies for targets in CNS, including MS and AD. Tonra et al. (2001) has reported that peripherally injected rabbit IgG showed significant penetration through a compromised BBB in EAE mice. Similar to results with the exogenous rabbit IgG, significantly increased levels of mouse albumin were detected in the cerebellum 11 days after induction of EAE. Further studies will be conducted to compare brain penetration of therapeutic antibodies in naive and neurological disease animal models by using qualitative immunohistochemical and quantitative enzyme-linked immunosorbent assay analyses.
In conclusion, CNS penetration for small-molecule therapeutic agents does not increase in MS- and AD-related animal models despite reported BBB disruption. The PK, CNS penetration, and side effect liability investigated in naive animals are still relevant to the disease conditions.

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