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Demystifying Brain Penetration in Central Nervous System Drug Discovery

Miniperspective

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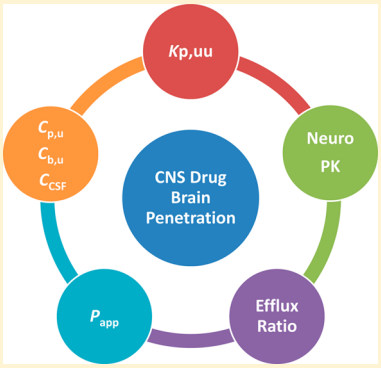
ABSTRACT: This Perspective provides important concepts about the blood–brain barrier (BBB) in drug discovery and how they should be applied effectively in designing successful CNS drugs. Key parameters for brain penetration are discussed, including unbound brain concentration, unbound brain-to-plasma ratio, BBB permeability, fraction unbound in brain and plasma, and transporters. Results from a retrospective analysis of 32 Pfizer CNS clinical drug candidates are described. Frequently encountered misconceptions about brain penetration in drug discovery programs are clarified. Strategies and guidance are provided to enhance or minimize brain exposure for CNS or peripheral targets, respectively. Recommendations for screening methodologies and a cascade in assessing brain penetration potential are presented.



INTRODUCTION

Neuroscience is the second largest therapeutic area in terms of sales, and yet the success rate of CNS drug candidates in the clinic is quite low (8%) compared to cardiovascular diseases (20%).[1] This difference is in part due to the lower predictability of animal models for human CNS diseases. As a result, many devastating brain diseases (e.g., Alzheimer's disease, stroke, Parkinson's disease) still do not have adequate treatment. In drug discovery, a major challenge of CNS therapy is the blood–brain barrier (BBB). It has been estimated that only 2% of CNS drug discovery compounds can cross the blood–brain barrier and reach the therapeutic targets.[2] This greatly limits the potential of a compound to become a successful CNS agent. Because of the great challenges and high risks associated with CNS drug discovery and development, several major pharmaceutical companies are exiting or deprioritizing CNS drug research in response to the investors' pressure.[3] This reaction can further delay the development of much needed CNS drugs for the patients.

The BBB consists of the endothelial cells that comprise the blood capillaries in the brain (Figure 1). It regulates brain exposure of drugs by having very tight intercellular junctions, leading to negligible paracellular transport, and it also has minimal pinocytosis.[4,5] Several transporters facilitate active uptake or efflux transport of drug molecules into and out of the brain.[6] Most small molecule drugs enter the brain by transcellular passive diffusion through the lipid membranes into the brain. Binding in blood and brain, and metabolism can also affect the disposition of a drug into the brain. The blood–CSF barrier (BCSFB) is the membrane that separates the blood from the CSF. The P-gp at the BCSFB pumps the substrates to the CSF rather than the blood. Systemically administered drugs can reach CSF either directly across the BCSFB or indirectly across the BBB followed by diffusion/convection transport from the interstitial fluid (ISF) to CSF.

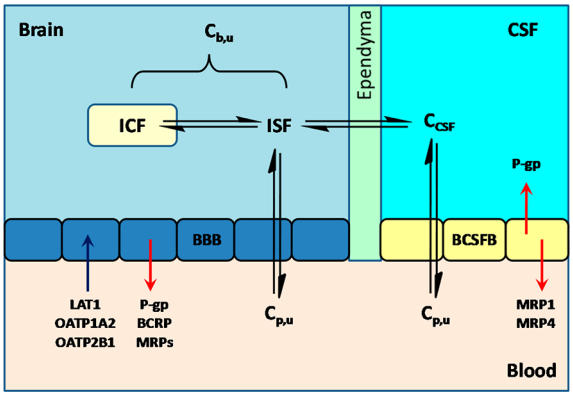


Figure 1. A three-compartment model illustrates unbound drug distribution equilibrium between plasma, brain (ISF and ICF), and CSF: BBB, blood–brain barrier; BCSFB, blood cerebrospinal fluid barrier; $C_{p,u}$, plasma unbound drug concentration; $C_{b,u}$, brain unbound drug concentration; C_{CSF}, CSF drug concentration. P-gp, BCRP, MRPs, LAT1, OATP1A2, and OATP2B1 are efflux and uptake transporters expressed at the BBB and BCSFB. Double arrows (⇆) represent drug distribution equilibrium across the various compartments in the absence of transporters. Single arrows indicate the direction that the drugs move toward by transporters across the membranes.

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Table 1. Parameters To Evaluate Brain Penetration

symbols	variables	comments
$C_{p,u}$, $C_{b,u}$, C_{CSF}	unbound plasma, brain, or CSF concentration	Unbound concentration at the site of action is the key for pharmacological activity in most cases.
$K_{p,uu}$ or $C_{b,u}/C_{p,u}$	unbound brain-to-plasma ratio	Measurement of brain penetration potential and if distribution equilibrium is achieved between blood and brain compartment.
B/P	total brain-to-plasma ratio	Based on total brain and plasma concentration or AUC. Mostly driven by nonspecific binding to proteins and lipids. Should not be use to guide SAR.
$f_{u,p}$, $f_{u,b}$	fraction unbound in plasma or brain	Useful for converting total drug to free drug. Has no impact on free drug concentration in vivo for oral drugs. No SAR should be developed to optimize f_u.
P_{app}	apparent BBB permeability	Rate, not the extent, of brain permeability. Can limit brain penetration when too low.
ER	efflux ratio of P-gp or BCRP	Efflux transporter substrates should not be developed as CNS drugs.

IMPORTANT CONCEPTS IN BRAIN PENETRATION

Many parameters are used to describe different aspects of brain penetration of drug candidates (Table 1). However, it is important to know which parameters are critical for guiding drug design, lead optimization, and candidate selection (i.e., unbound drug concentration in brain, unbound brain-to-plasma ratio, BBB permeability, transporter efflux ratio) and what parameters should not be used as selection criteria for compound advancement, since they have minimal impact on in vivo CNS efficacy (i.e., total brain-to-plasma ratio, fraction unbound in plasma or in brain). The common parameters for CNS drug discovery are discussed in the following sections.

Unbound Drug Concentration in the Brain. The free drug hypothesis is a fundamental principal for in vivo pharmacology, and it consists of two parts.[7] Part I states that the free (unbound) drug concentration at the site of action leads to pharmacological activity, and part II states that the free drug concentration at steady state is the same across any biomembrane (e.g., BBB) when drug transporters are not involved in the distribution process. It has been shown that the free drug hypothesis holds true for structurally diverse compounds in various disease targets and therapeutic areas.[7] Ideally, for intracellular targets, the unbound drug concentration in the intracellular fluid (ICF) should be used to elucidate the pharmacological effects. In practice, it is difficult to measure the intracellular free drug concentration in the brain. Instead, various surrogates are used to estimate the intracellular free drug concentration when there are no transporters involved in the disposition processes. These surrogates include drug concentration in extracellular fluid (ECF), unbound brain concentration, and unbound plasma concentration. It has been reported that for most compounds, the unbound brain concentration is within 3-fold of the ECF concentration.[8] This observation is consistent with free drug hypothesis part II, in that the free drug concentration is the same in ICF, ECF, brain, cerebrospinal fluid (CSF), and plasma at steady state when there is no transporter involvement. CSF can also be used as a surrogate for unbound brain, ECF, and ICF. For example, it has been demonstrated in a γ-secretase inhibitor drug discovery program for the treatment of Alzheimer's disease that inhibition of the generation of brain Aβx-42 and CSF Aβx-40 in 129/SVE mouse had good correlation between in vivo unbound brain IC_{50} and in vitro IC_{50}.[9] This suggested that the unbound brain concentration was reflective of the target site concentration. The in vitro/in vivo correlation (IVIVC) based on unbound drug concentration enabled the prediction of Aβ generation in vivo using in vitro IC_{50}. It is the unbound drug concentration in the brain (or CSF) rather than the total drug that leads to the development of PK/PD relationships.

Unbound Brain-to-Plasma Ratio. The ratio of unbound drug in brain to unbound drug in plasma ($K_{p,uu}$ or $C_{b,u}/C_{p,u}$) is a critical parameter to evaluate if distribution equilibrium between blood and brain compartments has been established. This term reflects the combined impact of BBB passive permeability and transporters. When $K_{p,uu}$ (typically based on unbound AUC of brain and of plasma) is near 1, the compound is at distribution equilibrium between the plasma and brain compartments. This is an ideal situation for drug development, where the unbound plasma concentration can be used to estimate the unbound brain concentration. CNS drug discovery compounds should be optimized for brain penetration based on $K_{p,uu}$. Typically, compounds that have good passive permeability and that are not substrates for transporters have $K_{p,uu}$ close to 1. An example is venlafaxine, an antidepressant with $K_{p,uu}$ of 0.98 in mouse,[10,11] suggesting distribution equilibrium between brain and plasma. When $K_{p,uu}$ is less than 1, a compound is a substrate for an efflux transporter (e.g., P-gp, BCRP) and/or the brain penetration is diffusion limited because of low passive permeability across the BBB. Examples are atenolol (ratio of unbound drug concentration in CSF to that in plasma, $K_{p,uu,CSF}$, is 0.038 in human because of low BBB passive permeability) and saquinavir ($K_{p,uu,CSF}$ is 0.0955 in human because of P-gp efflux and low BBB passive permeability).[12] Structural modification should be applied to improve the brain penetration of these types of compounds by enhancing BBB permeability or eliminating efflux transport. Advancement of compounds for CNS targets that possess distribution disequilibrium presents high risks in drug development, since the difficulty of estimating human brain unbound drug concentration for these compounds brings low confidence in human dose prediction. When the $K_{p,uu}$ is greater than 1, it suggests active uptake processes by influx transporters. Oxycodone ($K_{p,uu}$ = 3.1 in rats) and diphenhydramine ($K_{p,uu}$ = 5.5 in rats) are two examples of enhanced uptake into the brain, likely by influx transporters, though the uptake transporters have yet to be identified.[13] BBB uptake transporters are active research areas, and the goal is to utilize influx transporters to enhance brain uptake of CNS drugs that would otherwise not be able to enter the brain (e.g., pregabalin[14]).

Total Brain-to-Plasma Ratio. Even though $K_{p,uu}$ is one of the most important parameters to develop SAR and guide CNS compound optimization, total brain-to-plasma ratio [B/P ratio, from total (bound plus unbound) brain concentration or AUC divided by total plasma concentration or AUC] should not be used.[5,15] The B/P ratio is mostly governed by nonspecific binding to lipids and/or proteins in plasma and brain, and tends to be misleading toward biological activities. It is important to convert the total measured in vivo brain and plasma concentrations to unbound brain and plasma concentrations

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using the fraction unbound of brain and plasma measured in vitro (e.g., equilibrium dialysis). $K_{p,uu}$ can then be calculated and used for compound prioritization, rank ordering chemical series, or guiding structural optimization.

Fraction Unbound. Fraction unbound (f_u) in plasma or brain is useful to convert total drug concentration, obtained from neuroPK measurements, to unbound concentration in plasma or brain. However, fraction unbound itself should not be optimized through structural modification, since it has no impact on in vivo efficacy nor does it have any clinical relevance for orally administrated drugs.[7,16] Fraction unbound does not change the unbound drug concentration in vivo for oral drugs. A higher fraction unbound does not provide greater unbound concentration in the brain, and a lower fraction unbound does not give less unbound concentration in vivo. The strategies on how to optimize in vivo unbound concentration will be discussed later.

BBB Passive Permeability. BBB passive permeability (P_{app}) characterizes the rate across the BBB due to passive diffusion but not the extent of brain penetration. However, a low BBB passive permeability can reduce the extent of brain penetration due to diffusion-limited absorption into the brain. A high BBB permeability enables rapid establishment of a distribution equilibrium between plasma and brain compartments, but it does not mean that the unbound drug concentration will be high in the brain. In other words, high BBB passive permeability is beneficial for CNS drug candidates, but it does not necessarily translate to sufficient unbound drug concentration in the brain for achieving in vivo efficacy. This dichotomy is because the unbound concentration is also governed by intrinsic clearance and efflux transport in addition to BBB permeability. Excessively high passive permeability at the BBB can be counterproductive for CNS drugs, since high permeability usually requires high lipophilicity (log P), which will lead to nonproductive high nonspecific binding to lipids and proteins, promiscuous toxicity, rapid metabolism, and subsequent low unbound drug concentration in the brain.[7,17]

Efflux Ratio. Efflux ratio (ER) reflects the potential of a compound to be pumped out of the brain by BBB efflux transporters. Substrates of BBB efflux transporters have brain penetration impairment, as indicated by distribution disequilibrium between the plasma and the brain compartments. It is difficult to predict unbound drug concentration of efflux transporter substrates in the brains of humans and there are no good animal models. It is risky to advance P-gp or BCRP substrates to the clinic because the dose selected might be either too low to generate efficacy or too high and lead to toxicity. This is especially challenging for drug candidates with a narrow therapeutic index. Structural modifications should be made to circumvent P-gp or BCRP efflux, and resulting CNS drug candidates without efflux liabilities can then be considered for further evaluation.

LESSONS LEARNED FROM RETROSPECTIVE ANALYSIS OF PFIZER CNS CLINICAL DRUG CANDIDATES

A retrospective analysis of 32 structurally diverse Pfizer CNS drug candidates with human brain exposure data (either CSF or PET) was performed to understand the important factors influencing CNS drug disposition (Table 2).[18] The 32 Pfizer compounds were classified into three groups based on two criteria from in vitro and in vivo studies (Figure 2): (1) Does a compound have P-gp liability or low BBB permeability in vitro?

Table 2. Results from Retrospective Analysis of 32 Pfizer CNS Clinical Drug Candidates[18]

Classification	Characteristics	$K_{p,uu}$	Clinical CNS Penetration Outcomes
Group I (14 out of 32)	Good permeability	~1	Achieve distribution equilibrium in human
	Not P-gp substrate		Unbound $plasma_{human} \approx CSF \approx$ unbound $brain_{human}$
	Distribution equilibrium in animals		Unbound plasma can be used as a surrogate for unbound brain
			CSF has no added value compared to unbound plasma
			Single species neuroPK is sufficient, multiple species neuroPK has no added value
			Good CNS penetration in human
Group II (14 out of 32)	Low permeability or P-gp substrate or distribution disequilibrium in animals	varies	Conflicting data between in vitro and in vivo
			Low confidence in predicting human CNS penetration
			Might be due to species differences in efflux transporters
			Might be due to other efflux transporter involvement
			Might be due to unusual CNS distribution concentration time course
Group III (4 out of 32)	Low permeability or P-gp substrate and distribution disequilibrium in animals	<1	Distribution disequilibrium in human
			Unbound $plasma_{human} \neq CSF \neq$ unbound $brain_{human}$
			Difficult to predict unbound brain concentration in human
			Poor CNS penetration in human

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(2) Does a compound achieve distribution equilibrium in the preclinical species in vivo?

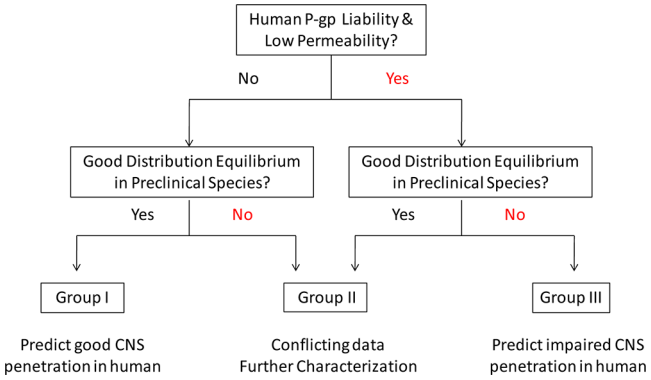

Figure 2. Retrospective analysis of Pfizer CNS drug candidates with human brain exposure data. P-gp was based on MDR1-MDCK data.[24] BBB permeability was determined using the MDCK-LE assay.[71]

Group I compounds demonstrated good BBB permeability, were not P-gp substrates in vitro, and also reached distribution equilibrium in vivo in the preclinical species. These were "well-behaved" CNS compounds, and all showed good CNS penetration in humans, meaning they reached distribution equilibrium ($K_{p,uu} \approx 1$) between CSF or free brain exposure (PET) and unbound plasma concentration (CSF or unbound brain concentration approximately equal to unbound plasma concentration). NeuroPK in multiple preclinical species did not provide additional values for this group of compounds, and single species neuroPK (typically in rat or mouse) was sufficient to determine if distribution equilibrium was established. For group I compounds, unbound plasma concentration can be used to estimate unbound brain concentration in humans and to develop PK/PD relationships at the CNS target sites in drug development. Measuring CSF drug concentration in humans does not provide added value over using unbound plasma concentration as a surrogate for unbound brain concentration. This suggests one can avoid CSF sampling in humans for group I compounds, which greatly reduces the costs of clinical studies and prevents any unnecessary safety risks associated with CSF sampling.

Group II compounds had conflicting in vitro and in vivo preclinical results, making it difficult to judge what the human outcome would be for CNS penetration. Greater than 60% of this group of compounds still demonstrated good CNS penetration in humans. Retrospective analysis showed that all the human P-gp substrates, based on in vitro MDR1-MDCK data, showed impaired brain penetration in humans, but not all the non-P-gp substrates had good human brain penetration. This is due to other factors impeding the prediction of brain exposure including involvement of efflux transporters other than P-gp (e.g., BCRP, MRPs), low passive permeability across the BBB, and the technical challenge in accurately measuring high plasma protein and brain binding. Not all the compounds that showed good brain penetration in rat had good human brain exposure, and not all compounds with rat brain exposure impairment had human brain exposure impairment. Dog had similar prediction as rat for human CNS penetration. Species differences in transporter expression and substrate specificity might account for the inaccurate prediction of human brain penetration using preclinical species (rat, mouse, or dog). Group II is a challenging group of compounds, further research in the BBB field is needed to enhance the predictability of human brain penetration for this group of compounds.

Group III had consistent in vitro and in vivo preclinical CNS penetration data suggesting impaired human brain penetration. Human clinical data showed significant impairment in brain penetration for all the group III compounds, which is consistent with in vitro and preclinical in vivo predictions. Cross-species comparison of CSF to unbound plasma ratio indicated different magnitudes of brain penetration impairment for different species, suggesting that preclinical species cannot be used to estimate human brain exposure for group III compounds. Since P-gp pumps substrates into the CSF at the BCSFB, the CSF concentration of a drug that is a P-gp substrate tends to overestimate unbound brain concentration.[19] In this case, both animal and human CSF data might not represent the unbound brain concentration for group III compounds. Therefore, even with human CSF concentration data, it is difficult to predict the unbound drug concentration in the brain.[20] For these reasons, it is challenging to project relevant clinical doses for group III compounds and this is especially risky for drug candidates with narrow therapeutic indexes. It is advisible to deprioritize group III compounds, particularly if they possess efflux activity.

TOOLS FOR ASSESSING BRAIN PENETRATION IN DRUG DISCOVERY

Many comprehensive reviews are available on in silico, in vitro, and in vivo methodologies in assessing BBB penetration.[5,18,21,22] Here we highlight a few key assays commonly applied in the pharmaceutical industry during the lead optimization and candidate selection stages in drug discovery.

NeuroPK. Because of the complexity of brain physiology, preclinical neuroPK is an efficient way to define temporal relationships of drug concentrations in CNS compartments (brain, CSF, and plasma).[21,23] Typically, the species selected is based on efficacy and toxicology models. NeuroPK studies may employ any dosing routes, but subcutaneous (sc) administration is the most preferred approach, since it bypasses first-pass liver metabolism and has low experimental variability. Oral dosing paradigms for programs looking for oral drugs are sometimes used, as all these parameters are optimized simultaneously. In a typical rodent neuroPK study, animals are euthanized at specific time points postdose. Then plasma, CSF, and brain are collected for exposure analysis. Unbound drug exposures in each of the compartments can be calculated by multiplying AUC-derived total exposure by unbound fraction plasma protein binding or brain tissue binding. CSF contains very little protein, and no protein binding is applied to CSF for unbound calculation. Assuming unbound brain concentration ($C_{b,u}$) represents free drug concentrations in brain ICF and ECF, the ratio $AUC_{b,u}/AUC_{p,u}$ ($K_{p,uu}$) reflects the concentration relationship across the BBB. The ratio $AUC_{CSF}/AUC_{p,u}$ gives an idea of the extent of membrane penetration at the BCSFB. These two parameters are important in defining whether the compound exhibits distribution equilibrium among CNS compartments. The ratio $AUC_{b,u}/AUC_{CSF}$ is particularly useful when a compound demonstrates distribution disequilibrium among the CNS compartments. This ratio reveals whether the drug concentration observed in CSF is reflective of unbound drug concentration in the brain.

In CNS drug discovery, knowledge derived from a well-conducted neuroPK study goes a long way for a drug candidate from discovery to clinical development. At the discovery stage,

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the unbound drug concentration in the brain, the time-course of drug distribution, and the elimination from the brain are all key to interpreting the exposure−pharmacodynamic response. During clinical development, data generated from preclinical neuroPK and transporter studies help project teams to predict human CNS penetration and hence to decide whether unbound plasma or CSF can be used as surrogates for unbound brain exposure in humans.

Transporters. *Efflux Transporters.* Efflux transporters at the BBB play a very critical role in preventing CNS penetration. The two most important efflux transporters at the BBB are P-gp and BCRP. They are ATP driven transporters responsible for the efflux of xenobiotic compounds. The two transporters have a large substrate overlap. Many BCRP substrates are also P-gp substrates, and very few compounds are pure BCRP substrates. P-gp has a broader substrate specificity and a very significant impact in limiting brain penetration of drugs in vivo. The protein expression level of BCRP is higher than P-gp at human BBB. BCRP substrates embrace both cations and anions, and P-gp is more inclined to cations. Dual substrates of P-gp and BCRP typically have very low brain penetration due to the added effect of the two transporters in eliminating drugs from the brain.[24]

Although primary and immortalized brain endothelial cells might seem to be most relevant for BBB studies, they are typically unsuitable for use on a regular basis to support drug discovery programs because of leaky junctions, low transporter expression, and high cost. Transfected cell lines with human or animal transporters are the most common tools in drug discovery to specifically assess transport effects (e.g, MDR1-MDCK, BCRP-MDCK).[10,24,25] High throughput 96-well formats of the assays are available to screen a large number of compounds to guide SAR and candidate selection.

Across multiple species, P-gp has high sequence homology (80−97%)[26] and similar functional activity.[24] However, for certain structural series, species differences in P-gp efflux activities have been observed.[27] Cell lines transfected with human and animal transporters are useful for diagnosing the disconnects between in vitro human transporter assays and in vivo animal neuroPK results.

Transporter knockout (KO) animals (e.g., Mdr1a/1b, Bcrp KO mice) are useful in vivo models to diagnose if transporters play a critical role in brain penetration of a compound in animals. However, the value of using KO animals to predict human transporter effects at the BBB is limited compared to cell lines transfected with human transporters. Human MDR1-MDCK has been shown to have a better prediction of P-gp effect on brain penetration in humans than P-gp KO mice,[10] due to the human specificity and high sensitivity of the MDR1-MDCK assay without the confounding factors of species differences in transporters and the additional in vivo complexity of KO mice.[18]

Uptake Transporters. Uptake transporters on the BBB can help facilitate brain uptake of compounds that would otherwise not be able to enter the brain on their own.[6] Examples of CNS drugs that are actively influxed into the brain by uptake transporters are L-DOPA, gabapetin, and pregabalin.[28] Recent reports of OATP1A2 transporter on the BBB generated a great deal of interest, since OATP1A2 has been shown to have a broad spectrum of substrates,[29] particularly positively charged amines.[30] Bidirectional transport studies with cell lines that express uptake transporters are the common approaches for in vitro screening of influx transporter substrates.[30,31] Uptake transporter KO mouse is also a valuable tool to understand in vivo contribution of influx transporter to CNS penetration. For example, Oatp1a4 is the major uptake transporter expressed at the BBB in rodents; thus, Oatp1a4 KO mice have been used successfully to study drug uptake.[32]

BBB Passive Permeability. BBB passive permeability measures the rate of passive diffusion across the BBB. When BBB passive permeability is too low, it can become rate limiting and affect the distribution equilibrium between the plasma and brain compartments. Many methodologies have been developed to measure BBB passive permeability. The gold standard in situ brain perfusion assay[33,34] is rarely used in the pharmaceutical industry, since many in vitro assays or calculated properties are quite reliable in estimating BBB permeability. In vitro, PAMPA-BBB[35,36] and cell monolayer transport assays[37] are most frequently applied to measure the rate of BBB permeation in drug discovery. Computational models based on physicochemical properties are quite effective in predicting BBB permeability.[38,39]

Plasma Protein Binding and Brain Tissue Binding. Fraction unbound of plasma protein binding and fraction unbound of brain tissue binding are critical parameters for converting total plasma and brain exposure, obtained from in vivo neuroPK studies, to unbound drug concentrations. Equilibrium dialysis, using plasma and brain homogenates, is the gold standard for measuring fraction unbound, since nonspecific binding to the devices has minimal impact on fraction unbound measurement.[40] High throughput methodologies are available for binding measurements, such as the EqD[41] and RED[42] devices. Care must be taken in binding experiments for highly bound compounds with high nonspecific binding. It might be necessary to take a longer time than for usual experimental conditions in order to achieve true equilibrium for these compounds. Other techniques, such as Transil[43] or stepwise equilibrium dialysis,[44] might be used to overcome the challenges of highly bound lipophilic compounds. Unlike plasma protein binding, brain tissue binding is independent of species and brain regions.[45,46] One can use brain tissue binding data from a single species (e.g., rat) to extrapolate to all the species in drug discovery. Since unbound drug concentration at the site of action is independent of plasma protein binding or brain tissue binding for orally administrated drugs, fraction unbound of compounds should not be optimized through structural modifications. One only needs to measure binding to plasma or brain when in vivo total concentration data are available to be converted to unbound drug concentration.

COMMON APPROACHES TO ESTIMATE UNBOUND DRUG CONCENTRATION IN THE BRAIN

Since unbound compound concentration in the brain is the key leading to in vivo pharmacological effects, various methodologies are available to obtain unbound drug concentration in the brain.

Rodents or Small Animals. NeuroPK can be conducted by taking brain and plasma samples at various time points and measuring total drug concentrations of the samples. The unbound drug concentration in the brain is derived by multiplying brain fraction unbound ($f_{u,b}$) by total brain concentration (C_b), i.e., $C_{b,u} = f_{u,b}C_b$. Unbound brain-to-plasma ratio is obtained from the study by dividing the unbound brain concentration with unbound plasma concentration [$K_{p,uu}$ =

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$C_{b,u}/C_{p,u} = (f_{u,b}C_b)/(f_{u,p}C_p)$, where $f_{u,p}$ is plasma fraction unbound and C_p is total plasma concentration].

Large Animals or Humans. Brain samples of large animals or humans are rarely available. Four different approaches can be applied to estimate unbound brain concentration of non-human primates (NHPs) or humans.

(1) The $K_{p,uu}$ is often preserved across species for non-transporter substrates. By use of $K_{p,uu}$ of rodents (e.g., rat), human $K_{p,uu}$ can be estimated ($K_{p,uu,human} \approx K_{p,uu,rat}$). Unbound drug concentration of human can be calculated by using the rat $K_{p,uu,\ rat}$ times the human unbound plasma concentration [$C_{b,u,\ human} \approx K_{p,uu,rat}C_{p,u,human}$].
(2) CSF concentration can be used as a surrogate for unbound brain concentration when there are no transporters involved in the disposition processes.
(3) When a compound reaches distribution equilibrium between brain and plasma, unbound plasma concentration can also be used as a surrogate for unbound brain concentration. In this case, CSF does not provide additional benefits compared to unbound plasma concentration.
(4) Receptor occupancy data from PET imaging can be used to estimate unbound brain concentration when used in combination with in vitro potency [RO = $C_{b,u}/(C_{b,u} + K_i)$].[47,48]

Transporter Substrates. It is often difficult to estimate human unbound drug concentration in the brain for transporter substrates based on in vitro data or in vivo brain exposure in preclinical species. There are no good translations to human unbound brain concentration from rodents, due to differences in expression level of transporters and substrate specificity. Rodents tend to significantly underestimate human brain exposure for P-gp substrates,[26] since rodents have higher P-gp expression at the BBB than humans. For example, GR205171 was about 9-fold higher in humans compared to rats.[26] Non-human primates have been suggested to translate better to human brain drug disposition when efflux transporters are involved,[26] but this is still not perfect. Human CSF or unbound plasma concentration tend to overestimate human unbound brain concentration of P-gp substrates.[19] The difficulties in predicting unbound human brain concentration of efflux transporter substrates lead to low confidence in projecting human dose and estimating therapeutic index. It is advisible not to advance efflux transporter substrates for CNS targets because of the high risk associated with low confidence in human translation.

STRATEGIES TO MINIMIZE BRAIN PENETRATION OF DRUGS FOR PERIPHERAL TARGETS

Minimizing brain penetration is an important strategy to reduce undesirable CNS side effects for drugs with targets in peripheral tissues. Several approaches have been demonstrated to be effective in restricting non-CNS compounds to the peripheral tissues in order to minimize the negative impacts on the CNS.[49]

Reducing Passive Permeability. Brain penetration potential decreases for drug candidates with low BBB passive permeability. Therefore, one strategy to minimize brain exposure is to reduce passive permeability, which may be accomplished by introducing polar functional groups, increasing molecular weight, increasing PSA, and adding intermolecular hydrogen bond donors and acceptors. When BBB permeability is low, brain penetration becomes diffusion rate limited and unbound drug concentration in the brain is low. The limitation of this approach is that these compounds might suffer from low oral absorption because of the low passive permeability. Especially for compounds with low solubility and are P-gp substrates, oral absorption can be challenging. However, this strategy can still be useful for iv drugs or for compounds that are very small and can be absorbed through the paracellular route in the intestine. Because the junctions between the endothelial cells at the BBB are very tight, these compounds would not be able to enter the brain by the paracellalar route and would have minimal brain penetration. Atenolol is an example with low passive permeability and small molecular size (266 Da). The drug is mostly absorbed through the paracellular pathway in the intestine and has good oral bioavailability in humans[50] (58%) but minimal brain penetration ($k_{p,uu,\ CSF}$ = 0.179 in human).[12]

Introducing Acidic Functional Groups. Another strategy is to take advantage of permeability−pH profiles for acids. Weak acids (e.g., carboxylic acids) tend to be more permeable at lower pH (intestine pH 6.5) than at higher pH (blood pH 7.4), since there are more neutral species at low pH. In systemic circulation, acid molecules are mostly ionized and have low permeability across the BBB, but they can still have good permeability for oral absorption due to the higher fraction of neutral species at the lower pH in the intestine. One example is indomeththacin (a carboxylic acid) with 98% oral bioavailability[50] in human but low brain penetration ($k_{p,uu,CSF}$ = 0.27 in human).[12] Therefore, introducing an acidic functional group is a potentially effective way to minimize brain exposure while maintaining good oral absorption.

Introducing P-gp Efflux Activity. P-gp efflux transport can limit brain penetration of its substrates and minimize CNS side effects. Because of the high unbound drug concentration in the intestine for most compounds, saturation of P-gp efflux is likely to occur, and this will not greatly reduce oral absorption. The P-gp transporter at the BBB, however, is difficult to saturate because of the low unbound drug concentration in systemic circulation. An example is loratadine, a second generation, nonsedating H_1 histamine antagonist used for the treatment of allergies. Loratadine and its active metabolite (desloratadine) are both P-gp substrates,[51,52] which prevents them from crossing the blood−brain barrier, and therefore, they do not have CNS side effects such as drowsiness. Loratadine has rapid oral absorption[53] and low brain penetration ($K_{p,uu}$ = 0.21 in mouse[10]).

Developing Dual Substrates of P-gp and BCRP Efflux Transporters. Dual substrates of P-gp and BCRP efflux transporters typically have very low brain penetration due to the added effect of the two transporters in eliminating drugs from the brain. This added effect has been demonstrated using P-gp and Bcrp knockout mice for several dual substrates.[25,54,55] The cooperative effect between P-gp and BCRP can be explained by their contributions to the net efflux at the BBB without any interactions between the two efflux transporters.[25] Developing dual substrates of P-gp and BCRP is an effective strategy to minimize brain penetration while maintaining acceptable oral bioavailability. Methotrexate is a P-gp and BCRP dual substrate with minimal brain penetration ($k_{p,uu,CSF}$ = 0.062 in human[12]) and reasonable oral bioavailability (36%).[56]

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CLARIFICATION OF MISCONCEPTIONS ABOUT THE BBB

There are quite a few misconceptions about the BBB in drug discovery and development, and they are frequently seen in publications and presentations. Here, a few of the most commonly encountered ones are discussed.

(1) *Some drug discovery project teams think that the higher the B/P ratio, the better the drug candidate is for CNS penetration.* B/P ratio is calculated based on total drug concentration and is mostly driven by nonspecific binding to proteins and lipids in brain and plasma. The fraction unbound in brain and plasma can be very different. Using B/P ratio to select compounds or guide SAR can be misleading. It is the unbound B/P ($K_{p,uu}$ or $C_{b,u}/C_{p,u}$) that should be used to evaluate the potential of a compound to cross the BBB and not the total B/P ratio. A $K_{p,uu}$ close to 1 suggests distribution equilibrium between brain and blood compartments, A $K_{p,uu}$ less than 1 indicates distribution disequilibrium due to efflux transporters or poor passive permeability across the BBB. A $K_{p,uu}$ greater than 1 is indicative of involvement of an uptake transporter.

(2) *Some people think that the higher the BBB permeability (P_{app}), the better is the CNS compound.* BBB permeability determines the rate of BBB membrane flux and gauges how fast a compound diffuses across the BBB. It is an important parameter for drugs that require rapid onset (e.g., antiepileptic drugs) and to determine if compounds have high potential to reach distribution equilibrium at steady state between the brain and blood compartments. However, it is less important for drugs intended for chronic dosing, where the extent of brain penetration (unbound drug concentration in the brain) at steady state drives CNS pharmacological effects.

(3) *In order to increase unbound drug concentration in the brain, many medicinal chemists think that one should reduce brain tissue binding by increasing brain fraction unbound ($f_{u,b}$).* Brain fraction unbound has no impact on unbound drug concentration in the brain for orally administrated drugs.[7,16] SAR should not be developed to optimize brain fraction unbound.

(4) *Because some successful CNS drugs are P-gp substrates (e.g., risperidone[10]), it is believed to be fine to advance strong P-gp or BCRP substrates for CNS targets.* If sufficient unbound brain concentration is achieved for P-gp/BCRP substrates, it is assumed that the compound can still be active in vivo. However, it will require much higher systemic unbound concentration to achieve the desirable unbound brain concentration due to the efflux pump activities, which could lead to potential toxicity or side effects owing to high systemic exposure. The challenge for developing an efflux transporter substrate is that it is difficult to predict human dose and therapeutic index because of distribution disequilibrium. Furthermore, efflux transporters are up- or down-regulated in disease states,[57−60] which adds another layer of complexity in estimating human brain drug exposure and additional risks in the drug development process. The recommendation is to not advance efflux transporter substrates for CNS targets. Structural modifications should be applied to circumvent efflux transport and identify drug candidates without efflux liabilities.[61]

(5) *There is a perception that P-gp efflux may be overcome by high BBB permeability.* An example that has been used to support this argument is verapamil, which is a P-gp substrate. Yet verapamil is able to penetrate the BBB, as shown by PET studies in humans.[62] The speculation has been that, in vivo, high BBB membrane permeability can overcome P-gp efflux observed in vitro. This speculation is incorrect, as it has been demonstrated that significant impairment of brain penetration for verapamil was observed in both rats and humans, even though it was able to enter the brain. $K_{p,uu}$ of verapamil was 0.13 in rats[63,64] and 0.15 in humans,[62,64] suggesting significantly impaired brain penetration in spite of its high passive BBB permeability. These data demonstrated that in both rat and human the effect of P-gp efflux was not overcome by high BBB permeability. Most compounds, including P-gp substrates, penetrate the brain to a certain extent. Compounds with P-gp efflux liability, even with high passive permeability, will likely lead to distribution disequilibrium between brain and plasma.

(6) *One argument frequently tried by project teams to rescue a P-gp/BCRP substrate for CNS indications is the saturation theory.* The thinking behind this is that when the dose is high enough, one should be able to saturate P-gp/BCRP transporters and achieve sufficient brain penetration, leading to efficacy. The fact is that P-gp/BCRP transporters at the BBB are very difficult to saturate because the unbound drug blood concentration is typically rather low (nanomolar) even at high doses and because P-gp/BCRP transporters are high capacity, low affinity transporters (high K_m, typically around 20−200 μM[65]). Using a P-gp/BCRP inhibitor is another strategy that has been considered to enhance the brain penetration of P-gp/BCRP substrates. Even though the effect has been demonstrated in animal studies,[66,67] P-gp/BCRP inhibitors have minimal effect on human brain penetration for P-gp/BCRP substrates[68,69] mainly because the inhibitors are not sufficiently potent or well-tolerated to achieve unbound systemic concentrations high enough to inhibit efflux transporters at the BBB. For the same reasons, significant human clinical DDI due to inhibition of efflux transporters at the BBB would not be anticipated to occur with known marketed P-gp/BCRP inhibitors.

(7) *Sometimes the CSF concentration is used to represent unbound drug concentration in the brain regardless of compound property.* CSF drug concentration can be used as a surrogate for unbound drug concentration in the brain for some compounds,[19] but it is not always an accurate measurement of unbound brain concentration, especially for compounds that are transporter substrates. For example, CSF drug concentration can be higher than unbound drug concentration in the brain for efflux transporter substrates. CSF drug concentration is also highly influenced by drugs in plasma that are transported through BCSFB, as BCSFB is more permeable than BBB. Studies have suggested that only one-third of CSF comes from the brain ISF and two-thirds of CSF is formed at the choroid plexus.[70] The localizations and functions of transporters at the BCSFB are different from those at the BBB.[18] Furthermore, CSF is not a well-mixed compartment unlike systemic circulation, so drug concentration in CSF can be varied significantly depending on the site

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of CSF sampling and the route of administration.[18] It is therefore important to conduct preclinical neuroPK experiments to establish the relationships between unbound brain, unbound plasma, and CSF drug concentration prior to design clinical studies to assess CNS target exposure using CSF drug concentration.

(8) *Some project teams think neuroPK in multiple species will be better than single species for predicting human CNS penetration.* This is not true. Retrospective analysis using Pfizer's clinical candidates indicated that in vitro human P-gp assay and rat neuroPK is effective for predicting CNS penetration in human.[18] There is no advantage of dog and rat over rat in terms of prediction accuracy. For compounds that are not substrates for efflux transporters, rat neuroPK-derived unbound brain-to-plasma ratio can be used with high confidence to project CNS penetration and estimate unbound brain exposure in human. NeuroPK using multiple animal species does not increase the confidence in predicting human brain exposure for nontransporter substrates but does add significantly to the cost. For P-gp substrates, a pronounced species difference was observed across multiple species: rat has been shown to underestimate human brain exposure; and monkey has been suggested to be better suited for human unbound brain exposure projection.[26]

CONCLUSIONS AND FUTURE PROSPECTS

Understanding of these important concepts about the brain exposure is critical for the success of CNS drug discovery. Optimization based on certain parameters, such as total brain-to-plasma ratio or fraction unbound, is inherently faulty and misleading and frequently leads to frustration and unsuccessful drug discovery programs. The recommended strategy to identify drug candidates with optimal CNS exposure is to reduce efflux transport by P-gp/BCRP, minimize metabolism and systemic clearance, and increase permeability and solubility. A screening paradigm for the key brain penetration properties has been developed to guide CNS compound optimization (Figure 3).

In the future, we expect to see more predictive PBPK models, which are being developed to guide CNS compound optimization and selection, as well as more imaging techniques (PET, SPECT) being applied to early drug discovery programs. The BBB transporter field will continue to evolve and new transporters will be discovered and utilized in drug discovery. Novel brain delivery technologies will be advanced to enhance brain exposure of biologics.

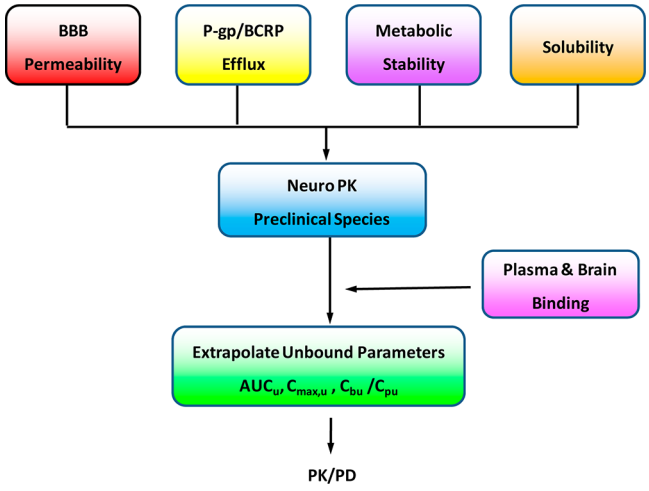

Figure 3. Screening cascade for CNS drug candidates in Drug Discovery (modified from refs 5 and 18).

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ABBREVIATIONS USED

AUC, area under the curve; BBB, blood–brain barrier; BCRP, breast cancer resistance protein; BCRP-MDCK, breast cancer resistance protein expressed in Madin–Darby canine kidney cell line; *B/P*, brain-to-plasma ratio based on total drug; $C_{b,u}$, unbound brain concentration; CNS, central nervous system;

CSF, cerebrospinal fluid; ECF, extracellular fluid; EqD, equilibrium dialysis device from HTDialysis; ER, efflux ratio; ICF, intracellular fluid; ISF, interstitial fluid; iv, intravenous; IVIVC, in vitro/in vivo correlation; KO, knockout; $K_{p,uu}$ or $C_{b,u}/C_{p,u}$ or $AUC_{b,u}/AUC_{p,u}$, ratio of unbound drug concentration in brain to that in plasma; $K_{p,uu,CSF}$, ratio of unbound drug concentration in brain CSF to that in plasma; f_u, fraction unbound; MDR1-MDCK, multidrug resistance protein 1 expressed in Madin–Darby canine kidney cell line; MRP, multidrug resistant protein; neuroPK, in vivo study of drug candidate pharmacokinetics in brain and blood; P_{app}, apparent passive permeability; PAMPA-BBB, parallel artificial membrane permeability assay for blood–brain barrier; PBPK, physiologically based pharmacokinetic modeling; PET, positron emission tomography; P-gp, P-glycoprotein; PK, pharmacokinetics; PK/PD, pharmacokinetics/pharmacodynamics; PSA, polar surface area; OATP1A2, organic anion transport protein 1A2; Oatp1a4, mouse organic anion transport protein 1a4; SAR, structure–activity relationship; SPECT, single photon emission computed tomography; RED, rapid equilibrium dialysis device; RO, receptor occupancy

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