17 Integrated Approaches to Blood–Brain Barrier

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17.1 INTRODUCTION

The central nervous system (CNS) is one of the most challenging therapeutic areas and many brain diseases still do not have adequate treatments. The major hurdles of discovering new CNS drugs, particularly in the area of neurodegeneration, are that there are few animal disease models and drugs must cross the blood–brain barrier (BBB) to reach the therapeutic targets.

The brain is highly protected anatomically to physical injury by the skull and to chemical insult by two major barriers, namely the BBB and the blood–cerebrospinal fluid barrier (BCSFB). The BBB is formed by the endothelium lining of the cerebral capillaries, while the BCSFB is formed by the epithelium of the choroid plexus (CP) [1]. CP consists of many capillaries, separated from the ventricles by choroid epithelial cells. The BBB and BCSFB represent physical and biological barriers that restrict and regulate the penetration of compounds from blood into and out of the brain intracellular fluid (ICF), interstitial fluid (ISF), and cerebrospinal fluid (CSF), respectively, and maintain the homeostasis of the brain and CSF microenvironments. Therefore, unlike in systemic circulation, the pharmacokinetics (PK) of a drug in brain is complicated by multiple compartments created by the BBB and the BCSFB. A great amount of research went into understanding the properties of BBB and BCSFB. The relationship between drug concentrations in blood, ISF, and CSF can be delineated into a three-compartment model (Fig. 17.1).

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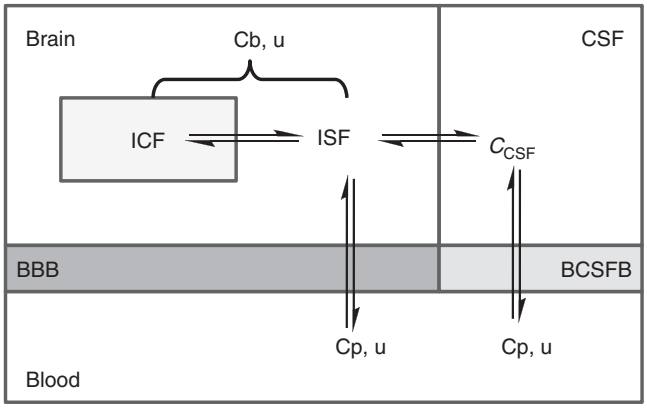

Figure 17.1 A three-compartment model illustrating unbound drug transfer between plasma, brain (ISF and ICF), and CSF. BBB, blood–brain barrier; BCSFB, blood–cerebrospinal fluid barrier; $C_{p,u}$, plasma unbound drug concentration; $C_{b,u}$, brain unbound drug concentration; C_{CSF}, CSF drug concentration.

For many physiological membranes, the unbound drug concentrations are equal on both sides at steady state. However, at the BBB and BCSFB, the contributions of active influx transporters, efflux transporters, and tight junctions between brain endothelial cells can cause, for some compounds, the unbound drug concentrations to be unequal across the BBB or the BCSFB. Metabolism within the CNS (very rare) and transport to CSF via ISF bulk flow may also contribute to disequilibrium of unbound drug concentrations in the blood, brain (ISF and ICF), and CSF compartments.

From a PK and pharmacodynamic (PD) perspective, the purpose of assessing brain penetration of a drug is to understand drug exposure at the target cellular locale. According to the free drug hypothesis, only unbound drug at the target cellular locale is available for the pharmacologic interaction [2]. The relative importance of unbound drug concentrations in different brain compartments depends on where the relevant target receptors are situated. If the drug is actively transported across the cell membrane, ICF concentrations could be expected to differ from brain ISF concentrations.

In this chapter, we discuss the critical elements for brain penetration and methodologies in assessing brain exposure. Retroanalysis on the prediction success for brain penetration of Pfizer CNS drug candidates and lessons learned, as well as screening strategies and future prospects in the BBB will be highlighted.

17.2 IMPORTANT PARAMETERS GOVERN BRAIN PENETRATION

The fact that CNS-targeted drugs require good brain penetration is unequivocal. However, what is unclear is which parameters should be used to define "good" brain penetration. Ultimately, it is the free drug concentration at the site of action in the brain that exerts pharmacological activity *in vivo* [2]. There are three important parameters that characterize brain penetration: rate, extent, and brain tissue binding [1]. Rate is how fast compound molecules enter the brain, extent is how many in the brain at steady state, and brain tissue binding is how many molecules are bound to brain tissue compared with unbound in solution [3].

For the extent of brain penetration, the total brain concentration-to-total plasma concentration ratio (also known as total B/P or K_p) is a common parameter used to describe and evaluate brain penetration. A compound is deemed "brain penetrant" if its total B/P is >0.04, because the cerebral blood volume is approximately 4% of the total brain volume. However, a ratio significantly >1.0 is often observed [4]. Does this indicate that drug molecules are being actively taken up into the brain? No, active uptake is not the case for a majority of drugs. Instead, the higher total B/P ratio is often owing to a significantly higher nonspecific binding of drug molecules to brain tissue than to plasma protein. An experiment conducted by Maurer *et al*. [5] explicitly revealed how nonspecific bindings can confound brain penetration assessment if it is based on the total B/P ratio. In the study, 32 structurally diverse CNS drugs were used. Their total B/P ratios were found to range from 0.06 to 24 after subcutaneous administration in mice [6]. Their nonspecific bindings in mouse plasma and brain homogenate were determined using equilibrium dialysis methodology, and not surprisingly, after corrected with binding factors, their free brain concentration-to-free plasma concentration ratios (also known as free B/P or C_{bu}/C_{pu} or $K_{p,uu}$) were significantly lower, ranging from 0.06 to 3.4. This indicated that nonspecific binding is a significant component of the total B/P parameter.

A high value of total B/P may mislead CNS researchers into the believing that a drug is being actively taken up into the brain and reaching high brain exposure levels, while, in reality, total B/P is merely driven by the different binding in plasma and brain, as indicated by fraction unbound in plasma ($f_{u,plasma}$) and fraction unbound in brain ($f_{u,brain}$).

Theoretically, free B/P would not exceed 1.0 when no active influx transporters are involved. Therefore, in CNS drug discovery, it is prudent to use the free B/P as the parameter to assess the extent of brain penetration, to avoid driving medicinal chemistry efforts down fruitless path of changing nonspecific binding. The free B/P represents the net extent of drug penetration across the BBB, which includes the effects of transporters, without being confounded by unspecific binding [1]. Therefore, free B/P is a better parameter to measure the extent of brain penetration compared with total B/P.

The rate of brain penetration is important for CNS drugs that require rapid onset. It is important to recognize that the rate of drug entering the brain across the BBB is not equal to the extent of transport/distribution to the brain, as the rate determines how fast the drug enters brain, not how much has entered at steady state. Rate across BBB affects whether distribution equilibrium among the various compartments can be established quickly. Rate is determined by permeability of the BBB, which is the composite of passive diffusion, efflux transport, and influx transport. The more permeable the BBB is to the drug, the quicker the drug will reach the brain tissue, but it is not permeability alone that is important regarding drug delivery to the brain. The brain PK of a centrally acting drug, and thereby its associated PD, are the result of a combination of influx and efflux processes and the extent of distribution of the drug within the brain tissue.

Another important parameter is unbound volume of brain ($V_{u,brain}$). $V_{u,brain}$ describes the relationship between the total drug concentration in the brain and the unbound drug concentration in brain ISF. It is a useful measure of drug distribution in the brain parenchyma. $V_{u,brain}$ is the ratio of total drug concentration in the brain and the unbound drug concentration in brain ISF. Since brain ISF concentration is often

converted from total brain concentration and fraction unbound of brain tissue ($f_{u,brain}$). $F_{u,brain}$ is inversely proportional to $V_{u,brain}$ [1].

17.3 METHODOLOGIES IN ASSESSING BRAIN PENETRATION

17.3.1 *In Vivo* Approaches

17.3.1.1 Preclinical Neuro PK Studies. On the basis of the understanding of multiple brain compartments, as discussed above, and the importance of nonspecific binding in the brain, efficient *in vivo* neuroPK (neuropharmacokinetics) (i.e., brain PK) studies are conducted in animals as part of CNS drug discovery [4,7]. NeuroPK helps define the relationships between drug concentrations in the CNS compartments and understand the mechanism-based pharmacology, the exposure (PK) and response (PD) relationships. The species selected for neuroPK is based on the species used for efficacy and toxicology studies. Plasma, brain, and CSF are collected to assess drug distribution in the various compartments. The neuroPK information provides temporal brain intercompartmental compound concentrations to guide dose and time point selection for pharmacology studies in preclinical species. Even though neuroPK studies may employ any dosing route, subcutaneous administration is the most preferred approach since it bypasses first-pass liver metabolism and has low experimental variability. Occasionally, for compounds with intrinsically low rates of CNS penetration, significant delay in reaching distribution steady state may be of concern, in which case an intravenous infusion regimen can be applied [8]. In a typical rodent neuroPK study, animals ($N = 3$/time point, preferably at least two dose levels, for example, 1 and 10 mg/kg) are euthanized at specific time points post dose, and plasma, CSF, and brain are collected for exposure analysis. Time-concentration profiles of the compound in plasma, CSF, and brain are generated.

These data reveal important PK parameters providing insight into the extent of compound CNS penetration and rate of elimination from each compartment. Knowing matrix-specific diffusion and elimination rates is important as compounds may have intercompartmental concentration-related delays and/or much longer half-lives in brain versus CSF or plasma. Such phenomena are key to understand the mechanism of hysteresis observed in exposure-PD response. Furthermore, free drug exposures in each of the compartment, plasma, CSF, and brain, can be calculated by combining area under the curve (AUC)-derived exposures for each neuromatrix and compound-specific $f_{u,plasma}$ and $f_{u,brain}$ values, determined by equilibrium dialysis studies using plasma and brain homogenate, respectively (Eqs. 17.1 and 17.2). Under normal physiological condition, CSF is low in protein. Therefore, the concentration measured in CSF is, in general, considered approximately free drug concentration:

$$\text{Free drug exposures in plasma: } AUC_{p,u} = AUC_p \cdot f_{u,plasma} \tag{17.1}$$

$$\text{Free drug exposure in brain: } AUC_{b,u} = AUC_b \cdot f_{u,brain} \tag{17.2}$$

Ratios between $AUC_{p,u}$, AUC_{CSF}, and $AUC_{b,u}$ provide important insights into the extent of compound distribution among these brain compartments. The ratio of $AUC_{b,u}$:$AUC_{p,u}$, presuming $C_{b,u}$ and C_{ISF} are equivalent, provides the concentration relationship across the BBB. The ratio of AUC_{CSF}:$AUC_{p,u}$, on the other hand, gives an

TABLE 17.1 Methodologies in Predicting Free Human Brain Concentration

Methods	Description	Comments
Human receptor occupancy (RO)	$RO = C_{bu}/(C_{bu} + K_i)$	Human RO can be obtained from imaging studies (e.g., PET)
Human CSF	Sampling CSF in human	CSF can overpredict free brain concentration for efflux transporter substrates
Animal neuroPK and human unbound plasma	$C_{bu} = [C_{bu}/C_{pu}]_{rat} \times C_{pu}$	C_{bu}/C_{pu} is often preserved across species, except for sheep
Human unbound plasma	$C_{pu} = C_p \times f_{u,p}$	When distribution equilibrium is established, typically for high permeable compounds that are not efflux transporter substrates

idea of the extent of partitioning at the BCSFB. These two parameters are important in defining whether there is distribution equilibrium among these neurocompartments, which is critical in understanding preclinical efficacy and safety and in predicting brain penetration in human. Whereas, the ratio of $AUC_{b,u}$:AUC_{CSF} defines the compound concentration relationship between ISF and CSF. This ratio is very important, particularly for a compound that demonstrated distribution disequilibrium at the BBB and/or at the BCSFB, because, for these compounds, plasma will overestimate drug exposure in brain ISF. If CSF exposure is determined in the clinic, human brain ISF exposure may be estimated from this using the rodent-derived relationship assuming an identical ratio across species (Table 17.1). For CNS compounds, careful characterization of these three exposure ratios increases the chances of adequate compound delivery to the pharmacologic target site and understanding cross-species exposure-response relationships.

17.3.1.2 CSF as a Surrogate for Brain Exposure. The use of CSF concentration as a surrogate marker for the unbound drug concentration in the CNS has become a more common practice for assessing CNS exposure. This is because there is a close relationship between the brain ISF and CSF, and CSF sampling is relatively simple and straightforward both in preclinical experiments and clinical settings. However, it is important to recognize, as aforementioned, that the brain consists of multiple compartments created by two distinct barriers (BBB and BCSFB) and that many factors are involved in the transport of drugs from plasma into the brain and the distribution within the brain. Systemically administered drugs can reach CSF either directly via passage across the BCSFB or indirectly by passage across the BBB followed by diffusion/convection transport from the ISF to CSF. Therefore, the usefulness of CSF as a surrogate for measurement of unbound drug concentration in the brain has been critically questioned [9]. From the PK/PD perspective, to determine whether CSF can serve as a surrogate for assessing the drug exposure at the pharmacological target site within the CNS and the dynamics of drug concentration–effect relationship, the critical issue is whether CSF concentration is in equilibrium with the target biophase concentration.

CSF, an essentially aqueous fluid free of drug binding proteins, circulates throughout the ventricles within the brain and the subarachnoid space surrounding the brain and

spinal cord. Because of its physically close proximity to the brain, drug concentration in the CSF is sometimes used as a surrogate of free drug concentration in the brain [10]. However, there are several important aspects of CSF worth special consideration when interpreting drug concentration in CSF as a surrogate exposure at the CNS target site.

First, drug distribution in CSF may be uneven, because CSF is not a well-stirred compartment. Unlike systemic circulation, where it takes <1 min to circulate blood throughout the body, the movement of CSF is much slower. The turnover rate of CSF is about 5–7 h. Drug concentration in CSF is expected to be significantly different depending on the site of CSF sampling and the route of administration. Remarkable differences (approximately 10-fold) in drug concentrations between ventricular and spinal CSF have been reported after intraventricular or lumbar injection [10]. The apparent differences in CSF drug kinetics between the ventricular and lumbar sites is not a surprise when one considers that the flow through the subarachnoid space receives only a fraction of the CSF flow originating from the ventricles. Furthermore, exchange of drug between CSF and the cord tissue bordering the descending spinal subarachnoid space probably exerts influence on the composition of the spinal CSF. These considerations definitely raise a serious question as to the usefulness of lumbar CSF as an indicator of drug availability and disposition in the brain.

Second, drug concentration in CSF is influenced by BCSFB at CP as well as drug concentration in ISF. The total volume of the CSF in adult humans is approximately 150–200 mL [11]. Various studies have suggested that about two-thirds of CSF is formed at the CP, and one-third of CSF enters the macroscopic spaces from brain ISF [12]. The BCSFB, separating blood from CSF, is formed by the epithelial cells of CP and their tight junctions. Unlike the BBB, the capillaries supplying the CP are fenestrated. Similar to the BBB, a number of influx and efflux transporters have been identified at the BCSFB. However, their locations and functions are somewhat different than those at BBB [13]. It has been reported that P-gp (MDR1 and ABCB1) localizes subapically at the CP epithelium, with drug transport in the direction from blood to CSF. In contrast, at the BBB, P-gp localizes at the luminal membrane of brain endothelial cells and pumps drugs out of brain. At BCSFB, other efflux transporters localize and function differently as well. Several groups of researchers showed MRP1 (ABCC1) localized basolaterally, conferring an opposing basolateral-to-apical drug-transport barrier. The localization of MRP1 suggests that MRP1 is an efflux transporter pumping drugs out of the CP, which is functionally different from P-gp at BCSFB. Understanding of the role of the BCSFB in transporting drugs into the brain is emerging and has been gradually gaining more attention over the years.

Despite the complexity of CSF physiology and PK, CSF sampling remains an important part of neuroPK study, which is a valuable tool for assessing drug CNS penetration in preclinical studies, and more importantly, projecting human CNS penetration (Table 17.1). Since CSF is not a homogeneous fluid, it is recommended that, in animal studies, CSF sampling be performed through a catheter inserted into the cisterna magna (in close proximity to the brain). In addition, drug distribution into CSF may be delayed from plasma due to low membrane permeability. Comparison of CSF and corresponding plasma unbound drug concentration based only on a single time point could be misleading. Therefore, it is highly desirable to assess CNS exposure in animals by comparing the AUCs of unbound drug concentration in CSF, brain, and plasma. As mentioned in the previous section, a well-conducted preclinical neuroPK

study will define drug distribution kinetics among CNS compartments and provide insights in designing a clinical study to assess human brain exposure. For compounds that exhibited distribution equilibrium among neurocompartments, free drug concentration in plasma can be used as a surrogate for brain exposure. For those that exhibited impaired brain penetration, CSF can be used as surrogate of drug brain concentration, as plasma will overestimate free drug exposures in brain. However, it is prudent to assess unbound drug concentration in the brain and use the ratio of $AUC_{b,u}$:AUC_{CSF} to define the relationship between ISF and CSF, as equilibrium may not always be established between these neurocompartments.

In summary, CSF drug concentration can be used as a useful surrogate for assessing unbound drug concentration at the target site within the brain. However, CSF drug concentration is not always an accurate surrogate for all drugs, especially for transporter substrates. Preclinical neuroPK can be used to design clinical studies to assess CNS target exposure in humans (Table 17.1).

17.3.1.3 Microdialysis. According to the free drug hypothesis, unbound drug in brain ISF is in direct contact or in equilibrium with that at the site of action [2]. *In vivo* intracerebral microdialysis is a valuable technique to determine biophase free drug concentrations and distribution in the brain as a function of time. The technique involves implantation of a thin dialysis probe into a selected area of the brain. The probe consists of an inlet tube, a semipermeable membrane, and an outlet tube. To mimic the function of a capillary blood vessel, the thin dialysis probe is continuously perfused with an artificial physiological solution. Drug molecules diffuse across the membrane along the concentration gradient (in the direction of the lower concentration) into or out of the selected tissue. A basic principle of microdialysis is that only unbound drug can freely diffuse through the semipermeable membrane [14]. Depending on the physicochemical properties of the drug, the concentration measured with the probe may not reflect the concentration in the ISF but is a function of the probe perfusion rate, blood flow, and diffusion in the tissue surrounding the probe. Therefore, *in vivo* recovery of the probe for a specific compound will need to be incorporated to translate the concentration measured in the dialysate to the unbound drug concentration in the tissue [15].

Microdialysis has been used to monitor biophase concentrations of antiepileptic drugs in animals and humans. Walker *et al*. [16] investigated the rate of lamotrigine penetration into plasma, CSF, and hippocampal and frontal cortex extracellular fluid compartments following systemic administration in nonepileptic rats. Following intraperitoneal injection, the ISF AUC of lamotrigine to total serum AUC ratio (0.4 ± 0.01) was similar to the free serum AUC to total serum AUC ratio (0.39 ± 0.01), and did not differ between hippocampus and frontal cortex. On the other hand, the CSF AUC to total serum concentration AUC ratio was 0.6 ± 0.05. Overall, the unbound concentrations of lamotrigine in three different compartments, serum, CSF, and ISF, were in a reasonably good agreement, supporting the concept of free drug hypothesis. In human, an intracerebral microdialysis study was also conducted during surgery by Scheyer *et al*. [17], who collected dialysate from the hippocampus of refractory epilepsy patients and determined phenytoin level in the dialysate. The steady-state extracellular phenytoin concentrations corresponded closely to the unbound plasma concentration, indicating free drug distribution equilibrium between brain ISF and plasma.

Although the microdialysis technique was developed more than two decades ago, it is primarily used for determination of neurotransmitters and not drug concentrations in the brain in the pharmaceutical industry. The main limitations of this technique include high resource requirements, low throughput, and special surgical skills to set up the experiment. In addition, many compounds in the discovery stage are very lipophilic, and it is difficult to apply microdialysis technique to study these compounds because of high nonspecific binding to the dialysis devices and tubing. Importantly, this method cannot be used routinely to measure the ISF concentration in the clinic for ethical reasons. Because of these constraints, alternative methods, such as CSF sampling and neuroPK studies combined with brain homogenate binding, have been used to estimate brain ISF exposure. The accuracy of using unbound brain concentration determined by a neuroPK/brain homogenate binding method and CSF exposure as a surrogate for brain ISF concentration was compared with those obtained by microdialysis in rat brain [8]. The results supported the use of neuroPK/brain homogenate approach or CSF as a surrogate for the ISF free drug concentration in drug discovery and also confirmed the distribution disequilibrium between brain, CSF, and plasma for efflux transporter substrates and low permeability drugs.

17.3.1.4 PET Imaging. Imaging is an attractive technique to obtain information on brain drug uptake in humans due to the noninvasive nature of the approach. There are a number of methods for imaging compounds in the brain, including positron emission tomography (PET), single photon emission computed tomography (SPECT), and magnetic resonance imaging (MRI). Before PET and SPECT, MRI was used to obtain PK information of drug in brain [18]. It suffered from low spatial resolution; therefore, its value for quantitative assessment was limited. PET and SPECT both use radioactive tracer materials and detect γ-rays. However, the tracer used in SPECT emits γ-radiation that is measured directly, whereas PET tracer emits positrons that annihilate with electrons up to a few millimeters away, causing two γ-photons to be emitted in opposite directions. A PET scanner detects these emissions "coincident" in time, which provides more localized information of radiation events and higher resolution images than SPECT. Therefore, PET is a better quantitative method for assessing drug brain distribution. SPECT scans, however, are significantly less expensive than PET scans, in part because they are able to use longer lived more easily obtained radioisotopes than PET. PET is a nuclear medicine imaging technique that produces a three-dimensional image or picture of functional processes in the body. The system detects pairs of γ-rays emitted indirectly by a positron-emitting isotopes (^{11}C, ^{13}N, ^{15}O, or ^{18}F), which is incorporated into the drug of interest. Typically, the isotopes are made in a cyclotron, and because of their short half-lives, they need to be rapidly incorporated into drug immediately before administration. Once administered to a patient, the label can be imaged noninvasively using a PET scanner. To study drug brain distribution, there are two main approaches using PET. The first is the direct approach, where the drug itself is labeled. The second is the indirect approach, where a labeled receptor ligand is administered and then unlabeled drug of interest is administered. The time course of receptor occupancy will be measured using the PET scanner as the drug displaces the ligand from the receptor.

Verapamil, a calcium channel blocker, has been used as a PET tracer to assess P-gp-mediated transport at the BBB in human, since it is a P-gp substrate and can also be straightforwardly labeled with ^{11}C. A human volunteer study utilizing [^{11}C]-verapamil

as a model P-gp substrate and cyclosporine A (CsA) as a P-gp inhibitor was conducted by Sasongko *et al*. [19]. Results showed that the inhibition of P-gp activity at the human BBB was modest, with ~90% increase in [^{11}C]-verapamil distribution into the brain in the presence of $\sim 2.9\,\mu M$ steady-state blood CsA concentration. These results are dramatically different from those obtained in the P-gp knockout (KO) or chemical KO mouse or in rats administered with a high dose (e.g., 50 mg/kg) of CsA, where ~10-fold increase in [^{11}C]-verapamil distribution into the brain was observed when compared with the wild-type mouse [20]. The observed discrepancy in the verapamil–CsA interaction at the BBB between rodents and human is likely due to differences in blood CsA concentrations. The PET imaging study provided evidence for the first time that P-gp activity at the human BBB is measurable and that it is only modestly inhibited by one of the most potent and Food and Drug Administration-approved P-gp inhibitors, cyclosporine. Kinetics of drug brain uptake must be interpreted with reference to concurrent blood drug concentrations and it is difficult to define the blood kinetics of a compound using PET alone. Solid-phase extraction (SPE) and high performance liquid chromatography (HPLC) analysis was used to determined the amount of radioactive verapamil and its metabolites in the plasma [19]. However, for a drug such as a verapamil, which has a $t_{1/2}$ in the range of 6–8 h, the plasma concentration–time profile of ^{11}C-labeled parent tracer would fail to predict the drug's PK parameters as the drug's elimination phase cannot be captured during the short time course of the PET experiment. For such drugs, a combination of PET imaging for assessment of drug tissue distribution with accelerator mass spectrometry (AMS) for plasma PK analysis can be particularly powerful. Verapamil brain distribution kinetics was determined in human in a microdosing study combined with PK profiling in plasma using AMS after administration of a mixture of (*R*)-[^{11}C]verapamil and (*R*/*S*)-[^{14}C]verapamil [21]. Each subject underwent PET scan of 60-min duration after receiving a microdose of labeled verapamil. The time–concentration profile of (*R*)-verapamil in human brain was demonstrated by reconstruction of the PET data, while concentration in plasma was profiled using HPLC with a radiometric detector, chromatographically separating parent drug and metabolites.

PET imaging also provided the opportunity to study species difference of P-gp transporter at the BBB by measuring brain concentrations and brain-to-plasma ratio of three labeled P-gp substrates, [^{11}C]verapamil, [^{11}C]GR205171, and [^{18}F]altanserin in rats, guinea pigs, monkeys, and humans [22]. Pronounced species differences were found in the brain and brain-to-plasma concentrations of these P-gp substrates, with higher brain distribution in humans, monkeys, and minipigs than in rats and guinea pigs. The brain-to-plasma ratio of [^{11}C]GR205171 was almost ninefold higher in humans compared with rats. These finding suggested species differences should be considered when extrapolating data obtained in animals to humans. Compounds found to be P-gp substrates in rodents are likely to be substrates in higher species as well, but the ratio of brain penetration, even corrected with binding factors, may not be reliably extrapolated from animals to humans. Despite these reported species differences, an in-depth study has revealed that the rodent is a suitable model for CNS penetration (Section 17.4.1).

The advantages of PET are obvious as it is not only noninvasive, which makes it attractive for clinical applications, but it also allows drug concentrations and/or receptor occupancy to be followed with high spatial (several millimeters) and time resolution. Thus, it is possible to monitor drug concentrations or effects in discrete structures in the brain. However, on a practical level, a disadvantage of PET is that it requires expensive

infrastructure (i.e., both a cyclotron and PET scanner in close proximity). PET also requires clever chemistry (i.e., dedicated personnel) to incorporate labels rapidly into compounds of interest before the label has decayed excessively. The other limitation of PET is that, in general, it is unable to discriminate between bound and unbound drug or parent compound and metabolites. Because of these reasons, there has been less use of PET for animal studies during the discovery stage, but there is now a growing trend for its use in drug development with the availability of commercial small animal PET scanners [23].

17.3.2 *In vitro* Methods

17.3.2.1 BBB Permeability. Passive permeability is an important parameter for assessing the rate of CNS penetration, since most small molecules enter the brain by transcellular passive diffusion. The rate of brain penetration plays a critical role for drug candidates that require rapid onset of pharmacological effects (e.g., anesthesia). It is also pivotal in terms of time necessary to achieve distribution equilibrium among the various neurocompartments (e.g., brain, CSF, and plasma).

In situ brain perfusion is the gold standard method for measuring BBB permeability *in vivo* [24,25], since it is quite reliable to determine the rate of brain penetration. In the brain perfusion studies, animals were anesthetized. The test compounds were infused in a perfusate to the external carotid artery for about 30 s [26]. At the end of the experiment, animals were sacrificed and the brains were homogenized and analyzed with liquid chromatography–mass spectrometry (LC-MS). Although it is useful, the *in situ* brain perfusion assay is not commonly applied in the pharmaceutical industry, because it does not provide the free drug concentration in the brain directly, which is most important for pharmacological activity. Instead, *in vitro* BBB permeability assays or calculated properties are more frequently employed to estimate the rate of brain penetration. There are two major *in vitro* BBB permeability methods: (i) parallel artificial membrane permeability assays (PAMPA-BBB) and (b) cell-based monolayer permeability assays [e.g., MDCK apparent permeability (P_{app}) from apical to basolateral].

PAMPA is a high throughput assay for passive diffusion originally developed to mimic intestinal membrane permeation and predict oral absorption [27–29]. The assay was further expanded to predict BBB permeability (PAMPA-BBB) using brain lipids as the artificial membrane [30,31]. PAMPA-BBB permeability values correlated well with *in situ* BBB permeation values obtained from brain perfusion experiments in rodents [32,33]. Because PAMPA-BBB has good predictability of the rate of brain penetration and is high throughput with low cost, it is a valuable tool as first-tier screening in drug discovery for BBB permeability.

Cell monolayer transport assays with primary cells or cell lines are also commonly used as models to assess BBB permeability, such as MDCK, LLC-PK1, Caco-2, and brain microendothelial cell (BMEC) [34]. Among the various cell-based *in vitro* assays, MDCK cells are most commonly used [26,34–36] to predict BBB permeability. Although MDCK cells are not of cerebral origin, the MDCK permeability assay has clear advantages over other cell types: easy cell culture, low maintenance, tight cellular junctions, low endogenous transporters, and low metabolizing enzymes. Recently, a

MDCK-LE (low efflux) cell line has been developed to further minimize the potential interferences from endogenous transporters on passive permeability measurement [37].

A comparison study on PAMPA and MDCK was conducted using 31 structurally diverse marketed CNS-active drugs, one active metabolite, and seven non-CNS-active compounds [38]. In general, the two assays gave a good correlation. Compounds with $P_{app} > 5 \times 10^{-6}$ cm/s in PAMPA or MDCK had a good BBB permeability *in vivo*. Importantly, most successful marketed CNS drugs have moderate to high passive permeability. Additionally, *in silico* models have been quite successful in predicting passive permeability across the BBB based on molecular properties and they can be applied effectively in early drug discovery [39,40].

17.3.2.2 Brain Tissue Binding. As discussed above, free drug concentration at the site of action is the species that exerts pharmacological activity and the free brain/plasma ratio ($C_{b,u}/C_{p,u}$) is an important parameter to evaluate the brain penetration potential of drug candidates. Several methods have been developed to measure free brain concentration, including microdialysis, CSF sampling, and a combination of neuroPK and brain tissue binding (Section 17.3.1). In drug discovery and development, it is a common approach to obtain free drug concentration in the brain by a combination of neuroPK and brain tissue binding studies, and the free C_{max} and AUC are obtained using Equation 17.2. The approach has been shown to have a good correlation with direct microdialysis and indirect CSF measurements in determining free drug concentration in the brain *in vivo* [5,41,42].

There are two methods that are commonly used for brain tissue binding measurements, which use (i) brain slice [41,43–45] or (ii) brain homogenate [46–49]. Brain slices preserve the cellular structures (cell membrane, influx and efflux transporters, and ICFs) and are considered physiologically more relevant. However, even with the difference between brain slice and brain homogenate, the two methods have a good correlation in measuring f_u values, especially when cytosolic pH partition is corrected for basic compounds [44,50]. It appears that the nonspecific binding to lipophilic components in the brain is the dominant mechanism for brain tissue binding, and that the presence of intact structural elements plays a less significant role in determining brain binding. One advantage of using brain homogenates over brain slices is that they are readily available from vendors and can be stored frozen and thawed right before experiments. High throughput 96-well format dialysis devices have been developed and can be used to determine f_u using brain homogenates (e.g., HTD 96 from HTDialysis [51] and RED from Thermo [52]). The devices are engineered to minimize nonspecific binding to the plastic wells and dialysis membranes to shorten the time required for compounds to reach equilibrium. The advantage of the equilibrium dialysis device is that nonspecific binding does not affect f_u determination, which is particularly important for highly lipophilic compounds. The good predictability of *in vivo* free drug concentration and the ease of use make brain homogenate binding one of the most widely employed methods for determining fraction unbound in brain tissue. While plasma protein binding is often species dependent, brain tissue binding has been shown to be species independent [53]. One can use a single species (e.g., rat) to estimate brain binding of all other species, including human in drug discovery. This greatly increases efficiency and reduces cost. Brain tissue binding has been shown to be independent of regional brain tissues [54], and the brain regions in the study included

CP, striatum, hippocampus, motor cortex, cerebellum, and thalamus [54], suggesting that no regional brain binding studies are necessary.

There are many misconceptions among drug discovery project teams regarding drug binding *in vivo*, including the perceived value of increasing fraction unbound through structure modification [2]. The rational of this misconception is to increase the free drug concentration at the site of action by manipulating fraction unbound. This approach is scientifically unsound. Changing fraction unbound will not affect free drug concentration *in vivo* for orally administrated drugs. Project teams should not attempt to increase fraction unbound to improve free drug exposure at the target or use fraction unbound to rank order compounds. Fraction unbound should only be used to calculate the free drug concentration by multiplying it to the total drug concentration obtained from *in vivo* neuroPK studies. For this reason, f_u data alone can be misleading and one does not need to measure f_u, unless *in vivo* total drug concentration or AUC exposure data has been measured.

17.3.2.3 Transporters. Measurement of the interaction of drugs with BBB transporters is critical in predicting the brain penetration potential of drug candidates. Many influx (solute carrier) and efflux (ATP-binding cassette, ABC) drug transporters in the BBB (Fig. 17.2) and the BCSFB have been reported [55]. More than 200 drug transporters in human BMECs were profiled using real-time polymerase chain reaction (PCR) [56]. The expression of human P-gp (MDR1, ABCB1) and breast cancer resistance protein (BCRP, ABCG2) are the highest in human BBB based on mRNA level [56]. Recently, 114 drug transporter protein levels were quantitatively determined in human brain microvessels using proteomics approaches with LC-MS/MS. The results showed that human P-gp and BCRP are the most abundant transporter proteins at the BBB, which is consistent with the mRNA levels [57]. On the basis of the protein level, human BCRP is slightly higher than P-gp in brain microvessels in the ratio of about 4:3. The human BCRP protein level is higher than mouse Bcrp, while human P-gp is lower than mouse P-gp in the BBB [57]. Transporter protein levels have also been reported for monkey with P-gp and Bcrp being the two major transporters in

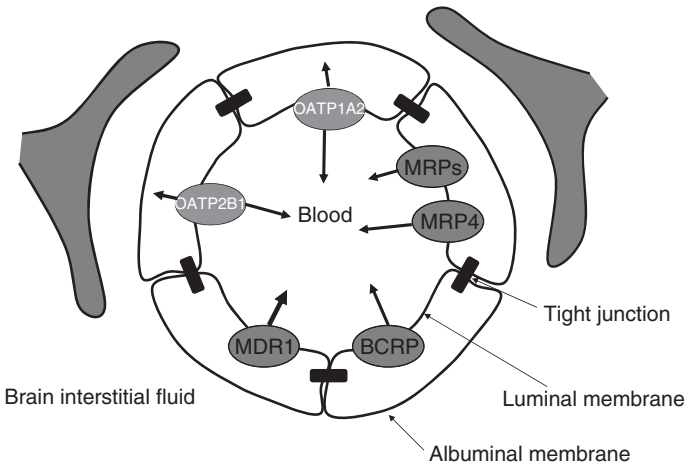

Figure 17.2 Drug transporters expressed at human blood–brain barrier.

the BBB. Monkey has slightly higher amounts of Bcrp compared with human [58]. Though human BBB BCRP has a slightly higher amount than P-gp, P-gp has been the dominant efflux transporter at the BBB to reduce compounds from entering the brain. P-gp and BCRP have a large substrate overlap and many BCRP substrates are P-gp substrates, and only a small number of compounds are BCRP-specific substrates. This may account for why P-gp seems to play a more important role than BCRP in limiting brain penetration of drugs *in vivo*. P-gp and BCRP transporters have a synergistic effect in restricting their dual substrates from entry into the brain, which is expected based on individual kinetic contribution to BBB efflux [59–62]. This has been applied in designing drugs that target peripheral tissues, where brain penetration can cause unwanted side effects. Besides P-gp and BCRP, other efflux transporters, such as MRP4, can pump certain compounds out of brain. However, the *in vivo* significance of other efflux transporters in the BBB is not well defined. Uptake transporters at the BBB, such as OATP1A2, OATP2B1, LAT1, and MCT1, can enhance transport of some drugs into the brain. L-DOPA, gabapentin, and pregabalin are examples of drugs that are active influxed into the brain by the LAT1 transporter [63]. It has been reported recently that OATP1A2 is a potential transporter to increase active uptake of certain drug candidates into the brain [64]. A positively charged amine atom is required for efficient OATP1A2-mediated uptake, and the uptake rate is in the order of tertiary $>$ secondary $>$ primary amines [64]. Uptake rate also improved with increased van der Waals volume, but not with Clog P [64]. The *in vivo* relevance of OATP1A2 in enhancing the CNS penetration has yet to be demonstrated.

Several *in vitro* BBB models with primary brain endothelial cells or immortalized brain endothelial cells have been developed. These models tend to have leaky junctions between the cells and downregulation of BBB transporters. Although they are of brain endothelial cell origin, the leaky junctions, low transporter expression, and high cost make them unsuitable for use on regular bases to support of drug discovery programs. Instead, the most common approach to assess transporter substrates and inhibitors is to use transfected cell lines that express selected transporters, for example, MDR1-MDCK, BCRP-MDCK, OATP1A2-HEK, and others. Cutoff values are established to differentiate substrates from nonsubstrates, typically based on empirical evaluation of compounds with known substrate properties. *In vitro* transporter data based on cell monolayer transport studies have a good correlation with *in vivo* results from transgenic animals. A study on P-gp efflux for a set of structurally diverse drugs showed that MDR1-MDCK data had a good correlation with *in vivo* efflux ratio obtained from P-gp KO mice versus wild type [6,38]. P-gp efflux activity is usually conserved across various species due to a high sequence homology of the protein (80–96%, rat, mouse, dog, and monkey). More than 3000 compounds were tested at both human MDR1-MDCK and mouse Mdr1a-MDCK transwell assays *in vitro*, and a good correlation ($R^2 = 0.92$) of efflux ratios was observed [38]. However, for certain structural classes, species difference in P-gp efflux has been demonstrated [65]. Cell lines transfected with transporters (e.g., P-gp and BCRP) from various animal species can be used to evaluate the different transporter activities from the different species. Human MDR1-MDCK showed to have a better prediction of human P-gp effect on BBB penetration than using P-gp KO mice, due to its human origin and high sensitivity without added complexity from various *in vivo* factors. Though the value of P-gp KO is to understand whether P-gp plays a major role in limiting brain penetration in mouse has been demonstrated, its predictive value to human P-gp interaction is limited compared with transfected

cell lines with human origin. Transporter information is widely used by project teams to guide structural modification, develop SAR (Structure-Activity Relationship), select compounds for *in vivo* studies, and diagnose *in vivo* observation.

P-gp found at the BBB reduces entry of toxic materials into the brain. Impaired P-gp function due to generic polymorphism, disease state, or aging may raise the levels of toxic compounds in the brain and increase the risk of developing certain neurodegenerative diseases. For example, decreased expression of P-gp in the BBB vasculature has been found in patients with advanced Parkinson's disease [66], Alzheimer's disease [67], and Creutzfeldt–Jakob disease [68]. In AD, P-gp upregulation in early pathogenesis was suggested as a compensatory mechanism to increase the capacity of detoxification, while at later disease stages, P-gp expression was lost due to brain cell degeneration [67]. Decreased BBB P-gp function seems to be a late event in neurodegenerative disorders and could enhance continuous neurodegeneration [66]. On the other hand, P-gp activity was found to increase in patients with chronic schizophrenia under treatment with antipsychotic drugs. The increased P-gp activity might be a factor for drug resistance in schizophrenia, induced by the use of antipsychotic agents [69]. Additionally, P-gp activity at certain BBB regions decreased during aging, which increased the risk of developing brain diseases [70,71].

17.3.3 *In Silico* Predictions and Simulations

17.3.3.1 Computational Models. A number of CNS models have been developed to predict brain penetration and they are most useful for library design and prediction before synthesis [72,73]. Models predicting passive permeability across the BBB have been developed using molecular descriptors related to lipophilicity, hydrogen bonding capacity, charge, and molecular weight (MW) [74], as well as descriptors [39], such as log D, van der Waals surface area of basic atoms, and polar surface area (PSA). Models for active or facilitative transporter processes are less mature owing to insufficient quality data necessary for model development. The rules for good CNS penetration are PSA $< 60–70\,\text{Å}^2$, log D between 1 and 3, and MW < 450, and rules for non-Pgp substrates are $\text{N} + \text{O} \leq 4$, MW < 400, and base $\text{p}K_\text{a} < 8$ [75–77].

17.3.3.2 CNS Multiparameter Optimization. To increase the survivability of CNS drug candidates, multiparameter optimization (MPO) was developed to shift the neuroscience medicinal chemistry to a design space with a higher probability of success [78]. Six calculated parameters were utilized to understand the interplay among the different physicochemical properties and to create a design tool focusing on aligning the CNS drug-like attributes. The six parameters in CNS MPO are lipophilicity (Clog P), distribution coefficient at pH 7.4 (Clog D), MW, topological polar surface area (TPSA), number of hydrogen bond donors (HBD), and basicity of the most basic center ($\text{p}K_\text{a}$) [79]. The CNS MPO scoring function offers great advantages over simple hard cutoff rules or using single parameter for optimization. The CNS MPO enables exploring medicinal chemistry design space through a holistic assessment approach to accelerate the identification of compounds with increased probability of success [80].

17.3.3.3 PBPK Models for BBB. Physiologically based pharmacokinetic (PBPK) models for BBB are based on the anatomical and physiological structure of the body and certain biochemistry. They are powerful tools to quantitatively predict brain exposure time course, brain-to-plasma ratio, and other important PK parameters *in vivo* [81]. Commercial BBB PBPK software is recently made available [82]. One of the challenges facing BBB PBPK models is to integrate transporter activity into the prediction. Several human BBB transporter proteins have been quantified using LC-MS [57]. The information, in conjunction with the level of transporter proteins in transfected cell lines, can provide a scaling factor to predict CNS penetration effectively.

Recently, *in vivo* disposition of 11 P-gp substrates in mouse brain was predicted using a PBPK model that incorporated the P-gp protein level in mouse brain capillaries and in transfected cells, P-gp *in vitro* activity, and drug unbound fractions in mouse plasma and brain [83]. For most of the compounds, the predicted brain-to-plasma concentration ratios and unbound brain-to-plasma concentration ratios were within three-fold of the observed *in vivo* values. With the progress in transporter proteomics in human BBB, the brain distributions of P-gp substrates in human would be predicted from the P-gp protein levels, *in vitro* activity, and drug unbound fractions as well. However, it is a challenge to validate the accuracy of the model in predicting human brain disposition, since the unbound human brain concentration is often unknown, and CSF and CNS pharmacology activity is unlikely to be in equilibrium with the free brain concentrations due to transporter interactions. The advances in imaging technology (e.g., PET) will be very helpful to validate the BBB PBPK models in humans.

17.4 RETROANALYSIS FOR PFIZER CLINICAL CANDIDATES

For drugs that have a site of action within the CNS, brain penetration is essential in accessing the site of action to deliver their pharmacological benefits and having a balanced efficacy and safety profiles. Even though all drugs do penetrate brain to some extent, drugs that have impaired brain penetration will have reduced exposures in brain and require proportionally higher doses to achieve therapeutic exposure at the CNS target site. Therefore, within CNS drug discovery, it has been recognized that having "good" CNS penetration is an added requirement for selecting promising drug candidates. However, in contrast to the established approaches of predicting systemic human PK parameters [84], it is not so obvious how to predict whether a compound will exhibit good CNS penetration in human.

Multiple factors contribute to the uncertainty around predicting human CNS penetration. First, how to measure brain penetration in human has always been a challenge. The easily accessible measurement site, blood, does not always reflect brain exposure. Despite close proximity of CSF to the brain, the value of CSF can be limited in predicting the drug concentration at the target site biophase in the CNS [12] (see above discussions). Intracerebral microdialysis and PET imaging are much improved techniques; however, their applications in CNS drug discovery and development are limited as intracerebral microdialysis is highly invasive, while suitable radiolabeled PET ligand may not be readily available for the novel drug targets being pursued. Furthermore, the limited human CNS exposure data have greatly hindered the progression

of predicting human CNS penetration, because multiple iterations of prediction refinement is the only way to advance predictive ADME science, as evidenced by success in the area of human PK prediction [85].

Nevertheless, the pharmaceutical industry has invested a significant amount of research in understanding factors influencing CNS drug disposition and, subsequently, in developing comprehensive tools to select candidates that have the best potential of being developed as successful CNS drugs. The preclinical tool box encompasses *in vitro* assays (e.g., membrane permeability, P-gp efflux transporter using MDR1-MDCK, and many others are being developed) and *in vivo* assays (e.g., neuroPK in rats, dog model for CSF sampling, and P-gp KO mouse). These are routinely used for CNS drug candidate selection. Questions have been raised regarding the predictability of these assays and confidence in using preclinical information in prediction of CNS penetration in humans. A retrospective analysis was conducted using 32 Pfizer proprietary clinical candidates, mostly with CNS indications, to address these important questions.

17.4.1 Preclinical and Clinical Data Used for Retrospective Analysis

To enable retrospective analysis, 32 Pfizer clinical candidates were selected that had either (i) CSF drug concentrations measured in humans or (ii) free drug concentrations in brain derived using receptor occupancy (from humans who had undergone clinical PET imaging studies), as well as *in vitro* receptor binding affinity, K_i. These 32 clinical candidates covered a wide range of CNS targets, including transmembrane G-protein-coupled receptors (GPCR), neurotransmitter gated channels, and ion channels. Their preclinical *in vitro* and *in vivo* data packages were collected, which included mainly membrane permeability, P-gp efflux, and brain penetration information from studies conducted in animals, mostly rats and mice, and a few guinea pigs or dogs. Compounds, which were shown as P-gp efflux substrates *in vitro* and/or exhibited brain penetration impairment *in vivo*, were also subjected to brain penetration studies in P-gp KO mice versus wild-type mice to confirm the role of P-gp in limiting their brain penetration [6].

These compounds were put into three groups (Fig. 17.3) based on their preclinical information by asking two questions: (i) Does the compound have a P-gp efflux liability and have low membrane permeability? (ii) Does the compound have good CNS penetration in animals? Since compounds in the data set are optimized clinical candidates, most of them have reasonably good membrane permeability. Therefore, the answer to the first question was essentially based on P-gp efflux liability. For the second question, free drug-based neuroPK methodology (see above) was applied to assess CNS penetration in animals. Compounds were considered as exhibiting interneurocompartmental distribution equilibrium when free drug exposures, in terms of AUC, were within 2.5-fold among plasma, CSF, and brain [5]. Compounds that showed both good *in vitro* and *in vivo* CNS penetration properties, meaning not having P-gp efflux liability and demonstrating good CNS penetration in animals, were categorized as group I compounds, also called *well-behaved* CNS compounds. On the other hand, compounds identified as having P-gp efflux liability and also preclinically showing impaired brain penetration were categorized as group III compounds. The remaining were put together as group II compounds, which had conflicting *in vitro* and *in vivo* findings, being either a P-gp efflux substrate or not showing distribution equilibrium among plasma, CSF, and brain compartments in preclinical species.

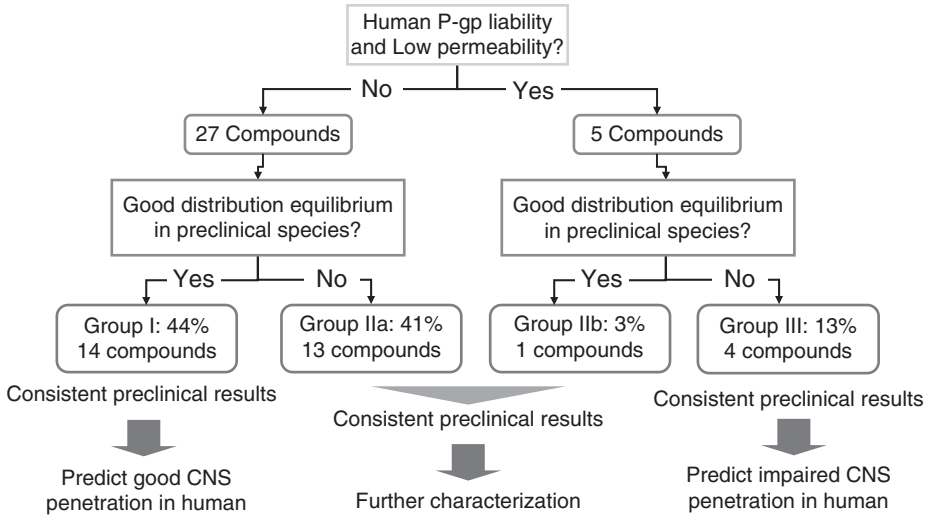

Figure 17.3 Road map of categorizing Pfizer proprietary clinical candidates ($n = 32$) into three groups based on their preclinical information. Group I ("well-behaved" CNS compounds): consistent *in vitro* and *in vivo* preclinical data suggesting good CNS penetration; group II: conflicting *in vitro* and *in vivo* preclinical CNS penetration data; and group III: consistent *in vitro* and *in vivo* preclinical indication impaired CNS penetration.

17.4.2 Human CNS Penetration Predicted from Preclinical Data

Human CNS penetration of these compounds revealed predictability of preclinical information (Fig. 17.4). For group I, or "well-behaved" CNS compounds, all of them exhibited good CNS penetration in human based on distribution equilibrium between CSF exposure and plasma or the fact that observed receptor occupancy could be predicted from unbound plasma in human and *in vitro* binding affinity. Furthermore, when

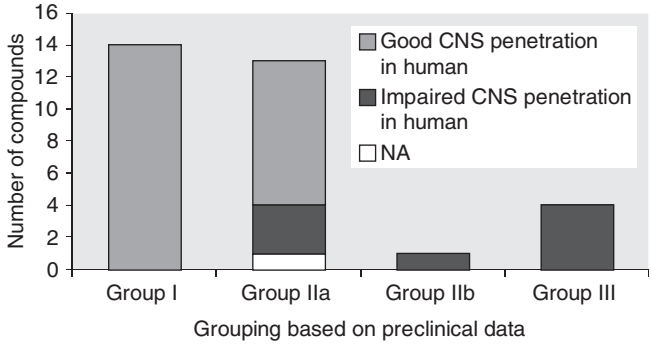

Figure 17.4 Correlation of preclinical and clinical CNS penetration of 32 Pfizer proprietary clinical candidates. Grouping was based on preclinical information (Fig. 17.3). Human CNS penetration assessment was based on human CSF/unbound plasma ratio using a cutoff of 2.5, human receptor occupancy data or efficacy outcome.

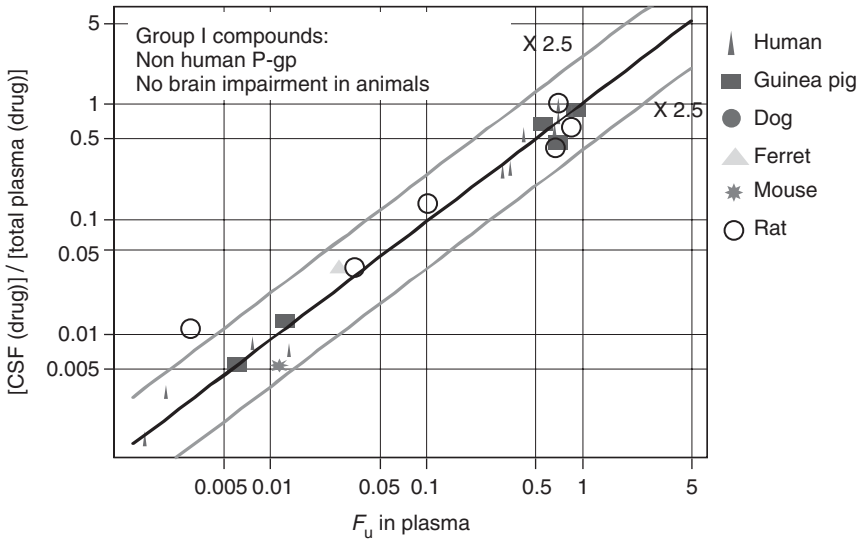

Figure 17.5 Correlation between $f_{u,plasma}$ and CSF/plasma ratio for group I compounds across multiple species. For compounds that exhibit free drug distribution equilibrium between CSF and plasma, unity line correlation is expected. The plot shows that majority of group I compounds are within 2.5 boundary of the unity line, indicating distribution equilibrium between CSF and plasma compartments.

plotting $f_{u,plasma}$ against the CSF/plasma ratio, essentially comparing CSF concentration with unbound plasma concentration, a majority of group I compound's data, across multiple species, are within the 2.5-fold boundary of the unity line (Fig. 17.5), indicating that for group I compounds, human CSF concentration data does not provide extra value over plasma free drug concentration data as a surrogate for CNS target exposure. This has a significant implication for CNS drug development, as this means for "well-behaved" CNS compounds, unbound plasma concentration is as good as CSF concentration in assessing PK/PD relationships at the CNS target site. Moreover, CSF sampling can be avoided, meaning significant clinical development cost saving and reducing unnecessary safety risk for clinical trial participants.

Group III compounds also had consistent *in vitro* data and *in vivo* preclinical CNS penetration data, with both data sets projecting unfavorable human CNS penetration. As expected, for these compounds, clinical data based on human CSF or receptor occupancy was consistent with preclinical prediction, all the group III compounds had impaired CNS penetration in human (Fig. 17.4). Furthermore, cross-species comparison of the CSF/unbound plasma concentration ratio indicated that a magnitude of disequilibrium between CSF and plasma compartments (i.e., CSF/unbound plasma ratio) is not consistent across species even when differences in plasma protein binding were accounted for. Though these compounds still penetrate brain, what makes their clinical development difficult is that it is very hard to get an accurate estimate of the actual concentration at the target biophase. As an example, one of the group III compounds, CP-615003, a potent subtype-selective $GABA_A$ partial agonist, was once brought into clinical development as a psychotherapy, but discontinued due to lack of efficacy [86].

The compound was known to have P-gp efflux liability, and preclinical CNS penetration studies also indicated impaired brain penetration. Nevertheless, the compound was brought forward into clinical development, because it was believed that sufficient target receptor occupancy could still be achieved based on projected CSF exposure in human. However, clinical PET imaging clearly demonstrated an underachieved receptor occupancy in human. Intracerebral microdialysis in rats conducted afterward revealed that this is due to a further distribution gradient from CSF to ISF. For the mechanism of the drug, exposure in the ISF drove the downstream pharmacological effect. It was shown that there was a 43-fold difference in unbound drug exposure between ISF and plasma, compared with only an 8-fold difference between CSF and plasma.

Similar to the preclinical observations, human CNS penetration of group II compounds has mixed results (Fig. 17.4). The large percentage of compounds in group II in the data set ($\sim$44%) reflects the difficulty of evaluating and predicting CNS penetration. It was noted that possible species difference in P-gp efflux contributed to the different outcomes between preclinical and clinical CNS penetration [65]. Also, transporters, other than P-gp, were found to play a role in uptake or efflux of compounds into or out of brain, respectively (e.g., BCRP [55,57]). From a technical perspective, accuracy in determining extremely high nonspecific binding ($>$99.9%) may also confound result interpretation from preclinical neuroPK studies. On the clinical side, for compounds that exhibit delayed distribution kinetics among neurocompartments, single time point CSF sampling may not represent steady-state CNS penetration in human. These findings underscore the necessity of further research in the area of CNS drug disposition. Furthermore, it is important to understand the time course of drug disposition in CSF, and its relevance to target biophase, when designing clinical trials incorporating a CSF sampling end point for assessing CNS target exposure in human.

17.4.3 Predictability of Physicochemical Properties and Preclinical *In Vitro* and *In Vivo* Studies

The predictability of the four most commonly used preclinical assays, namely *in vitro* human P-gp, rat neuroPK, dog CSF PK, and P-gp KO mice studies, were evaluated, based on their correlation with human CNS penetration observed in the clinic (Fig. 17.7). Results indicated that all the human P-gp substrates from the analysis had impaired CNS penetration in human, confirming the critical role of P-gp efflux in drug CNS disposition. In contrast to the expectation that the dog CSF model is a better predictor for human CNS penetration than rat neuroPK, comparable human predictability was observed between the two models, suggesting rat neuroPK as the choice for efficient preclinical evaluation. Interestingly, P-gp KO mice had some "false positive" and some "false negative" prediction for human CNS penetration. These were found to be due mostly to species differences in P-gp efflux between human and mouse and to involvement of other efflux transporters, such as MRP4. Nevertheless, the P-gp KO mice study is still a useful tool to identify whether observed brain distribution impairment in rodents is primarily driven by P-gp.

Brain-penetrant CNS drugs are known to have certain physicochemical attributes [7]. Physicochemical properties of the 32 drug candidates were profiled and their correlations with human CNS penetration were sought (Fig. 17.6). Results confirmed that physicochemical properties, such as MW, lipophilicity (log P and log D), and PSA,

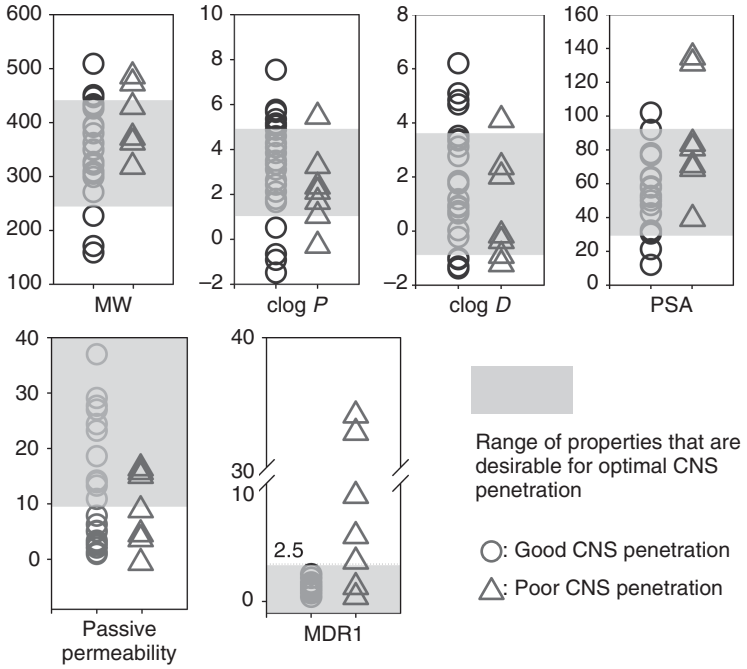

Figure 17.6 The roles of physicochemical properties and *in vitro* properties in predicting human CNS penetration. Clog *P*, Clog *D*, and PSA (polar surface area) were calculated using ACD Lab software (www.acdlabs.com). Passive permeability was assessed using a variant of MDCK cell line that does not express P-gp. MDR1 efflux ratio was obtained from transwell assay using MDR1-MDCK cell line.

are important determinants of CNS penetration. However, none of these should be used alone or as hard criteria when selecting CNS-penetrant compound (Section 17.3.3.2). One interesting observation is that the human P-gp assay appears to be the best assay to identify compounds that will have brain distribution impairment in human, as all the P-gp substrates in the data set exhibited distribution disequilibrium between plasma and brain/CSF ratios in human. However, the opposite is not true, as not being a P-gp substrate did not guarantee good CNS penetration in human (due to possible low passive permeability or efflux by other transporters).

17.4.4 Summary of Retroanalysis Results of Pfizer Proprietary Clinical Candidates

The outcome of retrospective analysis, wherein the predictability of preclinical assays for human CNS penetration was evaluated using 32 Pfizer proprietary clinical candidates, was encouraging (Fig. 17.7). When the human P-gp assay and rat neuroPK characterization (free drug partitioning between plasma, brain, and CSF) show consistent results, the two preclinical assays are effective in prediction of human CNS penetration with high confidence. When there is no human P-gp liability and good CNS penetration in rats (equilibrium of free drug partitioning between plasma, brain, and CSF), good CNS penetration in humans is predicted. More importantly, for

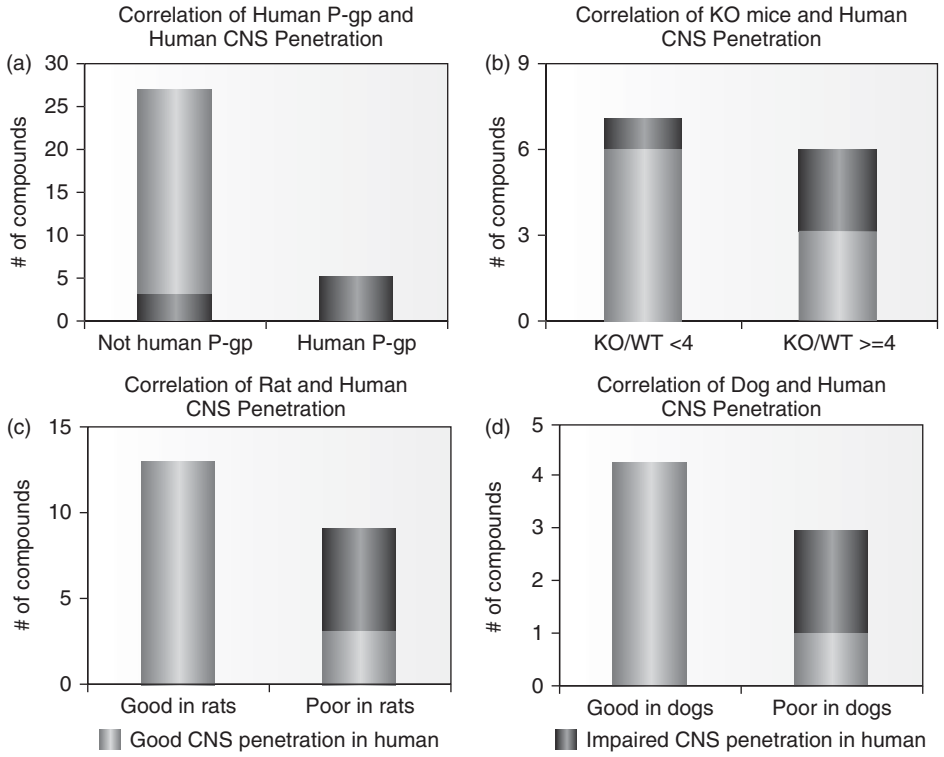

Figure 17.7 Predictability of preclinical assays to human CNS penetration, assessed by correlating results from human P-gp (MDR1/MDCK), P-gp KO mice model, rat neuroPK, and dog CSF model, respectively, to outcome of human CNS penetration observed in clinical trials by either human CSF exposure or receptor occupancy by PET. (a) Correlation of human P-gp and human CNS penetration, (b) correlation of KO mice and human CNS penetration, (c) correlation of rat and human CNS penetration, and (d) correlation of dog and human CNS penetration.

these compounds, the free drug exposure in plasma reflects the CNS target exposure in human. On the other hand, when there is human P-gp liability and impaired CNS penetration in rats (impaired drug partitioning between plasma, brain, and CSF), poor CNS penetration in humans is predicted. For these compounds, the free drug exposure in plasma will overestimate the CNS target exposure. In addition, the results of this retrospective analysis clearly underscore the need for further research in areas, such as transporters and brain PBPK, to better understand the physiological and molecular mechanisms influencing brain penetration and to predict human CNS penetration for compounds that exhibit less than favorable CNS penetration properties.

17.5 CONCLUSIONS

Integrated *in silico, in vitro*, and *in vivo* approaches have been developed to predict brain penetration of drug candidates. A BBB screening strategy is shown in Fig. 17.8

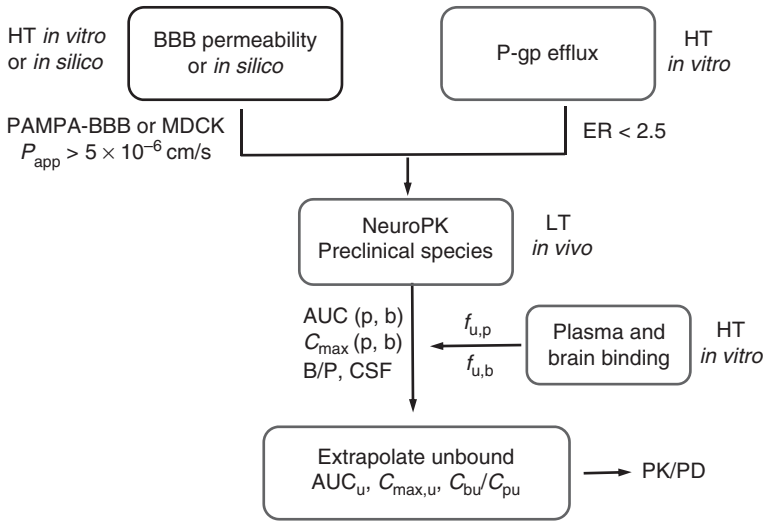

Figure 17.8 BBB screening paradigm in CNS drug discovery. *Source*: Modified from Ref. 3.

as an example to evaluate and predict brain exposure of drug discovery compounds. The key take-home messages and learning about BBB are summarized as follows:

- Use $K_{p,uu}$ (C_{bu}/C_{pu}) to evaluate the extent of brain penetration for drug candidates. Total brain/plasma ratio (i.e., B/P) is mostly due to nonspecific binding and does not reflect the potential of compounds to cross the BBB.
- $K_{p,uu}$ is usually preserved across species for nontransporter substrates, which enable prediction of human free brain drug concentration using unbound human plasma concentration.
- For compounds that exhibit distribution equilibrium in preclinical animals and have good passive permeability and no transporter efflux *in vitro*, free drug concentration in the plasma is a good surrogate for free drug concentration in the brain for humans. CSF measurement does not add extra values for the "well-behaved" (group I) compounds.
- CSF is a valuable surrogate for free drug concentration in the brain. However, for efflux transporter substrates, CSF tends to overestimate free drug concentration in the brain.
- Fraction unbound in brain tissue is useful to calculate free drug concentration in the brain. However, it will not affect the unbound brain concentration for orally administrated drugs. Fraction unbound should not be optimized through structural modification or be used to rank order compounds.
- P-gp and BCRP are two major efflux transporters in the BBB and they work synergistically to eliminate compounds from the brain. Substrates of efflux transporters demonstrate significant brain impairment. Rodent-based neuroPK studies tend to overestimate efflux activity of human BBB, and monkey is a better preclinical model to predict CNS penetration for human.

- Drug–drug interaction due to inhibition of P-gp or BCRP at the BBB is unlikely at clinical relevant doses, owing to the relatively low free drug concentration compared with K_i for most inhibitors.

17.6 FUTURE PROSPECTS

Our understanding of the BBB has advanced significantly over the years. Strategies and methodologies continue to evolve to help us discover new medicines to treat CNS diseases. We expect to see more imaging techniques applied to early drug discovery programs to guide in-depth understanding of drug–target interactions and mechanisms of actions. The BBB transporter field will continue to expand and the resulting data will be incorporated into building PK/PD models and developing predictive tools. Uptake transporters and carriers will be utilized effectively to enhance influx of drugs into brain. Novel brain delivery technologies will advance to bring impermeable compounds into the brain, such as innovations targeting transporters [87] and nanoparticles [88]. Biologics will be delivered to the brain and be available to treat brain diseases. An example of the current approaches is to use molecular Trojan horses to deliver biologics to the brain [89,90]. More BBB PBPK models will be developed and widely applied to accurately predict brain PK.

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ABBREVIATIONS

ABC	ATP-Binding Cassette
AUC	Area Under the Curve
AUC_b	Brain Area Under the Curve
$AUC_{b,u}$	Unbound Brain Area Under the Curve
AUC_p	Plasma Area Under the Curve
$AUC_{p,u}$	Unbound Plasma Area Under the Curve
AUC_u	Unbound Area Under the Curve
BBB	Blood–Brain Barrier
BCRP	Breast Cancer Resistance Protein
BCSFB	Blood–Cerebrospinal Fluid Barrier
$B/P = K_p$	Brain-to-Plasma Ratio Based on Total Drug
C_{max}	Maximum Concentration
$C_{max,u}$	Unbound Maximum Concentration
$C_{bu}/C_{pu} = K_{p,uu}$	Free Brain-to-Plasma Ratio
CSF	Cerebrospinal Fluid
CNS	Central Nervous System
CP	Choroid Plexus

f_u	Fraction Unbound
$f_{u,plasma} = f_{u,p}$	Fraction Unbound of Plasma
$f_{u,brain} = f_{u,b}$	Fraction Unbound of Brain Tissue
ICF	Intracellular Fluid
ISF	Interstitial Fluid
K_p = B/P	Brain-to-Plasma Ratio Based on Total Drug
$K_{puu} = C_{bu}/C_{pu}$	Unbound Brain-to-Plasma Ratio
MDCK	Epithelial Madin-Darby canine kidney cell line
MDR	Multidrug Resistance Protein
MPO	Multiparameter Optimization
MRI	Magnetic Resonance Imaging
MRP	Multidrug-Resistance-Associated Protein
NeuroPK	Neuropharmacokinetics
PAMPA	Parallel Artificial Membrane Permeability Assay
PD	Pharmacodynamics
PET	Positron Emission Tomography
PK	Pharmacokinetics
SPECT	Single Photon Emission Computed Tomography
V_u	Unbound Volume of Brain

REFERENCES

1. Hammarlund-Udenaes M, Friden M, Syvanen S, Gupta A, *et al*. On the rate and extent of drug delivery to the brain. Pharm Res 2008;25(8):1737–1750.
2. Smith DA, Di L, Kerns EH, *et al*. The effect of plasma protein binding on in vivo efficacy: misconceptions in drug discovery. Nat Rev Drug Discov 2010;9:929–939.
3. Di L, Kerns EH, Carter GT, *et al*. Strategies to assess blood-brain barrier penetration. Expert Opin Drug Discov 2008;3(6):677–687.
4. Liu X, Chen C. Strategies to optimize brain penetration in drug discovery. Curr Opin Drug Discov Devel 2005;8(4):505–512.
5. Maurer TS, DeBartolo DB, Tess DA, Scott DO, *et al*. Relationship between exposure and nonspecific binding of thirty-three central nervous system drugs in mice. Drug Metab Dispos 2005;33(1):175–181.
6. Doran A, Obach RS, Smith BJ, *et al*. The impact of P-glycoprotein on the disposition of drugs targeted for indications of the central nervous system: Evaluation using the MDR1A/1B knockout mouse model. Drug Metab Dispos 2005;33(1):165–174.
7. Hammarlund-Udenaes M, Bredberg U, Friden M, *et al*. Methodologies to assess brain drug delivery in lead optimization. Curr Top Med Chem 2009;9(2):148–162.
8. Liu X, Van Natta K, Yeo H, *et al*. Unbound drug concentration in brain homogenate and cerebral spinal fluid at steady state as a surrogate for unbound concentration in brain interstitial fluid. Drug Metab Dispos 2009;37(4):787–793.
9. de Lange ECM, Danhof M. Considerations in the use of cerebrospinal fluid pharmacokinetics to predict brain target concentrations in the clinical setting: implications of the barriers between blood and brain. Clin Pharmacokinet 2002;41(10):691–703.
10. Lin JH. CSF as a surrogate for assessing CNS exposure: an industrial perspective. Curr Drug Metab 2008;9(1):46–59.
11. Kohn MI, Radiology 1991. Vol. 178, p 115–122.

12. Shen DD, Artru AA, Adkison KK, *et al*. Principles and applicability of CSF sampling for the assessment of CNS drug delivery and pharmacodynamics. Adv Drug Deliv Rev 2004;56(12):1825–1857.
13. de Lange ECM. Potential role of ABC transporters as a detoxification system at the blood-CSF barrier. Adv Drug Deliv Rev 2004;56(12):1793–1809.
14. de Lange ECM, *et al*. Methodological issues in microdialysis sampling for pharmacokinetic studies. Adv Drug Deliv Rev 2000;45(2–3):125–148.
15. Verbeeck RK. Blood microdialysis in pharmacokinetic and drug metabolism studies. Adv Drug Deliv Rev 2000;45(2–3):217–228.
16. Walker MC, Tong X, Perry H, *et al*. Comparison of serum, cerebrospinal fluid and brain extracellular fluid pharmacokinetics of lamotrigine. Br J Pharmacol 2000;130(2):242–248.
17. Scheyer RD, During MJ, Hochholzer JM, *et al*. Phenytoin concentrations in the human brain: An in vivo microdialysis study. Epilepsy Res 1994;18(3):227–232.
18. Karson CN, Newton JE, Mohanakrishnan P, *et al*. Fluoxetine and trifluoperazine in human brain: a 19F-nuclear magnetic resonance spectroscopy study. Psychiatry Res 1992;45(2):95–104.
19. Sasongko L, Link JM, Muzi M, *et al*. Imaging P-glycoprotein transport activity at the human blood-brain barrier with positron emission tomography. Clin Pharmacol Ther 2005;77(6):503–514.
20. Hendrikse NH, Schinkel A H, Fluks E, *et al*. Complete in vivo reversal of P-glycoprotein pump function in the blood-brain barrier visualized with positron emission tomography. Br J Pharmacol 1998;124(7):1413–1418.
21. Wagner CC, Simpson M, Zeitlinger M, *et al*. Combined accelerator mass spectrometry-positron emission tomography human microdose study with 14 C- and 11 C-labelled verapamil. Clin Pharmacokinet 2011;50(2):111–120.
22. Syvaenen S, Lindhe O, Palner M, *et al*. Species differences in blood-brain barrier transport of three positron emission tomography radioligands with emphasis on P-glycoprotein transport. Drug Metab Dispos 2009;37(3):635–643.
23. Larobina M, Brunetti A, Salvatore M, *et al*. Small animal PET. A review of commercially available imaging systems. Curr Med Imaging Rev 2006;2:187–192.
24. Smith QR, David AD. In situ brain perfusion technique. Methods Mol Med 2003;89: 209–218.
25. Takasato Y, Rapoport SI, Smith QR, *et al*. An in situ brain perfusion technique to study cerebrovascular transport in the rat. Am J Physiol 1984;247(3 Pt 2):H484–H493.
26. Summerfield SG, Read K, Begley DJ, *et al*. Central nervous system drug disposition: the relationship between in situ brain permeability and brain free fraction. J Pharmacol Exp Ther 2007;322(1):205–213.
27. Kansy M, Senner F, Gubernator K, *et al*. Physicochemical high throughput screening: parallel artificial membrane permeation assay in the description of passive absorption processes. J Med Chem 1998;41(7):1007–1010.
28. Wohnsland F, Faller B. High-throughput permeability pH profile and high-throughput alkane/water log P with artificial membranes. J Med Chem 2001;44(6):923–930.
29. Avdeef A. The rise of PAMPA. Expert Opin Drug Metab Toxicol 2005;1(2):325–342.
30. Di L, Kerns EH, Fan K, *et al*. High throughput artificial membrane permeability assay for blood-brain barrier. Eur J Med Chem 2003;38(3):223–232.
31. Tsinman O, Tsinman K, Sun N, *et al*. Physicochemical selectivity of the BBB microenvironment governing passive diffusion-matching with a porcine brain lipid extract artificial membrane permeability model. Pharm Res 2011;28(2):337–363.
32. Di L, Kerns EH, Bezar IF, *et al*. Comparison of blood-brain barrier permeability assays: in situ brain perfusion, MDR1-MDCKII and PAMPA-BBB. J Pharm Sci 2009;98(6):1980–1991.

33. Tsinman O, Tsinman K, Sun N, *et al*. Physicochemical selectivity of the BBB microenvironment governing passive diffusion—matching with a porcine brain lipid extract artificial membrane permeability model. Pharm Res 2010;28(2):337–363.
34. Garberg P, Ball M, Borg N, *et al*. In vitro models for the blood-brain barrier. Toxicol In vitro 2005;19(3):299–334.
35. Doan KMM, Humphreys JE, Webster LO, *et al*. Passive permeability and P-glycoprotein-mediated efflux differentiate central nervous system (CNS) and non-CNS marketed drugs. J Pharmacol Exp Ther 2002;303(3):1029–1037.
36. Bachmeier CJ, Trickler WJ, Miller DW, *et al*. Comparison of drug efflux transport kinetics in various blood-brain barrier models. Drug Metab Dispos 2006;34(6):998–1003.
37. Di L, Whitney-Pickett C, Umland JP, *et al*. Development of a new permeability assay using low-efflux MDCKII cells. J Pharm Sci 2011;100(11):4974–4985.
38. Feng B, Mills JB, Davidson RE, *et al*. In vitro P-glycoprotein assays to predict the in vivo interactions of P-glycoprotein with drugs in the central nervous system. Drug Metab Dispos 2008;36(2):268–275.
39. Liu X, Tu M, Kelly RS, *et al*. Development of a computational approach to predict blood-brain barrier permeability. Drug Metab Dispos 2004;32(1):132–139.
40. Goodwin JT, Clark DE. In silico predictions of blood-brain barrier penetration: considerations to "Keep in Mind". J Pharmacol Exp Ther 2005;315(2):477–483.
41. Friden M, Gupta A, Antonsson M, *et al*. In vitro methods for estimating unbound drug concentrations in the brain interstitial and intracellular fluids. Drug Metab Dispos 2007;35(9):1711–1719.
42. Liu X, Smith BJ, Chen C, *et al*. Evaluation of cerebrospinal fluid concentration and plasma free concentration as a surrogate measurement for brain free concentration. Drug Metab Dispos 2006;34(9):1443–1447.
43. Kakee A, Terasaki T, Sugiyama Y, *et al*. Brain efflux index as a novel method of analyzing efflux transport at the blood-brain barrier. J Pharmacol Exp Ther 1996;277:1550–1559.
44. Becker S, Liu X. Evaluation of the utility of brain slice methods to study brain penetration. Drug Metab Dispos 2006;34(5):855–861.
45. Friden M, Ducrozet F, Middleton B, *et al*. Development of a high-throughput brain slice method for studying drug distribution in the central nervous system. Drug Metab Dispos 2009;37(6):1226–1233.
46. Kalvass JC, Maurer TS. Influence of nonspecific brain and plasma binding on CNS exposure: implications for rational drug discovery. Biopharm Drug Dispos 2002;23(8):327–338.
47. Mano Y, Higuchi S, Kamimura H, *et al*. Investigation of the high partition of YM992, a novel antidepressant, in rat brain - in vitro and in vivo evidence for the high binding in brain and the high permeability at the BBB. Biopharm Drug Dispos 2002;23(9):351–360.
48. Summerfield SG, Stevens AJ, Cutler L, *et al*. Improving the in vitro prediction of in vivo central nervous system penetration: integrating permeability, P-glycoprotein efflux, and free fractions in blood and brain. J Pharmacol Exp Ther 2006;316(3):1282–1290.
49. Wan H, Rehngren M, Giordanetto F, *et al*. High-throughput screening of drug-brain tissue binding and in silico prediction for assessment of central nervous system drug delivery. J Med Chem 2007;50(19):4606–4615.
50. Friden M, Bergstrom F, Wan H, *et al*. Measurement of unbound drug exposure in brain: modelling of pH partitioning explains diverging results between the brain slice and brain homogenate methods. Drug Metab Dispos 2011;39(3):353–362.
51. Available at http://htdialysis.com/.
52. Available at http://www.piercenet.com/Objects/View.cfm?type=ProductFamily&id=CF207C7A-34DA-4A16-BA83-5BB2F5D8F6AC.
53. Di L, Umland JP, Chang GC, *et al*. Species independence in brain tissue binding using brain homogenates. Drug Metab Dispos 2011;39(7):1270–1277.

54. Hong L, Jiang W, Pan H, *et al*. Brain regional pharmacokinetics of p-aminosalicylic acid and its N-acetylated metabolite: effectiveness in chelating brain manganese. Drug Metab Dispos 2011;39(10):1904–1909.
55. Giacomini KM, Huang S-M, Tweedie DJ, *et al*. Membrane transporters in drug development. Nat Rev Drug Discov 2010;9(3):215–236.
56. Warren MS, Zerangue N, Woodford K, *et al*. Comparative gene expression profiles of ABC transporters in brain microvessel endothelial cells and brain in five species including human. Pharmacol Res 2009;59(6):404–413.
57. Uchida Y, Ohtsuki S, Katsukura Y, *et al*. Quantitative targeted absolute proteomics of human blood-brain barrier transporters and receptors. J Neurochem 2011;117(2):333–345.
58. Ito K, Uchida Y, Ohtsuki S, *et al*. Quantitative membrane protein expression at the blood–brain barrier of adult and younger cynomolgus monkeys. J Pharm Sci 2011;100(9):3939–3950.
59. Zhou L, Schmidt K, Nelson FR, *et al*. The effect of breast cancer resistance protein and P-glycoprotein on the brain penetration of flavopiridol, imatinib mesylate (Gleevec), prazosin, and 2-methoxy-3-(4-(2-(5-methyl-2-phenyloxazol-4-yl)ethoxy)phenyl)propanoic Acid (PF-407288) in mice. Drug Metab Dispos 2009;37:946–955.
60. Polli JW, Olson KL, Chism JP, *et al*. An unexpected synergist role of P-glycoprotein and breast cancer resistance protein on the central nervous system penetration of the tyrosine kinase inhibitor lapatinib (N-\{3-Chloro-4-[(3-fluorobenzyl)oxy]phenyl\}-6-[5-(\{[2-(methylsulfonyl)ethyl]amino\}methyl)-2-furyl]-4-quinazolinamine; GW572016). Drug Metab Dispos 2009;37:439–442.
61. Zamek-Gliszczynski MJ, Kalvass JC, Pollack GM, *et al*. Relationship between Drug/Metabolite Exposure and Impairment of Excretory Transport Function. In 2009; 37:386–390.
62. Kodaira H, Kusuhara H, Ushiki J, *et al*. Kinetic Analysis of the Cooperation of P-Glycoprotein (P-gp/Abcb1) and Breast Cancer Resistance Protein (Bcrp/Abcg2) in Limiting the Brain and Testis Penetration of Erlotinib, Flavopiridol, and Mitoxantrone. In 2010; Vol. 333, pp 788–796.
63. Rautio J. Prodrugs and targeted delivery: towards better ADME properties. Wiley-VCH Verlag GmbH & Co; 2011. Weinheim, Germany.
64. Cheng ZZ. Blood-brain barrier transporters: friend or foe for CNS drug discovery and development. BioMedical Transporter 2011.
65. Hochman J, Mei Q, Yamazaki M, *et al*. Role of mechanistic transport studies in lead optimization. Volume 4, Optimizing the "Drug-Like" properties of leads in drug discovery (Biotechnology: pharmaceutical aspects). 2006:25–47.
66. Bartels A, Willemsen A, Kortekaas R, *et al*. Decreased blood brain barrier P-glycoprotein function in the progression of Parkinson's disease, PSP and MSA. J Neural Transm 2008;115(7):1001–1009.
67. Vogelgesang S, Cascorbi I, Kroemer H, *et al*. Deposition of amyloid is inversely correlated with the expression of P-glycoprotein—implications on the possibility of prevention of Alzheimer's disease. Acta Neuropathol 2001;102:545.
68. Vogelgesang S, Glatzel M, Walker LC, *et al*. Cerebrovascular P-glycoprotein expression is decreased in Creutzfeldt-Jakob disease. Acta Neuropathol 2006;111(5):436–443.
69. de Klerk OL, Willemsen ATM, Bosker FJ, *et al*. Regional increase in P-glycoprotein function in the blood-brain barrier of patients with chronic schizophrenia: A PET study with [11 C]verapamil as a probe for P-glycoprotein function. Psychiatry Res 2010;183(2):151–156.
70. Toornvliet R, van Berckel BNM, Luurtsema G, *et al*. Effect of age on functional P-glycoprotein in the blood-brain barrier measured by use of (R)-[11 C]verapamil and positron emission tomography. Clin Pharmacol Ther 2006;79(6):540–548.

71. Bartels AL, Kortekaas R, Bart J, *et al*. Blood brain barrier P-glycoprotein function decreases in specific brain regions with aging: a possible role in progressive neurodegeneration. Neurobiol Aging 2009;30(11):1818–1824.
72. Ajay, Bemis GW, Murcko MA, *et al*. Designing libraries with CNS activity. J Med Chem 1999;42(24):4942–4951.
73. Pardridge WM. Log(BB), PS products and in silico models of drug brain penetration. Drug Discov Today 2004;9(9):392–393.
74. Bickel U. How to measure drug transport across the blood-brain barrier. NeuroRx 2005;2(1):15–26.
75. Clark DE. In silico prediction of blood-brain barrier permeation. Drug Discov Today 2003;8(20):927–933.
76. Clark DE. Computational prediction of blood-brain barrier permeation. Annu Rep Med Chem 2005;40:403–415.
77. Didziapetris R, Japertas P, Avdeef A, *et al*. Classification analysis of P-glycoprotein substrate specificity. J Drug Target 2003;11(7):391–406.
78. Wager TT, Chandrasekaran RY, Hou X, *et al*. Defining desirable central nervous system drug space through the alignment of molecular properties, in vitro ADME, and safety attributes. ACS Chem Neurosci 2010;1(6):420–434.
79. Wager TT, Hou X, Verhoest PR, *et al*. Moving beyond rules: the development of a central nervous system multiparameter optimization (CNS MPO) approach to enable alignment of druglike properties. ACS Chem Neurosci 2010;1(6):435–449.
80. Wager TT, Villalobos A, Verhoest PR, *et al*. Strategies to optimize the brain availability of central nervous system drug candidates. Expert Opin Drug Discov 2011;6:371–381.
81. Liu X, Smith BJ, Chen C, *et al*. Use of a physiologically based pharmacokinetic model to study the time to reach brain equilibrium: an experimental analysis of the role of blood-brain barrier permeability, plasma protein binding, and brain tissue binding. J Pharmacol Exp Ther 2005;313(3):1254–1262.
82. http://www.simcyp.com/.
83. Uchida Y, *et al*. Blood-brain barrier (BBB) pharmacoproteomics (PPx): reconstruction of in vivo brain distribution of 11 P-glycoprotein substrates based on the BBB transporter protein concentration, in vitro intrinsic transport activity, and unbound fraction in plasma and brain in mice. J Pharmaco Expert Ther 2011;339(2):579–588.
84. Hosea NA, Collard WT, Cole S, *et al*. Prediction of human pharmacokinetics from preclinical information: comparative accuracy of quantitative prediction approaches. J Clin Pharmacol 2009;49(5):513–533.
85. Obach RS. Predicting clearance in humans from in vitro data. Curr Top Med Chem 2011;11(4):334–339.
86. Venkatakrishnan K, Tseng E, Nelson FR, *et al*. Central nervous system pharmacokinetics of the Mdr1 P-glycoprotein substrate CP-615,003: intersite differences and implications for human receptor occupancy projections from cerebrospinal fluid exposures. Drug Metab Dispos 2007;35(8):1341–1349.
87. van Weperen W, Gaillard P. Enhanced blood to brain drug delivery. Innovations Pharm Technol 2010;35:54–57.
88. Koffie RM, Farrar CT, Saidi L-J, *et al*. Nanoparticles enhance brain delivery of blood-brain barrier-impermeable probes for in vivo optical and magnetic resonance imaging. Proc Natl Acad Sci U S A 2011;108(46):18837–18842. S18837/18831-S18837/18836.
89. Pardridge WM. Molecular Trojan horses for blood-brain barrier drug delivery. Curr Opin Pharmacol 2006;6(5):494–500.
90. Pardridge WM. Molecular Trojan horses for blood-brain barrier drug delivery. Discov Med 2006;6(34):139–143.