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Review Article

Methods to assess drug permeability across the blood–brain barrier

Joseph A. Nicolazzo, Susan A. Charman and William N. Charman

Abstract

Much research has focussed on the development of novel therapeutic agents to target various central nervous system disorders, however less attention has been given to determining the potential of such agents to permeate the blood–brain barrier (BBB), a factor that will ultimately govern the effectiveness of these agents in man. In order to assess the potential for novel compounds to permeate the BBB, various in-vitro, in-vivo and in-silico methods may be employed. Although in-vitro models (such as primary cell culture and immortalized cell lines) are useful as a screening method and can appropriately rank compounds in order of BBB permeability, they often correlate poorly to in-vivo brain uptake due to down-regulation of some BBB-specific transporters. In-vivo models (such as the internal carotid artery single injection or perfusion, intravenous bolus injection, brain efflux index and intracerebral microdialysis) provide more accurate information regarding brain uptake, and these can be complemented with novel imaging techniques (such as magnetic resonance imaging and positron emission tomography), although such methods are not suited to high-throughput permeability assessment. This paper reviews current methods used for assessing BBB permeability and highlights the particular advantages and disadvantages associated with each method, with a particular focus on methods suitable for moderate- to high-throughput screening.

Introduction

The blood–brain barrier (BBB), formed by the endothelial cells lining the cerebral microvessels, has a pivotal role in protecting the brain parenchyma from blood-borne agents. While this protective function is essential for normal physiological function within the brain microenvironment, it results in a significant hindrance to the entry of drugs into the central nervous system (CNS). Unlike vascular endothelial cells in other regions of the body, the endothelial cells forming the BBB are characterized by tight intercellular junctions, minimal pinocytotic activity and the absence of fenestrations (Reese & Karnovsky 1967; Brightman & Reese 1969), thus limiting drug penetration into the brain parenchyma. In addition, the BBB expresses various efflux transporters, such as P-glycoprotein (P-gp) (Cordon-Cardo et al 1989), organic anion transporting polypeptide (Gao et al 1999), and breast cancer resistance protein (Eisenblätter & Galla 2002). These efflux proteins actively pump compounds out of the endothelial cells back into the blood (de Lange 2004), resulting in reduced CNS exposure. An additional barrier to the transport of drugs into the CNS is the presence of drug-metabolizing enzymes within the endothelial cells forming the BBB (Ghersi-Egea et al 1995).

Most of these barrier properties characteristic to brain capillary endothelial cells are partly induced and maintained by the close association of astrocytic foot processes with the endothelium (Bradbury 1985; Goldstein & Betz 1986; Bradbury 1993). These astrocytic foot processes invest close to 99% of the abluminal surface area of the capillary endothelium (Pardridge 1998). A schematic representation of the brain capillary endothelium and the close association with these astrocytic foot processes is shown in Figure 1.

Although most compounds must permeate the BBB in order to gain access to the brain, it is possible for compounds to circumvent the BBB and still reach the brain parenchyma. One such mechanism is the nose-to-brain route, where a compound may be directly transported to the brain via an olfactory pathway following absorption across the nasal mucosa (Illum 2004). Alternatively, compounds may permeate from the blood into the cerebrospinal fluid

Centre for Drug Candidate Optimisation, Monash University, 381 Royal Parade, Parkville, Victoria 3052, Australia

Joseph A. Nicolazzo, Susan A. Charman, William N. Charman

Correspondence: J. A. Nicolazzo, Centre for Drug Candidate Optimisation, Monash University, 381 Royal Parade, Parkville, Victoria 3052, Australia. E-mail: joseph.nicolazzo@vcp.monash.edu.au

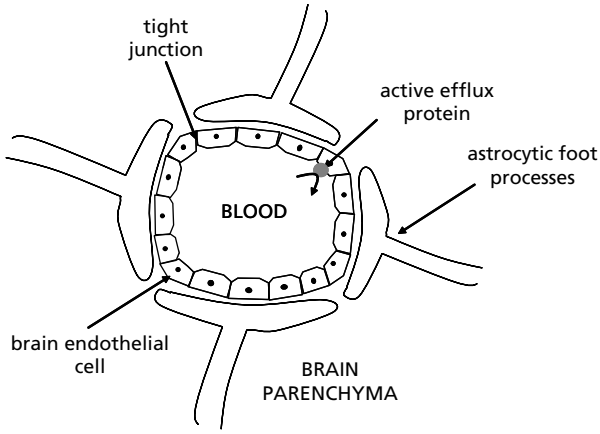

Figure 1 Schematic diagram of the blood–brain barrier, comprising the brain endothelial cell ensheathed by astrocytic foot processes.

(CSF), and subsequently permeate into the brain interstitial fluid. However, transport into the CSF is controlled by the choroid plexus (the epithelial barrier separating the blood from the CSF), and the capillaries perfusing the choroid plexus are quite porous, allowing normal access of compounds into the CSF (Brightman 1977). Therefore, transport across the choroid plexus is not an accurate measure of transport across the BBB, as these barriers are anatomically different. Even if a compound enters the CSF, its availability in the brain interstitial fluid (ISF) should not be assumed, since a functional barrier between these compartments exists, resulting from the difference between the bulk flow properties of CSF through the CSF flow tracks and diffusional flow rates in the brain parenchyma (Pardridge 1995). A schematic representation of the CSF and brain ISF compartments is shown in Figure 2. These alternative routes (nose-to-brain and CSF-to-brain), though important, are not discussed in this review, given that the focus of this review is on the BBB as a hindrance to effective CNS drug delivery.

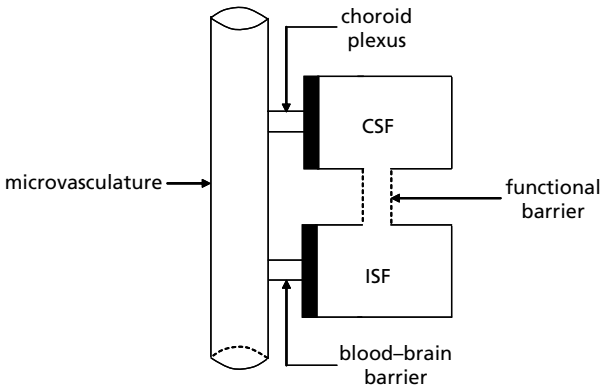

Figure 2 Schematic representation of the separation between the brain interstitial fluid (ISF) and cerebrospinal fluid (CSF). The endothelial barriers separating the ISF from the blood and the CSF from the blood are the blood–brain barrier and choroid plexus, respectively. Because of differences in the bulk flow properties of CSF and diffusional flow rates in brain parenchyma, a functional barrier between the CSF and ISF exists.

Over 98% of compounds intended for therapeutic use in the CNS never reach the market because of their inherent inability to cross the BBB (Pardridge 2001, 2002). Within the drug discovery and development process, it is commonplace to design chemical entities that act on particular CNS targets using in-vitro assays, without considering the physicochemical properties of such chemical entities and the consequent effect this may have on BBB permeability. Therefore, it is critically important to determine the BBB permeability of CNS drug candidates early in drug discovery, so that poor CNS candidates can be excluded or structurally modified, and promising CNS candidates can be accelerated through the development process.

There are various models that are used to assess the permeability of drugs across the BBB, including in-vitro, in-vivo and, more recently, in-silico methods. A summary of these methods, together with their inherent advantages and disadvantages is presented in this review, with a particular focus on methods that are suitable for moderate- to high-throughput screening of potential CNS drug candidates.

In-vitro models

The advantages associated with any in-vitro BBB model include lower compound requirement, the ability to assay compound directly in physiological buffer, greater throughput relative to in-vivo models, ability to assess transport mechanisms, the identification of early signs of cell toxicity, and, generally, lower cost (Lundquist & Renftel 2002). However, in order to appropriately mimic the BBB in-vivo there are some basic characteristics that an in-vitro model must possess. These are summarized in Table 1 (adapted from Reichel et al 2003). The in-vitro model that is chosen should possess as many of these characteristics as possible, while at the same time remaining practical and feasible for moderate- to high-throughput screening.

Isolated brain capillaries

It has been possible to isolate brain capillaries from various animal sources, however these are not well suited for permeability screening purposes. This is because of the inability to access the luminal surface of the isolated microvessels and, consequently, only drug loss from the abluminal (brain) compartment can be monitored.

Primary or low passage brain capillary endothelial cell cultures

Primary or low passage brain capillary endothelial cell cultures provide the closest phenotypic resemblance to the in-vivo BBB phenotype (Lundquist & Renftel 2002), although some features, such as BBB transporters and enzymes, can be down-regulated when the endothelial cells are removed from the brain and grown in culture (Pardridge 2004a). These capillary endothelial cells can be obtained from bovine, porcine, rat or human sources, although most researchers use bovine or porcine endothelial cells for the purposes of assessing drug transport because of the availability of such cells.

Table 1 Basic characteristics and requirements of in-vitro blood–brain barrier (BBB) models (modified from Reichel et al 2003)

Characteristic	Specific requirements for an acceptable in-vitro BBB model
Restricted paracellular pathway	TEER $\geq 2\,k\Omega\,cm^{2}$[a] Low paracellular permeability ($P_{sucrose}$ ~$3–12 \times 10^{-8}$ cm s^{-1})
Brain capillary endothelial cell characteristics	Morphology, endothelial cell and BBB markers (gamma-glutamyl transpeptidase, alkaline phosphatase), enzyme expression (monoamine oxidase, angiotensin converting enzyme)
Functional expression of BBB-specific transport mechanisms	Nutrient transfer (glucose transporter, L-amino acid transporter), efflux pumps (P-gp, OATP, BCRP), receptors (transferrin, insulin), low leucocyte adherence
In-vivo-like modulation	Permeability altered by bradykinin, interleukins, glial factors, dexamethasone
Practicality	Availability, convenience, predictability and reproducibility

[a]Although a transendothelial electrical resistance (TEER) of 2 kΩ cm^2 is considered ideal, it has been repeatedly demonstrated that a minimal TEER of more than 150–200 Ω cm^2 is sufficient for assessing drug permeability through the BBB in-vitro (Reichel et al 2003). $P_{sucrose}$, permeability coefficient of sucrose through the BBB; P-gp, P-glycoprotein; OATP, organic anion transporting polypeptide; BCRP, breast cancer resistance protein.

Bovine brain endothelial cell culture

The original bovine brain endothelial cell (BBEC) culture model was developed by Audus & Borchardt (1986), and has been used and characterized extensively (Pardridge et al 1990; Audus et al 1996; Rochat et al 1999; Bachmeier et al 2005). To obtain BBECs, the grey matter of bovine brains is isolated and treated either mechanically or enzymatically to yield around 100 million viable cells (from the grey matter of two bovine brains) (Gumbleton & Audus 2001). Once isolated, the cells can be stored at −80°C for up to 2 months (Audus et al 1996), thus avoiding the necessity to isolate cells each time an experiment is to be performed. The BBECs grow as primary cultures on standard plates or inserts (Transwell), which must first be treated with rat tail collagen to improve plating efficiency (Audus et al 1996). Once cells have reached confluence, the permeability of compounds can be assessed in both the apical-to-basolateral and basolateral-to-apical directions, and general mechanisms of transport can be elucidated. Due to the number of cells available, such an approach is well suited to high-throughput compound screening.

In order for an in-vitro model to be considered representative of the in-vivo situation, results obtained from the in-vitro model should be compared with those obtained in-vivo. Pardridge et al (1990) found an acceptable in-vitro–in-vivo correlation using BBECs ($r^2 = 0.7225$); however, it was found that the in-vitro permeability was about 150-fold greater than in-vivo permeability. This was attributed to the loss of expression of BBB-specific proteins and, consequently, the absence of various efflux mechanisms. In the same study, it was found that pre-treatment of the endothelial monolayers with astrocyte-conditioned medium resulted in a 30% reduction in sucrose transport through the BBB as a result of the increase in number and size of intercellular tight junctions induced by the astrocyte-conditioned medium (Tao-Cheng et al 1987; Pardridge et al 1990). Others have also shown an improvement in the barrier properties of BBEC cultures with the use of cAMP stimulants (Deli et al 1995), vasoactive peptides (Guillot & Audus 1991), and adrenergic agonists (Borges et al 1994).

The most common approach to improve the barrier properties of BBEC cultures is to co-culture the endothelial cells with primary astrocytes isolated from neonatal rats. This approach has been shown to maintain the characteristics of the BBB without the use of stimulants, in addition to up-regulating P-gp function (Gaillard et al 2000) and significantly increasing transendothelial electrical resistance (TEER) values (416 Ω cm^2 for BBECs compared with 661 Ω cm^2 for BBECs co-cultured with astrocytes) (Dehouck et al 1990). Since astrocytic foot processes invest more than 99% of the cerebral vasculature in-vivo and many features of the BBB in-vivo are induced by astrocytes (Pardridge 1998), it is not surprising that a more restrictive in-vitro BBB model results from the co-culturing of BBECs with astrocytes.

Once isolated from neonatal rat brains, astrocytes can either be grown on the underside of the Transwell filter (physical contact with BBECs) (Dehouck et al 1992) or on the bottom of multi-well plates (no contact with BBECs) (Dehouck et al 1995). This is schematically represented in Figure 3. Some debate still exists as to which method is preferable, as some researchers have shown a better correlation to in-vivo results when astrocytes are grown on the bottom of wells (no contact) (Dehouck et al 1995), whereas others have found more restrictive barrier properties when astrocytes are in contact with the BBECs (Gaillard et al 2001).

The major limitation associated with this co-culturing technique is that in addition to maintaining the growth of BBECs, there is the necessity to grow and culture rat astrocytes. Alternatively, C6 glioma cells may be co-cultured with BBECs, which reduces the need to isolate and culture rat astrocytes, and this approach has been shown to increase the TEER by 75% and reduce sucrose permeability by 50% (Raub 1996). The major disadvantage with the use of C6 glioma cells (or other cancer cells) is that they may result in a tumour-like BBB rather than a healthy BBB (de Boer et al 1999), which may lead to a poor correlation to brain uptake in a healthy individual.

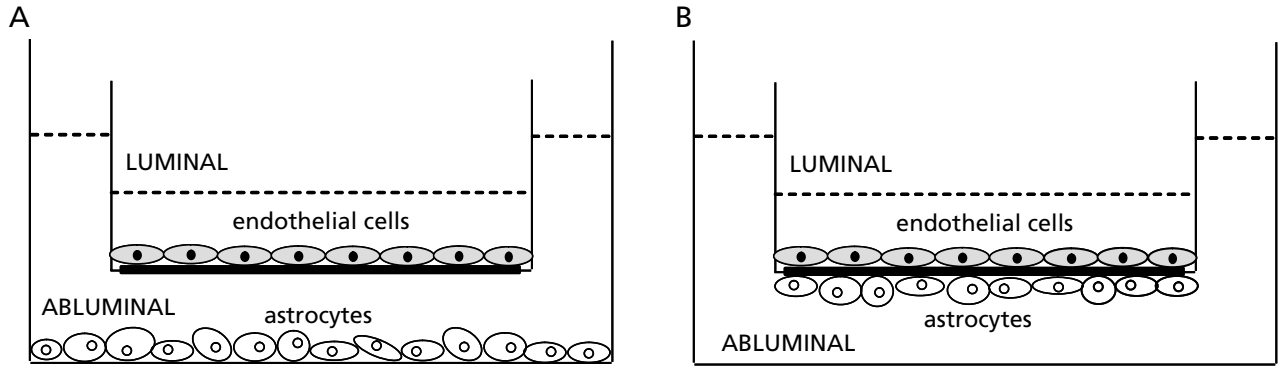

Figure 3 Schematic representation of bovine brain endothelial cells co-cultured with astrocytes using the no-contact method where the astrocytes are grown on the bottom of the multi-well plate (A), and the contact method where the astrocytes are grown on the underside of the Transwell filter (B).

Porcine brain endothelial cell culture

Although most research has focussed on the development and characterization of BBEC cultures as in-vitro models for the BBB, recent studies have shown that porcine brain endothelial cells may also serve as an appropriate model (Franke et al 1999, 2000). Some researchers have also co-cultured porcine brain endothelial cells with astrocytes in order to improve the restrictiveness of the culture system (Kido et al 2002), however further validation, particularly with respect to in-vitro–in-vivo correlations, may be required before this model becomes extensively utilized for the purposes of high-throughput compound screening.

Immortalized brain endothelial cells

Due to the problems associated with harvesting and maintaining primary cell cultures, various immortalized cell lines have been developed, most of which are derived from rats. All of these cell lines have one major disadvantage in that although they do form monolayers, they do not form complete tight junctions, resulting in a 'leaky' barrier (Reichel et al 2003). This has resulted in the generation of a number of immortalized, transformed, transfected and transduced cell lines. Some of the cell lines that have been generated by transfection of primary rat endothelial cells include the RBE4 cell line (Begley et al 1996), RBEC1 cell line (Nagasawa et al 2005), and TR-BBB13 cell line (Tetsuka et al 2003). However, the resulting TEER values of these cell lines are still fairly low, and are therefore not appropriate for BBB permeability screening but more suited to assessing endothelial cell uptake of compounds.

Cells of non-cerebral origin

Because of the insufficient barrier properties of immortalized brain endothelial cell lines, some researchers have focussed on using non-cerebral peripheral epithelial cell lines. One such cell line is the Madin-Darby canine kidney (MDCK) cell line, which is easy to grow and can be transfected with the multidrug resistance gene (MDR1), resulting in the polarized expression of P-gp (Pastan et al 1988). This transfected cell line has been used to assess the effect of P-gp on the permeability of various compounds through the BBB (Dai et al 2003; Polli et al 2003), and a recent collaborative study found that MDR1-transfected MDCK cells were the most representative of in-vivo BBB permeability compared with other in-vitro models, including BBEC/astrocytes, human brain endothelial cells/astrocytes, and Caco-2 cell lines (Garberg et al 2005). MDR1-transfected MDCK cells have also shown high absorptive transport for CNS-positive drugs and low absorptive transport for CNS-negative drugs (Wang et al 2005), and so may be a suitable model for BBB permeation.

Although this cell line has sufficient restrictive paracellular transport, the MDCK epithelial cells are morphologically different from brain endothelial cells, and would also differ with respect to transport properties, metabolism, and growth. The brain capillary endothelial cell is squamous with a large surface area, and so there is a lower cell density per unit surface area of endothelium (<1000 cells mm^{-2}), whereas the kidney cell is cuboidal in shape, resulting in a smaller surface area, and a consequent greater cell density per unit area of membrane (>10 000 cells mm^{-2}) (Gumbleton & Audus 2001). Therefore, MDCK cells produce a relatively higher transverse area of intercellular junction than what is present between brain endothelial cells and, consequently, paracellular transport (which is non-existent in BBB permeability) will be overestimated with this cell line. In addition, while P-gp is one of the most important efflux transporters at the BBB, and transfection of MDCK cells with the MDR1 gene compensates for this, there are also other efflux proteins such as breast cancer resistance protein (Eisenblätter & Galla 2002) and organic anion transporting polypeptide (Gao et al 1999) present in brain capillary endothelial cells, that may also play a role in overall CNS penetration. If using the MDR1-transfected MDCK cell line, one should be mindful not to disregard the potential effects of these other efflux transporters present in the in-vivo BBB.

Caco-2 cells have also been used to estimate BBB permeability; however, as with the MDCK cells, these are epithelial

cells and have different morphological characteristics to brain endothelial cells. A recent study comparing BBEC/astrocyte co-culture and Caco-2 cells with in-vivo studies demonstrated that Caco-2 cells were a poor model for BBB permeation studies (r^2 of 0.4624 and 0.8649 for Caco-2 cells and BBEC/astrocytes, respectively) (Lundquist et al 2002). An alternative cell line that has also been assessed as a model for the BBB is the ECV304 cell line, which is a bladder carcinoma cell with epithelial and endothelial properties. This cell line has also been co-cultured with C6 glioma cells or in C6-conditioned media; however, it was found to have low TEER values (indicative of poor paracellular restrictive properties) and a lack of P-gp expression (Hurst & Fritz 1996; Ramsohoye & Fritz 1998). Consequently, such an in-vitro model has limited applicability for assessing the permeability of compounds across the BBB.

Immobilized artificial membranes

Immobilized artificial membranes are a solid phase model of fluid membranes that have been proposed as an alternative for assessing drug permeability through cell membranes (Stewart & Chan 1998). These membranes, which are used as a chromatographic interface in high-performance liquid chromatography, consist of phosphatidylcholine residues covalently bound to silica propylamine and mimic a membrane lipid bilayer (Pidgeon et al 1995). There has been some work in attempting to correlate immobilized artificial membrane retention to brain penetration, however it is only useful for compounds that permeate the BBB via passive mechanisms. In one study, the brain uptake of 26 drugs (basic, neutral and acidic) appeared to correlate weakly to the immobilized artificial membrane retention factors, although an improvement in regression was observed when the effects of ionization and solute size were taken into account (Salminen et al 1997). While this method may be useful for predicting solute partitioning into membranes, it does not mimic diffusion across a membrane, and can have poor predictive power when brain uptake is affected by plasma protein binding, active transport, active efflux, or metabolism.

In-vivo models

While in-vitro models have many advantages and are useful for moderate- to high-throughput screening, BBB research should not only be performed using an in-vitro method. Rather, it is necessary to correlate observations made using an in-vitro BBB model to in-vivo studies (Pardridge 1999). There are various in-vivo methods that have been used to assess drug uptake into the brain, including the single carotid injection technique, in-situ perfusion technique, intravenous injection technique, brain efflux index, and intracerebral microdialysis. Each of these techniques are useful for the calculation of the BBB permeability–surface area (PS) product or logBB, where BB is the brain to blood ratio at some defined time. The best index of BBB permeability is the BBB PS product, which has units of $\mu L\ min^{-1}\ g^{-1}$ and is a measure of unidirectional clearance from blood to brain across the BBB (Pardridge 2004b). Although not suitable for high-throughput compound screening, various imaging techniques (such as quantitative autoradiography, magnetic resonance imaging, positron emission tomography, and single photon emission computed tomography) may be used to assess the transport properties of the BBB, and are also more useful in the diagnosis of various CNS diseases.

Carotid artery single injection technique (brain uptake index)

The carotid artery single injection technique or brain uptake index (BUI) involves cannulation of the carotid artery, followed by a single injection of compound in physiological buffer (Oldendorf 1970). A small volume of buffered Ringer's solution containing the compound of interest and a radiolabelled diffusible reference compound as an internal standard (such as ^{3}H-water) is rapidly injected (< 0.5 s) into the common carotid artery. The purpose of including the internal standard is to define the amount of injected material that actually distributes to the brain (Bonate 1995). The bolus passes through the brain within 2 s after the single injection, the animal is decapitated 5–15 s after injection, and the brain and injection solution are analysed to calculate the BUI (Pardridge 1995). The assumptions of the BUI are that the reference compound is freely diffusible across the BBB, that the drug does not back-diffuse from brain to blood, and that no metabolism occurs before decapitation (Bonate 1995).

The advantages of the BUI technique are that it is fast and many compounds can be evaluated in a short period of time, which is ideal in the high-throughput setting. The major disadvantage is that the capillary transit time is very short (1 s), and so brain extraction can only occur over a limited time, making it difficult to measure BBB PS products less than $10\ \mu L\ min^{-1}\ g^{-1}$ (Pardridge 1995). As the external arteries are not ligated, the compound may also diffuse throughout the whole body with only 10% of the compound reaching the brain (Bonate 1995).

In-situ perfusion technique

The in-situ perfusion technique is an extension of the carotid artery single injection technique, and was first developed by Takasato et al (1984). This involves a longer experimental period with carotid artery perfusion of the brain, followed by sampling of drug levels within the brain. In this method, the animal (usually a rat) is anaesthetized, and the perfusion catheter is placed in the external carotid artery just distal to the bifurcation of the common carotid artery, as shown schematically in Figure 4. The ipsilateral pterygopalatine, superior thyroid, and occipital arteries are ligated and cut, and the perfusion fluid is infused retrograde down the external carotid artery and up the internal carotid artery toward the brain (Smith 1996). Just prior to commencement of the perfusion, the common carotid artery is ligated to prevent mixing of the perfusion fluid with systemic blood at the carotid bifurcation. Following the perfusion, the animal is decapitated and the compound concentration is determined in order to calculate a BBB PS product. In addition to the compound of interest, a reference compound should be included for the measurement of brain plasma volume, such as radiolabelled sucrose (Taogoshi et al 2005) or inulin (Smith & Allen 2003; Garberg et al 2005).

One of the issues relating to this technique (and all in-vivo techniques for that matter) is that compound that is not within the brain microvasculature may either be within the brain

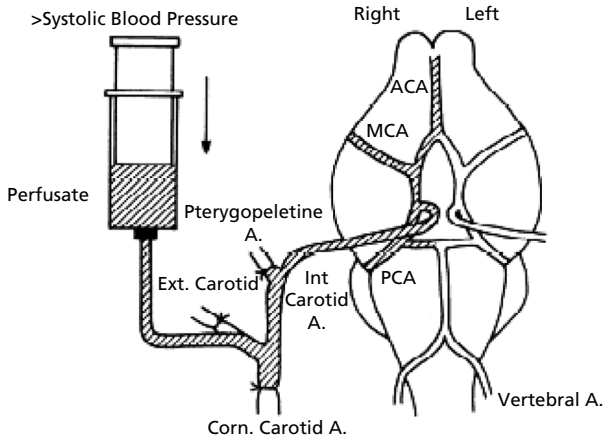

Figure 4 Schematic representation of the in-situ brain perfusion technique (taken, with permission, from Takasato et al 1984). ACA, anterior cerebral artery; MCA, middle cerebral artery; PCA, posterior cerebral artery.

parenchyma (transcytosis), bound to the endothelial lining, or present within the endothelial cells (endocytosis). To differentiate endothelial binding/endothelial endocytosis from actual transcytosis (uptake into brain parenchyma), a capillary depletion technique has been developed, where a dextran density centrifugation step is incorporated to deplete the brain homogenate of vasculature (Triguero et al 1990). However, this procedure can only be applied when the compound has high affinity binding to the microvasculature, because if it is non-specifically bound, it may become detached from the vasculature during the homogenization procedure and appear in the post-vascular compartment. Therefore, the capillary depletion technique should not be used when the test compound is not bound to the brain microvascular endothelium via a high affinity process (Pardridge 1995). For such compounds, it is preferred to use a post-perfusion wash to separate bound and transcytosed compound. This can be achieved by perfusing the vasculature with physiological buffer for a short period of time (10–30 s) at 4 mL min^{-1} (Samii et al 1994; Smith & Allen 2003).

The particular advantage of the in-situ perfusion technique is that there is no systemic exposure of the compound, and thus metabolism is avoided, except for that which occurs within the brain microcirculation (Pardridge 1995). The other major advantage is that there is total control over the perfusate solute concentration, and other constituents of the perfusion fluid can be varied, allowing ready characterization of saturable transport systems, plasma protein binding, and the effects of regulatory modifiers, hormones and neurotransmitters that can be presented to the brain at defined concentrations (Smith 2003). Also, the effect of pH, ionic content, and flow rate can be monitored (Bonate 1995). Recently, the BBB PS products of drugs were compared in rats and mice, and there was a good correlation between the BBB PS values obtained between the two species, indicating that this technique is amenable for use in mice (Murakami et al 2000). Consequently, the transport of drugs into the brain of genetically modified animals, such as P-gp knockout mice, can be assessed using this in-situ perfusion technique (Dagenais et al 2000; Cisternino et al 2001). The major disadvantages associated with this technique are that a complete kinetic analysis can involve a number of animals, requiring significant analytical time, in addition to the level of experimental difficulty (Smith 2003). This makes this technique unsuitable for high-throughput screening, however it can be used to provide mechanistic data, and provide information on factors that may be limiting brain uptake. Another disadvantage of this technique is that prolonged perfusion times (greater than 20 min) are impossible because of cerebral hypoxaemia (Bickel et al 1993).

Intravenous injection technique

In a recent review on BBB transport techniques, the intravenous injection technique was referred to as the 'gold standard' for assessing BBB permeability (Smith 2003). With this technique, a femoral vein of rats or mice is cannulated and the test compound is injected, or, alternatively, a tail-vein injection may be used. At various time points over the experiment, arterial blood is collected either by cannulation of a femoral artery in rats or by humanely killing the mice. In addition to the compound of interest, a plasma volume marker must also be administered, to correct for the amount of compound present in the brain microvasculature. Brain levels can be determined at the pre-determined time points (if animals are humanely killed over time) or at the end of the experiment (if arterial samples are being taken) (Pardridge 1995). To separate bound and transcytosed compound, only the capillary depletion technique can be used following intravenous dosing (Banks et al 2004), since the capillary drug-free rinse used in the in-situ perfusion technique is not possible.

One of the main advantages of this technique is that plasma and brain pharmacokinetics can be obtained, allowing for direct pharmacokinetic parameters to be calculated. Additionally, there is increased sensitivity (due to greater exposure to cerebral microvessels), and it is quite easy to measure BBB PS products less than 0.5 μL min^{-1} g^{-1} (Pardridge 1995). Other advantages of this technique are that the BBB is intact, all transporters, junctional proteins and enzymes are present at their physiological concentrations, the unique architecture of the blood vessels and perivascular cells is present and undisturbed, and cerebral metabolic pathways are not compromised (Smith 2003). Additionally, the degree of experimental difficulty is lower than that of the brain uptake index or in-situ perfusion technique (Bickel et al 1993). However, the major disadvantage with the intravenous technique is that there may be extensive metabolism by, and distribution into, peripheral organs, resulting in an inaccurate calculation of the BBB PS product, given the concentration within the brain microvasculature will be unknown (Pardridge 1995). In addition, at later time points, there is the possibility of back-diffusion from brain to plasma, which may confound BBB PS product calculations (Bickel et al 1993). Nevertheless, this technique provides a realistic evaluation of the brain levels that might be expected in humans, given it most closely resembles the human situation.

The intravenous technique described above is similar to the mouse brain uptake assay used by Raub (2004), where a single intravenous dose of a solute is administered, followed by blood and brain sampling at 5 min post-dose. The 5-min brain and plasma concentrations are used to calculate a permeability coefficient (P_{app}), with the presumption that metabolism,

back-flux, and tissue accumulation are negligible at that time point (Garberg et al 2005). However, the mouse brain uptake assay tends to underestimate the permeability of compounds with high P_{app} values, possibly due to back-flux and flow-dependent uptake of compounds, and overestimates the permeability of compounds with low P_{app} values, due to the problem of adequate vascular correction (using an impermeable marker) (Garberg et al 2005). Nonetheless, it is a useful screen for BBB penetration, and may be utilized in a high-throughput setting, to distinguish between poor and promising CNS candidates.

Brain efflux index

The brain efflux index technique was developed to determine mechanisms involved in brain-to-blood efflux, and involves direct microinjection of the compound of interest and an impermeable reference tracer into the brain (Kakee et al 1996). Following microinjection, the concentrations of test compound and reference tracer in the brain are determined over time. Although this technique does provide useful information on the involvement of various efflux transporters in the brain, such as P-gp (Kusuhara et al 1997), and the effect of inhibitors on brain efflux (Kitazawa et al 1998), it is not commonly used for permeability screening purposes. One of the major issues associated with this technique is that damage from needle tract injections may potentially alter the barrier properties of the BBB (Smith 2003), and so extreme caution must be taken when inserting the microinjections.

Intracerebral microdialysis

Intracerebral microdialysis involves direct sampling of brain interstitial fluid by implantating a dialysis fibre into the brain. The concentration of compound that has permeated into the brain following oral, intravenous or subcutaneous administration can be monitored over time within the same animal. The microdialysis probe consists of a semipermeable membrane and is perfused with a physiological solution, whereby compounds that are small enough to traverse the semipermeable membrane diffuse from higher to lower concentration (de Lange et al 1999). Therefore, any drug that enters the brain interstitial fluid will permeate into the physiological solution and may be subsequently assayed by an appropriate technique. Given the permeant is present in a physiological aqueous solution, quantification and separation from potential metabolites can be easily performed using high-performance liquid chromatography or capillary electrophoresis. The methodologies associated with dialysate sample separation and quantification have been recently reviewed (Dash & Elmquist 2003). Intracerebral microdialysis is the method of choice when interested in determining the local concentration of free drug as a function of time in individual freely moving animals (de Lange et al 1999).

The major advantage of this technique is that it provides pharmacokinetic profiles of compounds in the brain without the need to kill many animals at different time points (as would be necessary with all of the above-mentioned techniques) (Dai & Elmquist 2003). In addition, since both plasma and brain levels of compound can be determined over time, it is possible to determine the kinetics of influx and efflux from the brain (Dai & Elmquist 2003). More interestingly, the probe can be placed in any region of the brain, which may be useful when targeting a compound to a specific area of the brain (such as in a brain tumour or the substantia nigra in Parkinson's disease). However, if one is not interested in localized concentrations, this raises the issue of where to place the probe and whether multiple probes should be used in order to get an appropriate representation of drug levels throughout the brain (Bonate 1995). Another limitation of this technique is that it greatly depends on, and is limited by, the sensitivity of the assay method (de Lange et al 1999), since only low concentrations may be present in the dialysate. The other major disadvantage associated with intracerebral microdialysis is that insertion of the probe can result in chronic BBB disruption, as has been demonstrated by the passage of the normally impermeable inulin from blood to dialysate and extensive extravasation of serum albumin (Westergren et al 1995). An additional limitation of intracerebral microdialysis is that extraction across the fibre wall measured in-vitro is generally greater than that occurring in-vivo (Terasaki et al 1992). Even though in-vitro calibration of the probes is considered sufficient, in-vivo recovery may still deviate from in-vitro recovery and, consequently, complicated mathematical models and more complicated methods have been required (de Lange et al 1999).

Histochemistry

Although not appropriate for high-throughput screening purposes, the permeability of the BBB may be assessed using various histochemical techniques. The initial use of electron microscopy to visualize the distribution of horseradish peroxidase (Reese & Karnovsky 1967) has led others to use this technique to assess the transport of compounds through the BBB, in both healthy and pathological conditions, including hypertension (Nag et al 1977), brain trauma (Nag 1996), and seizures (Nitsch et al 1986). Although this technique has its obvious advantages, in that permeants can be detected visually within the brain, it is labour-intensive, involving animal studies, tissue collection and post-collection tissue staining, which is not conducive to high-throughput settings.

Imaging techniques

More recently, there has been some focus on the use of various imaging techniques to assess the permeability of compounds across the BBB, including quantitative autoradiography, magnetic resonance imaging, positron emission tomography, and single photon emission computed tomography. Although these techniques are not used in high-throughput drug discovery, they are non-invasive techniques that may be useful for assessing BBB permeability in pathological conditions. The major disadvantages associated with these techniques are their inherent costs, labour intensity, and inability to differentiate between parent compound and metabolites (in the case of labelled compounds).

Quantitative autoradiography involves administration of a radiolabelled compound into an animal, followed by blood sampling and brain removal. The brain is subsequently sectioned into 20-μm thick sections, placed in X-ray cassettes with a sheet of X-ray film and, following sufficient exposure, films are developed and analysed for the distribution-quantification of radioactivity by an image analysis system (Fenstermacher &

Wei 1998). Quantitative autoradiography has been a valuable tool in visualizing the brain uptake and distribution of various compounds (Hazai et al 1999; Coloma et al 2000; Plenevaux et al 2000), in addition to demonstrating the role of P-gp on the uptake of other compounds (Polli et al 1999).

Magnetic resonance imaging (MRI) is a sensitive method for the detection of CNS diseases and monitoring of their progression, and this technology has also been applied to qualitatively assess BBB permeability in both animals and humans. MRI involves administration of the contrasting agent, gadolinium-diethylenetriamine penta-acetic acid, whose appearance in the brain is related to the degree of BBB damage. Consequently, MRI cannot be used to assess drug permeability across the BBB, however it does provide useful information on the restrictive properties of the BBB and how such properties change in certain diseases, including stroke (Jiang et al 2005), multiple sclerosis (Wuerfel et al 2003), and encephalomyelitis (Floris et al 2004).

While MRI may provide qualitative insight into damage associated with the BBB, positron emission tomography (PET) has been shown to be a non-invasive, quantitative approach to measure the BBB PS product in humans under normal and disease-state conditions (Brooks et al 1984; Schlageter et al 1987). This technique involves the administration of a positron-emitting radionuclide or a compound labelled with an isotope that emits positrons. The subject is then placed in a counter that detects positrons emitted by the tracer and, with the use of computerized imaging techniques, two-dimensional images of the brain can be determined in real time, allowing for a kinetic evaluation of brain uptake (Bonate 1995). The two tracers that are more commonly used for PET are ^{82}Rb and ^{68}Ga-ethylenediaminetetra-acetic acid; however, the BBB permeability of compounds labelled with other positron-emitting labels, such as ^{11}C (Gulyas et al 2002) and ^{18}F (Langen et al 2005), has also been assessed using this technique. Most recently, PET has been used to measure the kinetics of ^{11}C verapamil and ^{11}C carvedilol brain uptake, and has been shown to be a sensitive tool for measuring in-vivo P-gp function at the BBB in both rodents and humans (Bart et al 2005; Luurtsema et al 2005; Sasongko et al 2005). This technique may provide a benefit in screening the brain uptake of other P-gp substrates using inhibition experiments (Bickel 2005).

Single photon emission computed tomography has also been useful as a non-invasive measure of BBB permeability. Following administration of a gamma-emitting compound, gamma scintigraphic images can be acquired using a gamma camera, and distribution of the compound throughout the body can be examined. Of major significance to BBB transport, it has been shown that ^{99m}Tc-sestamibi and other related compounds, such as ^{99m}Tc-tetrofosmin and a novel technetium (III) complex (^{99m}Tc-Q58), have higher brain uptake in P-gp knockout mice compared with wild-type mice (Luker et al 1997; Chen et al 2000; Dyszlewski et al 2002), indicating that such technetium-labelled compounds may be used to assess P-gp transport activity in-vivo. Although this technique may be useful in characterizing efflux transporters and BBB permeability in disease states, it will have a limited role in screening of compounds for potential brain uptake.

In-silico models

As a result of the increasing need for high-throughput drug discovery methods, in-silico models of BBB permeation are becoming more popular. However, most in-silico models have been based on in-vivo logBB values. As mentioned previously, BB is the brain to blood ratio at some defined time: it is a volume of distribution determined largely by cytoplasmic binding of drugs in the brain and much less by BBB permeability (Pardridge 2004b). The BBB PS product is a better index of BBB permeability as it predicts the level of free drug in the brain, since the level of free drug is determined by the total drug concentration in plasma, the PS product, and the fraction of drug in plasma that is available for transport into the brain (Pardridge 2004b). However, most in-silico models are based on logBB value determinations (Ecker & Noe 2004), and the lack of logPS data has limited the development and validation of models that predict BBB permeability (Liu et al 2004). Table 2 is a summary of some of the in-silico models developed on the basis of logBB data. From Table 2, some general concepts can be noted. The BB ratio is negatively correlated to molecular volume, molecular weight, polar surface area (PSA) and the number of N and O atoms (relating to H-bonding), but is positively correlated to lipophilicity. This has lead to two rules of thumb by Norinder & Haeberlein (2002): Rule 1: if N + O is five or less in a molecule, it has a high chance of entering the brain; Rule 2: if logP – (N + O) > 0, then logBB is positive.

The major disadvantage with these in-silico models is that the experimental logBB values are measured using different experimental procedures with large inter-animal variations, and the comparability of results obtained with such different experimental techniques has not been established (Bonate 1995). In addition, the experimental error in logBB measurements can be around 0.3 log units (Clark 2003), which questions the quality of the experimental data on which these models were based (Feher et al 2000).

Because of the limitations associated with the use of logBB when developing in-silico models of BBB permeation, it would be more appropriate to develop a model based on BBB PS data. One such model has been developed by Pfizer, which compares the logPS (determined by in-situ brain perfusions) with various parameters using 23 passively permeating drug-like compounds, and drugs that are thought to undergo active uptake and efflux (Liu et al 2004). This model included a data set of compounds that were considered representative of those that might be encountered in a drug discovery programme. Based on the compounds that were transported by passive diffusion alone, the following relationship was found:

$$\log PS = -2.19 + 0.262.\log D + 0.0583.VSA_{base} - 0.00897.PSA$$
$$(r^2 = 0.74,\ n = 23)$$

where logD is the logarithm of the partition coefficient between octanol and water at pH 7.4, and VSA_{base} is the van der Waals surface area of the basic atoms, a measure of the basicity of a compound. When an outlier was removed from their dataset, the r^2 increased from 0.74 to 0.80. Although this

Table 2 Some in-silico blood–brain barrier (BBB) models developed using logBB (brain to blood ratio at a defined time point) data

Model	r^2	Additional information	Reference
$LogBB = -0.485.\Delta logP + 0.889$ ($\Delta logP = logP_{octanol} - logP_{cyclohexane}$)	0.69	Using histamine H_2 antagonists dataset (n = 20) High brain concentrations achieved if H bonding is low Disadvantage is that you need a physical sample to obtain $logP_{oct}$ and $logP_{cyclohexane}$	Young et al (1988)
$LogBB = -0.002\ V_m - 0.024.PSA$	0.72	Using the same dataset as above When the authors tried to predict brain uptake of six new antihistamines, they were all overestimated by this model	Calder & Ganellin (1994)
$LogBB = -0.0057.MW + 0.309.logP_{cyclohexane} + 1.296$	0.845	Using the above dataset	Kaliszan & Markuszewski (1996)
$LogBB = -0.0145.PSA + 0.152.ClogP + 0.139$	0.787	Using 55 compounds (drug-like as well as gases) This model is not appropriate for compounds lacking polar groups	Clark (1999)
$LogBB = -0.218.(N + O) + 0.235.logP - 0.027$	0.803	Can be easily computed	Norinder & Haeberlein (2002)

$P_{octanol}$, experimentally determined partition coefficient between octanol and water; $P_{cyclohexane}$, experimentally determined partition coefficient between cyclohexane and water; V_m, molar volume; PSA, polar surface area; MW, molecular weight; ClogP, calculated logarithm of the partition coefficient between octanol and water; logP, logarithm of the experimentally determined partition coefficient between octanol and water.

Table 3 Selected examples of the multi-model approach used to assess blood–brain barrier (BBB) transport by various researchers in the field

Experimental purpose	Methods used	Reference
Effect of P-gp on cetirizine brain concentrations	MDR1-MDCK cell line and intravenous bolus in wild-type and P-gp-deficient mice	Polli et al (2003)
Transport of various drugs to the brain	Intravenous bolus (mice), intracerebral microdialysis (rats), BBECs and MDR1-MDCK cell line	Garberg et al (2005)
Transport of basic fibroblast growth factor to the brain	Binding to isolated rat capillaries, intravenous bolus, in-situ perfusion, and brain efflux index (in rats)	Deguchi et al (2000)
Efflux of GABA from the brain	Brain efflux index (in rats) and immortalized mouse cell line (MBEC4)	Kakee et al (2001)
Monocarboxylic acid transport across the BBB	Immortalized rat cell line (RBEC1), freshly cultured BBECs (transfected with MCT1) and BUI (in rats)	Kido et al (2000)
Role of transporters on adenosine transports across the BBB	BBECs co-cultured with astrocytes and in-situ brain perfusion (in rats)	Schaddelee et al (2003, 2005)
Effect of P-gp on brain transport	MDR1-MDCK cell line, oral/intravenous dosing in wild-type and P-gp-deficient mice, MDR1-transfected porcine kidney epithelial cells, and BBECs	Batrakova et al (2001); Dai et al (2003); Bachmeier et al (2005)
Brain flavonoid uptake	Immortalized rat cell line (RBE4), ECV/C6 glioma cell co-culture, and in-situ perfusion (in rats)	Youdim et al 2004

BBEC, bovine brain endothelial cells; BUI, brain uptake index; ECV, bladder carcinoma cell line; MCT1, monocarboxylic acid transporter; MDCK, Madin-Darby canine kidney; MDR1, multidrug resistance gene.

model was predictive of passively permeating compounds, it was found that logPS values of compounds that undergo active uptake were underpredicted and logPS values of efflux substrates were overpredicted. Nonetheless, it was found that the logPS was in the order of active uptake compounds > passive compounds > efflux compounds. This model was used to test two literature datasets where logPS had been determined experimentally, and the model predicted drug permeability well, with r^2 values of 0.77 and 0.94 for data sets of Murakami et al (2000) and Gratton et al (1997), respectively.

While this in-silico model appears to be the most appropriate, there are some general rules that have been developed (Clark 2003), which are an extension of Norinder and Haeberlein's two rules of thumb. These general rules are: (i) the sum of N+O should be 5 or less; (ii) ClogP – (N+O) should be greater than 0 (where ClogP is the calculated logarithm of the partition coefficient between octanol and water); (iii) PSA should be less than 60–90 $Å^2$; (iv) molecular weight should be less than 450 Da; and (v) logD in the range of 1–3 is recommended.

Conclusions

Various in-vitro, in-vivo and in-silico models are available that can be used to assess drug penetration across the BBB. Each model has its disadvantages and there needs to be a compromise between throughput potential and the limitations associated with the chosen model. However, in order to fully assess the brain uptake of new chemical entities and to completely understand the mechanisms involved in allowing or hindering BBB transport, one should employ both in-vitro and in-vivo techniques, and not rely solely on one method of screening. Some examples where researchers have used such a multi-model approach are shown in Table 3. In addition, in-silico models can be useful in reducing a large dataset of compounds into a smaller set of 'hit' compounds. The use of such a combined screening process involving in-vitro, in-vivo and in-silico models may confer greater reliability in predicting the potential CNS uptake of new chemical entities in humans.

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