Expert Opinion

Drug delivery across the blood–brain barrier: why is it difficult? how to measure and improve it?

Yaming Su & Patrick J Sinko†
†*Department of Pharmaceutics, Ernest Mario School of Pharmacy, Rutgers, State University of New Jersey, 160 Frelinghuysen Road, Piscataway, NJ 08854, USA*

The development of drugs that act in the CNS has been significantly impeded by the difficulty of delivering them across the blood–brain barrier (BBB). This article aims to provide the reader with a critical overview of important issues in the discovery and development of drugs that need to enter the brain to elicit pharmacological activity, focusing particularly on i) the role of drug transporters in brain permeation and how to manipulate them to enhance drug brain bioavailability; ii) the successful application, limitations and challenges of commonly used *in vitro* and *in vivo* methodologies for measuring drug transport across the BBB, and iii) a discussion of recently developed strategies (e.g., modulation of efflux transporters by chemical inhibitors and the employment of delivery vectors taking advantage of native transport systems at the BBB) for facilitating drug penetration into the brain.

Keywords: blood–brain barrier, brain capillary endothelial cells, Caco-2 cell, cell penetrating peptides, efflux, Madin-Darby canine kidney cell, P-glycoprotein, P-glycoprotein inhibitor, pegylated immunoliposome, receptor-mediated transport, transporter

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1. Introduction

People began to realise that a barrier existed between the blood and the brain > 100 years ago. In 1885, Paul Ehrlich, a German bacteriologist, observed that many dyes distributed widely into the body tissues but failed to stain the brain parenchyma. However, it remained unclear what tissue(s) or cell(s) were included in this barrier system until the late 1960s [1,2]. After decades of research, we now know that the blood–brain barrier (BBB) consists of densely packed brain capillary endothelial cells (BCECs) with tight junctions that form a daunting barrier, preventing many endogenous substances and xenobiotics from entering the brain. The thickness of this barrier, or the physical distance that separates the luminal or apical (i.e., facing the blood) and abluminal or basolateral (i.e., facing the brain tissue) capillary membranes was estimated to be 300 – 500 nm in human brain microvessels [3]. In addition to BCECs, at least three other types of cells (pericytes, astrocytes and neurons) were found that may directly or indirectly contribute to a functional BBB, as illustrated in Figure 1. Astrocytes are a class of large neuroglial (macroglial) cells in the CNS. A large body of *in vitro* [4-6] and *in vivo* [7,8] work has revealed that astrocytes maintain the integrity of the tight junctions and induce the expression of P-glycoprotein (Pgp, ABCB1) through cell-to-cell contact or the secretion of soluble factors. Another important component in the BBB microvasculature are pericytes, which are slender, contractile, mesenchymal-like cells that are found in close association with capillary walls. Pericytes are relatively undifferentiated and may become macrophages, smooth muscle cells or other cell types [9]. Compared with astrocytes, whose important role in the BBB is well characterised, the study of pericytes is

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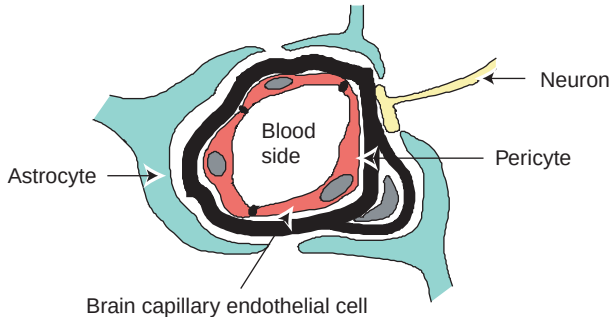

Figure 1. Schematic drawing of the blood–brain barrier microvasculature.

rather limited mainly due to the difficulty of purifying and culturing these cells. Available data suggest that pericytes play a role in brain angiogenesis, endothelial cell-tight junction formation and the expression of efflux transporters at the BBB (e.g., ATP-binding cassette [ABC] transporter) [10,11]. The BBB system is often referred to as a microvasculature consisting of BCECs, astrocytes and pericytes. However, the influence of neurons on the functions of the BBB should not be underestimated [12].

As a vital organ in the human body, the brain must maintain a stable microenvironment (i.e., brain homeostasis) and be free of inflammation or attack from harmful blood-carried substances generated endogenously or introduced exogenously. For this reason a nearly impenetrable BBB has evolved. However, this formidable barrier also imposes severe limitations on delivering therapeutic substances to the brain and CNS. For example, very few brain diseases such as depression, schizophrenia, epilepsy and chronic pain have been treated using small-molecule drugs. Other neurodegenerative diseases (e.g., Alzheimer's disease, Parkinson's disease and amytropic lateral sclerosis), brain cancer, brain or spinal cord trauma and stroke are still refractory to currently available therapeutic regimens. The BBB also significantly impedes brain penetration of many anti-HIV drugs such as azidodeoxythymidine, deoxythiacytidine and HIV protease inhibitors [13,14]. This presents serious challenges in the treatment of HIV infection as the virus residing in the brain escapes attack from drugs that are otherwise very effective in controlling it [15]. The replication of HIV in this sanctuary site remains one of the most significant challenges in anti-HIV therapy today.

A recently published market analysis report [16] showed that the global market for CNS drugs is currently at US$59 billion. Given the ever-increasing elderly population, the need for CNS-acting drugs is expected to continue to increase for the foreseeable future. Surprisingly, even though the overall market value for CNS drugs is large, the number of currently available CNS drugs is quite small comprising only ~ 5% of the > 70,000 known small molecule drugs [17,18]. The key reason for this imbalance is attributed to the fact that the majority of potential CNS drug candidates do not penetrate the BBB. The inability to deliver therapeutic agents across the BBB has significantly impeded the translation of many promising findings from the laboratory to the clinic.

Tremendous efforts have been undertaken to understand what types of molecules favour brain penetration. By structural comparison of marketed CNS and non-CNS drugs, van de Waterbeemd *et al.* [19] concluded that CNS drugs should have a molecular weight of < 450 Da and a total polar surface area < 90 Å in order to passively diffuse across the BBB. Others have suggested that lipophilicity was another crucial factor for brain uptake [20]. Computational predictions of brain penetration based on physicochemical properties have been shown to be useful for the early screening of brain-targeted drugs [21-23]; however, outliers are still frequently observed suggesting that other biological processes (e.g., drug efflux transporter mechanisms) may markedly influence drug transport across the BBB. Some models have attempted to include Pgp-mediated transport as an additional variable to enhance data fitting but the power of prediction was not optimal and requires further refinement [24].

With the expansion of our knowledge of human physiology and the advancement of drug delivery techniques, many molecules as large as oligonucleotides, peptides and antibodies have been successfully developed into drugs for the treatment of peripheral system diseases during the past several years. However, owing to the existence of the BBB, the delivery of large therapeutic compounds into the brain remains a significant challenge. The present review will elaborate several important issues in the discovery and development of drugs that need to enter the brain to elicit a pharmacological response, focusing particularly on i) active efflux across the BBB and the role of drug transporters; ii) *in vitro* and *in vivo* methodologies for measuring drug transport across the BBB, and iii) strategies for facilitating drug penetration into the brain.

2. Efflux across the blood–brain barrier: role of the drug transporters

There are a variety of membrane-bound proteins expressed at the BBB that are responsible for the translocation of neurotrophic factors, neurotransmitters and their metabolites between the brain and the blood. This section will focus on two drug transport-related transporter families: the ABC and the solute carrier family (SLC) transporters.

2.1 ATP-binding cassette transporters

The ABC transporter superfamily is the largest transporter gene family. The genes encoding ABC transporters are widely dispersed in the genome and show a high degree of amino acid sequence identity among eukaryotes [25,26]. Using phylogenetic analysis, the human ABC superfamily is divided into seven subfamilies with > 40 members identified so far. The topologies of ABC transporters have been proposed based on computational simulations and confirmed by experimental data due to the difficulty in crystallising membrane proteins.

In general, ABC transporters contain two ATP-binding sites located intracellularly, and ≥ 12 membrane-spanning α-helices, which associate with each other to become a specific membrane-spanning domain. As a typical ABC transporter, the three-dimensional architecture of Pgp has been proposed by Rosenberg *et al.* with the aid of electron microscopy and image analysis [27]. Of note, some transporters in the ABC superfamily such as the breast cancer resistance protein (BCRP; ABCG2) only contain one membrane-spanning domain and nucleotide-binding site. Recent studies suggest that BCRP is associated with other proteins or itself in order to become functional [28,29]. Therefore, BCRP is known as a half transporter.

Thus far, all identified ABC transporters have been reported to be efflux transporters, which means that they pump substrates out of cells into the extracellular environment. Therefore, membrane localisation of ABC transporters on polarised BCECs is critical for determining whether substrates are transported into or extruded from the brain. Pgp was originally identified in drug-resistant tumour cells [30]. Its expression has also been found in astrocytes [31], BCECs [32-34] and neurons [35]. Beaulieu *et al.* demonstrated that Pgp was mainly localised on the luminal side of BCECs by separating the luminal and abluminal membrane-enriched fractions by centrifugation [33]. This study group achieved this by coating the endothelial luminal membrane with cationic colloidal silica and a polyanion crosslinker, which changes the density of membrane fractions in the homogenates. *In situ* immunohistochemistry also showed that Pgp staining was intense on the luminal membrane of BCECs [34]. However, using different approaches, the studies revealed that Pgp is also localised in other cell types such as astrocytes and neurons [31,36], highlighting the complex functions of this transporter in the brain. Future studies need to elucidate the dynamic expression or localisation of Pgp in the BBB under different physiological or pathological conditions in order to achieve more efficient drug delivery into the brain by modulation of efflux transporters [35,36]. The data for other ABC transporters at the BBB is relatively scarce as compared with Pgp, and the results are often controversial. In a recent study, Nies *et al.* [37] investigated the expression and localisation of multi-drug resistance proteins (MRPs; ABCC) in the human brain using quantitative real-time PCR and confocal laser scanning microscopy. At the mRNA level, MRP1, 2, 3, 4 and 5 were detected but the immunohistochemical staining of brain cryosections showed no reactivity for MRP2 and MRP3 proteins, although the corresponding mRNA was detected. The results from the same study also demonstrated that MRP1, 4 and 5 were localised to the luminal side of BCECs. However, their findings were not totally consistent with previous observations that MPR1, 3 and 5 could not be detected at the protein level either in brain microvessels or the brain parenchyma [38]. The conflicting results may be due to variations across tissue specimens and the specificities of antibodies used by the different laboratories. In addition, the technical complications of immunohistochemistry itself could also lead to aberrant results. A comprehensive and conclusive statement about the expression of MRPs in the human BBB still cannot be made at this point in time. It should also be noted that orthologues of human MRPs seem to have different expression and localisation patterns at the BBB in other species. For example, immunostaining of rat brain capillaries clearly showed that Mrp2 was localised at the luminal membrane [39] but the data obtained from bovine brain capillaries suggested that Mrp2 was not expressed at the BBB, in agreement with human results [40].

BCRP was recently discovered from drug-resistant breast cancer cells [41,42] and in the placenta [43] by different research groups almost at the same time. BCRP is widely distributed in the small intestine, colon, liver, placenta [44] and the BBB [38]. Moreover, BCRP seems to play a key role in stem cell protection and differentiation [45]. The precise localisation of BCRP within brain capillary cells was investigated by Cooray *et al.* [38] using immunofluorescence confocal microscopy. After dual staining of BCRP and GLUT1 (the glucose transporter present at both sides of endothelium [46]) with anti-BCRP (BXP-21) and anti-GLUT1 antibodies tagged with different fluorescence colour, respectively, it was clearly observed that BCRP is localised at the luminal side of BCECs.

2.2 Solute carrier family transporters

The SLC family contains ~ 300 members in 43 subfamilies. The transporters within two subfamilies, SLCO (formerly SLC21A) and SLC22A, are of particular interest to pharmaceutical scientists, and have received a lot of attention during the past several years. A comprehensive review of SLC transporters at the BBB is available [47]. This section will focus on the well-studied SLC transporters that are associated with drug disposition in the brain.

Organic anion-transporting polypeptide (OATP)-1A2 (SLCO1A2, formerly SLC21A3; OATP-A or OATP1) is widely expressed in the small intestine, liver, kidneys, testes and brain [48]. Immunohistochemical staining revealed prominent reactivity against an OATP1A2 antibody in brain microvessels [49] but the membrane localisation of OATP1A2 at BCECs has not been clearly demonstrated thus far. OATP1A2 transports a variety of substances including bile acids, hormones and drugs such as fexofenadine [50] and saquinavir [51]. Due to overlapping substrate spectra between Pgp and OATP1A2, and the promiscuity of its membrane location at the BBB, the role of drug uptake by OATP1A2 needs further investigation.

Human OAT1 (SLC22A6) [52,53] and OAT3 (SLC22A8) [54] were originally cloned from the kidney and were found to be strongly expressed in this tissue. The transcripts of OAT1 and 3 were also detected in the brain by northern blot at a moderate level. Kikuchi *et al.* [55] suggested that rat Oat3 (Slc22a8, rOat3) is located on the abluminal and, possibly, luminal membrane of the BCECs. By using the brain efflux index (BEI) method (discussed in Section 3.2), this group found that the efflux of benzylpenicillin, which was microinjected into the

rat cerebrum, was saturable and could be inhibited by para-aminohippuric acid, a substrate of Oat transporters. Their data suggested that rOat3 could prevent the permeation of benzylpenicillin across the BBB considering the lack of expression of rat Oat1 (rOat1, Slc22a6) and Oat2 (rOat2, Slc22a7) at BCECs. Using similar strategies, it was also found that rOat3 could limit the brain uptake of thiopurine nucleobase analogues (e.g., 6-mercaptopurine and 6-tioguanine) [56] and statins (e.g., pravastatin and pitavastatin) [57]. However, it should be noted that there are no reports available that delineate the precise localisation of OAT1 and 3 in the human brain. Thus, the influence of these transporters in drug permeation across the human BBB remains unclear.

As the importance of membrane transporters at the BBB is increasingly recognised, robust molecular biological techniques, such as proteomics technology, have been employed to identify BBB-specific proteins, including membrane transporters or receptors [58,59]. Drugs and prodrugs can be designed with improved brain bioavailability by gaining a better understanding of the characteristics of membrane-bound proteins at the BBB. This can be achieved by taking advantage of absorptive influx transporters or receptors that are responsible for transport of their substrates or ligands from the blood to the brain, whilst avoiding secretory efflux transporters (e.g., Pgp) that extrude a wide variety of substances.

3. How to measure drug transport across the blood–brain barrier

Drug transport across the BBB can be experimentally measured *in vitro*, *in situ* and *in vivo*. *In silico* approaches are also employed to predict the brain uptake of chemical entities based on their physicochemical properties in the early stages of drug development. Bickel has summarised some frequently used techniques for measuring drug transport across the BBB [60]. In the present section, the authors elaborate on methodologies that were not discussed by Bickel, focusing on the advantages, limitations and potential pitfalls of each technique.

3.1 *In vitro* methologies

In vitro models of the BBB (e.g., BCECs culture) were developed with the goal of mimicking *in vivo* barrier properties. These models offer an alternative to *in situ* or *in vivo* animal studies and allow for the evaluation of drug transport across cell monolayers. Currently, there are few immortalised brain endothelial cell lines that are available from commercial vendors (e.g., bEND.3, mouse endothelioma cell from American tissue culture collection) or the academic community [61-64], and most of them are still in the early stages of characterisation, requiring further tests to determine whether they are suitable *in vitro* models of the BBB.

In the past decades, tremendous efforts have been undertaken to optimise BCECs culture systems to make them more physiologically relevant. It has been found that the presence of astrocytes in the vicinity of BCECs is crucial for the development of the BBB, by upregulating Pgp expression at the endothelial cells [11,65], enhancing the cell–cell tight junctions [6,66,67] and inducing the BBB-specific enzymes [68,69] or transporters [70-72]. Accordingly, newly developed *in vitro* BBB culture systems have been modified considering the influence of astrocytes: i) isolated primary BCECs can be seeded on the porous support materials of the insert of Transwell® (Corning) or Snapwell® (Corning), and the monolayer is supplied with astrocyte-conditioned medium (i.e., the medium harvested from astrocytes culture; Figure 2A) [6,66,73-75]. After BCECs polarise and the tight junctions are fully functional, the BCECs monolayers are ready for directional (basolateral → apical or apical → basolateral) drug transport studies; ii) BCECs are seeded on the porous membrane of the Transwell insert with astrocytes cultured on the bottom surface of the outer well (Figure 2B) [76]; iii) BCECs and astrocytes are grown on the uppermost or under surface of the same Transwell insert, respectively (Figure 2C) [77-79]. Although BCECs and astrocytes are not physically in contact with each other, they are separated by a semipermeable plastic membrane insert. In this co-culture BBB model, Gaillard *et al.* found that higher and less variable transendothelial

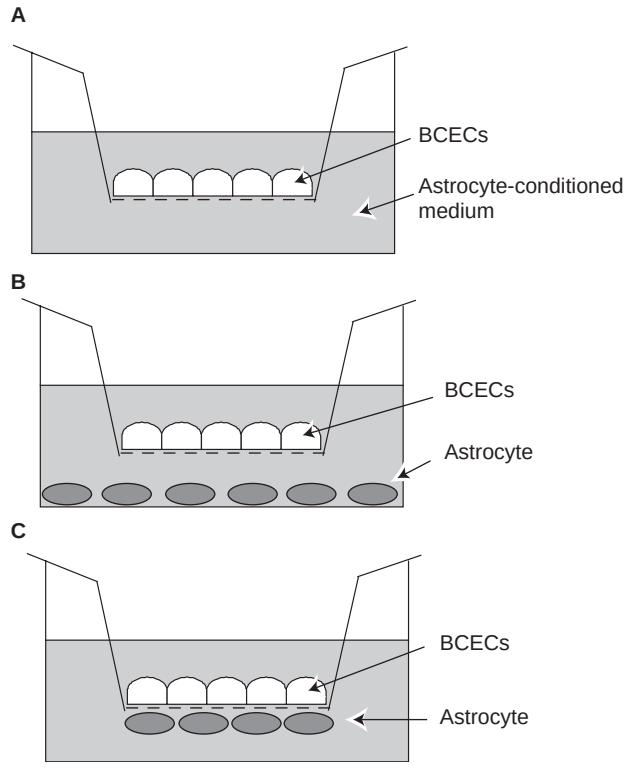

Figure 2. *In vitro* primary culture of BCECs for transcellular transport study. A) Single culture of BCECs with astrocytes-conditioned medium. **B)** Noncontact co-culture of BCECs and astrocytes. **C)** Contact co-culture of BCECs and astrocytes.
BCEC: Brain capillary endothelial cell.

electrical resistance (TEER) values were achieved when astrocytes were grown on the opposite side of the insert membrane as compared with the bottom surface of the outer well [80]. However, the need for astrocytes to be in close proximity to BCECs and the relationship to the magnitude of the effect on TEER values remains unclear.

Although primary brain endothelial cell culture provides a good *in vitro* model for the study of BBB development and mechanistic investigations of drug transport across the BBB, the preparation of BCECs is a time- and labour-intensive process. It is easy to introduce contamination by other cells, such as fibroblasts, smooth muscle cells or leptomeningeal cells into the preparations. A review of the current literature also reveals that the integrity of the BCECs monolayer varies remarkably between laboratories [81-83]. This is likely to be due to an inconsistent purity of the cell culture preparation. All of these disadvantages have limited the use of primary BCECs as a high-throughput screening tool in the early stages of drug development.

In addition to primary or immortalised BCECs, some epithelial cells of non-cerebral origin such as Caco-2 and Madin-Darby canine kidney (MDCK) cells have been exploited as potential *in vitro* BBB models. Caco-2 is a transformed human colon carcinoma cell line, and its polarised monolayer has been demonstrated to be a powerful tool for predicting the absorption, permeation and efflux transport properties of drug candidates in the gastrointestinal tract *in vivo*. Lohmann *et al.* found that the drug permeabilities obtained from Caco-2 cell monolayers correlated fairly well with results from primary cultured porcine BCECs [84]. However, the predictions of *in vivo* drug permeation across the BBB using Caco-2 culture was poor [85].

MDCK cells are an established dog renal epithelial cell line. It has two strains (i.e., MDCK-I and -II) that were possibly derived from the different parts of kidney tubules [86,87], and shows functional and biochemical disparities in experimental values including TEER [88]. Even though endogenous canine Pgp expression in the wild type MDCK-I or -II cells is low, the successful transfection of human multi-drug resistance 1 (*MDR1*) gene and preferred localisation of Pgp at the apical membrane in MDR1-MDCK-I or -II cells have made them a valuable *in vitro* permeability screening tool for the BBB. Using MDR1-MDCK-II cells, Doan *et al.* compared the passive permeability and Pgp-mediated efflux between 93 CNS (n = 48) and non-CNS (n = 45) drugs. These authors concluded that a drug candidate should have an *in vitro* $A \rightarrow B$ passive permeability ($P_{app\ A \rightarrow B}$) of $> 1.5 \times 10^{-5}$ cm/s (or 150 nm/s) and not be a good Pgp substrate (efflux ratio of $P_{app\ B \rightarrow A}/P_{app\ A \rightarrow B} < 2.5$) [89]. In another study carried out by Wang *et al.*, the passive permeabilities of 28 compounds were obtained using MDR1-MDCK-I cells, and then compared with *in vivo* brain penetration [90]. This group reported that compounds with $P_{app\ A \rightarrow B} > 3 \times 10^{-6}$ cm/s have a high brain uptake potential, and that the brain uptake of compounds with $P_{app\ A \rightarrow B} < 1 \times 10^{-6}$ cm/s as well as an efflux ratio of > 100 could be enhanced by inhibiting Pgp. It should be noted that the threshold values for high BBB permeation that have been suggested by the two different groups were markedly different. Numerous factors may account for these differences, including that the cell culture conditions differed across the laboratories resulting in variations in cell–cell tight junctions between MDCK-I and -II cell preparations. It has been reported that the TEER values of MDCK-II cells range from ~ 50 to 150 $\Omega \cdot cm^2$ [86,91,92], as compared with ≥ 2000 $\Omega \cdot cm^2$ for MDCK-I [88] or *in vivo* BCECs [93,94]. Therefore, it could be argued that the higher paracellular permeation in MDCK-II cells leads to the overestimation of small-molecule drug brain uptake *in vivo.* The variations in cell monolayers make the establishment of valuable *in vitro–in vivo* correlation models very difficult. Thus far, a quick and reliable *in vitro* BBB model is still unavailable and this has significantly impeded the high-throughput screening of CNS drugs by pharmaceutical companies.

3.2 *In vivo* methodologies

Several *in vivo* methods are now available for examining drug permeation across the BBB. Each method has its own advantages and limitations. In this section, the mouse brain uptake assay (MBUA) and brain uptake index (BUI) will be reviewed, whereas *in situ* brain perfusion and the BEI will be discussed in more detail.

MBUA was originally described by Ohno *et al.* [95]. In this method, a test compound is injected intravenously into the mouse as a single dose, then the animals are euthanised some time (typically 5 min) after administration, and the amount of compound in the blood and the brain are measured for the calculation of LogBB (i.e., the ratio of drug concentration in the brain and drug concentration in blood). The data obtained from this method can accurately reflect drug penetration into the brain under physiological conditions, assuming that the distribution between the blood and the brain at the sampling time point (e.g., 5 min) is at a steady state. Perhaps the most obvious difference between MBUA and BUI [96] is the site of drug administration, as the latter one employs a single rapid common carotid artery injection. Although the injection composition can be versatile, the sensitivity of BUI was found to be lower than that of MBUA (for a review see [60]).

The *in situ* brain perfusion method was originally developed in rats by Takasato *et al.* [97] and has been modified and adapted for the mouse [98,99]. In this technique, the brain is perfused with an oxygenated perfusion buffer containing test compounds through the cannulated carotid artery. The perfusion is terminated by decapitation at a fixed time point (typically 2 – 5 min), which should allow for sufficient drug accumulation in the brain to quantitate, whilst the integrity of the BBB remains intact. The concentration of the test compound in the brain is then determined by scintillation counting or liquid chromatography-tandem mass spectrometry for the calculation of the kinetic parameters of brain transport

[14]. Brain flux (J; nmol/g brain/min) can be calculated according to:

$$J = K_{in} C_{pf} \quad (1)$$

where C_{pf} is the compound concentration in the perfusate and K_{in} (ml/min/100 g) is the unidirectional transfer coefficient derived using the following relationship:

$$K_{in} = dX_{brain} / d_t / C_{pf} \quad (2)$$

where X_{brain} is the amount of compound in the brain and C_{pf} is the compound concentration in the perfusate. In a single time-point experiment, X_{brain}/T replaces dX_{brain}/d_t, where T is the perfusion time (min).

A critical experimental parameter in the mouse *in situ* brain perfusion is the perfusion rate. As the blood is carried to the brain by two paired arteries (i.e., internal carotid and vertebral arteries), if perfusion is too slow, the vertebral artery blood flow becomes the dominant blood supply to the brain, and the fluid in the brain arteries will not be identical to the perfusate. Consequently, the calculations described above are no longer valid. On the other hand, if the perfusion is too fast, the integrity of the BBB will be compromised. Perfusion rates of 1 – 2 ml/min are typically used in mouse *in situ* brain perfusion studies [14,99].

Several advantages of the *in situ* brain perfusion technique are: i) the mix of perfusate with systemic blood is minimised, and the metabolism of the tested compound and its plasma protein binding may be neglected. Therefore, the results more accurately reflect the interaction of compound with the BBB. However, this may also be considered a limitation if one is more interested in BBB permeation under physiological conditions; ii) as compared with the BUI method, a relatively longer perfusion time permits better interactions between the drug and the BBB. This becomes especially important if facilitated drug transport (e.g., receptor-mediated transport [RMT]) or active efflux are involved in brain permeation; and iii) by including inulin or sucrose in the perfusate, which do not significantly penetrate the BBB during the brief period of perfusion, the integrity of the BBB can be monitored in parallel to drug transport.

Kakee *et al.* developed a novel experimental approach (i.e., the BEI) to assess brain efflux transport processes [100]. As opposed to the brain uptake techniques discussed above (MBUA, BUI and *in situ* brain perfusion), which introduce the drug at the blood circulation side of the BBB and measure the appearance of drug in the brain, BEI involves intracerebral microinjection of a small amount of the test and reference compounds. At a predetermined time point, the animal is euthanised and the amount of compound remaining in the brain is quantitated. The percentage of the retained test compound (i.e., BEI) is normalised to a reference compound, which is relatively impermeable across the BBB, and calculated according to the following equation:

$$BEI\,(\%) = \left[1 - \frac{\left(\frac{amount \cdot of \cdot test \cdot drug \cdot in \cdot brain}{amount \cdot of \cdot reference \cdot in \cdot brain}\right)}{\left(\frac{amount \cdot of \cdot test \cdot drug \cdot injected}{amount \cdot of \cdot reference \cdot injected}\right)}\right] \times 100 \quad (3)$$

The BEI technique has been successfully used to characterise the efflux of a variety of endogenous substances and xenobiotics across the BBB, such as quinidine [101], dehydroepiandrosterone sulfate [102], GABA [103], pentazocin (a narcotic-antagonist analgesic) [104]. Recently, Raybon *et al.* employed the BEI method to study the brain efflux of aminoguanidine (a nitric oxide synthase inhibitor) [105], and found that the BEI value for aminoguanidine was > 100% after being normalised to inulin, which has limited BBB permeability. After eliminating the possibility of experimental error, they concluded that the unexpected high BEI value of aminoguanidine was possibly due to significant neuronal sequestration. The results from this work, if generalised, reveal an important constraint of the BEI method: if the drug of interest has a high nonspecific binding affinity to the brain parenchyma, or if it is significantly taken up by neuronal or glial cells, the efflux of the drug could be underestimated. Meanwhile, the metabolism of the drug in the brain after microinjection could also confound the results obtained from the BEI method. Indeed, when brain uptake techniques (e.g., MBUA, BUI and *in situ* brain perfusion) are employed, nonspecific association of the drug with brain endothelial cells could also potentially overestimate drug permeation to the brain if the penetration across the BBB can not be distinguished from the binding to the cell wall of brain capillaries.

4. Facilitating drug transport across the blood–brain barrier

It has been suggested that a CNS-targeted drug should have a molecular weight of < 400 or 500 Da and should not be a substrate for Pgp, based on a large data set obtained from *in vitro* and *in vivo* experiments. Computational analysis of quantitative structure–activity relationships has been used to define the constraints for CNS-targeted drug candidates in a more sophisticated manner using the polar surface area of < 90 Å, the molecular weight < 450 Da, and the ratio of a molecule's principal axes (a parameter that describe the molecular shape) should be < 5 [19,106]. Although these criteria have proven to be useful in identifying therapeutic compounds that exhibit satisfactory brain penetration in the early phase of drug development, they also eliminate some potentially potent drug candidates simply due to poor brain uptake. Although small-molecule drugs represent the majority of therapeutic entities on the market today, the past decade has witnessed an increasing number of large-molecule therapeutic agents such as oligonucleotides, peptides and proteins joining the drug development pipeline. Many recently developed

treatments for neurological disorders are based on biotechnology products, and require innovative delivery methods to insure successful clinical implementation.

A variety of strategies and technologies have been developed to manipulate or circumvent the BBB for drug delivery to the brain, including osmotic or chemical opening of the BBB [107], intracerebral implantation [108] (an invasive neurosurgical method), delivery via the nasal cavity (presumably bypassing the BBB) [109,110], carrier- or receptor-facilitated transcytosis [111], nanoparticle [112] or liposome formulations, inhibition of efflux transporters (e.g., Pgp) [113] and so on. The following sections will focus on strategies or technologies that have been extensively studied and have been demonstrated to result in improved brain permeation.

4.1 Modulation of efflux transporter

Although the role of efflux transporters such as Pgp in conferring the MDR phenotype to malignant tumour cells has been found to be far less important than other cellular proteins, it is evident from animal and clinical studies that Pgp at the BBB significantly restricts drug uptake into the brain. Classes of drugs whose brain penetration is compromised by Pgp include chemotherapeutics (e.g., doxorubicin, vinblastine, paciltaxel, etoposide), anti-HIV drugs (HIV protease inhibitors such as saquinavir and ritonavir; nucleoside analogues such as azidodeoxythymidine) and opioids [114]. Theoretically, the modulation of Pgp transport function can be achieved at several levels: i) downregulation of Pgp expression at the transcriptional or translational levels; ii) alteration of membrane targeting after the synthesis of the Pgp protein; and iii) inhibition of transport function using chemical inhibitors. The development of the first two approaches is still early (i.e., at the cell culture level). The last one has been extensively tested in the preclinical and clinical studies to assess whether concomitant administration of Pgp inhibitors can increase the effectiveness of the drug.

Pgp inhibitors can be classified into three generations based on the time sequence of their identification and the inhibitory mechanism. First-generation Pgp inhibitors include verapamil, ciclosporin A, tamoxifen and several calmodulin antagonists. Indeed, these inhibitors are also Pgp substrates, therefore, the inhibitory mechanisms have been attributed to competitive binding or transport. As inhibition requires a high blood concentration of the inhibitor (at least 3- to 5-times higher than the Michaelis constant [K_m]) chemical inhibition is inefficient. Because of the widespread availability (most of them are commercially available), the first-generation Pgp inhibitors have been extensively used to delineate the role of Pgp in drug transport across the cell membrane (e.g., drug-resistant tumour cells) or tissue barriers (e.g., intestine, liver, kidneys and BBB) where Pgp resides. Tsai *et al.* [115] examined the effect of Pgp modulators on the brain exposure of camptothecin and found that the coadministration of ciclosporin A (20 mg/kg) increased the blood-to-brain coefficient of distribution (AUC_{brain}/AUC_{blood}) by 1.5-fold, and significantly prolonged the mean residence time of camptothecin in the brain from 38.22 ± 2.05 to 46.29 ± 1.56 min. However, most first-generation Pgp inhibitors are themselves pharmacologically active and they inhibit Pgp efflux at concentrations much higher than those required for their own therapeutic activity. As a result, the application of the first-generation Pgp inhibitors in the clinic is limited. A few years later, analogues of the early Pgp inhibitors were synthesised with the goal of making the inhibitors more potent and less toxic. Representative second-generation Pgp inhibitors include biricodar (see Figure 3) and valspodar, a nonimmunosupressive derivative of ciclosporin D, which is 10- to 20-fold more potent than ciclosporin A in its ability to inhibit Pgp. Kemper *et al.* investigated the brain uptake of taxol derivatives in mice with or without the coadministration of valspodar, and found that the ratio of brain to plasma docetaxel concentration increased ~ 2-fold with valspodar cotreatment [116]. Pretreatment of mice with orally administered valspodar significantly boosted paclitaxel (intravenous injection) brain uptake as shown by the 6.5- and 3.0-fold increase of $AUC_{brain,0\text{-}24\ h}$ and $AUC_{plasma,0\text{-}8\ h}$, respectively, when compared with animals without Pgp inhibitor treatment [117].

During the past several years, more potent and specific Pgp modulators such as zosuquidar, elacridar, tariquidar and laniquidar have been developed as third-generation Pgp inhibitors (Figure 3). Cell culture experiments have showed that these agents typically inhibited Pgp activity at nanomolar concentrations with minimal inhibitory effects on the CYP450s [118-120]. In an *in vitro* study, Mistry *et al.* showed that the concentration of tariquidar that was required to fully reverse Pgp-mediated drug resistance was 20- to 48-fold lower than the concentration at which any toxicity was observed in the cell lines, and Pgp inhibition persisted for > 22 h even after tariquidar had been removed from the culture medium [120]. The inhibition specificity of tariquidar was demonstrated by the fact that the modulator, even at very high concentrations (50 µM), did not inhibit MRP function. Dantzig *et al.* systemically investigated the effect of zosuquidar on the transport activity of MRPs and CYP450-mediated metabolism [119], and found that zosuquidar had a much higher affinity with Pgp than that with CYP3A (apparent dissociation constant for an inhibitor [K_i] with CYP3A was 3.8 µM, ~ 60-fold higher than the affinity of Pgp for zosuquidar), and it also did not inhibit the transport activity of MRP1 or 2. These characteristics are considered to be superior to the second-generation Pgp inhibitors. For example, valspodar could inhibit CYP3A4-mediated metabolism of paclitaxel and vinblastine [121], and biricodar also affected MRP1 [122]. The working mechanisms of the third-generation Pgp inhibitors are still under investigation; however, it is clear that these inhibitors are not substrates of Pgp. Therefore, inhibition is not competitive. Indeed, third-generation Pgp inhibitors bind to Pgp at a site that is different than the drug-binding pocket [123]. Recently, animal studies using third-generation Pgp inhibitors to optimise pharmacokinetic profiles as well as to enhance the drug penetration into the brain have generated encouraging

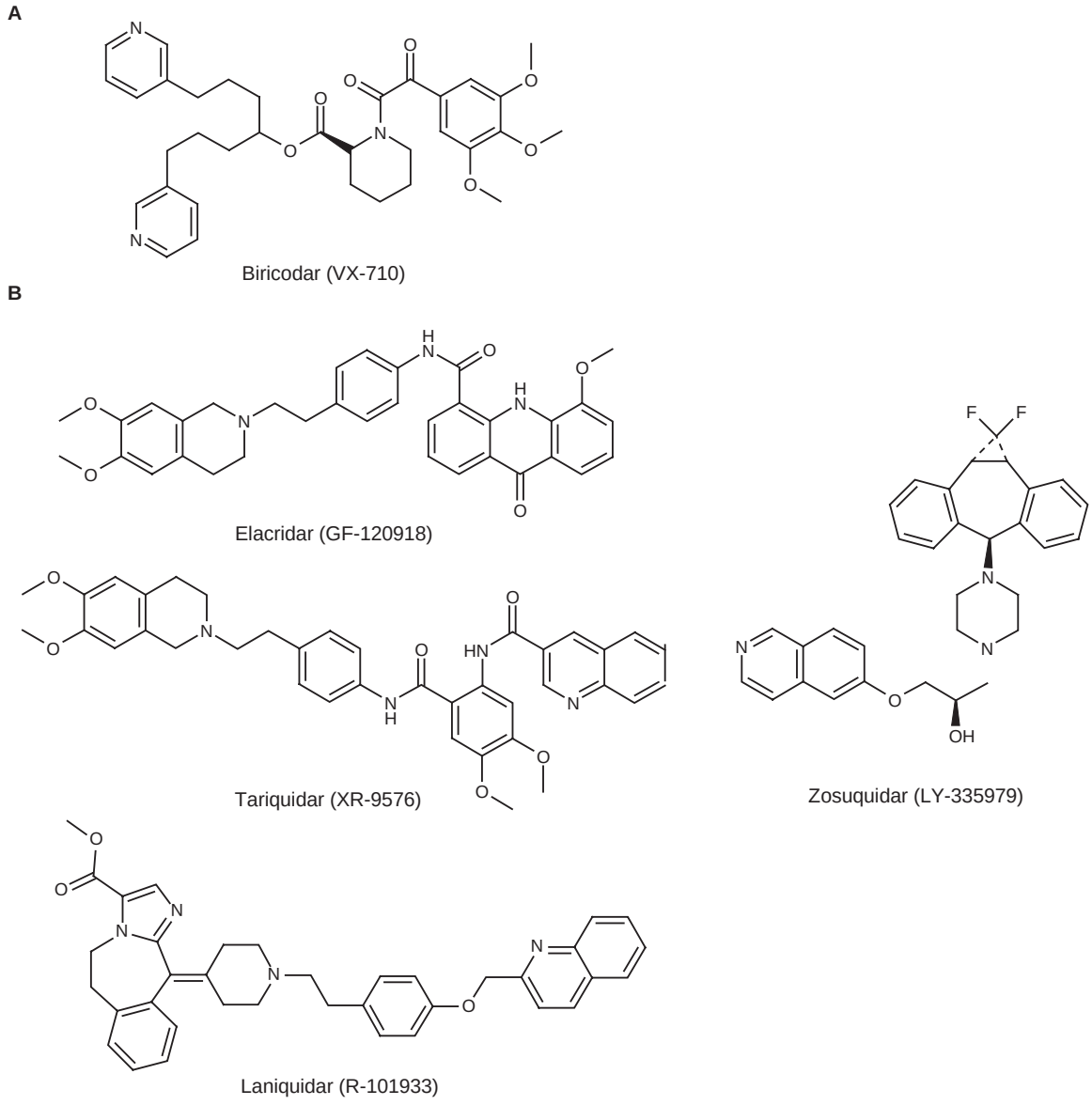

Figure 3. Structures of some potent P-glycoprotein inhibitors. A) Second-generation inhibitor. **B)** Third-generation inhibitors.

results, particularly in the area of HIV treatment. Polli *et al.* investigated the effect of elacridar on the brain exposure of amprenavir by using whole-body autoradiography [124]. These authors demonstrated a 13-fold increase in amprenavir concentrations in mice pretreated with elacridar. In a mouse *in situ* perfusion study, Park *et al.* showed that the inclusion of elacridar 10 µM in the perfusate resulted in a more than 7-fold increase in the brain distributional volume (i.e., uptake) of saquinavir, and the inclusion of MK571 100 µM (a specific Mrp family inhibitor) increased saquinavir apparent brain uptake by > 4.4-fold, suggesting that other efflux transporters at the BBB, such as Mrp, also play a significant role in the brain uptake and retention of saquinavir [14]. The other important efflux transporter identified at the BBB is BCRP. Breedveld *et al.* [125] compared the brain penetration of imatinib mesilate (imatinib, a tyrosine kinase inhibitor that effectively inhibits platelet-derived growth factor-induced glioblastoma) between the wild type and Bcrp1 knockout mice. It was also found that the brain penetration of intravenous imatinib significantly increased (2.5-fold) in knockout mice. Coadministration of elacridar, which is now known to inhibit both Pgp and BCRP [126], resulted in a 1.2-fold increase in imatinib brain penetration in Pgp knockout mice that have functionally competent Bcrp1. The importance of BCRP in restricting

brain permeation was recently questioned by Lee *et al.* [127] who found that elacridar significantly increased the brain uptake of dehydroepiandrosterone sulfate and mitoxantrone in Pgp knockout mice. However, the brain penetration of these two compounds in Bcrp1 knockout mice was comparable with that in wild type mice. The results of this group suggested that Bcrp1 did not make significant contributions to the drug efflux at the BBB. These authors also suggested that some other unknown elacridar-sensitive efflux transporters may exist at the BBB. At present, additional studies are needed before the importance of BCRP can be ascertained.

Coadministration of Pgp inhibitors with therapeutic agents has been tested in clinical cancer treatments aimed at increasing the oral bioavailability of poorly absorbed drugs, or to reverse efflux-mediated drug resistance in cancer patients [128,129]. Although most studies have demonstrated that blockade of Pgp remarkably enhanced the systemic exposure of drugs, the improvement of therapeutic outcomes in patients with recurrent or refractory malignancy was minimal. This suggests that multiple mechanisms may contribute to the clinical chemotherapeutic resistance [130]. With regard to Pgp efflux activity at the BBB, numerous animal studies have clearly demonstrated that Pgp inhibition resulted in enhanced brain permeation of anticancer drugs [116], although there are little data that are available to directly prove increased drug availability in the human brain by inhibition of efflux transporters at the BBB due to the lack of appropriate analysis methods [131].

Whilst the potency of Pgp inhibition is being tested *in vitro* or *in vivo*, the pharmacokinetics, pharmacology or toxicology of Pgp inhibitors should also be systemically investigated to facilitate their quick application in clinical trials. At present, many issues that are associated with the clinical use of Pgp inhibitors are unsolved. For example, one may wonder whether chronic dosing of Pgp inhibitors would upregulate other efflux mechanisms at the BBB as a compensatory mechanism. Indeed, this type of study is in progress [132].

4.2 Use of drug delivery vehicles

Under physiological conditions, carrier-mediated transport at the BBB is responsible for the translocation of circulating nutrients such as glucose, monocarboxylic acids, amino acids and choline into the brain [133-136], whereas RMT facilitates the brain uptake of circulating peptides such as insulin, transferrin and leptin [137-140]. One delivery strategy called the 'Trojan horse' strategy links therapeutic entities to a delivery vector in order to take advantage of these native transport systems at the BBB. During the past decade, the application of the Trojan horse concept has generated a variety of novel and efficient brain drug delivery systems.

4.2.1 *Receptor-mediated transport*

The RMT system is sometimes preferred as a route for drug delivery across the BBB presumably because carrier-mediated transport involves molecular movement through a small pore, whereas RMT uses receptor-mediated transcytosis. The transferrin receptor (TfR) and insulin receptor at the BBB facilitate the transport of their respective substrates, transferrin and insulin, by receptor-mediated transcytosis. However, the direct conjugation of ligands to a drug would compete with the endogenous ligands in the blood. This represents a formidable challenge to the successful implementation of this approach. Clearly, additional technological innovation will be required to overcome these challenges. Besides transferrin and insulin, peptidomimetic mAb for TfR or insulin receptor can bind to the exofacial epitopes on the receptors and initiate transcytosis without disturbing the natural ligand binding sites. As a consequence, any drugs that are attached to the mAb may also be transported into the brain. The linkage of the drug to the mAb can be achieved by several means. Huwyler *et al.* used pegylated immunoliposome (PIL) in which daunomycin was encapsulated in a poly(ethylene glycol)-modified liposome with anti-TfR mAb (OX26) tethered to the tips of the poly(ethylene glycol) strands, and successfully delivered daunomycin into the rat brain after intravenous injection [141]. With plasmid DNA encapsulated in the interior, the PIL delivery system has also been shown to be a useful tool for nonviral gene therapy in some chronic CNS diseases (for a review see [142]). Compared with conventional liposomes, PIL is associated with several advantages including increased stability and selective targeting (for a review see [143]). However, sophisticated formulation procedures pose the challenges to quality control in their scale-up and manufacture. The inherent thermodynamic and physical stabilities of PIL also demand very careful handling during transportation or storage. In order to quickly translate this encouraging technique from the laboratory to the clinic, future studies should focus on how to improve the stability of formulation. Nanoparticles may hold promise in overcoming these shortcomings associated with PIL (for a review see [112]). Nanoparticles themselves cannot freely diffuse across the BBB mainly due to their size (10 – 1000 nm); however, it was found that the coating of polybutylcyanoacrylate nanoparticles with polysorbate 80 can significantly transport the entrapped drugs into the brain [144]. The mechanisms of how these polysorbate 80-modified nanoparticles cross the BBB are still under discussion [145]. Olivier *et al.* has demonstrated that anti-TfR mAb could be successfully conjugated to the surface of peglaylated nanoparticles [146]. However, cell culture or animal studies showing the application of pegylated immunonanoparticles are still not available.

Another method of linking the drug and mAb is based on biotin–avidin technology. In this approach, the drug is biotinylated, whereas the mAb is fused with avidin or streptavidin. Owing to the high binding affinity between biotin and avidin, the drug and mAb can be easily linked via the bridge of biotin–avidin by simply mixing them in buffer before administration. In a recent study, Zhang *et al.* conjugated the 8D3 mAb (anti-mouse TfR Ab) and streptavidin through a

stable thiol ether linkage [147]. Meanwhile, the bacterial β-galactosidase was monobiotinylated with sulfo-*N*-hydroxy-succinimide-long chain-long chain-biotin. The double long-chain linker was designed to provide a 14-atom spacer between the biotin moiety and the ε-amino group of surface lysine residues on the enzyme and as such avoided the potential steric hindrance of the enzyme. The data obtained from this study demonstrate that the conjugation of the enzyme to TfR mAb via linkage of biotin–streptavidin significantly improved the brain uptake of β-galactosidase whose molecular weight is as large as 116 kDa.

In order to increase the specificity of brain targeting by taking advantage of RMT as described above, an exclusively BBB-localised receptor would be the optimal vector. However, such a receptor has so far not been identified. Indeed, a pharmacokinetic study showed that OX26-conjugated immunoliposomes also significantly distribute to other organs such as the kidneys and liver due to the abundance of the TfR on a broad variety of tissues [148]. The discovery of vectors at the BBB for specific brain delivery relies on a better understanding of the genomics and proteomics of BBB.

4.2.2 *Cell penetrating peptide-mediated transport*

Cell-penetrating peptides (CPPs), also known as protein transduction domains, are a collection of short peptides that enter cells by penetrating cell membranes. CPPs are derived from several cell-penetrating proteins (e.g., transcriptional activators and anti-DNA antibodies), signal sequence-based peptides, hydrophobic membrane translocating sequence peptides and designed peptides (for review see [149]). Although the membrane-penetrating mechanisms of CPPs are undergoing intensive investigations [150], the applications of CPPs as part of a Trojan horse strategy to facilitate brain permeation have showed some encouraging results. Rousselle *et al.* conjugated doxorubicin to SynB (CPPs derived from the antimicrobial peptide protegrin-1) through a chemical linker (succinate), and found that the conjugates achieved a 30-fold higher brain uptake than doxorubicin alone [151]. In another study, Mazel *et al.* identified similar brain permeabilities of SynB–doxorubicin conjugates between Pgp knockout and wild type mouse, indicating that conjugates may be able to bypass the Pgp-mediated efflux [152]. The chemical linkers coupling CPPs and drugs are typically biodegradable bonds such as amide or disulfide bonds [151,153]; therefore, the drug can be released after penetration across the BBB. Hence, the degradation rate of conjugates in the plasma must be controlled in order to have a sufficient amount of conjugates interacting with the BBB [151].

In order to load other large cargo such as proteins onto CPPs, the genes of the studied proteins can be ligated with coding sequences of CPPs by recombinant DNA techniques, then expressed in *Escherichia coli* or other systems as a fusion protein. For example, Cao *et al.* coupled Bcl-xL, a Bcl-2 family member with potent antiapoptosis activity, with transactivating transcriptional activator (TAT; CPPs derived from HIV-1 Tat protein), and demonstrated that intraperitoneal injection of the fusion protein into mice resulted in robust protein transduction in neurons in various brain regions, and the decreased cerebral infarction after the experimental focal ischaemia [154]. Similar strategies have been adopted for the delivery of other therapeutic proteins such as glial line-derived neurotrophic factor into the brain [155]. Of note, CPP-mediated transport is not brain specific. Schwarze *et al.* examined the body distribution of TAT-β galactosidase fusion protein using X-gal staining after intraperitoneal injection in mice, and observed a strong signal in the liver, kidneys, lung tissues, heart muscle, spleen and brain [156]. Therefore, tissue-targeting should be considered when a brain-specific drug delivery system is designed based on CPP-mediated transport.

5. Expert opinion and conclusions

The BBB represents a formidable barrier that drugs must overcome in order to enter the brain. The lack of fenestrations and the nearly impermeable cell–cell tight junctions in brain capillaries preclude paracellular penetration. Nonparacellular permeation is highly restricted by membrane transporters that have evolved as a detoxification barrier. The BBB can be best characterised as an extremely permeability-selective barrier allowing critical nutrients such as glucose into the brain whilst restricting entry to most other substances. In order to reach the brain parenchyma, the drugs must be small, fairly lipophilic and not a substrate for apical efflux transporters (e.g., Pgp) residing in the BBB. However, newly identified entities for the therapeutic intervention of CNS diseases such as oligonucleotides, peptides and proteins are quite large. The transport of these large biomolecules across nonfenestrated BCECs with tight junctions can no longer be achieved by simple diffusion. Thus, novel brain delivery technologies are desperately needed. It is now becoming evident that the lack of efficient means to facilitate drug permeation across the BBB has severely impeded translation of brain drug discovery from the laboratory to the clinic, and represents the primary reason why very few new CNS drugs were marketed during the past decade [157]. In addition, more powerful high-throughput screening tools for the *in vitro* assessment of drug transport across the BBB and better *in vitro–in vivo* correlation modelling will also help to accurately predict drug penetration into brain *in vivo*. This would significantly reduce the time for CNS drug development as well as increase the number of preclinical hits. Several clinical successes have been achieved by making prodrugs from relatively poorly absorbed drugs. For example, prodrugs such as valacyclovir improve the oral absorption of acyclovir several fold by using intestinal drug transporters such as the intestinal peptide transporter. However, valacyclovir is rapidly converted to acyclovir after absorption. Therefore, whilst blood concentrations are significantly increased, brain concentrations remain relatively low. This is representative of the difficulty of improving brain

exposure to therapeutic agents and clearly illustrates the complexity of the situation. The need for better brain delivery remains high but clinical successes are likely to rest in the hands of technological innovations in targeted drug delivery systems rather than the development of 'better' small-molecule drugs. It is highly unlikely that generic enhancement of BBB permeability (e.g., decreasing paracellular resistance) will ever be a safe and effective means for enhancing brain uptake as the target drug, and many other substances, will experience enhanced brain uptake. Therefore, it is abundantly clear that a drug-uptake pathway-specific targeting approach must be employed to enhance the delivery of a specific drug by means of a specific pathway without allowing other agents to access the brain.

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Bibliography

Papers of special note have been highlighted as either of interest (•) or of considerable interest (••) to readers.

1. REESE TS, FEDER N, BRIGHTMAN MW: Electron microscopic study of the blood-brain and blood-cerebrospinal fluid barriers with microperoxidase. *J. Neuropathol. Exp. Neurol.* (1971) 30(1):137-138.
2. REESE TS, KARNOVSKY MJ: Fine structural localization of a blood-brain barrier to exogenous peroxidase. *J. Cell Biol.* (1967) 34(1):207-217.
3. CORNFORD EM, HYMAN S, CORNFORD ME, LANDAW EM, DELGADO-ESCUETA AV: Interictal seizure resections show two configurations of endothelial Glut1 glucose transporter in the human blood-brain barrier. *J. Cereb. Blood Flow Metab.* (1998) 18(1):26-42.
4. TAO-CHENG JH, NAGY Z, BRIGHTMAN MW: Tight junctions of brain endothelium *in vitro* are enhanced by astroglia. *J. Neurosci.* (1987) 7(10):3293-3299.
5. HURST RD, FRITZ IB: Properties of an immortalised vascular endothelial/glioma cell co-culture model of the blood-brain barrier. *J. Cell. Physiol.* (1996) 167(1):81-88.
6. SOBUE K, YAMAMOTO N, YONEDA K *et al.*: Induction of blood-brain barrier properties in immortalized bovine brain endothelial cells by astrocytic factors. *Neurosci. Res.* (1999) 35(2):155-164.
7. JANZER RC, RAFF MC: Astrocytes induce blood-brain barrier properties in endothelial cells. *Nature* (1987) 325(6101):253-257.
- • A study providing direct evidence that astrocytes are capable of inducing BBB properties (e.g., tight junctions) in non-neural endothelial cells *in vivo*.
8. WILLIS CL, LEACH L, CLARKE GJ, NOLAN CC, RAY DE: Reversible disruption of tight junction complexes in the rat blood-brain barrier, following transitory focal astrocyte loss. *Glia* (2004) 48(1):1-13.
9. GERHARDT H, BETSHOLTZ C: Endothelial-pericyte interactions in angiogenesis. *Cell Tissue Res.* (2003) 314(1):15-23.
10. BALABANOV R, DORE-DUFFY P: Role of the CNS microvascular pericyte in the blood-brain barrier. *J. Neurosci. Res.* (1998) 53(6):637-644.
11. BEREZOWSKI V, LANDRY C, DEHOUCK MP, CECCHELLI R, FENART L: Contribution of glial cells and pericytes to the mRNA profiles of P-glycoprotein and multidrug resistance-associated proteins in an *in vitro* model of the blood-brain barrier. *Brain Res.* (2004) 1018(1):1-9.
12. TONTSCH U, BAUER HC: Glial cells and neurons induce blood-brain barrier related enzymes in cultured cerebral endothelial cells. *Brain Res.* (1991) 539(2):247-253.
13. WU D, CLEMENT JG, PARDRIDGE WM: Low blood-brain barrier permeability to azidothymidine (AZT), 3TC, and thymidine in the rat. *Brain Res.* (1998) 791(1-2):313-316.
14. PARK S, SINKO PJ: P-glycoprotein and mutlidrug resistance-associated proteins limit the brain uptake of saquinavir in mice. *J. Pharmacol. Exp. Ther.* (2005) 312(3):1249-1256.
- • In this study, *in situ* brain perfusion was performed to delineate the role of Pgp in saquinavir permeation across the BBB.
15. KANDANEARATCHI A, WILLIAMS B, EVERALL IP: Assessing the efficacy of highly active antiretroviral therapy in the brain. *Brain Pathol.* (2003) 13(1):104-110.
16. BARTON C: *CNS Drug Discoveries: What the Future Holds*. Espicom Business Intelligence (2005).
- • This is a market analysis that provides a comprehensive examination of major CNS therapy areas and analysis of the critical strengths, weaknesses, opportunities and threats of current and upcoming CNS products.
17. GHOSE AK, VISWANADHAN VN, WENDOLOSKI JJ: A knowledge-based approach in designing combinatorial or medicinal chemistry libraries for drug discovery. 1. A qualitative and quantitative characterization of known drug databases. *J. Comb. Chem.* (1999) 1(1):55-68.
18. LIPINSKI CA: Drug-like properties and the causes of poor solubility and poor permeability. *J. Pharmacol. Toxicol. Methods* (2000) 44(1):235-249.
19. VAN DE WATERBEEMD H, CAMENISCH G, FOLKERS G, CHRETIEN JR, RAEVSKY OA: Estimation of blood-brain barrier crossing of drugs using molecular size and shape, and H-bonding descriptors. *J. Drug Target.* (1998) 6(2):151-165.
20. CLARK DE: Prediction of intestinal absorption and blood-brain barrier penetration by computational methods. *Comb. Chem. High Throughput Screen* (2001) 4(6):477-496.
21. BASAK SC, GUTE BD, DREWES LR: Predicting blood-brain transport of drugs: a computational approach. *Pharm. Res.* (1996) 13(5):775-778.
22. FISCHER H, GOTTSCHLICH R, SEELIG A: Blood-brain barrier permeation: molecular parameters governing passive

diffusion. *J. Membr. Biol.* (1998) 165(3):201-211.

23. AJAY, BEMIS GW, MURCKO MA: Designing libraries with CNS activity. *J. Med. Chem.* (1999) 42(24):4942-4951.

24. ADENOT M, LAHANA R: Blood-brain barrier permeation models: discriminating between potential CNS and non-CNS drugs including P-glycoprotein substrates. *J. Chem. Inf. Comput. Sci.* (2004) 44(1):239-248.

25. DEAN M, HAMON Y, CHIMINI G: The human ATP-binding cassette (ABC) transporter superfamily. *J. Lipid Res.* (2001) 42(7):1007-1017.

26. JONES PM, GEORGE AM: Symmetry and structure in P-glycoprotein and ABC transporters what goes around comes around. *Eur. J. Biochem.* (2000) 267(17):5298-5305.

27. ROSENBERG MF, CALLAGHAN R, FORD RC, HIGGINS CF: Structure of the multidrug resistance P-glycoprotein to 2.5 nm resolution determined by electron microscopy and image analysis. *J. Biol. Chem.* (1997) 272(16):10685-10694.

•• This study provides the first experimental insight into the three dimensional structure of the ABC transporter.

28. XU J, LIU Y, YANG Y, BATES S, ZHANG JT: Characterization of oligomeric human half-ABC transporter ATP-binding cassette G2. *J. Biol. Chem.* (2004) 279(19):19781-19789.

29. HENRIKSEN U, GETHER U, LITMAN T: Effect of Walker A mutation (K86M) on oligomerization and surface targeting of the multidrug resistance transporter ABCG2. *J. Cell Sci.* (2005) 118(7):1417-1426.

30. JULIANO RL, LING V: A surface glycoprotein modulating drug permeability in Chinese hamster ovary cell mutants. *Biochim. Biophys. Acta* (1976) 455(1):152-162.

31. PARDRIDGE WM, GOLDEN PL, KANG YS, BICKEL U: Brain microvascular and astrocyte localization of P-glycoprotein. *J. Neurochem.* (1997) 68(3):1278-1285.

•• By using the immunocytochemical staining technique, the authors found that a principal site of immunoreactive Pgp in the brain microvasculature is localised to astrocyte foot processes. This finding argues with the statement that Pgp is localised at the apical membrane of BCECs.

32. CORDON-CARDO C, O'BRIEN JP, CASALS D *et al.*: Multidrug-resistance gene (P-glycoprotein) is expressed by endothelial cells at blood-brain barrier sites. *Proc. Natl. Acad. Sci. USA* (1989) 86(2):695-698.

•• Immunocytochemical staining showed the localisation of Pgp at BCECs.

33. BEAULIEU E, DEMEULE M, GHITESCU L, BELIVEAU R: P-glycoprotein is strongly expressed in the luminal membranes of the endothelium of blood vessels in the brain. *Biochem. J.* (1997) 326(Pt 2):539-544.

34. VIRGINTINO D, ROBERTSON D, ERREDE M *et al.*: Expression of P-glycoprotein in human cerebral cortex microvessels. *J. Histochem. Cytochem.* (2002) 50(12):1671-1676.

35. VOLK HA, BURKHARDT K, POTSCHKA H, CHEN J, BECKER A, LOSCHER W: Neuronal expression of the drug efflux transporter P-glycoprotein in the rat hippocampus after limbic seizures. *Neuroscience* (2004) 123(3):751-759.

36. VOLK H, POTSCHKA H, LOSCHER W: Immunohistochemical localization of P-glycoprotein in rat brain and detection of its increased expression by seizures are sensitive to fixation and staining variables. *J. Histochem. Cytochem.* (2005) 53(4):517-531.

37. NIES AT, JEDLITSCHKY G, KONIG J *et al.*: Expression and immunolocalization of the multidrug resistance proteins, MRP1-MRP6 (ABCC1-ABCC6), in human brain. *Neuroscience* (2004) 129(2):349-360.

• A comprehensive study assessing the expression and localisation of MRPs in the human brain.

38. COORAY HC, BLACKMORE CG, MASKELL L, BARRAND MA: Localisation of breast cancer resistance protein in microvessel endothelium of human brain. *Neuroreport* (2002) 13(16):2059-2063.

• This paper indicates that the expression of BCRP is clearly localised to the luminal side of BCECs by using immunofluorescence confocal microscopy.

39. MILLER DS, NOBMANN SN, GUTMANN H, TOEROEK M, DREWE J, FRICKER G: Xenobiotic transport across isolated brain microvessels studied by confocal microscopy. *Mol. Pharmacol.* (2000) 58(6):1357-1367.

40. ZHANG Y, HAN H, ELMQUIST WF, MILLER DW: Expression of various multidrug resistance-associated protein (MRP) homologues in brain microvessel endothelial cells. *Brain Res.* (2000) 876(1-2):148-153.

41. DOYLE LA, YANG W, ABRUZZO LV *et al.*: A multidrug resistance transporter from human MCF-7 breast cancer cells. *Proc. Natl. Acad. Sci. USA* (1998) 95(26):15665-15670.

42. MIYAKE K, MICKLEY L, LITMAN T *et al.*: Molecular cloning of cDNAs which are highly overexpressed in mitoxantrone-resistant cells: demonstration of homology to abc transport genes. *Cancer Res.* (1999) 59(1):8-13.

43. ALLIKMETS R, SCHRIML LM, HUTCHINSON A, ROMANO-SPICA V, DEAN M: A human placenta-specific ATP-binding cassette gene (ABCP) on chromosome 4q22 that is involved in multidrug resistance. *Cancer Res.* (1998) 58(23):5337-5339.

44. MALIEPAARD M, SCHEFFER GL, FANEYTE IF *et al.*: Subcellular localization and distribution of the breast cancer resistance protein transporter in normal human tissues. *Cancer Res.* (2001) 61(8):3458-3464.

45. BUNTING KD: ABC transporters as phenotypic markers and functional regulators of stem cells. *Stem Cells* (2002) 20(1):11-20.

46. GERHART DZ, LEVASSEUR RJ, BRODERIUS MA, DREWES LR: Glucose transporter localization in brain using light and electron immunocytochemistry. *J. Neurosci. Res.* (1989) 22(4):464-472.

47. KUSUHARA H, SUGIYAMA Y: Active efflux across the blood-brain barrier: role of the solute carrier family. *NeuroRx* (2005) 2(1):73-85.

• An updated review of SLC transporters at the BBB.

48. KULLAK-UBLICK GA, HAGENBUCH B, STIEGER B *et al.*: Molecular and functional characterization of an organic anion transporting polypeptide cloned from human liver. *Gastroenterology* (1995) 109(4):1274-1282.

49. GAO B, HAGENBUCH B, KULLAK-UBLICK GA, BENKE D, AGUZZI A, MEIER PJ: Organic anion-transporting polypeptides mediate transport of opioid peptides across blood-brain barrier. *J. Pharmacol. Exp. Ther.* (2000) 294(1):73-79.

- The experimental insight into OATP-mediated opioid peptides transport across the BBB.

50. CVETKOVIC M, LEAKE B, FROMM MF, WILKINSON GR, KIM RB: OATP and P-glycoprotein transporters mediate the cellular uptake and excretion of fexofenadine. *Drug Metab. Dispos.* (1999) 27(8):866-871.

51. SU Y, ZHANG X, SINKO PJ: Human organic anion-transporting polypeptide OATP-A (SLC21A3) acts in concert with P-glycoprotein and multidrug resistance protein 2 in the vectorial transport of saquinavir in Hep G2 Cells. *Mol. Pharm.* (2004) 1(1):49-56.

52. HOSOYAMADA M, SEKINE T, KANAI Y, ENDOU H: Molecular cloning and functional expression of a multispecific organic anion transporter from human kidney. *Am. J. Physiol.* (1999) 276(1 Pt 2):F122-F128.

53. RACE JE, GRASSL SM, WILLIAMS WJ, HOLTZMAN EJ: Molecular cloning and characterization of two novel human renal organic anion transporters (hOAT1 and hOAT3). *Biochem. Biophys. Res. Commun.* (1999) 255(2):508-514.

54. CHA SH, SEKINE T, FUKUSHIMA JI *et al.*: Identification and characterization of human organic anion transporter 3 expressing predominantly in the kidney. *Mol. Pharmacol.* (2001) 59(5):1277-1286.

55. KIKUCHI R, KUSUHARA H, SUGIYAMA D, SUGIYAMA Y: Contribution of organic anion transporter 3 (Slc22a8) to the elimination of p-aminohippuric acid and benzylpenicillin across the blood-brain barrier. *J. Pharmacol. Exp. Ther.* (2003) 306(1):51-58.

- The experimental insight into rat Oat3-mediated drug transport across the BBB.

56. MORI S, OHTSUKI S, TAKANAGA H, KIKKAWA T, KANG YS, TERASAKI T: Organic anion transporter 3 is involved in the brain-to-blood efflux transport of thiopurine nucleobase analogs. *J. Neurochem.* (2004) 90(4):931-941.

57. KIKUCHI R, KUSUHARA H, ABE T, ENDOU H, SUGIYAMA Y: Involvement of multiple transporters in the efflux of 3-hydroxy-3-methylglutaryl-CoA reductase inhibitors across the blood-brain barrier. *J. Pharmacol. Exp. Ther.* (2004) 311(3):1147-1153.

58. SHUSTA EV, BOADO RJ, PARDRIDGE WM: Vascular proteomics and subtractive antibody expression cloning. *Mol. Cell. Proteomics* (2002) 1(1):75-82.

59. PARDRIDGE WM: Blood-brain barrier genomics and the use of endogenous transporters to cause drug penetration into the brain. *Curr. Opin. Drug Discov. Devel.* (2003) 6(5):683-691.

60. BICKEL U: How to measure drug transport across the blood–brain barrier. *NeuroRx* (2005) 2(1):15-26.

•• A comprehensive review of the methodologies used for the assessment of drug transport across the BBB. The advantages and limitations of each technique are carefully discussed.

61. MURUGANANDAM A, HERX LM, MONETTE R, DURKIN JP, STANIMIROVIC DB: Development of immortalized human cerebromicrovascular endothelial cell line as an *in vitro* model of the human blood-brain barrier. *FASEB J.* (1997) 11(13):1187-1197.

62. TATSUTA T, NAITO M, OH-HARA T, SUGAWARA I, TSURUO T: Functional involvement of P-glycoprotein in blood-brain barrier. *J. Biol. Chem.* (1992) 267(28):20383-20391.

63. GREENWOOD J, PRYCE G, DEVINE L *et al.*: SV40 large T immortalised cell lines of the rat blood-brain and blood-retinal barriers retain their phenotypic and immunological characteristics. *J. Neuroimmunol.* (1996) 71(1-2):51-63.

64. KIDO Y, TAMAI I, OKAMOTO M, SUZUKI F, TSUJI A: Functional clarification of MCT1-mediated transport of monocarboxylic acids at the blood-brain barrier using *in vitro* cultured cells and *in vivo* BUI studies. *Pharm. Res.* (2000) 17(1):55-62.

65. FENART L, BUEE-SCHERRER V, DESCAMPS L *et al.*: Inhibition of P-glycoprotein: rapid assessment of its implication in blood-brain barrier integrity and drug transport to the brain by an *in vitro* model of the blood-brain barrier. *Pharm. Res.* (1998) 15(7):993-1000.

66. RIST RJ, ROMERO IA, CHAN MW, COURAUD PO, ROUX F, ABBOTT NJ: F-actin cytoskeleton and sucrose permeability of immortalised rat brain microvascular endothelial cell monolayers: effects of cyclic AMP and astrocytic factors. *Brain Res.* (1997) 768(1-2):10-18.

67. WOLBURG H, NEUHAUS J, KNIESEL U *et al.*: Modulation of tight junction structure in blood-brain barrier endothelial cells. Effects of tissue culture, second messengers and cocultured astrocytes. *J. Cell Sci.* (1994) 107(5):1347-1357.

68. ROUX F, DURIEU-TRAUTMANN O, CHAVEROT N *et al.*: Regulation of gamma-glutamyl transpeptidase and alkaline phosphatase activities in immortalized rat brain microvessel endothelial cells. *J. Cell. Physiol.* (1994) 159(1):101-113.

69. RAUH J, MEYER J, BEUCKMANN C, GALLA HJ: Development of an *in vitro* cell culture system to mimic the blood-brain barrier. *Prog. Brain Res.* (1992) 91:117-121.

70. O'DONNELL ME, MARTINEZ A, SUN D: Endothelial Na-K-Cl cotransport regulation by tonicity and hormones: phosphorylation of cotransport protein. *Am. J. Physiol. Cell Physiol* (1995) 269(6):C1513-C1523.

71. BOADO RJ, WANG L, PARDRIDGE WM: Enhanced expression of the blood-brain barrier GLUT1 glucose transporter gene by brain-derived factors. *Brain Res. Mol. Brain Res.* (1994) 22(1-4):259-267.

72. GAILLARD PJ, VAN DER SANDT IC, VOORWINDEN LH *et al.*: Astrocytes increase the functional expression of P-glycoprotein in an *in vitro* model of the blood-brain barrier. *Pharm. Res.* (2000) 17(10):1198-1205.

73. RUBIN LL, HALL DE, PORTER S *et al.*: A cell culture model of the blood-brain barrier. *J. Cell Biol.* (1991) 115(6):1725-1735.

74. EL HAFNY B, BOURRE JM, ROUX F: Synergistic stimulation of gamma-glutamyl transpeptidase and alkaline phosphatase activities by retinoic acid and astroglial factors in immortalized rat brain microvessel endothelial cells. *J. Cell. Physiol.* (1996) 167(3):451-460.

75. O'DONNELL ME, MARTINEZ A, SUN D: Cerebral microvascular endothelial cell Na-K-Cl cotransport: regulation by astrocyte-conditioned medium. *Am. J. Physiol. Cell Physiol* (1995) 268(3):C747-C754.

76. KIDO Y, TAMAI I, NAKANISHI T *et al.*: Evaluation of blood-brain barrier transporters by co-culture of brain capillary endothelial cells with astrocytes. *Drug Metab. Pharmacokinet.* (2002) 17(1):34-41.

77. KONDO T, KINOUCHI H, KAWASE M, YOSHIMOTO T: Astroglial cells inhibit the increasing permeability of brain endothelial cell monolayer following hypoxia/reoxygenation. *Neurosci. Lett.* (1996) 208(2):101-104.

78. ISOBE I, WATANABE T, YOTSUYANAGI T *et al.*: Astrocytic contributions to blood-brain barrier (BBB) formation by endothelial cells: a possible use of aortic endothelial cell for *in vitro* BBB model. *Neurochem. Int.* (1996) 28(5-6):523-533.

79. DEHOUCK MP, MERESSE S, DELORME P, FRUCHART JC, CECCHELLI R: An easier, reproducible, and mass-production method to study the blood-brain barrier *in vitro*. *J. Neurochem.* (1990) 54(5):1798-1801.

80. GAILLARD PJ, VOORWINDEN LH, NIELSEN JL *et al.*: Establishment and functional characterization of an *in vitro* model of the blood-brain barrier, comprising a co-culture of brain capillary endothelial cells and astrocytes. *Eur. J. Pharm. Sci.* (2001) 12(3):215-222.

81. KONDO T, IMAIZUMI S, KATO I, YOSHIMOTO T: Isolation and culture of brain endothelial cells and establishment of *in vitro* blood-brain barrier model. *Cell Transplant.* (1994) 3(Suppl. 1):S35-S37.

82. RAUB TJ, KUENTZEL SL, SAWADA GA: Permeability of bovine brain microvessel endothelial cells *in vitro*: barrier tightening by a factor released from astroglioma cells. *Exp. Cell Res.* (1992) 199(2):330-340.

83. DEHOUCK MP, JOLLIET-RIANT P, BREE F, FRUCHART JC, CECCHELLI R, TILLEMENT JP: Drug transfer across the blood-brain barrier: correlation between *in vitro* and *in vivo* models. *J. Neurochem.* (1992) 58(5):1790-1797.

84. LOHMANN C, HUWEL S, GALLA HJ: Predicting blood-brain barrier permeability of drugs: evaluation of different *in vitro* assays. *J. Drug Target.* (2002) 10(4):263-276.

85. LUNDQUIST S, RENFTEL M, BRILLAULT J, FENART L, CECCHELLI R, DEHOUCK MP: Prediction of drug transport through the blood-brain barrier *in vivo*: a comparison between two *in vitro* cell models. *Pharm. Res.* (2002) 19(7):976-981.

86. FULLER SD, SIMONS K: Transferrin receptor polarity and recycling accuracy in 'tight' and 'leaky' strains of Madin-Darby canine kidney cells. *J. Cell Biol.* (1986) 103(5):1767-1779.

87. VAN MEER G, STELZER EH, WIJNAENDTS-VAN-RESANDT RW, SIMONS K: Sorting of sphingolipids in epithelial (Madin-Darby canine kidney) cells. *J. Cell Biol.* (1987) 105(4):1623-1635.

88. STEVENSON BR, ANDERSON JM, GOODENOUGH DA, MOOSEKER MS: Tight junction structure and ZO-1 content are identical in two strains of Madin-Darby canine kidney cells which differ in transepithelial resistance. *J. Cell Biol.* (1988) 107(6 Pt 1):2401-2408.

89. DOAN KM, 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.

90. WANG Q, RAGER JD, WEINSTEIN K *et al.*: Evaluation of the MDR-MDCK cell line as a permeability screen for the blood-brain barrier. *Int. J. Pharm.* (2005) 288:349-359.

91. RICHARDSON JC, SCALERA V, SIMMONS NL: Identification of two strains of MDCK cells which resemble separate nephron tubule segments. *Biochim. Biophys. Acta* (1981) 673(1):26-36.

92. GUO A, MARINARO W, HU P, SINKO PJ: Delineating the contribution of secretory transporters in the efflux of etoposide using Madin-Darby canine kidney (MDCK) cells overexpressing P-glycoprotein (Pgp), multidrug resistance-associated protein (MRP1), and canalicular multispecific organic anion transporter (cMOAT). *Drug Metab. Dispos.* (2002) 30(4):457-463.

93. CRONE C, OLESEN SP: Electrical resistance of brain microvascular endothelium. *Brain Res.* (1982) 241(1):49-55.

94. BUTT AM, JONES HC: Effect of histamine and antagonists on electrical resistance across the blood-brain barrier in rat brain-surface microvessels. *Brain Res.* (1992) 569(1):100-105.

95. OHNO K, PETTIGREW KD, RAPOPORT SI: Lower limits of cerebrovascular permeability to nonelectrolytes in the conscious rat. *Am. J. Physiol.* (1978) 235(3):H299-H307.

96. OLDENDORF WH: Measurement of brain uptake of radiolabeled substances using a tritiated water internal standard. *Brain Res.* (1970) 24(2):372-376.

97. TAKASATO Y, RAPOPORT SI, SMITH QR: An *in situ* brain perfusion technique to study cerebrovascular transport in the rat. *Am. J. Physiol.* (1984) 247(3 Pt 2):H484-H493.

98. DAGENAIS C, ROUSSELLE C, POLLACK GM, SCHERRMANN JM: Development of an *in situ* mouse brain perfusion model and its application to mdr1a P-glycoprotein-deficient mice. *J. Cereb. Blood Flow Metab.* (2000) 20(2):381-386.

•• The development of an *in situ* mouse-brain perfusion model is described in detail in this paper.

99. MURAKAMI H, TAKANAGA H, MATSUO H, OHTANI H, SAWADA Y: Comparison of blood-brain barrier permeability in mice and rats using *in situ* brain perfusion technique. *Am. J. Physiol. Heart Circ. Physiol.* (2000) 279(3):H1022-H1028.

100. KAKEE A, TERASAKI T, SUGIYAMA Y: Brain efflux index as a novel method of analyzing efflux transport at the blood-brain barrier. *J. Pharmacol. Exp. Ther.* (1996) 277(3):1550-1559.

• This study describes the development of the BEI model for the assessment of drug transport across the BBB.

101. KUSUHARA H, SUZUKI H, TERASAKI T, KAKEE A, LEMAIRE M, SUGIYAMA Y: P-glycoprotein mediates the efflux of quinidine across the blood-brain barrier. *J. Pharmacol. Exp. Ther.* (1997) 283(2):574-580.

102. ASABA H, HOSOYA K, TAKANAGA H *et al.*: Blood-brain barrier is involved in the efflux transport of a neuroactive steroid, dehydroepiandrosterone sulfate, via organic anion transporting polypeptide 2. *J. Neurochem.* (2000) 75(5):1907-1916.

103. KAKEE A, TAKANAGA H, TERASAKI T, NAITO M, TSURUO T, SUGIYAMA Y: Efflux of a suppressive neurotransmitter, GABA, across the blood-brain barrier. *J. Neurochem.* (2001) 79(1):110-118.

104. MORIKI Y, SUZUKI T, FUKAMI T, HANANO M, TOMONO K, WATANABE J: Involvement of P-glycoprotein in blood-brain barrier transport of pentazocine in rats using brain uptake index method. *Biol. Pharm. Bull.* (2004) 27(6):932-935.

105. RAYBON JJ, BOJE KM: A critical evaluation of the brain efflux index method as applied to the nitric oxide synthase inhibitor, aminoguanidine. *Biopharm. Drug Dispos.* (2001) 22(9):391-401.
• This study points out the potential pitfall when the BEI model is used for the assessment of drug transport across the BBB.

106. KELDER J, GROOTENHUIS PD, BAYADA DM, DELBRESSINE LP, PLOEMEN JP: Polar molecular surface as a dominating determinant for oral absorption and brain penetration of drugs. *Pharm. Res.* (1999) 16(10):1514-1519.

107. NEUWELT EA, SPECHT HD, BARNETT PA *et al.*: Increased delivery of tumor-specific monoclonal antibodies to brain after osmotic blood-brain barrier modification in patients with melanoma metastatic to the central nervous system. *Neurosurgery* (1987) 20(6):885-895.
• This study describes a surgical method that can increase the drug permeability by opening tight junctions between BCECs

108. KREWSON CE, KLARMAN ML, SALTZMAN WM: Distribution of nerve growth factor following direct delivery to brain interstitium. *Brain Res.* (1995) 680(1-2):196-206.

109. FREY WH, LIU J, CHEN XQ *et al.*: Delivery of I-125-NGF to the brain via the olfactory route. *Drug Deliv.* (1997) 4(2):87-92.

110. SHIPLEY MT: Transport of molecules from nose to brain: transneuronal anterograde and retrograde labeling in the rat olfactory system by wheat germ agglutinin-horseradish peroxidase applied to the nasal epithelium. *Brain Res. Bull.* (1985) 15(2):129-142.

111. PARDRIDGE WM: Receptor-mediated peptide transport through the blood-brain barrier. *Endocr. Rev.* (1986) 7(3):314-330.

112. OLIVIER JC: Drug transport to brain with targeted nanoparticles. *NeuroRx* (2005) 2(1):108-119.

113. BEGLEY DJ: ABC transporters and the blood-brain barrier. *Curr. Pharm. Des.* (2004) 10(12):1295-1312.

114. KIM RB: Drugs as P-glycoprotein substrates, inhibitors, and inducers. *Drug Metab. Rev.* (2002) 34(1-2):47-54.

115. TSAI TH, LEE CH, YEH PH: Effect of P-glycoprotein modulators on the pharmacokinetics of camptothecin using microdialysis. *Br. J. Pharmacol.* (2001) 134(6):1245-1252.

116. KEMPER EM, VERHEIJ M, BOOGERD W, BEIJNEN JH, VAN TELLINGEN O: Improved penetration of docetaxel into the brain by co-administration of inhibitors of P-glycoprotein. *Eur. J. Cancer* (2004) 40(8):1269-1274.
• In this paper, the feasibility of using a Pgp inhibitor to boost the drug's brain bioavailability is demonstrated.

117. KEMPER EM, VAN ZANDBERGEN AE, CLEYPOOL C *et al.*: Increased penetration of paclitaxel into the brain by inhibition of P-glycoprotein. *Clin. Cancer Res.* (2003) 9(7):2849-2855.

118. TRAUNECKER HC, STEVENS MC, KERR DJ, FERRY DR: The acridonecarboxamide GF120918 potently reverses P-glycoprotein-mediated resistance in human sarcoma MES-Dx5 cells. *Br. J. Cancer* (1999) 81(6):942-951.

119. DANTZIG AH, SHEPARD RL, LAW KL *et al.*: Selectivity of the multidrug resistance modulator, LY335979, for P-glycoprotein and effect on cytochrome P450 activities. *J. Pharmacol. Exp. Ther.* (1999) 290(2):854-862.

120. MISTRY P, STEWART AJ, DANGERFIELD W *et al.*: *In vitro* and *in vivo* reversal of P-glycoprotein-mediated multidrug resistance by a novel potent modulator, XR9576. *Cancer Res.* (2001) 61(2):749-758.

121. WANDEL C, KIM RB, KAJIJI S, GUENGERICH P, WILKINSON GR, WOOD AJ: P-glycoprotein and cytochrome P450 3A inhibition: dissociation of inhibitory potencies. *Cancer Res.* (1999) 59(16):3944-3948.

122. ROWINSKY EK, SMITH L, WANG YM *et al.*: Phase I and pharmacokinetic study of paclitaxel in combination with biricodar, a novel agent that reverses multidrug resistance conferred by overexpression of both MDR1 and MRP. *J. Clin. Oncol.* (1998) 16(9):2964-2976.

123. MARTIN C, BERRIDGE G, HIGGINS CF, MISTRY P, CHARLTON P, CALLAGHAN R: Communication between multiple drug binding sites on P-glycoprotein. *Mol. Pharmacol.* (2000) 58(3):624-632.

124. POLLI JW, JARRETT JL, STUDENBERG SD *et al.*: Role of P-glycoprotein on the CNS disposition of amprenavir (141W94), an HIV protease inhibitor. *Pharm. Res.* (1999) 16(8):1206-1212.
•• By using whole-body autoradiography, the authors of this paper clearly demonstrated that Pgp residing at the BBB significantly restricted amprenavir exposure in the brain.

125. BREEDVELD P, PLUIM D, CIPRIANI G *et al.*: The effect of Bcrp1 (Abcg2) on the *in vivo* pharmacokinetics and brain penetration of imatinib mesylate (Gleevec): implications for the use of breast cancer resistance protein and P-glycoprotein inhibitors to enable the brain penetration of imatinib in patients. *Cancer Res.* (2005) 65(7):2577-2582.
• This study demonstrated that BCRP at the BBB might limit brain penetration of imatinib mesilate.

126. MALIEPAARD M, VAN GASTELEN MA, TOHGO A *et al.*: Circumvention of breast cancer resistance protein (BCRP)-mediated resistance to camptothecins *in vitro* using non-substrate drugs or the BCRP inhibitor GF120918. *Clin. Cancer Res.* (2001) 7(4):935-941.

127. LEE YJ, KUSUHARA H, JONKER JW, SCHINKEL AH, SUGIYAMA Y: Investigation of efflux transport of dehydroepiandrosterone sulfate and mitoxantrone at the mouse blood-brain barrier: a minor role of breast cancer resistance protein. *J. Pharmacol. Exp. Ther.* (2005) 312(1):44-52.
•• As the transport of two BCRP substrates (i.e., dehydroepiandrosterone sulfate and mitoxantrone) into the rat brain is not influenced by the modulation of BCRP activity, the author of this paper concluded that BCRP plays a minor role in restricting the drugs' brain bioavailability. More compounds need to be included in future studies to justify the efflux activity of BCRP at the BBB.

128. ADVANI R, FISHER GA, LUM BL *et al.*: A Phase I trial of doxorubicin, paclitaxel, and valspodar (PSC 833), a modulator of multidrug resistance. *Clin. Cancer Res.* (2001) 7(5):1221-1229.

129. BAUER KS, KARP JE, GARIMELLA TS, WU S, TAN M, ROSS DD: A Phase I and pharmacologic study of idarubicin, cytarabine, etoposide, and the multidrug resistance protein (MDR1/Pgp) inhibitor PSC-833 in patients with refractory leukemia. *Leuk. Res.* (2005) 29(3):263-271.

130. GREENBERG PL, LEE SJ, ADVANI R *et al.*: Mitoxantrone, etoposide, and

cytarabine with or without valspodar in patients with relapsed or refractory acute myeloid leukemia and high-risk myelodysplastic syndrome: a Phase III trial (E2995). *J. Clin. Oncol.* (2004) 22(6):1078-1086.

131. 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.
•• The authors of this article reported a novel non-invasive method to monitor the drug penetration into the human brain. This technique holds great promise in clinical study.
132. PLANTING AS, SONNEVELD P, VAN DER GAAST A *et al.*: A Phase I and pharmacologic study of the MDR converter GF120918 in combination with doxorubicin in patients with advanced solid tumors. *Cancer Chemother. Pharmacol.* (2005) 55(1):91-99.
133. MCALLISTER MS, KRIZANAC-BENGEZ L, MACCHIA F *et al.*: Mechanisms of glucose transport at the blood-brain barrier: an *in vitro* study. *Brain Res.* (2001) 904(1):20-30.
134. HALESTRAP AP, PRICE NT: The proton-linked monocarboxylate transporter (MCT) family: structure, function and regulation. *Biochem. J.* (1999) 343(Pt 2):281-299.
135. BOADO RJ, LI JY, NAGAYA M, ZHANG C, PARDRIDGE WM: Selective expression of the large neutral amino acid transporter at the blood–brain barrier. *Proc. Natl. Acad. Sci. USA* (1999) 96(21):12079-12084.
• A large neutral amino acid transporter (LAT) was identified from the bovine BBB.
136. ALLEN DD, SMITH QR: Characterization of the blood-brain barrier choline transporter using the *in situ* rat brain perfusion technique. *J. Neurochem.* (2001) 76(4):1032-1041.
137. KING GL, JOHNSON SM: Receptor-mediated transport of insulin across endothelial cells. *Science* (1985) 227(4694):1583-1586.
138. PARDRIDGE WM, EISENBERG J, YANG J: Human blood-brain barrier insulin receptor. *J. Neurochem.* (1985) 44(6):1771-1778.
139. PARDRIDGE WM, EISENBERG J, YANG J: Human blood-brain barrier transferrin receptor. *Metabolism* (1987) 36(9):892-895.
140. GOLDEN PL, MACCAGNAN TJ, PARDRIDGE WM: Human blood-brain barrier leptin receptor. Binding and endocytosis in isolated human brain microvessels. *J. Clin. Invest.* (1997) 99(1):14-18.
•• The data from this study provide evidence for a leptin receptor that mediates saturable, specific, temperature-dependent binding and endocytosis of leptin at the human BBB.
141. HUWYLER J, WU D, PARDRIDGE WM: Brain drug delivery of small molecules using immunoliposomes. *Proc. Natl. Acad. Sci. USA* (1996) 93(24):14164-14169.
142. PARDRIDGE WM: Tyrosine hydroxylase replacement in experimental Parkinson's disease with transvascular gene therapy. *NeuroRx* (2005) 2(1):129-138.
143. PARDRIDGE WM: Drug and gene targeting to the brain with molecular Trojan horses. *Nat. Rev. Drug Discov.* (2002) 1(2):131-139.
144. KREUTER J, ALYAUTDIN RN, KHARKEVICH DA, IVANOV AA: Passage of peptides through the blood-brain barrier with colloidal polymer particles (nanoparticles). *Brain Res.* (1995) 674(1):171-174.
145. KREUTER J: Nanoparticulate systems for brain delivery of drugs. *Adv. Drug Deliv. Rev.* (2001) 47(1):65-81.
146. OLIVIER JC, HUERTAS R, LEE HJ, CALON F, PARDRIDGE WM: Synthesis of pegylated immunonanoparticles. *Pharm. Res.* (2002) 19(8):1137-1143.
147. ZHANG Y, PARDRIDGE WM: Delivery of β-galactosidase to mouse brain via the blood–brain barrier transferrin receptor. *J. Pharmacol. Exp. Ther.* (2005) 313(3):1075-1081.
•• A large molecular weight enzyme was attached to an anti-TfR antibody through a biotin-avidin linker, and successfully delivered across the BBB via transcytosis.
148. HUWYLER J, YANG J, PARDRIDGE WM: Receptor mediated delivery of daunomycin using immunoliposomes: pharmacokinetics and tissue distribution in the rat. *J. Pharmacol. Exp. Ther.* (1997) 282(3):1541-1546.
• Daunomycin was encapsulated in immunoliposomes and delivered into brain. The tissue distribution study also revealed off-target delivery to other tissues such as the liver and kidneys.
149. DIETZ GP, BAHR M: Delivery of bioactive molecules into the cell: the Trojan horse approach. *Mol. Cell. Neurosci.* (2004) 27(2):85-131.
150. ZHANG X, WAN L, POOYAN S *et al.*: Quantitative assessment of the cell penetrating properties of RI-Tat-9: evidence for a cell type-specific barrier at the plasma membrane of epithelial cells. *Mol. Pharm.* (2004) 1(2):145-155.
151. ROUSSELLE C, SMIRNOVA M, CLAIR P *et al.*: Enhanced delivery of doxorubicin into the brain via a peptide-vector-mediated strategy: saturation kinetics and specificity. *J. Pharmacol. Exp. Ther.* (2001) 296(1):124-131.
152. MAZEL M, CLAIR P, ROUSSELLE C *et al.*: Doxorubicin-peptide conjugates overcome multidrug resistance. *Anticancer Drugs* (2001) 12(2):107-116.
153. ROUSSELLE C, CLAIR P, LEFAUCONNIER JM, KACZOREK M, SCHERRMANN JM, TEMSAMANI J: New advances in the transport of doxorubicin through the blood-brain barrier by a peptide vector-mediated strategy. *Mol. Pharmacol.* (2000) 57(4):679-686.
154. CAO G, PEI W, GE H *et al.*: *In vivo* delivery of a Bcl-xL fusion protein containing the TAT protein transduction domain protects against ischemic brain injury and neuronal apoptosis. *J. Neurosci.* (2002) 22(13):5423-5431.
•• Cell penetrating peptide (i.e., TAT) was linked to Bcl-xL as a fusion protein using recombinant DNA technology. Intraperitoneal injection of this fusion protein into mice resulted in significant delivery into the brain.
155. KILIC U, KILIC E, DIETZ GP, BAHR M: Intravenous TAT-GDNF is protective after focal cerebral ischemia in mice. *Stroke* (2003) 34(5):1304-1310.
156. SCHWARZE SR, HO A, VOCERO-AKBANI A, DOWDY SF: *In vivo* protein transduction: delivery of a biologically active protein into the mouse. *Science* (1999) 285(5433):1569-1572.
157. MILLER G: Drug targeting. Breaking down barriers. *Science* (2002) 297(5584):1116-1118.

Affiliation
Yaming Su[1] & Patrick J Sinko[†2] PhD, RPh
[†]Author for correspondence
[1]PhD candidate, Department of Pharmaceutics,
Ernest Mario School of Pharmacy, Rutgers,
State University of New Jersey,
160 Frelinghuysen Road, Piscataway,
NJ 08854, USA
[2]Parke-Davis professor of Pharmaceutics and
Drug Delivery, Chair, Department of
Pharmaceutics, Ernest Mario School of
Pharmacy, Rutgers,
State University of New Jersey,
160 Frelinghuysen Road, Piscataway,
NJ 08854, USA
Tel: +1 732 445 3831; Fax: +1 732 445 4271;
E-mail: sinko@rci.rutgers.edu