EXPERT REVIEW

Methodologies to Assess Drug Permeation Through the Blood–Brain Barrier for Pharmaceutical Research

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ABSTRACT The drug discovery process for drugs that target the central nervous system suffers from a very high rate of failure due to the presence of the blood–brain barrier, which limits the entry of xenobiotics into the brain. To minimise drug failure at different stages of the drug development process, new methodologies have been developed to understand the absorption, distribution, metabolism, excretion and toxicity (ADMET) profile of drug candidates at early stages of drug development. Additionally, understanding the permeation of drug candidates is also important, particularly for drugs that target the central nervous system. During the first stages of the drug discovery process, *in vitro* methods that allow for the determination of permeability using high-throughput screening methods are advantageous. For example, performing the parallel artificial membrane permeability assay followed by cell-based models with interesting hits is a useful technique for identifying potential drugs. *In silico* models also provide interesting information but must be confirmed by *in vitro* models. Finally, *in vivo* models, such as *in situ* brain perfusion, should be studied to reduce a large number of drug candidates to a few lead compounds. This article reviews the different methodologies used in the drug discovery and drug development processes to determine the permeation of drug candidates through the blood–brain barrier.

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ABBREVIATIONS

ADMET	Absorption, distribution, metabolism, excretion and toxicity
AJ	Adherens junctions
AMT	Adsorptive-mediated transcytosis
ATP	Adenosine triphosphate
BBB	Blood–brain barrier
BCRP	Breast cancer resistance protein
CNS	Central nervous system
CSF	Cerebrospinal fluid
GLUT	Glucose transporter
LAT	Large aminoacid transporter
LDL	Low density lipoprotein
MRP	Multidrug resistance associated protein
NCE	New chemical entities
OATP	Organic anion transporter protein
PAMPA	Parallel artificial membrane permeability assay
PEG	Polyethylene glycol
P-gp/ABCB1	P-glycoprotein
PK	Pharmacokinetics
PVDF	Polyvinylidene fluoride
RMT	Receptor-mediated transcytosis
TEER	Transendothelial resistance
TJ	Tight junctions
UWL	Unstirred water layer

INTRODUCTION

The high attrition rate of drug candidates during all stages of the drug development process is a critical issue for both economic and treatment reasons. It has been shown that

the major factor leading to the attrition of new chemical entities (NCEs) during drug development does not necessarily result from a lack of drug activity but is rather a result of inadequate pharmacokinetic (PK) properties (1). Particularly for central nervous system (CNS) diseases, the lack of permeation through the BBB prevents the active compound from reaching its target. Because of the huge costs associated with bringing a drug to market, it is important to fully characterise the ADMET (absorption, distribution, metabolism, excretion, toxicity) profile of candidate drugs as early as possible during the drug discovery process. A thorough ADMET evaluation decreases the risk of attrition during clinical phases or even possible withdrawal from the market (2). ADMET issues are even more important for drug candidates that target the central nervous system (CNS). Only 3 to 5% of CNS drug candidates that enter phase I clinical trials are successfully launched compared to approximately 10% for all compounds (2,3). The particular organisation of endothelial cells, which are connected by tight junctions and form the blood–brain barrier (BBB), is a further obstacle to the CNS penetration of drug candidates. Combined with numerous transporters, such as efflux and uptake transporters, and drug-metabolising enzymes present at the luminal side of the BBB, penetration of the BBB is a treatment issue for CNS diseases, such as Alzheimer's disease, Parkinson's disease and Huntington's disease. By contrast, when other organs are targeted, it is critical that BBB penetration is either null or reduced to limit adverse effects. Therefore, determining the distribution of a drug in and around the brain is important when developing a new compound.

Different methodologies exist to evaluate the permeation potential of new chemical entities. The choice of strategy relies primarily on the type of throughput (driven by the number of compounds that require testing) and the type of information needed. This implies that scientists should master these strategies to choose the proper methodology based on the information required, and they should be able to correctly interpret the results. Here, we review the different methods available for physicochemists, biologists and ADMET scientists at different stages of the drug discovery and development processes to select drug candidates that penetrate the BBB. After describing the physiology of the blood–brain barrier, in *silico*, *in vitro* and *in vivo* approaches to determine BBB permeation will be explored. Drawbacks and advantages will be critically examined, and key experimental and/or interpretation points will be highlighted. Finally, because an increasing number of strategies to enhance drug penetration require complex formulations, such as micro/nano-carriers, application of these different screening methods to modern drug development efforts will be discussed.

PHYSIOLOGY OF THE BLOOD–BRAIN BARRIER (BBB)

In 1885, the studies of Paul Ehrlich first highlighted the presence of the BBB. In his studies, Ehrlich intravenously injected various dyes, and he observed that almost the entire body was stained but not the brain (4,5).

Edwin Goldman, a student of Ehrlich's, continued this research using the dye trypan blue. He found that after intravenous injection of the dye, the choroid plexus and meninges were stained; however, no dye was recovered in either the brain or cerebrospinal fluid (CSF) (6). In another experiment, he injected the dye directly into the CSF and found that the entire brain was stained but not the rest of the body (7). These experiments demonstrated the existence of a biological barrier between the brain and the rest of the systemic circulation, the blood–brain barrier.

Over many years of research, our knowledge of the BBB has increased, and scientists are now aware that the BBB is a structure with complex cellular organisation that separates the brain parenchyma from the systemic circulation. It is of key importance for the maintenance of brain homeostasis, which is essential for good neuronal and synaptic activities (8–12), and represents the main route by which compounds reach the CNS. Moreover, the BBB also acts as a metabolic barrier due to the presence of numerous enzymes (13,14), including peptidases, γ-glutamyl transpeptidase (γ-GT), alkaline phosphatase (ALP), nucleotidases, cytochromes P450 (CYP450) and monoamine oxidase (MAO). These enzymes can either metabolise potentially harmful drugs to inactive CNS compounds, convert an inactive drug to its active CNS metabolite or degrade them into metabolites or substrates of specific efflux transporters, such as the P-glycoprotein or multidrug resistance proteins.

The BBB consists of brain capillaries that support endothelial cells and are surrounded by astrocytic end-foot processes (15). It is the central part of the neurovascular unit, which is responsible for communication between endothelial cells, astrocytes, pericytes and neurons (14).

Endothelial Cells and Tight Junctions

The specificity of the endothelial cells comprising the blood–brain barrier compared to the endothelial cells in the rest of the body is based on their organisation. Cerebral endothelial cells are connected by intercellular proteins. Occludins, claudins and junctional adhesion molecules, together with cytoplasmic accessory proteins, including zonula occludens-1 (ZO-1), ZO-2, ZO-3 and others, are transmembrane proteins that are responsible for the formation of tight junctions (TJs) (14) that seal the paracellular pathway (16–18) and make the brain nearly inaccessible to polar compounds that are not the substrates of specific transporters (19). Adherens

junctions (AJs) also contribute to the junction complex by joining the membrane proteins, cadherins, to the intermediary proteins, catenins, to form adhesive linkages between endothelial cells (20). The TJs in cerebral capillaries are approximately 50 to 100 times tighter than the TJs in peripheral capillaries (21) and lead to a high transendothelial electrical resistance (TEER) of approximately $1,500–2,000\,\Omega.cm^2$ compared to $3–33\,\Omega.cm^2$ for other tissues (22,23), which is due to the restriction of small ions, such as Na^+ and Cl^-, from passing through the TJs. Moreover, BBB endothelial cells differ from other endothelial cells in the low number of endocytotic invaginations at the luminal portion of the cell membrane, which leads to very limited pinocytic transcellular transport (12,20,24–28), a large number of mitochondria (29) and the polarised expression of transporters and receptors for active transport (30). Indeed, the brain endothelium has only 3–6 pinocytic vesicles *per* μm^3 compared to 82–93 *per* μm^3 for the peripheral endothelium (31,32).

Many transmembrane proteins are expressed on the luminal and abluminal membranes of the endothelium to transport nutrients that are essential for the brain and to eliminate waste products of metabolism. In particular, proteins, such as GLUT-1 (glucose transporters), transport polar nutrients; Na-ATPase and K-ATPase transport sodium and potassium ions respectively; insulin or transferrin receptors transport proteins (8,24,28,33,34), and organic anion transporting proteins (OATP) (35) transport hormones, opioids, steroids, statins, cardiac glycosides, anticancer drugs and antibiotics (15). These transporters all play important roles in the maintenance of cerebral equilibrium. Moreover, efflux transporters, such as P-gp/ABCB1(36) or BCRP (37) are highly expressed on the luminal side of endothelial cells.

Astrocytes, Pericytes and Basal Lamina

Astrocytes are important cellular constituents of the neurovascular unit. They are linked to interneurons and thus the entire cerebral microenvironment (19). Astrocytes represent approximately 50% of the total mammalian brain volume (38). Moreover, some studies indicate that astrocytes play a role in the upregulation of BBB properties, such as tighter tight junctions (39,40) and the expression of specific polarised transporters, such as P-gp/ABCB1(14) and the enzymatic system (14,19), due to astrocyte-endothelial cell interactions. This upregulation of BBB properties by astrocytes is synergistic with pericytes, neurons and perivascular macrophages (41). Astrocytes also have different functions, such as the formation of and activity at synapses, energetic and redox metabolism, intercellular communication, homeostasis (38) and glucose transport from the systemic circulation to the brain.

Similar to astrocytes and neurons, pericytes are part of the neurovascular unit and play a role in the maintenance of both BBB properties and cerebral homeostasis. Recent studies have also indicated a role for pericytes in haemostasis, as well as in immune and phagocytic processes (20,42). Pericytes surround endothelial cells and both cell types are supported by the basal lamina. The integrity of basal lamina allows the maintenance of BBB properties, due to its anchoring role, but the basal lamina does not have a significant impact on the permeability of the BBB. However, under specific pathological conditions or in response to an aggressive stimulus, its thickness can vary, which perturbs the normal function of the BBB (42).

MECHANISMS OF TRANSPORT THROUGH THE BBB

The BBB is one of the most important barriers in the body. The permeation of drugs through the BBB is subject to strong selection depending on the physicochemical properties of the compound, such as lipophilicity, molecular weight, permeability coefficient ($logP_e$), molecular volume, ionisation state, and/or their affinity to specific transporters(efflux or uptake transporters) that are present in the cellular space (43–45). Therefore, the BBB may not be as impermeable as indicated by the first experiments with dyes. The cellular organisation of the BBB and the presence of transmembrane proteins enable a selective regulation of the passage of molecules from the blood to the brain. This is of particular importance for the uptake of essential nutrients or active CNS drugs and protects the brain from undesirable compounds, which could be toxic to the CNS.

The specificities of brain capillaries makes of the BBB an effective and efficient barrier that limits the entry of xenobiotics into the brain. Molecules present in the blood stream can reach the CNS by two different pathways, the paracellular pathway, which is between 2 endothelial cells through the tight junctions, or the transcellular pathway, which is through an endothelial cell.

Molecules that reach the CNS *via* the transcellular pathway can diffuse passively, can be actively transported by specific transporters or can undergo endocytosis. For example, small lipophilic molecules, such as O_2 and CO_2, or very small compounds, such as ethanol, water or diverse lipophilic drugs, can freely diffuse through the lipid barriers of endothelial cells (19).

Paracellular Pathway

The paracellular pathway is a diffusion process that occurs between 2 cells. This pathway is limited to small hydrophilic molecules such as cimetidine, ranitidine, famotidine (46,47) and furosemide (48), which are hypothesised to be absorbed *via* the paracellular pathway in the intestinal track, due to the

aqueous surroundings of the cells (49). However, due to the presence of tight-junctions between two cerebral endothelial cells, this route is extremely limited and nearly non-existent at the BBB, although under some pathological conditions, tight junctions and adherens junctions between endothelial cells may be altered. This alteration enables leakage, which can allow passage of plasma proteins, fluids or immune cells into the brain (12,19,50–55). The selectivity of this route is limited to either size or shape features.

Transcellular Pathway

Complex tight junctions force therapeutic molecules to follow a transcellular pathway through the BBB rather than the paracellular pathway as in most endothelia (19).

Passive Diffusion at the BBB

Passive diffusion is one of the most straightforward mechanisms of permeation. This process requires a concentration gradient but no energy and no specific protein carriers. Diffusion requires physicochemical interactions between the compound and the membrane that must be crossed (43,44). Moreover, because there is no specific binding site, passive transport is not affected by stereochemistry and there is no saturation and no possible inhibition of the diffusion process (56). These observations indicate that passive diffusion is concentration-independent: the process occurs till equilibrium between the blood and the brain.

Passive diffusion through the BBB is highly affected by lipophilicity and the size of the compound (57–59). It was demonstrated that compounds with a molecular weight greater than 500–600 Da poorly permeated the BBB (60,61). However, when combined with good lipid solubility, molecules with molecular weights greater than 500 Da have interesting BBB permeability characteristics (62). Similarly, the lipophilicity of compounds should be high enough to allow for good affinity with lipidic membranes, but the lipophilicity should not be too high so as to avoid trapping of the compound in the membrane and bioaccumulation. By contrast, due to the hydrophobic nature of the membrane, ionisation will greatly impact diffusion because ionised compounds are highly hydrophilic and therefore have poor interactions with the membrane (63).

Carrier-Mediated Transport at the BBB

A certain number of uptake or efflux proteins are expressed at the BBB. These transporters are present on the luminal and abluminal membranes of the endothelium and regulate the entry of their specific substrates (19,64). Uptake proteins transport molecules from the blood to the brain. These transport systems allow the permeation of essential cerebral nutrients, such as glucose or amino acids, and either limit or prevent the passage of undesired or potentially toxic molecules. By contrast, efflux proteins, such as P-glycoproteins (P-gp/ABCB1), multidrug-resistance multidrug resistance associated proteins (MRP) or the breast cancer resistant protein (BCRP) (65), excrete their substrates out of the brain by pumping the substrates into the blood stream. At the BBB, P-gp/ABCB1 are highly expressed and a certain number of NCEs are substrates for this protein (56).

Carrier-Mediated Uptake. Carrier-mediated transport can be either active or facilitated. When the transport of a substrate needs either direct energy which requires ATP binding and hydrolysis to mediate the primary active transport process, such as transporters of the ABC superfamily, or indirect energy, which is driven by ion gradients that result from ATP-dependent primary transport, such as many transporters of the SCL superfamily, it is active carrier-mediated transport, whereas when transport requires only a concentration gradient and a transporter protein, it is facilitated carrier-mediated transport. Both types of transport are saturable, competitive and stereospecific (56). Moreover, these types of transport imply a specific interaction between the carrier proteins and the substrate.

Some examples of transporters are the glucose transporter (GLUT-1), the monocarboxylic acid transporter (MCT1), the large neutral amino-acid transporter (LAT1) and the organic anion transporters (OATP) (32,66). Specific transporters are also present for small ions, such as Na^+, K^+ or Cl^-, in both the blood to brain and brain to blood directions. These ion transporters maintain brain homeostasis because ionic disequilibrium between the blood and brain can have serious effects, such as brain oedema.

Efflux Transport. Efflux transport is an energy-dependant, active process, which pumps xenobiotics and metabolites out of the brain into the blood stream. The most well-known and studied efflux proteins belong to the ATP binding cassette (ABC) family, including the P-glycoproteins (P-gp/ABCB1), which are encoded by the multidrug resistance gene 1 (MDR1/ABCB1), the multidrug resistance associated protein (MRP) and the breast cancer resistance proteins (BCRP). These active transport processes are essential for brain protection because they prevent the cerebral penetration of potentially harmful drugs and also excrete waste products and metabolites. The expression of most efflux proteins is regulated by astrocytes or pericytes (67).

Trans- and Endocytosis Mechanisms

Brain penetration is not strictly limited to small lipophilic molecules or compounds shuttled by uptake proteins. Larger

molecules, such as peptides, proteins or even viruses, which are too large for a carrier-mediated process, can also penetrate the BBB *via* the few pinocytic vesicles that are present in endothelial cells. These large molecules can be transported either by receptor-mediated transcytosis (RMT), adsorptive-mediated transcytosis (AMT) (10) or fluid phase endocytosis (32).

During RMT, the ligand specifically binds to the receptor protein and is transported through the cell. The best characterised and utilised RMT protein is most likely the transferrin receptor, which has also been extensively studied for the delivery of immunoliposomes. Other well-known receptors include the low-density lipoprotein (LDL) receptor and the insulin receptor (32).

During AMT, a non-specific interaction occurs between the solute and the surface protein. Peptides, glycopeptides, glycoproteins, and viruses are transported by this pathway (32).

During a fluid phase endocytosis event, there is no contact between the solute and the protein. The substrate is situated close to the membrane, which deforms and encircles both the solute and some extracellular fluid and transports the entire vesicle to the abluminal side. Lucifer yellow is transported in this manner (32).

STRATEGIES FOR IMPROVING BRAIN PENETRATION

The BBB is a serious obstacle for the treatment of neurodegenerative diseases that require CNS action (43). Because of its physical organisation, the BBB prevents the passage of many drugs that target the CNS. Therefore, even if a potential drug has potent activity against its target, it may not be able to cross the BBB and will most likely be discarded during the drug development process. Moreover, metabolic features of the BBB may also prevent a CNS active drug from crossing the endothelial membrane because the therapeutic efficacy of the drug can be either inactivated or decreased by enzymes at the BBB. To circumvent the BBB and allow an active CNS compound to reach its target, many strategies exist, which may be either invasive or non-invasive with respect to the BBB.

Invasive Techniques

Direct Injection into the Cerebrospinal Fluid

Direct injection of drugs into the cerebrospinal fluid was the first strategy used to circumvent the BBB, primarily to target brain tumours. This technique is not very efficient because there is a poor diffusion between the cerebrospinal fluid and the brain and it is quite invasive (68). Nau *et al.* demonstrated a 3-fold increase in the mortality of infants with Gram-

negative meningitis treated with an intraventricular injection of aminoglycosides combined with intravenous injections of antibiotics compared to intravenous injection of antibiotics alone (68).

Therapeutic Opening of the BBB

Therapeutic opening of the BBB is a reversible process. Because of specific molecules which generate a hyperosmolar environment, the BBB loses its barrier properties, thus enabling passage of the therapeutics into the brain before the BBB regains its functions. A transient brain opening is generally obtained by intra-carotid injection of mannitol or alkyl glycerol, which creates hyperosmolar conditions on the systemic circulation side of the BBB and causes a reversible shrinkage of the endothelial cells and a loss of adherens and junctional proteins, leading to a paracellular opening between endothelial cells (69,70). However, depending on the mediator used to momentarily disrupt the BBB, an increase in transcellular permeability can also occur, such as with tumour necrosis factor α, which leads to the permeation of opportunistic toxic compounds. Moreover, the duration of the opening of the BBB will depend on the mediator used. Histamine provides a rapid and temporary opening, whereas thrombin causes drastic modifications of the endothelial cytoskeleton resulting in prolonged opening of the BBB with difficulties in returning to the basal state (51).

This difficult strategy must be handled with care and vigilantly monitored to prevent damage to the brain parenchyma and oedema, which may be fatal. However, when performed properly, therapeutic opening of the BBB allows for the delivery of active drugs into the CNS, which would not otherwise reach the brain. This strategy is primarily used for the treatment of brain tumours or life-threatening diseases that have not been cured with less invasive treatments.

Non-Invasive Techniques

Brain penetration can be improved either using an alternative administration route, inhibiting efflux transporters, chemically modifying or encapsulating the active compound.

The Nose-to-Brain Route

To circumvent the BBB and enter the brain parenchyma, alternative strategies for drug delivery, such as the nose-to-brain route, are useful. In the nose-to-brain pathway, the therapeutic compound can be directly transported to the brain by absorption in the nasal mucosa and transport *via* the olfactory routes (71,72). Therefore, localisation of the olfactory route close to a brain region that is exempt of BBB allows for the circumvention of the barrier, which allows the

drug to reach the CNS (73). This route has been evaluated for the permeation of cocaine (74), as well as formulations such as the alprazolam-loaded solid lipid nanoparticles (75) or even neuropeptides (76). This strategy suffers primarily from poor bioavailability, which ranges from 0.01% to 0.1% (72).

Inhibition of Efflux Transporters

The presence of numerous efflux transporters at the BBB prevents the entry of many CNS active compounds into the brain. In HIV treatment, the most efficient drugs, such as abacavir and efavirenz, are substrates of the ABC transporters. Therefore, an interesting strategy is to inhibit efflux transporter activity and saturate these transporters with substrates that have higher affinity than the drug (77). This strategy is efficient in HIV multi-therapy and improves the intracerebral concentration of HIV protease inhibitors (33). However, this strategy may have several drawbacks because inhibition of efflux transporters will allow the penetration of other xenobiotics, which may be potentially toxic in the CNS. Therefore, adverse side effects may occur using this strategy.

Use of Prodrugs

Pharmacology-based strategies are methods to either enhance the lipophilicity of a drug candidate to favour its passive permeation (78) or to mask the specific site recognised by efflux transporters. The primary goal of this strategy is to promote the permeation of compounds that have either low uptake or are substrates of efflux transporters in their native form. The addition of moieties to the drug, which are linked by covalent reversible bonds, allows for the physicochemical modification of the active compounds to cross the BBB. For example, dopamine, a treatment for Parkinson's disease, cannot cross the BBB and enter the central nervous system where its target is located. Therefore, carbonylation of dopamine allows for the active transport of the inactive prodrug form through the BBB. After the prodrug has entered the brain, DOPA decarboxylase activates L-Dopa into active dopamine. This strategy also permits the creation of a drug-reservoir, depending on the rate of liberation of the native active compound. This approach is, therefore, an asset for patient compliance. However, chemical modification of the native active drug may decrease its activity or bioavailability.

The Trojan Horses or the BBB Shuttles

The concept of a Trojan horse consists of coupling the drug of interest, which cannot penetrate the BBB, to a compound, such as a molecule, peptide, or transferrin, that crosses the BBB *via* an active process uptake transporter, such as the glucose transporter (GLUT-1) or transferrin receptor. The BBB-penetrating moiety is recognised by the specific receptor, leading to transport of the entire molecule, including the drug (78). An extension of this concept was proposed by Malakoutikhah and co-workers (79) who designed peptidic Trojan horses that were able to cross the BBB *via* passive diffusion. These compounds were defined as BBB shuttles. The challenge of both Trojan horses and BBB shuttles is to then liberate the active drug from the vector.

Drug Delivery with Nanocarriers

Liposomes, polymeric nanoparticles, solid lipid nanoparticles and micelles are all nanocarriers and have garnered great interest in recent pharmaceutical research. Because of the incorporation of a drug into the inside core of the nanocarrier, the drug bioavailability, physicochemistry and pharmacokinetics of the drug are changed (80,81). In pharmaceutical research, a well-known problem is the discovery and development of highly potent lead compounds, which are then found to be either insoluble or poorly soluble. In most cases, either the molecule will be discarded from the drug development process, or a ligand strategy will be used to enhance the solubility of the potential drug, with the risk of decreasing its potency.

Drug delivery is a method of bypassing poor solubility, poor permeability or poor bioavailability by incorporating the compound of interest into either phospholipidic, polymeric or inorganic vesicles (82).

Liposomes consist of a phospholipid bilayer; therefore, they allow for the incorporation of either hydrophilic molecules, on the inside core, or lipophilic molecules, inside the bilayer (82). These liposomes are extensively studied and highly promising nanocarriers, particularly for cancer therapy. Caelyx®, a pegylated liposomal formulation of doxorubicin that targets breast cancer cells, is a good representative of the success of these formulations (83,84).

Three generations of liposomes have now been developed. The first generation consists only of a vesicle formed by a phospholipid bilayer. These types of liposomes are rapidly recognised by the reticuloendothelial system and eliminated. Therefore, their efficacy is very limited and not applicable for pharmaceutical purposes. The second generation of liposomes is surrounded by polyethylene glycol, which is covalently linked to the outer part of the vesicle. These pegylated liposomes have a longer circulation time in the body because PEG is not recognised as a foreign body by the immune system. The third generation of liposomes is the most potent generation of liposomes. These liposomes consist of the same pegylated liposomes as the second-generation liposomes but are functionalised with specific moieties, such as monoclonal antibodies, added to the PEG chain. Therefore, the modified liposome is recognised by the

antibody-specific receptor and may be taken up by the cell. This strategy allows for specific targeting of cells. For example, immunoliposomes, grafted with OX26 monoclonal antibody are able to recognize transferrin receptor at the BBB, which transport it through the a rat BBB model *via* endocytosis (85). Up to now, many immune-conjugated nanocarriers are on clinical phases such as doxorubicin, anti-REH-2 (86), but none has launched the market yet. Future years will probably disclose numerous new formulations aiming at treating CNS pathologies.

The major drawback of liposomes is their poor stability, which is due to their tendency to aggregate and their sensitivity to oxidation and hydrolysis. Some of these problems can be reduced by formulation strategies, such as the addition of α-tocopherol to decrease oxidation (87). Other researchers have formulated liposomes as proliposomes, a dry granular product, which disperses to form multi-lamellar vesicles upon the addition of water (88).

Nanoparticles as drug carriers have also been extensively studied recently. Their uptake into the brain is hypothesised to occur *via* receptor-mediated endocytosis (89,90). Because unmodified nanoparticles have been shown to be rapidly cleared by the reticuloendothelial system, within 5 min in a mouse model, surfactants or covalent binding of polyethylene glycol on the polymeric core led to a prolonged circulation time and improved bioavailability (91). Only a few nanoparticle formulations of drugs are currently on the market (92), such as Rapamune®, an immunosuppressant drug. Promising results have been obtained in preclinical studies of a glioblastoma rat model, using doxorubicin-incorporated nanoparticles; however, no CNS-targeting nanoparticles are currently available in the market. Nanoparticles can either be polymeric, lipidic or inorganic. The safety profile of these vesicles is controversial, and much research is necessary to fully describe the mode of excretion, the possible accumulation of particles in organs and the side effects caused by these nanoparticles.

METHODS TO ASSESS BBB PERMEABILITY INFORMATION IN DRUG RESEARCH

In Silico Models That Predict BBB Permeability

In silico models are used during the early stages of the drug discovery process when thousands of compounds must be screened for either interactions with a specific target or for the appropriate physicochemical properties. For example, Lombardo *et al.* (93) succeeded in predicting the blood–brain partitioning of compounds (log BB) using the calculated solvation free energy. Others correlated log BB with a combination of the molar refraction, solute polarisability,

hydrogen bond donor or acceptor capacity and molecular volume (94,95). *In silico* strategies can filter large databases to preselect compounds of interest and can predict whether a compound will be prone to BBB penetration or not (96). These computational strategies can decrease the number of molecules to only few hit compounds, which are then tested with *in vitro* models to determine the pharmacokinetics and mechanism of action of the drug.

In silico models combine the measured brain penetration information that is available in the literature with molecular properties to build an algorithm that can predict BBB permeability. Using partial least squares regression, multiple regression analysis or neural networks, *in silico* models can generate ponderated regressions consisting of different physicochemical properties, such as the lipophilicity (log P), standard free energy, H-bond donating capacity, H-bond accepting capacity, and molecular weight of the drug.

In practice, the initial data, which are obtained from libraries, are divided into 2 subtest sets, a training set, which is used to build the algorithm, and a test set, which allows for determination of the predictive ability of the algorithm. The experimental permeabilities of the test set are statistically compared to the predicted values that are generated by the algorithm to determine this predictive power (96). The variety of algorithms that are able to build a predictive model is huge because many descriptors may be used to generate an equation. Some examples of *in silico* models to predict log BB are listed in a review of Abraham (96). In general, the chosen descriptors are related to the size of the molecules and their physicochemical properties. As Abraham noted, an increase in size-related descriptors leads to an increased log BB (higher brain penetration), whereas an increase in the polarity-related descriptors leads to a decreased log BB. This characteristic is linked to the physicochemistry of both compartments, with the brain being more lipophilic than the blood. Moreover, Didziapetris *et al.* (97) suggested that acids with a pKa > 4, containing greater than 8 oxygen and nitrogen atoms and a molecular weight greater than 400 Da were likely to be substrates of efflux proteins.

A major issue resulting from *in silico* models is the reliability of the chosen training sets and test sets. It is difficult to obtain experimental data that are homogeneous in terms of the experimental design, such as whether the perfusion was performed with whole blood, plasma or saline solutions, the reliability of descriptors, such as whether there was ionisation, and the experimental know-how. The ideal situation would be to obtain the experimental data from the same laboratory, under the same conditions; however, that situation is utopic because the amount of data would be either too low to build the model or not diverse enough. Therefore, attention should be paid when handling *in silico*-predicted data, and the user must also understand how the model was built.

In Vitro Models for Prediction of BBB Permeability

Different approaches have been proposed for the *in vitro* evaluation of whether a new chemical entity can cross the BBB. Significant differences exist from one method to another in terms of complexity and, consequently, cost and information obtained (Fig. 1). In this section, *in vitro* methods for predicting BBB permeability are ordered according to increasing complexity.

Immobilised Artificial Membrane

Lipophilicity (log P or log $P_{octanol/water}$) has long been reported to be a major parameter that influences CNS activity and the blood/brain concentration ratio (log BB) (98,99). Because of its lipophilic feature, n-octanol was widely used for pharmacokinetic predictions. The small polar head of n-octanol and its hydrophobic carbonylated chain make it appear similar to phospholipidic membranes. However, the relationship between log P and log BB is not strong enough for the needs of CNS researchers. Therefore, immobilised artificial membranes (IAMs) were proposed as an alternative to log P predicted with shake flask or liquid chromatography (100–103). Modified HPLC columns were prepared in which phospholipids were covalently bound to the silica (99). Experiments showed a linear relationship between the retention factors on IAMs and the partition between an

aqueous phase and liposomes (104). Moreover, IAM chromatographic retention factors were shown to generate information on membrane permeability (105). In some cases, these types of systems have shown reasonable results for permeability prediction, even if the retention time on the column does not reflect transport across the membrane (106,107). However, there are some limitations in terms of retention times for lipophilic compounds and stationary phase stability. Recently, short IAM columns appeared on the market. These columns (1–3 cm *versus* 10–12 cm) allow greater throughput but do not really offer any improvements in terms of reliability, and this approach is not widely used in drug discovery.

PAMPA Models

The parallel artificial membrane permeability assay (PAMPA) is a relatively recent technique developed in 1998 by Kansy *et al.* (108) to rapidly predict passive permeability through the gastrointestinal tract with high throughput efficiency. In this technique, a donor and an acceptor compartments are separated by a filter supporting a liquid artificial membrane. The drug to be tested, placed in the donor compartment, can then permeate between the donor and the acceptor compartments through the artificial membrane. The assay is performed in 96-well plates and thus enables high throughput screening. The permeability coefficient is then determined in a
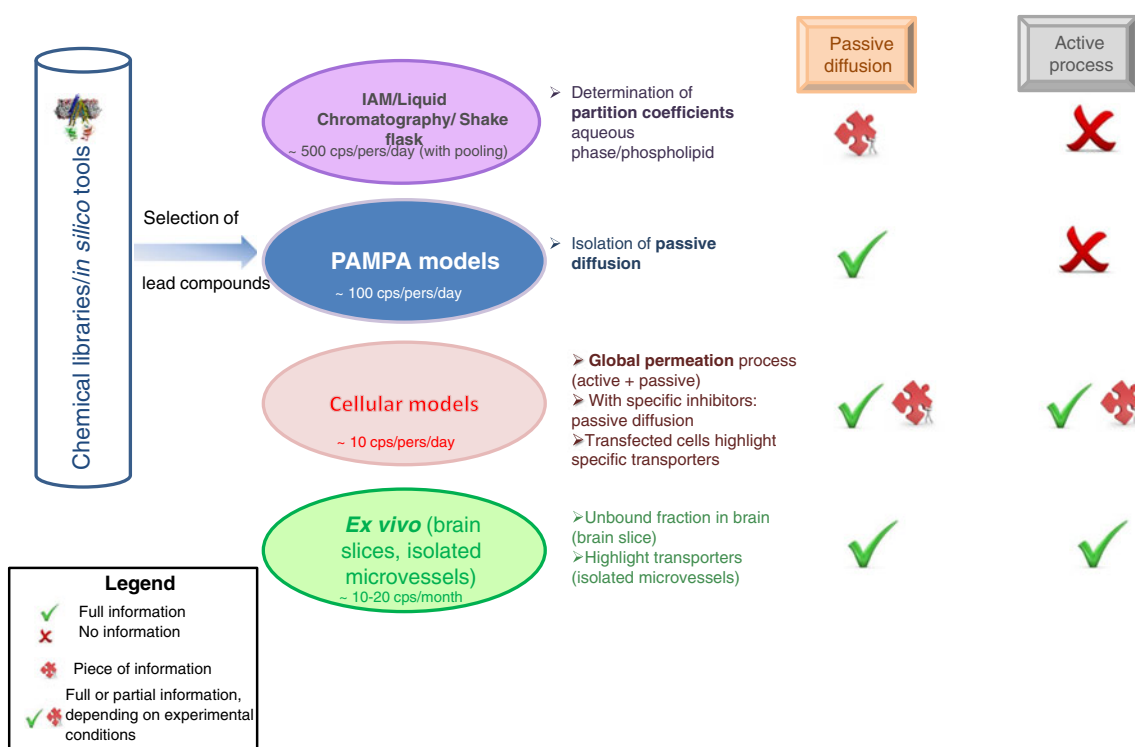

Fig. 1 Useful *in vitro* models to predict BBB permeability in early drug discovery process, and type of information generated.

straightforward manner with the permeability equation described by Avdeef (63). The artificial membrane that was originally made of phospholipids (56,109) can also be as simple as an organic solvent (110) or a mixture of solvents (111).

PAMPA is gaining interest in the early drug discovery process because it is possible to rapidly obtain the straightforward permeabilities of numerous compounds, it is cost efficient, it has high inter- and intra-laboratory reproducibility and it can target different biological membranes. The major drawback of the PAMPA technique is that it can only predict passive diffusion and is therefore unable to generate a full description of the permeability process at the BBB. It is well-known that numerous transporters and enzymes are expressed at the BBB level, greatly modifying the pharmacokinetics of the substrates of these transporters, such as verapamil, a P-gp/ABAB1 substrate. Therefore, for these specific compounds, PAMPA generates only a portion of the information regarding passive transcellular transport. PAMPA should not be used for compound selection purposes particularly for BBB penetration, because of the numerous transporters and metabolic enzymes that are present at the BBB. P-gp/ABCB1 substrates more permeate in a PAMPA model compared to *in vivo* or *in vitro* cellular models.

Phospholipid-Based Membrane. Developed in 2003 by Li Di *et al.* (112), PAMPA-BBB has shown good prediction of BBB penetration for CNS classes of drugs. The drugs are described as CNS+ for compounds that have a high penetration through the BBB, CNS- for compounds that have a low permeability or are unable to penetrate the BBB, and CNS+/− for compounds with a medium permeability coefficient. In this assay, the artificial membrane is composed of porcine polar brain lipid extracts and the incubation last for 18 h. A quantitative analysis correlating the permeability coefficients generated with PAMPA-BBB and *in situ* brain perfusion gave a poor correlation coefficient of $r^2 = 0.47$ (113,114) for a test set of 37 compounds. However, for 30 test compounds, this assay succeeded in predicting 25 compounds with the correct class of permeability, either CNS+ or CNS-. The only negative outlier was actively transported, whereas the 4 false positives were either substrates of efflux pumps or metabolised *in vivo*. One weak point of this assay is that it is performed under unstirred conditions, which maximises the unstirred water layer (UWL). UWL is a stagnant water layer at the two sides of the artificial membrane that has a distinct boundary with bulk water (63). This layer can highly modify the permeation of compounds, in particular hydrophobic compounds, because passage through the UWL is governed by diffusion laws. Because of the blood flow and the very small size of the cerebral capillary, the *in vivo* UWL is nearly null. However, this is not the case for the *in vitro* UWL, particularly for the unstirred *in vitro* assay in which the UWL can be as thick as 1.5 to 2.5 mm (115).

Solvent-Based PAMPA. HDC-NPOE PAMPA is a high throughput screening method that has recently been developed to predict the passive transendothelial permeability through the BBB and is currently under study (116). The innovation of this PAMPA technique is in the use of non-biological materials for the membrane, which allows for better reproducibility. In this new PAMPA model, the artificial membrane is composed of 75% hexadecane and 25% o-nitrophenyl octyl ether and incubation lasts for 7 h, enabling permeability determination within the working day. The experimental permeabilities determined by this new method demonstrated a good relationship with the permeabilities generated by a well-established cellular model BBCEC model from Cecchelli *et al.* (27,117) ($r^2 = 0.92$; $N = 13$) and the PAMPA-BBB (112) ($r^2 = 0.80$: $N = 14$). Furthermore, a correlation was also established with an *in vivo* model of rat brain perfusion (118) ($r^2 = 0.81$; $N = 6$) but only with a reduced number of compounds.

Compatibility of PAMPA with New Drug Delivery Strategies. In 2009, Han *et al.* (109) used PAMPA to determine the intestinal permeation of ginsenoside, a hydrophilic molecule with very low membrane permeation, that was incorporated into a water-in-oil microemulsion. The objective was to obtain information on the mechanism of permeability of this carrier system. The original protocol of Kansy (108) using a phospholipidic-based membrane was used. Han *et al.* demonstrated an increased permeation of ginsenoside due to good permeation of the water-in-oil microemulsion through the PAMPA membrane. These results were corroborated by rat everted intestinal sac studies.

In 2007, Mathot *et al.* (119) used the phospholipidic PAMPA developed by Kansy (120) and commercialised by pION Inc. to evaluate the passive diffusion of polymeric micelles formed from polymeric surfactants through the gastrointestinal tract. They had already shown in the Caco-2 model that the polymers were able to cross the cells but did not know the possible mechanism of passage. Analysis of the acceptor compartment demonstrated the passage of the polymeric micelles through the lipid artificial membrane with a permeability coefficient of 1.0×10^{-6} cm.s^{-1}. To the best of our knowledge, no current study has evaluated the prediction of BBB permeation of these materials using the solvent-based PAMPA technique. However, the results obtained for the phospholipid-based PAMPA models for gastrointestinal tract passive transport predictions indicate that these models can be used to obtain information on the permeation of chemicals loaded in specific carriers. However, testing a new material on PAMPA is not obvious and requires particular attention regarding the reciprocal impact of carriers on the artificial membrane and *vice versa*. Therefore, preliminary tests must be performed before the permeability coefficients obtained from PAMPA tests can be interpreted.

Cell Culture Models

Cellular models that predict BBB permeability are extensively used during the early drug discovery process. A large panel of cellular models exists that differ in origin, the type of expressed transporters, the tightness of the tight junctions and affiliation with a primary or immortalised cell line. These factors greatly influence the reproducibility of the permeability experiments and the capacity of these models to predict *in vivo* BBB permeability. Therefore, all cellular models are different and generate specific information on permeability through the BBB. Furthermore, none of the existing cellular models can fully predict the pharmacokinetic behaviour of drugs *in vivo*.

The main advantages of cellular models are the throughput rate, which allows for the evaluation of a reasonable number of compounds even if this rate is only moderate. Additionally, cellular models have the capacity to evaluate transport mechanisms, which depends on the type of expressed transporters and the possible evaluation of metabolism and cytotoxicity. Furthermore, pathological conditions can be investigated choosing an appropriate model (121). By contrast, homogeneity and reproducibility are difficult to obtain with cellular models, although these problems may be limited with the development of immortalised cell lines and standardised protocols.

To differentiate the wide variety of cellular models in existence since the 1970s, the following BBB parameters must be considered: the transendothelial electrical resistance (TEER), which indicates the tightness of transendothelial tight junctions and, therefore, restriction of the paracellular pathway; the endothelial permeability coefficient for paracellular markers such as sucrose, which indicates the integrity of the membrane (66); the expression of specific BBB transporters, such as the carrier-mediated transporters, including glucose transporters (GLUT1), monocarboxylic acid transporter (MCT1), large amino-acid transporters (LAT1), and cationic acid transporters (CAT); the active efflux transporters, including the ATP binding cassette (ABC) gene family or solute carrier (SLC) gene family (122); and the presence of BBB markers, such as factor VIII, γ-glutamyl transpeptidase (γ-GT), alkaline phosphatase (ALP) and monoamine oxidase (MAO) (123). Because the *in vivo* TEER of brain microvessels is approximately $1,000–2,000\,\Omega.cm^2$ (124,125) and the permeation of sucrose can be as low as 0.03×10^{-6} cm.s^{-1} (126), the ideal cellular model should provide values as close as possible to the known values for these parameters.

Primary Cultures. Cell biology research for the development of *in vitro* models of the human BBB began with primary bovine (40,127–137) and porcine (138–142) cultures (Table I) because the brain size of these animals is large;

leading to a high yield of cells per brain. However, rat (143–145), murine (146,147) and human (148–152) cell culture systems have also been developed. Compared to porcine or bovine cells, rat and mouse endothelial cells generate models with fewer BBB characteristics, such as a TEER value between 9 and $150\,\Omega.cm^2$ and a P_e (sucrose) of approximately 7.5×10^{-6} cm.s^{-1} (Table I). Therefore, mouse brain endothelial cells are difficult to culture and lead to poor development of the endothelium (67). Additionally, because the number of cells per brain is limited, the batches of cells are always different even when the same protocol is used for extracting and seeding cells. This variability causes reproducibility issues because the cells used for permeability determinations are not the same from one day to the next. This is one reason why researchers have very little interest in using mouse primary cells for BBB permeability studies even if the best murine models can compete with some of the bovine models (144,153).

Bovine endothelial cells were the first *in vitro* BBB model and were developed by Bowman *et al.* as soon as 1983 (130). However, one of the primary bovine models expressing sufficient TEER for the prediction of BBB penetration was developed by Zenker *et al.* in 2003 (154). In this model, the TEER value reached $1,350\,\Omega.cm^2$, but values this high were rare and were dependent on the batch of cells. Furthermore, no paracellular permeability verification was performed, which makes an appropriate discussion of this model difficult. In general, the average TEER value generated with primary bovine endothelial cells is $150–200\,\Omega.cm^2$, which is far from the *in vivo* TEER value of $1,000\,\Omega.cm^2$ (40,127–137).

Porcine models display the best barrier properties (Table I). These models exhibit high TEER values ranging from 70 to $1,800\,\Omega.cm^2$ depending on the culture conditions and medium supplementation. Porcine models also have a low paracellular permeation of sucrose, with values ranging between 0.2 and 25×10^{-6} cm.s^{-1} (138–142,155–162). Moreover, specific transporters, such as GLUT-1 or acetylated LDL, and brain enzymes, such as the γ-GT or ALP, display efficient metabolic activity (142) (Table I). The most efficient cellular model of the BBB was developed by Franke *et al.* (159,163,164) who grew primary porcine brain microcapillary endothelial cells in serum-free medium containing hydrocortisone. The result of this treatment was a monolayer of endothelial cells with very tight junctions, TEER values reaching $1,500\,\Omega.cm^2$ with an average of $700\pm100\,\Omega.cm^2$, and a sucrose permeation as low as 0.3×10^{-7} cm.s^{-1}. Further evaluations indicated that this model expressed several ATP-binding cassette (ABC) transporters, nutrient transporters and specific BBB receptors (165). However, this model has not been used for any further permeability determinations.

Table I Non Exhaustive List of Monoculture Models Aiming at Predicting BBB Permeability, Their Specificity and Information Provided

Author	Cells origins	TEER ($\Omega.cm^2$)	Paracellular marker permeability $P \times 10^{-6}$ cm.s^{-1}	Evidence of TJ	Pinocytic vesicles	Transporters	BBB receptors	BBB markers	In vitro/in vivo correlation
Ichikawa N. et al. (143)	rat BMEC					acetylated LDL	factor VIII	ALP, γ-GT	
Annunziata P. et al. (144)	rat BMEC	100					factor VIII		
Roux F. et al. (172)	immortalized rat RBE4		sucrose : 214±20				factor VIII	ALP, γ-GT	
Steiner O. et al. (11)	mouse BMEC		3 kDa dextran : 2.2±0.3	immunostaining, western blot					
Imaizumi S. et al. (147)	mouse BMEC	59.5±4.4					factor VIII	ALP, γ-GT	
Steiner O. et al. (11)	immortalized mouse BMEC		3 kDa dextran: 41±5	immunostaining, western blot					
Yang T. et al. (153)	mouse endothelial cell line bEnd5 + astrocyte conditioned media	121	sucrose: 8; mannitol: 8	immunostaining, western blot		P-gp/ABCB1, GLUT1, sodium, potassium, chloride cotransporters, protein kinase C isoforms			
Garberg P. et al. (123)	immortalized mouse BMEC	40–50	inulin: 11	immunostaining		P-gp/ABCB1		ALP, γ-GT	low correlation (N=10)
Culot M. et al. (232)	bovine brain EC		sucrose: 5.8±0.2	immunostaining		P-gp/ABCB1, MRP1, MRP4, MRP5 by RT-PCR; presence of P-gp/ABCB1 by western blot			
Raub T.J. et al. (134)	bovine brain EC	<9	sucrose: 25	transmission electron micrograph	few				
Bowman P.D. et al.(130)	bovine brain EC								
Audus K.L. et al. (129)	bovine brain EC							ALP, γ-GT	
Smith (135) K.R. et al.	bovine brain EC		sucrose: 0.08						
Rubin L.L. et al. (40)	bovine brain EC	61±2							
Rubin L.L. et al. (40)	bovine+ astrocyte conditionned medium	115±11							
Rubin L.L. et al. (40)	bovine EC+ cAMP	305±50							
Rubin L.L. et al. (40)	bovine EC+ astrocyte conditioned medium+ cAMP	625±82	restricted paracellular efflux		low rate of endocytosis				
Pirro J.P. et al. (133)	bovine brain EC	500–600						γ-GT	
Wang W. et al. (137)	bovine brain EC	160		immunostaining					
Letrent S.P. et al. (132)	bovine brain EC	72–184	morphine : 8.8–16	electron microscopy		P-gp/ABCB1		ALP, γ-GT	
Abbruscato T.J et al. (127)	bovine brain EC	200	sucrose: 8						
Abbruscato T.J. et al. (127)	bovine brain EC+ astrocyte	175	sucrose : 8						

Table I (continued)

Author	Cells origins	TEER ($\Omega.cm^2$)	Paracellular marker permeability $P \times 10^{-6} cm.s^{-1}$	Evidence of TJ	Pinocytic vesicles	Transporters	BBB receptors	BBB markers	In vitro/in vivo correlation
Yang T et al. (153)	Bovine brain EC+ astrocyte conditioned media / conditioned medium	>150	sucrose : 8; mannitol : 12	immunostaining, western blot		P-gp/ABCB1, GLUT1, sodium, potassium, chloride cotransporters, protein kinase C isoforms			
Rubin L. L. et al. (40)	Human EC+astrocyte conditioned medium+cAMP	339±107	restricted paracellular efflux		low rate of endocytosis				
Weksler B. et al. (174)	Immortalized human EC hCMEC/D3	<40	inulin : 12; sucrose : 27; lucifer yellow : 22			P-gp/ABCB1, BCRP, MDR1, MRP1, MRP3, MRP4, MRP5	human transferrin receptor		
Stins M. F. et al. (150)	Immortalized human EC	300–400				acetylated low density lipoproteins	factor VIII	γ-GT, carbonic anhydrase IV, lectin UEA I	
Sano Y. et al. (233)	Immortalized human EC (TY08)	35–43	inulin: 21			P-gp/ABCB1			
Fischer S et al. (140)	Porcine brain EC	89.4±3.1							
Franke (159,163) et al.	Porcine brain EC	700±100	Sucrose <1						$r^2 = 0.40$ (22 diverse compounds); $r^2 = 0.64$ (11 passive compounds)
Garberg P. et al. (123)	MDCKII-MDR1	120–140	inulin : 0.2; sucrose : 0.3			P-gp/ABCB1			$r^2 = 0.72$ (N=7), vs brain/blood ratio
Hakkarainen J. J. et al. (202)	MDCKII-MDR1		sucrose : 0.7±0.3						
Garberg P. et al. (123)	CACO-2	600–1,000	inulin : 0.08; sucrose : 1.4			P-gp/ABCB1, glut1, amino-acid transporters		ALP, γ-GT	$r^2 = 0.34$ (22 diverse compounds), $r^2 = 0.8611$ (11 passive compounds)
Hakkarainen J. J. et al.(202)	CACO-2		sucrose : 2.4±1.4						$r^2 = 0.83$ (N=7), vs brain/blood ratio

TEER transendothelial electrical resistance, *BMEC* brain microvascular endothelial cell, *EC* endothelial cell, *cAMP* cyclic adenosine monophosphate, *ALP* alkaline phosphatase, *γ-GT* gamma glutamyl transpeptidase, *P-gp/ABCB1* P-glycoprotein, *MRP* multiresistance drug protein, *RT-PCR* reverse transcription polymerase chain reaction, *BCRP* breast cancer resistant protein, *LDL* low density lipoprotein

Human brain cells are the gold standard for a human BBB *in vitro* model. However, for ethical reasons, the availability of this type of cell is very limited. The cells are generally obtained from biopsies of epileptic patients, and the number of cells obtained is very low. Rubin *et al.* and Bernas *et al.* developed primary human endothelial cell models with TEER values of $339 \pm 107 \Omega.cm^2$ and $>1,000 \Omega.cm^2$, respectively (40,166). No paracellular permeability was mentioned in these models, but the model developed by Bernas *et al.* was used in several studies to evaluate the effect of chemicals, such as cannabinoid receptor agonists, on the barrier function of BBB (167). Studies of these human models are limited and have instead focused on the generation of immortalised human cell lines, which should decrease the inter-individual, race, age and gender variations and increase the quality and reproducibility of the results obtained with human models.

Primary cell cultures may provide interesting information on human BBB permeability, but these models and culture conditions are not straightforward. Homogeneity and reproducibility of these models is not guaranteed because an animal brain cannot generate an infinite number of identical cells. Therefore, the variability of these models is due to inter-laboratory and inter-individual factors, among others, and leads to large standard deviations. Finally, primary monocultures of brain endothelial cells were shown to rapidly lose their BBB properties, including tight junctions and specific transporters (168).

Immortalised Cell Lines. To limit the drawbacks related to the handling of primary cells, researchers have immortalised their cultures to make cell lines. Immortalisation is generally achieved with either gene or virus transfection (148,169–171), such as the SV40 large T-gene antigen (67) or the E1A adenovirus gene (172) in the RBE4 model.

Few monoculture models of immortalised cell lines have been developed (Table I), however, bovine (173), human (148,150,174–181) mouse (182,183) and rat (172,183–188) endothelial cell lines have been established and tested for BBB properties. The model with the best BBB properties is a human brain endothelial cell line (150), with TEER values ranging from 300 to $400 \Omega.cm^2$; however, no sucrose permeation has been achieved, but inulin transport studies show low paracellular permeation. In 2005, Weksler *et al.* developed a model based in human cerebral cells: the hCMEC/D3 cell line, which displayed a TEER value below $40 \Omega.cm^2$ and a sucrose permeation of 27.10^{-6} cm.s^{-1} (174,189). Furthermore, many drug transporters, including most of the ABCB, ABCC and ABCG families found in the human BBB *in vivo* have been detected in the hCMEC/D3 cell line. Because of the existing BBB properties and its human origin, the hCMEC/D3 model has been used for many kinetic, pharmacological and permeability studies (190–193).

In order to understand the predictive ability of primary cells and immortalized cell line, Steiner *et al.* (11) compared primary mouse brain microvascular endothelial cells with immortalised mouse brain endothelial cell lines and determined that the two types of endothelial cells exhibited different cytoskeletal morphologies. Moreover, the protein occludin, which plays a role in tight junction formation, was localised in the primary endothelial cells but not in the cell line, indicating a divergence in the junctional organisation. This deviation leads to tighter junctions in the primary endothelial cells compared to the immortalised endothelial cells. Therefore, monocultures of immortalised cells are of limited interest for the prediction of BBB permeability, except the immortalised cell culture models derived from human cell lines.

Cocultures. Following the development of a variety of monoculture models and the determination that cerebral endothelial cells alone, whatever their origin, do not express the appropriate BBB properties and also lose their specific characteristics when isolated from their environment (27,145), cocultures became attractive models. In the human brain, there is constant communication between endothelial cells and other types of cerebral cells comprising the neurovascular unit, such as astrocytes, pericytes, neuroglia, and neurons (60,194). The action of surrounding cerebral cells on endothelial cells creates BBB properties and induces the production of junctional proteins and the expression of all the enzymes and transporters at the BBB (8,9,14,19,67,71,195). Moreover, it was shown that astrocytes were able to reinduce BBB properties (196).

Megard *et al.* (152) noted several interesting observations in their research on human brain endothelial cells cocultured with human astrocytes.

The resulting coculture exhibited specific barrier properties, such as a TEER value of $260 \pm 130 \Omega.cm^2$ (endothelial cells alone: $61 \pm 2 \Omega.cm^2$ and astrocytes alone: $37 \pm 5 \Omega.cm^2$) and a sucrose permeation of 17 ± 3.10^{-6} cm.s^{-1}. To validate this BBB model, the authors selected the lipophilicity of a compound as a good indicator of BBB permeability. They demonstrated a good relationship between the *in vitro* BBB permeability of this model that was corrected with the molecular weight and the partition coefficients ($r^2 = 0.88$). Using flow cytometry and PCR, they showed that their model expressed P-gp/ABCB1 mRNA. A permeability determination with known P-gp/ABCB1 substrates, such as vincristine, verapamil or vinblastine, revealed a higher permeability from the basal to the apical compartments than from the apical to the basal compartments, indicating an efficient efflux process. These results underline the beneficial effect of cocultures on the expression of specific BBB properties *in vitro* models. The upregulation of P-gp/ABCB1 and the higher TEER value in cocultures compared to monocultures were also observed in several other studies (9,154,184,195,197,198) (Table II).

Table II Non Exhaustive List of Coculture Models Aiming at Predicting BBB Permeability, Their Specificities and Information Provided

Author	Endothelial cell origin	Coculture cell type	TEER (Ω cm²)	Paracellular marker permeability × 10^{-6} cm.s^{-1}	Evidence of TJ	Pinocytic vesicles	Transporters	Receptors	Enzymes	In vitro/in vivo correlation
Roux et al. (172)	rat brain capillary EC (RBE4)	rat astrocytes	500 ± 20 (234)	sucrose: 38 ± 9	immunostaining		P-gp/ABCB1, GLUT1, LAT1 (235)	factor VIII, transferrin receptor	γ-GT, ALP	
Demeuse P. et al. (145)	rat brain capillary EC	rat astrocytes	438 ± 75			Few		factor VIII, transferrin receptor OX26	γ-GT	
Perrière N. et al. (15)	puromycin purified rat EC	rat astrocytes	>270	sucrose : <1.7	immunostaining and RT-PCR		P-gp/ABCB1, Bcrp, Mrps, GLUT1 abcg2, abcb1, abcc1, abcc4, abcc5			r² = 0.88
Lacombe O. et al. (203)	purified rat EC	rat astrocytes		sucrose: 3,28 ± 0.82	confocal microscopy and immunostaining					r² = 0.67
Lu W. et al. (205)	rat brain capillary EC	rat astrocytes	313 ± 23	sucrose :16	scanning electron microscopy and transmission electron microscopy					
Nakagawa S. et al. (194)	rat brain capillary EC	rat astrocytes and pericytes	354 ± 15	fluorescein : 3.9 ± 0.2	western blot, electron microscopy		P-gp/ABCB1, GLUT1, Mrp1 acetylated LDL	factor VIII	γ-GT	low correlation
Garberg P. et al. (123)	immortalized rat EC	immortalized rat astrocytes	50–70	inulin : 5.3; sucrose : 8.6						
Shayan G. et al. (236)	puromycin purified mouse EC	rat astrocytes	190	Na fluorescein: 3.5 ± 0.1	immunocytochemistry and western blot		P-gp/ABCB1, GLUT1			r² = 0.96 (N = 7)
Coisne C. et al. (237)	primary mouse brain microvascular endothelial cell	primary mouse glial cell	777.6 ± 14.8	sucrose: 4.5 ± 1.2; inulin: 1.2 ± 0.3	immunostaining		P-gp/ABCB1			
Gaillard PJ. et al. (195)	bovine EC	rat astrocytes	400–850	fluorescein: 6.0 ± 1.0	electron microscopy-presence of cadherin 5		P-gp/ABCB1 expression	αv integrin, P-selectin receptor; transferrin receptor	γ-GT	
Dehouck M.P. et al. (117)	Bovine EC	Rat astrocytes	700–800			Low rate	P-gp/ABCB1 (27,197); LDL (238)	Transferrin receptor (239)		r² = 0.86 (27,121) N = 20, with BUI
Stanness K. A. et al. (8) (3D model)	bovine aortic endothelial cells	astrocytes	736 ± 38	sucrose: 0.09						
Garberg P. et al. (123)	bovine EC	rat astrocytes	400–800	inulin: 0.7; sucrose: 4.0			P-gp/ABCB1	factor VIII	γ-GT, MAO	r² = 0.43 N = 22 (with log BB)
Raub T.J. et al. (134)	bovine EC	rat C6 astrocytes	160 ± 8	sucrose: 24						
Abbruscato T. J. et al. (127)	Bovine brain EC	rat C6 astrocytes	210	sucrose: 10						
Mabondzo A. et al. (3)	purified human EC	human astrocytes		lucifer yellow: 0.8; sucrose: 2.69 ± 0.25			GLUT1, LAT1, LAT2, ABCB1, ABCC1, ABCC4, ABCC5, ABCG2 LDL			r² = 0.90 (N = 6)
Garberg P. et al. (123)	human EC	human astrocytes		inulin: 12; sucrose: 22						no correlation
Garberg P. et al. (123)	human umbilical vein EC like cell line	rat C6 glioma cell line	100	inulin: 0.6; sucrose: 8.1			GLUT1, leucine amino acid carrier, P-gp/ABCB1		γ-GT	

Table II (continued)

Author	Endothelial cell origin	Coculture cell type	TEER (Ω.cm^2)	Paracellular marker permeability $\times 10^{-6}$ cm.s^{-1}	Evidence of TJ	Transporters	Pinocytic vesicles	Receptors	Enzymes	In vitro/in vivo correlation
Fischer S, et al. (142)	porcine brain microvascular EC	rat astrocytes	104–219			GLUT1, acetylated LDL			γ-GT, ALP	
Fischer S, et al. (142)	porcine brain microvascular EC	C6 glial cells	180 ± 12			GLUT-I, acetylated LDL			γ-GT, ALP	

TEER transendothelial electrical resistance, BMEC brain microvascular endothelial cell, EC endothelial cell, cAMP cyclic adenosine monophosphate, ALP alkaline phosphatase, γ-GT gamma glutamyl transpeptidase, P-gp/ABCB1 P-glycoprotein, MRP multiresistance drug protein, RT-PCR reverse transcription polymerase chain reaction, BCRP breast cancer resistant protein, LDL low density lipoprotein, MAO monoamine oxidase

These cocultures can be established with either primary cells or cell lines. As indicated in the review by Deli et al. (66), many different cocultures have been developed but are usually established with endothelial cells and astrocytes from various animals. When the BBB properties of these cocultures were compared to the barrier properties of the corresponding endothelial monoculture, the resulting TEER values were generally improved, whereas the permeation of a paracellular tracer was decreased. Consequently, the expression of tight junction proteins is upregulated under coculture conditions, resulting in an improved in vitro BBB model (197).

To determine the reason why BBB properties are reinduced when endothelial cells are cocultured with astrocytes, Bénistant et al. (168) evaluated the fatty acid composition of bovine brain capillary endothelial cells either monocultured or cocultured with rat astrocytes. They found that the phospholipid profiles of the endothelial cells were clearly different between the two culture conditions. The most significant differences were observed for palmitic acid, which was 13% of the total phospholipid proportion in the monoculture vs. 20% in the coculture, and for linoleic acid, which was 18% in the monoculture vs. 10% in the coculture. Therefore, these results indicated that the presence of astrocytes when culturing endothelial cells can modify the fatty acid composition of brain endothelial cells.

In 2009, Nakagawa et al. (194) established a coculture model with three different types of cerebral cells, endothelial cells, pericytes and astrocytes, to provide a more realistic representation of the in vivo BBB. The pericytes were shown to have a similar positive influence as astrocytes on the tightness of the tight junctions (199). The TEER value of this triple coculture reached $400\,\Omega$.cm^2, with a permeation of 3×10^{-6} cm.s^{-1} for the non-permeant dye fluorescein. Moreover, specific BBB transporters, such as P-gp/ABCB1, glucose-transporter (GLUT1) and ABCC1, were expressed in the brain endothelial cells (Table II).

The BBB model displaying the best barrier features was developed by Dehouck et al. (27,39,121,197,200) and was called the bovine brain capillary endothelial cells model (BBCEC). This model, consisting of primary bovine endothelial cells cultured on one side of the filter and rat glial cells on the other side of the filter, exhibits high TEER values due to the presence of complex tight junctions. The TEER values range between 700 and $800\,\Omega$.cm^2 (13), and the BBCEC model displays a low sucrose permeation between 5.4 and 32×10^{-6} cm.s^{-1}, a low rate of pinocytosis, the presence of P-gp/ABCB1 and metabolic enzymes, such as γ-GT, MAO, and the occurrence of LDL and transferrin receptors (27). This combination of characteristics leads to interesting BBB properties.

When correlating in vitro BBB data, which are corrected by the molecular weight and a logarithmic function, with the

corresponding BUI or *in vivo* BBB permeability, both of which are also corrected, excellent relationships were observed ($r^2 = 0.86$) (121).

In conclusion, with regard to cellular models consisting of cerebral cells, primary cell cocultures generate the best models, although the reproducibility may not be optimal. The upregulation of tight junction proteins under coculture conditions allows for increased TEER values and decreased paracellular transport, which are characteristics of the BBB. The gold standard model would be a coculture of primary human cells, but this requires a constant renewal of the donor brain tissue, which causes ethical concerns.

Cell Lines of Non-Cerebral Origin

The Madin-Darby Canine Kidney Cell line (MDCK). MDCK cells are cells of non-cerebral origin that are relatively easy to grow and can be transfected with specific gene transporters, particularly the MDR1 gene, which codes for polarised expression of P-gp/ABCB1 (71). These transfected MDCK cells can then be used for the MDR1-MDCK (I or II) assay. MDCK cells can generate TEER values as high as 1,800–2,200 $\Omega.cm^2$ (201), indicating the existence of robust barrier properties with high expression of tight junctional proteins. Moreover, the *in vitro-in vivo* correlation obtained with this model is better than some of the existing *in vitro* models with cerebral cells, with $r^2 = 0.64$ (123) or 0.72 (202) when passive compounds are selected for the correlation, depending on authors. However, *in vitro/in vivo* correlations with a diverse set of compounds (including compounds being actively transported or effluxed at the BBB) do not exhibit a significant correlation ($r^2 = 0.40$) (123). The greatest limitation of these cells is the absence of transporter proteins other than P-gp/ABCB1 and their different morphology compared to endothelial cells because MDCK cells are epithelial cells (71,203). Moreover, these cells are derived from dog kidney cells (201). However, transfection of the MDR1 gene allows for the determination of P-gp/ABCB1 substrates by measuring the permeability on both sides of the membrane. Another study conducted by Di Li *et al.* (114) did not generate an *in vitro-in vivo* correlation comparing the *in vitro* MDR1-MDCK permeation of a very diverse set of compounds with permeations obtained from *in situ* brain perfusion ($r^2 = 0.007$ (114)). This absence of correlation was most likely due to saturation of the P-gp/ABCB1 *in vivo*. The concentrations utilised for the *in situ* brain perfusion study were very high compared to the Michaelis-Menten constants of the compounds. These high concentrations lead to saturation of the efflux transport and use of the predominantly passive diffusion pathway, which overestimates the permeability *in vitro*. Therefore, attention should be paid to the experimental conditions when performing permeability assay with

saturable transport. Moreover, differences in membrane characteristics, such as epithelial kidney cells for the MDR1-MDCK assay and cerebral endothelial cells for the *in situ* brain perfusion study, lead to a significant discrepancy in the predicted BBB permeability. Moreover, the phospholipid composition of both membranes is fundamentally different, with a higher proportion of cholesterol in the cerebral membrane than the MDCK cells. This composition leads to an increased fluidity of MDCK cells, which facilitates passive permeability in this model.

Caco-2 Cells. Caco-2 cells are epithelial cells derived from a human colon carcinoma and are extensively used in the pharmaceutical industry to predict oral absorption through the intestinal epithelium. Moreover, the Caco-2 model is also used to predict BBB transport, even though the gastrointestinal tract is fundamentally different from the BBB, but as *in vitro* BBB models have evolved, the *in vivo* permeability predicted by the Caco-2 model became less and less useful (121,123,202). compared to other models. However, the Caco-2 assay remains an efficient method of identifying substrates of the P-gp/ABCB1 transporter from a set of test compounds because of the overexpression of P-gp/ABCB1 in cancer cells. More recently, Ball *et al.* (204) developed a physiologically based pharmacokinetics model to predict the fraction of unbound drug reaching the brain. They succeeded in generating an *in vitro* model that faithfully fits the *in vivo* unbound brain concentration of morphine, which is effluxed at the BBB, and oxycodone, which is taken up at the BBB. The $K_{p,uu}$ was determined in Caco-2 cells expressing the P-gp/ABCB1 efflux transporters for morphine and in TR-BBB13 cells, which enable active uptake, for oxycodone. Both resulting distributions were corrected by a relative activity factor, which was estimated by a comparison between the *in vitro* model predictions and the *in vivo* data in rats.

Adaptation of BBB Cell Models to New Delivery Strategies

Some models were used to obtain information on nanocarrier transport across the BBB.

Nanoparticles. In 2006, the transcytosis of polyethylene glycol-polylactide (PEG-PLA) nanoparticles across the BBB was evaluated by Lu *et al.* using a rat syngeneic coculture of brain capillary endothelial cells and astrocytes (205). The fluorescent compound 6-coumarin was incorporated inside these nanoparticles with a final size of 102.4 ± 6.8 nm. After transport study of the coumarin-loaded PEG-PLA nanoparticles across the coculture, a permeation of 4.8×10^{-6} cm.s^{-1} was obtained. However, the permeation of the paracellular marker sucrose was higher than the permeation of the nanoparticles. Therefore, either a

paracellular route or a transcytosis process may have occurred in this *in vitro* model.

Ragnaill *et al.* (206) later described the transport of 50 nm silicium dioxide nanoparticles through the *in vitro* hCMEC/D3 BBB model (174,189). They succeeded in showing that nanoparticles were effectively taken up by the cells with the membrane enveloping the nanoparticles. Moreover, they also observed the nanoparticles in both the endosomes and lysosomes and some on the basolateral side of the membrane. Therefore, they suggested that the silicium dioxide nanoparticles were endocytosed and that an exocytosis process also occurred.

Liposomes. The hCMEC/D3 cell line was also used to study the BBB penetration of an immunoliposome decorated with OX-26, an anti-transferrin monoclonal antibody (193). The objective of the study was to target the transferrin-receptor for transport of the immunoliposome by a receptor-mediated transcytosis process. The mean diameter of the immunoliposomes was assessed with dynamic light scattering and was less than 200 nm. They demonstrated that the immunoliposomes were transported through the endothelial monolayer by receptor-mediated endocytosis.

In vitro cellular models can therefore be used for the permeability determination of more complex formulations, such as nanocarriers. However, adaptation of the model may not be straightforward because of the potential cell toxicity of the carriers, and a full assessment of the validity of the models is required before a discussion of the results, but promising data regarding the distribution of nanocarriers are emerging.

Transport Across Isolated Brain Microvessels

Isolated brain microvessels are generally used to evaluate the gene expression of specific transporters at the BBB (207), but permeability experiments may also be conducted to identify specific transporters. Fluorescent probes are tested in the presence or absence of known substrates of the transporters. A variation in the permeability indicates the presence of the specific transporter that is being assessed (208,209). Miller *et al.* used confocal microscopy to detect fluorescent xenobiotics, such as daunomycin and fluorescein labeled dextrans, within the lumen of isolated brain microvessels (208). They succeeded in demonstrating a concentrative, specific and energy-driven transport process, indicating the expression of specific active transporters in isolated brain capillaries. Studies with specific substrates and immunostaining indicated that the active process was initiated by P-gp/ABCB1 and Mrps. The P-gp expression in isolated brain capillaries was corroborated by the studies of Durk *et al.* (209).

Transport across freshly isolated brain microvessels is a good alternative to cell culture because it is less time consuming; however, isolation of the brain microvessels requires much skill and very clean manipulations to obtain microvessels that are extremely pure. Moreover, reproducibility issues may be more important in this model than for cellular models because fresh microvessels must be freshly prepared for each experiment.

In Vivo Models

In vivo models are the best models available to predict human *in vivo* permeabilities because of the combination of all biological aspects in the same model, such as physiological barriers, transporters, and metabolic pathways. However, these models are expensive, time-consuming, require the mastering of animal-based assays and often necessitate radiolabelled compounds. These factors ensure that *in vivo* models are used during the later stages of the drug discovery process, just prior to clinical investigations, for a limited number of lead compounds. Different *in vivo* models are available, which allows for determination of the permeation of the molecules tested and in particular the logarithm of the BBB permeability-surface area (log PS) or the logarithm of the brain to plasma ratio (log BB) (Table III). These models include *in situ* brain perfusion, single carotid injection (brain uptake index; BUI), intravenous injection and intracerebral microdialysis (71). The log PS is considered the most relevant indicator of BBB permeability because it measures the clearance of a drug from the blood to the brain across the BBB (210) and is not altered by either metabolism or protein binding (114). The log PS is determined from the K_{in}, which is the clearance out of the brain. This determination better reflects BBB permeability..

Determination of the log BB requires several time point measurements, which requires animals a t each time point and is costly. Moreover, several factors, such as metabolism and binding, interfere with brain penetration in the log BB determination; therefore, log BB is not an accurate measurement of BBB permeability.

The Intravenous Administration Technique

Intravenous administration is one of the less invasive and most physiological methods of determining *in vivo* permeability. In this technique, the buffer containing radiolabelled solutes is injected inside a cannulated femoral vein or in the tail vein of the rat. The plasma concentration of the products is monitored for 10 s to several hours over various time points. The pharmacokinetic parameter obtained is the area under the curve of the drug concentration in the blood between time 0 and the time of the decapitation (211). Therefore, the measured permeability will include the effective permeability, which is influenced by protein binding and metabolism. This technique has numerous advantages, such

Table III Summary of the *In Vivo* Techniques in Late Drug Discovery Research

In vivo techniques	Characteristics	Experimental data
Brain/plasma ratio	Study of low to rapid penetrating compounds; choice of variable exposure times; possible metabolism, protein binding and active transport.	A partition coefficient between plasma (or blood) and brain is generated. Be careful to misleading caused by protein binding.
Brain uptake index	Study of moderate to rapid penetrating compounds; fast brain exposure (5 to 15 s between the injection and the sacrifice of the animal); no metabolism, no protein binding, no efflux transport.	log PS are generated. The extremely fast brain exposure time leads to sensitivity problems, due to very low concentration in the brain.
In situ brain perfusion	Suitable for low to rapid penetrating compounds; fast to long exposure times (5 s to 1 h); no metabolism, no protein binding, possible transport.	Data similar as Brain uptake index is generated. Sensitivity is much higher, thanks to a possible long brain exposure time.
Intravenous administration technique	Study of low to rapid penetrating compounds; monitoring of the plasma concentration; existence of *in vivo* metabolism, protein binding and transport.	A whole pharmacokinetics profile is generated, on an intact BBB. This is the less ethically invasive and the most physiological method.
Intracerebral microdialysis	More appropriate for medium permeant compounds. Drug monitoring of the unbound drug fraction after the choice of the mode of administration; existence of *in vivo* metabolism, protein binding and transport.	A whole pharmacokinetics profile is generated, but BBB can be damaged due to surgery. Monitoring is made on the same animal: decrease the number of experimental animals, no deviation due to variation in the origin, age, gender, weight of the animal.
Imaging	Visualisation of the distribution in the body. Appropriate for medium to rapid penetrating compounds.	Non-invasive technique, which can be employed in humans. Existence of sensitivity issues, require expensive equipment.

as the BBB remaining undisturbed and all transporters, enzymes and junctional proteins remaining intact, which enables metabolism. An entire pharmacokinetics profile can therefore be obtained. Moreover, because the evaluation can be performed for an extended period of time, both the plasma and brain pharmacokinetics can be determined. However, the study of specific influx transport is not possible with this intravenous administration technique.

The Brain/Plasma Ratio

The brain/plasma ratio, established by Ohno *et al.* (212), resulted in determination of the blood–brain distribution coefficient (BB), which is defined as the ratio between the concentration of the compound in the brain and in plasma. Therefore, this approach may be misleading because the partition parameter that is obtained is dependent on the affinity of the tested drug for circulating proteins in the blood stream (213). This method provides determination of the drug partition between the plasma and brain but does not provide a pharmacokinetic profile. Determination of the brain/plasma ratio can be performed either at steady state or at different time points and requires sacrificing the animal and determining the concentration of the unbound drug in both the brain homogenate and plasma.

A brain/plasma ratio ≥1 indicates that a compound is able to cross the BBB, whereas a brain/plasma ratio <1 reveals a poor distribution of the compound (213). However, due to plasma protein binding, a brain/plasma ratio <1 may also indicate a high affinity for circulating plasma proteins or an affinity for efflux proteins, both of which limit permeation of the compound through the barrier. Therefore, interpretation of the brain/plasma ratio must be performed cautiously.

Young *et al.* (214) used the brain/plasma ratio to design and select a CNS-active H_2 histamine receptor antagonist. More recently, Rohanova *et al.* used the brain/plasma ratio to study the brain penetration of a new illegal drug p-methoxymethamphetamine and its metabolites to better understand both the mode of action andas the toxicity profile of these new drugs (215).

The Unbound Brain/Plasma Ratio: $K_{p,uu}$

To correct the errors that may be introduced by measurement of the brain/plasma ratio, a recent tendency consist in determining the unbound brain to plasma ratio ($K_{p,uu}$). This parameter may be obtained directly by intracerebral microdialysis or with a combination of *in vivo* and *ex vivo* techniques. The last experiment is achieved measuring the quantity of drug that has reached the entire brain *in vivo* combined with an estimation of the unbound brain volume of distribution with *ex vivo* techniques such as the brain slice uptake experiment. The determination of $K_{p,uu}$ provides a better indication of the distribution of the active form of the drug. A $K_{p,uu}$ that is greater than unity indicates the presence of an active uptake process, whereas a $K_{p,uu}$ less than unity indicates the presence of an efflux process. A $K_{p,uu}$ near one indicates that the predominant pathway through the BBB is a passive diffusion process (216).

Intracerebral Microdialysis. Microdialysis allows for determination of the cerebral extracellular free drug concentration over a period of time with calculation of the $K_{p,uu}$. This free drug fraction represents the active form of the drug. Microdialysis requires the implantation of a dialysis probe in a selected area of the brain of the animal. This microdialysis probe consists of a semipermeable membrane that is continuously infused with physiological solution [217]. Therefore, the compounds that are able to permeate through the membrane will diffuse according to the concentration gradient. This procedure does not require a specific mode of administration, and the permeation of the tested compound can be monitored after oral, intravenous, subcutaneous or infusion administration. Therefore, any drug entering the brain, which is always the unbound fraction of the drug, will be monitored over time in the extracellular fluid of the same animal [218] using HPLC, UHPLC or capillary electrophoresis. This technique requires a reduced number of animals for the pharmacokinetics determination compared to other techniques [71]. Moreover, because the same animal is monitored, there is no deviation due to variation in the population, gender or age of the animal. However, implantation of the dialysis system may locally disrupt the BBB, leading to a possible misinterpretation of the results. Moreover, highly lipophilic compounds can be adsorbed onto the probe, leading to mass-balance errors. This technique is more appropriate for moderately permeable compounds.

Intracerebral microdialysis was used by Gupta *et al.* [219] to compare the BBB transport and CNS distribution of two cetirizine enantiomers, the R and the S forms, to evaluate the stereoselectivity that occurs at the BBB. They compared the K_p (total brain to total plasma concentrations), $K_{p,u}$ (total brain to unbound plasma concentrations) and $K_{p,uu}$ (unbound brain to plasma concentrations) for both enantiomers. Whereas the K_p value could have led to the interpretation that brain penetration was stereoselective for both isomers ($K_p = 0.22$ for S-cetirizine and 0.04 for R-cetirizine), the $K_{p,u}$ (0.44 for S-cetirizine and 0.22 for R-cetirizine) indicates that plasma protein binding greatly influences the biodistribution of both forms of the antihistamine. The unbound fraction was found to be 0.50 for S-cetirizine but only 0.15 for R-cetirizine. When considering both the plasma protein binding and brain tissue binding, they discovered that the $K_{p,uu}$ values were similar for both enantiomers ($K_{p,uu} = 0.17$ for S-cetirizine and 0.14 for R-cetirizine). These similar values indicate that both enantiomers are effluxed at the BBB ($K_{p,uu} < 1$) and that no significant difference occurs in their transport. The information generated by these *in vivo* methodologies that are able to distinguish between the active (unbound fraction) and inactive forms of the drugs are therefore able to reliably determine the biodistribution profile of a drug at the BBB.

The In Vitro Brain Slice Uptake Experiment Combined with the In Vivo K_p. Determination of the entire concentration of a drug in the brain *in vivo*, combined with the volume of distribution of the unbound drug determined by an *in vitro* brain slice uptake experiment, allows for calculation of the unbound brain to plasma concentration ratio $K_{p,uu}$ [220,221]. In the brain slice uptake experiment, the brain of a sacrificed animal is removed and immersed in ice-cold oxygenated pH 7.4 buffer [221]. Brain slices (300 µm) of striatal areas are cut with a microslicer and preincubated with an extracellular fluid buffer before addition of the drug. At specific incubation times, the brain slices are removed from the solution of buffer containing the drug, dried, weighed and homogenised for determination of the amount of drug recovered in the brain slice. The main advantage of this technique is in the determination of the pharmacologically active unbound fraction of the drug in the brain. Moreover, the brain slices contain intact cerebral endothelial cells with their functional transporters as well as the same extra-to-intra-cellular pH gradient found *in vivo* [220]. By contrast, the throughput of this model is limited due to the need for experimental animals and the minimum incubation time needed to reach drug equilibrium between the buffer and the brain slices, which is required to calculate $V_{u, brain}$ [220]. As the incubation time to reach equilibrium increases with the $V_{u, brain}$ and the $V_{u, brain}$ increases with lipophilicity, the majority of compounds during the drug discovery phases require longer and longer incubation timestime, which cannot be supported by the limited life expectancy of the brain slices. Friden *et al.* evaluated this brain slice uptake assay to rapidly determine the unbound drug concentration in the brain [221]. When they compared the results that were obtained for 15 diverse compounds with *in vivo* intracerebral microdialysis, they observed a reliable correlation between both techniques.

The Brain Uptake Index (BUI) Technique

The brain uptake index model is appropriate for compounds that moderately to rapidly penetrate the BBB and is generally performed in rats. A rapid bolus injection of 200 µl buffered solution containing a known concentration of a radiolabelled reference compound and the tested drug is administered directly into the carotid artery of the rat. The brain is then removed after 5–15 s and analysed for radiolabelled contents [71] by scintillation counting. BUI allows for determination of the log PS, which is the permeability multiplied by the surface area. This technique is based on the following three hypotheses: the time between injection and decapitation is so short that no metabolism occurs before the brain is removed; the compound can diffuse from the blood to the brain; and when the compound is in the brain, it cannot go back into the blood [222]. The main drawback of

this technique is that extremely low concentrations of drug are present in the brain because of the very short analysis time, which causes sensitivity problems for compounds with low permeability. This technique is more appropriate for compounds with a high permeability through the BBB. For example, this technique has been used by Oldendorf *et al.*, who demonstrated modulation of the transporter activity under different pH conditions (223).

The In Situ Brain Perfusion

In situ brain perfusion is suitable for low to high permeant compounds (43) or substrates of an endogenous transport system. This model provides the same information as the BUI but with higher sensitivity. *In situ* brain perfusion is performed on an artificial cerebral circuit, resulting from the ligature of some cerebral arteries and catheterisation of the external carotid artery. Therefore, the flow rate and duration of perfusion are known and can be controlled. The brain is then perfused with an oxygenated hydrogenocarbonate buffer containing a known amount of radiolabeled compound. Determination of the radioactivity at a predefined time, ranging from 5 s to 30 min,) allows for the direct determination of pharmacokinetics parameters, particularly the volume of distribution and the rate of compound transfer, K_{in} (43,224).

Because the composition of the perfusate is controlled, *in situ* brain perfusion allows for the determination of PS values without considering either metabolism or plasma and brain protein binding. Only the intact form of the drug reaches the brain. This method provides information regarding the time required for the compound to cross the BBB. Moreover, the amount of drug reaching the brain is fully controlled. Compared to the BUI, the sensitivity of *in situ* brain perfusion is much higher because of a longer exposition time of the brain (126). This method is particularly interesting for determination of the kinetics of saturable transport at the BBB.

In situ brain perfusion was used by Cannon *et al.* (225) to study the modulation of P-gp/ABCB1 activity. They found that activation of the sphingosine-1-phosphate receptor by a specific ligand such as sphingosine-1-phosphate led to a decrease in P-gp/ABCB1 activity. The activity returned to the basal state after the administration of sphingosine-1-phosphate receptor antagonist. They concluded that this strategy could be used to increase the CNS bioavailability of P-gp substrates, such as verapamil or loperamide.

The Brain Efflux Index

The brain efflux index has been developed to determine the presence of a potential efflux pathway at the BBB when the compound is postulated to be transported from the brain to the blood. This technique is generally used to understand why a sufficiently lipophilic compound fails to penetrate the brain (211).

Briefly, the test and reference compounds are microinjected directly into the brain tissue. After a predefined time, the brain is removed and the tissues are analysed to determine the concentration of the residual compounds.

This protocol requires the use of a highly sensitive method to determine the small amounts of compounds that may be present. Moreover, the researcher must be careful during the microinjection because a rapid or careless manipulation will irreversibly damage the BBB and confound the resulting measured permeability. The major drawback of this technique is the sacrifice of a large number of rats at different time points.

Kakee *et al.* (226) used this technique to highlight the saturable efflux transport of para-aminohippuric acid at the BBB. A comparison of the apparent efflux clearance that was obtained in this study using the brain efflux index method with the apparent influx clearance obtained by BUI confirmed the selective transport of the carboxylic acid under investigation from the brain to the blood.

Non-Invasive Imaging Techniques

Non-invasive imaging techniques have been developed to qualitatively and quantitatively determine the permeation of drugs *in vivo*. Moreover, imaging can also be used to identify P-gp/ABCB1 inhibitors or P-gp/ABCB1 substrates (227) or to determine the BCRP activity at the BBB (228). These techniques are primarily positron emission tomography (PET), magnetic resonance imaging or magnetic resonance spectroscopy. Because these techniques are non-invasive, they can be used in humans and allow for the determination of personalised pharmacokinetics. Imaging is also used to detect BBB damages in patients suffering from a stroke, brain tumours or multiple sclerosis. However, these techniques require expensive equipment and labelled radio-tracers. Sensitivity issues may be encountered, particularly with magnetic resonance imaging (126). Knight *et al.* used magnetic resonance imaging to localise and quantify the BBB opening in a rat model under ischaemic conditions after the intravenous infusion of gadolinium-diethylenetriamine pentaacetic acid to achieve a blood concentration (229). Under normal conditions, this substance should not penetrate the brain. Other researchers, such as Serres *et al.*, established a promising approach for the early diagnosis of brain tumors by targeting brain metastasis with iron oxide microparticles loaded with contrast agents (230). Magnetic resonance imaging allowed for the visualisation of brain metastases with 3-fold greater sensitivity than conventional tumour diagnostics. ^{13}C magnetic resonance spectroscopy was used to assess the relationship between brain and plasma concentrations of glucose in patients suffering from

diabetes type I during a hypoglycaemichypoglycemia event (231).

Therefore, imaging is an efficient approach to determine personalised permeabilities during or after a pathological event. The main advantage of this technique is that it can be conducted in human for specific purposes and with safe drugs or dyes. Moreover, the distribution profile can be visualised, identifying the targeted regions of the brain.

CONCLUSIONS

The specific organisation of the cerebral endothelial cells forming the BBB causes multiple problems for a drug that needs to reach the central nervous system. Additionally, the BBB is not only a physical barrier but also a pharmacological and a metabolic barrier due to the expression of numerous transporters and the presence of a variety of enzymes. These features are the reason why attrition rate is so high for CNS drug candidates. However, the population worldwide is aging and by 2050, elderly people over the age of 65 will represent 16% of the population (43). Along with this global aging, CNS disorders, such as Alzheimer's disease, increase exponentially, whereas treatments are lacking. Thus, there is a real need to either improve CNS drug discovery or to change the CNS drug delivery strategy by using colloidal vectorisation. The second option has garnered great interest during the last few decades. Vectorisation of active CNS drugs and mRNA or peptides with carriers that are able to recognise a specific target at the BBB are no longer impossibilities.

The main questions that remain to be answered in the early drug discovery and development processes are as follows: is the chemical CNS active? Is the drug a substrate of influx or efflux transporters? Is the drug bioavailable? Is it easily metabolised? As described in this review, many tools are available to predict and/or understand the mode of transport of a new chemical entity (NCE) through the BBB. These tools include computational sciences, *in vitro* and *in vivo* models and knowledge regarding new transporters. However, it is clear that a combination of methods, even *in vitro* models, should provide more relevant information regarding the potential of a drug to penetrate the BBB. Each method provides specific insight into the transport condition of NCEs, and only proper knowledge of the specificities of each model will help scientists to decrease the compound attrition rate. *In vivo* models provide much more global information regarding the BBB permeation of NCEs than other models. However, even simple models, such as artificial membranes, should not be neglected in the discovery process. Although these models provide neither a global view nor a realistic tool for the selection of compounds, they are useful for understanding the transport mechanism. The simplicity of these models isolates *de facto* unique or a limited number of aspects of the entire transport process. Similarly, although some cellular models do not provide sufficient relevancy in terms of BBB prediction, particularly regarding the tightness of the intercellular junctions as evaluated by TEER measurement and/or the permeation of sucrose, they constitute an extraordinarily predictive and sometimes selective tool for medicinal chemists if the data are well interpreted.

All of these models that are used for the evaluation of BBB penetration by NCEs may also provide information regarding the penetration of colloidal vectors and other nanocarriers. Currently during the drug discovery and development processes, an increasing number of delivery systems must be tested for efficacy; but, few models have been used and validated for the screening of new formulations. Moreover, kinetic parameters of carrier degradation that influence drug release should also be taken into consideration when evaluating a new model. Therefore, existing methods to predict or characterise BBB permeability need to adapt to these new drug delivery systems in order to fully understand their mode of action.

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