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JENNI J. HAKKARAINEN

In Vitro Cell Models in Predicting Blood-Brain Barrier Permeability of Drugs

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ABSTRACT

The blood-brain barrier restricts the passage of many drugs into the brain. This restrictive barrier is created by the presence of many features, such as the tight junctions between the brain capillary endothelial cells and efflux transporter proteins (*e.g.*, P-glycoprotein), all of which limit the transport of many compounds into the brain. In the early phase of drug development, cell based *in vitro* models are used to predict blood-brain barrier permeability of new drug candidates. In order to make appropriate predictions, it is important to be aware of the usefulness and limitations of these *in vitro* models. In addition, the relevance of the *in vitro* models needs to be assessed against their *in vivo* counterparts. Primary bovine brain microvessel endothelial cells (BBMECs) have been used as an *in vitro* blood-brain barrier model, since primary cells most closely represent the *in vivo* situation. However, the functionality of the efflux proteins and the *in vivo* relevance of the monocultured BBMECs have not been comprehensively assessed. Therefore, the general objective of this study was to evaluate the suitability of the monocultured BBMEC model for use in drug permeability studies.

The BBMEC model was confirmed as being leaky. One reason for this could be the partial perinuclear localization of the tight junction protein occludin. P-glycoprotein was found to be expressed and correctly localized in the monocultured BBMECs. However, P-glycoprotein expression was significantly higher in the BBMECs cultured on filter inserts. Although P-glycoprotein was shown to be functional in the BBMECs, no efflux was detected in the bidirectional transport studies. The molecular descriptors determining the passive drug permeability across the BBMEC model were similar with those present in epithelial cell models, suggesting that there are no clear differences between passive drug permeability in the endothelial and epithelial cell models when drugs need to be classified into different categories. In addition, no clear differences were found in the *in vitro-in vivo* correlations between BBMEC model and epithelial cell models, indicating that the predictive value of endothelial and epithelial cell models is similar when passive transport of drugs is being evaluated *in vitro*.

In conclusion, the monocultured BBMEC model was able to predict the *in vivo* brain entry of mainly passively transported drugs. In addition, undetected efflux suggests that the use of BBMEC model may pose a high risk of obtaining false negative results for drug candidates that are potential P-glycoprotein substrates. However, BBMECs are suitable for evaluating cellular mechanisms, such as in cellular uptake studies, where the tightness of the cell monolayer is not crucial.

National Library of Medicine Classification: WL 200, WG 700, QS 532.5.E7, QV 38
Medical Subject Headings: Blood-Brain Barrier; Tight Junctions; Capillary Permeability; Pharmaceutical Preparations; Endothelial Cells; Cells, Cultured; Microvessels; P-Glycoprotein; Models, Biological; Pharmacokinetics; *In Vitro*

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TIIVISTELMÄ

Veri-aivoeste rajoittaa useiden lääkeaineiden kulkua aivoihin. Tämä este muodostuu mm. aivojen kapillaariverisuonien endoteelisolujen välisistä tiiviistä liitoksista ja effluksipumppuproteiineista (esim. P-glykoproteiini), jotka rajoittavat useiden aineiden kulkua veri-aivoesteen läpi. Lääkekehityksen alkuvaiheessa uusien lääkeaine-ehdokkaiden veri-aivoeste läpäisevyyttä ennustetaan ns. *in vitro* -solumalleilla. On tärkeää tietää solumallien hyödyt ja rajoitukset. Solumallien antamaa tulosta täytyy verrata *in vivo* -eläinmallin antamaan tulokseen, jotta solumalleilla voidaan tehdä oikeita ennusteita. Naudan aivojen mikrosuonien primääriendoteelisoluja (BBMEC) on käytetty veri-aivoesteen solumallina, koska ne edustavat tarkimmin veri-aivoestettä. Yksisolukasvatetun BBMEC-mallin effluksipumppuproteiinien toiminnallisuutta ja relevanssia *in vivo* -eläinmallien antamiin tuloksiin ei ole arvioitu kattavasti. Tästä syystä tutkimuksen päätavoitteena oli arvioida yksisolukasvatetun BBMEC-mallin soveltuvuutta lääkeaineiden läpäisevyystutkimuksiin.

Tulokset varmistivat BBMEC-solumallin olevan vuotava. Mahdollinen syy vuotavuuteen voi olla tiivisliitosproteiini okkludiinin osittainen sijainti tuman läheisyydessä. P-glykoproteiini ilmentyi ja sijoittui oikein solukalvolle yksisolukasvatuksessa BBMEC-mallissa mutta P-glykoproteiinin ilmentyminen oli merkitsevästi korkeampi kasvatusinserttikalvolla kasvatetuissa BBMEC-soluissa. P-glykoproteiinin osoitettiin olevan toiminnallinen mutta sitä ei havaittu kaksisuuntaisissa kuljetuskokeissa. Molekyylin kemiallisia ominaisuuksia kuvailevat tuntomerkit, jotka määrittävät lääkeaineen passiivisen läpäisevyyden BBMEC-mallin yksisolukerroksen läpi, havaittiin olevan samanlaiset kuin epiteelisolumalleilla. Tämä viittaa siihen, että lääkeaineiden passiivisessa läpäisevyydessä ei ole selviä eroja endoteelisolumallin ja epiteelisolumallien välillä, kun lääkeaineet luokitellaan eri luokkiin. Tutkimuksessa havaittiin myös, että *in vitro-in vivo* korrelaatioissa ei ollut selviä eroja BBMEC-mallin ja epiteelisolumallien välillä. Endoteelisolumallin ja epiteelisolumallien ennustearvo on samankaltainen, kun arvioidaan pääasiassa lääkeaineiden passiivista kulkeutumista.

Yksisolukasvatetun BBMEC-mallin osoitettiin ennustavan pääasiassa lääkeaineiden passiivista aivokulkeutumista. Lisäksi BBMEC-mallin käyttäminen lääkeaine-ehdokkaiden, jotka ovat mahdollisesti P-glykoproteiinin substraatteja, veri-aivoesteläpäisevyyden ennustamiseen voi aiheuttaa riskin väärien negatiivisten tulosten saamiseen. BBMEC-malli soveltuu kuitenkin solumekanismien tutkimiseen, kuten soluunottokokeisiin, joissa solukerroksen tiiviys ei ole pääasiallinen vaatimus.

Luokitus: WL 200, WG 700, QS 532.5.E7, QV 38
Yleinen suomalainen asiasanasto: veri-aivoeste; mikroverisuonet; endoteeli; läpäisevyys; lääkkeet; lääkeaineet; soluviljely; farmakokinetiikka

Verba volant, scripta manent

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Kuopio, August 2013

Jenni Hakkarainen

List of the original publications

This dissertation is based on the following original publications, referred to in the text by Roman numerals **I-III**:

I Hakkarainen JJ*, Jalkanen AJ*, Kääriäinen TM, Keski-Rahkonen P, Venäläinen T, Hokkanen J, Mönkkönen J, Suhonen M and Forsberg MM. Comparison of *in vitro* cell models in predicting *in vivo* brain entry of drugs. *International Journal of Pharmaceutics 402: 27-36, 2010.*

II Hakkarainen JJ*, Pajander J*, Laitinen R, Suhonen M and Forsberg MM. Similar molecular descriptors determine the *in vitro* drug permeability in endothelial and epithelial cells. *International Journal of Pharmaceutics 436: 426-443, 2012.*

III Hakkarainen JJ, Rilla K, Suhonen M, Ruponen M and Forsberg MM. Re-evaluation of the role of P-glycoprotein in *in vitro* drug permeability studies with the bovine brain microvessel endothelial cells. *Xenobiotica, Early Online, DOI: 10.3109/00498254.2013.823529*

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* Authors with equal contribution

Contents

Abbreviations

A-B	apical-to-basolateral
ABC	ATP-binding cassette transporter
ACM	astrocyte conditioned medium
ALP	alkaline phosphatase
ATP	adenosine triphosphate
AUC	area under the concentration-time curve
Aβ	amyloid-β peptide
B-A	basolateral-to-apical
BBMECs	bovine brain microvessel endothelial cells
BCRP	breast cancer resistance protein
BSA	bovine serum albumin
BUI	brain uptake index
Caco-2	human epithelial colorectal adenocarcinoma cell line
calcein-AM	calcein acetoxymethyl ester
cAMP	cyclic adenosine monophosphate
CL_{in}, K_{in}	*in vivo* influx clearance
CNS	central nervous system
CNT	concentrative nucleoside transporter
COMT	catechol-*O*-methyl-transferase
CYP	cytochrome P450 enzyme
DAPI	4′,6-diamidino-2-phenylindole dihydrochloride
ECF	extracellular fluid
EDTA	ethylenediaminetetraacetic acid
ENT	equilibrative nucleoside transporter
ER	efflux ratio
GLUT1	glucose transporter 1
HBA	hydrogen-bond acceptor
HBD	hydrogen-bond donor
HBMECs	human brain microvessel endothelial cells
HBSS	Hank's balanced salt solution
hCMEC/D3	human brain endothelial cell line
HEPES	N-(2-hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid)
HPLC	high-performance liquid chromatography
K_p	*in vivo* partition coefficient
$K_{p,uu}$	*in vivo* unbound partition coefficient
LAT1	large neutral amino acid transporter 1
LC	liquid chromatography
LC-MS	liquid chromatography-mass spectrometry
LogBB	logarithm of the ratio of the total concentrations in brain and plasma
LogP	logarithm of the octanol/water partition coefficient
MCT1	monocarboxylic acid transporter 1
MDCK	Madin-Darby canine kidney cell line
MDCKII-MDR1	MDCK type II cell line transfected with the human *MDR1* gene

MDR1	multidrug resistance protein 1, P-glycoprotein
MRM	multiple reaction monitoring
mRNA	messenger ribonucleic acid
MRP	multidrug resistance-associated protein
MS	mass spectrometer
MW	molecular weight
OAT	organic anion transporter
OATPs	organic anion transporting polypeptides
OCT	organic cation transporter
P_{app}	apparent permeability coefficient
P_e	permeability across cell monolayer
$P_{in\ vivo}$	cerebrovascular permeability *in vivo*
PBMECs	porcine brain microvessel endothelial cells
PBS	phosphate buffered saline
PCA	principal component analysis
PSA	polar surface area
RBE4	rat brain endothelial cell line
RBMECs	rat brain microvessel endothelial cells
r	Pearson's correlation coefficient
r^2	coefficient of determination
SLC	solute-carrier transporter
TEER	transendothelial electrical resistance
TFA	trifluoroacetic acid
UPLC	ultra-performance liquid chromatography
UV/Vis	ultraviolet/visible spectroscopy
vWF	von Willebrandt factor/Factor VIII antigen
ZO-1	zonula occludens 1

1 Introduction

More than three hundred years ago, in 1695, the first experimental evidence for impermeability of cerebral blood vessels was observed by Humphrey Ridley, cited in Liddelow (2011). Ridley described that wax and mercury injected into bloodstream did not tint the nerves but had stained the ramifications of blood vessels in them (Liddelow, 2011). Almost two centuries later, Paul Ehrlich noted that an intravenously injected dye did not stain the brain, although other organs were dyed (Ehrlich, 1885). Ehrlich assumed that it was caused by weak binding affinity of the dye in the brain. Further experiments completed by Ehrlich's student Edwin Goldmann in 1913 revealed that trypan blue dye injected into cerebro-spinal fluid stained the brain but not the peripheral tissues, cited in Liddelow (2011). These results pointed to the existence of the barrier between the blood and central nervous system (CNS) (Hawkins and Davis, 2005, Liddelow, 2011).

Subsequently, two fundamental features of a blood-brain barrier were appreciated (Reese and Karnovsky, 1967). Firstly, the uniform formation of tight junctions between the endothelial cells. Secondly, the low frequency of vesicles in the endothelial cells of the blood-brain barrier. All the above experiments and several other investigations that were conducted in the elapsing years were significant in the discovery of the concept of the blood-brain barrier (Roy and Sherrington, 1890, Dermietzel and Krause, 1991, Liddelow, 2011).

In order to be effective, a drug needs to reach its target site and maintain an adequate therapeutic concentration to if it is to achieve its desired pharmacological response. Drug targeting into the brain is challenging due to the blood-brain barrier. The blood-brain barrier selectively regulates the transport of the compounds into and out of the brain (Risau and Wolburg, 1990). Conventional commonly used CNS drugs are almost exclusively small-molecular weight drugs (Pardridge, 2002). The majority of drugs, in some estimates as many as 98 % and none of the large-molecular drugs, are not able to permeate across the blood-brain barrier (Pardridge, 2002, Pardridge, 2005). This is one reason why many CNS diseases do not currently have effective drugs (Pardridge, 2005). However, the suggestion that only a few drugs enter the brain has been criticized as also large drugs are able to cross the blood-brain barrier *in vivo* (Fagerholm, 2007). Therefore, understanding the complex nature of the blood-brain barrier is an important part of the development of novel drugs for CNS diseases and disorders.

Drug development is time-consuming and costly (Stoner *et al.*, 2004, Paul *et al.*, 2010) and therefore in order to reduce the cost and time required for drug development, many technologies including *in vitro, in vivo* and *in silico* models can be applied to predict the properties of a drug candidate in the early drug development in the pharmaceutical industry (Stoner *et al.*, 2004). Recently, cell based *in vitro* models have been developed for use in drug permeability studies both to speed up early drug development and decrease the number of animal experiments. These *in vitro* models should closely resemble the brain endothelium and to exhibit relevant *in vivo* blood-brain barrier properties (*e.g.*, tight paracellular barrier and correct localization and functionality of the efflux proteins). In addition, the relevance of the cell models against *in vivo* counterpart needs to be assessed to allow reliable predictions based on *in vitro* data.

2 Review of the Literature

2.1 NEUROVASCULAR UNIT AND BLOOD-BRAIN BARRIER

2.1.1 Neurovascular unit

In the human brain, there are over 100 billion capillaries; the length of these capillaries stretches over 600 km (Pardridge, 2005). The brain capillaries are the smallest vessels in the vascular system with a diameter of only 3-7 μm (Figure 1) (Rodríguez-Baeza *et al.*, 2003). The surface area of the brain capillary endothelial cells that are creating the barrier between the blood and brain tissue has been estimated to be 12-20 m^2 in human (Pardridge, 2005, Krämer *et al.*, 2009).

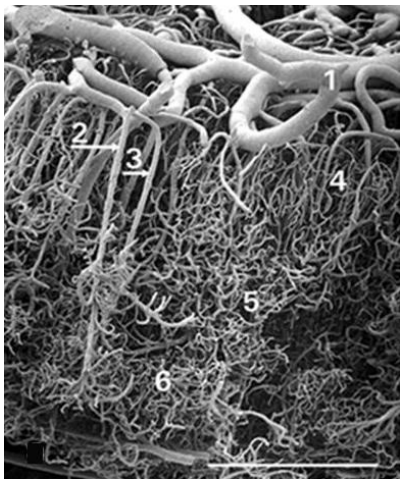

Figure 1. Scanning electron micrograph showing human brain vessels from the cerebral cortex (1) pial vessels, (2) long cortical artery, (3) middle cortical artery, (4) superficial capillary zone, (5) middle capillary zone, and (6) deep capillary zone. Scale bar 0.86 mm. (Rodríguez-Baeza *et al.*, 2003). Reprinted with the kind permission of John Wiley & Sons, Inc.

The brain capillary endothelial cells are encircled by several cell types that act as a secondary barrier around the endothelium. These cells include pericytes, astrocytes, neurons and together with the endothelial cells they form a structure called the neurovascular unit (Figure 2).

Astrocytes encircle 90 % of the abluminal side of endothelial cells, control the cerebral blood flow by constricting the cerebrovasculature (Mulligan and MacVicar, 2004) whereas neuronal activity will stimulate the astrocytes to evoke arteriole dilatation (Zonta *et al.*, 2003). In addition, cell culture studies have shown that factors released from astrocytes are able to upregulate the tight junction resistance (Rubin *et al.*, 1991a, Raub, 1996), induce enzyme activities such as alkaline phosphatase (ALP) and γ-glutamyl transpeptidase (DeBault and Cancilla, 1980, Hayashi *et al.*, 1997, Sobue *et al.*, 1999) and elevate messenger ribonucleic acid (mRNA) levels of relevant blood-brain barrier features, such as transferrin receptor, P-glycoprotein and glucose transporter 1 (GLUT1) (Hayashi *et al.*, 1997). In addition, upregulation of low density lipoprotein receptor was observed when the brain endothelial cells were cocultured with astrocytes *in vitro* (Dehouck *et al.*, 1994).

Pericytes are supporting cells located at the microvascular wall alongside the endothelial cells. Pericytes are the closest cell type adjacent to the brain endothelial cells and they share a

common basal lamina (Figure 2). The understanding of the biology of pericytes has increased in recent years (for review see Shepro and Morel, 1993, Dalkara *et al.*, 2011, Kamouchi *et al.*, 2011). The pericytes have contractile activity controlling the capillary diameter and thus, they can modulate the cerebral blood flow at the capillary level (Peppiatt *et al.*, 2006, Hamilton *et al.*, 2010, Fernández-Klett *et al.*, 2010). Pericytes also regulate endothelial cell differentiation and proliferation, *e.g.,* during angiogenesis (for review see Shepro and Morel, 1993). In addition, cell culture studies have shown that factors secreted by pericytes can increase claudin-5 expression in the brain endothelial cells, thus, enhancing the tightness of the blood-brain barrier (Shimizu *et al.*, 2012).

Neurons are closely associated with brain capillaries (Park *et al.*, 2003, Iadecola, 2004). The neuronal processes release several vasoactive agents, such as nitric oxide, acetylcholine, γ-aminobutyric acid, noradrenaline, dopamine and serotonin (Fergus and Lee, 1997, Iadecola, 1998, Iadecola, 2004, Lok *et al.*, 2007) which regulate local intracerebral blood flow.

The direct cell contacts and signaling pathways at the neurovascular unit modulate multiple brain microvascular functions, *e.g.*, cerebral blood flow and maintain the essential functions of the blood-brain barrier. It is very likely that there are several other interactions between the cells of the neurovascular unit but they are still poorly understood. The structure and regulation of the neurovascular unit have been reviewed in detail elsewhere (Ballabh *et al.*, 2004, McCarty, 2005, Correale and Villa, 2009, Abbott *et al.*, 2010).

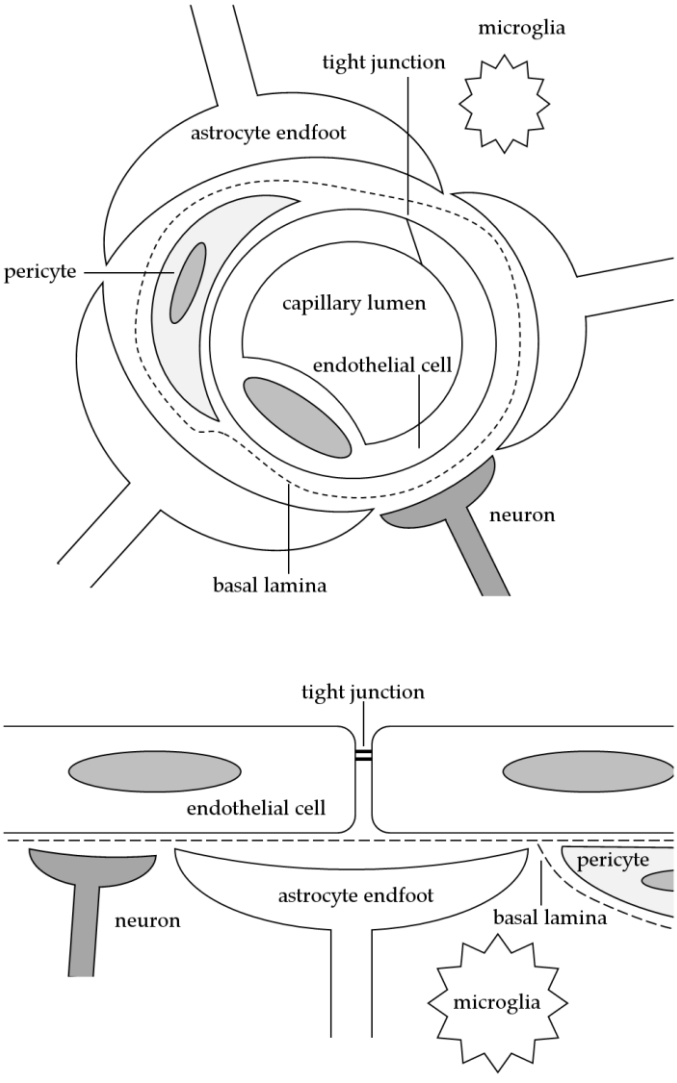

Figure 2. Structure of the neurovascular unit. Modified form Abbott *et al.*, 2006.

2.1.2 Structure and function of the blood-brain barrier

The blood-brain barrier is a feature of brain blood capillary vessels that are lined with a thin layer of endothelial cells. Since the primary role of the blood-brain barrier is to maintain the homeostasis between blood and brain by restricting cell, fluid and ionic transport, the brain endothelial cells are distinct from other endothelial cells in the body. This barrier is made up of three barriers; physical, transport and metabolic barrier, and it is controlling compounds to enter and leave brain by selectively supplying for essential nutrients and removing brain-borne metabolites (Risau and Wolburg, 1990, Abbott, 2005, Deli, 2007).

Physical barrier

One of the hallmarks of the blood-brain barrier is the restrictive paracellular barrier composed of a continuous network of tight junctions between the endothelial cells (Reese and Karnovsky, 1967, Nagy *et al.*, 1984). These are created by the presence of several tight junction specific proteins, such as occludin (Furuse *et al.*, 1993, Hirase *et al.*, 1997), zonula occludens 1 (ZO-1) (Watson *et al.*, 1991, Furuse *et al.*, 1994), claudin-1 and claudin-5 (Liebner *et al.*, 2000). In addition, adherens junctions composed of Ca^{2+}-dependent cadherin proteins promote also the adhesion between the endothelial cells (Schulze and Firth, 1993, Staddon *et al.*, 1995). The expression levels of occludin in the endothelial cells have been reported to vary between different tissues, with the highest expression being detected in the brain endothelial cells (Hirase *et al.*, 1997). Thus, the brain capillaries are substantially tighter than peripheral blood vessels (Abbott *et al.*, 2008) and paracellular transport of compounds between the brain endothelial cells is efficiently restricted. Tight junctions are able to prevent the transport of small ions, such as Na^+ and Cl^-. The transendothelial electrical resistance (TEER) of the blood-brain barrier *in vivo* has been shown to be 1000-2000 Ωcm^2 in rats and frogs (Crone and Olesen, 1982, Butt *et al.*, 1990) but it may be even much higher, 8000 Ωcm^2 in rats (Smith and Rapoport, 1986). For comparison, frog mesenteric blood capillaries have a resistance of only ~2 Ωcm^2 (Crone and Christensen, 1981) and frog muscle capillaries ~30 Ωcm^2 (Olesen and Crone, 1983) highlighting the fact that the resistance in the brain capillaries is dramatically higher than in the peripheral capillaries.

Transport barrier

Since the paracellular transport of compounds is efficiently restricted by the blood-brain barrier, there are many essential molecules that are needed by neurons but which cannot pass passively from blood to brain. These compounds require specific transporter proteins in order to gain access into the brain. The blood-brain barrier contains numerous transporter proteins and transcytosis mechanisms that mediate the efflux and uptake of various compounds across the brain capillary endothelial cells (see section 2.2.2).

Metabolic barrier

Many of the enzymes expressed in the mammalian blood-brain barrier, hinder the access of compounds into the brain. Several enzymes have been demonstrated to be highly expressed in the brain endothelial cells, *e.g.*, ALP, γ-glutamyl transpeptidase, cholinesterase, phosphoprotein phosphatase, aminopeptidases, carboxypeptidases, angiotensin converting enzyme, dipeptidyl peptidases, monoamine oxidase, dopa decarboxylase and cytochrome P450 enzymes (CYP) (for review see Brownlees and Williams, 1993, Dermietzel and Krause, 1991, Bertler *et al.*, 1966).

2.2 TRANSPORT ACROSS THE BLOOD-BRAIN BARRIER

There are many different transport mechanisms to help and hinder compounds to cross the blood-brain barrier 1) passive paracellular and transcellular diffusion, 2) active carrier mediated transport, 3) active efflux transport, 4) endocytosis (specific receptor-mediated and absorptive endocytosis) (Figure 3) for review see (Neuwelt, 2004, Pardridge, 2005, Abbott *et al.*, 2010).

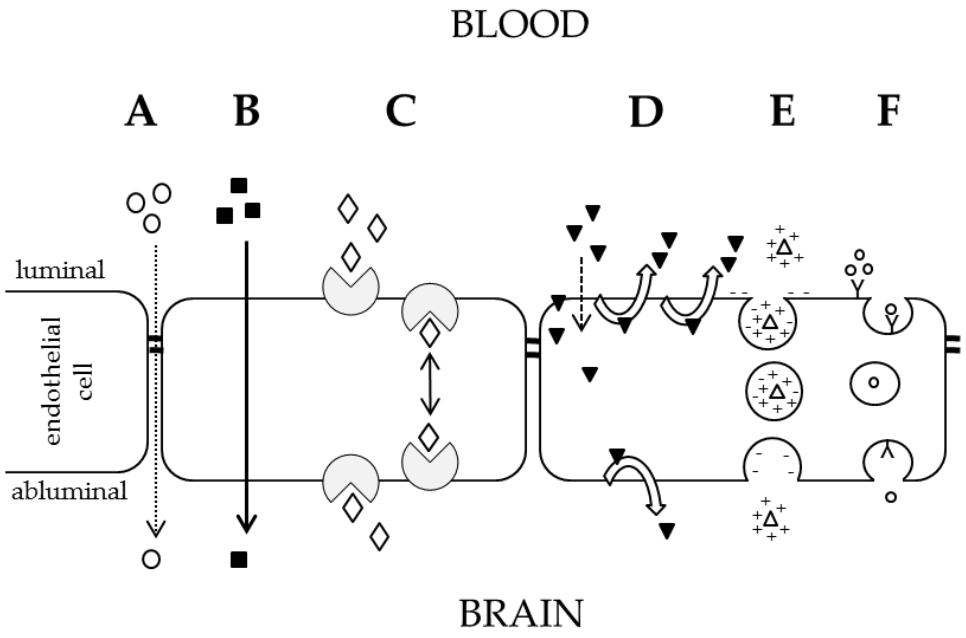

Figure 3. Transport routes across the blood-brain barrier. A) passive paracellular diffusion (small water soluble molecules), B) passive transcellular diffusion (lipid soluble, non-polar molecules), C) active carrier mediated transport (essential polar molecules; glucose, amino acids, nucleosides), D) active efflux transporters (lipid soluble, non-polar molecules and conjugates, drugs and xenobiotics), E) adsorptive endocytosis (cationized albumin), F) specific receptor-mediated endocytosis (transferrin, insulin). Modified from Neuwelt, 2004, Abbott *et al.*, 2010.

2.2.1 Passive permeability

Passive diffusion is a process where a compound moves down its concentration gradient and it does not require any expenditure of energy. The passive paracellular diffusion passes through the cellular tight junctions between the endothelial cells (*i.e.,* the paracellular pathway, Figure 3A) and passive transcellular diffusion occurs across the cell membrane (*i.e.,* the transcellular pathway Figure 3B). The paracellular pathway is negligible in the blood-brain barrier due to the occlusive tight junctions. Therefore, the brain permeability of low permeability compounds, *e.g.*, sucrose, mannitol and inulin (tracers for paracellular tightness) is negligible (Ferguson and Woodbury, 1969, Ohno *et al.*, 1978). Permeability coefficient of sucrose into the brain *in vivo* is ~3 × 10^{-8} cm/s (Ohno *et al.*, 1978). Whereas, high permeability compounds crossing the blood-brain barrier *in vivo* via passive transcellular diffusion, the permeability coefficients are several orders of magnitudes higher; *e.g.*, antipyrine 33 × 10^{-6} cm/s and ethanol >100 × 10^{-6} cm/s (Crone, 1965).

The basis of the molecule's possibility to cross the blood-brain barrier is strongly linked to its molecular properties, since the majority of the molecules capable of diffusing from the blood into the brain need to be transported across the endothelial cells. It is generally accepted that

four basic molecular properties are strongly associated with the capability to permeate across cell membranes; these are known as Lipiski's rule of five (Lipinski *et al.*, 1997).

In the blood-brain barrier, the physicochemical properties associated with the drug permeability across the blood-brain barrier are comparable to Lipinski's rule of five, which was developed for the estimation of oral absorption potential; lipophilicity (logarithm of the octanol/water partition coefficient, LogP), number of hydrogen-bond donor (HBD) groups (defined as hydrogen atom connected to electronegative atoms such as nitrogen or oxygen) and number of hydrogen-bond acceptor (HBA) groups (described as electronegative atoms with a lone pair of electrons), polar surface area (PSA) and molecular weight (MW) (Rapoport and Levitan, 1974, Abraham *et al.*, 1994, Gratton *et al.*, 1997, Fischer *et al.*, 1998, Kelder *et al.*, 1999, Abraham, 2004, Pardridge, 2005, Fu *et al.*, 2008) (Table 1). Physicochemical properties are commonly considered as interdependent (Hitchcock and Pennington, 2006). If a compound's ability to transport across blood-brain barrier is estimated based on physicochemical properties, the combination of the physicochemical properties should be considered instead of individual molecular properties alone.

Table 1. Physicochemical properties for increased potential of higher blood-brain barrier passive transcellular permeability and oral absorption.

Physicochemical property	**blood-brain barrier**	**oral absorption**
Octanol/water partition coefficient (LogP)	2-5[a], <3[b]	<5[c]
Hydrogen-bond donors (HBD)	<3[a], <4[b]	<5[c]
Hydrogen-bond acceptors (HBA)	<8[b]	<10[c]
Polar surface area (PSA, Å^2)	<90[a]	<140[d]
Molecular weight (MW, Da)	<500[a], <450[b]	<500[c]

[a] Hitchcock and Pennington, 2006; [b] Reichel, 2006; [c] Lipinski et al., 1997; [d] Veber et al., 2002.

Lipophilicity

The molecule needs to possess sufficient lipophilicity in order to be able to partition into the endothelial cell membranes (Oldendorf, 1974). The lipophilicity of the molecule can be quantified by LogP (Leo *et al.*, 1971). LogP is defined as the ratio of the concentration of neutral species of compound in octanol and water at equilibrium. Compounds with low LogP values (*e.g.*, LogP -1 = 1:10 octanol:water) are hydrophilic. Conversely, compounds with high LogP values (*e.g.*, LogP 3 = 1000:1 octanol:water) are lipophilic. Lipophilic molecules move readily from the blood to the endothelial cell membrane and, thus, LogP has been used as a general predictor of the blood-brain barrier permeability (Rapoport and Levitan, 1974). For example, the diffusion of lipophilic drug diazepam (experimental LogP 2.99 (Wang *et al.*, 1997)) into the brain is rapid and its movement across the blood-brain barrier does not appear to be restricted (Ramsay *et al.*, 1979). In contrast, sucrose is very hydrophilic (experimental LogP -3.67 (Leo *et al.*, 1971)) and its brain penetration is negligible (Ohno *et al.*, 1978). Lipophilicity depends also on the molecular forces between the drug and the phase into which it is partitioning, *e.g.*, the cell membrane, since the ionic attractive and repulsive interactions have also an impact; lipophilicity = hydrophobicity – polarity + ionic interactions (for review see Liu *et al.*, 2011). Traditionally, drug delivery into the brain has been improved by making the drug molecule more lipophilic by covering the hydrophilic parts with lipids and by adding hydrophobic groups (Abbott and Romero, 1996, Pardridge, 2002). There are limitations to increasing the

lipophilicity of the novel drug, *e.g.*, these may be its rapid distribution into the other organs, increased binding to plasma proteins and decreased biological activity.

Hydrogen-bonding

The capability to form hydrogen-bonds has been recognized as an important factor in brain penetration of drugs, and the brain penetration of a drug can be improved by reducing the overall hydrogen-bonding interactions (Young *et al.*, 1988). The hydrogen-bonding capability is known to decrease the *in vivo* brain distribution and to reduce the brain permeability of drugs (Abraham *et al.*, 1994, Gratton *et al.*, 1997).

Polarity

An increased polarity of compounds decreases the blood-brain partitioning (Norinder *et al.*, 1998) and blood-brain permeability (Abraham *et al.*, 1994, Gratton *et al.*, 1997, Abraham, 2004). PSA is the surface area occupied by polar atoms, mainly nitrogen and oxygen, and the hydrogen atoms attached to these polar atoms. PSA reflects also a drug's capability to form hydrogen bonds. The increase of PSA of drugs has been shown to decrease brain penetration (Kelder *et al.*, 1999).

Molecular weight and size

In general, small molecules with MW less than 400-500 Da have a better potential to cross the blood-brain barrier than larger molecules (Pardridge, 2005). In addition, the drug permeation across the blood-brain barrier decreases by 100-fold when the cross-sectional area of the drug is increased from 52 $Å^2$ (*e.g.*, drug with MW of 200 Da) to 105 $Å^2$ (*e.g.*, drug with MW of 450 Da) (Fischer *et al.*, 1998, Pardridge, 2005). Generally, passive permeation across the blood-brain barrier becomes reduced when the cross-sectional area of the drug molecule is ≥70-80 $Å^2$ (Fischer *et al.*, 1998, Seelig, 2007).

2.2.2 Active transport

Active transporters can be grouped into two classes, adenosine triphosphate (ATP)-binding cassette (ABC) and solute-carrier (SLC) transporters. ABC transporters are efflux membrane proteins transporting substrates from the intracellular compartment and/or lipid leaflets of the cell membrane back to the extracellular compartment of the cells (Higgins and Gottesman, 1992, Matheny *et al.*, 2001, Kimura *et al.*, 2007). The efflux transporters require energy from ATP hydrolysis to allow them to transport substrates across the cell membranes against a concentration gradient. Many SLC transporters are influx or bidirectional transporters that can either facilitate diffusion of substrates down the concentration gradients across the cell membranes, or they use energy originating from the inorganic or small ions to provide the driving force for the transport processes against the concentration gradient (Russel, 2010). Many transporters are highly expressed at the blood-brain barrier and they are intended to protect the brain from endogenous and exogenous toxins as well as supply nutrients into the brain, respectively (Pardridge, 2005).

ABC transporters

There are 49 ABC transporter genes in the human genome; these are grouped into seven subfamilies named from A to G (gene families *ABCA-ABCG*) (Vasiliou *et al.*, 2009). The first

characterized and the best studied glycosylated efflux protein is the multidrug resistance protein 1 (MDR1, gene *ABCB1*) designated as P-glycoprotein by Juliano and Ling (Juliano and Ling, 1976). In addition, multidrug resistance-associated proteins (MRPs, *ABCC* gene family) and breast cancer resistance protein (BCRP, gene *ABCG2*) were identified in multidrug resistant cells (Cole *et al.*, 1992, Krishnamachary and Center, 1993, Doyle *et al.*, 1998). Although the efflux proteins were initially found to contribute the multidrug resistance in tumor cells, the pioneering work in the field of multidrug resistance proceeded when Schinkel *et al.* generated P-glycoprotein knockout mice and demonstrated the protective role of P-glycoprotein in various tissues *in vivo* (Schinkel *et al.*, 1994, Schinkel *et al.*, 1995).

The efflux proteins are expressed in many human tissues, *e.g.*, liver, intestine, kidney and pancreas (Thiebaut *et al.*, 1987, Flens *et al.*, 1996, Maliepaard *et al.*, 2001a). The main role of the efflux transporters is to transport a wide variety of lipid soluble molecules out of the cells. The function of the efflux proteins has been related to many phases of the pharmacokinetics of the drugs; absorption from the gastrointestinal tract (Benet *et al.*, 1999, Floren *et al.*, 1997), distribution into different compartments in the body, such as brain and placenta (Schinkel *et al.*, 1994, Young *et al.*, 2003) and excretion from the liver and kidneys (Charuk *et al.*, 1994, Faber *et al.*, 2003).

The first observation of the expression of P-glycoprotein in the endothelial cells in the human blood-brain barrier was made by Cordon-Cardo *et al*. in 1989; these workers speculated that P-glycoprotein would have a physiological role in the regulation of drug transport into CNS (Cordon-Cardo *et al.*, 1989). It was found that luminally located P-glycoprotein in the blood-brain barrier acted as a protective mechanism restricting the penetration of harmful compounds, such as bilirubin and cortisol into the brain (Ueda *et al.*, 1992, Watchko *et al.*, 1998). Strong expression of P-glycoprotein has also been found in other blood-tissue barriers, such as the blood-testis barrier and the blood-placental barrier (Cordon-Cardo *et al.*, 1989, MacFarland *et al.*, 1994). At present, at least six efflux transporters have been detected in the blood-brain barrier, these being mainly located on the luminal side of the brain capillary endothelial cells. These efflux proteins are P-glycoprotein, MRP1, MRP2, MRP4, MRP5 and BCRP (Figure 4) (for review see Abbott *et al.*, 2010, Neuwelt *et al.*, 2011). The efflux transporters are not restricted only to capillary endothelial cells (Figure 4) since also other cell types of the neurovascular unit express several efflux transporters (Kim *et al.*, 2006, Kooij *et al.*, 2011, Gibson *et al.*, 2012, Chen *et al.*, 2013).

SLC transporters

Currently, 55 SLC gene families with at least 362 putatively protein-coding genes have been found in the human genome (He *et al.*, 2009). The SLC transporters at the blood-brain barrier are important in the transport of many essential polar molecules, since these molecules are not able to diffuse passively across the cell membranes (for review see Ohtsuki and Terasaki, 2007, Abbott *et al.*, 2010). SLC transporters expressed in the blood-brain barrier include energy transport systems, such as GLUT1 (gene *SLC2A1*), monocarboxylic acid transporter 1 (MCT1, gene *SLC16A1*) and amino acid transport systems, *e.g.*, large neutral amino acid transporter 1 (LAT1, genes *SLC3A2* and *SLC7A5*) (Figure 4). In addition, several other SLC transporters are expressed in the blood-brain barrier, *e.g.*, organic anion transport systems (organic anion transporters, OAT2-3, genes *SLC22A7,8*; organic anion transporting polypeptides, OATPs, *SLCO* gene family); organic cation transport systems (organic cation transporters, OCT2-3,

genes *SLC22A2,3*) and nucleoside transporters (concentrative nucleoside transporters, CNT2-3, genes *SLC28A2,3* and equilibrative nucleoside transporters, ENT1-2, genes *SLC29A1,2*) (Figure 4).

SLC transporters, such as LAT1 (Gynther *et al.*, 2008, Peura *et al.*, 2013) and GLUT1 (Gynther *et al.*, 2009), have been proposed as attractive transfer routes to allow drugs to gain access to the brain. LAT1 has higher transport capacity and it is less specific for its substrates than GLUT1 and, thus, LAT1 is more promising system for improved drug delivery into the brain (Abbott and Romero, 1996, Rautio *et al.*, 2013). However, competition of amino acids from consumed food may lead to some limitations in the use of LAT1 system (del Amo *et al.*, 2008). For example, the clinical response of levodopa which uses LAT system for transport across the intestinal wall and blood-brain barrier, is affected by the presence of amino acids in the plasma derived from dietary proteins (Carter *et al.*, 1989).

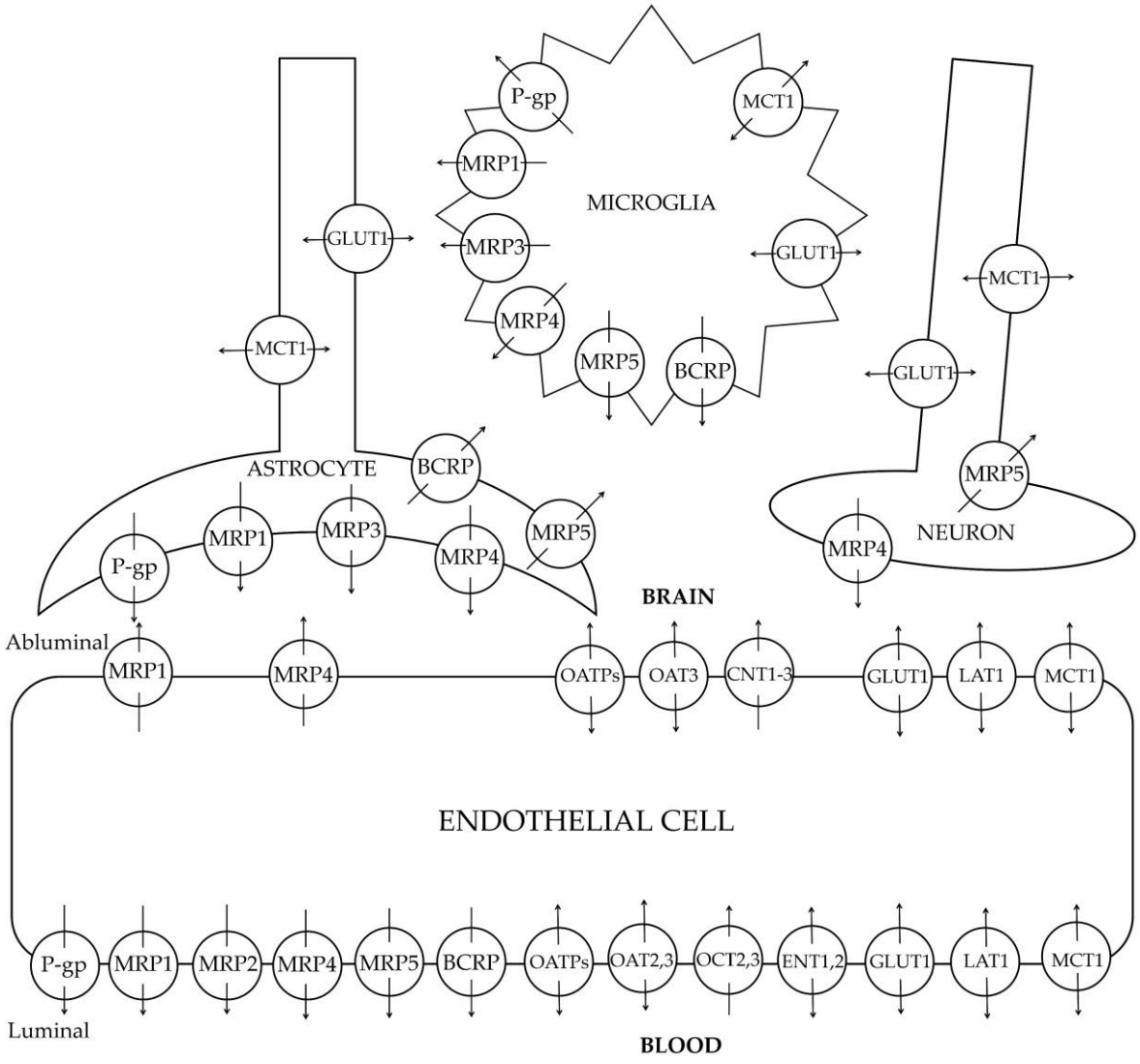

Figure 4. Transporters in the blood-brain barrier and in the neurovascular unit. P-gp, P-glycoprotein; MRP1-5, Multidrug resistance associated proteins 1-5; BCRP, Breast cancer resistance protein; OATPs, Organic anion transporting polypeptides; OAT2,3, Organic anion transporter 2,3; OCT2,3, Organic cation transporter 2,3; ENT1,2, Equilibrative nucleoside transporter 1,2; CNT1-3, Concentrative nucleoside transporter 1-3; GLUT1, Glucose transporter 1; LAT1, Large neutral amino acid transporter 1; MCT1, Monocarboxylic acid transporter 1. Modified form Ohtsuki and Terasaki, 2007, Abbott *et al.*, 2010, Neuwelt *et al.*, 2011. Arrows indicate the direction of transport (Abbott *et al.*, 2010, Zlokovic, 2011, Omidi and Barar, 2012).

Clinically important transporters in drug disposition

The understanding of the drug transporters has grown tremendously in recent years. The influence of efflux transporters in drug disposition in the body, their effect on multidrug resistance and drug-drug interactions related to inhibition of efflux transporters have been subjects of great interest for the past 10 years (Gottesman *et al.*, 2002, Marzolini *et al.*, 2004, Zhang *et al.*, 2006a, Giacomini *et al.*, 2010, Han, 2011, Maeda and Sugiyama, 2013). Today, several ABC and SLC transporters (*e.g.*, P-glycoprotein, BCRP, OATPs, OCTs, OATs) have been identified as clinically important key transporters related to the pharmacokinetics of different drugs. These clinically important transporters are also expressed in the blood-brain barrier (Figure 4). Therefore, the transport of drugs undertaken by these transporters (efflux and/or influx) may be more extensively affected in the tight blood-brain barrier than in the leakier organs, since paracellular transport is negligible in the blood-brain barrier.

Recently, the regulatory authorities have released guidelines for the pharmaceutical industry on the prediction of the transporter mediated drug-drug interactions of drug candidates (European Medicines Agency, Guideline on the Investigation of Drug Interactions, 2012; U.S. Food and Drug Administration, Guidance for Industry (draft), Drug Interaction Studies-Study Design, Data Analysis, Implications for Dosing, and Labeling Recommendations, 2012). Moreover, an International Transporter Consortium was established in 2007 to reach a consensus on the role of the clinically important transporters in drug safety and efficacy (Huang *et al.*, 2010). The consortium has published recommendations (Giacomini *et al.*, 2010), provided follow up commentaries, issued directions for the future recommendations and has discussed recent regulatory draft guidance documents (Tweedie *et al.*, 2013, Zamek-Gliszczynski *et al.*, 2012). These documents are important advances in the identification and assessment of clinically important drug-transporter interactions.

2.2.3 Endocytosis

A low level of endocytosis is a hallmark of the blood-brain barrier *in vivo* (Reese and Karnovsky, 1967, Goldstein *et al.*, 1986, Risau and Wolburg, 1990). This has also been demonstrated *in vitro* primary bovine endothelial cells (Guillot *et al.*, 1990, Raub and Audus, 1990). Therefore, only a small amount molecular trafficking across the blood-brain barrier is mediated by endocytosis. However, adsorptive endocytosis and specific receptor-mediated endocytosis (Figure 3) (Pardridge, 2002) may be important drug delivery pathways for polar and large molecule drugs and peptides into the brain (Abbott and Romero, 1996, Bickel *et al.*, 2001, Smith and Gumbleton, 2006).

2.3 EFFECTS OF CNS DISEASES ON THE FUNCTION OF THE BLOOD-BRAIN BARRIER

The tight junctions play an important role in the blood-brain barrier. Changes in the expression and localization of these proteins can have a strong influence on blood-brain barrier function and they have been suggested to be associated with the pathology of many neurological disorders (for review see Bednarczyk and Lukasiuk, 2011). There is also emerging evidence that also efflux transporters may play a role in the pathogenesis of neurodegenerative diseases (Cirrito *et al.*, 2005, Kortekaas *et al.*, 2005, Tai *et al.*, 2009). The altered functions of the blood-brain barrier and the neurovascular unit are both contributing factors to pathogenesis and the

consequence of CNS diseases (Hawkins and Davis, 2005). Currently, it is generally accepted that neuronal cell damage is not the only trigger in the pathogenesis of CNS diseases, but the damage at the level of neurovascularies may also contribute to the initiation and progression of the disease (Zlokovic, 2011, Stanimirovic and Friedman, 2012). At the moment, however, the molecular basis of the neurovascular link to the pathology is still poorly understood. In this connection, it is recommended that diagnostic and drug therapy strategies should be focused on the neurovascular unit in order to discover drug targets and develop novel drugs for CNS diseases and disorders (Stanimirovic and Friedman, 2012).

Brain ischemia has been shown to reduce the integrity of the blood-brain barrier (Albayrak *et al.*, 1997, Lorberboym *et al.*, 2006) and increased paracellular permeability contributes to the formation of brain edema (Castejón, 2012). An insufficient oxygen and glucose supply after an ischemic stroke is a trigger for multiple pathophysiological processes that cause injuries to endothelial cells, neurons and glia (Doyle *et al.*, 2008). The cell death is a result from complex chain of events, *e.g.*, acidosis, oxidative stress, periinfarct depolarization and apoptosis.

Multiple sclerosis is an inflammatory demyelinating disease whose origin is still unknown. A key factor may be a complex inflammatory cascade initiating the migration of activated leucocytes into the brain (for review see Minagar and Alexander, 2003). This inflammatory cascade is the most significant reason for the decrease in the numbers of tight junctions between the brain capillary endothelial cells and the loss of blood-brain barrier intergrity (for review see Minagar and Alexander, 2003, Bednarczyk and Lukasiuk, 2011). Recently, altered expression of the efflux transporters, such as decreased expression of P-glycoprotein in blood-brain barrier and increased expression of both P-glycoprotein and MRP1 in reactive astrocytes, in multiple sclerosis brain tissue was reported and interpreted as evidence for the pathological role of efflux transporters (Kooij *et al.*, 2011).

Alzheimer's disease is a progressive neurodegenerative disease characterized by neuronal loss, development of neurofibrillary tangles and deposition of amyloid plaques composed of amyloid-β peptides (Aβ) (for review see Skaper, 2012, Wilcock, 2012). Alzheimer's disease is age-dependent (for review see Candore *et al.*, 2006). There is evidence that the blood-brain barrier becomes leakier when the brain ages suggesting that a dysfunction of the blood-brain barrier may be present in the early stage of Alzheimer's disease (Starr *et al.*, 2009, Viggars *et al.*, 2011). Etiology of the Alzheimer's disease is complex; Aβ deposition is the major pathological feature but inflammation and oxidative stress also contribute to the pathogenesis of Alzheimer's disease (Skaper, 2012). Brain biopses from patients with Alzheimer's diseases have revealed that abnormalities at the blood-brain barrier (*e.g.*, diminished numbers of mitochondria in the endothelial cells, abnormal interendothelial junctions and pericytes adjacent but not surrounding the endothelium) can lead to a leakier blood-brain barrier (Stewart *et al.*, 1992). $A\beta_{1\text{-}42}$ has also been shown to weaken the blood-brain barrier integrity *in vitro* by disrupting tight junction localization and this phenomenon is believed to contribute to the development of Alzheimer's disease (Marco and Skaper, 2006). In addition, two efflux transporters, BCRP and P-glycoprotein, have been shown to be involved in the transport of Aβ across the blood-brain barrier both *in vitro* and *in vivo* (Vogelgesang *et al.*, 2002, Cirrito *et al.*, 2005, Tai *et al.*, 2009) indicating that increased accumulation of Aβ into the brain may have been attributable to reduced functionality of these efflux transporters.

Parkinson's disease is a progressive neurodegenerative disease associated with the loss of nigrostriatal dopaminergic neurons. However, the mechanisms underlying the

neurodegenerative process in Parkinson's disease are not fully understood (Kortekaas *et al.*, 2005). It has been hypothesized that inflammation, oxidative stress and disruption of the blood-brain barrier may all play their part in the pathogenesis of Parkinson's disease (Chen *et al.*, 2008, McGeer and McGeer, 2008). In addition, environmental toxins may have a role in the pathogenesis of Parkinson's disease. Their brain penetration because of the reduced activity or expression of P-glycoprotein has been postulated as contributing to the pathogenesis (Kortekaas *et al.*, 2005). P-glycoprotein function in the blood-brain barrier decreases with ageing (Toornvliet *et al.*, 2006, Bartels *et al.*, 2009, Bauer *et al.*, 2009). A dysfunction of P-glycoprotein with ageing could be a contributing factor for the development of neurodegenerative disorders, such as Parkinson's disease. In addition, genetic polymorphisms in the *MDR1* gene among ethnic populations may be one factor for increasing or decreasing the risk of Parkinson's disease (Lee *et al.*, 2004, Tan *et al.*, 2005).

2.4 MODELS FOR THE BLOOD-BRAIN BARRIER

Many different models are available for drug transport studies across the blood-brain barrier. These models can be classified into *in vitro, in vivo* and *in silico* models. All of these models have advantages and limitations that will be described in more detail in the subsequent sections.

2.4.1 *In vitro* primary cells

In an attempt to speed up the early drug development process and reduce the number of animal experiments in the characterization of drug candidates, cell based *in vitro* models have been developed. In order to predict reliably the *in vivo* blood-brain barrier permeability of drugs, an *in vitro* model should closely resemble the brain endothelium and exhibit relevant blood-brain barrier properties (*e.g.,* tight paracellular barrier, appropriate expression and functionality of efflux proteins and specific enzymes). Therefore, primary cells isolated from brain tissue have been considered to be the closest *in vitro* substitute for the *in vivo* blood-brain barrier (Gumbleton and Audus, 2001). The viable microvessels derived from the rat brain tissue can be considered to be the first *in vitro* model of blood-brain barrier (Joó and Karnushina, 1973). In the early 1980s, rat and bovine brain endothelial cells were successfully grown under cell culture conditions (Bowman *et al.*, 1981, Bowman *et al.*, 1983). Thereafter, primary brain endothelial cells have been isolated from several mammalian species, *e.g.*, rodents.

Table 2. Selected endothelial cell and blood-brain barrier markers. Modified from Deli, 2007.

Endothelial cell markers	**Blood-brain barrier specific markers**
von Willebrandt factor/Factor VIII antigen (vWF)	tight junction proteins (*e.g.*, ZO-1, occludin, claudins)
acetylated low-density lipoprotein uptake	tight junction functions (low permeability of paracellular markers, such as sucrose; high TEER)
vasoactive mediators (nitric oxide, endothelins, angiotensins)	enzymes (*e.g.*, ALP, γ-glutamyl-transpeptidase, monoamine oxidase A and B, COMT)
cell adhesion molecules (*e.g.*, vascular cell adhesion molecule-1)	active transporters (*e.g.*, GLUT1, LAT1, P-glycoprotein, MRPs, BCRP)
lectin binding	transport receptors (*e.g.*, insulin receptor, transferrin receptor)

ZO-1, zonula occludens 1; TEER, transendothelial electrical resistance; ALP, alkaline phosphatase; COMT, catechol-O-methyl-transferase; GLUT1, glucose transporter 1; LAT1, large neutral amino acid transporter 1; MRPs, multidrug resistance-associated proteins, BCRP, breast cancer resistance protein.

Bovine brain microvessel endothelial cells (BBMECs)

The BBMECs have been applied as an *in vitro* blood-brain barrier model (Baranczyk-Kuzma *et al.*, 1986, Audus and Borchardt, 1987, Audus *et al.*, 1990, Audus *et al.*, 1996). BBMECs have been extensively characterized in terms of endothelial cell and blood-brain barrier markers (Table 2) including ALP, catechol-*O*-methyl-transferase (COMT), γ-glutamyl transpeptidase and von Willebrandt factor/Factor VIII antigen (vWF) (Audus and Borchardt, 1986, Baranczyk-Kuzma *et al.*, 1986, Méresse *et al.*, 1989, Audus *et al.*, 1990) and uptake of acetylated low density lipoprotein (Tao-Cheng *et al.*, 1987, Stolz and Jacobson, 1991). In addition, expression of the tight junction proteins (occludin, ZO-1, claudin-1 and claudin-5) (Culot *et al.*, 2008) and efflux transporters (P-glycoprotein, MRP1, MRP4 and MRP5) have been characterized in the BBMECs at the protein level (Tsuji *et al.*, 1992, Beaulieu *et al.*, 1995, Fontaine *et al.*, 1996, Huai-Yun *et al.*, 1998, Zhang *et al.*, 2000, Zhang *et al.*, 2004). BBMECs have also been shown to express *MRP3*, *MRP6* and *BCRP* at the mRNA level but they lack *MRP2* (Zhang *et al.*, 2000, Warren *et al.*, 2009).

The functionality of efflux proteins has been demonstrated in the BBMECs with cellular uptake assays (Tsuji *et al.*, 1992, Joly *et al.*, 1995, Lechardeur and Scherman, 1995, Sun *et al.*, 2001, Silverstein *et al.*, 2004, Rice *et al.*, 2005, Bachmeier *et al.*, 2006, Iwanaga *et al.*, 2011). These aforementioned studies indicate that the BBMECs express functional proteins relevant to the function of the blood-brain barrier.

The permeability values for several model compounds as well as undisclosed molecules have been reported in the BBMEC model, but the tightness of the monocultured BBMECs has been shown to be highly variable (4 to 85 × 10^{-6} cm/s, Table 3) (Pardridge *et al.*, 1990, Eddy *et al.*, 1997, Glynn and Yazdanian, 1998, Cecchelli *et al.*, 1999, Johnson and Anderson, 1999, Polli *et al.*, 2000, Otis *et al.*, 2001, Karyekar *et al.*, 2003, Rice *et al.*, 2005). Monocultured BBMECs exhibit a rather leaky paracellular barrier which may limit their use in drug permeability studies.

BBMECs have also been cultured under an astrocyte conditioned medium (ACM) (Rubin *et al.*, 1991b), co-cultured with rat astrocytes (Tao-Cheng *et al.*, 1987) or in combination with agents that increase the cyclic adenosine monophosphate (cAMP) levels (*e.g.*, cAMP analogs, forskolin and cholera toxin) (Rubin *et al.*, 1991b), since the astrocytic factors and increased cAMP levels have been shown to enhance tight junctions *in vitro*. Therefore, co-cultures containing both BBMECs and rat astrocytes have also been used as an *in vitro* drug permeability model (Dehouck *et al.*, 1990, Dehouck *et al.*, 1992, Cecchelli *et al.*, 1999, Lundquist *et al.*, 2002). The tightness of the co-cultured BBMEC model has been shown to be better (permeability of sucrose 8.3 to 13 × 10^{-6} cm/s) than that of the monocultured BBMEC model (permeability of sucrose 32 × 10^{-6} cm/s) (Dehouck *et al.*, 1995, Lundquist *et al.*, 2002). In addition, it has been shown that P-glycoprotein expression is increased in the BBMECs when co-cultured with astrocytes (Fenart *et al.*, 1998, Gaillard *et al.*, 2000) indicating the inducible effect of astrocytic factors on P-glycoprotein expression. However, co-culture does not considerably increase the functionality of P-glycoprotein (Gaillard *et al.*, 2000). Recently, a combination of different culture medium supplements (ACM, cAMP derivative, dexamethasone, cAMP phosphodiesterase inhibitor) has been shown to improve the tightness of the BBMEC co-culture model (permeability of mannitol 0.5-0.9 × 10^{-6} cm/s) (Helms *et al.*, 2010, Helms *et al.*, 2012). This model seems to be the tightest *in vitro* blood-brain barrier model so far. However, this is still two orders of magnitude leakier than the blood-brain barrier *in vivo* (Ohno *et al.*, 1978).

Table 3. Permeability coefficient of sucrose across the selected *in vitro* models.

***In vitro* model**	**Experimental set-up**	**Permeability coefficient × 10^6 (cm/s)**	**Reference**
BBMEC	side-by-side	P_{app} 18-53	Eddy *et al.*, 1997, Johnson and Anderson, 1999, Otis *et al.*, 2001, Rice *et al.*, 2005
BBMEC	filter insert	P_{app} 4-39	Polli *et al.*, 2000, Johnson and Anderson, 1999, Karyekar *et al.*, 2003
BBMEC	filter insert	P_e 9.1-85	Pardridge *et al.*, 1990, Glynn and Yazdanian, 1998, Cecchelli *et al.*, 1999
PBMEC	side-by-side	P_{app} 39-80	Huwyler *et al.*, 1996, Zhang *et al.*, 2006b
PBMEC	filter insert	P_e 0.34$^{\#}$	Lohmann *et al.*, 2002
PBMEC	filter insert	P_{app} 4.0§-4.2	Hoheisel *et al.*, 1998, Franke *et al.*, 1999
PBMEC	filter insert	P_{app} 0.5$^{\#}$-1.0$^{\#}$	Hoheisel *et al.*, 1998, Franke *et al.*, 1999
HBMEC	filter insert	P_e 17*-50	Megard *et al.*, 2002
HBMEC	filter insert	P_{app} <8*-22*	Garberg *et al.*, 2005, Mabondzo *et al.*, 2010
hCMEC/D3	filter insert	P_e 28	Weksler *et al.*, 2005, Poller *et al.*, 2008
Caco-2	filter insert	P_{app} 1.4-1.7	Yazdanian *et al.*, 1998, Garberg *et al.*, 2005
MDCKII-MDR1	filter insert	P_{app} 0.3-0.4	Garberg *et al.*, 2005, Mashayekhi *et al.*, 2010
in vivo rat	intravenous injection	$P_{in\ vivo}$ 0.03	Ohno *et al.*, 1978

P_{app}, apparent permeability coefficient; P_e, permeability across cell monolayer ($1/P_e=1/P_{total}-1/P_{filter}$); * co-culture with astrocytes; $^{\#}$ serum free conditions supplemented with hydrocortisone; § serum containing conditions supplemented with hydrocortisone; $P_{in\ vivo}$, cerebrovascular permeability in vivo.

Porcine brain microvessel endothelial cells (PBMECs)

Few years after development of the BBMEC model, endothelial cells were also isolated from porcine brain (Tontsch and Bauer, 1989). Thereafter, PBMECs have been used as an *in vitro* blood-brain barrier model (Huwyler *et al.*, 1996, Drewe *et al.*, 1999), especially in Europe due to the issue of the bovine spongiform encephalopathy in cattle (Gumbleton and Audus, 2001). PBMECs display a tighter paracellular route after culturing under serum-free and hydrocortisone supplemented culture media (Table 3) (Hoheisel *et al.*, 1998, Tilling *et al.*, 1998, Franke *et al.*, 1999, Franke *et al.*, 2000, Lohmann *et al.*, 2002). Thus, the tightness of the PBMEC model appears to be promising. However, a limited amount of data is available describing the characteristics of PBMECs cultured under serum-free and hydrocortisone supplemented culture conditions. Drug permeability data is also scarce, only drug permeability of seven model drugs (Lohmann *et al.*, 2002), *MDR1* and *BCRP* gene expression at the mRNA level and functionality of BCRP have been demonstrated (Eisenblätter *et al.*, 2003).

PBMECs have also been cultured with ACM (Zhang *et al.*, 2006b) and co-cultured with rat astrocytes (Cohen-Kashi Malina *et al.*, 2009). Co-culturing with astrocytes has improved the tightness of the paracellular barrier (permeability of sucrose 0.2 × 10^{-6} cm/s, TEER >1000 Ωcm^2 were achieved in a filter insert system with a low stirring action) (Cohen-Kashi Malina *et al.*, 2009). Under well stirred conditions, however, PBMECs cultured with serum and ACM but without hydrocortisone have possessed a higher paracellular barrier (Huwyler *et al.*, 1996, Zhang *et al.*, 2006b) than PBMECs cultured under serum-free hydrocortisone supplemented medium (Table 3). The expressions of the transporters (*MDR1*, *LAT1*, *LAT2*, *BCRP*, *MRP1* and *MRP4*) have been detected in the PBMECs cultured with ACM at the mRNA level (Zhang *et al.*, 2006b). However, the functionality of the transporters has not been sufficiently assessed in PBMECs.

Rat brain microvessel endothelial cells (RBMECs)

The tightness of the monocultured RBMECs (permeability of sucrose 2-11 × 10^{-6} cm/s) (Parkinson and Hacking, 2005, Perrière *et al.*, 2007) is comparable to that of BBMEC or PBMEC models (Table 3). However, the disadvantage of the RBMECs is the low endothelial cell number per animal. In addition, the high abundance of pericytes in the primary RBMECs limits the use of RBMECs in comparison to those of BBMECs, PBMECs or human primary brain microvessel endothelial cells (HBMECs) (Parkinson and Hacking, 2005). The high amount of pericytes may physically interrupt the formation of endothelial cell monolayer and, thus, the tightness of the monolayer will increase. RBMECs have been used in drug uptake studies (Sun *et al.*, 2006, Qiang *et al.*, 2008) and drug transport studies (Perrière *et al.*, 2007, Li *et al.*, 2009).

In addition to co-cultures consisting of RBMECs with astrocytes (Perrière *et al.*, 2007) and RBMECs with astrocytes and pericytes (Nakagawa *et al.*, 2009) have been established. The permeability of sucrose has been reported to be as low as 1.4 × 10^{-6} cm/s when RBMECs were co-cultured with astrocytes in the presence of hydrocortisone and cAMP supplements (Perrière *et al.*, 2007). Recently, however, RBMECs co-cultured with astrocytes and pericytes did not display P-glycoprotein mediated efflux of known substrates (Hellinger *et al.*, 2012), suggesting that the tightness of the RBMEC model is inducible by astrocytic factors and different culture medium supplements, but the functionality of the active transport mechanisms is still difficult to reach.

Human brain microvessel endothelial cells

The first isolation of microvessel endothelial cells from human brains occurred in 1991 with samples from autopsy (Dorovini-Zis *et al.*, 1991). HBMECs have been characterized in terms of adhesion molecules (Wong and Dorovini-Zis, 1995, Wong and Dorovini-Zis, 1996, Stins *et al.*, 1997, Easton and Dorovini-Zis, 2001) and several endothelial cell markers (*e.g.*, vWF, strong ALP activity, uptake of acetylated low density lipoprotein, γ-glutamyl-transpeptidase, Table 2) (Stins *et al.*, 1997, Omari and Dorovini-Zis, 2001). In addition, the expressions of the transporter genes (*MDR1, MRP2, MRP1, MRP4, MRP5, MRP6, LAT1, GLUT1, MCT1*) were detected at the mRNA level in the HBMECs (Umeki *et al.*, 2002, Eilers *et al.*, 2008, Warren *et al.*, 2009). However, there is still some controversy concerning *MDR1* and *MRP2* expression at mRNA level in the HBMECs, since in some reports, gene expressions of *MDR1* (Umeki *et al.*, 2002) or *MRP2* (Warren *et al.*, 2009) were not detected. The presence of functional MRP mediated efflux and functional amino acid transporters has been reported in the HBMECs as assessed in uptake studies (Umeki *et al.*, 2002, Eilers *et al.*, 2008).

The permeability of sucrose was lower in the HBMECs co-cultured with human astrocytes (~20 × 10^{-6} cm/s) (Megard *et al.*, 2002, Garberg *et al.*, 2005) than in the monocultured HBMECs (50 × 10^{-6} cm/s) (Megard *et al.*, 2002) highlighting the important role of astrocytes in determining the tightness of human brain endothelial cells *in vitro*. Recently, the co-culture model of HBMECs has been further characterized; expression of *GLUT1*, *LAT1* and *MDR1*, *MRP1*, *MRP4*, *MRP5*, *BCRP* at mRNA level, good paracellular tightness (Table 3) and functionality of the efflux transporters have been demonstrated (Mabondzo *et al.*, 2010). The HBMEC model appears to be promising as an *in vitro* model for human blood-brain barrier. In addition, the application of the HBMEC model has been further developed by dynamic *in vitro* models with a hollow fibre technique to include flow shear stress (Siddharthan *et al.*, 2007, Cucullo *et al.*, 2011). However, the disadvantage of HBMEC model is the limited availability of the human brain tissue, ethical problems and high expense for screening of drug candidates.

2.4.2 *In vitro* cell lines

Human brain endothelial cell line (hCMEC/D3)

The immortal human brain endothelial cell line, hCMEC/D3, has been derived from HBMECs, and it possess many endothelial and blood-brain barrier markers, *e.g.*, tight junction proteins ZO-1 and claudin-5 (Table 2) (Weksler *et al.*, 2005). Furthermore, expression of the efflux transporters (*MDR1*, *MRP1-5*, *BCRP*) has been demonstrated in the hCMEC/D3 cells at the mRNA level and expression of P-glycoprotein, MRP1, MRP4, BCRP at the protein level (Weksler *et al.*, 2005, Poller *et al.*, 2008, Dauchy *et al.*, 2009, Ohtsuki *et al.*, 2013). The functionality of the efflux transporters P-glycoprotein, MRP and BCRP was demonstrated in the absence of astrocytes (Poller *et al.*, 2008, Dauchy *et al.*, 2009). At present, hCMEC/D3 cells form a rather leaky cell monolayer (permeability of sucrose 27 × 10^{-6} cm/s) and there are rather few studies reporting drug permeability values for some compounds (n=11, mainly passively transported drugs) in the hCMEC/D3 cell model (Weksler *et al.*, 2005, Poller *et al.*, 2008). Therefore, more drug permeability data is needed to characterize the drug permeability properties across hCMEC/D3 cell model. Attempts have also been made to develop a hCMEC/D3 based co-culture model (Hatherell *et al.*, 2011, Weksler *et al.*, 2013). The advantage of the hCMEC/D3 cells is that these cells are easy to grow. In addition, hCMEC/D3 cells have been reported to be suitable for use in the hollow fibre technique which incorporates flow shear stress and thus they may represent a more realistic *in vitro* system (Cucullo *et al.*, 2008). However, the drastic loss of the expression of several relevant blood-brain barrier proteins (*e.g.*, tight junction proteins and efflux transporters) as assessed at the mRNA level in hCMEC/D3 cells, as well as HBMECs, compared to freshly isolated brain endothelial cells (Urich *et al.*, 2012), and the low TEER values seem to be major challenges to the widespread use of hCMEC/D3 cells (Hatherell *et al.*, 2011, Weksler *et al.*, 2013).

Rat and mouse brain microvessel endothelial cell lines

There are several immortalized rat endothelial cell lines available (for review see Roux and Couraud, 2005) and one of the most commonly used is the rat endothelial cell line (RBE4) which has also been characterized in terms of the enzymatic activities of ALP and γ-glutamyl-transpeptidase (Roux *et al.*, 1994) and the functionality of P-glycoprotein via a cellular uptake assay (El Hafny *et al.*, 1997). The major limitation of RBE4 cells is the poor cell monolayer tightness (permeability of sucrose 214 × 10^{-6} cm/s) (Rist *et al.*, 1997) which is not adequate for drug permeability studies. This reduces the feasibility of this model for screening (Roux and Couraud, 2005) but it can be used for mechanistic studies.

In addition, several mouse brain endothelial cell lines have been established (Tatsuta *et al.*, 1992, Omidi *et al.*, 2003, Yang *et al.*, 2007). Similarly to the situation with rat brain endothelial cell lines, the mouse brain endothelial cell lines do not provide adequate tightness to permit drug permeability testing (Yang *et al.*, 2007). As a result, mouse brain endothelial cell lines are not widely used for screening purposes.

Human colorectal adenocarcinoma cell line (Caco-2)

Caco-2 cells are epithelial cells isolated from male colorectal adenocarcinoma (Fogh *et al.*, 1977). Caco-2 cells have been shown to represent a suitable epithelial model of intestinal transport studies (Hidalgo *et al.*, 1989). Passive absorption of drugs across Caco-2 cells has been studied reflecting the *in vivo* absorption characteristics in rats and humans (Artursson, 1990, Artursson

and Karlsson, 1991, Ranaldi *et al.*, 1992). Currently, Caco-2 cell model is widely used as an *in vitro* model for oral absorption both in academia and pharmaceutical industry (Hayeshi *et al.*, 2008). Caco-2 model has also been used for prediction of the blood-brain barrier permeability of drugs (Garberg *et al.*, 2005, Hellinger *et al.*, 2012). However, there is still only a limited number of reports investigating the use of Caco-2 as an *in vitro* model for blood-brain barrier.

P-glycoprotein mediated transport in the Caco-2 cells has been studied extensively by using known P-glycoprotein substrates such as paclitaxel, digoxin and vinblastine (Hunter *et al.*, 1993, Cavet *et al.*, 1996, Walle and Walle, 1998). The gene expression levels of efflux transporters (*e.g.*, *MDR1*, *MRP*s) in the Caco-2 cells were similar to those present in human intestine, with the exception of *BCRP* the levels of which were found to be ~100-fold lower in Caco-2 cells than in human intestine (Taipalensuu *et al.*, 2001). Despite promising results, the expression of P-glycoprotein varies as a function of cell passage, culture conditions and cell differentiation (Hosoya *et al.*, 1996, Anderle *et al.*, 1998, Goto *et al.*, 2003).

Caco-2 cells express many enzymes (Prueksaritanont *et al.*, 1996). ALP is a brush border enzyme localized at the apical membrane in the Caco-2 cells (Hidalgo *et al.*, 1989) and, thus, it is a widely used marker for Caco-2 enterocytic differentiation. The levels of CYP3A4 enzyme expression are very low in the Caco-2 cells when compared to those in human intestinal enterocytes (Prueksaritanont *et al.*, 1996, Nakamura *et al.*, 2003) and this is a well known limitation of Caco-2 cells. Therefore, genetically modified and chemically induced Caco-2 cells have been developed to increase the expression of drug metabolizing enzymes and P-glycoprotein (Schmiedlin-Ren *et al.*, 1997, Döppenschmitt *et al.*, 1999, Eneroth *et al.*, 2001, Korjamo *et al.*, 2005, Hellinger *et al.*, 2012).

One main drawback of Caco-2 cells is their long differentiation time (≥21 days) (Yuan *et al.*, 2009). In addition, Caco-2 cells are known to exhibit high variability between laboratories due to different origin of the cells and culture conditions (Behrens and Kissel, 2003, Hayeshi *et al.*, 2008). Thus, the inter-laboratory variability in the expression of transporters and enzymes has been studied confirming that the results emerging from different laboratories using Caco-2 cell are not identical (Hayeshi *et al.*, 2008). Due to the several limitations of Caco-2 cells in screening, also other cell models have been developed, such as Madin-Darby Canine Kidney (MDCK) cells, to be used as alternative *in vitro* models for prediction of drug absorption.

Madin-Darby Canine Kidney cell line

MDCK cells were isolated from a female cocker spaniel (for review see Dukes *et al.*, 2011). MDCK cells display clear apical-basolateral polarity with well-defined cell junctions and, thus, they are widely used as an *in vitro* epithelial cell model (Dukes *et al.*, 2011). Two sub-types (type I and II) have been isolated from parental MDCK strain; MDCKI cells from low passage (~60) and MDCKII cells from high passage (~113). The most striking difference between these two sub-types is that MDCKI form monolayers with high TEER (>4000 Ωcm^2) whereas MDCKII cell monolayers exhibit low TEER (<200 Ωcm^2) (Barker and Simmons, 1981). The enzymatic activities of ALP and γ-glutamyl-transpeptidase have been measured from MDCK cells (Veronesi, 1996). Since these enzymes are also expressed at the blood-brain barrier (Table 2) the MDCK cells have been proposed to represent a useful *in vitro* model for screen xenobiotics that disrupt the blood-brain barrier *in vivo* (Veronesi, 1996).

The advantage of the MDCK cells over Caco-2 cells is their shorter culture time (4 days vs. 21 days) (Irvine *et al.*, 1999). The drug permeability across the MDCK model has also been

compared with Caco-2 model showing that the permeability of drugs was comparable (Irvine *et al.*, 1999). In addition, the MDCK model and BBMEC model are able to place in a similar rank order drugs into low, medium and high permeability categories, although the leakiness was higher in the BBMEC model than in MDCK model (Polli *et al.*, 2000). These experiments indicate that MDCK cells may be valid substitute for Caco-2 or BBMEC cells for prediction of gut absorption or blood-brain barrier permeability.

Generally, MDCK type II cell line transfected with the human *MDR1* gene, encoding for human P-glycoprotein (MDCKII-MDR1), *MRP1* or *MRP2* genes have been used to evaluate the involvement of efflux transporters in intestinal drug absorption (Guo *et al.*, 2002, Luo *et al.*, 2002, Troutman and Thakker, 2003, Varma *et al.*, 2005, Shirasaka *et al.*, 2008, Thiel-Demby *et al.*, 2009). In addition, MDCK(I,II)-MDR1 cells have been used to study P-glycoprotein mediated transport (Lentz *et al.*, 2000, Polli *et al.*, 2001, Mahar Doan *et al.*, 2002, Troutman and Thakker, 2003, Tran *et al.*, 2004, Bentz *et al.*, 2005, Acharya *et al.*, 2006, Acharya *et al.*, 2008).

2.4.3 Comparison of *in vitro* models

Tightness

Sucrose is the most commonly used compound to represent as a low permeability molecule in the characterization of the tightness of the *in vitro* models for blood-brain barrier. A comparison between the tightness of the selected monocultured *in vitro* models is shown in Table 3. Other common characteristics of the *in vitro* models and advantages and limitations of the *in vitro* models based on an interpretation of the literature have been included in Table 4.

The paracellular tightness seems to be highly variable between the laboratories in the primary monocultured endothelial cell models (BBMECs and PBMECs), as well as between the endothelial and epithelial *in vitro* models. One of the tightest endothelial cell model available is PBMECs cultured under serum-free medium and supplemented with hydrocortisone (P_{app} of sucrose 0.5-1.0 × 10^{-6} cm/s) (Hoheisel *et al.*, 1998, Franke *et al.*, 1999). However, in serum containing culture medium, the P_{app} of sucrose in the PBMECs is extremely variable ranging from 4.2-8 × 10^{-6} cm/s in filter insert system (Franke *et al.*, 1999) to 39-80 × 10^{-6} cm/s in well-stirred conditions (Huwyler *et al.*, 1996, Zhang *et al.*, 2006b). A similar variation in the tightness of the BBMECs has been observed; 4-39 × 10^{-6} cm/s in filter insert system (Johnson and Anderson, 1999, Polli *et al.*, 2000, Karyekar *et al.*, 2003) to 18-53 × 10^{-6} cm/s in well-stirred conditions (Eddy *et al.*, 1997, Johnson and Anderson, 1999, Otis *et al.*, 2001, Rice *et al.*, 2005). These observations demonstrate that primary endothelial cell models are both sensitive to culture conditions affecting the paracellular barrier between the endothelial cells but they also suffer from high inter-laboratory variation.

The epithelial cell models are generally tighter than the endothelial cell models (Table 3). Apparently the higher P_{app} value of sucrose in the Caco-2 than in the MDCKII-MDR1 may partly be explained by the brush-border enzyme, sucrase-isomaltase, expressed in the Caco-2 cells (Pinto *et al.*, 1983). Metabolism of the [^{14}C]sucrose leads to the formation of [^{14}C]metabolites (*i.e.*, glucose and fructose) and in fact the P_{app} of [^{14}C]metabolites are being measured in the Caco-2 cells (Garberg *et al.*, 2005). Although rather tight *in vitro* models (P_{app} <1 × 10^{-6} cm/s) are available, the *in vitro* models are still several orders of magnitude leakier (Pardridge *et al.*, 1990, Avdeef, 2011) than the blood-brain barrier *in vivo* (~3 × 10^{-8} cm/s) (Ohno *et al.*, 1978) suggesting that the tightness of the blood-brain barrier *in vivo* is extremely difficult to mimic *in vitro*.

Efflux transporters

In the brain endothelial cells of many species, there is the apical (luminal) localization of P-glycoprotein, MRP1, MRP2, MRP4, MRP5 and BCRP and basolateral (abluminal) localization of MRP1 and MRP4. However, there may be species differences in the expression of MRP2 in the brain endothelial cells since no significant expression of *MRP2* was detected in human, rat, mouse, porcine or bovine brain endothelial cells at the mRNA level (Warren *et al.*, 2009). Whereas the apical localization of MRP2 has been demonstrated in brain capillaries isolated from rat (Miller *et al.*, 2000) or HBMECs at the mRNA level (Eilers *et al.*, 2008). Thus, the MRP2 expression in the blood-brain barrier is still controversial.

In comparison, Caco-2 model express *MDR1*, *BCRP*, *MRP1-5* at mRNA level (Taipalensuu *et al.*, 2001). Apically localized intestinal efflux transporters are P-glycoprotein, BCRP, MRP2 and MRP4, whereas the basolaterally localized intestinal efflux transporters are MRP1, MRP3-5 (Takano *et al.*, 2006, for review see Custodio *et al.*, 2008, Giacomini *et al.*, 2010). In addition, the MDCKII-MDR1 model overexpresses human P-glycoprotein and canine MRP2 at the protein level and canine MDR1, MRP1, MRP2, MRP5 at the mRNA level. The apically localized efflux proteins in kidney epithelial cells are P-glycoprotein, MRP2, MRP4 and BCRP, whereas MRP1, MRP3 and MRP5 localize basolaterally (Schinkel and Jonker, 2003).

The most notable differences between the localization of efflux transporters in the brain endothelial cell models and models of intestinal or kidney origin (Caco-2, MDCKII-MDR1) is the apical localization of MRP1 and MRP5 (MRP1 also basolaterally) in the brain endothelial cells, but there is basolateral localization in intestinal and kidney cells. In addition, *MRP3* has been detected only at low mRNA levels in the brain endothelial cells but instead MRP3 is localized basolaterally in the intestinal and kidney cells. The differences between endothelial and epithelial cells models highlight the fact that the non-brain originating cells may not express the transporters or they are not correctly localized and, thus, they do not model the *in vivo* blood-brain barrier adequately. Furthermore, all known efflux transporters have not been thoroughly characterized in the brain endothelial cell models from different species or epithelial cell models, at either the protein level or their subcellular localization (apical vs. basolateral). Therefore, it is not possible to conduct a detailed comparison of the localization of efflux transporters between the cell models. The differences in the expression and localization of efflux transporters may disturb the permeability of those drugs that are potential transporter substrates, when non-brain originating cell models are used in prediction of blood-brain barrier permeability of new drug candidates.

Anatomy and physiology of endothelial and epithelial cells

Endothelial and epithelial cells form different types of tissues and have distinct functions *in vivo*. The endothelial cells that form the inner lining of the blood vessels maintain vascular homeostasis, control the transfer of many molecules and have many metabolic and synthetic functions (Sumpio *et al.*, 2002). The epithelial cells in nephrons have a pivotal role in kidney function. Renal tubular epithelial cells participate in concentrating the glomerular filtrate into urine (Baud, 2003). The major function of the differentiated enterocytes (intestinal epithelial cells) *in vivo* is to digest and absorb nutrients and water from ingested food (Delie and Rubas, 1997) but also to protect the organism against luminal pathogens (Gibson *et al.*, 1996).

Brain endothelial cells, intestinal epithelial cells and renal tubular epithelial cells are morphologically very different. Brain endothelial cells are >100 μm long and spindle-shaped

with the height of the cells ranging from 0.2-2 μm and they does not have microvilli (Lechardeur and Scherman, 1995, Cecchelli *et al.*, 1999, Nakagawa *et al.*, 2009, Hellinger *et al.*, 2012). At confluency, Caco-2 cells are thin and tall (~6.4×30 μm) with long microvilli (~1.2 μm) (Hidalgo *et al.*, 1989). Recently, it was shown that Caco-2 cells, grown in a culture medium supplemented with vinblastine (to increase P-glycoprotein expression), were 8-15 μm high (Hellinger *et al.*, 2012) a value that is clearly shorter than that reported previously (Hidalgo *et al.*, 1989). In fact, the cellular dimensions may vary extensively due to the heterogeneity of the Caco-2 cells (Delie and Rubas, 1997). MDCKII cells are wider and shorter (~8×10 μm) with longer microvilli (~1-1.5 μm) (Barker and Simmons, 1981) than Caco-2 cells, but it was recently claimed that MDCKII-MDR1 cells are higher (10-20 μm) (Hellinger *et al.*, 2012) than the parental MDCKII cells.

There are also differences in the phospholipid composition of cell membranes between BBMECs, Caco-2 and MDCKII cells. The determinants of cell membrane fluidity are: 1) phosphatidylcholine to sphingomyelin ratio, 2) the unsaturated to saturated ratio of phospholipids, 3) the cholesterol to phospholipid ratio (Shinitzky and Barenholz, 1974, Williams *et al.*, 1988). Higher ratios increase the membrane fluidity and increase passive permeability. In the BBMECs, all of these three ratios are lower (Siakotos and Rouser, 1969, Bénistant *et al.*, 1995, Di *et al.*, 2009) than in the Caco-2 (Dias *et al.*, 1992) or MDCKII cells (Hansson *et al.*, 1986). Thus, the BBMECs cell membrane should be less fluid, more rigid and less permeable than the membrane in Caco-2 or MDCKII cells.

Table 4. Comparison of *in vitro* models used to assess blood-brain barrier permeability

Cell model	Cell type	Species	Characteristics	References	Advantages	Limitations
BBMEC	primary brain endothelial	bovine	vWF, tight junctions, a few pinocytic vesicles, ALP, γ-glutamyl transpeptidase, uptake of acetylated low density lipoprotein, occludin, ZO-1, claudin-1 and -5, P-glycoprotein	Bowman *et al.*, 1983, Audus and Borchardt, 1986, Tao-Cheng *et al.*, 1987, Stolz and Jacobson, 1991, Tsuji and Tamai, 1997, Cecchelli *et al.*, 1999, Culot *et al.*, 2008	brain origin, good tissue availability, good yield of cells	species differences, laborious and expensive, low dynamic range (permeability of transcellular marker / permeability of paracellular marker), does not demonstrate robust efflux
PBMEC	primary brain endothelial	porcine	vWF, tight junctions, ALP, γ-glutamyl transpeptidase, uptake of acetylated low density lipoprotein, ZO-1, occludin, claudin-5, P-glycoprotein	Tontsch and Bauer, 1989, Huwyler *et al.*, 1996, Hoheisel *et al.*, 1998, Drewe *et al.*, 1999, Nitz *et al.*, 2003, Zhang *et al.*, 2006b, Ott *et al.*, 2010	brain origin, good tissue availability, good yield of cells	species differences, laborious and expensive, low dynamic range, does not demonstrate robust efflux
RBMEC, MBMEC	primary brain endothelial	rodent	vWF, ALP, γ-glutamyl transpeptidase, uptake of acetylated low density lipoprotein, ZO-1, occludin, P-glycoprotein	Tatsuta *et al.*, 1992, Kis *et al.*, 1999, Parkinson and Hacking, 2005, Domoki *et al.*, 2008	brain origin, good tissue availability	species differences, low yield of cells, laborious and expensive, low dynamic range, does not demonstrate robust efflux
HBMEC	primary brain endothelial	human	vWF, ALP, γ-glutamyl transpeptidase, uptake of acetylated low density lipoprotein, *MDR1* mRNA	Dorovini-Zis *et al.*, 1991, Stins *et al.*, 1997, Omari and Dorovini-Zis, 2001, Megard *et al.*, 2002, Eilers *et al.*, 2008, Warren *et al.*, 2009	human brain origin	limited tissue availability, laborious and expensive, low dynamic range, currently limited amount of permeability data
hCMEC/D3	brain endothelial cell line	human	ZO-1, claudin-5, P-glycoprotein	Weksler *et al.*, 2005	human brain origin, easy to culture and unlimited number of cells	low dynamic range, currently limited amount of permeability data
RBE4	brain endothelial cell line	rat	vWF, ALP, γ-glutamyl transpeptidase, ZO-1, occludin, claudin-5, P-glycoprotein	Roux *et al.*, 1994, Begley *et al.*, 1996, Huwyler *et al.*, 1999, Kis *et al.*, 1999, Savettieri *et al.*, 2000, Balbuena *et al.*, 2011	easy to culture and unlimited number of cells	species differences, low dynamic range, not suitable for drug permeability studies due to high leakiness
Caco-2	epithelial cell line	human	ALP, ZO-1, occludin, claudin-1, claudin-4, human P-glycoprotein	Pinto *et al.*, 1983, Hosoya *et al.*, 1996, Yuan *et al.*, 2009, Kawauchiya *et al.*, 2011, Hellinger *et al.*, 2012, Yu *et al.*, 2013	human origin, easy to culture and unlimited number of cells, wide dynamic range, demonstrate robust efflux protein functionality	slow differentiation, colon origin, active efflux and influx transporters may not be expressed and localized similarly as in the brain endothelial cells
MDCK(I,II)-MDR1	epithelial cell line	dog	ALP, ZO-1, occludin, claudin-1, claudin-4, human P-glycoprotein	Veronesi, 1996, Hämmerle *et al.*, 2000, Polli *et al.*, 2001, Mahar Doan *et al.*, 2002, Kamau *et al.*, 2005, Hellinger *et al.*, 2012	easy to culture and unlimited number of cells, fast differentiation, wide dynamic range, demonstrate robust P-glycoprotein functionality	species differences, kidney origin, active efflux and influx transporters may not be expressed and localized similarly as in the brain endothelial cells

vWF, von Willebrand factor; ALP, alkaline phosphatase; ZO-1, zonula occludens 1; MDR1 multidrug resistance protein gene.

2.4.4 *In vivo* methods

Intravenous injection

An intravenous injection is considered the most physiological approach to study brain uptake since physiological conditions are maintained (for review see Bickel, 2005). The drug can be administered intravenously either as a bolus injection or as an infusion (Smith, 1989). Blood samples are collected at selected time points and plasma drug concentrations are determined. The total drug concentrations in the brain tissue and total blood or plasma concentrations are determined at the end of the experiment. The ratio between the total concentrations in brain and plasma, *i.e.*, the *in vivo* partition coefficient, $K_p = C_{brain}/C_{blood}$ or the logarithm of the partition coefficient ($LogBB = \log C_{brain}/C_{blood}$), is an estimate of the extent of drug delivery to the brain (Liu *et al.*, 2008, Hammarlund-Udenaes, 2010). Nonspecific binding in brain tissue and blood is a significant component of this parameter (Liu *et al.*, 2008). The *in vivo* influx clearance [CL_{in} or $K_{in} = (C_{total,brain} - C_{plasma} \times$ intravascular volume of the brain) / (study time $\times C_{plasma}$)] is an estimate of the rate of transport of drug across the blood-brain barrier (Hammarlund-Udenaes *et al.*, 2008). The disadvantages of this method are that different animals are needed for each data point and sensitive, specific and selective analytical methods are required (for review see Bickel, 2005, Kuhnline Sloan *et al.*, 2012).

Brain uptake index

In the brain uptake index (BUI) method (Oldendorf, 1970), the [^{3}H]-drug to be studied is administered as a rapid bolus injection simultaneously with the [^{14}C]-reference compound into the carotid artery. Concentrations of the drug and the reference compound are determined in the brain and in the injected solution at a single time point. The ratio of drug to reference compound is calculated [$BUI\% = ([^{14}C]_{brain}/[^3H]_{brain})/([^{14}C]_{injected}/[^3H]_{injected})$]. BUI% measures the rate of transport into brain based on the total concentrations of the drug in the brain at the early time points (5-15 sec) after drug injection (Cecchelli *et al.*, 2007, Hammarlund-Udenaes *et al.*, 2008). The advantage of the BUI method is that it is a rapid procedure which avoids peripheral metabolic artifacts. Generally, however, BUI is relatively insensitive *in vivo* method with which to differentiate between the compounds with low and high brain uptake (for review see Bickel, 2005).

In situ brain perfusion

The *in situ* brain perfusion technique, in the rat and in the mouse (Takasato *et al.*, 1984, Dagenais *et al.*, 2000, Murakami *et al.*, 2000), is considered as a gold standard for measuring blood-brain barrier permeability *in vivo*. The K_{in} can be estimated by the *in situ* brain perfusion technique and this parameter is commonly converted to permeability-surface area product (Chikhale *et al.*, 1994). The permeability-surface area product parameter relates to total brain and total blood concentrations describing the drug influx rate into the brain. In this technique, the peripheral metabolism of a drug is excluded by direct infusion of the compound into the blood vessels to the brain. This technique has a better ability to differentiate compounds in terms of their blood-brain barrier permeability than other techniques, *e.g.*, BUI method. The disadvantage of this method is that it is labor intensive (Kuhnline Sloan *et al.*, 2012). In addition, the high drug concentrations in the perfusion fluid may saturate the transport mechanism(s) and, thus, the

passive diffusion may be the predominant transport mechanism measured with this technique (Di *et al.*, 2009).

In vivo brain microdialysis

The microdialysis technique was originally developed for monitoring extracellular neurotransmitter levels in the brain (Benveniste and Hüttemeier, 1990). *In vivo* brain microdialysis has also been applied for pharmacokinetic studies of drug transport across the blood-brain barrier (for review see de Lange *et al.*, 1997). Microdialysis probes can be implanted in both the brain and peripheral tissues. The microdialysis probes are perfused with a physiological solution, and the driving force for drug diffusion across the semi-permeable membrane in the probe is the concentration gradient from brain tissue to perfusate (Elmquist and Sawchuk, 1997). *In vivo* brain microdialysis is a direct technique with which to assess unbound drug concentrations (Liu *et al.*, 2008).

The rate of transport (CL_{in}) can be estimated by the *in vivo* brain microdialysis technique (Hammarlund-Udenaes *et al.*, 1997, Hammarlund-Udenaes *et al.*, 2008). The extent of the drug concentration between brain and blood can be assessed by the unbound partition coefficient [$K_{p,uu}$ = the area under the unbound concentration-time curve $(AUC)_{unbound,brain,extracellular\ fluid\ (ECF)}$ / $AUC_{unbound,blood}$] at steady state (for review see Jeffrey and Summerfield, 2007, Hammarlund-Udenaes *et al.*, 2008). $K_{p,uu}$ can also reveal whether or not the drug undergoes active transport into the brain (Boström *et al.*, 2006, Jeffrey and Summerfield, 2007). Values less than unity are evidence of poor permeability, efflux transport, metabolism or extracellular fluid bulk flow which are all factors which can influence the unbound drug brain concentration. $K_{p,uu}$ values above unity would suggest that active influx transporters at the blood-brain barrier are enhancing the drug brain uptake. If the drug transport across the blood-brain barrier is dominated by passive diffusion, then the $K_{p,uu}$ value will be close to unity (Hammarlund-Udenaes *et al.*, 2008).

The *in vivo* brain microdialysis technique has also been used to compare overall drug exposure of new drug candidates in the brain after a single systemic dose (Jalkanen *et al.*, 2011). An *in vivo* unbound brain/blood ratio can be calculated from the unbound AUC in brain ECF and blood (Jalkanen *et al.*, 2011). The unbound brain/blood ratio is determined after a single dose. As a result, the unbound brain blood/blood ratio describes mainly the rate of the drug transport across the blood-brain barrier. In addition, in *in vivo* brain microdialysis, the AUC brain parameter describes also the distribution from the extracellular fluid into intracellular compartments, as well as the metabolism and elimination of the drug from the brain. Similarly, the AUC parameter in the blood takes into account the kinetics of absorption, distribution, metabolism and elimination throughout the body.

The advantage of the *in vivo* brain microdialysis method is that the unbound concentrations can be determined and individual concentration-time profiles can be constructed. The disadvantage of this method is the invasive nature of the insertion of the probe into the brain which may evoke blood-brain barrier damage (for review see de Lange *et al.*, 1997, Bickel, 2005). Furthermore, *in vivo* microdialysis is not suitable for highly lipophilic compounds due to compound adhesion to the perfusion tubing.

In vivo methods to assess the permeability of drugs across the blood-brain barrier

The intravenous injection approach, the BUI method, the *in situ* brain perfusion technique and the *in vivo* brain microdialysis method are able to estimate the permeability of drugs across the blood-brain barrier (Hammarlund-Udenaes *et al.*, 2008). One technique which measures only the blood-brain barrier permeability is the *in situ* brain perfusion technique. However, the weakness of the intravenous injection, BUI and *in situ* brain perfusion method is that the total brain and total blood concentrations are measured. It is generally accepted that the unbound drug is the form which is able to cross the blood-brain barrier and exert the pharmacological response (Hammarlund-Udenaes *et al.*, 2008). Therefore, the *in vivo* brain microdialysis method, which assesses the unbound drug concentrations on both sides of the blood-brain barrier, is currently considered the most relevant method.

2.4.5 *In silico* methods

Prediction of brain penetration of drugs by computational approaches is a fast and valuable tool in early drug discovery, since *in silico* methods are rather easy and quick to perform without even the need to synthesize the molecules. However, it is important to remember that a large amount of experimental data is needed before one can construct predictive *in silico* models and to validate them (van de Waterbeemd and Gifford, 2003). It is possible to predict blood-brain barrier permeation by assigning drugs into two classes (brain penetrating drugs or low/absent brain penetrating drugs) based on their molecular structure (Crivori *et al.*, 2000). The molecular properties that have been found to influence the blood-brain barrier permeability of drugs are mainly polarity (hydrophilic regions and polar regions), hydrogen-bonding capability and their distribution within the drug, with a smaller role for hydrophobic interactions (Crivori *et al.*, 2000).

Many *in silico* models are based on LogBB, which describes drug partitioning between blood and brain tissue (Young *et al.*, 1988). The *in silico* models based on LogBB data have been comprehensively reviewed earlier (for review see Norinder and Haeberlein, 2002, Clark, 2003). There is a consensus about the molecular properties that determine the partitioning between the blood and brain; polarity and hydrogen bonding capability correlate negatively with LogBB (*i.e.*, increasing polarity and hydrogen-bonding reduce brain penetration), whereas higher lipophilicity and small molecular size seems to correlate positively with LogBB. The correlation between LogBB and charge, molecular shape or flexibility is less clear (for review see Clark, 2003). There are several concerns associated with the use of LogBB value as an *in vivo* measure (Bickel, 2005, Goodwin and Clark, 2005). First, LogBB value is highly affected by drug binding to plasma and brain tissue proteins and, thus, it does not reflect the blood-brain barrier permeability process but partitioning between these tissues. Second, LogBB is quantified from total brain tissue concentrations which may not show a good correlation with the unbound concentration and, thus, pharmacologically active concentrations in the brain. Third, the LogBB should have been determined at steady state which is often violated in the data sets (Bickel, 2005). Therefore, the experimental data used for the development of the *in silico* models should be relevant and reflect the actual process which one intends to predict.

The lack of *in vivo* blood-brain barrier permeability data (*e.g.*, permeability-surface area product determined with *in situ* brain perfusion technique) has clearly limited the development of *in silico* predictive models based on this parameter, and only a few reports have generated these kinds of models (Gratton *et al.*, 1997, Abraham, 2004, Liu *et al.*, 2004). These studies have

revealed that the molecular properties related to polarity, hydrogen bonding and lipophilicity are important for blood-brain barrier permeability. In addition, Fridén's model (an *in silico* model based on the $K_{p,uu}$ parameter) has increased the understanding about unbound drug brain exposure; it highlighted that hydrogen bonding was the most important determinant for $K_{p,uu}$, demonstrating that addition of two HBAs to the molecule decreased the brain exposure by half (Fridén *et al.*, 2009). All *in silico* models together indicate that hydrogen bonding is the most important determinant for drug permeability across the blood-brain barrier. In contrast to previous *in silico* models based on LogBB, lipophilicity was not correlated with the unbound brain exposure *in vivo* (Fridén *et al.*, 2009). Since the unbound drug concentrations mainly determine the blood-brain barrier permeability *in vivo*, the kinds of approaches which take into account the unbound drug concentrations appear to be very promising.

In silico modeling of the P-glycoprotein interactions

P-glycoprotein is an important membrane transporter in *in vivo* drug disposition and its presence has a broad impact on absorption, distribution, metabolism and excretion. Therefore, attempts have been made to clarify the molecular attributes that are required for the P-glycoprotein interactions (Stouch and Gudmundsson, 2002). The interaction of compounds with the P-glycoprotein is a complex process. In the hydrophobic vacuum cleaner model, P-glycoprotein has been proposed to have a flexible drug binding pocket to which its substrates gain access from the membrane bilayer (Higgins and Gottesman, 1992). Therefore, P-glycoprotein substrates need to partition into the membrane before they can interact with the P-glycoprotein. As a result, its substrates are typically hydrophobic but in reality, the mechanism of the transport is not well understood.

Fixed spatial separation of two and three HBA groups of 2.5±0.3 Å and 4.6±0.6 Å have been suggested to be required for an interaction with P-glycoprotein (Seelig, 1998a, Seelig, 1998b). However, these rules based on the spatial separation of HBA are too simple to define P-glycoprotein substrates and inhibitors. Quantitative structure-activity relationship analysis has been employed to determine the descriptors that are important for P-glycoprotein substrates assayed by using *in vitro* MDCKII-MDR1 assay (Gombar *et al.*, 2004). It was claimed that the larger size of a molecule and its ability to partition into membranes increase its ability to act as a P-glycoprotein substrate. In addition, many studies have tried to develop the structure-based prediction models for P-glycoprotein substrates or inhibitors (for review see Chen *et al.*, 2012). However, the multiple binding sites of P-glycoprotein (Shapiro and Ling, 1997, Martin *et al.*, 2000, Aller *et al.*, 2009) and a broad variety of compounds known to interact with P-glycoprotein complicate the *in silico* prediction of the P-glycoprotein substrates and inhibitors. Thus, more studies and broader data are still needed to predict P-glycoprotein interactions *in silico*.

2.5 *IN VITRO-IN VIVO* CORRELATIONS

The cell models need to be assessed against *in vivo* counterpart to allow reliable predictions of blood-brain barrier permeability based on *in vitro* data. Therefore, several *in vitro-in vivo* correlation studies have been conducted for the BBMECs, Caco-2 and MDCK cell models (Table 5) and other endothelial cell-based cell models from different species (Table 6).

BBMEC model

Both monocultured BBMECs and BBMECs co-cultured with astrocytes have been used for drug permeability studies and the *in vitro* permeability values have been correlated with different *in vivo* counterparts (Table 5). Generally, good correlations have been obtained between *in vitro* and *in vivo* parameters. However, it is noteworthy that no correlation was found when a higher number of model drugs (n=22) including passively transported compounds and transporter protein substrates were incorporated into the evaluation of the *in vitro-in vivo* correlation between BBMECs co-cultured with astrocytes and the mouse brain uptake assay as the *in vivo* method (Garberg *et al.*, 2005). This indicates that no generalizations about the *in vitro-in vivo* correlation results can be made as yet.

PBMEC model

Drug permeability across PBMECs cultured under ACM has been correlated with *in vivo* brain permeability in the rat (Table 6) (Zhang *et al.*, 2006b). PBMECs cultured with ACM have shown very similar *in vitro* permeability properties and *in vitro-in vivo* correlation as monocultured BBMECs.

HBMEC model

The permeability properties of drugs in the HBMEC model were compared to human *in vivo* parameter determined by clinical positron emission tomography (Table 6) (Mabondzo *et al.*, 2010). The coefficient of determination (r^2) between *in vivo* and *in vitro* parameters was good (r^2=0.90, n=6) whereas a significantly lower *in vitro-in vivo* correlation (r^2=0.12, n=18) was found when mouse was used as the experimental animal in the *in vivo* determination (Table 6) (Garberg *et al.*, 2005).

Caco-2 model

The relevance of Caco-2 model for the prediction of blood-brain barrier permeability *in vivo* has been evaluated (Table 5) (Garberg *et al.*, 2005, Hellinger *et al.*, 2012). Recently, Caco-2 cells cultured with vinblastine supplementation (to increase P-glycoprotein expression) were found to yield a better *in vitro-in vivo* correlation (r^2=0.72) than the wild type Caco-2 model (r^2=0.61) (Hellinger *et al.*, 2012).

MDCKII-MDR1 model

MDCK(I,II)-MDR1 cells have been used to estimate the drug permeability across the blood-brain barrier *in vivo* (Table 5) (Wang *et al.*, 2005, Di *et al.*, 2009, Hellinger *et al.*, 2012). So far, however, there is no consensus about the suitability of these cells as an *in vitro* model for the blood-brain barrier. There are some workers who have claimed that MDCKII-MDR1 cells may not even be able to estimate the passive drug permeability across blood-brain barrier, although this model is excellent for studying P-glycoprotein mediated transport of drugs *in vitro* (Di *et al.*, 2009). Nonetheless, there are other reports stating that these cells could be used as a simple and fast model for the purpose of screening CNS drugs (Hellinger *et al.*, 2012, Wang *et al.*, 2005). More studies are needed to clarify the suitability of the MDCK-MDR1 model for prediction of drug permeability across blood-brain barrier *in vivo*.

Relatively good *in vitro-in vivo* correlations have been demonstrated between all *in vitro* cell models and different *in vivo* methods in drug permeability across the blood-brain barrier when a relatively limited number of model compounds (n<13) have been included in the *in vitro-in vivo* correlation analysis. Unfortunately, a trend toward lower correlations is observed when higher numbers of model compounds (n=22-46) are included in the analysis, suggesting that *in vitro* models may not be able to predict very well the permeability of drugs using multiple transport mechanisms to cross the blood-brain barrier *in vivo*. In addition, in the *in vitro-in vivo* correlation studies, usually the same model drugs are commonly used and many of them have no therapeutic CNS indications.

The reported correlations with a limited number of compounds are very similar but there is no consensus about whether endothelial or epithelial cell models are better. This suggests that the prediction power of the cell models is limited to the model drugs being chosen. However, the lack of standardized experimental conditions makes it difficult to compare different *in vitro* methods between laboratories. Furthermore, the different *in vivo* methods applied in the *in vitro-in vivo* correlation studies make it difficult to compare even the reported *in vitro-in vivo* correlations. Most of the *in vivo* methods measure the total drug concentrations at a single time point but with *in vivo* microdialysis it is possible to measure the unbound drug concentrations at several time points in the same animal.

Table 5. In vitro-in vivo correlations between the BBMEC, Caco-2 and MDCK cell models and several *in vivo* methods.

***in vitro* model (parameter)**	***in vivo* method (parameter)**	***in vitro-in vivo* correlation[¥]**	**Reference**
$BBMECs^{S\text{-}b\text{-}S}$ (P_e)	Rat, *in situ* brain perfusion ($P_{e,in\ vivo}$)	**r=0.85**, n=13,[*,#]	Pardridge *et al.*, 1990
$BBMECs^{co\text{-}culture}$, maximal extraction (%) compared to reference	Rat, BUI, maximal extraction (%) compared to reference	**r=0.88**, n=10,[§]	Dehouck *et al.*, 1992
$BBMECs^{FI}$ ($P_{trans,in\ vitro}$)	Rat, *in situ* brain perfusion ($P_{trans,in\ vivo}$)	*in vitro* reflects *in vivo*, n=5	Saheki *et al.*, 1994
$BBMECs^{FI}$ (P_e corrected to $P_{e,sucrose}$)	Rat, injection to left ventricle, single pass cerebral extraction (E)	**r=0.96**, n=7	Pirro *et al.*, 1994
$BBMECs^{FI}$ (P_e)	Rat, BUI, maximal extraction (%) compared to reference	**r=0.96**, n=9 **r=0.93**, n=9,[†,‡]	Dehouck *et al.*, 1995, Cecchelli *et al.*, 1999
$BBMECs^{co\text{-}culture}$ (P_e)	Rat, BUI, maximal extraction (%) compared to reference	**r=0.90**, n=9	Dehouck *et al.*, 1995, Cecchelli *et al.*, 1999
$BBMECs^{FI}$ (P_{app})	Rat, intravenous injection to tail vein (brain:plasma ratio)	**r=0.66**, n=10	Polli *et al.*, 2000
$BBMECs^{S\text{-}b\text{-}S}$ (P_{app})	Rat, microdialysis (CL_{in})	**good** correlation, n=8	Otis *et al.*, 2001, Hansen *et al.*, 2002
$BBMECs^{co\text{-}culture}$ (P_e)	Rat, BUI (%) Rat, *in situ* brain perfusion and intravenous injection technique ($P_{e,in\ vivo}$)	**r=0.93 BUI**, n=12 **r=0.95 $P_{e,in\ vivo}$**, n=13,[$]	Lundquist *et al.*, 2002
$BBMECs^{FI,ACM}$ (P_e)	Rat, *in situ* brain perfusion ($P_{e,in\ vivo}$)	**r^2=0.81** (**r=0.90**), n=10,[*]	Culot *et al.*, 2008
$BBMECs^{co\text{-}culture}$ (P_{app})	Mouse, intravenous injection to tail vein ($P_{app,in\ vivo}$)	**r^2=0.74** (**r=0.86**), n=10 (passive) **r^2=0.52**, (**r=0.72**), n=22 (all compounds)	Garberg *et al.*, 2005

(Continued.)

Table 5. In vitro-in vivo correlations between the BBMEC, Caco-2 and MDCK cell models and several *in vivo* methods. (Continued.)

***in vitro* model (parameter)**	***in vivo* method (parameter)**	***in vitro-in vivo* correlation[¥]**	**Reference**
BBMEC data from (Johnson and Anderson, 1999, Garberg *et al.*, 2005, Rice *et al.*, 2005, Lundquist *et al.*, 2002) (P_e)	Rodent, *in situ* brain perfusion (log $P_{c,in\ situ}$)	**r^2=0.09**, (**r=0.30**), n=46,[†] **r^2=0.58**, (**r=0.76**), n=19,[£]	Avdeef, 2011
Caco-2 (P_{app})	Rat, BUI (%)	**r=0.68**, n=10,[$]	Lundquist *et al.*, 2002
Caco-2 ($P_{e,AB}$/$P_{e,BA}$)	Human, positron emission tomography (plasma-brain exchange parameter)	**r^2=0.17**, (**r=0.41**), n=5	Mabondzo *et al.*, 2010
Caco-2 ($P_{app,BA}$/$P_{app,AB}$)	Rat, brain penetration (C_{brain}/C_{blood})	no correlation	Faassen *et al.*, 2003
Caco-2 (P_{app})	Mouse, intravenous injection to tail vein ($P_{app,in\ vivo}$)	**r^2=0.86**, (**r=0.93**), n=10 (passive) **r^2=0.34**, (**r=0.58**), n=22 (all compounds)	Garberg *et al.*, 2005
Caco-2 (P_{app})	Mouse, intravenous injection to tail vein ($P_{app,in\ vivo}$) corrected with tissue binding	**r^2=0.61**, (**r=0.78**), n=10	Hellinger *et al.*, 2012
Caco-2^{vinblastine} (P_{app})	Mouse, intravenous injection to tail vein ($P_{app,in\ vivo}$ corrected with tissue binding)	**r^2=0.72**, (**r=0.85**), n=10	Hellinger *et al.*, 2012
MDCK (P_{app})	Rat, *in situ* brain perfusion (K_{in})	**r=0.93**, n=16	Polli *et al.*, 2000
MDCK (P_{app})	Rat, injection to tail vein (brain:plasma ratio)	**r=0.80**, n=10	Polli *et al.*, 2000
MDCK (P_{app})	Mouse, intravenous injection to tail vein ($P_{app,in\ vivo}$)	**r^2=0.65**, (**r=0.81**), n=10 (passive) **r^2=0.46**, (**r=0.68**), n=22 (all compounds)	Garberg *et al.*, 2005
MDCK-MDR1 (P_{app})	Mouse, intravenous injection to tail vein ($P_{app,in\ vivo}$)	**r^2=0.64**, (**r=0.80**), n=10 (passive) **r^2=0.37**, (**r=0.61**), n=22 (all compounds)	Garberg *et al.*, 2005
MDCK-MDR1 (P_{app})	different *in vivo* methods (CNS+/-)	**good** correlation	Wang *et al.*, 2005
MDCKII-MDR1 (P_{app})	Rat, *in situ* brain perfusion (P)	**r^2=0.0071**, (**r=0.08**), n=37	Di *et al.*, 2009
MDCKII-MDR1 (P_{app})	Mouse, intravenous injection to tail vein ($P_{app,in\ vivo}$ corrected with tissue binding)	**r^2=0.78**, (**r=0.88**), n=10,[†]	Hellinger *et al.*, 2012

[¥] r^2 converted as r by equation $\sqrt{r^2}$; [S-b-S] side-by-side diffusion chambers; P_e, permeability across cell monolayer ($1/P_e=1/P_{total}-1/P_{filter}$); [*] parameters normalized for molecular weight $ln[P_e\times\sqrt{MW}]$ or $log[P_e\times\sqrt{MW}]$; [#] two molecules (L-dopa and glucose) were not included in the analysis due to their active transport mechanisms; [co-culture] endothelial cells co-cultured with astrocytes; BUI, brain uptake index; [§] diazepam was not included due to the rate-limiting step of filter permeability; [FI] filter inserts; [†] parameters log normalized; [‡] imipramine excluded from the analysis due to the sequestration into the BBMECs; P_{app}, apparent permeability coefficient; [$] parameters normalized for molecular weight $ln[BUI\times\sqrt{MW}]$ and $ln[P_e$ or $P_{app}\times\sqrt{MW}]$; [ACM] astrocyte conditioned medium; [£] corrected for paracellular and aqueous boundary layer; AB, apical-to-basolateral direction; BA, basolateral-to-apical direction; [vinblastine] cells cultured under 10 nM vinblastine.

Table 6. In vitro-in vivo correlations between endothelial cell-based cell models from different species and several *in vivo* methods.

***in vitro* model (parameter)**	***in vivo* method (parameter)**	***in vitro-in vivo* correlation[¥]**	**Reference**
HBMECs$^{co\text{-}culture}$ ($P_{e,AB}/P_{e,BA}$)	Human, positron emission tomography (plasma-brain exchange parameter)	$\mathbf{r^2=0.90}$, (**r=0.95**), n=6	Mabondzo *et al.*, 2010
HBMECs$^{co\text{-}culture}$ (P_e)	Mouse, intravenous injection to tail vein ($P_{app,in\ vivo}$)	$\mathbf{r^2=0.05}$, (**r=0.22**), n=9 (passive) $\mathbf{r^2=0.12}$, (**r=0.35**), n=18 (all compounds)	Garberg *et al.*, 2005
hCMEC/D3 (P_e)	Rat and mouse, *in situ* brain perfusion, (K_{in})	**r=0.94**, n=5	Weksler *et al.*, 2005
PBMEC (P_e)	Rat, *in situ* brain perfusion (logPS)	$\mathbf{r^2=0.60}$, (**r=0.77**), n=16 (all compounds) $\mathbf{r^2=0.89}$, (**r=0.94**), n=13,[*]	Zhang *et al.*, 2006b
RBMEC$^{co\text{-}culture}$ (P_e)	Rat and mouse, *in situ* brain perfusion, (K_{in})	**r=0.94**, n=10	Perrière *et al.*, 2007
RBMEC$^{co\text{-}culture}$ (P_{app})	Mouse, intravenous injection to tail vein ($P_{app,in\ vivo}$) corrected with $f_{u,brain}/f_{u,pasma}$ (*in vitro*)	$\mathbf{r^2=0.80}$, (**r=0.89**), n=10,[#]	Hellinger *et al.*, 2012
MBEC4 (P_{app})	Mouse, intravenous injection to tail vein ($P_{app,in\ vivo}$)	**low** correlation	Garberg *et al.*, 2005
PAMPA-BBB ($P_{e,pampa}$)	Rat, *in situ* brain perfusion (P)	$\mathbf{r^2=0.47}$, (**r=0.69**), n=37	Di *et al.*, 2009

[¥] r^2 converted as r by equation $\sqrt{r^2}$; [co-culture] cells co-cultured with astrocytes or with astrocytes and pericytes; P_e, permeability across cell monolayer ($1/P_e=1/P_{total}-1/P_{filter}$); AB, apical-to-basolateral direction; BA, basolateral-to-apical direction; P_{app}, apparent permeability coefficient; [*] phenylalanine, leucine and gabapentin were not included in the analysis due to the active transport mechanism; [#] parameters log normalized.

2.6 CONCLUSIONS FROM THE REVIEW OF THE LITERATURE

During the past decades, several *in vitro*, *in vivo* and *in silico* models have been introduced to permit the prediction of blood-brain barrier permeability. Clear progress in the development of the *in vitro* blood-brain barrier models has been gained during the last 10 years. However, all of these models have their own advantages but also limitations. Therefore, the choice of the model for prediction of blood-brain barrier permeability represents a compromise between high throughput with low predictive value and low throughput with high predictive value (Braun *et al.*, 2000). *In vitro* models have been considered to have a higher throughput in screening experiments but have a lower predictive value than *in vivo* models. Currently different *in vitro* models are being used for screening of drug candidates' abilities to permeate across blood-brain barrier. It seems that primary brain endothelial cells mimic best the *in vivo* blood-brain barrier, but expression levels of several proteins and their functionalities may be downregulated *in vitro*. In addition, primary cells are labor intensive and require regular isolation from fresh brain tissue. In contrast, immortal cell lines are readily available and easy to maintain but they may change their protein expression characteristics during passaging. At the moment, none of these models is able to predict adequately such a complex process as blood-brain barrier penetration of drugs in human. Therefore, at the moment, the combinations of the *in vitro*, *in vivo* and *in silico* technologies in the early drug development are still needed to construct an accurate picture about the blood-brain barrier traversing potential of a drug candidate.

3 Aims of the Study

In the early drug development, cell based *in vitro* models are commonly used to predict the blood-brain barrier permeability of new drug candidates. In order to make reliable predictions of the drug permeability across blood-brain barrier based on *in vitro* data, it is important to thoroughly characterize the *in vitro* models to assess the advantages and limitations of each of the *in vitro* models being used. In addition, the *in vitro* drug permeability data needs to be correlated against *in vivo* counterpart, to allow reliable predictions based on *in vitro* data. The general objective of this study was to evaluate the suitability of the monocultured BBMECs as an *in vitro* blood-brain barrier model for use in drug permeability studies.

The specific aims of the study were:

1. To optimize the monoculture conditions and characterize BBMEC model in terms of tightness, expression of tight junction proteins and specific brain endothelial cell markers and metabolic enzymes. The tightness, expression of tight junction proteins and metabolic enzyme activities in the monocultured BBMEC model were also compared with two generally used epithelial cell models, Caco-2 and MDCKII-MDR1.

2. To evaluate the expression, localization and functionality of P-glycoprotein in the monocultured BBMECs.

3. To examine the molecular descriptors determining the passive permeability of model drugs across the monocultured BBMEC model, and to compare these descriptors to those previously determined for epithelial cell models for assessing and classifying drugs.

4. To assess the *in vivo* relevance of the BBMEC, Caco-2 and MDCKII-MDR1 cell models with an *in vitro-in vivo* correlation analysis by using the brain microdialysis in the rat and unbound brain/blood ratio as the *in vivo* measure.

4 Materials and Methods

4.1 ENDOTHELIAL CELL ISOLATION AND CULTURE

4.1.1 Isolation of the BBMECs

Isolation of the BBMECs was performed as described previously (Audus and Borchardt, 1987, Audus *et al.*, 1996). Bovine brains were obtained from a local slaughterhouse (Atria Suomi Oy, Kuopio, Finland) and microvessel fragments from two brains were isolated as schematically described in Figure 5. The isolation protocol yielded 12 ml of microvessels from two bovine brains. The microvessels were stored in the liquid nitrogen for up to three months.

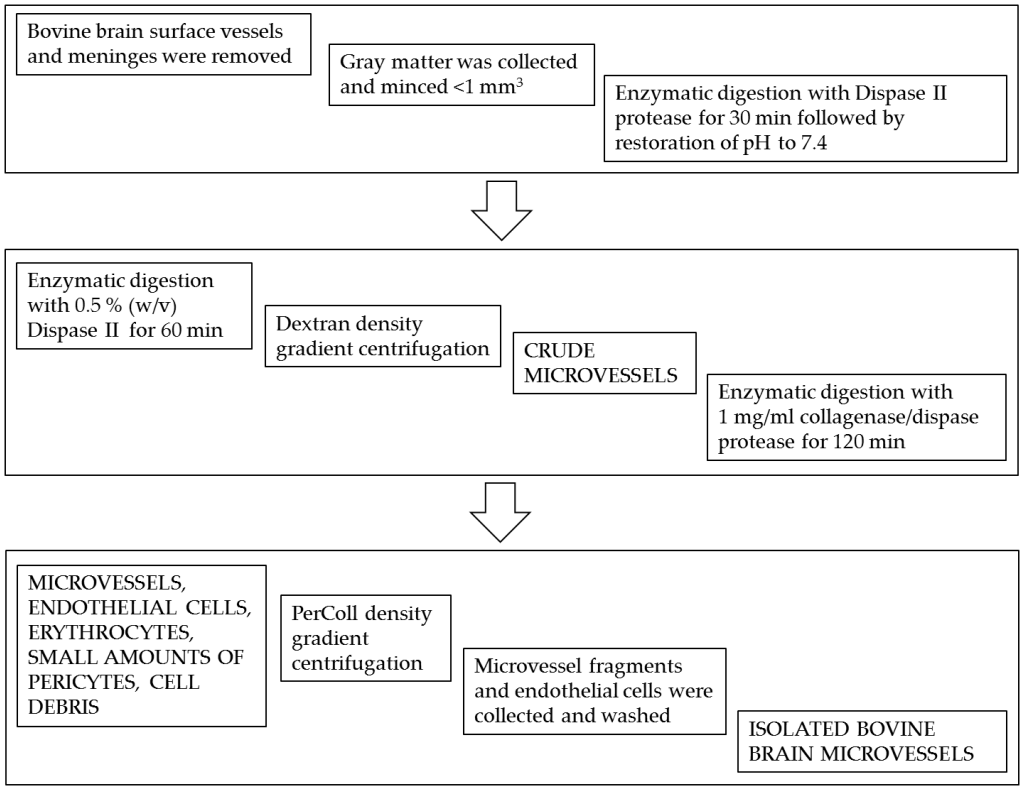

Figure 5. Schematic chart describing the main points of the isolation protocol Audus and Borchardt, 1987, Audus *et al.*, 1996.

4.1.2 BBMEC culture

Collagen-fibronectin coating - Tendons were removed aseptically from rat tails and sterilized with 70 % ethanol. Dried tendons were weighed and collagen was dissolved with 0.1 % acetic acid solution to give a final concentration of 3 mg/ml. The solution was stirred at 4 °C for 48-72 h. Collagen solution was centrifuged at 4000 × g at 4 °C for 2 h. The pellet was discarded and collagen solution was stored at 4 °C. All of the cell culture materials in use were coated (0.4 mg/cm^2) with 3 mg/ml collagen solution as described earlier (Audus and Borchardt, 1987). Collagen-coated cell culture materials were sterilized under UV light in laminar flow hood for two hours and stored at 4 °C and used within one week. Prior to seeding the BBMECs, collagen-coated cell culture materials were further coated with 50 µg/ml fibronectin (Sigma-Aldrich, St. Louis, MO, USA) (5 µg fibronectin/cm^2) in phosphate buffered saline (PBS, 129 mM NaCl, 2.5 mM KCl, 7.4 mM Na_2HPO_4, 1.3 mM KH_2PO_4) for 45 min.

Plating the brain microvessel fragments - Briefly, a sufficient number of cryovials were thawed (one cryovial per ~42 cm^2) in plating medium [45 % minimum essential medium (Invitrogen, Carlsbad, CA, USA), 45 % Ham's F12 nutrient mixture (Invitrogen), 10 % horse serum, 10 mM N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) (HEPES), 13 mM $NaHCO_3$, 50 μg/ml polymyxin B, 100 μg/ml penicillin G, 100 μg/ml streptomycin, 2.5 μg/ml amphotericin B, all supplied by Sigma-Aldrich] and centrifuged for 10 min at 1000 × g. The pellet of microvessel fragments was resuspended in plating medium and heparin (Sigma-Aldrich) was added to a final concentration of 0.15 mg/ml just prior to seeding the microvessel fragments onto collagen-fibronectin coated cell culture materials. The microvessel fragments were cultured for three days at 37 °C, 5 % CO_2. Thereafter, cultures were grown in culture medium (45 % minimum essential medium, 45 % Ham's F12 nutrient mixture, 10 % horse serum, 10 mM HEPES, 13 mM $NaHCO_3$, 100 μg/ml penicillin G, 100 μg/ml streptomycin, 50 μg/ml heparin, 20 μg/ml bovine endothelial cell growth factor (Roche, Basel, Switzerland). The culture medium was changed three times a week until the endothelial cells formed a confluent cell monolayer within 9-12 days. The experiments were performed with non-passaged primary monocultured BBMECs.

4.1.3 Tested cell culture medium supplements

The effects of different cell culture medium supplements and ACMs (Table 7) were tested to the P_{app} of [^{14}C]sucrose and the functionality of the efflux proteins assessed with calcein acetoxymethyl ester (calcein-AM) assay (see section 4.4.2) in the BBMECs.

Table 7. Tested cell culture medium supplements.

Supplement	Concentration	Source
ascorbic acid	30 μM	Sigma-Aldrich
hydrocortisone	550 nM	Sigma-Aldrich
rat C6 glioma medium	50 % (v/v)	in-house
adult mice astrocyte medium	50 % (v/v)	a kind gift from Cerebricon Ltd. (Kuopio, Finland)

4.2 EPITHELIAL CELL CULTURE

4.2.1 Caco-2

Caco-2 cells were obtained from American Type Culture Collection (Manassas, VA, USA) and cultured in Eagle's minimal essential medium (LGC Promochem, Teddington, UK) supplemented with 10 % heat-inactivated fetal calf serum (LGC Promochem), 100 IU/ml penicillin and 100 μg/ml streptomycin (LGC Promochem). Cells were cultured at 37 °C, 5 % CO_2 and subcultured twice a week at 80-90 % confluence with 0.25 % trypsin, 0.53 mM ethylenediaminetetraacetic acid (EDTA, LGC Promochem) and used between passages 45-49. Caco-2 cells were seeded at a density of 82 × 10^3 cells/cm^2 onto 12 well Transwell® permeable supports with 0.4 μm pore size polycarbonate (Corning, Corning, NY, USA). Cells were grown for 21 days before experiments and medium was changed three times a week. For immunostaining, the cells were seeded 50 × 10^3 cells/well onto 96 well plates (Nunc, Rochester, NY, USA) and cells were grown for 4 days.

4.2.2 MDCKII-MDR1

MDCKII-MDR1 cells were obtained from Netherlands Cancer Institute, Amsterdam. MDCKII-MDR1 cells were cultured in Gibco® Dulbecco's modified Earle's medium (Invitrogen)

supplemented with 10 % heat-inactivated fetal calf serum (LGC Promochem), 100 IU/ml penicillin and 100 μg/ml streptomycin (LGC Promochem). Cells were incubated at 37 °C, 5 % CO_2. Cells were subcultured twice a week at ~90 % confluence with 0.05 % trypsin, 1 mM EDTA (Lonza, Basel, Switzerland) and used between passages 33-59. MDCKII-MDR1 cells were seeded at a density of 39 × 10^3 cells/cm^2 onto Transwell® permeable supports (12 well, 0.4 μm pore size polycarbonate, Corning) or 50 × 10^3 cells/well onto 96 well plates (Nunc) and cells were grown for 4 days.

4.3 CHARACTERIZATION OF THE CELL MODELS

The cell-based *in vitro* model needs to exhibit appropriate blood-brain barrier properties, such as expression and functionality of tight junction proteins, active transporter proteins and specific enzymatic activities (Table 2). Therefore, selected proteins and enzyme activities were characterized from the BBMECs and compared with those from Caco-2 and MDCKII-MDR1 cells.

4.3.1 Protein expressions

*ZO-1, occludin and vWF immunostaining (**III**)* - The endothelial cell characteristics of the BBMECs were confirmed by assaying the vWF protein by immunostaining. In addition, the expressions of two tight junction proteins, ZO-1 and occludin, were determined by immunostaining. For immunostaining, the cells were fixed with methanol at -20°C for 10 min, blocked with 5 % normal goat serum in PBS for 10 min and permeabilized with 0.1 % Triton X-100 in PBS for 10 min. Primary antibodies were rabbit anti-human vWF (1/1000) (Dako, Glostrup, Denmark), rabbit anti-ZO-1 (1 μg/ml) (Zymed Laboratories, San Francisco, CA, USA) and rabbit anti-occludin (5 μg/ml) (Zymed Laboratories). Secondary antibody was goat anti-rabbit fluorescein isothiocyanate conjugate (10 μg/ml) (Zymed Laboratories). The endoplasmic reticulum was stained with concanavalin A Texas Red (100 μg/ml) (Molecular Probes, Eugene, OR, USA). The fluorescent images were obtained with a Nikon Eclipse Inverted Microscope (Nikon Corporation, Tokyo, Japan) with UltraVIEW confocal Imaging System (PerkinElmer, Waltham, MA, USA).

*P-glycoprotein immunoblotting (**III**)* - Cell lysates of the BBMECs cultured either on filter inserts or on filters on petri dishes, and MDCKII-MDR1 cells cultured on filter inserts were prepared as described earlier (Hamilton *et al.*, 2001). Total protein concentrations were determined with a protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA) using bovine serum albumin (BSA) as a standard. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed as described earlier (Laemmli, 1970). Proteins were transferred onto a nitrocellulose membrane by electroblotting (Towbin *et al.*, 1979). P-glycoprotein was detected with monoclonal antibody (C219) (1/50). Monoclonal anti-β-tubulin antibody (1/5000) (Sigma-Aldrich) was used as a loading control. Secondary antibody was anti-mouse-HRP (1/8000) (Pierce, Thermo Fisher Scientific, Rockford, IL, USA) and SuperSignal West Femto chemiluminescence substrate (Pierce) was used for antibody detection.

*P-glycoprotein immunostaining (**III**)* - P-glycoprotein localization was characterized from the BBMECs cultured either on filter inserts or on filters on petri dishes, and MDCKII-MDR1 cells cultured on filter inserts. For immunostaining, cells were fixed with 4 % paraformaldehyde, blocked with 1 % BSA and permeabilized with 0.1 % Triton X-100, 1 % BSA in PBS. P-

glycoprotein was recognized with primary monoclonal antibody against P-glycoprotein (1/5) (C219, Covance Research Products, Dedham, MA, USA). The secondary antibody was anti-mouse IgG Texas Red (1/500) (Vector Laboratories, Burlingame, CA, USA). Nuclei were labeled with 1 μg/ml 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) (Sigma-Aldrich). The fluorescent images were obtained with a Zeiss Axio Observer inverted microscope (40×NA 1.3 oil objective) equipped with LSM 700 confocal module (Carl Zeiss Microimaging, Jena, Germany). ZEN 2009 software (Carl Zeiss Microimaging) was used for image processing and 3D rendering. The intensity of the P-glycoprotein fluorescence was measured from four randomly selected images of 160 × 160 μm areas using ZEN 2009 software. The negative control images were measured identically. P-glycoprotein intensity was calculated as mean intensity × area after subtracting the intensity of the negative control sample.

4.3.2 Enzyme activities

ALP activity was measured as described earlier (Korjamo *et al.*, 2005). Briefly, cells were homogenized and cellular supernatant was used for the reaction. ALP from bovine intestinal mucosa (Sigma-Aldrich) was used as the standard. Absorbance was measured at 405 nm by Victor² microplate reader (PerkinElmer). The protein concentration of the cellular supernatant was determined as described above. ALP activities were expressed as pmol/min/μg protein.

The COMT activity assay was modified from the method described earlier (Forsberg *et al.*, 2003). Briefly, cells were homogenized and cellular supernatant was used for the reaction. The reaction products were quantified by using high-performance liquid chromatography (HPLC) with electrochemical detection. The total COMT activity (including both membrane bound and cytosolic COMT) was normalized with the protein concentration of cellular supernatant determined as described above. COMT activities were expressed as pmol/min/mg protein.

4.4 CELLULAR UPTAKE STUDIES

4.4.1 Cellular uptake in the BBMECs

BBMECs were cultured on 24 well plates until confluence. The uptake studies were carried out as previously described (El Hafny *et al.*, 1997, Silverstein *et al.*, 2004) with minor modifications. Briefly, cells were washed and pre-incubated with and without P-glycoprotein inhibitors GF120918 (3 μM) and quinidine (100 μM) (Table 8) in uptake buffer (129 mM NaCl, 0.63 mM $CaCl_2$, 2.5 mM KCl, 0.74 mM $MgSO_4$, 7.4 mM Na_2HPO_4, 1.3 mM KH_2PO_4, 5.3 mM D-glucose, 10 mM HEPES, pH 7.4) for 30 min. The cellular uptake of P-glycoprotein substrates [^{3}H]paclitaxel and [^{3}H]vinblastine (Table 9) were determined with and without the inhibitors for 120 min at 37 °C. After incubation, the cells were rapidly washed three times with ice-cold uptake buffer and then lysed with 0.1 M NaOH overnight on ice. The retained radioactivity was determined by liquid scintillation counting (see section 4.8.1) and the amount of drug was normalized with protein concentration and expressed as pmol/mg protein.

4.4.2 Calcein-AM assay

Calcein-AM assay was performed in the BBMECs and MDCKII-MDR1 cells as described earlier (Vellonen *et al.*, 2004). Efflux protein function was inhibited by using cyclosporine A (15 μM), progesterone (150 μM), verapamil (300 μM) and MK-571 (50 μM) (Table 8) containing 1 % dimethylsulfoxide in PBS. The fluorescence intensity was determined in a Victor² microplate reader (PerkinElmer) at wavelengths 485 nm (ex) and 550 nm (em). The retained fluorescence of

inhibitor treated wells was calculated and expressed as a percentage of fluorescence in the control wells (% of control).

Table 8. Efflux protein inhibitors.

Drug	Inhibitor for	Source	Reference
GF120918	P-glycoprotein, BCRP	a kind gift from GlaxoSmithKline (Research Triangle Park, NC, USA)	Hyafil *et al.*, 1993, Wallstab *et al.*, 1999, de Bruin *et al.*, 1999, Maliepaard *et al.*, 2001b
cyclosporine A	P-glycoprotein, Mrp2	Sigma-Aldrich	Jetté *et al.*, 1995, Giacomini *et al.*, 2010
MK-571	Mrp proteins	Cayman Chemical Company (Ann Arbor, MI, USA)	Gekeler *et al.*, 1995, Gutmann *et al.*, 1999, Weiss *et al.*, 2007, Reid *et al.*, 2003
progesterone	P-glycoprotein	Sigma-Aldrich	Yang *et al.*, 1989, Jetté *et al.*, 1995
quinidine	P-glycoprotein	Sigma-Aldrich	Kamimoto *et al.*, 1989, Jetté *et al.*, 1995, Giacomini *et al.*, 2010
verapamil	P-glycoprotein, Mrp1	ICN Biomedicals (Irvine, CA, USA)	Kamimoto *et al.*, 1989, Jetté *et al.*, 1995, Giacomini *et al.*, 2010, Potschka *et al.*, 2004, Goh *et al.*, 2002

4.5 DRUG PERMEABILITY STUDIES

4.5.1 Drugs

In this study, [^{14}C]sucrose and [^{14}C]diazepam were used as a low and high permeability reference compounds, respectively. The basic physicochemical properties (MW, LogP, PSA and number of hydrogen-bonding interactions) for sucrose (342.3, -4.49, 189.5 Å^2, 19, respectively) and diazepam (284.7, 2.80, 32.7 Å^2, 3, respectively) were calculated by the ACD/PhysChem Suite, version 12.01, 2009 (Advanced Chemistry Development, Toronto, ON, Canada). In the BBMEC model, 0.1 μCi/ml [^{14}C]sucrose (625 mCi/mmol, American Radiolabeled Chemicals, St. Louis, MO, USA) and 1 μCi/ml [^{3}H]diazepam (76.0 Ci/mmol, PerkinElmer) or 0.1 μCi/ml [^{14}C]diazepam (56.0 mCi/mmol, Amersham Biosciences, Uppsala, Sweden) were used. In the Caco-2 and MDCKII-MDR1 models, 2 μCi/ml [^{14}C]sucrose (625 mCi/mmol, American Radiolabeled Chemicals) and 0.1 μCi/ml [^{14}C]diazepam (56.0 mCi/mmol, Amersham Biosciences) were used.

The model drugs used in this study are listed in Table 9. The model drugs were selected to cover a wide range of physicochemical properties; MW (136.1 to 1202.6), LogP (-4.49 to 5.92), PSA (23.6 to 278.8 Å^2) and hydrogen-bonding interactions (3 to 28).

4.5.2 BBMEC model

The permeability experiment in the BBMECs cultured both on filters on petri dishes (**I-III**) and on filter inserts (**III**). The permeability experiments were conducted in an apical-to-basolateral (A-B) direction (**I-II**) but bidirectional transport studies (A-B direction and the basolateral-to-apical (B-A) direction) were also performed (**III**). Drugs were dissolved in transport buffer (129 mM NaCl, 2.5 mM KCl, 7.4 mM Na_2HPO_4, 1.3 mM KH_2PO_4, 0.63 mM $CaCl_2$, 0.74 mM $MgSO_4$, 5.3 mM D-glucose, 0.1 mM ascorbic acid, pH 7.4 (Borges *et al.*, 1994)) and the solutions were adjusted to pH 7.4 when applicable. The BBMECs cultured on filters were transferred to side-by-side diffusion chambers (PermeGear, Bethlehem, PA, USA) (Figure 6). Pre-warmed drug solution was introduced into the donor chamber (3 ml) and pure transport buffer solution was added into the receiver chamber (3 ml). Both chambers were stirred with magnetic stirring and external water circulation was used to maintain the temperature at 37 °C. Samples were

withdrawn from both receiver (100 µl) and donor (10 µl) chambers at 5, 10, 20, 30, 45 and 60 min time points. Fresh buffer was used to replace the fluid lost from the receiver chamber.

Bidirectional transport studies of [^{3}H]paclitaxel with or without P-glycoprotein inhibitor, GF120918, were conducted in the BBMECs cultured on filter inserts (**III**). The [^{3}H]paclitaxel was prepared either in transport buffer or in transport buffer supplemented with 5 µM GF120918 (Evers *et al.*, 2000) (Table 8). Prior to the transport studies, the BBMECs were pre-incubated with or without GF120918 for 30 min at 37 °C. The studies were performed in A-B and B-A directions at 37 °C as described above. The samples were taken from the receiver and donor side at 15, 30, 60, 90, 120 and 180 min time points and receiver samples were replaced with an equal volume of fresh transport buffer or transport buffer supplemented with GF120918. Sample drug concentrations were determined by using liquid scintillation counting, HPLC or liquid chromatography-mass spectrometry (LC-MS) analyses (see section 4.8.2).

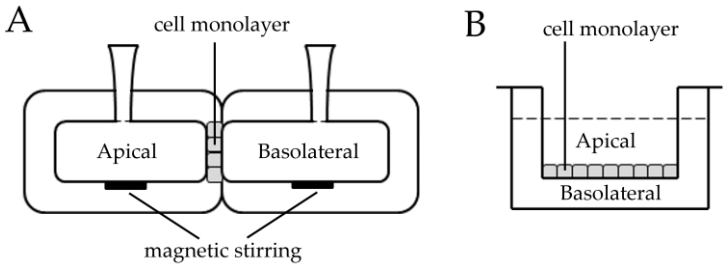

Figure 6. Schematic illustration of the side-by-side diffusion chamber system in the BBMEC model (A) and filter insert system in the Caco-2 and MDCKII-MDR1 cells (B) used for the permeability experiments. The apical and basolateral sides of the cell monolayers are shown.

4.5.3 Caco-2 and MDCKII-MDR1

The permeability studies of model drugs (alprenolol, atenolol, metoprolol, pindolol, entacapone, tolcapone, ondansetron, baclofen, midazolam, sucrose, diazepam) were conducted in the A-B direction in the Caco-2 and MDCKII-MDR1 cells grown on filter inserts (Figure 6) (**I**). Drug solutions were dissolved in Hank's balanced salt solution (HBSS; BioWhittaker, Lonza) supplemented with 25 mM HEPES, 0.02 % NaCl (BioWhittaker, Lonza) the pH values of the solutions were adjusted to 7.4 when applicable. Cells were washed twice with HBSS buffer and pre-incubated at 37 °C for 15 min. The transport studies were performed at 37 °C in an orbital shaker (Titramax 1000, Heidolph, Germany). In the A-B transport studies, pre-warmed drug solution was introduced into the donor chamber (0.5 ml) and pure HBSS-HEPES buffer was added into the receiver chamber (1.5 ml). Samples (100 µl) were withdrawn at 15, 30, 60, 120 and 180 min from receiver chambers. Receiver samples were replaced with an equal volume of fresh HBSS buffer. Samples (100 µl) from the donor solution were taken at the beginning and the end of the experiment.

In addition, the bidirectional transport studies of [^{3}H]paclitaxel in the absence and presence of 5 µM GF120918 were performed in the MDCKII-MDR1 model as described above with a few slight modifications (**III**). In the B-A transport studies, the basolateral chamber (1.5 ml) being the donor and apical chamber (0.5 ml) being the receiver. Samples were withdrawn at 15, 30, 60, 90, 120 and 180 min from the receiver chamber and samples were replaced with an equal volume of fresh HBSS buffer or buffer supplemented with GF120918 to replace the fluid loss from the receiver chamber.

Table 9. Physicochemical properties of the model drugs.

Model drug	*In vitro* concentr.	Drug class	MW[a]	LogP[a]	PSA (Å^2)[a]	H-bonding[a]	Original publ.	Source
alprenolol	20 µM	beta-adrenoceptor antagonist	249.4	2.91	41.5	5	**I**	Sigma-Aldrich
atenolol	100 µM	beta-adrenoceptor antagonist	266.3	0.33	84.6	9	**I**	Sigma-Aldrich
metoprolol	20 µM	beta-adrenoceptor antagonist	267.4	1.63	50.7	6	**I**	Sigma-Aldrich
pindolol	20 µM	beta-adrenoceptor antagonist	248.3	1.68	57.3	7	**I**	Sigma-Aldrich
entacapone	20 µM	COMT inhibitor	305.3	2.12	130.4	10	**I**	Orion Pharma (Espoo, Finland)
tolcapone	20 µM	COMT inhibitor	273.2	3.02	103.4	8	**I**	synthesized by Dr. Aino Pippuri, Orion Pharma
ondansetron	20 µM	antiemetic	293.4	1.55	39.8	4	**I**	Sigma-Aldrich
(±)-baclofen	20 µM	γ-aminobutyric acid analog	213.7	0.78	63.3	6	**I**	Sigma-Aldrich
midazolam	20 µM	benzodiazepine derivative	325.8	3.80	30.2	3	**I**	Roche
JTP-4819	20 µM	prolyl oligopeptidase inhibitor	359.4	0.72	90.0	9	Jalkanen *et al.*, 2011	synthesized by Dr. Elina Jarho, University of Eastern Finland
KYP-2047	20 µM	prolyl oligopeptidase inhibitor	339.4	2.17	64.4	5	Jalkanen *et al.*, 2011	synthesized by Dr. Elina Jarho, University of Eastern Finland
acyclovir	20 µM	antiviral	225.2	-1.48	144.8	12	**II**	Recordati industria chimica E Pharmaceutica (Milan, Italy)
allopurinol	20 µM	purine analog	136.1	-0.14	70.1	7	**II**	Sigma-Aldrich
antipyrine	20 µM	antipyretic, analgesic	188.2	0.44	23.6	3	**II**	Sigma-Aldrich
ibuprofen	20 µM	nonsteroidal anti-inflammatory	206.3	3.50	37.3	3	**II**	Sigma-Aldrich
metronidazole	20 µM	antibacterial	171.2	-0.14	83.9	7	**II**	Sigma-Aldrich
(±)-propranolol	20 µM	beta-adrenoceptor antagonist	259.3	2.90	41.5	5	**II**	Sigma-Aldrich
cephalexin	100 µM	antibacterial	347.4	0.35	138.0	11	**II**	ICN Biomedicals (Aurora, OH, USA)
(±)-verapamil	8 µM	calcium channel blocker	454.6	4.02	64.0	6	**II**	ICN Biomedicals
theophylline	20 µM	methylated xanthine derivative	180.2	-0.02	69.3	7	**II**	Orion Pharma
paracetamol	20 µM	antipyretic, analgesic	151.2	0.48	49.3	5	**II**	Orion Pharma
[^{3}H]cyclosporine A (9 Ci/mmol)	1 µCi/ml	immunosuppres-sant	1202.6	2.79	278.8	28	**II**	PerkinElmer
[^{3}H]digoxin (21.8 Ci/mmol)	0.5 µCi/ml	cardiac glycoside	780.9	1.29	203.1	20	**II, III**	PerkinElmer
[^{3}H]paclitaxel (54.6 Ci/mmol)	0.2 µCi/ml	antitumor	853.9	3.95	221.3	19	**III**	Moravek Biochemicals.
[^{3}H]vinblastine (10 Ci/mmol)	0.5 µCi/ml	antitumor	811.0	5.92	154.1	16	**III**	American Radiolabeled Chemicals

[a]*Values calculated by the ACD/PhysChem Suite, version 12.01, 2009 (Advanced Chemistry Development).*

4.6 DESCRIPTORS DETERMINING THE *IN VITRO* PERMEABILITY OF DRUGS

4.6.1 Conformational analysis and calculation of molecular descriptors

Twenty-five model drugs (acyclovir, allopurinol, alprenolol, antipyrine, atenolol, baclofen, cyclosporine A, cephalexin, diazepam, digoxin, entacapone, ibuprofen, JTP-4819, KYP-2047, metoprolol, metronidazole, midazolam, ondansetron, paracetamol, pindolol, propranolol, sucrose, theophylline, tolcapone and verapamil) were minimized using the ChemBioDraw Ultra, version 12.0 (CambridgeSoft, Cambridge, MA, USA). Conformational analysis was performed using MOE (Molecular Operating Environment, version MOE 2010.10, Chemical Computing Group, Montreal, Canada). The lowest found potential energy conformation was selected for use in the descriptor calculation. The molecular descriptors are numerical values that describe the chemical properties of the molecule. The molecular descriptors were calculated by VolSurf, version 4.1.4, (Molecular Discovery, Middlesex, UK).

4.6.2 Principal component analysis

Principal component analysis (PCA) was performed using SIMCA-P, version 12.0 (Umetrics, Umeå, Sweden) to define the molecular descriptors influencing the *in vitro* drug permeability across the BBMECs cultured on filters on petri dishes. The PCA model was created by using 102 calculated molecular descriptors. From the PCA model, possible strong outliers were determined by Hotelling's T^2 distribution at 95% confidence level. Permeability values of the model drugs were added into the PCA model. This PCA model was used to interpret which molecular descriptors correlate with the permeability of model drugs across the monocultured BBMEC model (Figure 3 in **II**).

4.7 *IN VIVO* BRAIN MICRODIALYSIS

Dual probe brain mircodialysis in the male Wistar rats was used to measure unbound drug concentrations in the blood and brain ECF and to determine the unbound brain/blood ratio. A rat was anesthetized, the intravenous microdialysis probe was inserted into the left femoral vein and the brain probe was placed into the striatum. The probes were perfused with Krebs Ringer at a flow rate of 2 μl/min for 80 min before drug administration. Nine model drugs (alprenolol, atenolol, metoprolol, pindolol, entacapone, tolcapone, baclofen, midazolam and ondansetron) were administered intraperitoneally at a dose of 50 μmol/kg. Dialysates were collected in 20 min fractions for 5 h. Drug concentrations were determined by using HPLC or LC-MS analyses (Table 10).

4.8 ANALYTICAL METHODS

4.8.1 Radiotracer samples

The radioactivity of the samples were analyzed by using liquid scintillation counting (1450 MicroBeta Trilux Liquid Scintillation Counter, Wallac, Finland) after the addition of 500 μl scintillation cocktail (PerkinElmer).

4.8.2 HPLC and LC-MS analyses

HPLC and LC-MS analyses (Table 10) were used for quantification of model drug concentrations in both the *in vitro* permeability studies and *in vivo* microdialysis.

Table 10. High-performance liquid chromatography and liquid chromatography-mass spectrometry analysis used for determination of *in vitro* permeability samples and *in vivo* microdialysis samples.

Drug	Method	Instrumentation	Column	Mobile phase	Detection	Reference
acyclovir paracetamol	HPLC	Unipoint™ LC system (Gilson, Middleton, WI, USA)	Inertsil ODS-3, 4.0 × 150 mm (GL Sciences, Tokyo, Japan)	isocratic elution: 0.03 % TFA in acetonitrile (A) and 0.03 % TFA in water (B); acyclovir A/B 5/95, paracetamol A/B 20/80	UV/Vis 254 nm	**II**
allopurinol metronidazole theophylline	HPLC	Unipoint™ LC system (Gilson)	Inertsil ODS-3, 4.0 × 150 mm (GL Sciences)	isocratic elution: 0.1 % TFA in acetonitrile (A) and 0.1 % TFA in water (B); allopurinol A/B 5/95, metronidazole A/B 10/90, theophylline A/B 15/85	UV/Vis, allopurinol 254 nm, metronidazole 315 nm, theophylline 272 nm	**II**
alprenolol pindolol	LC-MS	Surveyor HPLC system and LTQ linear ion trap MS (Thermo Scientific, Waltham, MA, USA).	Zorbax XDB C18, 2.1 × 100 mm, 3.5 µm (Agilent Technologies, Santa Clara, CA, USA)	gradient elution: 0.1 % formic acid in water (A) and 0.1 % formic acid in acetonitrile (B)	MS transitions, alprenolol *m/z* 250→116, pindolol *m/z* 249→116	**I**
antipyrine cephalexin ibuprofen	HPLC	Beckman System Gold (Beckman Coulter, Brea, CA, USA)	Xterra C18, 20 × 2.1 mm, 2.5 µm (Waters, Milford, MA, USA)	gradient elution: 0.05 % trifluoracetic acid (A) and 90 % acetonitrile, 10 % water, 0.038 % trifluoracetic acid (B)	UV/Vis 205 nm	Palmgrén *et al.*, 2004
atenolol	HPLC	HPLC 1100 system (Agilent Technologies)	Zorbax SB-phenyl-column, 2.1 × 100 mm, 3.5 µm (Agilent Technologies)	isocratic elution: 0.05 M potassium dihydrogen phosphate and 0.01 M 1-octanesulfonate, pH 3 (A) and methanol (B); (A/B 62/38)	fluorescence 230 nm (ex) 310 nm (em)	**II**
baclofen	LC-MS	Acquity UPLC and Quattro Premier triple quadrupole MS (Waters).	BEH C18 column, 2.1 × 50 mm, 1.7 µm (Waters)	gradient elution: 0.1 % formic acid (A) and (B) acetonitrile	MRM transitions *m/z* 214→116 and *m/z* 214→151	**I**
entacapone tolcapone	LC-MS	Surveyor HPLC system and LTQ linear ion trap MS (Thermo Scientific).	Zorbax XDB C18, 2.1 × 100 mm, 3.5 µm (Agilent Technologies)	isocratic elution: 50 % acetonitrile with 0.1% formic acid	MS transitions, entacapone *m/z* 306→233, tolcapone *m/z* 274→182	**I**
metoprolol propranolol verapamil	HPLC	Beckman System Gold (Beckman Coulter)	Xterra C18, 20 × 2.1 mm, 2.5 µm (Waters)	gradient elution: 0.05 % trifluoracetic acid (A) and 90 % acetonitrile, 10 % water, 0.038 % trifluoracetic acid (B)	fluorescence 230 nm (ex) 302 nm (em)	Palmgrén *et al.*, 2004
midazolam	LC-MS	Acquity UPLC, Quattro Premier triple quadrupole MS (Waters).	BEH C18 column, 2.1 × 50 mm, 1.7 µm (Waters)	gradient elution: 2 mM ammonium acetate (A) and acetonitrile (B)	MRM transitions *m/z* 326→291	**I**
ondansetron	LC-MS	Acquity UPLC, Quattro Premier triple quadrupole MS (Waters).	BEH C18 column, 2.1 × 50 mm, 1.7 µm (Waters)	gradient elution: 10 mM ammonia (A) and acetonitrile (B)	MRM transitions *m/z* 295→170 and *m/z* 295→184	**I**

HPLC, high-performance liquid chromatography; LC, liquid chromatography; UPLC, ultra-performance LC; TFA, trifluoroacetic acid; LC-MS, liquid chromatography-mass spectrometry; MS, mass spectrometer; UV/Vis ultraviolet/visible spectroscopy; MRM, multiple reaction monitoring, The analytical methods were partially validated with regard to specificity, selectivity, linearity precision and accuracy based on the U.S. Food and Drug Administration, Guidance for Industry, Bioanalytical Method Validation, 2001.

4.9 DATA ANALYSIS AND STATISTICS

Immunoblot analysis
The density of bands was quantified from three independent experiments with triplicate samples by using ImageJ software (Rasband WS, U.S. National Institutes of Health, Bethesda, MD, USA, http://imagej.nih.gov/ij/). Densitometry of P-glycoprotein bands was normalized with β-tubulin bands (loading control). Statistical significance of differences in the density of bands between the BBMECs cultured either on filters on petri dishes or on filter inserts was tested by unpaired Student's t-test with GraphPad Prism 5.03 software (San Diego, CA, USA). Statistical significance was set at $P<0.05$.

P-glycoprotein immunostaining
Statistical significance of differences in the P-glycoprotein intensities (mean intensity × area) between the BBMECs cultured either on filters on petri dishes, the BBMECs cultured on filter inserts and MDCKII-MDR1 cultured on filter inserts was tested by one-way ANOVA followed by Tukey's test by GraphPad Prism 5.03 software. Statistical significance was set at $P<0.05$.

Cellular uptake and calcein-AM assays
Statistical significance of differences in cellular uptake assays and calcein-AM assays was tested by one-way ANOVA followed by Dunnett's tests by using GraphPad Prism 5.03 software. Statistical significance was set at $P<0.05$.

Permeability experiments
The permeability experiments were used to study the transport of several model drugs (Table 9). The apparent permeability coefficient (P_{app}, cm/s) for the model drugs was calculated according to equation 1:

$$P_{app} = \frac{\Delta Qr / \Delta t}{A \times C_d} \qquad \text{(Equation 1)}$$

where $\Delta Qr/\Delta t$ is the steady state flux of drug, *i.e.*, the slope of the linear region of the cumulative amount of drug in receiver chamber versus time (h) plot; C_d is the drug concentration in the donor chamber; and A is the surface area (cm^2) available for transport. Sink conditions (receiver concentration <10% of the donor concentration) were taken into account.

Statistical significance of differences in the P_{app} values was tested by unpaired Student's t-test with GraphPad Prism 4.03 (**I**) and 5.03 software (**III**). Statistical significance was set at $P<0.05$.

The efflux ratio (ER) was calculated according to equation 2:

$$ER = \frac{P_{app}\ B-A}{P_{app}\ A-B} \qquad \text{(Equation 2)}$$

where P_{app} B-A is the P_{app} of drug in the basolateral-to-apical direction; P_{app} A-B is P_{app} of drug in the apical-to-basolateral direction.

Comparison between the P_{app} values in the BBMEC, Caco-2 and MDCKII-MDR1 models

The pair-wise linear regressions between the P_{app} values determined in the BBMEC, Caco-2 and MDCKII-MDR1 models were calculated by using GraphPad Prism 4.03 software. The relationship between the P_{app} values was analyzed with two-tailed Pearson correlation coefficients (r). The correlation was considered statistically significant when $P<0.05$.

Bi-directional transport assays

Statistical significance of differences in the P_{app} values were statistically compared by Student's unpaired t-test in GraphPad Prism 5.03 software. Statistical significance was set at $P<0.05$.

In vivo brain microdialysis

AUC, *i.e.*, area under the concentration-time curve from 0 to ∞, values were calculated from individual data for each of the model drugs both in brain ECF and blood with the trapezoidal rule by using GraphPad Prism 4.03 software. The unbound brain/blood ratio *in vivo* was determined according to equation 3:

$$unbound\ \ brain/blood\ ratio = \frac{AUC_{ECF_{0-\infty}}}{AUC_{blood_{0-\infty}}} \qquad \text{(Equation 3)}$$

where AUC_{ECF} is the AUC in brain extracellular fluid and AUC_{blood} is AUC in blood.

In vitro-in vivo correlations

The P_{app} values determined in the BBMEC ($\times 10^5$ cm/s), Caco-2 and MDCKII-MDR1 ($\times 10^6$ cm/s) models were log-normalized. The linear regression between the *in vitro* $\log(P_{app})$ and the *in vivo* unbound brain/blood ratio was calculated and two-tailed Pearson correlation coefficients were determined by using GraphPad Prism 4.03 software. The correlation was considered statistically significant when $P<0.05$.

5 Results

5.1 ISOLATION AND CULTURE CONDITIONS OF BBMECS

5.1.1 Tightness

The isolation protocol of the BBMECs used in this study has been described previously (Audus and Borchardt, 1987, Audus *et al.*, 1996). In order to obtain pure capillaries without any other cell types, such as pericytes, an optimization of the isolation process was needed. The concentration of enzymes and the incubation times for enzyme digestions were optimized to achieve a good dissociation of the brain capillaries and a high viability of the endothelial cells. Therefore, the isolation procedure and cell culture of the BBMECs needed refinement, *i.e.*, there had to be pure capillaries and low paracellular permeability before acceptable repeatability could be achieved.

The tightness of the BBMECs cultured on filters on petri dishes was assessed by determining the P_{app} of paracellular marker [^{14}C]sucrose. Steady and repeatable tightness was achieved from the isolation batch number eight onward (Figure 7). The paracellular tightness obtained was in line with the earlier studies with comparable culture and experimental conditions (Eddy *et al.*, 1997, Rice *et al.*, 2005). The cell isolation batches before batch number eight contained contaminating cells, presumably pericytes and their presence led to a loosening of the BBMEC monolayer.

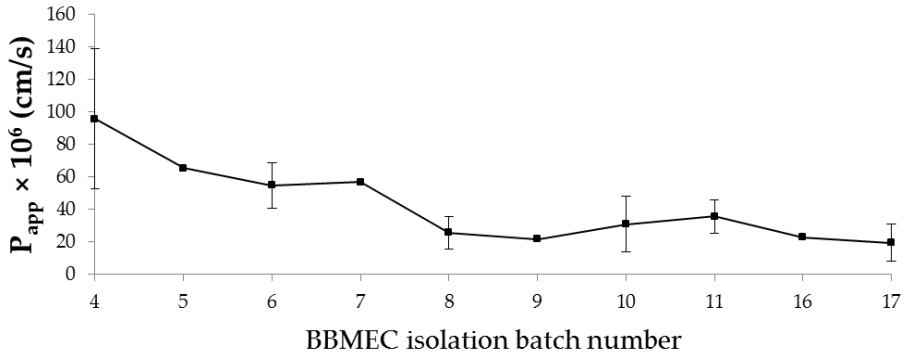

Figure 7. Tightness of the BBMEC monolayer determined with the apparent permeability coefficient (P_{app}) × 10^6 (cm/s) of [^{14}C]sucrose at different isolation batch numbers (mean±SD, n=2-10) (unpublished results).

5.1.2 Effects of different culture medium supplements

Tightness - Different cell culture supplements and ACMs were tested in the BBMECs cultured on filters on petri dishes (Figure 8). BBMECs cultured with ACM (Rubin *et al.*, 1991b) or co-cultured with astrocytes (Tao-Cheng *et al.*, 1987, Dehouck *et al.*, 1995) have previously been reported to enhance tight junctions. In addition, hydrocortisone has been shown to tighten primary porcine endothelial cell cultures (Hoheisel *et al.*, 1998) and ascorbic acid induces cell differentiation (Pasonen-Seppänen *et al.*, 2001). Unfortunately, in this study, neither supplements nor ACMs had any ability to improve but in fact, they seemed to loosen in some cases the tightness of the paracellular barrier in the monocultured BBMECs with the present culture conditions. It was observed that ascorbic acid accelerated the growth of the BBMECs, whereas BBMECs cultured in the presence of adult mice ACM seemed to deteriorate based on

the morphology of the BBMECs. The loosening of the paracellular barrier in the presence of adult mouse ACM may be due to the fact that it is an unsuitable culture medium for the composition of the BBMECs.

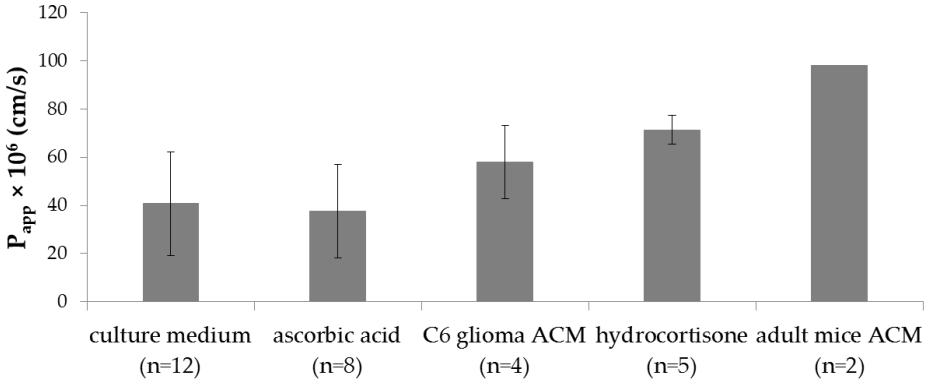

Figure 8. The effect of different culture medium supplements and ACMs to the apparent permeability coefficient (P_{app}) × 10^6 (cm/s) of [^{14}C]sucrose in the BBMECs cultured on filters on petri dishes. Data are mean±SD (unpublished results).

Efflux proteins - P-glycoprotein expression has been improved by co-culturing the BBMECs with astrocytes (Fenart *et al.*, 1998, Gaillard *et al.*, 2000). Therefore, the effects of the different culture medium supplements and ACMs on the functionality of the efflux proteins were tested by using calcein-AM assay. In general, no clear changes in the functionality of the efflux proteins were found when BBMECs grown in the presence of different culture medium supplements and ACMs were compared to those grown in normal culture medium (Figure 9). However, the BBMECs cultured with adult mice ACM exhibited a trend towards lower efflux protein functionality compared to BBMECs cultured in the normal culture medium ($P<0.05$). In our studies, neither the different cell culture supplements nor ACMs seemed to tighten the monocultured BBMECs or increase the functionality of efflux proteins in the BBMECs. For this reason, the BBMECs were cultured subsequently in the normal culture medium (Audus and Borchardt, 1987, Audus *et al.*, 1996).

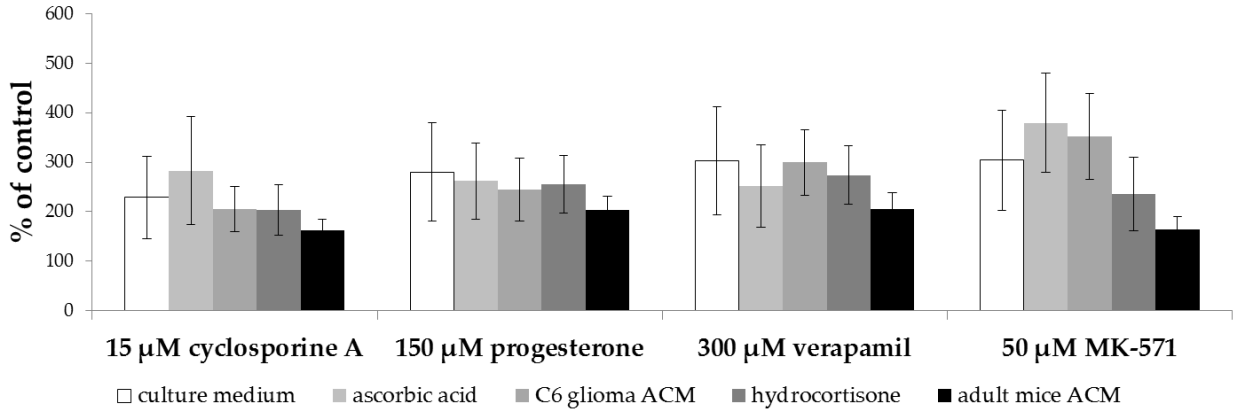

Figure 9. The effect of the different culture medium supplements and ACMs to the functionality of the efflux proteins. The functionality of the efflux proteins was assessed by using calcein-AM assay. Efflux protein inhibitors used were cyclosporine A (P-glycoprotein, Mrp 2), progesterone (P-glycoprotein), verapamil (P-glycoprotein, Mrp 1) and MK-571 (Mrp proteins). Different culture medium supplements and ACMs were compared to normal culture medium. The studies were conducted in quadruplicate and performed in three different experiments in the BBMECs. The results are expressed as a percentage of retained fluorescence of inhibitor treated wells vs. control wells (% of control). Data are mean±SD (unpublished results).

5.2 CHARACTERIZATION OF THE CELL MODELS

5.2.1 Morphology and protein expressions

Morphology

A spindle-shaped morphology is a characteristic for brain endothelial cells (Goldstein *et al.*, 1986, Isobe *et al.*, 1996). Thus, the spindle-shaped morphology was always checked during the cell culture by phase contrast microscopy. The morphology of the monocultured BBMECs was demonstrated to be spindle-shaped (Figure 10A) indicating that the isolation protocol of BBMECs produced relatively pure brain endothelial cells.

vWF

Endothelial cells are commonly characterized by the expression of vWF which is almost exclusively found in the endothelial cells (Goldstein *et al.*, 1986, Deli, 2007). The monocultured BBMECs were positively stained with vWF antibody (Figure 10B). This confirmed that the primary cells isolated from bovine brain gray matter were truly endothelial cells.

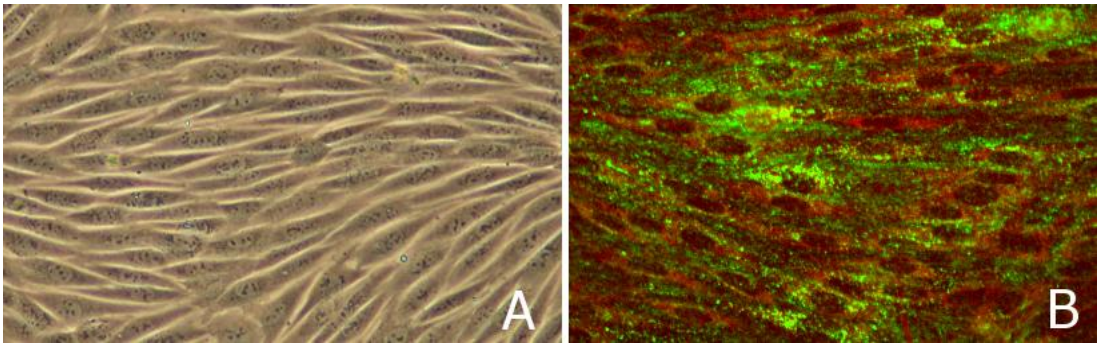

Figure 10. Phase contrast image of monocultured BBMECs (A) and the expression of vWF in the BBMECs by immunofluorescence staining (green) and endoplasmic reticulum stained with concanavalin A (red) (B), (unpublished results).

ZO-1 and occludin

The expression of ZO-1 and occludin were characterized from the BBMECs and the non-brain originating epithelial cells, Caco-2 and MDCKII-MDR1 (Figure 11). ZO-1 creates an important link between the cellular cytoskeleton and the transmembrane tight junction protein, occludin (Furuse *et al.*, 1994). ZO-1 is required for occludin to be localized correctly in the tight junction (Fanning *et al.*, 1998), while occludin is responsible for the paracellular barrier between the cells (Furuse *et al.*, 1993, Hirase *et al.*, 1997).

It was shown that ZO-1 formed a continuous band around the BBMECs (Figure 11A), Caco-2 (Figure 11B) and MDCKII-MDR1 cells (Figure 11C). This indicates that the foundation for occludin localization was present in all cells. In addition, occludin was observed to be located mainly between the cell interfaces in all cells (Figure 11D-F) but, interestingly, also intracellular perinuclear (near nucleus) staining in the BBMECs was observed (Figure 11D). This demonstrates that occludin may not be completely assembled in the tight junctions of the BBMECs.

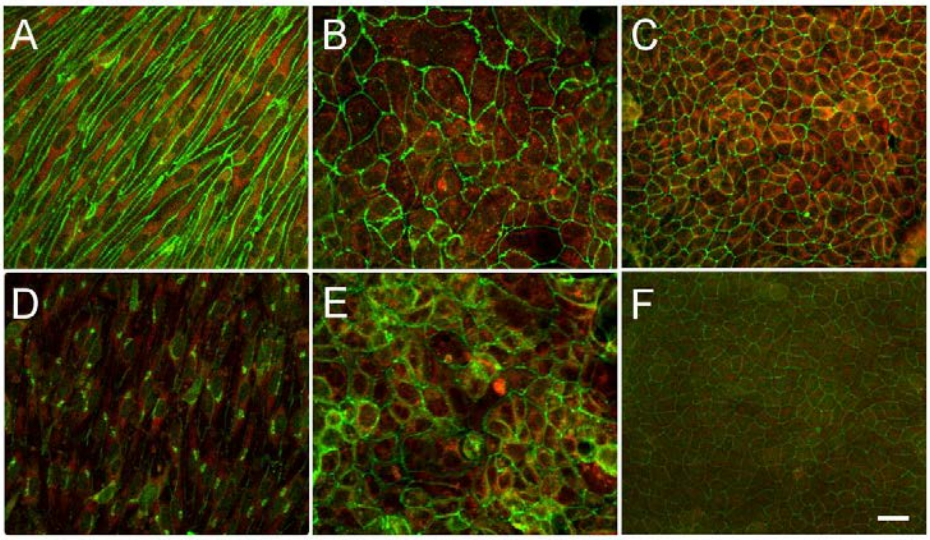

Figure 11. The expression of tight junction proteins ZO-1 (A-C) and occludin (D-F) in the BBMECs (A,D, **III**), Caco-2 (B,E, unpublished results) and MDCKII-MDR1 (C,F, **III**) detected by immunofluorescence staining (green). Endoplasmic reticulum was stained with concanavalin A (red). Scale bar 50 µm.

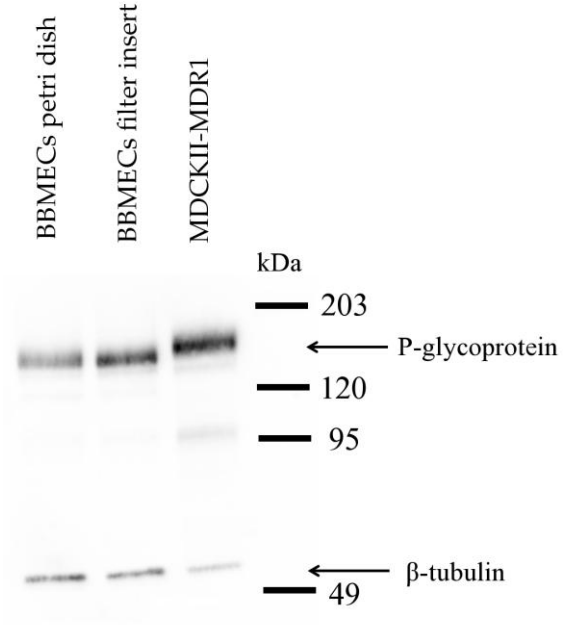

Figure 12. P-glycoprotein expression in the BBMECs cultured on filters on petri dishes and on filter inserts. P-glycoprotein overexpressing MDCKII-MDR1 cells were used as a positive control and β-tubulin was used as a loading control. P-glycoprotein was recognized at ~155 kDa in the BBMECs (Beaulieu *et al.*, 1995) and in the MDCKII-MDR1 cells. Modified from Figure 3 in **III**.

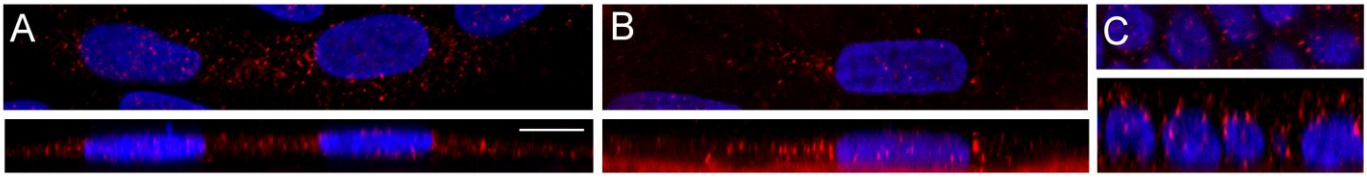

Figure 13. P-glycoprotein expression and localization in the BBMECs cultured on filter inserts (A), and on filters on petri dishes (B). MDCKII-MDR1 cells cultured on filter inserts were used as a positive control (C). The upper picture of each panel (A-C) represents the confocal optical section of the cells from the apical membrane (x-y plane) and the lower image is the stack of various x-y planes showing the vertical sections of the cells (x-z plane). Immunofluorescence staining of P-glycoprotein (red) and nuclei (blue). Scale bar 10 µm. Modified from Figure 4 in **III**.

P-glycoprotein

Expression and localization of P-glycoprotein were shown in the BBMECs cultured both on petri dishes and on filter inserts and MDCKII-MDR1 cells grown on filter inserts by using immunoblot analysis (Figure 12) and confocal microscopy (Figure 13).

The densitometry of the immunoblot bands revealed that P-glycoprotein expression was ~3-fold higher ($P<0.001$) in the BBMECs cultured on filter inserts (2.8±1.0, mean±SD) than those cultured on filters on petri dishes (1.0±0.4) highlighting the influence of culture conditions on P-glycoprotein expression in the BBMECs. In addition, the expression of P-glycoprotein in the monocultured BBMECs was nearly as high as the expression in the MDCKII-MDR1 cells overexpressing P-glycoprotein (estimated by visual inspection) (Figure 12).

Confocal microscopy studies revealed that P-glycoprotein was localized predominantly on the apical side in the BBMECs cultured on the filter inserts (Figure 13A), and on filters on petri dishes (Figure 13B) and in the MDCKII-MDR1 cells (Figure 13C) demonstrating the correct localization of P-glycoprotein in all of the studied cell models. In addition, a significantly, ~46-fold, higher ($P<0.001$) intensity of P-glycoprotein immunostaining was found in the BBMECs cultured on filter inserts than those on filters on petri dishes (Figure 4D in **III**). This again indicates that culture conditions have a clear influence on P-glycoprotein expression in agreement with the immunoblot data. In addition, the intensity of P-glycoprotein immunostaining was significantly ~2-fold higher ($P<0.05$) in the BBMECs cultured on filter inserts than in the P-glycoprotein overexpressing MDCKII-MDR1 cells (Figure 4D in **III**) indicating that P-glycoprotein is expressed at high level in the BBMECs cultured on filter inserts.

5.2.2 Enzyme activities

ALP enzyme activities were 1125, 290, 10 pmol/min/μg protein in the BBMECs, Caco-2 and MDCKII-MDR1 cells, respectively (unpublished results). ALP enzyme activity was 4- and 113-fold higher in the BBMECs than in the Caco-2 and MDCKII-MDR1 cells, respectively, revealing substantial differences in the ALP enzyme activities between the cells. ALP is a specific marker for the blood-brain barrier (Deli, 2007) and it has also been used as a biochemical characteristic of the BBMECs (Audus and Borchardt, 1987). In addition, the ALP enzyme has been used as a marker for cell differentiation in the Caco-2 cells (Matsumoto *et al.*, 1990). Furthermore, ALP has also been found in the MDCK cells (Veronesi, 1996).

Total COMT enzyme activities were 3.4, 151.0 and 68.5 pmol/min/μg protein in the BBMECs, Caco-2 and MDCKII-MDR1 cells, respectively (unpublished results). COMT activity was 20- and 44-fold lower in the BBMECs than in the MDCKII-MDR1 and Caco-2 cells, indicating clear differences in the COMT enzyme activities between the cells. It is known that COMT enzyme is expressed in various tissues, *e.g.*, liver, kidney, brain and intestine (Kaplan *et al.*, 1979, Nissinen *et al.*, 1988, Karhunen *et al.*, 1994) and it is also found in the BBMECs (Baranczyk-Kuzma *et al.*, 1986).

5.3 DRUG PERMEABILITY

5.3.1 P_{app} of the model drugs across the BBMEC model

The P_{app} values of low (sucrose) and high (diazepam) reference compounds were used to define the low and high permeability categories. In order to specify the permeability limit between the medium and high permeability categories previously defined limit was applied with BBMECs

in an identical experimental set-up (Eddy *et al.*, 1997). In this study, three categories for drug permeability across monocultured BBMEC model were used; low, P_{app} <40 × 10^{-6} (cm/s); medium, P_{app} 40-70 × 10^{-6} (cm/s) and high, P_{app} >70 × 10^{-6} (cm/s). The P_{app} values of the model drugs were determined from the A-B direction across the BBMECs cultured on filters on petri dishes (Figure 14).

Based on the P_{app} values, the model drugs could be divided into three categories; low, medium (Figure 14A) and high (Figure 14B). The drugs representing low permeability across the BBMEC model were cyclosporine A, JTP-4819, acyclovir, digoxin, entacapone, sucrose and baclofen. Cephalexin, atenolol, paclitaxel, allopurinol and paracetamol were categorized as drugs with medium permeability across the BBMEC model. Finally, pindolol, metronidazole, theophylline, vinblastine, verapamil, KYP-2047, metoprolol, antipyrine, ondansetron, midazolam, ibuprofen, propranolol, diazepam, alprenolol and tolcapone were categorized as drugs with high permeability across the BBMEC model. These results show that the monocultured BBMEC model is able to differentiate the model drugs into three different permeability categories.

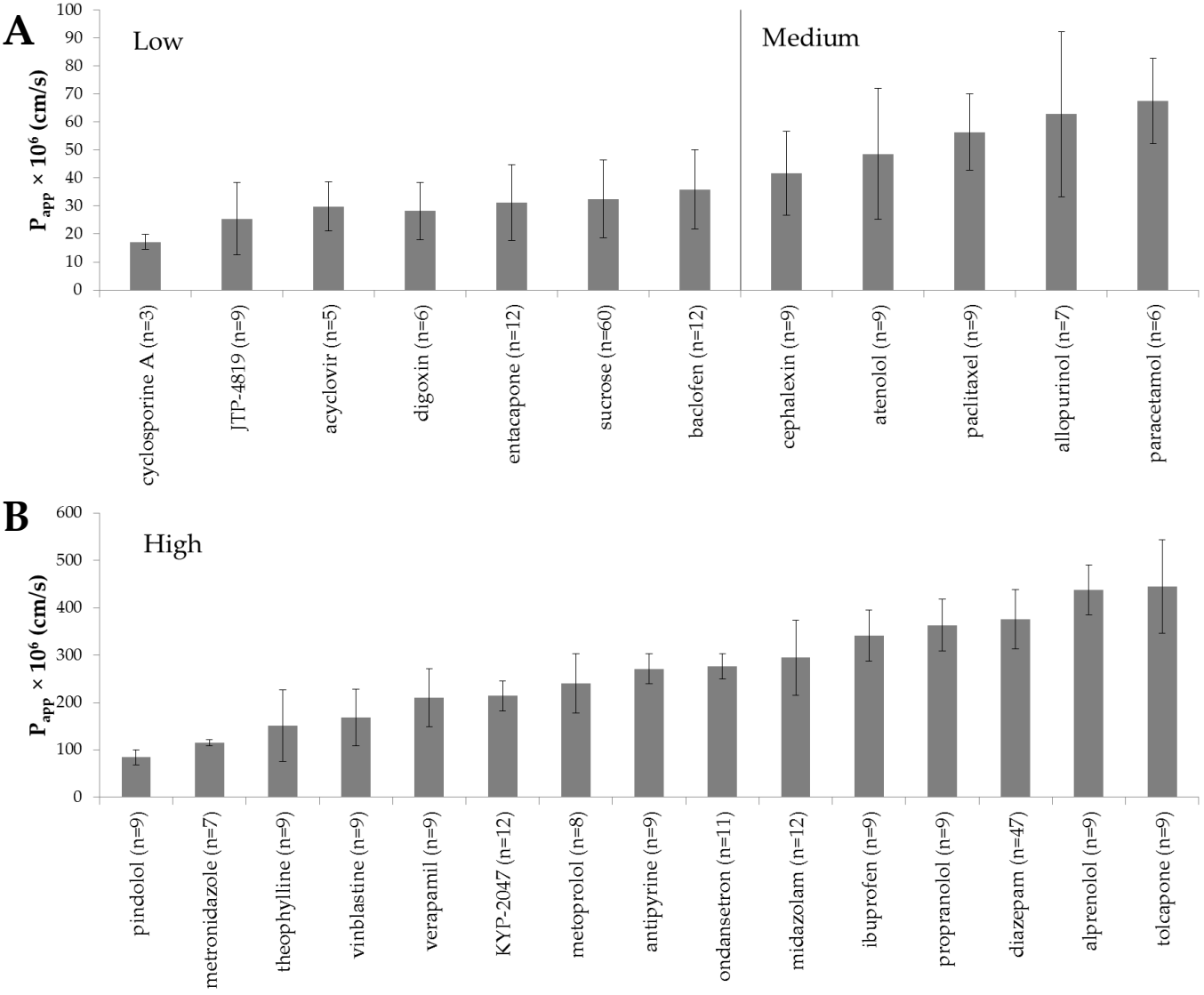

Figure 14. The apparent permeability coefficient (P_{app}) × 10^6 (cm/s) of model drugs from A-B direction across the BBMECs cultured on filters on petri dishes were classified as drugs with low and medium permeability (A) and drugs with high permeability (B). Categories for low, P_{app} <40 × 10^{-6} (cm/s); medium, P_{app} 40-70 × 10^{-6} (cm/s); high, P_{app} >70 × 10^{-6} (cm/s) were defined as described in section 5.3.1. Data are mean±SD.

5.3.2 Molecular descriptors determining the permeability of drugs across the BBMEC model

PCA analysis was used to determine the key molecular descriptors depicting the P_{app} of the model drugs across the monocultured BBMEC model (Table 11). The passive permeation of drugs across the cell membranes is highly dependent on their physicochemical properties (van de Waterbeemd *et al.*, 1996, Lipinski *et al.*, 1997, Abraham *et al.*, 1994, Kelder *et al.*, 1999, Palm *et al.*, 1997). High descriptor values for LogP, ratio between the hydrophobic/hydrophilic parts of a molecule, the amphiphilic moment, vectors pointing from the center of mass to the center of the hydrophilic regions, and hydrophobic regions all attribute to designation of high P_{app} values for the model drugs. In contrast, high descriptor values of ratio between the hydrophilic region/molecular surface, ratio between the hydrophilic/hydrophobic regions, hydrogen-bonding capabilities and polar volume of the molecule lead to low P_{app} values for the model drugs. This indicates that the key molecular descriptors determining the drug permeability across monocultured BBMECs were mainly related to hydrophobic and hydrophilic interactions, the balance between the hydrophilic and hydrophobic moieties of the drug and the potential for hydrogen bonding interactions.

It has also been demonstrated that the key molecular descriptors for monocultured BBMECs were in parallel to those previously described for Caco-2 and MDCK cells (Table 4 in **II**). Hydrophobic and hydrophilic interactions, the potential for hydrogen-bonding interactions, molecular size and flexibility were the most important physicochemical properties influencing the *in vitro* P_{app} across the epithelial cell models (Table 4 in **II**). In the monocultured BBMEC model, the molecular descriptors describing the molecular volume or surface properties were not clearly apparent from the set of molecular descriptors depicting the P_{app} of the model drugs, which may be result from the leakier paracellular route. Taken together, the molecular descriptors depicting the passive permeability of drugs across the monocultured BBMECs and epithelial cell models are similar.

Table 11. Twelve key molecular descriptors determining the apparent permeability coefficient (P_{app}) of model drugs across the BBMECs cultured on filters on petri dishes. Modified from Table 3 in **II**.

Molecular descriptor	**Attributes leading to high P_{app}**	**Attributes leading to low P_{app}**
1. Octanol/water partition coefficient (LogP)	↑	↓
2. Amphiphilic moment (vector pointing from the center of the hydrophobic region to the center of the hydrophilic region) (A)	↑	↓
3. Vectors pointing from the center of mass of a model drug to the center of the hydrophilic regions (IW1-6)	↑	↓
3. Hydrophobic interactions (D1-8)	↑	↓
5. Local minima of interaction energy between hydrophilic probe and the model drug (EW_{min})	↑	↓
6. Ratio between the hydrophobic and hydrophilic parts (CP)	↑	↓
7. Ratio between the hydrophilic regions and the molecular surface (CW1-8)	↓	↑
8. Ratio between the hydrophilic regions and the hydrophobic regions (HL1-2)	↓	↑
9. Hydrophilic regions (W1-8)	↓	↑
10. Hydrogen-bonding capabilities (HB)	↓	↑
11. Polar volume (W_p)	↓	↑
12. Vectors pointing from the center of mass of a model drug to the center of the hydrophobic regions (ID)	–	–

↑ *high descriptor value,* ↓ *low descriptor value, – does not differ from average values*

5.3.3 Comparison of the P_{app} values between the cell models

The permeability experiments were performed by using the apparatus typical for each cell model (*i.e.*, the BBEMC model in side-by-side diffusion chambers (Kuhnline Sloan *et al.*, 2012) or Caco-2 and MDCKII-MDR1 models in filter inserts system (Braun *et al.*, 2000) (Figure 6).

The permeability of the low permeability reference compound, [^{14}C]sucrose, in the BBMEC model was 13- and 21-fold higher than that in the Caco-2 and MDCKII-MDR1 cells, respectively (Table 12) demonstrating a leakier cell monolayer in the BBMEC model. The tight junctions, responsible for the paracellular barrier between the cells, are not so tightly closed in the paracellular route in the BBMECs as in the epithelial cells. The higher P_{app} values for drugs with low and medium permeability in the BBMEC model (Figure 14A) than in the Caco-2 and MDCKII-MDR1 models (Table 12) may result from the leakier paracellular route between the BBMECs. In addition, lower P_{app} values for efflux protein substrates in the Caco-2 and MDCKII-MDR1 cells than in the BBMECs are evidence of more robust efflux protein activity.

The P_{app} of the high permeability reference compound, [^{14}C]diazepam, in the BBMEC model was 5- and 6-fold higher than in the Caco-2 and MDCKII-MDR1 cells, respectively (Table 12). In addition, the P_{app} values of the model drugs (Table 12) show that the ranges of the determined P_{app} values were clearly different between the BBMEC and Caco-2 or MDCKII-MDR1 models, this being partially attributable to the different experimental set-ups (Figure 6). The wider unstirred water layer formed in the Caco-2 and MDCKII-MDR1 models may limit the exact measurement of P_{app} values for highly permeable drugs. Therefore, the P_{app} values for drugs with high permeability in the BBMEC model (Figure 14B) are clearly higher due to the well stirred system.

In addition, BBMECs are substantially thinner (0.2-2 μm) than Caco-2 (~30 μm) or MDCKII-MDR1 cells (~15 μm). As a result, diffusion distance across BBMECs is lower than in Caco-2 or MDCKII-MDR1 cells which may have an influence on the higher P_{app} values obtained in the BBMEC model (Brodin *et al.*, 2009). It should also be noted that the interlaboratory variation is evident in all cell models (Table 12).

The dynamic range describes how efficiently the cell model can discriminate between different P_{app} values, *i.e.*, a resolution power of the cell model. The dynamic range defined as $P_{app\ [^{14}C]diazepam}/P_{app\ [^{14}C]sucrose}$, was 11, 28 and 43 in the BBMEC, Caco-2 and MDCKII-MDR1 models, respectively. The dynamic range was 2.5- and ~4-fold greater in the Caco-2 and MDCKII-MDR1 models, respectively, than in the BBMEC model. This indicates that the BBMEC model possesses a lower dynamic range, although higher P_{app} values of high permeability compound can be achieved. The leakier paracellular barrier may explain the lower dynamic range in the BBMEC model.

Table 12. The apparent permeability values (P_{app}) × 10^6 (cm/s) A-B direction in the BBMECs cultured on filters on petri dishes, Caco-2 and MDCKII-MDR1 cell models. Data are mean±SD (n=3-60, **I-III**) highlighted with bold and supplemented with the reference data.

Model drug	Primary transport mechanism	BBMECs	Caco-2	MDCKII-MDR1
cyclosporine A	Transcellular/Efflux[a,b]	**17 ± 3 (II)**	0.7-3[c-e]	0.2-0.7[c,e]
JTP-4819	Paracellular[f]	25 ± 13[f]	n.a.	n.a.
acyclovir	Paracellular/Influx[g]	**30 ± 9 (II)**	0.3-2[h,i]	2[j]

(Continued).

Table 12. The apparent permeability values (P_{app}) × 10^6 (cm/s) A-B direction in the BBMECs cultured on filters on petri dishes, Caco-2 and MDCKII-MDR1 cell models. Data are mean±SD (n=3-60, **I-III**) highlighted with bold and supplemented with the reference data. (Continued).

Model drug	Primary transport mechanism	BBMECs	Caco-2	MDCKII-MDR1
digoxin	Paracellular/Efflux[b,k]	**31 ± 15 (II)**	0.5-2[c-e,l]	0.3-1.9[c,e,m]
entacapone	Paracellular[n]	**31 ± 14 (I)**	**2.5 ± 0.6 (I)**, 1[o]	**11 ± 3 (I)**
sucrose[Low]	Paracellular[b]	**33 ± 14 (I-III)**, 18-53[p-s]	**2.4 ± 1.4 (I)**, 1.4-1.7[c,h]	**1.5 ± 1.4 (I,III)**, 0.3-0.4[c,t]
baclofen	Paracellular/Influx[u]	**36 ± 14 (I)**	**0.9 ± 0.7 (I)**, 0.4[v,w]	**0.7 ± 0.4 (I)**, 0.9[w,*]
cephalexin	Paracellular/Influx[x]	**42 ± 15 (II)**	0.3[v,y]	0.5[y,§]
atenolol	Paracellular[k]	**49 ± 23 (I)**	**0.9 ± 0.2 (I)**, 0.2-3[h,z,v,y,å]	**0.7 ± 0.2 (I)**, 0.3-1[j,ä,ö]
paclitaxel	Paracellular/Efflux[a,b]	**56 ± 14 (III)**, 8.8[s]	0.8-4.4[e,l,aa]	**6.2 ± 1.9**[†], 0.5-1.5[e,m]
allopurinol	Transcellular[ab]	**63 ± 29 (II)**	n.a.	n.a.
paracetamol	Transcellular[ac]	**67 ± 15 (II)**	5-100[y,ad]	35[y,§]
pindolol	Paracellular[b]	**84 ± 16 (I)**	**29 ± 4 (I)**, 17-96[h,y]	**24 ± 4 (I)**, 27[j]
metronidazole	Transcellular[ae]	**115 ± 7 (II)**	n.a.	11[af,#]
theophylline	Paracellular[b]	**151 ± 76 (II)**, 40[r]	45-67[ag,ah]	30[j]
vinblastine	Paracellular/Efflux[a,b]	**169 ± 60 (III)**, 5.5[p]	1-5[c,e,aa,ai,aj]	<0.2-0.5[c,e,m,ai]
verapamil	Transcellular[a,b]	**210 ± 61 (II)**	16-155[c,e,z,v,ah]	16-59[c,e,j,ö,ak]
KYP-2047	Transcellular[f]	214 ± 31[f]	n.a.	n.a.
metoprolol	Paracellular[b]	**240 ± 63 (I)**	**55 ± 7 (I)**, 23-43[h,o,z,ă]	**63 ± 15 (I)**, 30-41[j,ä]
antipyrine	Transcellular[b,k]	**271 ± 31 (II)**, 40-73[r,al]	43-150[c,v,y]	53-79[c,ä,ö]
ondansetron	Transcellular/Efflux[am]	**276 ± 27 (I)**	**47 ± 5 (I)**, 18-110[y,an]	**38 ± 4 (I)**, 110[y,§]
midazolam	Transcellular[a,b]	**295 ± 79 (I)**	**39 ± 6 (I)**, 38[ao]	**42 ± 6 (I)**, 61-70[ä,ak]
ibuprofen	Transcellular/Influx[ap]	**341 ± 54 (II)**	10-53[z,i,v]	31[af,#]
propranolol	Transcellular[b]	**363 ± 55 (II)**, 80-147[p,r,al]	22-110[d,h,i,z,v,y,ă]	38-50[j,ä,ö,ak]
diazepam[High]	Transcellular[b]	**376 ± 62 (I-III),** 158[p]	**67 ± 23 (I)**, 33-71[c,h,i]	**65 ± 18 (I)**, 53[c]
alprenolol	Transcellular[aq]	**438 ± 53 (I)**	**52 ± 4 (I)**, 25-170[h,y,ă]	**53 ± 7 (I)**, 46[ä]
tolcapone	Transcellular[ar]	**445 ± 99 (I)**	**63 ± 12 (I)**	**64 ± 17 (I)**

[a] Polli et al., 2001; [b] Avdeef, 2011; [c] Garberg et al., 2005; [d] Crowe and Lemaire, 1998; [e] Troutman and Thakker, 2003; [f] Jalkanen et al., 2011; [g] Takeda et al., 2002; [h] Yazdanian et al., 1998; [i] Yee, 1997; [j] Thiel-Demby et al., 2009; [k] Hellinger et al., 2012; [l] Mease et al., 2012; [m] Taub et al., 2005; [n] Heimbach et al., 2003; [o] Heimbach et al., 2003; [p] Eddy et al., 1997; [q] Johnson and Anderson, 1999; [r] Otis et al., 2001; [s] Rice et al., 2005; [t] Mashayekhi et al., 2010; [u] van Bree et al., 1988; [v] Korjamo et al., 2005; [w] R-baclofen, Lal et al., 2009; [x] Dantzig and Bergin, 1990; [y] Irvine et al., 1999; [z] Yazdanian et al., 2004; [å] Artursson, 1990; [ä] Mahar Doan et al., 2002; [ö] Carrara et al., 2007; [aa] Walle and Walle, 1998; [ab] Turnheim et al., 1999; [ac] Duggin and Mudge, 1975; [ad] Khan et al., 2011; [ae] Land and Johnson, 1999; [af] Varma et al., 2012; [ag] Camenisch et al., 1998; [ah] Bergström et al., 2003; [ai] Lentz et al., 2000; [aj] Chan et al., 2005; [ak] Polli et al., 2001; [al] Shah et al., 1989; [am] Schinkel et al., 1996; [an] Gan et al., 1993; [ao] Tolle-Sander et al., 2003; [ap] Ogihara et al., 1996; [aq] Cogburn et al., 1991; [ar] Ceravolo et al., 2002; n.a. not available, permeability values have not been reported for Caco-2 or MDCK cells; [Low] low permeability reference compound; [*] MDCK cells; [§] MDCKII cells; [†] unpublished data; [#] low efflux transporter MDCKII cells; [High] high permeability reference compound.

Linear regressions of the P_{app} values of model drugs (sucrose, baclofen, entacapone, atenolol, pindolol, metoprolol, ondansetron, midazolam, alprenolol, tolcapone and diazepam) between the BBMEC, Caco-2 and MDCKII-MDR1 models are shown in Figure 15. Pearson correlation coefficients were r=0.91, r=0.93 and r=0.98 (P<0.001) for BBMEC vs. MDCKII-MDR1, BBMEC vs. Caco-2 and Caco-2 vs. MDCKII-MDR1, respectively. The fact that there were no clear differences in the Pearson correlation coefficients indicates that there is a linear correlation between P_{app} values of each cell model despite the clearly different P_{app} values and ranges.

In general, classification of the drugs based on their permeability is used in the early drug discovery (Amidon *et al.*, 1995, Li, 2005, U.S. Food and Drug Administration, Guidance for Industry, Waiver of *In Vivo* Bioavailability and Bioequivalence Studies for Immediate-Release Solid Oral Dosage Forms Based on a Biopharmaceutics Classification System, 2000). Based on the P_{app} values, the model drugs could be divided into three categories; low, medium and high (Figure 15). Since the P_{app} values were determined in the Caco-2 and MDCKII-MDR1 models with different permeability set-ups than that used in the BBMEC model, previously determined categories for drug permeabilities were used for these models; low, P_{app} <18 × 10^{-6} (cm/s); medium, P_{app} 18-40 × 10^{-6} (cm/s) and high, P_{app} >40 × 10^{-6} (cm/s) as presented earlier (Polli *et al.*, 2000). The P_{app} limits for the categories in the BBMEC model are described in section 5.3.1.

It was shown that the BBMEC model ranks the model drugs into the same categories as Caco-2 (Figure 15A) and MDCKII-MDR1 models (Figure 15B) with the exception of atenolol and pindolol that were categorized into low and medium categories, respectively, in the Caco-2 and MDCKII-MDR1 models but medium and high categories, respectively, in the BBMEC model. In addition, the Caco-2 and MDCKII-MDR1 models rank the model drugs into the same categories with the exception of the P-glycoprotein substrate, ondansetron, which had a significantly (P<0.001) lower P_{app} value in the MDCKII-MDR1 model than in the Caco-2 model (37.6 vs. 47.1 × 10^{-6} cm/s) (Figure 15C). This is caused by the stronger functionality of P-glycoprotein in the MDCKII-MDR1 cells than in the Caco-2 cells.

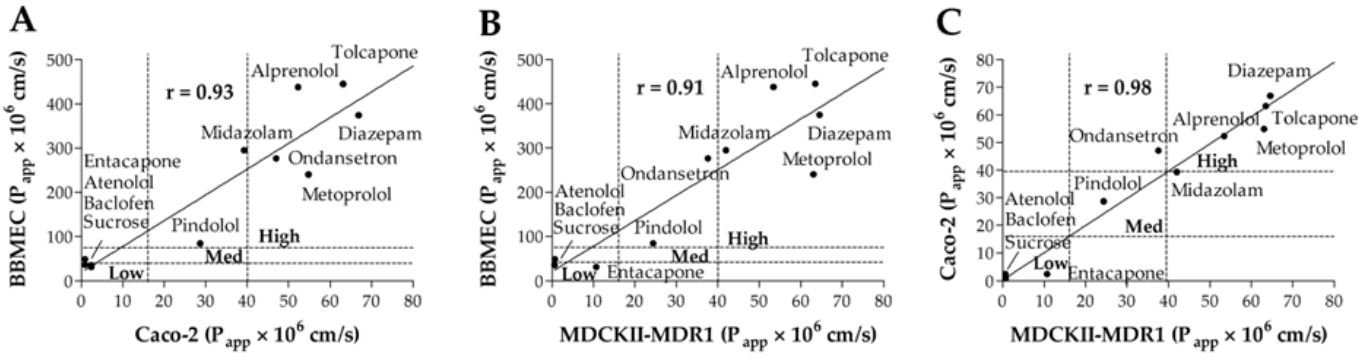

Figure 15. The apparent permeability coefficients (P_{app}) × 10^6 (cm/s) between the different cell models were compared by using pair-wise linear regressions between BBMEC vs. MDCKII-MDR1 (A), BBMEC vs. Caco-2 (B), Caco-2 vs. MDCKII-MDR1 (C). Relationships were analyzed with Pearson correlation coefficient (r). For BBMEC model, the limits for low, medium (Med) and high permeability categories were set as described in section 5.3.1. and for Caco-2 and MDCKII-MDR1, the limits were were set as described in section 5.3.3. Figure 3 in **I**.

5.4 P-GLYCOPROTEIN MEDIATED DRUG TRANSPORT

5.4.1 Functionality of P-glycoprotein in the BBMECs

In cellular uptake studies with two known P-glycoprotein substrates, paclitaxel and vinblastine, P-glycoprotein was shown to be functional in the BBMECs (Figure 16). Paclitaxel and vinblastine displayed a low cellular uptake in the absence of P-glycoprotein inhibitor, GF120918, but in the presence of the inhibitor the cellular uptake of paclitaxel and vinblastine was significantly increased by 9- and 3-fold, respectively, indicating that P-glycoprotein was functional in the BBMECs. In the presence of the second P-glycoprotein inhibitor, quinidine, the cellular uptake of paclitaxel was increased by 7-fold (Figure 16A), whereas the cellular uptake of vinblastine remained unchanged (Figure 16B). This indicates that quinidine is not able to inhibit the P-glycoprotein mediated efflux of vinblastine in the BBMECs, probably due to the different binding sites of vinblastine and quinidine in the P-glycoprotein (Martin *et al.*, 2000).

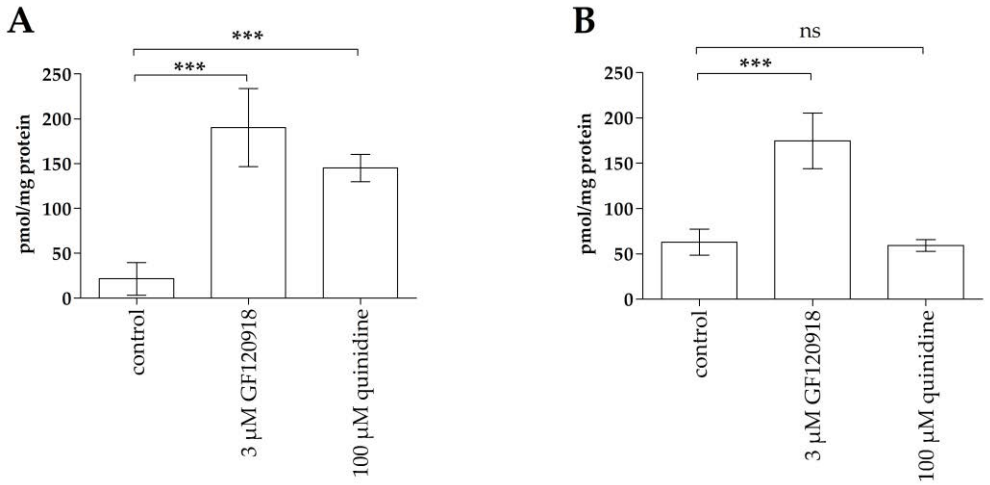

Figure 16. Functionality of P-glycoprotein was demonstrated by using cellular uptake studies of P-glycoprotein substrates paclitaxel (A) and vinblastine (B) in the BBMECs. P-glycoprotein was inhibited by using known inhibitors GF120918 (3 µM) and quinidine (100 µM). Cellular uptake studies were performed in three separate experiments and conducted in duplicate. Data are mean±SD. Statistical significance of differences vs. control was tested by one-way ANOVA followed by Dunnett's test, P<0.001 (***), ns=not significant. Figure 5 in **III**.

5.4.2 Functionality of efflux proteins in the BBMECs and MDCKII-MDR1 cells

The functionality of efflux proteins in the BBMECs and MDCKII-MDR1 cells was further confirmed by using the calcein-AM assay. Lipophilic non-fluorescent calcein-AM enters into the cells by passive transcellular diffusion. It is known that intracellular esterases metabolize calcein-AM to fluorescent calcein and calcein retention in the cells increases after inhibition of efflux proteins (Eneroth *et al.*, 2001).

In the BBMECs, the calcein retention was significantly increased by 2.4-, 2.8-, 3.4- and 3.2-fold when P-glycoprotein was inhibited with cyclosporine A, progesterone, verapamil and MK-571, respectively (Figure 17A). In the MDCKII-MDR1 cells, P-glycoprotein inhibition with cyclosporine A, progesterone or verapamil increased calcein retention by 6-fold or more, whereas MK-571 increased calcein retention by 3.9-fold (Figure 17B). Since all efflux protein inhibitors significantly increased the calcein retention, one can conclude that the efflux proteins are functional in both cell models. Similar results have also been obtained earlier in the BBMECs when P-glycoprotein was inhibited with verapamil (Iwanaga *et al.*, 2011), and in the MDCKII-

MDR1 cells when P-glycoprotein and MRP proteins were inhibited with verapamil or MK-571, respectively (Vellonen *et al.*, 2004).

The calcein-AM assay was used to test the efflux protein interactions of two model drugs, ondansetron and tolcapone, that were suspected to undergo efflux protein mediated transport. Ondansetron and tolcapone showed efflux protein inhibitory activity both in the BBMECs (~2-fold increase compared to control) and in the MDCKII-MDR1 cells (1.5 to 2-fold increase compared to control), but statistical significance of differences was not achieved (Figure 17). However, the slight increase in the calcein retention may indicate that ondansetron and tolcapone do exert some inhibitory activity against efflux protein(s) that are functional both in the BBMECs and MDCKII-MDR1 cells.

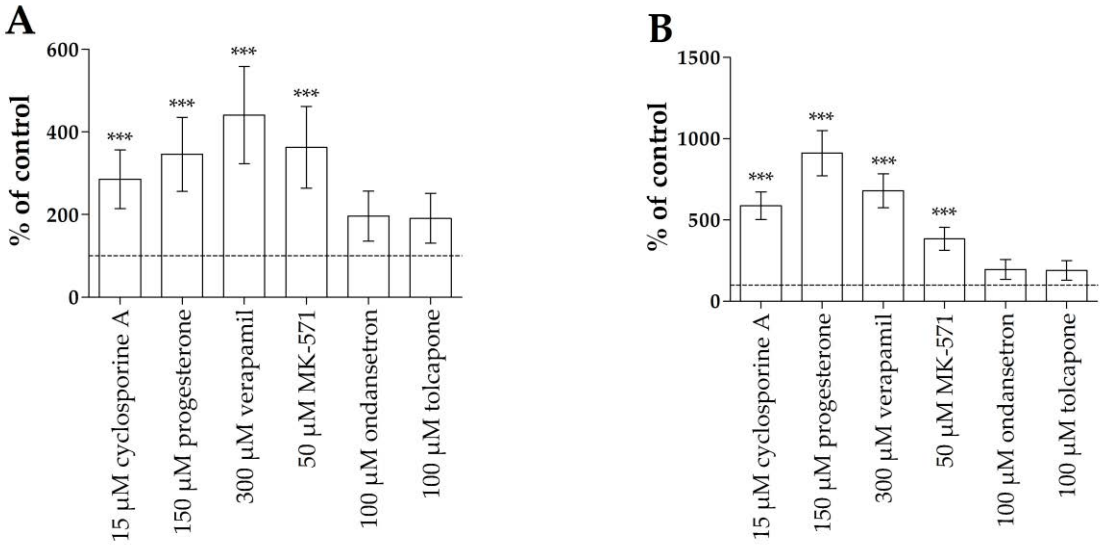

Figure 17. Calcein retention (% of control) in the BBMECs (A) and MDCKII-MDR1 (B) in the presence of known efflux protein inhibitors, cyclosporine A (15 µM), progesterone (150 µM), verapamil (300 µM) and MK-571 (50 µM). The studies were conducted in quadruplicate and performed in four different experiments in the BBMECs with the exception of ondansetron and tolcapone where one experiment was performed. One experiment in the MDCKII-MDR1 cells was performed. Data are mean±SD. Statistical significance of differences vs. control (dashed line) was tested by one-way ANOVA followed by Dunnett's test, $P<0.001$ (***). Note the differences in the scales on the y-axis. Modified from Figure 6 in **III**. MK-571, ondansetron and tolcapone are unpublished results.

5.4.3 P-glycoprotein mediated transport of drugs across the BBMECs

The functionality of P-glycoprotein as demonstrated in the uptake assays does not entirely reflect situation in the drug permeability assays, since both paracellular and transcellular permeability of drug across the cell monolayer have an impact on drug permeability assays. The P-glycoprotein mediated efflux in the drug permeability assays needs to exceed significantly the passive intrinsic permeability of drug in order to be detectable (Sugano *et al.*, 2010). Therefore, the functionality of P-glycoprotein was also assessed with the drug permeability assays.

P_{app} values of paclitaxel and digoxin were significantly lower ($P<0.001$ and $P<0.05$) in the BBMECs cultured on filter inserts in comparison to those cultured on filters on petri dishes (Figure 18A,B). This is explained by the higher expression of P-glycoprotein in the BBMECs cultured on filter inserts than those on filters on petri dishes observed by immunoblot experiments (Figure 12) and immunofluorescent staining (Figure 13).

The bidirectional transport assay is commonly used to estimate efflux protein functionality in *in vitro* models (Polli *et al.*, 2001) and is also approved by the regulatory authorities for identification of the involvement of transporters affecting the disposition of a novel drug (European Medicines Agency, Guideline on the Investigation of Drug Interactions, 2012). The criterion for functionality of the efflux proteins is normally accepted as ER≥2 (Giacomini *et al.*, 2010, Zhang *et al.*, 2006a).

The BBMECs cultured on filter inserts demonstrated ER values 1.7, 1.4 and ~1 for digoxin, paclitaxel and vinblastine, respectively (Figure 18). The corresponding ER values for BBMECs grown on filters on petri dishes were ~1 (Figure 18). Since the ER values in the BBMEC model did not reach criterion for efflux (ER≥2) for known P-glycoprotein substrates, it can be concluded that the functionality of P-glycoprotein is not detectable in the BBMEC model, if one considers the normal criteria for bidirectional transport applied.

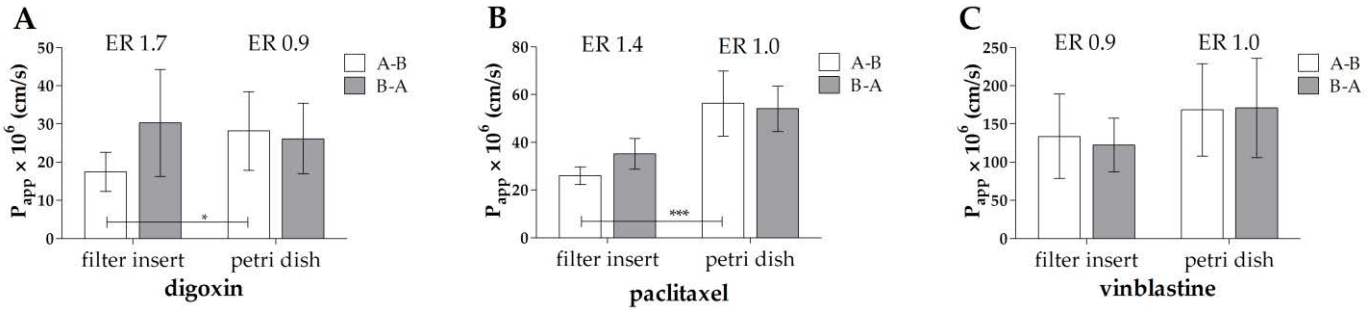

Figure 18. Apparent permeability coefficients (P_{app}) × 10^6 (cm/s) of three P-glycoprotein substrates digoxin (A), paclitaxel (B) and vinblastine (C) in the apical to basolateral (A-B) and in the basolateral to apical (B-A) directions in the BBMECs cultured on filter inserts and on filters on petri dishes. Permeation studies were conducted in triplicate and performed in at least three different experiments. Data are mean±SD (n=9-12). Efflux ratio (ER) was calculated by equation $P_{app,B-A}/P_{app,A-B}$. Statistical significance of differences was tested by Student's t-test, P<0.05 (*), P<0.001 (***). Figure 7 in **III**.

Bidirectional transport studies of [^{3}H]paclitaxel with or without GF120918 were conducted in the BBMECs cultured on filter inserts (**III**). It was shown that the ER value for paclitaxel decreased from ER 1.4 to 0.8 as a result of increased P_{app} in the A-B direction (Figure 19). This confirmed the hypothesis that the trend towards higher ER values in the BBMECs cultured on filter inserts (Figure 18) was attributable to the higher P-glycoprotein expression.

In the MDCKII-MDR1 cells overexpressing P-glycoprotein, the ER value for paclitaxel was 6 indicating that clear efflux was observed in the MDCKII-MDR1 cells. In addition, in the presence of GF120918 the ER value for paclitaxel did not decrease to unity but residual ER 2 was observed.

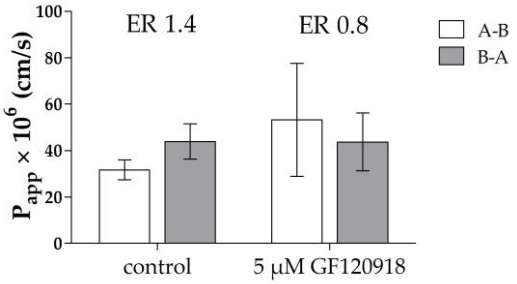

Figure 19. Apparent permeability coefficients (P_{app}) × 10^6 (cm/s) of paclitaxel in the apical to basolateral (A-B) and in the basolateral to apical (B-A) directions in the BBMECs cultured on filter inserts in the absence and presence of GF120918. Data are mean±SD (n=9). Efflux ratio (ER) was calculated by equation $P_{app,B-A}/P_{app,A-B}$. Figure 8 in **III**.

5.5 *IN VITRO-IN VIVO* CORRELATION OF THE CELL MODELS

5.5.1 *In vivo* unbound brain/blood ratio

The *in vivo* unbound brain/blood ratios are shown in Table 13. The extrapolated AUC portion was <20 % except in the case of tolcapone 35 % and baclofen 26 % in brain and atenolol 39 %, entacapone 21 % and baclofen 34 % in blood. After administration of a single dose, all model drugs were quantifiable in the rat brain ECF indicating that all these model drugs were able to cross the blood-brain barrier, at least to some extent. However, clear differences between the unbound brain/blood ratios can be found. The unbound brain/blood ratio was highest for alprenolol (0.77±0.15, mean±SD) and lowest for baclofen (0.08±0.10). These results indicate that the evaluation of *in vivo* unbound brain/blood ratios can help in the differentiation of drugs based on their ability to cross the blood-brain barrier and to enter into the brain (Table 13).

Table 13. Unbound brain/blood ratios were calculated using dual probe *in vivo* microdialysis AUC data. Data are mean±SD.

Model drug	Unbound brain/blood ratio
alprenolol	0.77 ± 0.15 (n=9)
metoprolol	0.64 ± 0.06 (n=6)
midazolam	0.61 ± 0.19 (n=6)
pindolol	0.34 ± 0.08 (n=8)
ondansetron	0.19 ± 0.07 (n=6)
tolcapone	0.17 ± 0.12 (n=6)
atenolol	0.16 ± 0.08 (n=5)
entacapone	0.14 ± 0.11 (n=10)
baclofen	0.08 ± 0.10 (n=9)

5.5.2 *In vitro-in vivo* correlation of the cell models

In vitro-in vivo correlation of the BBMEC, Caco-2 and MDCKII-MDR1 models were assessed by the linear regression between the *in vitro* log(P_{app}) and *in vivo* unbound brain/blood ratio (Figure 20). Significant *in vitro-in vivo* correlations were observed only if ondansetron and tolcapone were excluded from the data analysis, probably because they act as substrates for efflux transporters. The resulting Pearson correlation coefficients were r=0.99 (P<0.001) in the BBMEC model, r=0.91 (P<0.01) in Caco-2 model and r=0.85 (P<0.05) in MDCKII-MDR1 model. Apparent differences in the correlation coefficients between cell models may result from the fact that the paracellular leakiness of the BBMEC model restricts the differentiation between low permeability drugs. In addition, in a small model drug set (n=7), minor variations in the single data point exert a great impact on the r value. Therefore, no clear differences in the *in vitro-in vivo* correlations between the cell models were found, although the P_{app} values were substantially different in the BBMEC model than in the Caco-2 and MDCKII-MDR1 models. This indicates that the *in vitro-in vivo* correlations between the cell models are comparable when the rat brain microdialysis method is used as the *in vivo* measure.

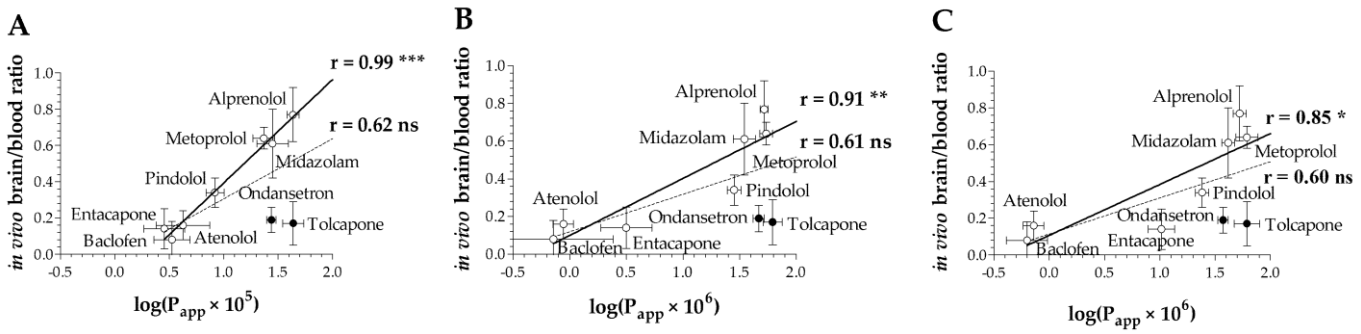

Figure 20. In vitro-in vivo correlations of the BBMEC (A), Caco-2 (B) and MDCKII-MDR1 (C) models. Each *in vitro* P_{app} values were log-normalized. *In vivo* brain/blood ratio represents the ratio of unbound drug concentration from the AUC from the rat brain ECF and unbound drug concentration from the AUC from the rat blood. Data are mean±SD. Dashed line (---) represents the linear regression with all data points and the solid line (—) represents the linear regression after exclusion of two data points (•). Pearson's correlation coefficients (r) were defined. P<0.001***, P<0.01**, P<0.05*, ns=not significant. Figure 4 in **I**.

5.5.3 Relationship between physicochemical properties, *in vitro* and *in vivo* parameters

The physicochemical properties of drugs have been shown to influence their blood-brain barrier permeabilities *in vivo* (Abraham *et al.*, 1994, Gratton *et al.*, 1997). In order to further evaluate the *in vitro-in vivo* correlations obtainable with the cell models, a third dimension of selected physicochemical properties of drugs (LogP, PSA and hydrogen bonding interactions) was added into the *in vitro-in vivo* correlations to create three dimensional plots (Figure 21).

The three dimensional scatter plots demonstrate the influence of different physicochemical properties of models drugs for *in vivo* and *in vitro* parameters. In general, low LogP value (Figure 21A-C) and high hydrogen bonding interaction capabilities (Figure 21G-I) are reflections of lower *in vivo* unbound brain/blood ratios and low P_{app} values. In contrast, high LogP, low PSA (Figure 21D-F) and a low amount of hydrogen bonding interactions translate into higher *in vivo* unbound brain/blood ratios and high P_{app} values.

Ondansetron and tolcapone are two model compounds that were excluded from the *in vitro-in vivo* correlation analysis in Figure 20; they are designated with black pins in the three dimensional scatter plots (Figure 21). There are differences in the physicochemical properties (LogP, PSA and hydrogen bonding interactions) between these two model drugs. Tolcapone displays higher values for LogP, PSA and hydrogen bonding interactions than ondansetron. The higher PSA and hydrogen bonding interactions of tolcapone may partly explain its low *in vivo* unbound brain/blood ratio, although the LogP value is rather high and, thus, for it the *in vitro-in vivo* correlation is poor.

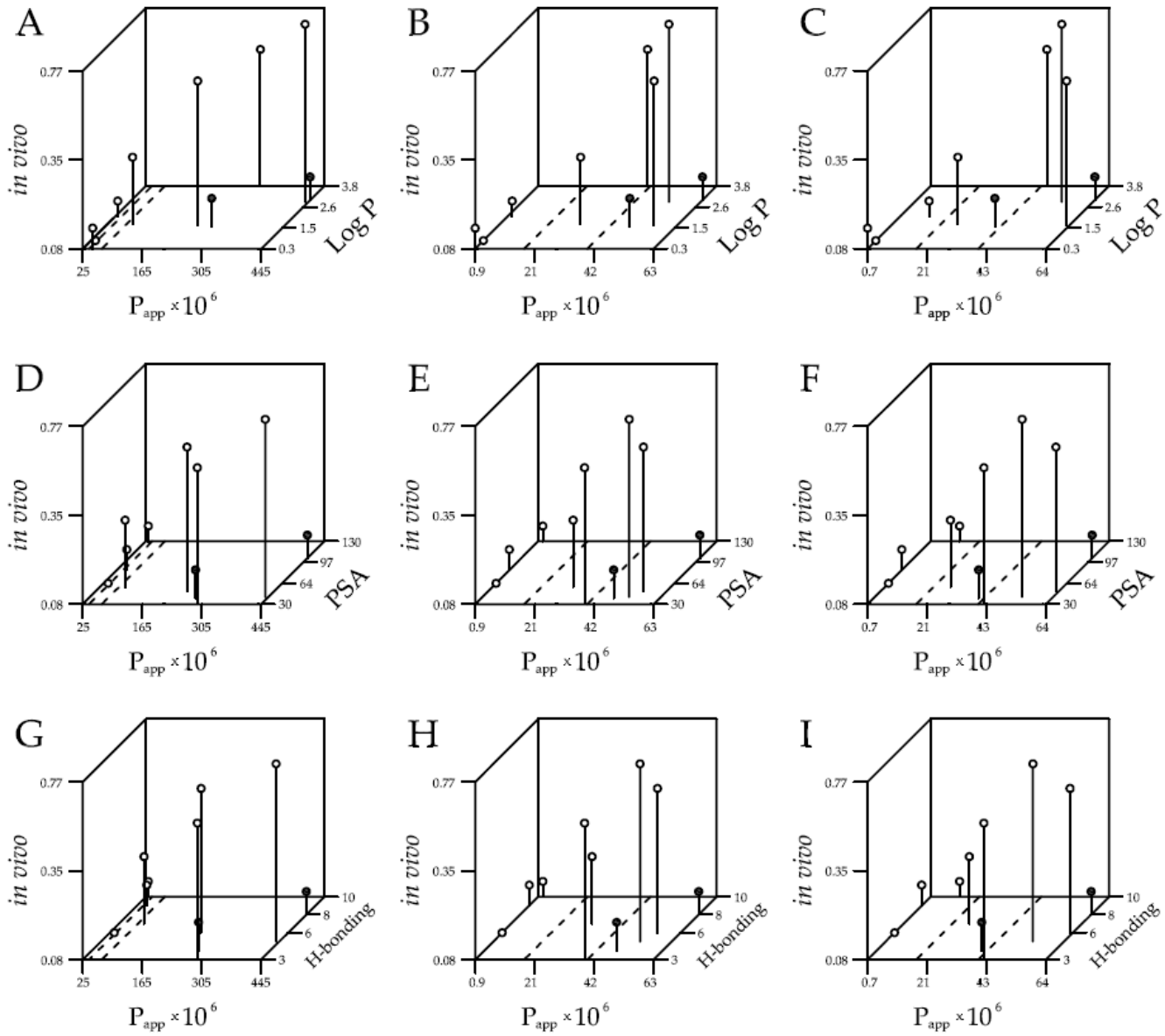

Figure 21. Influence of different physicochemical properties of model drugs for *in vitro* apparent permeability coefficients ($P_{app} \times 10^6$) and *in vivo* unbound brain/blood ratios (*in vivo*). Physicochemical properties; octanol/water partition coefficient (LogP, A-C), polar surface area (PSA, D-F) and hydrogen bonding interactions (H-bonding, G-I) in the BBMEC (A,D,G), Caco-2 (B,E,H) and MDCKII-MDR1 (C,F,I) cell models. *In vitro* and *in vivo* data are described in Table 12 and 13, respectively. Physicochemical properties of the model drugs are described in Table 9. Two model drugs (ondansetron and tolcapone that were excluded from the *in vitro-in vivo* correlation analysis in Figure 20) are marked with black pins. The dashed lines represent the limits for low, medium and high *in vitro* P_{app} categories. For BBMEC model, the limits were set as described in section 5.3.1. and for Caco-2 and MDCKII-MDR1 models, the limits were set as described in section 5.3.3.

6 Discussion

6.1 CHARACTERISTICS OF THE BBMEC MODEL

6.1.1 Tightness

The permeability of sucrose into the rat brain *in vivo* is extremely low (Ohno *et al.*, 1978), in fact to all intents and purposes, it is negligible, and, therefore, sucrose is the most commonly used low permeability compound in the characterization of the tightness of the *in vitro* models representing the blood-brain barrier. In this study, the P_{app} values for sucrose obtained with the monocultured BBMECs were in line with previous studies conducted with the monocultured BBMECs under similar culture and experimental conditions (Eddy *et al.*, 1997, Rice *et al.*, 2005). In general, however, the permeability of sucrose in the monocultured BBMECs has been shown to be very variable ranging from 4 to 85 × 10^{-6} cm/s (Pardridge *et al.*, 1990, Eddy *et al.*, 1997, Glynn and Yazdanian, 1998, Cecchelli *et al.*, 1999, Johnson and Anderson, 1999, Polli *et al.*, 2000, Otis *et al.*, 2001, Karyekar *et al.*, 2003, Rice *et al.*, 2005) evidence for the extensive inter-laboratory variability in evaluations of the tightness of monocultured BBMECs. One of the reasons for this variability may be the success or failure in the isolation of pure BBMECs, *i.e.*, contamination of BBMECs with other cells, such as pericytes, that are known to loosen the BBMEC monolayer as also observed in the present.

Since the permeability of sucrose was interpreted as a leaky paracellular barrier in the BBMEC model, different culture medium supplements (hydrocortisone, ascorbic acid and two different ACMs) were tested. However, it was found that these supplements did not improve the tightness of the monocultured BBMECs.

One possible explanation for the inability to achieve adequate tightness in the BBMECs cultured under ACMs may be that the ACM alone is not sufficient to tighten gaps between cells in the BBMEC model. In fact, it has been shown that astrocytes need to be in close contact with BBMECs in order to enhance the tight junctions (Tao-Cheng *et al.*, 1987). In addition, cultured endothelial cells might need synergistic enhancement from both astrocytic factors and agents that increase cAMP levels (Wolburg *et al.*, 1994). Recently, the BBMECs co-cultured in close contact with rat astrocytes and several different culture medium supplements (cAMP, phosphodiesterase inhibitor and dexamethasone) demonstrated increased TEER values which ranged from 760 to 1014 Ωcm^2 (Helms *et al.*, 2010, Helms *et al.*, 2012). In this co-culture BBMEC model, the permeability of mannitol was relatively low varying from 0.3 to 3.2 × 10^{-6} cm/s, suggesting that this novel BBMEC model may promote the development of tighter endothelial cell based *in vitro* models in the future.

One possible factor leading to leaky paracellular route in the BBMECs may be the present finding that the transmembrane tight junction protein, occludin, which is responsible for the paracellular barrier between the endothelial cells of blood-brain barrier (Hirase *et al.*, 1997) was found to be located in the perinuclear space in the BBMECs. As far as is known, this has not been previously demonstrated in the BBMECs. Incomplete localization of occludin may weaken the tight junctions between the BBMECs and, thus, may partly explain the leaky paracellular characteristics of the monocultured BBMEC model. One could speculate that occludin may be internalized, *e.g.*, by endocytosis, since also the adherens junction protein (VE-cadherin) has

been demonstrated to be internalized by endocytosis (Gavard and Gutkind, 2006). In addition, endocytic recycling of occludin has been indicated in epithelial cells (Morimoto *et al.*, 2005). In the future, this finding may also help in the development of tighter paracellular barrier between the BBMECs. In order to fully understand the association between possible internalization of occludin and paracellular transport of compounds across the BBMECs, however, further studies are needed.

In the present study, the tightness of the non-brain originating epithelial cell models, Caco-2 and MDCKII-MDR1, were compared with the monocultured BBMEC model. The paracellular permeability of sucrose in the BBMEC model was ~13-fold higher than the Caco-2 model and ~21-fold higher than the MDCKII-MDR1 model, which is in line with previous studies (Eddy *et al.*, 1997, Yazdanian *et al.*, 1998, Garberg *et al.*, 2005, Rice *et al.*, 2005, Mashayekhi *et al.*, 2010). This indicates that the epithelial cell models are able to form relatively tight junctions also *in vitro* but the brain endothelial cells may require more enhancement factors *in vitro*, such as astrocytic factors and compounds which increase the cAMP level, as discussed above. It should be remembered that brain endothelial cells at the blood-brain barrier *in vivo* are surrounded by other cell types present in the neurovascular unit that maintains the brain homeostasis by interactive regulatory mechanisms (Iadecola, 2004) and the isolation of the endothelial cells from this unit may attenuate the endothelial cell properties *in vitro*. Overall, however, in all *in vitro* models the tightness is still several orders of magnitude leakier than the *in vivo* blood-brain barrier. Therefore, achieving the tightness of the blood-brain barrier *in vivo* is an extremely demanding, perhaps even impossible, task *in vitro*.

6.1.2 Expression of P-glycoprotein

P-glycoprotein is one of the most extensively studied efflux protein. Since it is expressed at high levels in the blood-brain barrier (Cordon-Cardo *et al.*, 1989), it has an important role in the distribution of the drugs into the brain (Schinkel *et al.*, 1994). In addition, P-glycoprotein has been considered as being one of the most important transporters playing a role in pharmacokinetics in human (Fromm, 2004, Sasongko *et al.*, 2005, Wagner *et al.*, 2009). Therefore, P-glycoprotein was used as a model efflux transporter in this study; its expression and localization were confirmed before conducting functionality assays in the monocultured BBMECs.

Confocal images revealed that P-glycoprotein was localized predominantly in the apical cell membrane in the monocultured BBMECs cultured on filter inserts or on filters on petri dishes. This appears to be the first time that the apical localization of P-glycoprotein has been demonstrated in BBMECs by using confocal microscopy. The results correspond to the *in vivo* localization of P-glycoprotein at the blood-brain barrier, since apical localization of the P-glycoprotein in the fresh bovine brain tissue and BBMECs have been demonstrated previously with electron microscopy (Tsuji *et al.*, 1992).

In addition, these studies indicated that P-glycoprotein expression in the monocultured BBMECs was dependent on the culture conditions. When BBMECs are grown on filter inserts, the culture medium gains access to the cells also from the basolateral side of the BBMECs and this situation resembles more closely the *in vivo* situation. This most likely explains the higher P-glycoprotein expression of BBMECs cultured on filter inserts. Generally, the expression of P-glycoprotein in the BBMECs cultured on filter inserts seemed to be almost comparable with the P-glycoprotein overexpressing MDCKII-MDR1 cells when immunoblot assay (normalized to

protein concentration) was used, which has also supported by others (Zhang *et al.*, 2004). Furthermore, the immunofluorescence of P-glycoprotein was significantly higher (normalized to area) in the BBMECs cultured on filter inserts than in the MDCKII-MDR1 cells evidence also for high P-glycoprotein expression in the BBMECs.

6.1.3 Functionality of P-glycoprotein

The expression level of P-glycoprotein may not always correlate with protein functionality (Bailly *et al.*, 1995). Therefore, the functionality of P-glycoprotein was demonstrated in the BBMECs by using cellular uptake studies. The cellular uptake of known P-glycoprotein substrates was significantly increased when P-glycoprotein mediated efflux was inhibited by a P-glycoprotein inhibitor(s), which is in agreement with earlier cellular uptake studies which have investigated functional P-glycoprotein in the BBMECs (Tsuji *et al.*, 1992, Lechardeur and Scherman, 1995, Rice *et al.*, 2005, Joly *et al.*, 1995, Silverstein *et al.*, 2004).

The calcein-AM assay is generally used to demonstrate the functionality of P-glycoprotein, since calcein-AM, but not calcein, is good substrate for P-glycoprotein (Homolya *et al.*, 1993). Therefore, higher calcein retention in the presence of P-glycoprotein inhibitors is evidence for functional P-glycoprotein in the cells. However, also other efflux proteins such as MRP1 and MRP2 have an impact on the calcein-AM assay, since calcein-AM has been demonstrated to have interaction with MRP1 (Feller *et al.*, 1995, Holló *et al.*, 1996) and calcein undergoes interactions with both MRP1 and MRP2 (Evers *et al.*, 2000). In the present study, all efflux protein inhibitors used, including both P-glycoprotein and MRP inhibitors, significantly increased calcein retention in the BBMECs. Since P-glycoprotein and MRP1 (Zhang *et al.*, 2004) are known to be expressed in the BBMECs but MRP2 is absent (Zhang *et al.*, 2000), the higher calcein retention in the presence of P-glycoprotein or MRP inhibitors indicates that both P-glycoprotein and MRP1 are functional in the BBMECs. Similarly, all of the efflux protein inhibitors tested here significantly increased calcein retention in the MDCKII-MDR1 cells used as a control. In summary, the present results demonstrate that the P-glycoprotein, probably also MRP1, are functional in the BBMECs.

We also assessed the functionality of P-glycoprotein by using bidirectional transport assays in the monocultured BBMECs cultured on both filter inserts and on filters on petri dishes. P-glycoprotein mediated efflux was not observed in the monocultured BBMECs, even though the functionality of P-glycoprotein had been demonstrated. In fact, there is no consensus about whether the BBMECs should demonstrate sufficient efflux or not. The failure to detect P-glycoprotein mediated efflux is in line with the previous studies which have been performed with both monocultured BBMECs and BBMECs cultured with astrocytes for doxorubicin, rhodamine 123, zidovudine or paclitaxel (Masereeuw *et al.*, 1994, Rice *et al.*, 2005, Gaillard *et al.*, 2000). However, also contradictory results showing ER values between 2-4 for rhodamine 123 (Batrakova *et al.*, 2001, Karyekar *et al.*, 2003, Letrent *et al.*, 1999) and vinblastine (Eddy *et al.*, 1997, Otis *et al.*, 2001) have been published with monocultured BBMECs. The inconsistencies in the reported ER values may be due to differences in the P-glycoprotein expression as a result of different culture conditions as described in the present study (Figure 12 and 13). In addition, it should be noted that the ER values obtained in the BBMEC model, as well as other endothelial cell models based on RBMECs and PBMECs (ER<2.5) (Zhang *et al.*, 2006b, Hellinger *et al.*, 2012), are intrinsically clearly lower in the brain endothelial cell models than those in epithelial cell models, such as in the Caco-2 or MDCKII-MDR1. The Caco-2 model has produced ER values for

vinblastine of 11-17 and for digoxin of 5-24 (Troutman and Thakker, 2003, Garberg *et al.*, 2005, Hellinger *et al.*, 2012). In addition, ER values for vinblastine and digoxin have been reported as 23-370 and 53-60, respectively, in the MDCKII-MDR1 model (Garberg *et al.*, 2005, Hellinger *et al.*, 2012, Polli *et al.*, 2001) evidence that the ER values are significantly higher in the epithelial cell models. It may be difficult to evaluate the functionality of the active transport mechanisms in endothelial cells *in vitro*. Brain endothelial cells may rapidly lose their highly differentiated characteristics after isolation from the *in vivo* neurovascular environment and, thus, the functionality of several transport mecanisms may be lower *in vitro* than in the *in vivo* situation.

High passive permeability (including both paracellular and transcellular transport) may result in undetected efflux in bidirectional assays. Passive permeability can overwhelm the P-glycoprotein mediated efflux in the monocultured BBMECs; the passive transport processes have been reported to overcome P-glycoprotein mediated efflux in other cell models, such as MDCKII-MDR1 (Eytan *et al.*, 1997, Eytan, 2005, Lentz *et al.*, 2000, Varma *et al.*, 2005). The paracellular permeability of sucrose was relatively high in the monocultured BBMECs and, thus, it is most likely that the passive paracellular transport of compounds is dominant in drug permeability assays. In fact, the low ER value for rhodamine 123 (ER 1.7) has also been demonstrated in the PBMECs cultured in conjunction with ACM which has a leaky paracellular barrier (P_{app} of sucrose 80×10^{-6} cm/s) further confirming the suspicion that the leaky cell models may not be able to detect the efflux in bidirectional transport assays and, thus, they cannot be used for prediction of P-glycoprotein mediated efflux potential of drug candidates in early drug development. Taken together, since monocultured BBMECs were not able to reveal efflux in the bidirectional transport studies, their use in bidirectional transport studies may pose a real risk of obtaining false negative results in assessing ER values for drug candidates that are potential efflux transporter substrates.

6.2 DRUG PERMEABILITY

6.2.1 Dynamic range

The dynamic range describes how efficiently the cell model can discriminate between drugs with low and high P_{app} values. The lower dynamic range in the BBMEC model in comparison to the epithelial cell models seems to be a typical finding for the BBMEC model (Polli *et al.*, 2000) which can be explained partly by the leakier paracellular barrier. Similarly, a lower dynamic range has been observed earlier in the RBMECs co-cultured with astrocytes and pericytes (Hellinger *et al.*, 2012). Due to the low dynamic ranges, the leakier cell models may not be able to efficiently distinguish the P_{app} values of drugs.

6.2.2 Molecular descriptors determining passive drug permeability

The paracellular transport of drugs across the blood-brain barrier *in vivo* is negligible due to the fact that the tight junctions restrict passage through the paracellular route. Therefore, the drugs targeted into the brain need to be transported across the brain endothelial cells. The intrinsic molecular properties of a drug determine its ability to be transported via the transcellular pathway. The most important molecular properties influencing the passive brain penetration of drugs are lipophilicity (LogP), hydrogen bonding potential, polarity (*e.g.*, PSA) and MW (Rapoport and Levitan, 1974, Abraham *et al.*, 1994, Abraham, 2004, Gratton *et al.*, 1997, Kelder *et al.*, 1999, Pardridge, 2005, Fischer *et al.*, 1998). A better potential for blood-brain barrier permeability of drugs can be achieved when LogP is 2-5 or <3, hydrogen bonding interactions

(HBD <3-4 and HBA <8), PSA <90 Å^2 and MW less than 450-500 Da (Hitchcock and Pennington, 2006, Reichel, 2006). These limits are stricter than Lipinski's rule of five limits (*i.e.*, LogP <5, hydrogen bonding interactions (HBD <5 and HBA <10) and MW <500 Da (Lipinski *et al.*, 1997).

However, there are no comprehensive studies available which have evaluated the molecular properties which influence drug permeability across the blood-brain barrier in vitro, such as with the BBMEC model. Only a few molecular descriptors affecting the passive transport across the BBMEC model have been described; lipophilicity and molecular size (Usansky and Sinko, 2003), hydrogen bonding interactions of peptides (Chikhale et al., 1994) and computed free energy of solvation parameter (Lombardo et al., 1996). There are no other reports assessing the influence of descriptors in the permeability of drugs across BBMEC model. In the present study, twelve key molecular parameters of drugs from a set of 102 molecular parameters were identified as influencing the passive cell membrane permeability across the BBMEC model. These key molecular parameters were mainly related to lipophilic and hydrophilic interactions, the balance between the hydrophilic and lipophilic moieties of the drug and hydrogen bonding interactions, in parallel with those determining the passive brain penetration of drugs *in vivo* (Rapoport and Levitan, 1974, Abraham *et al.*, 1994, Gratton *et al.*, 1997, Fischer *et al.*, 1998, Kelder *et al.*, 1999, Abraham, 2004, Pardridge, 2005). The passive drug permeability *in vivo* and *in vitro* is determined with the comparable molecular properties of drugs. However, the brain entry of drugs *in vivo* is a complex process that is also influenced by active mechanisms, such as active transporters and metabolism.

At present, Caco-2 is the most widely used *in vitro* model for predicting the oral absorption of drug candidates in early drug development. Therefore, the molecular properties determining the permeability of drugs across Caco-2 monolayers have been rather extensively investigated (van de Waterbeemd *et al.*, 1996, Krarup *et al.*, 1998, Ertl *et al.*, 2000, Österberg and Norinder, 2000, Stenberg *et al.*, 2001, Guangli and Yiyu, 2006, Di Fenza *et al.*, 2007, Paixão *et al.*, 2010, Shinde *et al.*, 2011), whereas the permeability of drugs across MDCK cell monolayers is far less well understood (Chen *et al.*, 2005, Groenendaal *et al.*, 2008). The molecular properties determining the *in vitro* permeability of drugs in the Caco-2 and MDCK models are mainly lipophilicity, hydrophilicity, hydrogen bonding interactions, MW, molecular size and shape. In fact, these are in agreement with those determined for the BBMEC model in the present study with the exception of molecular size and shape properties that were not clearly apparent from the set of molecular descriptors in the BBMEC model. This may result from the paracellular leakiness of the BBMEC model. These results are in accordance with the molecular properties that have been associated with the passive permeability of drugs across the cell membranes (van de Waterbeemd *et al.*, 1996, Lipinski *et al.*, 1997, Abraham *et al.*, 1994, Kelder *et al.*, 1999, Palm *et al.*, 1997). The molecular descriptors determining the passive drug permeability across the endothelial and epithelial cells are similar, despite the cellular differences, *e.g.*, tightness, height of the cell monolayer and phospholipid composition of cell membranes (Siakotos and Rouser, 1969, Dias *et al.*, 1992, Hansson *et al.*, 1986, Di *et al.*, 2009, Hellinger *et al.*, 2012). For this reason, one could speculate that either an endothelial or an epithelial cell monolayer could be used when only passive transport is being evaluated *in vitro* and one is attempting to classify drugs into different categories.

6.2.3 Comparison of drug permeabilities between the *in vitro* models

The present study clearly demonstrated that the BBMEC model indicated generally higher P_{app} values for model drugs than Caco-2 or MDCKII-MDR1 models, but nonetheless the BBMEC model was able to rank the model drugs into the same categories as the Caco-2 and MDCKII-MDR1 models with a few exceptions, despite its leakiness and its lower dynamic range. This suggests that the BBMEC model and Caco-2 and MDCKII-MDR1 models, particularly when passive permeability is being evaluated, are able to classify the drugs and measure the drug permeation process similarly but the P_{app} scales are different. One explanation for this could be the different experimental set-ups. In this study, experimental set-ups typical for each cell model, *i.e.*, BBMECs in the well stirred side-by-side diffusion chambers (Hansen *et al.*, 2002) and Caco-2 or MDCKII-MDR1 cells in the filter insert system (Braun *et al.*, 2000) were applied. The side-by-side diffusion chamber system has more efficient stirring and, thus, the breadth of the unstirred water layer is narrower than in the filter insert system. The wider unstirred water layer formed in the filter insert systems is known to reduce the P_{app} values of lipophilic drugs (Korjamo *et al.*, 2008, Korjamo *et al.*, 2009) indicating that the higher unstirred water layer in the filter insert system, limits the exact measurement of P_{app} values for highly permeable drugs. Therefore, lipophilic drugs tend to have higher P_{app} values across the cell membranes when there is a narrow unstirred water layer and this does not cause a rate-limiting barrier. In this study, diazepam was used as high permeability reference compound and the P_{app} value of diazepam was observed to be higher in the BBMEC model than in the Caco-2 and MDCKII-MDR1 model, which is dependent on the thickness of the unstirred water layer in the different experimental set-ups. In a previous study by Zhang *et al.* 2006b, the permeability of lipophilic diazepam was substantially higher in stirred when compared to the unstirred conditions (Zhang *et al.*, 2006b). In addition, the thinner cell monolayer of the BBMECs and, thus, the lower diffusion distance may partially explain the higher P_{app} values obtained for both the low and high permeability model drugs.

Interlaboratory variation is a common problem in many *in vitro* models. Interlaboratory variation has been clearly demonstrated in Caco-2 cells (Walter and Kissel, 1995, Hayeshi *et al.*, 2008) and, thus, it complicates the comparison of the *in vitro* drug permeability values conducted in different laboratories. Therefore, the culture conditions and protocols for all cell models used for *in vitro* drug permeability testing should be standardized in an attempt to reduce the interlaboratory variation. Furthermore, the standardized protocols should include the relevant controls to reflect the entire dynamic range and also the robustness of active transport mechanisms before investigators will be able to systemically evaluate the properties of the *in vitro* model used. However, these kinds of standardized culture conditions or protocols for the endothelial cell models are still lacking.

6.3 *IN VITRO-IN VIVO* RELEVANCE

In vitro-in vivo correlation studies are generally undertaken to assess the relevance of *in vitro* models when they are used to predict *in vivo* parameters. The *in vitro-in vivo* correlation describes the mathematical relationship between the parameters determined *in vitro* and *in vivo*. In the present study, linear regressions between P_{app} values determined for nine model drugs in three *in vitro* models (BBMEC, Caco-2 and MDCKII-MDR1) and the *in vivo* unbound brain/blood ratios determined by the microdialysis method were calculated. Currently, the *in vivo* brain microdialysis method, which assesses the unbound drug concentrations from both sides of the blood-brain barrier, is considered as the most relevant method with which to assess

the unbound drug concentrations that are able to exert the pharmacological response (Hammarlund-Udenaes *et al.*, 2008). The unbound brain/blood ratio was determined after a single dose and it describes mainly the rate of the drug transport across the blood-brain barrier. However, the unbound brain AUC determined by *in vivo* brain microdialysis also illustrates the distribution of a drug from the extracellular compartment into the intracellular compartments and elimination from the brain similarly as the unbound AUC in blood takes into account absorption, distribution and elimination throughout the body. The present study indicated that the *in vitro-in vivo* correlations between BBMEC, Caco-2 or MDCKII-MDR1 model were similar when mainly passively transported compounds were examined in the analysis.

When model drugs known to undergo interaction with efflux proteins (ondansetron and tolcapone) were included, the *in vitro-in vivo* correlations were deteriorated. Ondansetron has previously been identified as a substrate for P-glycoprotein (Schinkel *et al.*, 1996) and the calcein-AM assay showed that tolcapone may undergo interactions with efflux protein(s) in the BBMECs and MDCKII-MDR1 cells as well. However, the specific efflux transport mechanism(s) involved in the transport of tolcapone remains to be clarified. On the other hand, the physicochemical properties of drugs also influence the brain penetration together with transporter mediated efflux. Therefore, the *in vitro-in vivo* correlations were evaluated further by using three dimensional plots that were generated by including physicochemical properties into the *in vitro-in vivo* correlations. The three dimensional plots showed general trends of the physicochemical properties differentiating between lower or higher *in vivo* and *in vitro* parameters. However, deviations from the *in vitro-in vivo* correlation trends, namely ondansetron and tolcapone, were only partially explained in terms of physicochemical properties. Thus, other restrictive mechanisms, such as functionality of efflux proteins, are the most likely reasons for their poor *in vitro-in vivo* correlations. This approach indicates that it is the multiple characteristics of drug, not only one individual physicochemical property, which determine a drug's potential to cross the blood-brain barrier *in vivo*.

Several *in vitro-in vivo* correlation studies utilizing different *in vitro* and *in vivo* models have been published (Table 5 and 6). However, the results are highly variable and there is neither consensus nor statistical evidence of whether endothelial or epithelial cell models are better. This argument is based on statistical analyses of the literature results (r) in Table 5 and 6; statistical significance of differences between cell models or between endothelia and epithelial cell models was tested by one-way ANOVA followed by Tukey's test by GraphPad Prism 5.03 software with statistical significance set at $P<0.05$. Most of the *in vivo* methods measure the total drug concentrations at a single time point. In this study, the *in vivo* microdialysis method was applied. The paracellular leakiness of the BBMEC model restricts the differentiation between low permeability drugs and, thus, may apparently improve the *in vitro-in vivo* correlation in the BBMEC model. In addition, in a small model drug set (n=7), a minor variation in single data point may have a great impact on r values. The present *in vitro-in vivo* correlation results indicate no clear difference between the cell models and are consistent with the previous literature findings (Table 5 and 6). No *in vitro-in vivo* correlation was found in a previous study when a higher number of model drugs (n=22 including passively transporting drugs, efflux and influx protein substrates) were used to study *in vitro-in vivo* correlations between co-cultured BBMEC, Caco-2 or MDCKII-MDR1 models (Garberg *et al.*, 2005). However, also better *in vitro-in vivo* correlations were achieved when only passively transported compounds (n=10) were examined (Garberg *et al.*, 2005). These poor *in vitro-in vivo* correlations of efflux protein

substrates can be explained by the fact that the efflux proteins are functioning in their natural rate *in vivo* but *in vitro* models may not be able to reproduce the actual efflux protein functionality corresponding to the *in vivo* situation. In summary, the similarities in the *in vitro-in vivo* correlations between different *in vitro* models indicate that *in vitro* models are able to classify drugs into categories and predict mainly passive drug entry into brain *in vivo*. This highlights the need to use *in vivo* methods to supplement the *in vitro* predictions in the early development of CNS drugs.

7 Conclusions and Future Prospects

This study aimed to clarify the relevance of the monocultured BBMEC model for use as an *in vitro* blood-brain barrier model in drug permeability studies. The BBMECs has been used already for several decades but there are still uncertainties about the reliability of monocultured BBMECs as a relevant *in vitro* blood-brain barrier model. The present study investigated the factors influencing passive drug permeability, role of the P-glycoprotein mediated efflux and the *in vivo* relevance of the monocultured BBMEC model. Furthermore, the properties of the BBMEC model were compared to two epithelial cell models (Caco-2 and MDCKII-MDR1) that are generally in use in academic laboratories and the pharmaceutical industry to assess drug permeability. On the basis of this study, the following specific conclusions can be drawn:

1. The tested cell culture conditions neither tightened the paracellular barrier nor enhanced the functionality of efflux proteins in the BBMECs. BBMECs were pure endothelial cells expressing several endothelial cell and blood-brain barrier markers. However, the BBMEC model was leakier and as a result it had a lower dynamic range than the Caco-2 or MDCKII-MDR1 cell models. One reason for this could be the partial perinuclear localization of the tight junction protein, occludin. The BBMEC model may be suitable for use in the studies of cellular mechanisms, such as in cellular uptake studies, where the tightness of the cell monolayer is not the principal requirement.

2. P-glycoprotein was expressed and correctly localized in the monocultured BBMECs. Culture conditions were found to affect P-glycoprotein expression in the BBMECs. P-glycoprotein expression was significantly higher in the BBMECs cultured on filter inserts. In cellular uptake studies, P-glycoprotein was shown to be functional, but no efflux was detected in the bidirectional transport studies. The failure to detect efflux suggests that the use of monocultured BBMECs may pose a real risk of obtaining false negative results for drug candidates that are potential P-glycoprotein substrates.

3. Lipophilicity, the balance between hydrophilic and lipophilic moieties, hydrophilic interactions and hydrogen bonding capabilities were the molecular descriptors determining the passive drug permeability across the BBMEC model and they were similar in both Caco-2 and MDCKII-MDR1 models, suggesting that there are no clear differences between passive drug permeability across the endothelial and epithelial cell models when one attempts to classify drugs into categories. Thus, either endothelial or epithelial cell monolayers could be used when passive transport of drugs needs to be evaluated *in vitro*.

4. The monocultured BBMEC model was able to predict the *in vivo* brain entry of mainly passively transported drugs as assessed by the *in vivo* unbound brain/blood ratio. However, no clear differences in the *in vitro-in vivo* correlations between BBMEC, Caco-2 and MDCKII-MDR1 models were found, indicating that the predictive value of endothelial and epithelial cell models is similar when it is mainly passive drug permeability that is being assessed and drugs are being classified into categories.

Despite much progress made during several decades in blood-brain barrier research, there is still no easily maintained and standardized cell culture model available. Ideal *in vitro* model should have the following characteristics:

- human brain endothelial cell origin
- stable genome without aneuploidy
- tightness comparable to *in vivo* situation
- functional expression of active transporters equivalent to *in vivo* situation
- correct cellular localization of the active transporters
- metabolic properties similar as *in vivo* blood-brain barrier
- realistic cellular and tridimensional blood-brain barrier architecture
- relevant *in vitro-in vivo* correlations for each transport mechanisms (*e.g.*, passive paracellular and transcellular diffusion, active efflux transport, active carrier mediated transport, endocytosis)
- easy to maintain and use, non-laborious, suitable for high-throughput screening

Despite recent developments, the currently available models are still far from ideal. However, recently a new approach in the development of *in vitro* models, *i.e.*, stem cell research, was introduced by developing endothelial cells which represent human blood-brain barrier cells derived from pluripotent stem cells (Lippmann *et al.*, 2012). The stem cell derived endothelial cells expressed the relevant tight junction and transporter proteins. In addition, the tightness of this blood-brain barrier model was clearly improved in comparison to other human brain endothelial cell-based models. Hence, this new sophisticated *in vitro* model might be the key to the development of next generation *in vitro* models for blood-brain barrier.

The number of people suffering from neurodegenerative disorders will grow substantially in the future, and they are becoming one of the leading causes of death (for review see Palmer, 2011). Therefore, there is a clear need to develop new drugs for the treatment of CNS disorders. One stumbling block is that the *in vitro* models especially those mimicking diseased blood-brain barrier do not exist at all. The lack of suitable models could well have been hampered the development of drugs for neurodegenerative disorders. However, the development of *in vitro* models for diseased blood-brain barrier will remain fraught with difficulties, as long as the pathogenesis, mechanisms and effects on blood-brain barrier dysfunctions of the neurodegenerative diseases are poorly understood.

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In Vitro Cell Models in Predicting Blood-Brain Barrier Permeability of Drugs

In the early phase of drug development, cell based *in vitro* models are often used to predict the blood-brain barrier permeability of new drug candidates. In order to make reliable predictions based on *in vitro* data, it is important to fully characterize the *in vitro* models and the *in vitro* drug permeability data needs to be correlated against an *in vivo* counterpart. In this thesis, the relevance of the bovine brain endothelial cell based *in vitro* blood-brain barrier model was assessed for use in drug permeability studies.

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