Evaluation of the hCMEC/D3 Cell Line, a New *In Vitro* Model of the Human Blood-Brain Barrier for Transport and Gene Regulation Studies

Inauguraldissertation

zur
Erlangung der Würde eines Doktors der Philosophie
vorgelegt der
Philosophisch-Naturwissenschaftlichen Fakultät
der Universität Basel

von

Birk Poller
aus Deutschland

Basel, 2009

Genehmigt von der Philosophisch-Naturwissenschaftlichen Fakultät

auf Antrag von

Prof. Dr. Jürgen Drewe

Prof. Dr. Jörg Huwyler

Basel, den 9. Dezember 2008

Prof. Dr. Eberhard Parlow

Dekan

Für Bianca und meine Eltern

Acknowledgements

In erster Linie bedanken möchte ich mich bei meinem Doktorvater Prof. Dr. Jürgen Drewe, in dessen Forschungsgruppe ich die Dissertation absolvieren durfte. Mit seinem grossen Optimismus sorgte er für ein gutes Arbeitsklima und Motivation bei seinen Mitarbeitern. Er war in all den drei Jahren ein hervorragender Chef, der immer hinter mir Stand.

Grosser Dank gilt Prof. Dr. Jörg Huwyler, der dieses Kooperationsprojektprojekt in die Wege geleitet hatte. Neben seiner fachlichen Kompetenz verstand er es hervorragend alle Kooperationspartner auf dem Laufenden zu halten und hatten bei auftauchenden Fragen oder Schwierigkeiten stets ein offenes Ohr.

Einen grossen Anteil am Gelingen dieses Projektes hatte unsere Laborleiterin Dr. Heike Gutmann. Ihre guten Ideen und ihr breites wissenschaftliches Wissen, halfen mir in vielen Situationen weiter.

Herzlicher Dank gilt auch Dr. Philippe Coassolo von F. Hoffmann-La Roche, dessen grosszügige finanzielle Unterstützung diese Dissertation ermöglichte. Bei zahlreichen Treffen konnte ich mit Ihm und seinen Mitarbeitern, insbesondere Dr. Cristoph Funk, Dr. Evelyn Hollnack-Pusch und Agnes Poirier, meine Resultate diskutieren und erhielt wichtige Anregungen und Informationen.

Bedanken möchte ich mich ebenfalls bei unserem Kooperationsparter in Paris, Prof. Dr. Pierre-Olivier Couraud, der uns die D3 Zelllinie zur Verfügung stellte. Während Besuchen seines Institutes und bei Telefonkonferenzen konnte ich grundlegende Techniken lernen, sowie aktuelle Ergebnisse austauschen.

Ursula Behrens, Dr. Angelika Maier und Dr. Felix Hammann waren während der längsten Zeit der Dissertation meine Kollegen im Labor 411. Ihnen danke ich ganz besonders für die gute Zusammenarbeit und die grosse Hilfsbereitschaft. Nicht zu vergessen sind die ehemaligen Doktoranden Dr. Manisha Kusch-Poddar, Dr. Petr Hruz, Dr. Philipp Schlatter sowie Dr. Christian Zimmermann, die die mir jederzeit mit Rat und Tat zur Seite standen. Sie alle sind dafür verantwortlich sind, dass ich die Zeiten im Labor, sowie bei unzähligen Mittags- und Kaffeepausen, in bester Erinnerung behalten werde.

Eine grosse Unterstützung erhielt ich für einige Monate durch die Master-Studentin Anne-Sophie Benischke, die sich mit grossem Engagement und Fleiss dem Projekt widmete und deren Resultate in die Dissertation mit eingeflossen sind.

Der Senglet-Stiftung und ihrem Präsidenten Dr. Dieter Tschan danke ich für die finanzielle Unterstützung.

Für die Übernahme des Prüfungsvorsitzes danke ich Herrn Prof. Dr. Peter C. Hauser.

Mein besonderer Dank gilt meinen Eltern. Sie unterstützten mich während meiner gesamten Ausbildung in allen Belangen und ermöglichten mir meine Pläne umzusetzen.

Meiner Partnerin Bianca danke ich für Ihre Unterstützung, und dass ich mit ihr in der Freizeit oder in gemeinsamen Ferien neue Energie tanken konnte.

Abbreviations

ATP	adenosine-5’-triphosphate
ABC	ATP binding cassette
ANOVA	analysis of variance
BBB	blood-brain barrier
BCEC	brain capillary endothelial cells
BCRP	breast cancer resistance protein
bFGF	basic fibroblast growth factor
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
cDNA	complementary DNA
cGMP	guanosine monophosphate
COX	cyclooxygenase
CNS	central nervous system
DCFH	2,7-dichlorofluorescin
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuklease
dNTP	deoxyribonucleotide
EGF	epidermal growth factor
FCS	fetal calf serum
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GDNF	glial-cell derived neurotrophic factor
GLP-1	glucagon-like peptide-1
GSH	glutathione
HBSS	Hank's balanced salt solution
hTERT	humane telomerase reverse transcriptase
hTfR	human transferrin receptor
HS	human AB serum
IFN-β1b	interferon-beta 1b
IGF	insulin-like growth factor
IL-1β	interleukin-1β
IL-6	interleukin-6
JAM	junctional adhesion molecules
mAB	murine antibody
MDR	multidrug resistance

MMP.....matrix metalloproteinase
MRP.....multidrug resistance protein
MX.....mitoxantrone
NO.....nitric oxide
NOS.....NO synthase
OCTN.....organic cation/carnitine transporter
OAT.....organic anion transporter
OATP.....organic anion transporting polypeptide
PBS.....phosphate buffered saline
PCR.....polymerase chain reaction
P_e.....permeability coefficient
PYY3-36.....peptide YY3-36
R123.....rhodamine 123
RNA.....ribonucleic acid
RT-PCR.....reverse transcitption PCR
SEM.....standard error of the mean
SLC.....solute carrier
SNP.....sodium nitroprusside
NAC.....N-acetylcystein
SV40.....simian vacuolating virus 40
TEER.....transendothelial electrical resistance
TJ.....tight junction
TNF.....tumor necrosis factor
TGF.....transforming growth factor
VEGF.....vascular endothelial growth factor
ZO.....zonula occludens

Table of contents

1 Summary

Brain endothelial capillary cells form the blood-brain barrier (BBB), a highly selective membrane between the peripheral blood and the central nervous system. The main functions of the BBB are to protect the brain tissue by preventing the entry of toxic compounds and to supply it with nutrients in order to assure proper function. Tight junctions are the key elements for the establishment of a tight barrier and seal the intercellular gaps against passive diffusion of hydrophilic compounds. A second important characteristic of the brain capillary endothelial cells are transport proteins that prevent brain penetration of their substrates by pumping them back in the blood. These compounds include a series of clinically used drugs. Important drug efflux transporters located at the BBB are P-glycoprotein (P-gp), the breast cancer resistance protein (BCRP) and the family of multidrug resistance proteins (MRP).

During drug development, the question of whether a drug candidate reaches the brain tissue is of great importance. Therefore, models are needed to predict the BBB permeability of new compounds. In the past, *in vitro* models have been developed to address this question. These models include isolated brain capillaries, isolated primary brain capillary endothelial cells and BBB cell lines of various origins. A major problem encountered with these cell lines was an insufficient paracellular resistance.

Recently, the hCMEC/D3 cell line was generated by immortalizing primary human brain endothelial cells. In culture this cell line shows a morphology that closely resembles to primary cells, forms tight monolayers and expresses BBB markers such as chemokine receptors, tight junctional molecules and ATP binding cassette (ABC)-transporters.

The aim of this thesis was to evaluate the hCMEC/D3 cell line as an *in vitro* model of the human BBB to study 1) permeability properties including para- and transcellular diffusion as well as active transport 2) the influence of endo- and exogenous factors on the paracellular permeability and 3) the regulation of breast cancer resistance protein and P-glycoprotein by pro-inflammatory cytokines.

The first study describes the characterization of the hCMEC/D3 cells as an *in vitro* model of the human BBB for permeability studies (section 5). The ability of the cells to allow discrimination between para- and transcellular diffusion was investigated by measuring the transport of a series of compounds with different physicochemical properties. A ratio of 2.8 was observed when comparing the permeabilities of the compounds with the highest and the lowest diffusion rate. The passive permeability of sucrose could be

reduced significantly by replacing fetal calf serum with human serum. Furthermore, quantitative mRNA expression of the ABC-transporters P-gp, BCRP, MRP1, MRP2, MRP3, MRP4, MRP5 as well as the human transferrin receptor (hTfR) was shown. Protein expression of P-gp, BCRP and the hTfR was detected and functional activity of P-gp, BCRP and the MRPs was investigated in efflux experiments. Furthermore, bidirectional P-gp transport activity was observed.

In a second project the impact of endo- and exogenous factors on the paracellular permeability of hCMEC/D3 monolayers was assessed, since it is know that the molecular assembly of tight junctions depends on the surrounding milieu (section 6). Based on reports in the literature, the cells were incubated with a variety of compounds that included anti-inflammatory drugs, growth factors and antioxidants. The effects on the monolayer tightness of hCMEC/D3 were investigated by measuring the transport of sucrose, a paracellular permeability marker. N-acetylcystein (NAC), atorvastatin and sodium nitroprusside (SNP) reduced the sucrose permeability significantly, and slightly increased zonula occludens protein (ZO-1) expression. Additionally, NAC and SNP reduced the generation of reactive oxygen species (ROS), which have been reported to disrupt the assembly of tight junctions.

The effect of the pro-inflammatory cytokines IL-1β, IL-6 and TNF-α on the expression and activity of the ABC-transporters BCRP and P-gp was investigated in the hCMEC/D3 cell line (section 7). IL-1β, IL-6 and TNF-α, which are know to be elevated during various diseases, suppressed significantly BCRP mRNA expression. In addition, BCRP activity was reduced under the influence of all tested cytokines, as shown by efflux experiments. P-gp mRNA levels were slightly reduced by IL-6 but significantly increased after TNF-α treatment. TNF-α also increased the protein expression of P-gp. This *in vitro* study indicates that expression levels of BCRP and P-gp at the BBB might be altered during acute or chronic inflammation, resulting in a changed brain penetration of their substrates.

In an isolated project, the pharmacokinetics and pharmacodynamics of increasing oral doses of the satiety peptides GLP-1 and PYY3-36 were assessed in healthy male volunteers. Oral administration of either peptide induced a rapid and dose-dependent increase in plasma drug concentrations. Oral administration of GLP-1 induced a potent effect on insulin release and both peptides suppressed ghrelin secretion. In conclusion, this study showed, for the first time, that satiety peptides such as GLP-1 and PYY3-36 can be orally delivered safely and effectively in humans.

2 Aim of the thesis

The main goal of the present thesis was a further characterization and improvement of a new *in vitro* model of the human blood-brain barrier. The blood-brain barrier is a selective barrier between the blood and the central nervous system limiting the brain penetration of xenobiotics as well as a series of clinically used drugs. While tight junctions prevent hydrophilic compounds from passively diffusing the expression of drug transporters enables active efflux of their substrates. During drug development it is of great importance to have an *in vitro* tool that allows prediction of the brain penetration of drug candidates. The recently developed human cell line hCMEC/D3, obtained from isolated human brain capillary endothelial cells is a promising model. Compared to primary cells, a blood-brain barrier cell line offers the advantage of a higher experimental throughput. However an extensive evaluation is needed regarding important blood-brain characteristics, such as the formation of a tight monolayer and the functional expression of drug efflux transporters. Our studies were focused on permeability properties including para- and transcellular diffusion as well as on the expression, functionality and regulation of ABC-transporters. Therefore this thesis is divided into the following sections:

- Characterization of the hCMEC/D3 cells with respect to passive and active transport.
- Improvement of the model towards lower paracellular permeability under influence of endo- and exogenous factors.
- Regulation of breast cancer resistance protein and P-glycoprotein by pro-inflammatory cytokines.

In an isolated project the oral pharmacokinetics and pharmacodynamics of the satiety hormones glucagon-like peptide-1 (GLP-1) and peptide YY3-36 (PYY3-36) using a novel application form was investigated.

3 Introduction

3.1 History of the blood-brain barrier

The discovery of the blood-brain barrier (BBB) dates to the end of the 19th century. Paul Ehrlich was the first to describe a barrier between the blood and the central nervous system (CNS). After injecting a dye into the systemic circulation of rats, he observed that the brain tissue and the spinal cord remained unstained (Ehrlich 1885). Ehrlich himself did not believe in the theory of a barrier and suggested an insufficient affinity of the dye to the CNS. Some years later his student Edwin Goldman demonstrated the existence of a barrier by staining the CNS with the same dyes injected into the subarachnoid space (Goldmann 1909). Lewandowski was the first to coin the term the “blood-brain barrier” (Lewandowski 1900). In 1967, Reese and Karnovsky revealed the basic structural characteristics of the BBB by electron microscopy. They showed that the capillary endothelial cells are responsible for the tightness of the barrier by forming intercellular tight junctions (TJ) (Reese and Karnovsky 1967). After Betz et al. had established the first *in vitro* system of the BBB using isolated brain capillary endothelial cells (BCEC) (Betz et al. 1980) many *in vivo* and *in vitro* models of the BBB were established.

3.2 Physiological functions of the BBB

The two main functions of the BBB are the protection of the brain from blood-bourne toxic compounds and the maintenance of CNS homeostasis. The BBB prevents almost all large hydrophilic compounds from entering the brain, whereas many lipophilic compounds and gases can freely diffuse across the membrane. Furthermore, BCEC express drug transporters which limit the brain penetration of their substrates (mainly lipophilic drugs and xenobiotics) by transporting them back into the peripheral blood (Banks 1999). Despite these protective functions, the BBB supplies the brain with the necessary nutrients, such as glucose and amino acids, by active transport processes. In addition, the BBB regulates the osmotic pressure in the CNS in order to ensure correct function (Drewes 2001).

3.3 Anatomy of brain capillaries

Brain capillaries are the smallest vessels of the human circulation (3-7 µm diameter) with a total length of 600-650 km and a surface area of 20 m^2 (Pardridge 2003). Different cell types, including endothelial cells, astrocytes pericytes and neurons build up these

capillaries. The cellular complex is named the “neurovascular unit” and regulates the cerebral bloodflow by interacting with smooth-muscle cells (Abbott et al. 2006).

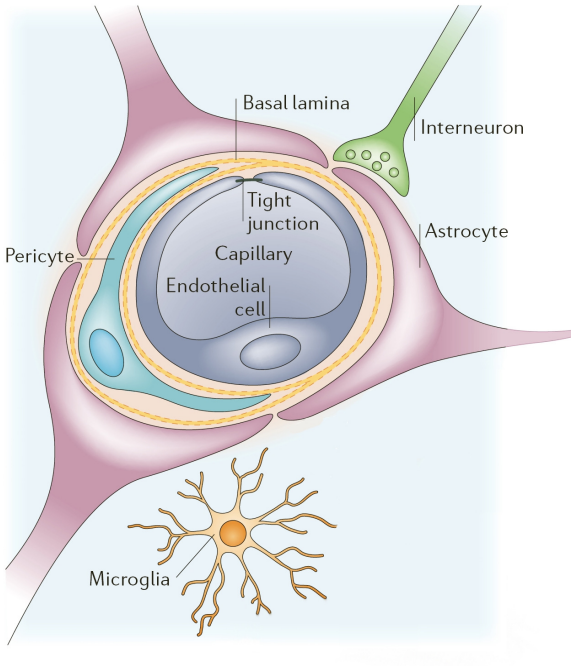

Figure 1 Cellular constituents of the blood–brain barrier. The barrier is formed by capillary endothelial cells, surrounded by basal lamina and astrocytic perivascular endfeet, pericytes and microglia (adapted from (Abbott et al. 2006)).

In comparison to peripheral endothelial cells they are characterized by a high mitochondrial content (Oldendorf et al. 1977), their lack of fenestration (Fenstermacher et al. 1988) and a minimal pinocytotic activity (Sedlakova et al. 1999). Furthermore, BCEC express TJ that seal the paracellular passage of hydrophilic compounds. The endothelial cells grow on a basal lamina, a membrane with a complex structure of collagen and proteins such as laminin, fibronectin and other extracellular-matrix proteins (Goldstein and Betz 1983). The endothelial cells share the basal lamina with surrounding pericytes, neurons and endfeet of the astrocytes.

Since pericytes and astrocytes are in close contact with the endothelial cells, it was assumed that they could induce specific features of the BCECs. In different *in vitro* models it was demonstrated that astrocytes improve the barrier function and alter the expression of drug transporters such as P-glycoprotein (P-gp) or the glucose transporter (GLUT1) by releasing soluble factors, which interact with the endothelial cells (Schinkel 1999; McAllister et al. 2001). An inducing effect towards more BBB-like properties was

shown for transforming growth factor (TGF), glial-derived neurotrophic factor (GDNF) and basic fibroblast growth factor (bFGF). As well as astrocytes, pericytes, which wrap around the endothelial cells, have more recently been shown to be responsible for the maturation of an intact BBB (reviewed in (Abbott et al. 2006)). It was suggested that pericytes stabilize the structure of endothelial brain capillaries by suppressing apoptosis of the endothelium (Ramsauer et al. 2002)

3.4 Tight junctions

The key structures of the BBB are the TJ. As seen by freeze-fracture images, TJ are assembled by a complex of intramembranous strands, which tighten the gaps between the cells (Nagy et al. 1984). The TJ consist of three integral membrane proteins (occludin, claudin and junctional adhesion molecules (JAM)), which are linked to different cytoplasmic accessory proteins, including the zonula occludens (ZO) proteins ZO-1, ZO-2, ZO-3 and cingulin. TJ are known to prevent the paracellular passage of small molecules and even ions such as Na^+ and Cl^-. This property leads to high transendothelial electrical resistances (TEER) of 1,500-2,000 Ω cm^2 *in vivo* (Butt et al. 1990).

The first integral membrane protein to be discovered was the 65 kDa occludin (Furuse et al. 1993). The structure of occludin consists of four transmembrane domains, a long cytoplasmic COOH terminus and a short cytoplasmic NH_2 terminus. Occludin is directly linked to the ZO proteins by its cytoplasmic domains (Hirase et al. 1997). Studies indicate that occludin does not directly exert paracellular tightness but regulates the permeability of TJ depending on its phosphorylation status (reviewed in (Wolburg and Lippoldt 2002)).

So far, 24 members of the claudin family are known (Ballabh et al. 2004). These 22 kDa phosphoproteins have four transmembrane domains and bind with their COOH terminus to cytoplasmic proteins such as ZO-1, ZO-2 and ZO-3 (Furuse et al. 1998). By binding homotypically to each other, claudins are assumed to seal the TJ (Furuse et al. 1999). At the BBB claudin-3 (formerly classified as claudin-1) and claudin-5 appear to be responsible for the low paracellular permeability (Morita et al. 1999; Liebner et al. 2000; Liebner et al. 2000; Lippoldt et al. 2000).

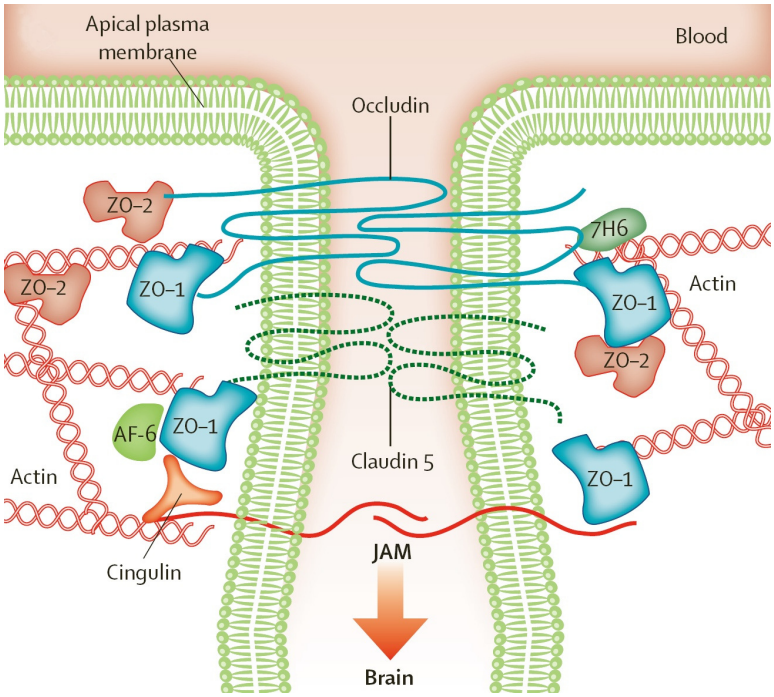

Figure 2 Molecular organization of a tight junction (adapted from (Neuwelt et al. 2008))

The JAM are 40 kDa proteins having a single transmembrane domain and an extracellular domain with two immunoglobulin-like loops formed by disulfide bonds (Martin-Padura et al. 1998). In brain endothelial cells JAM-1, JAM-2 and JAM-3 are present being involved in the maintenance of the TJ (Palmeri et al. 2000; Aurrand-Lions et al. 2001).

The integral membrane proteins are anchored to cytoplasmic accessory proteins, mainly ZO-1, ZO-2 and ZO-3. The ZO molecules and cingulin are membrane-associated guanylate kinase-like protein (MAGUKs). They share defined core regions (a SH_3, a guanylate cyclase and a PDZ domain), which enables them to be involved in signal transduction and anchoring the transmembrane TJ proteins to the cytoskeleton (Furuse et al. 1994; Itoh et al. 1999; Ebnet et al. 2000).

3.5 Transport at the blood-brain barrier

The expression of transport proteins is a major characteristic of the BCEC forming the BBB. Since the BBB is impermeable for nutrients, which are essential for the brain function, several transport systems are present in BCEC, including GLUT1, several amino acid transporters, nucleoside and monocarboxylic acid transporters (Drewes 2001).

Besides uptake transporters different families of drug efflux transport proteins are expressed in BCEC. These transporters limit their substrates including drugs, toxic metabolites or dietary toxins from brain penetration by an active efflux process (de Boer et al. 2003). The following sections are focused on the efflux transporters since they are an important characteristic of the BBB.

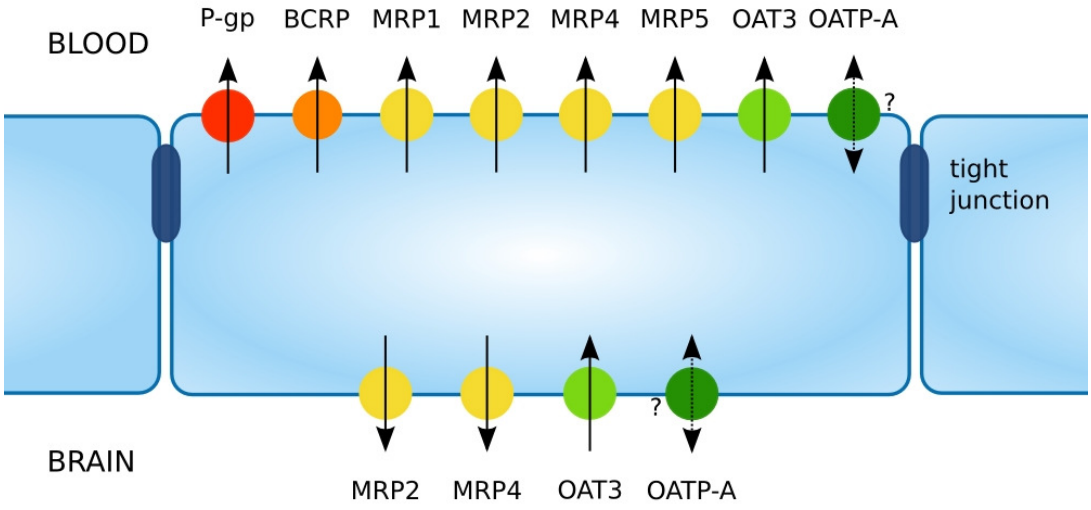

Figure 3 Schematic diagram of drug transporter expression in human brain endothelial capillary cells. The membrane localization of OATP-A is unclear.

3.5.1 ABC-transporter

ABC-transporters are one of the largest of all protein families, comprising 48 genes that are classified into seven subfamilies according to their gene structure and amino acid alignment (Dean et al. 2001). They are integral membrane proteins that use the energy of ATP hydrolysis for translocation of their substrates across membranes against high concentration gradients. The main function of ABC-transporters is the protection of the body from natural toxins or xenobiotics. Therefore, they are expressed at sites with excretory functions (liver and kidney) and at barriers that limit entry of toxins (BBB, blood-testis barrier, gastro-intestinal tract) (Schinkel and Jonker 2003; Loscher and Potschka 2005).

P-glycoprotein

P-glycoprotein (ABCB1, P-gp) is a 170 kDa phosphorylated glycoprotein with two homologous halves including 12 transmembrane domains and two ATP binding sites in total (Jones and George 2000). There exist two types of P-gp, coded by the MDR1 and

MDR2 genes. At the human BBB only P-gp encoded by MDR1 is present (Demeule et al. 2002).

P-glycoprotein was initially described in the field of cancer mediating multidrug resistance (MDR) by ATP-driven efflux of chemotherapeutics (Biedler and Riehm 1970). P-gp has a broad substrate specificity including anticancer drugs, immunosuppressive agents, corticoids, opioids, HIV protease inhibitors, antipsychotic drugs, antiepileptic drugs, etc (reviewed in (Loscher and Potschka 2005). It was also the first transporter to be discovered at the human BBB (Cordon-Cardo et al. 1989; Thiebaut et al. 1989). By confocal and electron microscopy several studies show an expression of P-gp at the luminal membrane of the BBB of various species (Jette et al. 1993; Demeule et al. 2002). In mdr1(-/-) knock-out mice 10- to 100-fold elevated brain levels of P-gp substrates such as ivermectin or vinblastine were found, demonstrating the impact of P-gp *in vivo* (Schinkel and Jonker 2003). Clinical drug-to-drug interactions mediated by P-gp at the BBB in humans were described for the P-gp substrates cyclosporine A and verapamil. Verapamil brain levels were significantly increased in the presence of cyclosporine A (Sasongko et al. 2005).

BCRP

The breast cancer resistance protein (ABCG2, BCRP) is a 72 kDa half-transporter with 6 transmembrane segments that forms functional homodimers. BCRP was first discovered in a therapy-resistant breast cancer cell line (Doyle et al. 1998). Only recently was it discovered to be expressed at the apical membrane in endothelial cells of the BBB of pigs and humans (Cooray et al. 2002; Eisenblatter and Galla 2002; Lee et al. 2005). In addition, BCRP is expressed in the placenta, colon and small intestine (Loscher and Potschka 2005). BCRP substrates include anticancer drugs such as mitoxantrone, daunorubicin, doxorubicin, topotecan and epirubicin, carcinogens and dietary toxins (van Herwaarden et al. 2003; Han and Zhang 2004). The role of BCRP *in vivo* is controversial. In mdr1 knock-out mice the brain uptake of mitoxantrone was increased in the presence of the P-gp and BCRP inhibitor elacridar, giving first functional evidence of BCRP mediated transport at the BBB (Cisternino et al. 2004). However, a different study reports no altered mitoxantrone brain levels in Bcrp knock-out mice (Lee et al. 2005).

MRPs

The proteins of the multidrug resistance protein (MRP, ABCC) family also belong to the superfamily of ABC-transporters. 12 MRPs are known to be members of the ABCC family. They are involved in the transport of anionic to neutral drugs such as anticancer drugs, anti-HIV drug and glutathione, glucuronide and sulphate conjugates. Although P-

gp substrates are generally cationic molecules there is a significant overlap of substrate specificity with MRPs (Borst et al. 2000; Loscher and Potschka 2005).

In bovine BCECs mRNA of MRP1, MRP4, MRP5 and MRP6 are present as well as low levels of MRP2 (Zhang et al. 2000; Berezowski et al. 2004). A different study also revealed the expression of MRP2 at the luminal side of brain capillaries of rat and porcien origin (Miller et al. 2000). However, only limited data is available on the expression of the MRPs at the human BBB. High mRNA levels of MRP1 compared to MRP2, MRP3 and MRP5 are reported (Dombrowski et al. 2001). A different study revealed MRP1, MRP4 and MRP5 expression at the protein level, whereas MRP2, MRP3 and MRP6 were not detectable (Nies et al. 2004). In bovine and human BCEC, MRP1 and MRP5 are expressed at the apical membrane, whereas MRP4 is equally expressed at both membranes (Zhang et al. 2000; Nies et al. 2004).

So far little is known about the impact of the MRPs *in vivo*. MRP1 knock-out mice showed no altered brain penetration of etoposide (Cisternino et al. 2003). However, the extrusion rate of an endogenous estradiol-glucuronide was reduced after intracerebral injection indicating MRP1 being involved in the excretion of metabolites from the brain, rather than in drug efflux from blood-derived compounds (Cisternino et al. 2003; Sugiyama et al. 2003). In rats lacking mrp2 phenytoin brain levels were increased, indicating that mrp2 prevents phenytoin from penetrating the brain (Potschka et al. 2003).

3.5.2 Solute carriers

The superfamily of solute carriers (SLC) contains a multitude of genes that are divided into 43 families coding transport proteins (Hediger et al. 2004). These proteins act passively as ion-coupled transporters, facilitated transporters or exchangers. At the BBB transporters of the SLCO/SLC21A and SLC22 family are involved in efflux processes.

Brain capillaries of the rat express the organic anion transporting polypeptides (Oatp) Oatp1a4 (Slco1a4), Oatp1a5 (Slco1a5) and Oatp1c1 (Slc1c1), at the luminal and abluminal membrane, where they are involved in the transport of amphiphatic organic anions such as bile acids and steroid conjugates (Kusuhara and Sugiyama 2005). Only one human isoform, OATP1A2 (SLCO1A2), with a high homology to the rat isoforms is known and expressed at the human BBB. However, its membrane localization as well as its impact *in vivo* remains unclear (Gao et al. 2000).

Members of the SLC22 family that are known to be expressed at the human BBB are the organic anion transporter (OAT3, SLC22A8) and the organic cation/carnitine transporter

(OCTN2, SLC22A3). OAT3 is expressed at the basolateral membrane of the rat BBB whereas its membrane localization at the human BBB remains unclear (Mori et al. 2004). Substrates of OAT3 include para-aminohippuric acid, benzylpenicillin and homovanillic acid (Kusuhara and Sugiyama 2005). OCTN2 is a sodium dependent carnitine transporter, which is expressed amongst others at the BBB of different species (Miecz et al. 2008). Since mice lacking this transporter show reduced brain concentrations of acetyl-carnitine a luminal localization is suggested (Kusuhara and Sugiyama 2005).

3.5.3 Receptor-mediated transcytosis

Compounds of high molecular weight, such as peptides of high molecular weight, are too large for transport by carrier proteins. Such molecules are translocated across the BBB by receptor-mediated transcytosis. Therefore, BCEC express receptors for ligands such as transferrin or insulin. The receptors are internalized by clathrin coated pits, forming early endosomes that are transported to the lysosomes, where their content can be released. The receptors are shuttled back to the plasma membrane by membrane vesicles (de Boer et al. 2003).

An important receptor at the BBB is the transferrin receptor (Jefferies et al. 1984). It is involved in the endo- and exocytosis of iron loaded transferrin. After binding of Fe^{2+}-transferrin to the TfR, endocytosis via clathrin-coated pits occurs (Li and Qian 2002). In order to deliver drugs that cannot cross the BBB to brain tissue, the transferrin system was used for targeting experiments. Liposomes loaded with daunomycin were coupled to antibodies directed against the TfR (Huwyler et al. 1996). After injecting these immunoliposomes into rats, significantly increased daunomycin brain levels were measured, compared to the free drug (Schnyder et al. 2005).

3.6 Models of the blood-brain barrier

During the process of drug development the question of whether new drug candidates cross the BBB is of great importance. CNS active compounds have to pass the BBB in order to reach their targets, whereas brain penetration might not be desirable for peripherally acting drugs that cause CNS-related adverse reactions. Therefore, there is a need for models showing the main BBB characteristics such as low paracellular permeability, expression and functional activity of transport systems in order to allow a precise and reliable prediction of drug permeability. During the past years many different *in vitro* models have been developed.

3.6.1 Isolated brain capillaries

Isolated brain capillaries of bovine origin were described as the first *in vitro* model of the BBB (Siakotos and Rouser 1969). Since then, many models have been reported using fragments of brain capillaries of different origins such as pigs, rat, mice or humans. These models were successfully used for drug accumulation or binding studies as well as for assessments of transporter activity and gene expression (Pardridge et al. 1985; Gutmann et al. 1999; Torok et al. 2003; Banks et al. 2005; Hartz et al. 2006). However, permeability studies are difficult to perform, as drugs cannot be applied to the apical side of the endothelial cells. Therefore, *in vitro* models using cultures of isolated primary BCEC were developed.

3.6.2 Isolated primary BCEC

By an isolation process endothelial BCEC are obtained, which can be seeded on permeable membranes, where the cells form a polarized monolayer (Cecchelli et al. 1999). This setup allows bidirectional permeability measurements. Depending on the origin of the cells, the culturing conditions and the measuring setup high electrical resistances can be observed, which indicate low ion fluxes. Transport of paracellular marker compounds such as inulin or sucrose gives more information about the tightness of the monolayer against small molecules. In addition, the functional activity of uptake and efflux transporters can be determined. By isolating cells of porcine and bovine origin, high yields of endothelial cells can be obtained, which meet the requirements for permeability studies (Bowman et al. 1983; Audus and Borchardt 1986; Franke et al. 1999; Gutmann et al. 2002). In addition, models of rat (Perriere et al. 2005) and human (Megard et al. 2002) origin have been published. Some groups developed co-culture systems using isolated astrocytes growing in the same well as BCEC. These studies demonstrated that astrocytes can induce the barrier properties of the BCECs (Cecchelli et al. 1999; Megard et al. 2002). The quantitative expression of drug transporters in isolated BCEC is a subject much debated. It has been shown that MRP1 is highly up-regulated compared to isolated capillaries in rat and porcine BCEC (Gutmann et al. 1999; Regina et al. 1999), whereas P-gp expression is reduced in cultured BCEC (Regina et al. 1999). A limitation of primary cell culture models is the laborious and time-consuming isolation process that does not allow high throughput studies. In addition, ethical considerations limit the availability of cells of human origin, and the problems of potential inter-species differences arise if experiments are performed using cells of non-human origin.

3.6.3 BBB cell lines

In order to obtain *in vitro* BBB models, which consume fewer resources and are easier to handle, cell lines of different origins were generated. These cell lines were obtained by transfection of primary BCEC with immortalizing genes, such as the SV40 T antigen, enabling cell division without degeneration of the phenotype for a certain number of passages. Among the cell lines used mostly are the rat cell lines RBE4 (Roux et al. 1994) and GPNT (Regina et al. 1999), the MBEC (Tatsuta et al. 1992) and the commercially available b.End3 (Omidi et al. 2003) of murine origin and the human cell lines NKIM-6 (Ketabi-Kiyanvash et al. 2007) and BB19 (Prudhomme et al. 1996; Kusch-Poddar et al. 2005). In contrast to cultures of primary BCEC, cell lines generally fail to form tight monolayers with sufficiently low paracellular tightness. Recently, the human brain capillary endothelial cell line hCMEC/D3 has been developed (Weksler et al. 2005). The hCMEC/D3 cells show a stable phenotype, the expression of endothelial cell markers, TJ molecules, chemokine receptors and ABC-transporters. Furthermore, the paracellular permeability is much lower compared to other cell lines. These characteristics make the hCMEC/D3 an interesting tool for permeability studies.

4 General Methods

4.1 Cell culture

Immortalized human brain capillary endothelial cells (the hCMEC/D3 cell line) were obtained under license from INSERM, France. The original brain endothelial cells of passage 0 used for the generation of the cell line were isolated from human brain tissue following surgical excision of an area from the temporal lobe of an adult female with epilepsy. The hCMEC/D3 cell line had been immortalized by lentiviral transduction of the catalytic subunit of human telomerase and SV40-T antigen into very early cultures of adult human brain endothelial microvascular cells (Weksler et al. 2005). The hCMEC/D3 cells used for the experiments were between passage 25 and 35. All culture ware was coated with rat-tail collagen type I solution at a concentration of 0.1 mg/ml and was incubated for 1 h at 37°C. For culturing, the cells were seeded in a concentration of 27000 cells/cm^2 and grown in EBM-2 medium supplemented with VEGF, IGF-1, EGF, basic FGF (bFGF), hydrocortisone, ascorbate, penicillin-streptomycin and 2.5% FCS, as recommended by the manufacturer. For functional assays the cells were grown in a growth-factor-depleted medium (EBM-2 supplemented with bFGF 1 ng/ml, 2.5% FCS, 0.55 µM hydrocortisone, 10 mM HEPES, and penicillin-streptomycin), in the following referred to as the assay medium. Cells were cultured in an incubator at 37°C with 5% CO_2, 95% fresh air and saturated humidity. Cell culture medium was changed every 2 to 3 days.

4.2 Quantitative RT-PCR TaqMan assay

Total RNA was isolated from confluent monolayers using the RNeasy Mini Kit. Total RNA was quantified with a NanoDrop-1000 photometer (Witec, Littau, Switzerland). The purity of the RNA preparations was high, as demonstrated by the 260 / 280 nm ratio (range 1.8-2.2). After DNase I digestion, 1 µg of total RNA was reverse transcribed by SuperScript II RT-Kit, according to the manufacturer's protocol, using random hexamers as primers.

TaqMan analysis was carried out on a 7900HT Sequence Detection System (Applied Biosystems). Polymerase chain reaction (PCR) conditions were 10 min at 95°C followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. Each TaqMan reaction contained 10 ng of cDNA in a total volume of 10 µl. qPCR Mastermix Plus from Eurogentec (Seraing, Belgium) was used. Primers and probes were used at concentrations of 900 and 225 nM, respectively. Primers were synthesized by Invitrogen (Basel, Switzerland) and

Eurogentec (Seraing, Belgium). For corresponding sequences of primers and probes for TaqMan analysis see Table 1. All samples were run in triplicates and not reverse-transcribed RNA served as a negative control. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression was measured to compare expression of the other genes with the absolute amount of a well-known house-keeping gene.

Gene	Accession No.	Sequence	
BCRP	AY333756	Forward:	5`-CAGGTCTGTTGGTCAATCTCACA-3`
		Reverse:	5`-TCCATATCGTGGAATGCTGAAG-3`
		Probe:	5`-CCATTGCATCTTGGCTGTCATGGCTT-3`
MDR1	M14758	Forward:	5`-CTGTATTGTTTGCCACCACGA-3`
		Reverse:	5`-AGGGTGTCAAATTTATGAGGCAGT-3`
		Probe:	5`-AAGCTGTCAAGGAAGCCAATGCCTATGACTT-3`
MRP1	NM_004996	Forward:	5`-GGGCTGCGGAAAGTCGT-3`
		Reverse:	5`-AGCCCTTGATAGCCACGTG-3`
		Probe:	5`-CCTCCACTTTGTCCATCTCAGCCAAGAG-3`
MRP2	NM_000392	Forward:	5`-ACTGTTGGCTTTGTTCTGTCCA-3`
		Reverse:	5`-CAACAGCCACAATGTTGGTCTCTA-3`
		Probe:	5`-CTCAATATCACACAAACCCTGAACTGGCTG-3`
MRP3	AF085690	Forward:	5`-GGTGGATGCCAACCAGAGAA-3`
		Reverse:	5`-GCAGTTCCCCACGAACTCC-3`
		Probe:	5`-CCAACCGGTGGCTGAGCATCG-3`
MRP4	AF071202 (Taipalensu)	Forward:	5`-AAGTGAACAACCTCCAGTTCCAG-3`
		Reverse:	5`-GGCTCTCCAGAGCACCATCT-3`
		Probe:	5`-CAAACCGAAGACTCTGAGAAGGTACGATTCCT-3`
MRP5	NM_005688	Forward:	5`-CTGCAGTACAGCTTGTTGTTAGTGC-3`
		Reverse:	5`-TCGGTAATTCAATGCCCAAGTC-3`
		Probe:	5`-CTGACGGAAATCGTGCGGTCTTGG-3`
GAPDH	M17851	Forward:	5`-GGTGAAGGTCGGAGTCAACG-3`
		Reverse:	5`-ACCATGTAGTTGAGGTCAATGAAGG-3`
		Probe:	5`-CGCCTGGTCACCAGGGCTGC-3`

Table 1 Sequences of TaqMan Primers and Probes.

For absolute quantification, external standard curves were used. Standards were gene-specific cDNA fragments that cover the TaqMan primer/probe area and they were generated by PCR. For sequences of the corresponding primers, see Table 2. The PCR products were purified by running a 1.5% agarose gel and a subsequent gel extraction (gel extraction kit, Qiagen). The standards were quantified using the PicoGreen reagent (Molecular Probes, Eugene, OR, USA) and were checked by sequencing (Microsynth GmbH, Balgach, Switzerland).

4.3 RT-PCR

As described in section 4.2 total RNA was isolated from D3 cells and reverse transcribed. PCR of the human transferrin receptor (hTfR) was performed with a thermocycler (Biometra, Göttingen, Germany). Each sample was amplified for 35 cycles (94°C for 30 s, 55°C for 30 s, 72°C for 60 s). The reaction mixture contained 2.5 µl of the cDNA template, 0.25 U AmpliTaq gold DNA polymerase, 2.5 µl 10x PCR buffer, $MgCl_2$ at a final concentration of 3 mM, 2 µl of dNTP reaction mixture (2.5 mM each), 7.5 pmol of each primer and water to a final volume of 25 µl. Primers for the hTfR were designed according to Milstone et al. (Milstone et al. 2006) and sequences are listed in Table 2. As negative control, not reverse transcribed RNA was used. The PCR products were separated by gel electrophoresis (1.5% agarose) and visualized by UV light in the presence of ethidium bromide.

Gene	Accession No.	Sequence	
hTfR	NM_003234	Forward:	5'-CTGCTATGGGACTATTGCTGTG-3'
		Reverse:	5'-CCGACAACTTTCTCTTCAGGTC-3'
BCRP	AY333756	Forward:	5'-TTTCAGCCGTGGAACTCTTT-3'
		Reverse:	5'-TGAGTCCTGGGCAGAAGTTT-3'
MDR1	M14758	Forward:	5`-ACAGTCCAGCTGATGCAGAGG-3`
		Reverse:	5`-CCTTATCCAGAGCCACCTGAAC-3`
MRP1	NM_004996	Forward:	5`-CACACTGAATGGCATCACCTTC-3`
		Reverse:	5`-CCTTCTCGCCAATCTCTGTCC-3`
MRP2	NM_000392	Forward:	5`-CCAATCTACTCTCACTTCAGCGAGA-3`
		Reverse:	5`-AGATCCAGCTCAGGTCGGTACC-3`
MRP3	AF085690	Forward:	5`-TCTATGCAGCCACATCACGG-3`
		Reverse:	5`-GTCACCTGCAAGGAGTAGGACAC-3`
MRP4	AF071202 (Taipalensu)	Forward:	5`-AAGTGAACAACCTCCAGTTCCA-3`
		Reverse:	5`-CCGGAGCTTTCAGAATTGAC-3`
MRP5	NM_005688	Forward:	5`-CTGCAGTACAGCTTGTTGTTAGTGC-3`
		Reverse:	5`-TCGGTAATTCAATGCCCAAGTC-3`
GAPDH	M17851	Forward:	5`-ACATCGCTCAGAACACCTATGG-3`
		Reverse:	5`-GCATGGACTGTGGTCATGAGTC-3`

Table 2 Sequences RT-PCR primers.

4.4 Western Blot

The presence of P-gp and BCRP was studied by Western Blot analysis using the murine antibody (mAb) C219 (Alexis) against P-gp and the mAb BXP-21 (Alexis) against BCRP. SDS-polyacrylamide gel electrophoresis was performed with a Mini-Protean II apparatus (Bio-Rad, Zürich, Switzerland). To hCMEC/D3 cell homogenates (0.7-0.9 mg/ml protein, obtained from cells cultivated under standard conditions) the same amount of Lämmli

buffer (Bio-Rad) was added. The samples were loaded onto an acrylamide/bisacrylamide gel (10% for BCRP, 6.5% for P-gp). After electrophoresis, the proteins were transferred electrophoretically (2 h at a constant amperage of 250 mA) to a 0.45 µm pore size nitrocellulose membrane using a Mini Transblot cell (Bio-Rad). The transfer buffer contained 192 mM glycine, 25 mM Tris–HCl, and 20% methanol. The membrane was blocked overnight at 4°C with 5% powdered skimmed milk in PBS containing 0.05% Tween 20 (PBS-T). Washed membranes were incubated with mAb C219 (1 µg/ml) or mAb BXP-21 (1.25 µg/ml) in PBS-T and 1% powdered skimmed milk for 2 h at 37°C. Washed membranes were incubated for 1 h at room temperature with horseradish peroxidase-conjugated rabbit anti-mouse IgG (1:500) in PBS-T containing 1% milk powder. Membranes were washed in PBS-T and P-gp or BCRP and were visualized using enhanced chemiluminescence detection (ECL-kit, Amersham Otelfingen, Switzerland).

4.5 Transport assay

For transport experiments hCMEC/D3 cells were seeded on type I collagen precoated Transwell® filters (polycarbonate 12 well, pore size 3.0 µm or polyester [clear], 12 well, pore size 0.4 µm) in a density of 50,000 cells/cm^2. Assay medium was changed after 4 and 7 days and transport assays were performed 7 to 10 days after seeding. Both sides of the chamber were washed with prewarmed HBSS supplemented with 10 mM HEPES, 1mM Na-Pyruvate, pH 7.4 at 37°C (HBSS-P). At time t=0, 0.3 µCi [^{3}H]-inulin, [^{14}C]-sucrose, [^{3}H]-morphine, [^{3}H]-propranolol, [^{14}C]-midazolam or 20 µM lucifer yellow were applied on the apical donor compartment. For propranolol transport, 10 µM of unlabeled substance were added. After 5, 10, 20, 30, 45 and 60 min the filters were moved to a new acceptor-well containing 1.5 ml prewarmed HBSS-P and samples were taken from the basolateral acceptor compartment of the well from the previous time point. Insta Gel plus scintillation liquid was added to the radioactive samples, and analysis was performed by scintillation counting (Packard TriCarb2000, Canberra Packard S.A.). Detection of lucifer yellow was carried out with a Perkin-Elmer HTS 7000 Bio Assay Reader using the following parameters: excitation at 430 nm, emission 535 nm.

Permeabilities of the test compounds are given as permeability coefficients. They take into account the relation between the permeability of the monolayer and the permeability of empty filters (precoated with rat-tail collagen, without cells). The slopes of the volume cleared vs. time represent the clearance for each condition. The clearance of each filter well is related to the clearance of empty filter wells and was used to calculate the

permeability coefficients (P_e) of the endothelial monolayer according to (Cecchelli et al. 1999):

$$\frac{1}{PS} = \frac{1}{m_e} - \frac{1}{m_f} \qquad \text{and} \qquad P_e = \frac{PS}{A}$$

where PS is the permeability-surface area product, A is the surface area of the filter and m_e and m_f are the volumes cleared vs. time corresponding to endothelial cells on filters and to empty filters, respectively.

For bidirectional transport experiments, cells were cultured as described above, but with 5% human serum instead of 2.5% FCS. After washing the cells with HBSS-P 5 µM rhodamine 123 (R123) and 0.6 µCi/ml [^{14}C]-sucrose were applied on the apical or basolateral compartment. The opposite compartment was filled with HBSS-P. After 10, 20, 40 and 60 min samples were drawn from the opposite compartment and were replaced with the same amount of HBSS-P. Fluorescence detection of R123 was carried out on a Perkin-Elmer HTS 7000 Bio Assay Reader using the following settings: excitation: 485 nM, emission: 535 nM. [^{14}C]-sucrose was analyzed as described above.

4.6 Drug accumulation assay

Drug accumulation assays were performed to test functional activity of P-gp, BCRP and MRPs. hCMEC/D3 cells were seeded on rat collagen type I precoated 24-well plates in a density of 37,000 cells/well. After 4 and 7 days the medium was changed. All assays were performed on a temperature-controlled shaker (THERMOstar, BMG labtech, Offenburg, Germany) at 37°C and 120 rpm. All monolayers were washed 3 times with prewarmed HBSS-P (37°C) and were preincubated for 15 min with corresponding inhibitors dissolved in HBSS-P (100 µM verapamil for P-gp, 50 µM prazosin for BCRP, 10 µM MK-571 for MRPs). The solutions were removed and the cells were incubated with HBSS-P containing the test-compounds (5 µM R123, 0.3 µCi [^{3}H]-digoxin, for P-gp, 0.3 µCi [^{3}H]-methotrexate for MRPs and 20 µM mitoxantrone for BCRP assay) and corresponding inhibitors for 30 min. To reach a final concentration of 5 µM digoxin and 1 µM methotrexate corresponding unlabeled substance was added to the radioactive tracers. After loading the plate was put on ice and the cells were washed twice with ice-cold HBSS-P with or without the test inhibitors. Subsequently HBSS-P alone and with

inhibitors was added to the cells, and the efflux took place for 40 min (mitoxantrone) or 60 min (all other substrates). The reaction was stopped by putting the plate on ice and rinsing 2 times with ice cold HBSS-P. Cells, which were treated with radioactive labeled compounds, were detached using trypsin and were transferred to scintillation vials. After adding Insta Gel plus, the samples were analyzed in a scintillation counter (Packard TriCarb2000). The cells loaded with fluorescent drugs were lysed with 0.8% (v/v) Triton X-100 in PBS for 15 min at 37°C. Samples were taken and fluorescence detection was carried out on a Perkin-Elmer HTS 7000 Bio Assay Reader using the following settings: R123 (excitation: 485 nm, emission: 535 nm), mitoxantrone (excitation: 610 nm, emission: 685 nm).

5 The human brain endothelial cell line hCMEC/D3 as a human blood-brain barrier model for drug transport studies

Birk Poller[1], Heike Gutmann[1], Stephan Krähenbühl[1], Babette Weksler[3], Ignacio Romero[4], Pierre-Olivier Couraud[2], Gerald Tuffin[5], Jürgen Drewe[1], Jörg Huwyler[5]

[1] Dept. of Clinical Pharmacology and Toxicology, University Hospital of Basel, 4031 Basel, Switzerland

[2] Institut Cochin, Centre National de la Recherche Scientifique UMR 8104, Institut National de la Santé et de la Recherche Médicale (INSERM) U567, Université René Descartes, Paris, France

[3] Weill Medical College of Cornell University, New York, NY, 10021, USA

[4] Department of Biological Sciences, The Open University, Walton Hall, Milton Keynes, United Kingdom

[5] University of Applied Sciences Northwestern Switzerland, Dept. of Pharmaceutical Technology, 4132 Basel, Switzerland

Journal of Neurochemistry

2008;**107:**1358-1368.

5.1 Abstract

The human brain endothelial capillary cell line hCMEC/D3 has been developed recently as a model for the human blood-brain barrier. In this study a further characterization of this model was performed with special emphasis on permeability properties and active drug transport. Para- or transcellular permeabilities (P_e) of inulin ($0.74*10^{-3}$ cm/min), sucrose ($1.60*10^{-3}$ cm/min), lucifer yellow ($1.33*10^{-3}$ cm/min), morphine ($5.36*10^{-3}$ cm/min), propranolol ($4.49*10^{-3}$ cm/min) and midazolam ($5.13*10^{-3}$ cm/min) were measured. By addition of human serum the passive permeability of sucrose could be reduced significantly by up to 39%. Furthermore, the expression of a variety of drug transporters (ABCB1, ABCG2, ABCC1-5) as well as the human transferrin receptor was demonstrated on the mRNA level. ABCB1, ABCG2 and transferrin receptor proteins were detected and functional activity of ABCB1, ABCG2 and the ABCC family was quantified in efflux experiments. Furthermore, ABCB1-mediated bidirectional transport of rhodamine 123 was studied. The transport rate from the apical to the basolateral compartment was significantly lower than that in the inverse direction, indicating directed P-glycoprotein transport. The results of this study emphasize the usefulness of the hCMEC/D3 cell line as an *in vitro* model to study drug transport at the level of the human blood-brain barrier.

5.2 Introduction

The ability or inability of a drug to penetrate into the brain is a key consideration in drug design. Drugs for treating central nervous system (CNS) disorders need to penetrate the blood-brain barrier (BBB). Conversely, non-penetration of the BBB is desired for peripherally acting drugs to minimize potential CNS-related side effects. The BBB is composed of brain capillary endothelial cells (BCEC). In contrast to other endothelial cells, BCEC are characterized by low passage rates for small hydrophilic molecules. This low permeability is caused by decreased transcytotic activity and by tight junctions and adherens junctions that limit paracellular passage (Wolburg and Lippoldt 2002). Furthermore, BCEC protect the brain from entry of potentially harmful endogenous and exogenous compounds by expression of non-specific transporter proteins which pump small hydrophobic molecules from the brain back into the blood. The first discovered export pump at the BBB was ABCB1 (formerly P-glycoprotein, P-gp, MDR1) (Cordon-Cardo et al. 1989) which is expressed at the apical membrane of BCEC where it limits the entry of drugs, toxic compounds and their metabolites into the brain (Schinkel 1999). The recently discovered ABCG2 (formerly breast cancer resistance protein, BCRP) was found to be expressed at the luminal surface of BCEC where it limits the entry of toxic

compounds to the brain (Cooray et al. 2002; Eisenblatter and Galla 2002; Cisternino et al. 2004). A further class of drug transporters at the BBB is the family of the MRPs (multidrug resistance proteins). MRP1 and MRP3-6 are expressed at the BBB on either the apical, basolateral or both sides of the BCECs, whereas the expression of MRP2 at the human BBB remains unclear (for review see (Loscher and Potschka 2005)). It should be noted that most drug transporting proteins are expressed constitutively in healthy brain tissue in line with their protective function.

The development of a model of the BBB that allows a reliable prediction of drug brain penetration *in vitro* has been a goal for a long time. In the past, efforts were made to develop cell culture models that mimic the *in vivo* situation. Isolated primary BCEC of various origins were used as models, sometimes co-cultured with astrocytes (Rowland et al. 1991; Huwyler et al. 1996; Cecchelli et al. 1999; Torok et al. 2003). This combination mimics the *in vivo* situation, where BCEC are surrounded by astrocytes and pericytes that are crucial for cell maturation and development of the tight junctions (Abbott et al. 2006). These models exhibited high electrical resistances, low permeabilities to small molecular weight compounds and functional expression of the most important drug transporters. Since isolation of BCEC is very laborious and time-consuming work, different immortalized rat or mouse cell lines, such as RBE4 (Roux et al. 1994), GPNT (Regina et al. 1999) and b.End3 (Omidi et al. 2003) have been developed. Drawbacks for their use as an *in vitro* model of the human BBB were their high paracellular permeabilities and animal origin. So far only three human immortalized cell lines have been developed: BB19 (Prudhomme et al. 1996; Kusch-Poddar et al. 2005), the recently described NKIM-6 (Ketabi-Kiyanvash et al. 2007) and the hCMEC/D3 (Weksler et al. 2005) cell line which was investigated in this study. Whereas the use of the BB19 cell line is limited by its high sucrose permeability, no permeability data for NKIM-6 are available. So far, active bidirectional transport has not been demonstrated in any human BBB cell line.

Recently, the hCMEC/D3 cell line has been developed by immortalization of primary human BCEC through co-expression of hTERT and the SV40 large T antigen via a lentiviral vector system. The hCMEC/D3 cells express typical endothelial markers such as CD31, VE-cadherin, and von Willebrand factor. Cells show a stable normal karyotype, maintain contact-inhibited monolayers in culture and form capillary tubes in matrix. Furthermore, this cell line expresses chemokine receptors and up-regulates adhesion molecules in response to inflammatory cytokines. Finally, cells demonstrate BBB characteristics such as the formation of tight junction proteins and the capacity to exclude

drugs. In culture, these cells functionally express important active transport proteins such as ABCB1, ABCC1 and ABCG2 (Weksler et al. 2005).

A recent study examined the hCMEC/D3 cell in a humanized dynamic *in vitro* BBB model under flow. In comparison to primary human brain microvascular endothelial cells in co-culture with human astrocytes, hCMEC/D3 cells exerted the same high transendothelial electrical resistance (TEER) even in absence of astrocytes (Cucullo et al. 2008). Other studies used the hCMEC/D3 as a model for studying BBB signaling and interaction with immune cells or pathogens (Levoye et al. 2006; Mairey et al. 2006; Viegas et al. 2006; Forster et al. 2008). Since there is a need for further evaluation of permeability properties, this study was performed.

The aim of the present study was to expand the evaluation of the hCMEC/D3 cell line as a predictive *in vitro* model of the BBB. Features which are important to predict the passage of molecules through the BBB are: a high tightness to hydrophilic low molecular weight compounds, a good permeability for hydrophobic low molecular weight compounds, and expression and function of transport proteins. Therefore we examined para- and transcellular transport rates of a series of compounds with different physicochemical properties and molecular weights in order to evaluate tightness and discrimination between high and low transport rates. In a further step it was our goal to further decrease paracellular permeability. The effect of human serum on sucrose permeability was investigated. To evaluate the transporter expression of our model we measured mRNA levels of P-gp, BCRP and MRP1-5 as well protein expression of P-gp and BCRP. We also measured the expression of the human transferrin receptor (hTfR) in the hCMEC/D3 cells. Functional activity of P-gp, BCRP and the MRP family in hCMEC/D3 cells, was tested by uptake assays using specific substrates and inhibitors. Furthermore, bidirectional transport of rhodamine 123 (R123), a P-gp substrate, was measured.

5.3 Materials and Methods

5.3.1 Materials

Ko-143, [^{14}C]-midazolam and TaqMan Mastermix were kindly provided by Roche Pharmaceuticals (Basel, Switzerland). All culture plasticware and Transwell® filters were purchased from Corning (Baar, Switzerland). Cell culture medium EBM-2 and supplements (vascular endothelial growth factor (VEGF), insulin-like growth factor (IGF-1), epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), fetal calf serum

(FCS) and hydrocortisone) were obtained from Lonza Bioscience (Verviers, Belgium). [^{3}H]-morphine, [^{14}C]-sucrose and [^{3}H]-digoxin were from Perkin Elmer (Schwerzenbach, Switzerland), [^{3}H]-inulin, [^{3}H]-propranolol, were from Amersham (Otelfingen, Switzerland) and [^{3}H]-methotrexate was purchased from Moravek (Brea, CA, USA). Hydrocortisone, lucifer yellow, mitoxantrone and verapamil, were purchased from Sigma (Buchs, Switzerland). Alexa 488 labeled secondary antibody, basic FGF, DNase I, Hank's balanced salt solution (HBSS), HEPES, penicillin-streptomycin, sodium pyruvate and SuperScript II RT-Kit were purchased from Invitrogen (Basel, Switzerland). Rat-tail collagen type I was obtained from Becton Dickinson (Allschwil, Switzerland). RNeasy Mini Kit was purchased from Qiagen (Hilden, Germany). qPCR Mastermix Plus was purchased from Eurogentec (Seraing, Belgium). Random hexamers and AmpliTaq gold DNA polymerase were obtained from Applied Biosystems (Rotkreuz, Switzerland). dNTPs were purchased from BioRad (Reinach, Switzerland). The murine antibody (mAb) C219 against P-gp and the mAb BXP-21 against BCR were purchased from Alexis. Rabbit anti-mouse IgG was purchased from Dako (Baar, Switzerland). Insta Gel plus scintillation liquid was obtained from Perkin-Elmer. All other chemicals were purchased from commercial sources at the highest quality available. Human AB serum (HS) was obtained from the blood donor bank (Blutspendezentrum, Universitätsspital Basel, Switzerland). A pool of 6 blood samples from healthy individuals with blood group AB was sterile filtered and heat inactivated for 1 h at 65°C.

5.3.2 Cell culture

For description see section 4.1.

5.3.3 Quantitative RT-PCR TaqMan assay

For description see section 4.2.

5.3.4 RT-PCR

For description see section 4.3.

5.3.5 Western blot

For description see section 4.4.

5.3.6 Flow cytometry analysis (FACS)

Cell suspensions of hCMC/D3 cells were prepared from confluent cell monolayers dispersed mechanically. The cells (0.5 x 10^6 cells in assay buffer: 0.17 M NaCl, 3.3 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) were incubated with 20 µg/ml of OX26 for 20 min at 4°C in the presence of 10% FCS. Control experiments (negative controls in absence of OX26 or secondary antibody only) were done in parallel. Cells were washed with ice-cold assay buffer. Cells were incubated with secondary antibody for 20 min at 4°C in 10% fetal calf serum in assay buffer using a 1:400 dilution of goat anti-mouse Alexa 488 labeled secondary antibody. Cells were washed twice with ice-cold assay buffer and were analyzed using a FACScan flow cytometer (Beckton Dickinson, Franklin Lakes, NJ, USA). Raw data were evaluated using Cytomation Summit software (Cytomation, Fort Collins, CO, USA).

5.3.7 Transport assay

For description see section 4.5.

5.3.8 Drug accumulation assay

For description see section 4.6.

5.3.9 Statistics

For statistical comparison, data from the treatment groups were compared with those from the controls by analysis of variance (ANOVA) with subsequent Dunnett's multiple comparison test. Where groups were compared, a pair-wise two-sided unpaired t-test was used. All comparisons were performed using SPSS for Windows software (version 14.0, SPSS Inc. Chicago, IL, USA).

5.4 Results

5.4.1 Transport assays

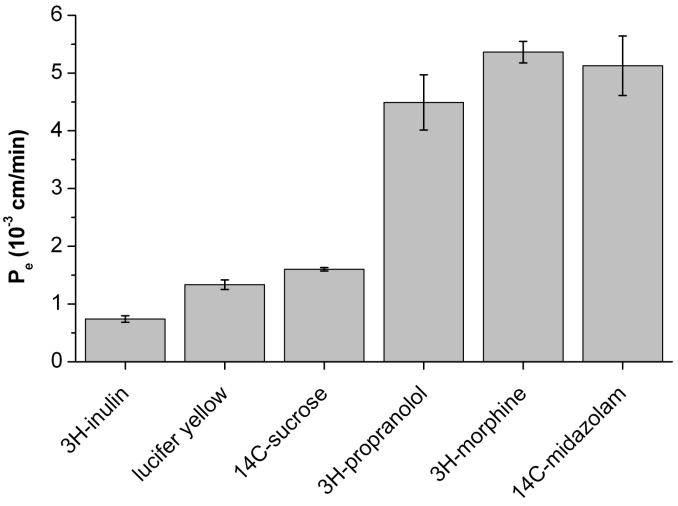

Figure 4 Permeability coefficients (P_e values $*10^{-3}$ cm/min) of model compounds with different molecular weight and hydrophilicity. Data represent means of n=6 ± SEM.

The BBB exhibits a very high tightness to hydrophilic compounds *in vivo* whereas lipophilic drugs can easily permeate the barrier. To assess the *in vitro* permeability of the hCMEC/D3 cell monolayer, a variety of test compounds with different physiochemical properties were investigated: inulin, lucifer yellow, sucrose, propranolol, morphine and midazolam. As shown in Figure 4, the lowest P_e value was measured for the large hydrophilic compound inulin ($0.74*10^{-3}$ cm/min). This value was comparable to the result of Weksler et al. where a P_e of $0.37*10^{-3}$ cm/min was measured. For the much smaller hydrophilic molecules sucrose ($1.60*10^{-3}$ cm/min) and lucifer yellow ($1.33*10^{-3}$ cm/min), higher permeabilities were measured, which are in agreement with previous data (Weksler et al. 2005). The highly lipophilic compounds midazolam ($5.13*10^{-3}$ cm/min) and propranolol ($4.49*10^{-3}$ cm/min), which were investigated for the first time in this cell line, had a 7-fold higher permeability compared to inulin. Morphine ($5.36*10^{-3}$ cm/min) also had 7-fold higher permeability compared to inulin (Figure 4). All transport experiments were performed 8-10 days after seeding of the cells. This was found to be the time frame where the monolayer had the lowest permeability with respect to apparent permeabilty of extracellular markers.

5.4.2 Influence of human serum on the sucrose permeability

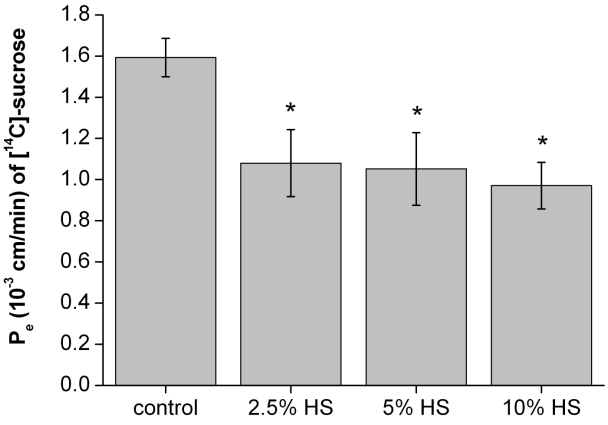

Figure 5 Effect of human serum on the [^{14}C]-sucrose permeability coefficients (P_e values *10^{-3} cm/min) of hCMEC/D3 monolayers. Data represent means of n=6 ± SEM (*: $p<0.05$).

As is known from literature, the tightness of the BBB depends on the correct assembly of the tight junctions. *In vivo* the formation of tight junctions is influenced by soluble factors of surrounding astrocytes and pericytes as well as by blood-derived compounds. It is the aim of an *in vitro* system to mimic the *in vivo* situation as closely as possible. Since the hCMEC/D3 cells are derived from human tissue, we replaced the FCS contained in the medium with HS, when the cells were growing on filters, in order to mimic the *in vivo* situation. Exposure of hCMEC/D3 cells to 2.5-10% HS resulted in a significant decrease of [^{14}C]-sucrose permeability (Figure 5). P_e values of [^{14}C]-sucrose were reduced by 32% (2.5% HS), 34% (5% HS) and 39% (10% HS) compared to control cells, grown under standard conditions (2.5% FCS) as shown in Figure 5.

5.4.3 ABC-transporter expression

The expression of MDR1, BCRP, MRP1, MRP2, MRP3, MRP4 and MRP5 in hCMEC/D3 cells was measured at the mRNA level by quantitative RT-PCR (Figure 6). The transcripts per µg RNA of MDR1, BCRP, and MRP1-5 range from 2*104 for MRP2 to 7*108 for MRP4. mRNA expression of BCRP, MDR1 and MRP1 lies in a similar range between 0.44–1.26*106 transcripts per µg RNA. The expression of MRP2 is very low, compared to the other transport proteins.

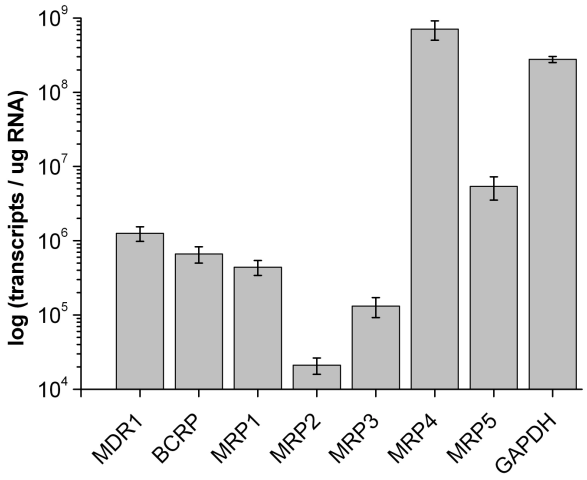

Figure 6 Absolute quantitative mRNA expression of ABC-transporters and GAPDH in hCMEC/D3 cells given as log(transcripts / µg RNA). Data represent means of n=6 ± SEM.

The expression of P-gp and BCRP proteins was assessed by Western Blot analysis using the C219 antibody against P-gp and the BXP-21 antibody against BCRP (Figure 7). P-gp with a molecular weight of 170 kDa was detected in hCMEC/D3 cell line (lane A) and in the human colon carcinoma cell line CACO-2 (lane B). CACO-2 cells are known to express the human P-gp at the protein level (Maier et al. 2007). Expression of BCRP with a molecular weight of 70 kDa was shown in the hCMEC/D3 cell line (lane C), and in a protein sample isolated from the human BBB cell line BB19 (lane D). The BB19 cell line was shown to express BCRP on protein level (Kusch-Poddar et al. 2005). BCRP as well as P-gp proteins were detected in the hCMEC/D3 cell line as shown in Figure 7, with expected molecular weights of 70 kDa and 170 kDa respectively.

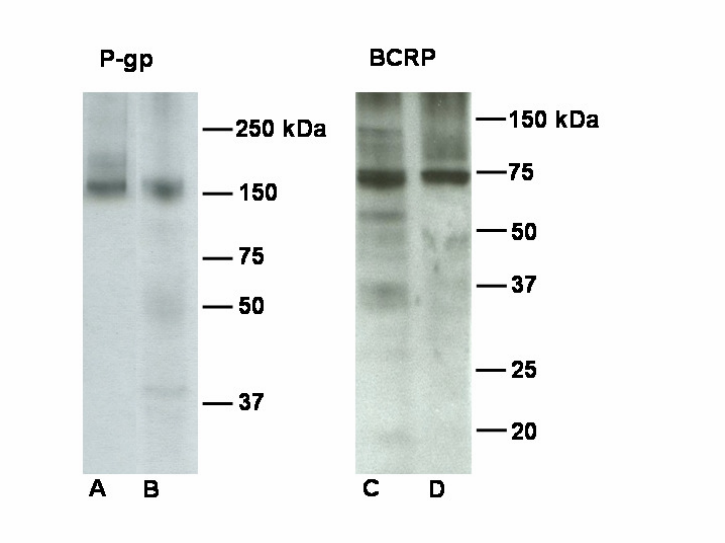

Figure 7 Western Blot analysis of P-gp (170kDa) and BCRP (70kDa) in hCMEC/D3 cells (D3). Bands represent protein of: hCMECD/3 cells (A and C), CACO-2 cells (B) and BB19 cells (D).

5.4.4 Expression of the transferrin receptor

The expression of hTfR was investigated using RT-PCR. The amplified 180 kb cDNA PCR product was detected in independent samples of hCMEC/D3 cells (data not shown), demonstrating the expression of the hTfR at the mRNA level.

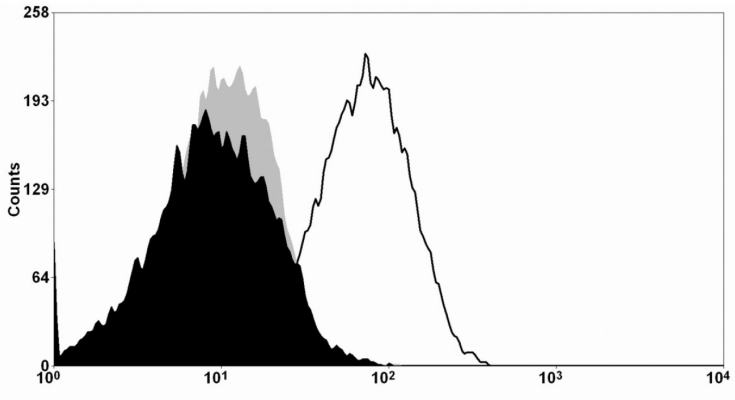

Figure 8 Transferrin receptor expression in hCMEC/D3: Immunolabeling and flow cytometry analysis using the anti-transferrin mAb OX26 (black: negative control; grey: second antibody only; white: OX26 immunostaining).

To detect the hTfR at the protein level flow-cytometry analysis was performed using the OX26 mAb against the hTfR. Huwyler et al. have described the OX26 mAb extensively (Huwyler et al. 1999). The data shown in Figure 8 clearly show that the hCMEC/D3 cell

line expresses the hTfR at the protein level, which is essential for the endocytotic uptake of iron-loaded transferrin into the brain.

5.4.5 Efflux assays

Transporter	Substrate	Inhibitor	% change compared to control	SEM	p-value
P-gp	R123	verapamil	+ 179.35	7.77	< 0.001
P-gp	R123	Ko-143	- 17.19	4.71	0.38
P-gp	digoxin	verapamil	+ 41.5	14.00	< 0.05
MRP	methotrexate	MK-571	+ 85.33	5.84	< 0.005
BCRP	mitoxantrone	prazosin	+ 50.37	21.21	0.15
BCRP	mitoxantrone	β-estradiol	+ 83.08	38.03	< 0.005
BCRP	mitoxantrone	Ko-143	+ 87.83	19.41	< 0.005
BCRP	mitoxantrone	verapamil	+ 101.77	25.48	< 0.001

Table 3 Effects of ABC-transporter inhibitors on the remaining substance in hCMEC/D3 cells after a defined efflux time. Data are given as percent change of intracellular remaining amount of the indicated substrate in the presence of an inhibitor compared to the amount in untreated control cells. Intracellular content of R123 (5 µM), digoxin (5 µM) and methotrexate (1 µM) was measured after 60 min, content of mitoxantrone (20 µM) after 40 min. Efflux was performed in the presence of the specific inhibitors verapamil (100 µM), Ko-143 (1 µM), MK571 (10 µM), prazosin (50 µM) and β-estradiol (20 µM). Two or more independent experiments were performed. Data are means of a representative experiment (n=3 ± SEM).

As shown in Table 3, the remaining amount of R123 and digoxin in the hCMEC/D3 cells was measured to investigate the functional expression of P-gp. The presence of the P-gp inhibitor verapamil increased the remaining amount of both compounds significantly, indicating that the drug export was decreased in the presence of the P-gp inhibitor. Verapamil increased R123 accumulation compared to its control by 179% whereas the accumulation of digoxin was increased by 41% in the presence of verapamil. The BCRP inhibitor Ko-143 did not influence the efflux of R123 significantly. In the presence of the MRP inhibitor MK571 an increase by 85% of the remaining MRP substrate methotrexate was observed.

BCRP function was investigated with mitoxantrone, a shared substrate of BCRP and P-gp. The mitoxantrone accumulation after 40 min was measured with and without prazosin (a BCRP and P-gp inhibitor), verapamil (a P-gp inhibitor), β-estradiol and Ko-143 (both specific BCRP inhibitors). The remaining substance was increased by 50%-

101% compared to control under addition of one of the listed inhibitors. Except for prazosin, the remaining mitoxantrone increase was highly significant in the presence of the inhibitors.

5.4.6 Bidirectional transport of the P-gp substrate R123

Bidirectional transport of the P-gp substrate R123 through hCMEC/D3 monolayers grown in standard medium containing 5% HS instead of FCS was measured. In Table 4 the permeabilities of sucrose and R123 through empty filters and hCMEC/D3 cells grown on filters are given. The permeabilities are given as slope of volume cleared (ml/min) as described in the section 4.5. The slopes (ml/min) of the empty filters for both compounds in both transport directions were at least twice as high as the slopes for the cells grown on filters.

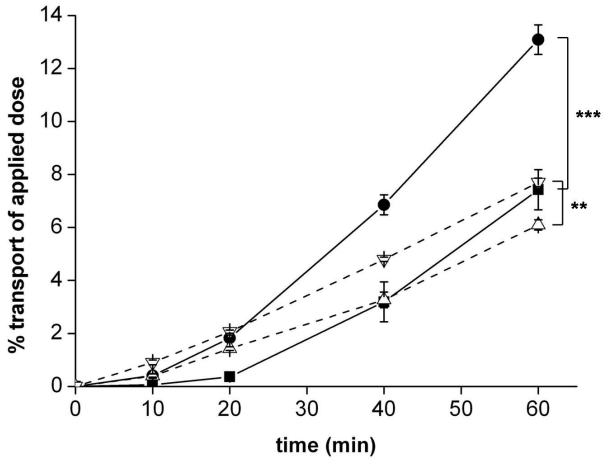

Figure 9 Bidirectional transport of R123 (solid lines) and sucrose (dashed lines) through hCMEC/D3 monolayers. Black squares and triangles indicate transport from apical to basolateral compartment. Black circles and down-pointing triangles indicate transport from basolateral to apical compartment. Data represent means of n=6 ± SEM (**: $p<0.005$, ***: $p<0.0005$).

As shown in Figure 9, R123 was transported to a significantly higher amount from basolateral (B) to apical (A) than from A to B. After 60 min the ratio of R123 transport A to B divided by B to A was 0.56. This difference was highly significant. [^{14}C]-sucrose was used as extracellular tightness marker. A higher transport from B to A compared to A to B was also observed for sucrose. However, the ratio of 0.79 was clearly higher than that observed for R123, indicating that R123 transport was restricted by P-gp. These findings demonstrate active bidirectional transport of the P-gp substrate R123 in hCMEC/D3 cells.

Compound	**sucrose**				**R123**			
Direction of Transport	A → B		B → A		A → B		B → A	
	cells	filter	cells	filter	cells	filter	cells	filter
m (ml/min)	0.52	1.08	0.65	2.05	0.56	1.69	1.07	3.04

Table 4 Permeabilities of R123 and sucrose from apical (A) to basolateral (B) and from basolateral to apical. Permeabilities are given as slopes (m) for hCMEC/D3 cells growing on filters (cells) and for empty filters (filters).

5.5 Discussion

The ability or inability of a drug to penetrate into the brain is a key consideration in drug design. Drugs for treating central nervous system (CNS) disorders need to be able to penetrate the BBB. BBB non-penetration is desired for peripherally acting drugs to minimize potential CNS-related side effects.

The aim of this study was to enlarge the characterization of the hCMEC/D3 cell line with regard to permeability against small molecular weight compounds and macromolecules, as well as transporter expression and functionality. Furthermore, directed P-gp transport was studied.

Since tightness to hydrophilic compounds is a main aspect of a good BBB, we investigated the passive paracellular permeabilities of sucrose, lucifer yellow and inulin as paracellular markers. Our sucrose ($1.60*10^{-3}$ cm/min) and inulin ($0.74*10^{-3}$) data are in concordance with the data generated by Weksler et al. (Weksler et al. 2005). In comparison with rat cell lines, such as RBE4 ($4.8*10^{-3}$ cm/min) (Rist et al. 1997), GPNT ($7.4*10^{-3}$ cm/min) (Regina et al. 1999) the hCMEC/D3 cell line exhibits a higher paracellular resistance. For the bovine co-culture model using primary BCEC and primary astrocytes, lower P_e values have been measured ($0.75*10^{-3}$ cm/min) (Cecchelli et al. 1999). A model using primary human BCEC and astrocytes in co-culture showed P_e values for sucrose ($1.0*10^{-3}$ cm/min) in a similar range compared to our results (Megard et al. 2002). The P_e of inulin in hCMEC/D3 cells was close to the reported values in the primary BCEC co-culture model ($0.37*10^{-3}$ cm/min) (Cecchelli et al. 1999).

Transcellular transport was investigated using the lipophilic low molecular weight compounds propranolol, morphine and midazolam. These molecules crossed the hCMEC/D3 monolayers to a much higher extent than the hydrophilic molecules. The ratio between propranolol and inulin permeabilities was 6.1, whereas the ratio between propranolol and sucrose was 2.8. This ratio allows discrimination between molecules with

a high or low permeation rate. Compared to a model using porcine BCEC, where the ratio between propranolol and sucrose was 5.7, the ratio in our model was lower. However, the rat cell line b.End3, which was investigated in the same study, was not able to dicriminate between different permeability marker compounds (Omidi et al. 2003).

As described above the sucrose permeability in the hCMEC/D3 cell line was good but still higher compared to the primary co-culture model of Cechelli et al. (Cecchelli et al. 1999). It is well-known that the maturation and tightness of BCECs can be modulated by different astrocyte or blood-derived factors. As the hCMEC/D3 cells are of human origin we investigated the effect of HS on hCMEC/D3 monolayers. Incubations of the hCMEC/D3 monolayers with different concentrations of HS (2.5, 5, 10%), replacing FCS, resulted in a decrease of sucrose permeability, which ranged between 32% and 39%. Already at a concentration of 2.5% the sucrose permeability was lower compared to cells treated with 2.5% FCS. At the highest concentration of 10% HS a P_e value of $0.97*10^{-3}$ cm/min was measured. This P_e value comes close to the sucrose permeability reported for the primary bovine co-culture model by Cecchelli et al., which is considered as a gold standard (Cecchelli et al. 1999). This finding is in contrast to other studies. In presence of ox serum decreased paracellular tightness was observed in primary porcine models of the BBB (Hoheisel et al. 1998; Nitz et al. 2003). However, endothelial cells of different origins and other types of serum compared to our experiments were used. The mechanism by which HS increases the tightness is unclear. Further studies are needed to clarify the involved processes.

Beside a morphology, which is similar to the *in vivo* situation and a good tightness mediated by tight junctions, the expression and functionality of ABC-transporters is a key feature of a good *in vitro* model. In addition to Weksler et al. (Weksler et al. 2005) who investigated mainly MDR1, MRP1, MRP5 and BCRP, we also tested MRP2, MRP3 and MRP4 expression at the mRNA level. The rank order of ABC-transporter mRNA expression in hCMEC/D3 cells was MRP4 > MRP5 > MDR1 > BCRP > MRP1 > MRP3 > MRP2, whereas the MRP2 expression level was around 100 fold lower compared to the other transporters. Similar results were obtained independently with the same cell line by other investigators (S Dauchy, X Declèves, JM Scherrmann, personal communication).

It is important to know that the expression of different ABC-transporters is highly variable among different species. Furthermore, differences are observed between brain tissue preparations, primary cultures of BCECs and cell lines: In a model of isolated primary rat BCECs a high expression of Mdr1a, Mrp1, Mrp4 and Bcrp mRNA was observed (Hori et al. 2004; Ohtsuki et al. 2007) as summarized in Table 5. These findings were supported

by Enerson and Drewes showing high expression of Bcrp, Mdr1 and Mrp5 in freshly isolated rat brain microvessels (Enerson and Drewes 2006). Higher expression levels of MRP1, MRP4, MRP5 and MRP6 were found in isolated primary BCEC from bovine origin (Zhang et al. 2000).

Ranking of ABC-transporter expression	Expression level	Tissue	Species	Reference
MRP4 > MRP5 > MDR1 > BCRP > MRP1 > MRP3 > MRP2	mRNA (qRT-PCR)	hCMEC/D3 cell line	human	this publication
Mdr1a > Mrp1 > Mrp4 > Bcrp >> Mrp5 > Mrp3 >> MRP6 > Mrp2	mRNA (qRT-PCR)	primary BCECs	rat	(Hori et al. 2004; Ohtsuki et al. 2007)
Bcrp > Mdr1 > Mrp5 > Mrp1 = Mrp3 = Mrp4 = Mrp6	mRNA (qRT-PCR)	freshly isolated brain microvessels	rat	(Enerson and Drewes 2006)
MRP4 = MRP5 = MRP6 > MRP1 > MRP3; MRP2 (nd)	mRNA (RT-PCR)	primary BCEC	bovine	(Zhang et al. 2000)
MRP1 >> MDR1 = MDR3 > MRP2 = MRP3 = MRP5	mRNA (qRT-PCR)	isolated primary BCECs	human (aneurysmic patients)	(Dombrowski et al. 2001)
MRP1 >> MDR1 > MRP2 > MDR3 = MRP5 = MRP3	mRNA (qRT-PCR)	isolated primary BCECs	human (epileptogenic tissue)	(Dombrowski et al. 2001)
MRP1, MRP4, MRP5 (nq); MRP2, MRP3, MRP6 (nd)	protein (immuno-fluorescene)	tissue sections	human	(Nies et al. 2004)}
MRP1 >> BCRP > MRP2 > MDR1	mRNA (qRT-PCR)	NKIM-6 cell line	human	(Ketabi-Kiyanvash et al. 2007)

Table 5 Ranking of ABC-transporter expression in cells or tissues of different origins (nd: not detectable, nq: not quantifiable).

Human BCECs of different origins (primary cultures) were compared previously (Table 5). Main differences can be summarized as follows: 1. there are qualitative differences with respect to ABC-transporter mRNA expression between cells from tissue obtained from aneurysm operations and epileptogenic tissue. In the epileptogenic tissue, MDR1, MRP2 and MRP5 were significantly increased (Dombrowski et al. 2001). Furthermore, the up-regulation of MDR1, MRP1 and MRP2 in epileptogenic tissue was confirmed on protein level (Sisodiya et al. 1999; Sisodiya et al. 2001; Aronica et al. 2005). Aronica et al. observed that BCRP expression was not significantly altered in epileptogenic tissue (Aronica et al. 2005). 2. in contrast to these primary cells, the hCMEC/D3 cells show a predominant expression of MRP4 and MRP5 and a reduced expression of MRP1. This is in agreement with data from Nies et al. investigating the expression of MRPs in human brain tissue. MRP1, MRP4 and MRP5 were found by immunostaining of human brain

tissue slices. MRP2, MRP3 and MRP6 were not expressed at detectable levels (Nies et al. 2004). Similar data were found in cells from rat and bovine origins (Nies et al. 2004). Recently, mRNA expression levels of a selection of different ABC-transporters were measured in the human BBB cell line NKIM-6 (Ketabi-Kiyanvash et al. 2007). The rank order of transporter expression was MRP1 >> BCRP > MRP2 > MDR1 (Table 5), which was different from primary cells of human origin. In particular, this cell line showed an extraordinarily high expression of MRP2 compared with MDR1. This is in contrast to our findings in the hCMEC/D3 cell line.

In addition to the transporter expression we also detected the hTfR at mRNA and protein level using RT-PCR and flow cytometry analysis. The transferrin receptor is known to be expressed at the human BBB, where it is involved in the endo- and transcytosis of the iron transport protein transferrin (Pardridge et al. 1987). The expression of the hTfR is the basis for endocytosis of transferrin in the hCMEC/D3 cells. In order to validate this process further investigations are required.

Besides mRNA expression of the ABC-transporters it is important to determine their expression at the protein level and to investigate whether they are functionally active. At the protein level P-gp and BCRP could be detected by Western blotting. For functional activity the efflux transporters P-gp, BCRP and members of the MRP family were examined by using different substrates and inhibitors. P-gp activity could be shown in uptake experiments using the known P-gp substrates R123 and digoxin (Schinkel et al. 1995). The efflux of these substrates could be inhibited with the P-gp inhibitor verapamil. Ko-143 a specific BCRP inhibitor had no influence on R123 efflux, indicating that only P-gp was responsible for the observed effect. Methotrexate efflux could be inhibited with the specific MRP inhibitor MK-571, indicating that members of the MRP family are functionally active (Gekeler et al. 1995; Zeng et al. 2001). It is difficult to investigate which specific MRP mediates this effect since there is a lack of MRP subtype specific inhibitors. In addition, we demonstrated that BCRP is expressed and functionally active in hCMEC/D3 cells. This was shown with mitoxantrone, a shared substrate of P-gp and BCRP. Mitoxantrone efflux could be inhibited with Ko-143, β-estradiol, which are two specific BCRP inhibitors, and verapamil (Allen et al. 2002; Pavek et al. 2005).

In a next step directed bidirectional P-gp transport was examined. Cells were cultured under the improved culture conditions with medium containing 5% HS replacing FCS as described before. We investigated the bidirectional transport of R123, a known P-gp substrate through hCMEC/D3 monolayers. The lower ratio of R123 flux from apical to basolateral compared to sucrose indicates active P-gp mediated transport. The effect

was moderate but corresponds to the observed data of R123 efflux experiments. It is also in line with the results of bidirectional transport data of vincristine in a bovine primary co-culture BBB model (Cecchelli et al. 1999) and digoxin in the murine b.End3 cell line (Omidi et al. 2003). In both models active P-gp transport was shown by a 2-fold lower transport rate from apical to basolateral than from basolateral to apical. It should be noted that the observed asymmetry in bidirectional transport is moderate. In contrast to P-pg overexpressing cells, the functional expression level of P-gp in hCMEC/D3 cells seems to be low. As a consequence, the ratio between apical to basolateral and basolateral to apical transport was in the range of 0.79 for R123. This is 50-fold lower compared to a study using P-gp over-expressing MDCK cells for the same substrate (Tang et al. 2004). In view of this situation, it is difficult to identify P-gp substrates reliably under the conditions used in the bidirectional transport assay of the present work. In particular, we could not observe statistically significant differences in Papp in presence or absence of P-gp inhibitors such as verapamil.

Up to now the hCMEC/D3 is the best characterized human BBB cell line. Many characteristics that are crucial for a good BBB model, such as specific BCEC markers and the expression of the most important junctional proteins have been proven. The tightness of the monolayer for hydrophilic high molecular weight compounds is high, and hydrophobic compounds are able to cross the artificial barrier by the transcellular way. The addition of HS significantly reduced the sucrose permeability. This improvement resulted in permeability values close to a primary bovine co-culture BBB model. Furthermore, many of the drug transporters, which are found in the human BBB *in vivo*, could be detected in this cell line. It could be shown that most of the drug transporters of the ABCB, ABCC and ABCG families and the hTfR are expressed. MDR1, BCRP and MRP1 are functionally active and could be inhibited by specific inhibitors. Moreover, it is the first time that active P-gp mediated bidirectional transport was shown in a human BBB cell line. This finding allows us to use the hCMEC/D3 cell line as a human BBB model to study passive and active transport processes.

We conclude therefore, that the hCMEC/D3 cell line is the most promising immortalized human BBB cell line available today. The model exhibits most of the characteristics that are essential for a good predictive BBB *in vitro* model.

5.6 Acknowledgments

Prof. Dr. Jürgen Drewe and Prof. Dr. Jörg Huwyler have contributed equally to the present work. We thank F. Hoffmann-La Roche Ltd. for helpful discussions and financial support.

6 Influence of endo- and exogenous factors on the permeability of the hCMEC/D3 cell line

Birk Poller[1], Anne-Sophie Benischke[1], Heike Gutmann[2], Jürgen Drewe[1]

[1] Dept. of Clinical Pharmacology and Toxicology, University Hospital of Basel, 4031 Basel, Switzerland

[2] Novartis Pharma AG, Klybeckstrasse 141, CH-4057 Basel, Switzerland

to be submitted

6.1 Abstract

The blood-brain barrier is a selective permeability barrier between the blood and the central nervous system. Brain endothelial cells form a tight monolayer sealed by tight junctions that prevent hydrophilic compounds from passive diffusion into the brain tissue. Therefore, a high paracellular resistance is a crucial characteristic for every *in vitro* model of the blood-brain barrier. Such a model is the human brain capillary endothelial cell line hCMEC/D3. It shows a low permeability against small hydrophilic compounds, yet higher than in models using primary brain capillary endothelial cells. Since the formation of tight junctions is depending on the surrounding milieu it was our aim to improve the tightness of hCMEC/D3 monolayers by medium supplements. The paracellular permeabilty was assessed by transport of the tightness marker sucrose through hCEMC/D3 monolayers. Based on literature reports the effect of N-acetylcysteine, atorvastatin, simvstatin, sodium nitroprusside, insulin, transforming growth factor, epidermal growth factor glial cell derived growth factor, interferon-β1b, minocycline, heparin, 8-Br-cyclic guanosine monophosphate, selenous acid, diclofenac, indomethacin and thalidomide on the sucrose permeability in hCMEC/D3 cells. N-acetylcysteine, atorvastatin and sodium nitroprusside reduced the sucrose permeability significantly. Additionally, the effect of the three compounds on the generation of reactive oxygen species and the expression of the junctional protein ZO-1 was determined. N-acetylcysteine and sodium nitroprusside significantly reduced the intracellular reactive oxygen species levels whereas atorvastatin had no effect. The ZO-1 levels detected by immunocytochemistry were slightly increased after treatment with any of the three compounds. However the expression was increased at the cell borders as well as within the cytosol.

Our study revealed evidence that the tightness of hCMEC/D3 monolayer can be increased by addition of N-acetylcysteine, atorvastatin and sodium nitroprusside to the culture medium. Furthermore the results suggest a potential connection between intracellular reactive oxygen species levels and the tightness of the monolayer.

6.2 Introduction

The blood-brain barrier is (BBB) is the permeability barrier between the blood and the central nervous system. Thereby, it prevents a broad range of compounds from the entry to the brain tissue. Tight junctions (TJ) seal the monolayer of endothelial brain capillary cells (BCEC) against the passage of small hydrophilic compounds. Therefore, an important characteristic of an *in vitro* model of the BBB is a high paracellular resistance.

Recently, the hCMEC/D3 cell line was developed as an *in vitro* model of the human BBB, by immortalizing primary human BCEC. This cell line shows important BBB characteristics such as the expression of tight junctional molecules, chemokine receptors and active drug efflux transporters (Weksler et al. 2005). Furthermore, in contrast to other cell lines the hCMEC/D3 cells form tight monolayers exerting high paracellular resistance (Poller et al. 2008). However, the permeability of paracellular marker compounds such as sucrose is lower in some *in vitro* models using primary cultures of BCEC of different origins (Cecchelli et al. 1999; Megard et al. 2002).

From literature, it is known that the molecular composition of TJ can be modulated by different factors resulting in an increased or reduced paracellular tightness of the BBB (Abbott et al. 2006). The BCEC are in close contact with glial cells namely pericytes or astrocytes. These cells release cell growth factors such as epidermal growth factor (EGF), transforming growth factor-β1 (TGF-β1) and glial-cell derived neurotrophic factor (GDNF), which were shown to increase the paracellular resistance of primary BCEC *in vitro* (Igarashi et al. 1999; Dohgu et al. 2005). It is also known that soluble inflammatory mediators such as tumor necrosis factor-alpha (TNF-α) cause a breakdown of the BBB barrier properties, which can be prevented by anti-inflammatory drugs (Candelario-Jalil et al. 2007). Interferon-beta 1b (IFN-β1b), used in the therapy of multiple sclerosis patients, is a drug that prevents the disruption of the BBB *in vitro* and *in vivo* possibly be reducing the release of pro-inflammatory cytokines and matrix metalloproteinases (MMP) (Kraus et al. 2004; Kraus and Oschmann 2006). The MMPs, mainly MMP-7 and MMP-9 are involved in the acute breakdown of the BBB during relapses of multiple sclerosis, by degrading collagen of the basal lamina (Yang et al. 2005). Furthermore, MMPs can cleave IFN-β1b, which can be inhibited by minocycline a potent inhibitor of the MMP-9 (Brundula et al. 2002; Nelissen et al. 2003).

Selenous acid and heparin are substances used in the culture medium of *in vitro* BBB models that report low paracellular permeabilities (Franke et al. 1999; Gaillard et al. 2001; Megard et al. 2002). However, no reports exist that indicate the importance of these supplements in order to reach low permeabilities. A recent study reported that reactive oxygen species (ROS) disrupt the BBB. This was shown in the GP8 and the hCMEC/D3 cell line (Schreibelt et al. 2007). Therefore, we invested four compounds that are described to reduce the generation of ROS, namely sodium nitroprusside (SNP), atorvastatin, simvastatin and N-acetylcystein (NAC). *In vitro* NO radical (NO(*)) releasing compounds decrease the permeability of isolated primary porcine BCEC monolayers (Winter et al. 2008). SNP being also a member of the NO(*) was shown to improve the

tightness of primary human BCEC monolayers (Wong et al. 2004). Statins also exert BBB stabilizing effects, which was shown for lovastatin, simvastatin and fluvastatin in monolyers of BCEC *in vitro* (Ifergan et al. 2006; Kuhlmann et al. 2006).

NAC is an antioxidant drug and acts as a radical scavenger, protecting cells against oxidative damage of OH radicals and H_2O_2. It is also a precursor of cysteine which is essential for the glutathione (GSH) sythesis. GSH maintains the cellular redox status in endothelial cells and protects the cells against ROS (Townsend et al. 2003; Atkuri et al. 2007).

The aim of the present study was to investigate the effect of different medium supplements on the paracellular tightness in the hCMEC/D3 cell line. Therefore, hCMEC/d3 cells were incubated with NAC, the statins atorvastatin and simvstatin, SNP, insulin, the growth factors TGF-β1, EGF and GDNF, INF-β1b, minocycline, heparin, 8-Br-cyclic guanosine monophosphate (8-Br-cGMP), selenous acid and with the anti-inflammatory drugs dicofenac, indomethacin and thalidomide. After the incubation period transport of sucrose through monolayers of hCMEC/D3 cell was measured to determine the effect of the supplements on the paracellular permeability. Since the integrity of TJ can be disturbed by ROS, their generation in the hCMEC/D3 cells was measured under influence of NAC, atorvastatin and SNP. Furthermore, immunolabelling of ZO-1 was performed after treatment with NAC, atorvastatin and SNP.

6.3 Materials and methods

6.3.1 Materials

The EBM-2 medium and supplements (vascular epidermal growth factor (VEGF), insulin-like growth factor-1 (IGF-1), epidermal growth factor (EGF), fibroblast growth factor (FGF), hydrocortisone) were from Lonza (Verviers, Switzerland). Fetal calf serum (FCS), HEPES, Hank's balanced salt solution (HBSS), insulin, phosphate buffered saline (PBS), penicillin-streptomycin, sodium pyruvate and TGF-β1 were from Invitrogen (Basel, Switzerland). All culture plasticware and Transwell® filters were from Corning (Baar, Switzerland). [^{14}C]-sucrose and Ultima Gold scintillation liquid was from Perkin Elmer (Schwerzenbach, Switzerland). Rat-tail collagen type I was from Becton Dickinson (Allschwil, Switzerland). Atorvastatin, bovine serum albumin (BSA), basic fibroblast growth factor (bFGF), cyclic adenosine monophosphate (cAMP), 2,7-dichlorofluorescin (DCFH), diclofenac, EGF, GDNF, heparin, hydrocortisone, indomethacin, minocycline, NAC, selenous acid, simvastatin, SNP and thalidomide were from Sigma (Buchs,

Switzerland). FluorSave was from Calbiochem (San Diego, CA, USA). Rabbit anti-ZO-1 polyclonal antibody was purchased from Zymed (Basel, Switzerland). Cy2 conjugated goat anti-rabbit andtibody and Cy3 conjugated goat anti-rabbit andtibody were obtained from Jackson Immuno Research Laboratries (West Grove, PA, USA). IFN-β1b (Betaferon©, Schering, Zurich, Switzerland) was a kind gift of Prof. Dr. Kappos (University Hospital, Basel). 8-Br-cGMP was kindly provided by Prof. Dr. Resink (University Hospital, Basel). All other chemicals were purchased at the highest quality available.

6.3.2 Cell culture

For description see section 4.1.

6.3.3 Incubation conditions for transport assays and immunocytochemistry

To investigate the effect of test compounds on the [^{14}C]-sucrose permeability and for immunostainings of ZO-1 the hCMEC/D3 cells were seeded on collagen coated Transwell® clear filters as described in section 4.5. Four and 7 days after the seeding the assay medium was changed and transport assays or immunostainings were performed after 10 days. The monolayers were incubated with the test compounds in different concentrations and for a defined time as summarized in Table 6.

Test compound	Concentrations	Time (h)
8-Br-cGMP	0.1, 0.5, 1 mM	24
atorvastatin	1, 10, 100 ng/ml	72
diclofenac	1, 5, 10 µM	72
EGF	0.5, 1, 5 µg/ml	72
GDNF ± cAMP	0.1, 1, 10 ng/ml	24
heparin	10, 100, 1000 µg/ml	72
IFN-β1b	50, 500, 1000 UI/ml	72
IFN-β1b + minocycline	IFN-β1b 500 UI/ml + minocycline 0.1, 100 µg/ml	72
indomethacin	1, 5, 10 µM	72
insulin	0.1, 1, 10 µg/ml	72
minocycline	0.1, 10, 100 µg/ml	72
NAC	5, 50, 100 µg/ml	72
selenous acid	5, 10 µg/ml	72
simvastatin	1, 10, 100 ng/ml	72
SNP	100, 250, 500 µM	24
TGF-β1	1, 5, 10 ng/ml	12
thalidomide	0.1, 1, 10 µM	72

Table 6 Concentrations and incubation times of test compounds. hCMEC/D3 cells were incubated as indicated for transport assays and immunocytochemistry.

6.3.4 Transport assay

For description see section 4.5.

6.3.5 Immunocytochemistry

For immunostainings of ZO-1 hCMEC/D3 cells were seeded on type I collagen coated Transwell® polyester membranes (12 well, pore size 0.4 µm) in a concentration of 50'000 cells/cm^2. ZO-1 stainings were performed 10 days after seeding. The monolayers were incubated with atorvastatin (1 ng/ml), SNP (250 µM) and NAC (100 µg/ml) as described in section 6.3.3.

Cells were washed twice with phosphate buffered saline (PBS) and the filter membranes were cut out. The cells were fixed with 4% paraformaldehyde for 10 min and were washed twice with PBS. Afterwards the cells were permeabilized in 0.5% Triton X-100 for 5 min. After blocking with PBS containing 3% BSA the filters were incubated with the primary antibody against ZO-1 (rabbit anti-ZO-1, 20 µg/ml dissolved in PBS containing 3% BSA) for 2 hours in a humid chamber at 37°C. Subsequently, the cells were washed 3 times for 5 min with PBS followed by the incubation with the secondary antibody (goat anti-rabbit Cy3 labeled or goat anti-rabbit Cy2 labeled) in a concentration of 15 µg/ml dissolved in PBS containing 3% BSA for 1 hour at 20°C. Stained cells were washed 3 times with PBS and the filter membranes were mounted on glass coverslides using FlourSafe. Fluorescence-stained cells were examined on a confocal Zeiss LSM 150 inverted laser scanning microscope (Carl Zeiss, Oberkochen, Germany). For detection of the secondary Cy2 labeled antibody an argon laser at an excitation wavelength of 488 nm was used and emission was measured at 510 nm. The detection of the Cy3 labeled secondary antibody was performed using an ArNe laser at an excitation wavelength of 543 nm. Emission was measured using a bandpass filter (505-550nm).

6.3.6 Reactive oxygen species (ROS) measurement

6.3.6.1 ROS assay

The generation of ROS was detected using the dye DCFH. DCFH is a non-fluorescent probe, which is oxidized by ROS to the fluorescent form 2,7-dichlorofluorescein (DCF). The cells were seeded on a type-I collagen precoated 96 well plate in concentration of 10000 cells per cm^2. Assay medium was changed every 3 or 4 days and 8 days after seeding the ROS assay was performed. After 4 hours of incubation with the supplements (see section 6.3.6.2) the medium was removed and cells were washed once with PBS. Thereafter, 100 µM DCF dissolved in assay medium were added and the plate was

incubated for 30 min at 37°C in an incubator. Subsequently the plate was measured using a HTS 7000plus plate reader with an excitation wavelength of 475 nm and an emission wavelength of 525 nm.

6.3.6.2 Incubation conditions for ROS assay

The hCMEC/D3 cells were incubated for 4 hours with assay medium containing atorvastatin (0.1, 1, 10, 100 ng/ml), NAC (5, 50, 100, 500, 1000 µg/ml), BSO (200 µM) and SNP (100, 250, 500 µM). H_2O_2 (0.1% in assay medium) was used as a positive control and assay medium containing 0.01% DMSO served as negative control. Previously the DCF fluorescence had been examined for assay medium alone and assay medium containing different DMSO concentrations (0.001, 0.01, 0.1%). No significant differences of DCF fluorescence intensity were observed (data not shown).

6.3.7 Statistics

For statistical comparison, data from the treatment groups were compared with those from the controls by analysis of variance (ANOVA) with subsequent Dunnett's multiple comparison test. Where groups were compared, a pair-wise two-sided unpaired t-test was used. All comparisons were performed using SPSS for Windows software (version 15.0, SPSS Inc. Chicago, IL, USA).

6.4 Results

6.4.1 Effects of test compounds on sucrose permeability

As it is known from literature the paracellular resistance of the BBB depends on the correct assembly of the tight junction proteins. *In vivo* the correct assembly of tight junctional proteins is influenced by soluble factors released by surrounding astrocytes and pericytes as well as by blood-derived compounds.

In order to increase the tightness of hCMEC/D3 monolayers the cells were incubated with the antioxidant drug NAC, the statins atorvastatin and simvastatin, SNP, the anti-inflammatory drugs diclofenac, indomethacin and thalidomide, IFN-β1b, the glial cell-derived growth factors (TGF-β1, GDNF, EGF), insulin, heparin, and selenous acid. After the incubation period transport of the radioactively labeled paracellular permeability marker [^{14}C]-sucrose through hCMEC/D3 monolayers was measured. All assays were performed in duplicates or triplicates. For all transport experiments P_e values of [^{14}C]-sucrose in the range of 1.6 - 2.0 x 10^{-3} cm/min were used as internal standard.

Test substance	Effect on P_e values of [^{14}C]-sucrose	n
NAC	Reduction by 12-18% (Figure 10)	9
atorvastatin	Reduction by 15% (Figure 11)	9
SNP	Reduction by 14% (Figure 12)	9
IFN-β1b	No significant change compared to control	9
IFN-β1b + minocycline	No significant change compared to control	6
insulin	No significant change compared to control	9
TGF-β1	No significant change compared to control	9
EGF	No significant change compared to control	6
GDNF ± cAMP	No significant change compared to control	9

Table 7 Effects of test compounds on P_e values of [^{14}C]-sucrose in hCMEC/D3 cells. n: number of measured filters.

Treatment of hCMEC/D3 monolayers with insulin (0.1, 1, 10 µg/ml) showed no change of [^{14}C]-sucrose permeability. Also the growth factors EGF (0.5, 1, 5 µg/ml), TGF-β1 (1, 5, 10 ng/ml) and GDNF (0.1, 1, 10 ng/ml) did not significantly improve the tightness of hCMEC/D3 monolayers against [^{14}C]-sucrose, when applied to the culture medium dose-dependently. The exposure of hCMEC/D3 cells to IFN-β1b alone (50, 500, 1000 UI/ml) or in combination with the metalloprotease inhibitor minocycline (0.1, 100 µg/ml) had no beneficial effect on the tightness of the cell monolayers.

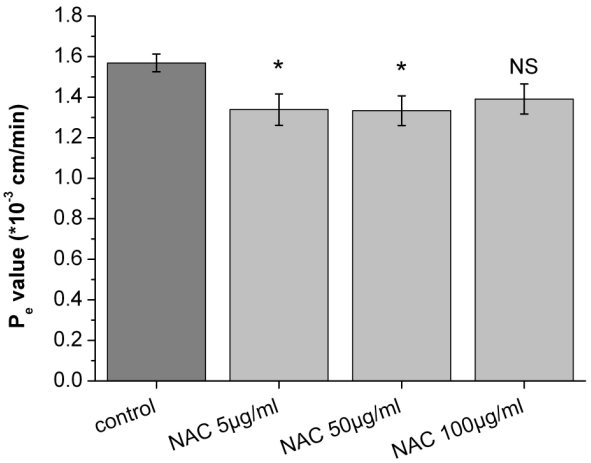

Figure 10 Effect of NAC (5, 50, 100 µg/ml) on the [^{14}C]-sucrose permeability of hCMEC/D3 monolayers. Data are means ± SEM of n=9 filters, *: $p<0.05$.

The effect of the antioxidant drug NAC on the paracellular permeability was also investigated in a dose-dependent manner (5, 50, 100 µg/ml). The P_e values of [^{14}C]-sucrose after treatment with 5 and 50 µg/ml were $1.33*10^{-3}$ and $1.34*10^{-3}$ cm/min

respectively (Figure 10), whereas the control filters showed a P_e of $1.56*10^{-3}$ cm/min. This corresponds to a statistically significant reduction of the permeability coefficient by 14% at both NAC concentrations. The treatment with 100 µg/ml NAC reduced the P_e value by 11% compared to the untreated control. However this decrease was statistically not significant.

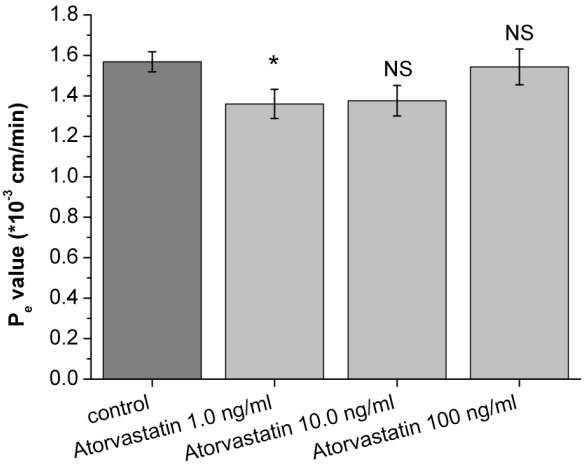

Figure 11 Effect of atorvastatin (1, 10, 100 ng/ml) on the [^{14}C]-sucrose permeability of hCMEC/D3 monolayers. Data are means ± SEM of n=9 filters, *: $p< 0.05$.

Atorvastatin was applied to the hCMEC/D3 cells in 3 concentrations (1, 10, 100 ng/ml). At the low concentrations of 1 and 10 ng/ml the P_e values of [^{14}C]-sucrose of $1.36*10^{-3}$ and $1.38*10^{-3}$ cm/min respectively were measured (Figure 11). At both concentrations the P_e values of [^{14}C]-sucrose permeability were reduced by 13% compared to the untreated control. However, only at the concentration of 1 ng/ml the sucrose permeability was statistically significant decreased. No altered permeability was observed after incubation with 100 ng/ml atorvastatin compared to the control.

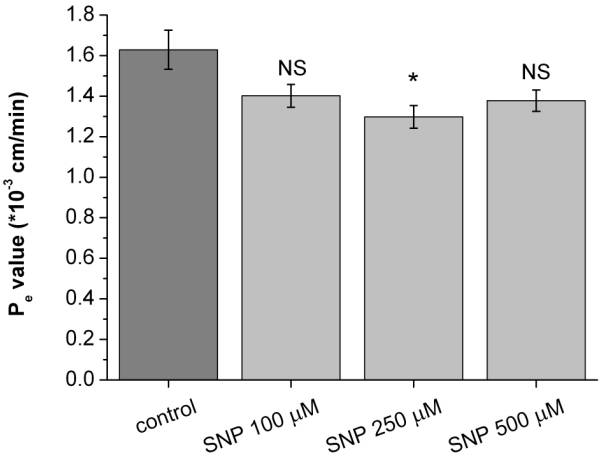

Figure 12 Effect of SNP (100, 250, 500 µM) on the [^{14}C]-sucrose permeability of hCMEC/D3 monolayers. Data are means ± SEM of n=9 filters, *: p< 0.05.

In another series of experiments hCMEC/D3 were grown on filters in the presence of different SNP concentrations (100, 250, 500 µM). P_e values after treatment with 100 µM and 500 µM SNP were $1.4*10^{-3}$ and $1.38*10^{-3}$ cm/min respectively (Figure 12). These values correspond to a reduction of [^{14}C]-sucrose permeability of 14% at 100 µM and of 15% at 500 µM SNP. However, these changes were not statistically significant. Only after treatment with 250 µM SNP the P_e value was statistically significant changed, where a P_e value of $1.3*10^{-3}$ cm/min was observed. This is a reduction of the [^{14}C]-sucrose permeability of 20% compared to the untreated control filters.

6.4.2 Preliminary results

Test compound	Effect on P_e values of [^{14}C]-sucrose	n
minocycline	No significant change compared to control	3
heparin	No significant change compared to control	3
8-Br-cGMP	No significant change compared to control	3
indomethacin	No significant change compared to control	3
simvastatin	No significant change compared to control	3
diclofenac	No significant change compared to control	3
thalidomide	No significant change compared to control	3
selenous acid	No significant change compared to control	3

Table 8 Preliminary results: Effects of test compounds on P_e values of [^{14}C]-sucrose in hCMEC/D3 cells.

In a series of experiments dose-dependent incubations with the three anti-inflammatory drugs indomethacin (1, 5, 10 µM), diclofenac (1, 5, 10 µM) and thalidomide (0.1, 1, 10 µM), 8-Br-cGMP (0.1, 0.5, 1 mM), selenous acid (5, 10 µg/ml), heparin (10, 100, 1000 µg/ml), simvastatin (1, 10, 100 ng/ml) and minocycline (0.1, 10, 100 µg/ml) were performed. None of these supplements did alter the [^{14}C]-sucrose permeability to a significant extent. However, these are only preliminary results and further experiments are necessary to validate these findings.

6.4.3 Intracellular ROS measurement

The effect of ROS generation was investigated in hCMEC/D3 cells, using DCFH. The non-fluorescent compound DCFH is oxidized by ROS to a fluorescent probe (DCF). The fluorescence intensity correlates with the intracellular ROS production. Since H_2O_2 is known to induce intracellular ROS generation it served as a positive control in a concentration of 0.1%. In our experiments H_2O_2 increased the ROS production by 275%. Additionally, the cells were incubated with 0.1% H_2O_2 in combination with NAC (500 µg/ml), atorvastatin (0.1 ng/ml) and SNP (250 µM). These three supplements did not decrease the H_2O_2 induced generation of ROS (Figure 13).

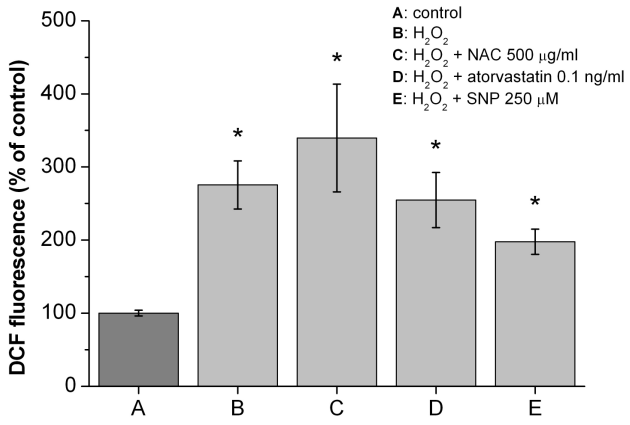

Figure 13 ROS production in hCMEC/D3 cells after 4 h of incubation with 0.1% H_2O_2. Data are means ± SEM of n=4; *: $p<0.05$.

Treatment of the cells with NAC (5, 50, 100, 500, 1000 µg/ml) for 4 hours resulted in a dose-dependent decrease of ROS production (Figure 14). The reduction of ROS generation was statistically significant at all tested NAC concentrations. Compared to the untreated control the DCF fluorescence was reduced by 46% after treatment with the lowest NAC concentration (5 µg/ml), whereas the highest NAC concentration (1000 µg/ml) reduced the DCF fluorescence by 65%.

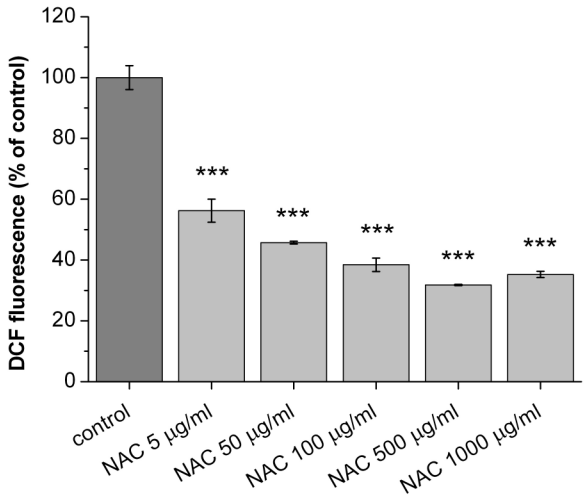

Figure 14 ROS production in hCMEC/D3 cells after 4 h of incubation with NAC (5, 50, 100, 500, 1000 µg/ml). Data are means ± SEM of n=4; *: p<0.0005.

Figure 15 shows a curve-fit and the calculated ED_{50} value (half maximal effect) of the reduced ROS generation after the dose-dependent NAC treatment. The ED_{50} of NAC was observed at a concentration of 1.91 µg/ml.

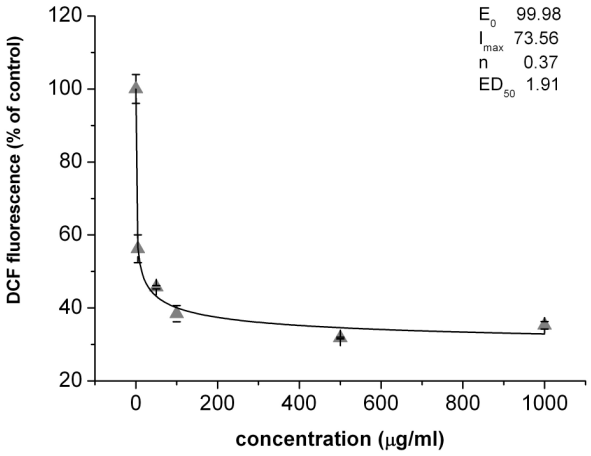

Figure 15 ED_{50} curve of ROS production in hCMEC/D3 cells. ED_{50} of NAC = 1.91 µg/ml. Data are means ± SEM of n=4.

Dose-dependent incubations with SNP (100, 250, 500 µM) decreased the ROS generation in hCMEC/D3 cells significantly (Figure 16). At a concentration of 250 µM SNP the DCF fluorescence intensity was reduced by 47% compared to the untreated control. In contrast, dose-dependent treatment of the cells with atorvastatin (0.1, 1, 10,

100 ng/ml) was not associated with a decrease in ROS generation (Figure 17).

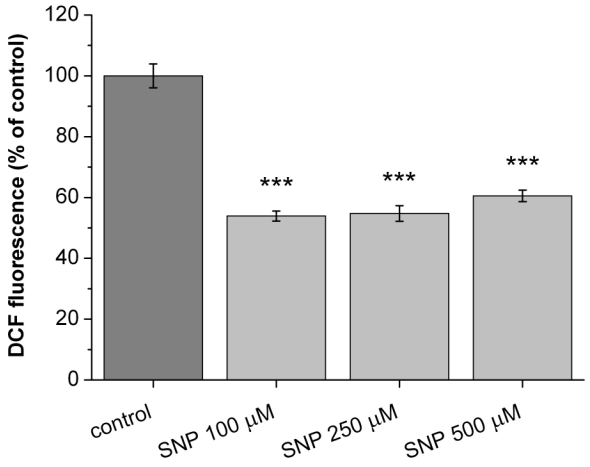

Figure 16 ROS production in hCMEC/D3 cells after 4 h of incubation with SNP (100, 250, 500 µg/ml). Data are means ± SEM of n=4; *: p<0.0005.

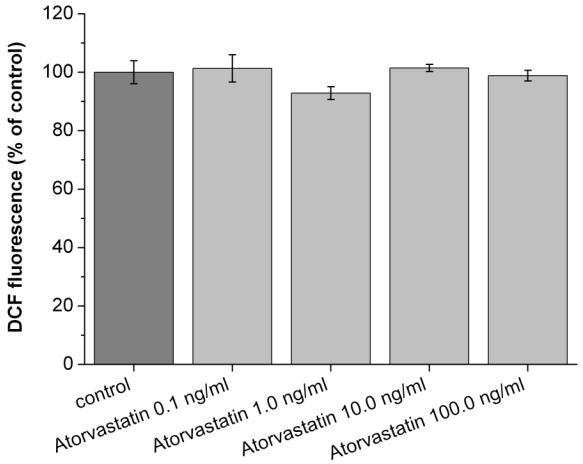

Figure 17 ROS production in hCMEC/D3 cells after 4 h of incubation with atorvastatin (0.1, 1, 10, 100 ng/ml). Data are means ± SEM of n=4.

6.4.4 Immunostaining of ZO-1 under influence of NAC, SNP and atorvastatin

Immunostainings of ZO-1 in hCMEC/D3 cells were performed in the presence of NAC, SNP and atorvastatin using the polyclonal antibody (rabbit anti ZO-1) against ZO-1. An intense staining was observed in the untreated control cells as shown in Figure 18 indicating the expression of ZO-1. The strongest intensity was found at the cell borders, but also a cytosolic staining of weaker intensity was observed. After treatment with NAC in a concentration of 100 µg/ml an altered ZO-1 expression pattern was observed. The

cells showed a generally stronger staining of ZO-1 compared to the control cells. ZO-1 expression was not only increased at the cell borders but also in the cytosol. Furthermore, the treated cells showed a more homogeneous morphological structure than the untreated control cells. Treatment of hCMEC/D3 cells with SNP (250 µM) and atorvastatin (1 ng/ml) caused also an increase in ZO-1 expression at the cells borders as well as within the cells.

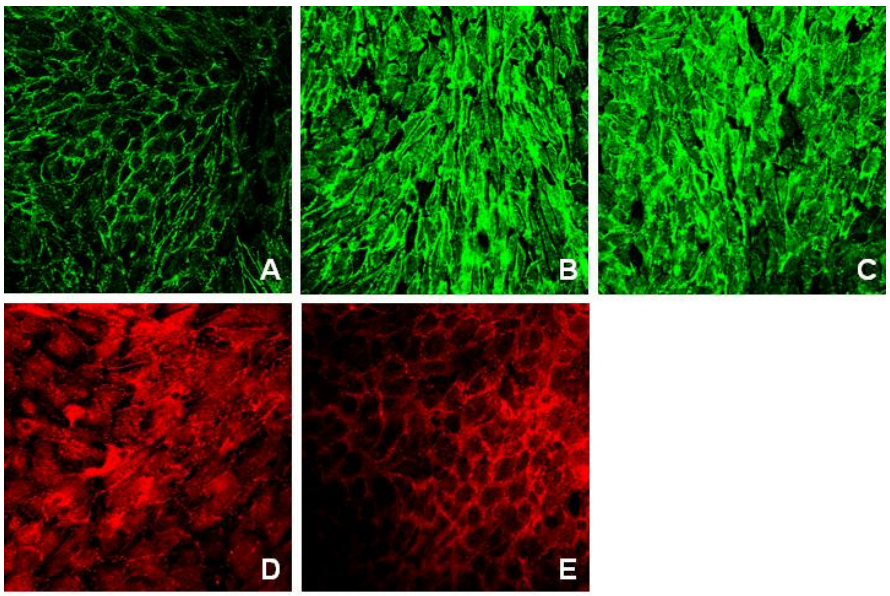

Figure 18 Confocal laser microscopy pictures of ZO-1 in hCMEC/D3 cells under different incubation conditions (40x magnification, A-C secondary Cy2 labeled AB goat anti-rabbit, D-E secondary Cy3 labeled AB goat anti-rabbit). A: untreated control of SNP and atorvastatin, B: 24h incubation with 250 µM SNP, C: 72h incubation with 1 ng/ml atorvastatin, D: untreated control of NAC, E: 72h incubation with 100 µg/ml NAC.

6.5 Discussion

The human BBB formed by endothelial brain capillary cells is characterized by a low paracellular permeability. TJs seal the intercellular gaps and restrict the passive diffusion of hydrophilic compounds. Therefore, low paracellular permeability is a crucial feature for every *in vitro* model of the BBB in order to perform transport studies. In this study the recently developed hCMEC/D3 cell line was used as an *in vitro* model of the human BBB. The hCMEC/D3 cells exhibit important BBB characteristics such as the expression of TJ molecules, chemokine receptors, drug efflux transporters and a low permeability (given as P_e) for the small hydrophilic tightness marker sucrose (Weksler et al. 2005; Poller et al. 2008). It was demonstrated that replacing FCS by human serum reduced significantly the sucrose permeability in the hCMEC/D3 (Poller et al. 2008). In addition it is known

that the molecular composition of TJs can be modulated under physiological and pathophysiological conditions leading to permeability changes (Abbott et al. 2006). Therefore, the aim of the present study was to reduce the paracellular permeability of hCMEC/D3 line monolayers by different medium supplements such as anti-inflammatory drugs, growth factor or antioxidants.

In an *in vivo* rat model a disruption of the BBB was observed after injection of the pro-inflammatory cytokine TNF-α. The barrier breakdown was accompanied by an induction of cyclooxygenase-2 (COX-2). The BBB disruption as well as the COX-2 up-regulation could be inhibited with the unspecific COX-inhibitor indomethacin (Candelario-Jalil et al. 2007). Therefore, we investigated the effect of indomethacin, the more COX-2 specific inhibitor diclofenac, and the TNF-α antagonist thalidomide on the tightness of hCMEC/D3 monolayers. The anti-inflammatory drugs were applied at pharmacological concentrations. In our preliminary experiments we did not observe an improvement of BBB tightness with any of the examined anti-inflammatory compounds. In our model the cells were maintained in absence of an inflammatory stimulus. This observation corresponds to the recently published data of Cucullo et al. In their dynamic *in vitro* BBB model using the hCMEC/D3 cell line, flow cessation and reperfusion caused a biphasic opening of the BBB. Pre-treatment with ibuprofen partially prevented BBB opening, whereas control cells under flow treated with ibuprofen showed no change in transendothelial resistances (Cucullo et al. 2008).

IFN-β1b is the standard treatment for patients with relapsing-remitting multiple sclerosis. During the relapses a breakdown of the BBB occurs followed by leukocyte infiltration into the brain and a demyelization process (Kraus and Oschmann 2006). The breakdown is likely to be caused by effects of soluble inflammatory mediators such as interferon-γ (IFN-γ), TNF-α, interleukin-1β, MMP-7, MMP-9 and nitric oxide. In contrast, IFN-β1b has a stabilizing effect on the BBB and leads to a down-regulation of pro-inflammatory cytokines and to an up-regulation of anti-inflammatory cytokines. Furthermore, it could be demonstrated that IFN-β1b reduces the permeability of inulin and sucrose through monolayers of primary bovine BCEC *in vitro* (Kraus et al. 2004). In our experiments IFN-β1b was applied to the hCMEC/D3 cells at pharmacological concentrations. No reduction of sucrose permeability at any of the used concentration was observed. None of the concentrations of IFN-β1b did significantly lower the sucrose P_e values in comparison to the control cells. This might be caused by the absence of an inflammatory stimulus in the hCMEC/D3 cells. Further investigations are needed to clarify this question.

MMPs, mainly MMP-7 and MMP-9 are involved in the acute breakdown of the BBB during relapses of multiple sclerosis. They degrade the collagen of the basal lamina, which is the basement of the endothelial cells (Yang et al. 2005). Furthermore, MMPs can cleave IFN-β1b, which is used for the therapy of multiple sclerosis, resulting in a reduced efficacy of the therapy. Minocycline a member of the tetracycline antibiotics is an inhibitor of the MMPs with high potency for MMP-9 (Brundula et al. 2002; Nelissen et al. 2003). In our experiments, we did not observe reduced sucrose permeability after incubating the hCMEC/D3 cells with minocycline. Also sucrose P_e values of cells treated with the combination of minocycline and IFN-β1b were not significantly different from controls. These findings might indicate that the MMPs had no negative effect on the monolayer or were not released at sufficient levels.

In vivo the BCEC are surrounded by astrocytes and pericytes. These cells release growth factors that modulate formation of the TJs (Abbott et al. 2006). It was shown that TGF-β1 a soluble factor produced by pericytes, could reduce sodium fluorescin permeability in the MBEC4 mouse cell line (Dohgu et al. 2005). GDNF is another soluble growth factor, which is released by glial cells that surround BCEC. In a previous publication GDNF induced an immediate increase in transendothelial resistance of primary porcine BCEC monolayers (Igarashi et al. 1999). The effect was only observed when GDNF was applied in the presence of the cAMP analogue CPT-cAMP in combination with the phosphodiesterase inhibitor, RO20-1724. In the hCMEC/D3 cell line TGF-β1 and GDNF were investigated at the same concentrations as described by Dohgu et al. and Igarashi et al. However, no reduction of sucrose permeability was observed in the hCMEC/D3 cells in our experiments. Furthermore, no decrease of sucrose permeability compared to the control was observed when GDNF was added either alone or in combination with the cAMP analogue.

Recently, it has been found that the BBB in rats became leaky for small molecular weight molecules during a streptozotocin-induced period of diabetes. The observed disruption could partly be prevented under insulin treatment (Huber et al. 2006; Hawkins et al. 2007). However, our study revealed no permeability decreasing effects of insulin on hCMEC/D3 monolayers. For our experiments the cells were not maintained under hyperglycemic conditions. This fact might explain why the supplementation with insulin did not improve the tightness of the hCMEC/D3 cells.

Based on a publication of Franke et al. (Franke et al. 1999) we also investigated the effect of selenous acid and the growth hormone EGF on the hCMEC/D3 cells. In the model of Franke et al., using isolated primary porcine BCEC, high electrical resistances

were reported while EGF and selenous acid were contained in the culture medium. However, it was not shown if these supplements were necessary in order to reach high electrical resistances. Although applied at the same concentration range as published, selenous acid and EGF had no beneficial effect on the monolayer tightness in our model. The data obtained from selenous acid experiments are preliminary.

Heparin is also a common medium supplement which has been used in different models of the BBB (Gaillard et al. 2001; Megard et al. 2002). So far there are no studies that investigated the effect of heparin on the paracellular permeability of BCECs. In our preliminary experiment, no beneficial effect on the monolayer tightness was observed, when heparin was added to the culture medium.

Recently, the generation of ROS was described to disrupt the integrity of the BBB *in vitro*. Schreibelt et al. observed a reduced expression of the TJ molecules occludin and claudin-5 and a decreased transendothelial resistance of confluent monolayers. The human and rat cell lines hCMEC/D3 and GP8 respectively served as models of the BBB (Schreibelt et al. 2007). Our aim was to investigate if antioxidant compounds can improve the integrity of hCMEC/D3 monolayers. Therefore, the cells were treated with compounds that are described to reduce the generation of ROS such as SNP, atorvastatin, simvastatin and NAC.

In endothelial cells NO is generated by the nitric oxide synthase (NOS), where it regulates the blood flow through the activation of NO-sensitive guanylyl cyclases and the generation of cycliyc guanosin monophosphate (cGMP). Furthermore, based on its structure as a free radical, NO can scavenge ROS by reactions of unpaired electrons (Chiueh 1999). By binding to iron complexes NO also reduces the production of ROS that are generate during redox reaction in the respiratory chain (Chiueh 1999; Foster et al. 2003).

Effects of NO releasing compounds on permeability properties have been investigated in different models of the BBB. The changes of permeability depend on the redox state of NO (NO(*), NO(+), NO(-)) released by the donor compound. Whereas NO(+) and NO(-) cause a disruption of the BBB in rats, no permeability changes are reported for the NO(*) radical (Boje and Lakhman 2000). *In vitro* NO(*) releasing compounds had decreased the permeability of isolated primary porcine BCEC monolayers (Winter et al. 2008). SNP being also a member of the NO(*) radical releasing compounds was reported to increase the tightness of monolayers of primary human BCEC. It was postulated that the

production of cGMP by the guanylyl cyclase and activation of cGMP-dependent protein kinase were involved in the observed process (Wong et al. 2004).

For that reason, we investigated the effect of SNP in our hCMEC/D3 model. After treatment with SNP in a concentration of 250 µM the sucrose permeability was significantly reduced. To investigate if this effect was cGMP-depended the hCMEC/D3 cells were incubated with 8-Br-cGMP, a cGMP-analogue. However, 8-Br-cGMP did not affect the tightness in our model.

Based on the antioxidant effects of NO(*) we investigated the effect of the NO-releaser SNP on the generation of ROS in the hCMEC/D3 cells. After treatment with SNP (250 µM) a significantly reduced DCF fluorescence was measured compared to control cells, indicating reduced intracellular ROS levels compared to the control. This result supports the thesis that NO exerts protective effects on the BBB based on its antioxidant properties.

In a next step the expression of ZO-1 under influence of SNP was investigated by immunocytochemistry. A generally more intense staining of ZO-1 was observed compared to the untreated control cells. However, ZO-1 was not only increased at the cell borders but also in the cytosol.

Recent studies revealed evidence that statins stabilize the BBB. It was shown that lovastatin, simvastatin and fluvastatin decreased the tightness of BCEC monolyers *in vitro* (Ifergan et al. 2006; Kuhlmann et al. 2006). Furthermore, statins exert anti-inflammatory effects by reducing the expression of cytokines such as TNF-α and interleukin-6 (Youssef et al. 2002). Statins also show antioxidant properties such as reducing lipoprotein oxidation and the generation of ROS (Vaughan and Delanty 1999). In our study the influence of atorvastatin and simvastatin on the monolayer tightness was investigated. In addition, the effect of atorvastatin on the generation of ROS and the protein expression of ZO-1 was assessed. When applied at pharmacological concentrations atorvastatin significantly decreased the sucrose permeability in the hCMEC/D3 cell line. In contrast sucrose P_e values were not affected by treatment with simvastatin.

Therefore, we investigated the generation of ROS in the hCMEC/D3 cells after treatment with atorvastatin. In a preliminary experiment no statistically significant decrease of ROS generation was observed compared to the untreated control. This result indicates that reduced paracellular permeability caused by atorvastatin is not dependent on antioxidant effects. However, these preliminary results need to be confirmed.

In a next step, the expression of ZO-1 was investigated after atorvastatin treatment using immunocytochemistry. Compared to untreated control cells atorvastatin caused a generally stronger staining of ZO-1, indicating an increased protein expression. However, ZO-1 expression was increased at the cell border as well as in the cytosol. An increased ZO-1 expression at the cells borders might lead to a higher paracellular resistance of the model.

Another known antioxidant agent and radical scavenger is NAC, which protects cells against OH radicals and H_2O_2. NAC is a derivative of the amino acid cysteine, which is an essential precursor for the synthesis of glutathione (GSH). Consequently deacetylation of NAC increases the intracellular level of cysteine and GSH, which in turn is responsible for maintaining the cellular redox status in endothelial cells and provides antioxidant protection against ROS. Therefore, NAC has a highly antioxidant potential, which was shown in several studies (Townsend et al. 2003; Atkuri et al. 2007). An structure analogue of NAC was shown to reduce the ROS generation in brain endothelial cells (Price et al. 2006).

Based on these properties, we investigated the effect of NAC on the paracellular tightness in the hCMEC/D3 cell line by determining the sucrose permeabilty. The P_e values for sucrose after NAC treatment in a concentration of 50 µg/ml and 5 µg/ml were significantly reduced as compared to the control.

We also investigated the hypothesis that ROS production is reduced after NAC treatment. Therefore, we determined the intracellular ROS generation in hCMEC/D3 cells. Treatment with NAC resulted in a dose-dependent decrease of DCF production. The greatest reduction was observed at a NAC concentration of 1000 µg/ml. The ED_{50} value of NAC was calculated at a concentration of 1.91 µg/ml. H_2O_2, a well-known ROS producer, served as a positive control for the generation ROS measurement the cells (Arbogast and Reid 2004). Compared to the control H_2O_2 induced ROS generation in our experiment by 275%. However, this induction was not reversible by adding NAC, atorvastatin or SNP. Possibly, the H_2O_2 induced intracellular ROS generation was too high to be suppressed by the NAC.

ZO-1 immunostainings were performed in order to assess the effect of NAC on the molecular assembly of tight junctions. At a concentration of 100 µg/ml NAC the staining of ZO-1 was more intense indicating higher expression levels. As observed for treatment with atorvastatin the ZO-1 expression was increased at cell borders as well as in the cytosol, may giving an indication for a higher resistance of the model.

In conclusion, we could demonstrate that the antioxidant compounds NAC, SNP and atorvastatin reduced the permeability of the paracellular marker sucrose in the hCMEC/D3 cell line. Furthermore, we showed a suppressed generation of ROS and increased levels of ZO-1 under influence of NAC and SNP, which indicates that intracellular ROS levels might influence the composition of TJ. However, information on other tight junctional components is needed to conclusively determine this question.

All other tested compounds including the anti-inflammatory drugs diclofenac, indomethacin and thalidomide, IFN-β1b, the glial cell-derived growth factors (TGFβ1, GDNF, EGF), insulin, heparin, and selenous acid, had no effect on the permeability of hCMEC/D3 monolayers.

7 Regulation of BCRP (ABCG2) and P-glycoprotein (ABCB1) by cytokines in a model of the human blood-brain barrier

Birk Poller[1], Jürgen Drewe[1], Stephan Krähenbühl[1], Jörg Huwyler[2], Heike Gutmann[3]

[1] Dept. of Clinical Pharmacology and Toxicology, University Hospital of Basel, 4031 Basel, Switzerland

[2] University of Applied Sciences Northwestern Switzerland, Dept. of Pharmaceutical Technology, 4132 Basel, Switzerland

[3] Novartis Pharma AG, Klybeckstrasse 141, CH-4057 Basel, Switzerland

submitted to

Biochemical Pharmacology

7.1 Abstract

Brain capillary endothelial cells form the blood-brain barrier (BBB), a highly selective permeability membrane between the blood and the brain. Tight junctions that prevent small hydrophilic compounds from passive diffusion into the brain tissue and endothelial cells express different families of drug efflux transport proteins that limit the amount of their substrates that can penetrate the brain. Two prominent efflux transporters are the breast cancer resistance protein (BCRP) and P-glycoprotein (P-gp). During inflammatory reactions, which are associated with various diseases, pro-inflammatory cytokines are present in the systemic circulation. In this study the effect of the pro-inflammatory cytokines interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) on the expression and activity of BCRP and P-gp was investigated in the human hCMEC/D3 cell line. BCRP mRNA levels were significantly reduced by IL-1β, IL-6 and TNF-α. The strongest BCRP suppression at the protein level was observed after IL-1β treatment. IL-1β, IL-6 and TNF-α significantly reduced the BCRP activity as assessed by mitoxantrone uptake experiments. P-gp mRNA levels were slightly reduced by IL-6 but significantly increased after TNF-α treatment. TNF-α also increased the protein expression of P-gp.

This *in vitro* study indicates that expression levels of BCRP and P-gp at the BBB might be altered during acute or chronic inflammation, resulting in changes in the extent to which their substrates can penetrate the brain.

7.2 Introduction

The brain capillary endothelial cells (BCEC) are the main constituents the blood-brain barrier (BBB), a highly selective permeability barrier that protects the brain tissue from infiltration of potentially toxic compounds and xenobiotics from the bloodstream. The passive diffusion of hydrophilic molecules into the brain is thereby prevented by tight junctions, which seal the monolayer of the endothelial cells. Furthermore, various drug efflux-transporters are located at the BBB and extrude their substrates from the brain by an active transport process. These transporters belong to the superfamilies of ATP-binding cassette transporters (ABC-transporters) and solute carriers (SLC). Two prominent and well characterized representatives of the ABC-transporters are the breast cancer resistance protein (BCRP or ABCG2) and P-glycoprotein (P-gp or ABCB1) (Schinkel and Jonker 2003).

BCRP is expressed at the apical plasma membrane of the BBB, as documented for the human, porcine and mouse BBB (Cooray et al. 2002; Eisenblatter and Galla 2002; Eisenblatter et al. 2003; Cisternino et al. 2004). This transporter has been shown to be involved in the transport of drugs such as mitoxantrone, prazosin and topotecan, anthracyclines and food carcinogens (reviewed in (Loscher and Potschka 2005)). The relevance of BCRP transport *in vivo* was shown in a mdr1a(-/-) knock-out mouse model. By blocking BCRP with specific inhibitors, the brain uptake of mitoxantrone and prazosin was significantly increased, demonstrating that BCRP, and not P-gp, was involved (Cisternino et al. 2004).

P-gp was the first efflux transporter to be described at the human BBB (Cordon-Cardo et al. 1989). It is expressed at the apical membrane of BCECs, where it limits the brain uptake of a broad range of clinically used compounds such as anticancer drugs, corticosteroids or antiepileptic drugs (Schinkel et al. 1995; Loscher and Potschka 2005). The relevance of P-gp at the BBB was shown in mdr1a(-/-) knock-out mice. The absence of the transporter leads to highly increased brain levels of drugs such as digoxin, vinblastine, cyclosporine A or domperidone (Schinkel et al. 1996). The clinical relevance of this transporter is well documented (Lin and Yamazaki 2003).

Inflammation is associated with a variety of diseases, including central nervous system disorders such as multiple sclerosis, bacterial meningitis, Alzheimer's disease and Parkinson's disease. During these diseases, several changes in the morphology and permeability of the BBB have been described (de Vries et al. 1997; Huber et al. 2001; Reale et al. 2008). Cytokines are the mediators of the inflammation and high levels of tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and interleukin-6 (IL-6) are present in the brain tissue or in the systemic blood circulation during acute inflammation. In cell culture models, these cytokines were shown to disrupt the integrity of the BBB (de Vries et al. 1996; Forster et al. 2008).

The influence of inflammation or single cytokines on the expression of ABC-transporters has been investigated in tissue samples from patients and in *in vitro* cell culture systems. P-gp and BCRP, for example, are expressed at significantly lower levels during active inflammation in colonic epithelium from patients with ulcerative colitis, while expression levels of IL-1β and IL-6 mRNA were higher in biopsies from actively inflamed tissue compared to healthy controls (Englund et al. 2007; Gutmann et al. 2008). In primary placental trophoblasts, TNF-α and IL-1β decreased the mRNA and protein expression of P-gp and BCRP by 40-50% (Evseenko et al. 2007). At the BBB, there are only very limited data concerning BCRP expression and controversial reports on the regulation of

P-gp under influence of TNF-α exist. There are reports showing P-gp mRNA or protein up-regulation, whereas other studies observed a decreased P-gp function. However, the systems used for the investigations varied greatly (Theron et al. 2003; Bauer et al. 2007; Seelbach et al. 2007).

The human hCMEC/D3 cell line, which shows a very similar morphology to primary human BCECs and expresses important BBB markers such as junctional molecules, chemokine receptors and ABC-transporters served as an *in vitro* model of the human BBB (Weksler et al. 2005; Poller et al. 2008).

It was the aim of the present work to investigate the impact of the pro-inflammatory cytokines IL-1β, IL-6 and TNF-α on the expression of the ABC-transporters P-gp and BCRP. Modulatory effects were determined on the mRNA and protein level. Furthermore, the functional activity of BCRP was investigated by measuring cellular efflux experiments using BCRP substrate mitoxantrone.

7.3 Materials and methods

7.3.1 Materials

The EBM-2 medium and supplements (vascular endothelial growth factor (VEGF), insulin-like growth factor (IGF-1), epidermal growth factor (EGF), basic fibroblast growth factor (bFGF) and hydrocortisone) were purchased from Lonza (Verviers, Switzerland). All culture plasticware and Transwell® filters were from Corning (Baar, Switzerland). Rat-tail collagen type I was from Becton Dickinson (Allschwil, Switzerland). Fetal calf serum (FCS), bFGF, DNAse I, Hanks' balanced salt solution (HBSS), HEPES, penicillin-streptomycin, sodium pyruvate and SuperScript II RT-Kit were from Invitrogen (Basel, Switzerland). RNeasy Mini Kit was purchased from Qiagen (Hilden, Germany). qPCR Mastermix Plus was from Eurogentec (Seraing, Belgium). Random hexamers were from Applied Biosystems (Rotkreuz, Switzerland). dNTPs were from BioRad (Reinach, Switzerland). The murine antibody (mAb) C219 against P-gp and the mAb BXP-21 against BCR were from Alexis (Lausen, Switzerland). Rabbit anti-mouse IgG was from Dako (Baar, Switzerland). bFGF, hydrocortisone, IL-1β, Il-6, mitoxantrone and TNF-α were from Sigma (Buchs, Switzerland). FluorSave was from Calbiochem (San Diego, CA, USA). All other chemicals were of analytical grade and purchased from commercial sources.

7.3.2 Cell culture

For description see section 4.1.

7.3.3 Incubation conditions for the pro-inflammatory cytokines TNF-α, IL-1β, IL-6

The cell culture medium was replaced with FCS-reduced assay medium (0.25% instead of 2.5%) 24 h before the beginning of the incubation period. Then the hCMEC/D3 cells were treated with TNF-α (2, 20 ng/ml), IL-1β (0.2, 2 ng/ml), IL-6 (2, 20 ng/ml) in FCS-reduced assay medium for 72 h. Control cells were maintained in FCS-reduced assay medium. The medium for all treatments was replaced every 24 h. Toxicity was tested in advance for all applied substances using sulforhodamine B staining according to Skehan et al. (Skehan et al. 1990).

7.3.4 Quantitative RT-PCR TaqMan assay

For description see section 4.2.

7.3.5 Western Blot

For description see section 4.4.

7.3.6 Drug accumulation assay

For description see section 4.6.

7.3.7 Statistics

Data from the treatment groups were compared with those from the controls by analysis of variance (ANOVA) with subsequent Dunnett's multiple comparison test. All comparisons were performed using SPSS for Windows software (version 15.0, SPSS Inc., Chicago, IL, USA).

7.4 Results

7.4.1 Influence of IL-1β, IL-6 and TNF-α on BCRP and P-gp mRNA expression

hCMEC/D3 cells were treated with the pro-inflammatory cytokines IL-1β, IL-6 and TNF-α for 72 h. As shown in Figure 19, BCRP mRNA expression was reduced significantly in cells treated with IL-1β, IL-6 and TNF-α. The strongest down-regulation of BCRP mRNA

(by 45%) was observed under IL-6 treatment. IL-1β and TNF-α exhibited a reduction of BCRP gene expression by 33% and 31% compared to the untreated control cells.

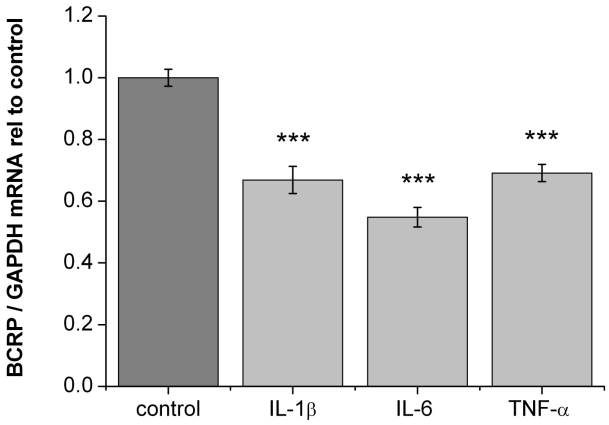

Figure 19 Relative expression of BCRP mRNA normalized to GAPDH in hCMEC/D3 cells. Data are given as means of n=6-9 ± SEM (***: p<0.0005).

The P-gp mRNA expression was measured after 72 h of incubation with IL-1β, IL-6 and TNF-α in hCMEC/D3 cells as shown in Figure 20. P-gp was down-regulated by 14% after treatment with IL-1β and by 21% after treatment with IL-6. In contrast incubation of the cells with TNF-α resulted in a highly significant increase in P-gp mRNA expression by 49% compared to the control cells.

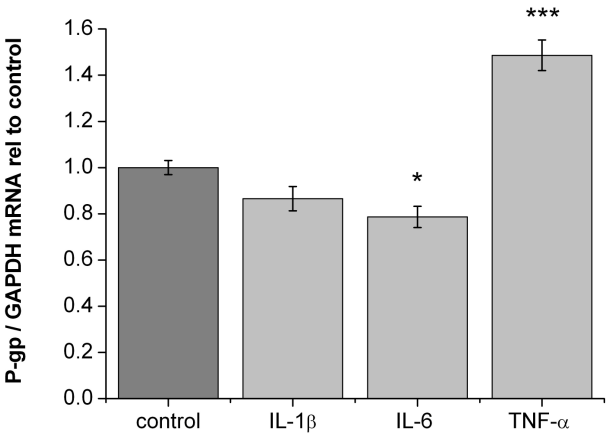

Figure 20 Relative expression of P-gp mRNA normalized to GAPDH in hCMEC/D3 cells. Data are given as means of n=6-9 ± SEM (*: p<0.05, ***: p<0.0005).

7.4.2 Influence of IL-1β, IL-6 and TNF-α on BCRP and P-gp protein expression

Expression levels of BCRP and P-gp in hCMEC/D3 cells were determined on protein level by Western blot analysis, shown in Figure 21. Immunoblotting was performed after a 72 h incubation of hCMEC/D3 cells with IL-1β, IL-6 and TNF-α. Equal protein loading was checked by Ponceau red staining after the transblot and only samples with equal protein content were compared. For BCRP, a very slight down-regulation compared to the control was observed after treatment with IL-6. The down-regulation was more pronounced with IL-1β and TNF-α. The protein levels of P-gp were not changed under influence of IL-6 and IL-1β. TNF-α exerted a clear increase in P-gp protein expression.

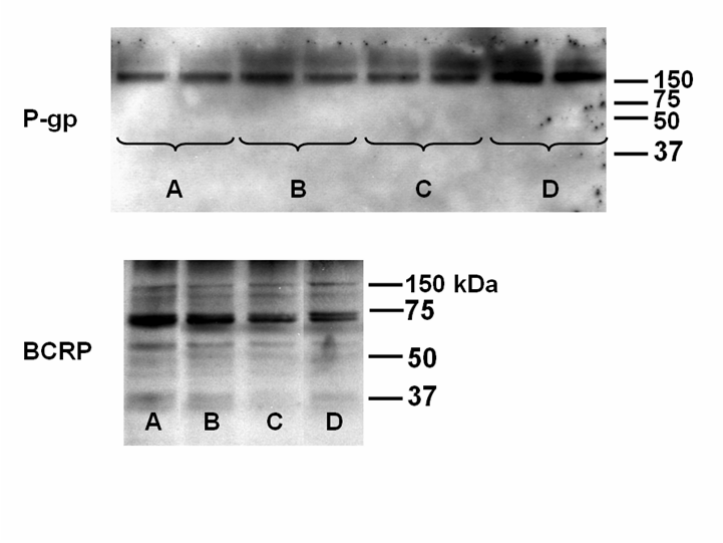

Figure 21 Protein expression of BCRP and P-gp in hCMEC/D3 cells under influence of reference treatment (A), IL-6 (B), IL-1β (C) and TNF-α (D).

7.4.3 Mitoxantrone (MX) accumulation under influence of cytokines

To confirm that the cytokine-induced changes in BCRP mRNA expression are also reflected in an altered transport function, efflux studies were performed using the BCRP substrate mitoxantrone. Prior to the drug accumulation experiments the hCMEC/D3 cells were incubated for 72 h with IL-1β, IL-6 and TNF-α. The BCRP inhibitor prazosin was used as a positive control. An increase in the MX accumulation by 11% was observed in the presence of prazosin as shown in Figure 22. Pretreatment with IL-1β at a concentration of 2 ng/ml caused a statistically significant increase in intracellular MX amount by 57% in hCMEC/D3 cells compared to the control. At a concentration of 0.2 ng/ml the intracellular MX showed a not statistically significant elevation of 22% as compared to control.

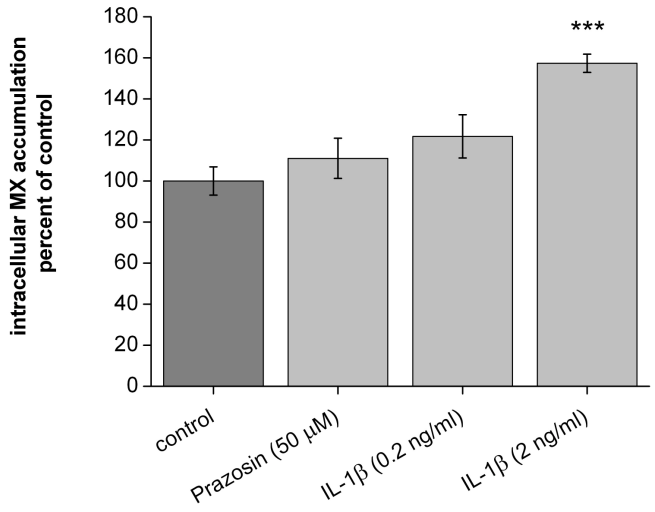

Figure 22 Accumulation of MX in hCMEC/D3 cells after 72 h pretreatment with IL-1β (0.2, 2 ng/ml), in percent of control. Data are given as means of n=8 ± SEM (***: p<0.0005).

Pretreatment of the hCMEC/D3 cells with IL-6 resulted in significantly higher intracellular MX amounts as compared to untreated control cells (Figure 23). At a concentration of 2 ng/ml IL-6 the accumulation was increased by 120% and at a concentration of 20 ng/ml IL-6 by 96%. Acute prazosin inhibition reduced the BCRP-mediated MX efflux in untreated hCMEC/D3 cells and resulted in a significantly higher intracellular MX accumulation (80%) as compared to the control.

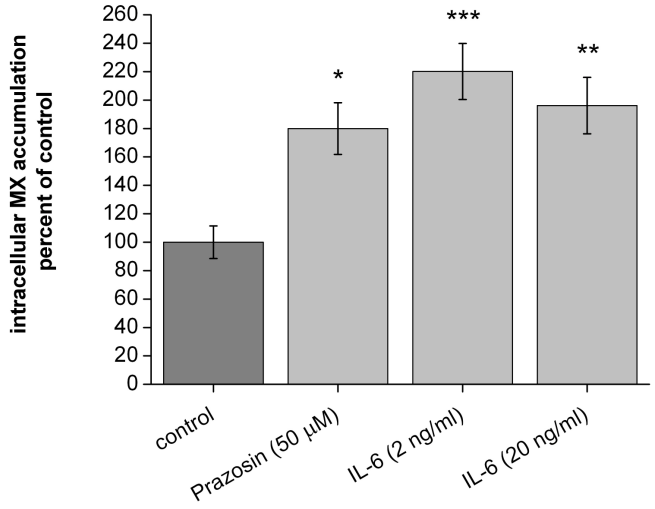

Figure 23 Accumulation of MX in hCMEC/D3 cells after 72 h pretreatment with IL-6 (2, 20 ng/ml), in percent of control. Data are given as means of n=7-9 ± SEM (*: p<0.05, **: p<0.005, ***: p<0.0005).

After incubation of hCMEC/D3 cells with TNF-α at concentrations of 2 and 20 ng/ml a significantly higher MX accumulation was observed as compared to the control cells (Figure 24). At a TNF-α concentration of 2 ng/ml and 20 ng/ml the intracellular MX amount was increased by 96% and 69%, respectively. Only at a concentration of 2 ng/ml was a statistically significant increase observed. Prazosin inhibited the MX efflux in untreated cells, resulting in a 53% higher intracellular accumulation as compared to the control.

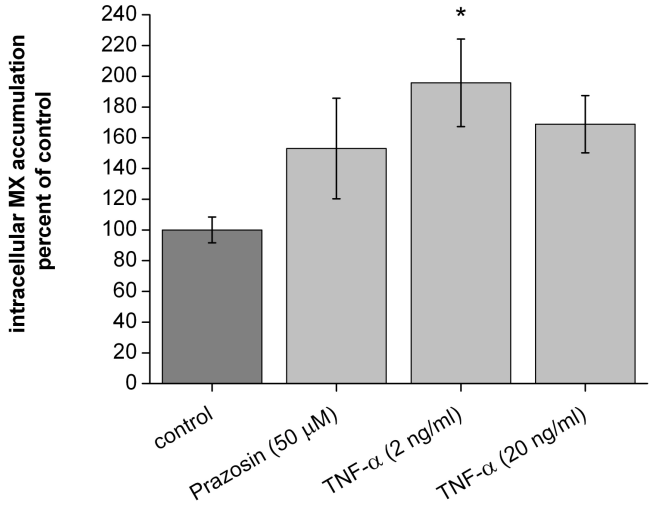

Figure 24 Accumulation of MX in hCMEC/D3 cells after 72 h pretreatment with TNF-α (2, 20 ng/ml), in percent of control. Data are given as means of n=7-9 ± SEM (*: p<0.0005).

7.5 Discussion

In this study, the effect of the pro-inflammatory cytokines IL-1β, IL-6 and TNF-α on expression and function of the ABC-transporters BCRP and P-gp was investigated using an *in vitro* model of the human BBB, namely the immortalized human brain endothelial cell line hCMEC/D3. These cells were shown previously to preserve many characteristics of the BBB. In particular, functional expression of several ABC-transporters was shown including the well characterized BCRP and P-gp (Poller et al. 2008).

Up to now, little was known about the influence of the pro-inflammatory cytokines on the expression of BCRP, especially at the level of the BBB. In two studies, mRNA and protein expression of BCRP and P-gp was measured in colon biopsies from patients with ulcerative colitis (Englund et al. 2007; Gutmann et al. 2008). Both studies found a significant down-regulation of BCRP and P-gp in inflamed tissue. Englund et al. also reported markedly increased mRNA levels of IL-1β and IL-6 in inflamed tissue. The

authors of both studies concluded that the suppression of BCRP and P-gp expression was likely to be caused by the inflammatory process. In primary placental trophoblasts the same concentrations of IL-1β and TNF-α as used in our study reduced the BCRP mRNA expression (Evseenko et al. 2007). Similar findings were obtained in the present study, which showed a significant down-regulation of BCRP and P-gp in a cell-culture model of the BBB. In the latter study, down-regulation of mRNA by TNF-α resulted in a reduced functional activity of BCRP as demonstrated by an increased accumulation of MX. The authors showed, in addition that IL-6 caused a weak but insignificant down-regulation of BCRP (Evseenko et al. 2007). At the BBB level, there exists a diverging report that TNF-α does not change the Bcrp protein expression in freshly isolated rat brain capillaries (Bauer et al. 2007). In contrast to Bauer et al. we observed a down-regulation of BCRP expression in the hCMEC/D3 cells. However, compared to Bauer et al. the TNF-α concentration used for our studies was higher (1 ng/ml vs. 20 ng/ml TNF-α in this study) and the incubation period was longer (3-6 h vs. 72 h in this study), which may explain the difference. The reduction in BCRP mRNA expression in the hCMEC/D3 cells was reflected in the protein level even though it was not very pronounced. However, a significantly reduced intracellular accumulation of the BCRP substrate MX was observed under influence of each of the tested cytokines, indicating reduced BCRP activity. MX efflux was reduced to a similar magnitude in cells pre-treated with the cytokines as in untreated cells inhibited with prazosin during the efflux period. Compared to findings in primary trophoblasts, 20 ng/ml TNF-α increased the intracellular MX accumulation in the hCMEC/D3 cells to a greater extent (70% vs 15%) (Evseenko et al. 2007). This might be explained by a higher basal expression level of BCRP at the BBB compared to primary trophoblasts.

P-gp mRNA and protein expression were significantly up-regulated after treatment with TNF-α, whereas IL-1β and by IL-6 did not change P-gp expression levels. The effect of the pro-inflammatory cytokines, in particular TNF-α, on the expression of P-gp is discussed controversially. In the study of Englund et al. the down-regulation of BCRP was accompanied by a suppression of P-gp mRNA and protein, while IL-1β and IL-6 mRNA levels were elevated (Englund et al. 2007). In isolated primary placental trophoblasts a down-regulation of P-gp under the influence of TNF-α and IL-1β was also reported (Evseenko et al. 2007). In BCEC several studies observed an increased P-gp expression after exposure to TNF-α corroborating our finding. In the GPNT mouse BBB cell line, 10 ng/ml TNF-α caused an increase in mdr1a after 6 h and of mdr1b after 24 h. In contrast, the P-gp activity was decreased after TNF-α treatment for 48 and 72 h (Theron et al. 2003). This controversial finding might be caused by differences between

species. For P-gp, Bauer et al. found an up-regulated expression in isolated rat brain capillaries after treatment with low concentrations of TNF-α. Whereas P-gp activity was reduced for the first 4 h of the treatment, a 2-fold increase in the activity was measured after 6 h of TNF-α exposure, accompanied by elevated P-gp protein levels. The authors (Bauer et al. 2007) postulated an endothelin-dependent regulation of P-gp and showed the involvement of the nitric-oxide synthase (NOS), protein kinase C (PKC) and nuclear factor-κB (NFκB) in the down-stream regulation pathway. The induction of P-gp mRNA and protein was accompanied by a 2- to 3-fold increase in luminal cyclosporine accumulation. Together with these reports our findings indicate that TNF-α may increases the expression of P-gp at the BBB.

TNF-α, IL-1β and IL-6 are early mediators during the inflammatory response. Elevated systemic cytokine levels are linked to many diseases during which inflammation occurs, such as multiple sclerosis, bacterial meningitis, Alzheimer's disease and Parkinson's disease. The cytokines can either be released by cells of the CNS or can reach the BCECs from the systemic circulation.

In a cell BBB culture model using primary rat BCECs, TNF-α, IL-1β and IL-6 were shown to disrupt the integrity of the BBB in a dose-dependent manner. However, those cytokines did not disturb the monolayer tightness in their model, when doses were used as in our experiments (de Vries et al. 1996; Forster et al. 2008). Chronically increased levels of cytokines could therefore lead to an altered expression of BCRP and P-gp without an opening of the BBB.

Most interestingly, we observed a distinct down-regulation of BCRP mRNA expression after treatment with each of the investigated cytokines. Although the effect was not very pronounced on the protein level, the accumulation of MX was significantly increased after cytokine treatment, indicating a lower transport activity of BCRP. In this study we showed for the first time a down-regulation of BCRP expression in an *in vitro* model of the human BBB under influence of IL-1β, IL-6 and TNF-α. Treatment of hCMEC/D3 cells with these cytokines decreased BCRP mRNA and protein expression and resulted in a decreased BCRP activity. The expression of P-gp was not altered by IL-1β and IL-6 but mRNA and protein levels were increased by TNF-α.

7.6 Acknowledgements

We thank F. Hoffmann-La Roche Ltd. and the Senglet foundation for financial support.

8 Isolated project: Pharmacokinetics and pharmacodynamic effects of oral GLP-1 and PYY3-36: a proof of concept study in healthy subjects

C Beglinger[1,2], B Poller[1,3], E Arbit[1], C Ganzoni[1], S Gass[1], I Gomez-Orellana[4] and J Drewe[1,3].

[1]Clinical Research Center, Department of Research, University Hospital, Basel, Switzerland

[2]Division of Gastroenterology, Department of Medicine, University Hospital, Basel, Switzerland

[3]Department of Clinical Pharmacology and Toxicology, University Hospital, Basel, Switzerland

[4]Emisphere Technologies, Tarrytown, New York, USA

Clinical Pharmacology & Therapeutics

2008;**84**,4:468–474.

8.1 Introduction

A number of peptides released from the gastrointestinal tract have recently been shown to regulate appetite and food intake. Peptide YY3-36 (PYY3-36) has been shown to induce satiety and to reduce food intake in human and rodents (Batterham et al. 2002; Batterham et al. 2003). Glucagon-like peptide-1 (GLP-1) acts mainly as an incretin hormone, promoting postprandial insulin release and improving pancreatic β-cell function, but it has also been reported to inhibit food intake in humans (Gutzwiller et al. 1999). Current therapeutic routes of application (subcutaneous injections or nasal sprays) do not mimic the physiological release of these hormones. New technologies have made it possible to use the oral route as a pathway to deliver large peptides into the systemic circulation (Fix 1996; Goldberg and Gomez-Orellana 2003; Ding et al. 2004). Up to now, GLP-1 and PYY3-36 have not been administered orally. The aim of this study was to evaluate the pharmacological profile and the insulin-releasing activity of increasing single oral doses of GLP-1 in healthy male volunteers. We also obtained a dose–response curve relating to oral administration of PYY3-36 in healthy male subjects.

8.2 Methods

The study was conducted as a phase I, open, placebocontrolled, dose-escalating study. Six healthy male subjects were given oral doses of either a placebo or GLP-1 in a dose-escalating schedule (doses of 0.5, 1.0, 2.0, and 4.0 mg). Next, another group of six healthy male subjects were given oral doses of either a placebo or PYY3-36 in the same pattern of escalating doses (doses of 0.25, 0.5, 1.0, 2.0, and 4.0 mg). Peptide tablets were prepared mixing either GLP-1 or PYY3-36 with 200 mg of a new delivery agent, sodium N-[8-(2-hydroxybenzoyl)amino] caprylate (SNAC).

8.3 Results

The lowest dose of GLP-1 did not produce a relevant pharmacokinetic profile (Figure 25) Higher oral doses of GLP-1 induced a dose-dependent increase in plasma drug concentrations; an exception was the 2 mg dose. Mean plasma drug concentrations peaked at between 15 and 30 min after oral administration. For PYY3-36, the absorption was rapid, with mean plasma drug concentrations peaking between 15 and 17.5 min after administration Figure 25. For GLP-1, the absolute bioavailability could be calculated from the oral and intravenous routes of administration and ranged from 1.0 to 9.8%; the bioavailability was not dose dependent.

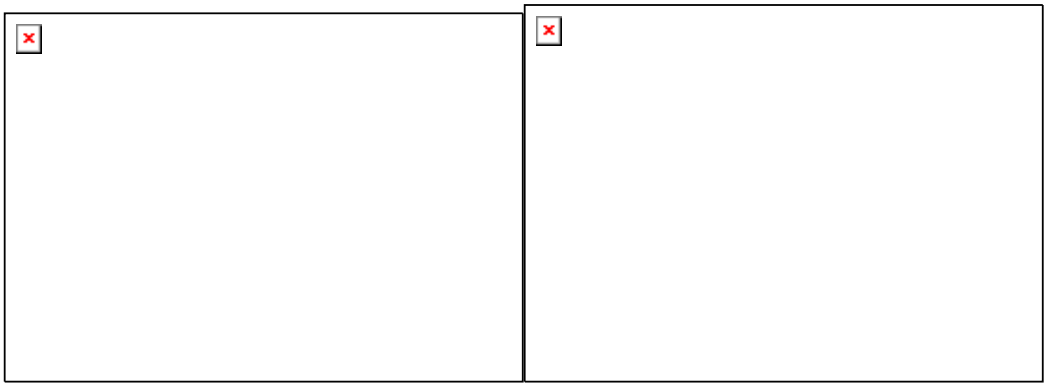

Figure 25 Plasma kinetics of glucagon-like peptide (GLP-1) (left figure). Plasma concentrations of GLP-1 after oral administration of increasing doses of GLP-1. n = 6. Plasma concentrations of peptide YY3-36 (PYY3-36) (right figure) after oral administration of increasing doses of PYY3-36. n = 6. Data represent means ± SEM.

Furthermore, orally administered GLP-1 at doses higher than 0.5 mg stimulated insulin release significantly. Insulin release peaked at ~45–77 min after oral drug administration. Orally administered GLP-1 also exhibited a dose-dependent trend for inhibition of ghrelin secretion. The inhibition of ghrelin release was observed to last for ~60 min after oral administration of the GLP-1 peptide at doses higher than 0.5 mg. Orally administered PYY3-36 exhibited a dose-dependent clear trend for inhibition of ghrelin secretion. As in the case of GLP-1, for PYY3-36 also, the trend of inhibition of ghrelin secretion appears to correlate well with the plasma concentrations of the administered peptide.

Beside brief phases of nausea and/or abdominal discomfort and 2 episodes of vomiting at higher doses, both peptides were well tolerated.

8.4 Discussion

The pharmacokinetics of both peptides, reveal several important aspects. The peptides are readily absorbed from the gastrointestinal tract, with peak concentrations achieved on average within 15–35 min after the oral dose. A mean absolute bioavailability of 4% (range 1–9.8%) was reached relative to intravenous administration of GLP-1. The mean peak concentrations in plasma after the highest dose of GLP-1 (4.0 mg) were several times higher after oral administration than after intravenous administration. The plasma concentrations obtained after higher oral doses of GLP-1 are therefore clearly pharmacological concentrations and should be further explored therapeutically. The mean peak PYY concentrations in plasma after oral doses of PYY3-36 >0.5 mg produced plasma levels that were above those seen after a 1,500 kcal meal in a cohort of 50 healthy subjects (CB, JD, unpublished data). The plasma concentrations obtained after these oral doses of PYY3-36 are therefore clearly pharmacological concentrations with potential for therapeutic application. The potential clinical applications of oral GLP-1 include therapy for type 2 diabetes and obesity, whereas PYY3-36 could lead to reduction in food intake and promote weight loss.

Macromolecules, especially peptides, have a low oral bioavailability because of their molecular size and physicochemical characteristics and the rapid degradation by digestive enzymes in the gastrointestinal tract (Woodley 1994; Bernkop-Schnurch and Walker 2001; Gomez-Orellana 2005). Recently, the delivery agent–based approach has been proposed to overcome some of these problems. The technology is based on the development of small organic molecules, termed “carriers”, that interact noncovalently with macromolecules to enable their oral absorption. In this study, the peptides were mixed with the delivery agent SNAC to transport the peptides across the intestinal epithelium. SNAC binds noncovalently to the two peptides, thereby increasing their lipophilicity. These interactions are reversible, enabling the carrier molecule and the drug to dissociate readily after absorption by dilution in the bloodstream (Goldberg and Gomez-Orellana 2003).

In summary, both intravenous GLP-1 and PYY3-36 have been shown to reduce appetite and food intake in healthy volunteers and in patients with obesity and/or type 2 diabetes. However, intravenous or subcutaneous administration is cumbersome and impractical for long-term treatment regimens; the pharmacokinetic profiles of the two peptides obtained in this study suggest that the oral route of administration is very promising and could overcome the problems associated with intravenous administration. Whether oral GLP-1

or oral PYY3-36 administrations are beneficial in different clinical applications remains to be shown. Protocols are being developed that will specifically address these questions.

9 Conclusion and outlook

CNS-acting drugs represent a considerable part of all drugs today. During drug development the distribution of a drug into the brain needs to be assessed. The BBB forms the main obstacle and prevents a broad range of compounds from entering the brain tissue. Small hydrophobic compounds are unable to cross the barrier and depend on active uptake processes whereas many lipophilic drugs, which could cross the membrane passively, are the substrates of efflux transporters. Therefore, in order to predict brain penetration of new drug candidates an *in vitro* model of the human BBB would be of great use.

In this thesis the human brain capillary endothelial cell line hCMEC/D3 was investigated in terms of its permeability properties and gene regulation. We found that the hCMEC/D3 cell line allows to discriminate between compounds with low paracellular and such with high transcellular permeability. Under improved conditions the paracellular flux of comounds could even be reduced. Furthermore, the expression and functional activity of important ABC-transporters was shown in the hCMEC/D3 cells. In addition, it could also be demonstrated that the permeability of hCMEC/D3 monolayers could be modulated by endo- and exogenous factors. In further studies, altered expression and activity of the ABC-transporters BCRP and P-gp in response to pro-inflammatory cytokines was observed.

Based on these findings, the hCEMC/D3 cell line is an interesting *in vitro* model of the human BBB, offering a variety of possible applications:

- The transport of drugs through the BBB *in vitro* can be studied using the hCMEC/D3 cell line. Compared to other BBB *in vitro* cell lines, the paracellular tightness of the hCMEC/D3 cells is higher. However, bidirectional active transport was only observed under improved culture conditions. Therefore, the use of the hCMEC/D3 cell line is limited to mechanistic studies rather than for high throughput screenings.

- This model can be used for drug accumulation and efflux studies since important transport systems are expressed and active in the hCMEC/D3 cell line. Compared to transporter-over-expressing cell lines, the activity of the transporters in the hCMEC/D3 cells is low. However, the cells not only express single but a multitude of transporters, simulating the complex situation of the BBB. Therefore, this system might allow detecting interactions between different transport processes.

- The influence of exo- and endogenous factors on the barrier functions of the hCMEC/D3 cells can be studied. Since a breakdown of the BBB is observed during a multitude of diseases, the hCMEC/D3 cell line can serve as a mechanistic tool in order to simulate pathological alterations.

- Gene regulation can be studied in the hCMEC/D3 cell line. This offers the opportunity to determine the expression levels and activities of target genes and proteins during simulated states of diseases or under influence of applied drugs. Furthermore, signaling experiments can be performed.

- The hCMEC/D3 cells as model of the human BBB in order to apply targeting techniques. As it was shown that the hCMEC/D3 cells express the human transferrin receptor, it could be possible to transfer drugs by a targeting vector across hCMEC/D3 monolayers. However, systems suited for targeting experiments need to be further evaluated in these cells.

We conclude that the hCMEC/D3 cell line is a versatile *in vitro* model of the human BBB that can be used for numerous applications as shown in this thesis. Therefore, we hope that the hCMEC/D3 cell will be a helpful tool in the field of drug discovery.

10 References

Abbott N. J., Ronnback L. and Hansson E. (2006) Astrocyte-endothelial interactions at the blood-brain barrier. *Nat Rev Neurosci* **7,** 41-53.

Allen J. D., van Loevezijn A., Lakhai J. M., van der Valk M., van Tellingen O., Reid G., Schellens J. H., Koomen G. J. and Schinkel A. H. (2002) Potent and specific inhibition of the breast cancer resistance protein multidrug transporter in vitro and in mouse intestine by a novel analogue of fumitremorgin C. *Mol Cancer Ther* **1,** 417-425.

Arbogast S. and Reid M. B. (2004) Oxidant activity in skeletal muscle fibers is influenced by temperature, CO2 level, and muscle-derived nitric oxide. *Am J Physiol Regul Integr Comp Physiol* **287,** R698-705.

Aronica E., Gorter J. A., Redeker S., van Vliet E. A., Ramkema M., Scheffer G. L., Scheper R. J., van der Valk P., Leenstra S., Baayen J. C., Spliet W. G. and Troost D. (2005) Localization of breast cancer resistance protein (BCRP) in microvessel endothelium of human control and epileptic brain. *Epilepsia* **46,** 849-857.

Atkuri K. R., Mantovani J. J., Herzenberg L. A. and Herzenberg L. A. (2007) N-Acetylcysteine--a safe antidote for cysteine/glutathione deficiency. *Curr Opin Pharmacol* **7,** 355-359.

Audus K. L. and Borchardt R. T. (1986) Characteristics of the large neutral amino acid transport system of bovine brain microvessel endothelial cell monolayers. *J Neurochem* **47,** 484-488.

Aurrand-Lions M., Johnson-Leger C., Wong C., Du Pasquier L. and Imhof B. A. (2001) Heterogeneity of endothelial junctions is reflected by differential expression and specific subcellular localization of the three JAM family members. *Blood* **98,** 3699-3707.

Ballabh P., Braun A. and Nedergaard M. (2004) The blood-brain barrier: an overview: structure, regulation, and clinical implications. *Neurobiol Dis* **16,** 1-13.

Banks W. A. (1999) Physiology and pathology of the blood-brain barrier: implications for microbial pathogenesis, drug delivery and neurodegenerative disorders. *J Neurovirol* **5,** 538-555.

Banks W. A., Kumar V. B., Franko M. W., Bess J. W., Jr. and Arthur L. O. (2005) Evidence that the species barrier of human immunodeficiency virus-1 does not extend to uptake by the blood--brain barrier: comparison of mouse and human brain microvessels. *Life Sci* **77,** 2361-2368.

Batterham R. L., Cohen M. A., Ellis S. M., Le Roux C. W., Withers D. J., Frost G. S., Ghatei M. A. and Bloom S. R. (2003) Inhibition of food intake in obese subjects by peptide YY3-36. *N Engl J Med* **349,** 941-948.

Batterham R. L., Cowley M. A., Small C. J., Herzog H., Cohen M. A., Dakin C. L., Wren A. M., Brynes A. E., Low M. J., Ghatei M. A., Cone R. D. and Bloom S. R. (2002) Gut hormone PYY(3-36) physiologically inhibits food intake. *Nature* **418,** 650-654.

Bauer B., Hartz A. M. and Miller D. S. (2007) Tumor necrosis factor alpha and endothelin-1 increase P-glycoprotein expression and transport activity at the blood-brain barrier. *Mol Pharmacol* **71,** 667-675.

Berezowski V., Landry C., Dehouck M. P., Cecchelli R. and Fenart L. (2004) Contribution of glial cells and pericytes to the mRNA profiles of P-glycoprotein and multidrug

resistance-associated proteins in an in vitro model of the blood-brain barrier. *Brain Res* **1018,** 1-9.

Bernkop-Schnurch A. and Walker G. (2001) Multifunctional matrices for oral peptide delivery. *Crit Rev Ther Drug Carrier Syst* **18,** 459-501.

Betz A. L., Firth J. A. and Goldstein G. W. (1980) Polarity of the blood-brain barrier: distribution of enzymes between the luminal and antiluminal membranes of brain capillary endothelial cells. *Brain Res* **192,** 17-28.

Biedler J. L. and Riehm H. (1970) Cellular resistance to actinomycin D in Chinese hamster cells in vitro: cross-resistance, radioautographic, and cytogenetic studies. *Cancer Res* **30,** 1174-1184.

Boje K. M. and Lakhman S. S. (2000) Nitric oxide redox species exert differential permeability effects on the blood-brain barrier. *J Pharmacol Exp Ther* **293,** 545-550.

Borst P., Evers R., Kool M. and Wijnholds J. (2000) A family of drug transporters: the multidrug resistance-associated proteins. *J Natl Cancer Inst* **92,** 1295-1302.

Bowman P. D., Ennis S. R., Rarey K. E., Betz A. L. and Goldstein G. W. (1983) Brain microvessel endothelial cells in tissue culture: a model for study of blood-brain barrier permeability. *Ann Neurol* **14,** 396-402.

Brundula V., Rewcastle N. B., Metz L. M., Bernard C. C. and Yong V. W. (2002) Targeting leukocyte MMPs and transmigration: minocycline as a potential therapy for multiple sclerosis. *Brain* **125,** 1297-1308.

Butt A. M., Jones H. C. and Abbott N. J. (1990) Electrical resistance across the blood-brain barrier in anaesthetized rats: a developmental study. *J Physiol* **429,** 47-62.

Candelario-Jalil E., Taheri S., Yang Y., Sood R., Grossetete M., Estrada E. Y., Fiebich B. L. and Rosenberg G. A. (2007) Cyclooxygenase inhibition limits blood-brain barrier disruption following intracerebral injection of tumor necrosis factor-alpha in the rat. *J Pharmacol Exp Ther* **323,** 488-498.

Cecchelli R., Dehouck B., Descamps L., Fenart L., Buee-Scherrer V. V., Duhem C., Lundquist S., Rentfel M., Torpier G. and Dehouck M. P. (1999) In vitro model for evaluating drug transport across the blood-brain barrier. *Adv Drug Deliv Rev* **36,** 165-178.

Chiueh C. C. (1999) Neuroprotective properties of nitric oxide. *Ann N Y Acad Sci* **890,** 301-311.

Cisternino S., Rousselle C., Lorico A., Rappa G. and Scherrmann J. M. (2003) Apparent lack of Mrp1-mediated efflux at the luminal side of mouse blood-brain barrier endothelial cells. *Pharm Res* **20,** 904-909.

Cisternino S., Mercier C., Bourasset F., Roux F. and Scherrmann J. M. (2004) Expression, up-regulation, and transport activity of the multidrug-resistance protein Abcg2 at the mouse blood-brain barrier. *Cancer Res* **64,** 3296-3301.

Cooray H. C., Blackmore C. G., Maskell L. and Barrand M. A. (2002) Localisation of breast cancer resistance protein in microvessel endothelium of human brain. *Neuroreport* **13,** 2059-2063.

Cordon-Cardo C., O'Brien J. P., Casals D., Rittman-Grauer L., Biedler J. L., Melamed M. R. and Bertino J. R. (1989) Multidrug-resistance gene (P-glycoprotein) is expressed by endothelial cells at blood-brain barrier sites. *Proc Natl Acad Sci U S A* **86,** 695-698.

Cucullo L., Couraud P. O., Weksler B., Romero I. A., Hossain M., Rapp E. and Janigro D. (2008) Immortalized human brain endothelial cells and flow-based vascular modeling: a marriage of convenience for rational neurovascular studies. *J Cereb Blood Flow Metab* **28,** 312-328.

de Boer A. G., van der Sandt I. C. and Gaillard P. J. (2003) The role of drug transporters at the blood-brain barrier. *Annu Rev Pharmacol Toxicol* **43,** 629-656.

de Vries H. E., Kuiper J., de Boer A. G., Van Berkel T. J. and Breimer D. D. (1997) The blood-brain barrier in neuroinflammatory diseases. *Pharmacol Rev* **49,** 143-155.

de Vries H. E., Blom-Roosemalen M. C., van Oosten M., de Boer A. G., van Berkel T. J., Breimer D. D. and Kuiper J. (1996) The influence of cytokines on the integrity of the blood-brain barrier in vitro. *J Neuroimmunol* **64,** 37-43.

Dean M., Hamon Y. and Chimini G. (2001) The human ATP-binding cassette (ABC) transporter superfamily. *J Lipid Res* **42,** 1007-1017.

Demeule M., Regina A., Jodoin J., Laplante A., Dagenais C., Berthelet F., Moghrabi A. and Beliveau R. (2002) Drug transport to the brain: key roles for the efflux pump P-glycoprotein in the blood-brain barrier. *Vascul Pharmacol* **38,** 339-348.

Ding X., Rath P., Angelo R., Stringfellow T., Flanders E., Dinh S., Gomez-Orellana I. and Robinson J. R. (2004) Oral absorption enhancement of cromolyn sodium through noncovalent complexation. *Pharm Res* **21,** 2196-2206.

Dohgu S., Takata F., Yamauchi A., Nakagawa S., Egawa T., Naito M., Tsuruo T., Sawada Y., Niwa M. and Kataoka Y. (2005) Brain pericytes contribute to the induction and up-regulation of blood-brain barrier functions through transforming growth factor-beta production. *Brain Res* **1038,** 208-215.

Dombrowski S. M., Desai S. Y., Marroni M., Cucullo L., Goodrich K., Bingaman W., Mayberg M. R., Bengez L. and Janigro D. (2001) Overexpression of multiple drug resistance genes in endothelial cells from patients with refractory epilepsy. *Epilepsia* **42,** 1501-1506.

Doyle L. A., Yang W., Abruzzo L. V., Krogmann T., Gao Y., Rishi A. K. and Ross D. D. (1998) A multidrug resistance transporter from human MCF-7 breast cancer cells. *Proc Natl Acad Sci U S A* **95,** 15665-15670.

Drewes L. R. (2001) Molecular architecture of the brain microvasculature: perspective on blood-brain transport. *J Mol Neurosci* **16,** 93-98; discussion 151-157.

Ebnet K., Schulz C. U., Meyer Zu Brickwedde M. K., Pendl G. G. and Vestweber D. (2000) Junctional adhesion molecule interacts with the PDZ domain-containing proteins AF-6 and ZO-1. *J Biol Chem* **275,** 27979-27988.

Ehrlich P. (1885) Das Sauerstoff-Bedürnis des Organismus. Eine Farbanalytische Studie. *Berlin: Hirschwald*.

Eisenblatter T. and Galla H. J. (2002) A new multidrug resistance protein at the blood-brain barrier. *Biochem Biophys Res Commun* **293,** 1273-1278.

Eisenblatter T., Huwel S. and Galla H. J. (2003) Characterisation of the brain multidrug resistance protein (BMDP/ABCG2/BCRP) expressed at the blood-brain barrier. *Brain Res* **971,** 221-231.

Enerson B. E. and Drewes L. R. (2006) The rat blood-brain barrier transcriptome. *J Cereb Blood Flow Metab* **26,** 959-973.

Englund G., Jacobson A., Rorsman F., Artursson P., Kindmark A. and Ronnblom A. (2007) Efflux transporters in ulcerative colitis: decreased expression of BCRP (ABCG2) and Pgp (ABCB1). *Inflamm Bowel Dis* **13,** 291-297.

Evseenko D. A., Paxton J. W. and Keelan J. A. (2007) Independent regulation of apical and basolateral drug transporter expression and function in placental trophoblasts by cytokines, steroids, and growth factors. *Drug Metab Dispos* **35,** 595-601.

Fenstermacher J., Gross P., Sposito N., Acuff V., Pettersen S. and Gruber K. (1988) Structural and functional variations in capillary systems within the brain. *Ann N Y Acad Sci* **529,** 21-30.

Fix J. A. (1996) Strategies for delivery of peptides utilizing absorption-enhancing agents. *J Pharm Sci* **85,** 1282-1285.

Forster C., Burek M., Romero I. A., Weksler B., Couraud P. O. and Drenckhahn D. (2008) Differential effects of hydrocortisone and TNFalpha on tight junction proteins in an in vitro model of the human blood-brain barrier. *J Physiol* **586,** 1937-1949.

Foster M. W., McMahon T. J. and Stamler J. S. (2003) S-nitrosylation in health and disease. *Trends Mol Med* **9,** 160-168.

Franke H., Galla H. J. and Beuckmann C. T. (1999) An improved low-permeability in vitro-model of the blood-brain barrier: transport studies on retinoids, sucrose, haloperidol, caffeine and mannitol. *Brain Res* **818,** 65-71.

Furuse M., Sasaki H. and Tsukita S. (1999) Manner of interaction of heterogeneous claudin species within and between tight junction strands. *J Cell Biol* **147,** 891-903.

Furuse M., Fujita K., Hiiragi T., Fujimoto K. and Tsukita S. (1998) Claudin-1 and -2: novel integral membrane proteins localizing at tight junctions with no sequence similarity to occludin. *J Cell Biol* **141,** 1539-1550.

Furuse M., Hirase T., Itoh M., Nagafuchi A., Yonemura S., Tsukita S. and Tsukita S. (1993) Occludin: a novel integral membrane protein localizing at tight junctions. *J Cell Biol* **123,** 1777-1788.

Furuse M., Itoh M., Hirase T., Nagafuchi A., Yonemura S., Tsukita S. and Tsukita S. (1994) Direct association of occludin with ZO-1 and its possible involvement in the localization of occludin at tight junctions. *J Cell Biol* **127,** 1617-1626.

Gaillard P. J., Voorwinden L. H., Nielsen J. L., Ivanov A., Atsumi R., Engman H., Ringbom C., de Boer A. G. and Breimer D. D. (2001) Establishment and functional characterization of an in vitro model of the blood-brain barrier, comprising a co-culture of brain capillary endothelial cells and astrocytes. *Eur J Pharm Sci* **12,** 215-222.

Gao B., Hagenbuch B., Kullak-Ublick G. A., Benke D., Aguzzi A. and Meier P. J. (2000) Organic anion-transporting polypeptides mediate transport of opioid peptides across blood-brain barrier. *J Pharmacol Exp Ther* **294,** 73-79.

Gekeler V., Ise W., Sanders K. H., Ulrich W. R. and Beck J. (1995) The leukotriene LTD4 receptor antagonist MK571 specifically modulates MRP associated multidrug resistance. *Biochem Biophys Res Commun* **208,** 345-352.

Goldberg M. and Gomez-Orellana I. (2003) Challenges for the oral delivery of macromolecules. *Nat Rev Drug Discov* **2,** 289-295.

Goldmann E. (1909) Die äussere und innere Sekretion des gesunden und kranken Organismus im Licht der vitalen Färbung. *Beitr Klin Chir* **64,** 192-265.

Goldstein G. W. and Betz A. L. (1983) Recent advances in understanding brain capillary function. *Ann Neurol* **14,** 389-395.

Gomez-Orellana I. (2005) Strategies to improve oral drug bioavailability. *Expert Opin Drug Deliv* **2,** 419-433.

Gutmann H., Torok M., Fricker G., Huwyler J., Beglinger C. and Drewe J. (1999) Modulation of multidrug resistance protein expression in porcine brain capillary endothelial cells in vitro. *Drug Metab Dispos* **27,** 937-941.

Gutmann H., Bruggisser R., Schaffner W., Bogman K., Botomino A. and Drewe J. (2002) Transport of amentoflavone across the blood-brain barrier in vitro. *Planta Med* **68,** 804-807.

Gutmann H., Hruz P., Zimmermann C., Straumann A., Terracciano L., Hammann F., Lehmann F., Beglinger C. and Drewe J. (2008) Breast cancer resistance protein (BCRP) and P-glycoprotein (P-gp) expression in newly diagnosed and therapy refractory ulcerative colitis. *Digestion* **in press**.

Gutzwiller J. P., Drewe J., Goke B., Schmidt H., Rohrer B., Lareida J. and Beglinger C. (1999) Glucagon-like peptide-1 promotes satiety and reduces food intake in patients with diabetes mellitus type 2. *Am J Physiol* **276,** R1541-1544.

Han B. and Zhang J. T. (2004) Multidrug resistance in cancer chemotherapy and xenobiotic protection mediated by the half ATP-binding cassette transporter ABCG2. *Curr Med Chem Anticancer Agents* **4,** 31-42.

Hartz A. M., Bauer B., Fricker G. and Miller D. S. (2006) Rapid modulation of P-glycoprotein-mediated transport at the blood-brain barrier by tumor necrosis factor-alpha and lipopolysaccharide. *Mol Pharmacol* **69,** 462-470.

Hawkins B. T., Lundeen T. F., Norwood K. M., Brooks H. L. and Egleton R. D. (2007) Increased blood-brain barrier permeability and altered tight junctions in experimental diabetes in the rat: contribution of hyperglycaemia and matrix metalloproteinases. *Diabetologia* **50,** 202-211.

Hediger M. A., Romero M. F., Peng J. B., Rolfs A., Takanaga H. and Bruford E. A. (2004) The ABCs of solute carriers: physiological, pathological and therapeutic implications of human membrane transport proteinsIntroduction. *Pflugers Arch* **447,** 465-468.

Hirase T., Staddon J. M., Saitou M., Ando-Akatsuka Y., Itoh M., Furuse M., Fujimoto K., Tsukita S. and Rubin L. L. (1997) Occludin as a possible determinant of tight junction permeability in endothelial cells. *J Cell Sci* **110 (Pt 14),** 1603-1613.

Hoheisel D., Nitz T., Franke H., Wegener J., Hakvoort A., Tilling T. and Galla H. J. (1998) Hydrocortisone reinforces the blood-brain properties in a serum free cell culture system. *Biochem Biophys Res Commun* **247,** 312-315.

Hori S., Ohtsuki S., Tachikawa M., Kimura N., Kondo T., Watanabe M., Nakashima E. and Terasaki T. (2004) Functional expression of rat ABCG2 on the luminal side of brain capillaries and its enhancement by astrocyte-derived soluble factor(s). *J Neurochem* **90,** 526-536.

Huber J. D., Egleton R. D. and Davis T. P. (2001) Molecular physiology and pathophysiology of tight junctions in the blood-brain barrier. *Trends Neurosci* **24,** 719-725.

Huber J. D., VanGilder R. L. and Houser K. A. (2006) Streptozotocin-induced diabetes progressively increases blood-brain barrier permeability in specific brain regions in rats. *Am J Physiol Heart Circ Physiol* **291,** H2660-2668.

Huwyler J., Wu D. and Pardridge W. M. (1996) Brain drug delivery of small molecules using immunoliposomes. *Proc Natl Acad Sci U S A* **93,** 14164-14169.

Huwyler J., Drewe J., Klusemann C. and Fricker G. (1996) Evidence for P-glycoprotein-modulated penetration of morphine-6-glucuronide into brain capillary endothelium. *Br J Pharmacol* **118,** 1879-1885.

Huwyler J., Froidevaux S., Roux F. and Eberle A. N. (1999) Characterization of transferrin receptor in an immortalized cell line of rat brain endothelial cells, RBE4. *J Recept Signal Transduct Res* **19,** 729-739.

Ifergan I., Wosik K., Cayrol R., Kebir H., Auger C., Bernard M., Bouthillier A., Moumdjian R., Duquette P. and Prat A. (2006) Statins reduce human blood-brain barrier permeability and restrict leukocyte migration: relevance to multiple sclerosis. *Ann Neurol* **60,** 45-55.

Igarashi Y., Utsumi H., Chiba H., Yamada-Sasamori Y., Tobioka H., Kamimura Y., Furuuchi K., Kokai Y., Nakagawa T., Mori M. and Sawada N. (1999) Glial cell line-derived neurotrophic factor induces barrier function of endothelial cells forming the blood-brain barrier. *Biochem Biophys Res Commun* **261,** 108-112.

Itoh M., Furuse M., Morita K., Kubota K., Saitou M. and Tsukita S. (1999) Direct binding of three tight junction-associated MAGUKs, ZO-1, ZO-2, and ZO-3, with the COOH termini of claudins. *J Cell Biol* **147,** 1351-1363.

Jefferies W. A., Brandon M. R., Hunt S. V., Williams A. F., Gatter K. C. and Mason D. Y. (1984) Transferrin receptor on endothelium of brain capillaries. *Nature* **312,** 162-163.

Jette L., Tetu B. and Beliveau R. (1993) High levels of P-glycoprotein detected in isolated brain capillaries. *Biochim Biophys Acta* **1150,** 147-154.

Jones P. M. and George A. M. (2000) Symmetry and structure in P-glycoprotein and ABC transporters what goes around comes around. *Eur J Biochem* **267,** 5298-5305.

Ketabi-Kiyanvash N., Herold-Mende C., Kashfi F., Caldeira S., Tommasino M., Haefeli W. E. and Weiss J. (2007) NKIM-6, a new immortalized human brain capillary endothelial cell line with conserved endothelial characteristics. *Cell Tissue Res* **328,** 19-29.

Kraus J. and Oschmann P. (2006) The impact of interferon-beta treatment on the blood-brain barrier. *Drug Discov Today* **11,** 755-762.

Kraus J., Ling A. K., Hamm S., Voigt K., Oschmann P. and Engelhardt B. (2004) Interferon-beta stabilizes barrier characteristics of brain endothelial cells in vitro. *Ann Neurol* **56,** 192-205.

Kuhlmann C. R., Lessmann V. and Luhmann H. J. (2006) Fluvastatin stabilizes the blood-brain barrier in vitro by nitric oxide-dependent dephosphorylation of myosin light chains. *Neuropharmacology* **51,** 907-913.

Kusch-Poddar M., Drewe J., Fux I. and Gutmann H. (2005) Evaluation of the immortalized human brain capillary endothelial cell line BB19 as a human cell culture model for the blood-brain barrier. *Brain Res* **1064,** 21-31.

Kusuhara H. and Sugiyama Y. (2005) Active efflux across the blood-brain barrier: role of the solute carrier family. *NeuroRx* **2,** 73-85.

Lee Y. J., Kusuhara H., Jonker J. W., Schinkel A. H. and Sugiyama Y. (2005) Investigation of efflux transport of dehydroepiandrosterone sulfate and mitoxantrone at the mouse blood-brain barrier: a minor role of breast cancer resistance protein. *J Pharmacol Exp Ther* **312,** 44-52.

Levoye A., Dam J., Ayoub M. A., Guillaume J. L., Couturier C., Delagrange P. and Jockers R. (2006) The orphan GPR50 receptor specifically inhibits MT1 melatonin receptor function through heterodimerization. *Embo J* **25,** 3012-3023.

Lewandowski M. (1900) Zur Lehre von der Cerebrospinalflüssigkeit. *Zeitschrift für Klinische Medicin* **40,** 480-494.

Li H. and Qian Z. M. (2002) Transferrin/transferrin receptor-mediated drug delivery. *Med Res Rev* **22,** 225-250.

Liebner S., Kniesel U., Kalbacher H. and Wolburg H. (2000) Correlation of tight junction morphology with the expression of tight junction proteins in blood-brain barrier endothelial cells. *Eur J Cell Biol* **79,** 707-717.

Liebner S., Fischmann A., Rascher G., Duffner F., Grote E. H., Kalbacher H. and Wolburg H. (2000) Claudin-1 and claudin-5 expression and tight junction morphology are altered in blood vessels of human glioblastoma multiforme. *Acta Neuropathol* **100,** 323-331.

Lin J. H. and Yamazaki M. (2003) Clinical relevance of P-glycoprotein in drug therapy. *Drug Metab Rev* **35,** 417-454.

Lippoldt A., Liebner S., Andbjer B., Kalbacher H., Wolburg H., Haller H. and Fuxe K. (2000) Organization of choroid plexus epithelial and endothelial cell tight junctions and regulation of claudin-1, -2 and -5 expression by protein kinase C. *Neuroreport* **11,** 1427-1431.

Loscher W. and Potschka H. (2005) Blood-brain barrier active efflux transporters: ATP-binding cassette gene family. *NeuroRx* **2,** 86-98.

Loscher W. and Potschka H. (2005) Role of drug efflux transporters in the brain for drug disposition and treatment of brain diseases. *Prog Neurobiol* **76,** 22-76.

Maier A., Zimmermann C., Beglinger C., Drewe J. and Gutmann H. (2007) Effects of budesonide on P-glycoprotein expression in intestinal cell lines. *Br J Pharmacol* **150,** 361-368.

Mairey E., Genovesio A., Donnadieu E., Bernard C., Jaubert F., Pinard E., Seylaz J., Olivo-Marin J. C., Nassif X. and Dumenil G. (2006) Cerebral microcirculation shear stress levels determine Neisseria meningitidis attachment sites along the blood-brain barrier. *J Exp Med* **203,** 1939-1950.

Martin-Padura I., Lostaglio S., Schneemann M., Williams L., Romano M., Fruscella P., Panzeri C., Stoppacciaro A., Ruco L., Villa A., Simmons D. and Dejana E. (1998) Junctional adhesion molecule, a novel member of the immunoglobulin superfamily that distributes at intercellular junctions and modulates monocyte transmigration. *J Cell Biol* **142,** 117-127.

McAllister M. S., Krizanac-Bengez L., Macchia F., Naftalin R. J., Pedley K. C., Mayberg M. R., Marroni M., Leaman S., Stanness K. A. and Janigro D. (2001) Mechanisms of glucose transport at the blood-brain barrier: an in vitro study. *Brain Res* **904,** 20-30.

Megard I., Garrigues A., Orlowski S., Jorajuria S., Clayette P., Ezan E. and Mabondzo A. (2002) A co-culture-based model of human blood-brain barrier: application to active transport of indinavir and in vivo-in vitro correlation. *Brain Res* **927,** 153-167.

Miecz D., Januszewicz E., Czeredys M., Hinton B. T., Berezowski V., Cecchelli R. and Nalecz K. A. (2008) Localization of organic cation/carnitine transporter (OCTN2) in cells forming the blood-brain barrier. *J Neurochem* **104,** 113-123.

Miller D. S., Nobmann S. N., Gutmann H., Toeroek M., Drewe J. and Fricker G. (2000) Xenobiotic transport across isolated brain microvessels studied by confocal microscopy. *Mol Pharmacol* **58,** 1357-1367.

Milstone L. M., Adams B. D., Zhou J., Bruegel Sanchez V. L. and Shofner J. (2006) Stratum-specific expression of human transferrin receptor increases iron in mouse epidermis. *J Invest Dermatol* **126,** 648-652.

Mori S., Ohtsuki S., Takanaga H., Kikkawa T., Kang Y. S. and Terasaki T. (2004) Organic anion transporter 3 is involved in the brain-to-blood efflux transport of thiopurine nucleobase analogs. *J Neurochem* **90,** 931-941.

Morita K., Sasaki H., Furuse M. and Tsukita S. (1999) Endothelial claudin: claudin-5/TMVCF constitutes tight junction strands in endothelial cells. *J Cell Biol* **147,** 185-194.

Nagy Z., Peters H. and Huttner I. (1984) Fracture faces of cell junctions in cerebral endothelium during normal and hyperosmotic conditions. *Lab Invest* **50,** 313-322.

Nelissen I., Martens E., Van den Steen P. E., Proost P., Ronsse I. and Opdenakker G. (2003) Gelatinase B/matrix metalloproteinase-9 cleaves interferon-beta and is a target for immunotherapy. *Brain* **126,** 1371-1381.

Neuwelt E., Abbott N. J., Abrey L., Banks W. A., Blakley B., Davis T., Engelhardt B., Grammas P., Nedergaard M., Nutt J., Pardridge W., Rosenberg G. A., Smith Q. and Drewes L. R. (2008) Strategies to advance translational research into brain barriers. *Lancet Neurol* **7,** 84-96.

Nies A. T., Jedlitschky G., Konig J., Herold-Mende C., Steiner H. H., Schmitt H. P. and Keppler D. (2004) Expression and immunolocalization of the multidrug resistance proteins, MRP1-MRP6 (ABCC1-ABCC6), in human brain. *Neuroscience* **129,** 349-360.

Nitz T., Eisenblatter T., Psathaki K. and Galla H. J. (2003) Serum-derived factors weaken the barrier properties of cultured porcine brain capillary endothelial cells in vitro. *Brain Res* **981,** 30-40.

Ohtsuki S., Yamaguchi H., Asashima T. and Terasaki T. (2007) Establishing a method to isolate rat brain capillary endothelial cells by magnetic cell sorting and dominant mRNA expression of multidrug resistance-associated protein 1 and 4 in highly purified rat brain capillary endothelial cells. *Pharm Res* **24,** 688-694.

Oldendorf W. H., Cornford M. E. and Brown W. J. (1977) The large apparent work capability of the blood-brain barrier: a study of the mitochondrial content of capillary endothelial cells in brain and other tissues of the rat. *Ann Neurol* **1,** 409-417.

Omidi Y., Campbell L., Barar J., Connell D., Akhtar S. and Gumbleton M. (2003) Evaluation of the immortalised mouse brain capillary endothelial cell line, b.End3, as an in vitro blood-brain barrier model for drug uptake and transport studies. *Brain Res* **990,** 95-112.

Palmeri D., van Zante A., Huang C. C., Hemmerich S. and Rosen S. D. (2000) Vascular endothelial junction-associated molecule, a novel member of the immunoglobulin superfamily, is localized to intercellular boundaries of endothelial cells. *J Biol Chem* **275,** 19139-19145.

Pardridge W. M. (2003) Molecular biology of the blood-brain barrier. *Methods Mol Med* **89,** 385-399.

Pardridge W. M., Eisenberg J. and Yang J. (1985) Human blood-brain barrier insulin receptor. *J Neurochem* **44,** 1771-1778.

Pardridge W. M., Eisenberg J. and Yang J. (1987) Human blood-brain barrier transferrin receptor. *Metabolism* **36,** 892-895.

Pavek P., Merino G., Wagenaar E., Bolscher E., Novotna M., Jonker J. W. and Schinkel A. H. (2005) Human breast cancer resistance protein: interactions with steroid drugs, hormones, the dietary carcinogen 2-amino-1-methyl-6-phenylimidazo(4,5-b)pyridine, and transport of cimetidine. *J Pharmacol Exp Ther* **312,** 144-152.

Perriere N., Demeuse P., Garcia E., Regina A., Debray M., Andreux J. P., Couvreur P., Scherrmann J. M., Temsamani J., Couraud P. O., Deli M. A. and Roux F. (2005) Puromycin-based purification of rat brain capillary endothelial cell cultures. Effect on the expression of blood-brain barrier-specific properties. *J Neurochem* **93,** 279-289.

Poller B., Gutmann H., Krähenbühl S., Weksler B., Romero I., Couraud P.-O., Tuffin G., Drewe J. and Huwyler J. (2008) The human brain endothelial cell line hCMEC/D3 as a human blood-brain barrier model for drug transport studies. *Journal of Neurochemistry* **107,** 1358-1368.

Potschka H., Fedrowitz M. and Loscher W. (2003) Multidrug resistance protein MRP2 contributes to blood-brain barrier function and restricts antiepileptic drug activity. *J Pharmacol Exp Ther* **306,** 124-131.

Price T. O., Uras F., Banks W. A. and Ercal N. (2006) A novel antioxidant N-acetylcysteine amide prevents gp120- and Tat-induced oxidative stress in brain endothelial cells. *Exp Neurol* **201,** 193-202.

Prudhomme J. G., Sherman I. W., Land K. M., Moses A. V., Stenglein S. and Nelson J. A. (1996) Studies of Plasmodium falciparum cytoadherence using immortalized human brain capillary endothelial cells. *Int J Parasitol* **26,** 647-655.

Ramsauer M., Krause D. and Dermietzel R. (2002) Angiogenesis of the blood-brain barrier in vitro and the function of cerebral pericytes. *Faseb J* **16,** 1274-1276.

Reale M., Iarlori C., Thomas A., Gambi D., Perfetti B., Di Nicola M. and Onofrj M. (2008) Peripheral cytokines profile in Parkinson's disease. *Brain Behav Immun*.

Reese T. S. and Karnovsky M. J. (1967) Fine structural localization of a blood-brain barrier to exogenous peroxidase. *J Cell Biol* **34,** 207-217.

Regina A., Romero I. A., Greenwood J., Adamson P., Bourre J. M., Couraud P. O. and Roux F. (1999) Dexamethasone regulation of P-glycoprotein activity in an immortalized rat brain endothelial cell line, GPNT. *J Neurochem* **73,** 1954-1963.

Rist R. J., Romero I. A., Chan M. W., Couraud P. O., Roux F. and Abbott N. J. (1997) F-actin cytoskeleton and sucrose permeability of immortalised rat brain microvascular endothelial cell monolayers: effects of cyclic AMP and astrocytic factors. *Brain Res* **768,** 10-18.

Roux F., Durieu-Trautmann O., Chaverot N., Claire M., Mailly P., Bourre J. M., Strosberg A. D. and Couraud P. O. (1994) Regulation of gamma-glutamyl transpeptidase and alkaline phosphatase activities in immortalized rat brain microvessel endothelial cells. *J Cell Physiol* **159,** 101-113.

Rowland L. P., Fink M. E. and Rubin L. L. (1991) Cerebrospinal fluid: blood-brain barrier, brain edema, and hydrocephalus, in *Principles of neural science* (Kandel E. R., Schwartz J. H. and Jessell T. M., eds), pp 1050-1060. Elsevier Science, New York.

Sasongko L., Link J. M., Muzi M., Mankoff D. A., Yang X., Collier A. C., Shoner S. C. and Unadkat J. D. (2005) Imaging P-glycoprotein transport activity at the human

blood-brain barrier with positron emission tomography. *Clin Pharmacol Ther* **77,** 503-514.

Schinkel A. H. (1999) P-Glycoprotein, a gatekeeper in the blood-brain barrier. *Adv Drug Deliv Rev* **36,** 179-194.

Schinkel A. H. and Jonker J. W. (2003) Mammalian drug efflux transporters of the ATP binding cassette (ABC) family: an overview. *Adv Drug Deliv Rev* **55,** 3-29.

Schinkel A. H., Wagenaar E., Mol C. A. and van Deemter L. (1996) P-glycoprotein in the blood-brain barrier of mice influences the brain penetration and pharmacological activity of many drugs. *J Clin Invest* **97,** 2517-2524.

Schinkel A. H., Wagenaar E., van Deemter L., Mol C. A. and Borst P. (1995) Absence of the mdr1a P-Glycoprotein in mice affects tissue distribution and pharmacokinetics of dexamethasone, digoxin, and cyclosporin A. *J Clin Invest* **96,** 1698-1705.

Schnyder A., Krahenbuhl S., Drewe J. and Huwyler J. (2005) Targeting of daunomycin using biotinylated immunoliposomes: pharmacokinetics, tissue distribution and in vitro pharmacological effects. *J Drug Target* **13,** 325-335.

Schreibelt G., Kooij G., Reijerkerk A., van Doorn R., Gringhuis S. I., van der Pol S., Weksler B. B., Romero I. A., Couraud P. O., Piontek J., Blasig I. E., Dijkstra C. D., Ronken E. and de Vries H. E. (2007) Reactive oxygen species alter brain endothelial tight junction dynamics via RhoA, PI3 kinase, and PKB signaling. *Faseb J* **21,** 3666-3676.

Sedlakova R., Shivers R. R. and Del Maestro R. F. (1999) Ultrastructure of the blood-brain barrier in the rabbit. *J Submicrosc Cytol Pathol* **31,** 149-161.

Seelbach M. J., Brooks T. A., Egleton R. D. and Davis T. P. (2007) Peripheral inflammatory hyperalgesia modulates morphine delivery to the brain: a role for P-glycoprotein. *J Neurochem* **102,** 1677-1690.

Siakotos A. N. and Rouser G. (1969) Isolation of highly purified human and bovine brain endothelial cells and nuclei and their phospholipid composition. *Lipids* **4,** 234-239.

Sisodiya S. M., Heffernan J. and Squier M. V. (1999) Over-expression of P-glycoprotein in malformations of cortical development. *Neuroreport* **10,** 3437-3441.

Sisodiya S. M., Lin W. R., Squier M. V. and Thom M. (2001) Multidrug-resistance protein 1 in focal cortical dysplasia. *Lancet* **357,** 42-43.

Skehan P., Storeng R., Scudiero D., Monks A., McMahon J., Vistica D., Warren J. T., Bokesch H., Kenney S. and Boyd M. R. (1990) New colorimetric cytotoxicity assay for anticancer-drug screening. *J Natl Cancer Inst* **82,** 1107-1112.

Sugiyama D., Kusuhara H., Lee Y. J. and Sugiyama Y. (2003) Involvement of multidrug resistance associated protein 1 (Mrp1) in the efflux transport of 17beta estradiol-D-17beta-glucuronide (E217betaG) across the blood-brain barrier. *Pharm Res* **20,** 1394-1400.

Tang F., Ouyang H., Yang J. Z. and Borchardt R. T. (2004) Bidirectional transport of rhodamine 123 and Hoechst 33342, fluorescence probes of the binding sites on P-glycoprotein, across MDCK-MDR1 cell monolayers. *J Pharm Sci* **93,** 1185-1194.

Tatsuta T., Naito M., Oh-hara T., Sugawara I. and Tsuruo T. (1992) Functional involvement of P-glycoprotein in blood-brain barrier. *J Biol Chem* **267,** 20383-20391.

Theron D., Barraud de Lagerie S., Tardivel S., Pelerin H., Demeuse P., Mercier C., Mabondzo A., Farinotti R., Lacour B., Roux F. and Gimenez F. (2003) Influence

of tumor necrosis factor-alpha on the expression and function of P-glycoprotein in an immortalised rat brain capillary endothelial cell line, GPNT. *Biochem Pharmacol* **66,** 579-587.

Thiebaut F., Tsuruo T., Hamada H., Gottesman M., Pastan I. and MC. W. (1989) Immunohistochemical localization in normal tissues of different epitopes in the multidrug transport protein P170: evidence for localization in brain capillaries and crossreactivity of one antibody with a muscle protein. *J Histochem Cytochem* **37,** 159-164.

Torok M., Huwyler J., Gutmann H., Fricker G. and Drewe J. (2003) Modulation of transendothelial permeability and expression of ATP-binding cassette transporters in cultured brain capillary endothelial cells by astrocytic factors and cell-culture conditions. *Exp Brain Res* **153,** 356-365.

Townsend D. M., Tew K. D. and Tapiero H. (2003) The importance of glutathione in human disease. *Biomed Pharmacother* **57,** 145-155.

van Herwaarden A. E., Jonker J. W., Wagenaar E., Brinkhuis R. F., Schellens J. H., Beijnen J. H. and Schinkel A. H. (2003) The breast cancer resistance protein (Bcrp1/Abcg2) restricts exposure to the dietary carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine. *Cancer Res* **63,** 6447-6452.

Vaughan C. J. and Delanty N. (1999) Neuroprotective properties of statins in cerebral ischemia and stroke. *Stroke* **30,** 1969-1973.

Viegas P., Chaverot N., Enslen H., Perriere N., Couraud P. O. and Cazaubon S. (2006) Junctional expression of the prion protein PrPC by brain endothelial cells: a role in trans-endothelial migration of human monocytes. *J Cell Sci* **119,** 4634-4643.

Weksler B. B., Subileau E. A., Perriere N., Charneau P., Holloway K., Leveque M., Tricoire-Leignel H., Nicotra A., Bourdoulous S., Turowski P., Male D. K., Roux F., Greenwood J., Romero I. A. and Couraud P. O. (2005) Blood-brain barrier-specific properties of a human adult brain endothelial cell line. *Faseb J* **19,** 1872-1874.

Winter S., Konter J., Scheler S., Lehmann J. and Fahr A. (2008) Permeability changes in response to NONOate and NONOate prodrug derived nitric oxide in a blood-brain barrier model formed by primary porcine endothelial cells. *Nitric Oxide* **18,** 229-239.

Wolburg H. and Lippoldt A. (2002) Tight junctions of the blood-brain barrier: development, composition and regulation. *Vascul Pharmacol* **38,** 323-337.

Wong D., Dorovini-Zis K. and Vincent S. R. (2004) Cytokines, nitric oxide, and cGMP modulate the permeability of an in vitro model of the human blood-brain barrier. *Exp Neurol* **190,** 446-455.

Woodley J. F. (1994) Enzymatic barriers for GI peptide and protein delivery. *Crit Rev Ther Drug Carrier Syst* **11,** 61-95.

Yang G., Li L., Volk A., Emmell E., Petley T., Giles-Komar J., Rafferty P., Lakshminarayanan M., Griswold D. E., Bugelski P. J. and Das A. M. (2005) Therapeutic dosing with anti-interleukin-13 monoclonal antibody inhibits asthma progression in mice. *J Pharmacol Exp Ther* **313,** 8-15.

Youssef S., Stuve O., Patarroyo J. C., Ruiz P. J., Radosevich J. L., Hur E. M., Bravo M., Mitchell D. J., Sobel R. A., Steinman L. and Zamvil S. S. (2002) The HMG-CoA reductase inhibitor, atorvastatin, promotes a Th2 bias and reverses paralysis in central nervous system autoimmune disease. *Nature* **420,** 78-84.

Zeng H., Chen Z. S., Belinsky M. G., Rea P. A. and Kruh G. D. (2001) Transport of methotrexate (MTX) and folates by multidrug resistance protein (MRP) 3 and MRP1: effect of polyglutamylation on MTX transport. *Cancer Res* **61,** 7225-7232.

Zhang Y., Han H., Elmquist W. F. and Miller D. W. (2000) Expression of various multidrug resistance-associated protein (MRP) homologues in brain microvessel endothelial cells. *Brain Res* **876,** 148-153.

Curriculum vitae

Personal Data

Name:	Birk Poller
Date of birth:	June 2nd, 1981
Place of birth:	Basel, CH
Nationality:	German
Address:	Alemannengasse 11, 4058 Basel, Switzerland
Phone (home):	+41 61 222 27 73
Phone (mobile):	+41 79 774 44 69
Email:	birk.poller@unibas.ch

Education

1988 – 1992	Elementary School, Steingrubenweg, Riehen, Switzerland
1992 – 2000	Grammar school, Gymnssium Bäumlihof, Basel, Switzerland
2000 – 2005	Studies in Pharmaceutical Science, University of Basel, Switzerland, Swiss Federal Diploma in Pharmacy
2004	Diploma thesis 'Influence of St. John's wort extracts and constituents on P-glycoprotein and CYP3A4 expression in an intestinal cell culture model' Division of Clinical Pharmacology and Toxicology, University of Basel, Switzerland (thesis advisor: Prof. Dr. J. Drewe)
2006-2008	PhD Thesis, Division of Clinical Pharmacology and Toxicology, University Hospital of Basel, University of Basel, Switzerland (doctoral advisor: Dr. Jürgen Drewe)

Personal Training and Experience

2005:	3 Months of Deputy Pharmacist at the Community Pharmacies 'Engel-Apotheke', Basel, Switzerland and 'Seeland-Apotheke', Biel, Switzerland
2006-2008	Therapeutic Drug Monitoring (TDM) and Clinical Pharmacology Service (KLIPS), University Hospital, Basel, Switzerland
Since 2006:	Deputy Pharmacist at the Community Pharmacy 'Notfall Apotheke Basel', Basel, Switzerland
Since 2007	Author in the Framework of 'i.m@il-Offizin'

Personal Management

2006-2008	Assisting in Undergraduate Practical Classes
2008	Training and Supervising of a Diploma Thesis

Publications

Beglinger C., **Poller B.**, Arbit E., Ganzoni C., Gass S., Gomez-Orellana I. and Drewe J. (2008) Pharmacokinetics and Pharmacodynamic Effects of Oral GLP-1 and PYY3-36: A Proof-of-concept Study in Healthy Subjects. Clin Pharmacol Ther.

Gutmann H., **Poller B.**, Buter K. B., Pfrunder A., Schaffner W. and Drewe J. (2006) Hypericum perforatum: which constituents may induce intestinal MDR1 and CYP3A4 mRNA expression? Planta Med 72, 685-690.

Poller B., Gutmann H., Krähenbühl S., Weksler B., Romero I., Couraud P.-O., Tuffin G., Drewe J. and Huwyler J. (2008) The human brain endothelial cell line hCMEC/D3 as a human blood-brain barrier model for drug transport studies. Journal of Neurochemistry 107, 1358-1368.

Posters

10. Blut-Hirnschranken-Expertentreffen, May, 2008, Bad Herrenalb, Germany

Attended meetings

8. Blut-Hirnschranken-Expertentreffen, May, 2006, Bad Herrenalb, Germany

9. Blut-Hirnschranken-Expertentreffen, May, 2007, Bad Herrenalb, Germany

10. Blut-Hirnschranken-Expertentreffen, May, 2008, Bad Herrenalb, Germany

Languages:

German (first language), French, English

During my studies I attended courses of the following lecturers:

Arber S., Beier K., Betz G., Bickle T.A., Bienz K.A., Boelsterli U., Boller Th., Brenner A., Brenner H.R., Drewe J., Eberle A., Engel J., Erb P., Ernst B., Folkers G., Gehring J., Gescheidt G., Grzesiek S., Guentert T., Güntherodt H.-J., Hauri H.-P., Hauser P., Hersberger K., Huwyler J., Im Hof H.-C., Imanidis G., Jenal U., Keller W., Kessler M., Kiefhaber T., Körner C., Krähenbühl S., Kunz D., Leuenberger H., Lüdin E., Lüthi A., Mayans O., Meier B., Meier C., Meier T., Melchers F., Meyer J., Meyer U., Monard D., Mühlebach S., Müller H., Müller J., Neri D., Otten U., Pfleiderer G., Rehmann-Sutter C., Reichert H., Rüegg M., Schaffner W., Schirmer T., Schlienger R., Schmid B., Schmid V., Scholer A., Schönenberger C., Seelig J., Séquin U., Sick I., Spiess M., Spornitz U., Stoeckli E., Strazewski P., Vedani A., Wessels H.-P., Zaugg C., Zuberbühler A.,