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TRANSPORT OF NSAIDs ACROSS A BLOOD-BRAIN BARRIER *IN VITRO* MODEL BASED ON CELL LINE PBMEC/C1-2

Rigorous thesis in cooperation with

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ABSTRACT

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The blood-brain barrier (BBB) has a prominent role in regulation of the transport of substances into and out of the central nervous system (CNS). Partly, the BBB inhibits the entrance of substances harmful for the brain, it regulates the delivery of needed substances and it takes part in efflux of useless substances as well. The equilibrium of these regulation systems is essential for the correct function of the CNS, without which the homeostasis would be disturbed.

Non-steroidal anti-inflammatory drugs (NSAIDs) are very well known for their anti-inflammatory effect, for reduction of fever and pain. Due to their bright, everyday usage, some side effects on the brain were observed (sleepiness, giddiness, nausea). This has evoked the question, how NSAIDs can cross the BBB.

PBMEC/C1-2 cell monolayer was used as an *in vitro* model of the BBB. The transport of following NSAIDs was investigated: celecoxib, diclofenac, ibuprofen, lornoxicam, meloxicam, piroxicam and tenoxicam. The experiments were carried out using only one NSAID (single transport studies) or more substances (group transport studies) at once. Different conditions (diverse transport mediums, serum contest, adding of transport inhibitors verapamil or probenecid, excluding of some NSAIDs substances) during group studies were simulated to observe a possible influence on the transport properties of NSAIDs. Internal standards diazepam (transcellular marker) and carboxyfluorescein (CF) (paracellular marker) were added to normalize the obtained data.

Ranking of single substance studies were similar to ranking of corresponding group study. Rankings of group studies were mostly influenced by using serum-free medium, by adding transport inhibitors verapamil or probenecid or by using astrocyte-conditioned medium. Serum-free study confirmed a strong binding ability of some NSAIDs to the serum proteins and its influence on the transport abilities. Group studies with transport inhibitors pointed to transport proteins, which are involved in permeation properties of the chosen substances. And finally, the suggestion that all substances can interact with each other was also proven.

Explicitly it was confirmed that most NSAIDs could cross the BBB significantly and consequently can influence the function of the CNS.

ABSTRAKT

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Hematoencefalická bariéra (HEB) má významnou roli v regulaci transportu látek do centrálního nervového systému (CNS) a opět z něj ven. HEB částečně zabraňuje průchodu látkám škodlivým pro mozek, zčásti reguluje dodávání potřebných látek a také se podílí na odstraňování odpadních látek. Vyváženost těchto regulačních systémů je nezbytná pro správnou funkci CNS, bez které by došlo k narušení homeostázy.

Nesteroidní antiflogistika (NSA) jsou velmi dobře známa pro svůj protizánětlivý účinek, pro schopnost snižovat horečku a pro tišení bolesti. Díky jejich každodennímu užívání byly pozorovány určité vedlejší účinky na mozek (ospalost, závratě, nevolnost). To vyvolalo otázku, jak vlastně NSA prostupuji hematoencefalickou bariéru.

Monovrstva vytvořená z buněk PBMEC/C1-2 byla použita jako *in vitro* model hematoencefalické bariéry. Transportní vlastnosti byly zkoumány u následujících NSA látek: celekoxib, diklofenak, ibuprofen, lornoxikam, meloxikam, piroxikam a tenoxikam. Pokusy byly prováděny s použitím pouze jedné látky NSA (jednoduché transportní pokusy) nebo s více látkami najednou (skupinové transportní pokusy). Během skupinových pokusů byly simulovány odlišné podmínky (různá transportní media, přítomnost séra, přidání transportních inhibitorů verapamilu nebo probenecidu, vyloučení některých NSA látek), aby bylo možno pozorovat případný vliv na transportní vlastnosti NSA. Pro standardizaci získaných dat byly přidány vnitřní standardy diazepam (transcelulární marker) a carboxyfluorescein (paracelulární marker).

Pořadí studií jednotlivých látek bylo podobné pořadí odpovídající skupinové studii. Skupinové studie byly nejvíce odlišné při použití transportního media bez séra, po přidání transportních inhibitoru verapamilu nebo probenecidu a po použití media k podpoře růstu astrocytů. Studie s mediem bez séra potvrdila u některých NSA silnou vazebnou schopnost na plazmatické bílkoviny a tím i jejich vliv na transportní schopnosti. Skupinové studie s transportními inhibitory poukázaly na transportní proteiny, které jsou zapojeny do transportních vlastností vybraných NSA. Nakonec i domněnka, že se všechny látky mohou navzájem ovlivňovat, byla také potvrzena.

Nepochybně bylo prokázáno, že většina NSA může značně prostupovat hematoencefalickou bariéru a tudíž ovlivňovat funkce centrálního nervového systému.

Prohlášení

Prohlašuji, že tato práce je mým původním autorským dílem, které jsem vypracovala samostatně. Veškerá literatura a další zdroje, z nichž jsem při zpracování čerpala, jsou uvedeny v seznamu použité literatury a v práci řádně citovány. Práce nebyla využita k získání jiného nebo stejného titulu.

Contents

Abbreviations

a-b/b-a	Apical to basolateral side/basolateral to apical side
ABC	ATP binding cassette
ACM	Astrocyte-conditioned medium
AJs	Adherens junctions
AMT	Adsorption mediated transcytosis
ATP	Adenosine-5'-triphosphate
BBB	Blood-brain barrier
BCRP	Breast cancer resistance protein (also ABCG2)
BM	Basement membrane (= basal lamina)
C6	Glioma cell line C6
Caco-2 cell line	Human adenocarcinoma cell line
CF	Carboxyfluorescein
cAMP	Cyclic adenosine-3′, 5′-monophosphat
CNS	Central nervous system
COX	Cyclooxygenase
DMSO	Dimethylsulfoxid
ECV304 cell line	Human umbilical vein cell line
EDTA	Ethylenediaminetetraacetic acid
Fib	Fibronectin
GLUT-1	Glucose transporter-1
Ham's F12	Ham reg. nutrient mixture with L-glutamine
IL-1/IL-2	Interleukin 1/Interleukin 2
IMDM	Iscove's modified Dulbecco's medium
JAMs	Junctional adhesion molecules
LDL	Low density lipoprotein
MAGUKs	Membrane-associated guanylate kinase-like proteins
MCTs	Monocarboxylate transporters
MeOH	Methanol
MDR	Multidrug resistance
MRPs	Multidrug resistance related proteins
NBS	Newborn calf serum
NSAIDs	Non-steroidal anti-inflammatory drugs
OATs	Transporters for organic anions
OCTs	Transporters for organic cations
OTC	Over the counter
PBMEC/C1-2	Porcine brain microvascular endothelial cells/C1-2
PBS	Phosphate buffer saline
PGE_2	Prostaglandin E_2
PGG_2	Prostaglandin G_2

PGH_2	Prostaglandin H_2
PGH_2-synthase	Prostaglandin H_2 synthase
P-gp	P-glycoprotein (MDR1, ABCB1)
PE	Permeability coefficient
PS	Permeation/surface coefficient
qPCR	Quantitative real time polymerase chain reaction
RMT	Receptor mediated transcytosis
RP-HPLC	Reversed-phase high performance liquid chromatography
RT	Room temperature
r_s	Spearman's correlation coefficient
SLC family	Solute carrier family
SNRI	Serotonin–norepinephrine reuptake inhibitor
TEER	Transendothelial electrical resistance
TJs	Tight junctions
TNF-α	Tumor necrosis factor α
UEA-1 receptor	Ulex europaeus agglutinin-1 receptor
UV	Ultraviolet light
vWF	Von Willebrand factor
γ-GT	Gama-glutamyl transpeptidase
ZO-1/-2/-3	Zonula occludens 1/2/3
$5HT_3$ receptor	5-hydroxytryptamine receptor

1 INTRODUCTION

1.1 Historical background

The first mention about the blood-brain barrier (BBB) is leading to the German scientist Paul Ehrlich (1854 - 1915), who received the Nobel Prize in Physiology and Medicine in 1908. His experiments with water soluble dyes injected into the peripheral circulation stained all animal organs except the brain and the spinal cord. He just assumed that there was a different binding affinity to the dye substance (Ribatti et al., 2006; Joó, 1993).

Edwin Goldmann (1878 - 1956) continued with the experiments and injected the dye into the cerebro-spinal fluid directly. He could observe the dyed brain but no color was present in the peripheral body parts (Goldmann, 1913). These experiments supported the idea of some unknown barrier.

The term 'blood-brain barrier' was used for the first time by the Berlin physician Lewandowski in 1900s.

However, the Latvian biochemist and physiologist Lina Stern proposed the very first concept of the hematoencephalic barrier (1921). As one of the first women in science, she carried out the pioneering experiments with the intention of regulatory function of the BBB, the effect of neurotransmitters (e.g. acetylcholine and adrenalin) and insulin on the CNS and the significance of the BBB for the treatment of some infectious diseases of the brain. Deeper understanding of the mechanism and properties of the BBB brought Stern to an idea to administrate the medications directly into the ventricles of the brain. Thereby she contributed to an increased effectiveness of cure of diseases such as encephalitis, tetanus, tuberculous meningitis, etc. In 1943 she got the Stalin Prize for achievement in the research of the BBB (Jewish Women's Archive, 2005).

Later in the 1960s due to the development of the scanning electron microscope, it was possible to visualize the blood-brain barrier and their tight junction chains.

But still since then, all experiments were made only *in vivo* or *in situ* (Abbott, 2005a). Finally, isolation of brain capillaries and cells in 1970s made *in vitro* studies possible (Joó and Karnishina, 1973). Further, experiments with isolated primary endothelial cells were improved, followed by generations of clone cell lines derived from different animal primary

cells to develop *in vitro* models, which had the most identical characteristics to the human blood-brain barrier.

1.2 Structure of the blood-brain barrier

The blood-brain barrier (BBB) belongs besides the choroid plexus epithelium and the arachnoid epithelium to the protective barrier layers of the central nervous system (CNS). It protects the brain from xenobiotics and it maintains the homeostasis of the brain (Dash and Elmquist, 2003). The largest contact area (approximately 20 m^2 per 1.3 kg brain), the shortest distance between the blood and the brain (8-20 μm) and the tight arrangement of the tight junctions of the BBB allow the greatest control over the brain microenvironment (Abbott, 2005a; Abbott et al., 2006). The BBB forms a kind of 'physical barrier', which consists mainly of endothelial cells that encircle the cerebral microvessels. The paracellular transport route is restricted due to the tight junctions, thus, the molecules are forced to cross the BBB transcellularly (Abbott, 2002; Abbott, 2005b). Only small lipophilic or gaseous molecules can pass the barrier freely (Abbott et al., 2006). The presence of different specific transport mechanisms is required by any other molecules (according to their lipid solubility), which manage the exact exchange between the blood and the CNS (Stannes et al., 1997). Besides supplying functions with essential nutrients, the transport system has an irreplaceable role by effluxing and excluding waste or harmful compounds. Hereby it is clear that the prevailed function is protective, which under special circumstances can complicate treatment of CNS diseases (e.g. epilepsy, CNS inflammations). However, the importance of the BBB is herewith not fully depleted. The BBB covers a number of static and dynamic properties that ensure a stable composition of the microenvironment of the CNS by preventing fluctuations of ions, it separates the pools of neurotransmitters that act centrally and peripherally, and finally it precisely controls the entrance of larger molecules such as proteins and peptides (Abbott and Romero, 1996; Abbott, 2005a).

As mentioned above, the main components of the BBB are the endothelial cells. The absence of fenestrations, more extensive tight junctions and sparse pinocytic vesicular transport differentiate the endothelial cells from those in the peripheral parts of the body. Since the restriction of the ionic movement is so significant, the transendothelial electrical resistance (TEER) can reach more than 1000 $\Omega*cm^2$ compared to the peripheral capillaries (usually 2-20 $\Omega*cm^2$) (Butt et al., 1990). In addition, the BBB is composed of the capillary

basement membrane (BM) (= basal lamina), astrocyte's end-feet and pericytes. These compounds together (Fig. 1.1) with neurons interactions and smooth muscles around the blood capillaries are also known as 'neurovascular unit', which mainly regulate the local blood flow and their blood supply (Abbott, 2005b; Abbott et al., 2006).

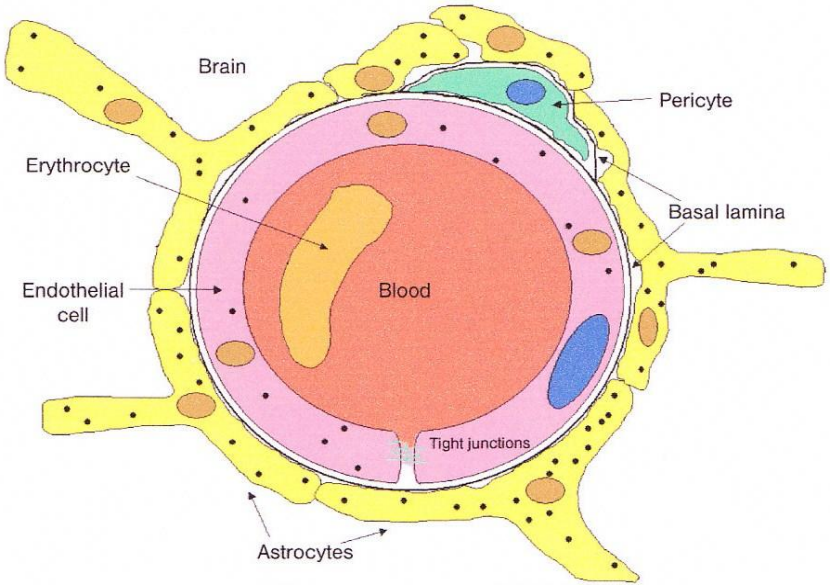


Fig 1.1: Brain microvessel in a schematic cross section. Source: Neuhaus and Noe, 2009.

Nevertheless, there are two parts of the brain where the protective BBB is lacking. These parts are the area postrema and the posterior pituitary area (= neurohypophysis). These areas regulate the autonomic nervous system and endocrine glands of the body (Abbott, 2005b).

1.3 Parts of the blood-brain barrier

The junctional complex is composed of tight junctions (TJs) and adherens junctions (AJs). A cadherin-catenin complex and its associated proteins are building the adherens junctions. Compared to that, the tight junctions consist of three integral membrane proteins (claudin, occludin and junction adhesion molecules) and of many cytoplasmic accessory proteins (e.g. zonula occludens proteins, cingulin) (Abbott, 2005b; Ballabh et al., 2004).

The phosphoproteins claudins are the main building blocks of the TJs and meanwhile 24 members have been identified. The dominant representation passes to claudins-1 and -5

whose major function is to form the primary seal of the TJs in the human brain. These proteins with four transmembrane domains bind via carboxyl terminals to the cytoplasmatic proteins (ZO-1, ZO-2 and ZO-3).

A larger phosphoprotein than claudin is occludin, which identically has also four transmembrane domains. The long COOH-cytoplasmatic domains is directly associated with ZO-proteins. The paracellular barrier of TJs is created from two extracellular loops of occludin and claudin. The main purpose of occludine seems to be altering the paracellular permeability and to contribute to the high TEER (Abbott, 2005b; Abbott et al., 2006; Ballabh et al., 2004).

Further, the third type of TJ-proteins is the junctional adhesion molecules (JAMs) with a single transmembrane domain. Since they belong to an immunoglobulin superfamily, their extracellular parts have two immunoglobulin-like loops formed by disulfide bonds. It is assumed that their main role is the cell-to-cell adhesion and monocyte transmigration through the BBB (Ballabh et al., 2004). Nevertheless, this knowledge seems to be incomplete and requires further investigations.

Additionally, other membrane proteins namely adherens junctions consist of cadherin, which further joins the actin cytoskeleton via catenins. This connection helps to form the adhesive contacts between cells.

Besides proteins taking part in the extracellular connections, there are some proteins located only in cytoplasm. These accessory proteins involve zonula occludens proteins (ZO-1, ZO-2 and ZO-3), cingulin, 7H6 and others. Mostly investigated are the ZO-proteins. They belong to membrane-associated guanylate kinase-like proteins (MAGUKs), which connect with claudins, occludines and JAMs. Additionally, the primary cytoskeleton protein actin binds to ZO-1 and ZO-2 and thus provides structural support to the endothelial cells (Abbott, 2006; Ballabh et al., 2004). The paracellular space is depicted in Fig. 1.2.

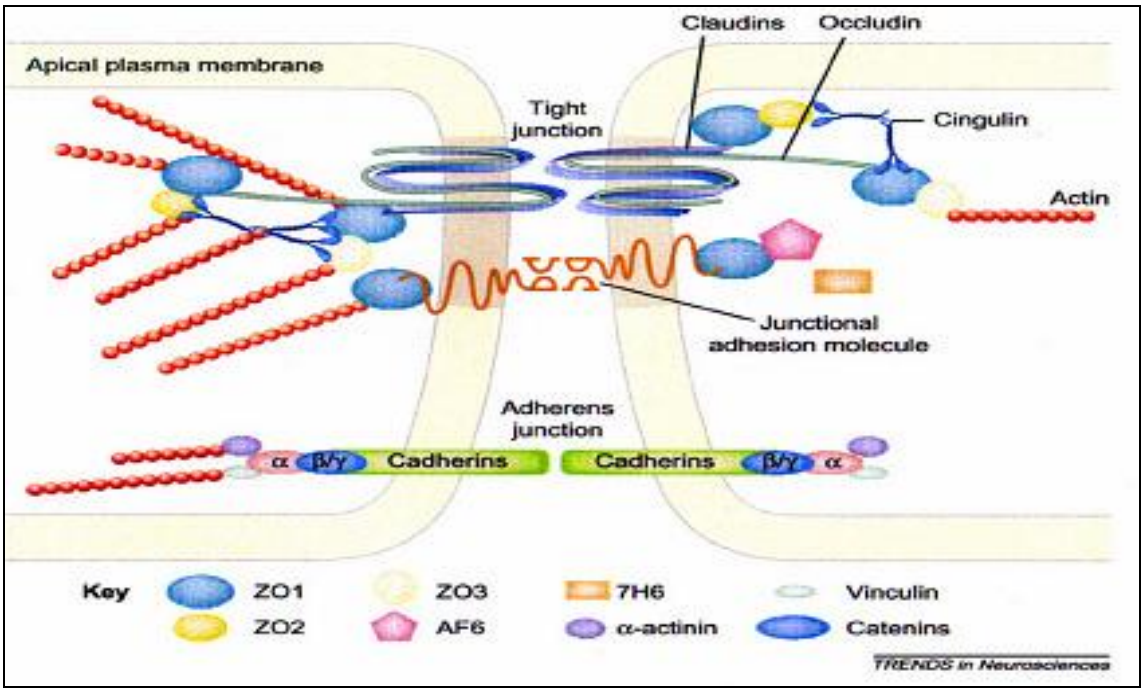


Fig. 1.2: Picture of paracellular space
The paracellular space between two endothelial cells of the brain capillary is restricted by tight junctions (TJs), junctional adhesion molecules (JAMs) and adherens junctions (AJs). Claudins and occludins of the TJs are connected to zonula occludens (ZO-1, ZO-2 and ZO-3), cingulin and actin. JAM proteins are also connected to ZO-proteins. Adherens junctions (AJs) connect cadherins to catenins and to actin fibers. Source: Huber et al., 2001.

Several studies have suggested that the ability to form a functional BBB is not possible through CNS endothelial cells only. The surrounding cells may have inducing influence on their property too, which was experimentally demonstrated by several studies (Ballabh et al., 2004). It is assumed that astrocytes and endothelial cells can change their information between each other and hence regulate each other's functions. Since the astrocytes can reach inducing influence due to secretion of several chemical agents (transforming growth factor-β, basic fibroblast growth factor, angiopoetin-1, etc.) (Abbott et al, 2006). Neuhaus et al. (2008) suggested to culture human or bovine endothelial cell-monolayer in astrocyte-conditioned media (ACM), which can induce the BBB's tightness.

Suggestion that BBB properties could be induced by astrocytes support also the fact, that Neuhaus et al. (2008) was observing the difference between BBB mimicking cells co-cultured with astrocytic media and without. Already Stanness et al. (1997) observed the same effect by establishing a new cell line suitable as a model of BBB *in vitro*.

However, another contribution of astrocytes was observed on the homeostasis of water, ions, amino acids and neurotransmitters of the brain. The process of recycling of several substances or the removing of the water is directly influenced by astrocytes because of their strategic position between neurons and capillaries (Abbott et al., 2006).

Microvessel cells pericytes are wrapping the endothelial cells, and mainly providing their structural stability. It is assumed that pericytes could be involved in the process of cerebral auto-regulation because they possess numerous varied receptors (e.g. receptors for vasopressin, for angiotensin II, for catecholamines, etc.) (Abbott et al., 2005b). Also the corporation between pericytes and blood vessels assumes regulation of endothelial cells´ proliferation, survival, migration, differentiation and vascular branching (Dohgu et al., 2005). Ramsauer et al. (2002) showed the increased resistance to apoptosis of endothelial cells while they were cultured with pericytes and astrocytes. This finding supported the concept that pericytes may play an important role in the genesis of the BBB (Balabanov and Dore –Duffy, 1998; Hirsche and D´Amore, 1997).

1.4 Influence on the BBB

As described above, the BBB has an irreplaceable role in the protection of the CNS. Abbott (2004b) suggested that the BBB should not be seen as a static barrier, but more as a barrier which can adapt to current needs of the brain. Generally, there are several substances, which have a direct influence on the BBB and can modulate its tightness. Some of them can increase BBB permeability (e.g. bradykinin, histamine, serotonin, glutamate, adenosine, ATP, interleukins, TNF-α), others can cause BBB tightening (e.g. steroids, cAMP, noradrenergic agents).

If the tight junctions open, an inflammation and a consequent brain edema can occur. Further, any changes by transport mechanisms can lead to brain 'starvation' and hypoxia because of decreased supply of required nutrients (Abbott, 2005b). On the other hand, increased influx of any compounds can change the exact composition of the microenvironment and disturb the normal functions of the brain.

As mentioned above, astrocytes can modify the BBB function through secretion of specific chemical agents, which are essential for the normal function of the BBB. Any abnormalities or disequilibrium in chemical composition of brain liquids can result in neuropathology (Abbott et al., 2006).

Some brain tumors are characterized by the down-regulation of protein claudin-3. The claudin-3 down-regulation and the BBB disruption were observed by multiple sclerosis or by the Alzheimer's disease (Abbott, 2005b). Accumulation of beta-amyloid caused intoxication on the neighborhood endothelium, which is the first attribute of the Alzheimer's

disease. On the contrary in epilepsy, the P-gp transporters are upregulated and ABC transporter expression is changed, which occurs mostly during seizure activity. In addition, also Parkinson's disease is connected with changed expression of the P-gp (Kortekaas, et al., 2005).

The range of diseases, which are linked with BBB dysfunction or disruption, is enormous. Generally, we can say that any pathological agent can influence the BBB function. Consequently, there are many studies investigating these issues. Already many chemical substances are known und used for treatment of brain diseases. On the one hand, it is required to stabilize the BBB permeability. For example, dexamethasone is successfully used in brain edema because it can reduce the inflammation and it can improve the barrier functions (mainly increasing the tightness and up-regulation of P-gp) (Cucullo et al., 2004). Also calcium-channel blockers can reduce brain damages in hypoxia and hypertension. On the other hand, the controlled barrier opening is necessary for treatment of some brain diseases to deliver chemical substances directly to the point of origin of the illness.

It was confirmed that various vitamins and other substances could have neuroprotective functions. Fish oil, vitamin C and E, red wine, garlic, fruit and soya may be beneficial because they can reduce the incidence of systemic vascular disease generally (Abbott et al., 2006). Flavonoids and polyphenolic agents derived from different plants may have a positive effect on the endothelium as well (Youdim et al., 2002).

1.5 Transport mechanisms across the BBB

To maintain homeostasis it is necessary to control the transport of nutrients into the brain. Hence, the paracellular route is restricted by the tight junctions, a number of several transport mechanisms are expressed on the BBB surface (Fig. 1.3).

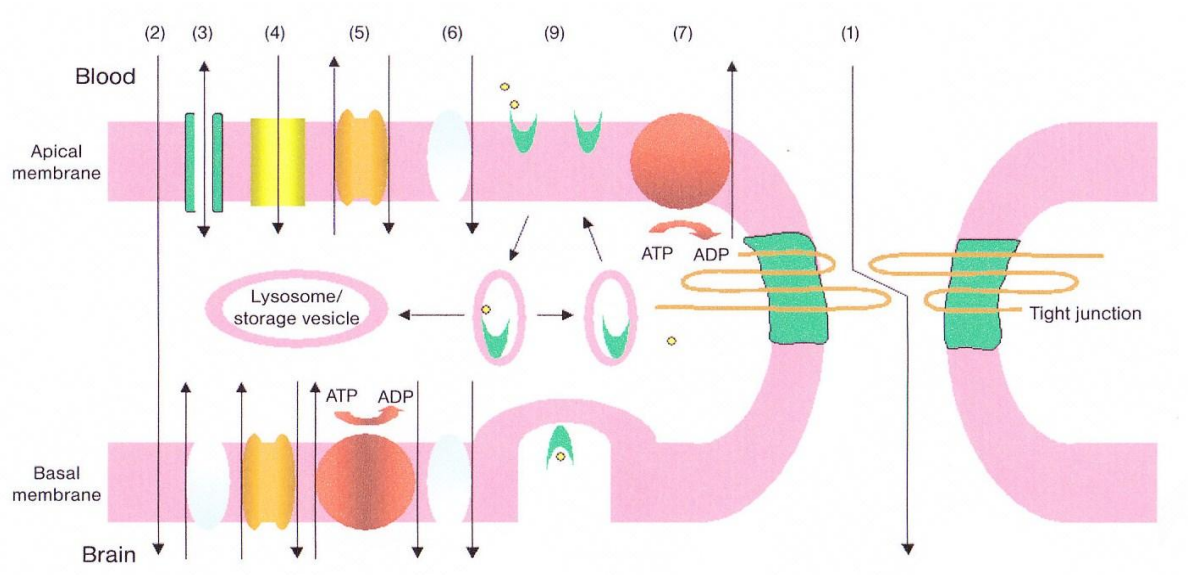


Fig. 1.3: Types of transport mechanisms: (1) paracellular transport, (2) transcellular diffusion, (3) cation channels, (4) ion symports, (5) ion antiport, (6) facilitated diffusion, (7) active transport, (8) active antiport transport, (9) endocytosis. Source: Neuhaus and Noe, 2009.

The freely transcellular diffusion, driven by a concentration gradient, is possible only for small lipophilic molecules such as oxygen, carbon dioxide and ethanol. The higher lipophilicity of a substance results in increased permeation ability (Abbott, 2004a).

Other substances are crossing the BBB via passive transport mechanisms such as channels or carrier-proteins, which are still energy independent. Ions are carried by ion channels (K^+ ion), ion symports ($NA^+/K^+/Cl^-$ co-transporter) or by ion antiports (NA^+/H^+ exchange). These carrier-proteins are specialized and require binding of a substrate, which changes the protein conformation and that starts the transport activity. They can possess only one binding receptor (uniport) or two receptors for different molecules, which go in the same way (symport) or in an opposite way (antiport).

The facilitated diffusion is typical for glucose, which is carried by GLUT-1 transporter, from high to low concentration. Other carrier-mediated mechanisms contribute to the transport of many other substances such as monocarboxylates, hexoses, amino acids, nucleosides, glutathione, small peptides, etc. (Abbott, 2002).

For other substances there are more than 10 different active transport systems, which are mostly energy dependent and against the concentration gradients. Na^+/K^+ ATPase is an example for active antiport transport, which is located generally on the abluminal side of the BBB, specialized for moving solutes out of the brain (O'Kane et al., 1999).

P-glycoprotein (P-gp) is an ATP-dependent efflux pump with localization on the luminal side of brain capillary endothelial cells and it is specialized for the transport of lipophilic molecules (Loscher and Potschka, 2005). P-gp ensures efflux of xenobiotics from cells and thus contributes mainly to the protection of organs (Neuhaus et al., 2010).

Larger substances such as peptides or proteins have to be transported across the BBB by endocytosis. There are two different types of the endocytosis – transcytosis and pinocytosis. The receptor mediated transcytosis (RMT) requires a specific interaction with a cell membrane receptor and it is energy dependent. RMT ensures transport of substances such as transferrin, insulin, leptin, insulin-like growth factors, etc. (Pardridge, 2005). The other type of endocytosis is less specific and requires only binding of cationic molecules to the negatively charged membrane surface (Abbott, 2005a; Pardridge, 2005). However, the adsorption mediated transcytosis (AMT) is less active in the brain than in the periphery. Pinocytosis is responsible for uptake of extracellular fluids, but is significantly less abundant in the brain endothelium than in the peripheral blood capillaries.

1.5.1 The efflux pump system

The BBB possesses an efflux transport system, which is important for protecting the brain from xenobiotics and to remove waste products from the CNS. Most of these transporters belong to the solute carrier family (SLC) or to the ATP binding cassette (ABC) transporter family. An overview of the most important transporters is presented in the Fig. 1.4.

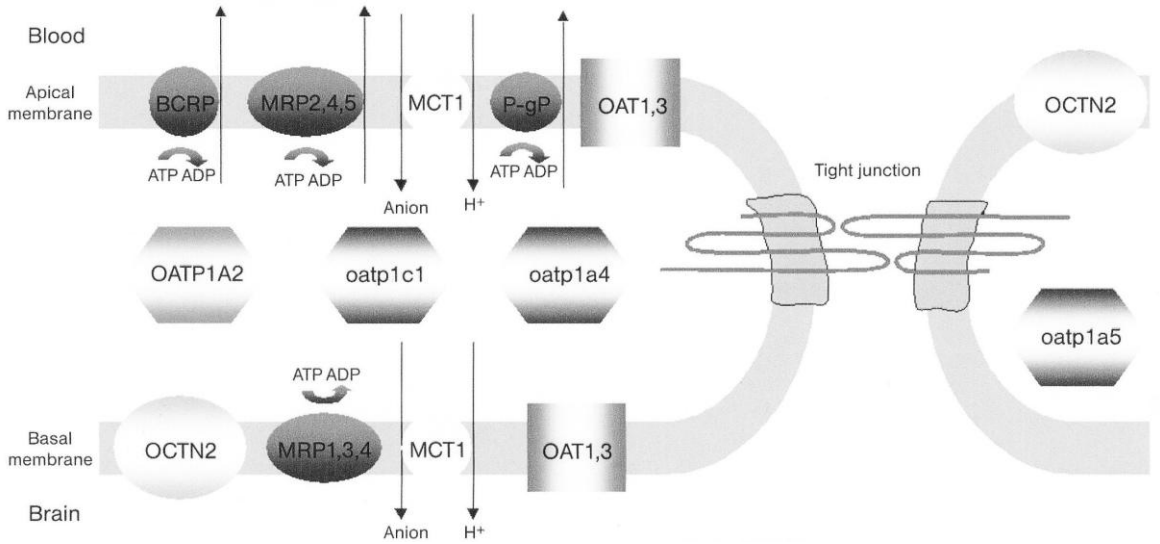


Fig 1.4: Localization of main drug transporters at the BBB. Source: Neuhaus and Noe, 2009.

Members of the SLC transporter family

Monocarboxylate transporters (MCTs) are members of the solute carrier family (SLC) and consist of 14 members, called MCT1-9, MCT11-13 and TAT-1. The major role for the BBB has the most studied MCT-1, distributed at the luminal as well as at the abluminal side of the brain microvessels. The expression itself 'monocarboxylate transporters' already reveals the typical substrate, which is transported to or out of the brain. Usually, glucose is the main energy substrate for the brain. But under pathological conditions (e.g. diabetes, prolonged starvation), the brain can also import other substances such as lactate, pyruvate and ketone bodies acetoacetate and β-hydroxybutyrate, which afterwards are converted into energy (Nehlig et al., 1993). It was observed that oligodendrocytes or astrocytes are able to utilize lactate for neoglucogenesis or glycogen synthesis (Dringer et al., 1993). On the contrary, during ischemia or brain injury, efflux of lactate from the brain into the bloodstream was increased by MCTs (Frerichs et al., 1990).

Other transporter groups belonging to the SLC transporter family are organic ion transporters (human OATs, rodent oats) and transporting peptides (human OATPs, rodent oatps). Generally, these transporters are responsible for active influx or efflux of drugs and xenobiotics, neuroactive peptides, thyroid hormones, bile salts and steroid conjugates (Meier et al., 1997). For example, OATP1A2 was found in brain capillaries and is responsible for transport of bile acids, organic anions, cations and steroids from the blood into the brain and vice versa (Hagenbuch and Meier, 2004). In contrast, the similar transporters OAT1 preferring organic anions, and OAT3 preferring dicarboxylates, are more specific. Also OCTN2 seems to be polyspecific for mainly organic cations and L-carnitine and further for antibiotic cephaloridine, L-lysine, L-methionine, verapamil, choline and quinidine (Koepsell and Endou, 2004).

Members of the ABC transporter family

P-glycoprotein (ABCB1, MDR1) was reported to be the most important member of the ATP binding cassette (ABC) transporter family localized on the BBB (Lorscher and Potschka, 2005) and it is a member of the MDR/TAP subfamily (subfamily B, member 1). Hence, it is responsible for the multidrug resistance of cells. P-gp is a membrane-associated glycoprotein with six transmembrane domains. P-glycoprotein could be found in different

human tissues as intestinal epithelium, brain capillary endothelium, and in cells in kidney, liver and testis, in adrenal gland and placenta (Lincová, Farghali et al., 2007). Nevertheless, P-gp is also present in cancer cells, which results in inadequate reply to a chemotherapeutical treatment or an absolute resistance (Seeber et al., 1982; Lehne, 2000). P-gp can efflux substances with diverse molecular weight. Examples of P-gp substrates are listed in the Table 1.1.

Examples of substrates for human P-glycoprotein

Substance	Drug class
Aldosterone	Endogenous substrate
Asimadoline	Antianalgetic, κ-opioid receptor antagonist
Cortisol	Endogenous substrate
Cyclosporin A	Immunosuppressant
Daunorubicin	Chemotherapeutic anthracyclines
Digoxin	Cardiac glycoside
Doxorubicin	Chemotherapeutic anthracyclines
Etoposide	Chemotherapeutic epipodophyllotoxin
Fexofenadine	H1-antihistamine
Glucuronides	Conjugates of phase II metabolized drugs
Gramicidin-D	Antibacterial
Ivermecin	Anthelmintic drug
Loperamide	Antidiarrheal agent, opioid
Loratadine	H1-antihistamine
Methotrexate	Dihydrofolate reductase inhibitor
Morphine	Analgesic drug
Nifedipine	Ca^{2+} channel blocker
Olanzapine	Antipsychotic drug
Ondansetron	5-HT_3 receptor antagonist
Phenytion	Antiepileptic drug
Quetiapine	Antipsychotic drug
Ranitidine	H2-antihistamine
Rapamycin	Immunosuppressant
Risperidone	Antipsychotic drug
Trimipramine	Tricyclic antidepressant
Venlafaxin	Antidepressant, SNRI
Vinblastine	Chemotherapeutic vinca alkaloid, antimitotic drug
Vincristine	Chemotherapeutic vinca alkaloid, antimitotic drug

Table 1.1: Examples of substrates for P-glycoprotein. Source: adopted from Neuhaus and Noe, 2009.

P-gp presents the first line defense of the brain. Therefore there is a big interest to discover a possibility how to bypass this efflux pump. A specific P-gp blocker commonly used in the laboratory practice is verapamil, a Ca^{2+}-channel blocker. It belongs to the first generation of the P-gp inhibitors (along with cyclosporine, amiodarone, tamoxifen) (Pechandová et al., 2006). Unfortunately, it showed cardiotoxicity at the effective dosages

(Ficková et al., 2002). However, there are more than 40 substances identified with a P-gp blocking function, e.g. quinidine, clarithromycin, doxorubicin, pyronaridin, valproats (Ficková et al., 2002).

There are other efflux pumps, which are involved in the multidrug resistance and belong to the ABC transporter family. It is known that P-gp is encoded by the gene named multidrug resistance 1 (MDR1) and meanwhile there are already nine **multidrug resistance-associated proteins** (MRPs) discovered (MRP1-9; called also ABCC1-6 and ABCC10-12). It was found out that MRP2, MRP4 and MRP5 are present in the brain endothelium and MRP1 prevails in the choroid plexus (Wijnholds, 2005). Because of a low expression of MRP3 and MRP6 in the brain, it is assumed a minor role for them at the BBB. Their main source of transport substances are negatively charged acidic anions (purine- and pyrimidine-based nucleotide analogues), natural compounds and drugs bound to glutathione, glucuronate and sulfate and neutral drugs cotransported with glutathione (Borst et al., 2000). Probenecid and sulfinpyrazone are typical transport inhibitors used for studies.

The third member of the ATP binding cassette family is **breast cancer resistance protein** (BCRP; called also ABCG2), located on the luminal side of the BBB (Doyle and Ross, 2003). It is believed that BCRP has to dimerize to be activated. Several substrates for the BCRP are the same as for P-gp or MRPs, which means that BCRP contributes to the multidrug resistance phenomenon. Following exogenous substances are transported by BCRP: doxorubicin, daunorubicin, etoposide, topotecan, mitoxantrone, methotrexate, irinotecan, prazosine, azidothymidine, lamivudine (Doyle and Ross, 2003), but also endogenous substrates such as estrogens are involved.

1.6 Non-steroidal anti-inflammatory drugs

The non-steroidal anti-inflammatory drugs (NSAIDs) are symptomatic drugs, which play an important role in the suppression of pain, fever and inflammation. Nowadays NSAIDs are widely used especially against pain generally, muscle ache and for its antipyretic effect. The easy access to these drugs is mainly caused by their placement into 'over-the-counter' (OTC) medicine category. The various routes of administration (oral, rectal, local, parenteral application, etc.) allow also the treatment of children (e.g. ibuprofen).

Pain is influenced mainly peripherally, but partly also on the CNS level. NSAIDs do not influence visceral pain at all. The antipyretic effect comes fast. Nevertheless, there is no influence on the regular body temperature. Naturally, the basic effect of the NSAIDs is the suppression of an inflammation. NSAIDs can intervene into the acute phase of the inflammation, but they cannot influence the chronic phases. Some of the NSAIDs have also an anti-aggregation effect, which coxibs fully lack (Lincová and Farghali et al., 2007).

The NSAIDs reversibly inhibit the cyclooxygenase enzyme (COX) (also known as PGH_2-synthase), which is important for the transformation of prostanoids to prostaglandins, thromboxanes and prostacyclins. The inhibitions take place in the first part of the transformation where COX converts the arachidonic acid into endoperoxid PGG_2. The following process where PGG_2 is converted into PGH_2 is not influenced (Fig 1.3) (Lincová and Farghali et al., 2007).

Until recently two main isoforms of COX were known. COX-1 was believed to be a constitutional form, which leads to formation of prostanoids that ensure physiological functions of the organism (e.g. gastric protection, vascular homeostasis, platelet aggregation, kidney function) (Nanau and Neuman, 2010). On the other hand, the inducible form COX-2 catalyzes production of prostanoids, which have a local inflammatory effect and cause fever and pain. The division of the function is not that easy as it seems at the beginning. It was already found out, that also COX-1 plays some role in the inflammation and contrary COX-2 shows some physiological functions (e.g. in CNS, in kidneys, in digestive tract) (Mutschler et al., 2013).

Lately, a third type of COX was discovered. COX-3 exists in heart and CNS and probably also in other tissues (Lincová and Farghali et al., 2007).

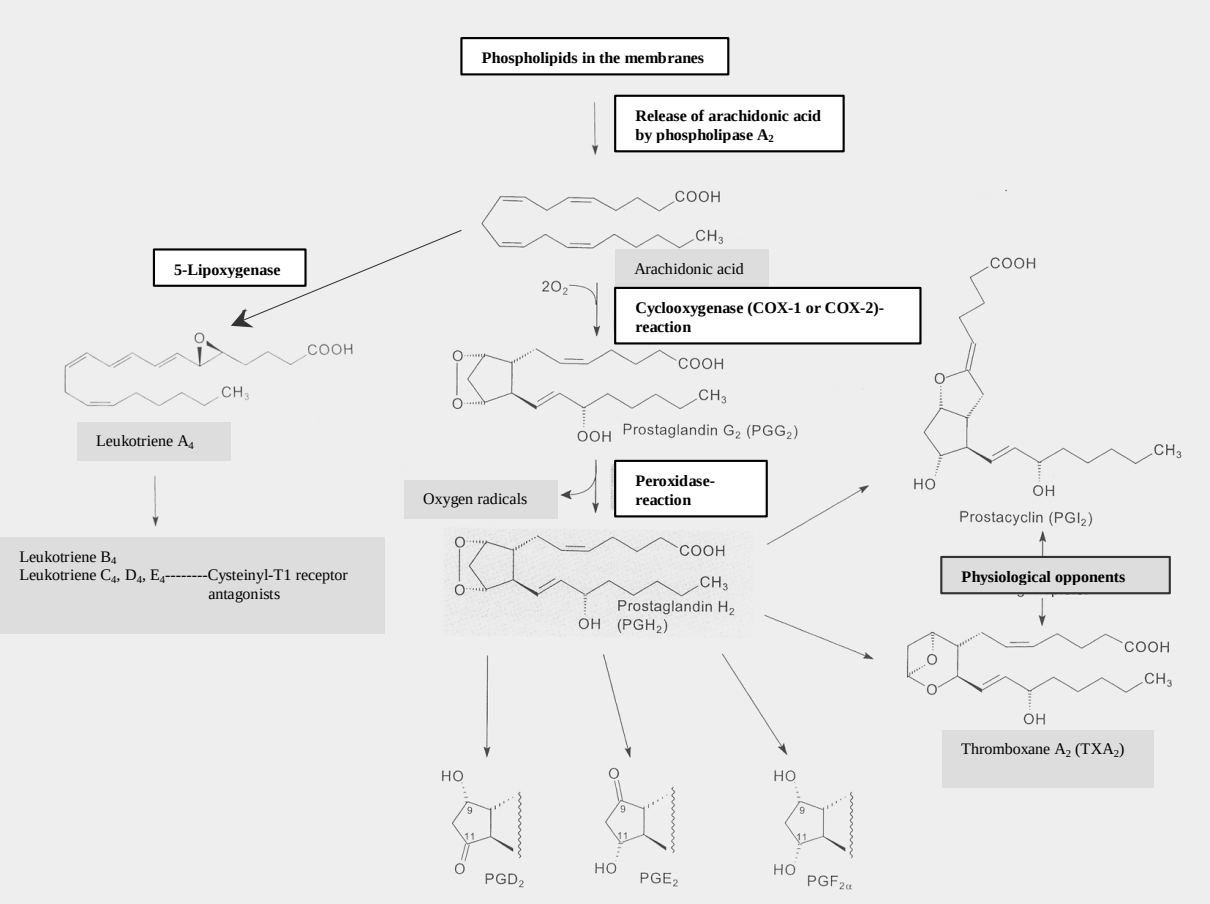


Figure 1.3: Scheme of conversion of arachidonic acid
The arachidonic acid and its conversion into leukotrienes, prostaglandins and thromboxanes with their belonging enzymes. Source: Steinhilber et al., 2005.

There are some other effects of the NSAIDs. Some of them can inhibit also the formation of leukotrienes (e.g. indometacin, diclofenac, nimesulid, ketoprofen) and concurrently they reduce the production of bradykinin. Some of them can reduce the production of free oxygen radicals, they suppress the migration of macrophages and leukocytes in the place of inflammation, they influence the production of cytokines, they inhibit the release of histamine, etc. The newest studies, based on randomized controlled trials, showed that long-time treatment with acetylsalicylic acid (≥ 75 mg daily) might reduce the incidence of cancer metastasis (Rothwell et al., 2012).

Side and adverse effects

It is obvious that besides their positive effects these chemical substances have also side effects. The common side effects on the skin (e.g. urticaria, rash, itchiness or pruritus) may just be temporary and should disappear among a longer therapy (Mutschler et al., 2013). On the contrary, an allergic reaction is a serious reaction of the body and may lead

under dramatic circumstances to anaphylaxis, angioedema and even to death. On the other hand, a specific side effect was reported for ibuprofen. The hypersensitivity syndrome is characterized by fever, rash and internal organ involvement, which occur with delay until 12 week after the onset of the ibuprofen therapy (Nanau and Neuman, 2010).

In this case, bronchial spasm and induction of asthmatic attack are classified as pseudoallergic reaction (Mutschler et al., 2013). However, people with asthmatic problems may avoid using NSAIDs at all.

A long time therapy especially of higher dosage may lead to auditory disorder, tinnitus and vertigo or even to loss of hearing (Lincová and Farghali et al., 2007).

It is generally known that NSAIDs negatively influence the gastrointestinal tract. Through the blockade of prostaglandins production the gastric wall loses its protective effect and the disruption of mucosa caused by gastric juices is started. It leads to gastrointestinal bleeding, inflammation and to gastric ulcer. Generally, stomach disorder (nausea, emesis, cramps, flatulence, absence of appetite, stomach upset, etc.) accompanies those diseases (Lincová and Farghali et al., 2007). It was observed that ibuprofen is safer than acetylsalicylic acid with regard to gastrointestinal bleeding (Rainsford, 1999).

The inhibition of production of thromboxanes could cause unexpected, inner bleeding. This has to be taken into account especially if the patient is treated with anti-aggregation medicine (Lincová and Farghali et al., 2007; Mutschler et al., 2013).

Last but not least, using of NSAIDs may disrupt the physiological function of kidney and may cause hepatitis (Lincová and Farghali et al., 2007; Mutschler et al., 2013).

There are numerous possible interactions with other drugs. Many drug groups, such as coumarin anticoagulants, antihypertensive drugs, sulfonamide, oral antidiabetics, certain antibiotics (ampicillin), etc. change or lose their effectiveness or increase their side effects.

Pharmacokinetic characteristics were already discussed in detail in the diploma thesis (Nováková, 2009).

Although NSAIDs have many adverse side effects in the recent time, there are several studies that pointed to positive effects, which have not been described yet. For example, Dokmeci (2004) published a report, in which he pointed at a possible connection between using ibuprofen in a long-term therapy by patients suffering from rheumatoid arthritis and less occurrence of Alzheimer's disease by the same patients compared to a group of people, who did not use any analgetics for longer time. That uncovered an ability of ibuprofen to

reduce the beta-amyloid plaques in the brain, which were connected with the genesis of Alzheimer's disease. Another study showed ibuprofen (but also indomethacin and sulindac) in combination with lipoic acid as beneficial by reducing beta-amyloid plaques in rats as well (Di Stefano et al., 2010).

Another report including observation of early phases of schizophrenia and major depression showed that ill patients produced a higher number of inflammatory factors caused by PGE_2 as a maker of cytokines (IL-4, IL-5, IL-6, IL-10, etc.). Therefore, COX-2 inhibitors (celecoxib) or mixed COX-1/COX-2 inhibitors (acetylsalicylic acid) were add-on to risperidone or olanzapine and showed better results in the treatment (Műller et al., 2008) compared to antidepressant as monotherapy or to placebo. The same observations were confirmed by rofecoxib, which can increase serotonin levels in rat brains and so reduce the manifestation of the illness (Műller, 2010).

These are just a few examples for NSAIDs, which can show their beneficial effect in co-treatment therapies. Further studies might bring new knowledge into standards therapies.

2 AIM

The non-steroidal anti-inflammatory drugs (NSAIDs) are commonly used for treatment of headache, fever, different kind of pain or against inflammation. However, many studies have proven that NSAIDs are able to cross the BBB more than it was expected and currently are able to influence further uptake of other substances.

The fact that there was no systematic study including more than three substances, gave us the idea to create a compact study including several NSAID substances.

1) Seven different NSAIDs (celecoxib, diclofenac, ibuprofen, lornoxicam, meloxicam, piroxicam and tenoxicam) should be investigated in this study.
2) Comparison of single and group transport studies (except lornoxicam) across a BBB *in vitro* model based on the cell line PBMEC/C1-2.
3) Different conditions during the transport studies (serum protein free medium, influence of inhibition of some transport mechanisms, influence of astrocytic factors) may affect the results
4) Influence of transport inhibitors to elucidate a possible role of transporters in NSAID transport since interaction of NSAIDs and transport inhibitors are known (Angelini at al., 2008).
5) Gained data should be compared with already published data across a BBB *in vitro* model based on cell line ECV304 (Nováková, 2009).

3 MATERIAL AND METHODS

3.1 Material

3.1.1 Cell line PBMEC/C1-2

Thanks to isolation and immortalization of porcine brain microvascular endothelial cells (PBMEC) by Teifel and Friedl (1996), the new cell line PBMEC/C1-2 (Fig. 3.1) was established, which retained several BBB properties. The glucose transporter (GLUT-1), γ-glutamyltranspeptidase (γ-GT) and apolipoprotein A-1 belong to the specific blood-brain barrier features exhibited by the PBMEC/C1-2. Furthermore, this cell line has been distinguished from other cell lines with the presence of the von Willebrand factor (vWF), lectin binding receptors for UEA-1 and uptake of acetylated LDL (Neuhaus et al., 2006). The presence and functionality of P-gp was later verified on different levels (western blotting, immunofluorescence, qPCR) (Neuhaus et al., 2009; Neuhaus et al., 2010). The cell tightness, measurable as TEER value, can be induced by astrocytic factors (Neuhaus et al., 2010). One major advantage is the good stability (cultivation for nearly one year without loss of any main properties). Thanks to its features, the immortalized cell line PBMEC/C1-2 was recommended as a useful *in vitro* model of the BBB (Neuhaus et al., 2008).

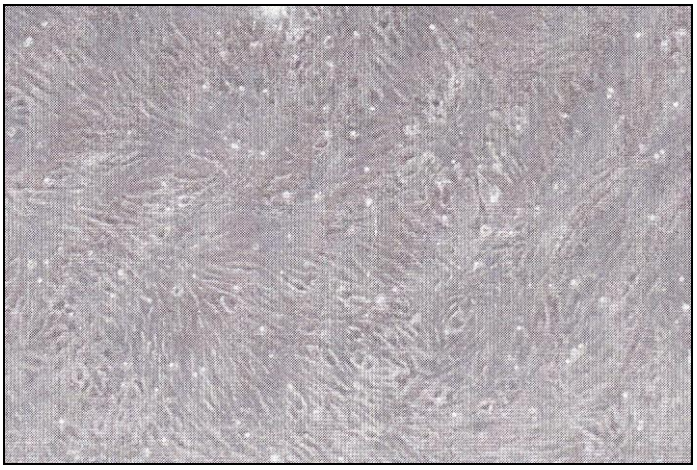


Fig. 3.1: Confluent PBMEC/C1-2 under the light microscope. An elongated and stretched morphology is typical for this cell line. Source: Photo made by cultivation.

3.1.2 Media

Three types of media were used for all experiments. All media were always prepared under sterile conditions, they were stored at 4°C and they were prewarmed to reach 37°C before use.

PBMEC medium without fibronectin

This medium was used for common treatment of the cells (cultivating, media changing, spitting and seeding).

Medium composition: Ham's F12 and IMDM 1:1, 200 mM L-glutamine, 10 mg/ml holo-transferrin, 1,000 U/ml heparin, NCS, 10,000 U/ml penicillin; 10 mg/ml streptomycin, 250 μg/ml amphotericin B, ACM (collected supernatant of C6 medium, sterile).

C6 medium

Transport experiments were carried out using this C6 medium except two experiments, one, where PBMEC medium without fibronectin was used and another one, where C6 medium was prepared without serum.

C6 medium without serum

The composition of this medium was identical to C6 medium except the NCS was not added. It was used only for one group study, where binding ability of NSAIDs to plasma proteins was investigated.

Exact compositions of all media were listed in the diploma thesis (Nováková, 2009).

3.1.3 RP-HPLC

All samples were analyzed using the reversed-phase high performance liquid chromatography (RP-HPLC). For the RP-HPLC were following components used:
DGV - 20A5/prominence Degasser (Shimadzu), SIL – 20AC/prominence Auto Sampler (Shimadzu), CTO – 20AC/prominence Column Oven (Shimadzu), SPD – 20A/prominence UV detector (Shimadzu), RID – 10A/Shimadzu Refractive Index Detector (Shimadzu), CMB

– 20A/prominence Communications Bus Module (Shimadzu), 820975-906 Norrowbore HPLC Column 2.1 x 50 mm packed with Zorbax SB-C8 5 µm (INULA Instrumentelle Analytik Löwenburggasse 2, A-1082 Wien), 821125-915 HPLC Column 2.1 x 12.5 mm packed with Zorbax SB-C8 5 µm (INULA Instrumentelle Analytik Löwenburggasse 2, A-1082 Wien), Lichrospher column 100 Rp-18, 250 x 4 mm, 5 µm pore size LichroCART 4-4 precolumns (Merck KGaA, Darmstadt, Germany).

3.1.4 Substances

Following substances were used for the experiments:

Celecoxib, diclofenac, lornoxicam and diazepam were kind gifts from Dr. Maierhofer (AGES PharmMed, Vienna, Austria).

Ibuprofen (I1892), meloxicam (M3935), piroxicam (P5654), tenoxicam (T0909) and probenecid (P8761) were bought from Sigma-Aldrich, Vienna, Austria.

5(6)-Carboxyfluorescein (21877) and verapamil (94837) were bought from Fluka, Buchs, Switzerland.

3.2 Methods

3.2.1 Treatments of the cell culture's flask

Every manipulation with cells took place in the laminar air flow cabinet to insure the sterile conditions. All media and solutions used for the treatment were always preheated at 37°C in a warming chamber. The flasks were placed into the incubator for cultivation at 37°C, 95% air/5% CO_2 and 96% humidity.

Coating of T25 flasks

The whole surface area of a new sterile T25 flask was covered with 2 ml of 1% sterile gelatin solution and incubated for at least 20 minutes at room temperature (RT). After the gelatin solution was removed, the flask was ready for cell seeding.

Cell subcultivation

The surface of a T25 flask was washed with 2 ml sterile PBS twice, after that, 2 ml of trypsin/EDTA solution were added and incubated for 3 - 5 minutes at 37°C and finally the solution was removed. A new growth medium was added (5 ml) to get a homogenous cell suspension. Required volume of the cell suspension was transferred into a new, gelatin coated T25 flask, usually in a ratio 1:5.

For more details see the diploma thesis Nováková (2009).

3.2.2 Transwell model

The transwell model – a six well plate - was used for cell cultivation and experiments. It consisted of six chambers (acceptors of the substances) and six inserts (donors of the substances) with a semi-permeable membrane with 1 μm pore size at the bottom and a total growth area of 4.2 cm^2 (Fig. 3.2).

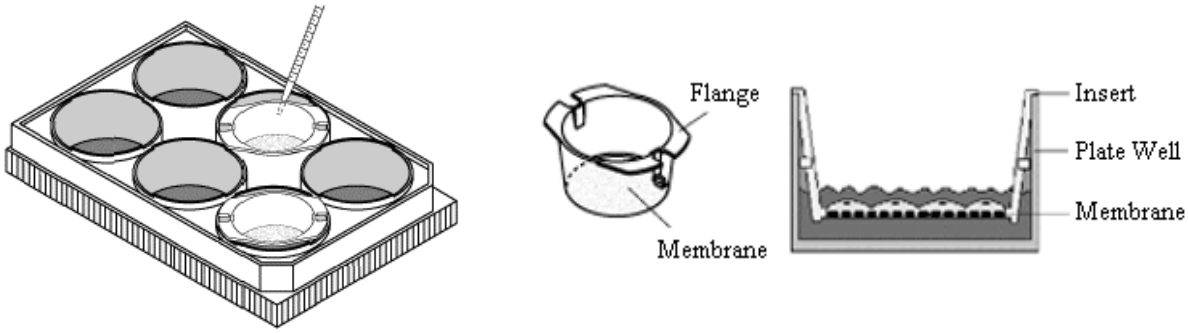


Fig. 3.2: Transwell model
The 6-well plate with six chambers and inserts, detail of an insert and a side view.
Source: Beckton and Dickinson, 2009.

Coating of inserts

The inserts coating was made with 150 µl of 0.14 mg/ml collagen (dissolved in sterile PBS). After the plate was incubated for 1 hour by 37°C, the supernatants of collagen were removed. Next, the coating with 150 µl of 100 µg/ml fibronectin solution followed in the same way as with the collagen solution. When the supernatants of fibronectin were removed, the plate was prepared for cell seeding.

Cell seeding

Via cell counting using the Thoma chamber, the required amount for the experiment was fix on 80,000 cells/cm^2. Every well was filled with 3 ml of preheated growth medium and inserts with the total volume of 2 ml, where 3 inserts were filled with the precalculated amount of the cell suspension.

The cells growing time was five days until they were tight enough for an experiment. Usually, the medium was changed every day.

The conditions for growing in the incubator were 37°C, 5% CO_2 and 96% humidity.

3.2.2.1 Measurement of the Transendothelial Electrical Resistance

Transendothelial electrical resistance (TEER) is a significant characteristic of the compactness of the cell layer where the higher TEER value of the cell monolayer reported a higher restriction of the paracellular space.

The values of TEER were measured via a Millipore Millicell Electrical Resistance System (ERS, Millipore Vienna) every day since the second day.

The electrodes were equilibrated with the same growth medium for at least 30 minutes before measurement. Every measurement was preceded by medium change in the 6-well plate and incubation time by room temperature for 1 hour.

The real resistance of the cell monolayer was gained by subtracting the average value of the blank inserts ($TEER_{blank}$) from the value of the well with cells ($TEER_{cell}$) and the whole result was multiplied by the surface area to obtain the TEER values in Ohm*cm^2 (see the formula below).

TEER [Ω*cm^2] = ($TEER_{cell}$ – average $TEER_{blank}$) * surface area (4.2 cm^2)

3.2.3 Transport studies

General procedures by transport experiments

The experiment was carried out on the 5th day. Before the experiment was started, it was important to confirm the usability of the cells via TEER measuring as describe before (3.2.2.1).

Detailed procedures can be found in the diploma thesis (Nováková, 2009).

All inserts, filled with experimental solution, were placed into the incubator where they stayed the whole time of the experiment. Just shortly before the end of the experiment they were taken out. The inserts were replaced six times (every 40 minutes) during the four hours experiments.

The supernatants from the inserts, the medium samples from all chambers, a sample of pure C6 medium and of an experimental stock solution were collected and used for further analyses.

Internal standards

Internal standards were used to be able to normalize the permeability data of NSAIDs and to minimize the influence of cell layer's variability. Two substances were chosen: diazepam and carboxyfluorescein (CF).

Diazepam as a transcellular marker can pass the BBB via passive diffusion because of its very high lipophilicity (Fig. 3.3 right). The sterile filtered stock solution of diazepam dissolved in methanol had a concentration of 10 mM.

Carboxyfluorescein is known as a paracellular fluorescent marker (Fig. 3.3 left). It was dissolved in water with final concentration of 400 µM.

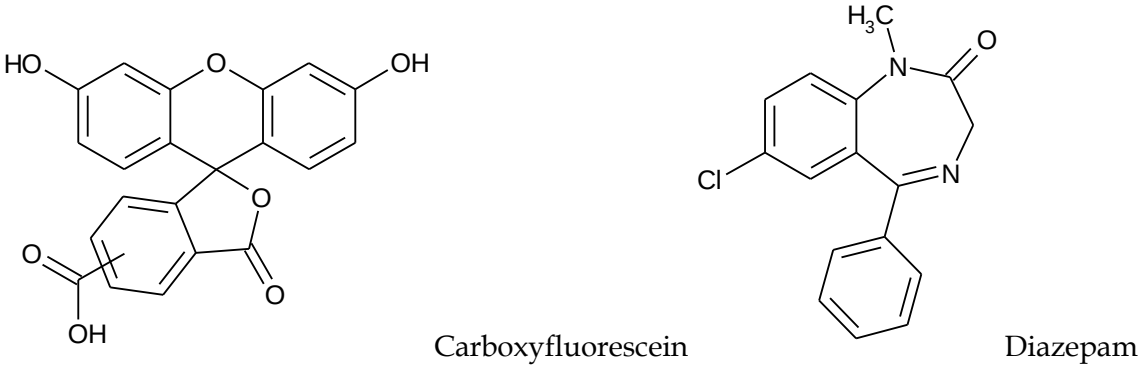


Fig. 3.3: Formula of carboxyfluorescein (left) and diazepam (right) used as internal standards
Source: http://www.chemexper.com/

Stock solutions with test substances

Depending on their physical-chemical characteristics the tested substances were dissolved either in water (Aqua purificata) or in dimethylsulfoxide (DMSO). All water solutions were sterile filtered.

Diclofenac (3 mM solution), ibuprofen and meloxicam (10 mM solution each) were dissolved in water (Fig. 3.4 shows chemical formulas).

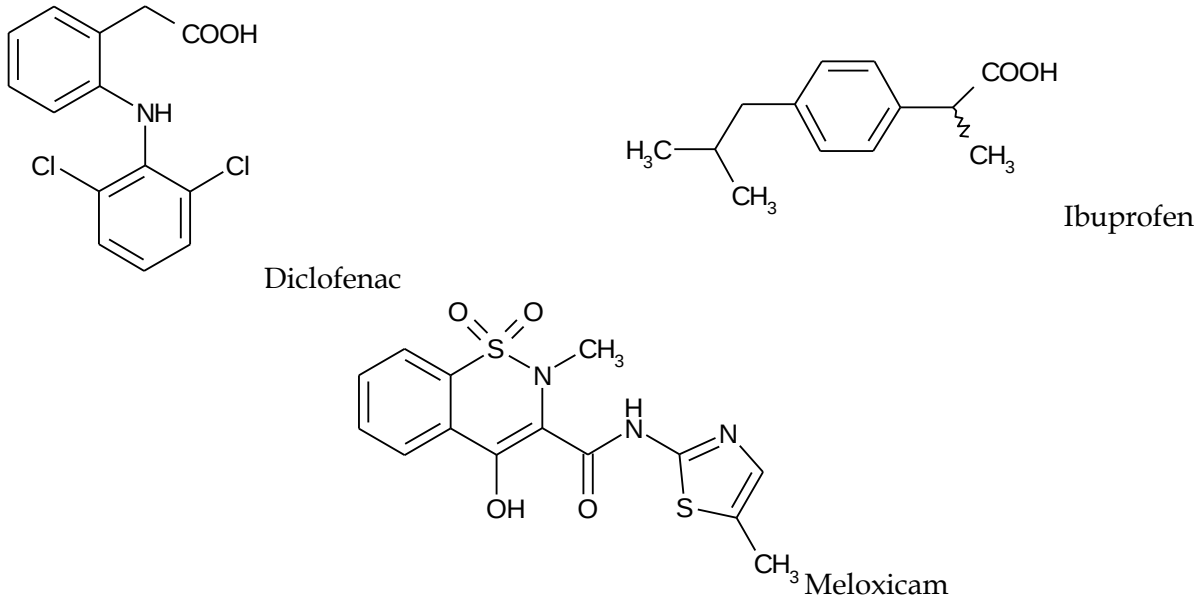


Fig. 3.4: Formula of diclofenac, ibuprofen and meloxicam. Source: http://www.chemexper.com/

Lornoxicam (10 mM solution), tenoxicam (10 mM solution) and piroxicam (10 mM solution) were dissolved in DMSO (Fig. 3.5 shows chemical formulas). A further solution of tenoxicam and piroxicam was prepared where the substances were mixed together in DMSO (100 mM each). This solution was used for the group studies only.

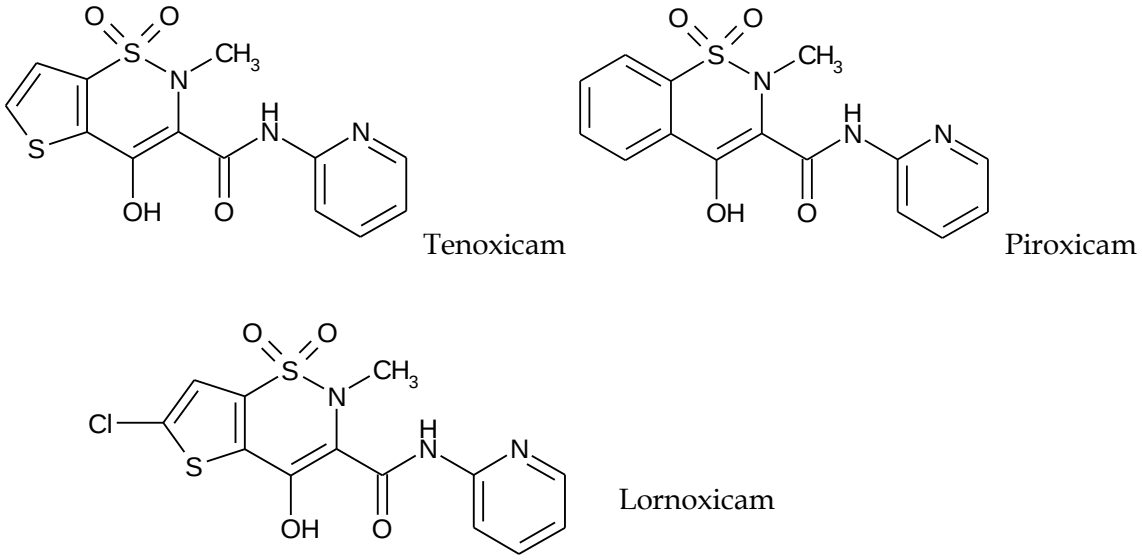


Fig. 3.5: Formula of tenoxicam, piroxicam and lornoxicam. Source**:** http://www.chemexper.com/

Celecoxib was dissolved in DMSO in two different concentrations, one used for single studies (10 mM) and the second one for group studies (100 mM) (Fig. 3.6 shows chemical formulas).

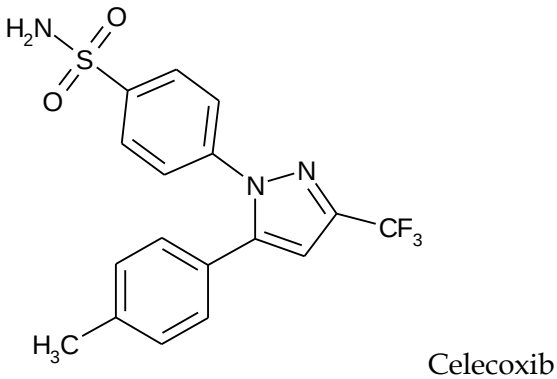


Fig. 3.6: Formula of celecoxib. Source: http://www.chemexper.com/

The sterile test solutions consisted of a vehicle medium (usually C6 medium, if not mentioned otherwise) and stock solutions: 100 µM of the respective NSAIDs, 1% DMSO, diazepam (100 µM) and carboxyfluorescein (5 µM) (except one group study without CF).

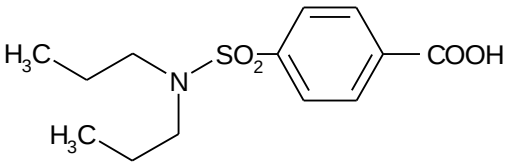
Specific blockers used for transport group studies

Into two group studies specific transport blockers – probenecid or verapamil – were added.

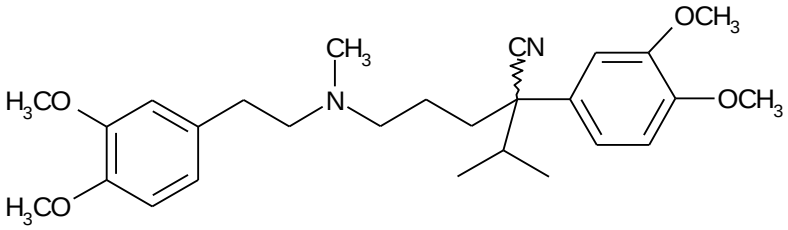
Probenecid as an organic acid is a blocker for the transport of organic anions by OATs or MRP transporter systems. In the humane medicine it is used for gout treatment (Lincová and Fargali et al., 2007).

In another study verapamil - an L-type calcium channel blocker - was added. Inhibition of the voltage dependent Ca^{2+} channel leads to smooth muscles relaxation that causes negative ionotropic and chronotropic effects on the heart. That is the reason, why verapamil is used for the treatment of angina pectoris, hypertension and cardiac arrhythmia and as a vasodilatator generally (Lincová and Fargali et al., 2007). It is also known the inhibiting influence on the P-glycoprotein efflux system (Pechandová et al., 2006). Verapamil can be also used as an alternative medicine for treatment of mania and hypomania in pregnant patients (Giannini et al., 1984; Giannini et al., 2000).

Probenecid and verapamil were dissolved in DMSO (100 mM of each). The Fig. 3.7 shows formulas of probenecid and verapamil.



Probenecid



Verapamil

Fig. 3.7: Formula of probenecid and verapamil. Source: http://www.chemexper.com/

3.2.3.1 Fluorescence measurement

To analyze the permeability of carboxyfluorescein (CF), the fluorescence of the samples was measured by a microplate reader (BMG, excitation: 485 nm, emission: 520 - 535 nm).

The stock solution of mixed substances, the pure medium and each sample (100µl) were pipetted into the fluorescence plate and measured. The gained data were used for determination of the cleared volume. The principle of the calculation will be mentioned in section 3.2.3.2.

3.2.3.2 Reversed-phase high performance liquid chromatography

List of the RP-HPLC apparatus is in section 3.1.3.

Methanol (MeOH) mixed with potassium phosphate buffer pH = 2.5 (except single study with celecoxib, where pH was 3.5) were used as eluents, which were degassed by ultrasonic treatment and they were tested before each measurement. The injection needles were washed with methanol and water mixture (1:1).

Before measuring, samples were purified with methanol as described in the diploma thesis (Nováková, 2009) and were measured three times and its average was use for calculations of the permeability coefficients.

Ultraviolet (UV) detection of the RP-HPLC was for diazepam, ibuprofen and diclofenac 220 nm and for tenoxicam, meloxicam and piroxicam 370 nm during both types of studies. Only celecoxib was detected by two different wavelengths: at 254 nm during single study (Zarghi et al., 2006) and at 220 nm during the group studies.

The injection volume was changed in relation to the used column and to the type of study. The injection volume was 20 µl by the mainly used column 150 x 4 mm (Zorbax). Only once, for analysis of the single study with piroxicam, a column of 250 x 4 mm (Lichrospher) was used because the shorter column (150 x 4 mm) did not allow an exact detection of the peak areas. In this case the injection volume was 50 µl. The time of the

analyses varied from 8 to 18 minutes. The pre-column of the Zorbax column was changed regularly after every 10 - 20 bar increase of pressure.

3.2.3.3 Single studies

Single studies of each substance helped to understand the general permeation abilities how they can cross the BBB *in vitro* model.

The composition of each solution are mentioned in Table 3.1.

No	Composition of the solution
1	100 µM diclofenac, 100 µM diazepam, 5 µM CF, 1% DMSO in C6 medium
2	100 µM ibuprofen, 100 µM diazepam, 5 µM CF, 1% DMSO in C6 medium
3	100 µM piroxicam, 100 µM diazepam, 5 µM CF, 0.98 % DMSO* in C6 medium
4	100 µM tenoxicam, 100 µM diazepam, 5 µM CF, 1% DMSO in C6 medium
5	100 µM meloxicam, 100 µM diazepam, 5 µM CF, 1% DMSO in C6 medium
6	100 µM lornoxicam, 100 µM diazepam, 5 µM CF, 1% DMSO in C6 medium
7	100 µM celecoxib, 100 µM diazepam, 5 µM CF, 1% DMSO in C6 medium

Table 3.1: Composition of the solutions
Seven single studies and their exact solutions are described here.
* Solution with piroxicam was prepared with concentration of DMSO 0.98%.

3.2.3.4 Group studies

Generally, the group studies were conducted in the same way as the single studies. Table 3.2 describes the compositions of experimental solutions used for each study in detail.

Different conditions of experiments gained a new data pool, which was then compared to each other. In all group studies lornoxicam was missing because of analytical issues.

The main study was made by using all substances except celecoxib and involving C6 medium, which was used as a basis for all following experiments.

The second study was carried out in serum used for cell cultivation, in PBMEC medium without fibronectin.

The third study in serum free C6 medium outlined the relationship between substances and their abilities to bind to plasma proteins.

In the study without carboxyfluorescein (4) the influence of CF on the transport abilities of NSAIDs was investigated.

Into the fifth study celecoxib was added additionally, to examine at least one COX-2 blocker.

Next group of studies was compared while using two different transport blockers. In two of the group studies probenecid or verapamil were added. The studies were without meloxicam because of impossibility to separate chromatograms of blockers and meloxicam. The special preparation way of these studies (cultivation already with blocker before the study was started) was already described in the diploma thesis (Nováková, 2009).

The last comparable study involved no meloxicam and no blockers.

No	Composition of the solution
1	100 µM diclo, ibu, tenox, melox, pirox 100 µM diazepam, 5 µM CF 1% DMSO in C6 medium
2	100 µM diclo, ibu, tenox, melox, pirox 100 µM diazepam, 5 µM CF 1% DMSO in PBMEC -Fib medium
3	100 µM diclo, ibu, tenox, melox, pirox 100 µM diazepam, 5 µM CF 1% DMSO in C6 medium without serum
4	100 µM diclo, ibu, tenox, melox, pirox 100 µM diazepam, without 5 µM CF 1% DMSO in C6 medium
5	100 µM celec, diclo, ibu, tenox, melox, pirox 100 µM diazepam, 5 µM CF 1% DMSO in C6 medium
6	100 µM celec, diclo, ibu, tenox, pirox 100 µM diazepam, 5 µM CF 1% DMSO in C6 medium with 100 µM probenecid
7	100 µM celec, diclo, ibu, tenox, pirox 100 µM diazepam, 5 µM CF 1% DMSO in C6 medium with 100 µM verapamil
8	100 µM celec, diclo, ibu, tenox, pirox 100 µM diazepam, 5 µM CF 1% DMSO in C6 medium

Table 3.2: Composition of the solutions.
Group studies are listed with their exact test solutions.

3.2.3.5 The permeability coefficient and its calculation

To determine the permeability coefficients (PE), cleared volume had to be calculated, which was derived from clearance parameter. Clearance [µl/min] is a pharmacokinetic dimension defining the amount of biological fluids totally cleared from a substance in defined time.

The following equation was used for calculation (1) (Neuhaus et al., 2006).

(1) $$\text{Cleared volume [µl]} = (C_A * V_A)/C_D$$

C_A... concentration in acceptor chamber

V_A....total volume in acceptor chamber

C_D....concentration in donor insert

To be able to use the integrated peak areas for the calculation, the equation was altered. The peak area for defined time ($peakarea_t$) was multiplied by the total volume in the well (3,000 µl) and divided by the peak area of the substance in the stock solution ($3000/peakarea_{STL}$) (2). Because the concentration in the donor insert was reducing during every transferring step, the sum of the peak areas from the preceding steps was used to be subtracted from the $peakarea_{STL}$ and multiplied by 1.5 (the ratio between the two different volumes in the well and in the insert (3,000/2,000 = 1.5)) (3). The final cleared volume was a sum of all cleared volumes at different times. The calculation of the cleared volume was made for every study.

(2) $$\text{Cleared volume [µl]} = peakarea_t * (3{,}000/peakarea_{STL})$$

(3) $$\text{Cleared volume [µl]} = peakarea_t * (3{,}000/ (peakarea_{STL} - \sum peakarea_{t-1})) * 1.5$$

3.2.3.6 Ratios

Although the treatment of all 6-well plates during the cell cultivation and the experiments was always under the same conditions, variations could have been caused by the collagen-fibronectin-coated membrane or by the cell monolayer. To minimize such variety, the permeation coefficients were expressed as ratios normalized to the internal standards – diazepam and carboxyfluorescein. Herewith, a new pool of data was created, which was further used for comparisons of substances.

PE_{cell} values for substance and diazepam expressed in ratios the slowness factor of the substance to cross be BBB compared to diazepam.

The same ratio was calculated for substance and CF relationship.

Ratios were calculated for each time interval in all types of studies.

3.2.3.7 Statistics

For the statistics, the Spearman´s rank correlation coefficient (r_s) was calculated. This coefficient showed as a nonparametric measure the statistical dependence between two variables. Ranking orders of NSAIDs according to their permeability ratios to diazepam under the compared conditions were generated and used as basis to calculate the Pearson correlation coefficient. Pearson correlation coefficient is defined between the ranked variables for a sample of size *n* as:

$r_s = 1 - [(6^* \Sigma_i d_i^2)/ (n^*(n^2-1))]$

$d_i = rk(x_i) - rk(y_i)$ rk...difference of ranking of both variables

For the calculation of the statistical significances (Software Sigma Stat 6.0) between the groups, which differed in the substance compositions, a one-way ANOVA was used. To compare the groups with same substance compositions under different experimental transport conditions (in C6 medium, PBMEC -Fib; without serum; without meloxicam; without meloxicam and with probenecid; without meloxicam and with verapamil) a two-

way ANOVA was accomplished followed by an all pairwise multiple comparison procedure (Holm-Sidak method) with an overall significance level of 0.05.

4 RESULTS

4.1 Single studies

Seven single studies were carried out, each with one NSAID as well as diazepam and CF as internal standards (for the exact composition of solutions see Table 3.1). During these studies, two different time intervals were observed (0 to 40 minutes and 40 to 240 minutes). Results showed no significant differences in data normalized to diazepam comparing these two time intervals. Consequently data presented in the results focused on the time interval 40 – 240 minutes. For data presentation and interpretation only the most important data - PE_{cell} values, ratios substance/diazepam and TEER values - are summarized in this part. All obtained single data are listed in the section 'Appendix' at the end of this thesis.

The Table 4.1 summaries the gained values from the single studies, followed by graphical representation in the Fig. 4.1.

PE_{cell} [µm/min]	piroxicam	tenoxicam	ibuprofen	meloxicam	lornoxicam	celecoxib	diclofenac	CF
NSAIDs	23.82 ± 3.81	22.04 ± 0.78	16.95 ± 1.54	12.34 ± 0.68	15.27 ± 1.18	13.44 ± 1.69	10.60 ± 1.15	-
diazepam	43.82 ± 7.64	44.95 ± 2.24	36.47 ± 3.03	32.29 ± 10.20	46.12 ± 11.88	42.30 ± 7.61	58.93 ± 4.14	-
CF	16.67 ± 0.67	17.99 ± 1.28	17.25 ± 0.49	16.95 ± 0.46	16.53 ± 0.51	19.66 ± 0.83	18.56 ± 0.58	-
Ratio PEcell subst/diaz	0.55 ± 0.05	0.49 ± 0.01	0.46 ± 0.01	0.41 ± 0.13	0.35 ± 0.13	0.32 ± 0.03	0.18 ± 0.02	0.40 ± 0.03*
Average - TEER [Ohm*cm²]	67.2 ± 2.42	50.4 ± 2.42	67.2 ± 4.20	50.4 ± 2.42	51.8 ± 8.40	50.4 ± 2.42	63.0 ± 2.42	-

Table 4.1: PE_{cell} values for all NSAID substances, diazepam and CF in [µm/min] gained by single studies, ratio PE_{cell} values of substance/diazepam for every NSAID, CF value was gained as an average from all single studies (*) and average TEER values. (n = 3, mean ± SD)

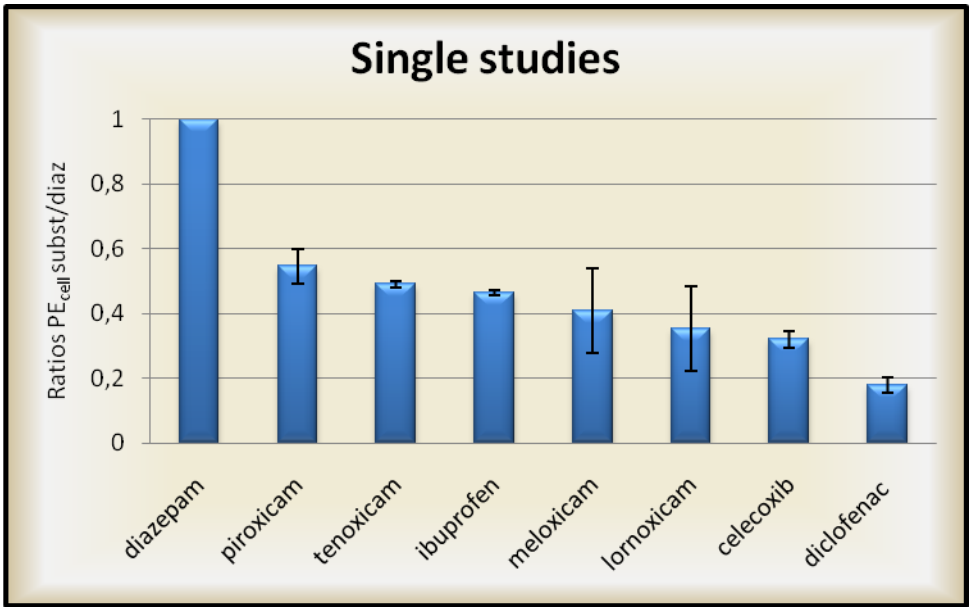


Fig 4.1: Graph shows substances ranking according to their ratio PE_{cell} values. (n=3, mean ± SD)

Diazepam was the fastest substance to cross the barrier in the time interval 40 – 240′, followed by piroxicam and tenoxicam. Surprisingly, CF was faster than ibuprofen in the ibuprofen single study according to PE_{cell} values. But the ratio PE_{cell} CF/diaz placed CF in the ratio ranking behind ibuprofen. Lornoxicam, celecoxib and meloxicam followed them. Finally, diclofenac seemed to be the slowest substances of all (Fig. 4.1 and Table 4.1).

By observing the ratios to diazepam, the ranking corresponded similarly to the PE_{cell} ranking (Table 4.1). Here, meloxicam appeared directly behind ibuprofen, which resulted from the lowest PE_{cell} value of diazepam in this study (32.29 ± 10.20 µm/min) and so the minor ratio (2.60 ± 0.73) was responsible for the placement.

Transport could also be influenced by different paracellular permeability of each single monolayer, reflected in its TEER. TEER values proved tightness and suitability of the cell layers for the transport studies. A correlation between TEER and permeability seemed not to be adequate (Table 4.1), since several substances probably permeated transcellular and TEER is a parameter for paracellular tightness.

Generally, the most important conclusion from all single studies was that piroxicam was able to cross the BBB *in vitro* as the fastest substance from all chosen NSAIDs. Diclofenac was the slowest substance from all.

4.2 Group studies

In total, eight group studies were carried out in order to observe several NSAIDs within the same experiment to reduce the influence of cell layer's variability. Each study offered the possibility to observe the influence of different circumstances and added substances on the transport ability of NSAIDs. For this purpose the gained data were compared within every study and with other studies. Nevertheless, for data interpretation, it had to be kept in mind that the substances interact with each other and influence the transport abilities of each other.

The studies were divided into two groups under which they can be compared. As the main study for the first part the study with C6 medium was chosen. Five studies were included in this part. The second half of the studies was compared with the study included celecoxib but no meloxicam. Meloxicam had to be left out because it was not able to separate it from other substances properly by RP-HPLC after addition of two different transport blockers verapamil and probenecid. Anyway, the second group of studies, which was compared, was created by three studies. Substance permeability during all studies was investigated for the time intervals 0 - 40 and 40 - 240 minutes. Here again, only time interval 40 - 240′ was presented because the other time interval did not show any significant differences. All data are listed in the ´Appendix´.

A summary of the studies with C6 medium, PBMEC –Fib medium, C6 medium without CF, C6 medium without serum and C6 medium with celecoxib is presented in the Table 4.2.

PE_{cell} [µm/min]	Substances							
	diazepam	piroxicam	tenoxicam	ibuprofen	meloxicam	diclofenac	celecoxib	CF
C6 medium	24.91 ± 2.57	25.23 ± 5.10	16.69 ± 1.74	12.46 ± 1.95	9.39 ± 0.33	10.25 ± 0.80	-	16.02 ± 1.80
PBMEC -Fib medium	39.18 ± 2.32	29.86 ± 5.84	25.87 ± 9.41	29.33 ± 2.45	24.32 ± 1.18	19.82 ± 1.45	-	18.01 ± 1.38
C6 med. without CF	33.96 ± 3.53	30.27 ± 2.61	20.74 ± 1.52	24.70 ± 2.68	19.97 ± 2.72	17.22 ± 1.63	-	-
C6 med. without serum	36.65 ± 2.71	24.96 ± 2.08	21.67 ± 0.92	32.74 ± 1.59	23.71 ± 1.20	30.20 ± 2.45	-	17.67 ± 0.70
C6 med. incl. celecoxib	40.04 ± 2.99	41.98 ± 3.89	23.10 ± 1.18	25.85 ± 2.66	22.87 ± 2.64	19.50 ± 1.59	13.26 ± 1.07	18.67 ± 1.56

Table 4.2: PE_{cell} values for all NSAID substances, diazepam and CF in [µm/min] used in appropriate group study mentioned in the left column during the time interval 40 - 240 minutes. (n=3, mean ± SD)

Group study with C6 medium

Analyzing the study with C6 medium it surprised that piroxicam seemed to be even faster than diazepam. Incidentally, the values of piroxicam (25.23 ± 5.10 µm/min) and diazepam (24.91 ± 2.57 µm/min) were very close. Taking the overlapping standard deviations into account, the difference between permeability of piroxicam and diazepam between 40 - 240′ was considered not significant.

Group study in PBMEC –Fib medium

Comparing the first two studies – study with C6 medium and study with PBMEC medium without fibronectin (-Fib) – all PE_{cell} values were increased in PBMEC medium without fibronectin (Table 4.2). PBMEC -Fib medium consists of a 1:1 mixture of C6 medium and C6 conditioned medium containing astrocytic factors. A significant jump was made by ibuprofen, which moved from fifth position in the C6 medium study (12.46 ± 1.95 µm/min) to third place in the other study (29.33 ± 2.45 µm/min). It is interesting that the values for ibuprofen and piroxicam were very close to each other (ibu: 29.33 ± 2.45 µm/min; pirox: 29.86 ± 5.84 µm/min). Meloxicam also speeded up about 2-fold (from 9.39 ± 0.33 to 24.32 ± 1.18 µm/min) and so did diclofenac (from 10.25 ± 0.80 to 19.82 ± 1.45 µm/min). However, diclofenac was still placed behind meloxicam. PE_{cell} values for CF seemed to be more or less fixed (16.02 ± 1.80 µm/min; 18.01 ± 1.38 µm/min).

Group study in C6 medium without carboxyfluorescein

In the study without CF, ibuprofen was still in third place behind diazepam and piroxicam, followed by tenoxicam, meloxicam, and diclofenac. All PE_{cell} values were increased again (Table 4.2), which was mostly significant for ibuprofen (from 12.46 ± 1.95 to 24.70 ± 2.68 µm/min) and meloxicam (from 9.39 ± 0.33 to 19.97 ± 2.72 µm/min).

Group study in C6 medium without serum

Not surprisingly the most dramatic changes in the permeability ranking were observed in the study using medium without serum compared to the main C6 medium study. This time, diazepam was followed by ibuprofen and diclofenac that speeded up considerably (ibu: from 12.46 ± 1.95 to 32.74 ± 1.59 µm/min; diclo: from 10.25 ± 0.80 to 30.20 ± 2.45 µm/min). Finally, piroxicam lined up, but with a very similar PE_{cell} value as by the C6 medium study (25.23 ± 5.10 µm/min; 24.96 ± 2.08 µm/min), which evoked the question

about the intensity of its plasma binding. Tenoxicam was the last substance from all chosen NSAIDs, although its value was still increased. Again CF had very stabile data (16.02 ± 1.80 µm/min; 17.67 ± 0.70 µm/min) (Table 4.2).

Group study in C6 medium with celecoxib

The final study of this first group of studies included celecoxib, which showed similar results as the main study with C6 medium. Also here, piroxicam permeated faster than diazepam. In this case, the values were 41.98 ± 3.89 µm/min for piroxicam and 40.04 ± 2.99 µm/min for diazepam. Again, considering the standard deviations, the change in the ranking was not relevant. Furthermore, ibuprofen overtook tenoxicam and placed in third position. Meloxicam and diclofenac followed by CF occupied the subsequent places. The slowest substance was celecoxib. Generally, all values here were increased in comparison to the C6 medium study (Table 4.2).

Type of study	Substances						
	piroxicam	tenoxicam	ibuprofen	meloxicam	diclofenac	celecoxib	CF
C6 medium	1.18 ± 0.23	0.67 ± 0.01	0.51 ± 0.13	0.14 ± 0.08	0.42 ± 0.07	-	0.64 ± 0.01
PBMEC –Fib medium	0.76 ± 0.13	0.66± 0.24	0.75 ± 0.05	0.62 ± 0.02	0.51 ± 0.03	-	0.46 ± 0.02
C6 med. without CF	0.89 ± 0.02	0.61 ± 0.02	0.73 ± 0.02	0.60 ± 0.13	0.51 ± 0.01	-	-
C6 med. without serum	0.68 ± 0.06	0.59 ± 0.03	0.90± 0.05	0.65 ± 0.03	0.82 ± 0.03	-	0.48 ± 0.02
C6 med. incl. celecoxib	1.05 ± 0.11	0.58 ± 0.01	0.64 ± 0.02	0.57 ± 0.04	0.49 ± 0.01	0.33 ± 0.01	0.47 ± 0.04

Table 4.3: Ratios substance/diazepam. (n=3, mean ± SD)

Looking at the ratio data after normalization to diazepam, the ranking was mostly confirmed (Table 4.3).

Finally, three more group studies were investigated (group study without meloxicam, group study without meloxicam and with probenecid, group study without meloxicam and with verapamil). A summary of these studies is presented in the Table 4.4.

PE cell [µm/min]	Substances						
	diazepam	piroxicam	tenoxicam	ibuprofen	diclofenac	celecoxib	CF
C6 med. without melox	52.96 ± 5.83	38.50 ± 3.96	26.81 ± 1.88	27.43 ± 3.06	20.46 ± 1.91	15.47 ± 1.02	20.60 ± 1.52
C6 med. without melox/with probenecid	39.12 ± 4.26	30.26 ± 5.85	25.32 ± 1.36	32.51 ± 5.51	25.80 ± 2.77	16.65 ± 2.20	17.41 ± 0.41
C6 med. without melox/with verapamil	58.03 ± 5.10	40.07 ± 8.39	29.18 ± 0.65	42.14 ± 2.11	32.33 ± 1.65	21.06 ± 0.91	24.60 ± 1.00

Table 4.4: PE_{cell} values for all NSAID substances, diazepam and CF in [µm/min] used in appropriate group study mentioned in the left column during the time interval 40 - 240 minutes. (n=3, mean ± SD)

Group study in C6 medium without meloxicam and with probenecid

The differences between the study without meloxicam and the study without meloxicam/with probenecid were remarkable (Table 4.4). Ibuprofen switched position with piroxicam when probenecid was used and jumped to the second place (27.43 ± 3.06 µm/min; 32.51 ± 5.51 µm/min). Moreover, diclofenac speeded up to the fourth position (from 20.46 ± 1.91 to 25.80 ± 2.77 µm/min). Surprisingly, passing the barrier was easier for CF than for celecoxib, which occupied the last position. Compared to the study without meloxicam and without blockers, all values decreased, when probenecid was added.

Group study in C6 medium without meloxicam and with verapamil

When verapamil was added, changes in the ranking were the same as in the probenecid study. Ibuprofen overtook piroxicam that was followed by diclofenac and tenoxicam. Finally, celecoxib was closing the sequence. A notable shift was detected for diclofenac, which jumped ahead of tenoxicam with a value of 32.33 ± 1.65 µm/min. Generally, this time all values were increased compared to the main study without meloxicam and without any blockers.

Correspondence with the ratio data was confirmed again (Table 4.5).

Type of study	Substances					
	piroxicam	tenoxicam	ibuprofen	diclofenac	celecoxib	CF
C6 med. without melox	0.73 ± 0.03	0.51 ± 0.02	0.52 ± 0.01	0.39 ± 0.01	0.29 ± 0.03	0.48 ± 0.04
C6 med. without melox/with probenecid	0.72 ± 0.13	0.65 ± 0.04	0.83 ± 0.05	0.66 ± 0.01	0.67 ± 0.07	0.45 ± 0.05
C6 med. without melox/with verapamil	0.69 ± 0.14	0.50 ± 0.03	0.73 ± 0.05	0.56 ± 0.02	0.36 ± 0.03	0.37 ± 0.08

Table 4.5: Ratios substance/diazepam. (n=3, mean ± SD)

Averages of TEER values before the beginning of experiments were recorded (Table 4.6) and had to be taken into consideration in terms of ranking and migration abilities of the substances. Probably, the absence of plasma proteins and CF resulted in less stimulation of the BBB tightness, which was presented by lower TEER values.

Type of study	Average - TEER [Ohm*cm²]
C6 medium	60.20 ± 4.20
PBMEC –Fib medium	57.40 ± 2.42
C6 medium without CF	54.60 ± 4.20
C6 medium without serum	56.00 ± 2.42
C6 medium incl. celecoxib	57.40 ± 2.42
C6 medium without melox	56.00 ± 2.42
C6 medium without melox/with probenecid	67.20 ± 2.42
C6 medium without melox/with verapamil	58.80 ± 4.85

Table 4.6: Average TEER values for every group study. (n=3, mean ± SD)

Fig 4.2 shows a summary of the NSAID transport ranking normalized to diazepam including all eight experimental settings.

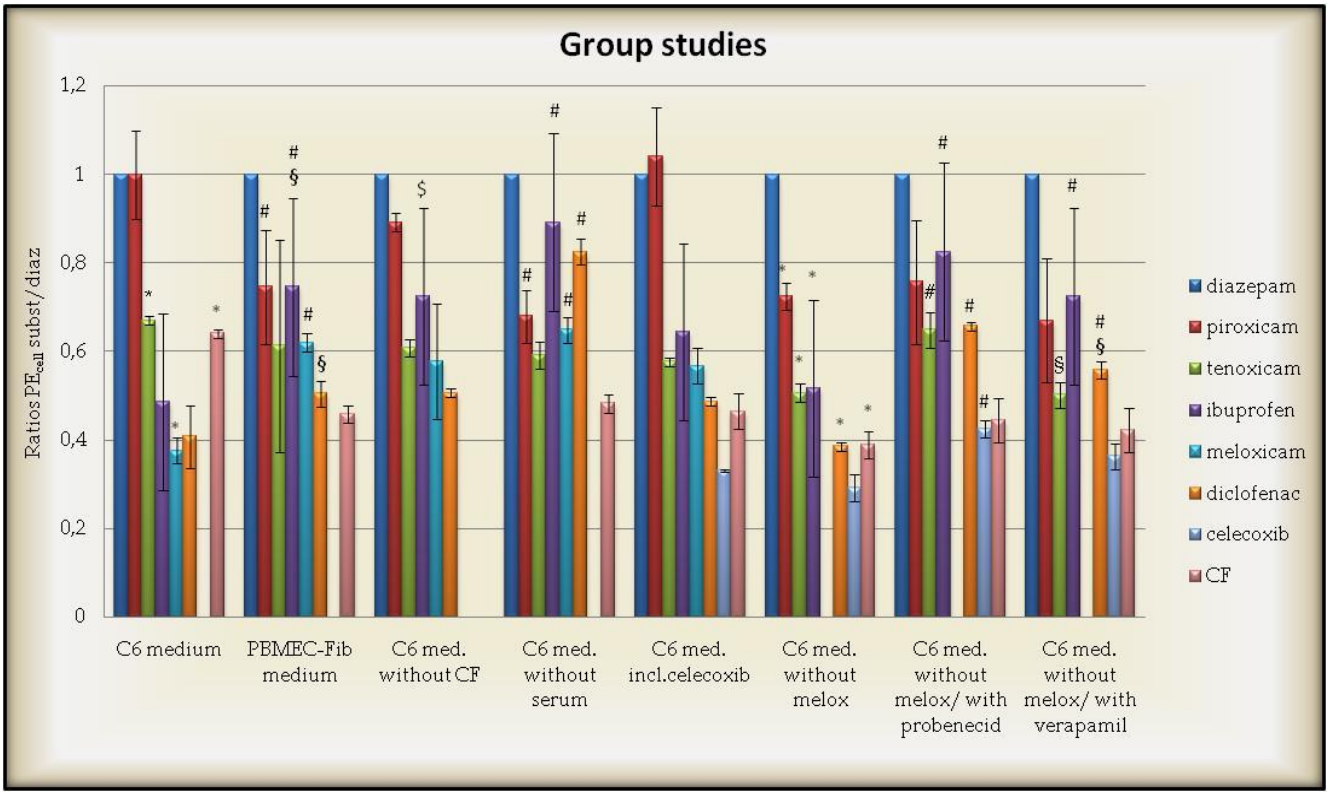


Fig. 4.2: Group studies by the time interval 40 – 240 min. depicted with statistical significances (explanation in the text below). Numerical data (ratios PE_{cell} subst/diaz) were presented in the table before (Table 4.5 and 4.7). (n=3, mean ± SD)

For the calculation of statistical significances (Software Sigma Stat 6.0) between the groups, which differed in the substance compositions, a one-way ANOVA was used. To compare the groups with same substance compositions under different experimental transport conditions (in C6 medium, PBMEC -Fib; without serum; without meloxicam; without meloxicam and with probenecid; without meloxicam and with verapamil) a two-way ANOVA was accomplished followed by an all pairwise multiple comparison procedure (Holm-Sidak method) with an overall significance level of 0.05. Statistical significance ($p<0.05$) for each substance is indicated in the figure by * (incl. celecoxib vs. C6 medium, incl. celecoxib vs. without melox and without blockers), by # (C6 medium vs. without serum or minus Fib medium; without melox and blockers vs. without melox/with probenecid or without melox/with verapamil), by § (without serum vs. minus Fib medium; without meloxicam/with probenecid vs. without meloxicam/with verapamil) or by $ (incl. celecoxib vs. without CF).

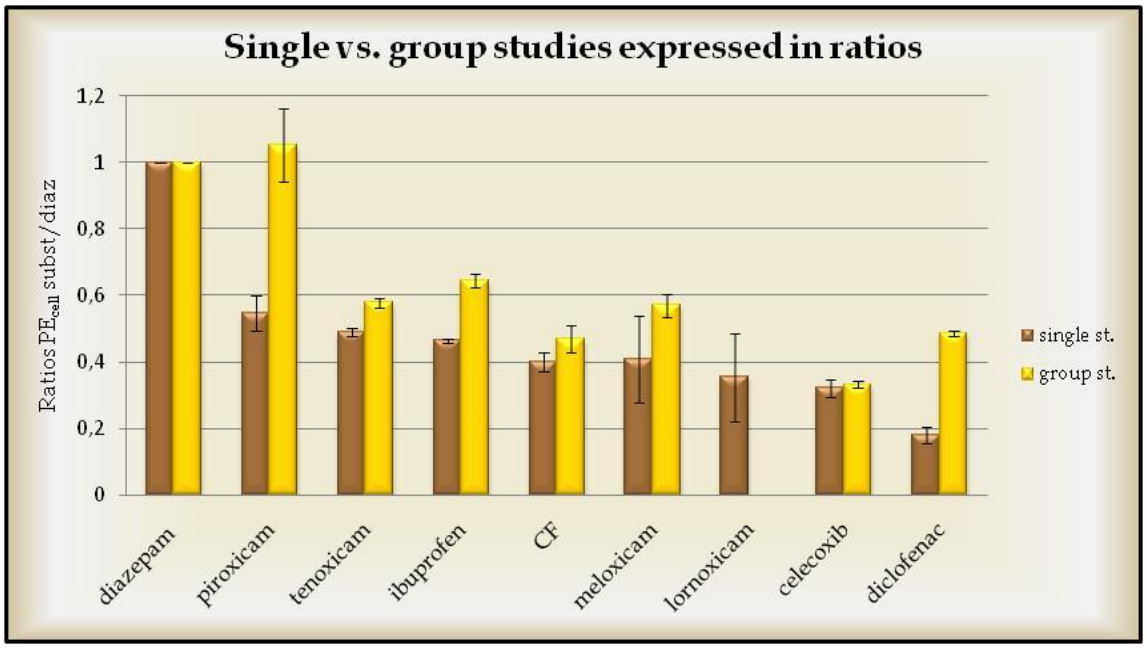
The results of the group study with all investigated substances (diazepam, piroxicam, ibuprofen, meloxicam, tenoxicam, diclofenac, celecoxib, carboxyfluorescein) were compared to the study without celecoxib (C6 medium), C6 medium accomplished in serum-free C6

medium (without serum), C6 medium accomplished in PBMEC-Fib medium (minus Fib medium) and C6 medium and carboxyfluorescein (without CF). Furthermore, the results of the group study with all investigated substances (diazepam, piroxicam, ibuprofen, meloxicam, tenoxicam, diclofenac, celecoxib, carboxyfluorescein) were compared to the study without meloxicam and any blockers (without melox and blockers), without meloxicam and with probenecid (without melox/with probenecid) and without meloxicam and with verapamil (without melox/with verapamil) (Fig. 4.2).

4.3 Comparison between single and group studies

During the single studies, the fastest substance to pass the barrier was diazepam and its value was about 2-fold higher compared to the subsequent substance piroxicam. In contrast to that, values of diazepam and piroxicam were very close to each other within the group study.

Because PE_{cell} values of ibuprofen and meloxicam were rising in the group study, CF was shifted in the ranking down to seventh place. Here again, ibuprofen was behind tenoxicam in the single studies but ahead of it in the group study. Diclofenac, on the last position in single studies, was in front of celecoxib in the group study, which ended in the last place. Surprisingly, values of tenoxicam, celecoxib and CF during both types of studies were very similar. Graphical illustration is shown in the following Figure 4.3.



Substances	diazepam	piroxicam	tenoxicam	ibuprofen	CF	meloxicam	lornoxicam	celecoxib	diclofenac
single studies	1.00	0.55 ± 0.05	0.49 ± 0.01	0.46 ± 0.01	0.40 ± 0.03*	0.41 ± 0.13	0.35 ± 0.13	0.32 ± 0.03	0.18 ± 0.02
group studies	1.00	1.05 ± 0.11	0.58 ± 0.01	0.64 ± 0.02	0.47 ± 0.04	0.57 ± 0.04	-	0.33 ± 0.01	0.49 ± 0.01

Fig. 4.3: Above, a graphical figuration is representing the ratios PE_{cell} subst/diaz values and its dependence on the type of the study. Below, the numerical table is giving the exact ratio values of all substances. The value for CF for the single studies was calculated as an average from all values obtained within all single studies (*). (n=3, mean ± SD)

Investigating the validity of the obtained data based on comparison of PE_{cell} ranking and ratio subst/diaz ranking brought differences especially for the single studies (Fig. 4.3).

The rankings were totally identical for the first two substances piroxicam and tenoxicam and for the last substance, which was diclofenac. The distinct difference was caused by meloxicam, which was in fifth place in the PE_{cell} ranking (including only NSAID substances and excluding lornoxicam) but in the ratio ranking in fourth place, in which it overtook celecoxib and CF. This might be caused by the fact that the PE_{cell} value for diazapem during the meloxicam single study was the lowest from all single studies (32.29 ± 10.20 µm/min). Although the PE_{cell} for meloxicam was not so low (12.34 ± 0.68 µm/min), the ratio caused its drift forward in the ratio ranking. Comparing the ranking and ratio, another observation was made for the substances CF and ibuprofen during the single studies where they switched their places. The Spearman's rank order correlation coefficient was here 0.893 ($p<0.05$).

In contrast to that, PE_{cell} value ranking and ratio ranking for the group study showed total correspondency. This is another example for the fact that an experiment carried out under the same conditions shows less fluctuation of the data.

Finally, the nominal rankings are summarized in Table 4.7.

	Ranking of substances	
	single study	group studies
1.	diazepam	piroxicam
2.	piroxicam	diazepam
3.	tenoxicam	ibuprofen
4.	CF	tenoxicam
5.	ibuprofen	meloxicam
6.	lornoxicam	diclofenac
7.	celecoxib	CF
8.	meloxicam	celecoxib
9.	diclofenac	-

Table 4.7: Ranking of substances within both time intervals based on PE_{cell} values.

Generally, it can be stated that diazepam and piroxicam belonged to the fastest substances to pass the barrier. Tenoxicam was more or less stable somewhere in the middle of the ranking. Diclofenac and celecoxib shared the last positions. Consequently, ibuprofen, meloxicam and diclofenac showed the biggest numerical fluctuations of all NSAIDs. Generally, values were increased within the group study compared to single studies. Only a slight decrease was observed for celecoxib.

5 DISCUSSION

Non-steroidal anti-inflammatory drugs are commonly used for treatment of headache, fever, pain of muscles or inflammation. Moreover, Dokmeci (2004) described other interesting observations. He pointed at a possible correlation of ibuprofen use in a long-term therapy in patients suffering from rheumatoid arthritis and a lower occurrence of Alzheimer's disease compared to a group of people who did not use analgetics on a regular basis for a longer time period. That revealed the ability of ibuprofen to reduce the beta-amyloid plaques in the brain, which are connected with the genesis of Alzheimer's disease. This suggested that ibuprofen was transported across the blood-brain barrier and acting in the brain. Naturally, questions about the transport abilities of other NSAIDs across the blood-brain barrier gained importance.

The fact that there was no comprehensive systematic study about the transport of NSAIDs across the BBB including more than three substances led us to the idea to carry out a compact study including seven NSAID substances (celecoxib, diclofenac, ibuprofen, lornoxicam, meloxicam, piroxicam and tenoxicam) to investigate their ability to cross the BBB in an *in vitro* model in single studies as well as in group studies (except lornoxicam) under different conditions. This has become the main aim of the thesis.

Single transport studies across PBMEC/C1-2

Starting with the single studies, the ranking according of the PE_{cell} values (time interval 40 - 240 min.) was the following:

diazepam → piroxicam → tenoxicam → carboxyfluorescein (CF) → ibuprofen → lornoxicam → celecoxib → meloxicam → diclofenac

As expected, diazepam was the fastest substance to cross the BBB (PE_{cell} values within all single studies were between approx. 32 and 59 μm/min). Diazepam was followed by two oxicams, piroxicam and tenoxicam. CF landed in the fourth place and thereby it overtook ibuprofen. The end of the ranking belonged to lornoxicam, celecoxib, meloxicam and diclofenac. Observing the data normalized to diazepam, the ranking was almost identical (lornoxicam switched place with meloxicam).

It was a surprise that CF passed the barrier faster than five NSAIDs (ibuprofen, lornoxicam, celecoxib, meloxicam and diclofenac) since CF is partly used as marker for paracellular leakage (Poetsch et al., 2010). This might be caused by the leaky PBMEC/C1-2 cell layers. For example, CF was the slowest substance in similar experiments in a previous study using cell line ECV304 (Nováková, 2009), which average TEER value was 131 Ω*cm² in comparison to 57 Ω*cm² of PBMEC/1-2 layers. As a result of less restriction of the paracellular route for CF, its permeability increased and thereby CF was shifted into the middle of the permeability ranking using PBMEC/C1-2 cells.

Another interesting point was the rank of tenoxicam. It was commonly believed that ibuprofen could cross the BBB very easily. However, during our studies it was shown that even tenoxicam passes the barrier faster than ibuprofen. In this case a higher P-gp expression on the barrier surface could play an important role. Already Yazdanian and colleagues (2004) reported ibuprofen as a substrate for the P-gp efflux system (see below).

A further point of interest arose by celecoxib, which ranked ahead of meloxicam and diclofenac. In this case, the P-gp system could play a crucial role also, especially for diclofenac. A possible explanation for the higher influx of celecoxib was the fact that celecoxib could pass the barrier partly paracellularly. Looking at the ratio data from ECV304 (0.23 ± 0.02) and PBMEC/C1-2 (0.32 ± 0.03) studies for celecoxib, this idea might be supported. Anyway, there is no experimental report for this speculation.

Group studies across PBMEC/C1-2

Comparing the group studies across PBMEC/C1-2 to each other, several conclusions could be drawn. In the main study with C6 medium an interesting observation was directly found for the first two positions in the permeability ranking. It was surprising to find piroxicam even ahead of diazepam, although it was commonly known that diazepam as a small molecule with high lipophilicity (log P 3.08; source: www.drugbank.ca) could cross the BBB transcellular very easily. Compared to that piroxicam is not as lipophilic (log P 1.31; source: www.drugbank.ca) but its PE_{cell} values were almost the same as diazepam in this study (pirox: 25.23 ± 5.10 µm/min; diaz: 24.91 ± 2.57 µm/min). Since the standard deviation of PE_{cell} values for piroxicam was higher than for diazepam, and the values were very close to each other, it can be concluded that both substances crossed the barrier almost equally. This interesting effect occurred again in another group study which included celecoxib (pirox: 41.98 ± 3.89 µm/min; diaz: 40.04 ± 2.99 µm/min).

Furthermore, a group study was carried out, where the PBMEC –Fib medium was used instead of the C6 medium. This medium contained astrocytic factors (ACM), which were essential for inducing BBB properties in the *in vitro* model (Neuhaus, 2007). This medium was commonly used for everyday treatment of the cells. Data clearly showed an increase of PE_{cell} values compared to the C6 medium study. The most significant drift was observed by ibuprofen, but also by tenoxicam, meloxicam, diclofenac and even by CF. It is interesting that the PE_{cell} increase also influenced the ranking and changed it completely. Although the ACM medium should induce the BBB properties, obviously it did not tighten the barrier for the investigated substances. Conversely, the obtained data were higher than expected. This finding is difficult to explain, maybe the ACM containing medium causes a change in the transporter functionalities or in general the basal tightness of the seeded cells was weaker than during the other study. However, the data normalized to diazepam can be used to compare the different rankings as done in the results part.

The group study without serum confirmed the theory that the crossing ability of substances across the BBB is dependent on their free serum fraction. Compared to the C6 medium study, PE_{cell} values increased dramatically for ibuprofen and especially for diclofenac. This pointed at a high protein binding ability of these two substances. Surprisingly, this study showed that presence or absence of plasma proteins did not influence the passage of piroxicam.

When using two transport blockers, the influence onto the permeability of ibuprofen was significant. As reported, probenecid was a blocker of OATs and MRP2 transporters and verapamil blocked Ca^{2+} channels as well as P-gp (Cantz et al., 2000; Cihler et al., 2000). These results suggested that ibuprofen was a substrate of some transporter proteins that were blocked by both probenecid and verapamil. The increased ibuprofen migration could either be caused by some unknown transporter or by inhibiting the P-gp efflux system expressed especially on the used PBMEC/C1-2 cell line (Neuhaus et al., 2010). Similar observations were made for diclofenac because its PE_{cell} values were increased during these two studies as well. Interestingly, these two studies including transport blockers did not show any significant effect on piroxicam.

Addition of verapamil influenced celecoxib and CF as well. Both substances were suspected to be a substrate of P-gp or some other specific transport mechanism. This was observed in the verapamil study but not in the probenecid study. Also the permeability of tenoxicam was more or less stable during many studies, but in the verapamil study it showed a slight increase. Despite the blocking effects of verapamil on P-gp, it was reported

that verapamil can also decrease the tightness of blood-brain barrier in *in vitro* models (Deli et al., 2005), which could be an additional reason for the increased permeability of celecoxib and the paracellular marker CF.

Angelini et al. (2008) were investigating the multidrug resistance (MDR) by uterine sarcoma cells (MES-SA/Dx-5), which express high levels of P-gp. They explored the effect of doxorubicin in combination with different NSAIDs (ibuprofen, curcumin, sulindac, NS-398) compared to verapamil, as a P-gp standard blocker at two different concentrations. Ibuprofen was shown as a useful competitor to doxorubicin for binding on P-gp and hence it increased the intracellular concentration of doxorubicin. The benefit of co-treatment with ibuprofen, used in low concentrations, could lead to reduction of doxorubicin concentration und hence to decrease its side effects while the cytotoxic activity was still maintained. Thus, the possibility to use several NSAIDs as chemosensitizing agents could by a huge step forward in the whole tumor treatment strategy.

Another possible co-treatment with a P-gp blocker (verapamil) was observed by drug-resistant seizures (Pirker et al., 2011).

Comparison of single vs. group transport studies across PBMEC/C1-2

Comparing the group study with all NSAIDs to single studies, several correspondences were found out. The ranking based on the ratio substance/diazepam values was following:

Single studies: piroxicam → tenoxicam → ibuprofen → meloxicam → CF → lornoxicam → celecoxib → diclofenac

Group study: piroxicam → ibuprofen → tenoxicam → meloxicam → diclofenac → CF → celecoxib

Diazepam was the fastest substance although a small paradox occurred by the group study. The piroxicam PE_{cell} value was slightly higher than diazepam's value (diaz: 40.04 ± 2.99 µm/min; pirox: 41.98 ± 3.89 µm/min). Taking the deviation in account, the differences were statistically insignificant. Which means that piroxicam was the fastest NSAID substance in both studies. On the next position a switch between tenoxicam and ibuprofen was observed. A bigger change occurred by CF, which dropped in the ranking in the group study

to the second last position. In contrast, diclofenac was in the group study faster than CF and celecoxib. Observing the numerical values, the smallest variations were observed by the celecoxib (single study: 0.32 ± 0.03; group study: 0.33 ± 0.01), CF (single study: 0.40 ± 0.03; group study: 0.47 ± 0.04) and by tenoxicam (single study: 0.49 ± 0.01; group study: 0.58 ± 0.01). Spearman's rank correlation coefficient calculated for these two rankings was 0.893 ($p < 0.05$).

Earlier, studies with ECV304 cell line and the same NSAIDs substances were made (Nováková, 2009). The comparison between single and group studies was also observed. The ranking based on the ratio values to diazepam was following (time interval 40 – 240 min.):

Single studies: piroxicam → ibuprofen → tenoxicam → lornoxicam → celecoxib → meloxicam → diclofenac

Group study: piroxicam → ibuprofen → meloxicam → tenoxicam → diclofenac → celecoxib

Also here, piroxicam was the fastest NSAID substances of all, followed by ibuprofen. Diclofenac was in both cell lines the slowest substances in the single studies and celecoxib the slowest in the group studies. The Spearman's ranking correlation coefficient for the ECV304 cell line in this comparison was 0.821 ($p < 0.05$).

Comparison between PBMEC/C1-2 and ECV304 cell line

In a recently published diploma thesis (Nováková, 2009) we investigated the permeability of the same NSAID substances using another cell line called ECV304. This human cell line was introduced as a spontaneously transformed human umbilical vein cell line, which exhibited increased TEER values, the P-gp efflux system and up-regulated BBB markers as transferrin receptor, glucose transporter GLUT-1 and gamma glutamyl transpeptidase when co-cultured with glioma C6 cells (Hurst and Fritz, 1996). Obtained data gave us the opportunity to compare the two studies, to look for generalizations and to discover new relations between substances and different conditions.

Single studies

First of all, results of the single studies based on the ratios PE_{cell} values to diazepam during the time interval 40 - 240 minutes were compared. The Spearman's ranking correlation factor was 0.929 ($p<0.05$), which showed significant correspondence for single studies by different cell lines. PE_{cell} values for diazepam were in the single studies using PBMEC/C1-2 cell line between 32.29 and 58.93 µm/min. and by ECV304 between 19.93 and 39.95 µm/min. The permeability coefficients for CF were by PBMEC/C1-2 between 16.67 and 19.66 µm/min. and by ECV304 between 3.89 and 5.67 µm/min. This corresponded to findings made by Neuhaus et al. (2008) that ECV304 cell line forms higher tight junctions expressed by studies as higher TEER values.

PE_{cell} values were normalized to diazepam to be able to compare them under different conditions. The ranking of substances was following:

PBMEC/C1-2: piroxicam → tenoxicam → ibuprofen → meloxicam → lornoxicam → celecoxib → diclofenac

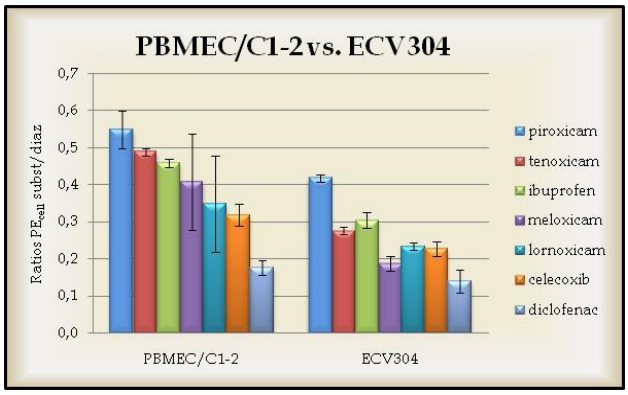
ECV304: piroxicam → ibuprofen → tenoxicam → lornoxicam → celecoxib → meloxicam → diclofenac

In both rankings piroxicam was the fastest substance. This supports the theory that piroxicam was less influenced by the tightness of the tight junctions and the efflux system P-gp. The first changes were shown at the substance pair ibuprofen and tenoxicam. Using the PBMEC/C1-2 cell line, tenoxicam was faster (tenox: 0.49 ± 0.01; ibu: 0.46 ± 0.01). Compared to that in ECV304 studies ibuprofen overtook tenoxicam (ibu: 0.31 ± 0.02; tenox: 0.28 ± 0.001). This raised the question if ibuprofen was a substrate for P-gp, which was less expressed by the ECV304 cell line compared to the PBMEC/C1-2 cells (Neuhaus at al., 2010; Yazdanian at al., 2004).

Lornoxicam and celecoxib had similar ranking in both cell lines and also the ratios PE_{cell} to diazepam were close to each other in each cell line (PBMEC/C1-2: lornoxicam: 0.35 ± 0.13, celecoxib: 0.32 ± 0.03; ECV304: lornoxicam: 0.24 ± 0.01, celecoxib: 0.23 ± 0.02).

Compared to the ECV304 study meloxicam was faster than lornoxicam, celecoxib and diclofenac across PBMEC/C1-2 layers. In this study, it is possible that meloxicam could pass the barrier partly through the paracellular space. Another explanation might be involvement of some type of transport mechanism, which is not present on the ECV304 cell line.

Furthermore, in this case diclofenac was the slowest substance to cross the BBB. This pointed at the fact that diclofenac – as well as ibuprofen – are possible substrates for P-gp. Another reason might be the presence of an unknown transport mechanism missing on PBMEC/C1-2 but not on ECV304. The ratio-ranking overview is summarized in the following graph and table (Fig. 5.1).



Substances	reciprocal ratios PE_{cell} diaz/subst	
	PBMEC/C1-2	ECV304
piroxicam	0.55 ± 0.05	0.42 ± 0.01
tenoxicam	0.49 ± 0.01	0.28 ± 0.01
ibuprofen	0.46 ± 0.01	0.31 ± 0.02
meloxicam	0.41 ± 0.13	0.19 ± 0.02
lornoxicam	0.35 ± 0.13	0.24 ± 0.01
celecoxib	0.32 ± 0.03	0.23 ± 0.02
diclofenac	0.18 ± 0.02	0.14 ± 0.03

Fig. 5.1: Comparison of single studies made on PBMEC/C1-2 and ECV304 cell line, time interval 40 – 240 min. Graph left shows values of ratio PE_{cell} subst/diaz, which is numerically expressed in the table right. On this graph meloxicam overtook lornoxicam and celecoxib in the ranking, which was caused by to low PE_{cell} value of diazepam in the study. Data are presented as means ± SD (n=3).

Single vs. group studies

Also the ranking of NSAIDs in single studies was compared to ranking gained from group studies using both cell lines. Without regard to different type of study (single or group) and different type of cell line, piroxicam permeated always as the fastest of the chosen NSAID. Also interesting finding wad observed by celecoxib. Although its place in the ranking was changing according to other substances, its ratio value was very similar within cell line type (ECV304: single study 0.23 ± 0.02, group study 0.29 ± 0.02; PBMEC/C1-2: single study 0.32 ± 0.03, group study 0.33 ± 0.01). A small switch in the PBMEC/C1-2 ranking was by tenoxicam and meloxicam. But the numerical different was not that remarkable. Only transport property for diclofenac was increased by the group study (from 0.18 ± 0.02 by single study to 0.49 ± 0.01 during group study). The correlation coefficient due Spearman was 0.893 ($p<0.05$) for PBMEC/C1-2 layers. Also ECV304 cell layer revealed a high Spearman's rank order correlation coefficient of 0.821 ($p<0.05$). The correspondence indicated that the studies' results showed similar rankings. The main changes in the placing during the ECV304 cell line appeared by meloxicam and diclofenac, which overtook celecoxib in the group study compared to single studies and so rearranged the ranking. The numerical values were not that significant, but generally higher in the group study.

Group studies

Comparing the group studies (PBMEC/C1-2 vs. ECV304 cell lines), many correspondences but also some differences were found. To be able to compare the results, all data were normalized to diazepam, the internal standard used in both studies.

C6 medium group studies

Already in the study with C6 medium (without celecoxib) some variations occurred. A significant switch in the ranking was created by tenoxicam, which ranked in second place across PBMEC/1-2 layers, but in fourth place using cell line ECV304 (Fig. 5.2). Also, the numerical difference of tenoxicam compared in both cell lines was notable (PBMEC/C1-2: 0.67 0.01; ECV304: 0.41 ± 0.04). Even more interesting movement evoked meloxicam, which was on the last, fifth place across PBMEC/C1-2 (0.14 ± 0.08) but on the third place across ECV304 (0.45 ± 0.03). The coefficient according to Spearman was here 0.5, which indicates no significant correlation of the ranking.

The results for both cell lines corresponded perfectly in the group studies where celecoxib was added. The ranking was following: piroxicam, ibuprofen, tenoxicam, meloxicam, diclofenac and celecoxib (for PBMEC/C1-2). The only difference was the ranking of tenoxicam and meloxicam, which switched positions by ECV304 cell line. The numeral difference was minimal (PBMEC/C1-2: tenox: 0.58 ± 0.01; melox: 0.57 ± 0.04 / ECV304: tenox: 0.44 ± 0.06; melox: 0.45 ± 0.04), so the result could be considered identical. Piroxicam was even faster than diazepam in the PBMEC/C1-2 group study but consideration of the numeral values (pirox: 1.05 ± 0.11) made it permeate almost equally. The Spearman's ranking coefficient was very high, 0.929 ($p<0.05$).

Influence of CF

Carboxyfluorescein was used as a paracellular marker. Its absence should have proved a possible influence on the transport properties of the NSAIDs. The only significant difference was in the PBMEC C1-2 study where permeability for ibuprofen (0.51 ± 0.13 to 0.73 ± 0.02) and meloxicam (0.14 ± 0.08 to 0.60 ± 0.13) was increased and piroxicam (1.18 ± 0.23 to 0.89 ± 0.02) decreased compared to the group study without celecoxib. In contrary to that, by the ECV304 cell line no significant changes were observed.

Influence of astrocyte-conditioned medium

Neuhaus et al. (2008) have already published a positive influence of astrocyte-conditioned medium (PBMEC -Fib) on the growth properties of the BBB. Therefore a group study where this medium was used also during the experiment itself was carried out.

Comparing the group study without celecoxib and with using PBMEC –Fib medium by the ECV304 cell line, just a small ranking rearrangement appeared by meloxicam and tenoxicam although they permeated almost equally. Therefore, the Spearman's correlation coefficient was here 0.964 ($p<0.05$). Generally all values were decreased in PBMEC –Fib medium.

Compared to that, the PBMEC/C1-2 cell line did not show such a correlation at all. The ranking changed totally also with different values for all substances except tenoxicam.

Interestingly, the comparison of the group studies in the astrocyte-conditioned medium between the two cell lines revealed to the similar ranking of NSAID substances (piroxicam, ibuprofen, tenoxicam, meloxicam and diclofenac). The correspondence was also confirmed by the value of Spearman's ranking order correlation coefficient of 0.964 ($p<0.05$).

Influence of serum

The possible influence of medium consisting serum was investigated. One study was carry out in serum free medium, which showed a total correspondence in the ranking, which lead to the Spearman's rank correlating value of 1.00. By both cell lines the influence of serum free medium on the transport ability of ibuprofen and diclofenac was very significant indicating the role of free fraction for these two substances.

Parepally and colleagues (2006) investigated the ability of ibuprofen to cross the BBB in dependence on its free plasma fraction. Their studies were carried out with increasing plasma protein amount and showed that the transport of ibuprofen decreased with increased plasma concentration. Furthermore, they showed a saturated transport profile for ibuprofen when no plasma proteins were added indicating the involvement of an active transport system for ibuprofen across the BBB. This was probably overlooked in previous studies where the experimental solutions contained plasma proteins. Our studies with C6 medium without serum clearly confirmed these data and the theory about the important role of plasma binding for the transport of ibuprofen across the BBB.

Influence of transport blockers verapamil and probenecid

To be able to compare group studies with and without transport blockers, meloxicam was excluded due to analytical reasons.

First of all, in the study, in which no blocker was added, using PBMEC/C1-2 cells piroxicam was followed by ibuprofen and tenoxicam and celecoxib was the slowest substance. Using cell line ECV304, the first three places were the same as well but in this case diclofenac was the slowest substance. Generally, the ranking changed only in the last two positions and the Spearman's ranking value was 0.857 ($p<0.05$).

In the next two studies with transport inhibitors probenecid or verapamil, ibuprofen was the first in the ranking when PBMEC/C1-2 cells were used. Relevant differences were observed for tenoxicam, which was rapidly slowed down by probenecid (0.35 ± 0.02 to 0.12 ±0.03) and increased by verapamil (0.35 ± 0.02 to 0.65 ± 0.06) in the ECV304 cell model but not by PBMEC/C1-2 cells. That might point to a fact that tenoxicam was a substrate of an unknown transport mechanism, which might be present on ECV304, but not on the other cell line and might be blocked by probenecid and verapamil. Ibuprofen and diclofenac permeated significantly faster in the PBMEC/C1-2 during both transport blockers, where the same effect was not observed by ECV304 cell line. Interestingly, ibuprofen permeated even faster than piroxicam over the PBMEC/C1-2 cell line. Additionally, also the permeability of celecoxib was influenced especially by probenecid in the PBMEC/C1-2 study (0.29 ± 0.03 to 0.67 ± 0.07) compared to study without meloxicam and any blockers. The Spearman's ranking correlating value for the probenecid studies comparing the two different cell lines was 0.821 ($p<0.05$) and for the verapamil studies 0.786 ($p<0.05$).

Earlier transport studies with NSAIDs across other cell models provided very helpful results for further interpretation of our obtained data.

For example, Yazdanian et al. (2004) carried out experiments using the Caco-2 cell line (human adenocarcinoma cell line) and measured transport permeabilities of several substances including some NSAIDs in two directions – from the apical to the basolateral (a-b) side and from the basolateral to the apical (b-a) side. The aim of his study was to test the affinity of substances to some transporters, which are located only on one side of the barrier. Also, P-gp belongs to these transporters, which was prominently expressed by Caco-2 cells and was mainly located on the apical side. In this study the NSAIDs ibuprofen, diclofenac,

meloxicam and piroxicam were investigated next to other substances. The ranking in the direction apical to basolateral was as follows:

Caco-2: piroxicam → diclofenac → meloxicam → ibuprofen

Compared to our obtained ranking:

PBMEC/C1-2: piroxicam → ibuprofen → meloxicam → diclofenac *

ECV304: piroxicam → ibuprofen → meloxicam → diclofenac *

* PE_{cell} value ranking with omitted substances according to Caco-2 study.

The first observation pointed at piroxicam, which was the fastest NSAID in the Caco-2 study as well as in our BBB studies. The a-b/b-a ratios, which were established to observe any relevant affinity to P-gp, did not show any influence (ratio around 1). On the other hand, the ratio for ibuprofen was about 2 and was a significant hint for the involvement of an active transporter in its transport, possibly P-gp (apical-basolateral: 10.1; basolateral-apical: 19.8). This again confirmed the theory of ibuprofen being actively effluxed. The last conclusion was based on the ranking where in our studies ibuprofen followed piroxicam but in the Caco-2 study ibuprofen was the last substance. This also could be caused by presence or absence of some specific transport system for ibuprofen by the Caco-2 cell line.

In respect of the study results, piroxicam was usually the fastest substance of all NSAIDs, mostly followed by ibuprofen. Dependence of ibuprofen and diclofenac on the plasma protein amount was confirmed. In most cases celecoxib was the slowest substance. Addition of probenecid or verapamil influenced ibuprofen, diclofenac, CF and celecoxib to the highest extent. There seemed to be some unknown transport mechanism for tenoxicam, which was present on the ECV304 cell line. In contrast to studies with ECV304 cells, the influence of CF on the transport ability of ibuprofen and diclofenac was not confirmed by experiments with PBMEC/C1-2 cells.

In conclusion, many studies with NSAIDs have been carried out, which indicated promising and positive effects on brain-related diseases. There are just few mentioned here.

As described by Müller (2008; 2010), inflammation is involved in the pathogenesis of both depression and schizophrenia. In those neurological disorders COX-2 expression as well as PGE_2 production is increased. In animal models using COX-2 inhibitors surprisingly showed beneficial results compared to placebo.

Another study (Hakan et al., 2010) showed neuroprotective effects of meloxicam using another relevant disease model – rat´s traumatic brain. The rats treated with meloxicam showed less edema and more preserving BBB permeability compared to traumatic brains without any chemical treatment.

Earlier published in Neuropharmacology (van Vlient et al., 2010), sub-chronic treatment with COX-2 inhibitors of rat epilepsy models reduced the expression of P-glycoprotein that normally is increased by epileptical seizures. This enhanced P-gp expression decreased phenytoin delivery to the brain, which was used as an antiepileptic drug in this study. A significant increase of phenytoin in the brain was achieved by COX-2 inhibitor treatment. Also Schlichter and colleagues (2010) carried out a similar study, using celecoxib and phenobarbital. They drew the same conclusion that chronic treatment with celecoxib increased the activity of the antiepileptic substance phenobarbital in rat's brains due to increased BBB permeability.

This shows that NSAIDs have an even bigger potential range of application. However it is still unclear whether NSAID administration reduces the inflammating peripheral components of these diseases and this reduces blood-brain barrier breakdown by inflammation and finally restores brain homeostasis and decreases disease's outcome or whether NSAIDs act directly within the CNS after BBB permeation.

In this context, several studies confirmed the ability of NSAIDs to penetrate the CNS (Novakova et al., 2014) and consequently gaining knowledge about the permeation properties of NSAIDs across the blood-brain barrier will get increasingly important.

6 CONCLUSION

Investigation of the blood-brain barrier (BBB) is a huge challenge for many scientists because the BBB still raises many questions. This study, which has dealt with the transport of non-steroidal anti-inflammatory drugs (NSAIDs) across the BBB, was initiated by an unremitting increasing usage of these drugs in our everyday life. NSAIDs are massively used against pain, inflammation and fever, but little is known about their ability to cross the BBB and their potential to cause CNS side effects. Thus, in this study we investigated the relation between NSAIDs and their transport across the BBB with *in vitro* models.

As a BBB *in vitro* model porcine immortalized cell line PBMEC/C1-2 was chosen to be used for testing the permeability properties of several NSAIDs (celecoxib, diclofenac, ibuprofen, lornoxicam, meloxicam, piroxicam and tenoxicam,) across a Transwell model. Several experiments showed that piroxicam had the ability to cross the barrier as the fastest NSAID, which in some causes competed with the internal standard for the transcellular route diazepam. Piroxicam seemed to be a substance, which permeability was stable under several circumstances. Furthermore, the theory of the strong binding to plasma proteins and its influence on permeability of ibuprofen and diclofenac was confirmed. As a result of the increased free plasma fraction, substances passed the BBB *in vitro* even faster than piroxicam. Celecoxib was the slowest substance passing the barrier, even slower than CF, the second internal standard that was used as a paracellular marker. Probably because of lower tightness in the model using PBMEC/C1-2 cells and respectively lower TEER values, CF crossed the barrier easily, so it turned out to be not suitable as a paracellular marker under these conditions.

Our experiments present only a few of many possibilities that could be investigated. The cell line PBMEC/C1-2 exhibits not only advantages (high functionality of transporters as P-gp) but also disadvantages (low tightness) for BBB modeling. Therefore, usage of other cell lines or primary cells, methods and also BBB models (static or dynamic, *in vitro* vs. *in vivo*), could certainly result in additional information of high value to obtain a more comprehensive view about the transport of NSAIDs across the BBB. Although, our data tell us a lot about permeation properties of chosen NSAIDs, these findings should not be applied generally. On the contrary, they can be combined with data from other models *in vivo* as well as *in vitro* to obtain a complete and summarized overview of the passage of NSAIDs across the blood–brain barrier.

7 ZÁVĚR

Zkoumání hematoencefalické bariéry (HEB) je velkou výzvou pro mnoho vědců, neboť tato bariéra vyvolává spoustu nezodpovězených otázek. Studie, která se zabývala transportem nesteroidních antiflogistik (NSA) přes HEB byla iniciována stále rostoucí spotřebou těchto látek v běžném životě každého z nás. NSA jsou masivně užívána k tišení bolesti, proti zánětu i horečce, přesto jen málo je známo o jejich schopnostech přecházet přes HEB. Tato studie měla tedy za cíl prozkoumat vztahy mezi NSA a jejich transportem přes HEB za použití *in vitro* modelu.

Jako model hematoencefalické bariéry *in vitro* byla vybrána prasečí buněčná linie PBMEC/C1-2, aby byla použita k testování vlastností průchodnosti různých NSA (celekoxib, diklofenak, ibuprofen, lornoxikam, meloxikam, piroxikam a tenoxikam) přes Transwell model. Jak z několika experimentů vyplývá, má piroxikam schopnost prostupovat tuto bariéru jako nejrychlejší NSA, někdy dokonce konkuruje vnitřnímu standardu pro transcelulární cestu diazepamu. Piroxikam se ukázal být látkou, jehož průchodnost byla velmi stabilní za různých podmínek, jimž byl vystaven. Dále i teorie, o silné vazbě na plazmatické bílkoviny a jejím vlivu na transportní vlastnosti ibuprofenu a diklofenaku, byla potvrzena. V důsledku zvýšené volné plazmatické frakce přestupovaly tyto substance přes HEB *in vitro* dokonce rychleji než piroxikam. Celekoxib byl látkou procházející přes bariéru nejpomaleji, dokonce pomaleji než CF, druhý vnitřní standard, který byl používán jako paracelulární marker. Pravděpodobně kvůli nižší těsnosti modelu při použití buněk PBMEC/C1-2, respektive v důsledku nižších hodnot TEER, se CF přes bariéru dostával snadněji, a tak se nezdá být jako paracelulární standard v této souvislosti příliš vhodným.

Naše získaná data jsou jen zlomkem možností, které lze zkoumat. Buněčná linie PBMEC/C1-2 ukazuje jak své výhody (vysokou funkčnost transportních mechanizmů jako P-gp), tak i nevýhody (nižší těsnost) pro vytváření HEB modelů. Proto použití jiných buněčných linií nebo primárních buněk, jiných metod a HEB modelů (statických nebo dynamických, *in vitro* nebo *in vivo*) by jistě přineslo další poznatky značného významu, které by přispěly k získání komplexnějšího obrazu o transportu NSA přes HEB. Ačkoli nám naše data napovídají mnohé o vlastnostech průchodnosti vybraných NSA, nelze tyto poznatky aplikovat univerzálně. Spíše naopak, mohou být kombinována s daty získanými z jiných modelů jak *in vivo*, tak *in vitro* k vytvoření kompletního a souhrnného přehledu o průchodnosti nesteroidních antiflogistik přes hematoencefalickou bariéru.

8 APPENDIX

In this section all numerical results and tables (PS values, PE values, effects of correction, ratios) are listed.

Initially, tables with TEER values are presented. At first, TEER values for the single studies and then for group studies are listed.

On the following pages tables with all other values are presented. The sequence starts with single studies, with the time interval 0-40 minutes followed by time interval 40-240 minutes, and ends with the group studies with the time intervals in the same order.

Substances	Average - TEER [Ohm*cm²]
tenoxicam	50.4 ± 2.42
celecoxib	50.4 ± 2.42
meloxicam	50.4 ± 2.42
lornoxicam	51.8 ± 8.40
diclofenac	63.0 ± 2.42
piroxicam	67.2 ± 2.42
ibuprofen	67.2 ± 4.20

Table 8.1: Single studies.
TEER values of PBMEC/C1-2 cell monolayer used during single studies for NSAIDs single substance experiments.

Type of study	Average - TEER [Ohm*cm²]
C6 medium without meloxicam/with probenecid	67.20 ± 2.42
C6 medium	60.20 ± 4.20
C6 medium without meloxicam/with verapamil	58.80 ± 4.85
PBMEC -Fib medium	57.40 ± 2.42
C6 medium incl. celecoxib	57.40 ± 2.42
C6 medium without melox	56.00 ± 2.42
C6 medium without serum	56.00 ± 2.42
C6 medium without CF	54.60 ± 4.20

Table 8.2 Group studies.
TEER values of PBMEC/C1-2 cell monolayer used during group studies for NSAIDs substances experiments.

Single studies

Time interval: 0-40 min.

Substances	PS_{blank} [µl/min]	PS_{all} [µl/min]	Ratio PE_{cell} CF/subst	PS_{cell} [µl/min]	Ratio PE_{all} diaz/subst	PE_{all} [µm/min]	Ratio PE_{cell} diaz/subst	PE_{cell} [µm/min]	Ratio PE_{all} diaz/CF	Ratio PE_{cell} diaz/CF	EoC [%]
piroxicam	9.44 ± 1.03	4.34 ± 0.57	0.66 ± 0.12	8.04 ± 1.92	1.14 ± 0.26	10.34 ± 1.37	1.33 ± 0.53	19.49 ± 4.56	1.42 ± 0.20	1.95 ± 0.55	186.70 ± 20.30
diazepam	9.50 ± 0.97	4.86 ± 0.81	0.96 ± 0.07	9.94 ± 3.51	1.37 ± 0.19	11.56 ±1.92	1.68 ± 0.44	24.63 ± 8.36	1.34 ± 0.23	1.77 ± 0.57	208.96 ± 36.97
CF	9.77 ± 1.15	3.42 ± 0.23	1.50 ± 0.53	5.26 ± 0.54	1.59 ± 0.52	8.14 ± 0.54	2.31 ± 1.29	12.55 ± 1.29	1.17 ± 0.09	1.47 ± 0.28	153.95 ± 5.53
tenoxicam	8.89 ± 0.89	3.40 ± 0.33	1.58 ± 0.24	5.51 ± 0.84	1.77 ± 0.12	8.10 ± 0.78	2.43 ± 0.34	13.21 ± 2.00	1.23 ± 0.18	1.57 ± 0.41	162.39 ± 9.46
diazepam	9.60 ± 0.97	4.65 ± 0.75	1.44 ± 0.22	9.02 ± 3.10	1.83 ± 0.25	11.07 ± 1.79	2.69 ± 0.73	22.23 ± 7.38	1.34 ± 0.31	1.95 ± 0.80	197.24 ± 32.29
CF	10.13 ± 1.09	3.48 ± 0.27	1.97 ± 0.39	5.29 ± 0.61	2.26 ± 0.45	8.28 ± 0.63	3.38 ± 1.06	12.65 ± 1.45	1.30 ± 0.09	1.69 ± 0.24	152.45 ± 6.00
ibuprofen	8.31 ± 0.54	2.66 ± 0.25	2.85 ± 0.64	3.91 ± 0.56	2.94 ± 0.85	6.33 ± 0.60	5.22 ± 2.29	9.34 ± 1.33	1.25 ± 0.20	1.79 ± 0.60	147.21 ± 6.71
diazepam	9.47 ± 0.57	4.72 ± 0.61		9.40 ± 2.38		11.23 ± 1.46		22.86 ± 5.68			201.37 ± 25.17
CF	10.40 ± 0.33	3.85 ± 0.14		6.12 ± 0.36		9.18 ± 0.34		14.59 ± 0.85			158.92 ± 3.45
meloxicam	8.89 ± 0.65	2.68 ± 0.61		3.83 ± 1.31		6.37 ± 1.45		9.32 ± 3.13			144.00 ± 14.76
diazepam	8.48 ± 0.61	4.10 ± 0.69		7.93 ± 2.70		9.76 ± 1.64		19.54 ± 6.42			196.77 ± 31.78
CF	9.64 ± 0.60	3.48 ± 0.44		5.45 ± 1.05		8.29 ± 1.06		13.10 ± 2.50			157.07 ± 10.87
lornoxicam	8.56 ± 0.87	2.63 ± 0.03		3.79 ± 0.07		6.26 ± 0.08		9.04 ± 0.16			144.32 ± 0.79
diazepam	9.36 ± 0.98	4.82 ± 0.70		9.92 ± 2.86		11.47 ± 1.66		24.32 ± 6.82			209.15 ± 30.59
CF	11.08 ± 0.37	3.64 ± 0.35		5.43 ± 0.76		8.68 ± 0.83		12.98 ± 1.81			149.20 ± 6.86
diclofenac	8.83 ± 1.02	2.10 ± 0.19		2.75 ± 0.33		5.00 ± 0.45		6.57 ± 0.80			131.23 ± 3.78
diazepam	9.87 ± 1.01	4.69 ± 0.57		8.94 ± 2.01		11.17 ± 1.37		21.65 ± 4.79			192.15 ± 20.37
CF	11.05 ± 1.18	3.59 ± 0.24		5.33 ± 0.52		8.56 ± 0.56		12.71 ± 1.24			148.30 ± 4.70
celecoxib	7.17 ± 0.99	1.75 ± 0.33		2.32 ± 0.57		4.17 ± 0.79		5.57 ± 1.35			132.62 ± 7.90
diazepam	9.15 ± 1.30	4.98 ± 0.89		10.93 ± 4.35		11.85 ± 2.11		27.47 ± 10.35			226.16 ± 47.55
CF	10.53 ± 1.26	3.99 ± 0.15		6.41 ± 0.39		9.49 ± 0.36		15.28 ± 0.93			160.97 ± 3.71

Table 8.3 Single studies. Time interval 0–40 minutes.
Left: All single studies are ordered in ranking from the fastest NSAIDs substance to the slowest.
Right: Ratios values according to diazepam and CF.

Single studies

Time interval: 40-240 min.

Substances	PS_{blank} [µl/min]	PS_{all} [µl/min]	PS_{cell} [µl/min]	PE_{all} [µm/min]	PE_{cell} [µm/min]	EoC [%]
piroxicam	8.69 ± 0.72	4.63 ± 0.36	9.91 ± 1.60	11.02 ± 0.87	23.82 ± 3.81	215.13 ± 18.43
diazepam	8.94 ± 0.72	5.99 ± 0.35	18.15 ± 3.21	14.27 ± 0.84	43.82 ± 7.64	305.76 ± 35.86
CF	9.42 ± 0.94	4.01 ± 0.09	7.00 ± 0.28	9.56 ± 0.22	16.67 ± 0.67	174.33 ± 2.98
tenoxicam	7.47± 0.31	4.13 ± 0.07	9.25 ± 0.33	9.84 ± 0.16	22.04 ± 0.78	223.93 ± 4.41
diazepam	9.17 ± 0.47	6.17 ± 0.10	18.86 ± 0.94	14.69 ± 0.24	44.95 ± 2.24	305.91± 10.27
CF	10.16 ± 0.47	4.33 ± 0.18	7.54 ± 0.54	10.31 ± 0.42	17.99 ± 1.28	174.39 ± 5.29
ibuprofen	6.72 ± 0.12	3.45 ± 0.15	7.10 ± 0.65	8.22 ± 0.36	16.95 ± 1.54	205.94 ± 9.65
diazepam	9.25 ± 0.49	5.76 ± 0.18	15.27 ± 1.27	13.71 ± 0.43	36.47 ± 3.03	265.60 ± 13.77
CF	11.69 ± 0.30	4.47 ± 0.08	7.24 ± 0.21	10.65 ± 0.19	17.25 ± 0.49	161.95 ± 1.76
lornoxicam	7.42 ± 0.94	3.44 ± 0.14	6.40 ± 0.50	8.18 ± 0.33	15.27 ± 1.18	186.50 ± 6.71
diazepam	7.88 ± 0.16	5.54 ± 0.47	18.71 ± 4.99	13.20 ± 1.11	46.12 ± 11.88	345.79 ± 63.33
CF	11.28 ± 0.74	4.30 ± 0.08	6.94 ± 0.22	10.23 ± 0.20	16.53 ± 0.51	161.54 ± 1.92
celecoxib	6.07 ± 0.45	2.92 ± 0.19	5.62 ± 0.71	6.95 ± 0.45	13.44 ± 1.69	192.99 ± 11.67
diazepam	8.64 ± 0.44	5.79 ± 0.36	17.50 ± 3.20	13.78 ± 0.86	42.30 ± 7.61	305.50 ± 36.96
CF	12.09 ± 0.36	4.91 ± 0.12	8.25 ± 0.35	11.68 ± 0.30	19.66 ± 0.83	168.30 ± 2.89
meloxicam	8.03 ± 1.21	3.15 ± 0.10	5.18 ± 0.28	7.50 ± 0.25	12.34 ± 0.68	164.53 ± 3.53
diazepam	7.28 ± 0.77	4.66 ± 0.56	12.96 ± 4.28	11.10 ± 1.33	32.29 ± 10.20	286.25 ± 58.84
CF	10.09 ± 1.18	4.17 ± 0.07	7.12 ± 0.19	9.94 ± 0.16	16.95 ± 0.46	170.55 ± 1.93
diclofenac	5.83 ± 0.26	2.52 ± 0.16	4.44 ± 0.48	6.00 ± 0.38	10.60 ± 1.15	176.41 ± 8.31
diazepam	8.00 ± 0.12	6.04 ± 0.10	24.69 ± 1.74	14.39 ± 0.25	58.93 ± 4.14	409.27 ± 21.73
CF	10.07 ± 0.41	4.39 ± 0.08	7.79 ± 0.24	10.46 ± 0.18	18.56 ± 0.58	177.40 ± 2.43

Substances	Ratio PE_{all} CF/subst	Ratio PE_{cell} CF/subst	Ratio PE_{all} diaz/subst	Ratio PE_{cell} diaz/subst	Ratio PE_{all} diaz/CF	Ratio PE_{cell} diaz/CF
piroxicam	0.87 ± 0.09	0.72 ± 0.15	1.30 ± 0.05	1.84 ± 0.18	1.49 ± 0.10	2.63 ± 0.48
tenoxicam	1.05 ± 0.06	0.82 ± 0.09	1.49 ± 0.01	2.04 ± 0.05	1.43 ± 0.08	2.51 ± 0.27
ibuprofen	1.30 ± 0.08	1.03 ± 0.12	1.67 ± 0.02	2.15 ± 0.03	1.29 ± 0.06	2.12 ± 0.23
meloxicam	1.33 ± 0.03	1.38 ± 0.05	1.48 ± 0.16	2.60 ± 0.73	1.12 ± 0.12	1.90 ± 0.55
lornoxicam	1.25 ± 0.03	1.09 ± 0.05	1.62 ± 0.19	3.07 ± 0.96	1.29 ± 0.13	2.80 ± 0.76
celecoxib	1.68 ± 0.07	1.47 ± 0.13	1.98 ± 0.05	3.13 ± 0.25	1.18 ± 0.04	2.14 ± 0.30
diclofenac	1.75 ± 0.15	1.77 ± 0.26	2.41 ± 0.16	5.61 ± 0.74	1.38 ± 0.03	3.18 ± 0.25

Table 8.4 Single studies. Time interval 40–240 minutes.
Left: All single studies are ordered in ranking from the fastest NSAIDs substance to the slowest.
Right: Ratios values according to diazepam and CF.

Group studies

Time interval: 0-40 min.

Type of study	Substances	PS_{blank} [µl/min]	PS_{all} [µl/min]	PS_{cell} [µl/min]	PE_{all} [µm/min]	PE_{cell} [µm/min]	EoC [%]
C6 medium	diazepam	9.32 ± 0.82	4.76 ± 0.33	9.75 ± 1.38	11.34 ± 0.77	23.37 ± 3.28	205.38 ± 14.80
	piroxicam	9.68 ± 0.65	4.69 ± 0.38	9.08 ± 1.41	11.16 ± 0.91	21.80 ± 3.35	194.57 ± 14.52
	tenoxicam	9.45 ± 0.69	3.74 ± 0.20	6.19 ± 0.56	8.91 ± 0.49	14.78 ± 1.33	165.72 ± 5.93
	CF	10.84 ± 0.89	3.75 ± 0.20	5.74 ± 0.46	8.93 ± 0.48	13.68 ± 1.11	153.01 ± 4.28
	ibuprofen	8.44 ± 1.04	3.10 ± 0.37	4.89 ± 0.94	7.38 ± 0.89	11.76 ± 2.25	158.50 ± 11.18
	meloxicam	9.19 ± 0.51	2.80 ± 0.33	4.03 ± 0.71	6.68 ± 0.79	9.66 ± 1.69	144.16 ± 7.71
	diclofenac	8.26 ± 0.53	2.56 ± 0.05	3.71 ± 0.11	6.10 ± 0.12	8.84 ± 0.25	144.92 ± 1.29
PBMEC -Fib medium	diazepam	9.26 ± 0.27	5.22 ± 0.29	11.94 ± 1.53	12.42 ± 0.68	28.61 ± 3.63	229.75 ± 16.48
	piroxicam	9.94 ± 0.25	5.01 ± 0.46	10.09 ± 1.82	11.92 ± 1.08	24.28 ± 4.34	202.65 ± 18.33
	tenoxicam	9.44 ± 0.28	4.36 ± 0.69	8.11 ± 2.44	10.39 ± 1.65	19.85 ± 5.81	188.30 ± 25.83
	ibuprofen	9.00 ± 0.19	4.26 ± 0.18	8.10 ± 0.63	10.15 ± 0.41	19.32 ± 1.50	190.17 ± 7.00
	meloxicam	9.69 ± 0.23	4.14 ± 0.15	7.24 ± 0.45	9.86 ± 0.35	17.25 ± 1.06	174.79 ± 4.61
	CF	10.27 ± 0.36	3.91 ± 0.04	6.31 ± 0.11	9.30 ± 0.10	15.02 ± 0.26	161.44 ± 1.06
	diclofenac	8.46 ± 0.23	3.29 ± 0.17	5.39 ± 0.45	7.84 ± 0.39	12.86 ± 1.07	163.87 ± 5.33
C6 medium without CF	diazepam	9.81 ± 0.46	5.17 ± 0.11	10.93 ± 0.49	12.31 ± 0.26	26.04 ± 1.16	211.50 ± 4.96
	piroxicam	10.52 ± 0.50	5.07 ± 0.09	9.78 ± 0.34	12.07 ± 0.22	23.31 ± 0.80	193.02 ± 3.21
	ibuprofen	9.56 ± 0.47	4.22 ± 0.11	7.55 ± 0.37	10.04 ± 0.27	17.98 ± 0.87	179.02 ± 3.84
	tenoxicam	10.34 ± 0.47	4.13 ± 0.07	6.89 ± 0.20	9.84 ± 0.17	16.40 ± 0.48	166.59 ± 1.93
	meloxicam	9.76 ± 0.30	3.66 ± 0.29	5.87 ± 0.75	8.73 ± 0.68	14.02 ± 1.78	160.32 ± 7.65
	diclofenac	9.07 ± 0.44	3.20 ± 0.10	4.95 ± 0.24	7.62 ± 0.24	11.78 ± 0.57	154.57 ± 2.64
	CF	-	-	-	-	-	-
C6 medium without serum	diazepam	10.43 ± 1.02	6.11 ± 0.12	14.79 ± 0.71	14.56 ± 0.29	35.24 ± 1.69	241.96 ± 6.79
	ibuprofen	10.41 ± 0.89	5.73 ± 0.12	12.75 ± 0.60	13.64 ± 0.29	30.37 ± 1.42	222.60 ± 5.75
	diclofenac	10.42 ± 1.00	5.25 ± 0.06	10.59 ± 0.24	12.50 ± 0.14	25.22 ± 0.57	201.69 ± 2.28
	piroxicam	9.74 ± 1.12	4.59 ± 0.13	8.70 ± 0.47	10.94 ± 0.31	20.72 ± 1.12	189.41 ± 4.82
	meloxicam	10.14 ± 1.07	4.45 ± 0.06	8.22 ± 0.21	10.81 ± 0.15	19.58 ± 0.49	181.11 ± 2.04
	tenoxicam	9.97 ± 1.05	4.34 ± 0.05	7.69 ± 0.17	10.33 ± 0.13	18.31 ± 0.40	177.14 ± 1.67
	CF	10.13 ± 1.23	3.85 ± 0.11	6.21 ± 0.29	9.17 ± 0.27	14.80 ± 0.70	161.33 ± 2.90

Table 8.5 Group studies. Time interval 0–40 minutes.
First four group studies and their obtained data. Substances within every study are ordered from the fastest substance to the slowest one.

Group studies

Time interval: 0-40 min.

Type of study	Substances	PS_{blank} [µl/min]	PS_{all} [µl/min]	PS_{cell} [µl/min]	PE_{all} [µm/min]	PE_{cell} [µm/min]	EoC [%]
C6 medium incl. celecoxib	diazepam	8.99 ± 0.53	5.42 ± 0.07	13.62 ± 0.42	12.89 ± 0.16	32.44 ± 1.00	251.59 ± 4.69
	piroxicam	8.29 ± 0.61	4.50 ± 0.10	9.86 ± 0.45	10.72 ± 0.23	23.49 ± 1.08	218.98 ± 5.48
	ibuprofen	8.54 ± 0.41	4.05 ± 0.13	7.71 ± 0.47	9.64 ± 0.31	18.37 ± 1.13	190.38 ± 5.54
	tenoxicam	9.33 ± 0.61	4.12 ± 0.01	7.39 ± 0.04	9.81 ± 0.03	17.59 ± 0.09	179.21 ± 0.42
	meloxicam	9.17 ± 0.56	4.01 ± 0.08	7.13 ± 0.24	9.55 ± 0.18	16.98 ± 0.57	177.76 ± 2.60
	CF	9.88 ± 0.26	3.70 ± 0.23	5.92 ± 0.60	8.82 ± 0.55	14.14 ± 1.42	160.09 ± 6.04
	diclofenac	8.02 ± 0.46	3.20 ± 0.10	5.32 ± 0.27	7.62 ± 0.23	12.68 ± 0.63	166.37 ± 3.32
	celecoxib	6.41 ± 0.37	1.61 ± 0.05	2.14 ± 0.09	3.82 ± 0.12	5.10 ± 0.21	133.42 ± 1.40
C6 medium without meloxicam	diazepam	10.58 ± 0.71	5.66 ± 0.12	12.17 ± 0.56	13.47 ± 0.29	29.00 ± 1.34	215.16 ± 5.32
	piroxicam	11.13 ± 0.79	5.16 ± 0.13	9.62 ± 0.45	12.29 ± 0.30	22.93 ± 1.06	186.53 ± 4.01
	ibuprofen	10.15 ± 0.65	4.10 ± 0.24	6.87 ± 0.69	9.75 ± 0.57	16.40 ± 1.65	167.86 ± 6.82
	tenoxicam	10.62 ± 0.81	4.13 ± 0.07	6.75 ± 0.19	9.83 ± 0.17	16.08 ± 0.45	163.55 ± 1.77
	CF	11.89 ± 0.87	3.89 ± 0.08	5.79 ± 0.17	9.27 ± 0.19	13.78 ± 0.41	148.70 ± 1.45
	diclofenac	9.64 ± 0.59	3.27 ± 0.16	4.96 ± 0.38	7.79 ± 0.39	11.81 ± 0.91	151.48 ± 3.95
	celecoxib	8.37 ± 0.55	1.90 ± 0.08	2.46 ± 0.13	4.53 ± 0.19	5.86 ± 0.31	129.43 ± 1.57
	meloxicam	-	-	-	-	-	-
C6 medium without meloxicam and with probenecid	diazepam	7.95 ± 0.56	5.78 ± 0.52	21.10 ± 7.44	13.75 ± 1.23	53.02 ± 17.72	380.01 ± 93.58
	ibuprofen	7.85 ± 0.59	4.96 ± 0.48	13.45 ± 3.41	11.81 ± 1.14	32.91 ± 8.12	275.99 ± 43.44
	piroxicam	6.71 ± 1.48	4.39 ± 0.38	12.66 ± 3.05	10.44 ± 0.91	30.94 ± 7.25	293.62 ± 45.37
	diclofenac	7.44 ± 0.54	4.14 ± 0.37	9.36 ± 1.85	9.86 ± 0.87	22.60 ± 4.41	227.66 ± 24.90
	tenoxicam	7.82 ± 0.66	4.19 ± 0.32	9.00 ± 1.44	9.96 ± 0.75	21.63 ± 3.43	216.14 ± 18.39
	CF	8.60 ± 0.02	3.84 ± 0.26	6.94 ± 0.86	9.14 ± 0.62	16.60 ± 2.05	181.04 ± 9.99
	celecoxib	5.98 ± 0.43	2.23 ± 0.29	3.56 ± 0.74	5.32 ± 0.69	8.57 ± 1.77	160.22 ± 12.43
	meloxicam	-	-	-	-	-	-
C6 medium without meloxicam and with verapamil	diazepam	8.70 ± 0.33	5.92 ± 0.21	18.50 ± 2.15	14.09 ± 0.51	44.31 ± 5.11	313.81 ± 24.65
	ibuprofen	8.48 ± 0.36	5.00 ± 0.20	12.18 ± 1.24	11.90 ± 0.49	29.12 ± 2.94	244.28 ± 14.57
	piroxicam	8.40 ± 0.47	4.84 ± 0.37	11.42 ± 2.01	11.52 ± 0.89	27.53 ± 4.79	237.69 ± 23.95
	diclofenac	8.22 ± 0.49	4.37 ± 0.12	9.33 ± 0.54	10.41 ± 0.28	22.25 ± 1.28	213.63 ± 6.56
	tenoxicam	8.74 ± 0.35	4.44 ± 0.11	9.00 ± 0.46	10.56 ± 0.26	21.46 ± 1.10	203.09 ± 5.27
	CF	9.03 ± 0.42	4.27 ± 0.08	8.10 ± 0.28	10.17 ± 0.18	19.30 ± 0.66	189.77 ± 3.09
	celecoxib	6.68 ± 0.58	2.20 ± 0.15	3.28 ± 0.33	5.24 ± 0.35	7.83 ± 0.78	149.23 ± 4.92
	meloxicam	-	-	-	-	-	-

Table 8.6 Group studies. Time interval 0–40 minutes.
Second part of group studies and their obtained data. Substances within every study are ordered from the fastest substance to the slowest one.

Group studies

Time interval: 0-40 min.

Type of study	Substances	Ratio PE_{all} CF/subst	Ratio PE_{cell} CF/subst	Ratio PE_{all} diaz/subst	Ratio PE_{cell} diaz/subst	Ratio PE_{all} diaz/CF	Ratio PE_{cell} diaz/CF
C6 medium	piroxicam	0.80 ± 0.02	0.63 ± 0.05	1.02 ± 0.03	1.07 ± 0.05	1.27 ± 0.03	1.70 ± 0.12
	tenoxicam	1.00 ± 0.02	0.93 ± 0.02	1.27 ± 0.02	1.58 ± 0.08		
	ibuprofen	1.23 ± 0.20	1.20 ± 0.30	1.56 ± 0.24	2.05 ± 0.53		
	meloxicam	1.35 ± 0.14	1.44 ± 0.21	1.71 ± 0.13	2.44 ± 0.26		
	diclofenac	1.47 ± 0.07	1.55 ± 0.12	1.86 ± 0.10	2.64 ± 0.32		
PBMEC -Fib	piroxicam	0.79 ± 0.08	0.63 ± 0.13	1.04 ± 0.04	1.19 ± 0.08	1.34 ± 0.09	1.91 ± 0.27
medium	ibuprofen	0.92 ± 0.05	0.78 ± 0.07	1.22 ± 0.02	1.48 ± 0.07		
	tenoxicam	0.91 ± 0.15	0.80 ± 0.25	1.21 ± 0.13	1.49 ± 0.26		
	meloxicam	0.94 ± 0.04	0.87 ± 0.07	1.26 ± 0.03	1 .65 ± 0.11		
	diclofenac	1.19 ± 0.07	1.17 ± 0.11	1.58 ± 0.05	2.22 ± 0.16		
C6 medium	piroxicam	-	-	1.02 ± 0.01	1.12 ± 0.02	-	-
without CF	ibuprofen	-	-	1.23 ± 0.06	1.45 ± 0.13		
	tenoxicam	-	-	1.25 ± 0.01	1.59 ± 0.03		
	meloxicam	-	-	1.41 ± 0.09	1.87 ± 0.18		
	diclofenac	-	-	1.62 ± 0.04	2.21 ± 0.09		
C6 medium	ibuprofen	0.67 ± 0.02	0.49 ± 0.03	1.07 ± 0.02	1.16 ± 0.04	1.59 ± 0.03	2.38 ± 0.08
without	diclofenac	0.73 ± 0.02	0.59 ± 0.03	1.16 ± 0.02	1.40 ± 0.05		
serum	piroxicam	0.84 ± 0.05	0.72 ± 0.07	1.33 ± 0.05	1.70 ± 0.14		
	meloxicam	0.85 ± 0.03	0.76 ± 0.04	1.35 ± 0.02	1.80 ± 0.07		
	tenoxicam	0.89 ± 0.02	0.81 ± 0.03	1.41 ± 0.01	1.92 ± 0.05		

Table 8.7 Group studies. Time interval 0–40 minutes.
Ratio values according to diazepam and CF for the first four group studies.

Group studies

Time interval: 0-40 min.

Type of study	Substances	Ratio PE_{all} CF/subst	Ratio PE_{cell} CF/subst	Ratio PE_{all} diaz/subst	Ratio PE_{cell} diaz/subst	Ratio PE_{all} diaz/CF	Ratio PE_{cell} diaz/CF
C6 medium incl.celecoxib	piroxicam	0.82 ± 0.05	0.60 ± 0.06	1.20 ± 0.04	1.38 ± 0.10	1.47 ± 0.11	2.31 ± 0.29
	ibuprofen	0.92 ± 0.07	0.77 ± 0.09	1.34 ± 0.05	1.77 ± 0.14		
	tenoxicam	0.90 ± 0.06	0.80 ± 0.08	1.31 ± 0.01	1.84 ± 0.05		
	meloxicam	0.92 ± 0.07	0.84 ± 0.11	1.35 ± 0.01	1.91 ± 0.01		
	diclofenac	1.16 ± 0.08	1.12 ± 0.13	1.69 ± 0.06	2.56 ± 0.18		
	celecoxib	2.31 ± 0.16	2.77 ± 0.30	3.37 ± 0.13	6.37 ± 0.41		
C6 medium without meloxicam	piroxicam	0.75 ± 0.03	0.60 ± 0.04	1.10 ± 0.01	1.27 ± 0.03	1.45 ± 0.06	2.11 ± 0.16
	ibuprofen	0.93 ± 0.05	0.85 ± 0.10	1.38 ± 0.06	1.78 ± 0.12		
	tenoxicam	0.94 ± 0.03	0.86 ± 0.05	1.37 ± 0.01	1.80 ± 0.04		
	diclofenac	1.19 ± 0.08	1.17 ± 0.12	1.73 ± 0.05	2.46 ± 0.09		
	celecoxib	2.05 ± 0.12	2.36 ± 0.19	2.98 ± 0.08	4.95 ± 0.15		
	meloxicam	-	-	-	-		
C6 medium without meloxicam and with probenecid	ibuprofen	0.78 ± 0.03	0.52 ± 0.08	1.17 ± 0.03	1.59 ± 0.17	1.50 ± 0.03	3.14 ± 0.67
	piroxicam	0.88 ± 0.05	0.55 ± 0.10	1.32 ± 0.09	1.72 ± 0.45		
	diclofenac	0.93 ± 0.02	0.74 ± 0.06	1.39 ± 0.02	2.31 ± 0.33		
	tenoxicam	0.92 ± 0.01	0.77 ± 0.04	1.38 ± 0.03	2.41 ± 0.44		
	celecoxib	1.73 ± 0.11	1.96 ± 0.16	2.60 ± 0.10	6.08 ± 0.78		
	meloxicam	-	-	-	-		
C6 medium without meloxicam and with verapamil	ibuprofen	0.85 ± 0.02	0.67 ± 0.04	1.18 ± 0.01	1.52 ± 0.02	1.39 ± 0.02	2.29 ± 0.18
	piroxicam	0.89 ± 0.06	0.71 ± 0.11	1.23 ± 0.06	1.62 ± 0.16		
	diclofenac	0.98 ± 0.01	0.89 ± 0.02	1.35 ± 0.01	1.99 ± 0.11		
	tenoxicam	0.96 ± 0.01	0.90 ± 0.02	1.33 ± 0.01	2.06 ± 0.13		
	celecoxib	1.94 ± 0.09	2.47 ± 0.16	2.69 ± 0.09	5.65 ± 0.23		
	meloxicam	-	-	-	-		

Table 8.8 Group studies. Time interval 0–40 minutes.
Ratio values according to diazepam and CF for the second part of group studies.

Group studies

Time interval: 40-240 min.

Type of study	Substances	PS_{blank} [µl/min]	PS_{all} [µl/min]	PS_{cell} [µl/min]	PE_{all} [µm/min]	PE_{cell} [µm/min]	EoC [%]
C6 medium	piroxicam	8.88 ± 0.17	4.80 ± 0.44	10.44 ± 2.14	11.43 ± 1.06	25.23 ± 5.10	219.26 ± 24.09
	diazepam	8.31 ± 0.61	4.62 ± 0.21	10.42 ± 1.08	11.01 ± 0.50	24.91 ± 2.57	225.94 ± 13.01
	tenoxicam	8.21 ± 0.39	3.78 ± 0.21	6.99 ± 0.73	8.99 ± 0.51	16.69 ± 1.74	185.38 ± 8.91
	CF	10.70 ± 0.57	4.12 ± 0.28	6.71 ± 0.76	9.81 ± 0.67	16.02 ± 1.80	162.90 ± 7.08
	ibuprofen	6.28 ± 1.27	2.84 ± 0.24	5.20 ± 0.82	6.77 ± 0.57	12.46 ± 1.95	183.37 ± 13.03
	diclofenac	6.03 ± 0.46	2.51 ± 0.12	4.30 ± 0.34	5.97 ± 0.28	10.25 ± 0.80	171.42 ± 5.61
	meloxicam	7.63 ± 0.58	2.60 ± 0.06	3.94 ± 0.14	6.19 ± 0.14	9.39 ± 0.33	151.66 ± 1.79
PBMEC -Fib medium	diazepam	7.93 ± 0.37	5.35 ± 0.10	16.43 ± 0.97	12.74 ± 0.24	39.18 ± 2.32	307.51 ± 12.26
	piroxicam	8.57 ± 0.30	5.06 ± 0.43	12.34 ± 2.45	12.04 ± 1.02	29.86 ± 5.84	246.41 ± 28.63
	ibuprofen	7.01 ± 0.24	4.46 ± 0.14	12.28 ± 1.03	10.62 ± 0.32	29.33 ± 2.45	275.78 ± 14.68
	tenoxicam	8.04 ± 1.16	4.53 ± 0.66	10.37 ± 3.95	10.78 ± 1.58	25.87 ± 9.41	235.15 ± 49.17
	meloxicam	8.24 ± 0.27	4.56 ± 0.10	10.20 ± 0.50	10.85 ± 0.23	24.32 ± 1.18	224.01 ± 6.03
	diclofenac	6.26 ± 0.20	3.57 ± 0.11	8.31 ± 0.61	8.50 ± 0.27	19.82 ± 1.45	232.90 ± 9.71
	CF	9.78 ± 0.67	4.26 ± 0.18	7.55 ± 0.58	10.15 ± 0.43	18.01 ± 1.38	177.32 ± 5.93
C6 medium without CF	diazepam	8.98 ± 0.34	5.50 ± 0.22	14.20 ± 1.48	13.10 ± 0.52	33.96 ± 3.53	258.83 ± 16.52
	piroxicam	9.98 ± 0.47	5.58 ± 0.21	12.68 ± 1.09	13.29 ± 0.50	30.27 ± 2.61	227.44 ± 10.97
	ibuprofen	8.02 ± 0.36	4.51 ± 0.21	10.33 ± 1.12	10.75 ± 0.51	24.70 ± 2.68	229.37 ± 14.02
	tenoxicam	9.30 ± 0.38	4.49 ± 0.17	8.70 ± 0.64	10.70 ± 0.40	20.74 ± 1.52	193.62 ± 6.84
	meloxicam	8.18 ± 0.36	4.13 ± 0.27	8.34 ± 1.14	9.83 ± 0.64	19.97 ± 2.72	202.59 ± 13.96
	diclofenac	7.20 ± 0.35	3.60 ± 0.17	7.21 ± 0.68	8.58 ± 0.40	17.22 ± 1.63	200.49 ± 9.51
	CF	-	-	-	-	-	-
C6 medium without serum	diazepam	8.90 ± 0.57	5.64 ± 0.16	15.35 ± 1.14	13.42 ± 0.37	36.65 ± 2.71	272.85 ± 12.78
	ibuprofen	8.96 ± 0.39	5.42 ± 0.10	13.74 ± 0.67	12.91 ± 0.24	32.74 ± 1.59	253.56 ± 7.44
	diclofenac	8.62 ± 0.50	5.13 ± 0.17	12.65 ± 1.03	12.20 ± 0.40	30.20 ± 2.45	247.21 ± 11.96
	piroxicam	7.94 ± 0.60	4.51 ± 0.16	10.46 ± 0.87	10.75 ± 0.38	24.96 ± 2.08	232.00 ± 10.99
	meloxicam	8.44 ± 0.55	4.57 ± 0.10	9.95 ± 0.50	10.88 ± 0.25	23.71 ± 1.20	217.95 ± 5.97
	tenoxicam	8.16 ± 0.48	4.30 ± 0.09	9.10 ± 0.39	10.24 ± 0.20	21.67 ± 0.92	211.60 ± 4.75
	CF	8.97 ± 0.44	4.06 ± 0.09	7.42 ± 0.29	9.67 ± 0.21	17.67 ± 0.70	182.75 ± 3.27

Table 8.9 Group studies. Time interval 40–240 minutes.
First part of group studies and their obtained data. Substances within every study are ordered from the fastest substance to the slowest one.

Group studies

Time interval: 40-240 min.

Type of study	Substances	PS_{blank} [µl/min]	PS_{all} [µl/min]	PS_{cell} [µl/min]	PE_{all} [µm/min]	PE_{cell} [µm/min]	EoC [%]
C6 medium incl. celecoxib	piroxicam	6.69 ± 0.08	4.84 ± 0.13	17.56 ± 1.63	11.53 ± 0.30	41.98 ± 3.89	363.56 ± 24.42
	diazepam	8.31 ± 0.17	5.56 ± 0.14	16.78 ± 1.25	13.23 ± 0.33	40.04 ± 2.99	302.36 ± 15.09
	ibuprofen	7.14 ± 0.16	4.30 ± 0.18	10.81 ± 1.12	10.23 ± 0.42	25.85 ± 2.66	252.18 ± 15.68
	tenoxicam	8.32 ± 0.21	4.48 ± 0.11	9.69 ± 0.50	10.66 ± 0.26	23.10 ± 1.18	216.62 ± 5.97
	meloxicam	8.43 ± 0.21	4.48 ± 0.25	9.56 ± 1.11	10.66 ± 0.60	22.87 ± 2.64	213.95 ± 13.16
	diclofenac	6.51 ± 0.10	3.62 ± 0.13	8.17 ± 0.67	8.63 ± 0.31	19.50 ± 1.59	225.74 ± 10.28
	CF	9.56 ± 0.03	4.30 ± 0.20	7.82 ± 0.66	10.24 ± 0.47	18.67 ± 1.56	182.01 ± 6.87
	celecoxib	5.00 ± 0.18	2.63 ± 0.10	5.56 ± 0.45	6.27 ± 0.24	13.26 ± 1.07	211.37 ± 9.01
C6 medium without meloxicam	diazepam	8.84 ± 0.18	6.32 ± 0.20	22.11 ± 2.45	15.04 ± 0.47	52.96 ± 5.83	351.59 ± 27.71
	piroxicam	9.84 ± 0.26	6.11 ± 0.24	16.10 ± 1.66	14.54 ± 0.58	38.50 ± 3.96	264.36 ± 16.90
	ibuprofen	7.27 ± 0.26	4.45 ± 0.20	11.46 ± 1.29	10.60 ± 0.47	27.43 ± 3.06	258.39 ± 17.68
	tenoxicam	8.26 ± 0.26	4.76 ± 0.14	11.24 ± 0.79	11.33 ± 0.33	26.81 ± 1.88	236.37 ± 9.56
	CF	11.08 ± 0.15	4.85 ± 0.20	8.64 ± 0.64	11.56 ± 0.47	20.60 ± 1.52	178.09 ± 5.75
	diclofenac	6.68 ± 0.33	3.75 ± 0.16	8.56 ± 0.80	8.93 ± 0.37	20.46 ± 1.91	228.62 ± 12.02
	celecoxib	5.21 ± 0.28	2.89 ± 0.09	6.49 ± 0.43	6.88 ± 0.20	15.47 ± 1.02	224.67 ± 8.26
	meloxicam	-	-	-	-	-	-
C6 medium without meloxicam and with probenecid	diazepam	8.36 ± 0.43	5.53 ± 0.21	16.34 ± 1.79	13.17 ± 0.50	39.12 ± 4.26	296.45 ± 21.38
	ibuprofen	7.95 ± 0.44	5.00 ± 0.32	13.49 ± 2.31	11.91 ± 0.77	32.51 ± 5.51	271.84 ± 29.11
	piroxicam	6.45 ± 1.01	4.26 ± 0.28	12.50 ± 2.46	10.13 ± 0.66	30.26 ± 5.85	296.95 ± 38.07
	diclofenac	7.48 ± 0.36	4.42 ± 0.20	10.78 ± 1.16	10.52 ± 0.47	25.80 ± 2.77	244.84 ± 15.55
	tenoxicam	7.82 ± 0.55	4.51 ± 0.10	10.62 ± 0.57	10.73 ± 0.25	25.32 ± 1.36	235.87 ± 7.31
	CF	9.60 ± 0.06	4.15 ± 0.06	7.31 ± 0.17	9.88 ± 0.13	17.41 ± 0.41	176.18 ± 1.80
	celecoxib	6.05 ± 0.27	3.24 ± 0.20	6.95 ± 0.93	7.70 ± 0.47	16.65 ± 2.20	215.55 ± 15.30
	meloxicam	-	-	-	-	-	-
C6 medium without meloxicam and with verapamil	diazepam	8.01 ± 0.19	6.02 ± 0.14	24.28 ± 2.14	14.34 ± 0.33	58.03 ± 5.10	404.31 ± 26.77
	ibuprofen	7.20 ± 0.18	5.11 ± 0.07	17.68 ± 0.89	12.18 ± 0.17	42.14 ± 2.11	345.98 ± 12.33
	piroxicam	7.54 ± 0.43	5.17 ± 0.33	16.50 ± 3.52	12.32 ± 0.80	40.07 ± 8.39	323.33 ± 46.76
	diclofenac	7.04 ± 0.20	4.63 ± 0.08	13.56 ± 0.69	11.03 ± 0.20	32.33 ± 1.65	292.91 ± 9.87
	tenoxicam	7.85 ± 0.15	4.78 ± 0.04	12.25 ± 0.27	11.39 ± 0.10	29.18 ± 0.65	256.21 ± 3.48
	CF	8.77 ± 0.17	4.74 ± 0.09	10.33 ± 0.42	11.29 ± 0.21	24.60 ± 1.00	217.83 ± 4.78
	celecoxib	5.44 ± 0.08	3.37 ± 0.05	8.84 ± 0.38	8.01 ± 0.13	21.06 ± 0.91	262.72 ± 7.05
	meloxicam	-	-	-	-	-	-

Table 8.10 Group studies. Time interval 40–240 minutes.
Second part of group studies and their obtained data. Substances within every study are ordered from the fastest substance to the slowest one.

Group studies

Time interval: 40-240 min.

Type of study	Substances	Ratio PE_{all} CF/subst	Ratio PE_{cell} CF/subst	Ratio PE_{all} diaz/subst	Ratio PE_{cell} diaz/subst	Ratio PE_{all} diaz/CF	Ratio PE_{cell} diaz/CF
C6 medium	piroxicam	0.86 ± 0.02	0.64 ± 0.06	0.97 ± 0.05	1.00 ± 0.10	1.12 ± 0.03	1.56 ± 0.03
	tenoxicam	1.09 ± 0.02	0.96 ± 0.02	1.22 ± 0.01	1.49 ± 0.01		
	ibuprofen	1.46 ± 0.22	1.32 ± 0.34	1.64 ± 0.21	2.05 ± 0.52		
	diclofenac	1.65 ± 0.18	1.58 ± 0.30	1.85 ± 0.16	2.45 ± 0.43		
	meloxicam	1.59 ± 0.08	1.70 ± 0.14	1.78 ± 0.19	2.65 ± 0.19		
PBMEC -Fib	ibuprofen	0.95 ± 0.01	0.61 ± 0.01	1.20 ± 0.03	1.34 ± 0.09	1.26 ± 0.04	2.18 ± 0.10
medium	piroxicam	0.85 ± 0.08	0.62 ± 0.13	1.06 ± 0.08	1.34 ± 0.23		
	meloxicam	0.93 ± 0.02	0.74 ± 0.02	1.17 ± 0.01	1.61 ± 0.05		
	tenoxicam	0.96 ± 0.15	0.76 ± 0.26	1.20 ± 0.16	1.63 ± 0.50		
	diclofenac	1.19 ± 0.02	0.91 ± 0.01	1.50 ± 0.04	1.98 ± 0.11		
C6 medium	piroxicam	-	-	0.99 ± 0.01	1.12 ± 0.03	-	-
without CF	ibuprofen	-	-	1.22 ± 0.02	1.38 ± 0.04		
	tenoxicam	-	-	1.22 ± 0.01	1.64 ± 0.05		
	meloxicam	-	-	1.34 ± 0.13	1.73 ± 0.37		
	diclofenac	-	-	1.53 ± 0.01	1.97 ± 0.02		
C6 medium	ibuprofen	0.75 ± 0.01	0.54 ± 0.01	1.04 ± 0.02	1.12 ± 0.07	1.39 ± 0.02	2.07 ± 0.10
without	diclofenac	0.79 ± 0.01	0.59 ± 0.02	1.10 ± 0.02	1.21 ± 0.04		
serum	piroxicam	0.90 ± 0.02	0.71 ± 0.04	1.25 ± 0.04	1.47 ± 0.13		
	meloxicam	0.89 ± 0.002	0.75 ± 0.01	1.23 ± 0.02	1.54 ± 0.06		
	tenoxicam	0.94 ± 0.003	0.82 ± 0.01	1.31 ± 0.02	1.69 ± 0.07		

Table 8.11 Group studies. Time interval 40–240 minutes.
Ratio values according to diazepam and CF for the first four group studies.

Group studies

Time interval: 40-240 min.

Type of study	Substances	Ratio PE_{all} CF/subst	Ratio PE_{cell} CF/subst	Ratio PE_{all} diaz/subst	Ratio PE_{cell} diaz/subst	Ratio PE_{all} diaz/CF	Ratio PE_{cell} diaz/CF
C6 medium incl.celecoxib	piroxicam	0.89 ± 0.02	0.45 ± 0.01	1.15 ± 0.04	0.96 ± 0.10	1.29 ± 0.05	2.15 ± 0.17
	ibuprofen	1.00 ± 0.04	0.72 ± 0.06	1.29 ± 0.02	1.55 ± 0.05		
	tenoxicam	0.96 ± 0.04	0.81 ± 0.06	1.24 ± 0.004	1.73 ± 0.04		
	meloxicam	0.96 ± 0.07	0.82 ± 0.11	1.24 ± 0.04	1.76 ± 0.11		
	diclofenac	1.19 ± 0.04	0.96 ± 0.06	1.53 ± 0.02	2.05 ± 0.04		
	celecoxib	1.64 ± 0.06	1.41 ± 0.11	2.11 ± 0.03	3.02 ± 0.03		
C6 medium without meloxicam	piroxicam	0.80 ± 0.03	0.54 ± 0.05	1.03 ± 0.02	1.38 ± 0.05	1.30 ± 0.04	2.57 ± 0.18
	ibuprofen	1.09 ± 0.04	0.75 ± 0.07	1.42 ± 0.02	1.93 ± 0.05		
	tenoxicam	1.02 ± 0.03	0.77 ± 0.03	1.33 ± 0.003	1.97 ± 0.08		
	diclofenac	1.29 ± 0.05	1.01 ± 0.08	1.68 ± 0.02	2.59 ± 0.06		
	celecoxib	1.68 ± 0.02	1.33 ± 0.03	2.19 ± 0.06	3.42 ± 0.30		
	meloxicam	-	-	-	-		
C6 medium without meloxicam and with probenecid	ibuprofen	0.83 ± 0.05	0.55 ± 0.09	1.11 ± 0.03	1.21 ± 0.08	1.33 ± 0.05	2.25 ± 0.23
	piroxicam	0.98 ± 0.07	0.59 ± 0.12	1.30 ± 0.09	1.32 ± 0.26		
	diclofenac	0.94 ± 0.04	0.68 ± 0.07	1.25 ± 0.01	1.52 ± 0.01		
	tenoxicam	0.92 ± 0.02	0.69 ± 0.04	1.23 ± 0.02	1.54 ± 0.09		
	celecoxib	1.29 ± 0.08	1.06 ± 0.14	1.71 ± 0.05	2.35 ± 0.11		
	meloxicam	-	-	-	-		
C6 medium without meloxicam and with verapamil	ibuprofen	0.93 ± 0.03	0.59 ± 0.05	1.18 ± 0.02	1.38 ± 0.09	1.27 ± 0.05	2.37 ± 0.28
	piroxicam	0.92 ± 0.05	0.63 ± 0.11	1.17 ± 0.08	1.49 ± 0.33		
	diclofenac	1.02 ± 0.04	0.76 ± 0.07	1.30 ± 0.01	1.79 ± 0.07		
	tenoxicam	0.99 ± 0.03	0.84 ± 0.05	1.26 ± 0.02	1.99 ± 0.13		
	celecoxib	1.41 ± 0.05	1.17 ± 0.10	1.79 ± 0.03	2.75 ± 0.19		
	meloxicam	-	-	-	-		

Table 8.12 Group studies. Time interval 40–240 minutes.
Ratio values according to diazepam and CF for the second part of group studies.

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