CHARLES UNIVESITY IN PRAGUE
FACULTY OF PHARMACY IN HRADEC KRÁLOVÉ

Department of pharmacology and toxicology

Determination of permeability and active transport of selected butyrylcholinesterase inhibitors in vitro

MASTER'S THESIS

Thesis supervisor: PharmDr. Lukáš Červený, Ph.D.

Hradec Králové, 24th April 2016 **Radek Machan**

„I declare that this thesis is of my original production. All literature and other sources I was drawing upon during elaboration are listed in list of references and are properly quoted. This work has never been used to achieve either different or same academic degree”.

In Hradec Králové, 24th April 2016 Radek Machan

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Abstrakt

Univerzita Karlova v Praze

Farmaceutická fakulta v Hradci Králové

Katedra farmakologie a toxikologie

Student: Radek Machan

Školitel: PharmDr. Lukáš Červený, Ph.D.

Název diplomové práce: Stanovení permeability a aktivního transportu vybraných inhibitorů butyrylcholinesterázy in vitro

Evropská léková agentura (European Medicine Agency; EMA) a Úřad pro potraviny a léčiva (Food and Drug Administration; FDA) kladou důraz na prozkoumání permeability léčiv skrz biologické membrány a jejich interakcí s ABC transportéry, které jsou fyziologickými bariérami produkovány, ať už v rámci preklinického testování léčiv nebo u molekul, které jsou již klinicky užívány, avšak chybí u nich důkazy o těchto parametrech. Cílem této práce bylo zhodnotit schopnost několika experimentálních inhibitorů butyrylcholinesterázy, které byly vytvořeny za účelem léčby demence, prostupovat skrze hematoencefalickou bariéru a objasnit význam transportních proteinů ABC rodiny (ATP-binding cassette/ABC transporters) v tomto přenosu.

Při této práci byla využita *in vitro* obousměrná transportní studie přes monovrstvy tvořené polarizovanými a vysoce diferenciovanými Caco-2 buňkami. Hodnoty permeability získané z těchto měření byly podobné hodnotám několika běžně užívaných léčiv pro terapii CNS nemocí (např. antidepresiv, antiepileptik). Molekuly také vykazovaly hodnotu efluxního poměru (basolaterální – apikální/ apikální – basolaterální) přibližně jedna, což naznačuje žádný či zanedbatelný vliv aktivního transportu.

Abstract

Charles University in Prague

Faculty of Pharmacy in Hradec Králové

Department of Pharmacology & Toxicology

Student: Radek Machan

Supervisor: PharmDr. Lukáš Červený, Ph.D.

Title of diploma thesis: Determination of permeability and active transport of selected butyrylcholinesterase inhibitors in vitro

European Medicine Agency (EMA) and Food and Drug Administration agency (FDA) emphasise drug membrane permeability and drug-drug interactions on ABC transporters expressed in physiological barriers should be investigated for compounds in preclinical studies or for those already clinically used but evidence free. In this work we aimed to assess the capability of several experimental butyrylcholinesterase inhibitors that had been designed to treat dementia to permeate blood-brain barrier and to elucidate role of ATP-binding (ABC) cassette transporters in this transport.

For this purpose, we employed *in vitro* bidirectional transport study across monolayers formed by polarized and highly differentiated Caco-2 cells. The permeability values gained from measurements were similar to values of several commonly used drugs for treatment of CNS disorders (e.g. antidepressants, antiepileptics). In addition, the compounds showed values of efflux ratio (basolateral-to-apical/apical-to-basolateral) approximately one which suggest none or negligible involvement of active transport.

Contents

1. Introduction

With the aging population, the neural diseases affecting the central nervous system (CNS), such as dementia, are steadily increasing. Therefore, there is a need for development of neuropharmaceuticals, which are capable of treating or at least slowing down the neural damage. To reach site of actions neuropharmaceuticals must pass through the blood-brain barrier (BBB). *In vitro* cell-based models represent a useful screening tool to anticipate drug BBB permeability and interactions with drug transporters (Vastag *et al.* 2011).

2. Theoretical part

2.1.Cholinesterase inhibitors

Alzheimer's disease is one of most prevalent types of dementia in elderly population (around 60 – 80% cases). As the condition progresses, due to degeneration of neural cells followed by decrease of cholinergic transmission, thus cognitive function and memory of patient are impaired. Unfortunately, only symptomatic treatment currently exists targeting cholinesterase, an enzyme responsible for acetylcholine (ACh) degradation and in more severe cases also NMDA (N-methyl-D-aspartate) receptors.

Human brain has two types of cholinesterase: acetylcholinesterase (AChE), which is more substrate specific and highly efficient, and butyrylcholinesterase (BChE). In healthy person both enzymes contribute to ACh biodegradation with AChE representing around 80% of total activity. As the illness progresses, AChE activity gradually decreases, while BChE activity remains unchanged or is even increased. Furthermore, due to the presence of BChE in neuritic plaques, it may play a role in formation of β-amyloid (Giacobini 2004).

Currently used drugs for treatment of Alzheimer's disease either specifically target AChE (donepezil, galantamine) or bind to AChE and BChE in equal manner (e.g. rivastigmine). In more severe cases, there may be a need for selective inhibitors of BChE due to its steadily increasing importance in ACh metabolism in central nervous system (Lane *et al.* 2006).

During the development of new potential drugs, many preclinical experimental procedures have to be conducted, including those assessing the ability of molecule to penetrate physiological barriers, which in case of BChEi also means transport through BBB.

2.2.The blood-brain barrier

The BBB consists of endothelial cells of microvessels, basal lamina, pericytes and astrocyte end-feet. Endothelial cells forming the BBB are connected with tight junctions and lack fenestrations. Tight junctions (along with the adherens junctions) form the junction complex, which restricts the paracellular transport of molecules (Ballabh *et al.*2004).

BBB regulates the influx and efflux of various substances into the brain thus maintains its stable environment and functionality. Large surface area of BBB (approximately 12-20 m^2 /1.3 kg brain) and short diffusion distance from capillaries to neural cells (< 10-15 μm) make it important transport pathway into brain tissue (Abbot 2005).

The BBB is not present in every part of the brain. The circumventricular organs, i.e. *area postrema* or neurohypophysis, have capillaries with fenestrations, which allow those parts of central nervous system to communicate with the rest of body by detecting signals from peripheral tissues (i.e. inflammatory markers like interleukins) or contribute on maintaining the homeostasis (via hormones) (Ganong 2000)

2.3. Mechanism of transport across blood-brain barrier

The junction complex ensuring the tightness of the BBB seals the paracellular pathway, restricting movement of polar molecules through the intercellular space of endothelium and forcing the compounds to take the trancellular route.

Large molecules (i.e. polypeptides or proteins) may be carried across the BBB using vesicles, which can be formed by two mechanisms. The first one, demanding specific interaction of membrane receptor with the macromolecule is called receptor mediated transcytosis (RMT). The latter one, adsorptive mediated transcytosis (AMT), utilizes noncovalent

bond between positively charged molecules and negatively charged endothelial glycocalyx (Abbot 2005).

Small molecules like oxygen or carbon dioxide can diffuse freely across the lipid bilayer, ensuring the oxidative metabolism and regulation of pH in brain. Nutrients, which are able to move via the paracellular route in peripheral tissues, are prevented to use this way of permeation across the BBB. In order to ensure the delivery of those compounds into CNS, BBB expresses several uptake transmembrane transporters that are able to transfer molecules of MW <1000. Nowadays, more than 20 specific carriers have been identified for endogenous molecules including glucose, amino acids, nucleosides, nucleobases etc. Carriers may function independently on energy mediating facilitative diffusion driven by the concentration gradient (energy independent, e.g. GLUT-1, nucleoside transporters), while some transport their substrates only uni-directionally, often against concentration gradient, which requires direct supply of energy in form of ATP (primary active transport) or coupling to symport or antiport of another molecule (secondary active transport). The localization of carriers can be either on apical or basal side of barrier or even on both (Abbot 2005, Pardridge 2007).

Primary active transporters are predominantly represented by family of ATP-binding cassette (ABC) efflux transporters with broad substrate spectrum that pump compounds out of the endothelial cells. The most widely expressed are P-glycoprotein (P-gp, ABCB1), the Multidrug Resistance-Related Proteins (MRPs, ABCC) and the Breast Cancer Resistance Protein (BCRP, ABCG2). These transporters are able to reduce the passage of their substrates (either drugs or toxic compounds) across membranes and thus protect sensitive tissue of brain (Pardridge 2007).

2.4.P-glycoprotein

As a member of ABC family, P-gp encoded by *ABCB1/MDR1* gene in mammals, is found in many different body tissues (mostly on apical side of membrane), especially in those forming physiological barriers (i.e. renal tubules, hepatocytes, intestine or BBB), and plays important part in pharmacokinetics of several (mostly lipophilic) drugs. Efflux mediated by P-

gp is also frequent cause of multi-drug resistance of various tumour cells to anticancer drugs (Adachi *et al.* 2001, Zhou 2008)).

P-gp interacts with wide range of compounds, which differ in structure and molecule size (from small molecules to macromolecules like peptides). Activity of P-gp can be inhibited by competitive binding of substrates (verapamile, tamoxifen) or by drugs blocking ATP hydrolysis (steroids, disulfiram), or induced by compounds causing higher transcription activity (phenytoin) (Sharom 2011, Zhou 2008).

2.5.Breast Cancer Resistance Protein

Another member of ABC family is BCRP, which was discovered later than P-gp and was first cloned from doxorubicin-resistant breast cancer cell line. BCRP can not only be found in breast cancer, localization is similar to P-gp. BCRP is mostly expressed in biological barriers as well as in tumorigenic pathological tissues.

BCRP also plays role in drug pharmacokinetics in similar way to P-gp. BCRP has wide substrate specifity, frequently overlapping with P-gp, recognizing molecules both positively and negatively charged. Activity of this efflux transporter can also be modulated like P-gp, especially in terms of inhibition, which is sought in order to increase the drug disposition in targeted area. (Štaud, Pávek 2005)

2.6.In vitro models for blood-brain barrier permeability

Predicting the level of brain penetration is important part in drug development. Thus, there are several models used to simulate BBB permeability; i.e. *in silico* prediction, parallel artificial membrane permeability assay (PAMPA), cell-culture based models and animal models (Vastag, Keserü 2009)

PAMPA was first developed to estimate the rate of gastrointestinal absorption and was furthermore employed as means of permeability assessment early in the drug discovery process by pharmaceutical industry. It involves use of artificial, lipid infused membrane, therefore is focused only on prediction of passive transport due to the lack of proteins integrated in cell membrane. However, by modifying the lipid composition of membrane,

one can achieve a system capable of recognizing the compounds as BBB permeable or non-permeable, which correlates well with cell-based models (Mensch *et al.* 2010, Vastag, Keserü 2009).

Favourable tools to anticipate the BBB penetration are cell-based *in vitro* models due to the higher complexity of information (than PAMPA can provide) regarding the passive and active transport (Hellinger *et al.* 2012). To make the cell models viable for BBB permeability prediction, there are two critical features to follow:

a) presence of functional efflux transporters, especially P-gp, can furthermore make prediction of permeability more accurate. P-gp is involved in brain distribution of a drug and can severely reduce the rate of penetration. P-gp functionality can be described as the fold difference of basolateral-to-apical and apical-to-basolateral drug transport across monolayer formed by polarized cells
b) absence of leakage; intercellular integrity of cells can be measured with transendothelial electrical resistance (TEER) or by tracer permeability. The threshold value of TEER should be 150-200 Ωcm^2 (although the brain endothelium TEER is greater than 1000 Ωcm^2) to consider the data gained from the model as reliable (Deli *et al.* 2005). Permeability of solutes through paracellular pathway is estimated with fluorescent or isotope markers (e.g. fluorescein, Lucifer yellow) (Vastag *et al.* 2011).

There are two types of *in vitro* cell culture models used to predict BBB permeability for drugs: brain capillary endothelial cell-based and epithelial cell-based BBB models (Hellinger *et al.* 2012)

2.7.Endothelial cell culture models

Endothelial cell culture models are based on cells of brain capillary endothelium cultured alone or along with astrocytes and/or pericytes. These include primary cell cultures such as porcine brain microvessel endothelial-cell model, bovine brain capillary endothelial-cell model and co-culture of rat brain capillary model with astrocytes and pericytes (Zhang *et al.* 2006; Culot *et al.* 2008; Nakagawa *et al.* 2009).

Other types of endothelial cells include immortalized brain cell lines, which are easier to handle and are less expensive. However, there have been some drawbacks, which make them less suitable for drug permeability analysis, e.g. incomplete tight junctions (Vastag, Keserü 2009).

Brain capillary cells are considered to imitate best the properties of BBB due to the lipid composition of membrane, complexity of tight junctions and composition of BBB enzymes (Dehouck *et al.* 1994). On the other hand, due to high cost and intensive handling, these models are not suitable for industrial scale.

2.8.Epithelial cell culture models

Surrogate BBB models, Madin-Darby Canine Kidney (MDCK) and human colon carcinoma (Caco-2) cell lines, have showed highly comparable *in vitro - in vivo* BBB permeability correlations, despite their different origin, similar transporter spectrum and membrane composition (Garberg *et al.* 2005).

2.8.1. Human colon carcinoma cell line

Caco-2 cell line originates from human colon adenocarcinoma. Despite being derived from large intestine, Caco-2 cells exhibit many features similar to cells of small intestine, such as microvilli, intercellular junctions, enzymes, uptake and efflux transporters (Ward *et al.* 2000). This model represents a useful tool for investigation of transcellular and paracellular permeability, vesicular transport, active transport, facilitated transport and efflux systems. Therefore it is commonly used for prediction of intestinal absorption of drug after oral administration. Best correlation is gained for molecules transported mostly by passive diffusion and minimally influenced by active uptake (Artursson *et al.* 2012). Due to the expression of efflux transporters similar to BBB (Table 1), the Caco-2 cell line results obtained can be also extrapolated to BBB (Hellinger *et al.* 2012).

There are several reasons, why the Caco-2 cell line is widely used. They are easily cultivated and the growth can be performed on many plastic surfaces, nitrocellulose filters and on Transwell® membrane made from polycarbonate. The cultivation takes about 21 days until the cells are

polarized and fully differentiated. When growing, the cells display the contact inhibition, thus resulting in monolayer formation (Ward *et al.* 2000).

Integrity check by TEER results in different values depending on the passage number and equipment used for measurement (Briskey-Anderson *et al.* 1997). Another option is assaying the monolayer permeability for solutes using paracellular transport (e.g. mannitol or fluorescein). The epithelial permeability of Caco-2 cell monolayers appears to be close to permeability of human or rat intestinal epithelia regarding the junction complex throughput (Artursson *et al.* 2012).

Transporters/Enzymes	Brain capillary endothelial cells	Caco-2 cells
ABC Family	MDR1/P-gp MRP1 MRP2 MRP4 MRP5 BCRP	MDR1/P-gp MRP1 MRP2 MRP3 MDR3 BCRP
SLC Family	OATP1A2 OATP1C1	OATP-B OCTN1 OCTN2
CYP Family	CYP1B1 CYP2U1 CYP2D6 CYP2E1 CYP2J2 P450 reductase	CYP2A13 CYP2C18 CYP51 CYP27 P450 reductase

Table 1: *Comparison of several common transporters and enzymes involved in drug transport and metabolisation in brain capillary endothelium and Caco-2 cell line (Kusuhara, Sugiyama 2005; Sun et al. 2002; Neuhoff 2005; Giacomni et al. 2010)*

3. Aim of the thesis

The objective of this thesis was to determine the permeability of several butyrylcholinesterase inhibitors (BChEi) with the use of Caco-2 cell monolayers and to find out any signs of excessive active transport in either direction across the cell monolayers.

4. Materials and methods

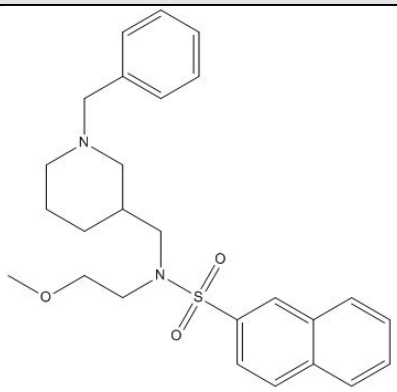
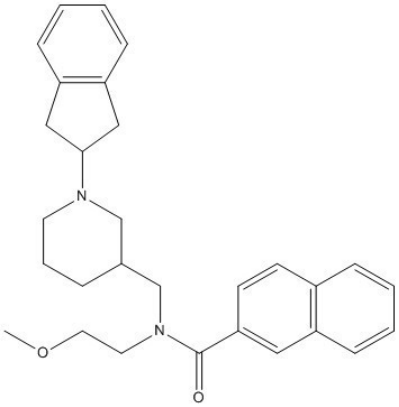
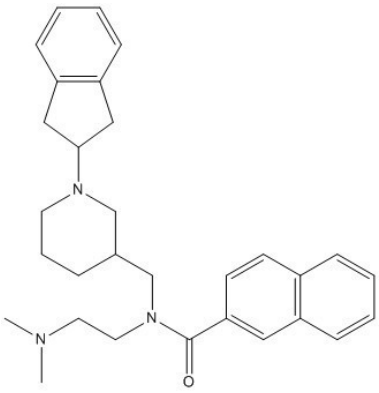
4.1.Materials

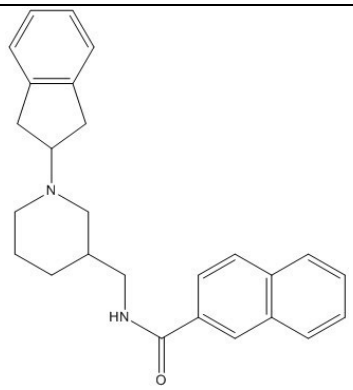
Complete growth medium used for cultivation consisted of Dulbecco's Minimum Essential Medium (DMEM; Sigma-Aldrich Co., USA), 20% FBS (Fetal Bovine Serum; Gibco®, USA), 1% Penicillin/Streptomycin (Sigma Aldrich Co., USA) and 1% L-glutamine (Sigma Aldrich Co., USA). DPBS (Dulbecco's Phosphate Buffer Saline; used for rinsing the tissue) and Trypan Blue solution (used as staining) originated from Sigma Aldrich Co. (USA). TrypLE™ Express Enzyme used for detaching the cells from surface of culture flasks came from Gibco® (Grand Island NY, USA).

All experiments were performed using 10mM solution of glucose in Ringer for the basolateral side of membrane and 10mM solution of mannitol in Ringer for the apical side. The Ringer solution consisted of 140.6 Na^+,5 K^+, 1.2 Ca^{2+}, 1.2 Mg^{2+}, 121.8 Cl^-, 25 HCO_3^-, 0.4 $H_2PO_4^-$, 1.6 HPO_4^{2-} in mM (Žakelj *et al.* 2004)

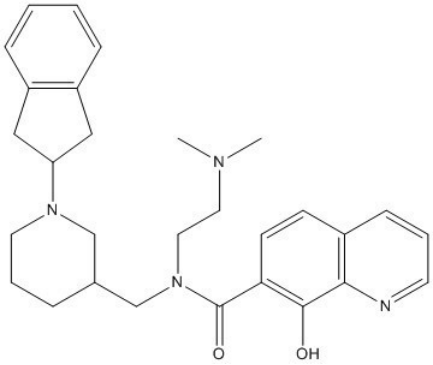
Compounds tested in the experiments were provided by University of Ljubljana, Faculty of Pharmacy (Table 2).

Table 2: *The list of tested compounds with corresponding labels and molecular weight.*

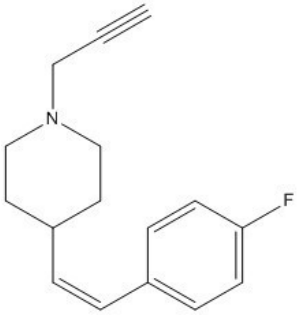
Structure	Codename and molecular weight
	GUK-901 MW = 452.609 logP = 4.39
	GUK-982 MW = 442.592 logP = 4.72
	GUK-987 MW = 455.634 logP = 4.88



GUK-1007
MW = 384.513
logP = 4.64



GDK-375
MW = 472.63
logP = 3.58



SDH-381
MW = 243.33
logP = 3.38

4.2.Cell cultivation

Caco-2 cells used for experiments were obtained from American Tissue Culture Collection (ATCC) HTB-37. After unfreezing and removing the cryoprotectant (DMSO) by adding 8ml of medium and centrifugation (800 RPM, 8 min), the cells were resuspended in appropriate amount of growth medium, transferred in culture flask (TPP Techno Plastic Products, Trasadingen, Switzerland) and put into incubator (37°C, 5% CO_2). The medium was changed every two days.

After reaching 80-90% confluence, which occurred usually once in seven days, the medium was removed from the flask and the cells were rinsed with 5ml of DPBS. Afterwards, 5ml of trypsin solution (TrypLE^{TM}) was added and

the flask was incubated at 37°C for 1 minute. Next, the flask was shaken in order to detach the cells from the surface. To interrupt the enzymatic action of trypsine, 5ml of growth medium were added. The suspension was centrifuged (800 RPM, 8 min). The supernatant was removed and the cells were resuspended in 3ml of fresh medium and counted. Counting was performed by mixing 10µl of cell suspension and 40µl of trypan blue solution and transferring 10µl of the mixture on hemacytometer.

Cells (passage 18-25) were seeded on 12 well plates polycarbonate Transwell® Permeable Support (Corning Incorporated, Tewksbury, USA) at density 0.1 x 10^6/ml) reaching 0.5ml of medium in upper, apical side. Then 1.5ml of medium was added to lower, basolateral (Figure 1). Cells were grown for 3 weeks with the medium changed every two days.

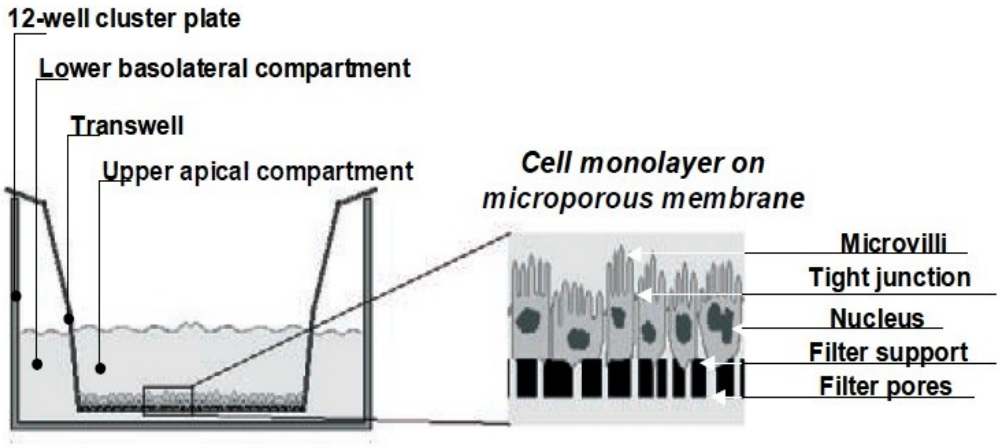


Figure 1: *Schematic illustration of an epithelial monolayer grown on a permeable support, e.g. Transwell® (Neuhoff 2005)*

4.3.Monolayer integrity check

The integrity of monolayers on inserts was checked by TEER using electrode Millipore Millicell® against blank wells filled with medium. Next, the resistance of monolayer was multiplied by effective growing area (1.1 cm^2) and expressed as $\Omega \cdot cm^2$. The average TEER value was 727 ± 96 $\Omega \cdot cm^2$. The TEER was measured before the experiment.

Fluorescein (FLU), added to the donor solution, was used to check cell monolayer integrity after experiment. From samples collected in different intervals (every 20 minutes up to 120 minutes), 40 µl were transferred to

microtiter plate and supplemented with 160 µl of 0.025M NaOH. The samples were analyzed on fluorescence and UV well plate reader Tecan Safire^{2}™. Then the permeability was calculated from calibration curve and should not exceed $1 \cdot 10^{-6}$ cm·s^{-1}.

4.4.In vitro transport studies

Prior to experiment, solutions used for transport experiments were prepared. Donor solutions contained 100µM of tested compound, 20µM of FLU and 10mM of either mannitol for apical to basolateral transport (AP-BL) or glucose for basolateral to apical experiment (BL-AP). Acceptor solutions were composed of either 10mM mannitol for apical compartment or 10mM glucose for basolateral compartment

All basolateral solutions, both for apical to basolateral transport and vice versa, were added to plates and pre-warmed in Labnet Vortemp 56 EVC Shaking Incubator (37°C, 5% CO_2, 20 RPM). Inserts with the cell monolayer were disposed of growth medium and rinsed with Ringer solution (pH 7.4). Next, apical solutions were pipetted to apical side of inserts and those were transferred to pre-incubated wells.

The samples from acceptor site were collected every 20 minutes for 2 hours, 100µl from apical side for BL-AP measurements and 300µl from basolateral side for AP-BL measurements, then the missing volume was refilled with either mannitol or glucose solution, depending on side from which the samples were taken. In the end of experiment, samples from donor solutions were taken and diluted twentyfold (Žakelj *et al.* 2004).

4.5.HPLC

All samples were analyzed with an Agilent 1100 series HPLC system (degasser, binary pump, temperature-regulated well plate sampler, column thermostat and UV-VIS diode array detector) using Zorbax Eclipse XDB-C18 column (4.6 x 75mm, 3.5µm) at 55°C. The mobile phase was composed of diluted phosphoric acid (pH 2.5) and acetonitrile (ACN). Linear gradient was used in method development and isocratic methods were used for sample analysis.

4.6.Data analysis

The apparent permeability coefficient (P_{app}) was calculated using equation 1 (Žakelj *et al.* 2004):

$$P_{app} = \frac{dQ}{dt} \times \frac{1}{AC_0}, \qquad \textit{Eq. 1}$$

where d*Q*/d*t* is the amount, which diffused through the membrane per time unit, *A* is the surface area of monolayer (1.12 cm^2) and C_0 is the initial concentration of compound in donor solution (100μM).

All data obtained from HPLC analysis were processed using calibration curve for each compound. Calibration curves with Pearson coefficient with at least R^2= 0.963 were used for calculations. Results are presented as means ± standard deviation (SD) of at least four parallel permeability measurements.

Data were statistically evaluated using non-parametric Mann-Whitney test with two-tailed P values using GraphPad Prism 5.0 software.

5. Results

Permeability coefficients of five tested compounds through the Caco-2 cell monolayers were calculated in AP-BL and BL-AP directions, then efflux ratio was determined for each compound.

5.1.HPLC methods development

First, using 2μM solution of compound dissolved in water, all tested compounds underwent HPLC-UV analysis using gradient elution with ACN percentage steadily increasing in order to determine approximate ratio of mobile phase components. Next, using isocratic elution ideal H_3PO_4: ACN ratio was established to lower the retention time, but not to affect chromatogram resolution. Wavelength at which the substances absorbed the most was also acquired. The method setup was performed before the measuring of permeability. The methods used for each compound are listed in Table 3.

Table 3: *The composition of mobile phase with corresponding percentage, wavelength and retention time used in HPLC analysis.*

Compound	Diluted H_3PO_4 (pH 2.5) (%)	Acetonitrile (%)	Wavelenght (nm)	Retention time (min)
GUK-901	63	37	231	1.38
GUK -982	66	34	224	1.45
GUK - 987	79	21	224	1.03
GUK -1007	67	33	234	1.60
GDK - 375	83	17	251	1.20
SDH - 381	79	21	240	2.18

5.2.Transport studies of butyrylcholinesterase inhibitors

Nearly every compound, that the experiments were conducted with, showed transport across cell monolayers, but not affected by active transport (Figure 2). There was slight increase in amount transported from basolateral to apical compartment compared to opposite direction (Table 4); however the efflux ratio, which was acquired as a quotient of $P_{app\ (BL\text{-}AP)}/P_{app\ (AP\text{-}BL)}$ and ranged from 0.97 to 1.65, showed no considerable efflux transporter involvement. Average results of measurements performed are shown in Table 5 Table 4and illustrated in Figure 3.

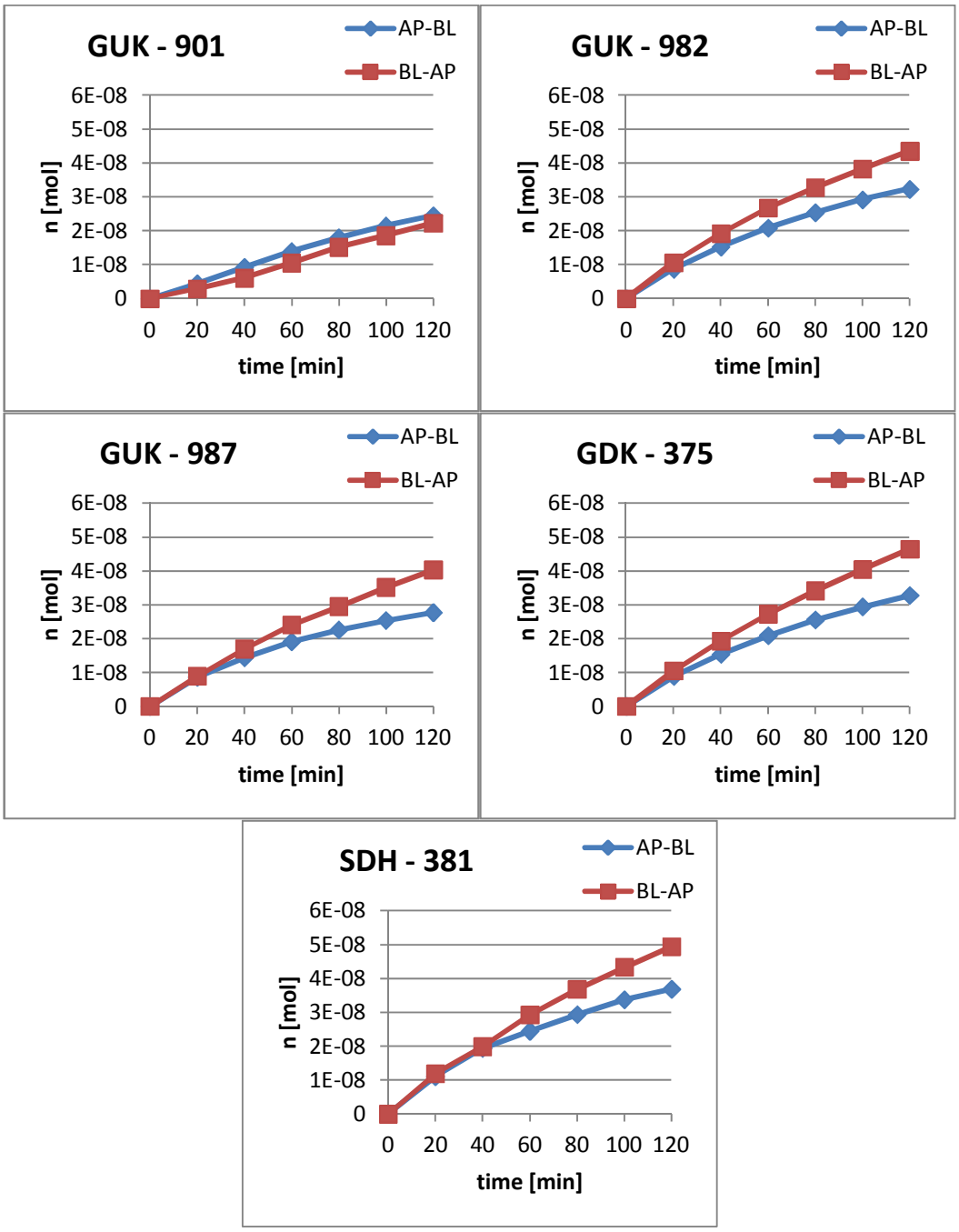


Figure 2: *Graphic representation of cumulative amount curves over time; blue line represents amount of selected compounds transported across monolayer in apical-to-basolateral direction, red line amount transported in basolateral-to-apical direction.*

Table 4: *Apparent permeability coefficient values for each measurement and filter (P_{app}; cm · s^{-1} x 10^{-6}). In the case of BL-AP direction measurements for SDH-381, the sixth filter used for this compound showed low TEER value before the experiment, and the fluorescein check confirmed the leakiness of that monolayer, therefore it was not included in the average result.*

	P_{app}									
	GUK -901		GUK-982		GUK-987		GDK-375		SDH-381	
No.	AP-BL	BL-AP	AP-BL	BL-AP	AP-BL	BL-AP	AP-BL	BL-AP	AP-BL	BL-AP
1	45.55	28.38	33.68	45.60	28.63	40.22	32.54	50.54	40.46	43.21
2	36.88	26.59	36.77	48.87	26.86	46.82	36.07	52.53	39.00	45.33
3	34.90	34.41	36.44	50.35	28.89	47.49	36.36	49.51	58.21	47.50
4	21.06	29.75	33.63	43.66	27.35	39.52	36.70	54.73	40.66	47.21
5	21.44	29.16	34.51	50.10	27.47	50.41	38.92	56.13	47.68	46.80
6	19.84	26.53	34.92	52.25	28.83	52.21	31.42	55.64	36.82	108.12

Table 5: *Results of transport experiments showing average apparent permeability (P_{app}; cm · s^{-1} x 10^{-6}) for both directions with corresponding standard deviation (SD; n=6, for SDH-381 in BL-AP direction n=5) both in absolute and relative values (RSD; %). Efflux ratio between average BL-AP and AP-BL P_{app} values also included.*

	AP-BL			**BL-AP**			
Label	***P_{app}***	**SD**	**RSD**	***P_{app}***	**SD**	**RSD**	**Efflux ratio**
GUK-901	29.95	10.67	35.64	29.14	2.90	9.94	0.97
GUK-982	34.99	1.35	3.86	48.47	3.23	6.66	1.39
GUK-987	28.00	0.88	3.16	46.11	5.22	11.32	1.65
GDK-375	35.34	2.81	7.95	53.18	2.76	5.18	1.50
SDH-381	43.80	7.94	18.13	46.01	1.78	3.86	1.05

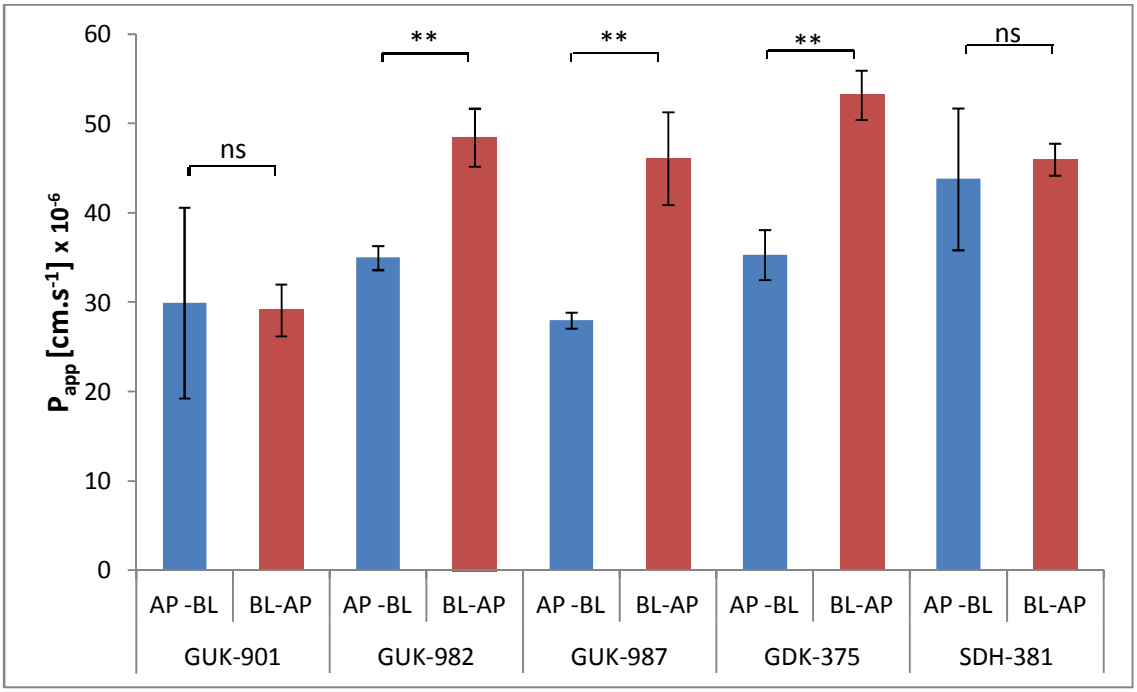


Figure 3: *Graphic representation of average P_{app} values for both directions across Caco-2 cell monolayers. Values gained were almost equivalent for AP-BL and BL-AP transport. Each bar indicates mean ± SD (n=6); ns $p > 0.05$, ** $p \leq 0.01$.*

5.3.Monolayer tightness fluorescein measurements

The simultaneous monolayer leakiness check conducted using fluorescein as marker showed no extreme values of permeability (P_{app} = 5.03 ± 2.48 ·10^{-7} cm·s^{-1}; Table 6). However, the apparent permeability values gained from BL-AP transport experiments exceeded the opposite direction measurement's values by one order of magnitude. Therefore, an experiment was carried out to discover possible influence of tested compounds on transport of fluorescein across the Caco-2 cell monolayers.

In this experiment, the apparent permeability of fluorescein alone and with GUK-987 as model compound either on apical or basolateral side was being determined using the same conditions as for previous bidirectional measurements.

After calculating the apparent permeability coefficient and efflux ratio, the results showed that the amount transported in BL-AP direction with GUK-987 on either side greatly exceeds the amount of FLU transported in same direction without GUK-987 (Table 7Table 6, Figure 4).

Table 6: *Apparent permeability coefficient values of fluorescein for each measurement and filter (P_{app}; cm · s^{-1} x 10^{-6}). Numbers of filters correlate with Table 3. It is noticeable how the transport from basolateral side in the presence of several compounds is highly exceeding the transport in opposite direction.*

	FLU P_{app}									
	GUK -901		GUK-982		GUK-987		GDK-375		SDH-381	
No.	AP-BL	BL-AP	AP-BL	BL-AP	AP-BL	BL-AP	AP-BL	BL-AP	AP-BL	BL-AP
1	0.06	0.84	0.07	1.04	0.09	0.97	0.06	0.34	0.14	0.29
2	0.09	2.52	0.04	0.98	0.12	1.43	0.07	0.44	0.15	0.45
3	0.05	0.91	1.18	1.07	0.09	1.31	0.05	0.28	0.14	0.57
4	0.10	0.77	0.08	1.07	0.32	1.24	0.07	0.32	0.08	0.47
5	0.10	0.97	0.08	1.25	0.21	1.36	0.07	0.38	0.13	0.43
6	0.10	0.73	0.22	3.94	0.17	1.40	0.06	0.35	0.17	42.88

Table 7: *Results of fluorescein transport experiment showing average apparent permeability (P_{app}; cm · s^{-1} x 10^{-6}) for both directions (with and without GUK-987) with corresponding standard deviation (SD; n=4) both in absolute and relative values (RSD; %). Efflux ratio between average BL-AP and AP-BL P_{app} values also included.*

	AP-BL			**BL-AP**			
Label	***P_{app}***	**SD**	**RSD**	***P_{app}***	**SD**	**RSD**	**Efflux ratio**
FLU	0.14	0.05	32.05	0.24	0.01	6.01	1.72
FLU + GUK-987 (A)	0.10	0.02	20.40	1.31	0.30	23.13	13.51
FLU + GUK 987 (B)	0.11	0.02	13.44	1.25	0.15	12.04	10.92

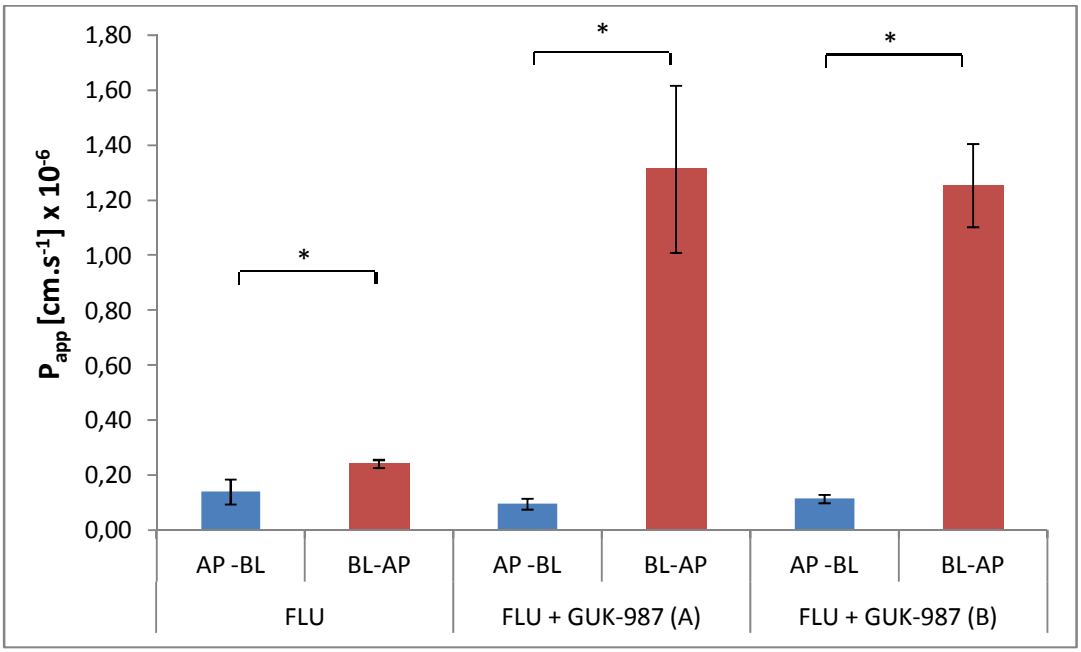


Figure 4: *Graphic representation of average P_{app} values for FLU without GUK and with GUK on either apical or basolateral side, including both directions of FLU diffusion across Caco-2 cell monolayers. BL –AP values gained from experiments with GUK present on either side highly exceed those gained from both AP-BL transport measurements and measurements without GUK. Each bar indicates mean ± SD (n=4); * p ≤ 0.05*

5.4.GUK-1007 transport measurement

Compound labelled GUK-1007 was excessively metabolised and only very limited amount was able to permeate across the monolayer in unchanged form. HPLC-UV analysis (Figure 5) discovered that no significant amount passed through in either direction. Therefore the samples were also analyzed by LC-MS (MS data not shown). This analysis resulted in recovery of around eleven possible fragments and metabolites.

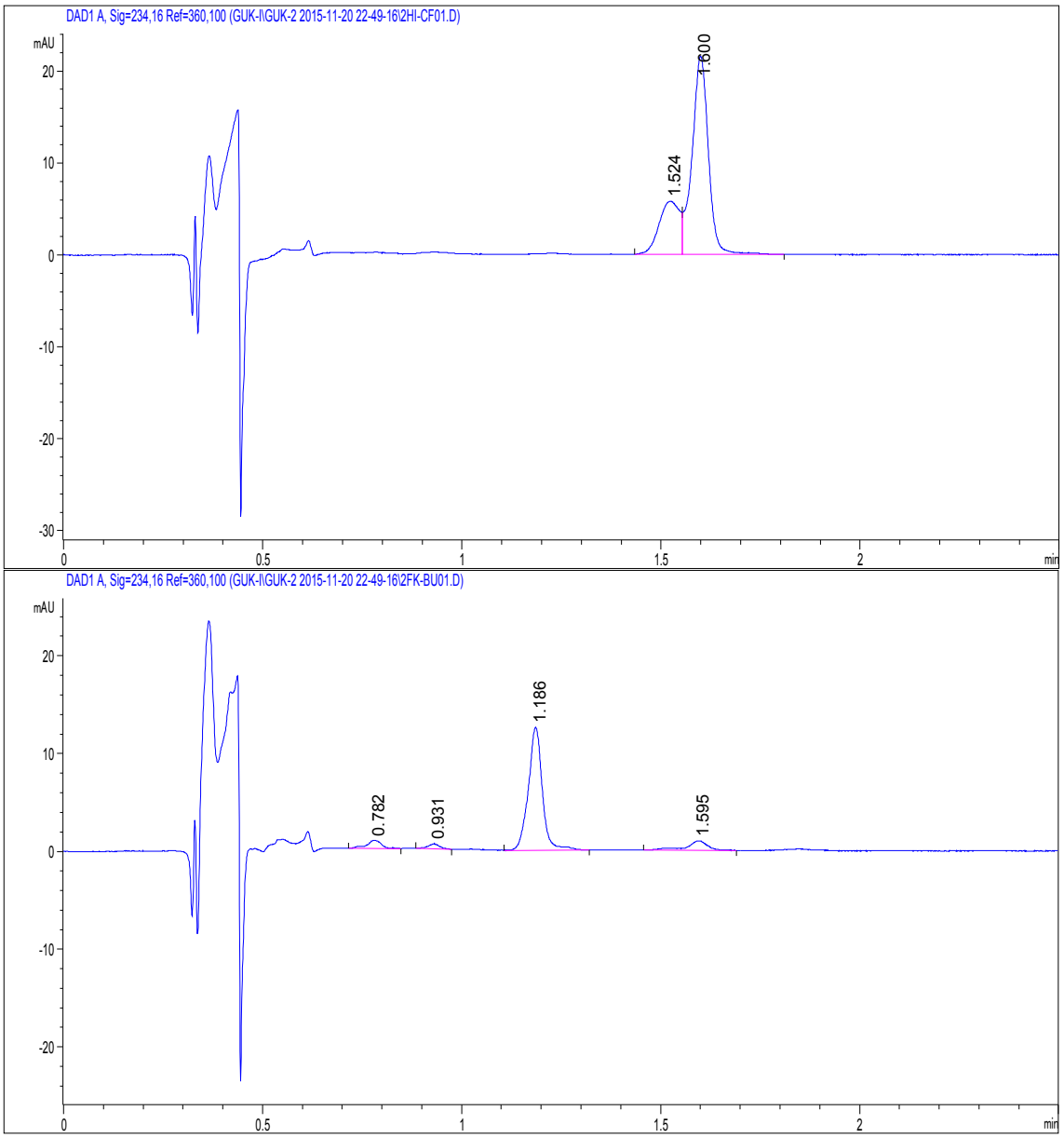


Figure 5: *HPLC-UV chromatograms depicting GUK-1007 calibration sample (the former one) and GUK-1007 acceptor side sample (the latter one). There is small amount of parent compound noticeable in acceptor side sample; however compared to the metabolites it is negligible.*

6. Discussion

The reason for utilization of Caco-2 cell line, which is commonly used for prediction of intestinal absorption, as a model for BBB permeability and active efflux has risen from the properties of the cells. Although its origin differs from BBB, due to presence of tight junctions and limitation of paracellular transport, the permeability for compounds transported can be comparable to the permeability of brain capillary endothelial models, which are most reliable in the manner of data gained (Vastag, Keserü 2009). Caco-2 cells reveal certain disadvantages. First, the growth rate is three-time slower compared to MDCK, other type of cell line for BBB permeability prediction. Furthermore, the expression of efflux transporters may not reach the levels in BBB (use of transfected models, such as VB-Caco-2 or MDCK-MDR1 may be more suitable) (Hellinger *et al* 2012). On the other hand, Caco-2 cell line is well established *in vitro* approach to predict intestinal absorption and role of transporters as well. In comparison to MDCK cells, when cultured properly, Caco-2 cells show more reliable level of growth in manner of monolayer cohesion (Volpe 2008).

In order to identify the compound as a substrate of apical efflux transporters (P-glycoprotein, BCRP etc.) with clinical impacts, the efflux ratio (coefficient of permeability B→A to A→B direction) value should be equal or higher than two (Sun *et al*. 2008). Molecules, such as GUK-901 and SDH-381 with their P_{app} coefficients for both directions being almost equal, can be practically transported without any influence of unidirectional active membrane transporters. Other compounds (GUK-982, 987 and GDH-375) showed efflux ratio ranging from 1 to 2, however this asymmetry in transport was insignificant. Moreover, molecules with such low ratio value usually tend to exhibit good CNS penetration *in vivo* (Summerfield *et al.* 2006). In order to determine the degree of active transport, another experiment with the use of efflux transporter inhibitors, such as verapamil which inhibits both P-glycoprotein and BCRP (Breedveld *et al.* 2006), can be performed.

Regarding fluorescein permeability as simultaneous leakiness check, all compounds proved enhanced diffusion of FLU through cell monolayer in BL-AP direction. FLU is commonly recognized by two classes of transporters

expressed in BBB and Caco-2 cells: MRPs and organic anion transport proteins (OATPs) (Sun, Miller and Elmquist, 2001). Interestingly, the MRP-2 mediated efflux of FLU is stimulated by higher concentration of glucose (Hawkins *et al.* 2007); however this is not the case because of glucose level being same in all measurements. Another example of modification of FLU transport across membrane may be by inhibition of either MRP or OATP (using e.g. probenecid) (Sun, Miller and Elmquist, 2001). Therefore, it is safe to assume that these chemical substances somehow might activate transporters on luminal side of membrane, which leads to increased efflux of FLU. However, the exact mechanism of activation is unknown.

GUK-1007, which chemical structure differs from others by amide derived from primary amine, was highly metabolised and therefore was not detectable.

Before the compound reaches BBB, it has to overcome many obstacles (intestinal barrier, liver, kidneys), in which the presence of ABC transporters can affect pharmacokinetics of drugs and therefore influence anticipated drug absorption, distribution and excretion. Our tested compounds have been designed for oral administration and in this regard our results indicate good intestinal absorption. With the permeability rate ranging from 28.00 to 43.80 cm $\cdot$ s^{-1} x 10^{-6} we hypothesize that the tested compounds will be quantitatively absorbed from the intestine. (Breemen, Li 2005). However, not always the *in vitro* and *in vivo* results correlate; *in vivo* CNS penetration is determined not only by permeability and transporter activity, but also by unbound fraction in plasma (Summerfiel *et al*. 2006). Therefore, *in vivo* testing is needed in order to fully evaluate the CNS penetration and other pharmacokinetic properties of the tested compounds.

7. Conclusion

The purpose of this thesis was to investigate the ability of several compounds, which may serve as new butyrylcholinesterase inhibitors to treat certain types of dementia in elderly patients, to penetrate through cell barriers *in vitro*. Our results suggest reliable transport ability across physiological barriers, such as intestinal and BBB, with permeability coefficients comparable to drugs commonly used in treatment of different CNS disorders such as epilepsy or depression. The involvement of ABC efflux transporters was also assed in order to predict the fate of compound after administration. Efflux ratios obtained indicate negligible or none efflux transporter influence on compound transport. However, the physiological rate of transport may be further affected by the degree of protein binding and also the rate of metabolism.

8. List of abbreviations

ABC – ATP binding cassette family of proteins

ACN – acetonitrile

ACh – acetylcholine

AChE - acetylcholinesterase

AMT – adsorptive mediated transcytosis

AP - BL – apical to basolateral direction

ATCC – American tissue culture collection

ATP – adenosine triphosphate

BBB – blood-brain barrier

BChE - butyrylcholinesterase

BChEi – butyrylcholinesterase inhibitors

BCRP – breast cancer resistance protein

BL - AP – basolateral to apical direction

Caco-2 – human colon adenocarcinoma cell line

CNS – central nervous system

DMEM – Dulbecco's minimum essential medium

DMSO – dimethyl sulfoxide

DPBS – Dulbecco's phosphate buffer saline

FAAH – Fatty Acid Amide Hydrolase

FBS – fetal bovine serum

FLU – fluorescein

HPLC – high-performance liquid chromatography

LC-MS – analytical system comprised of liquid chromatography and mass spectrometry

MDCK – Madin-Darby canine kidney cell line

MRP – multidrug resistance-related protein

MW – molecular weight

NMDA – N-methyl – D aspartate

OATP – organic anion transport protein

P-gp – P-glycoprotein

PAMPA – parallel artificial membrane permeability assay

P_{app} – apparent permeability coefficient

RMT – receptor mediated transcytosis

RPM – revolutions per minute

RSD – relative standard deviation

SD – standard deviation

TEER – transendothelial electrical resistance

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