University of Szeged
Faculty of Science and Informatics
Ph.D. School in Biology

NEW AGENTS TO OPEN THE BLOOD-BRAIN BARRIER: THE EFFECTS OF TESMILIFENE AND SHORT-CHAIN ALKYLGLYCEROLS ON BRAIN ENDOTHELIAL CELLS

Summary of the Ph.D. thesis

Fruzsina Walter

Supervisors
Dr. Mária Deli, scientific adviser
Dr. Szilvia Veszelka, research associate

Hungarian Academy of Sciences
Biological Research Centre, Institute of Biophysics
Biological Barriers Research Group

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1. Introduction

The blood-brain barrier (BBB) forms a dynamic interface separating the systemic circulation from the central nervous system (CNS). Brain capillary endothelial cells constitute the anatomical and functional basis of the BBB, which selectively regulates the transcellular and paracellular transport of molecules and passage of cells between the blood and brain. The BBB restricts the penetration of drugs to brain tissue mainly *via* tight junctions and efflux transporters and prevents effective treatment of several neurological diseases, including gliomas, the most common form of brain tumors in adults. Glioblastoma multiforme evolves from astroglial or astroglial precursor cells and is the most severe form of glioma with very poor prognosis. The current therapy of malignant gliomas or brain metastases is unsatisfactory because of the low penetration of drugs to tumor tissue due to efflux transporters and tight junctions expressed at brain capillaries. Therefore, microvessels in glioma are in the focus of intensive research and the search for new adjuvants to improve drug delivery is highly relevant from clinical point of view.

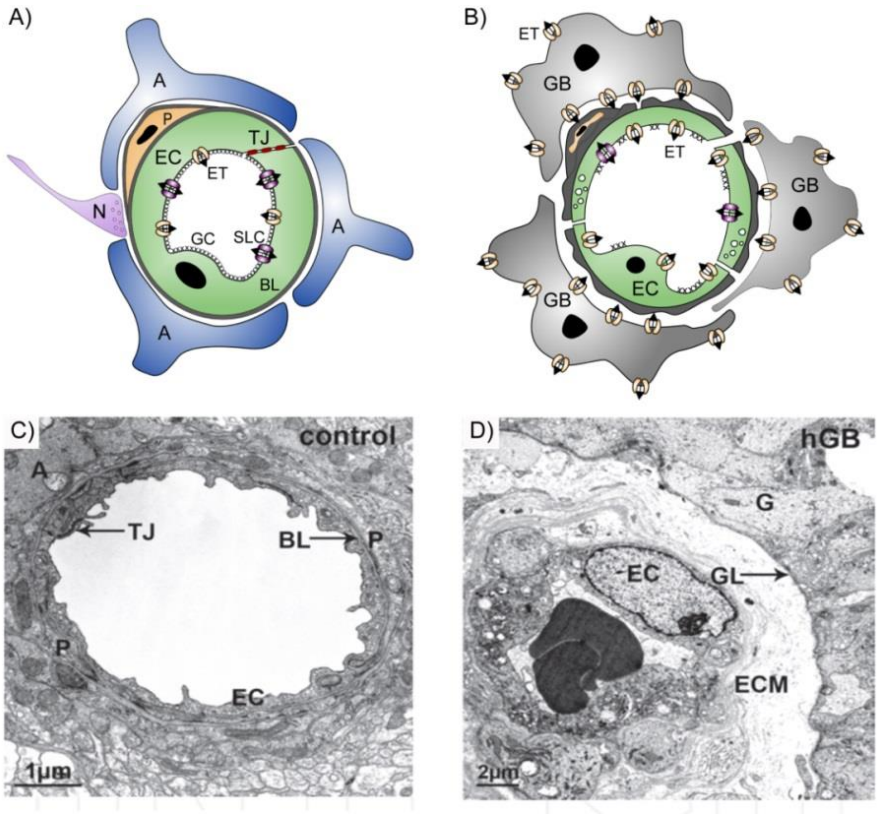

Figure 1. Functional and morphological differences at the blood-brain barrier in glioma. **A and C:** Morphology of the blood-brain barrier: tight junctions (TJs) and transporters; well established connections between the members of the neurovascular unit. **B and D:** Brain capillary in glioma. Electronmicrographs from Wolburg et al, 2012. A: astroglia; BL: basal lamina; EC: endothelial cell; ECM: extracellular matrix; ET: efflux transporter; GC: glycocalyx; hGB: human glioblastoma; N: neuron; TJ: tight junction; SLC: solute carrier transporter proteins.

Main changes in capillaries in gliomas are: (i) elevated endothelial cell number, (ii) endothelial hyperplasia leading to loss of function, (iii) decrease in endothelial cell volume, (iv) cell shape changes, (v) disruption of interendothelial tight junctions, (vi) increased number of vesicles, caveolae and fenestrations, (vii) thickened and irregular basal membrane, (viii) enlarged perivascular space, (ix) necrosis of microvascular cells and (x) the coverage of microvessels by astroglial endfeets is replaced by glioma cells (Figure 1). These morphological changes result in blood-brain barrier dysfunction.

One of the strategies to increase the CNS penetration of drugs is to change the functions of cerebral endothelial cells either by opening interendothelial tight junctions (Table 1) or by inhibiting the activity of drug efflux pumps. In this study two agents which open the BBB, tesmilifene and short-chain alkylglycerols (AGs) were selected for examination.

Table 1. Opening tight junctions at the blood-brain barrier to enhance delivery of drugs to the CNS (Deli 2011, modified)

Method	**Drug, molecules**	**Stage of developement**	**Reference**
Osmotic BBB disruption by mannitol	Anticancer drugs (intraarterial administration for brain tumor treatment)	Clinical trials	Doolittle, 2014
Short-chain alkylglycerols and oligoglycerolipids	Fluorescein, albumin, anticancer drugs, antibiotics	Preclinical development, animal experiments	Erdlenbruch 2003a,b Genzyme, 2010
Cereport (bradykinin analog)	Anticancer drugs: carboplatin	Phase I-II. clinical trials terminated	Packer, 2005
Zonula occludens toxin	Anticancer drugs: methotrexate, paclitaxel (intracarotid administration)	Animal experiments	Menon, 2005
Nitric oxide donors	Sucrose, γ-aminobutiric acid (intracarotid administration and in situ brain perfusion)	Animal experiments	Weyerbrock, 2012
High frequency focused ultrasound	Antibodies, doxorubicin	Animal experiments	Burgess, 2014
Tesmilifene	Small molecular weight tracers, paracellular opening	Preclinical development, animal experiments	Deli, 2000, 2003 Walter, 2015

In animal studies intraarterial injection of short-chain AGs 1-*O*-pentylglycerol and 2-*O*-hexyldiglycerol transiently open the BBB and increase the delivery of small or large molecules to brain parenchyma of rodents in a concentration dependent way. Tesmilifene, a tamoxifen-related compound, has chemopotentiating properties in both experimental and clinical cancer studies. Treatment with tesmilifene caused temporary, acute CNS side-effects in patients when used as a chemotherapeutic adjuvant, indicating the opening of the BBB. Previous studies from our laboratory demonstrated that tesmilifene increased the permeability of the BBB in rats and also decreased the tightness of endothelial monolayers in culture. However, there were no data on the direct effect of AG or tesmilifene on brain endothelial cells and the underlying mechanism of the modification of BBB permeability. The aim of our studies was to test the direct effects of AGs and tesmilifene on cultured brain microvascular endothelial cells, and to investigate the effects of tesmilifene on the permeability of the glioma vasculature.

2. Materials and methods

2.1. Animals

Animal studies were performed following the regulations of the 1998. XXVIII. Hungarian law and the EU Directive 2010/63/EU about animal protection and welfare. For our experiments Wistar rats (both sexes, Harlan Laboratories, United Kingdom) were used, which were kept in the conventional animal house of the Biological Research Centre, Szeged.

2.2. Materials and reagents

Drugs and chemicals used in the study were purchased from Sigma-Aldrich Ltd., Hungary, unless otherwise indicated. Tesmilifene (HCl salt, mw: 319,9 Da) was synthesized as described previously (Brandes and Hermonat 1984). The short-chain AGs, 2-*O*-HG and 1-*O*-PG, were obtained from Genzyme Corporation (Cambridge, MA, USA).

2.3. Glioma implantation and dye extravasation measurements

Effects of tesmilifene on the microvessel permeability in healthy brain and glioma tissue were investigated in 3-month-old sham operated (n=12) or glioma implanted (n=12) Wistar rats. The implantation procedure of RG2 rat glioblastoma cells was performed based on a previous method (Aas et al. 1995). Two weeks after the implantation extravasation of permeability markers sodium fluorescein (SF, 376 Da, 2 %) and Evans blue-labeled albumin (EBA, 67 kDa, 2 %) was tested with or without tesmilifene treatment (5 mg/kg, iv., n=6 or

saline solution n=6). After the permeability experiment tissue samples from cerebellum, midbrain, left and right cortex, glioma or implantation site on the right cortex were collected. Concentration of the tracers in samples was measured using a PTI spectrofluorometer (Photon Technology International Inc., USA).

2.4. Cell culture

2.4.1. RG2 glioblastoma cell line

Glioblastoma tumor cell line RG2 was purchased from ATCC (American Tissue Culture Collection, USA). RG2 cells were kept in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal bovine serum (Pan Biotech, Germany) and 50 µg/ml gentamycin. In all culture conditions, plastic surfaces were coated with rat tail collagen.

2.4.2. Preparation of primary cultures and establishment of the *in vitro* BBB model

Experiments with AGs and tesmilifene were performed on primary rat brain endothelial cell monocultures and double or triple co-culture models of the BBB consisting of primary rat brain endothelial cells and glial cells, with or without pericytes. These three cell types were isolated and cultured according to the method described in our previous studies (Nakagawa et al, 2009; Veszelka et al, 2013; Hülper et al, 2013; Jaehne et al, 2014; Walter et al, 2015; Figure 2).

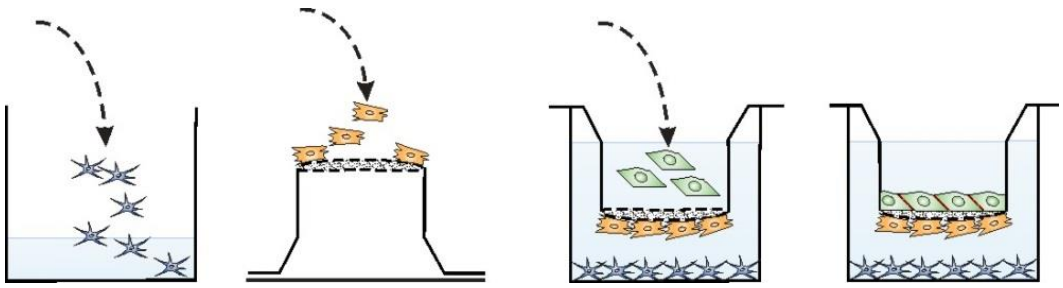

Figure 2. Establishment of the triple co-culture model of the blood-brain barrier. 1. culture of glial cells; 2. passage of pericytes to the lower surface of the tissue culture insert; 3. passage of brain endothelial cells to the top of the tissue culture insert; 4. the triple blood-brain barrier co-culture model.

In the first four days the culture medium for primary rat brain endothelial cells contained puromycin (4 µg/ml) to eliminate contaminating cell types. Brain endothelial cells were plated on surfaces coated with collagen type IV and fibronectin (100 µg/ml for both). Endothelial cell culture medium consisted of DMEM/F12, plasma-derived bovine serum (15 %; First Link, UK), heparin (100 µg/ml), insulin (5 µg/ml), transferrin (5 µg/ml), sodium

selenite (5 ng/ml), basic fibroblast growth factor (1 ng/ml; Roche, Switzerland) and gentamycin (50 μg/ml). After brain endothelial cells became confluent hydrocortisone (550 nM; experiments with tesmilifene) or cyclic AMP (250 μM CPT-cAMP + 17,5 μM phosphodiesterase inhibitor RO 201724, Roche; experiments with AGs) were added to the culture medium to tighten junctions.

2.5. Treatments

During our experiments AGs were used at 15 – 60 mM concentrations for a short-term, 5 minute exposure. After the administration of AGs in addition to acute effects a 30 min recovery phase was also tested on cells by the re-addition of cell culture medium in case of resistance measurement, permeability experiments and immunohistochemistry. Mannitol was used as a reference molecule to open tight junctions at a concentration relevant for clinical conditions (1.4 M). The effect of tesmilifene on brain endothelial cells was tested in the 1 – 200 μM concentration range for 1, 16 and 24 hours. All treatments on primary brain endothelial cells were performed in endothelial culture medium. In the AG experiments viability tests were performed using saline solution to mimic the experimental conditions during in vivo tests.

2.6. Cell viability assays

Changes in cell viability after AG and tesmilifene treatments were tested with several assays. In the colorimetric MTT and WST tests living, metabolically active cells reduce tetrazolium dyes to formazans, therefore the reduction of the dyes decreases in parallel with cellular toxicity. To measure apoptotic cell death after AG treatment an enzyme linked immunosorbent assay (ELISA) was used according to the protocol of the manufacturer (Roche). Kinetics of the viability of primary rat brain endothelial cells was monitored by real-time measurement of impedance (RTCA-SP, ACEA Biosciences, USA). Impedance values of cultures correlate linearly with cell number, adherence, growth and viability. In this assay cells are culture in a 96-well plate with integrated golden microelectrodes on the bottom of the wells (E-plates). In the case of tesmilifene toxicity was also studied using double fluorescent staining of cell nuclei. For the assessment of metabolic activity the production of ATP was measured in brain endothelial cells after tesmilifene treatment using the CellTiter-Glo luminescence kit (Promega, WI, Madison, USA) according to the description of the manufacturer.

2.7. Evaluation of barrier integrity

2.7.1. Transendothelial electrical resistance measurement

Transendothelial electrical resistance (TEER) was measured by an EVOM voltohmmeter (World Precision Instruments, USA) combined with STX-2 electrodes. Recorded resistance was expressed to the surface of the Transwell filters ($\Omega \times cm^2$) and TEER of cell-free inserts (130 $\Omega \times cm^2$) was subtracted from these values.

2.7.2. Permeability measurement

During permeability experiments two fluorescent tracers, sodium fluorescein and Evans blue labeled albumin were used, which penetrate the blood-brain barrier poorly in physiological conditions, similarly to the *in vivo* experiments. The concentration of the fluorescent dyes was measured in the lower (acceptor) compartment of the co-culture models using a multiwell plate reader. Endothelial permeability coefficients (P_e) were calculated from clearance values of tracers as described in detail in previous publications (Deli et al, 2005; Hülper et al, 2013).

2.8. Immunohistochemistry

Brain endothelial cells were stained for the integral membrane tight junction protein claudin-5 and for junctional associated proteins β-catenin and zonula occludens protein-1 (ZO-1) after AG and tesmilifene treatments. Cells were incubated with primary antibodies: anti-claudin-5 (mouse monoclonal antibody, Invitrogen, Life Technologies, USA), anti-β-catenin and anti-ZO-1 (rabbit polyclonal antibodies, Invitrogen, Life Technologies); and with secondary antibodies Alexa Fluor-488-labeled anti-mouse (Life Technologies, Invitrogen, USA) and Cy3-labeled anti-rabbit and Hoechst dye 33342 for nucleus staining. Cells were examined by a NikonEclipse TE2000 fluorescent microscope.

2.9. Freeze fracture electron microscopy

To investigate the morphology and strand complexity of tight junctions transmission electron microscopy was performed on freeze fracture replicas of brain endothelial cells after AG treatment according to a previously described protocol (Wolburg et al, 1994). Cells were fixed for electron microscopy after acute treatment by AGs with or without 30 min recovery. TJ strands are visible on freeze fracture samples as continuous pearl-chain like strands either on the P-face (protoplasmic side) or on the E-face (exoplasmic side). The P-face/E-face ratio of tight junction strands is an important parameter for the functional quality of the BBB.

2.10. Evaluation of the activity of efflux pumps: P-glycoprotein (Pgp), breast cancer resistant protein (Bcrp) and multidrug resistance associated protein-1 (Mrp-1)

To determine the activity of Pgp and Bcrp efflux pumps rhodamine 123 (R123) accumulation in brain endothelial cells was measured. Two well-known pump inhibitors were also tested: cyclosporine A (1.6 μM), inhibitor of Pgp and Bcrp and verapamil (2 μM), inhibitor of Pgp pump. The efflux of glutathione bimane, ligand of the Mrp-1 pump was measured after treatment with tesmilifene. To block Mrp-1 probenecid (100 μM) was applied.

2.11. Quantitative real-time PCR

For this analysis primary rat brain endothelial cells were grown in 10 cm dishes. The confluent cultures were treated with 100 μM tesmilifene for 1 h and were collected by cell scrapers for total RNA isolation. Gene expression analysis was performed as described in our recent paper (Tóth et al. 2014). The expression of the selected rat BBB genes was analyzed by quantitative PCR using TaqMan Low Density Array 384-well microfluidic cards preloaded with inventoried TaqMan Gene Expression Assays (Applied Biosystems, USA). Expression of all genes was normalized to the 18S rRNA as endogenous control, highly expressed in eukaryotic cells. Expression values of studied genes were determined by the correlation of normalized expression of genes calculated by $2^{-\Delta Ct}$ formula to the lowest expression level which can be safely detected by this RT-PCR method.

2.12. Statistical analysis

For statistical analysis GraphPad Prism 5.0 software (GraphPad Software Inc., USA) was used. Data are presented as means ± S.E.M., except for the tesmilifene *in vivo* measurements. Changes were considered statistically significant at $p < 0.05$. Data were tested for Gaussian distribution by the D'Agostino-Pearson normality test. After the normality test ANOVA analyses and Kruskal-Wallis, Dunn's, Bonferroni or Dunnett tests were performed. All measurements were repeated at least three times, the number of parallel samples was minimum three.

3. Results

3.1. The effects of alkylglycerols on cultured brain endothelial cells

High treatment concentration (60 mM) of AGs decreased the viability of brain endothelial cells therefore this concentration was not used in further experiments. Acute PG and HG treatments caused a significant resistance drop compared to the control group. Recovery of resistance 30 min after PG treatment did not reach the level of control monolayers, but an increase in TEER during the recovery phase showed the reversibility of the effect of PG (Figure 3A). A decrease in resistance was also measured for acute HG treatment, but TEER was completely recovered in 30 minutes (Figure 3B). Mannitol also caused a resistance drop, but the effect was not reversible in the examined recovery phase.

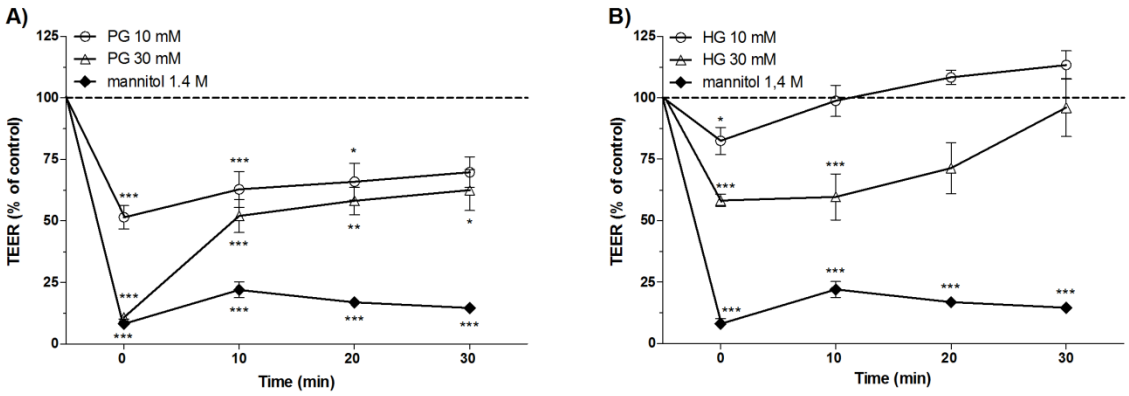

Figure 3. Transendothelial electrical resistance (TEER) measurement: the effect of alkylglycerols on the resistance of brain endothelial monolayers. A) PG: pentilglycerol, B) HG: hexildiglycerol. *, $p < 0,05$; **, $p < 0,01$; ***, $p < 0,001$ compared to the untreated control cells.

To further analyze the effects of AGs on the barrier integrity of brain endothelial cells, permeability experiments for fluorescein and Evans blue labeled albumin was performed on the double BBB co-culture model (Figure 4). Short-term PG and HG treatment (5 min, 30 mM) significantly increased the flux of sodium fluorescein, while the albumin transcytosis was not changed. In the recovery phase permeability values returned to the level of the control for both PG and HG (Figure 4). These results are in accordance with the resistance measurements and indicate that AGs elevate BBB permeability via the paracellular pathway. These results were also confirmed by changes in the immunostaining pattern for junctional proteins claudin-5 and β-catenin. However the E-face/P-face ratio of junctional strands in brain endothelial cells did not change after acute PG or HG treatment or in the recovery phase. The pearl chain-like structure of TJs was continuous and regular, suggesting that AGs do not change the ultrastructure or complexity of TJ strands.

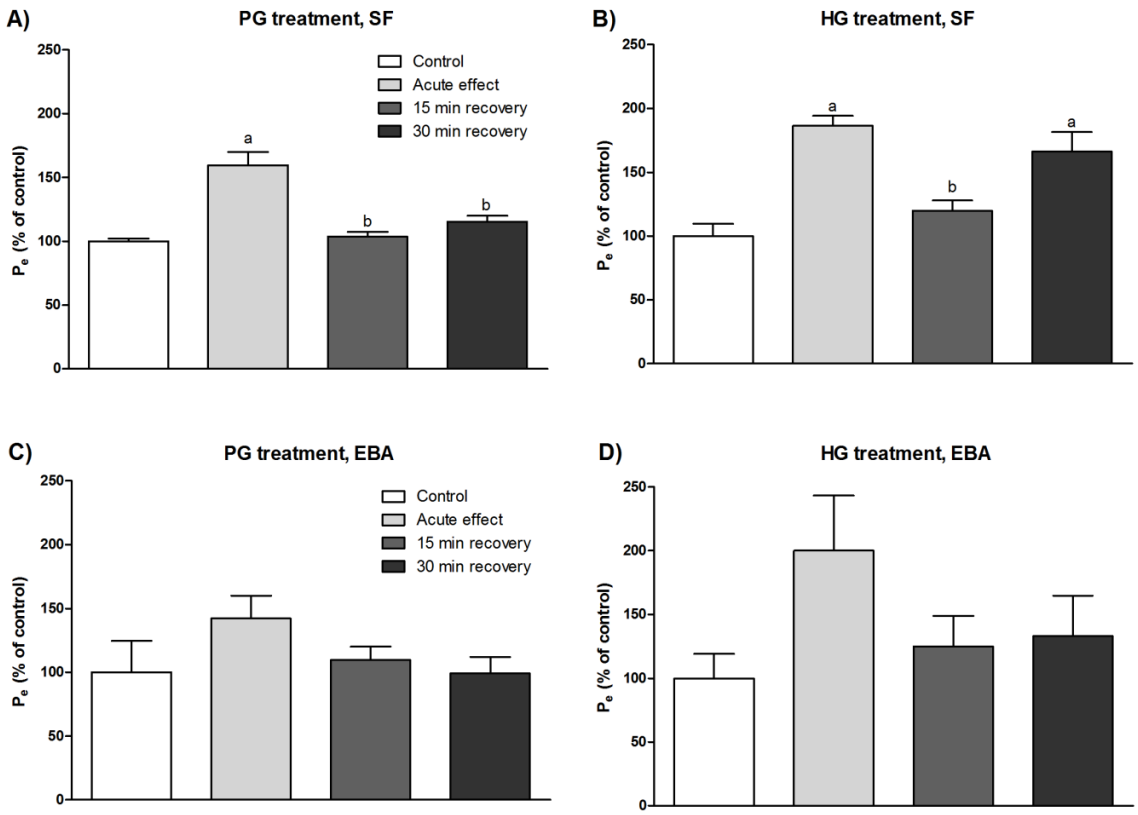

Figure 4. Permeability experiments: the effect of alkylglycerols on the permeability of brain endothelial monolayers for sodium fluorescein (SF) and Evans blue labeled albumin (EBA). PG: pentilglycerol, HG: hexildiglycerol. a, $p < 0.05$ compared to the control group; b, $p < 0.05$ compared to the acute effect.

3.2. The effects of tesmilifene on the blood-glioma barrier and on cultured brain endothelial cells

Using the RG2 glioma model, tesmilifene highly and selectively increased the extravasation of fluorescein to the tumor tissue as compared to the surrounding brain tissue in rats (Figure 5).

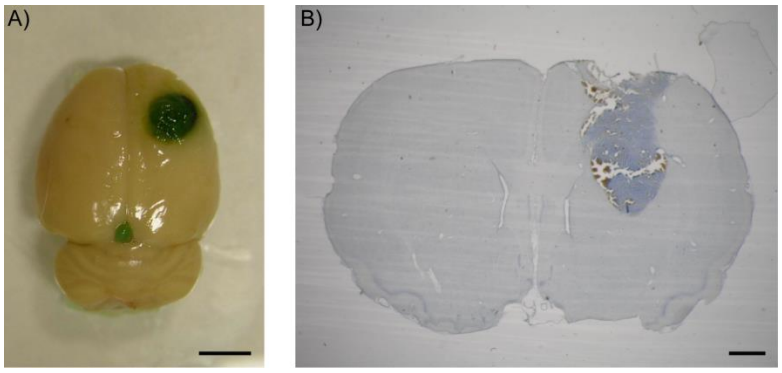

Figure 5. Blood-brain/blood-glioma permeability experiment in rats. A) Photo of an RG2 glioma implanted brain after the permeability experiment. Bar: 5 mm. B) Brain slice demonstrating the size and anatomical position of the implanted glioma. Bar: 1 mm.

Viability tests on cultured brain endothelial cells showed a toxic effect of tesmilifene when applied at 200 µM therefore this concentration was excluded from the study. Tesmilifene decreased the resistance of brain endothelial monolayers at 100 µM concentration in the barrier integrity tests on the triple BBB co-culture model. Tesmilifene (50 and 100 µM) increased the permeability for fluorescein, a marker of paracellular flux and the transcellular transport of albumin, while the lowest concentration (1 µM) decreased the permeability of both markers, similarly to cyclic AMP (25 µM) treatment (Figure 6). These results were in accordance with tesmilifene induced changes in the junctional morphology of brain endothelial cells visualized by immunolabeling of junctional proteins.

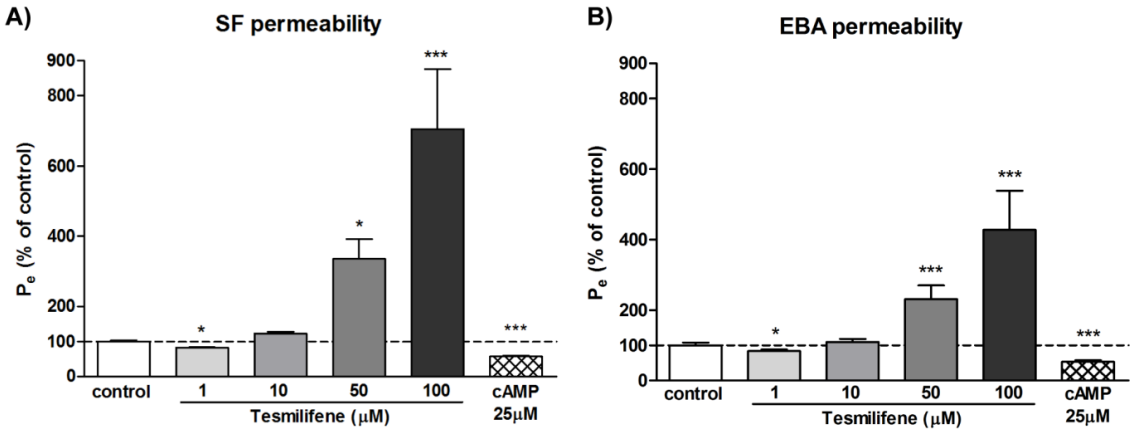

Figure 6. Permeability tests on the triple BBB co-culture model. The effects of tesmilifene on the permeability of A) sodium fluorescein (SF) and B) Evans blue labeled albumin (EBA). *, $p < 0.05$; ***, $p < 0.001$ compared to the control group.

The activity of three efflux pumps, Pgp, Bcrp and Mrp-1 was also tested after tesmilifene treatment (Figure 7).

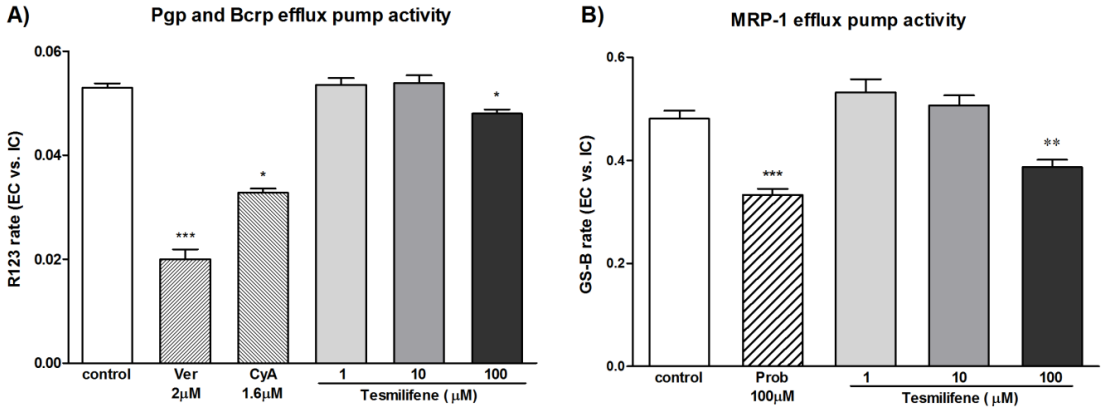

Figure 7. Effects of tesmilifene on the efflux pump activity in brain endothelial cells. A) P-glycoprotein and Bcrp pump activity. CyA: cyclosporine A; Ver: verapamil. B) Mrp-1 pump activity. Prob: probenecid. EC: extracellular, IC: intracellular, R123: rhodamine 123, GS-B: glutathione-bimane. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ compared to the control group.

Cyclosporin A, verapamil and probenecid, well-known inhibitors for these efflux pumps effectively reduced the efflux of fluorescent ligands from brain endothelial cells indicating the presence of functional Pgp, Bcrp and Mrp-1 transporters in the cultures (Figure 7). Tesmilifene decreased the activity of these efflux pumps in brain endothelial cells.

Early effect of tesmilifene on the impedance of brain endothelial cells could be partially blocked by inhibitors of the signaling pathways MAPK/ERK (U0126) and PI3K/Akt (LY294002), which are involved in the regulation of BBB permeability, and by β-estradiol, which is well-known for its beneficial effects on vascular endothelial cells (Figure 8).

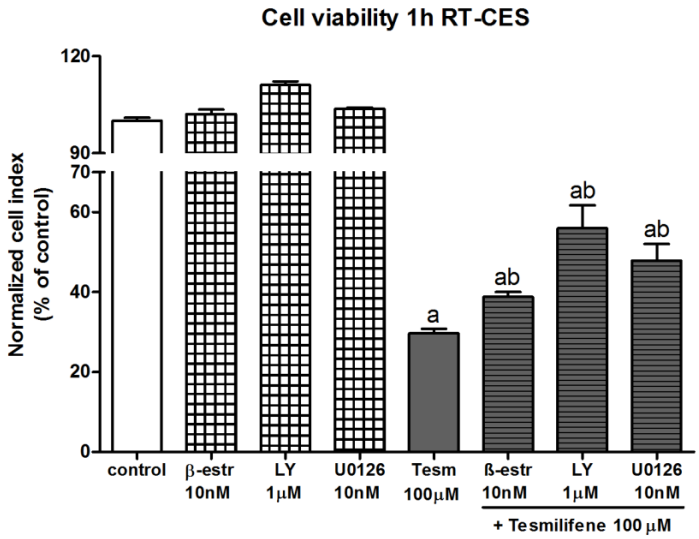

Figure 8. Inhibitors of different receptors and signal pathways modify the effect of tesmilifene (100 μM) on brain endothelial cells. β-estr: β-estrogen; LY: LY294002. a, $p < 0.05$, compared to control group and b, $p < 0.05$, compared to the tesmilifene treated group.

Tesmilifene (100 μM, 1 h) decreased the expression of several genes important in BBB function compared to untreated endothelial cells. Changes in mRNA expression higher than 2-fold are shown in Figure 9. Downregulated genes were: (i) endothelial cell selective adhesion molecule (Esam) and claudin-1, -3, -5, -11, -19 junctional proteins, (ii) Bcrp, Mrp-4, organic anion transporter-1 (Oatp1) and excitatory amino acid transporter-2, -3 (Eaat-2,-3) efflux pumps, (iii) solute carriers for amino acids (Sat-1, Snat-5), creatin (Crt), ascorbic acid (Asct2), glycine (Glyt1), taurine (Taut), thyroid hormones (Mct8), and (iv) sulfotransferase (Sult1A1), Cyp2D4 and γ-glutamyl transferases (Ggt-1, -5, -7) metabolic enzymes. Tesmilifene completely inhibited the expression of several genes, namely: claudin-2, glucose transporter-5, dopamine, noradrenalin and GABA transporters, Mrp-8 and Eaat-4. The expression level of neither Abcc1 nor Abcb1b coding for Mrp-1 and Pgp, respectively, was affected by tesmilifene.

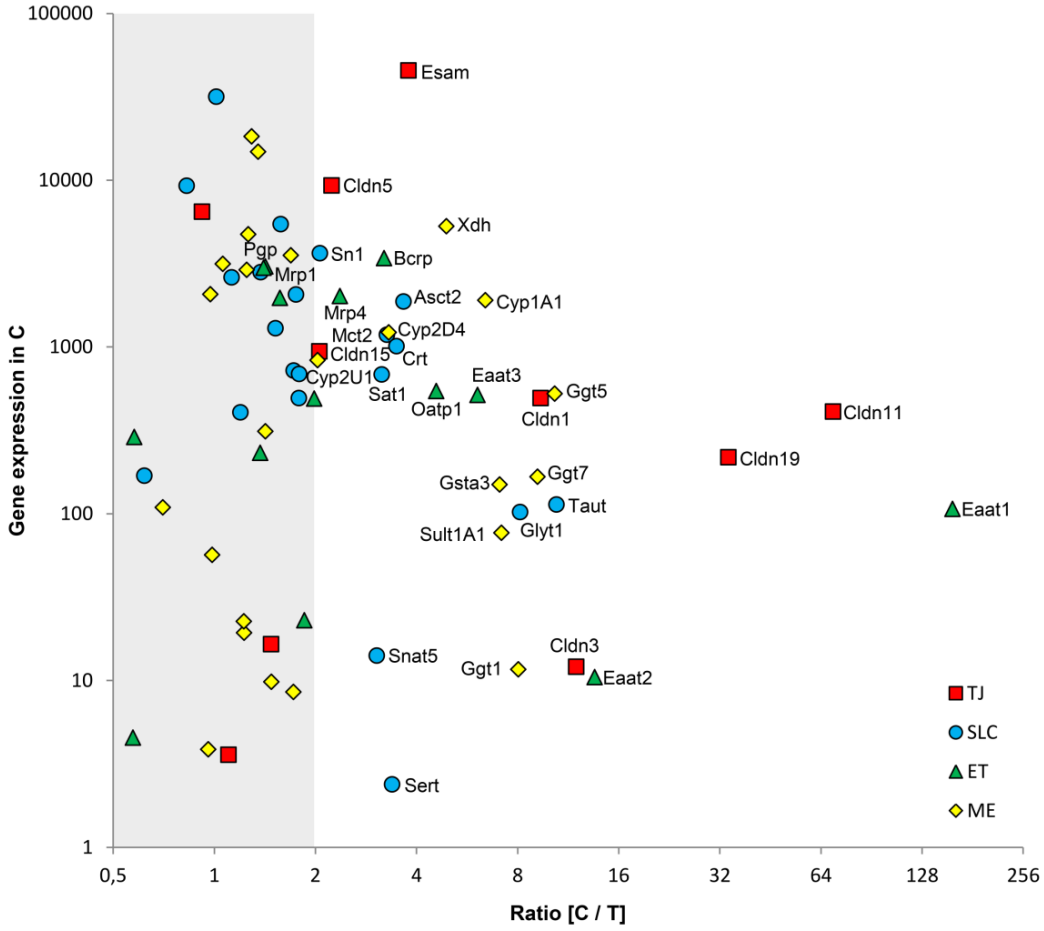

Figure 9. The effects of tesmilifene on gene expression of selected blood-brain barrier genes in rat brain endothelial cells. The figure shows the ratio between gene expression of tight junction proteins (TJ, red square), SLC transporters (SLC, blue circle), efflux transporters (ET, green triangle) and selected metabolic enzymes (ME, yellow diamond) in control (C)/tesmilifene treated (T) brain endothelial cells. White part: down-regulated genes, grey part: no significant regulation. The graph, scaled on the y axis, also shows the gene expression values in the control sample to demonstrate the level of expression of studied genes.

4. Summary

The discovery of new molecules and techniques to increase the efficiency of drug delivery to the nervous system for the treatment of malignant brain tumors is an important and relevant research area. Because the BBB is a key interface regulating the transport of molecules between the systemic circulation and the central nervous system, two new potential agents to open the BBB were tested in our studies.

It was demonstrated for the first time, that acute treatment of cultured brain endothelial cells with short-chain AGs increased the permeability of the monolayers via the paracellular route, which effect was reversible without causing viability changes in endothelial cells. The opening of the paracellular pathway was not accompanied by the reorganization of tight

junction strands. Our experiments showed that the recovery of brain endothelial cells after treatment with AGs was complete and neither the functions, nor the morphology of the BBB model was affected. Our data fully support the previous findings of animal experiments. To better understand the mode of action of AGs on brain endothelial cells further experiments including lipidomics are planned.

We were the first to describe that the chemotherapeutic adjuvant tesmilifene increased the penetration of a small molecule tracer to the glioma more significantly, than to the surrounding brain tissue. Tesmilifene acts via multiple signaling pathways in brain endothelial cells, which regulate the permeability of the BBB. Since tesmilifene also inhibits the activity of efflux pumps (Figure 10), it could be potentially used for the delivery of chemotherapeutics to brain tumors.

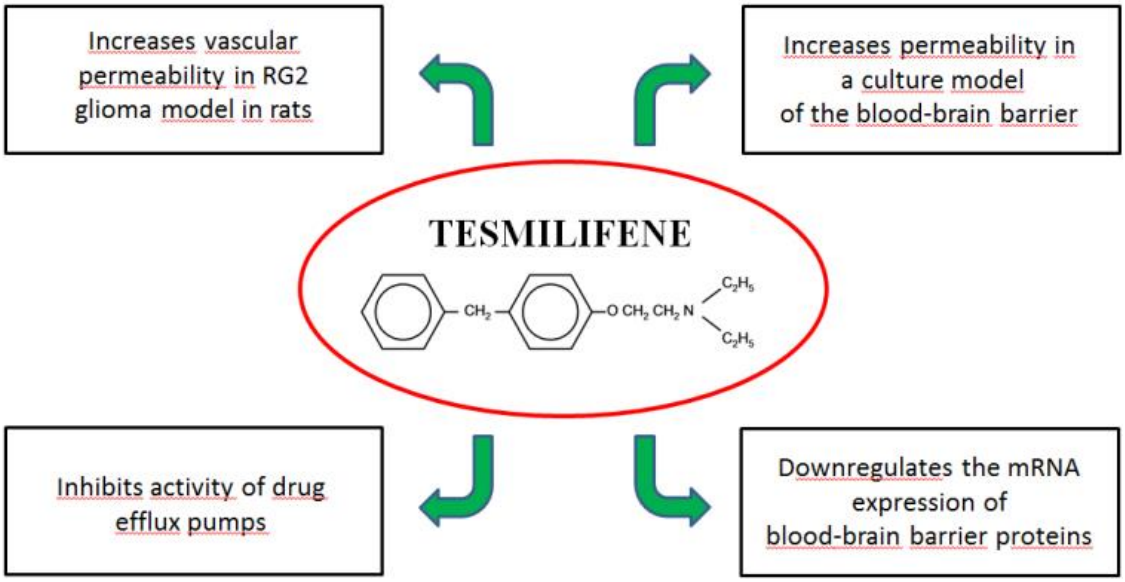

Figure 10. Summary of the main effects of tesmilifene on brain endothelial cells.

Our data support previous clinical observations and the results of animal experiments, and indicate that AGs and tesmilifene increase the permeability of the BBB by directly acting on brain endothelial cells. Based on the presented findings, it would be of interest to test the effect of AGs and tesmilifene on brain delivery of chemotherapeutic drugs and their efficacy as adjuvants in the treatment of brain tumors.

Publication list:

ΣIF: 39.242

Publications related to the subject of the thesis:

I. Hülper P, Veszelka S, **Walter FR**, Wolburg H, Fallier-Becker P, Piontek J,Blasig IE, Lakomek M, Kugler W, Deli MA. Acute effects of short-chain alkylglycerols on blood-brain barrier properties of cultured brain endothelial cells. *British Journal of Pharmacology*, 169: 1561-73 (2013) IF: 4,99

II. **Walter FR**, Veszelka S, Pásztói M, Péterfi ZA, Tóth A, Rákhely G, Cervenak L, Ábrahám CS, Deli MA. Tesmilifene modifies brain endothelial functions and opens the blood-brain/blood-glioma barrier. *Journal of Neurochemistry*, közlés alatt, (2015) IF: 4,281

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I. Hellinger E, Veszelka S, Tóth AE, **Walter F**, Kittel A, Bakk ML, Tihanyi K, Háda V, Nakagawa S, Duy TD, Niwa M, Deli MA, Vastag M. Comparison of brain capillary endothelial cell-based and epithelial (MDCK-MDR1, Caco-2, and VB-Caco-2) cell-based surrogate blood-brain barrier penetration models. *European Journal of Pharmaceutics and Biopharmaceutics*. 82: 340-51 (2012) IF: 3,826

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V. Tóth AE, **Walter FR**, Bocsik A, Sántha P, Veszelka S, Nagy L, Puskás LG, Couraud PO, Takata F, Dohgu S, Kataoka Y, Deli MA. Edaravone protects against methylglyoxal-induced barrier damage in human brain endothelial cells. *PLoS One*. 9:e100152 (2014) IF: 3,234

VI. Schilling-Tóth B, Sándor N, **Walter FR**, Bocsik A, Sáfrány G, Hegyesi H. Role of GDF15 in radiosensitivity of breast cancer cells. *Central European Journal of Biology*. 9: 982-992 (2014) IF: 0,710

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*, authors contributed equally to the manuscript