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REŠETANJE ZAVIRALCEV AKVAPORINA 1 IN 3 NA HUMANIH ERITROCITIH Z METODO MERJENJA SIPANE SVETLOBE V ZAUSTAVLJENEM TOKU

SCREENING FOR AQUAPORIN 1 AND 3 INHIBITORS IN HUMAN RED BLOOD CELLS WITH STOPPED-FLOW LIGHT SCATTERING METHOD

DIPLOMSKA NALOGA

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This Master's Thesis was performed at the Faculty of Pharmacy, Lisbon, Portugal with the collaboration of Faculty of Pharmacy, Ljubljana, Slovenia. I worked under the mentorship of Professor Dr. Darko Černe, and co-mentorship of Associate Professor Dr. Graça Soveral.

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STATEMENT

I hereby declare that I have performed and written this Master's Thesis solely by myself under the mentorship of Professor Dr. Darko Černe, and co-mentorship of Associate Professor Dr. Graça Soveral.

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ABSTRACT

Aquaporins (AQPs) are small proteins, located in cell membranes of numerous tissues permeating water and other small solutes, such as glycerol, and providing facilitation of their transport across plasma membrane. They represent quite novel, yet a promising area in pharmacologic researches. Although there is not much known about AQP modulators thus far, studies more and more reveal physiological roles of AQPs and potential use of AQP modulators in the therapy of various diseases.

From pharmaco-therapeutical aspect, a special interest is dedicated to their involvement in cancer progress knowing that AQPs are overexpressed in many types of tumor cells. A therapy preventing or inhibiting tumorigenesis and metastasis formation is one of the most promising concerning AQP1 and AQP3. Inhibitors of AQP1, in particular, would reduce growth and spread of several tumors with AQP1 up-regulation and inhibitors of AQP3 would serve as medications of skin cancer.

To date, there are not many inhibitors known, only few suggested but none included in clinical trial neither we have a lead compound. Screening of compounds, which may discover new AQP inhibitors, is thus of great importance because it may offer us new possibilities and perspectives.

A determinant chosen to define AQPs' activity was cell permeability using stopped-flow light scattering method. Experiments are based on measuring cell volume changes and detecting divergent intensity of scattered light after exposing the cells to osmotic shock. Then, water and glycerol membrane permeability can be calculated. Decreased cell permeability indicates inhibitory effect on AQPs' activity.

Results show that compounds FS6, FS10, FS12 and FS72 are the new inhibitors of AQPs. They inhibit AQP3 in 70-80% (IC_{50} values ranging between 100 and 200 μM) and three of which, namely FS10, FS12 and FS72, show also inhibitory effect on AQP1 in 20-30%.

To summarize, the results described in my thesis offer an interesting insight into compounds' effectiveness and may lead to a development of a lead compound as mentioned compounds present some similar molecular features. Certainly, we would need to proceed with further researches in cell cultures and *in vivo* studies to confirm inhibitory effect and to find out more about mechanisms of action.

RAZŠIRJENI POVZETEK

Akvaporini predstavljajo dokaj novo, vendar zelo obetavno področje farmakoloških raziskav. Kljub temu, da še ni veliko znanega, pa študije vse bolj odkrivajo fiziološke vloge akvaporinov in morebitno uporabo njihovih modulatorjev v terapiji različnih bolezni. Akvaporini so majhni membranski proteini, ki jih lahko najdemo v vseh živečih organizmih, od sesalcev in ostalih vretenčarjev, do nevretenčarjev, rastlin in mikroorganizmov. V človeškem telesu se nahajajo v številnih tkivih in skrbijo za olajšan prehod vode in drugih majhnih molekul preko celične membrane.

Poznamo 13 različnih vrst akvaporinov (0-12). Po funkciji jih delimo v dve skupini: pravi akvaporini 0, 1, 2, 4, 5, 6 in 8 prepuščajo le vodo, z izjemo akvaporina 6, ki je dodatno prepusten tudi za nekatere anione; akvagliceroporini 3, 7, 9 in 10 pa poleg vode prepuščajo tudi ostale manjše molekule, kot sta glicerol in sečnina. Prepustnost ter biološka vloga akvaporinov 11 in 12 še nista povsem razjasnjeni.

Pravi akvaporini so kot vodni kanalčki izraženi v epiteliju in endoteliju tistih tkiv, ki so povezana s transportom vode. V žleznem in ledvičnem tkivu so udeleženi v fizioloških funkcijah izločanja tekočin in koncentriranja urina. Pomembno vlogo imajo tudi v drugih tkivih, kot so rdeče in bele krvne celice ter astroglia. Udeleženi so v procesu otekanja tkiv ter pri uravnavanju možganskega in očesnega pritiska.

Akvagliceroporini uravnavajo raven glicerola v maščobnem tkivu in kožni povrhnjici, njihovo vlogo lahko zaznamo v presnovi maščob in uravnavanju vsebnosti vode v koži.

Spreminjanje funkcije ali izraženosti akvaporinov bi lahko imelo mnogo uporabnih aplikacij s terapevtskega stališča. Nove učinkovine bi služile za terapije številnih bolezni, kot so obolenja ledvic, možganski edem, glavkom, okužba, debelost in epilepsija.

S farmakološko-terapevtskega vidika je najbolj zanimiva povezava akvaporinov z napredovanjem bolezni raka, saj je v mnogih vrstah tumorskih celic njihova izraženost povečana. Včasih se določena vrsta akvaporina pojavi v tumorskih celicah, četudi v normalnih celicah ni prisotna.

Študije predlagajo, da akvaporini omogočijo hiter vstop vode v maso tumorja, le-ta pa povzroči edem ter večanje tumorja. Poleg tega akvaporini pospešujejo proces celične migracije, celične delitve in posledično, nastajanja novih krvnih žil. Proces celične

migracije v tumorskih celicah poveča možnost nastanka metastaz zaradi povišane hitrosti celic, ki prečkajo stene krvnih žil in potujejo proti zdravemu tkivu.

V diplomskem delu smo se osredotočili predvsem na vlogo akvaporina 1 in 3, saj sta ti dve vrsti izraženi tudi v eritrocitih, ki smo jih v krvnih vzorcih uporabili za preučevanje zaviralnega učinka spojin.

Akvaporin 1 je najbolj izražen v endotelijskih celicah krvnih žil po celem telesu, z izjemo centralnega živčevja. V splošnem se ta vrsta nahaja v tkivih z izraženo absorptivno ali izločevalno vlogo, kot na primer v ledvičnem tkivu, očesnem epiteliju, žolčnih celicah in horoidnem pleksusu, kjer skrbi za nastanek in izločanje cerebrospinalne tekočine. Zaviralci akvaporina 1 bi torej lahko predstavljali diuretične učinkovine, učinkovine za zniževanje očesnega pritiska v primeru glavkoma in učinkovine za zdravljenje hidrocefalusa (vodenoglavosti).

Akvaporin 1 se nahaja tudi v žilnem endoteliju številnih tumorjev, kar nakazuje možno vlogo pri njihovem nastanku, rasti in širjenju. Pretekle študije so pokazale, da tumorske celice brez akvaporina 1 migrirajo počasneje, samo tumorsko tkivo pa ima manjšo gostoto krvnih žil, kar hitreje povzroči odmiranje tkiva. Nasprotno pa se krvne žile v tumorskem tkivu z izraženim akvaporinom 1 razraščajo hitreje, kar privede do povečanega nastanka in širjenja rakavih zasevkov.

Akvagliceroporin 3 je poleg vode prepusten tudi za glicerol. Izražen je v epitelijskih celicah roženice in očesne veznice, v ledvičnem tkivu in mehurju, v črevesnih celicah in v koži, natančneje v keratinocitih. V očesu, črevesnih celicah in koži skrbi za večjo regeneracijo in hitrejše celjenje tkiva po poškodbi. Povezava med akvaporinom 3 in nastankom tumorjev pa je v njegovi vlogi privzema glicerola v celico. Glicerol je namreč pomemben dejavnik pri nastajanju energetske molekule ATP (adenozin trifosfat), ki zagotavlja energijo celici in njenim presnovnim potrebam. V primeru tumorskega tkiva so energetske potrebe povišane in zaviralci akvaporina 3 bi posledično znižali raven ATP-ja, kar bi ustavilo ali vsaj upočasnilo procese celične delitve in razrast tumorja. Poleg tega pretekle študije kažejo, da so miši brez akvaporina 3 celo odporne na razvoj kožnega raka.

Protitumorna terapija, ki bi preprečevala ali zaustavila razvoj raka, je ena najbolj perspektivnih v povezavi z akvaporinom 1 in akvagliceroporinom 3. Zaviralci akvaporina 1 bi namreč zmanjšali rast in širjenje številnih tumorjev s povečano izraženostjo akvaporina 1, zaviralci akvaporina 3 pa bi služili kot zdravila proti kožnemu raku.

V tem trenutku je poznanih le malo zaviralcev akvaporinov, vendar od teh spojin niti ena ni vključena v klinične študije. Poleg tega ne poznamo nobenega naravnega modulatorja akvaporinov, ki bi pripomogel k razvoju podobnih spojin. Zato je torej rešetanje spojin, s katerim bi odkrili nove zaviralce akvaporinov, zelo pomembno, saj nam lahko ponudi nove možnosti.

Za ugotavljanje aktivnosti akvaporinov smo izbrali preučevanje celične prepustnosti. Uporabili smo metodo merjenja sipane svetlobe v zaustavljenem toku. Metoda temelji na izpostavitvi celic osmotskemu šoku, merjenju sledečih sprememb celičnega volumna in detekciji sipane svetlobe. Iz sprememb volumna (krčenje in ponovno širjenje celice) ter različne intenzitete sipane svetlobe lahko izračunamo prepustnost za vodo in glicerol. Zmanjšana celična prepustnost za vodo in/ali glicerol predstavlja zaviralni učinek na aktivnost akvaporinov. To pomeni, da se bodo določeni procesi v celici, kot na primer celična migracija in nastajanje ATP-ja, ustavili ali upočasnili, posledično pa privedli do upočasnitve rasti in širjenja tumorja.

Testirali smo tri različne družine spojin. Vse spojine, uporabljene v diplomskem delu, so bile sintetizirane na Kemijskem oddelku Fakultete za farmacijo v Lizboni. Za pozitivno kontrolo smo uporabili $HgCl_2$, znanega zaviralca akvaporinov, ki zmanjša tako prepustnost vode kot tudi glicerola, vendar je neselektiven in zelo toksičen. Kot najbolj obetavna se je izkazala skupina holinov (FS spojine). Vse spojine iz te družine so topne v vodi, kar je velika prednost tako pri laboratorijskih poskusih, kot tudi za nadaljnje študije v bioloških sistemih. Rezultati kažejo, da so spojine FS6, FS10, FS12 in FS72 novi zaviralci akvaporinov. Zavirajo akvaporin 3 v 70-80% (IC_{50} vrednosti so med 100 in 200 μM). Tri od teh, natančneje FS10, FS12 in FS72, pa kažejo zaviralni učinek tudi na akvaporin 1 v 20-30%.

Rezultati v diplomski nalogi ponujajo vpogled v učinkovitost spojin in lahko vodijo v razvoj spojine vodnice, glede na to, da pri primerjavi molekularnih struktur potencialnih zaviralcev lahko opazimo nekaj podobnosti (dolg hidrofobni rep, hidroksilne skupine). Vsekakor pa bi potrebovali nadaljnje raziskave na celičnih kulturah in *in vivo* študijami, da bi potrdili zaviralni učinek omenjenih spojin in izvedeli več o mehanizmih delovanja.

ABBREVIATIONS

AQP	Aquaporin
Arg	Arginine
Asn	Asparagine
ATP	Adenosine triphosphate
Auphen	[Au(1,10-phenatroline)Cl2]Cl
DMSO	Dimethyl sulfoxide
fl	Femtoliter
HCl	Hydrochloric acid
$HgCl_2$	Mercury chloride
His	Histidine
H_2O	Water
H_3O^+	Hydronium ion
hRBC	Human red blood cells
IC	Inhibitory concentration
IC_{50}	Half maximal inhibitory concentration
KCl	Pottasium chloride
KH_2PO_4	Potassium dihydrogen phosphate
MCV	Mean cell volume
MeOH	Methanol
MM	Molecular mass
NaCl	Sodium chloride
nm	Nanometer
Na_2HPO_4	Disodium hydrogen phosphate
Osm	Osmoles
PBS	Phosphate buffered saline
RBC	Red blood cells
RNAi	RNA interference
SAR	Structure activity relationship
SC	Stratum corneum
TGN-020	2-(nicotinamoyl)-1,3,4-thiadiazole

1. INTRODUCTION

1.1. Cell membrane water permeability

Water represents approximately 70% of human body mass and proper water permeability regulation through cell membranes is necessary for living. Water needs to be correctly distributed in our tissues in order to sustain fluid balance. (1)

First it was believed that water transport through cell membranes occurred only by simple diffusion through the lipid bilayer, but experimental observations implicated that some cells or tissues have very high water permeability (e.g. mammalian erythrocytes, amphibian bladder) which implied that simple diffusion was not the only way for water crossing the lipid bilayer. Today we can confirm the existence of specific proteic water channels, called aquaporins, which mediate water movement. Besides simple diffusion aquaporins provide a facilitated path for water permeation across the membrane.

Simple diffusion of species and solutes across biological membranes is a relatively slow process. In contrast, aquaporins are found in certain membranes (they are widely present in tissues all over human body) and their capacity for permeating water is 10- to 100-fold higher than simple diffusion. Being bi-directional water pores through lipid bilayer, water movement in and out from the cell through aquaporins is driven by osmotic gradients. (2)

1.2. Aquaporins

1.2.1. Structure and function

Aquaporins (AQPs) are small (~30 kDa/monomer) intrinsic membrane proteins which can be widely found in all living organisms: mammals and other vertebrates, invertebrates, plants and microorganisms. (2) They are located in plasma membranes where they behave as water channels facilitating water transport. (3)

The first aquaporin found was aquaporin 1 (AQP1) and it was discovered coincidentally in the early 1990s when investigating RH antigens in red blood cells. (2) To date, 13

homologous members can be distinguished in mammals and they are differentially expressed in several cells and tissues. (4)

By their function, AQPs can be divided into 2 groups: the first group (orthodox aquaporins) transport water selectively and includes AQP 0, 1, 2, 4, 5, 6 and 8, whereas AQP6 showed to be additionally permeable to anions, namely nitrate and chloride (4,5); and the second group, called aquaglyceroporins, includes AQP 3, 7, 9, and 10, which transport not only water but also glycerol and other small solutes such as urea (Table I). (5,6) All the isoforms have similar protein sequence, more accurately 25%-60% of homology. (6)

Table I. Permeability of aquaporins (4,5,7)

AQP isoform	Permeability
AQP0	Water
AQP1	Water, (CO_2, NO)
AQP2	Water
AQP3	Water, glycerol
AQP4	Water
AQP5	Water
AQP6	Water and anions (nitrate, chloride)
AQP7	Water, glycerol and urea
AQP8	Water, NH_3, H_2O_2
AQP9	Water, glycerol and other small solutes
AQP10	Water, glycerol and urea
AQP11	?
AQP12	?

In membranes AQPs are stably present as tetramers and each of the monomer forms a pore that allows water to pass (Figure 1). (6) They are highly selective as water channels and even ions (OH^-, H_3O^+) are not able to pass through. Presently, there are eleven atomic AQP structures defined of which six mammalian: AQP0, AQP1, AQP2, AQP4, AQP5, and AQP9. (8,9)

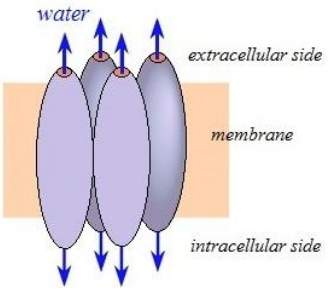


Figure 1. Structure of AQP. In membranes aquaporin is present as tetrameric assembly in which each monomer forms a water pore. Based on (4)

Studies demonstrated that each aquaporin subunit consists of 6 α-helices linked together. The inside of the pore is lined with negatively charged residues allowing the formation of hydrogen bonds between water molecules and amino acid residues. The center of aquaporin is narrow (approximately 2.8 Å in diameter) and it contains positively charged residues (Arg, His, Asn) that behave as a selectivity filter. As water molecule moves through the channel it is flipped midway by strong positive charge and leaves the channel on the other side.

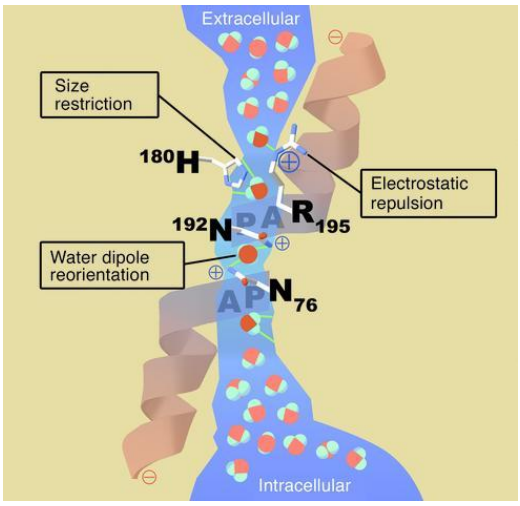


Figure 2. Aquaporin monomer from the inside. The factors for high water selectivity of aquaporin water channels: 1 Size restriction. 2 Electrostatic repulsion. 3 Water dipole reorientation. Reprinted with permission from (10)

We have thus more reasons why only water molecules fit through the aquaporin channel making it a highly specific channel. First, the size restriction in the narrow part of the aquaporin prevents larger solutes to pass. Secondly, positively charged residues play a big role in proton repulsion. Arg-195 has a powerful positive charge plus it is located in the narrowest part of the channel. His-180 provides a second functionally very important positive charge. At neutral pH is uncharged but at lower pH becomes protonated. Last is the reason of changed water orientation while inside the channel because it interrupts possible hydrogen bonding, between one water molecule passing the channel and those below and above it, essential for passage of hydronium ions (H_3O^+). Reorientation together with positively charged residues in the narrow midway of aquaporin provides an explanation why passage of hydronium ions is repulsed. (1,2)

Aquaglyceroporins are wider in the middle of the channel therefore the molecule of glycerol is able to pass through. Orthodox aquaporins hence represent a size filter within their structure assuring only water to cross cell membrane. (9)

1.2.2. Expression and physiological roles

AQPs are expressed in epithelium and endothelium of tissues associated with fluid transport, such as glandular epithelium and kidney tubules where they are included in physiological functions, as in fluid secretion of exocrine glands and urinary concentrating process. They have significant function also in other tissues, such as skin, adipose tissue, red and white blood cells and astroglia, where they have important role in tissue swelling, as in the brain after the stroke, injured cornea and infection. They regulate formation of brain edema and intracranial as well as intraocular pressure. AQPs that transport water and glycerol regulate glycerol content in fat and epidermis, influences of which can be seen in fat metabolism and skin hydration. Studies suggest that AQPs might also have important role in cell volume regulation and neural function (seizures, sensory signaling). (3,4,6,11)

Table II. Tissue expression of aquaporins. (4,6,11)

AQP isoform	Expression of aquaporins in different tissues
AQP0	Eye lens fiber cells (4), liver (12)
AQP1	Kidney tubules, endothelia of body vascular system (except in central nervous system), erythrocytes, brain (choroid plexus), corneal endothelium, ciliary epithelium, intestine, cholangiocytes
AQP2	Kidney collecting duct
AQP3	Skin (epidermis), erythrocytes, enterocytes and stomach, airway epithelium, conjunctiva and cornea, urinary bladder and kidney collecting duct; pancreas, liver, spleen and prostate (13)
AQP4	Asterocytes in brain and spinal cord, inner ear, kidney collecting duct, glandular epithelia, airways, skeletal muscle, stomach, retina and ciliary epithelium; cholangiocytes (14)
AQP5	Glandular epithelia (salivary glands, lacrimal glands and airway submucosal glands), gastrointestinal tract, corneal epithelium, alveolar epithelium
AQP6	Kidney collecting duct (intercalated cells) (4), cerebellum, inner ear, parotid cells, gastrointestinal tract (15)
AQP7	Adipocytes, testis, kidney proximal tubule; gastrointestinal tract, skeletal muscle, inner ear (16)
AQP8	Liver, salivary gland, pancreas, intestine, heart, kidney tubules and collecting duct; reproductive system- male and female (17)
AQP9	Liver, white blood cells, brain, testis; kidney, spleen (18)
AQP10	Small intestine; adipocytes (16)
AQP11	Kidney proximal tubule, liver; brain, testis
AQP12	Pancreatic acinar cells

Another major role of AQPs is the possible involvement in tumor biology. AQPs are usually over-expressed in different tumor cells, where they help tumor to grow and spread, but we are still not sure whether this is a common phenomenon to all types of cancer.

Where studied, higher tumor level is in strong correlation with AQP expression (Table III). (3,19) Sometimes some of the AQP isoforms appear in tumor cells even if they are originally not present in the normal cells. Because of AQPs' role in transporting water through the membrane, most studies suggest that AQPs allow water to enter into the tumor mass rapidly, causing edema and tumor expansion. In addition, AQPs accelerate tumor cell migration, proliferation and consequently, angiogenesis. (3)

Table III. AQP expression in human tumors. (3,7)

Type of tumor	AQP expression
Brain tumor	↑ AQP1
	↑ AQP3
	↑ AQP4
	↑ AQP5
	↑ AQP9
Cholangiocarcinoma	↑ AQP1
Choroid plexus tumor	↑ AQP1
Colorectal cancer	↑ AQP1
	↑ AQP3
	↑ AQP5
	↓ AQP8
Epithelial Ovarian tumor	↑ AQP5
Esophageal and oral squamous cell carcinoma	↑ AQP3
Follicular Thyroid tumor	↑ AQP7
Hemangioblastoma	↑ AQP1
Hepatocellular cancer	↓ AQP8
	↓ AQP9
Laryngeal cancer	↑ AQP1
Multiple myeloma	↑ AQP1 (20)
Myelogenous leukemia	↑ AQP5
Pancreatic cancer	↑ AQP5
Prostate cancer	↑ AQP3
Pulmonary adenocarcinoma	↑ AQP1
	↑ AQP3
	↑ AQP5
Renal cell tumor	↓ AQP1
	↑ AQP3
Skin squamous cell carcinoma	↑ AQP3

Consequently, by modulating AQP function or AQP expression we would be able to discover new drug therapies and treatments for several pathologies such as kidney diseases, brain edema, glaucoma, inflammatory disease, obesity, epilepsy and cancer. (3,4,6)

1.2.3. AQP-dependent cell migration

AQP-dependent cell migration is associated not only to angiogenesis, but also to other physiological and pathophysiological processes where rapid cell movement is required, such as organogenesis in the embryo, inflammatory response, wound healing, glial scarring and tumor metastasis. (19) AQPs as water channels facilitate cell migration by accelerating transmembrane water movement and this process is not specific for special AQP isoform or special type of cell. (4,19)

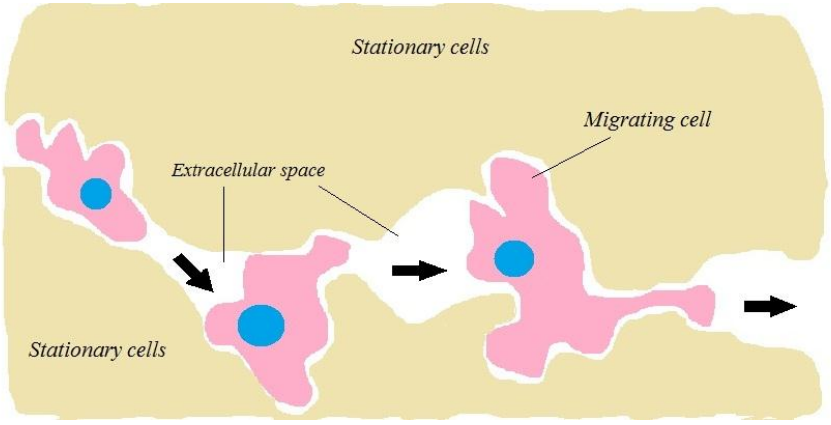


Figure 3. Cell migration. Schematic demonstration of cell undergoing the changes in cell shape during cell migration through the extracellular space. Based on (19)

If AQP-dependent cell migration happens in tumor cells, as a consequence metastatic potential raises because of increased speed of migrating cells crossing blood vessel barriers toward normal tissue. (21) Cell migration appears to be very important determinant in tumor progress (angiogenesis, spread and metastasis) and wound healing. These facts generate an explanation why AQPs are normally up-regulated in numerous tumor types and why AQP expression strongly correlates with higher tumor level. (11)

Water permeability in tumor cells and AQP expression are likely to be meaningful determinants for cell migration and tumor spread. (21)

There are two proposed mechanisms that can explain how AQPs (in particular AQP1) participate in tumorigenesis by enhancing water transport through the cell membrane.
First assumption says that AQPs facilitate rapid cell volume changes. Consequently, cell shape changes as migrating cells go through the extracellular space which is irregularly shaped. Migrating cells also create hydrostatic forces that move apart adjoining stationary cells. However, this theory cannot explain why AQPs polarize at the front part of migrating cells.

According to the second assumption, which considers also AQP polarization, osmolality changes are responsible for rapid water influx through membrane. Because of actin de-polymerization and ion influx osmolality is increased in the front part of migrating cells. Ion transporters most likely cause that water enters into the cell through the membrane at the front part, which results in localized swelling of the cell, and flows out from the back part, which results in localized shrinkage of the cell. According to this theory we can inhibit or accelerate cell migration by extracellular osmolality changes. Water influx, facilitated by AQP polarization at the front part of the cell, increases local hydrostatic pressure and causes expansion of cell membrane, which creates a protrusion. Then actin re-polymerizes for stabilization of protrusion. All these processes help the cell to move forward (Figure 4). (3,19)

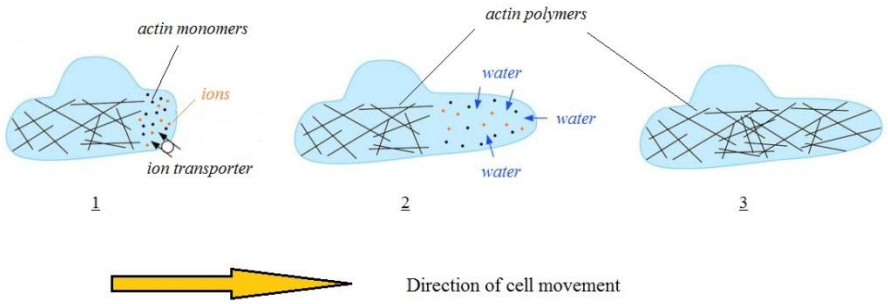


Figure 4. Proposed mechanism of AQPs being involved in cell migration. 1 Actin de-polymerization and ion influx through ion transporter cause increase of osmolality in the front part of migrating cell. 2 AQP-facilitated water influx increases local hydrostatic pressure, forming a cell protrusion. 3 Actin re-polymerization to stabilize the protrusion. Based on (3,19)

It should be stressed out that AQPs only help in cell migration by accelerating it with rapid changes in cell volume. Water crosses cell membrane also through the lipid bilayer even when AQP pores are not present in the membrane. Cell migration happens anyway but the process is slower. (19)

Another proposed mechanism for aquaporins being involved in tumor progression is related to their capacity of transporting glycerol, such as AQP3. Novel studies established a connection between glycerol transport, facilitated by AQP3 aquaglyceroporin, and cell proliferation along with tumor formation. The data implicate involvement of glycerol in cellular metabolism as a meaningful determinant for ATP generation.

Aquaglyceroporin 3 mediates uptake of glycerol into the cell which is then metabolized to glycerol-3-phospate (G3P) and according to studies, G3P is essential intermediate for production of ATP, main molecule for cellular energy. ATP level is in close correlation with cell proliferation and tumorigenesis as reduced energy capacity disables cell proliferation, migration and tumor formation (Figure 5).

Not only AQP3-deficient cells have decreased glycerol content, but they have also reduced content of its metabolite glycerol-3-phosphate together with ATP. Additionally, AQP3-deficient cells were resistant to develop tumors (skin tumor in particular), indicating that absence of AQP3 in cells relates to damaged cell metabolism.

This implicates the importance of glycerol on cellular level as a significant regulator of ATP energy. (22)

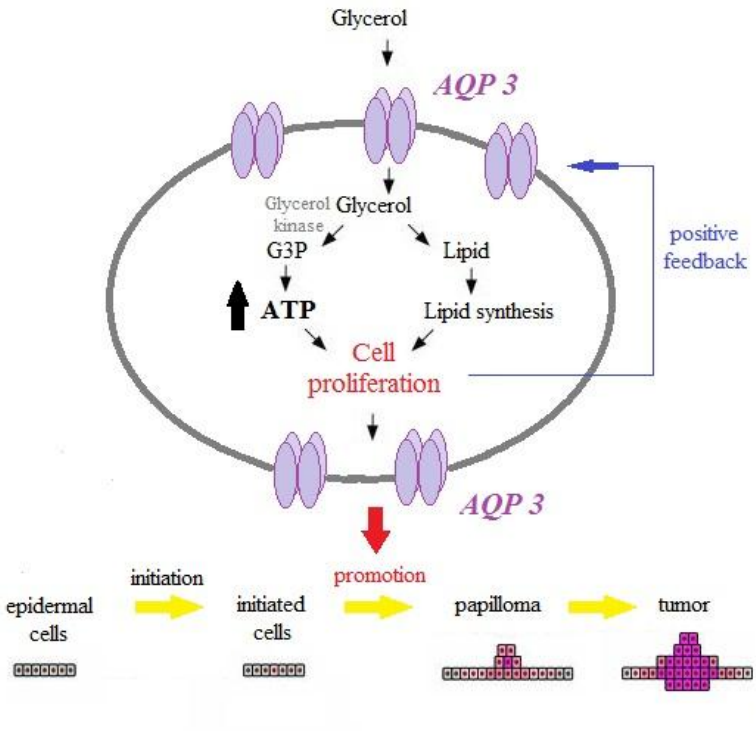


Figure 5. Proposed mechanism of cell proliferation facilitated by AQP3 and its involvement in tumor progression. The mechanism presents glycerol inclusion in biosynthesis and positive feedback in which cell proliferation raise expression of AQP3. Based on (3,22)

In this work, aquaporin 1 and aquaporin 3, the two isoforms expressed in erythrocytes (4), were experimentally used to screen for molecules with inhibitory effect on aquaporin function. Thus, some aspects of AQP1 and AQP3 will be presented more thoroughly.

1.2.4. Aquaporin 1

AQP1 is expressed in endothelial cells of blood microvessels all over our body leaving out our central nervous system. (3) In general, this isoform of water channel exists in organs with absorptive or secretory function. In detail, it is present in renal proximal tubules, descending limb of loop of Henle and vasa recta endothelium in order to perform urinary concentration. It is also present in non-pigmented ciliary epithelium to maintain intraocular pressure; in cholangiocytes for bile secretion and in epithelium of choroid plexus for production and secretion of cerebrospinal fluid. (2,6) Inhibitors of AQP1 may be thus effective as diuretic drugs; they can be used in treatment of glaucoma by lowering intraocular pressure and in treatment of hydrocephalus. (6)

AQP1 is also expressed in vascular endothelia of numerous tumors, which implicates a possible role of AQP1 in tumor growth and spread. (6) In studies to verify this hypothesis, tumor cells were used in *in vitro* tests for investigating cell migration and *in vivo* for exploring metastatic potential and tumor growth. There are two methods in which we can study influence of AQP expression or deficiency. One is preparation of mice, genetically lacking of certain AQP, in this case AQP1 (AQP1-null mice). Tumor cell lines are afterwards injected or implanted subcutaneously into this group of mice as well as into normal mice expressing AQP (wild-type mice). (20) Another method is when mouse tumor cells with or without AQP1 are either injected intravenously or implanted subcutaneously into normal healthy mice. (21)

Mice that received tumor cell lines without AQP1 have shown defective endothelial cell migration, which causes reductive proliferation and angiogenesis. With slowed cell migration, tumor angiogenesis is impaired and density of blood microvessels becomes lower which is observable in larger tumor necrosis. Therefore, tumor growth and spread are limited in AQP1-deficient mice. Invasiveness of tumor is reduced and potential to form metastases is decreased. (6,19,21)

Conversely, in wild-type mice expressing AQP1, we can notice enhanced cell migration. Tumor cells expressing AQP1 migrated more rapidly *in vitro*, causing greater extravasation of tumor cells across microvessel barriers. AQP1 expression in culture increased plasma membrane permeability for water by 5- to 10-fold. Also, AQP1 expression enlarged the number as well as the shape of lamellipodia, wide part of protrusion formed during cell migration. To consider all the factors *in vitro*, we can understand why AQP1 has possibly important function in tumor spread and local invasiveness *in vivo*. AQP1-expressing tumor cells show greater metastatic ability by crossing barriers of vascular endothelia and the data of studies reveal that wild-type mice expressing AQP1 develop more metastases comparing to AQP1-null mice. (3,19,21)

1.2.5. Aquaporin 3

AQP3 is one of the aquaglyceroporins, transporting not only water but also glycerol. AQP3 is expressed in epithelial cells of cornea and conjunctiva, in kidney collecting duct and urinary bladder, in enterocytes, and in skin, more precisely in keratinocytes. (3,19) AQP3 expression in the eye facilitates cell migration and proliferation of epithelial cells causing greater regeneration and re-epithelialization of cornea after a corneal injury. (23) In mice, whose colitis has been chemically induced, AQP3-null mice had impaired regeneration and proliferation of enterocytes in colon because of limited cell migration. Colitis in wild-type mice was less severe and surviving rate was higher. (24) In mammalian skin, AQP3-deficient mice exhibit slower wound healing because of impaired cell migration and proliferation. In the case of cancer, mouse models lacking AQP3, are resistant to development of skin cancer. (3)

The aquaglyceroporin AQP3 is normally present in keratinocytes, the basal layer of epidermis. Due to glycerol transport it has an important role in biosynthesis and hydration of stratum corneum (SC), which is the most external skin layer. It consists of corneocytes, which develop of proliferating keratinocytes in epidermis beneath. SC hydration depends on various determinants, such as skin structure, composition of lipids and proteins, barrier properties, external humidity and the concentration of substances which help to retain water, e.g. ions and free amino acids.

AQP3-deficient mice express reduced hydration of SC with deferred biosynthesis after SC removal, slowed wound healing and decreased skin elasticity. As an aquaglyceroporin, AQP3 is responsible for glycerol transport from blood through keratinocyte basal layer into the epidermis and SC, therefore AQP3 deficiency can be observed as reduced glycerol permeability and diminished glycerol content in epidermal cells and SC (Figure 6). Lower SC hydration and skin elasticity correlates with ability of glycerol to retain water, whether delayed wound healing is associated to glycerol role in biosynthesis. Interestingly, exposing AQP3-null mice to high external humidity does not increase skin hydration, contrary to wild-type mice where external humidity helps to adjust hydration of SC. Differently, systemic or topical preparations for glycerol substitution improves skin abnormalities, indicating the significance of glycerol for skin hydration purpose. (4)

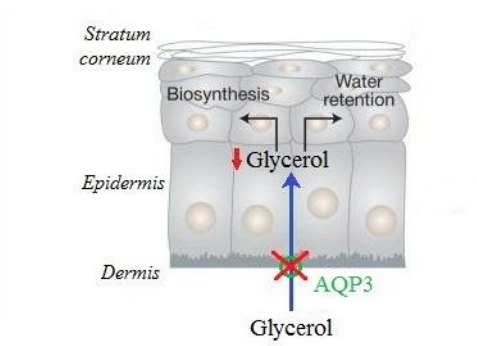


Figure 6. Proposed mechanism of glycerol involvement in skin hydration. In AQP3-deficiency decreased glycerol level in skin causes reduced skin hydration. Based on (4,25)

AQP3 is strongly expressed in basal cells of human squamous cell carcinoma. Studies of cutaneous papilloma formations also describe that AQP3-null mice do not develop papillomas while wild-type expressing AQP3 mice produce numerous tumors under the same conditions.

Epidermal cells, lacking AQP3, show decreased levels of glycerol, its metabolite G3P and ATP, a key energy source for supplying tumor's aggressive metabolic necessities. Therefore AQP3 presents an important element in formation of skin tumors and hence offers an interesting insight into novel strategy of preventing and treating skin tumors as AQP3-deficient mice are resistant to skin tumorigenesis. (3)

According to all the facts listed, there is a strong basis for researching possible AQP inhibitors. In the case of AQP1, inhibitors would serve as tumor medications, for suppressing angiogenesis and reducing tumor growth. (3,6) Likewise, inhibition of AQP3 water and glycerol channel may also have an important role in tumor therapy by preventing formation of skin tumors, as well as other tumors with AQP3 up-regulation. (3)

1.2.6. Aquaporin-based therapeutics

As already presented, altering the function of AQP can have wide spectrum of possible implications in therapeutic use. We can affect transepithelial fluid movement (glandular secretion, urinary concentrating process), cell migration (wound healing, angiogenesis, cancer metastasis), neural signal transduction and other water transport processes (e.g. brain swelling). In case of aquaglyceroporins, modulating AQPs' roles can be recognized in skin hydration, fat metabolism and tumorigenesis.

Consequently, AQP modulator medications are thought to be of wide utility in the therapy of edema, wound healing, kidney disorders, glaucoma, epilepsy, obesity and cancer. (3,4,6,11)

My work was primarily focused into finding an AQP inhibitor with possible later application in cancer therapy considering there are no documented AQP inhibitors acceptable for clinical development to date. (11) Merely few suitable compounds have been noted and a fact that there is no natural AQP modulator known is a considerable factor in this unfortunate statistics. (7)

Nevertheless, sulfhydryl-reactive compounds with heavy metals, e.g. $HgCl_2$, can inhibit multiple AQPs by interaction with residues of cysteine-189 generating a blockage and preventing water to pass. (1,4,26) However, mercurial substances are not selective and extremely toxic. Therefore, there is a significant concern in finding non-toxic AQP-inhibitors which would also be AQP-selective. (4)

Studies some years ago proposed a few candidates, such as acetoazolamide, tetraethylammonium and DMSO but subsequent measurements could not confirm true inhibition of these compounds. (11)

Recent studies (years 2009 and 2010) suggested compound TGN-020 (2-(nicotinamoyl)-1,3,4-thiadiazole) as well as bumetanide and its derivates (furosemide and a compound named AqB013) as inhibitors of non-selective AQP1 and AQP4 water transport. (7)

Additionally, four more molecules (NSC670229, NSC164914, NSC301460, NSC168597) were recognized as potential AQP1 and AQP4 inhibitors using a high-throughput screening of the National Cancer Institute's chemical library. (27) There is a reasonable interest in researching compound NSC670229 because its structure is small and simple. However for now, *in vivo* studies have not yet been reported. (7)

Recent developments described gold(III) complexes as possible inhibitors of AQP3 channels. (28) The most promising compound, Auphen ([Au(1,10-phenatroline)Cl_2]Cl), is a water soluble substance and highly selective to inhibit AQP3 dependent glycerol transport. Together with minor toxicity and low concentration needed for inhibitory effect it represents a leading compound acceptable for promising *in vivo* studies. (29)

Another strategy besides chemical modulators is targeting AQPs' regulatory proteins in order to suppress expression of certain AQP. (7) Effective technology for gene silencing is called RNA interference (RNAi) which causes protein down regulation. There are already positive results of studies focused on targeting AQP1 and AQP4 expression, respectively and consequently inhibiting AQP's activity. This indirect method is thought to be useful approach in disorders where AQP up-regulation occurs in a long term (e.g. angiogenesis). (26)

In conclusion, modulation of AQP function or expression could have therapeutic potential in several pathologies and further exploration of AQPs as drug targets seems urgent to disclose lead compounds for new therapies.

AQPs and their potential use in therapy are listed in Table IV. Biological roles of AQP6 (30), AQP11 and AQP12 (31) have not been completely established yet. Roles of AQP9 and AQP10 are roughly clarified but it remains to stay unclear how relevant they are in pathology of certain diseases and to what extent they can represent a target for pharmacotherapy. (7)

Table IV. Possible implications of AQP modulators. (4,6,7,11,19)

	INHIBITORS	ACTIVATORS
AQP0		➢ Inhibiting caractogenesis
AQP1	➢ Reducing urine concentration – diuretic drugs in edematous conditions (e.g. congestive heart failure); ➢ Reducing tumor growth and spread by limiting angiogenesis; ➢ Treatment of hydrocephalus ➢ Topical application: reducing intraocular pressure in glaucoma	
AQP2	➢ Reducing urine concentration and reducing water retention – diuretic drugs in a therapy of hyponatremia, congestive heart failure, hypertension and also during pregnancy	➢ Treating polyuria of nephrogenic diabetes insipidus
AQP3	➢ Prevention/therapy of skin cancer and other cancers related to AQP3 overexpression	➢ Wound healing, tissue regeneration after injury; ➢ Treating dry eye syndromes
AQP4	➢ Reducing brain swelling, intracranial pressure and glial scar formation after stroke or injury of brain or spine (causing cytotoxic edema); ➢ Reducing seizures induced by different stimuli (acoustic, light, chemical) – antiepilepsy drugs; ➢ Reducing intraocular pressure in glaucoma	➢ Reducing brain swelling in vasogenic edema and hydrocephalus
AQP5	➢ Treating hypersecretions of airway fluid in rhinitis and bronchitis	➢ Treating reduced and hyperviscous gland secretions in certain conditions (e.g. cystic fibrosis and Sjogren's syndrome); ➢ Treating dry eye syndromes
AQP7		➢ Reducing fat mass in obesity
AQP8	➢ Increasing female fertility (17)	➢ Female birth control (17)

2. OVERALL AIM OF THE STUDY

Considering the majority of aquaporin inhibitors remain undiscovered and the fact that there is a positive link between aquaporins' extensive expression and various tumor-associated processes (tumorigenesis, growth, spread, metastasis), potential inhibitors would be a great contribution to the development of new anticancer therapies.

My work will be primarily focused into finding an AQP inhibitor with possible later application in cancer therapy. The most efficient approach for searching and testing many new compounds with unexpected impact is screening.

Therefore, my principal goal will be to screen many compounds in order to get results of their impact on cell membrane permeability in human red blood cells. All compounds, tested in our study, were synthesized in Chemistry Department of Faculty of Pharmacy in Lisbon. Method used to determine cell permeability will be stopped-flow light scattering and subsequently data analysis will be done to define the results of the tested compounds. Decreased cell permeability for water and glycerol correlates with inhibitory effect on AQPs' activity.

In the interest of evaluation we will determine half-maximal inhibitory concentration (IC_{50}) and describe structure activity relationship (SAR) for compounds showing potential inhibitory effect. Volume measurements will be performed in order to assure that initial volumes of hRBCs in solutions with compounds are the same in the beginning of stopped-flow experiments. We will measure mean cell volume (MCV) in solutions of hRBCs and our compounds using a coulter counter.

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Samples

- Venous blood samples in citrate anticoagulant (2.7% of citric acid, 4.5% of trisodium citrate and 2% of glucose)

3.1.2. Chemicals and reagents

- Dimethyl sulfoxide (DMSO), *Sigma-Aldrich, St. Louis MO USA*
- Disodium hydrogen phosphate (Na_2HPO_4), *Merck, Darmstadt, Germany*
- Glycerol 87%, *Sigma-Aldrich, St. Louis MO USA*
- Hydrochloric acid (HCl), *Merck, Darmstadt, Germany*
- Methanol, *Merck, Darmstadt, Germany*
- Mercury chloride ($HgCl_2$), *BDH AnalaR, Poole, England*
- Potassium chloride (KCl), *Merck, Darmstadt, Germany*
- Potassium dihydrogen phosphate (KH_2PO_4), *BDH, Poole, England*
- Sodium chloride (NaCl), *Merck, Darmstadt, Germany*
- Sucrose, *BDH, Poole, England*

3.1.3. Buffers and solutions

- <u>PBS buffer (300 mOsm/L)</u>

NaCl……..8g
KCl……...0.20g
Na_2HPO_4……..1.44g
KH_2PO_4……0.24g
Distilled water……..to 1L

} adjust pH to 7.4 with HCl

- Sucrose solution (200 mOsm/L)

Sucrose…….17.115g

PBS…….to 250 mL

- Glycerol solution (200 mOsm/L)

Glycerol (87%)……5.354g

PBS…….to 250 mL

3.1.4. Compounds

All compounds were synthesized in the Chemistry Department of Faculty of Pharmacy in Lisbon.

3.1.4.1. NAP compounds

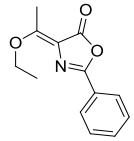
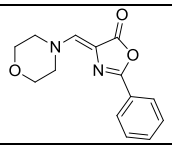
Table V. Characteristics of NAP compounds.

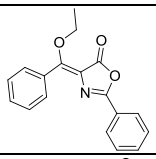
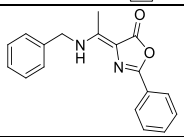
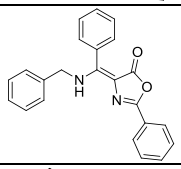
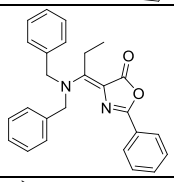
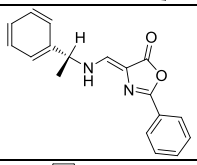
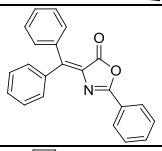
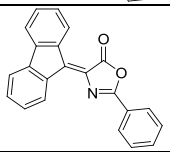
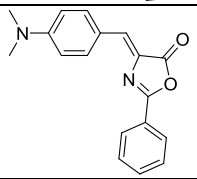
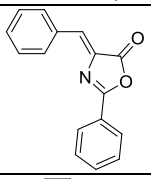
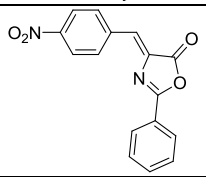
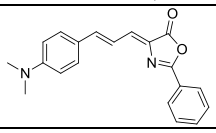
Compound	Molecular formula	MM (g/mol)	Solubility
NAP 20	$C_{17}H_{15}NO_2$	265,33	Methanol (also AcOEt, CH_2Cl_2, Et_2O)
NAP 78	$C_{23}H_{19}NO_2$	341,43	Methanol (also AcOEt, CH_2Cl_2, Et_2O)
NAP 80	$C_{17}H_{15}NO_2$	265,33	Methanol (also AcOEt, CH_2Cl_2, Et_2O)
NAP 15.1	$C_{20}H_{18}N_2O_2$	318,42	Methanol (also AcOEt)

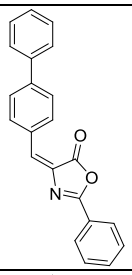
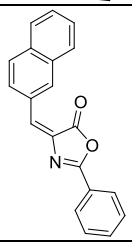
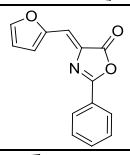
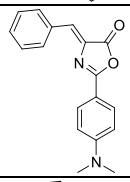
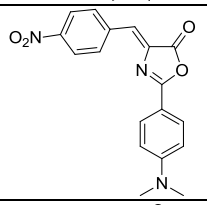
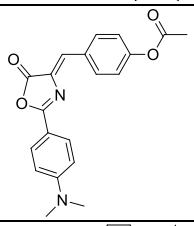
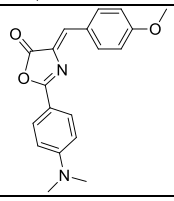
Structures are concealed for research purposes.

3.1.4.2. Oxazolone compounds

Table VI. Characteristics of oxazolone compounds.

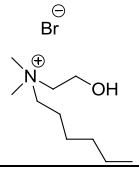
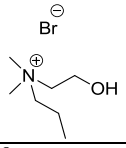
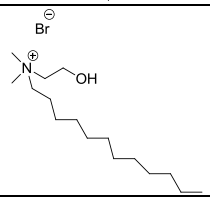
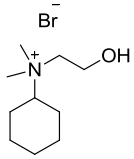
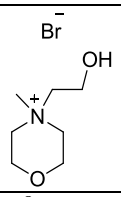
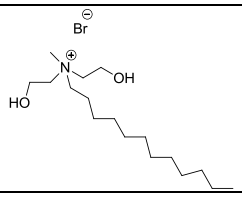
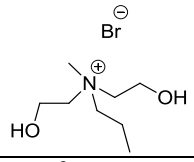
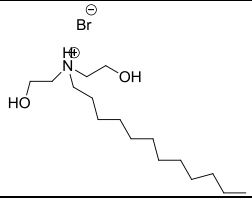
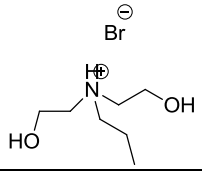
Compound	Structure	MM (g/mol)	Solubility
PGE37		231,09	DMSO
PGE24		258,27	DMSO

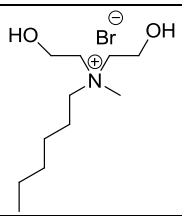
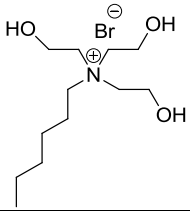
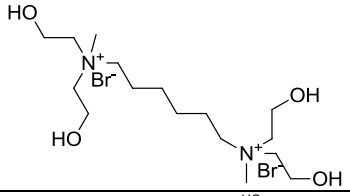
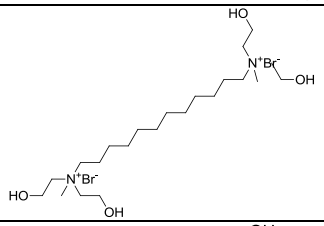
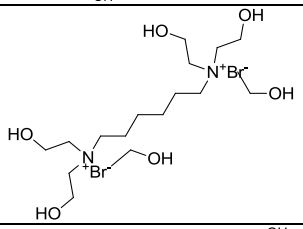
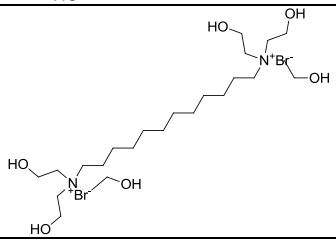
PGE15-A		293,32	DMSO
R35		292,33	DMSO
MNM – R31		354,14	DMSO
MNM – R43		396,18	DMSO
MNM – R45		292,33	DMSO
OXA001		325,36	DMSO
OXA002		323,34	DMSO
OXA003		292,33	DMSO
OXA004		249,26	DMSO
OXA005		294,26	DMSO
OXA007		318,37	DMSO

OXA009		325,36	DMSO
OXA010		299,09	DMSO
OXA011		239,23	DMSO
OXA020		292,33	DMSO
OXA022		337,33	DMSO
OXA024		350,37	DMSO
OXA025		322,36	DMSO

3.1.4.3. Choline compounds

Table VII. Characteristics of choline compounds.

Compound	Structure	MM (g/mol)	Solubility
FS3		254,21	H_2O
FS5		212,13	H_2O
FS6		338,37	H_2O
FS7.2		252,18	H_2O
FS8		226,11	H_2O
FS10		368,39	H_2O
FS11		242,15	H_2O
FS12		354,37	H_2O
FS13		228,13	H_2O

FS49		239,78	H_2O
FS51		314,26	H_2O
FS65		482,29	H_2O
FS68		566,45	H_2O
FS69.2		542,34	H_2O
FS72		626,50	H_2O

3.1.5. Laboratory equipment

- **Centrifuge**: Heraeus Sepatech Labofuge Ae 0-5000, *Heraeus, Hanau, Germany*
- **Coulter Counter:** CASY-1 Cell Counter, *Schärfe System GmbH, Reutlingen, Germany*
- **Osmometer**: Semi-micro osmometer, *Knauer, Berlin, Germany*
- **pH meter**: 744 pH Meter, *Metrohm, Herisau, Switzerland*

- **Pipettes**: Tipor-VL 0.5-10 μL, 2-20 μL, 20-200 μL, 100-1000 μL, *Orange Scientific, Braine-l'Alleud, Belgium*
- **Scale**: Analytical balance Sartorius Research R200D, *Sartorius, Goettingen, Germany*
- **Stopped-flow device**: Hi-Tech Scientific PQ/SF-53, *TgK Scientific, Bradford-on-Avon, United Kingdom*
- **Ultrasonic water bath**: Branson B2200 E4, *Branson Ultrasonics, Danbury, USA*

3.2. Methods

3.2.1. Preparation of blood samples

Serum of fresh blood samples was removed and PBS buffer was added to the test tube. Samples were centrifuged at 4 °C, 1500 × g for 5 min and washed in PBS three times. Afterwards solution in PBS buffer with 0.5% of hematocrit was prepared and put on ice. Prepared samples were used for experiments within one day.

3.2.2. Preparation of stock solutions and solutions for experiments

3.2.2.1. NAP compounds

Firstly, we prepared stock solutions with final concentration of 0.05 M for NAP20, NAP78 and NAP80. Compound NAP15.1 was prepared at lower final concentration of 0.01 M due to its poor solubility.

Solutions for experiments were prepared with 3 ml of blood sample in three different concentrations: 100 μM, 250 μM and 500 μM.

3.2.2.2. Oxazolone compounds

All compounds except OXA002 and OXA022 were prepared in 0.01 M stock solutions. Compounds OXA002 and OXA022 were prepared at lower concentration (0.005 M) due to their poor solubility.

Solutions for experiments were prepared with 3 ml of blood sample only in one concentration: 50 μM.

Few compounds were then re-tested, therefore we prepared different stock solutions. MNM-R31, MNM-R43, OXA001 and OXA011 were prepared in 0.05 M stock solutions. Compounds OXA003 and OXA024 were prepared in 0.025 M stock solutions.
Solutions for experiments were as well prepared in 50 μM regarding the 3 ml of blood sample.

3.2.2.3. Choline compounds

Stock solutions for all compounds were prepared in final concentration of 0.01 M for preliminary experiments.

Solutions for experiments were prepared with 3 ml of blood sample only in one concentration. For most of the compounds this concentration was 500 μM, except for compounds FS12 and FS72, where we used 250 μM solutions. In 500 μM solutions they induced hemolysis.

For consequent tests compounds FS6, FS10, FS12 and FS72 were prepared in another stock solution: 0.05 M of final concentration for FS6, FS10 and FS12. Compound FS72 was prepared in a stock solution of 0.025 M.

Experiments for determination of concentration dependence were done for FS6, FS10, FS12 and FS72 in several concentrations ranging from 10 μM to 750 μM.

3.2.3. Stopped-flow light scattering method

Stopped-flow light scattering is one of the several possible screening methods for searching AQP modulators. It is a simple method where a small sample aliquot is needed to determine membrane permeability. Experiments are based on measuring cell volume changes and detecting scattered light dependence on cell volume after exposing the cells to osmotic shock. (11,32)

Samples we used in stopped-flow light scattering were human red blood cells (hRBC). We chose hRBC because of simple and relatively quick preparation, their large AQP1 and AQP3 expression and valuable sensitivity to osmotic changes.

As AQP1 permeates only water and AQP3 permeates also glycerol beside water, membrane permeability was tested for water and glycerol. Human RBCs incubated in PBS were mixed quickly with hypertonic solution to generate an osmotic gradient. For testing osmotic water permeability cells were challenged with hypertonic sucrose solution (considering sucrose an impermeant solute) which caused cell shrinkage. For examining membrane glycerol permeability hRBCs were exposed to hypertonic glycerol solution (considering glycerol a permeant solute) which at first led to cell shrinkage but the process was followed by re-swelling as a result of glycerol entrance accompanied by water.
Water and glycerol membrane permeability can be calculated from changes of cell volume (shrinking and re-swelling) and consequently, intensity of scattered light. (29,32)

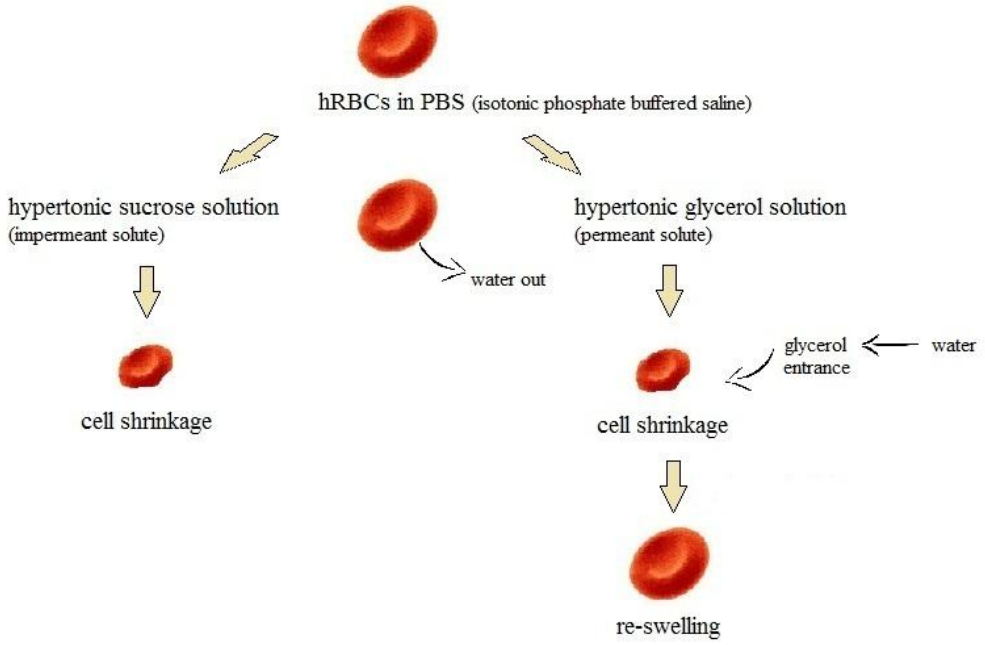


Figure 7. The principle of stopped-flow light scattering method. Imposing hRBCs to an osmotic shock.

Stopped-flow experiments start when small volumes of solutions are quickly pushed from syringes into a mixer to initiate a reaction. Fresh mixture of solutions enters into an observation cell and then fills a stopping syringe which causes immediate stoppage of the flow thus the technique is called "stopped-flow" (Figure 8). This process is just milliseconds long and is also established as the dead time of the stopped-flow system. In our system dead time was 2ms. (29)

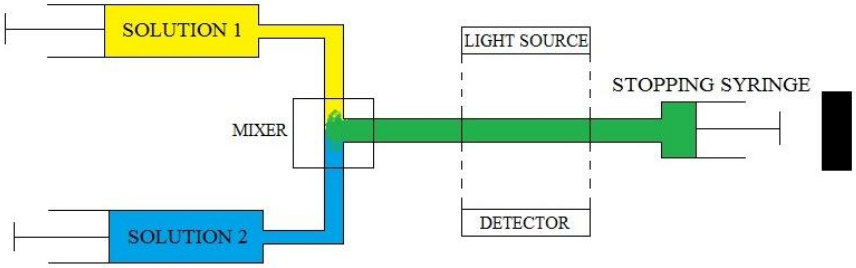


Figure 8. Stopped-flow technique.

Our experiments were performed at 23°C for glycerol permeability and at 10°C for water permeability. Temperature was controlled with a microcomputer. All solution osmolarities were determined from freezing point depression on a semi-micro osmometer using standards of 400 mOsm/L.

The most practical parameter to define properties of water transport is the osmotic water permeability coefficient (P_f). It is determined from the volume flux across a membrane after imposing it to an osmotic gradient. P_f presents helpful information about water transport and whether it is facilitated by AQPs. (32)

Measuring the osmotic water permeability (P_f)

100 µL of a suspension of fresh erythrocytes (hematocrit 0.5%) was mixed with an equal volume of hyperosmotic PBS containing 200 mOsm/L sucrose as a non-permeable osmolyte to produce a 100 mOsm/L inwardly directed sucrose gradient. The kinetics of cell shrinkage was measured from the time course of 90° scattered light intensity at 400 nm until a stable light scatter signal was attained. To calculate P_f, the light scatter signal was fitted to one exponential function, and the rate constant k was used in the following equation:

Equation *1*

$$P_f = k\,(V_o / A)\,(1 / V_w(osm_{out})_\infty)$$

V_w…… molar volume of water

V_o / A…….. initial cell volume to area ratio

$(osm_{out})_\infty$…….. final medium osmolarity after the applied osmotic gradient

k…… single exponential time constant fitted to the light scattering signal of erythrocyte shrinkage

Measuring the glycerol permeability (P_{gly})

100 µL of erythrocyte cell suspension (hematocrit 0.5%) was mixed with an equal volume of hyperosmotic PBS containing 200 mOsm/L glycerol, creating a 100 mOsm/L inwardly directed glycerol gradient. After the first fast cell shrinkage due to water outflow, glycerol influx in response to its chemical gradient was followed by water influx with subsequent cell re-swelling. To calculate P_{gly}, the light scatter signal of cell re-swelling was fitted to one exponential function, and the rate constant k was used in the following equation:

Equation *2*

$$P_{gly} = k\,(V_o / A)$$

V_o / A…….. initial cell volume to area ratio

k…….. single exponential time constant fitted to the light scattering signal of glycerol influx in erythrocytes

For inhibition experiments cells were incubated with different concentrations of compounds, from freshly prepared stock aqueous or methanol or DMSO solution, for various times (30 min – 90 min) at room temperature before stopped-flow experiments. As a positive control we used $HgCl_2$, a well-known aquaporin inhibitor, but incubation time in this case was 10 min due to its high toxicity.

The inhibition concentration necessary to achieve 50% inhibition (IC_{50}) was calculated by nonlinear regression of dose-response curves (Graph Pad Prism, Inc) to the equation 3:

Equation *3*

$$y = y_{min} + (y_{max} - y_{min})/(1 + 10^{((LogIC50-[Inh])\,H)})$$

y…… percentage inhibition obtained for each concentration of inhibitor [Inh]

H……..… Hill slope (29)

4. RESULTS AND DISCUSSION

Water and glycerol permeability measurements were performed on erythrocytes in freshly prepared blood samples. Results are mostly represented in graphs, where blue color presents water features and orange color presents glycerol features. Generally graphs show water or glycerol permeability and the effect of incubation of hRBCs with our compounds. As a positive control we used $HgCl_2$, a well-recognized and prominent AQP inhibitor, which decreases water as well as glycerol permeability.

Diminished water permeability indicates AQP1 inhibition whereas diminished glycerol permeability indicates AQP3 inhibition.

4.1. NAP compounds

4.1.1. Influence of the solvent

Firstly, we tested the influence of methanol itself as a solvent. With this aim we prepared methanol controls in five different concentrations. We chose methanol volumes equal to the volumes of stock solutions later added to red blood cells in order to get a specific concentration (100 µM, 250 µM and 500 µM).

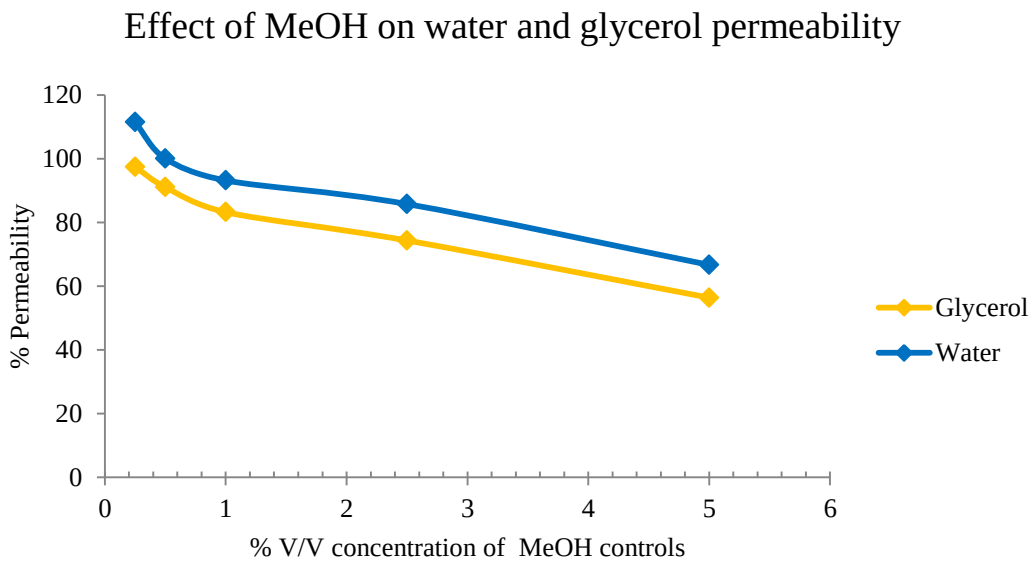


Figure 9. Effect of different methanol concentrations on water and glycerol permeability.

Solubility in methanol can be questionable which is especially evident in solutions with high methanol concentration. Values of water and glycerol permeability drop as concentration of methanol rises.

At first glance our results are just the opposite of literature facts. According to several studies methanol decrease rigidity and thickness of lipid bilayer causing increase in fluidity of membrane thus becoming more permeable and leakier. (33,34) Therefore, percentage of permeability should increase with higher methanol concentration.

However, it is possible that methanol has similar effect to ethanol which itself inhibits aquaporins in yeast. (35) Despite fluidifying the lipid membrane and consequently stimulating diffusion transport, the inhibitory effect of methanol on transportation process through aquaporins may result in decrease of water and glycerol permeability in stopped-flow experiments also in hRBCs.

Due to this reason the results of compounds which would show potential inhibition (60%-80% permeability) in high concentration of methanol solutions cannot be considered as certain.

4.1.2. Effect of the compounds

We tested four compounds of the NAP family, all of them soluble in methanol.
Compound NAP15.1 was tested at 500 μM but the influence of methanol was extremely significant thus the result could not have been considered as relevant.

In Figure 10 we can see that none of the compounds has significant effect on water or glycerol permeability. Based on these results, we can conclude that compounds do not show any important inhibitory effect on AQP1 or AQP3 in hRBCs.

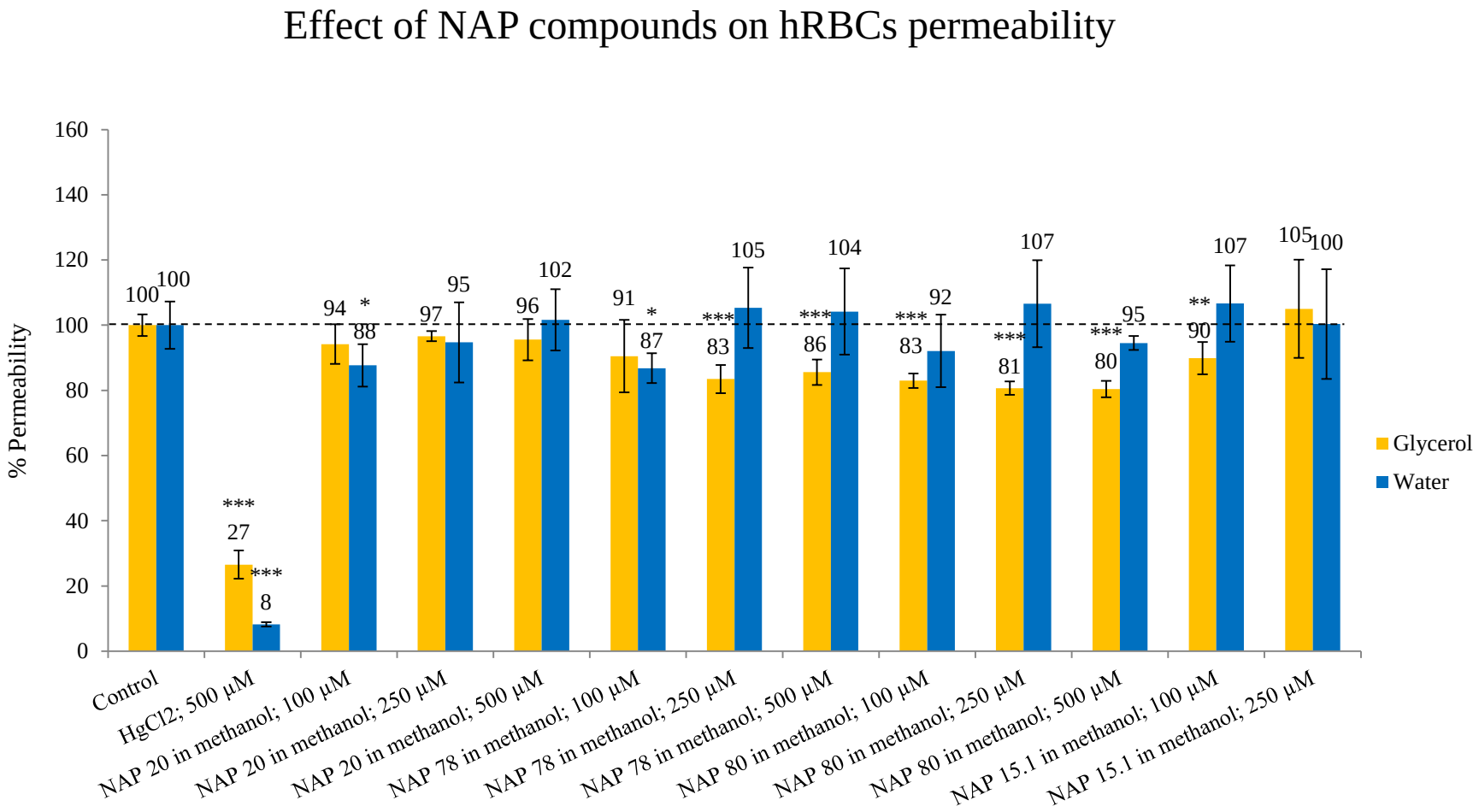


Figure 10. Results of compounds presented in a graph in comparison to proper methanol control. (*P<0.05, **P<0.01, ***P<0.001)

4.2. Oxazolone compounds

4.2.1. Influence of the solvent

First of all we tested the influence of solvent itself, in this case DMSO. With this aim we prepared DMSO controls in five different concentrations for testing glycerol permeability and in four different concentrations for testing water permeability. We chose DMSO volumes equal to the volumes of stock solutions later added to red blood cells in order to get a specific concentration (50 μM).

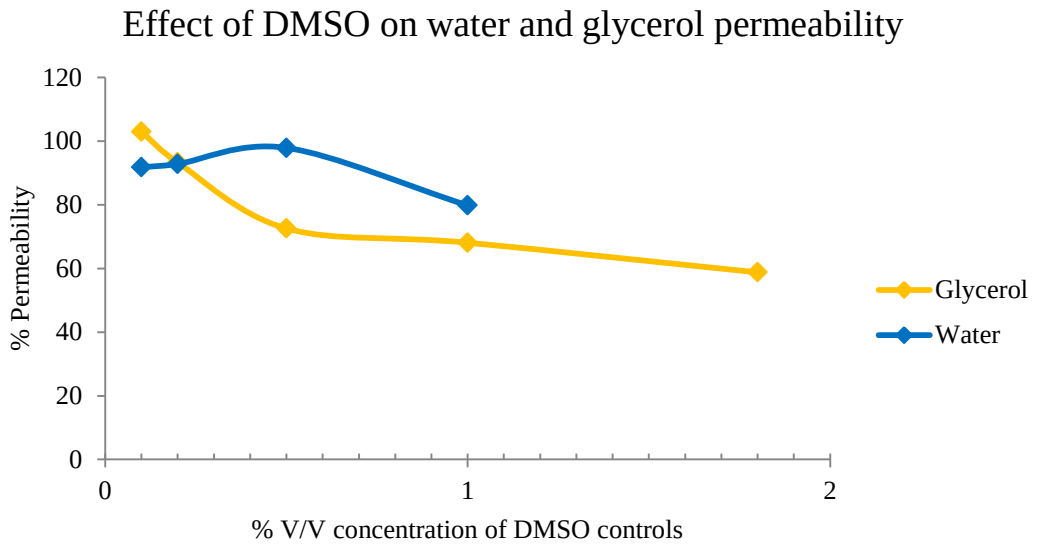


Figure 11. Effect of different DMSO concentration on water and glycerol permeability.

DMSO in the past has been proposed as an inhibitor of aquaporins, primarily of AQP1, because it lowers cell water permeability. Further studies showed decelerated osmotic equilibrium, however the reason is an 'osmotic clamp' effect instead of true AQP1 inhibition. Consequences of its high osmolality and rapid membrane permeation were formerly misinterpreted as inhibition of aquaporins. (36)
We can thus understand the influence of DMSO on water permeability in the Figure 11.

On the other hand, impact of DMSO on AQP3 water and glycerol channel has not yet been investigated but the influence cannot be completely excluded. As we can see in Figure 11, DMSO affects glycerol permeability even more than water permeability, especially in high

concentrations. The cause remains unexplained whereas it can affect either AQP3 channel or membrane of the cell.

Therefore, we have to be careful interpreting the results of compounds in DMSO solutions. In solutions with large DMSO volumes the results of compounds, which would show potential inhibition (60%-80% permeability), cannot be regarded as conclusive.

4.2.2. Effect of the compounds

When testing compounds of oxazolone family, concentration of 50 μM was chosen. We acquired good and significant signals at this concentration aside from results obtained at higher concentrations which were not possible to analyze. Perhaps the compounds themselves are toxic in higher concentration and in some way damage cell membrane although they do not cause hemolysis in a tube during incubation. The other potential reason is the effect of DMSO as a solvent. To avoid the influence of DMSO we would need to prepare another, more concentrated stock solution if the compound solubility allowed it. The significance of DMSO in solution with hRBCs would thus be as minimized as possible.

Some compounds were re-tested in more concentrated stock solutions. Results are combined with others and presented in the same graph.

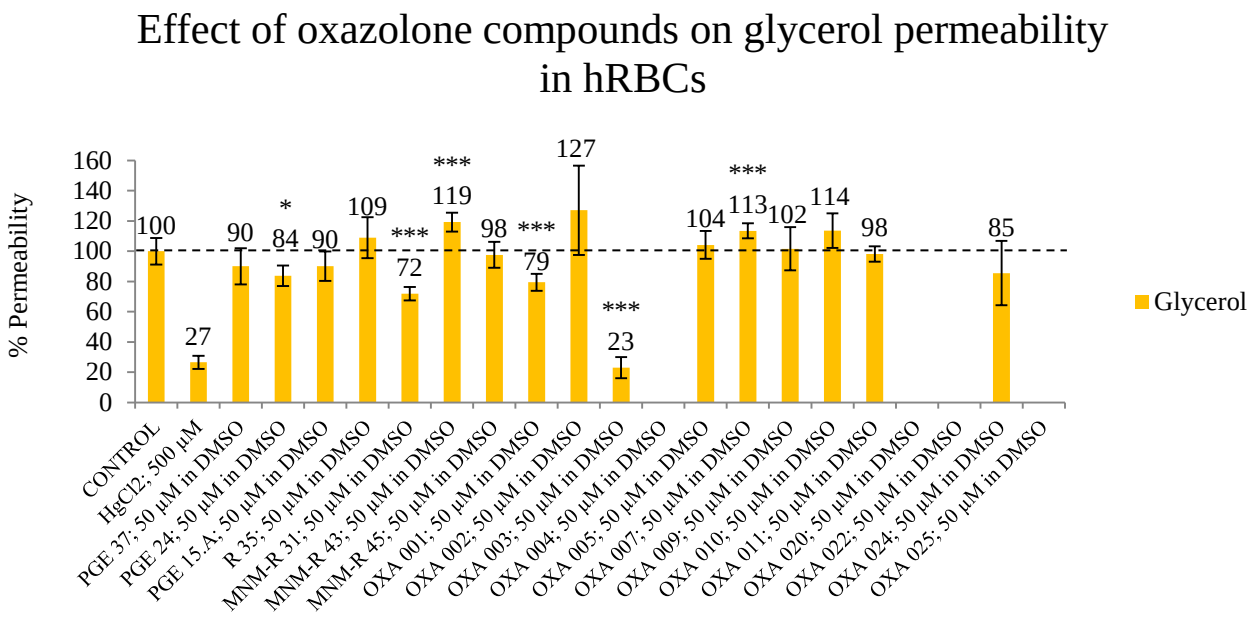


Figure 12. Results of oxazolone compounds on glycerol permeability in hRBCs, presented in a graph in comparison to proper DMSO control. (*P<0.05, **P<0.01, ***P<0.001)

As seen in Figure 12, from all compounds only one had a statistic significant effect on glycerol permeability. Compound OXA003 displayed 23% of glycerol permeability compared to the control, which means 77% of AQP3 inhibition. Nevertheless, handling with OXA003 should be cautious considering the risk of causing hemolysis in a tube. Human RBCs were incubated with OXA003 with three times shorter incubation time (10 min) than other compounds (30 min) because 30 min of incubation time resulted in hemolysis of red blood cells at OXA003 concentration of 50 µM.

It was not possible to obtain results for compounds OXA004, OXA020, OXA022 and OXA025 because signals in stopped-flow were extremely peculiar. The reason may be their possible harmful effect on AQP3 water and glycerol channels or DMSO influence; however same volumes of DMSO added to hRBCs in case of other compounds did not affect cells in such extent that we would not be able to obtain results. We can eliminate the impact of these compounds on cell membrane because in case of water permeability acquiring signals and attaining the results were not presenting difficulties.

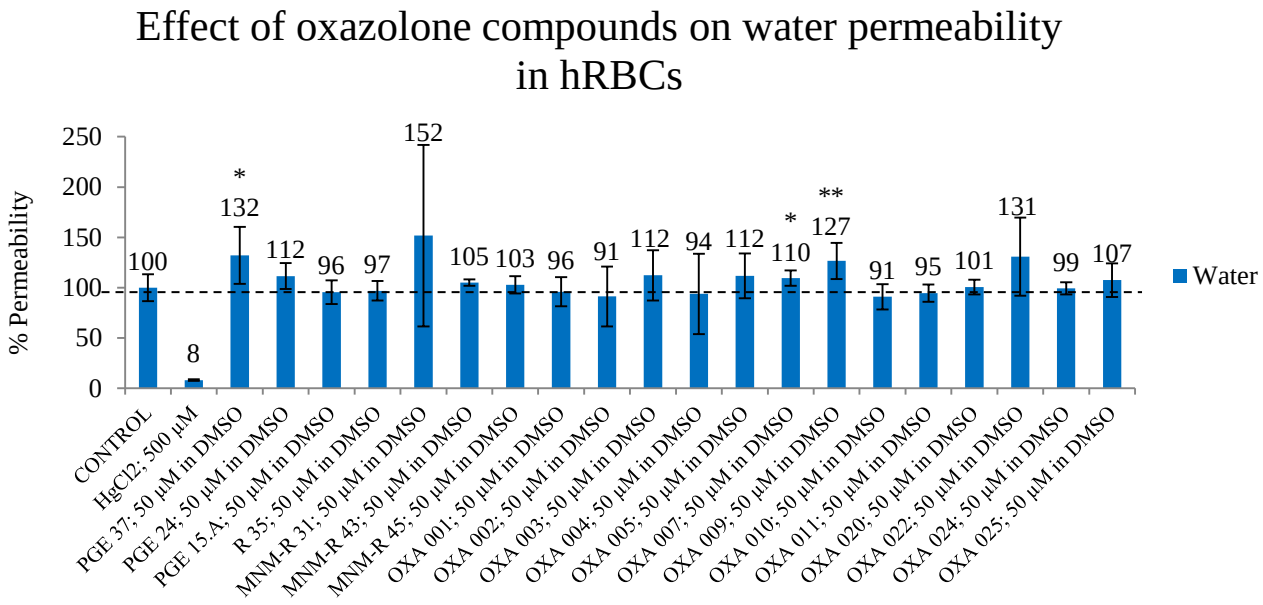


Figure 13. Results of oxazolone compounds on water permeability in hRBCs, presented in a graph in comparison to proper DMSO control. (*P<0.05, **P<0.01, ***P<0.001)

According to the results in Figure 13, none of the compounds of oxazolone group has significant effect on water permeability therefore compounds do not show any important inhibitory effect on AQP1 in hRBCs. Compound MNM-R31 has a huge standard deviation because only 2 measurements were obtained due to its peculiar signals in stopped-flow.

4.2.3. Structure activity relationship (SAR) and comparison of the structures

All compounds contain an oxazolone ring in their structure (Figure 14) and on the C2 carbon atom an aromatic benzene ring is attached.

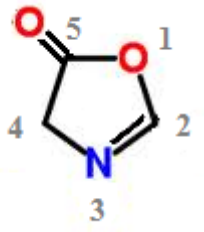


Figure 14. Chemical structure of oxazolone ring. (37)

Dimethylamine functional group is added on the respective aromatic ring in compounds OXA020, OXA022, OXA024 and OXA025. Interestingly, this may be in connection with the fact that in case of OXA020, OXA022 and OXA025 signals for glycerol permeability in stopped-flow were not possible to acquire. Although, we could not as well obtain signals for OXA004, that has no dimethylamine functionality in its structure. On the other hand, we could acquire signal for compound OXA024 despite having a dimethylamine substituent on the benzene ring. The reason may be in a long side chain where we have acetate group in OXA024 molecule. Of all functional groups in side chains of compounds OXA020, OXA022, OXA024 and OXA025 this one is the most polar. We have no certain proof of connection between functional group polarity and potential damaging effect on AQP3 thus no signals but it may be an idea to justify this occurrence and a helpful suggestion when searching a lead compound or synthesizing completely new compounds.

In C4-side chains, bound on the fourth carbon atom of oxazolone ring, there are usually aromatic systems (at least one benzene ring is present) and frequently nitrogen included in the molecules forming secondary or tertiary amine.

Possible explanation for inhibitory effect of compound OXA003 is the presence of dimethylamine group on a benzene ring in C4 side chain (the one bound on the fourth carbon atom). In comparison to OXA004 this functional group is the only difference between the two molecules and for OXA004 we could not acquire signals. Moreover, C4 side chain has to be short enough because longer side chains, like in OXA007, apparently represent a steric obstacle.

4.3. Choline compounds

4.3.1. Effect of the compounds

When testing choline compounds, since these are water soluble, we used hRBCs without compounds as control.

Figure 15 shows the effect on AQP1 and AQP3 caused by choline compounds (250 μM or 500 μM concentration). The results, obtained in our experiments, show that choline family includes four compounds which effectively influence glycerol permeability, FS6, FS10, FS12 and FS72. All four compounds significantly lower glycerol permeability to 20%-

30%, which means they inhibit AQP3 in 70-80% at concentration 500 μM for FS6 and FS10, and 250 μM for FS12 and FS72. In 500 μM solutions FS12 and FS72 induced hemolysis.

As demonstrated in Figure 15, compound FS6 did not show significant effect on diminishing water permeability (91%), while compounds FS10, FS12 and FS72 revealed also a small effect on water permeability; in case of FS10 and FS12, 78% (nearly 20% of AQP1 inhibition) and in case of FS72, 66% (nearly 35% of AQP1 inhibition) at given concentrations.

Considering that these four compounds certainly inhibit aquaglyceroporin 3, which permeates glycerol as well as water, the smaller decrease in water permeability can be attributed to the inhibition of AQP3. Its contribution to water flow in total is, though, of smaller extent in opposition to AQP1, which mainly transports water through membranes of hRBCs. (29) Percentage of lower water permeability therefore consists of AQP1 inhibitions, plus smaller effect of AQP3 inhibition.

In addition to AQP1 channel, water crosses the membranes also through lipids by simple diffusion and this process cannot be stopped by inhibitors. As a result, a decrease of total water permeability value due to AQP1 inhibition is less noticeable.

As for AQP3, glycerol cannot pass through lipids. In experiments we are only observing glycerol passing through AQP3, therefore a reduction of glycerol permeability shows the actual inhibition of AQP3.

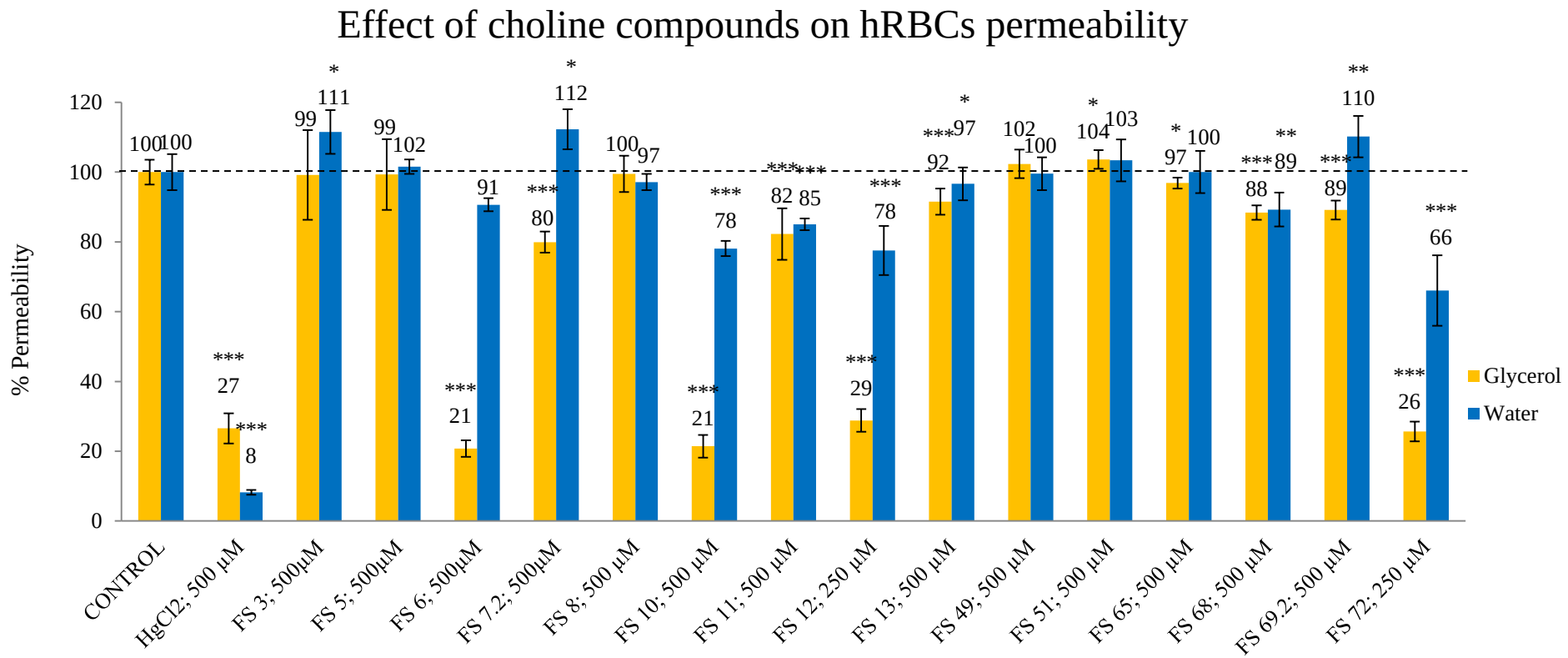


Figure 15. Results of compounds presented in a graph in comparison to control, which was blood solution in PBS. (*P<0.05, **P<0.01, ***P<0.001)

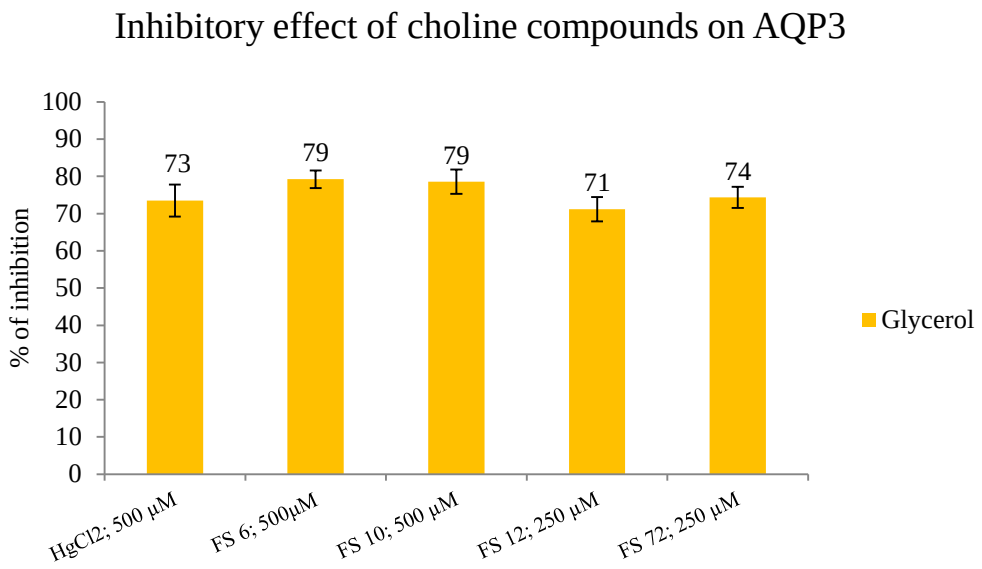


Figure 16. Percentage of AQP3 inhibition for compounds FS6, FS10, FS12 and FS72. All compounds are similarly effective in comparison to $HgCl_2$ (with a statistical significance of $P<0.001$)

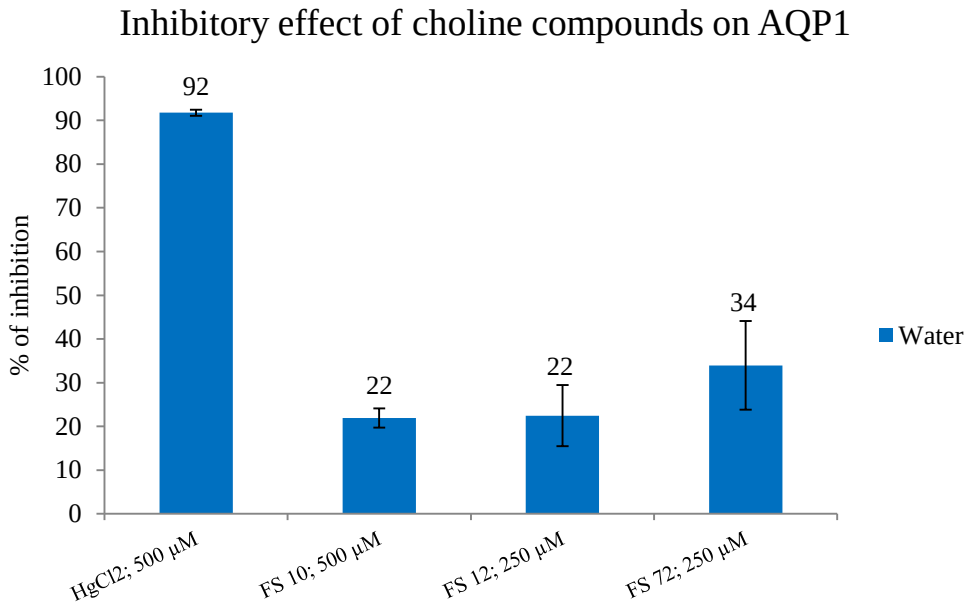


Figure 17. Percentage of AQP1 inhibition for compounds FS10, FS12 and FS72.

4.3.2. Traces from stopped-flow experiments

Representative traces of experiments on stopped-flow apparatus are shown in Figure 18 and 19. Compound FS10 was taken as an example to exemplify an inhibition signal in stopped-flow experiments. Human RBCs incubated with FS10 (30 min at room temperature), reached steady state (final equilibrium volume) later in comparison to the control. This means that the process after incubation with FS10 is slower and has a lower time constant.

Steady state in case of glycerol permeability corresponds to the point when cells reach their initial volume after re-swelling process. In case of water permeability, since the solute is impermeant, water moves out and cells gain a new osmotic equilibrium and thus do not re-swell.

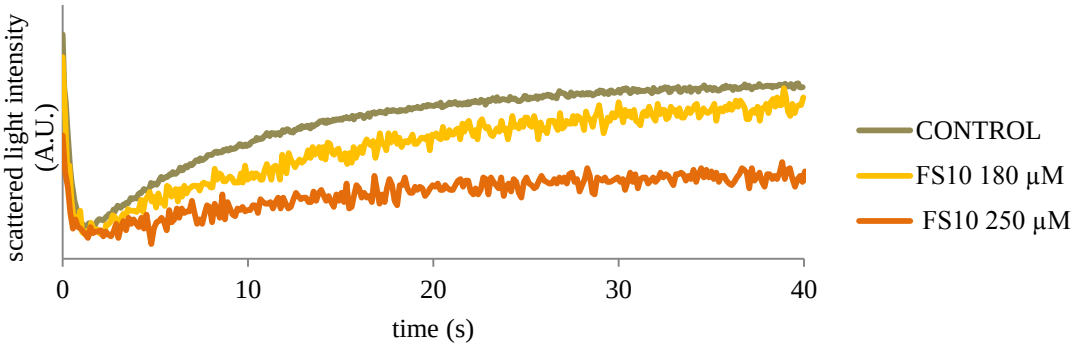


Figure 18. Representative traces of glycerol permeability (control and after incubation with 180 µM and 250 µM FS10, 30 min at room temperature). FS10 has higher effect on glycerol permeability. As we increase concentration of compound, we decrease time constant even more and decelerate the process.

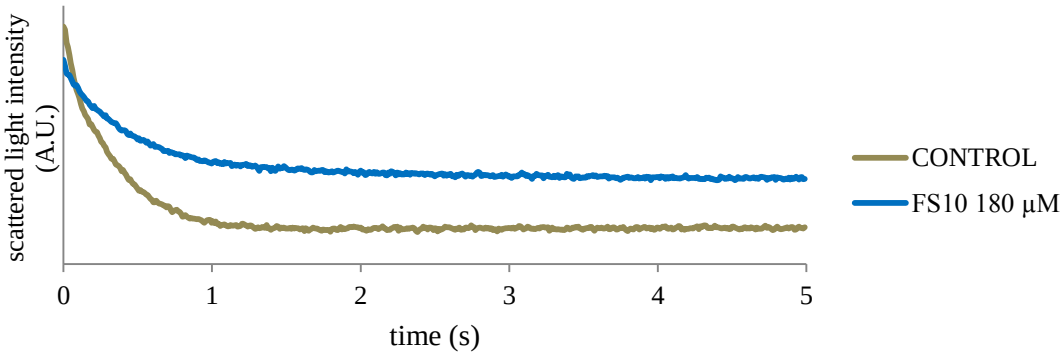


Figure 19. Representative traces of water permeability.

4.3.3. Determining half maximal inhibitory concentration (IC_{50})

For compounds showing inhibitory effect on AQP3 we determined half maximal inhibitory concentration (IC_{50}) from dose-response curves. All compounds were incubated with hRBCs for 30 min at room temperature. All experiments were performed twice at 23°C. The results in the Table VIII are the calculated average of two experiments.

Table VIII. Values of half maximal inhibitory concentrations.

Compound	IC_{50} (µM)
FS6	182
FS10	184
FS12	172
FS72	129

Comparing the IC_{50} values, all of them are quite similar, in low micromolar range between 100 and 200 µM.

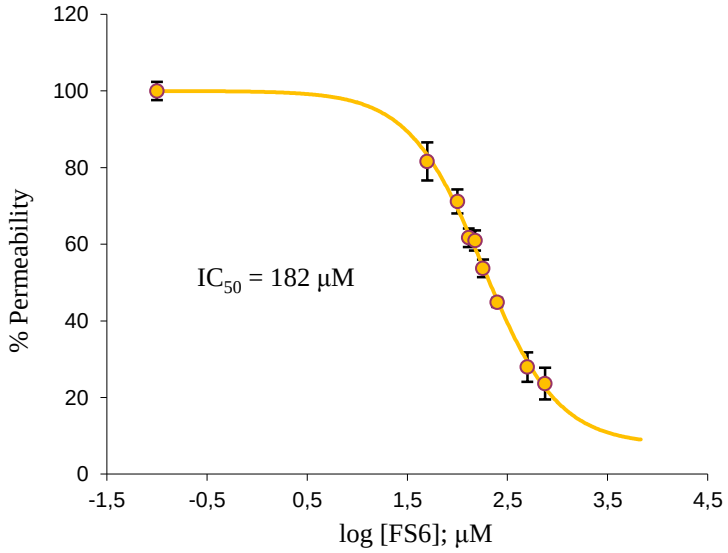


Figure 20. A dose-response curve, obtained for FS6 after incubation time of 30 min, room temperature.

4.3.4. Structure activity relationship (SAR) and comparison of the structures

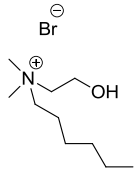
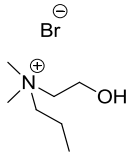
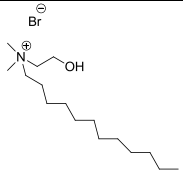
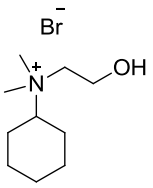
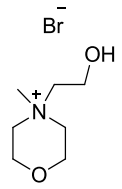
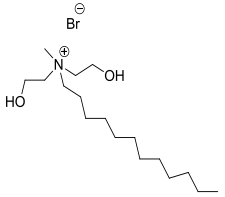
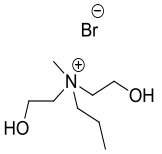
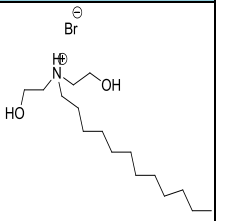
All compounds are cholines in the form of salt (N^+Br^-). They all have quaternary ammonium cation meaning they are permanently charged and a functional group of R-CH_2CH_2-OH. With an exception of FS12 and FS13, where there is a proton (H^+) bound on ammonium, they all have alkyl groups (R-CH_3 or R-CH_2CH_2-OH) bound on quaternary ammonium cation.

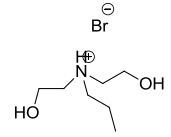
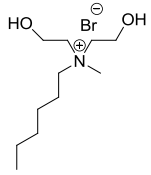
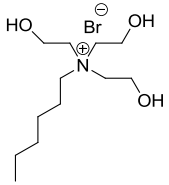
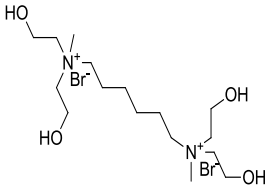
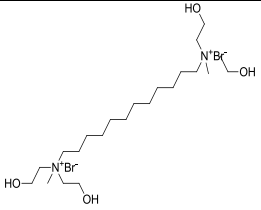
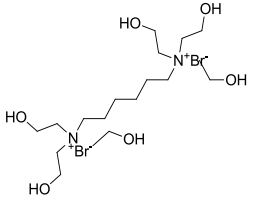
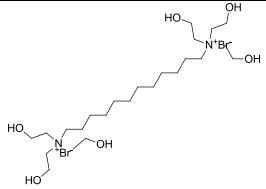
We propose that a hydrophobic tail is crucial for effectiveness. In set of our compounds it is necessary that a compound possess a hydrophobic tail of twelve carbon atoms to show inhibition activity.

All compounds as cholines in their structure include a hydrophilic hydroxyl group (-OH) bound on quaternary nitrogen via a spacer consisting of two carbon atoms. Extra hydroxyl groups correlate with higher effectiveness, supposing that a compound includes a long hydrophobic tail. Influence of hydroxyl group is especially seen in double cholines, where impact is even more significant, e.g. comparison of FS68 and FS72.

If we compare structures of our inhibitory compounds, FS6 and FS10 have similar structures and effect, although one extra hydroxyl group in FS10 increases efficiency. Between FS10 and FS12 there is only a slight difference in structure. FS10 has one methyl group bound on quaternary ammonium cation instead of proton H^+ in FS12, but according to the results, this substitution has no important influence on inhibitory effect.
As for double cholines, for example in FS72, it is necessary to have two extra hydroxyl groups in a molecule to have an effect (in comparison to FS68).

Table IX. Comparison of the choline compounds' structures and effectiveness.

Compound	**FS 3**	**FS 5**	**FS 6**	**FS 7.2**	**FS 8**	**FS 10**	**FS 11**	**FS 12**
Glycerol permeability %	99 %	99 %	21 %	80 %	100 %	21 %	82 %	29 %
Water permeability %	111 %	102 %	91 %	112 %	97 %	78 %	85 %	78 %
Structure								

Compound	**FS 13**	**FS 49**	**FS 51**	**FS 65**	**FS 68**	**FS 69.2**	**FS 72**
Glycerol permeability %	92 %	102 %	104 %	97 %	88 %	89 %	26%
Water permeability %	97 %	100 %	103 %	100 %	89 %	110 %	66 %
Structure							

4.3.5. Volume measurements

When measuring water and glycerol permeability we have to be aware of initial cell volume as an important determinant in calculations. Cell membrane permeability depends on initial volume/area ratio (V_0/A) which is supposed to be the same in all the experiments. Some of the compounds can cause damage to cell membrane during incubation, which may result in different initial cell volumes before stopped-flow experiments. In order to assure that no changes in cell initial volumes occurred and that the transient of volume changes after the osmotic shock can be compared, we determined initial RBCs volumes using a coulter counter. In diluted blood samples incubated with our compounds we measured mean cell volume (MCV).

Table X. Results of volume measurements for FS6, FS10, FS12 and FS72.

	MCV (femtoliters)	
	Concentration of compound = IC_{50}	Concentration of compound = $IC_{50}\times10$
CONTROL	68	67
FS6	67	79
FS10	68	80
FS12	68	73
FS72	67	68

From the results in Table X there are no significant differences in mean cell volumes at compound concentration of IC_{50}. We chose as well concentrations ten times higher than IC_{50} values, but again no significant differences were observed.

A meaningful obstacle, which cannot be overlooked, is a fact that samples used in stopped-flow experiments were much more diluted (0.5% of hematocrit) than samples needed for coulter counter (50% of hematocrit). In order to compare influence of our compounds on RBCs as in stopped-flow experiments, we would have to incubate the cells with concentration hundred times higher than IC_{50} values (10000 μM) in order to keep the same relation (number of cells to compound mass) before coulter counter measurements. On one hand we could not use such high concentration of compounds due to their scarce availability and solubility; on the other hand samples for coulter counter experiments are

not possible to dilute more because the device is not sensitive enough to detect RBCs or their volume change in highly diluted samples.

However, a positive aspect from volume measurements is that at IC_{50} or $IC_{50}\times10$ we observed no alteration in the initial cell volume. Normally, when incubating hRBCs with our compounds before stopped-flow experiments in high concentration as $IC_{50}\times10$ (room temperature) hemolysis would be induced, but in coulter counter hemolysis did not occur. It means the cells were healthy and the cause of the decreased permeability in the stopped-flow is really the effect of compounds.

5. CONCLUSIONS

Summarizing all the data gathered, choline family is the most promising one of the three compound families, investigated in this study. NAP compounds are not promising, however we only tested four compounds of this family, which may have not been enough to reach a certain conclusion. In addition, methanol itself may have inhibitory effect on aquaporin channels as seen in comparison of control solutions which establishes influence of the solvent as an important determinant when investigating inhibition of new compounds on aquaporins.

Set of oxazolone compounds have not proven themselves as potential, with an exception of one single compound, OXA003. The main obstacle in this family of compounds represented DMSO as a solvent, which has a big influence on membrane permeability even in control solutions. Even few signals were not possible to obtain, which may have been a consequence of DMSO or toxicity of compounds. Nevertheless, also OXA003 in incubation time of 30 min, which was chosen as incubation time for all compounds, induced hemolysis at concentration of 50 μM, therefore we had to shorten the incubation time to 10 min. Given these considerations, OXA003 is probably not the best candidate to proceed with.

The choline family is the most promising, mainly the compounds FS6, FS10, FS12 and FS72. All compounds are soluble in water, which is a great advantage not only for experiments but also for further research in biological systems. The above mentioned four compounds show inhibitory effect on AQP3 in 70-80%, of which three, namely FS10, FS12 and FS72, cause also a slight inhibition of AQP1 in 20-30% (FS6 and FS10 at 500 μM, and FS12 and FS72 at 250 μM concentration). The smaller effect obtained on water permeability is because water can pass through the lipids in addition to the AQP1 channel; a reduction of AQP1 would be less detectable because water is still crossing the lipid bilayer, and we are looking at the total water permeability, AQP1 plus lipids. Whereas for AQP3, the lipids are impermeable to glycerol and we are only seeing glycerol through AQP3. The inhibition of AQP3 is immediately seen as a decrease in glycerol permeability.

Based on results of IC_{50} for glycerol permeability, all four compounds have quite similar values, between 100 and 200 μM.

Comparing the structures of compounds showing inhibitory effect, we can see a presence of certain similar features (a long hydrophobic tail, hydroxyl groups) which may be useful in further studies when synthesizing new compounds or searching for a lead compound.

Volume measurements in coulter counter showed that compounds caused no damage to the hRBCs in tested concentrations and no changes in initial cell volume were detected. Therefore, signals of decreased water and glycerol permeability in stopped-flow were truly induced by selected compounds.

To confirm the inhibitory effect of these compounds we would have to pursue them into further studies, starting experiments on cell cultures, researching effectiveness in tumor cell lines and proceeding with the evaluation of their potential and efficiency in observations *in vivo*.

A lot of questions appear regarding my research work, including the mechanisms of action and technological development of proper pharmaceutical form for tested compounds. All these inquiries remain to stay unanswered and may be topics for future work.

In conclusion, there are a lot of challenges still waiting before we discover highly selective and non-toxic AQP inhibitor with high affinity for a particular isoform of AQP. My thesis, presenting some definite results, is one step forward in approaching the goal and can offer suggestions for further investigation.

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