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Aquaporin Water and Solute Channels from Malaria Parasites and Other Pathogenic Protozoa

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Search for Novel Antimalarial Drugs: Shift to the Parasite–Host Interface

Current and former treatments of malaria are aimed at intracellular targets of the plasmodial cell. The major principle of action of antimalarial chemotherapeutics such as chloroquine[1] is to inhibit heme polymerization in the digestive food vacuole. Heme is a product of hemoglobin degradation by the parasite, and if it is not detoxified by polymerization, it will result in the generation of reactive oxygen species that irreversibly damage the cell. Other compounds such as pyrimethamine and cycloguanyl block folate biosynthesis in the parasite's cytosol.[2] Promising clinical trials have been conducted with fosmidomycin,[3] an inhibitor of deoxyxylulose 5-phosphate reductoisomerase that blocks isoprene biosynthesis in a parasite-specific organelle, the apicoplast.[4] Other recent approaches include inhibitors of plasmodial enzymes: farnesyl transferases[5] and hemoglobin-degrading proteases.[6]

Despite the availability of potent compounds, however, there remains the challenging problem of overcoming the selection for and spread of drug-resistant parasite strains. Drug resistance in plasmodia usually evolves by an accumulation of mutations in target enzymes which decrease the affinity of inhibitors such as pyrimethamine,[7] or by mutations that render drug-export proteins hyperactive. The latter mutations thus keep the intracellular drug levels at subtoxic concentrations which, for example, is the mechanism of chloroquine resistance.[8] Recently, parasites have been identified that exhibit resistance against artemisinin,[9] which is currently the last treatment option for cases of multidrug-resistant plasmodia. Thus, a novel and promising approach may be to attack the parasite cell from the outside, that is, at the interface with the host.[10]

The parasite–host interface is made up of channels and transporters for 1) the establishment of ionic and pH gradients,[10] 2) the import of nutrients and biosynthetic precursors such as glucose[11] and glycerol,[12] and 3) the export of waste metabolites such as lactate.[10] Blockage of such processes should severely affect the parasite by deprivation of its energy supply or by a build-up of cytotoxic metabolites.

Genome analyses of various *Plasmodium* species indicate that the parasite–host interface is limited to only the essential components, probably as a result of an enormous selective pressure that demands the minimum number of antigenic surface structures.[13] For instance, only six members of the major facilitator superfamily for various nutrients and metabolites have been identified in the *Plasmodium* genomes, whereas similarly complex yeast strains express 60–80 such channels.[13] A member of this subset of *P. falciparum* facilitators is a single aquaglyceroporin (PfAQP), a channel protein for water and small uncharged solutes.[12]

Potential Physiological Functions of Protozoan Aquaporins

Although water and solute channels constitute an ancient protein family for which more than 450 isoforms from all kingdoms of life have been described,[14] it was not before the early 1990s that their first member, human aquaporin 1 (AQP1), was discovered.[15] Functionally, the aquaporin protein family is divided into two major subfamilies: water-specific channels (orthodox aquaporins) and channels that are also permeable to small uncharged solutes, such as glycerol and urea (aquaglyceroporins). Aquaporin-facilitated diffusion is driven by an osmotic or chemical gradient.[16]

Physiological functions of water and solute channels have been intensively studied in humans.[17] Accordingly, human aquaporins are critically involved in the regulation of bodily water homeostasis (AQP1–4), lung moistening (AQP1), tear and saliva secretion (AQP5), and body glycerol and fat metabolism (AQP7). The study of knockout mice revealed additional and quite unexpected roles for which a relation to water or solute facilitation is not directly evident;[18] These include the maintenance of eye lens transparency (AQP0), tumor angiogenesis (AQP1), wound healing (AQP3), and neural signal transduction (AQP4). Hence, aquaporins are considered as attractive drug targets,[19] and compounds that modulate aquaporin function could be used as diuretics (aquaretics), and as treatments for edema, glaucoma, epilepsy, obesity, and cancer.[18] However, potent and specific inhibitors for human aquaporins are not yet available.

The role of water and solute channels in unicellular human pathogenic parasites is less well established. Nevertheless,

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based on their permeability properties and expression profiles during development, the metabolic activity of the parasite, and the changing ionic, osmotic and nutritional conditions provided by the human and insect hosts, highly individual scenarios are emerging for different parasites.

Plasmodia and *Toxoplasma*

Parasites of the phylum *Apicomplexa* are strictly intracellular and thus live in a well-balanced environment. Malaria parasites (*Plasmodium* spp.) invade liver cells and erythrocytes,[20] whereas toxoplasmosis parasites (*Toxoplasma gondii*) infect a broad range of cells.[21] Both parasites express a single aquaglyceroporin with intermediate (*T. gondii*, TgAQP[22]) to excellent (*P. falciparum*, PfAQP[12]) water permeability and good glycerol permeability (Table 1). The solute-permeability profile of both aquaglyceroporins is particularly well characterized and includes nitrogen metabolites, polyols of up to five carbon atoms in length, carbonyl compounds, and arsenite (Figure 1 A). Charged compounds, including protons and longer or cyclic polyols, do not pass (Figure 1 B). PfAQP is constitutively expressed at all developmental stages.[12]

Table 1. Water and solute permeability of protozoan aquaporins.

Species	Isoform	P_f [$\mu m\,s^{-1}$][a]	P_s [$\mu m\,s^{-1}$][b]	Reference
P. falciparum	PfAQP	276	0.91	[12]
T. gondii	TgAQP	108	0.86	[22]
T. brucei	TbAQP1	172	1.02	[24]
	TbAQP2	139	1.09	
	TbAQP3	164	0.95	
T. cruzi	TcAQP	30	0	[28]
	TcAQPβ	n.d.	n.d.	
	TcAQPγ	n.d.	n.d.	
	TcAQPδ	n.d.	n.d.	
L. major	LmAQP1	n.d.	n.d.[c]	[31]
	LmAQPα	n.d.	n.d.	
	LmAQPβ	n.d.	n.d.	
	LmAQPγ	n.d.	n.d.	
	LmAQPδ	n.d.	n.d.	
E. cuniculi	EcAQP	87	0	[34]

[a] Permeability to water; n.d. = not determined. [b] Permeability to glycerol. [c] Permeability to $Sb(OH)_3$ was observed.

Apicomplexan aquaporins have been proposed to play physiological roles in 1) the protection of the parasites from osmotic stress during kidney passages or during transmission between human and insect hosts,[12] 2) glycerol uptake as a precursor for membrane lipid biosynthesis,[12] 3) the mitigation of oxidative stress by increasing the $NADH/NAD^+$ ratio,[12] and 4) the release of toxic metabolites.[23] Apicomplexan aquaglyceroporins seem to be highly integrated multifunctional channels. Consequently, inhibition may severely affect parasite proliferation.

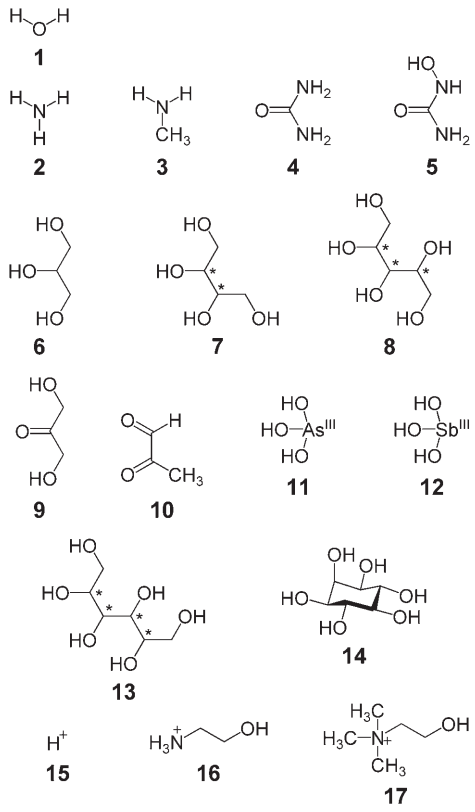

Figure 1. Compounds used in permeability assays of aquaglyceroporins. A) Permeants: water **1**, ammonia **2**, methylamine **3**, urea **4**, hydroxyurea **5**, glycerol **6**, C_4-polyols **7** such as erythritol, C_5-polyols **8** such as D-arabitol, ribitol and xylitol, dihydroxyacetone **9**, methylglyoxal **10**, arsenite **11**, and antimonite **12**. B) Impermeants: C_6-polyols **13** such as mannitol and sorbitol, myoinositol **14**, protons **15**, ethanolamine **16**, and choline **17**.

Trypanosoma brucei

Parasites that cause African sleeping sickness swim freely in the host's blood. They encounter osmotic stress during each kidney passage, during transmission, and especially inside the gut of the tse-tse fly vector, which may call for a functional water channel that allows membrane-protecting water exchange.[24] In addition, an efficient glycerol exit pathway is vital to the parasite. This is due to a unique glucose metabolic pathway in a parasite-specific organelle: the glycosome. Here, under anaerobic conditions, glucose is converted into equimolar amounts of glycerate 1,3-bisphosphate and glycerol phosphate. The phosphate moiety from the latter metabolite is then transferred to ADP to form ATP through catalysis by glycerol kinase.[25] This energetically unfavorable reaction can only be maintained when dephosphorylated glycerol is readily removed from the glycosome by facilitated diffusion. Indeed, the application of 5 mM external glycerol leads to accumulation of glycerol in the glycosome, arrest of ATP generation, and cell death.[26] Accordingly, aquaglyceroporin inhibitors that prevent glycerol from diffusion out of the glycosome would have great potential as a treatment for sleeping sickness.

The *T. brucei* genome encodes three aquaglyceroporins (TbAQP1–3) with good water and glycerol permeability[24] (Table 1). Expression profiling has shown that TbAQP1 predom-

inates at the transition between human and insect parasite forms, TbAQP2 is constitutively expressed in the parasite, and TbAQP3 is expressed only in blood-stage parasites in the human host.

Trypanosoma cruzi

A peculiar mechanism to cope with hypo-osmotic stress has been proposed for the causative agent of Chagas disease, *T. cruzi*, a protozoan from the same order (*Kinetoplastida*) as *T. brucei*. This involves vacuolar H^+ ATPases and aquaporins in a two-compartment contractile system.[27] H^+ ATPases generate an electrochemical gradient in one vacuole of the complex which drives water influx through the aquaporin water channels.[28] The second vacuole fuses periodically with the plasma membrane to release water. Consequently, the cell volume is decreased.[27] However, water permeability of TcAQP, an aquaporin that is associated with the contractile system, is surprisingly low, as determined in an in vitro assay based on *Xenopus* oocytes[28] (Table 1). It is possible that analogous to human AQP6, which is expressed concomitantly with H^+ ATPases in cytosolic vesicles of kidney intercalated cells,[29] acid is needed to gate the pore, or that another as-yet uncharacterized isoform (TcAQPβ-δ,[30] Table 1) is the actual water channel of the system.

Leishmania

Antimonials are still the first-line treatment of leishmaniasis.[31] The active compound in vivo is thought to be Sb^{III} after reduction of the applied Sb^{V} in the form of sodium stibogluconate or meglumine antimonate. The aquaglyceroporin LmAQP1 (Table 1) is directly linked to drug resistance of *Leishmania major* parasites.[31] Loss of the LmAQP1 gene as a result of natural selection or targeted gene knockout renders the parasites 10-fold more resistant to Sb^{III} treatment. Overexpression of the gene, in turn, leads to sensitization of the parasites to Sb^{III}, and can decrease EC_{50} values by two orders of magnitude.[31,32] This identifies LmAQP1 as a major influx route for trivalent antimony into *Leishmania* parasites and thus, as the first aquaglyceroporin that facilitates the uptake of a drug into a cell.

Based on sequence comparisons, the LmAQP1 protein is expected to be a typical solute channel.[30] However, this still needs to be directly established. The remaining four aquaporin genes in the *L. major* genome (LmAQPα-δ) are not yet characterized in terms of their permeability profile[32] (Table 1).

Microsporidia

Contrary to the apicomplexan and kinetoplastid protozoa described above, parasites from the phylum microsporidia are not actively transmitted by a vector, but in the form of an infectious, environmentally resistant spore.[33] Upon contact with a putative host, the spore extrudes a polar filament (germination) and inoculates its contents into a host cell. The current view holds that the germination process is initiated by the cleavage of disaccharides, resulting in an increase in intrasporal osmolarity.[34] Subsequently, the influx of water, most likely facilitated by aquaporins, leads to the extrusion of the filament.[35] A single aquaporin was recently identified in the genome of the microsporidia *Encephalozoon cuniculi* and characterized as a water-specific channel[33] (Table 1). It is envisioned that aquaporin inhibitors may block germination and provide a novel regime in the treatment of microsporidia infections.

Orthodox Aquaporins versus Aquaglyceroporins

Despite different permeants and evolutionary adaptations, all aquaporins share a common architecture, as deduced from sequence analysis and structure elucidation by cryoelectron microscopy and X-ray crystallography (Table 2).

Table 2. Available aquaporin structural data.

PDB Code	Isoform	Type	Resolution [Å]	Comment
1FQY	AQP1, human	water	3.8	–
1H6I	AQP1, human	water	3.54	–
1IH5	AQP1, human	water	3.7	–
1J4N	AQP1, bovine	water	2.2	–
1FX8	GlpF, *E. coli*	glycerol	2.2	with substrate
1LDA	GlpF, *E. coli*	glycerol	2.8	–
1LDF	GlpF, *E. coli*	glycerol	2.8	W48F, F200T mutations
1LDI	GlpF, *E. coli*	glycerol	2.7	–
1RC2	AQPZ, *E. coli*	water	2.5	–
2ABM	AQPZ, *E. coli*	water	3.2	open/closed state
1YMG	AQP0, human	water	2.24	–
2B6O	AQP0, human	water	1.9	closed state
2B6P	AQP0, human	water	2.4	open state
2C32	AQP0, human	water	7.01	loosely packed 3D crystals
2EVU	AQPM, *M. thermoautotrophicum*	glycerol	2.3	–
2F2B	AQPM, *M. thermoautotrophicum*	glycerol	1.68	–

Accordingly, aquaporins form homotetramers in the cell membrane with one individual pore in each monomer; unlike ion channels, the center of the quaternary structure is impermeable. The channel-like pore interior is 20 Å long, and is characterized by a ladder of evenly spaced backbone carbonyl oxygen atoms ($\approx$3.2 Å, Figure 2A) in an otherwise hydrophobic environment[36] (Figure 2B). The carbonyl oxygen atoms act as hydrogen bond acceptors and guide water or solute molecules through the channel. The ladder is interrupted in the center of the channel by the side-chain amide nitrogen atoms of two invariant asparagine groups, which are part of two canonical Asn-Pro-Ala motifs (Figure 2A). Another feature is a positively charged arginine residue (Figure 2A) in juxtaposition with two aromatic residues (ar/R region) at the pore constriction (Figure 2C and D).

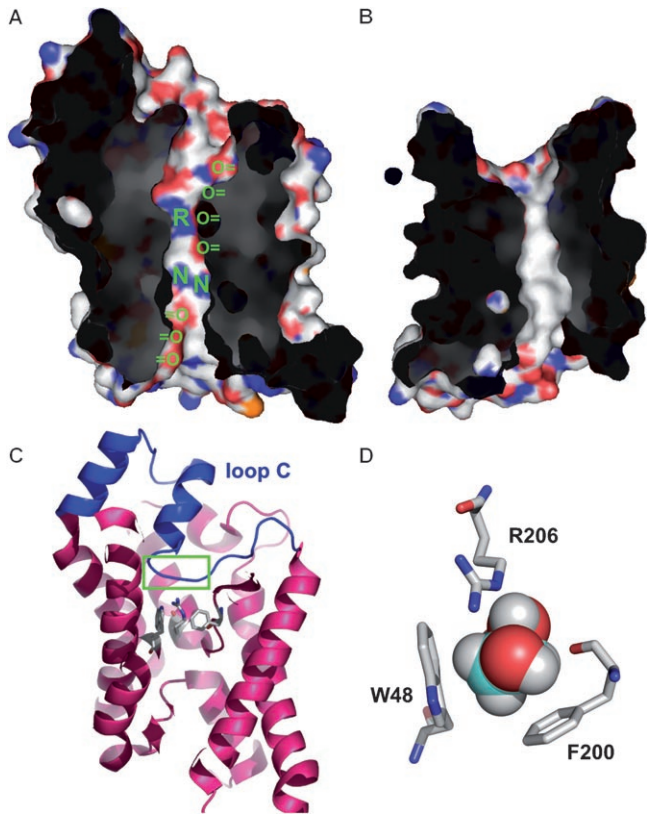

Figure 2. Crystal structure of the prototypical *E. coli* aquaglyceroporin GlpF. A) Section along the channel axis with view on the polar side. The extracellular space is on the top, the cytosolic side, at the bottom. The ladder of backbone carbonyl oxygen atoms is labeled (=O) as well as the conserved asparagine residues of the two central Asn-Pro-Ala motifs (N, see text) and the arginine at the ar/R constriction (R, see text). B) Section along the channel axis with a view of the opposite, lipophilic side. C) Diagram of the GlpF structure in the same orientation as shown in part A). Transmembrane spans 1 and 6 are omitted for a clear view of the ar/R constriction, shown in stick representation (Trp 48, Phe 200, and Arg 206). The extracellular connecting loop C is labeled in blue; the green box denotes the region of a conserved amino acid triad (Phe-Ser-Thr, positions 135–137) that is close to the ar/R region in aquaglyceroporins. D) View of the ar/R constriction from the extracellular side. Displayed is one of three glycerol molecules in the crystal structure that is located in the ar/R constriction (PDB code: 1FX8).

This channel architecture compensates almost ideally for the energetic cost of hydrogen-bond breakage for the isolation of a water molecule from bulk water as it enters the aquaporin pore. In fact, the activation energy (< 5 kcal mol^{-1}) and the diffusion rate ($> 10^9$ s^{-1}) for water passage through aquaporin is similar to that of a free water molecule in solution. At the same time, aquaporins employ highly efficient selectivity mechanisms against larger molecules and ions, including protons, to maintain the electrochemical gradient of the cell.[36]

The selectivity of orthodox aquaporins for water against larger molecules can easily be rationalized by size exclusion at the ar/R constriction, which is about 2.8 Å in diameter and perfectly matches the size of a water molecule.[37] Aquaglyceroporins have pore constrictions of about 3.5 Å[38] (Figure 2D) to accommodate the passage of glycerol and other uncharged solutes (Figure 1A). Indeed, our research group could show that the water-specific orthodox AQP1 can be turned into an aquaglyceroporin that allows passage of urea and glycerol by introducing point mutations that widen the pore diameter in the ar/R region.[39] This implies that the aquaporin channel interior is generally suitable for both water and solute passage and that the discrimination occurs at the ar/R constriction.

According to molecular dynamics/quantum mechanics simulations, the exclusion of charged compounds is based on a strong positive electrostatic field that emanates from the center of the aquaporin pore where the positive ends of two short α helices meet.[40] As the capping amino acids of these helices, the asparagine residues of both Asn-Pro-Ala motifs mark this site in the channel structure (Figure 2A) and build the peak of an energy barrier of about 15 kcal mol^{-1} for protons.[40] Another somewhat lower peak is located around the ar/R region. Exchange of the arginine at the ar/R constriction of mammalian AQP1 for valine results in proton leakage of the aquaporin mutant (≈ 50 H$^+$ s^{-1}) indicating that this region significantly contributes to the exclusion of protons.[39] The AQP1 R195V mutant is currently the only available aquaporin for the experimental study of proton conductance and respective exclusion mechanisms. In a first experiment, we compared proton permeation with that of deuterons.[39] In H_2O, the mobility of H^+ is high owing to the Grotthuss mechanism, which is based on rapid hydrogen-bond flipping rather than H_3O^+ diffusion.[39] In D_2O, the mobility of D^+ is 1.5-fold less than that of H^+ in H_2O, whereas the mobilities of H_3O^+ and D_3O^+ are similar. The proton currents observed through AQP1 R195V in acidic H_2O and D_2O solutions were equal, indicating that H_3O^+ and D_3O^+ are the respective permeating species.[39] This confirms the theoretical models of aquaporin proton exclusion that favor electrostatic repulsion over interruption of the Grotthuss mechanism.[40]

Connecting Loop C

A puzzling observation was the dramatic variation in water permeability of various aquaglyceroporins. For instance, the *E. coli* aquaglyceroporin GlpF has a water permeability that is two orders of magnitude lower than that of the *Plasmodium falciparum* aquaglyceroporin PfAQP. This cannot be explained by peculiarities in the pore layout itself, because the residues of the ar/R constriction (Figure 2D) are identical in both solute channels, and only two residues differ in the subsequent selective part down to the central Asn-Pro-Ala filter region. Mutation of the two varying amino acids, however, as well as mutations in the second sphere around the pore residues did not alter the water permeability of PfAQP.[36]

What, then, is the chemical basis of the excellent water permeability of PfAQP? Whereas the protein cores of GlpF and PfAQP appear to be highly similar, it is the connecting loops, in particular loop C (Figure 2C, in blue), that are markedly different.[36] In GlpF, this loop dips deep into the protein core. Here, an amino acid triad, Phe-Ser-Thr (positions 135–137), is located in close proximity to the ar/R constriction (Figure 3, top row). Generally, a Phe-Ser/Ala-Thr triad in loop C is well conserved among aquaglyceroporins from bacteria to humans.[36] The amino acid sequence at the corresponding site

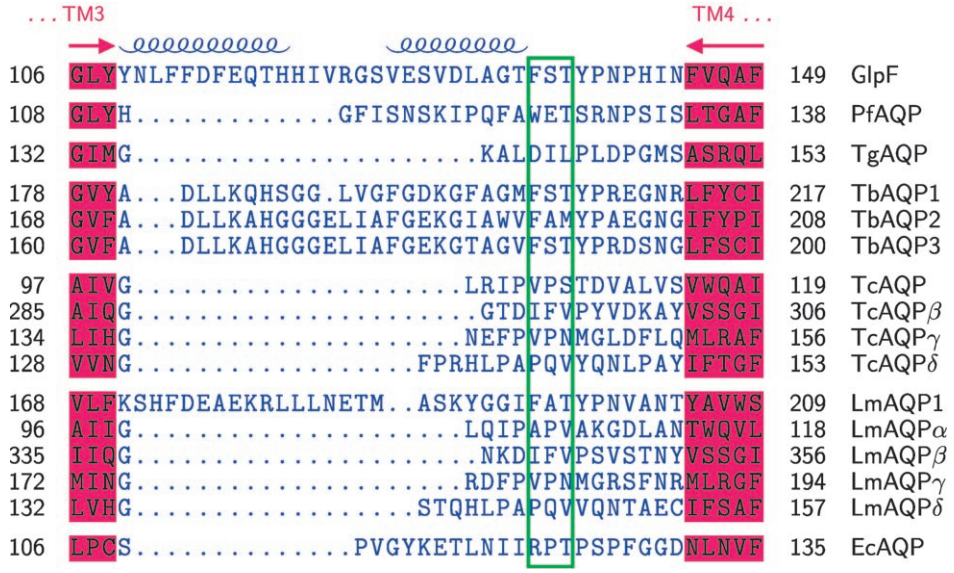

Figure 3. Section of a multiple sequence alignment showing the connecting loop C of various protozoan aquaporins. Parts of the adjacent transmembrane (TM) spans 3 and 4 are shaded red. Helical domains of the *E. coli* aquaglyceroporin GlpF are labeled above the sequence. The green box marks a conserved amino acid triad that was shown to be located close to the ar/R constriction in GlpF and PfAQP. The alignment was set and labeled with TeXshade.[41] Abbreviations: Pf = *Plasmodium falciparum*, Tg = *Toxoplasma gondii*, Tb = *Trypanosoma brucei*, Tc = *Trypanosoma cruzi*, Lm = *Leishmania major*, Ec = *Encephalitozoon cuniculi*.

in PfAQP, however, clearly deviates and carries a glutamate (position 125) instead of a serine in the center of the triad (Figure 3, second row). Introduction of point mutations at position 125 indeed modulated PfAQP water permeability (E125Q, E125D; 50% decrease) or decreased it to the background level of the oocyte assay (E125S). However, glycerol permeability was only marginally affected in all mutants.[36] Together, the data show that the connecting loop C is part of the aquaglyceroporin water/solute selectivity filter.

As a structure that spans the extracellular side of an aquaporin, loop C markedly shapes the protein surface. Furthermore, as a result of the dip into the pore at the ar/R site, loop C forms a vestibule at the outer channel mouth (Figure 2A). Sequence comparison of aquaporin loops C from human pathogenic parasites reveals a high degree of variability (Figure 3). The length deviation already indicates major structural differences. Only the *T. brucei* aquaporins and the *L. major* aquaporin 1 possess enough residues to assume a fold similar to the *E. coli* aquaglyceroporin. Further, these protozoan isoforms contain the Phe-Ser/Ala-Thr motif, which clearly shows their relation to GlpF. The remaining protozoan aquaporins carry much shorter loops C without a Phe-Ser/Ala-Thr motif and any clear sequence similarity (Figure 3). Such structural specificities could possibly be used for the design of selective inhibitors.

Current Status

Human pathogenic protozoan parasites are heterogeneous in various aspects such as vector selection, life style, metabolism, and assembly of the parasite–host interface. They express between one and five aquaporins, mostly aquaglyceroporins, in a developmental-stage-dependent manner. The aquaporins appear to be involved in osmotic protection and in lipid and glucose metabolism. Structurally, protozoan aquaporins exhibit differences in the extracellular connecting loop C, which is part of the selectivity filter for water and solutes and which forms a vestibule at the pore entry.

However, the study of protozoan aquaporins in terms of suitability as novel targets for chemotherapy is still at the stage of target evaluation. Parasite knockout strains are needed to test the role of aquaporins in proliferation and infection; furthermore, the elucidation of protozoan aquaporin protein structures is a prerequisite to start a medicinal chemistry approach with the aim to design inhibitors. Respective studies are underway.

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