Metallodrugs as protein modulators
Batista de Almeida, Andreia Filipa

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A4. Exploring the gating mechanisms of aquaglyceroporins: new clues for inhibitors design?

This chapter is adapted from two submitted manuscripts:

Andreia de Almeida*, Ana P. Martins*, Andreia Mósca, Hein J. Wijma, Catarina Prista, Graça Soveral, Angela Casini
Exploring the gating mechanisms of aquaporin-3: new clues for inhibitors’ design?
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Human aquaporin-7 expressed in yeast reveals pH gating
(submitted)

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4.1. Introduction

Aquaporins (AQPs) are a family of small membrane proteins belonging to the Major Intrinsic Protein (MIP) superfamily. They are expressed in almost every organism and are involved in the bidirectional transfer of water and small solutes across cell membranes in response to osmotic and hydrostatic pressure gradients, having important roles in physiology and homeostasis.

The presently known mammalian AQPs (AQP0-AQP12) can be divided into three groups according to their primary structure and permeability [1]: i. orthodox or classical AQPs, considered to be water selective (AQPs 0, 1, 2, 4, 5, 6 and 8). Although AQP6 has been proved to be a pH-sensitive chloride channel or possibly a nitrate channel and AQP8 has been found permeable to urea, they are both classified under the classical AQP group; ii. aquaglyceroporins (AQP3, 7, 9, 10) permeable to glycerol and other small solutes, in addition to water; and iii. superaquaporins (AQP11, 12), with lower sequence homology to the other mammalian AQPs and unique subcellular localization [2], whose permeability specificity has been difficult to establish, although AQP11 was recently reported to transport both water and glycerol [3].

AQPs are considered crucial to maintain the water homeostasis in many epithelia and endothelia involved in fluid transport [4]. In addition, due to their unique ability to transport glycerol, aquaglyceroporins have important roles in glycerol metabolism in non-fluid transport tissues such as in skin, fat and liver [5-7]. In particular, AQP7, a member of aquaglyceroporin subgroup, has been associated with the adipose tissue, where is the major source of glycerol efflux from adipocytes [7-9] but it is also involved in maintenance of sperm quality and motility in human...
testis [10]. Remarkably, AQP7 is also found in cardiac tissue, where information about the role of aquaporins is scarce [11]. A recent study reported that glycerol transported intracellularly by AQP7 could be used as an alternative substrate for energy production in cardiomyocytes, under cardiac stress [12].

Interestingly, AQP3s, as many other channels and transporters, can be subjected to regulation. Indeed, it has been shown that the function of various eukaryotic water selective aquaporins are regulated by post-translational modifications, such as phosphorylation [13-16], pH [17, 18], divalent cations [19, 20], or membrane surface tension [14, 21-23].

Less is known about the regulation of aquaglyceroporins and glycerol transport. Only a few studies have shown regulation of permeability of AQP3 by pH, copper and nickel [24-26], indicating that aquaglyceroporins may also be subjected to pH gating. In general, we believe that a better understanding of human aquaglyceroporin regulation in biological environments would facilitate the identification of mechanisms of water/glycerol fluxes modulation, which may lead to the design of novel inhibitors with potential therapeutic applications for human health.

The other gated mammalian aquaporins, AQP0, expressed in the lens, and AQP6, expressed in the intercalated cells of the kidney collecting ducts, appear to have low permeability at physiological pH, increasing below pH 7 and with a maximum of permeability at about and below pH 6.5, respectively [20, 27]. On the other hand, rat AQP3 showed the opposite behavior, with an overall maximum of permeability for water and glycerol above pH 6.5, decreasing with lower pH, until completely closed at pH 5 [28, 29]. As AQP0 and AQP6 are both orthodox aquaporins and AQP3 permeates glycerol, these differences may be correlated to protein function.

In this context we decided to focus our investigation on two isoforms: AQP3 and AQP7. AQP3 has a wide tissue distribution in the epithelial cells of kidney, airways and skin, suggesting a role in water reabsorption, mucosal secretions, skin hydration, and cell volume regulation [30]. Moreover, recent studies demonstrated an aberrant AQP3 expression in tumor cells of different origins, particularly aggressive tumors [31], suggesting this enhanced protein expression to be of diagnostic and prognostic value. On the other hand, AQP7 is expressed in tissues related to metabolism, such as liver and adipocytes, which indicates an important role of this isoform in energy metabolism [32].

Thus, in collaboration with the group of Prof. G. Soveral (University of Lisbon) two different models were used: i) human red blood cells (hRBC), expressing human AQP3, and ii) yeast cells expressing hAQP7 and rAQP3 - to investigate the pH gating of the two aquaglyceroporin isoforms.

It is worth mentioning that due to its intrinsic low water plasma membrane permeability, yeast has been reported as a suitable system for heterologous individual aquaporin expression [33]. Yeasts are easily and permanently transformed (an advantage to transient transfection in *Xenopus laevis* oocytes and mammalian cell lines), have short doubling times, can be maintained in diverse environments and even the composition of the intracellular medium can be controlled, creating a variety of conditions to study aquaporin regulation [18, 21, 23]. On the other hand, hRBC have been shown to be a very good natural model to assess AQP3 function since they only express one orthodox aquaporin (AQP1) and one aquaglyceroporin isoform (AQP3) [34].

Therefore, we selected *Saccharomyces cerevisiae* strains, silenced on endogenous orthodox aquaporins (Aqy1 and Aqy2) to overexpress hAQP7. In our experimental conditions, the endogenous aquaglyceroporins YFL054c and Fps1 are inactive since the first does not seem to
mediate glycerol entry and the second is in a closed state induced by high external osmolarity in the permeability assays [35]. In addition, we used hRBC to assess hAQP3 function. Water and glycerol permeability was assessed in both cases by stopped flow spectroscopy. It is worth mentioning that the activity of heterologous aquaporins anchored in the membrane of aquaporin-null strains has been powerfully characterized by this method [18, 23, 36]. Moreover, the use of a fluorescence self-quenching methodology for assessing cell volume changes in yeast walled cells [37] induced by solute gradients represents an added value for the study of aquaglyceroporins. This methodology opens new perspectives to measure aquaglyceroporin activity in minimally disturbed cells that are quite stable during a rather long experiment.

Overall, we were able to unravel for the first time the gating of hAQP7 by external pH, to confirm previously reported studies on rAQP3, and to study the gating of glycerol transport in hAQP3, described here for the first time. Our results show that all three isoforms (hAQP3, rAQP3 and hAQP7) are gated by pH, important differences are observed among AQP3 and AQP7 isoforms. Through molecular modeling studies, we investigated the pH dependent closure/opening of both human channels at a molecular level, allowing us to predict gating mechanisms of the two isoforms and possibly other aquaglyceroporins. The obtained results are discussed in terms of the putative physiological roles of pH gating in aquaglyceroporins.

4.2. Results and Discussion

4.2.1. pH gating of AQP3

Rat aquaporin-3 (rAQP3) has been described to be gated by pH, when expressed in Xenopus oocytes [29] and other authors identified the key residues for pH gating in human AQP3 [28]. However, the details of the pH gating of the human isoform of AQP3 are not known, as well as there is no information if the gating affects the glycerol transport, in this isoform, in the same manner. Thus, we started by evaluating the gating of rat AQP3, in a yeast cell model, well characterized by our group [36], followed by the characterization of the pH gating of human AQP3, in a human red blood cell model (hRBC), also well characterized by us as a model for AQP3 function [34].

Following the evaluation of AQP3 function upon pH changes, we used in silico approaches to investigate, in detail, the mechanism of pore closure.

4.2.1.1. pH gating of rat AQP3 in yeast

In our study we first evaluated rAQP3 gating, in a yeast model, using stopped-flow spectroscopy. Functional aquaporin studies, performed using heterologous expression of aquaporins in an aqp-null strain of Saccharomyces cerevisiae, have been previously described by our group [38]. This yeast strain expresses also two endogenous aquaglyceroporins, which were not silenced: Fps1 and YFL054c. Fps1 is crucial for yeast osmotic adaptation being inactivated within seconds after a hyper-osmotic shock to ensure intracellular retention and accumulation of glycerol [39]. Thus, in our experimental conditions it remains in a closed state induced by high external osmolarity in the permeability assays. Additionally, YFL054c is not permeated by glycerol under normal conditions or when subjected to hyper or hypo osmotic stress [35]. Moreover, deletion of the two aquaglyceroporins can cause changes in cell membrane content, lead to cell wall stress and
increased temperature sensitivity, which could influence the output in our experimental setup.[35] Therefore, we optimized an expression system where only the orthodox aquaporins were silenced.

Yeast cells were transformed with either the empty plasmid (control cells) or the plasmid containing the rat AQP3 gene (mentioned as rAQP3 cells, for clarity). The expression of rAQP3 in the S. cerevisiae model was assessed by fluorescence microscopy, using GFP tagging. In transformed cells, rAQP3-GFP is localized at the cellular membrane, while cells with empty plasmid have a homogeneous cytoplasmic distribution (Figure 1).

Figure 1. Localization of GFP-tagged rAQP3 expressed in S. cerevisiae aqy-null strain. Epifluorescence (left panel) and phase contrast (right panel) images of S. cerevisiae aqy-null strains transformed with rAQP3.

Stopped-flow technique allows volume monitoring of cells subjected to hypo and hyperosmotic stress: when cells are exposed to hyperosmotic shock with impermeant solutes, water outflow induces cell shrinkage. Conversely, when the osmotic shock is provided by a permeable solute as glycerol, cells first shrink due to water outflow and afterwards swell again due to glycerol passage. Thus, water and glycerol permeability is then evaluated according to cell swelling or shrinkage monitored by 90° light scattering, detected by the stopped-flow. In the case of the yeast cell model, the cells are pre-loaded with carboxyfluorescein, and the fluorescence intensity reflects volume changes.

At first, in order to evaluate if the observed effect was due to AQP3 being expressed in the yeast cells, both groups of control and rAQP3 were incubated at two different pH values, namely pH 5 and 7. These pH conditions were chosen based on previous literature [28, 29], to have closed (pH 5) and open (pH 7) AQP3.

In Figure 2 the water and glycerol permeability (P_f and P_gly, respectively) of control and rAQP3 cells are shown. It is possible to observe that, while control cells have no glycerol permeability, they do show basal water permeability at both tested pH conditions, due to the intrinsic water permeability of the membrane lipid bilayer. Interestingly, from panel C of Figure 2 it is evident that, at pH 5, there is no permeation by glycerol, with a significant increase at pH 7, which clearly demonstrate the close and open states of rAQP3.

Since the control cells present a basal water permeability that is not altered by the expression of rAQP3 when incubated at pH 5, it is possible to normalize the P_f that corresponds to the permeability of rAQP3 alone. Knowing from these results (Figure 2) and previous studies that hAQP3 is in a closed state at low pH (ca. 5),[29] the normalized water permeability via rAQP3 was obtained by subtracting the permeability values of control cells at each pH value. For P_gly, this subtraction was not necessary since the control cells show no glycerol permeability at any pH. The
A4

rAQP3 permeability for both water and glycerol are shown in Figure 3. We can observe that the channel is closed for both water and glycerol between pH 5 and 6 and has maximum permeability at pH 6.5 (glycerol) and pH 7 (water), respectively. This behaviour and Hill slope values found for water and glycerol in the rAQP3 isoform (see Table 1) are similar to those reported previously [29].

4.2.1.2. pH gating of human AQP3

Afterwards, we evaluated hAQP3 gating in hRBC. hRBC co-express hAQP1 (selective for water) and hAQP3 (permeating water and glycerol) and thus both isoforms contribute for water permeability. Previous studies showed that human AQP1 is not gated by pH [20, 29], and thus any pH-dependent effect on hRBC water permeability would be due to individual gating of hAQP3. Knowing that pH does not influence water permeation via lipid bilayer nor via hAQP1, water permeability corresponding exclusively to hAQP3 was obtained by subtracting the total cell permeability at pH 5 (where...
AQP3 is in the closed state [28, 29]) from the total permeability at each pH value (Figure 4).

In accordance with previous studies [28, 29], we observed a maximum permeability for both water and glycerol between pH 6.5 and 7.5, and a decreased permeability and pore closure at lower pH, with the pore completely closed at pH 5.

The calculated pK\textsubscript{a} values for both water and glycerol were found to be approximately the same, ca. 6.1, with Hill slopes of about 2 and 4, respectively. While the pK\textsubscript{a} for glycerol permeability is in accordance with our data on rAQP3, the pK\textsubscript{a} value for water is slightly lower (6.1 vs 6.8). Notably, while the Hill coefficients vary from those calculated for rAQP3 - which may be due to both differences in protein sequence or in the selected cellular model - they have the same 2-fold difference (Table 1). It is worth mentioning that in spite of the strong sequence homology (ca. 95%) between the two isoforms (see Figure S3 in supplementary information) still the 5% difference in sequence may account for a different mechanism of inhibition, as will be discussed further.

Hill coefficients, as black boxes parameters, may be subjected to different interpretations. One explanation found in literature for this difference of half the value for water, when compared to glycerol, is based on the Eyring energy barrier model [40], and explained by the differences in activation energy (E\textsubscript{a}) of both solutes. Interestingly, the measured activations energies for water and glycerol in hRBC evidenced a two-fold value for glycerol permeability [41]. It was hypothesized that, as E\textsubscript{a} for water permeability is low, water molecules cross the channel by forming a single line of hydrogen bonds, while glycerol, with higher E\textsubscript{a}, and having three OH groups, will establish more hydrogen bonds than water molecules when passing through the channel [40]. In fact, such hydrogen bond network for both water and glycerol is evidenced by X-ray studies of the bacterial glycerol facilitator (bGlpF) channel [42] and of the Plasmodium falciparum isoform (pfAQP) [43] (Figure 5). Moreover, glycerol molecules have their OH groups pointing towards the hydrophilic side of the channel, favouring such hydrogen bond network. In the case of hAQP3, we can also observe this phenomenon in molecular dynamics (MD) simulations.[44] Remarkably, in this latter study the number of hydrogen bonds in the crystal structures, as well as in the MD simulations, is similar to the Hill slopes found by us for hAQP3 in hRBC, approximately 1.5 for water and 4 for

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### Table 1. pK\textsubscript{a} and Hill slope values for water and glycerol, of human and rat AQP3. Obtained by fitting the data presented in Figures 3 and 4.

<table>
<thead>
<tr>
<th>AQP3 variant</th>
<th>pK\textsubscript{a}</th>
<th>Hill Slope</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Water</td>
<td>Glycerol</td>
</tr>
<tr>
<td>rat</td>
<td>6.80 ± 0.15</td>
<td>6.40 ± 0.20</td>
</tr>
<tr>
<td>human</td>
<td>6.08 ± 0.01</td>
<td>6.12 ± 0.01</td>
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glycerol.

Although the importance of H-bonding interactions between substrates and amino acid residues inside the AQP3 channel cannot be underestimated, and certainly plays a role in determining the activation energies of each substrate, recent experimental findings from our groups on the pH gating of aquaglyceroporin-7 (hAQP7) (described in the following sections) indicate that the Hill slope is similar for water and glycerol. Therefore, other factors may influence the overall pH gating mechanisms of AQPs, in addition to the number of H-bonds between substrates and the protein channel.

A second explanation for the observed difference in Hill coefficient values is the amount of titrable residues inside the channel, as postulated previously by Zeuthen et al. [29] This theory is based on the possible competition between the H\(^+\) and glycerol molecules for the protonable side-chains. A phenomenon of non-competitive inhibition of glycerol binding, by two protons, has been observed in hRBC [45]. The limitation of this theory is the fact that the titrable residues would be located in the channel lining, where they could affect glycerol H-bond formation. Later work on aquaporin sequencing and structure showed that hydrophilic and hydrophobic sides constitute the aquaporin lining and few to no residues are actually titrable.

**Figure 5.** H-bond network of (A) water and (B) glycerol permeation. X-ray structure of bacterial glycerol facilitator (bGlpF) with water (A), pdb1LDA, and glycerol, pdb1FX8.

**4.2.1.3. Investigation of the pH gating mechanism of hAQP3 by molecular modelling**

In order to investigate the molecular mechanism of pH gating of hAQP3, a molecular modelling approach previously developed by our group was used [9]. However, in the case of the present study, a homology model of human AQP3 in the tetrameric form was built, instead of the monomeric form, based on the available structure of the bacterial glycerol facilitator (GlpF, pdb code 1LDI) [46]. The final model was obtained by averaging 50 individual models, using MOE software (MOE 2012.10; CCG 2012) [47], as described in the experimental section.

Analysis of the model shows the common fold, shared by the aquaporin family, containing six transmembrane helices and two half-helices, for each monomer. The two half-helices are located inside of the pore of each monomer and contain the typical NPA (Asp-Pro-Ala) motif that constitutes one of the aquaporin’s selectivity filters. The residues in these two NPA motifs are Asn83-Pro84-Ala85, and Asn215-Pro216-Ala217 (Figure S2 in supplementary information). Another selectivity filter, the narrowest part of the channel lining, is located near the extracellular entrance and is named ar/R SF (aromatic/arginine selectivity filter). This selectivity filter is an important structural feature of aquaporins, where the arginine is fully conserved in all mammalian aquaporins (Figure S2). The ar/R SF also serves as a distinctive feature among aquaporins, as the composition in aminoacids
may vary in water and glycerol channels: classical aquaporins have an ar/R SF formed by 4 residues, including, commonly a phenylalanine and histidine, while aquaglyceroporins’ ar/R SF comprises only three residues. Thus, these differences account for pore size and selectivity among aquaporin isoforms. All these features are observed in our model of hAQP3, where Phe63, Tyr212 and Arg218 constitute the ar/R SF (Figure S2).

According to the previously reported site-directed mutagenesis studies, the molecular mechanism behind the gating of AQP3 involves four titrable residues, namely His53, Tyr124, Ser152 and His154 [28]. However, the lack of structural information about this isoform led the authors only to speculate on the type of interactions these residues could possibly establish with unknown surrounding residues, based on possible similar behaviours of histidines, tyrosines and serines in enzymes. Using our homology model of the tetrameric form of hAQP3, it is possible to locate the pointed residues at the interface of the monomers, closer to the extracellular side of the protein (Figure 6). These residues may be involved in important monomer-monomer interactions and their protonation/deprotonation may affect the overall assembly of the tetramer and, consequently, of the water and glycerol permeability. In detail, at pH 7 in our model His53 is located at the central pore lining and its side-chain appears to have the possibility to form H-bonds with residues Thr58, Thr52 and Gln45 in the same monomer while interacting also with the aromatic ring of Phe56, located in an adjacent monomer. These interactions are different in each monomer. Interestingly, mimicking the protonation state of the protein at pH 5 leads to the formation of new H-bonds, namely a second H-bond with Thr52 (this time with its side-chain) Thr204, Gly51 and Thr62. The formation of the new H-bonds may cause loop A to move closer to the monomer pore and cause structural modifications in transmembrane-helix 5 (TM5).

On the other hand, Tyr124 does not appear to have a clear role or to be particularly sensitive to pH changes. Due to its very high pKₐ (typical range for a Tyr side-chain in proteins is 9-12 [48]), it is unlikely that its side-chain is affected by changes in the pH range from 5 to 8. In addition, the side-chain of Tyr124 appears to be pointing out in the direction of the membrane, not participating in any interaction with other residues. The only apparent interactions of this residue are between its backbone and the backbones of Trp128 and Phe120, contributing to the maintenance of the helical structure. Interestingly, at pH 5, in one monomer is possible to see the formation of a new H-bond with the backbone of Ile127. This cannot explain the influence of pH on Tyr124 and the possible changes it may induce.

Regarding Ser152 and His154, these residues are located in the region between two adjacent monomers. At pH 7, while the backbone of His154 is forming an H-bond with the backbone of Ser152, located in the same loop (Loop C), the His154 side-chain is forming an H-bond with the side-chain of His129, in opposite end of loop C of another monomer (Figure 6B).

At lower pH, the same interactions appear to be maintained and a new H-bond may be formed with the backbone of Gly153. The formation of this new bond in the same loop may weaken the interaction between the two histidines, leading to a movement of loop C towards the channel opening. This disruption, together with the above-described movement of loop A due to protonation of His53, may be the cause for blockage of the channel for water and glycerol permeability. This structural change of movement of loop C was also observed in the MD studies on mercury inhibition of hAQP3, which leads to a collapse of the ar/R SF [44]. This movement may not be simultaneous as, due to neighbouring amino acid side-chains, the pKₐ of His53 and His154
Figure 6. Homology model of tetrameric human AQP3. A: Extracellular top view of the tetrameric form of hAQP3 and position of residues involved in pH regulation. (B) Positions of His129 from monomer A and His154 from monomer D, as well as Ser152. The dashed blue line represents the H-bond formed between the two histidines at pH 7. C: Scheme of the interactions of His154 with neighboring residues, at pH 7, for each of the 4 monomers.

may be subjected to small variation, causing a gradual conformational change with pH decrease (or increase).

Previous studies by Zelenina et al. show that a mutation of His129 to an alanine residue does not affect water permeability or change the pH sensitivity range [28]. However, glycerol permeability was not measured and the contribution of this residue for the mechanism of inhibition of hAQP3 by pH, regarding glycerol permeability, cannot be excluded.

Interestingly, loop movement upon pH changes was also observed for the orthodox water channel bovine AQP0 (bAQP0). This isoform has a maximum of permeability similar to that of hAQP3 at pH 6.5, however it is closed at pH 8.5 [49]. The residues responsible for pH sensibility were identified by site-directed mutagenesis as two histidines: His40 and His122, in loops A and C, respectively. While His40 in bAQP0 is in a similar position as His53 in our model of hAQP3, His122 is in the position corresponding to Ser152 (and close to His154) in hAQP3 (Figure S4 in supplementary information). Overall, as described for bAQP0, we propose that key histidines in loops A and C that span the outer vestibule contribute to pH sensitivity in hAQP3. Moreover, insertion of two histidines in similar positions in hAQP1, a non pH-gated aquaporin, induced pH sensitivity in the same range as bAQP0 [49], further confirming the key role of these residues in pH gating.
As observed for the MD study on Hg$^{2+}$ inhibition of hAQP3, by Spinello et al. [44], the closed state of bAQP0 involves movement of a loop (in this case loop A) and a collapse of the ar/R SF (Figure S5, in supplementary information), shown in the X-ray structures of the open and closed bAQP0 [50]. This collapse in the SF appears to be different than the one described for hAQP3, most likely due to differences in amino acid composition and diameter of the channel. Mutations in the histidine of loop A - His40 in AQP0 [49] and His53 in AQP3 [28] - showed a shift of the pH sensitivity towards a more alkaline range. This effect supports the idea that the pK$_a$ of histidine residues in different regions of the same protein may be very different, leading to different levels of channel regulation.

Other studies reported that the orthodox water channel AQP4 also shows pH-sensitivity, which was recently attributed to a particular histidine residue, His95, predicted by in silico methodologies [51]. His95, located inside the channel and facing the intracellular side, is conserved in all aquaporins, including those that do not show pH-sensitivity, such as AQP1. Therefore, it is difficult to conclude that it is the only one responsible for the observed pH gating mechanism.

4.2.1.4. rAQP3 vs hAQP3

Molecular modelling was useful also to explain observed differences among the Hill slope values of hAQP3 with respect to rAQP3 (see Table 1). Human AQP3 shares a sequence identity higher than 80% with most mammalian AQP3 isoforms. Nonetheless, changes in key residues may change permeability and regulatory features. When compared human and rat AQP3 isoforms, although a sequence similarity of about 95% is observed, they do not share one of the residues that may be involved in the pH gating of the hAQP3, namely His129, which is substituted by an alanine (Figure 7). Even though this mutation does not seem to affect water permeability of hAQP3 [28], its effect on glycerol permeability is unknown. Moreover, a mutation of the same residue on a human and rat isoform may not have the same effect, as a network of hydrogen bonds is a very delicate system and is highly dependent on the neighbouring residues. Therefore, we can

![Figure 7. Sequence alignment of rat and human aquaporin-3. The black boxes highlight the pH-sensitive residues, while the grey box highlights His129 in hAQP3. * represents the residues that are not conserved in the two isoforms.](image-url)
only conclude that the mutation H129A is able to produce a functional rAQP3 glycerol and water channel. Additionally, it is possible that the differences in the observed pK_a and Hill slope for the human and rat isoforms are due to species differences, even though we cannot exclude the possibility of cell-model differences.

4.2.2 Gating of human AQP7

Following our studies on the gating of human AQP3, we proceeded by investigating the possible pH gating of another aquaglyceroporin, human AQP7. Just as hAQP3, hAQP7 also has an important role in human physiology, namely in glycerol metabolism in adipocytes and liver [32]. Thus, here we investigate the pH gating of hAQP7, expressed in a yeast model and following the same approaches as described in section 4.2.1. Even though both isoforms have similar permeability properties, their mechanism of gating may be different, as observed for other aquaporins [16, 52-54]. Thus, we investigated the molecular mechanism of pH gating, using in silico methodologies, and described, for the first time, the gating of hAQP7 by pH.

4.2.2.1. Cloning and heterologous expression of hAQP7

Expression and subcellular localization of hAQP7 in yeast transformants was confirmed by cytoplasmic fluorescence (Figure 8A), while yeast cells expressing hAQP7-GFP show membrane-localized fluorescence, confirming hAQP7 localization at the plasma membrane (Figure 8B). In addition, GFP-tagged hAQP7 was also observed in internal membranes, probably endoplasmic reticulum or in vesicles of the secretory pathway in an early stage of the protein trafficking to the cell membrane. Because proper folding of GFP fused to the C-terminus of a target protein depends on the correct folding of the latter, only folded fusion GFP will become fluorescent [55]. The observed fluorescence shows that GFP is properly folded.

Figure 8. Localization of GFP-tagged hAQP7 expressed in S. cerevisiae aqy-null strain. Epifluorescence (left panels) and phase contrast (right panels) images of S. cerevisiae aqy-null strains transformed with (A) the empty plasmid pUG35 and (B) hAQP7. Localization at the yeast plasma membrane is depicted for hAQP7-yeast strain.
4.2.2.2. Functional characterization of hAQP7 expressed in yeast

The activity of hAQP7 expressed in S. cerevisiae aqy-null strains was assessed through stopped flow fluorescence spectroscopy by challenging cells equilibrated in isosmotic solution at pH 7.4 (mammalian physiological pH) with sorbitol (impermeant solute, inducing water fluxes) or glycerol gradients.

As expected, in the case of sorbitol hyperosmotic shock, the relative cell volume decreases till cells reach a new osmotic equilibrium volume (Figure 9A). At low temperature (11 ºC), it is clear that hAQP7-expressing cells (named hAQP7 cells for clarity) show a faster volume equilibration (P < 0.05) with higher osmotic permeability coefficient P_f ((2.0 ± 0.1) x 10^{-4} cm s^{-1}) than cells transfected with the empty plasmid (named pUG35 cells) ((1.50 ± 0.08) x 10^{-4} cm s^{-1}). However, at higher temperatures (37 ºC), the two traces almost overlap and similar P_f values were obtained ((1.15 ± 0.07) x 10^{-3} cm s^{-1}) for hAQP7 cells and ((1.05 ± 0.09) x 10^{-3} cm s^{-1}) for pUG35 cells, P > 0.1). Such temperature dependent behaviour is better analysed in the Arrhenius plot (ln P_f vs 1/T) shown in Figure 9B, used to calculate the activation energy E_a for water transport, a valuable parameter indicating the contribution of protein-channels for permeation. Here, it is observed that at a higher temperature range (23 to 36 ºC) the E_a values are similar (15.2 ± 0.85 kcal mol^{-1}), while for the lower temperature range (11 to 23 ºC) E_a decreases (10.3 ± 0.74 kcal mol^{-1}) only for hAQP7.
expressing cells, unmasking the channel contribution to water permeation.

The undetectable increase in water transport and concomitant decrease in $E_a$ at a high temperature range can be explained by the relatively high contribution of the lipid bilayer to the total water permeation across the cell membrane. Conversely, at lower temperatures the lipid pathway becomes almost impermeable and water permeation through aquaporins can be discriminated. Thus, yeast cells expressing hAQP7 show a significant increase in $P_f$ in parallel with a 36% decrease in $E_a$ at low temperatures confirming the contribution of the channel pathway to water permeation [56].

Regarding glycerol permeability, a clear difference between the control pUG35 and hAQP7 strain is well recognised. Figure 9C shows the relative volume changes caused by glycerol gradients used to calculate $P_{gly}$ and $E_a$ for glycerol transport. Since glycerol is a permeant solute, upon a glycerol osmotic shock both water and glycerol fluxes take place concomitantly. After the initial cell shrinkage due to the faster water outflow in response to the initial osmotic gradient, cells re-swell caused by the glycerol influx in response to its chemical gradient. pUG35 and hAQP7 expressing cells show a large difference in the rate of volume changes. Using the $P_f$ value previously estimated for each yeast strain, the calculated $P_{gly}$ of AQP7 cells ($8.59 \pm 0.06 \times 10^{-7}$ cm s$^{-1}$) was eight-fold the control ($1.1 \pm 0.10 \times 10^{-4}$ cm s$^{-1}$). Accordingly, we obtained an $E_a$ value of 23.2 ± 3.1 kcal mol$^{-1}$ for control pUG35 cells and 11.5 ± 0.97 kcal mol$^{-1}$ for hAQP7 expressing cells (Figure 9D), thus confirming hAQP7 glycerol channel activity in the yeast expression system.

### 4.2.2.3. Inhibition of hAQP7 by Auphen

Previous studies by our group have detected the strong and selective inhibitory effect of the gold(III) coordination compound Auphen [Au(phen)Cl$_2$]Cl (phen=1,10-phenantroline) on hAQP3 and hAQP7 expressed in human erythrocytes and mammalian cultured cells [57-60]. To evaluate the inhibitory effect of Auphen on hAQP7 activity in our yeast model, water and glycerol permeability of pUG35 and hAQP7 strains were estimated at pH 7.4 in the presence and absence of the gold compound. Thus, yeast cells were incubated for 30 min with Auphen (at 70 µM, a concentration higher than that reported to saturate hAQP7 in adipocytes [60]) prior to the permeability assays and compared with non-treated cells. In the case of pUG35 control cells, Auphen did not induce any significant inhibition ($P > 0.1$) in water and in glycerol permeability (Figure 10A and 10B). Instead, for cells expressing hAQP7, a marked inhibitory effect of the compound (70 µM) was detected, with a decrease of 34% and 84% for $P_f$ and $P_{gly}$, respectively. It is worth mentioning that the measured permeabilities represent the total water or glycerol fluxes across the yeast cell membrane, i.e., through the lipid bilayer plus hAQP7 and yeast endogenous glycerol facilitators. To discriminate the fluxes across the hAQP7 channel, $P_f$ and $P_{gly}$ values of pUG35 control cells were further subtracted from those obtained for hAQP7 expressing cells ($P_{Channel} = P_{AQP7} - P_{pUG35}$) giving only the contribution of the hAQP7 channel. Therefore, it can be concluded that Auphen inhibited water and glycerol transport via hAQP7 by approximately 92% (as depicted in Figure 10C for the highest Auphen concentration).

A dose-response curve for $P_{gly}$ inhibition was obtained by incubation of cells with increasing concentrations of Auphen (from 0 to 70 µM) and showed a half-maximal effective concentration (EC$_{50}$) of 12.95 ± 0.35 µM (Figure 10C). Interestingly, this calculated EC$_{50}$ value is slightly higher
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than the one previously calculated for hAQP7 in the adipocyte model (6.5 ± 3.7 µM) [60]. This discrepancy may be due to differences in cell membrane constitution of each cell model, which may have an effect on Auphen’s uptake. In fact, as previously postulated [60] the most favourable Auphen binding sites in hAQP7 are methionine residues facing the cytoplasmic side of the channel, which implies that the gold compound needs to enter the cell before reaching its putative binding site in the protein channel. Moreover, we cannot exclude differences due to the distinct methodologies applied, i.e., stopped-flow for cells suspensions vs fluorescence microscopy in cultured adipocytes.

4.2.2.4. AQP7 permeability is dependent on external pH

In a second series of experiments, we investigated the pH dependence of hAQP7 permeability in the selected yeast model. Thus, water and glycerol permeability experiments were performed at 23 °C varying pH from 5 to 7.5. Permeability of the hAQP7 channel (P\text{Channel}) is represented in Figure 11A as a function of external pH. Remarkably, a strong pH dependence of both water and glycerol permeability was detected, showing that at pH 5 hAQP7 is completely inactive, while the maximal activity is reached when pH is raised to 6.5.

The pH dependence of hAQP7 channel activity was analysed by fitting the experimental data (P\text{W} and P\text{gly channel corrected values}) to a to a Hill equation from where the Hill coefficients and p\text{K}a values for channel gating were estimated. The obtained p\text{K}a value (corresponding to 50% channel activity) of 5.9 and the Hill coefficient around 3, suggest the involvement of 3 proton-binding residues or, alternatively, 3 protein subunits in the pH dependent gating mechanism.
Figure 11. pH dependence of hAQP7 activity. (B) Permeability normalized for each data set and expressed as % of their maximal values ($P_{\text{Channel},W}$ and $P_{\text{Channel},G}$). Hill coefficient = 3.09 and 2.89 and $pK_s$ = 5.87 and 5.9, for water and glycerol transport, respectively. (C) Activation energy $E_a$ of glycerol transport of hAQP7 (calculated as $P_{\text{Channel}} = P_{\text{AQP7}} - P_{\text{pUG35}}$) at pH 5 (17.4 ± 2.4 Kcal mol$^{-1}$), pH 6.5 (11.0 ± 1.5 Kcal mol$^{-1}$), and pH 7.5 (10.2 ± 1.2 Kcal mol$^{-1}$).

To further confirm the pH dependence of hAQP7 channel activity, the $E_a$ values for glycerol permeation were estimated at three distinct pH values (pH 5, 6 and 7.5) at which the channel is expected to be on fully closed and open configurations. As shown in Figure 11B, the $E_a$ for glycerol permeation significantly decreased from pH 5 (17.4 ± 2.4 kcal mol$^{-1}$) to pH 6.5 (11.0 ± 1.5 kcal mol$^{-1}$) and pH 7.5 (10.2 ± 1.1 kcal mol$^{-1}$), corroborating the proposed channel closure/opening transition.

The effect of pH on hAQP7 has been assessed in a recent study [61] that explored the kinetic features of hAQP7 expressed in Madin-Darby canine kidney (MDCKII) cells. By measuring cellular $^{14}$C-glycerol uptake in the range of pH 5 to 8, the authors did not observe any meaningful change in solute transport via hAQP7 activity although the uptake was significantly reduced at the lowest pH of 5, and thus concluded that $H^+$ was not important for AQP7 operation. Conversely, taking advantage of the yeast cell system to measure glycerol influx through hAQP7, we could demonstrate that pH-gating mechanisms take place for this isoform.

### 4.2.2.5. Investigation of the pH gating mechanism of hAQP7 by molecular modelling

As for hAQP3, we developed a homology model of hAQP7 in a tetrameric form (see detailed information in the methodology section), which allowed once more to visualize the common fold shared by the aquaporin family, comprising six transmembrane helices and two half-helices, with their N-terminal ends located in the center of the pore. These two half-helices contain distinctive central motifs, which in the case of hAQP7 are NAA (Asn94, Ala95, Ala96) and NPS (Asn226, Pro227, Ser228) instead of the classical NPA (asparagine-proline-alanine) sequence (Figure 13).

Moreover, three residues, Phe74, Tyr223 and Arg229, constitute the ar/R SF of hAQP7 as for hAQP3. As previously mentioned, in the case of AQP7, we obtained a Hill coefficient of approximately 3 for pH gating for both water and glycerol transport. This suggests that there are, at least, three protonatable sites that are cooperatively involved in regulation and that the same mechanism inhibits transport of both permeants. Previous research by Zelenina et al. [25] using site directed mutagenesis, demonstrated that four residues might be involved in the regulation of human AQP3.
Figure 12. Homology model of human AQP7, showing the conserved aromatic/Arginine selectivity filter (ar/R SF) and NPA motifs. In magenta are highlighted the residues corresponding to those previously identified as pH-sensitive in hAQP3 [28] and in red is shown the His140, hypothesized to be interacting, via H-bonds, with His165.

Figure 13. Homology model of human AQP7. (A) Tetrameric form of hAQP3, showing the residues, in the four monomers, corresponding to the pH-sensitive residues in hAQP3. (B) Hypothesized interaction of His140 from monomer A and His165 from monomer D. (C) Localization of Arg170 from monomer A and Tyr135 from monomer D. Different colours correspond to the four different monomers in the hAQP7 tetramer.
Interestingly, this mechanism appears to be different from the one described for hAQP3 in section 4.2.1. In the case of hAQP3, water and glycerol are not gated in the same manner upon pH changes. Nonetheless, sequence alignment of hAQP3 and hAQP7 shows that hAQP7 shares most of the pH-sensitive residues of hAQP3 (His53, Tyr124, Ser152 and His154), namely in hAQP7 Tyr64, Tyr135, Pro163 and His165 (Figure 12). Analysing the hAQP7 monomer, it is hard to predict how these residues alone can be so important for pH sensitivity, as they do not appear to share any interaction with other neighbour residues (Figure 12). Thus, we decided to further analyse these residues in the context of the hAQP7 overall tetrameric assembly, as performed for hAQP3 described earlier in this subchapter. Notably, in our model, all the residues corresponding to those indicated by Zelenina et al. for hAQP3 [25], are located in extracellular loops or helices and may have contact with other monomers (Figure 13A).

As we observe an effect of pH on the gating of hAQP7, that is similar for water and glycerol, the first theory described by previous authors for hAQP3, where the H-bonding network is higher for glycerol, explaining the differences in Hill slope can be applied. In fact, this theory can explain the behavior of hAQP3 in the permeation of water and glycerol, with Hill slopes of approximately 2 and 4, respectively, but fails at explaining why this Hill slope is the same for both solutes in the case of hAQP7, as both channels transport both solutes in a similar fashion. Instead, our attempt to explain the Hill slope differences in hAQP3 may serve to elucidate the similarities in hAQP7. Even though both channels transport both water and glycerol, structural changes induced by pH may be different, as the involved residues are not exactly the same.

Thus, we hypothesize that the protonation of a residue in one or more of the protein subunits may trigger a change in the structure of the entire tetramer. In the most simple model, if the change is symmetric over all subunits, there would be two states of the tetramer, one in which the protonated state is favored and one in which the deprotonated state in all four subunits is favorable. This would explain the Hill coefficient of 3, which nearly corresponds to the number of subunits or to the number of identified titrable residues.

In detail, His165 (corresponding to His154 in AQP3) is located in loop C and interacts via a hydrogen bond with His140, which itself is located in transmembrane-helix 3 from the next monomer (Figure 13B). At physiological pH (7.4), the software predicted His165 to be protonated, while His140 is in a deprotonated state. Given a possible interaction between these two side-chains, it is hypothesized that protonation of the highly accessible His140 would cause these side-chains to repel each other, causing loop C to move closer to the channel entrance. Interestingly, movement of loop C in hAQP3, and consequent closure of the channel, was described for hAQP3 inhibition by mercury, using molecular dynamics simulations [44]. Thus it is possible that the mechanism of gating by extracellular pH and inhibition by metals share some structural features.

Similarly to the described histidines, Tyr135 in AQP7 (the crucial Tyr124 in AQP3) can contribute to inter-monomer interactions, as it may form an H-bond with the positively charged Arg170 of the next monomer (Figure 13C). This H-bond is expected to strongly stabilize the deprotonated state of Tyr135, thereby possibly lowering its $pK_a$ to the observed value of 6.1. It is well established that strong H-bonding can shift the $pK_a$ of protein sidechains outside their usual window [62]. Indeed, the commonly observed $pK_a$ range for tyrosine is 9-12, while for histidine it is 5-8 [63]. None of the other tyrosines studied in the homology model had similar H-bonding
features that could lower the pK_a so strongly.

Another residue involved in pH sensitivity in AQP3 is His53. In that position, AQP7 does not have a histidine residue, but a tyrosine residue instead (Tyr64) (Figure 13A). Tyr64 in AQP7 is located in an accessible position from the extracellular side in Loop A, and appears to be contributing to monomer-monomer interactions in the central pore. However, its environment does not present any feature that could account for an unusually low pK_a of this residue. Ser152 in AQP3 was also shown to be involved in pH gating, but it is not present in hAQP7 where it is substituted by a non-protonatable residue (Pro163). Hence, this residue is not suitable to be involved in the pH gating of hAQP7.

4.3. Conclusions

The main objective of the present studies was to assess regulation the permeability properties of two human aquaglyceroporins, hAQP3 and hAQP7, by pH, as well as to investigate the pH gating of rat AQP3, expressed in a yeast cell-model. This was achieved in cellular models where analysis was unlikely to be compromised by the co-expression of other aquaporin isoforms. In the case of hAQP3, we used a hRBC model, well-described as a good model for functional studies of this aquaglyceroporin. Furthermore, due to their easy molecular genetics as well as availability of a wide range of mutants, we selected the yeast S. cerevisiae devoided of endogenous aquaporins to express hAQP7 and rAQP3.

In our yeast cells, hAQP7 and rAQP3 localized at the plasma membrane and it was possible to measure increased water permeability at low temperature where the fluidity of the lipid bilayer is reduced [56]. Moreover, compared to control cells, yeast expressing hAQP7 and rAQP3 displayed considerably higher glycerol permeability at mammalian physiological pH (pH 7.4), confirming their function as a glycerol channel. The concomitant lower $E_a$ for glycerol transport corroborated this observation and assured that the yeast cell system can be used for assessment of aquaporin regulation. Additionally, the inhibitory effect of Auphen towards hAQP7 was also confirmed in this model. Importantly, the yeast cell model herein optimized allowed disclosing for the first time the pH dependence of hAQP7 activity, showing that this channel changes from an open to closed state when pH drops from 7.5 to 5.

In the present study we investigated the pH gating of rat and human AQP3 by stopped flow spectroscopy. For the first time we were able to fully characterize not only the effects of pH gating on water, but also on glycerol permeability in this human isoform. In the case of water, the obtained results confirm the previous observations of hAQP3 gating in oocytes [29]. Interestingly, previous reports on rAQP3 pH gating were confirmed in our yeast model, which highlighted differences with the human isoform. In fact, while hAQP3 shows the same pK_a for both water and glycerol, the pK_a values are similar for water, but different for glycerol in the rAQP3 system. These differences may be due to species differences, even though we cannot exclude that the selected investigational system itself may partly lead to this variation.

In the light of the experimental Hill slope values for water and glycerol, a few theories on differences in the pH gating mechanisms of aquaporin permeation have been postulated. Current knowledge about aquaporin sequence and structure allows us to discard the hypothesis of protonation of residues inside the channel.
According to our data the maximum of protein function, for both aquaglyceroporins, is achieved at the expected mammalian physiological pH range of 6 to 7.5. Reflecting upon the possible reasons for such phenomenon, the cellular and tissue distribution of these aquaporins may shed some light on the physiologic meaning of its regulation. For example, glycerol metabolism and hAQP7 regulation is well documented in adipose tissue and liver [6, 7], while in the kidney AQP7 is important for glycerol reabsorption preventing glyceroluria [64]. However, less is known about AQP7 regulation in tissues where it is also moderately expressed, such as pancreatic β-cells controlling insulin secretion [65] and in skeletal muscle and heart importing glycerol for energy production [12, 66, 67]. In the male reproductive system, AQP7 is present particularly in the spermatids, as well as in the testicular and epididymal spermatozoa, suggesting that AQP7 has some role in late spermatogenesis [10, 68].

The role of hAQP3, in post-copulatory sperm osmoadaptation and migration has also been recently suggested [69]. Notably, a number of reports indicate that pH gradients and the concentration of bicarbonate in the lumen of the extratesticular ducts are involved in the regulation of sperm metabolism and motility [70] and thus have a direct physiological role in reproductive function. It is tempting to speculate that the pH gating of human AQP7 and AQP3 has biological relevance for sperm maturation and quiescence.

Notably, metal compounds have also been shown to modulate the function of AQPs. For example, among the endogenous transition metal ions, Cu$^{2+}$ and Ni$^{2+}$ ions, in the form of CuSO$_4$ and NiCl$_2$, have been demonstrated to cause water and glycerol permeability decrease in cells expressing human AQP3-GFP in a dose-dependent manner and the effect was rapid and reversible, Pb$^{2+}$ and Zn$^{2+}$ ions had no effect in AQP3 permeability [28, 71]. Moreover, the effect of Ni$^{2+}$ was pH-dependent: at neutral and acidic pH, the AQP3-mediated water permeability was completely inhibited by 1 mM NiCl$_2$. At pH 7.4 and 8.0, the $P_f$ in transfected cells was decreased by Ni$^{2+}$, but remained significantly higher than that in non-transfected cells. Site-directed mutagenesis studies identified three residues, Trp128 and Ser152 in the second extracellular loop and His241 in the third extracellular loop of AQP3, as determinants of Ni$^{2+}$ inhibition effects [28]. These Ni$^{2+}$-sensitive residues are the same as for Cu$^{2+}$, which suggests the same binding site and mechanism of inhibition [71]. Interestingly, Ser152 was identified as a common determinant of both Ni$^{2+}$ and pH sensitivity.

These findings confirm our idea that knowledge of the physiological mechanisms of AQPs gating may open the way to new strategies to selectively target different AQPs and to achieve optimization of inhibitors, such as the recently reported gold-based compounds [34, 58, 59] potentially active also as His binders. Finally, considering the importance of glycerol in multiple vital physiological processes, regulation of its permeation across hydrophobic cell membranes via AQPs may be crucial for cell proliferation, adaptation and survival, and future research to untangle the biological relevance of aquaglyceroporins’ pH gating in health and disease conditions ought to be conducted.

### 4.4. Experimental section

**Strains, plasmids and growth conditions:** Plasmid with human aquaporin-7 (hAQP7) cDNA (pWPi-DEST-AQP7) [9] was used for AQP7 cDNA amplification.

The centromeric plasmid pUG35 was used for cloning human AQP7, conferring C-terminal GFP tagging, MET25.
promoter and CYC1-T terminator.

*Escherichia coli* DH5α [72] was used as host for routine propagation of the plasmids. *E. coli* transformants were maintained and grown in Luria-Bertani broth (LB) at 37 °C; ampicillin (100 µg/ml) [73]. Plasmid DNA from *E. coli* was isolated using a GenElute™ Plasmid Miniprep Kit (Sigma-Aldrich).

Saccharomyces cerevisiae, 10560-6B MATa leu2::hisG trp1::hisG his3::hisG ura352 aqy1D::KanMX aqy2D::KanMX (YSH1770, further indicated as aqy-null) was used as a host strain for heterologous expression of human AQP7 and for functional studies. Yeast strains were grown and maintained at 28 °C with orbital shaking in YNB (yeast nitrogen base) without amino acids (DIFCO), with 2% (w/v) glucose (and 2% (w/v) agar for solid medium) supplemented with the adequate requirements for prototrophic growth [74]. For stopped-flow assays, the same medium was used.

**Cloning of human AQP7 gene in uG35:** *E. coli* DH5α was transformed with (pWPi-DEST_lnAQP7) and used for propagation of the plasmid. Plasmidic DNA was isolated and purified.

hAQP7 specific primers modified to incorporate restriction sites for SpeI (underlined) and ClaI (underlined) (5'-GGACTAGTCTATGGTTCAAGCATCCGGGCACAG-3' and 5'-CCATCGATGGAGATGCTCTAGGGCCATGGATTCTAG-3' respectively) were designed and used for PCR amplification of hAQP7 cDNA. PCR amplification was carried out in an Eppendorf thermocycler with Taq Change DNA polymerase (NZYTech). A temperature gradient PCR was previously performed to determine the optimum annealing temperature. The PCR product was digested with SpeI and ClaI restriction enzymes (Roche Diagnostics™), purified using Wizard® SV Gel and PCR Clean-Up System kit (Promega) and cloned into the corresponding restriction sites of pUG35 digested by the same restriction enzymes, behind MET25 promoter and in frame with GFP sequence and CYC1-T terminator, using T4 DNA Ligase (Roche). Cloning was performed according to standard protocols [73] to construct the expression plasmid pUG35-hAQP7. The plasmid was used to transform DH5α *E. coli* strain, propagated and subjected to extraction and purification. Fidelity of constructs and correct orientation were verified by PCR amplification, restriction analysis and DNA sequencing. Agarose gel electrophoresis and restriction site mapping were performed according to standard methods [73, 75].

**Transformation of the S. cerevisiae aqy-null strain:** Transformation of the aqy-null strain with pUG35-AQP7 was performed by the lithium acetate method described in [75]. The same strain was also transformed with an empty pUG35 vector (which does not contain hAQP7 cDNA) to be used as a control (further indicated as pUG35 cells). Transformants were selected on YNB medium without uracil as auxotrophic marker.

**Subcellular localization by fluorescence microscopy:** For subcellular localization of GFP-tagged AQP7 in *S. cerevisiae*, yeast transformants in mid-exponential phase were observed with a Zeiss Axiovert 200 fluorescence microscope, at 495 nm excitation and 535 nm emission wavelengths. Fluorescence microscope images were captured with a digital camera (CoolSNAP EZ, Photometrics, USA) and using the Metaffluor software (Molecular Devices, Sunnyvale, CA).

**Fluorophore loading:** Yeast transformants were grown up to OD660nm=1, harvested by centrifugation (5000 x g; 10 min; 4 °C), washed and re-suspended in ice cold sorbitol (1.4 M) K+-citrate buffer (50 mM, pH 7.4) up to a concentration of 0.33 g ml⁻¹ wet weight and kept on ice for at least 90 minutes. Prior to the osmotic challenges the cell suspension was pre-loaded with the non-fluorescent precursor 5-and-6-carboxyfluorescein diacetate (CFDA, 1 mM for 10 min at 30 °C) that is cleaved intracellularly by nonspecific esterases and generates the impermeable fluorescent form known to remain in the cytoplasm [37].

**Stopped-flow fluorescence assays:** Stopped-flow was used to monitor cell volume changes of cells loaded with a concentration-dependent self-quenching fluorophore [37]. Experiments were performed on a HI-TECH Scientific PQ/SF-53 stopped-flow apparatus, which has a 2 ms dead time, temperature controlled, interfaced with an IBM PC/AT compatible 80386 microcomputer. Experiments were performed at temperatures ranging from 7 to 38 °C. Five runs were usually stored and analysed in each experimental condition. In each run 0.1 ml of cell suspension (1:10 dilution in resuspension buffer) was mixed with an equal amount of iso (baseline) or hyperosmotic solutions (of sorbitol or glycerol) of 1.25 tonicity ((Δ = (osmol∞)/(osmol∞))). Fluorescence was excited using a 470 nm interference filter and detected using a 530 nm cut-off filter and the changes in fluorescence due to carboxyfluorescein (CFDA) fluorescence quenching were recorded.

**Cell volume measurements:** Equilibrium cell volumes were obtained by loading cells with CFDA under a fluorescent microscope equipped with a digital camera as previously described [37]. Cells were assumed to have a spherical shape with a diameter calculated as the average of the maximum and minimum dimensions of each cell.

**Calibration of the fluorescence signals into relative volume:** The fluorescence traces obtained were corrected by subtracting the baseline trace that reflects the bleaching of the fluorophore. The calibration of the resulting traces for the two strains followed our previous strategy [21], where a linear relationship between relative volume and F was obtained.
(v_{rel} = a F/F_o + b); the values of a and b were estimated individually for each sorbitol osmotic shocks, considering the initial and final fluorescence values and the correspondent relative volumes obtained previously by our group for the same toxicity shock. These values were then used for the calibration of the traces in the glycerol osmotic shock preformed under the same experimental conditions, toxicity and temperature.

**Permeability and activation energies evaluation:** The experimental protocols to assess aquaporin function were designed to keep the membrane surface tension to a minimum in order to maintain aquaporin activity at its maximum as previously found in our laboratory [21]. This was accomplished by equilibrating cells in 1.4 M sorbitol solution (considering sorbitol a non-diffusible solute) followed by the application of low toxicity hyperosmotic shocks (A = 1.25) with sorbitol or glycerol. Under these experimental conditions and immediately prior (t=0) the osmotic shocks the intracellular non-diffusible species (C_{ND_in,0}) and glycerol (C_{Gly_in,0}) concentrations were 1.4 M and 0 M respectively. Upon the introduction of the hyperosmotic shocks (t=0+) the extracellular medium osmolality changes to 1.75 M and the initial extracellular solute concentrations and the concentration gradients (ΔC=C_{in}-C_{out}) change accordingly: i) for the sorbitol shock, the extracellular sorbitol (C_{Sor_out}) concentration is 1.75 M and the initial gradient of non-diffusible species (ΔC_{Total}=C_{ND_in}-C_{Sor_out}) is -0.35M and ii) for the glycerol shock, the extracellular media is composed by a mixture of sorbitol 0.7 M (C_{Sor_out}) and glycerol 1.05 M (C_{Gly_out}) and the initial gradients are ΔC_{ND,0}=0.7 M and ΔC_{Gly,0}=-1.05 M, ΔC_{Total}=-0.35 M. Using the analysis described in [9] that incorporates all these osmotic and concentration gradients and their respective water and glycerol fluxes, together with the value of 0.4 for the relative non-osmotic volume previously determined [21], the permeability coefficients for water (P_w) and glycerol (P_{gly}) transport were evaluated.

For this end, the calibrated experimental curves v_{flu} were fitted to their theoretical curves, considering the water and glycerol fluxes and the resulting changes in cell volume and intracellular concentrations of solutes. Optimization of permeability values was accomplished by numerical integrations using the mathematical model implemented in the Berkeley Madonna software (http://www.berkeleymadonna.com/). The activation energy (Ea) of water transport was evaluated from the slope of the Arrhenius plot (ln P_w as a function of 1/T).

**Inhibition with Auphen:** Inhibition experiments were carried out in the same way as above. Intact cell suspensions equilibrated in isotonic solution (sorbitol 1.4 M) were incubated with fluorescent probe (CFDA) in the absence or presence of Auphen (70 μM), at room temperature for 30 minutes.

Similarly to above, cell suspension is confronted with a hypertonic solution by the addition of sorbitol or glycerol, to characterize the water and glycerol transport, respectively. Acquisition and calibration of fluorescence signals was as described above, at 23 ºC. Estimation of P_w and P_{gly} was done by fitting the theoretical curves following the same method described above. Inhibitory effect of Auphen was evaluated by comparing values of P_w and P_{gly} at 23 ºC with and without incubation with Auphen.

**External pH dependence:** In order to further characterize the pH dependence on hAQP7 channel status (open/closed), yeast transformants cells were grown up as previously described and incubated in isotonic solution (sorbitol 1.4 M) under different pH values (varying from 5 to 7.5) at least 90 minutes. In these conditions, cells deprived of carbon source and incubated in ice for a long period, are considered in starvation and unable to maintain an internal pH gradient. Thus, the internal pH equals the external pH [76].

After the incubation with the fluorescence probe, stopped-flow experiments were performed at 23 ºC for both water and glycerol transport at different external pH. For each pH buffer the osmotic challenges, isotonic (to access baseline), and hyper-osmotic with sorbitol (for water) and glycerol (for glycerol) were performed sequentially, in order to secure that data points for water and glycerol transport through the AQP7 channel were obtained under the same temperature and pH conditions for both control cells and expressing hAQP7 cells.

**Statistical Analysis:** The results were expressed as mean ± SEM of n individual experiments. Statistical analysis between groups was performed by the unpaired t-test. P values < 0.05 were considered statistical significant. Statistical analyses were performed using the Prism software (GraphPad Software Inc., San Diego, CA).

**Molecular modeling:** The 3D structure of hAQP7 and hAQP3 were obtained by homology modeling using Molecular Operating Environment (MOE 2012.10) (CCG 2012) [47]. The choice of a template structure was based on the sequence identity between the isoforms and the sequence of the AQPs with available resolved structures from human, bacteria and Plasmodium falciparum (UniProt 2013 codes O14520, C8TK05 and Q8WPZ6, respectively). The isoform that has the highest sequence similarity with hAQP7 and hAQP3 is the bacterial isoform Glycerol Facilitator (GlpF), which was then chosen as a template structure. Three resolved structures for bGlpF, crystalized either with or without glycerol and solved by X-Ray diffraction, were retrieved from the Protein Data Bank [46]. Among them, the template was selected that had the best resolution (2.70 Å) without any substrate (pdb 1LDI). The tetrameric form was assembled according to directions given in the pdb file and the structure was prepared and protonated at pH 7 under forcefield...
Amber12EHT. Thus, the tetrameric form of human AQP7 model was built: 50 intermediate models were generated and averaged to obtain the final homology model.

The obtained models were checked for more realistic rotamers of side chains in the regions of Ar/R SF and NPA, by comparison with the available crystal structures of all the other AQP isoforms (pdb codes 1H6I, 3D80, 3DS9, 1RLD1 and 3C02). The structures were protonated at pH 7 and an energy minimization refinement was performed, also under the Amber12EHT force field, during which the Ca atoms were fixed. After identification of the residues of interest for the mechanism of pH gating, the same energy minimization procedure was used to further refine them.

References
Exploring the Gating Mechanisms of Aquaglyceroporins: New Clues for Inhibitor’s Design?


Part B
Gold Compounds as Anticancer Agents