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Identification of arylsulfonamides as Aquaporin 4 inhibitors

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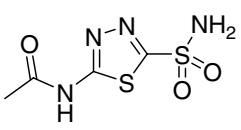
Abstract—Carbonic anhydrase inhibitors AZA, EZA, and 4-acetamidobenzsulfonamide were found to inhibit human AQP4-M23 mediated water transport by 80%, 68%, and 23%, respectively, at 20 μM in an in vitro functional assay. AZA was found to have an IC_{50} against AQP4 of 0.9 μM. Phloretin was inactive under the same conditions.

Aquaporin 4 (AQP4)[1] is a water specific channel protein belonging to the Aquaporin family of transmembrane water and water/glycerol transporters.[2,3] This protein is present in high concentrations in mammalian brain tissues,[4] particularly glial astrocytes, and is widely distributed in kidney, lung, muscle, and gastrointestinal tissues.[5] It is generally considered that AQP4 plays a significant auto-regulatory role in the brain because of its significant presence in glial cells, and hence might be a suitable target for drug discovery.

The precise role played by AQP4 in human physiology and pathology is not known. However, in addition to the auto-regulatory transport of intercellular water, evidence suggests that AQP4 is involved in the pathologies of edema,[6] epilepsy,[7,8] schizophrenia,[9,10] and possibly abnormal cytoskeletal morphology.[11] Modulators of this protein might be useful as therapeutic agents for any of these diseases. However, the study of the physiological roles played by AQP4, and its role in these and other pathologies has undoubtedly been hindered by the lack of any identified ligands or modulators for this protein.

Currently, only the tetraethylammonium cation (TEA) has been shown to inhibit AQP4 mediated water transport.[12] Unfortunately, the lack of SAR that could be developed around TEA suggested that it was not a viable starting point for our study.[13] Instead we turned to known ligands for other AQP isozymes. Acetazolamide (**1**, AZA) is a widely used pan-carbonic anhydrase (CA) inhibitor[14] and was recently shown to be a potent inhibitor of AQP1.[15,16] Phloretin (**2**) is a flavoid known to possess broad inhibitory activity toward a variety of ion channels,[17,18] and has also been shown to modulate several AQP isoforms, in particular the aquaglyceroporins, specifically AQP3, AQP7, and AQP9.[19–21] While AQP1 is closer to AQP4 in terms of sequence homology,[22] the lack of information regarding what types of binding interactions are available to AQP4 made it impossible to exclude either compound *a priori*.



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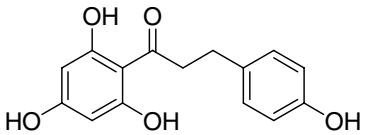

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During the initial phases of this project, the electron diffraction structure of the rat Aquaporin 4-M23 isozyme (rAQP4b) was generously made available to us by professor Fujiyoshi, Kyoto University.[23] Like all members of the Aquaporin family, AQP4 forms a protein homotetramer, where each protein monomer unit has a viable

Keywords: Aquaporin 4; AQP4; Inhibitors; Acetazolamide; AZA; 6-Ethoxybenzothiazole-2-sulfonamide; EZA; Phloretin.
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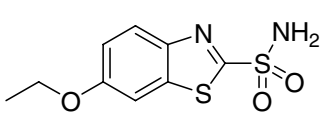
and presumably functional pore (Figure S1). The lack of an obvious gating mechanism or protein bridged binding site capable of blocking the water channel led us to consider modeling only a single protein monomer in lieu of the entire biological unit. The coordinates of one protein monomer were then imported into the BioMedCAChe/ActiveSite software environment for our virtual screening study.[24,25]

The virtual docking studies indicated that the preferred conformation for both compounds was quite similar. Moreover, the docking scores for **1** and **2** with rAQP4b were found to be essentially identical, −63.666 and −62.293 kcal/mol, respectively. In its final docked conformation (Fig. 1a), **1** was stabilized by electrostatic interactions between the sulfonamide group of **1** and guanidyl group of Arg-216 and the backbone carbonyl of Gly-209, as well as the acetamide group of **1** and carboxyl group of Asp-69. Additional hydrophobic interactions between **1** and Trp-59, Thr-56 and Ile-73 were also indicated. These interactions were consistent with that described between **1** and AQP1.[16] Likewise, the final docked structure of **2** (Fig. 1b) also indicated the presence of electrostatic interactions between the phenol and Arg-216. Additional electrostatic interactions were found between the ligand's trihydroxyphenone group and Asp-69 and His-151. Hydrophobic interactions were also identified between **2** and Thr-56, Ile-73 and Ile-205.

Compounds **1** and **2** were assayed for inhibition of AQP4 mediated hypoosmotic water transport in an in vitro functional assay using *Xenopus* oocytes expressing the human Aquaporin 4-M23 isozyme (hAQP4b).[26,27] Oocytes were generally handled and prepared as previously described,[28] and the assay was conducted using a modification of existing procedures.[29] The untransformed results, Figure 2, clearly indicated that **1** was able to inhibit hAQP4b mediated water transport, while **2** was not. A more detailed analysis of the data indicated that AZA showed 80 ± 4% inhibition, and was statistically relevant; whereas, **2** had no statistically relevant effect on the rate of water transport.[30]

The dose dependency of hAQP4b inhibition by **1** at ligand concentrations between 0.01 and 10 μM was also investigated. Statistically significant inhibition of water transport was found at 10 and 1 μM, in addition to that at 20 μM. No inhibition was observed for oocytes incubated with either 0.1 or 0.01 μM of **1**. Using those data, the apparent IC_{50} for **1** was found to be 0.9 μM with a maximum inhibition of 85%, Figure 3.

Despite the similar results of the in silico study, and the nearly identical sequences between the human and rat isozymes (approximately 97% identical), only **1** was found to inhibit AQP4 mediated hypoosmotic water transport. The drastic differences found for **1** and **2** lead to a persistent question about the validity of our model, and its utility as a predictive tool. Indeed, **1** and **2** were quite different in their overall structures and chemical properties; therefore, we were interested to compare the modeling results and inhibitory activities of similar compounds, in particular those related to **1**.



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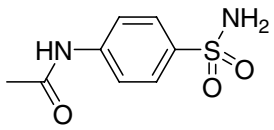

4

The potential of additional pan-CA inhibitors to block AQP4 mediated water transport was investigated. Compounds **3** (EZA) and **4** were well-known pan inhibitors of various CA subtypes.[31] Generally, **3** was shown to have inhibitory activity across a range of CA isozymes that was similar to **1**, while **4** was weaker. The docking

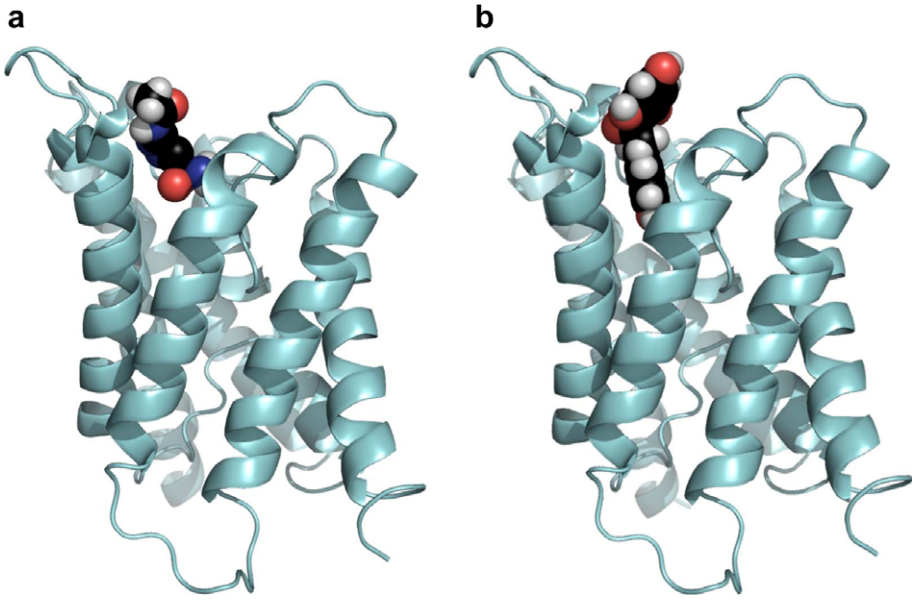

Figure 1. (a) Acetazolamide (**1**) and (b) phloretin (**2**) docked to the rAQP4b protein monomer. The ligands are shown as space-filling models, while the protein is shown as a ribbon schematic structure.

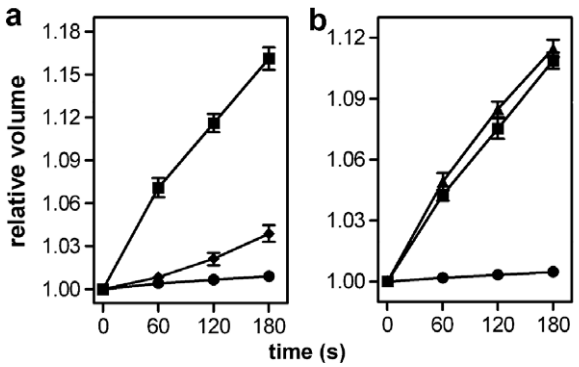

Figure 2. Relative volumes of hAQP4b cRNA transfected oocytes in hypoosmotic medium as a function of time. Relative volumes are reported for sham injected oocytes incubated with 0.1% DMSO (●); cRNA injected oocytes incubated with 0.1% DMSO (■); and cRNA injected oocytes incubated in the presence of 0.1% DMSO plus 20 μM (a) **1** (♦) or (b) **2** (▲). Error bars represent the standard error determined from $n = 7$ oocytes at each point.

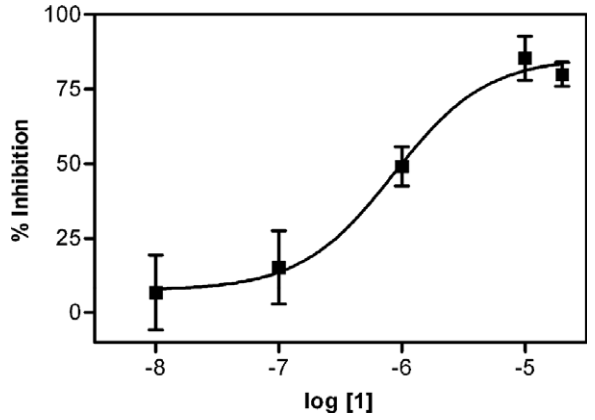

Figure 3. Osmotic water permeability inhibition of hAQP4b cRNA injected oocytes as a function of (**1**). Percent inhibition is given for $n = 5$ oocytes incubated with 0.01, 0.1, 1, 10, and 20 μM **1**, respectively, all with 0.1% DMSO present. Error bars indicate the cumulated standard error at that concentration. The curve fit from the non-linear least squares analysis using a sigmoidal function is shown as a solid line, apparent $IC_{50} = 0.86$ μM.

of **3** and **4** into the pore-active site of AQP4 showed preferred binding geometries similar to that of **1**. The docking score of EZA (−67.042 kcal/mol) was quite similar to that of AZA, while that of **4** (−53.963 kcal/mol) indicated it was less well stabilized within the binding pocket.

Subsequently, **3** and **4** were studied in the hAQP4b transfected oocyte functional assay. Both compounds were assayed at 20 μM as previously described, and the untransformed results are shown in Figure 4. Under those conditions, EZA showed a 68 ± 6% inhibition of AQP4; whereas, the 23 ± 5% inhibition found for **4** was clearly weaker. Both results were shown to be statistically significant ($P < 0.01$).

Despite the fact that this research is still in its initial phase, the results obtained thus far suggest that CA inhibitors may be particularly useful as a pool of potential ligands for AQP4. Moreover, at least within a class

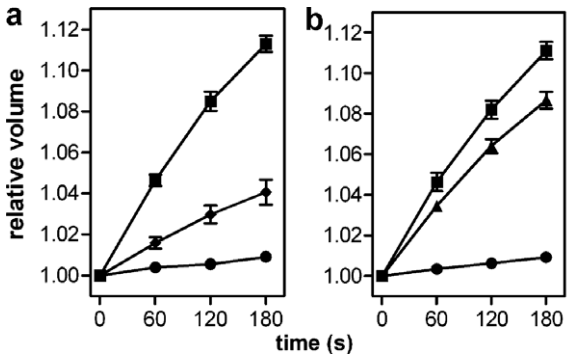

Figure 4. Relative volumes of hAQP4b cRNA transfected oocytes in hypoosmotic medium as a function of time. Relative volumes are reported for sham injected oocytes incubated with 0.1% DMSO (●); cRNA injected oocytes incubated with 0.1% DMSO (■); and cRNA injected oocytes incubated in the presence of 0.1% DMSO plus 20 μM (a) **3** (♦) or (b) **4** (▲). Error bars represent the standard error determined from $n = 7$ oocytes at each point.

of related compounds, a virtual screening approach might also be useful for prioritizing ligands for screening. As suggested by the virtual docking studies, electrostatic interactions between the ligand and residues within the water channel may be important for the sulfonamide-based CA inhibitors. However, it is impossible at this point to ascertain the relative importance of that interaction compared to other factors, such as the steric size and stereoelectronic properties of the ligand, in the inhibition of AQP4.

The failure of phloretin to show inhibitory activity in this assay is somewhat disappointing, particularly given its promising result in the virtual docking study. The degree to which the lack of inhibition results from poor binding properties, or in its ability to gain access to the channel binding site is impossible to assess at this time. Further results will be necessary to understand the reason behind this failure, and to incorporate that information into our modeling and compound prioritization process.

Based on these preliminary results, it is clear that AQP4 function can be modulated by AZA, as well as other sulfonamide-based CA inhibitors. Our goals are to further explore the nature of these inhibitory interactions and try to determine if there is a more fundamental relationship between AQP4 and CA inhibition.

Acknowledgments

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Supplementary data

An image of the AQP4 protein homotetramer and detailed experimental conditions are provided. Supplemen-

tary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2006.12.010.

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25. The procedure is described in greater detail in the supplemental materials section. However, in brief, the extracellular surface and pore domains of the rAQP4b protein monomer were examined for potential binding sites. An energy-minimized model of **1** was then manually aligned into those potential binding sites. The energy of the resulting structure was then minimized using the MM3 force field, where the protein coordinates were fixed while the ligand structure was not restrained. The lowest energy structures provided the basis for defining a protein binding site for use in the virtual docking experiment. Energy-minimized models of **1** and **2** were then docked into the protein using BioMedCache's ActiveSite docking module and the PMF energy function.
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27. The experimental protocols are described in greater detail in the supplemental materials section: however, in brief, oocytes from an adult *Xenopus laevis* were manually prepared to expose the inner membrane. Denuded oocytes were injected within 24 h of their isolation with hAQP4b cRNA[26] and allowed to incubate for an additional 48 h prior to use. Prior to the assay, oocytes were transferred to a 96-well microplate and were allowed to incubate with the inhibitor in MBS supplemented with a fixed concentration of DMSO for 2 h prior to the assay. Images of the oocytes were captured at regular intervals after the dilution of the incubation medium and then analyzed to determine the relative volume of the oocyte. Those relative volumes were compared to determine inhibition of the AQP4 mediated water transport.
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30. %inh is the percent inhibition of water transport of AQP4 cRNA injected oocytes incubated with any of **1**–**4**, compared to those incubated with blank solution, and is given by the relationship $\%\mathrm{inh} = 100(1 - \{[(\mathrm{Pf} \pm \mathrm{SE}) - (\mathrm{Pf}_{\mathrm{sham}} \pm \mathrm{SE}_{\mathrm{sham}})]/[(\mathrm{Pf}_{\mathrm{blank}} \pm \mathrm{SE}_{\mathrm{blank}}) - (\mathrm{Pf}_{\mathrm{sham}} \pm \mathrm{SE}_{\mathrm{sham}})]\})$, the errors reported for %inh are the cumulated standard errors. Calculation of Pf is described in greater detail in the supplemental materials section.
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