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The aquaporin-4 water channel as a potential drug target in neurological disorders

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Abstract

Introduction—Aquaporin-4 (AQP4) is a water transporting protein expressed at the plasma membrane of astrocytes throughout the central nervous system (CNS). Analysis of AQP4 knockout mice has suggested its broad involvement in brain water balance, neuroexcitation, glial scarring, neuroinflammation, and even neurodegenerative and neuropsychiatric disorders. Broad clinical utility of AQP4 modulators has been speculated.

Area covered—This review covers the biology of AQP4, evidence for its roles in normal CNS function and neurological disorders, and progress in AQP4 drug discovery.

Expert opinion—Critical examination of available data reduces the lengthy potential applications list to AQP4 inhibitors for early therapy of ischemic stroke and perhaps for reduction of glial scarring following CNS injury. Major challenges in identification and clinical development of AQP4 inhibitors include the apparent poor druggability of AQPs, the many homologous AQP isoforms with broad tissue distribution and functions, technical issues with water transport assays, predicted undesired CNS and non-CNS actions, and the need for high blood-brain barrier permeation. To date, despite considerable effort, validated small-molecule AQP4 inhibitors have not been advanced. However, biologic (‘aquaporumab’) is in development for neuromyelitis optica, an autoimmune inflammatory demyelinating disease where CNS pathology is initiated by binding of anti-AQP4 autoantibodies to astrocyte AQP4.

Keywords

AQP4; astrocyte; brain edema; epilepsy; glia scarring; stroke; neuromyelitis optica; spinal cord injury

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Declaration of Interest

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1. Introduction

There has been considerable interest in aquaporin-4 (AQP4) as a drug target for neurological disorders, with much speculation about potential indications of AQP4 modulators. AQP4 was identified by homology cloning and originally named MIWC (Mercurial-Insensitive Water Channel) because of its insensitivity to inhibition by mercury-containing compounds [1], which contrasted with other AQPs known at the time. AQP4 is expressed in astrocytes throughout the central nervous system (CNS) in brain, spinal cord and optic nerve, and is particularly concentrated in foot-processes adjacent to microvessels at the blood-brain barrier [2]. AQP4 is also expressed outside of the CNS, in skeletal muscle (sarcolemma), kidney (inner medullary collecting duct), stomach (parietal cells) and exocrine glandular epithelia [3]. As AQP4 is a water-selective membrane transport protein, it was reasonable to anticipate, as first demonstrated in 2000 using knockout mice [4], its involvement in water movement across the blood-brain barrier; subsequently, a number of unanticipated roles of AQP4 were discovered. Herein, we review and opine on evidence on the roles of AQP4 in normal physiology and disease, potential clinical indications of AQP4 modulators, and progress and challenges in AQP4 drug discovery.

2. AQP4 structure and function

AQP4 is one of ~13 homologous mammalian aquaporins, with many of the family members having a broad tissue distribution and a diverse set of functions (reviewed in refs. [5,6]). Like other aquaporins, AQP4 monomers have molecular size of ~30 kDa and contain six membrane-spanning helical segments (labeled S1, S2, S4, S5, S6 and S8 in Fig. 1A) and two shorter helical segments (labeled S3 and S7) that only partly span the membrane. There are two major isoforms of AQP4, a longer ‘M1’-AQP4 isoform with translational initiation at M1 and shorter ‘M23’-AQP4 isoform with translational initiation at M23, which are generated by alternative gene splicing [7]. There may be additional minor isoforms in some species [8], and leaky translation has been reported in some culture systems [9]; however, these observations have uncertain biological significance. AQP4 monomers form stable homotetramers (all M1 or M23 isoforms) and hetero-tetramers (mixed M1 and M23 isoforms) in membranes, with each monomer containing a separate water-selective pore.

A high resolution X-ray crystal structure of human AQP4 (pdb=3GD8) was solved, showing membrane-spanning helical domains surrounding cytoplasmic and extracellular vestibules that are connected by a narrow aqueous pore (Fig. 2B) [10]. The water-conducting channel contains a narrow selectivity filter including Arg216 and His201, which reduces pore diameter to 1.5 Å and prevents the passage of other solutes such as urea and glycerol. Residues Asn213 and Asn97 of two conserved Asn-Pro-Ala (NPA) motifs reside near the center of the pore. Molecular dynamics simulations indicate that water molecules move through a narrow aqueous pore in each AQP4 monomer, with steric and electrostatic factors responsible for water selectivity, and identify energetically favorable positions for water molecules at the NPA motifs and in the center of the channel [10]. Functional studies show that AQP4 is selective for the transport of water in response to transmembrane osmotic gradients, and has a relatively high single-channel water permeability compared to other aquaporins [11]. The water permeability of the M1 and M23 isoforms of AQP4 is similar,

and plasma membrane AQP4 is constitutively active and probably not subject to acute regulation at the channel level.

In contrast to other aquaporins, AQP4 tetramers further assemble in crystal-like supramolecular clusters in membranes called orthogonal arrays of particles (OAPs) [12], which have a cobblestone-like appearance by freeze-fracture electron microscopy (Fig. 2C, top). AQP4 supramolecular clusters have more recently been visualized by super-resolution optical imaging (Fig. 2C, bottom), which has provided a wealth of information about their structure, function and regulation [13,14]. The original discovery that AQP4 is the OAP protein was motivated by the visualization of OAPs in tissues where AQP4 is expressed, which was proven by the appearance of OAPs following AQP4 transfection into cells and their absence in tissues from AQP4 knockout mice [15,16]. OAPs are stabilized by the association of AQP4 tetramers through M23–M23 interactions involving hydrophobic residues just downstream of methionine 23 [17]. OAP size depends on the proportion of M1 and M23 AQP4 isoforms [18,19], which, as supported by mathematical modeling [20], is explicable by M23–M23 inter-tetramer interactions. Although the biological significance of OAPs is not completely understood, there is evidence that small, mobile OAPs facilitate astrocyte migration, whereas larger, less mobile OAPs are involved in AQP4 polarization to astrocyte foot-processes [21] through interactions with anchoring proteins including α-syntrophin and the dystroglycan complex [22,23]. These anchoring complexes may also facilitate co-localization of AQP4 with swelling sensitive ion channels including Kir4.1 and TRPV4, as discussed further below. A clinically important finding is that AQP4 OAPs are the primary target of immunoglobulin G anti-AQP4 autoantibodies in the inflammatory demyelinating disease neuromyelitis optica [24].

3. Roles of AQP4 in the central nervous system

3.1 Water transport into and out of the brain

The high expression of AQP4 at the blood-brain barrier and in ependymal cells lining ventricles motivated the original studies of brain swelling in experimental mouse models of cytotoxic (cellular) edema. Reduced brain swelling and improved survival in AQP4 null *vs.* wildtype mice were found after acute water intoxication (Fig. 2A), and reduced hemispheric swelling was found in ischemic stroke produced by transient middle cerebral artery occlusion (Fig. 2B) [4]. Subsequent work demonstrated improved long-term outcome in AQP4 null mice in a stroke model [25). AQP4 null mice also showed improved survival and outcome in models of global cerebral ischemia produced by 2- or 4-vessel occlusion [26,27]. Transgenic mice overexpressing AQP4 showed greater brain swelling and worse outcome in water intoxication [28]. The water transporting role of AQP4 provides a simple explanation for these findings – in cytotoxic edema excess water moves from the vasculature into the brain parenchyma through an intact blood-brain barrier driven osmotically in water intoxication by reduced plasma osmolality, and in ischemia by Na^+/K^+ pump failure. These observations have suggested the utility of pharmacological AQP4 inhibition in stroke.

However, in models of vasogenic (leaky-vessel) brain edema AQP4 deletion in mice appears to have an opposite, deleterious effect. In experimental models of blood-brain barrier disruption produced by tumor or focal cortical-freeze injury, water moves from the

vasculature into the brain in an AQP4-independent manner and excess water is eliminated primarily through the glia limitans into cerebrospinal fluid. Increased brain water accumulation and intracranial pressure were found in AQP4 null *vs.* wildtype mice with brain tumor, brain abscess, focal cortical-freeze injury, and following direct infusion of saline into the brain [29,30]. These findings suggested that excess brain water in vasogenic edema is eliminated by an AQP4-dependent route. Also, AQP4 null mice had greater ventricular enlargement and worse outcome in an experimental model of obstructive hydrocephalus produced by intracisternal kaolin injection [31]. It remains unclear, however, how AQP4, a water-selective transporter, could facilitate net removal of fluid, which contains both solute and water, from the brain. A recently proposed 'glymphatic' mechanism posits that solute clearance from brain parenchyma occurs by AQP4-dependent, hydrostatically driven fluid flow from para-arterial to para-venous spaces through the extracellular space of the brain parenchyma [32]. However, mathematical modeling has argued against AQP4-driven convective water and solute transport in parenchymal extracellular space [33], and recent experiments have challenged key predictions of the glymphatic mechanism [34]. Perhaps the deleterious effect of AQP4 deletion in vasogenic edema is related to compensatory changes in mouse knockout models and changes in brain structure, such as the baseline extracellular space expansion in AQP4 null mice [35]. Alternatively, AQP4 may be required for remodeling of the extracellular space caused by elevated intracranial pressure to facilitate clearance of edema fluid.

Various other conditions associated with brain or spinal cord edema likely have mixed cytotoxic and vasogenic edema mechanisms that vary over time and depend on the precise injury sustained. For example, AQP4 deletion in mice is beneficial in a crush model of spinal cord injury where edema might be primarily cytotoxic [36], but deleterious in a contusion model where edema is primarily vasogenic [37]. AQP4 deletion in mice was mildly protective in one model of traumatic brain injury [38], though complex kinetics of region-specific changes in brain water were seen. The possible use of AQP4 modulators thus requires attention to the complex spatial and kinetic aspects of edema fluid accumulation and clearance.

3.2 Astrocyte migration

Migration of reactive astrocytes occurs in various brain injuries to isolate the injured tissue and form a glial scar; migration of astrocyte-derived tumor cells (glioblastoma) occurs with local extension and invasion. AQP4 expression is high in reactive astrocytes and in high-grade glioblastoma. Following the discovery that aquaporins, including AQP4, facilitate cell migration [39], impaired migration was found in astrocyte cultures from AQP4 null *vs.* wild-type mice in wound healing and scratch assays, and reduced glial scarring was found in AQP4 null mice following cortical stab injury [40]. Further in vivo work showed impaired astrocyte migration to a site of injury in brains injected with fluorescently labeled astrocytes from AQP4 null mice compared to labeled astrocytes from wild-type mice [41]. Mechanistic studies suggested that AQP water transport is responsible for AQP-facilitated cell migration in which water influx occurs at the leading edge of a migrating cell in response to rapid local changes in intracellular osmolality. AQP4 was found to polarize in lamellipodia at the front end of migrating astrocytes [40]. Also, AQP4 might facilitate rapid changes in cell shape

that are required for migrating cells to squeeze through the narrow and irregular extracellular space in brain, as supported by the marked elongation seen in migrating astrocytes [41]. These findings suggest the possibility of pharmacological modulation of AQP4 water permeability to alter astrocyte migration and glial scarring, and perhaps to reduce cell migration and invasion in glioblastoma. AQP4 knockdown in human glioblastoma cell cultures has been reported to reduce their migration [42]; however, it is difficult to interpret the data because the knockdown also caused apoptosis and changes in cellular signaling.

3.3 Neural signal transduction

Another somewhat unexpected effect of AQP4 deletion in mice is altered neural signal transduction. AQP4 is expressed in supportive cells adjacent to electrically excitable cells, including astrocytes *vs.* neurons in brain and spinal cord, and Müller *vs.* bipolar cells in retina. AQP4 deletion in mice was found to impair auditory, visual and olfactory signal transduction as studied by evoked potential and/or behavioral methods [43–45]. Remarkably, seizure susceptibility in response to the GABA antagonist pentylenetetrazol was increased in AQP4 null *vs.* wild-type mice [46], and electrically induced seizures following hippocampal stimulation were much more prolonged [47].

Mechanistic studies suggested that impaired K^+ uptake from brain extracellular space in AQP4 deficiency might account for the seizure phenotype in AQP4 null mice. Measurements of K^+ concentration in brain cortex in AQP4 null mice using K^+-sensitive microelectrodes showed slowed K^+ clearance following local stimulation by brief electrical pulses [47]. Slowed K^+ clearance was also seen in a model of cortical spreading depression in AQP4 null mice using a triazacryptand K^+-sensitive fluorescent dye [48]. More recent microelectrode studies showed reduced velocity and frequency in cortical spreading depression in AQP4 null mice as produced by KCl application, and ascribed the effect to slowed increase in extracellular K^+ during neuronal depolarization [49].

Though several studies thus implicate altered extracellular K^+ dynamics in AQP4 deficiency, how this occurs remains unclear. One suggestion has been interaction of AQP4 with the inwardly rectifying K^+ channel Kir4.1, though patch-clamp studies provided evidence against functionally significant AQP4-Kir4.1 interactions [50]. Mathematical modeling of coupled K^+ and water transport in brain extracellular space supports the conclusion that reduced astrocyte water permeability in AQP4 deficiency can account for the altered K^+ dynamics by altering the rate and extent of changes in extracellular space volume following neuroexcitation [51]. Microelectrode measurements in hippocampal slices suggested the involvement of AQP4 in gap junction coupling and long-range K^+ buffering [52], which might also explain some phenotype observations in AQP4 null mice. Recent experiments have found impairment of neurotrophin-dependent forms of hippocampal synaptic plasticity in AQP4 null mice [53], where a related behavioral impairment in location-specific object memory was also observed. With regard to AQP4 therapeutics, the complex effects of AQP4 deletion on neuroexcitatory phenomena and their limited mechanistic understanding dampens prospects for AQP4-targeted therapy of seizure and related disorders.

3.4 Other potential roles of AQP4

Studies in AQP4 null mice have suggested various other AQP4 functions, though the data are largely descriptive with limited or no mechanistic information about whether the phenotype finding are a primary effect of AQP4 loss of function or an indirect effect of the gene knockout. Studies utilizing AQP4 null mice generated in Nanjing reported a variety of neurochemical and neurobehavioral abnormalities [54]; however, the data are difficult to interpret because the mice manifest marked baseline abnormalities such as a leaky blood-brain barrier that are not seen in mice generated by other groups. A possible role of AQP4 in neuroinflammation was reported in which AQP4 deletion in mice was associated with reduced severity of experimental autoimmune encephalomyelitis compared to wild-type mice, as well as reduced inflammation following intracerebral LPS administration [55]; studies in astrocyte cultures suggested a mechanism involving AQP4-dependent cell swelling and cytokine release. AQP4 has been shown to activate astrocyte Ca^{2+} signaling via TRPV4 in response to osmotic stimulation [56,57], raising the possibility that it may participate in some forms of neurovascular coupling that are regulated by Ca^{2+} elevation in endfeet. Recently, attention has focused on a proposed role for AQP4 in clearance of toxic protein aggregates from the brain by a ‘glymphatic’ mechanism [32], as mentioned above; however, these findings are controversial and it remains unclear if AQP4 plays a major role in regulating transport of solutes from the interstitium under physiological CSF pressure.

4. AQP4 drug discovery

There has been considerable interest in aquaporin-targeted therapeutics, but limited progress. Some aquaporins are inhibited by sulfhydryl-reactive heavy metal ions, such as mercury and gold, though heavy metal-containing compounds are generally not suitable for use in live cells because of their toxicity and non-selective protein reactivity. Of note, AQP4 is not inhibited by heavy metal-containing compounds because it lacks a key cysteine residue found in most other aquaporins [58]. Various compounds have been reported as inhibitors of a related aquaporin, AQP1, including tetraethylammonium (TEA^+), azetazolamide, various loop diuretic analogs, and others (reviewed in ref. [59]); however, attempts to replicate AQP1 inhibition by these compounds have failed [60]. As discussed [59], challenges in identification of aquaporin inhibitors include assay methods for compound screening that can be inaccurate and produce false positive results. The Xenopus oocyte swelling assay, which has been used in most studies of water transport modulators, is particularly prone to artifact because apparent changes in oocyte volume can be affected by many factors besides water transport.

Several of the putative AQP1 inhibitors mentioned above were also reported to inhibit AQP4 (Fig. 3), including TEA^+ [61], acetazolamide and related carbonic anhydrase inhibitors [62], and bumetanide and its analog AqB013 [63]. Also, surprisingly, multiple chemically unrelated anti-epileptic drugs were reported as AQP4 inhibitors, including zonisamide, lamotrigine, phenytoin and topiramate [64]. A calcein quenching screening assay of 3,575 compounds reported identification of three low-potency inhibitors, NSC168597, NSC164914 and NSC670229 [65], though the structures are non-drug-like and contain unusual and likely toxic trialkyl lead and tin functionalities. The molecule TGN-020 was

identified as an AQP4 inhibitor by a virtual screening approach based on similarity in structural features to previous reported carbonic anhydrase inhibitors and anti-epileptics [66]. Intraperitoneal injection of TGN-020 at high dose (200 mg/kg) was reported to reduce ischemic cerebral edema as seen by magnetic resonance imaging [67], and reduced infarct volume was reported in a rat model of ischemic stroke treated with TGN-020 [68]. A patent filed by Aeromics, Inc. (Cleveland, OH) describes a compound, IMD-0354, which was previously characterized as an inhibitor of the kinase IKKβ [69], that inhibits AQP4 and reduces intracranial pressure in mice following acute water intoxication [70]; a phenol phosphate pro-drug of IMD-0354 reduced brain swelling and improved neurological outcome in a mouse model of ischemic stroke. Aeromics and Cleveland Clinic investigators reported a molecule AER271 as a pro-drug of AQP4 inhibitor AER270 that blocks AQP4-induced activation of T cells [71] and reduces ischemia reperfusion injury following heart transplant surgery in mice [72]. While the structures of AER270 and AER271 have not been disclosed, they are likely to be in the same class of molecules as IMD-0354 and its phosphate pro-drug.

It is a priori quite unexpected that a wide variety of common, chemically unrelated carbonic anhydrase inhibitors, diuretics, anti-epileptics and other drugs could inhibit AQP4. Attempts to corroborate AQP4 inhibition by the carbonic anhydrase inhibitors, anti-epileptics and related compounds were unsuccessful [73], suggesting artifact in the oocyte swelling assays reported by Huber et al. Also, as will be reported separately, we could not corroborate AQP4 inhibition in cell-based assays using high concentrations of AqB013, TGN-020 or IMD-0354, as well as the earlier proposed AQP4 inhibitors. In our own search for AQP4 inhibitors, several candidates were identified from a docking screen of ~10^6 compounds from the UCSF-ZINC library against the extracellular surface of the 1.8Å X-ray crystal structure of human AQP4. An example shown in Fig. 4 is a 2-phenyl-4-carboxamido-quinoline, which achieved a very good complementary fit with the extracellular opening of the pore, surrounded by hydrophobic residues Pro65, Val 68, Phe77, Ile205, as well as positively charged Arg216 that is critical to the AQP4 selectivity filter. However, experimental testing of this compound and ~2000 other compounds with highest docking scores in a cell-based functional assay did not reveal useful AQP4 inhibitors, nor did testing of ~10^5 diverse, drug-like synthetic small molecules (unpublished results).

5. Aquaporumab: an AQP4-targeted biologic for neuromyelitis optica

An AQP4-targeted therapeutic is in development whose function is not to modulate AQP4 water transport activity or expression, but to block the binding of an anti-AQP4 autoantibody to AQP4. The autoimmune disease is neuromyelitis optica (NMO), an inflammatory demyelinating disease of the central nervous system that causes pathology in spinal cord and optic nerve, and to a lesser extent in brain, and can produce progressive motor, sensory and visual deficit (reviewed in ref. [74]). Most patients with NMO are seropositive for polyclonal immunoglobulin G anti-AQP4 antibodies, called AQP4-IgG, which are thought to be pathogenic by a mechanism involving their binding to AQP4 on astrocytes resulting in complement- and cell-mediated cytotoxicity, inflammation, blood-brain barrier disruption, demyelination and neurological deficit. Current therapies for NMO include immunosuppressants, plasma exchange and B cell depletion, and there is an acknowledged

need for therapies with improved efficacy and reduced side-effects especially for long-term suppression of disease exacerbations.

The therapeutic approach under consideration is blockade of the binding of pathogenic, polyclonal AQP4-IgG with extracellular epitope(s) on AQP4. As the initiating event in CNS pathogenesis in seropositive NMO is AQP4-IgG binding to astrocyte AQP4, a blocking molecule could provide a highly selective, non-immunosuppressive therapy for seropositive NMO. Initial screening to identify small molecule blockers of AQP4-IgG binding to AQP4 produced several low-affinity natural products, including arbidol, tamarixetin and berbamine [75], whose potential binding sites on AQP4 have been deduced by molecular modeling [76]. However, these molecules have relatively low potency and blood-brain barrier permeability, and it is unclear whether additional screening efforts would yield suitable drug candidates, in part because of the challenges in blocking of protein-protein interactions by small molecules.

An alternative approach is the development of a biologic to block AQP4-IgG binding to AQP4. In one strategy, a high-affinity anti-AQP4 antibody (‘aquaporumab’) was generated in which the antibody Fc portion was mutated to eliminate effectors functions involved in complement- and cell-mediated cytotoxicity (Fig. 5A). A human antibody was selected by sequence analysis of plasma cells from cerebrospinal fluid of NMO patients; after effector-neutralizing Fc mutations the antibody blocked binding of polyclonal, patient-derived AQP4-IgG to AQP4 and prevented NMO pathology in cell culture (Fig. 5B) and in vivo experimental animal models of seropositive NMO [77]. In order for the aquaporumab blocking approach to be successful in human NMO, the affinity of the mutated aquaporumab antibody to an extracellular epitope AQP4 must be sufficiently high, the antibody must have access to astrocyte AQP4, and the antibody should not be toxic. The ability of the aquaporumab antibody to block binding of polyclonal human AQP4-IgG to AQP4 is probably a steric effect due to its large size compared the AQP4 molecule. In one example shown in Fig. 5C, a partially affinity-matured aquaporumab prevented the killing of AQP4-expressing cell cultures by serum from a seropositive NMO patient, which was also found for sera from multiple different NMO patients. Further optimization and clinical testing will be needed to establish the efficacy of the aquaporumab blocking approach to treat NMO.

6. Expert opinion

Is AQP4 is a valid target for drug development, and if so, can useful AQP4-targeting therapeutics can be developed? Regarding the first question, the data from AQP4 knockout mice implicate AQP4 involvement in a wide range of CNS functions including water accumulation and clearance, neuroexcitatory processes, glial reaction to injury, neuroinflammation and others. While individual findings have motivated speculation about the broad utility of AQP4 modulators in stroke, CNS injury, edema associated with brain tumors and infection, epilepsy, multiple sclerosis and even Alzheimer’s disease and neuropsychiatric disorders, the available data and understanding of AQP4 biology mandate a more realistic view. It is difficult from data in AQP4 knockout mice to deduce whether phenotype findings are a primary consequence of absence of AQP4 function or a secondary consequence of altered expression and/or function of other proteins in the CNS and

elsewhere. While AQP4 knockdown experiments have provided some confirmatory data, they are also subject to concerns about secondary and off-target actions. Recently generated AQP4 knockout rats may provide confirmatory evidence in a second species, but the concern of secondary effects remains and the rat is just another rodent.

An important issue in the interpretation of phenotype effects of AQP4 deletion is whether the findings are plausible mechanistically, and if not, acknowledge the need for further research before extrapolation of a phenotype observation to a human clinical indication. Perhaps the most clear-cut and mechanistically plausible phenotype is reduced brain water accumulation in cytotoxic edema produced by water intoxication in which acute serum hyponatremia drives water osmotically into the brain across an intact, AQP4-containing blood-brain barrier. An AQP4 inhibitor should be efficacious in acute water intoxication if it penetrates well into brain, but this indication would have minimal clinical significance. However, as cytotoxic edema is important in the pathogenesis of ischemic stroke, at least early on, AQP4 inhibition may be of benefit. Edema in other acute CNS disorders, such as traumatic brain or spinal cord injury, is produced by a variable and time-dependent mix of cytotoxic and vasogenic edema mechanisms due to microvascular injury, and hence AQP4 inhibition may be ineffective or even worsen edema because of inhibition of fluid clearance. A much less clear-cut edema-related phenotype produced by AQP4 deficiency is reduced clearance of excess brain water in vasogenic edema, in which water accumulates because of blood-brain barrier disruption and excess water is cleared from the brain into the CSF or the venous circulation. How a water-selective transporter could facilitate clearance of excess fluid, which contains solutes and water, remains unexplained, as does whether the phenotype data in AQP4 knockout mice represent a primary effect of AQP4 loss of function. As the recent glymphatic hypothesis remains controversial and unsubstantiated, there lacks ample rationale for AQP4-based therapy of neurodegenerative diseases that might target A-beta or tau clearance. Further, though AQP4 upregulation may seem to have utility in neurological disorders associated with primarily vasogenic or interstitial edema, such as brain tumor, certain infections and hydrocephalus, there is insufficient mechanistic evidence at present to support the development of drugs to increase AQP4 expression or function for these indications.

Other phenotype findings raise additional concerns for AQP4 modulator therapy. AQP4-dependent neuroexcitation, which involves K^+/water coupling in brain ECS, would be a double-edged sword in contemplating AQP4-targeted therapy for epilepsy. The complex effects of AQP4 deletion or downregulation on seizure threshold and severity, though robust and even modeled mathematically, remain incompletely understood at the mechanistic level and would predict a very narrow therapeutic window for AQP4-targeted therapy. A related concern is whether AQP4 inhibition, as might be used to reduce brain edema early in ischemic stroke, might produce seizures or other undesired side effects within or outside of the CNS, such as inhibition of placental AQP4 in pregnancy. Another double-edged sword is AQP4 modulator therapy to inhibit or enhance astrocyte responses to injury; increased gliosis may be beneficial early after injury to isolate injured CNS tissue but deleterious in later phases where axonal regeneration occurs. Nonetheless, the involvement of AQP4 in migration of reactive astrocytes following injury, and perhaps in cancerous astrocytes in glioblastoma, along with a reasonably well-substantiated mechanism of AQP4-facilitated

cell migration, suggest the potential utility of AQP4 inhibition in late-phase CNS injury, and perhaps as adjunctive therapy to reduce cellular invasion in glioblastoma. Lastly, consideration of AQP4 as a target for neuroinflammatory and neuropsychiatric conditions is not well-supported by the data, with the notable exception of NMO as discussed above.

The second central question is whether AQP4-targeted therapeutics can be developed. There are a series of challenges for development of small molecule therapeutics. AQP4 is a member of a family of at least a dozen homologous proteins expressed in humans, many of which have broad tissue distribution where they are involved in critical cellular and organ functions such as urinary concentration, exocrine glandular secretion, angiogenesis and metabolism. An AQP4-selective therapeutic would thus be needed, which poses a challenge because of conserved amino acid sequences in the pore region of the various AQP isoforms. In addition to usual pharmacological considerations, an AQP4 therapeutic would require blood-brain barrier penetration; for acute therapy of ischemic stroke very rapid permeation is needed to obtain therapeutic levels in brain over the short window of predicted efficacy. Finally, whether AQPs in general, and AQP4 in particular, is druggable remains uncertain. As discussed in this review considerable efforts have not produced verified small molecule inhibitors of AQP4, raising questions about its druggability, perhaps that may be related to its narrow pore structure that excludes molecules other than water. There have been difficulties as well in making reliable, artifact-free measurements of cellular water transport. Finally, identification of an AQP4 activator is unrealistic because it is probably constitutively fully active, and the identification of AQP4-selective transcriptional activators (or inhibitors) is uncertain. Notwithstanding the many challenges, advances in screening and computational methods may yield bona fide small molecule AQP4 inhibitors for testing in experimental animal models and for advancement to the clinic. With regard to AQP4-targeted antibody therapeutics such as aquaporumab, while selectivity and obtaining functional inhibition are not concerns, the challenge will be in delivery into the CNS at sustained therapeutic concentrations.

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Abbreviations

AQP4	aquaporin-4
CNS	central nervous system
CSF	cerebrospinal fluid
ECS	extracellular space
IgG	immunoglobulin G
NMO	neuromyelitis optica

OAP	orthogonal arrays of particles
TRPV4	transient receptor potential cation channel subfamily V member 4

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Article highlights

- Aquaporin-4 (AQP4) is a water-selective plasma membrane water channel expressed in astrocytes throughout the central nervous system, where it is concentrated in foot-processes at the blood-brain barrier.
- Mice lacking AQP4 show less brain swelling following water intoxication and ischemic stroke as a consequence of slowed water movement across the blood-brain barrier.
- AQP4 facilitates migration of reactive astrocytes and glial scarring.
- AQP4 is the target of pathogenic autoantibodies in the inflammatory demyelinating disease neuromyelitis optica.
- Other functions of AQP4 in the central nervous system have been reported, including involvement in clearance of excess brain water, neuroexcitatory phenomena and neuroinflammation, though mechanisms are not certain.
- Though the data support several clinical indications for pharmacological modulators of AQP4 function, there has been little progress in identification and validation of AQP4 modulators.

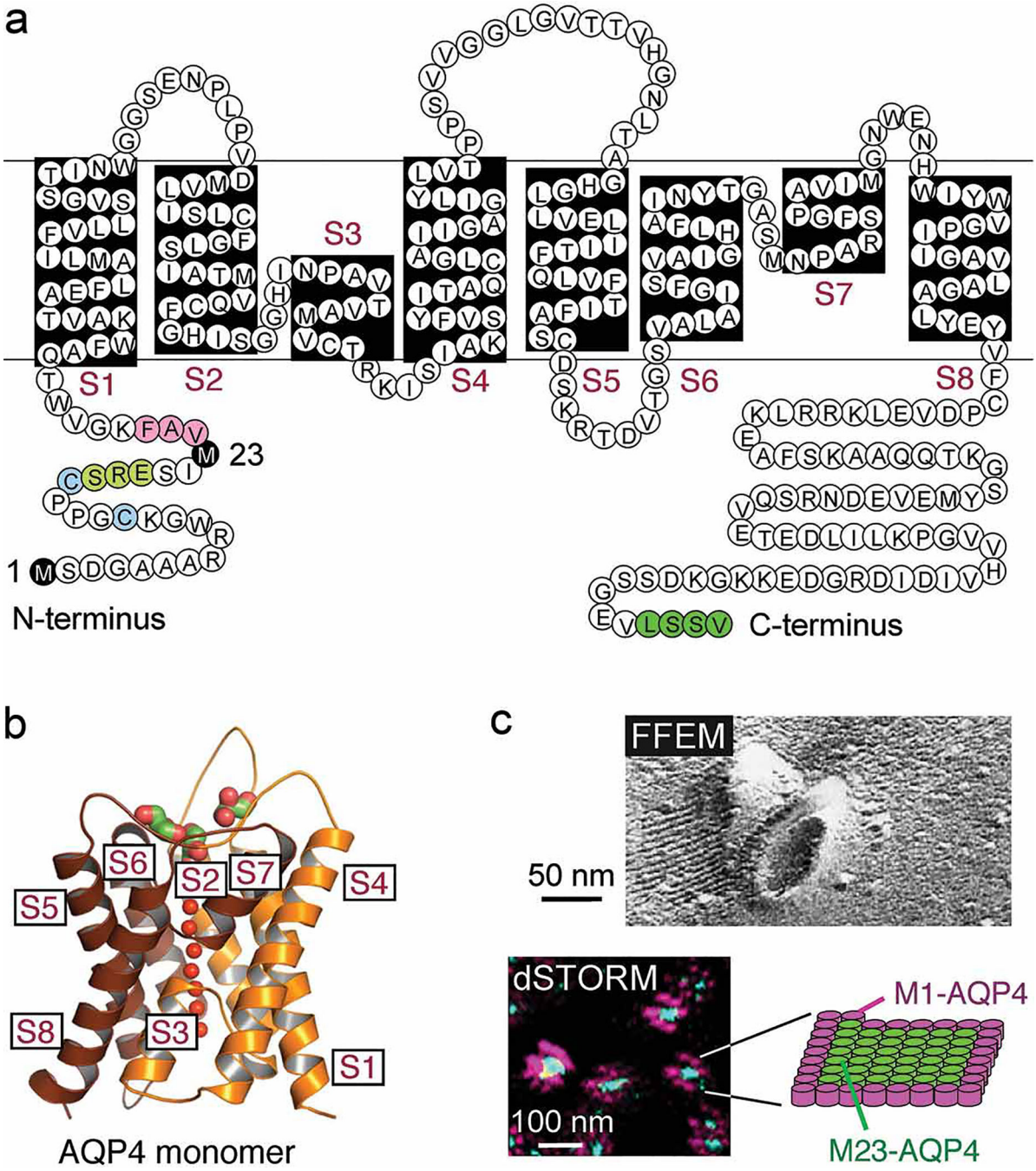

Figure 1. Sequence and structure of AQP4

A. AQP4 amino acid sequence showing eight membrane-embedded helical segments (labelled S1-S8). Met1 and Met23 translation initiation sites shown of the two AQP4 isoforms (black). AQP4 tetramers can form orthogonal arrays of particles (OAPs) through intermolecular N-terminal associations between M23 molecules involving the residues highlighted in pink. The residues in light green prevent N-terminal associations between M1 AQP4 molecules. Cysteine residues (in blue) are sites of palmitoylation involved in regulated OAP assembly. The C-terminus of AQP4 contains a PDZ domain (in green) that

may be involved in protein-protein interactions. A single chain of water molecules (red spheres) shown in the aqueous pore. **B.** X-ray crystal structure of human AQP4 (Protein Data Bank ID: 3GD8) showing the eight membrane-embedded helical segments (labeled S1-S8). **C.** Freeze-fracture electron micrograph of M23-AQP4-expressing CHO cells showing AQP4 OAPs as cobblestone-like array structures (top). Super-resolution micrograph, obtained by direct stochastic optical reconstruction microscopy (dSTORM), in cells co-expressing a green fluorescent M23-AQP4 and red fluorescent M1-AQP4. Diagram shows OAP structure deduced from dSTORM, with M23-AQP4-enriched core and M1-AQP4 periphery. Adapted from refs. [7,8].

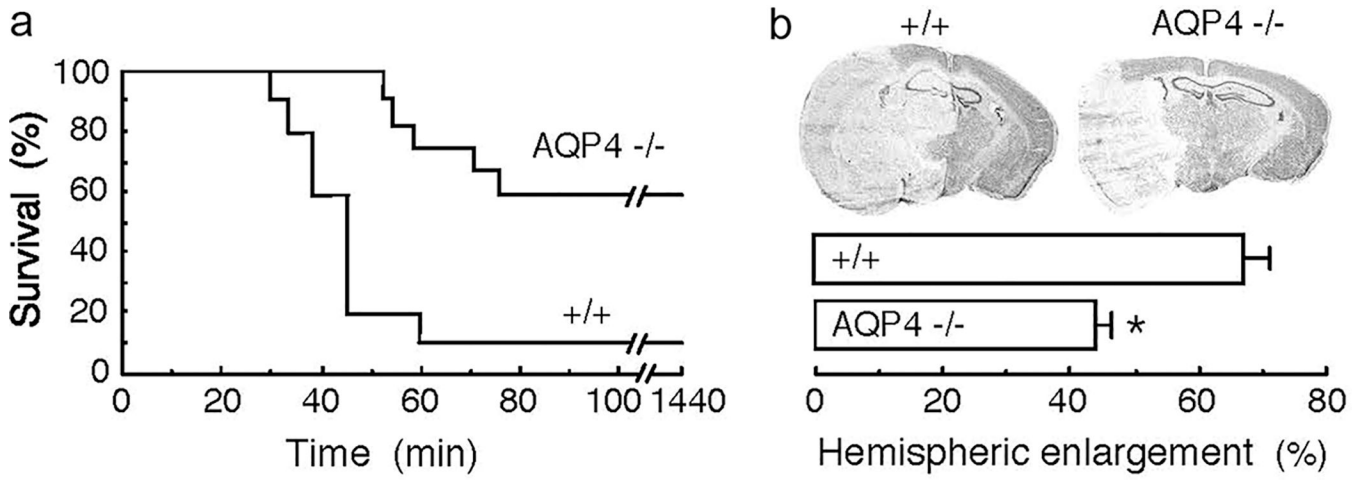

Figure 2. AQP4 deletion in mice reduces brain water accumulation in cytotoxic edema caused by water intoxication and ischemic stroke

A. Water intoxication model. Survival of wildtype and AQP4 knockout mice (12 mice per group) after acute water intoxication produced by intraperitoneal water injection (20 % body weight). **B.** Ischemic stroke model. Brain sections of mice at 24 hours after ischemic stroke produced by permanent middle cerebral artery occlusion (top). Average hemispheric enlargement expressed as a percentage determined by image analysis of brain sections (bottom). Adapted from ref. [4].

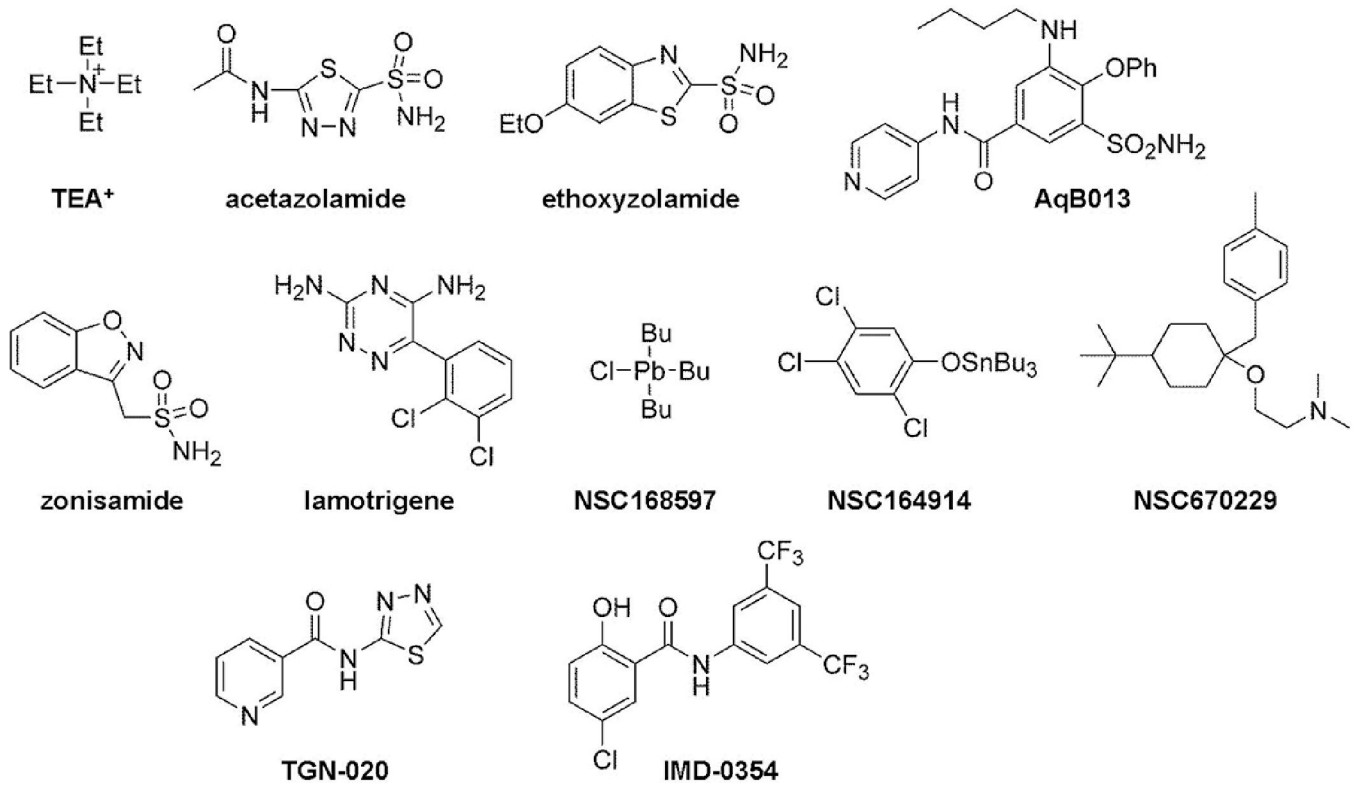

Figure 3. Chemical structures of putative small-molecule AQP4 inhibitors
See text for explanations.

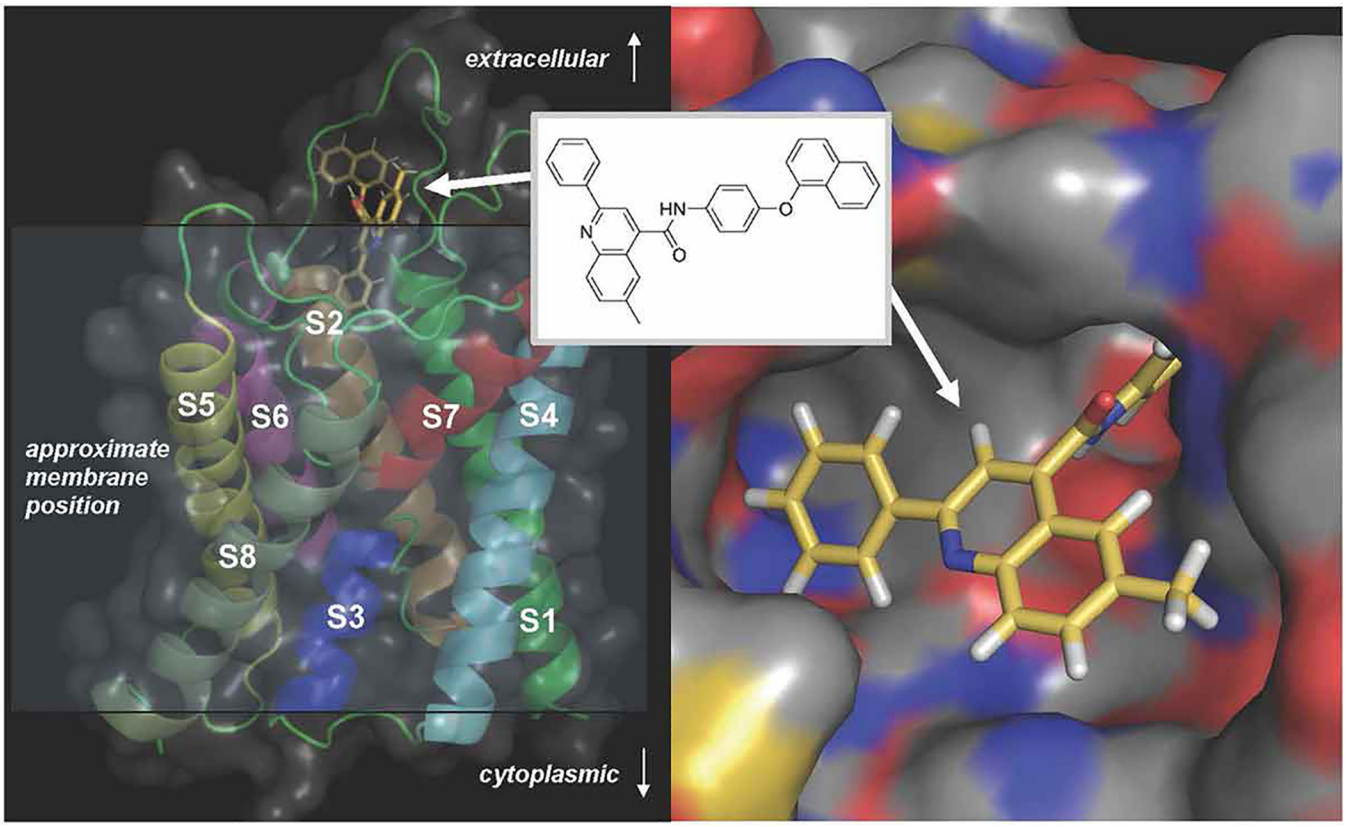

Figure 4. Docking of a small molecule to human AQP4

X-ray crystal structure of human AQP4 showing helical segments S1-S8, and docked with a 2-phenyl-4-carboxamidoquinolone inhibitor candidate (left). Zoomed-in representation of the AQP4-ligand complex, showing complementary fit between the two molecules (right).

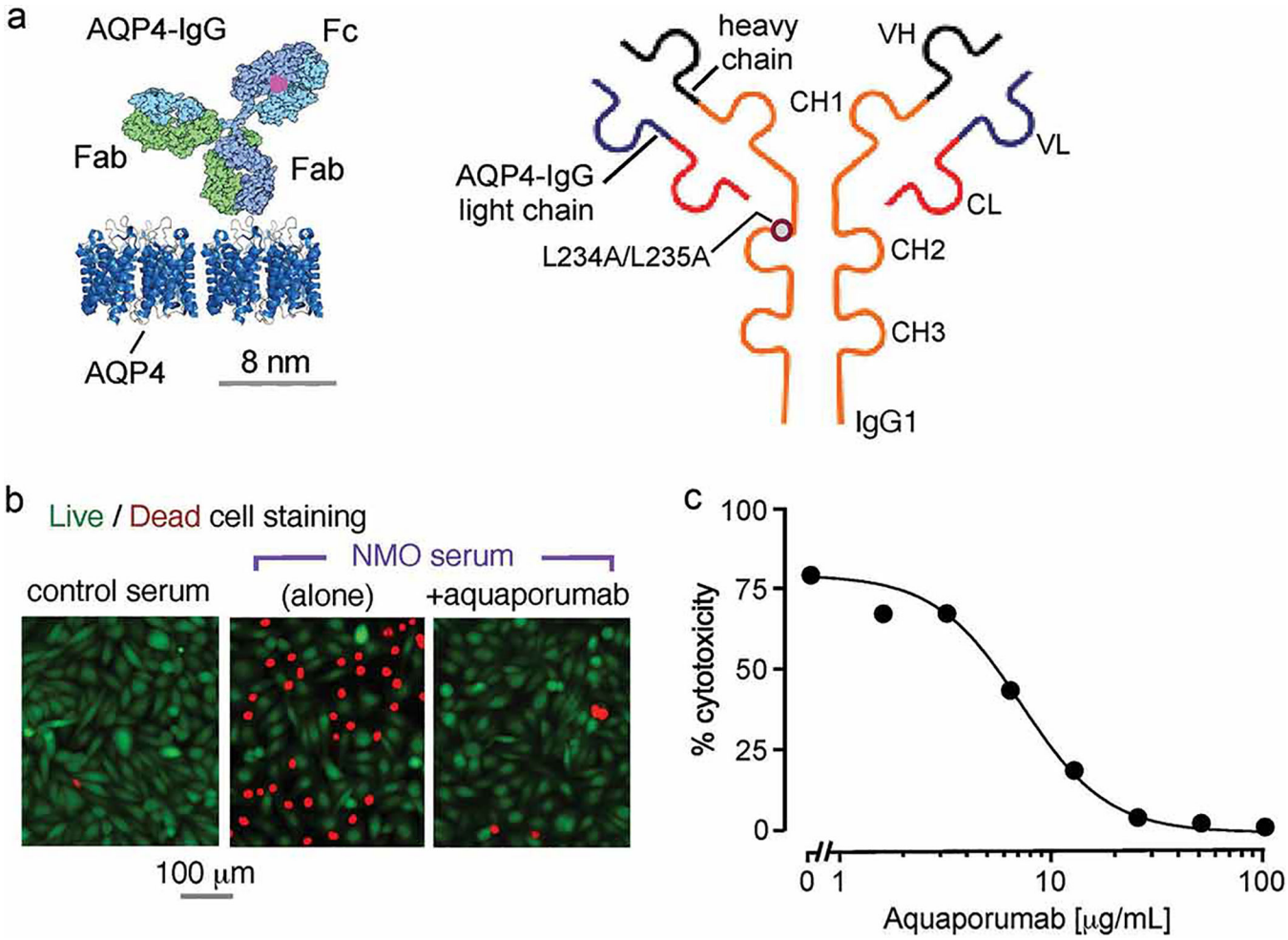

Figure 5. 'Aquaporumab' anti-AQP4 antibody for therapy of seropositive neuromyelitis optica

A. To-scale diagram of AQP4-IgG binding to membrane-associated AQP4 (left). Anti-AQP4 IgG1 antibody with L234A/L235A mutations in the Fc region that eliminate complement and cellular effector functions (right). **B.** Live/dead (green/red) staining of AQP4-expressing cells exposed to human control or NMO serum, showing protection by aquaporumab (adapted from ref. [76]). **C.** Percentage cytotoxicity of AQP4-expressing cells incubated with NMO patient serum and human complement, with different concentrations of aquaporumab added.