Astrocytic aquaporin 4 subcellular translocation as a therapeutic target for cytotoxic edema in ischemic stroke

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Brain edema is a common feature of several brain diseases (e.g., stroke, traumatic brain injury, hydrocephalus, brain cancer, and brain infections). Brain edema leads to increased intracranial pressure and worsens outcomes in ischemic stroke patients. Conventional treatments to control brain edema, thus reducing intracranial pressure include different osmotherapeutics, hyperventilation, tromethamine, hypothermia, and barbiturate coma. However, level 1 evidence of efficacy is lacking for these treatments, with some being harmful rather than beneficial (Bardutzky and Schwab, 2007). It has been proposed aquaporin 4 (AQP4) can be a novel drug target for treating brain edema (Vandebroek and Yasui, 2020). AQP4 is a small integral membrane protein and is strongly expressed in the brain. It has a highly polarized expression towards the abluminal side of astrocytic endfeet that surrounds the brain vasculature and is also expressed on the subpial and subependymal astrocyte processes, as well as basolateral membranes of ependymal cells (Patabendige et al., 2021). AQP4 is primarily involved in bidirectional water flux, but also has diverse roles such as Ca^{2+} signaling, K^+ buffering, neuroinflammation, and waste clearance (Verkman et al., 2017). Astroglial water movements induced by AQP4 have been shown to be a driving force contributing to the paravascular clearance of interstitial solutes like amyloid-β, thus participating in the so-called "glymphatic system" (Iliff et al., 2012).

The expression and polarization of AQP4 on astrocytic endfeet are altered during cerebral ischemia, resulting in swelling of astrocytes due to water movement from microvessels to the brain parenchyma across the blood-brain barrier (BBB) (Patabendige et al., 2021). AQP4 has been implicated in cytotoxic edema formation and dissolution following neurological injury when the BBB is intact. Evidence for a major role in cytotoxic edema for AQP4 has been shown in experimental studies using AQP4 knockout mice, where focal cerebral ischemia led to a 35% reduction in cerebral edema in AQP4 deficient mice 24 h after middle cerebral artery occlusion (MCAO) compared with controls (Manley et al., 2000). Furthermore, glial-conditional AQP4 knockout mice have been shown to have a 31% reduction in BBB water uptake compared with controls after systemic hypo-osmotic stress (Haj-Yasein et al., 2011).

Astrocytes form the "tripartite synapse" in the brain and play an essential role in neurotransmitter homeostasis and brain energy metabolism (Patabendige et al., 2021). During ischemic stroke, ATP levels fall due to the blockage/reduction in blood flow to the brain, leading to the inhibition of ATP-dependent transporters, such as Na^+/K^+ ATPase. This results in the influx of osmolytes, such as Na^+ that generate an osmotic force, driving water into cells of the central nervous system (CNS) leading to cellular swelling. As perivascular AQP4 allows bidirectional water flow, it is reasonable to assume that this is most likely the rate-limiting step for both water influx and efflux after ischemic stroke. Several studies have shown that AQP4 expression is altered following ischemic stroke, but with some capacity for recovery after injury. Frydenlund et al. (2006) have shown a biphasic change in perivascular AQP4 expression in the ischemic cortex, with an initial reduction at 24 hours of reperfusion that reduces water influx, then a partial recovery of AQP4 expression at 72 hours following transient MCAO in mice. The recovery of AQP4 expression at 72 hours would support the reabsorption of excess fluid accumulated due to edema formation. However, there was no recovery of AQP4 expression in the ischemic core, while the cortical border showed an increase in AQP4 expression. These findings suggest that AQP4 expression is subjected to varying regional changes, and therefore the expression of AQP4 on astrocytic endfeet is crucial for controlling cerebral edema following neuronal injury. AQP4 deletion has different impacts in edema formation with mixed cytotoxic and vasogenic edema mechanisms. AQP4 deletion is beneficial in a mouse crush model of spinal cord injury (primarily cytotoxic edema), but deleterious in a mouse contusion model (primarily vasogenic edema) (Verkman et al., 2017). Complex kinetics of region-specific changes in brain water is seen in a mouse model of traumatic brain injury (Verkman et al., 2017). Thus, the complex spatial and kinetic features of edema fluid build up and clearance must be considered before using AQP4 inhibitors to treat edema. Furthermore, there is a high level of structural conservation of amino acid sequences in the pore region, and the water selective narrow pore structure in AQP4 (pore diameter is reduced to 1.5 Å due to Arg216 and His201) excludes the passage of other solutes, such as glycerol. All of which leads to difficulties in identifying selective AQP4 inhibitors to prevent water flux (Verkman et al., 2017).

AQP4 can be modulated by targeting endogenous pathways and using pharmacological means. Four main pathways of AQP4 regulation have been described: (1) translational regulation via microRNAs that targets AQP4; (2) phosphorylation of AQP4 to target AQP4 trafficking and subcellular localization, as well as channel gating; (3) metal ions, which bind directly to AQP4 to inhibit its function, but can also increase AQP4 expression on astrocytes via indirect means when present at high levels in the cellular environment; and (4) small molecule inhibitors (Vandebroek and Yasui, 2020). These small molecule inhibitors include tetraethylammonium (TEA^+), acetazolamide and related carbonic anhydrase inhibitors such as bumetanide (an inhibitor of sodium-potassium-chloride cotransporter 1) and its analogue, AqB013, as well as anti-epileptic drugs (e.g., lamotrigine, phenytoin, and topiramate) and the selective AQP4 blocker, 2-(nicotinamide)-1,3,4-thiadiazole (TGN-020) (Verkman et al., 2017) (**Table 1**). A single dose of TGN-020 has been shown to reduce brain edema in a rat MCAO model when administered after the onset of ischemia (Pirici et al., 2018). Nevertheless, as off-target actions of TGN-020 are currently unknown, further investigations are warranted. Despite compelling evidence from experimental studies suggesting the potential of AQP4 modulators as a treatment strategy for reducing cerebral edema after brain ischemia, finding suitable drugs has been challenging. So far, none of the potential AQP4 modulators have been approved for human use. Furthermore, questions regarding whether some of these small molecule inhibitors can effectively inhibit AQP4 have been raised. Several issues including artefacts in oocyte swelling

assays, inability to reliably reproduce these inhibitory effects in cell-based assays and potential AQP4 independent actions on water transport by these molecules leading to confounding interpretations of animal studies are some of the concerns (Verkman et al., 2017). Given that AQP4 is responsible for driving cytotoxic edema formation in the acute phase of ischemic injury, while helping to clear vasogenic edema at later stages, complete inhibition of AQP4 is not a viable strategy for resolving cerebral edema.

A new strategy is to target AQP4 subcellular translocation rather than its inhibition/expression, given the recent evidence demonstrating the implications of AQP4 polarization to the abluminal membrane of perivascular astrocytic endfeet during cerebral edema. Steiner et al. (2012) have shown that following transient MCAO in mice, polarized expression of AQP4 on astrocytic endfeet was lost, and AQP4 was redistributed over the entire astrocytic cell surface. A recent study by Kitchen et al. (2020) has demonstrated that calmodulin-dependent phosphorylation of AQP4 led to an increased expression of AQP4 at the plasma membrane of astrocytes in hypoxia-induced edema. The mechanism involves transient receptor potential vanilloid type 4 (TRPV4)-facilitated Ca^{2+} influx that activates calmodulin, leading to cAMP-dependent protein kinase A (PKA) activation. The phosphorylation of AQP4 at Ser276 causes AQP4 to relocalize to the plasma membrane. Calmodulin also directly interacts with AQP4 and drives the AQP4 subcellular relocalization. This translocation of AQP4 from the astrocytic endfeet to the cell surface leads to an increase in water flux. However, inhibition of calmodulin with trifluoperazine (TFP, a calmodulin antagonist) significantly reduced AQP4 translocation, CNS edema, and accelerated functional recovery compared with untreated animals. TFP is approved by the UK National Institute for Health and Care Excellence, and the US Food and Drug Administration as an antipsychotic. The study used a dose in rats that was equivalent to its licensed use for humans. Therefore, these findings demonstrate the potential of TFP as a therapeutic strategy for reducing cerebral edema by preventing the subcellular relocalization of AQP4 to the plasma membrane of astrocytes, a strategy that is preferable to a complete inhibition of AQP4 (**Figure 1**). Further evidence for using TFP for reducing cerebral edema has been provided by a recent study by Sylvain et al. (2021) using a photothrombotic stroke model in mice. They demonstrated that treating mice with TFP 1 hour after stroke leads to a reduction in brain water content at 24 hours post-stroke, accompanied by AQP4 inhibition at the mRNA and protein levels. However, treatment with TFP 30 minutes before stroke did not lead to a significant reduction in brain water content. Furthermore, TFP treatment led to an increase in glycogen levels in the ischemic penumbra, and the time of TFP administration was irrelevant. This increase in glycogen levels could provide a beneficial effect on brain energy metabolism in the penumbra during the acute phase of stroke and may support neuroprotective ischemic preconditioning. A recent study on cultured astrocytes exposed to oxygen-glucose deprivation has demonstrated the potential of KN-62, a selective inhibitor of the Ca^{2+}/calmodulin-dependent protein kinase II in reducing astrocytic swelling and decreasing AQP4 upregulation associated with ischemia compared with untreated astrocytes (Li et al., 2021). However, the researchers did not investigate whether KN-62 treatment inhibited translocation of AQP4 from astrocytic endfeet to cell surface as demonstrated by TFP treatment.

Table 1 | **Selected aquaporin 4 modulators and their effects on brain edema or water permeability**

AQP4 modulator	Model	Effect on brain edema or water permeability	Reference
Tetraethylammonium (TEA^+)	Oocytes	Reduced water permeability	Detmers et al., 2006
Acetazolamide	Oocytes	Reduced water permeability	Huber et al., 2007
Bumetanide	Oocytes	Reduced water permeability	Migliati et al., 2009
	Mouse MCAO	Reduced brain edema	Migliati et al., 2010
AqB013	Oocytes	Reduced water permeability	Migliati et al., 2009
Anti-epileptic drugs	Oocytes	Reduced water permeability	Huber et al., 2009
2-(Nicotinamide)-1,3,4-thiadiazole (TGN-020)	Mouse MCAO Rat MCAO	Reduced brain edema	Igarashi et al., 2011 Pirici I et al., 2017
Trifluoperazine (TFP)	Rat crush injury Mouse PT	Reduced brain edema	Kitchen et al., 2020 Sylvain et al., 2021

Several aquaporin 4 (AQP4) modulators have been described in the literature. However, none have been approved for human use despite promising experimental data that demonstrate a reduction in water permeability or brain edema. The main experimental models used in these studies include the Xenopus oocyte model, rodent middle cerebral artery occlusion (MCAO) or photothrombotic (PT) stroke model or rodent crush injury model.

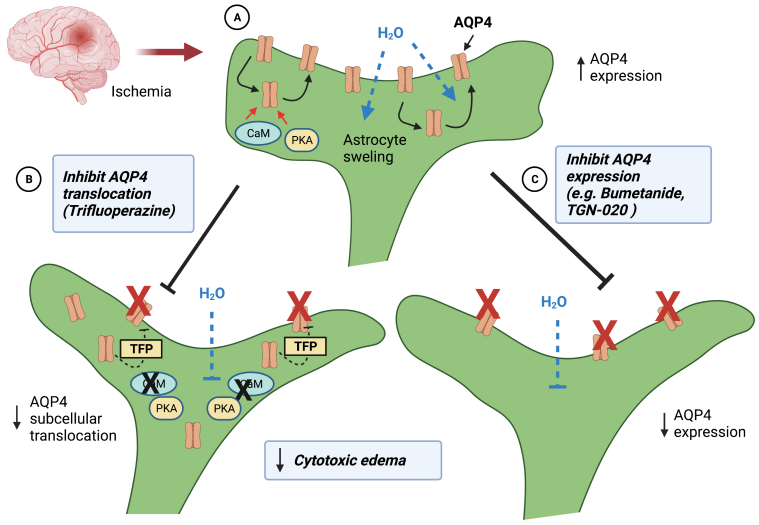

Figure 1 | **Targeting astrocytic aquaporin 4 expression and translocation in cytotoxic oedema.** During acute ischemia, reduced adenosine triphosphate (ATP) levels lead to the failure of ATP-dependent transporters such as Na^+/K^+ ATPase, driving water (H_2O) into cells due to the influx of osmolytes, and causing cellular swelling. Astrocytes responds to ischemic insult by increasing the expression of aquaporin 4 (AQP4), the main water channel in the brain that is highly expressed on the abluminal surface of the astrocytic endfeet. This leads to an increase in AQP4-mediated influx of water into astrocytes, in a calmodulin (CaM)-dependent manner, causing astrocyte swelling (cytotoxic edema). CaM also activates adenosine monophosphate (cAMP)-dependent protein kinase A (PKA) that phosphorylates AQP4, leading to the relocalization of AQP4 to the plasma membrane (A). The Emerging evidence from experimental studies demonstrate that targeting this CaM-mediated AQP4 subcellular relocalization using the CaM inhibitor, trifluoperazine (TFP), leads to a reduction in cytotoxic edema following ischemia (B) (Kitchen et al., 2020). This is a promising strategy to reduce cytotoxic edema without the need for pharmacological inhibition of AQP4 with drugs such as bumetanide or 2-(nicotinamide)-1,3,4-thiadiazole (TGN-020) (C), which also reduces cytotoxic edema by reducing AQP4 expression, but may have important implications due to its broad distribution and functions within and outside of the central nervous system.

Developing effective drugs for treating cerebral edema following ischemic stroke has been a major challenge. Despite evidence from experimental studies suggesting the potential of AQP4 inhibition as a potential treatment strategy for reducing cytotoxic edema, none of the candidate drugs have succeeded in being approved for human use. Major hurdles include the apparent poor druggability – the likelihood of being able to modulate AQP4 with a small-molecule drug, the ability to cross the BBB, broad tissue distribution (expression within and outside of CNS) and diverse functions of AQP4, and the potential for undesired actions.

For example, using AQP4 inhibitors during the early phase of ischemic stroke may lead to seizures because of AQP4-dependent neuroexcitation, as this involves K^+/water coupling in brain extracellular fluid, and therefore, limits the use of AQP4 modulators in epileptic patients. Another concern is the inhibition of placental AQP4 in pregnancy and the implications for AQP4-mediated maternal-fetal fluid exchange. Furthermore, AQP4 modulators that inhibit or enhance astrocytic responses to injury need careful consideration, as increased gliosis can be beneficial in forming the glial scar to surround the lesion site, but can have detrimental effects during the chronic phase, preventing axonal regeneration and CNS recovery (Patabendige et al., 2021). In addition, as discussed earlier, the issues surrounding the use of oocyte swelling assays can be a hindrance to AQP4 drug discovery. To overcome this methodological issue, Kitchen et al. (2020) have described a novel method to quantify AQP-mediated water transport across cells using Calcein – a dye that is quenched in a concentration-dependent manner. This concentration-dependent fluorescence quenching can be used as a probe of cell volume on short timescales, and therefore, allowing the measurement of plasma membrane water flux.

Recent advances which include *in silico* approaches to design novel drugs for target validation and optimization could provide new avenues for AQP4 modulation as a treatment strategy for reducing brain edema (Verkman et al., 2017). Another approach, which has shown potential is to target AQP4 subcellular translocation to the cell surface for reducing cerebral edema. Further studies on these aspects will provide an improved understanding of the underlying molecular mechanisms of brain water flux regulation by AQP4 that can be pharmacologically targeted to develop an effective treatment strategy for reducing cytotoxic edema. If successful, this could lead to a reduction in neurological damage associated with ischemic stroke by potentially creating an environment conducive for neuroprotection and neuroregeneration.

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