Is astrocytic aquaporin subcellular translocation a better therapeutic target for cytotoxic oedema than its inhibition in ischaemic stroke?

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Brain oedema is a common feature of several brain diseases (e.g., stroke, traumatic brain injury, hydrocephalus, brain cancer and brain infections). Brain oedema leads to increased intracranial pressure (ICP) and worsens outcomes in ischaemic stroke patients. Conventional treatments to control brain oedema, thus reducing ICP 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 oedema (Vandebroek and Yasui, 2020). AQP4 is a small integral membrane protein, and is strongly expressed in the brain. It has a highly polarised expression towards the abluminal side of astrocytic endfeet that surround 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+} signalling, 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 polarisation of AQP4 on astrocytic endfeet are altered during cerebral ischaemia, 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 oedema formation and dissolution following neurological injury when the BBB is intact. Evidence for a major role in cytotoxic oedema for AQP4 have been shown in experimental studies using AQP4 knockout (KO) mice, where focal cerebral ischaemia led to a 35% reduction in cerebral oedema in AQP4 deficient mice 24 h after middle cerebral artery occlusion (MCAo) compared with controls (Manley et al., 2000). Furthermore, glial-conditional AQP4 KO 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 plays an essential role in neurotransmitter homeostasis and brain energy metabolism (Patabendige et al., 2021). During ischaemic 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 ischaemic stroke. Several studies have shown that AQP4 expression is altered following ischaemic stroke, but with some capacity for recovery after injury. Frydenlund et al (2006) have shown a biphasic change in perivascular AQP4 expression in the ischaemic cortex, with an initial reduction at 24 h of reperfusion that reduces water influx, then a partial recovery of AQP4 expression at 72 h following transient MCAo in mice. The recovery of AQP4 expression at 72 h would support reabsorption of excess fluid accumulated due to oedema formation. However, there was no recovery of AQP4 expression in the ischaemic 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 expression of AQP4 on astrocytic endfeet is crucial for controlling cerebral oedema following neuronal injury. AQP4 deletion has different impacts in oedema formation with mixed cytotoxic and vasogenic oedema mechanisms. AQP4 deletion is beneficial in a mouse crush model of spinal cord injury (primarily cytotoxic

oedema), but deleterious in a mouse contusion model (primarily vasogenic oedema) (Verkman et al., 2017). Complex kinetics of region-specific changes in brain water are seen in a mouse model of traumatic brain injury (Verkman et al., 2017). Thus, the complex spatial and kinetic aspects of oedema fluid accumulation and clearance must be considered before using AQP4 inhibitors to treat oedema. 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 localisation, 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, bumetanide (sodium-potassium-chloride cotransporter 1 (NKCC1) inhibitor) and its analogue AqB013, as well as anti-epileptic drugs (e.g. lamotrigine, phenytoin and topiramate) and TGN-020 (Verkman et al., 2017) (Box 1). A single dose of TGN-020 has been shown to reduce brain oedema in a rat MCAo model, when administered after the onset of ischaemia (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 oedema after brain ischaemia, finding suitable drugs have 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 oedema formation in the acute phase of ischaemic injury, while helping to clear vasogenic oedema at later stages, complete inhibition of AQP4 is not a viable strategy for resolving cerebral oedema.

A new strategy is to target AQP4 subcellular translocation rather than its inhibition/expression, given the recent evidence demonstrating the implications of AQP4 polarisation to the abluminal membrane of perivascular astrocytic endfeet during cerebral oedema. Steiner et al (2012) have shown that following transient MCAo in mice, polarised 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) have demonstrated that calmodulin-dependent phosphorylation of AQP4 led to an increased expression of AQP4 at the plasma membrane of astrocytes in hypoxia-induced oedema. 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 relocalise to the plasma membrane. Calmodulin also directly interacts with AQP4 and drives the AQP4 subcellular relocalisation. 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 oedema, and accelerated functional recovery compared with untreated animals. TFP is approved by the UK National Institute for Health and Care Excellence (NICE), and the US Food and Drug Administration (FDA) as an antipsychotic. The study used a dose in rats that was equivalent to its licenced use for humans. Therefore, these findings demonstrate the potential of TFP as a therapeutic strategy for reducing cerebral oedema by preventing the subcellular relocalisation of AQP4 to the plasma membrane of astrocytes, a strategy that is preferable than a complete inhibition of AQP4 (Figure 1). Further evidence for using TFP for reducing cerebral oedema 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 h after stroke leads to a reduction in brain water content at 24 h post-stroke, accompanied by AQP4 inhibition at the mRNA and protein levels. However, treatment with TFP 30 min 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 ischaemic 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 ischaemic pre-conditioning. 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 (CaMKII) in reducing astrocytic swelling and decreasing AQP4 upregulation associated with ischaemia 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.

Developing effective drugs for treating cerebral oedema following ischaemic 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 oedema, 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 ischaemic 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-foetal fluid exchange. Furthermore, AQP4 modulators that inhibit or enhance astrocytic responses to injury needs 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 hinderance 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 optimisation could provide new avenues for AQP4 modulation as a treatment strategy for reducing brain oedema (Verkman et al., 2017). Another approach, which has

shown potential is to target AQP4 subcellular translocation to the cell surface for reducing cerebral oedema. 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 oedema. If successful, this could lead to a reduction in neurological damage associated with ischaemic stroke by potentially creating an environment conductive for neuroprotection and neuroregeneration.

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Box 1: Selected aquaporin 4 (AQP4) modulators.

Several 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 oedema. 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.

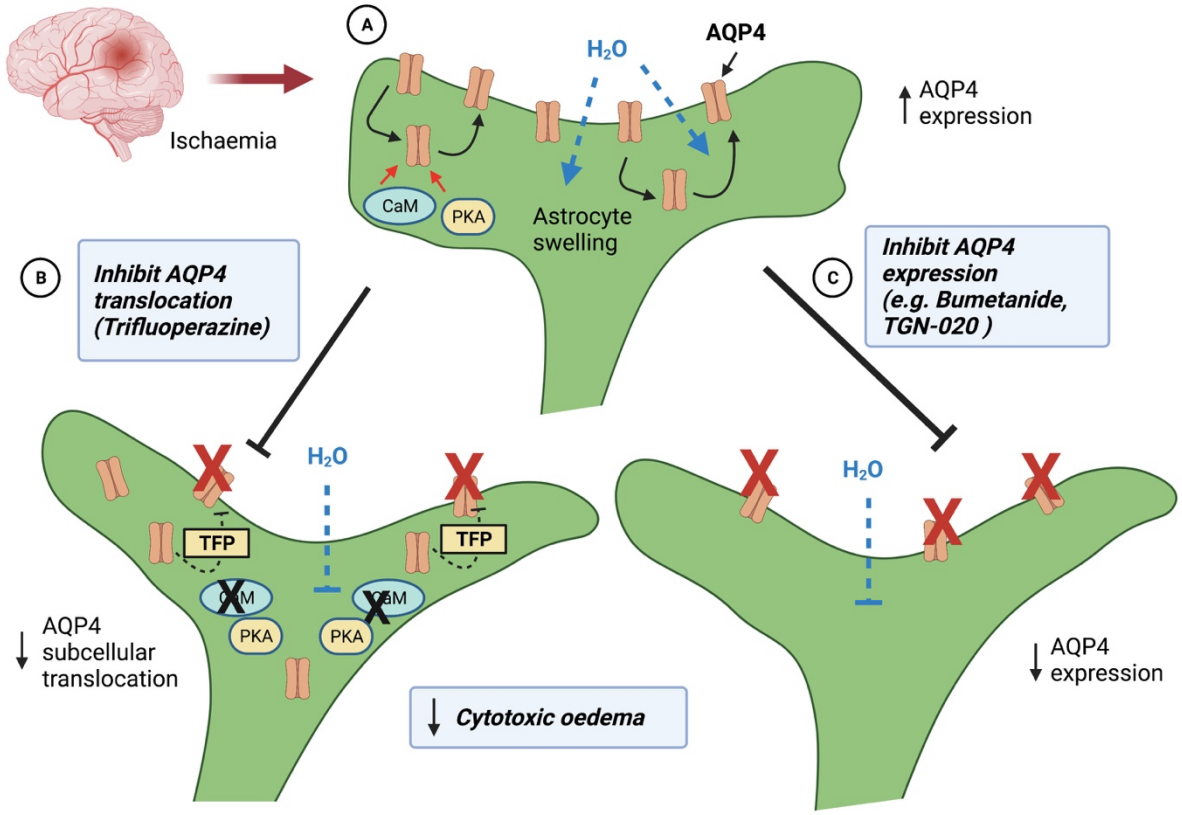
Figure 1 Targeting astrocytic aquaporin 4 (AQP4) expression and translocation in cytotoxic oedema

During acute ischaemia, reduced adenosine triphosphate (ATP) levels lead to the failure of ATP-dependent transporters such as Na^+/K^+ ATPase, driving water into cells due to the influx of osmolytes, and causing cellular swelling. Astrocytes responds to ischaemic insult by increasing the expression of 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 oedema). CaM also activates adenosine monophosphate (cAMP)-dependent protein kinase A (PKA) that phosphorylates AQP4, leading to the relocalisation of AQP4 to the plasma membrane (A). The Emerging evidence from experimental studies demonstrate that targeting this CaM-mediated AQP4 subcellular relocalisation using the CaM inhibitor, trifluoperazine (TFP), leads to a reduction in cytotoxic oedema following ischaemia (B) (8,9). This is a promising strategy to reduce cytotoxic oedema without the need for inhibition of AQP4 (C), which also reduces cytotoxic oedema by reducing AQP4 expression, but may have important implications due to its broad distribution and functions within and outside of the central nervous system.

Box 1

AQP4 Modulator	Model	Effect on brain oedema/ water permeability	Reference
Tetraethylammonium (TEA+)	Oocyte	Reduced water permeability	Detmers FJM et al. (2006) J Biol Chem. 281:14207–14214.
Acetazolamide	Oocyte	Reduced water permeability	Huber VJ et al. (2007) Bioorg Med Chem Lett. 17:1270–1273.
Bumetanide	Oocyte Mouse MCAo	Reduced water permeability Reduced brain oedema	Migliati E et al. (2009) Mol Pharmacol. 76:105–112. Migliati ER et al. (2010) Neurocrit Care. 13:123-31.
AqB013	Oocyte	Reduced water permeability	Migliati E et al. (2009) Mol Pharmacol. 76:105–112.
Anti-epileptic drugs	Oocyte	Reduced water permeability	Huber VJ et al. (2009) Bioorg Med Chem. 17:418–424.
TGN-020	Mouse MCAo Rat MCAo	Reduced brain oedema	Igarashi H et al. (2011) Neurol Sci. 32:113–116. Pirici I et al. (2017) Int J Mol Sci. 19:46
Trifluoperazine (TFP)	Rat crush injury Mouse PT	Reduced brain oedema	Kitchen P et al. (2020) Cell. 181:784-799.e19. Sylvain NJ et al. (2021) Biochim Biophys Acta Biomembr. 1863:183573.

Figure 1


A
Ischaemia
H_2O
AQP4
AQP4 expression
CaM
PKA
Astrocyte swelling
B
Inhibit AQP4 translocation (Trifluoperazine)
C
Inhibit AQP4 expression (e.g. Bumetanide, TGN-020)
H_2O
TFP
TFP
CaM
PKA
PKA
AQP4 subcellular translocation
Cytotoxic oedema
H_2O
AQP4 expression