Trends in Pharmacological Sciences

CellPress OPEN ACCESS

Review

Recent breakthroughs and future directions in drugging aquaporins

Mootaz M. Salman [1,2,*] Philip Kitchen,[3,*] Andrea J. Yool,[4,*] and Roslyn M. Bill [3,*]

Aquaporins facilitate the passive transport of water, solutes, or ions across biological membranes. They are implicated in diverse pathologies including brain edema following stroke or trauma, epilepsy, cancer cell migration and tumor angiogenesis, metabolic disorders, and inflammation. Despite this, there is no aquaporin-targeted drug in the clinic and aquaporins have been perceived to be intrinsically non-druggable targets. Here we challenge this idea, as viable routes to inhibition of aquaporin function have recently been identified, including targeting their regulation or their roles as channels for unexpected substrates. Identifying new drug development frameworks for conditions associated with disrupted water and solute homeostasis will meet the urgent, unmet clinical need of millions of patients for whom no pharmacological interventions are available.

Highlights

Aquaporins are attractive targets for therapeutic intervention in the diverse conditions associated with water and solute dyshomeostasis that affect millions of patients worldwide every year.

Aquaporin drug discovery has made little progress, possibly due to a range of assumptions including the intrinsic non-druggability of the aquaporin pore, compounded by issues with the reproducibility of current assays. We challenge the persistent idea that aquaporins are not druggable; the field is still in its infancy and much progress is yet to be made.

Viable routes to inhibition of aquaporin function have recently been identified, including targeting their regulation as well as their pores. Identifying new aquaporin-targeted drugs for conditions associated with disrupted water and solute homeostasis will meet an urgent, unmet clinical need as no pharmacological interventions are currently available.

Inhibition of hydrogen peroxide permeation through AQP1 provides a new approach to treating hypertrophic cardiomyopathies.

Inhibition of AQP4 localization with the licensed drug trifluoperazine provides compelling evidence for a new approach to treating CNS edema.

Diseases of aquaporin dysfunction

Water is essential for life. Consequently, its abundance within the cells and tissues of all organisms is tightly controlled. Although the pathways that maintain fluid volume and internal electrolyte concentration remain incompletely understood, aquaporin water channels play key roles [1].

Thirteen **aquaporin** (see Glossary) isoforms are found throughout the human body, where they facilitate a range of functions including the secretion of cerebrospinal fluid (CSF), tears, saliva, sweat, and bile, and the concentration of urine (Table 1) [2]. Perturbation of aquaporin function can result in diverse disorders including loss of vision, loss of skin barrier function, kidney diseases, xerostomia (dry mouth), and the **edema** that follows stroke or trauma to the brain or spinal cord (Table 1) [2]. In the case of xerostomia (which is associated with reduced aquaporin 5 (AQP5) function in the salivary gland), pharmacological activation of AQP5 has been suggested as a treatment, either by direct channel agonism or by activation of a signaling pathway controlling AQP5 surface expression [3]. In the brain, AQP4 facilitates cytotoxic edema; relocalization of AQP4 to the perivascular astrocyte membrane exacerbates this [4] by increasing astrocyte membrane water permeability and possibly by regulating the endfoot membrane localization of ion channels (e.g., Kir4.1, TRPV4, or SUR1-TRPM4) via colocalization. In a vasogenic edema model, *Aqp4*$^{-/-}$ mice developed significantly increased intracranial pressure compared with wildtype mice, also confirming a role for AQP4 in edema resolution [5]. Several other aquaporins are overexpressed in a variety of cancer types (Table 1). Given the crucial role of water transport in the migration of tumor cells and of angiogenic endothelial cells, aquaporin inhibition has been suggested as a novel anticancer strategy [6]. Notably, no pharmacological interventions currently exist for any pathologies of aquaporin dysfunction, which instead rely on **symptom management**.

Aquaporin structure, function, and classification

The identification of molecules that inhibit transport through specific aquaporin isoforms is not a simple proposition for drug discovery on account of the high level of structural conservation within the family [7]. The small diameter of all aquaporin pores, together with the chemical properties of

[1]Department of Physiology, Anatomy and Genetics, Kavli Institute for NanoScience Discovery, University of Oxford, Oxford OX1 3PT, UK
[2]Oxford Parkinson's Disease Centre, University of Oxford, Oxford, UK
[3]School of Biosciences, College of Health and Life Sciences, Aston University, Birmingham B4 7ET, UK
[4]University of Adelaide, School of Biomedicine, Adelaide, South Australia 5005, Australia

*Correspondence:
mootaz.salman@dpag.ox.ac.uk
(M.M. Salman),
p.kitchen1@aston.ac.uk (P. Kitchen),
andrea.yool@adelaide.edu.au
(A.J. Yool), and
r.m.bill@aston.ac.uk (R.M. Bill).

the pore-lining amino acid side-chains, mean that finding drug-like compounds that can enter and block them is a challenging prospect [8,9].

Aquaporins are tetrameric transmembrane proteins that facilitate the passive movement of water, small neutral solutes, and some ions across biological membranes. The driving force is an osmotic or hydrostatic gradient for water transport, or a concentration gradient for solutes. Each tetramer is comprised of four functional monomers in which an hour-glass-shaped pore is formed from six transmembrane helices and two helical re-entrant loops (Figure 1A).

Aquaporins are typically categorized as either being selective for water alone (the 'orthodox' aquaporins, AQP0, AQP1, AQP2, AQP4, AQP5, AQP6, and AQP8) or for water and small uncharged solutes, including glycerol and urea (the 'aquaglyceroporins', AQP3, AQP7, AQP9, and AQP10). The most recently discovered family members are in a separate grouping designated as 'superaquaporins' (AQP11 and AQP12). However, this functional categorization does not capture the diversity within the subgroups, with some orthodox isoforms having a broader substrate selectivity (e.g., urea permeates AQP8; Table 1) [10]. Distinct members of all three groups facilitate hydrogen peroxide transport; the superaquaporins facilitate both water and glycerol transport when purified and reconstituted into polymersomes (although their intracellular localization makes cell-based assays challenging) [11]. The superaquaporins also share less than 35% homology with other family members [12].

Amino acid sequence alignments also support the classification of aquaporins into three groups (Figure 2). The largest group comprises the orthodox aquaporins and non-mammalian aquaporins with experimentally determined ion channel function [big brain (BIB), AtPIP2;1, and Nod26]. Known mammalian aquaporin ion channels (AQP0, 1, and 6) are also included in this group (Figure 2). AQP8 is the most distantly related member of the orthodox group, in line with its unusual permeability properties, although other phylogenetic analyses suggest it clusters with the superaquaporins (AQPs 11,12A, and 12B; Figure 2) [13]. The group that includes the aquaglyceroporins (AQPs 3, 7, 9, and 10) captures the well-characterized glycerol-permeable channels (Figure 2). Residues in loops B and D influence channel gating. Weblogo diagrams showing relative amino acid identity (Figure 2) reveal that these domains are well conserved, apart from loop D in the aquaglyceroporins. Deeper insight into the structure–function relationships across the family will facilitate the development of new modulators for all three groups.

Controversies around current aquaporin modulators

The size and shape of aquaporin pores are understood in precise atomic detail (Figure 1A). This has enabled a molecular-level understanding of how aquaporins allow water molecules (but not protons) to permeate cell membranes [7] and how aquaglyceroporins facilitate the passage of larger molecules such as glycerol [14]. Although no structural evidence exists [15], isoforms such as human AQP6 have also been shown experimentally to mediate the flow of ions [16]. It has been proposed that ions might be conducted either via the central pore within the homotetramer (e.g., AQP1), through intra-subunit pores (e.g., AQP6) [17] or via cotrafficking of aquaporins and ion channels (e.g., AQP2-TRPV4) [18] (Figure 1B). However, despite a wealth of knowledge and intense efforts in industry and academia, no clinically useful pore-blocking drugs for any aquaporin have yet been discovered [9]. A key issue contributing to this situation may be the limited number of *in vitro* assays suitable for **high-throughput screening** and validating the pharmacological regulation of aquaporin function (Figure 3). Methodological differences in executing these assays mean that attempts to replicate prior work may also result in equivocal outcomes. The status of many aquaporin modulators is therefore controversial, as we discuss in the following text.

Orthodox aquaporins

Pharmacological blockers for orthodox aquaporins are proposed to act by occluding the intra-subunit water-conducting pore at either the extracellular or intracellular vestibule (Figure 1B). For example, aquaporin isoforms that contain accessible cysteine residues in the pore region (e.g., human AQP1-Cys189) can be reversibly modified by mercury chloride ($HgCl_2$; or other cysteine-reactive heavy metal compounds) to inhibit water transport [8]. Despite their undisputed importance in laboratory-based assays, heavy metal compounds are not viable leads for clinical application because of their cytotoxicity.

The data supporting the status of other aquaporin blockers are less clear. Tetraethylammonium (TEA; Box 1) ions are proposed to block aquaporin pores reversibly by interacting with pore-lining tyrosine residues, but the *in vivo* value of TEA is limited by its inhibition of other channel proteins, incomplete block of water channel function at maximal doses, and toxicity [9]. Using *Xenopus laevis* oocytes (Figure 3A), TEA reduced water fluxes of human AQP1 (Tyr186), AQP2 (Tyr178) and AQP4 (Tyr185) with IC_{50} values of 1.4, 6.2, and 9.4 μM, respectively [19,20]. Human AQP3 and AQP5, which lack a tyrosine residue in this position, were not blocked by TEA [20]. However, in erythrocytes (which also express AQP3), inhibition by Hg^{2+}, Au^{2+}, and Ag^{2+}, but not TEA, was observed [21]. In another oocyte study, TEA was found not to block AQP1 [22]. Notably, the erythrocyte study measured cell shrinking (net water efflux; Figure 3B), rather than oocyte swelling (net water influx; Figure 3A), as done in the experiments that first identified TEA-mediated block. While the current gold-standard assay for studying aquaporin function is the *Xenopus laevis* oocyte swelling assay, a survey of the literature suggests that it is especially susceptible to false positives, with many of the reports of aquaporin inhibitors discovered using this assay not reproduced in mammalian expression systems. Although no-one has yet offered a plausible explanation for this, it is crucial that this potential shortcoming is addressed experimentally.

We propose that studies of mammalian aquaporin inhibitors should, as a minimum, use two different mammalian expression systems, one exogenous to allow for a clear negative control, and one endogenous to control for overexpression artifacts prior to any *in vivo* work. An example from our own work is AQP4 stably overexpressed in Madin–Darby Canine Kidney (MDCK) cells with a non-transfected MDCK control and primary astrocytes endogenously expressing AQP4. As most mammalian aquaporins are recycled between the plasma membrane and endosomal compartments (and this process is tightly regulated by a variety of intracellular signaling pathways), we also propose that aquaporin plasma membrane abundance should also be measured (e.g., by cell surface biotinylation [23]) to ensure that any measured changes in aquaporin function are due to inhibition of channel function and not to changes in aquaporin membrane abundance.

Using oocytes, AqB013 (an analog of bumetamide, a loop diuretic that inhibits Na-K-Cl cotransporters), was shown to inhibit AQP1 and AQP4 [25]. Bumetanide reduced cerebral edema and AQP4 expression in a rodent stroke model [26]. Whether the effect was mediated by direct blockage of AQP4, alteration of AQP4 expression, or inhibition of Na-K-Cl cotransporters is unclear. A furosemide derivative (AqF026) increased AQP1 water channel activity in oocytes and *in vivo* accelerated AQP1-dependent peritoneal dialysis in a rodent model. This effect was not seen in the AQP1 null animal, suggesting AqF026 specificity [27]. The AQP1 modulatory function of both molecules has been challenged [28], although data for synthesis of AqF026 were not provided. Overall, differences in the experimental setup make it difficult to directly compare these studies.

3,3′-(1,3-Phenylene)bis(2-propenoic acid) is marketed as AQP1 inhibitor, TC AQP1 1, on the basis that it inhibits AQP1-mediated water flux in oocytes (IC_{50} 8 μM) [29]. However, independent

Glossary

Aquaporin: these are found in all organisms and share a common structural architecture. The functional aquaporin unit is a homotetramer in which each aquaporin monomer is composed of six transmembrane α-helices connected by alternating intracellular and extracellular loops. The transmembrane domains form a right-handed bundle around the central pore of each monomer through which water, solute, and in some cases, ion transport occurs. Water transport is facilitated by direct hydrogen bonding between a single file of water molecules and the family's signature Asn-Pro-Ala (NPA) motif and pore-lining hydrogen-bonding sites. In the water-selective orthodox aquaporins, exclusion of molecules larger than water is aided by the aromatic/arginine constriction site. In the neutral solute permeable aquaglyceroporins, solute discrimination (e.g., between glycerol and urea) depends on a complex interplay between the solute chemistry and the size and chemistry of the pore. While the structural biology of the aquaporin family is well established, the mechanisms that regulate the physiological function of aquaporins are less well understood.

Edema: refers to swelling, usually as a result of injury or inflammation, associated with excessive fluid accumulation. Cytotoxic cerebral edema is the accumulation of fluid in intact cells in the brain or spinal cord. It can be rapidly triggered when control of energy-dependent solute homeostasis is disrupted, often by ATP depletion as a result of hypoxia. Loss of control of transmembrane solute gradients can lead to the development of transmembrane osmotic gradients, which drive water influx through aquaporins, leading to a net swelling of brain tissue. There are no pharmacological interventions for cytotoxic cerebral edema, which is life-threatening due to the physical constraints on brain volume imposed by the inflexible skull.

High-throughput screening: screening using high-throughput (HTP) assays is a drug discovery process that involves automated equipment to allow testing of large numbers of chemical and/or biological compounds for a specific biological target. HTP assays accelerate target analysis, as large-scale

evaluation of this molecule in human erythrocytes demonstrated that it did not inhibit AQP1 water permeability at 50 μM when assayed by stopped flow spectrophotometry [8]. Despite this uncertainty, several chemical vendors currently market this compound as a validated AQP1 inhibitor.

Bacopaside II (from the traditional medicinal herb *Bacopa monnieri*) was characterized as a selective blocker of AQP1 expressed in oocytes. The results for mice *in vivo* and human cardiac cells *in vitro* showed that hypertrophic remodeling of the heart was caused by AQP1-mediated transport of hydrogen peroxide, which could be prevented by treatment with bacopaside II [30]. These findings suggest that inhibitors of AQP1 represent new possibilities for treating hypertrophic cardiomyopathies [30].

The arylsulfonamide, acetazolamide, is a carbonic anhydrase inhibitor (IC_{50} 10 nM) that is used clinically as a diuretic and to treat glaucoma and some convulsive disorders. It blocked AQP1 [29] (IC_{50} 5.5 μM) and AQP4 (IC_{50} 0.9 μM) [31] in *Xenopus* oocytes, although some investigators could not replicate this effect [32]. N-(5-sulfamoyl-1,3,4-thiadiazol-2-yl) acetamide is a derivative of acetazolamide that is active against carbonic anhydrase (IC_{50} 72 nM), and a candidate AQP4 inhibitor [33]. Ethoxyzolamide, another carbonic anhydrase inhibitor, was reported to inhibit human AQP4-M23-mediated water transport when tested at 20 μM [31] in oocytes. Other studies have described acetazolamide inhibition of aquaporin-mediated water transport due to the downregulation of expression, rather than a direct inhibition of channel function [34].

N-1,3,4-Thiadiazol-2-yl-3-pyridinecarboxamide is sold as AQP4 inhibitor, TGN-020; it inhibited water flux in oocytes expressing the M23 isoform of human AQP4 with an IC_{50} of 3.1 μM [35] and reduced ischemia-induced brain edema in mice pretreated 15 min before middle cerebral artery occlusion [36]. However, others report being unable to reproduce these results in cell-based assays [37] or have challenged the interpretation of the data [38]. Indeed, in our hands, TGN-020 does not inhibit AQP4 function in primary astrocytes expressing endogenous AQP4 or mammalian cell-lines overexpressing exogenous AQP4. There are also discrepancies between experiments with TGN-020 and those using *Aqp4*$^{-/-}$ animals. TGN-020 had no effect on stimulus-induced extracellular space (ECS) volume dynamics in the *ex vivo* CA1 stratum radiatum of *Aqp4*$^{+/+}$ rats [39], whereas in *Aqp4*$^{-/-}$mice (with no TGN-020 treatment), the same ECS volume changes were enhanced [40]. To our knowledge, the effect of TGN-020 has not been evaluated in *Aqp4*$^{-/-}$ mice. Recent *in vivo* findings suggesting that TGN-020 administration impairs glymphatic tracer distribution in mice [41] and slows ^{17}O-labeled H_2O distribution in rats [42] do not clarify the mode of action of TGN-020. In the glymphatic system, the clearance of brain waste occurs through paracellular flow. Classic tracer studies measure paracellular flow, while the use of $H_2{}^{17}O$ captures both paracellular flow and diffusive transcellular exchange of water. Importantly, both are AQP4-dependent, the former indirectly and the latter directly [43]. Acetazolamide has also been shown to inhibit glymphatic tracer distribution [44]. While its mechanism of action remains to be established, it could be via inhibition of AQP4 and/or carbonic anhydrase, which reduces CSF secretion (acetazolamide is used clinically to reduce CSF secretion in patients with intracranial hypertension). This highlights the need for a comprehensive understanding of all the potential targets of a drug before interpreting the results of *in vivo* experiments.

AER-270 is sold as a selective AQP4 inhibitor (IC_{50} 240 nM). Pharmacological inhibition of AQP4 by AER-270 was suggested to reduce central nervous system (CNS) edema and AER-271, a pro-drug of AER-270, is the subject of a phase I trial in healthy volunteers (NCT03804476; this trial completed enrollment in August 2019, but is yet to report any data). However, only 20%

compound libraries containing thousands of molecules can quickly be screened in a cost-effective way. There are no currently validated high-throughput assays of aquaporin function.

Symptom management: management of symptoms without addressing the underlying pathological cause(s). For example, osmotherapy using hyperosmotic saline or mannitol is often used in an attempt to reduce cerebral edema, despite limited evidence of efficacy.

Table 1. The sites of expression, associated diseases, and established permeabilities of the 13 human aquaporins. There are no pharmacological interventions to treat pathologies arising from their dysregulation

AQP isoform	Main expression site(s)	Single-channel water permeability [$x10^{-14}$ cm^3 s^{-1}]	Other known permeabilities	Major associated diseases
AQP0	Lens fiber cells	0.25	None	Cataracts
AQP1	Red blood cells Kidney: proximal tubule Brain: choroid plexus Lung: alveolar epithelial cells Liver: biliary tract	6.0	Monovalent cations Nitric oxide Hydrogen peroxide	Hyposthenuria Hydrocephalus Glioblastoma Lung cancer
AQP2	Kidney: collecting ducts	3.3	None	Nephrogenic diabetes insipidus (NDI)
AQP3	Skin Eye Respiratory tract: trachea, bronchial epithelium, epithelial cells	2.1	Glycerol Ammonia Hydrogen peroxide	Skin diseases Psoriasis Atopic dermatitis Eczema Colorectal cancer
AQP4	Brain: astrocyte, ependymal cells Kidney: collecting ducts Lung: bronchial epithelium	24	Nitric oxide	Brain and spinal cord edema Neuromyelitis optica Glioblastoma Epilepsy Alzheimer's
AQP5	Salivary glands Lacrimal gland Sweat gland Lung Eye: cornea	5.0	Hydrogen peroxide	Sjögren's syndrome Xerostomia Breast cancer
AQP6	Kidney	Low; no quantitative data	Ammonia Nitrate Monovalent anions	None yet reported
AQP7	Adipose tissue Kidney Testis	No quantitative data	Glycerol Urea Ammonia Arsenite	Adipocyte hypertrophy Type 2 diabetes mellitus Non-alcoholic fatty liver disease Metabolic syndrome
AQP8	Colon and small intestine Pancreas Liver	No quantitative data	Urea Ammonia Hydrogen peroxide	Colorectal and liver cancers Inflammatory bowel diseases
AQP9	Liver Brain Testis	No quantitative data	Glycerol Urea Hydrogen peroxide Arsenite, Carbamides Polyols Purines Pyrimidines Lactic acid Ammonia	Cystic liver disease Liver cirrhosis
AQP10	Small intestine Adipose tissue	No quantitative data	Glycerol	Obesity
AQP11	Kidney: proximal tubules Testis Liver	~2	Glycerol	Chronic kidney disease
AQP12	Pancreas	No quantitative data	None	Pancreatitis

maximal inhibition of mouse and human AQP4 was reported, compared with 70% maximal inhibition of rat AQP4. Despite this, AER-270 had the same effect on the water content in rat and mouse stroke models, suggesting that the effect may be AQP4-independent [45]. AER-270 is a known nuclear factor κB (NF-κB) inhibitor [usually under the name IMD-0354; N-(3,5-bis

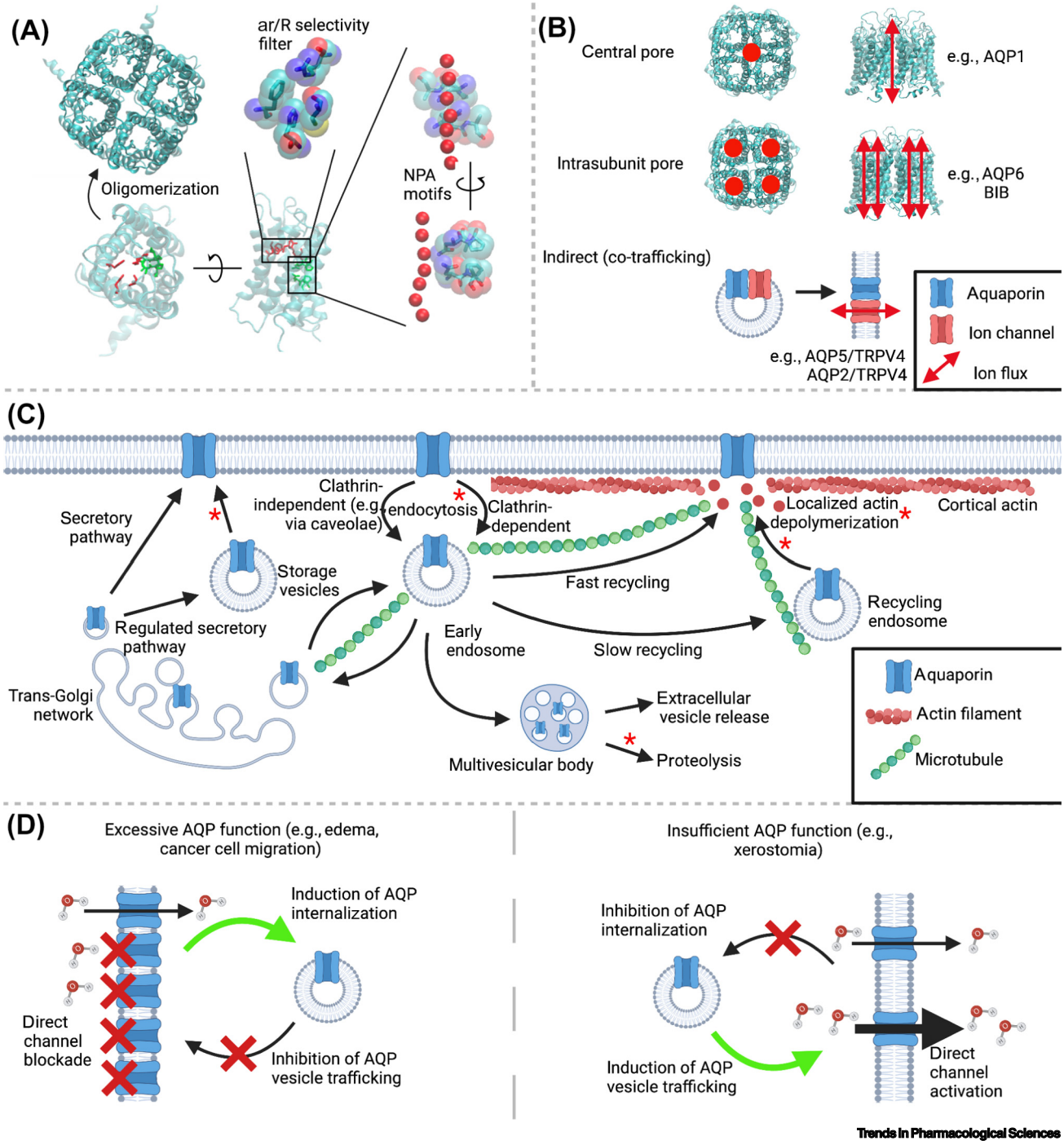

Figure 1. Aquaporin-mediated flow of water, small neutral solutes and ions and regulation by dynamic aquaporin subcellular relocalization. (A) Overview of aquaporin structural biology. Water molecules (red spheres) traverse the pore in single file. Two key regions facilitate water transport capacity [Asn-Pro-Ala (NPA) motifs, green], and solute exclusion (ar/R selectivity filter, red). For some isoforms, small neutral solutes such as glycerol traverse the pore in a similar fashion. (B) Three models of aquaporin-mediated transmembrane ion transport – direct ion transport through the aquaporin central (fifth) or monomeric (water channel) pores or cotrafficking of ion channels to the membrane. Double-headed red arrows indicate that passive, bidirectional ion flux is possible; its direction depends upon the ion concentration gradient. (C) Schematic representation of trafficking and recycling of aquaporins between the plasma membrane and vesicle pools. Red asterisks indicate processes that can be modulated by aquaporin posttranslational modification (e.g., phosphorylation) and/or aquaporin–protein interactions (e.g., AQP2-actin and AQP4-calmodulin). (D) Schematic representation of pharmacological strategies to treat conditions in which aquaporin-mediated conductance is dysregulated. Created with BioRender.com.

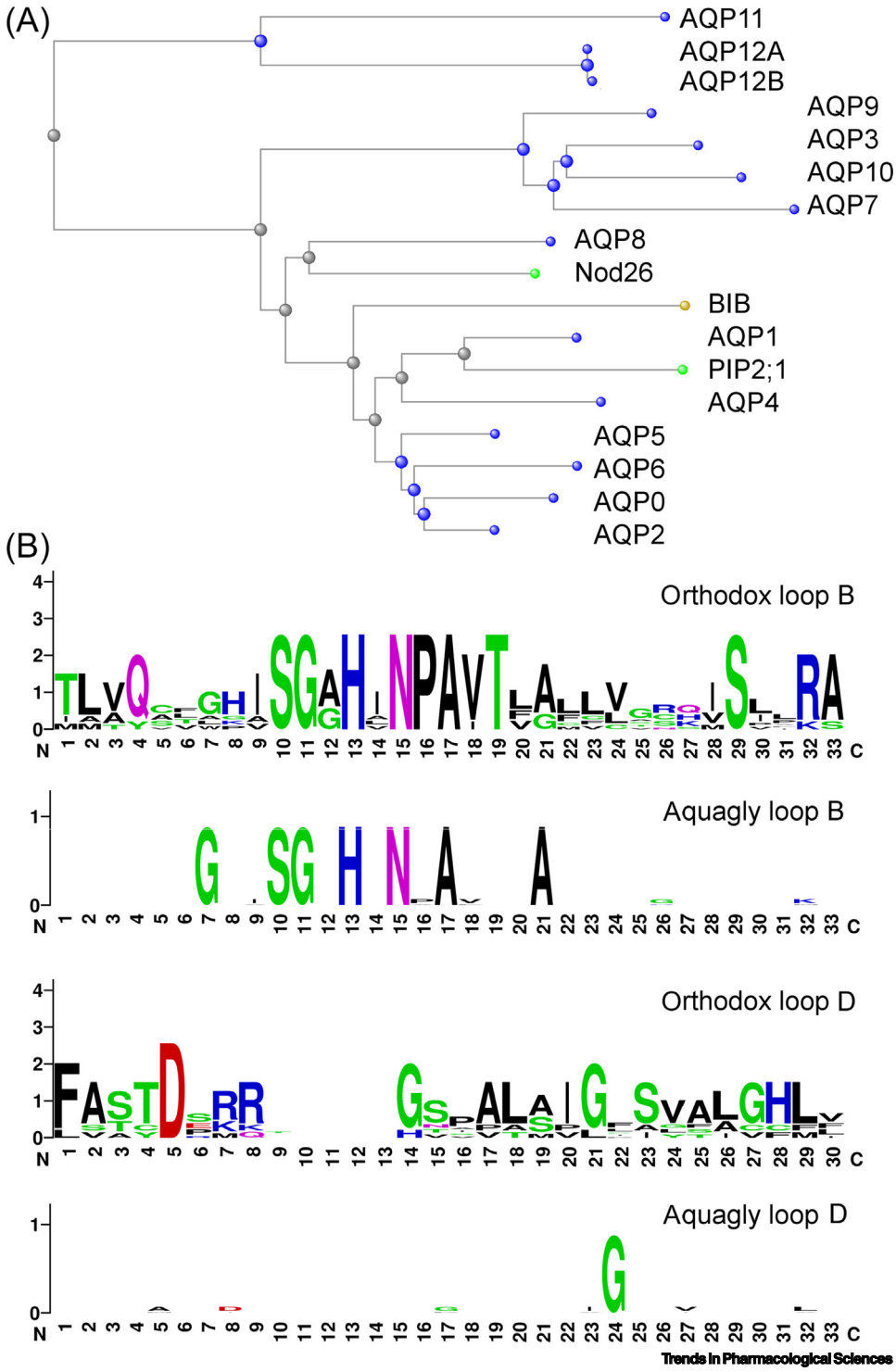

Figure 2. Human aquaporin amino acid sequence homologies, generated using COBALT (Constraint-based Multiple Alignment Tool)[i]. (A) Phylogenetic tree alignments were done for the complete coding sequences of proteins AQP0 (NP_036196), AQP1 (NP_000376), AQP2 (NP_000477), AQP3 (NP_004916), AQP4 (NP_001641), AQP5

(Figure legend continued at the bottom of the next page.)

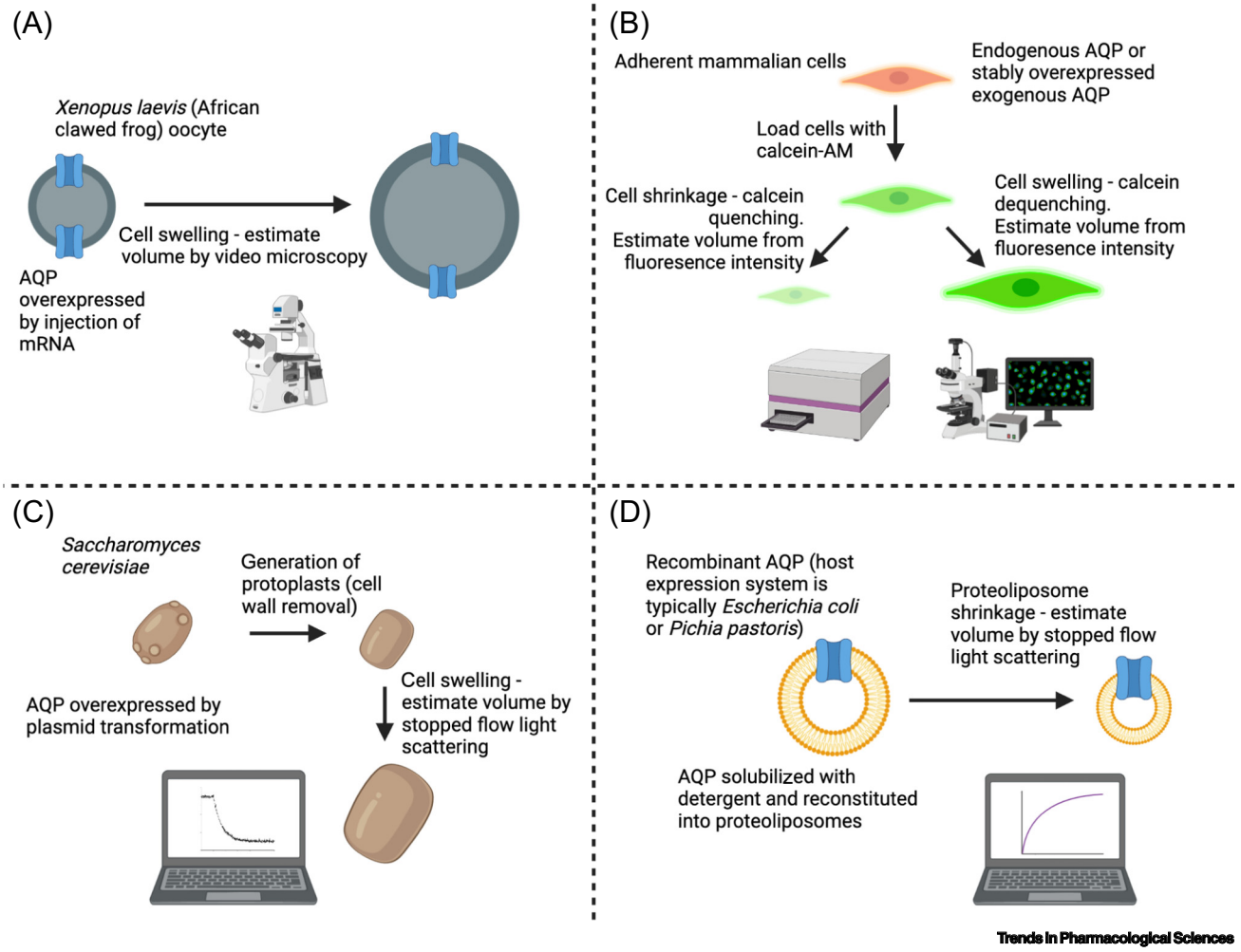

Figure 3. The lack of reliable *in vitro* assays suitable for screening and validating the pharmacological regulation of aquaporin function hinders drug discovery. Overview of four common functional assays for aquaporin water transport. (A) The *Xenopus laevis* oocyte assay is regarded as the gold-standard assay in the field, although it suffers from a lack of inter-laboratory reproducibility and is not amenable to high-throughput formats. Cell shrinkage and/or swelling can be measured by monitoring (B) changes in calcein fluorescence in adherent mammalian cells or by stopped flow light scattering in (C) yeast protoplasts or (D) proteoliposomes. Cell shrinkage/swelling assays have the potential to be adapted to 96- or 384-well plates, but this has yet to be robustly demonstrated on a large screen. Created with BioRender.com.

(trifluoromethyl)phenyl)-5-chloro-2-hydroxybenzamide], and NF-κB inhibition can reduce CNS water content [46], highlighting the need to consider off-target effects in *in vivo* studies.

The identification of clinically useful 'aquaretics', selective blockers of aquaporin water pores, would present an exciting advance for the therapeutic management of edema and fluid volume disorders. However, the inhibitory actions of TGN-020, AER-270, and other molecules discussed

(NP_001642), AQP6 (NP_001643), AQP7 (NP_001161), AQP8 (NP_001160), AQP9 (NP_066190), AQP10 (NP_536354), AQP11 (AAH40443), AQP12A (Q8IXF9), and AQP12B (AAI39749), with three non-mammalian AQPs known to have ion channel function, Arabidopsis AtPIP2;1 (P43286), Drosophila Big Brain (NP_001260313), and soybean Nodulin-26 (NP_001236728). (B).WebLogo (version 2.8.2)[ii] summary diagrams illustrating amino acid sequence patterns in loop B (two upper panels) for orthodox aquaporin channels (AQPs 1,2,4,5,6, PIP2;1 and BIB) and aquaglyceroporins (AQPs 3,7,9,10), and the sequence patterns for loop D for the same sets of AQP (two lower panels).

Box 1. Structures of putative small molecule aquaporin inhibitors.

Several structurally unrelated molecules have been proposed as aquaporin inhibitors, but in many cases the supporting evidence is equivocal. Examples of some of the most well-known inhibitors are given here (see Figure I). References in bold are supportive, while those in italics challenge the molecule's inhibitory function: **[A]** Migliati *et al.* [26]; **[B]** Farr *et al.* [45]; **[C]** Huber *et al.* [35]; **[D]** Gao *et al.* [24]; **[E]** Seeliger *et al.* [29]; **[F]** Brooks *et al.* [19]; **[G]**; Nakada and Huber [33]; **[H]** Huber *et al.* [31]; **[I]** Pei *et al.* [66]; *[J]* Esteva-Font *et al.* [28]; *[K]* Yang *et al.* [32] *[N]* denotes that inhibitory function has not been directly determined, but rather questioned in the reviews of the field on the basis of similarities with other tested agents. Carbon atoms (C) are shown in grey; oxygen (O) in red; sulfur (S) in yellow; nitrogen (N) in blue, and fluorine (F)/chlorine (Cl) in green.

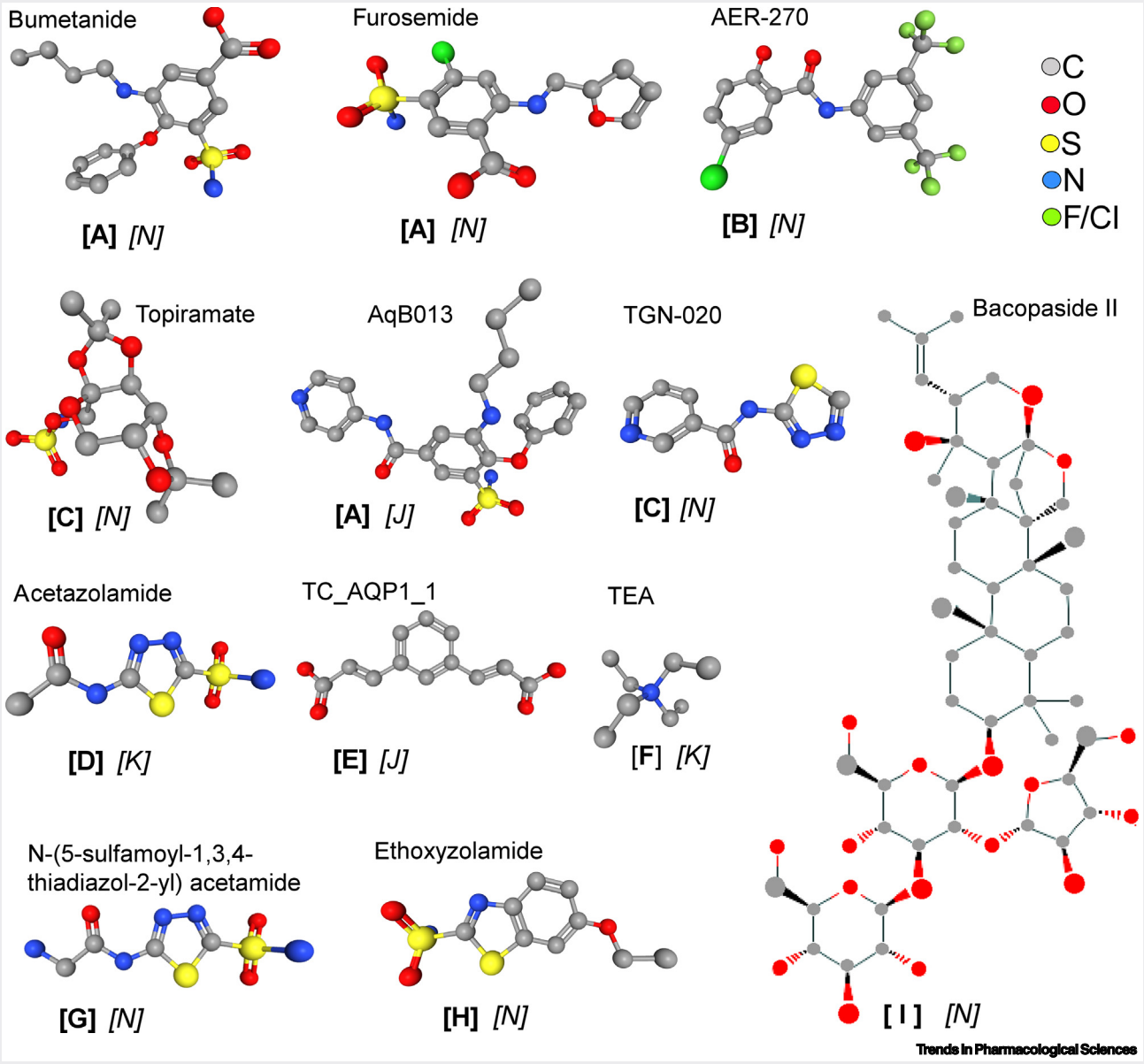

Figure I. Structures of putative small molecule aquaporin inhibitors.

in the preceding text have not always been reproduced in other assays and many may have AQP4-independent effects on brain water transport, potentially confounding the interpretation of *in vivo* studies. Indeed, the proposed AQP4 inhibitor topiramate [35] has anticonvulsant and

antiepileptic properties that are attributed to its combined effects in blocking excitatory voltage-gated sodium channels and kainate/AMPA glutamate receptors, potentiating the inhibitory activity of $GABA_A$ receptor signaling, and inhibiting carbonic anhydrase.

Aquaglyceroporins

A series of Au(III) compounds, including Auphen [Au^{III}(1,10-phenanthroline)Cl_2], potently inhibit glycerol permeation in human erythrocytes [47]. Auphen blocked NLRP3-inflammasome activation to a level comparable to knockdown of AQP3 by shRNA silencing [48]. Inhibition of AQP7 by Auphen in an adipocyte cell model implicated specific methionine residues as likely candidate binding sites [49]. Other Au(III) compounds have been reported to have excellent anticancer activity, low toxicity (LD_{50} 30 mg/kg), and low nephrotoxicity [50].

Earlier work showed that AQP3 water permeability is inhibited by 1 mM Ni^{2+} [47], and water and glycerol permeability of AQP3 is inhibited by 1 mM **Cu^{2+}** [49]. Polyoxotungstates were found to inhibit AQP3 function and impaired migration of an AQP3-expressing melanoma cell line, with P2W18, P2W12, and P5W30 identified as the most potent [51]. Screening of a library of drug-like small molecules using a calcein fluorescence quenching assay (Figure 3B) [52] identified new candidate inhibitors for AQPs 3, 7, and 9 [49].

Overall, there is much promise in the rapidly expanding portfolio of new inhibitors for orthodox aquaporins and aquaglyceroporins. However, a lack of clarity on the status of published molecules, some of which are marketed as aquaporin inhibitors, needs to be addressed in order to move the field forward, to prevent the generation of spurious *in vivo* data and to identify leads that can be translated to the clinic.

Targeting aquaporin regulation in the search for new modulators

In many physiological processes, cells need to modulate their plasma membrane water and solute permeabilities. Control via gene expression can alter water or solute permeability in the long term, but is too slow for a rapid response. Instead, conformational changes of individual aquaporin pores to change single-channel permeabilities (gating, observed mainly in plant and microbial aquaporins) or regulation of the number of pores in the membrane (more pores resulting in higher permeability) are strategies for a more rapid change in membrane permeability. AQP0 is gated via a protein–protein interaction with calmodulin [53], and AQPs 6 [16] and 10 [54] are reported to be gated by large changes in pH. While the induction of large pH changes *in vivo* is not feasible, the use of peptide-mimetic drugs that mimic the relevant histidine residues could be explored pharmacologically.

Mammalian aquaporins are much more commonly regulated by the dynamic control of their subcellular localization (Figure 1C) than they are by gating. This is best characterized for AQP2 in the kidney-collecting duct, where activation of the G_s-coupled V2 vasopressin receptor leads to phosphorylation of AQP2 at a C-terminal serine residue (Ser256) by protein kinase A, leading to redistribution of AQP2-bearing vesicles to the plasma membrane [55]. Although this has traditionally been considered an idiosyncrasy of AQP2, there is a now a large body of literature describing subcellular relocalization of most mammalian aquaporins, including AQP1, AQP3, AQP4, AQP5, AQP7, AQP8, AQP9, and AQP10 [56]. Indeed, a static view of aquaporin localization is not consistent with the need for homeostatic control in response to dynamic extracellular environments [57] and it is increasingly clear that subcellular relocalization is a ubiquitous regulatory mechanism across the mammalian aquaporin family.

Despite the ubiquity of aquaporin membrane insertion/removal and recycling through vesicle pools (Figure 1C), the underlying molecular mechanisms appear to be distinct for different

aquaporin isoforms, and in some cases, even for the same aquaporin in different cell types. As an example of this variation on a biological theme, in kidney cells phosphorylation of AQP4 at Ser276 enhanced lysosomal targeting of AQP4 upon internalization [57], reducing the amount of AQP4 recycled back to the membrane through the recycling endosome pathway. In contrast, phosphorylation of AQP4 in astrocytes led to an increase in plasma membrane abundance [4,23]. From a drug development perspective, this cell-type specificity is attractive, as it reduces the potential for side effects. However, it also emphasizes that care must be taken to choose appropriate *in vitro* models before proceeding to *in vivo* studies.

Direct aquaporin phosphorylation [56] (with different kinases acting on different aquaporins) has been demonstrated for protein kinases A and C, and casein kinase II [57]. Further kinases, including extracellular signal-regulated kinase, p38 mitogen-activated protein kinase, and Ca^{2+}/calmodulin-dependent protein kinase II have been implicated in aquaporin subcellular relocalization, but clear evidence that the aquaporin is the direct target of phosphorylation is awaited [58,59]. The initial signals that activate these kinases to phosphorylate aquaporins are also diverse, but typically involve activation of a G protein-coupled receptor [55] or a gated ion channel. The latter are typically from the transient receptor potential family [4], members of which can facilitate Ca^{2+} influx in response to a variety of signals, including changes in membrane tension caused by cell swelling or shrinkage [60].

As these mechanisms are subtly different for different aquaporins in different tissues, there is an unexploited opportunity for the development of specific and selective modulators of aquaporin function (Figure 1D). We recently demonstrated that relocalization of APQ4 in astrocytes depends upon a direct interaction with calmodulin. Using the calmodulin-inhibiting antipsychotic drug, trifluoperazine, AQP4 localization to the blood–spinal cord barrier was reduced after spinal cord injury in rats, CNS edema was ablated, and functional recovery was accelerated compared with untreated animals [4]. This study provides a blueprint for drug discovery for other aquaporin-mediated pathologies, as was recently demonstrated in a stroke model [61].

Targeting ion channel function in the search for new modulators

The current view of AQP1-mediated conduction of ions is that ions and water are transported through separate pathways [62], with water going through the individual pores of the subunits, and ion transport occurring through the central pore (Figure 1B) and blocked by Cd^{2+}, 5-hydroxymethyl furfural, bacopside I, and the bumetanide derivative, AqB011 [63].

AqB011 caused dose-dependent inhibition of the AQP1 ion pore, with an estimated IC_{50} of 14 μM, but did not interfere with water transport [64]. Molecular docking predicted the site of interaction to be intracellular loop D at the conserved arginine residues needed for AQP1 ion channel gating [15]. This was confirmed by mutagenesis and electrophysiology [65]. AqB011 holds promise as a possible adjunct treatment for controlling cancer metastasis, by blocking the AQP1 ionic conductance that facilitates cancer cell migration in AQP1-positive cancers [25,66]. The medicinal plant component, bacopaside I, similarly blocked the AQP1 ion channel in loop D, and inhibited migration of AQP1-expressing colon cancer cells [63]. Based on the amino acid sequences of the loop D gating region, AqB011 might be expected to be selective; for example, the plant channels AtPIP2;1 and AtPIP2;1 with different loop D domains were insensitive [67].

Ubiquitous modulation by divalent cations is a theme seen across many types of ion channels and receptors, including N-methyl-D-aspartate (NMDA) glutamate receptors, cation channels, K^+ channels, Na^+ channels, and others as reviewed previously [68]. Tyrosine phosphorylation in the C-terminal domain increased the activation of AQP1 by cGMP [69]. When key C-terminal

residues were altered by site-directed mutagenesis, activation of the AQP1 ionic current was impaired, but water permeability was retained [69], suggesting that intramolecular interactions between loop D and the C-terminus might add another layer of gating control [65].

Concluding remarks and future perspectives

Aquaporins support a wide range of physiological processes; their dysregulation leads to diverse diseases. Although they are validated drug targets, it is still unclear whether their pores are intrinsically undruggable or whether they can be blocked with small drug-like molecules (see Outstanding questions). Targeting the molecular mechanisms of aquaporin subcellular relocalization provides a compelling alternative approach for drug development, as demonstrated for AQP4 in models of CNS edema. The definition of signature motifs and molecular mechanisms of ion transport also provide new avenues for future drug discovery. Development of robust assays and the validation of potential aquaporin modulators across different assay systems will underpin the field. This will give hope to the millions of patients worldwide each year for whom no pharmacological intervention is available.

Outstanding questions

Are aquaporin pores intrinsically undruggable or can they be blocked with sufficient specificity by small drug-like molecules?

Is aquaporin subcellular localization a viable alternative target for drug discovery?

Can we define signature motifs and molecular mechanisms of ion transport in relevant classes of aquaporins?

Can a reproducible, standard assay be established to validate aquaporin function that has the potential to be miniaturized for high-throughput screening?

Acknowledgments

The authors acknowledge grants from the Biotechnology and Biological Sciences Research Council (to R.M.B. and P.K. through BB/P025927/1); Aston University (to P.K. through a 50th Anniversary Prize Fellowship); and the Australian Research Council Grant ARC DP190101745 (to A.J.Y.).

Declaration of interests

No interests are declared.

Resources

[i]www.ncbi.nlm.nih.gov/tools/cobalt/re_cobalt.cgi

[ii]http://weblogo.berkeley.edu/logo.cgi

References

1. Kourghi, M. *et al.* (2018) Fundamental structural and functional properties of aquaporin ion channels found across the kingdoms of life. *Clin. Exp. Pharmacol. Physiol.* 45, 401–409
2. Azad, A.K. *et al.* (2021) Human aquaporins: functional diversity and potential roles in infectious and non-infectious diseases. *Front. Genet.* 12, 654865
3. Soyfoo, M.S. *et al.* (2018) Involvement of aquaporins in the pathogenesis, diagnosis and treatment of Sjogren's syndrome. *Int. J. Mol. Sci.* 19, 3392
4. Kitchen, P. *et al.* (2020) Targeting aquaporin-4 subcellular localization to treat central nervous system edema. *Cell* 181, 784–799.e19
5. Salman, M.M. *et al.* (2021) Emerging roles for dynamic aquaporin-4 subcellular relocalization in CNS water homeostasis. *Brain* awab311. https://doi.org/10.1093/brain/awab311
6. Dajani, S. *et al.* (2018) Water transport proteins-aquaporins (AQPs) in cancer biology. *Oncotarget* 9, 36392–36405
7. Bill, R.M. and Hedfalk, K. (2021) Aquaporins - expression, purification and characterization. *Biochim. Biophys. Acta Biomembr.* 1863, 183650
8. Tradtrantip, L. *et al.* (2017) Aquaporin-targeted therapeutics: state-of-the-field. *Adv. Exp. Med. Biol.* 969, 239–250
9. Abir-Awan, M. *et al.* (2019) Inhibitors of mammalian aquaporin water channels. *Int. J. Mol. Sci.* 20, 1589
10. Brown, D. (2021) Aquaporin function: seek and you shall find! *Function (Oxf)* 2, zqaa041
11. Bjorkskov, F.B. *et al.* (2017) Purification and functional comparison of nine human aquaporins produced in Saccharomyces cerevisiae for the purpose of biophysical characterization. *Sci. Rep.* 7, 16899
12. Ishibashi, K. *et al.* (2021) The role of mammalian superaquaporins inside the cell: an update. *Biochim. Biophys. Acta Biomembr.* 1863, 183617
13. Gonen, T. and Walz, T. (2006) The structure of aquaporins. *Q. Rev. Biophys.* 39, 361–396
14. de Mare, S.W. *et al.* (2020) Structural basis for glycerol efflux and selectivity of human aquaporin 7. *Structure* 28, 215–222 e3
15. Yu, J. *et al.* (2006) Mechanism of gating and ion conductivity of a possible tetrameric pore in aquaporin-1. *Structure* 14, 1411–1423
16. Warshel, A. (2005) Inverting the selectivity of aquaporin 6: gating versus direct electrostatic interaction. *Proc. Natl. Acad. Sci. U. S. A.* 102, 1813–1814
17. Yool, A.J. and Campbell, E.M. (2012) Structure, function and translational relevance of aquaporin dual water and ion channels. *Mol. Asp. Med.* 33, 553–561
18. Pizzoni, A. *et al.* (2021) Release of ATP by TRPV4 activation is dependent upon the expression of AQP2 in renal cells. *J. Cell. Physiol.* 236, 2559–2571
19. Brooks, H.L. *et al.* (2000) Inhibition of aquaporin-1 water permeability by tetraethylammonium: involvement of the loop E pore region. *Mol. Pharmacol.* 57, 1021–1026
20. Detmers, F.J. *et al.* (2006) Quaternary ammonium compounds as water channel blockers. Specificity, potency, and site of action. *J. Biol. Chem.* 281, 14207–14214
21. Yang, B. *et al.* (2006) Comparative efficacy of HgCl2 with candidate aquaporin-1 inhibitors DMSO, gold, TEA+ and acetazolamide. *FEBS Lett.* 580, 6679–6684
22. Sogaard, R. and Zeuthen, T. (2008) Test of blockers of AQP1 water permeability by a high-resolution method: no effects of tetraethylammonium ions or acetazolamide. *Pflugers Arch.* 456, 285–292
23. Kitchen, P. *et al.* (2015) Identification and molecular mechanisms of the rapid tonicity-induced relocalization of the aquaporin 4 channel. *J. Biol. Chem.* 290, 16873–16881

24. Gao, J. *et al.* (2006) Acetazolamide inhibits osmotic water permeability by interaction with aquaporin-1. *Anal. Biochem.* 350, 165–170
25. De Ieso, M.L. and Yool, A.J. (2018) Mechanisms of aquaporin-facilitated cancer invasion and metastasis. *Front Chem.* 6, 135
26. Migliati, E.R. *et al.* (2009) Inhibition of aquaporin-1 and aquaporin-4 water permeability by a derivative of the loop diuretic bumetanide acting at an internal pore-occluding binding site. *Mol. Pharmacol.* 76, 105–112
27. Tsai, C.M. (2020) AqF026 may act as a cancer therapeutic agent via inducing cancer cell oncosis. *Med. Hypotheses* 140, 109685
28. Esteva-Font, C. *et al.* (2016) Experimental evaluation of proposed small-molecule inhibitors of water channel aquaporin-1. *Mol. Pharmacol.* 89, 686–693
29. Seeliger, D. *et al.* (2013) Discovery of novel human aquaporin-1 blockers. *ACS Chem. Biol.* 8, 249–256
30. Montiel, V. *et al.* (2020) Inhibition of aquaporin-1 prevents myocardial remodeling by blocking the transmembrane transport of hydrogen peroxide. *Sci. Transl. Med.* 12, eaay2176
31. Huber, V.J. *et al.* (2007) Identification of arylsulfonamides as aquaporin 4 inhibitors. *Bioorg. Med. Chem. Lett.* 17, 1270–1273
32. Yang, B. *et al.* (2008) Lack of aquaporin-4 water transport inhibition by antiepileptics and arylsulfonamides. *Bioorg. Med. Chem.* 16, 7489–7493
33. Nakada, T. and Huber, V.J. Inhibitors of aquaporin 4, methods and uses thereof, 2010, US Patent US7659312B2 https://patents.google.com/patent/US7659312B2/en.
34. Han, M. *et al.* (2020) Collateral augmentation treatment with a combination of acetazolamide and head-down tilt in a rat ischemic stroke model. *J. Clin. Neurosci.* 73, 252–258
35. Huber, V.J. *et al.* (2009) Identification of aquaporin 4 inhibitors using in vitro and in silico methods. *Bioorg. Med. Chem.* 17, 411–417
36. Igarashi, H. *et al.* (2011) Pretreatment with a novel aquaporin 4 inhibitor, TGN-020, significantly reduces ischemic cerebral edema. *Neurol. Sci.* 32, 113–116
37. Verkman, A.S. *et al.* (2017) The aquaporin-4 water channel as a potential drug target in neurological disorders. *Expert Opin. Ther. Targets* 21, 1161–1170
38. Choi, Y.H. *et al.* (2021) Molecular mechanisms of neuroimmune crosstalk in the pathogenesis of stroke. *Int. J. Mol. Sci.* 22, 9486
39. Toft-Bertelsen, T.L. *et al.* (2021) Clearance of activity-evoked K(+) transients and associated glia cell swelling occur independently of AQP4: a study with an isoform-selective AQP4 inhibitor. *Glia* 69, 28–41
40. Haj-Yasein, N.N. *et al.* (2012) Aquaporin-4 regulates extracellular space volume dynamics during high-frequency synaptic stimulation: a gene deletion study in mouse hippocampus. *Glia* 60, 867–874
41. Harrison, I.F. *et al.* (2020) Impaired glymphatic function and clearance of tau in an Alzheimer's disease model. *Brain* 143, 2576–2593
42. Alshuhri, M.S. *et al.* (2021) Direct imaging of glymphatic transport using H217O MRI. *JCI Insight* 6, e141159
43. Salman, M.M. *et al.* (2021) Aquaporin 4 and glymphatic flow have central roles in brain fluid homeostasis. *Nat. Rev. Neurosci.* 22, 650–651
44. Zhang, C. *et al.* (2018) Characterizing the glymphatic influx by utilizing intracisternal infusion of fluorescently conjugated cadaverine. *Life Sci.* 201, 150–160
45. Farr, G.W. *et al.* (2019) Functionalized phenylbenzamides inhibit aquaporin-4 reducing cerebral edema and improving outcome in two models of CNS injury. *Neuroscience* 404, 484–498
46. Gong, Y. *et al.* (2021) Inhibition of the NKCC1/NF-κB signaling pathway decreases inflammation and improves brain edema and nerve cell apoptosis in an SBI rat model. *Front. Mol. Neurosci.* 14, 641993
47. Wenzel, M.N. *et al.* (2019) Insights into the mechanisms of aquaporin-3 inhibition by gold(iii) complexes: the importance of non-coordinative adduct formation. *Inorg. Chem.* 58, 2140–2148
48. da Silva, I.V. *et al.* (2021) Aquaporin-3 is involved in NLRP3-inflammasome activation contributing to the setting of inflammatory response. *Cell. Mol. Life Sci.* 78, 3073–3085
49. Sonntag, Y. *et al.* (2019) Identification and characterization of potent and selective aquaporin-3 and aquaporin-7 inhibitors. *J. Biol. Chem.* 294, 7377–7387
50. Altaf, M. *et al.* (2019) Potent in vitro and in vivo anticancer activity of new bipyridine and bipyrimidine gold (iii) dithiocarbamate derivatives. *Cancers (Basel)* 11, 474
51. Pimpao, C. *et al.* (2020) The aquaporin-3-inhibiting potential of polyoxotungstates. *Int. J. Mol. Sci.* 21, 2467
52. Kitchen, P. *et al.* (2020) Calcein fluorescence quenching to measure plasma membrane water flux in live mammalian cells. *STAR Protoc.* 1, 100157
53. Fields, J.B. *et al.* (2017) Calmodulin gates aquaporin 0 permeability through a positively charged cytoplasmic loop. *J. Biol. Chem.* 292, 185–195
54. Gotfryd, K. *et al.* (2018) Human adipose glycerol flux is regulated by a pH gate in AQP10. *Nat. Commun.* 9, 4749
55. Vukicevic, T. *et al.* (2016) The trafficking of the water channel aquaporin-2 in renal principal cells-a potential target for pharmacological intervention in cardiovascular diseases. *Front. Pharmacol.* 7, 23
56. Nyblom, M. and Törnroth-Horsefield, S. (2016) Regulation of eukaryotic aquaporins. In *Aquaporins in Health and Disease: New Molecular Targets for Drug Discovery* (Soveral, G. and Nielsen, S. and Casini, A., eds), pp. 53–71, CRC Press
57. Nesverova, V. and Törnroth-Horsefield, S. (2019) Phosphorylation-dependent regulation of mammalian aquaporins. *Cells* 8, 82
58. Salman, M.M. *et al.* (2017) Transcriptome analysis suggests a role for the differential expression of cerebral aquaporins and the MAPK signalling pathway in human temporal lobe epilepsy. *Eur. J. Neurosci.* 46, 2121–2132
59. Salman, M.M. *et al.* (2017) Transcriptome analysis of gene expression provides new insights into the effect of mild therapeutic hypothermia on primary human cortical astrocytes cultured under hypoxia. *Front. Cell. Neurosci.* 11, 386
60. Salman, M.M. *et al.* (2017) Hypothermia increases aquaporin 4 (AQP4) plasma membrane abundance in human primary cortical astrocytes via a calcium/transient receptor potential vanilloid 4 (TRPV4)- and calmodulin-mediated mechanism. *Eur. J. Neurosci.* 46, 2542–2547
61. Sylvain, N.J. *et al.* (2021) The effects of trifluoperazine on brain edema, aquaporin-4 expression and metabolic markers during the acute phase of stroke using photothrombotic mouse model. *Biochim. Biophys. Acta Biomembr.* 1863, 183573
62. Rivera, M.A. and Fahey, T.D. (2019) Association between aquaporin-1 and endurance performance: a systematic review. *Sports Med. Open* 5, 40
63. De Ieso, M.L. *et al.* (2019) Combined pharmacological administration of AQP1 ion channel blocker AqB011 and water channel blocker Bacopaside II amplifies inhibition of colon cancer cell migration. *Sci. Rep.* 9, 12635
64. Kourghi, M. *et al.* (2016) Bumetanide derivatives AqB007 and AqB011 selectively block the aquaporin-1 ion channel conductance and slow cancer cell migration. *Mol. Pharmacol.* 89, 133–140
65. Kourghi, M. *et al.* (2018) Identification of Loop D domain amino acids in the human aquaporin-1 channel involved in activation of the ionic conductance and inhibition by AqB011. *Front Chem.* 6, 142
66. Pei, J.V. *et al.* (2016) Drug discovery and therapeutic targets for pharmacological modulators of aquaporin channels. In *Aquaporins in Health and Disease: New Molecular Targets For Drug Discovery* (Soveral, G. *et al.*, eds), pp. 275–297, CRC Press
67. Kourghi, M. *et al.* (2017) Divalent cations regulate the ion conductance properties of diverse classes of aquaporins. *Int. J. Mol. Sci.* 18, 11
68. Phillips, M.B. *et al.* (2020) Interplay between gating and block of ligand-gated ion channels. *Brain Sci.* 10, 928
69. Ozu, M. *et al.* (2018) Aquaporins: more than functional monomers in a tetrameric arrangement. *Cells* 7, 209