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### Permalink

<https://escholarship.org/uc/item/92k5n43x>

### Journal

Nature Reviews Drug Discovery, 13(4)

### ISSN

1474-1776

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### Publication Date

2014-04-01

### DOI

10.1038/nrd4226

Peer reviewed



Published in final edited form as:

*Nat Rev Drug Discov.* 2014 April ; 13(4): 259–277. doi:10.1038/nrd4226.

## Aquaporins: important but elusive drug targets

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### Abstract

The aquaporins (AQPs) are a family of small, integral membrane proteins that facilitate water transport across the plasma membranes of cells in response to osmotic gradients. Data from knockout mice support the involvement of AQPs in epithelial fluid secretion, cell migration, brain oedema and adipocyte metabolism, which suggests that modulation of AQP function or expression could have therapeutic potential in oedema, cancer, obesity, brain injury, glaucoma and several other conditions. Moreover, loss-of-function mutations in human AQPs cause congenital cataracts (AQP0) and nephrogenic diabetes insipidus (AQP2), and autoantibodies against AQP4 cause the autoimmune demyelinating disease neuromyelitis optica. Although some potential AQP modulators have been identified, challenges associated with the development of better modulators include the druggability of the target and the suitability of the assay methods used to identify modulators.

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Aquaporins (AQPs) are a class of membrane water channels whose primary function is to facilitate the passive transport of water across the plasma membrane of the cell in response to osmotic gradients that are created by the active transport of solutes. Aquaglyceroporins, which form a subset of the 13 mammalian AQPs, also facilitate the passive transport of glycerol and possibly other small solutes such as urea and carbon dioxide. As we discuss below, the water-selective AQPs are involved in many biological functions, including transepithelial fluid transport, cell migration, brain oedema and neuroexcitation. The aquaglyceroporins are involved in cell proliferation, adipocyte metabolism and epidermal water retention.

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#### Competing interests statement

The authors declare competing interests: see Web version for details.

#### DATABASES

**PDBePISA tool:** [http://www.ebi.ac.uk/msd-srv/prot\\_int/pistart.html](http://www.ebi.ac.uk/msd-srv/prot_int/pistart.html)

**Protein Data Bank:** <http://www.pdb.org>

**ZINC Database:** <http://zinc.docking.org>

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As highlighted here, data from AQP-knockout mice and from humans with loss-of-function mutations in AQPs suggest that modulators of AQP function may have broad clinical indications, including in nephrology (for the treatment of oedema and hypertension), neurology (for the treatment of brain swelling and epilepsy), oncology (for the treatment of tumour angiogenesis and proliferation), ophthalmology (for the treatment of corneal and lens transparency as well as glaucoma) and in the treatment of obesity and dermatological indications (namely, epidermal hydration and proliferation). In addition, two human diseases that are linked to aquaporins (known as aquaporinopathies) present drug development opportunities, including potential therapies: nephrogenic diabetes insipidus (NDI), which is caused by loss-of-function *AQP2* mutations; and neuromyelitis optica (NMO), which is caused by the presence of auto-antibodies against AQP4.

Here, we review the structure and function of AQPs, the evidence in support of AQPs as drug targets, as well as progress and challenges in the discovery of AQP-targeted small molecules, biologics and gene therapies. Although there is compelling evidence from studies using knockout mice that AQPs are drug targets, progress in the discovery of AQP modulators has been slow, in part because current efforts to identify inhibitors are hampered by challenges in screening assays and in targeting the compact, pore-containing AQP molecule.

## AQP structure and function

There is a large amount of information available about the molecular structure of AQPs (reviewed in REFS 1,2), which could potentially facilitate the discovery of AQP-targeted small molecules. AQPs are organized as tetramers on membranes (FIG. 1a). At least one of the AQPs, AQP4, can associate into higher-order supramolecular assemblies known as orthogonal arrays of particles, in which AQP4 tetramers form square arrays that are stabilized by the interactions of aminoterminal residues in the monomeric units<sup>3-5</sup>. Early, low-resolution AQP structures solved by electron crystallography include AQP0 (also known as MIP; Protein Data Bank (PDB) codes: 1SOR and 2B6O), AQP1 (PDB codes: 1IH5, 1FQY and 1H6I) and AQP4 (PDB code: 2D57). High-resolution X-ray crystal structures are available for AQP0 (PDB codes: 1YMG and 2B6P), AQP1 (PDB code: 1J4N), AQP4 (PDB code: 3GD8) and AQP5 (PDB code: 3D9S). Although the low-resolution structures garnered an understanding of the general topology of AQPs, the high-resolution structures provided greater insight into the atomic-level mechanisms of water and solute conduction and of proton and/or ion exclusion, and provide a basis for virtual screening and molecular dynamics simulations. Electron and X-ray crystal diffraction structures for several non-mammalian AQPs have also been solved, including the bacterial aquaporin AqpZ (PDB codes: 1RC2 and 2ABM) and the glycerol facilitator GlpF (PDB codes: 1LDA, 1LDI and 1FX8), as well as malarial *Plasmodium falciparum* AQP (PfAQP; PDB code: 3C02).

The monomeric units of AQPs are ~30 kDa and consist of six transmembrane  $\alpha$ -helices (known as M1, M2, M4–M7 and M8), two half helices (M3 and M7) and five connecting loops (loops a–e) (FIG. 1b,c). The N- and carboxyterminal domains are located in the cytoplasm. Although AQPs facilitate water transport (and aquaglyceroporins additionally

transport glycerol) by a mechanism involving an aqueous pore, they prevent the conduction of protons, which is crucial to avoid dissipation of proton gradients.

Each AQP monomer consists of an extracellular and cytoplasmic vestibule that is connected by a central amphipathic pore region of 20–25 Å containing hydrophilic and hydrophobic surfaces, as exemplified by the X-ray structure of bovine AQP1 (REF. 6) (FIG. 1d). The hydrophilic surface consists of  $\alpha$ -carbonyl groups from the polypeptide backbone, and the hydrophobic residues of the amphipathic pore prevent the permeation of larger molecules<sup>6</sup>. There are two conserved Asn-Pro-Ala (NPA) motifs in the half-helices M3 and M7, which contain inward-facing asparagine polar side chains. These motifs are part of the hydrophilic surface of the amphipathic pore and are important for preventing proton conduction<sup>7</sup>. Together with the backbone  $\alpha$ -carbonyl groups, the NPA motifs act as hydrogen-bond donors and acceptors that coordinate the transport of water (or glycerol for the aquaglyceroporins) through the pore<sup>6,8</sup>.

The extracellular vestibule<sup>6</sup> contains a constriction region that is made up of aromatic and arginine residues (known as the ar/R constriction or the selectivity filter), which is also important for preventing proton conduction<sup>7,9</sup>. The ar/R constriction makes up the narrowest region of the pore, and has a diameter of ~2.8 Å in human AQP1, ~1.5 Å in human AQP4 and ~3.4 Å for the less selective aquaglyceroporins<sup>6,8,10</sup>. The narrow interior pore region of AQPs presents a potential challenge in the identification of small molecules that target the aqueous pore region.

Molecular dynamics simulations have been useful in elucidating the mechanisms of water and glycerol transport through AQPs. An interesting conclusion from these studies is that water molecule dipoles orient symmetrically on opposing sides of the NPA motif, which prevents proton transfer through a Grothuss-type ‘proton-wire’ mechanism<sup>11</sup>. One theory for the mechanism of proton blockade emerging from molecular dynamics simulations is that hydronium ions need to be de-solvated, which is energetically unfavourable in the protein environment<sup>12,13</sup>. Free-energy profiles for water permeation through the aqueous pore region<sup>13,14</sup>, obtained from molecular dynamics simulations, have also been used to investigate the potential water- and solute-transporting function of the central region of AQP tetramers<sup>15</sup>.

Recent molecular dynamics simulations have suggested that a novel gating mechanism operates in AQP5, in which monomeric channels are in distinct open or closed conformations<sup>16</sup>. This finding adds to the knowledge of the structural basis of AQP gating<sup>17</sup>; previous experimentally and computationally derived mechanisms proposed that AQP gating involved mechanosensitivity and phosphorylation in yeast AQPs<sup>18</sup>, and membrane tension in both mammalian and yeast AQPs<sup>19–21</sup>.

The combination of molecular dynamics and other computational methods, such as virtual screening, has been used to identify possible AQP inhibitors (BOX 1). The abundance of X-ray crystal structure data suggests that virtual screening and computationally biased high-throughput screening will aid the identification of inhibitors.

**Box 1****The use of computational methods in AQP drug discovery**

The growing knowledge of aquaporin (AQP) structure, particularly the atomic-level geometry of specific hydrophobic and hydrophilic residues in the pore region, makes AQPs amenable to computational methods of drug discovery. High-resolution X-ray diffraction crystal structures are now available for the major mammalian AQPs: AQP0, AQP1, AQP4 and AQP5. Owing in part to recent improvements in parallel computing, which have been facilitated by graphical processor units, and techniques such as coarse-grained simulations for biological membranes<sup>178</sup>, it has become practical to perform molecular dynamics simulations on physiologically relevant timescales of up to 1 millisecond.

Protein–ligand docking has been used to propose binding modes of small-molecule AQP inhibitors<sup>132,135,136,145,179</sup> and, in some cases, the docking results have been studied using molecular dynamics simulations to propose the dynamics of inhibitor binding<sup>132,179</sup>. In one study, docking was used for the virtual screening of AQP1 inhibitors, then molecular dynamics was used to confirm that the orientation of the inhibitors remained static under equilibration conditions<sup>142</sup>. However, in most cases the inhibitors identified by the computational screen were later found to be inactive by definitive functional assays (as discussed in the main text). AQP binding and the selectivity of the AQP3-targeting gold (III) inhibitors was investigated using homology modelling and docking, with inhibitor binding characterized by density functional theory calculations. It was hypothesized that the inhibitors interact with the accessible Cys40 residue on the extracellular side of AQP3, with the traditional metal-binding residue Cys189 in AQP1 not favourably positioned to interact with these compounds<sup>126,127</sup>.

Although the extensive structural information available for AQP makes virtual screening promising in principle, the use of computational technology to identify bona fide inhibitors is challenging, as the binding of small molecules does not necessarily translate to channel blocking activity. This may be especially true for AQPs because of their small molecular size and narrow aqueous pore. This issue is highlighted by the gold-containing inhibitor Au(phen), which blocks glycerol transport by AQP3 but not water transport<sup>126,127</sup>, perhaps owing to insufficient channel occlusion. More sophisticated molecular dynamics-based approaches have been explored to model AQP inhibition, which involve the calculation of water permeability coefficients<sup>145</sup>; however, these approaches were computationally intensive and water permeability coefficients did not correlate with inhibitory efficacy. Improvements in the computational methodology used in molecular dynamics could lead to more reliable virtual screening for the discovery of AQP inhibitors.

As mentioned above, the major cellular function of AQPs is to facilitate the osmotic transport of water — and glycerol for the aquaglyceroporins — across plasma membranes of cells. Below, we discuss how these basic functions translate into many biological activities that have potential clinical applications. There is also evidence (some of which is

conflicting) to suggest that in addition to the transport of water and glycerol, AQPs could potentially transport gases such as carbon dioxide<sup>22–24</sup>, ammonia<sup>24,25</sup> and nitric oxide<sup>26,27</sup>, as well as larger polar solutes such as sugars<sup>28</sup>, hydrogen peroxide<sup>22,29,30</sup> and even some ions<sup>31</sup>. However, it is not known whether the transport of molecules other than water and glycerol is biologically important.

## Biological function of AQPs in mammals

AQPs are widely expressed in the body (FIG. 2), particularly in cell types that are involved in fluid transport, such as epithelial cells in several organs, as well as in some cell types that do not have an obvious role in fluid transport, such as adipocytes. Because there is a lack of verified inhibitors, studies of the phenotypes of AQP-knockout mice have provided most of the information on AQP physiology and on the potential indications of AQP-targeted modulators (TABLE 1). Below, we further discuss the results from those phenotypic studies that, in our view, have the greatest relevance to clinical indications of AQP modulators.

### Epithelial fluid transport

AQPs facilitate transepithelial water transport in response to osmotic gradients that are created by the transport of active ions and neutral solutes (FIG. 3a). AQP1 is expressed in the epithelium of the renal proximal tubule and the thin descending limb of the loop of Henle, as well as in the endothelium in the descending vasa recta; mice that are deficient in AQP1 have a greatly impaired ability to concentrate urine<sup>32</sup>. In these mice, the reduced permeability of the renal proximal tubule to water impairs the normal absorption of water filtered by the glomerulus<sup>33</sup>; reduced water permeability in the thin descending limb of the loop of Henle and the vasa recta impairs the renal counter-current multiplication and exchange systems that are responsible for concentrating urine<sup>34,35</sup>. Thus, inhibition of AQP1 is predicted to produce water diuresis by a mechanism that is different from conventional salt transport-blocking diuretics, which suggests that AQP1 blockers could have clinical potential in the refractory oedema that is associated with congestive heart failure and cirrhosis.

AQP2, AQP3 and AQP4 are expressed in kidney collecting duct epithelial cells. Fluid retention by the kidney (also known as antidiuresis) requires a high transepithelial permeability to water in the collecting duct, which is accomplished by vasopressin-induced trafficking of AQP2 from an intracellular vesicular compartment to the apical (that is, lumen-facing) plasma membrane of the cell<sup>36</sup>; AQP3 and AQP4 are constitutively active as water transporters on the basolateral (that is, blood-facing) cell membrane. Mice lacking AQP2, AQP3 or AQP4 have defects in urine-concentrating function (albeit to varying levels), and hence inhibition of these AQPs would produce an aquaretic response similar to that produced by vasopressin V<sub>2</sub> receptor antagonists<sup>37–40</sup>. Interestingly, when AQP11 (whose function is not fully understood) is deleted, mice develop renal and hepatic cysts through as yet unknown mechanisms<sup>41</sup>.

AQPs facilitate the secretion of water into acini of glands following the creation of an osmotic gradient by the active secretion of solutes (FIG. 3b). Mice lacking AQP5, which is expressed in salivary and airway sub-mucosal glands, have defective secretion of saliva<sup>42</sup>

and airway mucus<sup>43</sup>. Namely, the secretions are hyperosmolar and occur at lower levels because the low water permeability of the gland acini in the absence of AQP5 prevents osmotic equilibration across the epithelium. In addition, AQP1 facilitates the secretion of aqueous fluid by the ocular ciliary epithelium<sup>44</sup> and of cerebrospinal fluid by the choroid plexus<sup>45</sup>. Therefore, AQP1 inhibition might reduce intraocular pressure in glaucoma and intracranial pressure in brain trauma or stroke, whereas inhibition of AQP5 could reduce salivation and airway mucus production during anaesthesia.

As suitable AQP inhibitors or neutralizing antibodies targeting AQPs are not available, and informative gene knockdown studies have not been undertaken, the current knowledge about the role of AQPs in epithelial fluid transport comes from studies carried out in knockout mice.

### CNS functions

AQP4 is the principal water channel expressed in astrocytes throughout the central nervous system (CNS), particularly at astrocyte end-feet at the blood–brain barrier and the ependymal–cerebrospinal fluid barrier<sup>4,46</sup>. There is evidence that AQP4 is involved in the transport of water into and out of the brain and spinal cord, in neuroexcitation and in astrocyte migration following injury (FIG. 3d,e). It has been proposed that AQP4 modulators have therapeutic potential in the treatment of brain oedema (of several aetiologies), epilepsy and neural regeneration, as well as brain and spinal cord trauma<sup>47</sup>. As AQP4 facilitates water movement across the blood–brain barrier, AQP4-deficient mice have better survival and reduced accumulation of water in the brain than wild-type mice in models of cytotoxic brain oedema (that is, models that involve cell swelling). These mouse models of cytotoxic oedema include water intoxication, focal and global ischaemia and bacterial meningitis<sup>48–51</sup>.

AQP4 also facilitates water exit from the brain in vasogenic oedema. In this process, water moves into the brain by a bulk fluid flow mechanism through a leaky blood–brain barrier and exits through the AQP4-rich glia limitans that lines the brain ventricles and the surface of the brain<sup>52</sup>. AQP4-deficient mice have greater accumulation of water in models of vasogenic brain oedema, including models of intraparenchymal fluid infusion, cortical-freeze injury, brain tumour, brain abscess and subarachnoid haemorrhage<sup>52–55</sup>, as well as in obstructive hydrocephalus<sup>56</sup>. AQP4 probably has a similar role in the spinal cord; deletion of AQP4 reduces cytotoxic swelling and improves clinical outcomes in a mouse model of spinal cord compression injury<sup>57</sup>, but increases vasogenic swelling in a mouse model of spinal cord contusion injury<sup>58</sup>. To validate AQP4 as a drug target, it will be important to investigate verified, selective AQP4 inhibitors when they become available.

AQP4-deficient mice also have altered neuroexcitation: namely, prolonged seizure duration<sup>59</sup> and cortical spreading depression<sup>60</sup>. AQP4 deficiency is associated with a slower rate of potassium reuptake by astrocytes following neuroexcitation<sup>59,61</sup>, which — as supported by mathematical modelling — may be related to a reduced electrochemical driving force for potassium reuptake caused by slowed astrocyte water uptake and contraction of the extracellular space<sup>62</sup>. AQP4-deficient mice also have impaired glial scar formation, which may be related to defective astrocyte migration<sup>63,64</sup>. Reduced glial

scarring caused by AQP4 inhibition may facilitate neural regeneration following brain or spinal cord injury, or a stroke.

AQP1 is expressed in dorsal root ganglion neurons and nociceptive C-fibres<sup>65–67</sup>. An early study reported that AQP1-null mice had reduced pain sensitivity<sup>65</sup>, but another study found no effect of AQP1 deletion on pain perception<sup>67</sup>. It is now known that AQP1 deletion in mice causes a selective reduction in thermal inflammatory pain and cold-induced pain, which may involve an interaction between AQP1 and the voltage-gated sodium channel Nav1.8 (also known as SCN10A)<sup>68</sup>. These findings raise the possibility of using AQP1 inhibitors as novel, non-narcotic analgesics.

### Tumour angiogenesis and spread

AQPs are also involved in tumour angiogenesis, invasion, metastasis and growth. AQP1 is highly expressed in tumour-associated microvascular endothelial cells<sup>69</sup>, and numerous studies have shown that AQPs are highly expressed in several different tumour cell types. In many tumours, the same AQPs are also expressed in the non-tumour cells from which the tumour originates<sup>70</sup>. For example, AQP1 (REF. 71), AQP4 (REFS 72,73) and AQP9 (REF. 74), which are expressed in normal astrocytes, are often also expressed in human astrocytomas, with AQP4 expression correlating with tumour severity<sup>72</sup>. Mice lacking AQP1 have reduced growth of implanted and spontaneously generated tumours as a consequence of defective tumour angiogenesis<sup>75,76</sup>. Also, implanted AQP1-expressing tumours have greater local invasiveness and more metastases than tumours lacking AQPs<sup>77</sup>.

AQP-dependent cell migration, in which the polarization of AQPs to the leading edge of migrating cells facilitates the entry of water into extending lamellipodia in the direction of cell movement (FIG. 3c), is one mechanism that has been proposed to facilitate AQP1-dependent tumour angiogenesis and metastasis<sup>78</sup>. Several studies show that AQP facilitates the proliferation of some types of tumour cells through mechanisms that may involve altered signalling pathways<sup>79–83</sup>. Remarkably, AQP3-deficient mice are resistant to the formation of skin tumours following the administration of chemical stimuli; this resistance may result from impaired glycerol uptake by tumour cells<sup>79</sup>.

These compelling observations suggest that AQP inhibitors have therapeutic potential in oncology. Current treatments for glioblastoma either target rapidly dividing tumour cells (such as radiotherapy, temozolomide or the combination of procarbazine, lomustine and vincristine) or they inhibit angiogenesis (for example, bevacizumab). AQP inhibitors could be unique in targeting infiltrating tumour cells, which might convert a tumour that diffusely infiltrates into normal tissue — and that is not surgically excisable — into a tumour with a distinct border. Whether AQP4 inhibitors could reduce the infiltration of glioblastoma cells into normal brain tissue could be tested by the implantation of glioblastoma cells with inducible AQP4 expression into the mouse brain. Switching off AQP4 expression, which is equivalent to administering an AQP4 inhibitor, would be predicted to reduce tumour cell infiltration into the normal brain tissue.



## Functions of AQP-facilitated glycerol transport

Mouse phenotype studies have clarified the functional significance of glycerol transport by the aquaglyceroporins (FIG. 3f–h): namely, AQP3, AQP7 and AQP9.

AQP3 is expressed in the basal layer of proliferating epidermal keratinocytes. AQP3-deficient mice have reduced hydration of the stratum corneum and reduced skin elasticity<sup>84</sup>. Deficiency of AQP3 results in reduced permeability of epidermal cells to glycerol, which consequently reduces the glycerol content of the stratum corneum and epidermis, in which AQP3 normally acts as a water-retaining ‘humectant’. Normalization of the glycerol content in the skin of AQP3-deficient mice by systemic replacement corrects skin dehydration and related defects<sup>85</sup>. Several cosmetic companies have marketed anti-ageing formulations that claim to contain natural-product enhancers of epidermal AQP3 expression<sup>86</sup>. However, increasing AQP3 expression<sup>87</sup> in the skin should be carried out with caution as AQP3 is highly expressed in skin tumours, and AQP3 deficiency in mice greatly reduces the formation of these tumours. Therefore, increasing AQP3 expression could increase the formation of skin tumours. Moreover, AQP3 deficiency is associated with impaired corneal<sup>88</sup> and cutaneous<sup>89</sup> wound healing in mice, and with impaired colonic epithelial cell proliferation in a mouse model of colitis<sup>181</sup>. AQP3 is also expressed in immune cells, and its deletion from these cells in mice impairs the function of macrophages<sup>91</sup> and T cells<sup>29</sup>. Because AQP3 has several roles, both beneficial and deleterious, establishing an acceptable therapeutic window for an AQP3 modulator would be challenging.

The aquaglyceroporins AQP7 (expressed primarily in adipocytes) and AQP9 (expressed in hepatocytes) are involved in adipose metabolism. AQP7-null mice have a progressive age-related increase in adipose mass and adipocyte hypertrophy, with the accumulation of glycerol and triglycerides in adipocytes<sup>92,93</sup>. AQP7 deficiency leads to reduced permeability of the plasma membrane to glycerol, which causes cellular accumulation of glycerol and triglyceride as well as upregulation of glycerol kinase expression. In humans, AQP7 expression in adipocytes is downregulated in obesity<sup>94</sup>. These results suggest that increasing adipocyte AQP7 expression and/or function may be a novel approach for treating obesity<sup>95</sup>; however, there is a lack of critical proof-of-concept data, such as the effects of AQP7 overexpression (induced by gene transfer) on adipocyte hypertrophy in obese mice. There is evidence that AQP9 is involved in the hepatic uptake of glycerol and perhaps urea<sup>96,97</sup>, and that AQP7 and AQP9 act as metabolic regulators in diabetes and obesity<sup>95</sup>. Nevertheless, further work using clinically relevant animal models is needed to validate AQP9 as a drug target.

## AQP mutations in humans

Data from individuals with rare loss-of-function mutations in AQPs have provided important information about the functions of AQPs in humans (TABLE 1). Humans and mice lacking functional AQP0 have congenital cataracts<sup>90,98</sup>. The primary functions of AQP0 in the lens may be in cell–cell adhesion of lens fibres and in the regulation of gap junction channels, rather than in water transport<sup>99</sup>.

A report of two individuals who lacked functional AQP1 showed that they had defective urine-concentrating function<sup>100</sup>, as seen in AQP1-knockout mice<sup>32</sup>, but without other gross abnormalities. As discussed further below, humans lacking functional AQP2 have the rare genetic disorder NDI<sup>101</sup>. There are reports of individuals with an apparent lack of functional AQP3 (REF. 102), but this study provided no information on their phenotype. Three children with an AQP7 mutation were reported to manifest hyperglyceroluria and a defect in ATP secretion by platelets<sup>103</sup>. Two recent studies showed that AQP5 mutations were associated with the development of palmoplantar keratoderma, but it is unclear whether these mutations affect the water permeability of AQP5 (REFS 104,105). Genetic studies have sought to identify disease-relevant AQP polymorphisms in disorders including stroke, migraine, Menière's disease, NMO, temporal lobe epilepsy, cataracts, diabetes and obesity, but compelling associations have not been found.

### Aquaporin-targeted drugs: state of the art

Assays that measure the functions of AQPs are crucial for the screening and optimization of small-molecule modulators. TABLE 2 summarizes the key features of assays that are used for the primary screening of AQP modulators and assays of water transport function that are used for the screening of AQP modulators.

### Functional assays

Functional analysis of AQPs requires the measurement of membrane water (or glycerol) permeability. One approach to determine water permeation is the measurement of water flux across a tight (that is, non-leaky) cell layer, typically an epithelial cell cultured on a porous support (FIG. 4a). In this approach, water flux is driven by a transepithelial osmotic gradient using a membrane-impermeant solute such as sucrose or mannitol. The flux of water is measured by the dilution of an indicator dye in the fluid volume on the small apical surface<sup>106</sup>, or by measuring the height of the fluid in an Ussing chamber using electrical or optical methods<sup>107</sup>. The measurement of transepithelial water transport is useful for secondary assays but not for primary high-throughput screening because of the technical complexity, the long-time needed for each measurement (tens of minutes), and the relatively imprecise data generated.

Measuring the kinetics of the cell volume (or the membrane vesicle or reconstituted proteoliposome volume) in response to a rapidly imposed osmotic gradient is a useful approach for quantifying the permeability of cell membranes to water in primary screening assays (FIG. 4b). Several approaches have been developed for following cell volume kinetics, including image analysis, light scattering and interferometry in unlabelled cells<sup>108,109</sup> and reporter dye intensity in fluorescently labelled cells, as measured by total internal reflection<sup>110</sup>.

Several approaches are suitable for primary functional screens of AQPs. In the calcein method, AQP-expressing cells are grown as monolayer cultures on a solid support, such as a multi-well plate, stained with calcein and then subjected to an osmotic gradient by the addition of an anisomolar (that is, hypo-osmolar or hyperosmolar) solution. Cytoplasmic calcein fluorescence measures cell volume, as the fluorescence of calcein is quenched by

cytoplasmic proteins whose concentrations change as cells shrink or swell<sup>111</sup>. A similar method, which does not require dye loading, uses the genetically encoded, cytoplasmically expressed yellow fluorescent protein YFP-H148Q–V163S, whose fluorescence is quenched by chloride<sup>112</sup>. The fluorescence of YFP-H148Q–V163S provides a read-out of cell volume because the cytoplasmic chloride concentration changes as cells shrink or swell<sup>113,114</sup>. Both the calcein- and YFP-based approaches rely on kinetic measurements of fluorescence following the addition of anisomolar solution; hence, the osmotic response of the cell must be sufficiently slow (approximately a few seconds) for accurate analysis, and the time taken to mix the solution must be short compared to the osmotic equilibration time.

A simple method that relies on a single read-out of cell volume, rather than a kinetic analysis, involves measuring cell lysis in response to an osmotic gradient of a permeant solute (FIG. 4c). In this method, erythrocytes, which natively express AQP1 and urea transporter B (UTB; also known as SLC14A1), are preloaded with the urea analogue acetamide, which is transported by UTB and equilibrates across the erythrocyte membrane over a time period slightly slower than that of water. Dilution of erythrocytes into an acetamide-free solution results in rapid, AQP1-dependent cell swelling and lysis. Reduced water permeability of AQP1 (for example, caused by AQP1 blockers) prevents cell lysis, as water influx is slower than the dissipation of the osmotic gradient by acetamide efflux. The extent of cell lysis is quantified by a single read-out of near-infrared light absorbance. The erythrocyte lysis assay has been used to identify UTB inhibitors that had nanomolar potency and increased cell lysis<sup>115</sup>. From a screening of 100,000 small molecules for AQP1 inhibitors, preliminary results produced a few weakly active compounds (A.S.V., unpublished observations).

Each of the above methods has potential limitations (TABLE 2). Not discussed here are possible methods using glycerol transport assays that could be used for functional screens of aquaglyceroporin modulators. Measurements of transepithelial water permeation are useful for the verification of compounds that were active in primary screens (as discussed above), although stopped-flow measurements of cell volume kinetics (measured by a light scattering read-out) in response to rapidly imposed osmotic gradients are, in our view, better. Stopped-flow measurements can be made in plasma membrane vesicles from AQP-expressing cells<sup>116</sup>, in reconstituted proteoliposomes<sup>117</sup> or in small cells such as erythrocytes<sup>115</sup>.

Swelling kinetics in *Xenopus laevis* oocytes that express AQPs (induced by the injection of complementary RNA) has been used in several studies of AQP modulators. Swelling kinetics is deduced by image analysis of a cross-sectional area of an oocyte using low magnification and low numerical aperture objectives<sup>118,119</sup>. Although oocyte swelling is useful for detecting large differences in water permeation, as used in the original identification of AQP1 as a water channel<sup>120</sup>, it is of limited value for the study of AQP modulators as it produces artefacts. As discussed<sup>116</sup>, apparent oocyte swelling depends not only on water permeability but also on other factors such as solute transport, oocyte geometry, the physical properties of the plasma membrane as well as cytoplasmic and extracellular unstirred layers (that is, aqueous layers in which solute transport is diffusion-limited), each of which can be influenced by a putative AQP modulator. In addition, the

strict technical details about how oocyte swelling measurements should be performed (as discussed below) have not been adhered to in most published reports.

### AQP inhibitors

As discussed below, the identification of AQP modulators has turned out to be unexpectedly challenging. Four classes of AQP-targeted small molecules have been described: cysteine-reactive heavy metal-based inhibitors; small-molecule scaffolds that are reported to inhibit water conductance; small molecules that target the interaction between AQP4 and the NMO autoantibody; and agents that act as chemical chaperones to facilitate the cellular processing of NDI-causing AQP2 mutants (FIG. 5).

The inhibition of water permeation into erythrocytes (which express AQP1) by sulphhydryl-reactive heavy metal ions and organomercury compounds, such as pCMBS (*p*-chloromercuribenzenesulphonate), has been appreciated for over 40 years<sup>121</sup>. The inhibition of AQP1 by mercury(II) chloride involves a covalent interaction with Cys189, which is thought to sterically occlude the water pore<sup>122,123</sup>. A mutation in the Cys189 residue of AQP1 prevents its inhibition by mercury, and other AQPs (such as AQP4) that lack a cysteine residue at this position are resistant to inhibition by mercury<sup>124</sup>. Other heavy metals such as silver (in silver nitrate and silver-sulphadiazine)<sup>125</sup> and gold(III) (in the compounds Au(phen), Au(bipy), Au(bipy)Me, Au(bipy)NH<sub>2</sub> and Au(terpy))<sup>126</sup> also inhibit some AQPs (FIG. 5). It was suggested that these inhibitors might interact with a cysteine residue near the conserved NPA domain<sup>125</sup>.

Gold-containing compounds were reported to inhibit AQP3, with Au(phen) being the most potent in the series<sup>126,127</sup>. Interestingly, Au(phen) inhibits glycerol permeation through AQP3 to a greater extent than water permeation, which suggests that steric occlusion of the channel by Au(phen) is insufficient to block water transport. Computational modelling suggested that gold-based inhibitors interact with Cys40, which is present in the extracellular domain of AQP3 (REF. 127). Although heavy metal-containing drugs are generally toxic, there are drugs with clinically acceptable toxicity, such as the chemotherapy drug cisplatin. In addition, other organometallic compounds that contain gold<sup>128,129</sup> and ruthenium<sup>130</sup> have been described as potential anti-cancer agents with minimal toxicity. It is notable that mercury(II) chloride was used historically to treat syphilis before the discovery of penicillin, and as a diuretic before the discovery of thiazides and loop diuretics.

Several studies have described small-molecule inhibitors of water permeability in AQP-expressing *X. laevis* oocytes, which do not contain metals. One such class is the non-selective tetraalkylammonium ion channel blockers such as tetraethylammonium (TEA<sup>+</sup>). TEA<sup>+</sup> was reported to be a reversible inhibitor of AQP1 (REF. 131), and later found to be a blocker of AQP1, AQP2 and AQP4 (REF. 132). A tentative TEA<sup>+</sup> binding site was proposed based on the resistance of a mutant AQP1 (with a mutation at the Tyr186 site) to TEA<sup>+</sup> (REFS 131,132). The carbonic anhydrase inhibitor acetazolamide has been reported to inhibit AQP1 expressed in *X. laevis* oocytes<sup>133</sup> and in AQP1-transfected human embryonic kidney 293 (HEK293) cells<sup>134</sup>. Acetazolamide and a related sulphonamide<sup>135</sup>, as well as several anti-epileptic drugs<sup>136</sup>, were reported to inhibit AQP4 expressed in *X. laevis* oocytes. The loop diuretic bumetanide was reported to be a weak inhibitor of AQP4

expressed in *X. laevis* oocytes (with an  $IC_{50}$  value of  $>100 \mu\text{M}$ ), and a synthesized analogue (AqB013) had an  $IC_{50}$  value of  $\sim 20 \mu\text{M}$  for AQP1 and AQP4 (REF. 137). The same group found that another analogue (AqF026) slightly increased AQP1-mediated oocyte swelling<sup>138</sup>. The loop diuretic furosemide was also shown to weakly inhibit AQP1 expressed in *X. laevis* oocytes<sup>139</sup>. In our view, it is unexpected that many common drugs that target various structurally and functionally unrelated membrane proteins were reported to inhibit or increase AQP-mediated water transport.

Unfortunately, follow-on studies of these putative AQP modulators — using alternative functional assays that are less prone to artefacts — have failed to verify AQP inhibition. For example, in an assay using purified human AQP4 reconstituted into liposomes,  $\text{TEA}^+$  was inactive at concentrations of up to  $10 \text{mM}$ <sup>10</sup>. There was no inhibition of AQP1 by acetazolamide (at concentrations of up to  $1 \text{mM}$ ) when measured by pressure-induced haemolysis of erythrocytes<sup>140</sup>. In addition, acetazolamide and  $\text{TEA}^+$  were inactive when tested at concentrations of up to  $10 \text{mM}$  by stopped-flow light scattering in erythrocytes, which natively express AQP1, and in epithelial cells that were stably transfected with AQP1 (REF. 141). Another report re-examined the activity of putative AQP1 inhibitors in *X. laevis* oocytes (the cells that were originally used to show the activity of the inhibitors) using a high-resolution method to measure osmotic water permeation<sup>142</sup>. This study found that acetazolamide and  $\text{TEA}^+$  (at concentrations of up to  $100 \mu\text{M}$  and  $500 \mu\text{M}$ , respectively) did not inhibit AQP1 (REF. 142). Although the authors considered several explanations for this result, they posited that earlier studies that used lower-sensitivity techniques could have been susceptible to secondary effects related to the distribution of ions across the plasma membrane<sup>142</sup>.

Preliminary work re-examining the effects of various loop diuretics and their analogues in several systems showed no AQP-mediated inhibition or augmentation of water transport (A.S.V., unpublished observations). The series of aryl sulphonamides and anti-epileptics that were reported to inhibit AQP4 in *X. laevis* oocytes<sup>135</sup> were inactive at concentrations of up to  $100 \mu\text{M}$  when they were re-evaluated using plasma membrane vesicles from AQP4-transfected mammalian cells and primary cultures of human glial cells<sup>143</sup>. Finally, a study using AQP4-expressing *X. laevis* oocytes found that AQP4-IgG (immunoglobulin G) autoantibodies in NMO inhibited AQP4-mediated water transport<sup>140</sup>, although this finding was not repeated in studies that used assays that are less prone to artefact<sup>116,141</sup>.

Recently, several AQP1 inhibitors were identified<sup>142</sup> in a virtual screen of 1,000,000 compounds from the University of California, San Francisco (UCSF)'s ZINC database<sup>143</sup>; the screen was carried out to identify compounds that bound to the extracellular vestibule of AQP1. Out of 14 hit compounds that were tested further, three structurally unrelated compounds were reported to inhibit the osmotic swelling of AQP1-expressing *X. laevis* oocytes; these compounds included *m*-benzene diacrylic acid (BZDA), which had an  $IC_{50}$  value of  $\sim 8\text{--}18 \mu\text{M}$ . However, none of these compounds inhibited AQP1-mediated water permeation when it was tested in a definitive erythrocyte stopped-flow assay<sup>142</sup>. This result mirrors the concerns mentioned above about potential artefacts that can occur when the oocyte swelling assay is used.

A medium-throughput assay that used calcein fluorescence in cells expressing AQP1 or AQP4 (REF. 144) screened 3,575 small molecules, including 418 US Food and Drug Administration (FDA)-approved drugs, for inhibition of water transport. This yielded four distinct scaffolds, including NSC168597, NSC670229 and NSC164914, which had IC<sub>50</sub> values of ~ 27–49 μM in water transport assays in rat erythrocytes. In another recent study that used homology modelling, molecular dynamics, computational docking and site-directed mutagenesis to identify AQP9 inhibitors<sup>145</sup>, an initial screen of ~1,000,000 compounds identified six AQP9 candidate inhibitors. These included the representative compound ID4, which had an IC<sub>50</sub> value of ~ 4–10 μM in calcein fluorescence assays in AQP9-expressing Chinese hamster ovary (CHO) cells. Independent verification of AQP inhibition by these compounds is awaited.

### AQPs are challenging drug targets

Progress in the identification of agents that modulate water permeation through AQPs has been remarkably slow compared to modulators of other channel proteins. This is partly due to the challenges of water permeation assays, which are fraught with potential artefacts; furthermore, unlike for electrophysiological assays of ion transport, robust gold-standard assays of AQP activity are not available. We think that the over-reliance on *X. laevis* oocyte and calcein-based assays has produced candidate AQP inhibitors that have not been verified by subsequent measurements because there are multiple, water-transport-independent determinants of oocyte swelling rates and calcein fluorescence signal.

The hit rate for the identification of small-molecule AQP inhibitors appears to be very low compared to that for other membrane proteins such as ion channels, solute transporters and membrane receptors. Perhaps the small size of the functional AQP monomer and its very small pore diameter, which prevents the access of small molecules, translates to poor druggability. As AQPs are simple passive pores, they lack sophisticated gating and transport mechanisms that can be targeted by small molecules. Furthermore, because AQPs transport small, neutral molecules, electrostatic interactions are limited to those between hydrogen-bond donors and acceptors, rather than interactions involving charges. Last, mutations in the extracellular and cytoplasmic domains of AQPs generally have little effect on water permeability through the channel, which suggests that the binding of an inhibitor should occur deep in the narrow AQP pore to physically prevent water conduction.

Further large-scale screening of random and computationally biased libraries is needed, as well as rigorous validation of compounds that have apparent activity using definitive assays of AQP-mediated water (or glycerol) transport. In addition, as several human AQP homologues exist, many with a wide tissue distribution, functional testing of verified inhibitors against one AQP isoform should be carried out against a panel of the major AQPs.

### AQP gene transfer

AQP gene transfer has been considered for treating salivary gland hypofunction, which is a common side effect of radiation therapy for head and neck cancer. Radiation damages the acinar cells that secrete saliva, largely sparing salivary duct cells, so drugs that stimulate saliva secretion by targeting acinar cells are generally ineffective. In irradiated salivary

glands, the osmotic pressure of the duct lumen is greater than that of the interstitium, which has provided the rationale for increasing water movement across the duct cells into the lumen by *AQP1* gene transfer. This idea is supported by the observation that adenovirus-mediated transfer of human *AQP1* cDNA into the parotid glands of rats<sup>146</sup> and miniature pigs<sup>147</sup> by retrograde infusion into the parotid duct partially restored saliva secretion after radiation therapy for head and neck cancer. A Phase I clinical trial of adenovirus-mediated *AQP1* cDNA transfer to previously irradiated parotid glands in eleven participants<sup>148</sup> showed that there was an objective increase in saliva flow in six individuals and a subjective improvement in five individuals. However, there are major challenges associated with AQP gene transfer, including targeting the correct cell type, achieving efficient transfection, producing a sustained therapeutic effect and avoiding immune activation. In the Phase I clinical trial, parotiditis was observed in eight of the eleven participants.

## Aquaporinopathies as targets for AQP modulators

Here, we discuss two aquaporinopathies — NMO and NDI — in which progress has been made in developing AQP-based therapeutics.

### NMO: an autoimmune disease caused by AQP4-specific antibodies

NMO is an autoimmune inflammatory disease of the CNS that leads to paralysis and loss of vision. Most cases of NMO are associated with the presence of an IgG1 antibody, termed AQP4–IgG (or NMO–IgG)<sup>149</sup>, which binds to conformational epitopes on the extracellular surface of AQP4 (REFS 150–153) and, when injected in mice, produces the histological features of human NMO lesions<sup>154</sup>. The working hypothesis for NMO pathogenesis involves the entry of AQP4–IgG into the CNS and its binding to AQP4 on perivascular astrocyte end-feet, which leads to activation of the classical complement cascade, an inflammatory response that is accompanied by marked granulocyte and macrophage infiltration, as well as secondary oligodendrocyte damage, demyelination and neuron death<sup>152</sup>. Current NMO treatments include immunosuppression (that is, administration of corticosteroids, azathioprine, methotrexate, cyclophosphamide, mycophenolate or mitoxantrone), depletion of CD20<sup>+</sup> B cells (using rituximab) and plasma exchange<sup>155</sup>.

Based on progress in understanding the mechanisms that underlie NMO pathogenesis, new NMO therapeutics are being investigated that target AQP4, AQP4–IgG and various mediators of cytotoxicity and inflammation (FIG. 6a). One AQP4-targeted strategy involves using monoclonal antibodies to block the binding of AQP4–IgG to AQP4, which is the initiating event in NMO pathogenesis. A recombinant monoclonal antibody against AQP4–IgG has been engineered, called aquaporumab, which binds tightly to AQP4 but lacks cytotoxic effector functions<sup>156</sup>. Because of the small extracellular footprint of AQP4, aquaporumab competes sterically with pathogenic, polyclonal AQP4–IgG. Aquaporumab prevented the formation of NMO lesions in *ex vivo* spinal cord slices and in mice *in vivo*<sup>156</sup>. As an alternative approach, a small-molecule screen identified compounds that bind to AQP4 and inhibit AQP4–IgG binding without affecting the surface expression or water permeability of AQP4. However, the compounds that were identified in the initial screen, including the antiviral arbidol, the flavonoid tamarixetin and several plant-derived berbamine alkaloids, have relatively low potency and CNS penetration<sup>157</sup>.

Another approach for reducing AQP4–IgG binding involves neutralizing the pathogenic AQP4–IgG with bacteria-derived endoglycosidase<sup>158</sup> or with the *Streptococcus pyogenes* IgG-degrading enzyme IdeS<sup>159</sup>. Endoglycosidases cause selective deglycosylation of the IgG heavy chain, which eliminates the complement-dependent cytotoxicity and antibody-dependent cell-mediated cytotoxicity effector functions of the antibody. IdeS cleaves IgG antibodies into Fc and F(ab')<sub>2</sub> fragments. The resulting non-pathogenic AQP4–IgG (that is, deglycosylated AQP4–IgG or AQP4–IgG–F(ab')<sub>2</sub>) binds to AQP4 in cultured cells with an affinity that is comparable to that of the intact pathogenic AQP4–IgG, thus competing with pathogenic AQP4–IgG for AQP4 binding. These AQP–IgG-targeted enzymatic therapeutics might be applied by therapeutic apheresis. As NMO is a rare disease, funding the development of novel treatments is difficult, as is carrying out clinical trials.

Other potential therapeutic strategies for NMO include reducing the expression of AQP4 on astrocytes or preventing its supramolecular assembly in orthogonal arrays<sup>160</sup>, upregulating complement inhibitor proteins such as CD59 or reducing the entry of AQP4–IgG into the CNS (FIG. 6a). Tocilizumab, a humanized monoclonal antibody that targets the interleukin-6 receptor (IL-6R), reduces the survival of AQP4–IgG-secreting plasmablasts *in vitro*<sup>161</sup>, and case reports show that patients treated with this antibody have reduced NMO symptoms<sup>162,163</sup>. As AQP4-targeted therapies are more selective than general immunosuppressive agents or complement- or leukocyte-targeted therapies, they may have fewer side effects.

#### **NDI: a genetic disorder caused by loss-of-function AQP2 mutations**

Loss-of-function mutations in *AQP2* cause NDI, an autosomal hereditary disease that is characterized by severe polyuria and polydipsia, which — if inadequately treated — lead to recurrent dehydration in early life and cognitive deficits. NDI that is caused by an *AQP2* mutation is very rare, with an incidence of ~1 in 20 million births<sup>164,165</sup>. Normally, regulation of water permeation in the renal collecting duct epithelium is mediated by the binding of vasopressin to V<sub>2</sub> receptors at the basolateral membrane, which causes exocytic insertion of intracellular AQP2-containing vesicles into the apical plasma membrane of the cell, primarily through cyclic AMP signalling<sup>36</sup> (FIG. 6b). Water then moves from the tubule lumen into the bloodstream across constitutively active AQP3 and AQP4 on the basolateral membrane, and through AQP2 on the apical membrane, producing concentrated urine. Hereditary NDI is also caused by a mutation in the gene encoding the V<sub>2</sub> receptor (*AVPR2*), with an incidence of ~1 in 2 million births. Fluid replacement is the mainstay of therapy for NDI caused by mutations in *AQP2* or *AVPR2*; thiazide diuretics are used to impair the maximal ability to dilute urine and reduce urine output<sup>164</sup>. Novel therapeutic approaches that are under consideration for NDI caused by loss-of-function *AVPR2* mutations include pharmacological chaperones (that is, V<sub>2</sub> receptor antagonists)<sup>166</sup> and the targeting of alternative G protein-coupled receptors in the collecting duct to restore cAMP signalling<sup>167</sup>.

The treatment of NDI caused by *AQP2* mutations presents a greater challenge. Mutations in *AQP2* have been described that lead to an autosomal recessive trait involving defective cellular processing or functioning of AQP2, or an autosomal dominant trait in which mutant



AQP2 prevents the targeting of wild-type AQP2 on the plasma membrane<sup>165</sup>. The AQP2<sup>T126M</sup> mutation causes misfolding of AQP2 and leads to its retention at the endoplasmic reticulum<sup>168</sup>, similarly to the F508 mutation in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene that causes cystic fibrosis. Incubation of AQP2<sup>T126M</sup>-expressing cells with non-selective chemical chaperones, including glycerol and trimethylamine oxide (TMAO) (FIG. 5d), rescued the cellular processing defect, allowing the insertion of AQP2<sup>T126M</sup> into the plasma membrane and the restoration of cellular water permeability. A small screen of approved and investigational drugs identified the heat shock protein 90 (HSP90) inhibitor 17-allylamino-17-demethoxy-geldanamycin (17-AAG) as a corrector of AQP2<sup>T126M</sup> processing, which partially corrected NDI in transgenic mice with the AQP2<sup>T126M</sup> mutation<sup>40</sup>. As NDI caused by an AQP2 mutation is very rare, repurposing of existing drugs is a more practical approach than the development of a new chemical entity.

## Non-mammalian aquaporins as drug targets

AQPs are found in disease-causing bacteria (AqpZ and GlpF in *Escherichia coli*)<sup>169,170</sup>, protozoan parasites that cause malaria (PfAQP in *P. falciparum*)<sup>171</sup> and toxoplasmosis (TgAQP1 in *Toxoplasma gondii*)<sup>172</sup>, as well as in parasitic worms that cause Chagas disease (TcAQP1, TcAQP2 and TcAQP3 in *Trypanosoma Cruzi*)<sup>173</sup>, sleeping sickness (TbAQP1, TbAQP2 and TbAQP3 in *Trypanosoma brucei*)<sup>174</sup> and schistosomiasis (SmAQP in *Schistosoma mansoni*)<sup>175</sup>. In addition to facilitating water transport, many parasitic AQPs are permeable to glycerol and lactate. In general, AQP deletion in parasites is not lethal, but it reduces parasite growth and virulence, and hence these attenuated parasitic forms could be used in vaccines. However, parasitic AQPs seem to be the major entry routes of cytotoxic drugs into the parasite<sup>172-176</sup>, and AQP deletion renders the parasites relatively resistant to treatments with hydroxyurea, dihydroxyacetone and the trivalent metalloids arsenic and antimony.

Host AQPs are potential drug targets for the treatment of parasitic infections. For example, the human malarial parasite that resides within erythrocytes relies on AQP3 and AQP9 in the erythrocyte plasma membrane to obtain glycerol. The possibility of using AQP3 and AQP9 inhibitors as antimalarial agents has been proposed<sup>177</sup>, but further work is needed to understand the role of host versus parasite AQPs in the metabolic processes within the parasite.

## Further directions and challenges

The *in vitro* data and results from studies in animal models indicate compelling opportunities for AQP-targeted therapeutics. Perhaps the best validated indications for AQP inhibitors include refractory oedema, tumour angiogenesis and spread, neuroprotection and glaucoma. Enhancement of AQP function, by upregulating AQP expression or possibly by gene transfer, may be beneficial in obesity, tumour-associated vasogenic brain swelling, salivary and/or lacrimal gland dysfunction and wound healing. An as yet unexplored possibility is the application of an AQP-targeted antibody conjugated to a toxin for tumour

therapy, such as aquaporin–toxin conjugates for the treatment of AQP4-expressing glioblastomas. Additional potential indications of AQP modulators are listed in TABLE 1.

However, progress in this field has been slow. As discussed above, challenges in developing small-molecule AQP inhibitors include conceptual and technical issues associated with screening assays, as well as the apparent poor druggability of AQPs. The existence of 13 homologous human AQP isoforms, many of which have broad tissue distributions, presents a further challenge in minimizing the off-target effects of AQP modulators. The topical application of AQP modulators for the treatment of glaucoma or cutaneous diseases may reduce this challenge, as might targeting renal tubules that are in contact with drug-enriched luminal fluid. Notwithstanding these challenges, the risk–reward ratio seems to be favourable for further exploration of AQPs as drug targets, which may include large-scale functional screens of diverse compound collections as well as computationally biased screening driven by high-resolution AQP crystal structure data.

## Acknowledgments

The authors' research is funded by the US National Institutes of Health (A.S.V. and M.O.A.), the Guthy-Jackson Charitable Foundation (A.S.V. and M.C.P.) and the Cystic Fibrosis Foundation (A.S.V.).

## Glossary

<b>Aquaglyceroporin</b>	A class of the aquaporins (AQP3, AQP7 and AQP9) that transport glycerol in addition to water
<b>Virtual screening</b>	A computational technique that is used to identify small-molecule modulators of a drug target. This is typically conducted in a structure-based manner (using protein–ligand docking) or in a ligand-based manner (using similarity searching or through the use of pharmacophore models)
<b>Molecular dynamics simulations</b>	Computational methods that simulate the physical motion of atoms and molecules. When applied to biomolecules, the results are typically trajectories of the atoms of the protein, solvent, ions, bound ligands and so on, over timescales that range from nanoseconds to microseconds
<b>Grotthuss-type ‘proton-wire’ mechanism</b>	A hypothesis for proton movement in bulk water, where a proton can ‘hop’ along a continuous line of water molecules in a hydrogen-bonded network, with the resultant reorientation of water molecules after the transfer has occurred
<b>Free-energy profiles</b>	A term that is used to describe the estimated energetics of a molecule passing through the span of a channel protein; it is usually calculated by molecular dynamics simulations
<b>Salt transport-blocking diuretics</b>	Drugs that inhibit the absorption of Na <sup>+</sup> , K <sup>+</sup> and Cl <sup>−</sup> ions in the kidney and therefore secondarily inhibit water absorption. For example, amiloride inhibits the epithelial sodium channel in

	the distal tubule, and furosemide inhibits the $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ symporter in the thick ascending limb of the loop of Henle
<b>Aquaretic response</b>	The urinary elimination of water without electrolyte loss (as opposed to a diuretic response in which urinary elimination of water is secondary to the elimination of salt)
<b>Astrocyte end-feet</b>	Astrocyte processes that surround microvascular endothelial cells in the central nervous system
<b>Vasogenic oedema</b>	Oedema in the central nervous system where water accumulates in extracellular spaces
<b>Cortical spreading depression</b>	A wave of depolarization that spreads in the brain and is followed by the suppression of brain activity
<b>Glial scar</b>	A scar in the central nervous system that is formed in response to damage and involves reactive astrocytes and microglia
<b>Density functional theory calculations</b>	A computational method that uses quantum-mechanical theory to model the energy and chemical structure of molecules
<b>Infiltrating tumour cells</b>	Tumour cells that spread into normal tissue such that there is no clear border between the tumour and the normal tissue
<b>Stratum corneum</b>	The outermost layer of the epidermis that is composed of dead cells (that is, keratinocytes)
<b>Lens fibres</b>	Long, thin transparent cells that form the bulk of the lens, arranged in concentric layers
<b>Gap junction channels</b>	Intercellular channels composed of connexin proteins that allow the passage of small molecules (typically <1 kDa) between cells
<b>Palmoplantar keratoderma</b>	A disease that is characterized by abnormal thickening of the skin on the palms of hands and soles of feet
<b>Ussing chamber</b>	An apparatus in which a cell layer separates two solution compartments; it is used to measure ion transport
<b>Stopped-flow measurements</b>	Assays that are carried out using an apparatus in which two solutions are mixed together rapidly (in <1 millisecond) and have an optical read-out
<b>IC<sub>50</sub> value</b>	The concentration of a compound that produces 50% inhibition of a target function
<b>Parotiditis</b>	Inflammation of the parotid salivary gland
<b>Therapeutic apheresis</b>	The passage of blood through a filtering apparatus to remove or inactivate a pathogenic substance

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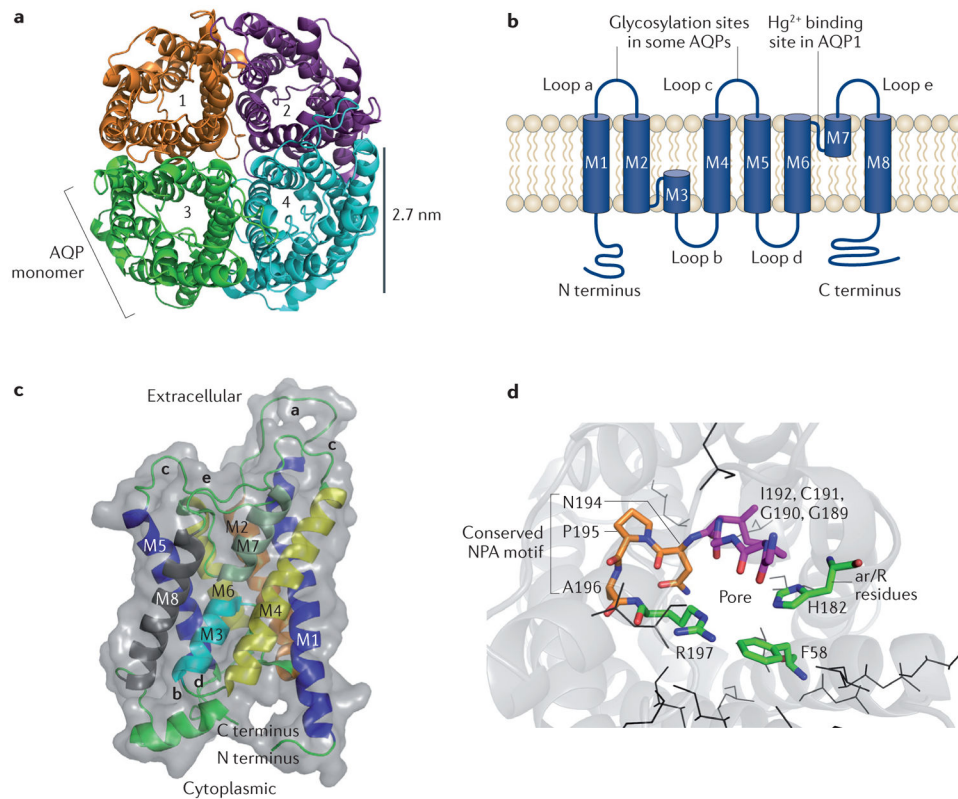
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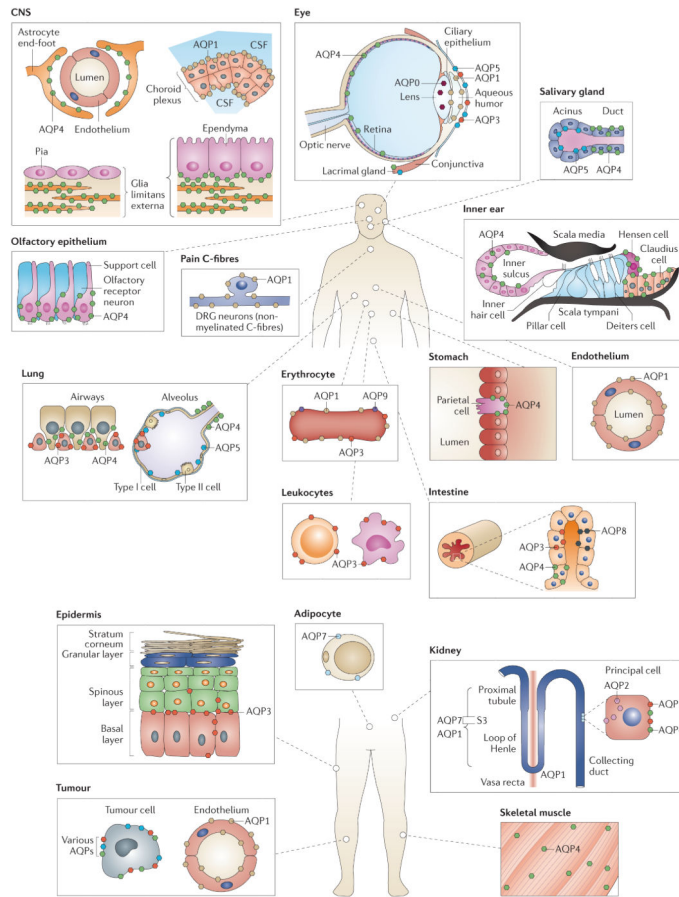
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### Figure 1. Structure of aquaporins

**a** | A top view of the extracellular face of an aquaporin 1 (AQP1) homotetramer, with monomers labelled 1–4, based on the X-ray structure of bovine AQP1 (Protein Data Bank (PDB) code: 1J4N). The tetrameric structure was modelled by the interactive PDBePISA (proteins, interfaces, structures and assemblies) tool. **b** | A schematic of AQP membrane topography. **c** | Structure of the bovine AQP1 monomeric unit, which shows key helical domains (labelled M1–M8) and connecting linkers (labelled a–e). **d** | A view into the extracellular vestibule of bovine AQP1. The constriction region (in green) is made up of aromatic and arginine residues (known as the ar/R constriction; residues Phe58, His182 and Arg197); extracellular Asn-Pro-Ala (NPA) residues (Asn194, Pro195 and Ala196) are shown in orange; backbone  $\alpha$ -carbonyl hydrogen-bond acceptors (Ile192, Cys191, Gly190 and Gly189) are shown in violet; and hydrophobic side chains comprising nonpolar amphipathic surface are shown in black.

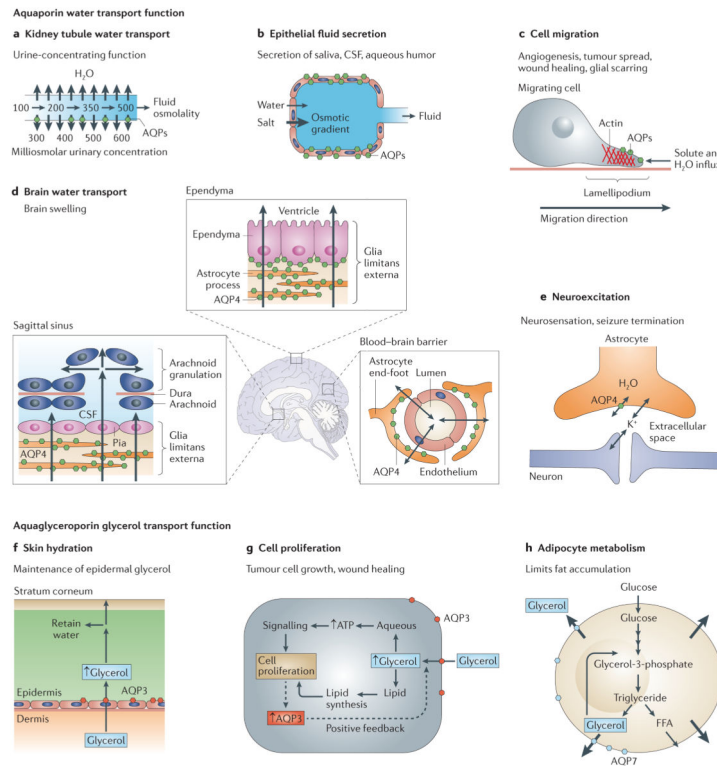


**Figure 2. Tissue distribution of mammalian aquaporins**

In the central nervous system (CNS), aquaporin 1 (AQP1) is expressed in the choroid plexus, and AQP4 is expressed on astrocyte end-feet and glia limitans. In the eye, AQP0 is expressed in the lens, AQP1 is expressed in the corneal endothelium, AQP3 is expressed in the conjunctiva and corneal epithelium, AQP4 is expressed in the retinal Muller cells and lacrimal gland, and AQP5 is expressed in the corneal epithelium and lacrimal gland. In salivary glands, AQP4 is found in the duct cells and AQP5 is expressed in the acini. In the inner ear, AQP4 is expressed in the Claudius, Hensen and inner sulcus cells. AQP4 is expressed in olfactory epithelial cells. AQP1 is expressed in the pain-processing C-fibres of the spinal cord dorsal horn. AQP1 is expressed in all vascular endothelial cells except in the CNS. AQP4 is expressed in the parietal acid-secreting cells of the stomach. In the lung, AQP3 is expressed in the submucosal glands, AQP4 is expressed in the airway cells, and AQP5 is expressed in the alveolar type I cells. Erythrocytes express AQP1, AQP3 and AQP9. In the kidney, AQP1 is expressed in the proximal tubule, thin descending limb of the loop of Henle and vasa recta, AQP2 is expressed in the luminal side of collecting duct principal cells, AQP3 and AQP4 are found in the basolateral side of collecting duct principal cells, and AQP7 is expressed in the S3 segment of the proximal tubule. AQP3 is expressed in macrophages and T cells. In the skin, AQP3 is expressed in epidermal cells, primarily in the stratum basale. Adipocytes express AQP7. Tumour cells express several types of AQPs; for example, glioblastoma cells express AQP1, AQP4 and AQP9, whereas AQP1 is

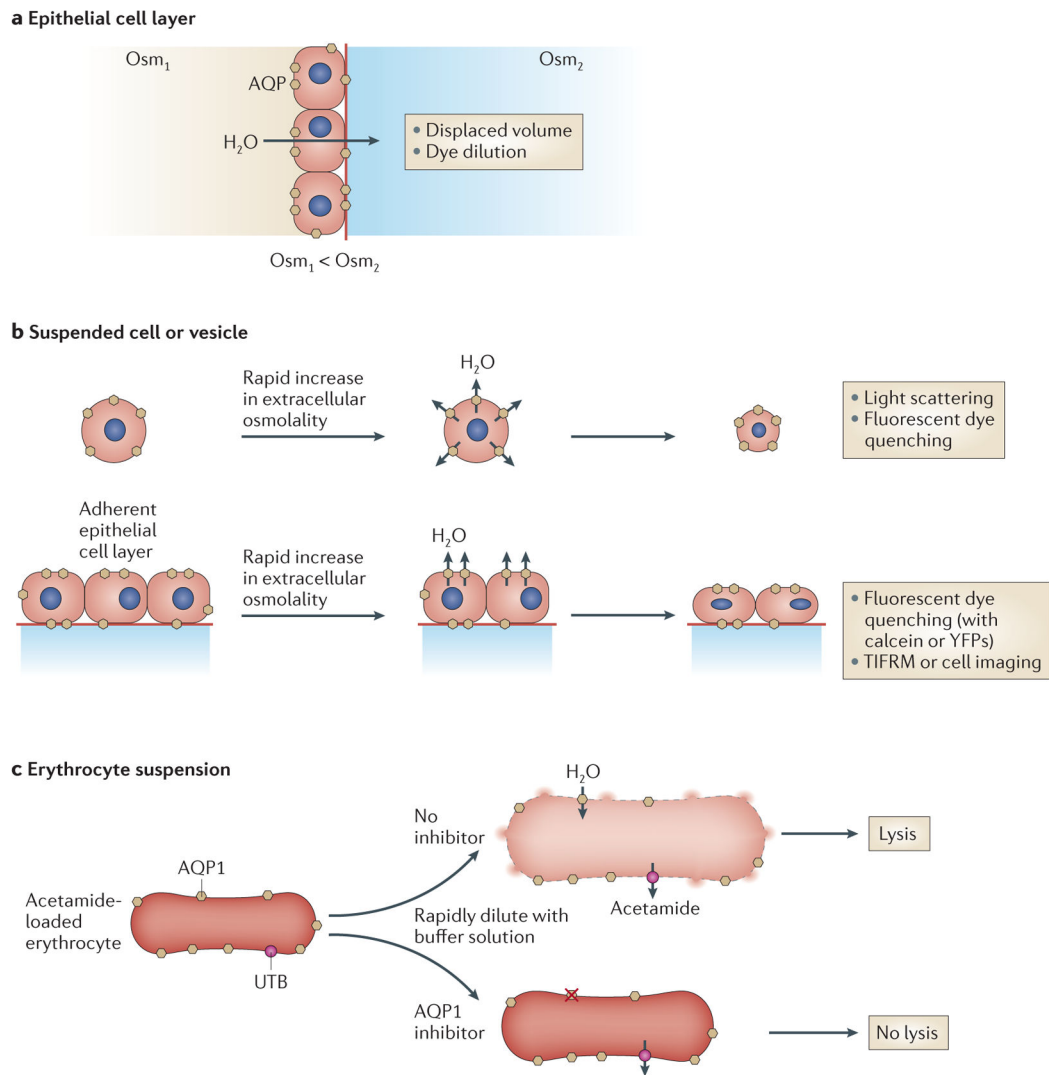
expressed in the microvascular endothelium of tumours in general. AQP4 is expressed on the fast twitch fibres of skeletal muscle. AQP3, AQP4 and AQP8 are expressed in the epithelial cells of the intestine. CSF, cerebrospinal fluid; DRG, dorsal root ganglion.





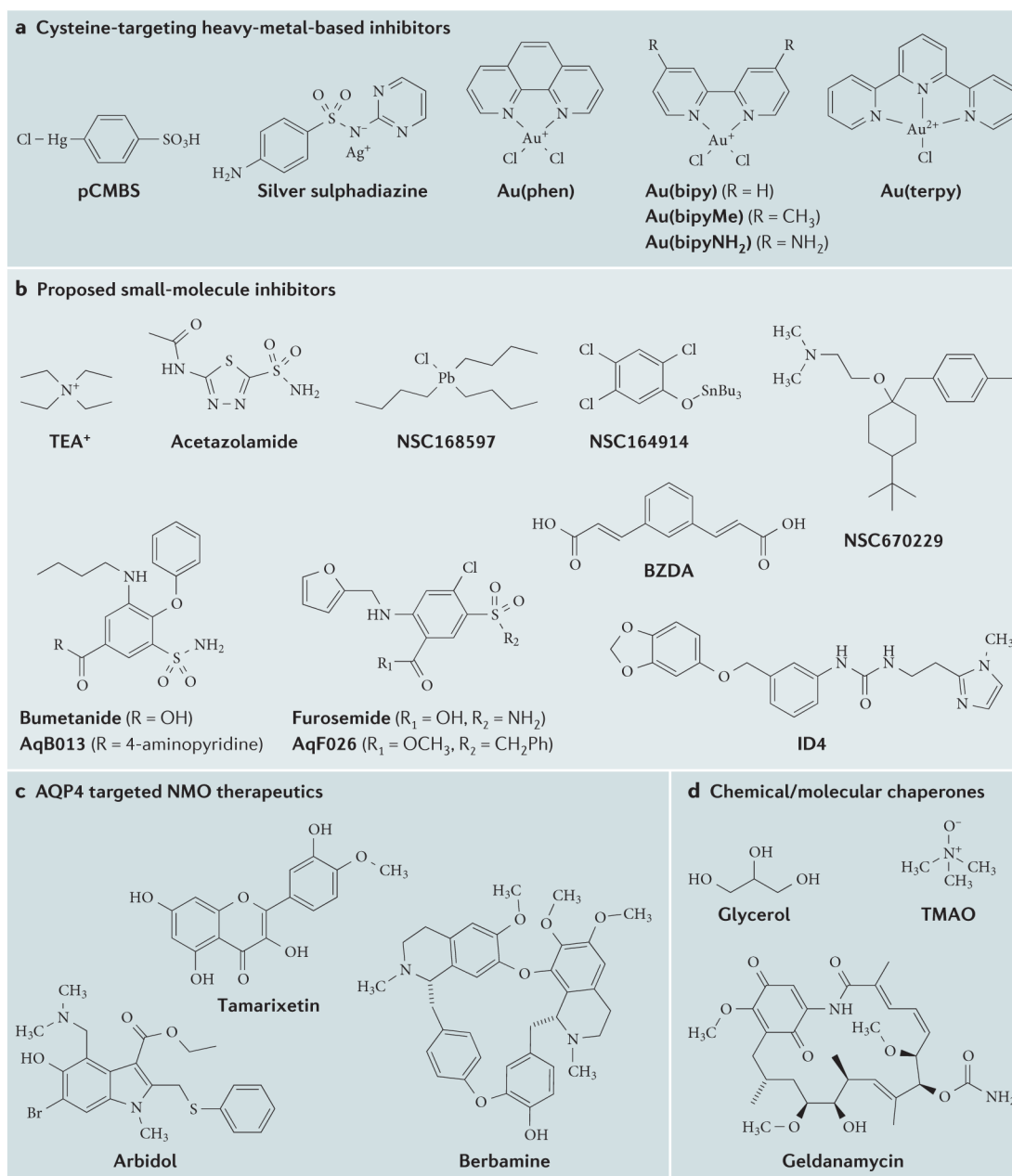
**Figure 3. Major physiological functions of aquaporins**

**a** | In kidney tubules and microvessels, a high transepithelial water permeability in the proximal tubule, thin descending limb of the loop of Henle, vasa recta and collecting duct is required for urine-concentrating function. **b** | During fluid secretion by epithelial cells, a high transepithelial water permeability facilitates the active secretion of near-isosmolar fluid. **c** | Aquaporin (AQP)-facilitated cell migration involves the entry of water into protruding lamellipodia in migrating cells. **d** | Water permeation across the blood–brain barrier and the blood–CSF (cerebrospinal fluid) barrier (that is, the ependyma and sagittal sinus) facilitates the movement of water into and out of the brain. **e** | AQP4-facilitated water transport in astrocytes during potassium reuptake following neuroexcitation causes contraction of the extracellular space, maintaining the driving force for potassium reuptake. **f** | AQP3 facilitates skin hydration by maintaining high glycerol levels in the stratum corneum, which acts as a humectant to retain water. **g** | AQP3 maintains high cellular glycerol levels for the generation of ATP and lipid biosynthesis, which leads to cellular proliferation. **h** | AQP7 facilitates the exit of glycerol from adipocytes, preventing the intracellular accumulation of glycerol and triglyceride. FFA, free fatty acid.



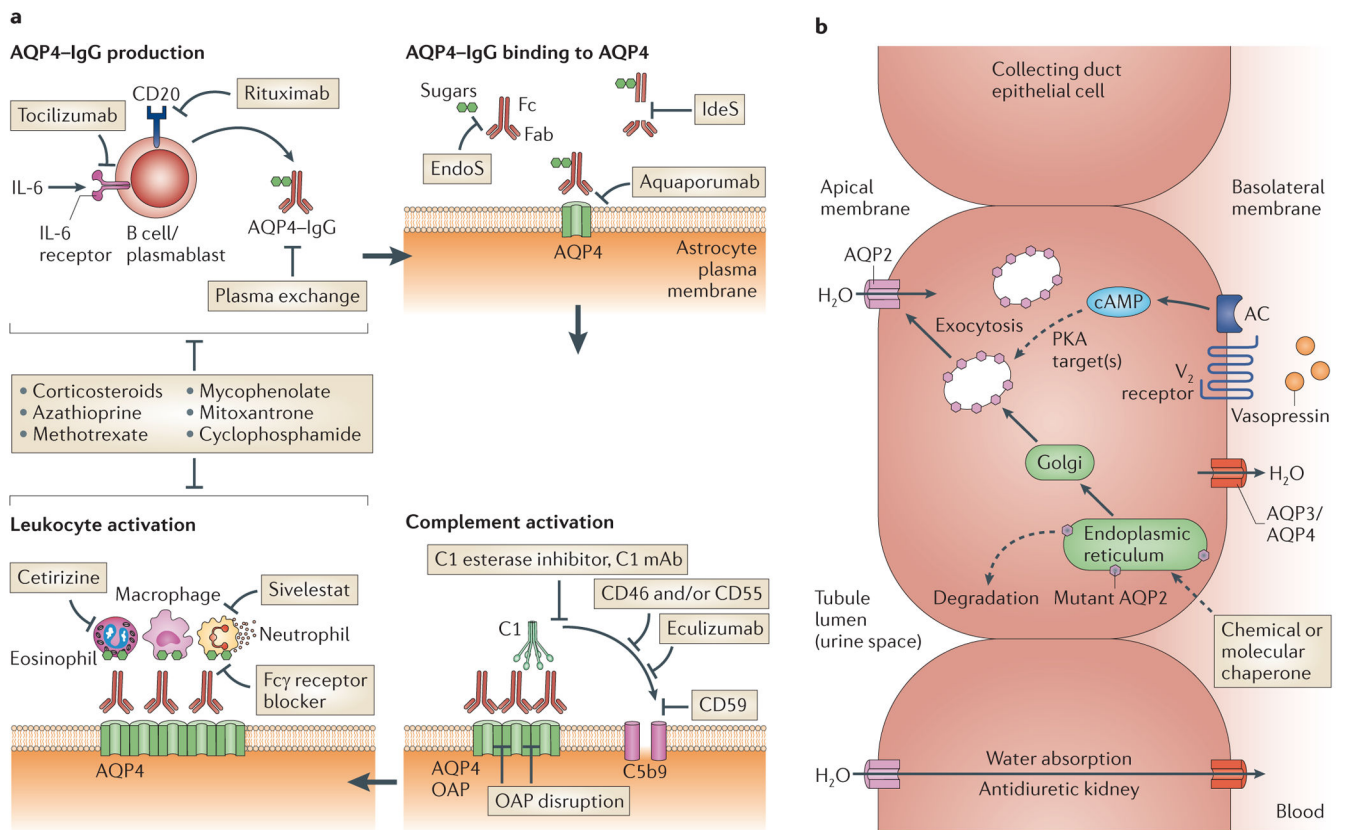
**Figure 4. Assays of aquaporin-mediated water transport**

**a** | An assay of water transport across a tight epithelial cell monolayer in response to a transepithelial osmotic gradient. **b** | Methods based on changes in cell volume in response to an osmotic gradient. Water efflux in response to an osmotic gradient, which causes cell shrinkage, can be measured in suspended cells, vesicles or liposomes (top panel) or an adherent cell layer (bottom panel). **c** | An assay that produces a single-point read-out of aquaporin 1 (AQP1)-mediated water transport in erythrocytes, which natively express AQP1 and urea transporter B (UTB). The dilution of acetamide-loaded erythrocytes into an acetamide-free solution causes a rapid water influx, cell swelling and lysis; AQP1 inhibition prevents lysis. Osm, osmolality; TIRFM, total internal reflection fluorescence microscopy; YFP, yellow fluorescent protein.



**Figure 5. Putative aquaporin-targeted small-molecule modulators**

**a** | Cysteine-reactive, heavy-metal-based aquaporin (AQP) inhibitors<sup>121,123,125–127</sup>. **b** | Proposed small-molecule AQP inhibitors and activators<sup>131,132,134,137,139,142–145</sup>. **c** | Small molecules that inhibit the binding of an AQP4 IgG (immunoglobulin G) autoantibody to AQP4 in neuromyelitis optica<sup>157</sup>. **d** | Chemical chaperones that facilitate trafficking of misfolded AQP2 mutants to the plasma membrane in nephrogenic diabetes insipidus<sup>40,168</sup>. BZDA, *m*-benzene diacrylic acid; pCMBS, *p*-chloromercuribenzenesulphonate; TEA<sup>+</sup>, tetraethylammonium; TMAO, trimethylamine N-oxide.



**Figure 6. AQP4-targeted therapies for neuromyelitis optica and nephrogenic diabetes insipidus**

**a** | An illustration of the mechanism that underlies neuromyelitis optica (NMO) pathogenesis, in which aquaporin 4 (AQP4)–IgG (immunoglobulin G) production and subsequent binding to AQP4 is followed by the activation of complement and leukocyte infiltration. NMO therapeutics are shown in boxes. These include drugs and therapies that target AQP4 production (namely, rituximab, tocilizumab and plasma exchange; currently used treatments), the binding of AQP4–IgG to AQP4 (aquaporumab, endoglycosidase S (EndoS) and the *Streptococcus pyogenes* IgG-degrading enzyme IdeS; potential treatments), complement activation (eculizumab, a complement C1-targeted monoclonal antibody (mAb) and the C1 esterase inhibitor; potential treatments), molecules that disrupt the formation of orthogonal arrays of particles (OAPs; a potential treatment), complement inhibitors (CD46, CD55 and CD59; potential treatments) and inhibitors of leukocyte activity (sivelestat and cetirizine; potential treatments). General immunosuppressive agents are shown as well. The Fc $\gamma$  receptor on immune cells binds to the constant part of IgG. The Fc $\gamma$  receptor on immune cells (neutrophils, eosinophils and macrophages) binds to the constant part of AQP4–IgG. **b** | A schematic showing the cellular processing of AQP2 in epithelial cells of the renal collecting duct. In response to vasopressin, which causes intracellular cyclic AMP elevation and protein kinase A (PKA)-induced phosphorylation, AQP2-containing vesicles are targeted to the plasma membrane and inserted into the apical plasma membrane of the cell. Nephrogenic diabetes insipidus (NDI) can be caused by AQP2 mutations; some mutations cause AQP2 misfolding and endoplasmic reticulum-dependent degradation. The action of chemical or molecular chaperones on the mutant AQP2 can correct mutant AQP2

misfolding. AC, adenylyl cyclase; C5b9, complement C5b9 membrane attack complex; IL-6, interleukin-6; V<sub>2</sub> receptor, vasopressin V<sub>2</sub> receptor.

Table 1

Key features of mammalian aquaporins

AQP	Expression sites	Phenotypes of AQP-null mice	Phenotype of individuals with AQP mutations	Potential therapeutic effects	Refs
AQP0	Lens	Cataract	Cataract	• Not known	90
AQP1	Renal tubules and microvessels, choroid plexus, ciliary epithelium, corneal endothelium, pain-processing C-fibres, vascular endothelium, tumour vessels and red blood cells	Diuresis, reduced tumour angiogenesis, reduced intraocular pressure, reduced CSF secretion, reduced nociception	NDI	• Inhibition: diuretic, reduced tumour angiogenesis, reduced ocular pressure in glaucoma	32,33,44, 45,68,75,148
AQP2	Renal collecting duct	NDI	NDI	• Inhibition: diuretic	39,101,168
AQP3	Renal collecting duct, epidermis, conjunctiva, corneal epithelium, immune cells, intestinal epithelium and red blood cells	Diuresis, dry skin, reduced growth of skin tumours, impaired skin wound healing, impaired regeneration of colonic epithelium and impaired leukocyte function	Unclear	• Activation or upregulation: enhanced skin hydration, enhanced wound healing • Inhibition: diuretic, antitumour effects, anti-inflammatory effects	29,37,84, 89,180,181
AQP4	Astrocytes, retinal Muller cells, lacrimal gland, salivary duct, inner ear, olfactory epithelium, gastric parietal cells, airways, renal collecting duct, placenta, muscle, gut epithelium and glioblastomas	Reduced cytotoxic or increased vasogenic CNS oedema, accelerated obstructive hydrocephalus, increased seizure threshold and duration, deafness and anosmia	None identified	• Activation or upregulation: reduced vasogenic CNS oedema • Inhibition: reduced cytotoxic CNS oedema, anti-epileptic effects, promotion of axonal regeneration, glioblastoma therapy	38,48,52, 72,182–187
AQP5	Corneal epithelium, sweat glands, lacrimal glands, salivary glands, airway submucosal glands, alveolar type I cells and epidermis	Reduced saliva secretion, reduced airway submucosal secretion, thin cornea and reduced tear volume	Palmoplantar keratoderma	• Activation or upregulation: restoration of salivary and lacrimal gland hypofunction • Inhibition: reduced salivation and airway mucus secretion	42,43,188
AQP6	Intracellular vesicles in renal collecting duct intercalated cells	Not reported	None identified	• Not known	189
AQP7	Fat cells, renal proximal tubule (S3 segment), testis and myocardium	Obesity, insulin resistance and hyperglyceroluria	Hyperglyceroluria, platelet defect	• Activation or upregulation: anti-obesity effects	92–95

AQP	Expression sites	Phenotypes of AQP-null mice	Phenotype of individuals with AQP mutations	Potential therapeutic effects	Refs
AQP8	Intestinal epithelium	No major abnormality	None identified	• Not known	190
AQP9	Hepatocytes, erythrocytes and possibly some brain cells	Hypoglyceroemia and reduced red cell glycerol permeability	None identified	• Not known	95,97,177,191
AQP10	Intestinal enterochromaffin cells	<i>Aqp10</i> is reported to be a pseudogene in mice	None identified	• Not known	192
AQP11	Liver, testis and intracellular membranes in renal proximal tubule	Polycystic kidneys and hepatocyte vacuolization	None identified	• Not known	41
AQP12	Exocrine pancreas	No major abnormalities	None identified	• Not known	193

AQP, aquaporin; CNS, central nervous system; CSF, cerebrospinal fluid; NDI, nephrogenic diabetes insipidus.

**Table 2**

## Assays of AQP water transport function

Cellular system	Assay	Throughput	Advantages	Limitations
Suspensions of proteoliposomes, vesicles or small cells	Stopped-flow (measured by light scattering or fluorescence quenching)	Low	Quantitative, low variability in data, good for all transport rates	Technically challenging, requires specialized instrumentation
AQP-expressing <i>Xenopus laevis</i> oocytes	Cell swelling following osmotic challenge (measured by image analysis)	Low	Applicable to any AQP that can be expressed in oocytes	Technically challenging, high variability of data, fraught with artefact because there are many determinants of oocyte swelling
Cell monolayers grown on a porous filter	Transepithelial water flow in response to osmotic gradient (measured by indicator dilution)	Low	Not subject to artefact	Labour intensive, requires cell lines with AQP expression on all plasma membranes, highly variable data
Cell monolayers grown on a solid support	Volume kinetics following osmotic challenge (measured by cytoplasmic calcein or YFP fluorescence)	High	Technically simple	Requires stable expression of AQP at an appropriate level, potential artefacts due to the effects of the compound on indicator fluorescence
Cell monolayers grown on a solid support	Volume kinetics following an osmotic challenge (measured by cytoplasmic fluorescent dye)	High	Reliable, relatively artefact-free	Requires stable AQP expression at appropriate level, requires total internal reflection fluorescence optics
Erythrocytes	Lysis of acetamide-loaded erythrocytes following an osmotic challenge	High	Technically simple, single-point read-out, relatively artefact-free	Only applicable for AQP1, semi-quantitative read-out of cellular lysis

AQP, aquaporin; YFP, yellow fluorescent protein.