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Urea transporter proteins as targets for small-molecule diuretics

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Abstract

Conventional diuretics such as furosemide and thiazides target salt transporters in kidney tubules, but urea transporters (UTs) have emerged as alternative targets. UTs are a family of transmembrane channels expressed in a variety of mammalian tissues, in particular the kidney. UT knockout mice and humans with UT mutations exhibit reduced maximal urinary osmolality, demonstrating that UTs are necessary for the concentration of urine. Small-molecule screening has identified potent and selective inhibitors of UT-A, the UT protein expressed in renal tubule epithelial cells, and UT-B, the UT protein expressed in vasa recta endothelial cells. Data from UT knockout mice and from rodents administered UT inhibitors support the diuretic action of UT inhibition. The kidney-specific expression of UT-A1, together with high selectivity of the small-molecule inhibitors, means that off-target effects of such small-molecule drugs should be minimal. This Review summarizes the structure, expression and function of UTs, and looks at the evidence supporting the validity of UTs as targets for the development of salt-sparing diuretics with a unique mechanism of action. UT-targeted inhibitors may be useful alone or in combination with conventional diuretics for therapy of various oedemas and hyponatraemias, potentially including those refractory to treatment with current diuretics.

Introduction

Urea is the end-product of nitrogen metabolism in mammals; it is generated mainly in the liver, excreted by the kidney, and highly concentrated in urine compared with levels in blood. A central role for urea and urea transport in the urinary concentrating mechanism was first proposed by Gamble and colleagues in 1934,[1] based on the observation that increased urine concentration in rats resulted from urea loading. Urea transporter (UT) proteins, which facilitate the passive transport of urea driven by a concentration gradient across some cell plasma membranes, are now known to be essential in the urinary concentrating mechanism.

It has long been appreciated that urea permeability (P_{urea}) varies widely between different cell membranes;[2,3] the high P_{urea} of human erythrocyte membranes ($4–10 \times 10^{-4}$ cm/s)[4] compared with artificial lipid bilayers ($\sim 4 \times 10^{-6}$ cm/s)[5,6] suggested the existence of facilitated urea transport. Similarly, studies of rabbit kidneys have shown high transepithelial P_{urea} ($\sim 2 \times 10^{-5}$ cm/s) in isolated perfused cortical collecting ducts,[7] and very high P_{urea} ($\sim 4 \times 10^{-4}$ cm/s) in the inner medullary collecting duct (IMCD).[8]

Early molecular studies suggested the existence of UT proteins because urea transport in *Xenopus* oocytes increased after the cells were injected with mRNA from toad urinary bladder, a tissue that has high P_{urea}.[9] The first UT protein was identified in rabbit kidneys in 1993 by using expression cloning;[10] subsequent work has identified and characterized homologous UTs from other mammals and lower organisms, greatly advancing our understanding of UT biology, particularly in the kidney. This Review discusses these discoveries and looks at emerging evidence from experiments with UT knockout mice and small-molecule UT inhibitors, which show that UT inhibitors have clinical potential as salt-sparing diuretics, or 'urearetics', that have a unique mechanism of action. This mechanism is the disruption of the countercurrent multiplication mechanism for urinary concentration that leads to a diuretic response.

UT proteins

Molecular genetics

Mammalian UT proteins are encoded by two genes that are arranged in tandem: *SLC14A1* and *SLC14A2*. In humans, these genes are located ~50 kb apart on chromosome 18.[11,12] The *SLC14A1* gene consists of 11 exons and encodes two variants of UT-B, UT-B1 and UT-B2, which are splice variants of the *SLC14A1* gene[13,14] that show 100% homology except for an additional 55 amino acids in the N-terminus of UT-B2.[15] In this Review, we use the term UT-B to refer to both splice variants, and only distinguish between the splice variants when their expression or functions differ.

The *SLC14A2* gene consists of 26 exons and encodes six UT-A isoforms, which are under the control of two distinct promoters: UT-Aα and UT-Aβ.[16,17] UT-A1, UT-A3, UT-A4, UT-A5 and UT-A6 are transcribed from the UT-Aα promoter, which is located upstream of exon 1, whereas UT-A2 is transcribed from the internal UT-Aβ promoter.[18] The entire length of the *SLC14A2* gene encodes UT-A1, which consists of 930 amino acids; the other five isoforms share different regions of this coding sequence (Figure 1).[19–21] UT-A1, UT-A2 and UT-A3 have all been identified in mice, rats and humans, whereas UT-A4 has only been identified in rats, UT-A5 only in mice and UT-A6 only in humans.[19,22,23] UT-B has >60% homology with UT-A2.[24]

Molecular structure

The first X-ray crystal structure of a UT that was solved was that of the bacterial protein dvUT, a functional homologue of mammalian UTs from *Desulphovibrio vulgaris*.[25] Structures were determined for dvUT both alone and co-crystallized with the urea analogue dimethylurea (Protein Data Bank IDs 3K3G and 3K3F, respectively). The same group subsequently solved X-ray crystal structures of the mammalian UT-B from *Bos taurus*, both

alone and co-crystallized with selenourea (Protein Data Bank IDs 4EZC and 4EZD, respectively; Figure 2).[26] These studies showed that the quaternary structures of dvUT and bovine UT-B are similar, with both forming homotrimers (Figure 2a); size-exclusion chromatography suggested that human UT-B also exists as a homotrimer. Whether or not the UT-A isoforms also exist as homotrimers is still unknown. A dimeric structure of UT-A1 was proposed on the basis of chemical crosslinking, but the identity of the species as a homodimer or hetero-oligomer was not established.[27] Although UT-A1 and UT-A3 are coexpressed in the IMCD, immunoprecipitation suggests that they do not oligomerize.[28]

Bovine UT-B and dvUT each contain two homologous halves that fold to form the complete channel (Figure 2b), and it has been postulated that the homologous halves descend from a common ancestral gene that duplicated at a time near to the divergence of eukaryotes from prokaryotes.[29] Each UT-B half contains five transmembrane helices (T1a–T5a and T1b–T5b) and a tilted helix (Pa and Pb) that is approximately half the length of the membrane-spanning helices. The N-termini and C-termini of dvUT and UT-B are cytoplasmic, as confirmed by topogenic signal analysis[30] and immunoadsorption experiments with human UT-B.[31]

The UT-A isoforms UT-A2, UT-A3 and UT-A4 are each predicted to have a similar molecular structure to UT-B: 10 transmembrane domains with two internal hydrophobic repeats, linked by an extracellular connecting loop and with intracellular N-termini and C-termini. The largest UT-A isoform, UT-A1, consists of this structure duplicated.[13,32]

UT-B contains an amphipathic pore region formed by the association of the two homologous halves (Figure 2c). The UT-B selectivity filter is thought to involve three regions that line the central pore: the S_0, S_i and S_m sites.[26] The S_0 and S_i sites, which are on the extracellular and cytoplasmic sides respectively, form a ladder of main-chain and side-chain oxygen atoms that are able to form hydrogen bonds and that interact with the polar urea molecule. The S_m site serves as a central constriction of the channel.

When co-crystallized with bovine UT-B, the urea analogue selenourea interacts with the selectivity filter by hydrogen bonding with Asn227 at the S_0 site, and with Asn63 at the S_i site (Figure 2c). The S_m site is relatively narrow and lined by the hydrophobic side chains of Thr172 and Thr334, which, in addition to serving as a steric constriction, might desolvate urea as it passes through the pore. A Thr172Val mutation increases steric hindrance at the constriction site and results in reduced P_{urea}, whereas Thr172Ser, Thr334Ser, and Thr172Ser–Thr334Ser mutations reduce steric hindrance and slightly increase P_{urea}.[26] Interestingly, despite the narrow and desolvating S_m constriction site, UT-B can conduct water, whereas the UT-A isoforms cannot.[33–35]

Other small-molecule analogues of urea are also able to traverse the UT-B channel, including formamide, acetamide, methylurea, methylformamide, ammonium carbonate and acrylamide.[36] Molecular dynamics simulations that used the technique of umbrella sampling concluded that urea traverses the channel with minimal rotation, allowing it to continuously maintain hydrogen bonding interactions with the ladder of hydrogen-bond-accepting groups, and preventing substantial alteration of the UT-B structure.[26]

Tissue expression

Most UTs are expressed in the kidney (Figure 3), where their role in the urinary concentrating mechanism has been well studied. UT-A1 is expressed on the apical membranes and UT-A3 on the basolateral membranes of cells in the IMCD, which is the most urea-permeable segment of the nephron.[22,37,38] One study reported that UT-A3 is also localized to the apical membranes of cells in the IMCD after vasopressin administration.[39] UT-A2 is expressed in the thin descending limbs of the short loop nephrons in the outer medulla and in the distal part of long loop nephrons in the inner medulla.[40] UT-A4 has been reported in renal medulla only in rats, and its precise cellular localization is not known.[22] UT-B, including both splice variants, is expressed in endothelial cells of the descending vasa recta.[41,42]

Some UTs are also expressed outside of the kidney (Box 1); knowledge of extrarenal UT expression sites and function is important in drug discovery to identify potential off-target, extrarenal effects of UT inhibition.

Box 1

Extrarenal expression of mammalian UT proteins

- UT-B1: bladder, brain, colon, ear, erythrocyte, heart, liver, lung, pancreas, prostate, skeletal muscle, small intestine, testis, thymus, spleen

- UT-B2: brain (human), rumen (bovine), thymus (mouse)

- UT-A1: cochlea

- UT-A2: heart, liver

- UT-A3: cochlea, inner medullary collecting duct

- UT-A4: renal medulla (rat)

- UT-A5: testis (mouse)

- UT-A6: colon (human)

Abbreviation: UT, urea transporter.

Extrarenal expression of UT-B splice variants differs between tissues; UT-B1 is expressed more widely than UT-B2, which has only been detected in the brain of humans.[15,41] Immunocytochemistry has demonstrated the localization of UT-B1 to villous epithelium in the small intestinal, gastric glandular epithelium[43] and colonic crypt epithelium[44,45] in the digestive tract, ependymal cells and astrocytes in the brain, aortic endothelia cells,[12,41,46] and Sertoli cells in the testes.[47] UT-A isoforms have limited extrarenal expression, but UT-A1,[48] UT-A2,[49] UT-A3[48] and UT-A6[21] have been detected in extrarenal tissues in humans, whereas the UT-A5 transcript is expressed in peritubular myoid cells in the seminiferous tubules in mouse testes.[19,50]

Potential roles of extrarenal UTs have been proposed. UT-A2 in liver and heart may facilitate efflux of metabolically generated urea,[49,51,52] and UTs in the inner ear have been

postulated to facilitate urea transport between the endolymph and perilymph, contributing to fluid homeostasis.[48] Phenotype studies on UT-B knockout mice have revealed neuropsychiatric,[53] cardiac conduction,[54,55] bladder[56] and testicular[57] abnormalities, although the relevance of these phenotype findings is unclear, both because they lack plausible mechanisms, suggesting secondary effects, and because there are no studies of humans with loss-of-function mutations in UT-B that report extrarenal abnormalities.

Regulation

Vasopressin regulation of P_{urea} in the collecting duct was described before the identification of UTs;[58–60] vasopressin is now known to be a major regulator of UT-A1 and UT-A3 in the IMCD. Vasopressin binding to V2 receptors causes elevation of cAMP, activation of protein kinase A, and phosphorylation of UT-A1 and UT-A3.[28,61–63] Serine phosphorylation of UT-A1 and UT-A3 is involved in their accumulation in the plasma membrane and is associated with an increase in IMCD P_{urea}.[64–66] Independently of vasopressin, hypertonicity also increases IMCD P_{urea} by UT-A1 and UT-A3 phosphorylation and membrane targeting,[39,67] via mechanisms involving a calcium-dependent protein kinase C.[68]

Recent data also suggest the involvement of caveolin, snapin and actin in apical membrane UT targeting.[69] UT-A1 degradation, which involves the lysosome–proteasome system, might regulate UT-A1 expression at a post-transcriptional level.[70]

Non-mammalian UTs

UTs are found not only in mammals, but also in non-mammalian organisms including bacteria, plants and fish. A brief discussion of non-mammalian UTs is informative in understanding the wide-ranging functions of UTs, some of which are relevant in mammals.

Bacterial UTs can be divided into three distinct functional classes: pH-independent, proton-gated and ATP-activated. The bacterial UT protein Yut, found in *Yersinia,* is pH-independent and homologous to mammalian UTs, showing 22% sequence identity to human UT-A1;[71,72] for comparison, dvUT shows 35% sequence identity to human UT-B.[25] UreI from *Helicobacter pylori*, a bacterium that survives in the highly acidic gastric environment, is a H^{+}-gated UT. *H. pylori* uses UreI to transport urea into the cytoplasm, where it is then hydrolysed to form NH_3 and CO_2;[71,73] the NH_3 and CO_2 are transported to the periplasm, where NH_3 neutralizes acid, and CO_2 (upon conversion to bicarbonate) buffers periplasmic pH to ~6.1.[73] Some bacterial UTs belong to the ATP-binding cassette (ABC) class of transporters, meaning that urea transport by these transporters is accompanied by ATP hydrolysis. The ABC class of UTs is used by the soil bacterium *Corynebacterium glutamicum* and various species of cyanobacteria, in which imported urea is hydrolysed by urease and used as a catabolic nitrogen source.[71]

One class of plant aquaporins called tonoplast intrinsic proteins, which were identified in *Arabidopsis*, show pH-independent and phloretin-sensitive urea transport.[74] *Arabidopsis* also expresses a high-affinity H^{+}–urea symporter called AtDUR3, which belongs to a family of Na^{+}–solute symporters.[75] Although only limited information is available, increased

expression of AtDUR3 has been observed in *Arabidopsis* seedlings, suggesting that urea transport might be involved in early plant development.[75]

Of non-mammalian vertebrates that express UTs, fish have received the most attention.[76,77] An interesting difference between fish and mammals is that fish lack an erythrocyte UT.[76] In teleosts, UTs, together with ammonia transporters, seem to be involved in nitrogen excretion through the gills,[76] whereas in elasmobranches, UTs in the kidney and gill help to retain urea against the concentration gradient set up by the surrounding water.[76,78] As would be expected, fish UTs have greater homology than do bacterial UTs to mammalian UTs; for example, the UT of *Danio rerio* (zebrafish) has 63% sequence identity to human UT-A2. In 2005, a new UT (named UT-C) was identified in the proximal tubule of kidneys in seawater eel, which is the first example of a UT expressed in proximal tubule.[79]

UTs in kidney function

UTs and the urinary concentrating mechanism

Concentration of urine by the kidney involves a counter-current multiplication mechanism, in which the anatomical configuration of the tubules and vasa recta permits countercurrent flow and intrarenal urea recycling, resulting in a hypertonic inner medulla. Urea has a central role in this mechanism.[80,81] The IMCD, which expresses UT-A1 and UT-A3, is responsible for urea reabsorption during antidiuresis; this reabsorption is needed to maintain a hypertonic medullary interstitium. The fenestrated ascending vasa recta, which is highly permeable to urea, facilitates transfer of urea from the inner to the outer medulla, where its concentration is lower. Interstitial urea coming from the ascending vasa recta is partially reabsorbed by the non-fenestrated descending vasa recta, which expresses UT-B, and the thin descending limb of Henle, which expresses UT-A2, by a countercurrent exchange mechanism in which urea recycling conserves renal urea.[80–82]

Although these basic processes are probably correct (at least qualitatively), many questions remain about the role of urea in countercurrent multiplication and exchange mechanisms, as mathematical models are unable to predict the actual magnitude of urinary concentration in mammals. Current understanding of the role of urea and UTs in the urinary concentrating mechanism is discussed in a recent review.[83]

Lessons from UT knockout mice

The first UT knockout mouse was generated in 2002 by targeted UT-B gene disruption.[84] UT-B knockout mice show a modest, ~50% increase in fluid intake and urinary output compared with wild-type mice, and manifest a mild reduction in maximum urinary osmolality (1,500 mOsm/kg H_2O versus 2,000 mOsm/kg H_2O in wild-type mice). The knockout mice show a more than twofold reduction in the urine-to-plasma ratio of urea compared with that of wild-type mice, indicating a reduced capacity of the kidney to concentrate urea above its blood level. This defect suggests that urea recycling in the descending vasa recta is impaired; the greater quantities of urea returned to the general circulation result in reduced inner medullary hypertonicity and urinary concentration.[84,85]

Mice that lack both UT-A1 and UT-A3 show a marked defect in urinary concentrating function.[86] With a normal diet, UT-A1/3 knockout mice have more than a twofold increase in fluid intake and an approximately threefold increase in urine volume, accompanied by a marked reduction in maximal urinary osmolality compared with wild-type mice (800 mOsm/kg H_2O versus 2,300 mOsm/kg H_2O in wild-type mice). As predicted, the concentration of urea in the inner medulla is reduced by more than threefold, which supports the proposed requirement for UTs in the IMCD for urea accumulation in the inner medullary interstitium.

Interestingly, UT-A1/3 deletion did not affect urine concentration in mice that were on a low-protein diet, indicating that the impaired urinary concentrating function in UT-A1/3 knockout mice depends on the excretion of large amounts of urea.[86] UT-A1 also seems to be more important than UT-A3 in urinary concentration, as the impaired urinary concentration in UT-A1/3 knockout mice was corrected by transgenic replacement of UT-A1 even without replacement of UT-A3.[87]

Unexpectedly, UT-A2 knockout mice do not manifest defective urinary concentration under normal conditions.[88] However, these mice do show a mild decrease in maximal urine osmolality when on a low-protein diet (2,500 mOsm/kg H_2O versus 3,500 mOsm/kg H_2O in wild-type mice), with a >1.5-fold reduction in urea concentration in the inner medulla compared with wild-type mice.[88] Intriguingly, double-knockout mice that lack UT-B and UT-A2 show lesser impairment in urinary concentration ability than do mice that lack only UT-B.[85] One speculative explanation put forward for this unexpected observation is that deletion of UT-A2 reduces the loss of urea from the descending limb to the general circulation; however, the precise function of UT-A2 in the urinary concentrating mechanism remains unclear.

We note that interpretation of phenotype data from knockout mice is subject to the caveat that knockout mice generally manifest chronic altered expression of many proteins, as has been reported for UT knockout mice,[89,90] so that findings can be the result of primary or secondary effects. Selective, rapidly acting UT inhibitors are useful in distinguishing between primary versus secondary effects.

Lessons from humans with UT mutations

The UT-B gene locus *SLC14A1* determines an individual's Kidd blood group, and contains two alleles: Jk[a] and Jk[b]. Rare individuals that lack both of these alleles, termed Jk[null], show no clinical abnormality except for reduced maximal urinary concentration. Healthy adults concentrate their urine to ~1,200 mOsm/kg H_2O, whereas individuals with the Jk[null] genotype concentrate urine to ~800 mOsm/kg H_2O.[91,92] Jk[null] individuals manifest loss of UT-B function, demonstrated by the finding that their erythrocytes are resistant to lysis at high urea concentration.[92] Humans with loss-of-function mutations in UT-A isoforms have not been identified.

Since 2011, gene association studies have identified single nucleotide polymorphisms (SNPs) of the *SLC14A1* gene that show it to be a susceptibility locus for bladder carcinogenesis.[93–95] An association between urinary bladder cancer and urine concentration

or frequency of urination was proposed. Various human SNPs of UT-A2 have been linked to reduced diastolic blood pressure in Chinese men.[96] Other UT-A2 SNPs have been associated with development of metabolic syndrome in Asians,[97] although the responsible mechanisms and relevance of these observations are not clear.

UT inhibitors

Until recently, available UT inhibitors included the non-selective membrane-intercalating agent phloretin and chemical analogues of urea, such as dimethylthiourea (DMTU), that are effective at millimolar concentrations.[4] The identification of selective, small-molecule UT inhibitors, some of which have an IC_{50} (concentration that causes 50% inhibition) in the low nanomolar range, has arisen from the development of new assays of UT function that are suitable for high-throughput screening of diverse collections of drug-like molecules.

Assaying UT function

As the exclusive function of UT proteins is to facilitate the transport of urea (and some urea analogues), assaying UT function involves the measurement of urea transport from one side of a cell membrane, or an epithelium, to the other side (Figure 4). In one version of the assay, urea is added on one side of an epithelial cell layer, such as an epithelial cell culture grown on a porous filter, and its accumulation on the opposite side of the layer is measured by either an enzymatic, urease-based colorimetric assay, or by using radiolabelled ^{14}C–urea (Figure 4a). This type of epithelial cell assay has been used to test UT inhibitors in UT-A1-transfected MDCK cells.[98] Measurement of transepithelial urea transport has been performed in isolated microperfused kidney tubules[8] and other *ex vivo* epithelia such as toad bladder.[99]

Urea transport across individual cell membranes can be measured in large cells by assaying urea in extracellular fluid samples, which has been done in *Xenopus* oocytes.[9] In smaller cells, urea transport is usually measured indirectly through the secondary effects of urea movement on water transport and hence on cell (or membrane vesicle or liposome) volume. This technique is used because urea equilibration occurs over seconds or tens of seconds, which makes the physical separation of cells from extracellular solution difficult.

Stopped-flow light scattering has been widely used to measure P_{urea} in erythrocytes.[4,100] In this method, erythrocytes in suspension are subjected to an inwardly directed urea gradient, which results in initial osmotic water efflux and cell shrinkage, detected as an increase in scattered light intensity, followed by urea and water influx and cell reswelling, detected as a phase of decreasing scattered light intensity (Figure 4b). Inhibition of the erythrocyte UT-B slows the reswelling phase. Other methods that follow cell volume kinetics have also been applied to measurement of urea transport, such as methods that use the volume-sensing fluorescent dye calcein.[101] Although the methods described above provide accurate data on P_{urea}, they are not easily adapted to automated high-throughput screening platforms.

The first high-throughput screening assay for the identification of UT inhibitors used erythrocytes, which natively express UT-B.[98] The assay uses a read-out of erythrocyte lysis measured by near-infrared light absorbance at single time points (Figure 4c). Erythrocytes

are preloaded with acetamide, a urea analogue that is transported by UT-B at a rate such that its equilibration time is similar to the equilibration time for osmotic water transport. This preloading imposes a large, outwardly directed gradient of acetamide that causes cell swelling, but this swelling is limited by UT-B-facilitated acetamide efflux. UT-B inhibition slows acetamide efflux, which leads to increased cell lysis. Once acetamide concentration was optimized for identification of UT-B inhibitors, the assay was found to be robust and sensitive, giving a statistical Z-factor of ~0.6, and to correlate well with UT-B inhibition data from stopped-flow light scattering measurements. As discussed below, the erythrocyte lysis assay has been used in several screening studies to identify UT-B inhibitors.

Development of a similar UT-A urea transport assay was challenging, because available cell lines do not natively express UT-A isoforms, UT-A-facilitated urea equilibration across cell membranes is rapid, and robust measurement of cell volume using an automated screening platform is difficult. One assay for UT-A1 inhibitors involves the measurement of cell volume changes that result from extracellular addition of urea to transfected MDCK cells that stably express UT-A1 (Figure 4d).[102] Cell volume is measured indirectly using the fluorescence of the chloride-sensing, genetically encoded fluorescent protein YFP–H148Q–V163S;[103] cell shrinkage increases cytoplasmic chloride concentration, which subsequently reduces YFP fluorescence. The MDCK cells are also transfected with the water channel aquaporin-1 to make osmotic water equilibration faster than urea equilibration. Addition of urea to the extracellular solution drives osmotic water efflux and cell shrinkage, which is followed by urea (and water) entry and a return to the original cell volume. The assay produces a robust difference between the fluorescence curve shapes with and without UT-A1 inhibition, and correlates well with transepithelial urea transport measurements. This assay has been used in screening studies to identify UT-A1 inhibitors.

Discovery of small-molecule UT inhibitors

An initial screening of 50,000 drug-like small molecules that used the high-throughput erythrocyte lysis assay with human erythrocytes (Figure 4c) identified ~30 UT-B inhibitors from the phenylsulphoxyoxozole, benzenesulphonanilide, phthalazinamine, and aminobenzimidazole chemical classes.[98] Analysis of ~700 chemical analogues of these four scaffolds identified many active compounds, the most potent of which inhibited human UT-B urea transport with an IC_{50} of ~10 nM and caused ~100% inhibition at concentrations over 1 μM. The compounds were characterized and used to confirm water transport through UT-B.

A subsequent screening of 100,000 compounds that used mouse erythrocytes aimed to identify potent inhibitors of rodent (and human) UT-B, because the inhibitors that were identified in the original screening of human UT-B urea transport had relatively weak activity against mouse and rat UT-B.[104] The most potent UT-B inhibitor identified in this screening was the triazolothienopyrimidine 3-(4-ethyl-benzenesulphonyl)-thieno[2,3-e][1,2,3] triazolo[1,5-a]pyrimidin-5-yl]-thiophen-2-ylmethylamine (UTB$_{inh}$-14; Figure 5). UTB$_{inh}$-14, which was synthesized and purified in five steps, fully and reversibly inhibited urea transport with an IC_{50} of 10 nM for human UT-B (Figure 5b) and 25 nM for mouse UT-B.[104] UTB$_{inh}$-14 is highly selective, with no inhibition of UT-A1 even at high

concentration. Competition studies showed reduced potency of inhibition with increased urea concentration, which suggests that UTB_{inh}-14 binds to the UT-B protein near the urea binding site of UTB_{inh}-14, in a cleft near the cytoplasmic pore region of the UT-B molecule (Figure 5c). As described below, UTB_{inh}-14 has been used in mice to test the effect of acute UT-B inhibition on urinary concentrating function.

Medicinal chemistry has been used to optimize triazolothienopyrimidine properties and increase the metabolic stability of UTB_{inh}-14.[105] Analysis of structure–activity relationships indicated that CH_2 hydroxylation of the ethyl substituent in UTB_{inh}-14 is a major determinant of the molecule's metabolic stability. Replacement of the CH_2 hydrogen atoms with fluorine atoms to produce the compound {3-[4-(1,1-difluoroethyl)-benzenesulphonyl]-thieno[2,3-e][1,2,3]triazolo[1,5-a] pyrimidin-5-yl}-thiophen-2-ylmethylamine resulted in a ~40-fold improvement of *in vitro* metabolic stability over UTB_{inh}-14 with little effect on UT-B inhibition potency. The optimized UT-B inhibitor accumulated in the kidneys and urine in mice, and reduced maximum urinary concentration.

High-throughput screening has also identified small molecules that inhibit UT-A proteins (Figure 6). Screening of 100,000 synthetic small molecules for UT-A1 inhibition using the assay described in MDCK cells (Figure 4d) identified four classes of compounds, each with a low-micromolar IC_{50}; the most potent of these compounds was UTA_{inh}-A1 (Figure 6a).[102] The UT-A1 isoform was used for the screening because it is predicted to be the most important isoform for urinary concentration. UT-A1 inhibition by each of the UT-A1 inhibitors was reversible and largely non-competitive. An interesting finding was the identification of compounds that have a wide range of selectivities for UT-A1 and UT-B; even within the same chemical class, relatively minor chemical modifications produced compounds with very high selectivity and others with little UT-A1 versus UT-B selectivity.

Subsequent screening was performed to identify UT-A1-selective inhibitors that have suitable pharmacological properties for efficacy testing in rodents.[106] This screening identified one compound—the arylthiazole UTA_{inh}-E02 (Figure 6a)—that reversibly inhibited UT-A1 urea transport with an IC_{50} of 1–2 μM via a non-competitive mechanism, and that has ~100-fold greater selectivity for UT-A1 than for UT-B (Figure 6b). Liquid chromatography–mass spectrometry was used to predict therapeutic inhibitor concentrations in rats following intravenous bolus administration.

Interestingly, screening of approved and investigational drugs and natural products also identified nicotine, sanguinarine and an indolcarbonylchromenone as UT-A1 inhibitors, albeit with relatively weak IC_{50} values of 10–20 μM.[107]

Diuretic action of UT inhibitors in rodents

The UT-B-selective inhibitor UTB_{inh}-14 was studied in mice after pharmacological studies had been used to establish the optimal compound administration vehicle, dose, route and timing to maintain concentrations in the kidneys and urine that are predicted to be therapeutic.[104] Mice were administered intraperitoneal UTB_{inh}-14 or a vehicle control, followed by the vasopressin receptor agonist dDAVP. Urine osmolality was mildly reduced in UTB_{inh}-14-treated mice compared with vehicle-treated mice; this reduced osmolality was

similar to the urine osmolality observed in UT-B knock-out mice, in which UTB$_{inh}$-14 had no significant effect (Figure 7a). UTB$_{inh}$-14 also mildly increased urine output and reduced urine osmolality in mice that were given free access to water. The fact that the diuretic effect of selective UT-B inhibition was relatively minor was not unexpected, as tubule epithelial UT-A is predicted to be the major target for diuretic effect. A subsequent study of a non-selective UT inhibitor in rats also reported diuretic action.[108] Acute inhibition of UT-B by selective inhibitors should be useful in studying the potential extrarenal effects of UT-B inhibition, as mentioned above.

The effect of the UT-A1-selective inhibitor UTA$_{inh}$-E02 on urinary concentration has been studied by intraperitoneal injection of UTA$_{inh}$-E02 into rats that were also administered dDAVP.[106] In vehicle-treated animals, dDAVP reduced urine volume and increased urine osmolality, but UTA$_{inh}$-E02 prevented these changes. At 3–6 h after UTA$_{inh}$-E02 administration, little residual difference remained between UTA$_{inh}$-E02-treated and vehicle-treated rats, indicating inhibitor reversibility (Figure 7b). In hydrated rats, increased urine volume and reduced urine osmolality were seen at 0–3 h after UTA$_{inh}$-E02 administration; urine volume and osmolality returned to baseline in the 3–6 h collection. These studies support the hypothesis that selective UT-A inhibition has a diuretic effect; this effect is advantageous for drug development because UT-A isoforms are primarily expressed in kidney, whereas UT-B is relatively ubiquitous, so off-target effects are likely to be less problematic with UT-A inhibition than with UT-B inhibition.

A 2012 study examined the diuretic action of the non-selective UT inhibitor DMTU, which is a urea analogue that has been used at high doses in experimental animal models as a hydroxyl radical scavenger, where it inhibited urinary concentration.[109] DMTU fully and reversibly inhibited rat UT-A1 and UT-B by a non-competitive mechanism with an IC$_{50}$ of 2–3 mM.[107] Following a single intraperitoneal injection of 500 mg/kg DMTU, peak plasma concentration of DMTU was 9 mM with a half-life of ~10 h, and urine concentration of DMTU was 20–40 mM. Chronic treatment with high-dose DMTU resulted in a sustained reduction in urine osmolality from 1,800 mOsm/kg H$_2$O to 600 mOsm/kg H$_2$O and a threefold increase in urine output, both of which fully reversed after DMTU discontinuation. DMTU did not further impair the already reduced urinary concentrating function in rats on a low-protein diet. Rats that were given chronic DMTU treatment showed mild hypokalaemia, but no abnormalities in serum chemistry. Hypokalaemia is usually seen after diuretic administration, and its likely cause in the DMTU-treated rats is, at least in part, an increased distal delivery of urinary fluid. Short-term DMTU administration indicated a salt-sparing diuretic action. The DMTU data further support the diuretic efficacy of UT inhibition.

Indications for UT inhibitors

UT inhibitors, particularly inhibitors of UT-A1, have several potential clinical indications. UT-blocking diuretics may be useful, either alone or in combination with conventional diuretics that block salt transport, in oedema caused by fluid overload (congestive heart failure, nephrotic syndrome and cirrhosis) and in hyponatraemia caused by chronic elevation in vasopressin level (syndrome of inappropriate antidiuretic hormone secretion [SIADH]). Their unique mechanism of action on renal countercurrent mechanisms means that UT-A1

inhibitors might be effective in refractory oedema, for which conventional diuretics such as furosemide and thiazides have limited effectiveness, and could produce a relatively salt-sparing diuresis. After optimization of their pharmacological properties, testing of UT-A1 inhibitors in clinically relevant animal models of oedema and hyponatraemia will be important to define their clinical indications.

Conclusions

Our understanding of urea transport mechanisms and the role of urea transport in the urinary concentrating mechanism has been greatly advanced by the identification of UTs and studies in transgenic mice and rodents treated with UT inhibitors. However, unresolved issues remain concerning UT physiology and the translation of UT biology into therapeutics. The precise roles and regulation of the different mammalian UT isoforms in the urinary concentrating mechanism remain incompletely understood, as does the validity of translating findings in animal models to humans. Nevertheless, the available results provide a compelling rationale for advancing UT inhibitors, particularly inhibitors of UT-A1, as salt-sparing diuretics with a mechanism of action that differs from that of existing salt-blocking diuretics. Target properties of UT inhibitors for clinical applications include high UT-A1 inhibition potency and selectivity, good pharmacological properties such as oral bioavailability, and few off-target effects. Ultimately, clinical trials will be needed to assess the efficacy and safety of UT inhibitors alone and together with conventional diuretics, the occurrence of hypokalaemia with UT use, and the clinical indication profile of UTs.

Acknowledgments

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Key points

- Urea transporter (UT) proteins facilitate the passive transport of urea driven by a concentration gradient across the cell plasma membrane in some kidney and extrarenal cells

- The kidney expresses UT-A1 and UT-A3 in inner medullary collecting duct epithelium, UT-A2 in thin descending limbs of the loop of Henle, and UT-B in descending vasa recta endothelium

- Kidney UTs are required for the generation of concentrated urine

- High-throughput screening has identified potent and selective small-molecule inhibitors of UT-A and UT-B that produce a salt-sparing diuresis in rodent models

- UT inhibitors may be useful alone or in combination with conventional diuretics for therapy of various oedemas and hyponatraemias

Review criteria

We searched PubMed (all years) using the search terms "urea transport", "renal concentrating mechanism" and "urea transporter knockout". All articles identified were full-text papers. We also read abstracts from the American Society of Nephrology and Experimental Biology meetings (2011–2014) to identify further relevant studies.
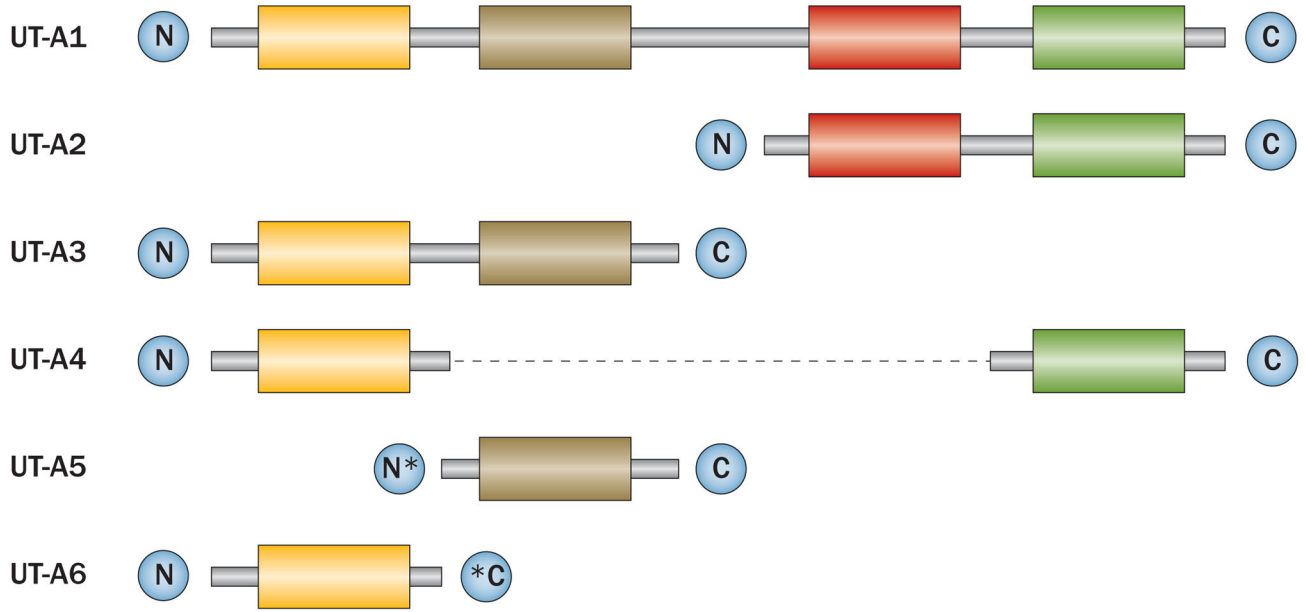
Figure 1.
Schematic representation of the primary structures of mammalian UT-A isoforms. UT-A1 comprises four hydrophobic regions. UT-A2, UT-A3 and UT-A4 each comprise two hydrophobic regions, which are identical to regions in UT-A1, as indicated by matching coloured boxes. UT-A5 and UT-A6 are identical to UT-A3 except for a unique N-terminus in UT-A5 and a unique C-terminus in UT-A6. Coloured boxes represent hydrophobic regions, grey lines represent regions with common DNA coding sequences, the dashed black line connects regions that are continuous with one another, asterisks represent regions that have unique coding sequences. Abbreviation: UT, urea transporter. Permission obtained from Wiley © Smith, C. P. Mammalian urea transporters. *Exp. Physiol*. **94**, 180–185 (2009).
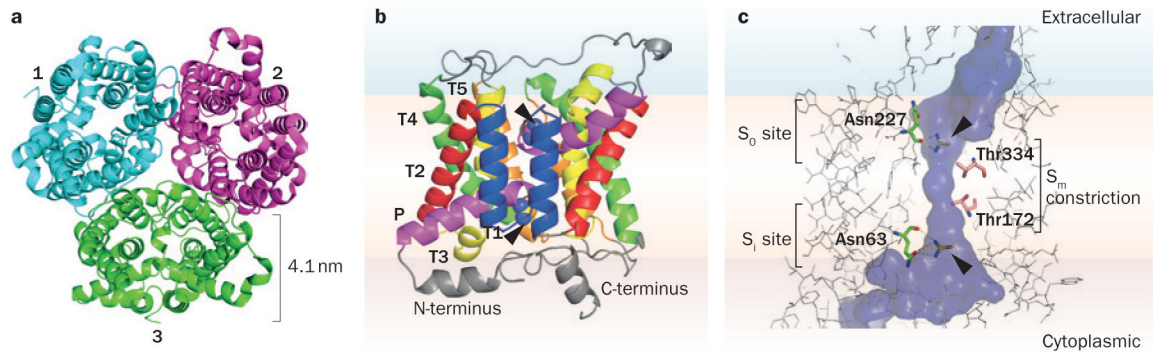
Figure 2.
Molecular structure of bovine UT-B. **a** | Top view of the extracellular face of the bovine UT-B homotrimer. Monomers are labelled 1–3. Based on the X-ray crystal structure data (Protein Data Bank ID 4EZD).[26] **b** | Structure of a bovine UT-B monomer viewed from its side, with the position of the membrane approximated, and with two co-crystallized selenourea molecules indicated in spherical form at the putative urea binding sites (arrowheads). **c** | Magnified view of a UT-B monomer and narrow channel surface. The co-crystallized selenourea molecules (arrowheads) are depicted interacting through hydrogen bonds with Asn227 at the S_0 site and Asn63 at the S_i site. Thr334 and Thr172 form the narrow S_m constriction at the centre of the channel. Abbreviations: P, tilted helix; T, transmembrane helix.
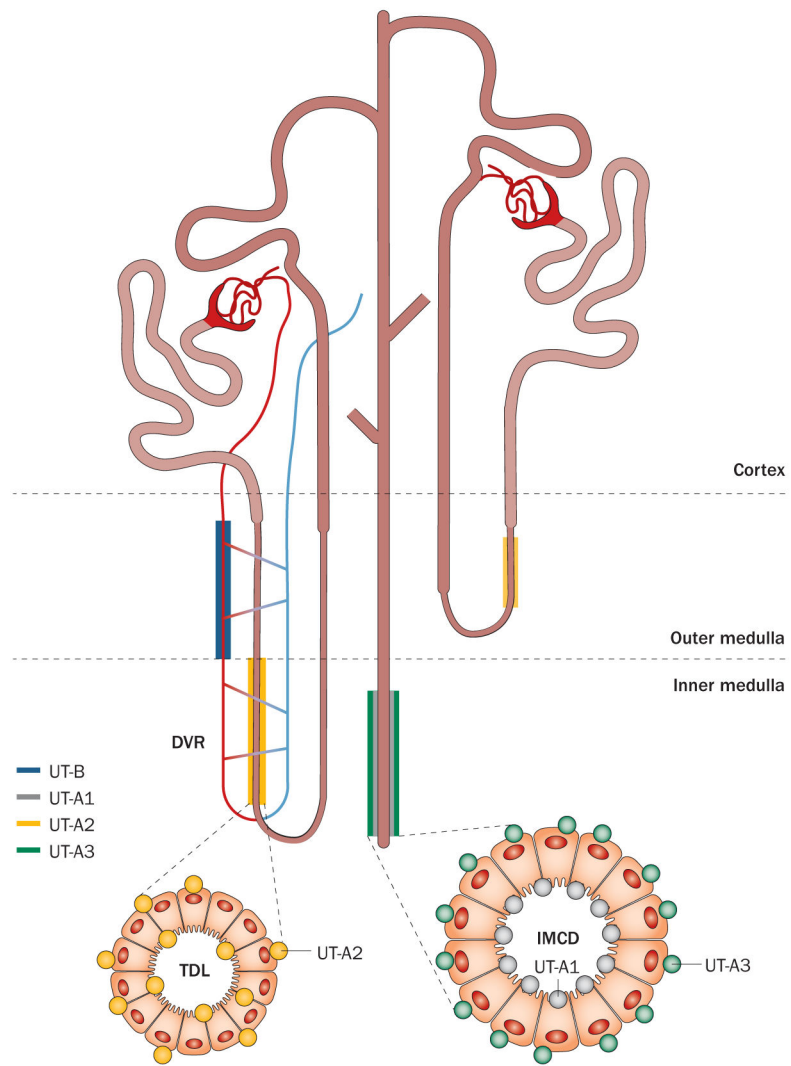
Figure 3.
Expression of the major mammalian UT isoforms in the kidney. Abbreviations: DVR, descending vasa recta; IMCD, inner medullary collecting duct; TDL, thin descending limb of Henle; UT, urea transporter.
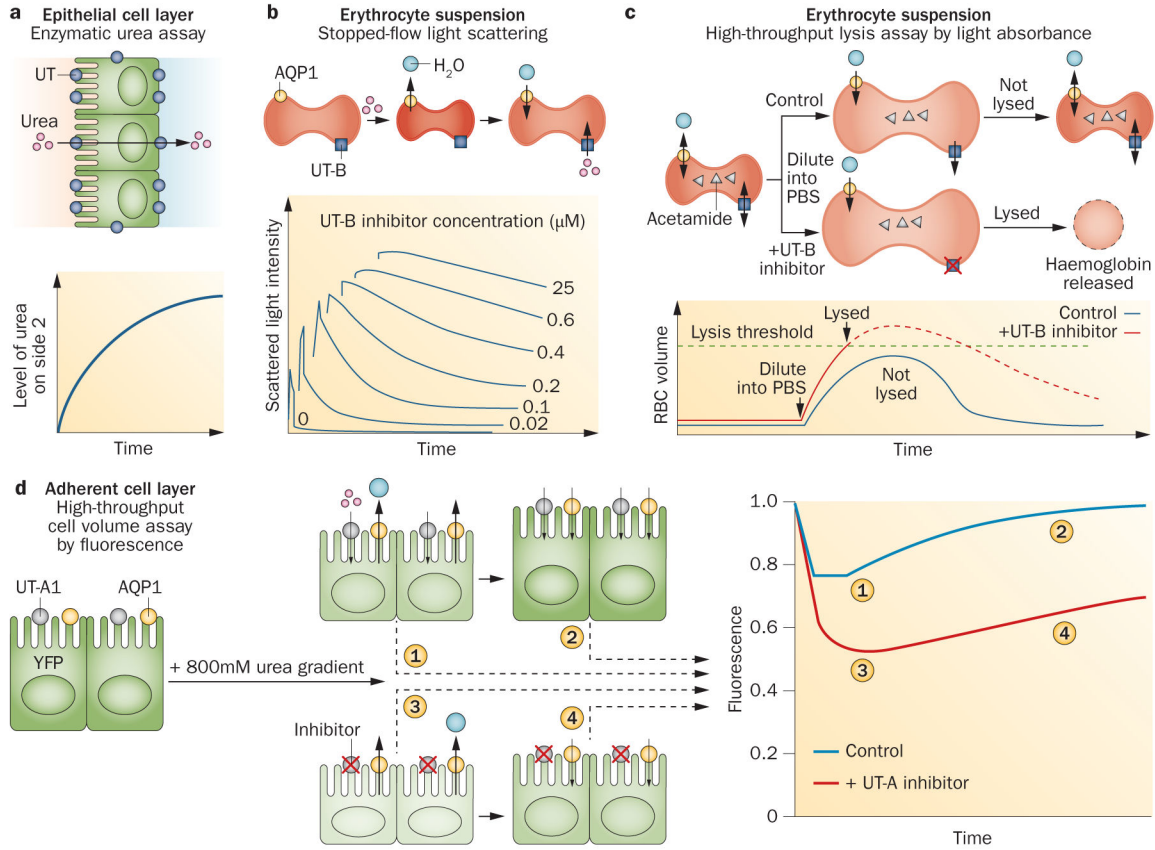
Figure 4.
Assays of UT-mediated urea transport. **a** | UT-mediated transport increases urea levels on side 2 over time. **b** | UT-B inhibition prevents cell reswelling after shrinkage. Scattered light intensity decreases as cells swell (bottom), and swelling kinetics are slowed with UT-B inhibition. **c** | UT-B inhibition impairs acetamide exit in an isosmolar external buffer, leading to lysis when water entry increases erythrocyte volume beyond a threshold (bottom). Dashed curve shows the hypothetical progression of erythrocyte volume without lysis. **d** | An inwardly directed urea gradient causes water efflux and cell shrinkage, detected as reduced YFP fluorescence. UT-A1 inhibition slows subsequent cell swelling, detected as an increase in YFP fluorescence. Abbreviations: AQP1, aquaporin-1; PBS, phosphate-buffered saline; RBC, red blood cell; UT, urea transporter; YFP, yellow fluorescent protein. Permission obtained for parts b and c from the Federation of American Societies for Experimental Biology © Levin, M. H. *et al. FASEB J.* **21**, 551–563 (2007). Part d reprinted from *Chemistry & Biology* **20**, Esteva-Font, C. *et al.*, A small molecule screen identifies selective inhibitors of urea transporter UT-A. 1235–1244, © 2013, with permission from Elsevier.
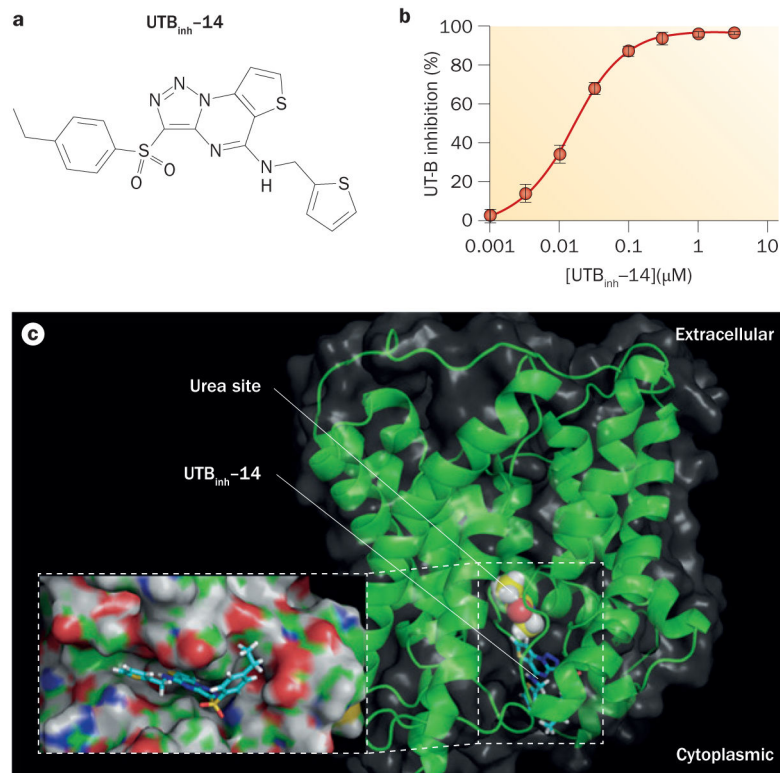
Figure 5.

UTB$_{inh}$-14 inhibition of UT-B. **a** | The chemical structure of UTB$_{inh}$-14. **b** | Concentration–inhibition data for UTB$_{inh}$-14 inhibition of human UT-B that shows an IC$_{50}$ of 10 nM when fitted to a single site inhibition model. **c** | Docking of UTB$_{inh}$-14 shown in a homology structural model of human UT-B, showing UTB$_{inh}$-14 binding at the cytoplasmic surface. The computed binding site of urea analogue dimethylurea (yellow) is shown. The inset shows a magnified view of UTB$_{inh}$-14 bound in a groove at the UT-B channel region. Abbreviations: inh, inhibitor; UT, urea transporter. Permission for part b obtained from the American Society of Nephrology © Yao, C. *et al. J. Am. Soc. Nephrol.* **23**, 1210–1220 (2012).
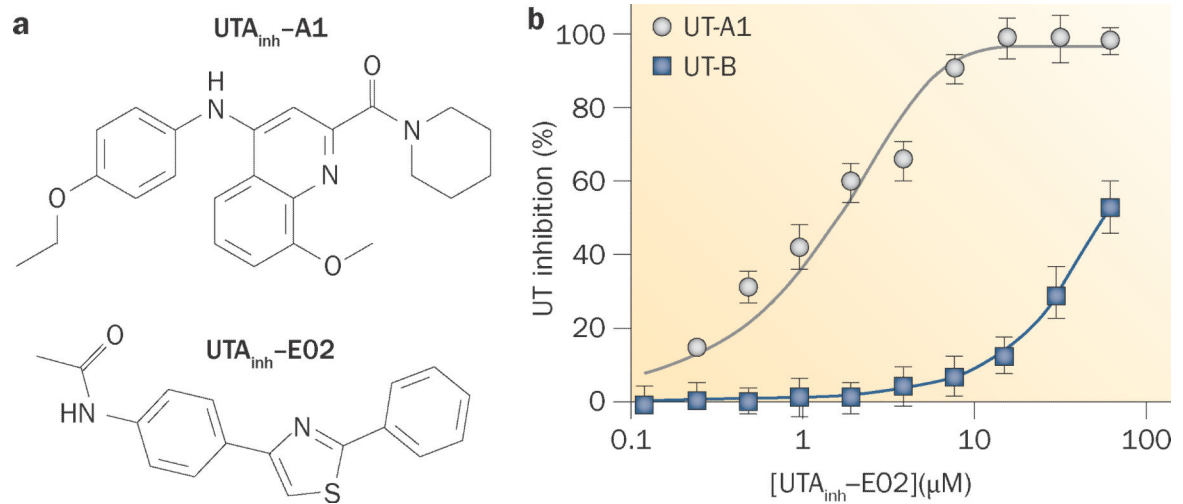
Figure 6.
Small-molecule inhibitors of UT-A. **a** | The chemical structure of UTA $_{inh}$-A1 and the chemical structure of UTA$_{inh}$-E02. **b** | Concentration–inhibition data for UTA$_{inh}$-A1 inhibition of UT-A1 and UT-B urea transport. UT-A1 inhibition exhibits an IC$_{50}$ of 1–2 μM, approximately 100-fold higher than the IC$_{50}$ it exhibits for UT-B inhibition. Abbreviations: inh, inhibitor; UT, urea transporter. Permission for part b obtained from the Federation of American Societies for Experimental Biology © Esteva-Font, C. *et al. FASEB J*. **28**, 3878–3890 (2014).
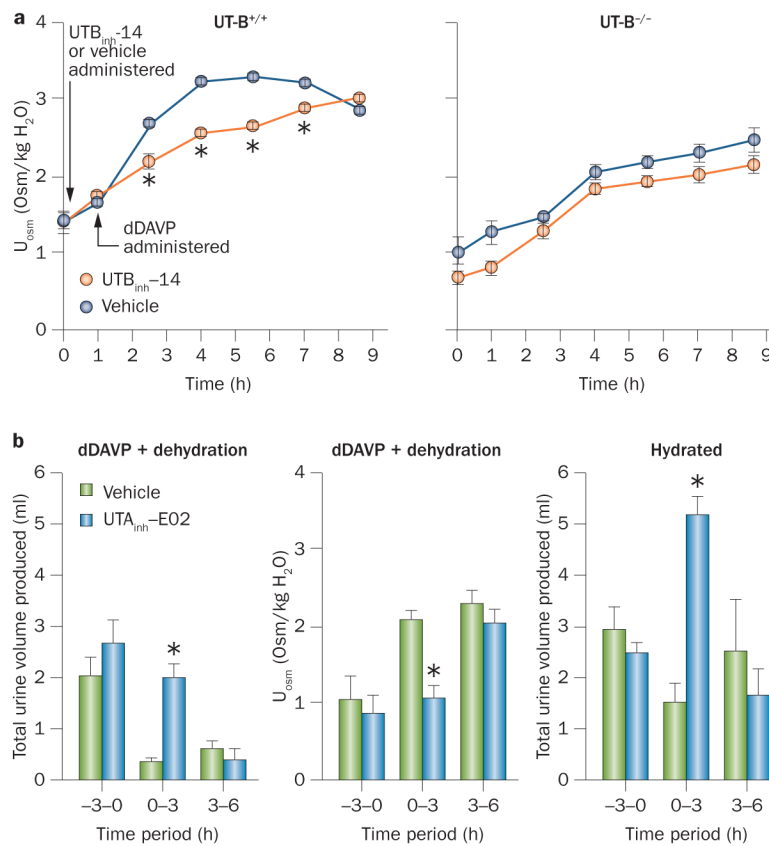
Figure 7.

UT inhibitor diuretic action in rodents. **a** | UTB$_{inh}$-14 (300 μg) mildly reduces urine osmolality in wild-type mice after dDAVP administration (left graph; mean ± S.E., 6 mice per group). dDAVP and UTB$_{inh}$-14 have no effect in UT-B knockout mice (right graph). **b** | dDAVP (intraperitoneal, 4 μg/kg every 3 h) and dehydration produces maximal urinary concentration. UTA$_{inh}$-E02 (5 mg) administered by intravenous injection at time 0 prevents the effects of dDAVP on urine volume (left graph) and osmolality (centre graph) (mean ± S.E., 4 rats per group). The same effect is seen in hydrated rats (right graph; mean ± S.E., 3 rats per group. * indicates P <0.01. Abbreviations: dDAVP, desmopressin; U$_{osm}$, urine osmolality; UT, urea transporter. Permission obtained for part a from the American Society of Nephrology © Yao, C. *et al. J. Am. Soc. Nephrol.* **23**, 1210–1220 (2012) and for part b from the Federation of American Societies for Experimental Biology © Esteva-Font, C. *et al. FASEB J.* **28**, 3878–3890 (2014).