Triazolothienopyrimidine Inhibitors of Urea Transporter UT-B Reduce Urine Concentration

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ABSTRACT

Urea transport (UT) proteins facilitate the concentration of urine by the kidney, suggesting that inhibition of these proteins could have therapeutic use as a diuretic strategy. We screened 100,000 compounds for UT-B inhibition using an optical assay based on the hypotonic lysis of acetamide-loaded mouse erythrocytes. We identified a class of triazolothienopyrimidine UT-B inhibitors; the most potent compound, UTBinh-14, fully and reversibly inhibited urea transport with IC50 values of 10 nM and 25 nM for human and mouse UT-B, respectively. UTBinh-14 competed with urea binding at an intracellular site on the UT-B protein. UTBinh-14 exhibited low toxicity and high selectivity for UT-B over UT-A isoforms. After intraperitoneal administration of UTBinh-14 in mice to achieve predicted therapeutic concentrations in the kidney, urine osmolality after administration of 1-deamino-8-D-arginine-vasopressin was approximately 700 mosm/kg H2O lower in UTBinh-14–treated mice than vehicle-treated mice. UTBinh-14 also increased urine output and reduced urine osmolality in mice given free access to water. UTBinh-14 did not reduce urine osmolality in UT-B knockout mice. In summary, these data provide proof of concept for the potential utility of UT inhibitors to reduce urinary concentration in high-vasopressin, fluid-retaining conditions. The diuretic mechanism of UT inhibitors may complement the action of conventional diuretics, which target sodium transport.


Urea is generated by the liver as the major end product of nitrogen metabolism, released into the blood, and excreted by the kidneys. The processing of urea by the kidney is complex, involving countercurrent multiplication and exchange mechanisms that greatly increase urea concentration in the renal medulla compared with plasma. In the maximally concentrating (antidiuretic) kidney, urea concentration in the urine can reach >1000 mM in mammals,1,2 much greater than the serum urea concentration of 4–10 mM. The renal countercurrent mechanisms involve intrarenal urea recycling facilitated by urea transporters (UTs) expressed in renal tubule epithelial cells (UT-A, encoded by the Slc14A2 gene) and renal vasa recta microvessels (UT-B, encoded by the Slc14A1 gene).3–7 Phenotype analysis of knockout mice lacking UT-B8,9 or various UT-A isoforms10–12 has provided evidence for the involvement of UTs in the urinary concentrating mechanism, subject to the caveat that gene knockout may produce off-target effects such as compensatory changes in the expression of non-UT transport proteins.13,14 Although UT function has been studied mainly in the kidney, UTs are also expressed in erythrocytes as well as the testis, brain, heart, and urinary bladder.15

Defective urinary concentrating function in UT knockout mice suggests the potential utility of UT inhibitors as diuretics that would impair urinary concentrating function by a mechanism different from that of salt-transport inhibitors such as...
furosemide, or aquaretics such as V2-receptor antagonists. Until recently, available UT inhibitors included the nonselective membrane intercalating agent phloretin and various urea analogs with IC50 of tens of millimolars.16 By high-throughput screening of 50,000 compounds, we previously identified phenylsulfoxylxooxazole inhibitors of human UT-B with an IC50 of <100 nM.17 However, the inhibitors identified against human UT-B were much less potent for mouse UT-B and had poor metabolic stability, precluding proof-of-concept studies of their action in rodent models.

We report the screening of a large collection of diverse, drug-like small molecules to identify potent inhibitors of mouse UT-B for proof-of-concept testing in mice in vivo. Screening of 100,000 compounds and optimization by testing of structural analogs yielded a novel triazolothienopyrimidine class of UT inhibitors with low nanomolar potency for inhibition of urea transport of both mouse and human UT-B. Testing in mice supported their predicted in vivo diuretic action.

RESULTS

UT-B Inhibitor Identification by High-Throughput Screening

We screened 100,000 chemically diverse small molecules to identify potent and selective inhibitors of UT-B that were suitable for efficacy studies in mice. Screening was done using mouse erythrocytes, which strongly express UT-B and are highly water permeable because they also express aquaporin-1 (AQP1) water channels. The screening method involved assay of erythrocyte lysis in response to a large, outwardly directed gradient of acetamide, a urea analog that is transported efficiently by UT-B. A large, outwardly directed gradient of acetamide causes transient cell swelling, but little cell lysis, because UT-B–facilitated acetamide efflux limits water influx (Figure 1A). UT-B inhibition prevents acetamide efflux, allowing unopposed cell swelling and consequent cell lysis, which was recorded by reduced near-infrared light absorption at 710 nm. Acetamide, rather than urea or other urea analogs, was selected because its efflux occurs over a time comparable with osmotic equilibration in mouse erythrocytes, which increases assay sensitivity. The acetamide loading concentration to best resolve UT-B inhibition was determined empirically as 1.25 M, giving a Z' factor for UT-B inhibitor screening of >0.6. Screening was done at a 25-μM concentration of test compounds based on initial studies showing a low percentage of active compounds.

Primary screening yielded active inhibitors of mouse UT-B (>75% inhibition at 25 μM) in three chemical classes, which included two novel classes (triazolothienopyrimidines and phthalazinamines) and benzenesulfanilides (Figure 1B). A related benzenesulfanilide (UTBinh-201) was identified in our prior screen of 50,000 compounds (unrelated to the 100,000 compounds screened here) for inhibitors of human UT-B.17 Screening of approximately 900 commercially available analogs of the three chemical classes revealed triazolothienopyrimidines with nanomolar potency for inhibition of UT-B. We focused on compound UTBinh-14 for further in vitro and in vivo characterization because of its low nanomolar potency for inhibition of mouse and human UT-B, and its high UT-B versus UT-A selectivity (see below). A focused study of triazolothienopyrimidine structure-activity relationships and metabolic stability will be reported separately.

UTBinh-14 was synthesized as a highly pure (>99%) by HPLC crystalline powder (Figure 1C). The synthesis involved reaction of 3-bromothiophene-2-carbaldehyde 1 with sodium azide in DMSO to generate an azidothiophene carbaldehyde,18 which was oxidized using the Lindgren reaction19 to generate 3-azidothiophene-2-carboxylic acid 2. Steglich esterification20 gave the azido ester intermediate 3. Arylsulfonylacetanitride intermediate 4 was generated by alkylation of 4-ethylenzethiol with bromoacetanitride followed by oxidation with mCPBA. Lactam 5 was then synthesized by base-mediated (2+3) cycloaddition of 3 and 4, and converted to UTBinh-14 using the benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate coupling reagent in the presence of thiophene-2-methylamine21 by microwave irradiation.

UT-B inhibition potency was assayed quantitatively by stopped-flow light scattering from the kinetics of urea influx in response to an inwardly directed urea gradient. Rapid mixing of an erythrocyte suspension with an equal volume of a hyperosmolar, urea-containing solution to give a 100-mM inwardly directed urea gradient produced rapid cell shrinking due to osmotic water efflux, followed by cell swelling as a consequence of urea (and water) influx. Figure 2A shows light scattering kinetics at different UTBinh-14 concentrations, which was present in both the erythrocyte- and urea-containing solutions. Increasing UTBinh-14 concentration greatly slowed the kinetics of urea efflux, UTBinh-14 at 1 μM produced near 100% inhibition, as evidenced by swelling kinetics that were comparable with those measured in erythrocytes from UT-B knockout mice (lower curves). UTBinh-14 did not inhibit urea transport in UT-B null erythrocytes, which is mediated by transport across the lipid bilayer by a solubility diffusion mechanism. Concentration-inhibition data gave IC50 values of 25.1 nM for mouse UT-B and 10.3 nM for human UT-B (Figure 2B).

UT-B Inhibition Mechanism and Specificity

Figure 3A shows reversible UT-B inhibition by UTBinh-14, as expected from the absence of reactive functional groups in the UTBinh-14 chemical structure. Incubation of mouse erythrocytes with 100 nM UTBinh-14—which reduced urea transport by approximately 75%—followed by washout, restored the original high urea permeability. To investigate the sidedness of UT-B inhibitor action, erythrocytes were exposed externally to UTBinh-14 at a final concentration of 100 nM just at the time of stopped-flow measurement by inclusion of 200 nM UTBinh-14 only in urea-containing solution. For comparison, measurements were made in which 100 nM UTBinh-14 was included in both
erythrocyte- and urea-containing solutions, and in which 100 nM UTBinh-14 was included only in the erythrocyte-containing solution. Figure 3B shows urea transport inhibition only when UTBinh-14 was present in the erythrocyte-containing solutions, providing evidence for an intracellular site of action.

UTBinh-14 permeation into erythrocytes was rapid, although not instantaneous, as shown in the right panel in Figure 3B.

To investigate whether UTBinh-14 competes with urea at a common binding site on the UT-B transporter, UTBinh-14 inhibition efficacy was measured as a function of urea gradient. Measurements were done at UTBinh-14 concentrations of 0 and 50 nM. Figure 3C shows urea concentration-dependent reduction in UTBinh-14 inhibition potency, suggesting a competitive mechanism for UTBinh-14 inhibition of UT-B urea transport. To study transport inhibition symmetry, urea efflux was induced by preloading erythrocytes with 100 mM urea, which were then mixed in the stopped-flow apparatus with urea-free PBS. Figure 3D shows slowed kinetics of urea efflux with increasing UTBinh-14 concentration, with an IC50 value of 26.7 nM, similar to that of 25.1 for UTBinh-14 inhibition urea influx in Figure 2B.

The putative site of UTBinh-14 binding to the UT-B protein was determined by docking computations after homology modeling of the human UT-B structure based on crystal structure data for a bacterial urea transporter homolog (Figure 4A).22 The predicted UTBinh-14 binding site was in a centrally located groove in the channel used for urea transport (Figure 4B). This location is consistent with the data above showing urea competition and an intracellular site of action of UTBinh-14. Figure 4C summarizes the key interactions predicted by the model. Most of the interactions involve hydrophobic and neutral-polar amino acids in the binding pocket, including Leu-121, Leu-364, Phe-71, Thr-368, Leu-116, Asn-165, Ser-169, and Ser-365.

UT-B selectivity and cytotoxicity were studied. Because of sequence similarities between UT-B and UT-A isoforms, we measured UT-A inhibition. Transepithelial urea transport was measured in MDCK cells stably expressing UT-A1, UT-A3, and UT-B. UT-A1 and UT-A3 are cAMP-regulated urea transporters expressed in kidney tubule epithelia.23–25 MDCK cells were cultured on collagen-coated porous filters for measurement of urea accumulation in the apical solution (basolateral to apical transport) after addition of urea to the basolateral solution. The top panel in Figure 5A show increased urea permeability in each of the transfected cell lines. Increased urea permeability by UT-A1
osmotic water permeability was measured by stopped-transporters often colocalize with urea transporters. Erythrocyte and TMEM16A) transport were also measured, because these panel in Figure 5A shows little inhibition of UT-A1 and UT-A3 facilitated urea transport was inhibited by phloretin. The bottom and UT-A3 required forskolin, whereas UT-B did not. UT-facilitated urea transport was inhibited by phloretin. The bottom panel in Figure 5A shows light scattering in erythrocytes from UT-B knockout mice. (B) erythrocyte- and urea-containing solutions. Bottom two curves show urea transport in erythrocytes from UT-B knockout mice. (B) Concentration-inhibition data showing percentage inhibition (mean ± SEM, n=3–5) with fitted IC50 values (single-site binding model) of 25.1 and 10.3 nM for mouse and human UT-B, respectively.

and UT-A3 required forskolin, whereas UT-B did not. UT-facilitated urea transport was inhibited by phloretin. The bottom panel in Figure 5A shows little inhibition of UT-A1 and UT-A3 facilitated urea transport by UTBinh-14 under conditions in which UT-B facilitated urea transport was strongly inhibited, indicating high specificity for UT-B. A possible explanation for this specificity is that UT-B encodes a Leu residue at position 116, whereas UT-A1 encodes an Ala at the corresponding position, 176, as do UT-A2 and UT-A3. As seen in the docking model (Figure 4C), Leu-116 presents a surface at the binding site of the central sulfone functional group in the inhibitor, which might account for the specificity of UTBinh-14 for UT-B over UT-A isoforms.

Effects of UTBinh-14 on water (AQP1) and chloride (CFTR and TMEM16A) transport were also measured, because these transporters often colocalize with urea transporters. Erythrocyte osmotic water permeability was measured by stopped-flow light scattering. We previously reported that approximately 90% of water transport in erythrocytes is mediated by AQP1, approximately 8% by UT-B, and the remainder through the lipid bilayer.26 The left panel in Figure 5B shows no significant inhibition of water transport in erythrocytes from wild-type mice, indicating that UTBinh-14 does not inhibit AQP1. However, UTBinh-14 inhibited water transport significantly in AQP1-null erythrocytes (Figure 5B, right), supporting our prior conclusion that erythrocyte water transport is mediated, in part, by UT-B.22,27 Figure 5C shows no significant inhibition of CFTR or TMEM16A chloride conductance by 10 μM UTBinh-14, as measured by short-circuit current. Last, by MTT assay, incubation of MDCK cells with UTBinh-14 up to 10 μM for 24 hours (where it remains soluble) showed no cytotoxicity (Figure 5D).

UTBinh-14 Reduces Urinary Concentration in Mice
To investigate the efficacy of UTBinh-14 in reducing maximum urinary concentration in mice in vivo, a formulation to deliver UTBinh-14 was established that gave predicted therapeutic UTBinh-14 concentration in mouse kidneys for several hours after a single administration. After testing several vehicles and administration routes, we found that intraperitoneal administration of UTBinh-14 in 5% DMSO, 2.5% Tween-80, and 2.5% PEG-400 gave the target UTBinh-14 concentration in kidneys and urine. A quantitative LC/MS assay was developed to measure UTBinh-14 concentration in kidney homogenates in which UTBinh-14 was extracted in an organic phase, concentrated, separated by reverse-phase HPLC, and detected by gated electrospray mass spectrometry. The left panel in Figure 6A shows ion current chromatograms and the deduced linear assay response, in which known amounts of UTBinh-14 were added to kidney homogenates. The right panel in Figure 6A shows the kinetics of UTBinh-14 in kidney after a single intraperitoneal injection of UTBinh-14 using the above-described formulation. Peak UTBinh-14 concentration was approximately 10 μM (normalized to kidney water volume) and remained >1 μM for at least 4 hours, well above the IC50 for UT-B inhibition in vitro. UTBinh-14 was similarly assayed in blood and urine after intravenous administration (Figure 6, B and C). UTBinh-14 concentration was >1 μM in blood and urine for several hours after a single intraperitoneal administration.

UT-B gene disruption in mice reduces maximum urinary concentrating ability.9 To study UTBinh-14 effects on maximum urinary concentration, mice were administered 300 μg of UTBinh-14 (or vehicle control) followed 1 hour later by the V2-receptor agonist 1-deamino-8-D-arginine-vasopressin (dDAVP). Figure 7A shows a stable, high urine osmolality of >3000 mosm/kg H2O over several hours after dDAVP administration. Figure 7B shows a similar, although slower, response in mice receiving vehicle before dDAVP, probably because of the osmotic load. Urine osmolality and urea were significantly reduced by inclusion of UTBinh-14 in the formulation. The inset in Figure 7B shows urine volume in 4-hour collections from a separate set of studies. UTBinh-14 did not significantly reduce urine osmolality or urea concentration in UT-B knockout mice (Figure 7C), with values similar to those in UTBinh-14-treated wild-type mice. Under baseline conditions, in mice given free access to food and water and in the absence of dDAVP, UTBinh-14 significantly increased urine volume and reduced urine osmolality and urea concentration (Figure 7D). Last, the effects of UTBinh-14 on
DAVP-stimulated urinary concentration were measured in mice placed for 1 week on low-, normal, and high-protein diets. Figure 7E shows that there was little effect of UTBinh-14 in mice on a low-protein diet, in which urine concentration was low, but there was a robust effect of UTBinh-14 in mice on a high-protein diet.

DISCUSSION

We identified by high-throughput screening a class of triazolothienopyrimidine compounds that inhibit urea transport of both mouse and human UT-B with low nanomolar potency. Our lysis-based screen relied on opposing water and urea transport processes in erythrocytes, which are highly urea and water permeable because of their expression of UT-B and AQP1, respectively. The most potent UT-B inhibitor, UTBinh-14, reversibly inhibited UT-B by competition with urea at a putative site at a tight cleft at the intracellular surface of the UT-B protein. UTBinh-14 was selective for inhibition of UT-B over the UT-A isoforms, and showed little toxicity. Intraperitoneal administration of UTBinh-14 in mice, under conditions that produced predicted therapeutic concentrations in kidney, reduced dDAVP-dependent urine osmolality to that found in UT-B knockout mice. The similar results of “chemical” versus genetic knockout of UT-B suggest that UT-B gene deletion in mice is not associated with compensatory effects that normalize maximum urinary concentrating ability. The inhibitor studies thus support conclusions from genetic knockout on the role of UT-B in urinary concentrating function in mice. UTBinh-14 should be useful, as well, in investigating the potential involvement of UT-B in extrarenal organs in which it is expressed, including the heart, testis, brain, and urinary bladder, where the potential involvement of UT-B in cardiac conduction, sperm maturation, and behavior has been reported.28–30

The triazolothienopyrimidine UTBinh-14 has drug-like properties, including the presence of multiple hydrogen bond acceptors, molecular weight of 455, and computed cLogP of 4.55 and polar surface area of 87 square Å.31 The closest literature precedent on the triazolothienopyrimidine scaffold was a report by Westerlund32 involving the use of cycloaddition chemistry to generate the core heterocycle. Recently, this general scaffold has been reported to show antagonist activity against 5-HT6 receptors in vitro,33 although no pharmacology or in vivo data were reported. In
As found in mice, humans lacking UT-B manifest a modest urinary concentrating defect, supporting the potential diuretic efficacy of UT-B inhibitors. However, based on mouse knockout data, a greater diuretic effect might be conferred by urea transport inhibitors targeting UT-A isoforms as well. Structure-activity analysis of triazolothienopyrimidine and the alternative UT-B inhibitors in Figure 1B may yield compounds with reduced UT-B selectivity. In this study, the high UT-B selectivity of UTBinh-14 allowed its study in mice without confounding effects on UT-A isoforms, allowing unambiguous interpretation of urinary concentrating data in UT-B knockout mice.

Notwithstanding the expression of UTs outside of the kidney and hence potential off-target actions, inhibitors of the various urea transporter isoforms may be useful in modulating fluid handling by the kidney. Because UT inhibitors have a fundamentally different mechanism of action from conventional diuretics (which target kidney tubule salt transporters such as the Na⁺/K⁺/2Cl⁻ cotransporter) or aquaretics (which block vasopressin V₂ receptors), urea transport inhibitors might act in synergy with existing agents. Diuretics are widely used to increase renal salt and water clearance in conditions associated with total body fluid overload such as congestive heart failure and cirrhosis, as well as normovolemic states such as hypertension and syndrome of inappropriate secretion of antidiuretic hormone. By disrupting countercurrent mechanisms and intrarenal urea recycling, inhibitors of UT, alone or in combination with loop diuretics, may induce a diuresis in states of refractory edema in which conventional diuretics are ineffective.

**CONCISE METHODS**

**Collection of Mouse and Human Blood**

Whole blood was collected from 8- to 12-week-old (25–35 g) wild-type or UT-B–null mice or AQP1-null mice in a CD1 genetic background by orbital puncture after subcutaneous injection of sodium heparin. Procedures were approved by the Committee on Animal Research at the University of California, San Francisco. Human venous blood was collected into heparinized tubes, stored at 4°C, and used within 48 hours.

**UT-B Inhibitor Identification by High-Throughput Screening**

Screening was carried out using a Beckman Coulter (Fullerton, CA) integrated system equipped with liquid handling robot (Biomek FX) and plate readers (FLUOstar Optima; BMG, Durham, NC), as previously described. Primary screening was done using a collection of 100,000 diverse, drug-like compounds from commercial sources (ChemDiv, San Diego, CA; Asinex, Winston-Salem, NC). For assay of UT-B inhibition, whole blood was diluted to a hematocrit of approximately 1% in PBS containing 1.25 M acetamide and 5 mM glucose. We added 100 μL of the erythrocyte suspension to each well of a 96-well round-bottom microplate, to which test compounds were added (1 μL, 25 μM compound concentration, 1% final concentration in PBS).
DMSO). After 10 minutes of incubation, 20 μL of the erythrocyte suspension was added rapidly to each well of a 96-well black-walled plate (Costar, Corning, NY) containing 180 μL of isosmolar buffer (PBS containing 1% DMSO). Vigorous mixing was achieved by repeated pipetting. Erythrocyte lysis was quantified by absorbance at a 710-nm wavelength, made within 5 minutes after hypo-osmolar shock. Each assay plate contained eight negative “no-lysis” controls (isotonic buffer; PBS plus 1.25 M acetamide with 1% DMSO) and eight positive “full-lysis” controls (distilled H2O). The percentage of erythrocyte lysis was calculated using control values from the same plate as follows: % lysis = 100% (A_{test} – A_{pos})/(A_{neg} – A_{pos}), where A_{neg} is the absorbance value from a test well.

For analysis of structure-activity relationships, 900 commercially available analogs (ChemDiv and Asinex) were tested.

**Figure 5.** UTB\textsubscript{inh-14} urea transport specificity and toxicity. (A) UT. The top panels show the kinetics of transepithelial urea transport across monolayers of MDCK cells expressing indicated urea transporters (mean ± SEM, n=3). Where indicated, forskolin (10 μM) and/or phloretin (100 μM) were present. Data are also shown for non-transfected MDCK cells in the left-most panel. The bottom panel shows inhibition of urea transport (measured at 60 minutes) by indicated concentrations of UTB\textsubscript{inh-14} (mean ± SEM, n=3). (B) AQP1 water transport. Osmotic water permeability measured in wild-type (left) and AQP1-null erythrocytes (right) in response to a 100-mM inwardly directed sucrose gradient at 10°C. (C) CFTR and calcium-activated chloride channels. Short-circuit current was measured in T84 cells. The left panel shows that CFTR was activated by 10 μM forskolin, with 1 and 10 μM UTB\textsubscript{inh-14} added as indicated (solid curve; dashed curve, no inhibitor added). CFTR chloride current was blocked by 20 μM CFTR\textsubscript{inh-172}. CFTR was inhibited by pretreatment with 20 μM CFTR\textsubscript{inh-172}. The right panel shows that TMEM16A chloride current was activated by 100 μM ATP. UTB\textsubscript{inh-14} was added as indicated. ATP-induced current was blocked by 100 μM tannic acid. (D) MTT cytotoxicity assay. MDCK cells were incubated with indicated concentrations of UTB\textsubscript{inh-14} for 24 hours (mean ± SEM, n=4). DMSO and phenol toxicity controls shown.

**Stopped-Flow Measurement of Erythrocyte Urea and Osmotic Water Permeabilities**

Erythrocyte urea and water permeabilities were measured by stopped-flow light scattering using a Hi-Tech SF-51 instrument (Wiltshire, UK). For measurement of urea permeability, dilutions of whole blood (mouse or human) in PBS (hematocrit approximately 0.5%) were incubated with test compounds for 5 minutes and then subjected to a 100-mM inwardly directed urea gradient. After an initial osmotic shrinking phase, the kinetics of increasing cell volume caused by urea influx was measured as the time course of 90° scattered light intensity at 530 nm, with increasing cell volume producing reduced scattered light intensity. Urea permeability and percentage inhibition were computed as previously described. In some experiments, different urea gradients (25–200 mM) were used or inhibitor was added only to the erythrocyte-containing or urea-containing solutions. In some experiments, an outwardly directed urea gradient was used in which erythrocytes were incubated for 30 minutes with PBS containing specified urea and mixed in the stopped-flow apparatus with PBS. In reversibility experiments, erythrocytes were incubated for 30 minutes with inhibitor, washed three times to remove the inhibitor, and UT was assayed. Osmotic water permeability was measured using a 100-mM gradient of sucrose.

**Synthesis Procedures**

3-Azidothiophene-2-Carbaldehyde (2)

We treated 3-bromothiophene-2-carbaldehyde (1) (2.00 g, 10.5 mmol) with sodium azide (1.99 g, 30.7 mmol) in DMSO (27 ml). The reaction was heated to 65°C for 48 hours in a sealed 40-ml vial. The reaction mixture was then taken up into H2O (35 ml) and extracted with diethyl ether (3 x 20 ml). The organic product layer was washed with 25 ml of saturated NaCl solution, dried over Na2SO4, and concentrated in vacuo to yield compound 2 (1.22 g, 76% yield).
3-Azidothiophene-2-Carboxylic Acid (3)

Compound 2 (1.22 g, 8.0 mmol) was dissolved in 30 ml of acetone: H2O (2:1) and treated with sulfamic acid (1.16 g, 12.0 mmol). Sodium chlorite (1.27 g, 17.1 mmol) was added and stirred at 0°C for 30 minutes. The mixture was alkalinized to pH 9 with sodium carbonate, and the product was washed with diethyl ether (10 ml). The aqueous layer was carefully treated with aqueous concentrated HCl to pH 1. The product was extracted with diethyl ether (3 × 10 ml), dried over Na2SO4, and concentrated in vacuo to yield compound 3 (1.01 g, 75% yield).

Isobutyl-3-Azidothiophene-2-Carboxylate (4)

Compound 3 (5.8 g, 34.3 mmol) was treated with 1-bromo-2-methylpropane (4.47 ml, 1.2 equivalent) and cesium carbonate (7.82 g, 0.7 equivalent) in anhydrous N,N-dimethylformamide (0.1 M) and heated to 80°C for 6 hours under argon. The mixture was cooled, dissolved in H2O (150 ml), and extracted with 1:1 diethyl ether/ethyl acetate (3 × 300 ml). The organic layer was washed with distilled H2O (5 × 100 ml), dried over Na2SO4, and concentrated in vacuo to yield compound 4 as an oil (6.77 g, 77% yield).

3-(4-Ethyl-Benzesulfonyl)-4H-Thieno[2,3-e][1,2,3]Triazolo[1,5-a]Pyrimidin-5-one (5)

Sodium ethoxide (1.54 g, 22.6 mmol, 10 equivalent) was added to a solution of compound 4 (0.71 g, 3.4 mmol, 1.5 equivalent) in anhydrous ethanol (22 ml). The reaction solution was stirred for 15 minutes followed by addition of azido ester (0.51 g, 2.3 mmol) and the reaction mixture was further stirred for 3 hours. Solid NaHCO3 (4.75 g, 56.5 mmol) was added and the reaction mixture was taken up in CHCl3 (100 ml), washed with aqueous HCl (1 M), concentrated in vacuo, and crystallized to yield compound 5 (568 mg, 70%) as a white solid.

3-(4-Ethyl-Benzesulfonyl)-Thieno[2,3-e][1,2,3]Triazolo[1,5-a]Pyrimidin-5-one [3-(4-Ethyl-Benzesulfonyl)-Thieno[2,3-e][1,2,3]Triazolo[1,5-a]Pyrimidin-5-one]-Thiophen-2-ylmethylamine (UTBinh-14)

Pyrimidin-5-one compound 5 (560 mg, 1.55 mmol) was treated with 2-thiophenemethylamine (0.48 ml, 4.66 mmol, 3 equivalent), DBU (0.70 ml, 4.66 mmol, 3 equivalent), and benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (2.43 g, 4.66 mmol, 3 equivalent) in anhydrous acetonitrile (5.1 ml), and placed in a microwave vessel. The mixture was heated in a microwave synthesizer at 100°C for 30 minutes. The reaction mixture was treated with aqueous HCl (1 M) and stirred for 30 minutes before Na2CO3 was added to adjust pH to approximately 9. The reaction mixture was dissolved in CHCl3 (75 ml) and washed with aqueous HCl (1 M, 3×75 ml). The organic product layer was concentrated in vacuo and purified by flash column chromatography as follows: dichloromethane +1% acetic acid → dichloromethane/methanol (100:1+1% acetic acid). The residue was re-crystallized from ethanol to yield UTBinh-14 (392 mg, 55% yield) as a white solid. 1H NMR (CDCl3, 500 MHz): δ 1.21 (t, 3H, J=10 Hz), 2.67 (q, 2H, J=10 Hz), 5.15 (d, 2H, J=10 Hz).
Urea Transport across MDCK Cell Monolayers
cDNAs encoding mouse UT-A3 and UT-B were cloned into pCDNA3.1 and used to generate stably transfected MDCK cell lines. MDCK cells stably expressing UT-A123 were used as described.17 cDNAs encoding mouse UT-A3 and UT-B were cloned into pcDNA3.1 and used to generate stably transfected MDCK cell lines. MDCK cells stably expressing rat UT-A123 were used as described.17 Cells were cultured in DMEM containing 10% FBS and 0.5 mg/ml G418. Cells were grown on 12-mm collagen-coated Transwell inserts (0.4 μm pore size; Costar) in 12-well plates and used after culture for 4 days (transepithelial resistance 500–600 Ω-cm²). Urea flux in the basolateral to apical direction across unstimulated and forskolin-stimulated cell layers was measured in response to a 15-mM urea gradient. Cultures were preincubated for 30 minutes at 37°C with DMSO vehicle or forskolin, with or without UT-B inhibitor, in both the apical-facing (0.2 ml) and basal-facing (1 ml) solutions. The basolateral solution was replaced with PBS containing 15 mM urea. Five-microliter aliquots of apical fluid were collected at specified times for assay of urea concentration (Quantichrome Urea Assay Kit; BioAssay Systems, Hayward, CA).

Liquid Chromatography/Mass Spectrometry
Kidneys from UTBinh-14–treated (or control) mice were rapidly removed after renal arterial perfusion with PBS. Kidneys were weighed, mixed with acetic acid (100 μl/g tissue), and ethyl acetate (10 ml/g tissue), and homogenized. The homogenate was centrifuged

Figure 7. UTBinh-14 reduces urinary concentration in mice. (A) Urine osmolality in wild-type mice (mean ± SEM, 6 mice per group, *P<0.01). Mice received dDAVP (1 μg/kg, intraperitoneal) where indicated. (B) Urine osmolality and urea concentration in wild-type mice after dDAVP (1 μg/kg) and UTBinh-14 (300 μg) (or vehicle) (mean ± SEM, 6 mice per group, *P<0.01). The inset shows urine volume in 4-hour urine collections without or with UTBinh-14 (mean ± SEM, 5 mice per group). (C) Same protocol as in B, but in UT-B knockout mice (mean ± SEM, 5 mice per group). (D) Urine collected from mice given free access to food and water (no dDAVP) without or after administration of UTBinh-14 (300 μg) (mean ± SEM, 5 mice per group, *P<0.05). Mice were placed on indicated low-, normal or high-protein diets for 1 week before 4-hour urine collections without or after administration of UTBinh-14 (300 μg) (mean ± SEM, 5 mice per group, *P<0.05).
at 3000 rpm for 15 minutes at room temperature. Calibration standards were prepared in kidney homogenates from control mice to which known amounts of UTBinh-14 were added. The ethyl acetate-containing supernatant was dried under nitrogen and the residue was reconstituted in acetonitrile/H₂O (3:1) containing 0.1% formic acid. UTBinh-14 recovery from kidney homogenates was approximately 85%. Reverse-phase HPLC was carried out using an Xterra MS C18 column (2.1×100 mm, 3.5 μm particle size; Waters, Milford, MA) connected to a solvent delivery system (model 2690; Waters). The solvent system consisted of a linear gradient from 5% to 95% acetonitrile containing 0.1% formic acid over 16 minutes (0.2 ml/min flow). UTBinh-14 was detected by absorbance at 262 nm. Mass spectra were acquired on a mass spectrometer (Alliance HT 2790 + ZQ; Waters) using positive ion detection. For analysis of blood and urine, fluids were diluted with an equal volume of water and extracted with ethyl acetate.

**UTBinh-14 Influx Kinetics in Erythrocytes**

UTBinh-14 (2 μM) was added to a 2% suspension of erythrocytes in PBS. At specified times, 600-μl aliquots were removed and layered over 350 μl of silicone oil (density 1.05 g/ml) overlying 100 μl of 0.55 M sucrose in PBS (density 1.075 g/ml). Samples were spun for 30 seconds at 12,000 rpm to rapidly separate cells from supernatant. UTBinh-14 was assayed in the cell pellet by liquid chromatography/mass spectrometry after ethyl acetate extraction as described above.

**Molecular Docking Computations**

A homology model of human UT-B was generated using the SWISS MODEL online utility (http://swissmodel.expasy.org) in automated mode, using the sequence of the full human UT-B1 protein (accession code, CAB60834). Residues 43–378 were modeled, based on a urea transporter from Desulfofribrio vulgaris (PDB code 3M6E). UTBinh-14 was drawn in ChemDraw (Cambridge Software, Cambridge, MA), converted to a SMILES strings, transformed to a three-dimensional conformation, and minimized using PIPELINE PILOT (Accelrys, San Diego, CA). The single conformation was passed through MOLCHARGE (OpenEye, Santa Fe, NM) to apply AM1BCC charges, and through OMEGA (OpenEye) to generate a multiconformational library for UTBinh-14. The UT-B protein was prepared for docking using the FRED-RECEPTOR (OpenEye) utility, using the homology model of human UT-B. The receptor site was generated using a 12 cubic Å box. Docking was performed using FRED (v2.2.5) (OpenEye), which was configured to use consensus scoring functions ChemGauss3, OECChemScore, ScreenScore, PLP, and ZapBind. Docking of the inhibitor was carried out free of pharmacophore restraint. Each final pose was minimized by FRED by molecular dynamics using the MMFF94 force field within the active site. The final protein-ligand complex was visualized using PYMOL (Schrödinger, San Diego, CA).

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**DISCLOSURES**

None.

**REFERENCES**