Thienoquinolins exert diuresis by strongly inhibiting UT-A urea transporters

Huiwen Ren, Yanhua Wang, Yongning Xing, Jianhua Ran, Ming Liu, Tianluo Lei, Hong Zhou, Runtao Li, Jeff M. Sands, and Baoxue Yang

1State Key Laboratory of Natural and Biomimetic Drugs, Department of Pharmacology, School of Basic Medical Sciences, Peking University, Beijing, China; 2Renal Division, Departments of Medicine and Physiology, Emory University School of Medicine, Atlanta, Georgia; 3Department of Anatomy, Neuroscience Research Center, Basic Medical College, Chongqing Medical University, Chongqing, China; and 4Key Laboratory of Molecular Cardiovascular Sciences, Ministry of Education, Beijing, China

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DIURETICS ARE USED CLINICALLY to treat a variety of diseases, including edema, hypertension, and heart failure (18, 34), and are often used for long-term therapy. However, long-term use of conventional diuretics, such as furosemide, thiazide, and spironolactone, can have several adverse effects, including hypokalemia (36), hypernatremia (20), hyperuricemia (8), hyperlipidemia (1), and a decrease in glucose tolerance (44). These adverse effects result from the mechanism by which they induce a diuresis since water excretion follows salt excretion. Therefore, it would be desirable to discover novel diuretic targets and diuretics that do not cause electrolyte disturbances.

Several urea transporter (UT) proteins have been cloned (23). UT-B is expressed in erythrocytes and descending vasa recta. UT-A1 and UT-A3 are expressed in the inner medullary collecting duct (IMCD). Phenotype analysis of knockout mice lacking UT-B (51) or UT-A1 and UT-A3 (10) has provided evidence for the involvement of UTs in the urinary concentrating mechanism. Functional deletion of UT-B or UT-A isoforms caused significant polyuria and a urea-selective reduction in urine concentrating ability (50). However, deletion of UT-B or UT-As did not affect GFR or the clearance rate of the principal solutes (Na+, K+, Cl−) in urine, except for urea (10–12, 51). Therefore, these findings suggest that UT inhibitors might be useful as novel diuretics to cause the excretion of water without disturbing electrolyte balance and metabolism (9, 25, 38, 47, 50).

Our previous study discovered a class of potent small-molecule UT inhibitors, thienoquinolins, using an erythrocyte osmotic lysis assay (30). Structure and activity relationship analysis showed a thienoquinolin analog 1-(3-amino-6-methylthieno[2,3-b]quinolin-2-yl)ethanone, named PU-14, had inhibition activity on human, rabbit, rat, and mouse UT-B with an IC50 in the micromolar range. In vivo testing showed that PU-14 caused a dose-dependent diuresis in rats. PU-14-treated rats had normal levels of blood Na+, K+, Cl−, and lipids. All results suggest that PU-14 caused a urea-selective diuresis without significant adverse effects and that thienoquinolin might be developed into a novel diuretic.

PU-14 decreased urine osmolality by ∼60% in rats (30). A UT-B specific inhibitor, UTBinh-14, developed by Verkman and colleagues (53), decreased urine osmolality by only ∼25% in mice. UT-B null mice had a 25.5% decrease in urine osmolality and an increase in blood urea levels (51). PU-14 significantly decreased urine urea but did not increase blood urea, which is similar to the phenotype of UT-A knockout mice. All these data suggest that thienoquinolins exert their diuretic effect by inhibiting more than UT-B alone. The IMCD urea transporters UT-A1 and UT-A3 are attractive diuretic drug targets since they are located in the last portion of the nephron. Thus they should have maximal effects on renal urea reabsorption and minimal downstream effects on electrolyte excretion (41, 42), in contrast to conventional diuretics that act in more proximal portions of the nephron (45).

In this study, we used structure and activity relationship analysis of thienoquinolin analogs to optimize the compound. We identified a novel UT inhibitor, PU-48, that inhibits UT-A activity more than UT-B. Rat IMCD perfusion experiments showed that PU-48 significantly inhibited vasopressin-stimulated urea transport. PU-48 significantly increased urine output and decreased urine osmolality in an in vivo rat model without affecting electrolyte metabolism. These findings indicate that

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the diuretic activity of thienoquinolin is mainly based on inhibiting the IMCD UT-A urea transporters.

METHODS

Animals. CD-ICR mice (18–22 g body wt, equal numbers of males and females) and adult male Sprague-Dawley (SD) rats (body weight: 200–220 g) were supplied by the Experimental Animal Center, Peking University. UT-B knockout mice in a C57BL/6 background were generated by targeted gene disruption as reported previously (51).

For tubule perfusion, male SD rats (Charles River Laboratories, Wilmington, MA), weighing 50–75 g, were injected with furosemide (5 mg/kg body wt) 30 min before death. Kidneys were dissected to remove the inner medulla, and single terminal IMCDs were micro-dissected at 17°C. All animal protocols were approved by the Ethics Committee of Peking University or the Emory University Institutional Animal Care and Use Committee.

Blood samples. Human venous blood was taken from healthy adult volunteers into heparinized tubes and kept at 4°C. Rat blood was collected from 8-wk-old (~220 g) male SD rats by orbital venous plexus puncture using the tip of a blood collection tube after peritoneal injection with sodium heparin (0.5 mg/kg). Mouse blood was collected from 10-wk-old (~25 g) male wild-type or UT-B-null mice on a C57BL/6 genetic background by orbital puncture following peritoneal injection with sodium heparin (0.5 mg/kg). All blood samples were used within 48 h of collection.

Compounds. Compounds were purchased from a commercial source (Asinex, Moscow, Russia) and were dissolved in DMSO. PU-48, used in the in vivo experiments, was home synthesized with purity at >99% as determined by HPLC.

Erythrocyte lysis assay for determining UT-B inhibition activity. The erythrocyte lysis assay was modified from a method described previously (29). Erythrocytes were diluted to a hematocrit value of 2% in hyperosmolar PBS containing 1.25 M urea and 5 mM glucose. Erythrocyte suspensions were preserved at room temperature for 2 h by periodic pipette mixture. One-hundred microliters of the erythrocyte suspension were added to a 96-well round-bottom microplate, and then 1 μl of test compound was added and mixed. After a 6-min incubation, 20 μl of the compound-treated erythrocyte suspension was rapidly added to a 96-well black wall microplate that contained 180 μl PBS. Following vigorous mixing, erythrocyte lysis was quantified by absorbance at 710 nm. The percentage of erythrocyte lysis in each test well was calculated using control values from the same plate as: %lysis = 100% (A_test − A_time0)/(A_well − A_time0), where A_well is the absorbance value from a test well.

Assay of UT-facilitated urea flux. UT-A1-expressing type I Madin-Darby canine kidney (MDCK) cells or control type I MDCK cells (ATCC, No. CCL-34) were grown on 12-mm-diameter collagen-coated Transwell inserts (0.4-μm pore size; Costar; Corning, Corning, NY) to form tight monolayers (resistance >1 kΩ/cm²), as described previously (30). After a washing with PBS, PBS containing forskolin (10 μM), with or without PU-14 or PU-48, was added to both the apical-facing (0.2 ml) and basal-facing (1.2 ml) surfaces. After a 30-min incubation, the basal-facing solution was replaced by PBS containing 15 mM urea with or without PU-14 or PU-48. Apical fluid samples (5 μl) were collected at specified times for enzymatic assay of urea (QuantiChrom Urea Assay Kit; BioAssay Systems, Hayward, CA). Inhibition refers to the percentage of inhibition of forskolin-stimulated transport (after control transport has been subtracted from the forskolin-stimulated component) at 60 min. The assay of UT-B-facilitated urea flux was performed in UT-B-expressing type I MDCK cells with the same procedure as the assay of UT-A1-facilitated urea flux but without forskolin stimulation.

Measurement of UT-A-mediated urea transport in collecting duct. Single rat terminal IMCDs were dissected, mounted on glass pipettes, and perfused as described previously (39, 49). The tubule was warmed to 37°C in 1 ml of bath solution for 45 min. Next, three 1-min collections of 30–50 nl/min were made to measure basal (control) urea permeability. Next, 10 μM of PU-48 were added to the bath (basolateral) solution for 30 min and urea permeability was measured again to determine whether PU-48 can inhibit basal urea permeability. To test whether PU-48 can inhibit vasopressin-stimulated urea permeability, basal urea permeability was measured, and then 20 pM vasopressin was added to the bath, and then with vasopressin still present, there was sequential addition of PU-48 at different concentrations (1, 5, and 10 μM). Urea permeability was measured in each condition, with 20–30 min separating the collection periods. All five periods were tested in each individual IMCD. Urea was assessed using an ultramicromfluorometer as described previously (39, 49). Urea permeability was calculated as described previously (39, 49).

Measurement of diuretic activity. Male UT-B null mice, wild-type mice, or SD rats were adapted in metabolic cages (Harvard Apparatus, Holliston, MA) for 3 days. Water and food were provided ad libitum during the whole study. Bladder was emptied by gentle abdominal massage. Then, urine was collected every 2 h. PU-48 in corn oil was administered by subcutaneous injection. Corn oil was used as a vehicle control. Urine volume was measured by gravimetry, assuming a density of 1 g/ml. Urinary osmolality was measured by freezing point depression (Micro-osmometer; Fisker Associates, Norwood, MA). Urea concentration was measured with the QuantiChrom urea assay kit as above.

For studying long-term diuretic activity, 50 mg/kg PU-48 (the first dose was double) in corn oil were administered to rats by subcutaneous injection every 8 h. Corn oil was used as a vehicle control and 5 mg/kg hydrochlorothiazide (HCTZ; the first dose was double) in corn oil as a positive control, respectively. Urine was collected and water intake was recorded every 24 h. At 2.5 h after the last administration, a blood sample was collected by heart puncture (while rats were under anesthesia with pentobarbital at 40 mg/kg body wt). The animals were euthanized after collection of blood by cardiac puncture while they were under anesthesia. Inner medulla tissue homogenates were obtained by homogenizing ~20 mg of papillary tissue in a 10-fold excess of distilled water, and the supernatant after centrifugation was assayed for solute concentration and osmolality (28, 40, 51). Serum and urinary urea and urea concentration were measured as Na⁺, K⁺, Cl⁻, cholesterol, triglyceride, high-density lipoprotein, and low-density lipoprotein were measured in a clinical chemistry laboratory. Serum aldosterone was measured by enzyme-linked immunosorbent assay kit (CEA911Ge; Cloud-Clone).

Immunoblot analysis. Kidney medullary tissues were homogenized in RIPA lysis buffer containing a protease inhibitor cocktail (Roche Diagnostics). Total protein was separated by sodium dodecyl sulfate-polyacrylamide gelelectrophoresis. Proteins were blotted to polyvinylidene difluoride membranes (Amersham Biosciences, Arlington Heights, IL). Blots were incubated with primary antibodies against UT-B (51) (a kind gift from Dr. Trinh-Trang-Tan, INSERM, Paris, France); UT-A2 (54) and UT-A3 (6) (generated by Abgent, San Diego, CA); aquaporin (AQP)1 (32), AQP3 (31), AQP4 (32), Na⁺-K⁺-Cl⁻ cotransporter (NKCC2) (Santa Cruz Biotechnology, Santa Cruz, CA); AQP1 (32) (a kind gift from Dr. Dennis Brown, Harvard Medical School, Boston, MA); or β-actin (Santa Cruz Biotechnology). Goat anti-rabbit IgG horseradish peroxidase or goat anti-mouse IgG horseradish peroxidase (Zhang-shan, Beijing, China) was added, respectively, and the blots were developed with an ECL plus kit (Amersham Biosciences). Relative protein expression levels were quantified by optical density analysis and normalized to β-actin.

Histology. Kidneys and liver were fixed with formalin and embedded in paraffin, and 5-μm paraffin sections were cut and stained with hematoxylin and eosin.

Cytotoxicity assay. Type I MDCK cells were cultured at 37°C in a humidified 95% air-5% CO₂ atmosphere in Dulbecco’s modified Eagle’s medium (GIBCO) supplemented with 10% fetal bovine serum (Hyclone, South Logan, UT), 100 U/ml penicillin, and 100 mg/ml
streptomycin. Cytotoxicity was assayed using a cell counting kit-8 (CCK-8 kit; Dojindo, Kumamoto, Japan) as described previously (30).

**Acute toxicity test.** An acute toxicity test was performed with 40 CD-ICR mice at 4 wk old, male and female in equal numbers. The animals were housed with the same gender and maintained on a commercial pellet diet, given deionized water ad libitum, and kept in plastic cages in a 20 ± 2°C, 50–70% relative humidity room with a 12-h light-dark cycle. After 1 wk for acclimation, the mice were randomly divided into two groups: the PU-48 group and the control group. Each group had 10 male and 10 female mice. The mice were fasted overnight before treatment. Mice were gavage administered with a PU-48 suspension in corn oil at a dose of 14 g/kg body wt. The control group was given 1% sodium carboxy methyl cellulose solution instead of PU-48. After administration, the mice were observed daily for a total of 14 days (48). The mice were observed for any changes in skin and fur, eye membranes, respiratory, circulatory, autonomic and central nervous systems, and behavior pattern. Special attention was given to tremors, convulsions, salivation, nausea, vomiting, diarrhea, lethargy, sleep, and coma. The body weight of the mice was recorded before and every day after the administration. Two weeks later, the mice were killed after being anesthetized by ether. Liver and kidneys were excised and weighed accurately.

**Electrophysiology.** Cardiac hERG (Kv11.1), NaV1.5 (sodium channel), and CaV1.2 (calcium channel, voltage-dependent, L type, alpha 1C subunit) channels were recorded using whole cell patch-clamp technology. Briefly, HEK293 cells stably expressing hERG, NaV1.5, or CaV1.2 channels were cultured on the coverslips. The electrophysiological recordings were obtained under visual control using a microscope. The amplifier EPC10 was used for recording of the electrophysiological signal. Offset potentials were nulled directly before formation of a seal. No leak subtraction was made. Cell capacitance (in pF) was made from whole cell capacitance compensation. The data were stored and analyzed with Patchmaster and Igor Pro. All experiments were performed at room temperature.

**Statistical analyses.** Data were analyzed using a paired Student’s t-test or repeated measures ANOVA, followed by Fisher’s least significant difference analysis for multiple comparisons. *P* < 0.05 was considered statistically significant.

**RESULTS**

Identification of more potent thienoquinolin UT inhibitors. Our previous study found that with thienoquinolin as the scaffold, substitution of Me or MeO as R1 and Me as R4 was favorable for the improvement of UT-B inhibition activity. To further explore the structure-activity relationship of this novel kind of compound and discover more potent thienoquinolin UT inhibitors, 34 commercially available thienoquinolin analogs were selected and evaluated for their UT-B and UT-A inhibition activity, as assessed by assay of transmembrane urea transport. A modified erythrocyte lysis assay was used for measuring the inhibition of UT-B-mediated transmembrane urea transport. Urea-loaded erythrocytes showed similar UT-B transport kinetics as acetamide-loaded erythrocytes. The optimal urea loading concentration was determined to evaluate UT-B transport kinetics. Figure 1A shows effect of urea concentration on erythrocyte osmotic lysis. We used 1.25 M urea for determining inhibition activity on UT-B-mediated urea transport. The structure-activity relationships are summarized in Fig. 2. Based on the previous structure-activity relationships, all of the selected compounds contained an Me, MeO, or EtO group as R1, and various groups at R4, such as OH, NHR, and OR. As shown in Fig. 2, all the compounds with MeO as the R4 group (PU-31; 48, 55) exhibited excellent activity, which was over 10 times more potent than PU-14, especially PU-48. However, the compounds with other types of R4 groups gave an IC50 of >10 μM. These results indicate that MeO as the R4 group in the compound is necessary for UT inhibition activity, which inspired us to test whether instead of MeO with RO as the R4 might lead to more potent compounds.

![Figure 1](http://ajprenal.physiology.org/)

**Fig. 1.** Inhibition activity of PU-48 on human, rat, and mouse urea transporter UT-B. **A:** erythrocyte osmotic lysis assay for UT-B inhibition. Rat erythrocytes were preloaded with urea at concentrations from 0 to 3 M in the absence (○) or presence (●) of 20 μM PU-48. After replacement of the external buffer with a urea-free isosmolar solution, erythrocyte lysis was quantified from a single time point measurement of absorbance at 710 nm wavelength. **B:** chemical structure of PU-48, methyl(3-amino-6-methoxythieno[2,3-b] quinoline-2-yl) carbamate. **C:** dose-dependent inhibition activity of PU-48 on human and rat UT-B determined by the osmotic lysis assay in erythrocytes. **D:** dose-dependent inhibition activity of PU-48 on urea permeability in erythrocytes from wild-type (+/+), UT-B null (−/−), and wild-type (+/+; UT-B) mice. Data are means ± SE, *n* = 3.
PU-48 has much stronger inhibition activity on UT-A than UT-B.

Inhibition activity of PU-48 on UT-A-mediated urea transport in IMCD. Figure 4A shows a perfused rat terminal IMCD mounted on pipettes. PU-48 (10 μM) significantly inhibited basal urea transport by 32.6% in perfused rat terminal IMCDs (n = 6; P < 0.01; Fig. 4B). When PU-48 was washed out of the bath for 30 min, urea permeability was returned to nearly basal levels (n = 3; P < 0.01 vs. PU-48; P = NS vs. basal), indicating that the inhibitory effect of PU-48 is reversible. In two tubules, the washout phase was continued up to 80 min and there was no change in urea permeability during this prolonged washout/time control phase (data not shown), indicating intactness of tubule function over this time period. Vasopressin (20 pM) significantly increased urea permeability from 22.0 ± 3.4 cm/s (n = 6; P < 0.01; Fig. 4A). With vasopressin still present, adding 1 μM PU-48 to the bath decreased urea permeability to 21.9 ± 2.0 × 10^{-5} cm/s (n = 6; P < 0.01). There was no further change when PU-48 was washed out of the bath (for 2 h before administration of PU-48, urine output was higher than control). As indicated, PU-48 (4 μM; ○) or PU-14 (4 μM; ●) was present. B: dose-dependent inhibition activity of PU-48 on UT-A (●) or UT-B (○) in MDCK cells stably expressing rat UT-A or UT-B. C: IC_{50} of PU-48 calculated from experiments in B. Data are means ± SE; n = 3.

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> ">" indicates the IC_{50} was greater than 10 μM
wild-type mice (Fig. 5, A and B). Urine output significantly increased, and urinary osmolality (Fig. 5A), urea concentration, and nonurea solute concentration (Fig. 5, B–D) significantly decreased after subcutaneous administration of PU-48 at 100 mg/kg in both UT-B null mice and wild-type mice. PU-48 significantly increased urea excretion in both wild-type mice and UT-B-null mice (Fig. 5E). Notably, the excretion of nonurea solutes did not change (Fig. 5F) in both wild-type and UT-B null mice, indicating no significant loss of Na⁺, K⁺, or Cl⁻. PU-48 caused a urea selective diuresis without disturbing electrolyte metabolism by acting on UT-A.

**Diuretic activity of PU-48.** The diuretic activity of PU-48 in rats was assessed using metabolic cages. Urine output significantly increased in a dose-dependent manner after subcutaneous administration of PU-48 at 12.5 and 50 mg/kg, compared with vehicle controls (Fig. 6, left; P < 0.05). Urinary osmolality (Fig. 6, middle; P < 0.05) and urea concentration (Fig. 6, right; P < 0.05) were significantly reduced by PU-48. PU-48 at 3.125 mg/kg did not significantly affect urine output, urinary osmolality, or urinary urea concentration. The peak changes of urine output, urinary osmolality, and urinary urea concentration occurred at 2 h after PU-48 administration. Levels of urine output, urinary osmolality, and urinary urea concentration returned to the basal level at 10 h after PU-48 administration.

To investigate the long-term effect of PU-48 on urea handling and urinary concentrating ability, rats were treated with PU-48 at 50 mg/kg every 8 h for 6 days. PU-48 caused continuous diuresis (Fig. 6, left) and low urinary osmolality and urea concentration (Fig. 6, middle and right). The diuretic efficacy of PU-48 was similar to that of a conventional diuretic, HCTZ, which was used as a positive control. However, urinary urea concentrations were higher in the PU-48-treated group than in the HCTZ-treated group. There was no significant difference in blood Na⁺, K⁺, Cl⁻, or urea (Table 1) after the 6-day treatment with PU-48, compared with the vehicle control group. Blood glucose, cholesterol, and triglyceride were not changed after the 6-day treatment with PU-48 either. These data indicate that the diuretic effect of PU-48 does not cause electrolyte imbalance or abnormal metabolism.

Figure 7 shows the composition of the aqueous component of the inner medulla as measured using the supernatants of centrifuged homogenates of inner medullas. Total osmolalities

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**Fig. 4.** Inhibition activity of PU-48 on urea transport in rat terminal inner medullary collecting ducts (IMCDs). A: photomicrograph of a perfused rat terminal IMCD mounted on pipettes. B: PU-48 (10 μM) inhibits urea permeability in perfused rat terminal IMCDs. PU-48 (10 μM) significantly inhibited basal urea transport by 32.6% (n = 6; P < 0.01). When PU-48 was washed out of the bath for 30 min, urea permeability was returned to nearly basal levels (n = 3; P > 0.05 vs. PU-48; P = NS vs. basal), indicating that the inhibitory effect of PU-48 is reversible. Number in parentheses indicates number of tubules. C: PU-48 (1, 5, and 10 μM) inhibits vasopressin-stimulated urea permeability in perfused rat terminal IMCDs (n = 6; P < 0.01). Vasopressin (20 μM) significantly increased urea permeability (n = 6; P < 0.01). With vasopressin still present, adding 1 μM PU-48 to the bath significantly decreased urea permeability (n = 6; P > 0.05). There was no further change when PU-48 was increased to 5 or 10 μM. Data are means ± SE. *P < 0.01 by ANOVA.

**Fig. 5.** Effect of PU-48 on urinary concentrating activity and renal handling of solutes in wild-type and UT-B null mice. Wild-type and UT-B null mice were observed in metabolic cages and subcutaneously injected with or without 100 mg/kg of PU-48 just after a 2-h urine collection (time 0). Urine samples were collected every 2 h. A: urine output. B: urinary osmolality (Uosm). C: urine urea concentration (Uurea). D: urine nonurea concentration (Unon-urea). E: urine excretion. F: excretion of nonurea solutes. ○: Wild-type mice without PU-48; □: wild-type mice with PU-48; ●: UT-B null mice without PU-48; ■: UT-B null mice with PU-48. Data are means ± SE; n = 6. *P < 0.05 compared with control UT-B null mice; #P < 0.05 compared with control wild-type mice.
were significantly reduced in inner medullary tissue of PU-48-treated rats, compared with those in vehicle control rats and HCTZ-treated rats (Fig. 7A), which may be due to a reduction in inner medullary urea concentration (Fig. 7B). There was no significant difference in Na\(^+\) and Cl\(^-\) concentrations in inner medullary tissues between the three groups (Fig. 7, C and D).

**Effect of PU-48 on expression of channel proteins related to urine concentration**. AQP1, AQP2, AQP3, AQP4, UT-A1, UT-A2, UT-A3, NCC, and NKCC2 are involved in the urine concentrating mechanism. Expression levels of these proteins in the cortex (Fig. 8A), outer medulla (Fig. 8B), and inner medulla (Fig. 8C) were determined by Western blot analysis to check if the expression of these proteins is regulated by treatment with 50 mg/kg PU-48 for 6 h. No significant difference was found in the expression levels of these proteins between PU-48-treated rats and control rats (Fig. 8D).

**Effect of PU-48 on sodium, potassium, and calcium channels**. Figure 9 shows that 10 \(\mu\)M PU-48 does not significantly inhibit the potassium channel hERG (Kv11.1), sodium channel protein NaV1.5, or L-type calcium channel CaV1.2, as determined by electrophysiology.

**Toxicity analysis of PU-48**. Cytotoxicity of PU-48 was evaluated using a cell counting kit-8 (CCK-8) assay in MDCK cells. Treatment with PU-48 at 80 \(\mu\)M for 72 h did not significantly reduce MDCK cell viability (Fig. 10A). Next, we tested for any acute toxicity of PU-48 in mice. No visible symptoms of lethargy, anorexia, vomiting, or diarrhea were

**Table 1. Blood and urinary chemistry in control or PU-48-treated rats**

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<td>Serum HDL-C, mM</td>
<td>0.6 ± 0.2</td>
<td>0.7 ± 0.2</td>
</tr>
<tr>
<td>Serum LDL-C, mM</td>
<td>0.2 ± 0.1</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>Serum Glu, mM</td>
<td>9.3 ± 0.9</td>
<td>8.7 ± 1.7</td>
</tr>
<tr>
<td>Serum Chol, mM</td>
<td>1.4 ± 0.3</td>
<td>1.7 ± 0.4</td>
</tr>
<tr>
<td>Serum ALDO, pg/ml</td>
<td>75.1 ± 12.1</td>
<td>78.7 ± 5.8</td>
</tr>
<tr>
<td>Urinary Na, mM</td>
<td>142.5 ± 34.3</td>
<td>114.5 ± 14.4</td>
</tr>
<tr>
<td>Urinary K, mM</td>
<td>276.5 ± 61.0</td>
<td>206.2 ± 57.5</td>
</tr>
<tr>
<td>Urinary Cl, mM</td>
<td>207.9 ± 49.0</td>
<td>168.9 ± 18.1</td>
</tr>
<tr>
<td>Urinary urea, mM</td>
<td>1,265.1 ± 81.1</td>
<td>1,043.8 ± 80.1*</td>
</tr>
<tr>
<td>Urinary excretion of Na, mmol·kg(^{-1})·day(^{-1})</td>
<td>6.2 ± 1.4</td>
<td>7.9 ± 0.9</td>
</tr>
<tr>
<td>Urinary excretion of K, mmol·kg(^{-1})·day(^{-1})</td>
<td>12.0 ± 2.4</td>
<td>14.3 ± 0.4</td>
</tr>
<tr>
<td>Urinary excretion of Cl, mmol·kg(^{-1})·day(^{-1})</td>
<td>9.0 ± 1.7</td>
<td>11.7 ± 0.9</td>
</tr>
<tr>
<td>Urinary excretion of urea, mmol·kg(^{-1})·day(^{-1})</td>
<td>55.2 ± 7.3</td>
<td>73.1 ± 13.7*</td>
</tr>
</tbody>
</table>

TG, triglyceride; HDL-C and LDL-C, high- and low-density lipoprotein; Glu, glucose; Chol, cholesterol; ALDO, aldosterone. Data are means ± SE; *P < 0.05 compared with control rats.
found after gastrointestinal administration with 14 g/kg PU-48 for 14 days. There was no significant difference in body weight between the PU-48-treated mice and the control mice (Fig. 10B). Histological examination showed no significant morphological abnormality in kidney or liver tissues in PU-48-treated rats (Fig. 10C).

**DISCUSSION**

Recently, we found a novel UT inhibitor thienoquinolin analog PU-14, which significantly inhibited both UT-B- and UT-A-mediated transmembrane urea transport (30). However, PU-14 had an IC$_{50}$ at the micromolar level and inhibited UT-B more than UT-A. In this study, we screened thienoquinolin analogs to develop compounds that have a stronger inhibitory activity on IMCD urea transporter UT-A isoforms and better pharmacological characteristics as diuretics. We also addressed the mechanism by which thienoquinolins work as diuretics and their adverse effects.

According to the structure activity analysis of analogs of thienoquinolin, we identified the compound PU-48 with an IC$_{50}$ at 0.90 μM on UT-B and IC$_{50}$ at 0.32 μM on UT-A in MDCK cells. The IC$_{50}$s of PU-48 in human, rat, and mouse
UT-B are 10-fold stronger than those of PU-14. PU-48 has methoxyl groups at both the R1 position and R4 position in its chemical structure.

To confirm the effect of PU-48 on IMCD UT-As, urea transport in the last portion of collecting duct, the terminal IMCD, was measured using the tubule perfusion technique. PU-48 significantly reduced basal urea transport by 31.1% at 10 μM. PU-48 completely inhibited the vasopressin-stimulated component of urea transport at 1, 5, and 10 μM. The PU-48-treated UT-B null mice showed greater urine output increase and urine osmolality decrease than those observed in wild type mice. The diuretic effect of PU-48 was urea selective and did not change nonurea solute excretion in either wild-type or UT-B null mice. These data suggest that PU-48 exerts its diuretic effect by strongly inhibiting the IMCD UT-As.

Treatment with PU-48 at 50 mg/kg for 6 days caused continuous diuresis and low urine concentrating ability in rats. UT-A1 and UT-A3 are located in the IMCD (4). In IMCD cells, UT-A1 is mainly located at the apical membrane region (35), while UT-A3 is mainly located at the basolateral membrane region (43, 46). However, UT-A1 is capable of going to the basolateral membrane (13) and UT-A3 has been reported in one study at the apical membrane after vasopressin treatment (6). Therefore, both UT-A1 and UT-A3 increase urea transport in the last portion of the IMCD and are regulated by vasopressin (5, 17, 21, 24). Genetic deletion of the urea transporters UT-A1 and UT-A3 completely abolished vasopressin-regulated urea transport across the IMCD (3, 7). The deletion of these UTs resulted in a marked defect in inner medullary urea accumulation and a marked limitation in the ability of the kidneys to conserve water caused by a urea-dependent osmotic diuresis.

In this study, an analysis of solute concentrations in the inner medulla was performed to assess the effect of PU-48 on corticomedullary solute gradients. PU-48 significantly decreased inner medullary tissue osmolality and urea concentration, which indicates that PU-48 reduced urea accumulation in the inner medulla by inhibiting UT-A-mediated IMCD urea transport. Sodium and chloride concentrations in the inner medulla were unaffected by PU-48, which agrees with studies in UT-A1/UT-A3 null mice. With a similar diuretic activity as HCTZ, PU-48 caused a lower inner medullary urea concentration and higher urinary urea concentration than HCTZ did, which indicates lower urea transport between the IMCD lumen and the medullary interstitium.

The greater urine flow rate in PU-48-treated rats can be explained by a urea-dependent osmotic diuresis. To evaluate if the expression of other channels and transporters that may, in part, compensate for the observed diuresis was altered, we examined the abundance of the major sodium transporters, UTs, and AQPs in kidneys from PU-48-treated rats and control rats. PU-48 had no significant effect on the expression of UT-A1, UT-A2, UT-A3, UT-B, AQP1, AQP2, AQP3, AQP4, NCC, or NKCC2 as determined by Western blot analysis, indicating that PU-48 is a highly selective UT inhibitor, especially for UT-A1 (22). Inhibition of cardiac channels, such as hERG or NaV1.5, which induce a prolongation of QT interval, is the reason for cardiac repolarization toxicity. PU-48 did not affect the hERG, NaV1.5, or CaV1.2 channels, suggesting that PU-48 is not hazardous to cardiac repolarization. In addition, the safety of PU-48 has been initially substantiated by our acute toxicity test in which no toxic effects on mice were detected up to a dose as high as 14 g/kg.

It has been reported that analogs of thienoquinolin have multiple biological activities. 2-ary-1,2,3,4-tetrahydropyridol[2’,3’:4,5]thienol[2,3-b]quinolin-4-ones, an antitumor ellipticine, is supposed to serve as an heat shock protein 90 (HSP90) inhibitor (IC50 ranging from 2 to 32 μM) and kill SKBr3 cells, which have a high level of expression of HSP90 (37). In addition, these compounds have been reported to manifest analgesic activity (2) and function as an antibacterial (27, 33), antivirus, antifungal, and miticidal effect (15, 16, 19, 26). Here we report that PU-48, as a member of thienoquinolin analogs, can produce a marked effect as a diuretic, a function of such compounds that has not been discovered before.

In conclusion, PU-48 is a specific UT inhibitor with an IC50 at a submicromolar level. It does not have notable toxicity in either in vitro or in vivo conditions. The diuretic activity that PU-48 displays is mainly based on inhibiting the IMCD urea transporter UT-A isoforms, and it does not cause loss of electrolytes. PU-48, or its further chemically modified analogs, may be developed as a novel class of diuretics that do not have the adverse effects caused by classical diuretics. This class of diuretics might be used to treat hyponatremic states associated with volume expansion, such as congestive heart failure, hepatic cirrhosis, nephrotic syndrome, and the syndrome of inappropriate antidiuresis.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


