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Review Article

Urea Transporter Inhibitors as Novel Diuretics

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Abstract

Urea transporter (UT) proteins, including isoforms of UT-A in kidney tubule epithelia and UT-B in vasa recta endothelia and erythrocytes, play an important role in the urine concentration mechanism by mediated an intra renal urea recycling, suggesting that functional inhibition of these proteins could have therapeutic use as a novel class of diuretic. Recently several classes of UT inhibitors have been identified and found to functionally inhibit UTs. A class of thienoquinolins, which specifically inhibit both UT-A and UT-B, exhibit diuretic activity. It is predicated that UT inhibitors have potential clinical applications as sodium-sparing diuretics in edema from different etiologies, such as congestive heart failure and cirrhosis.

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INTRODUCTION

Diuretics are used clinically to treat a variety of diseases, including edema, hypertension, and heart failure [1,2] and are often used for long-term therapy. However, long-term use of conventional diuretics, such as loop diuretics, thiazides, amiloride, acetazolamide can have several adverse effects, including hypokalaemia, hyperkalaemia, hyponatraemia, hyperuricemia, hyperlipidemia, and glucose tolerance decrease [3-5]. The adverse effects result from the mechanism by which these diuretics act on the specific targets to reduce sodium reabsorption. Increased water excretion follows the salt excretion. Therefore, it would be desirable to discover novel diuretic targets and develop diuretics that do not cause electrolyte disturbances.

Intrarenal urea recycling is involved in the urinary concentrating mechanism [6,7]. The phenotype analysis on mice with urea transporter (UT) gene knockout shows that UTs play important role in the intrarenal urea recycling (Figure 1) and the urinary concentrating mechanism [8-11]. A short and a long loop of Henle are depicted within the 4 kidney zones, along with an arterial (descending) and venous (ascending) vasa recta (DVR and AVR, respectively). The pathways allowing urea to recycle in kidney are indicated by black arrows. In normal condition, concentrated urea is delivered to the tip of the papilla by the terminal part of the collecting ducts expressing the vasopressin-regulated urea transporters UT-A1/3. Urea is taken up by ascending blood in AVR, and a significant fraction of it is returned to the inner medulla by being reintroduced either in the DVR (expressing UT-B). A significant amount of urea is assumed to be secreted by an active transport in the pars recta and be transferred from descending limb of loops of Henle via UT-A2-mediated fluxes. The UT null mice have polyuria and low urine osmolality without electrolyte loss. *In vivo* effect of UT inhibitors causes significant diuresis with normal Na^+, K^+, Cl^- excretion [12,13]. These data indicate that UT inhibitors may be developed as a novel class of diuretics. This review will summarize the information on the molecular characteristics of UTs, the renal phenotypes of UT null mice, the discovery of UT inhibitors and the diuretic effect of UT inhibitors.

Molecular characteristics, localization and function of UTs

Urea transporters are a family of proteins facilitates the

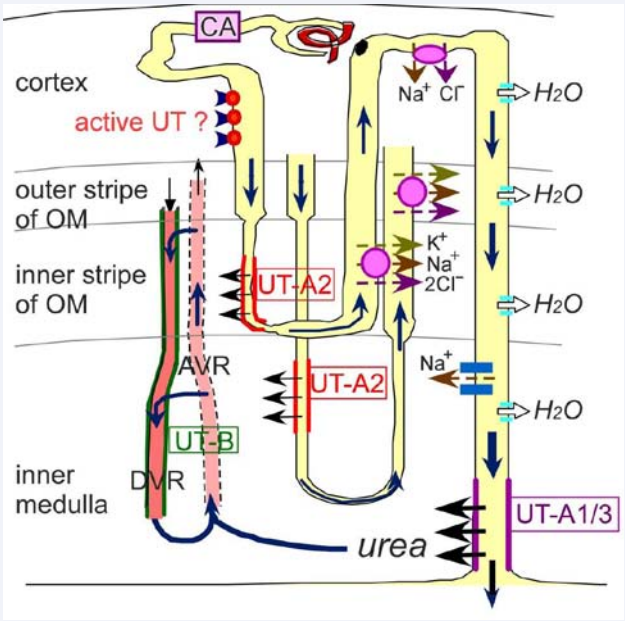

Figure 1 Diagram depicting the vascular and tubular routes of intra renal urea recycling.

passive transport of urea across the plasma membrane in certain cell types. Two urea transporter genes have been cloned, *Slc14a1* encoding UT-B)[14-17] and *Slc14a2* encoding several UT-A members (UT-A1-A6) via alternative splicing and alternative promoters [18-24].

UT-B is a glycosylated protein, which molecular weight is between 37~ 65 kDa [25,26]. UT-B is localized in the non-fenestrated endothelial cells that are characteristic of descending *vasa recta* (Figure 1) [27-30]. UT-B has also been identified in bladder, colon, testis, brain, heart, aorta, cochlea, mesenteric artery, etc. [31-40]. UT-B also transports several chemical analogues of urea, including methyl urea, formamide, acetamide, acrylamide, methylformamide, ammonium carbamate, etc [41]. Previous studies show that rat and mouse UT-B can function as a water channel, in addition to its role as a urea transporter[26,42].

UT-A subfamily includes 6 members. UT-A1 is the largest protein (97~117 kD) of the members and is expressed in the apical plasma membrane and in cytoplasm of the inner medullary collecting ducts (IMCD) (Figure 1) [43-48]. UT-A2 and UT-A1 have the same C-terminal amino acid sequence [49, 50]. UT-A2, with a 55 kDa molecular weight, is expressed in the thin descending limb of the loop of Henle [51-54]. UT-A3 and UT-A1 have the same N-terminal amino acid sequence [55]. UT-A3, having glycosylated protein bands at 44 and 67 kDa and a 40 kDa non-glycosylated UT-A3 protein band by Western blot, is expressed in inner medullary tip [56,57]. UT-A3 protein is most abundant in the inner medullary tip, weakly detected in the inner medullary base and outer medulla, and absent in cortex [56]. UT-A4 and UT-A1 have the same N- and C-terminal amino acid sequences, but UT-A4 is smaller and essentially consists of the N-terminal quarter of UT-A1 spliced into the C-terminal quarter of UT-A1. UT-A4 mRNA is detected in rat kidney medulla. UT-A5 is expressed in testis ([58] and UT-A6 is expressed in colon but not in kidney.

Studies on UT knockout mouse models revealed and confirmed the physiological functions of UTs in urine concentration mechanism. The UT-B null mice were polyuric, consuming and excreting approximately 50% more fluid than litter-matched wild-type mice [59]. The urine osmolality in UT-B null mice was significantly lower than that in wild-type mice. Plasma urea concentration was significantly higher, and urinary urea concentration was significantly lower in the UT-B null mice. These opposing changes suggest that plasma urea increased because the kidney was less able to recycle urea and concentrate urea in the urine. The urine-to-plasma ratio of urea concentration reflects the capacity of the kidney to concentrate urea above its level in body fluids. This ratio was markedly decreased in UT-B null mice. UT-B deficiency also produced a 2-fold reduction in inner medullary urea concentration with little effect on inner medullary Na^+, K^+ and Cl^- concentrations. These observations suggest that UT-B functional defect caused urea selective diuresis.

Another transgenic mouse model with UT-A1/A3 deletion provided evidence that UT-A1 and UT-A3 play important role in urine concentrating mechanism, and the functional inhibition of UT-A1 and UT-A3 cause a urea-dependent osmotic diuresis [60]. In water-restricted UT-A1/A3 null mice there was a significantly reduced concentration of urea in the inner medullary interstitium compared to wild-type mice (Fenton et al. 2004) [60], but no reduction in the Na^+, Cl^- or K^+ concentrations. UT-A1/A3 null mice had a substantially attenuated corticomedullary osmolality gradient and no urea gradient, yet the corticomedullary sodium gradients were almost identical to wild-type mice. These data indicate that NaCl accumulation in the inner medulla is not reliant on either IMCD urea transport or the accumulation of urea in the IMCD interstitium. Taken together, these data suggest that the functional inhibition of urea transporters may cause diuresis without electrolyte loss. Urea transporter inhibitors may be developed as a novel class of diuretics.

Urea transporter inhibitors

A high throughput UT inhibitor screening assay, using human erythrocytes based on UT-B-facilitated acetamide transport, was set up in Verkman group [61]. From a small molecule library, several classes of chemical compounds phenylsulfoxyoxozole, benzenesulfonanilide, phthalazinamine, and aminobenzimidazole were identified as selective UT-B inhibitors [61]. Some active compounds have the potent inhibition activity on UT-B urea transport with IC_{50} ~10 nM, with ~100% inhibition at higher concentrations. Though the potency of the best compound was

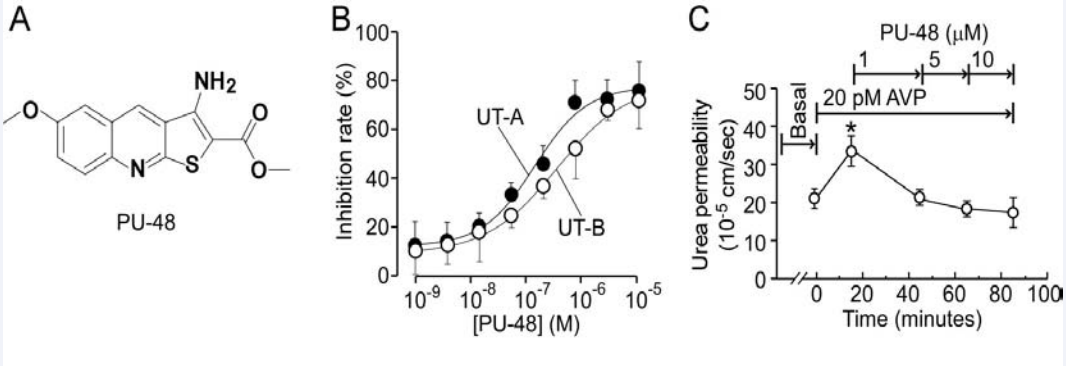

Figure 2 Inhibition activity of PU-48 on urea transporters. **A.** Chemical structure of PU-48. **B.** Inhibition activity of PU-48 on UT-A1 or UT-B mediated urea transport in epithelial cells. Rat UT-A1 or UT-B stably transfected MDCK cells were treated with PU-48 at different concentrations. **C.** PU-48 (1, 5, and 10 μmol/l) inhibits vasopressin-stimulated urea permeability in perfused rat terminal inner medullary collecting ducts. (reproduced from Ren et al. 2014) [13].

excellent, it was not further developed because of their relatively low potency for rodent UT-B, low UT-A inhibition activity, and low metabolic in hepatic microsomes, making them difficult to perform diuresis *in vivo*. In a follow-on [62-64] screen produced several other classes of UT-B inhibitors. *In vitro* studies showed that UTB_{inh}-14 fully and reversibly inhibited urea transport with IC_{50} of ~10 nmol/l for human UT-B and ~25 nmol/l for mouse UT-B. UTB_{inh}-14 was highly selective against UT-B *vs.* UT-A isoforms. Intraperitoneal administration of UTB_{inh}-14 in mice to achieve therapeutic concentrations in kidney, urine osmolality following dDAVP in UTB_{inh}-14-treated mice was significantly lower than in vehicle-treated mice. UTB_{inh}-14 also increased urine output and reduced osmolality in mice given free access to water. Though these data provided proof-of-concept for the potential utility of UT-B inhibition to reduce urinary concentration in a high-vasopressin state, the reduction in urine osmolality was relatively modest.

UT-A isoform is the most important for urinary concentration as it is the rate-limiting step in apical membrane urea transport in the inner medullary collecting duct and hence required to establish the hyper molar renal medullary interstitium. Several classes of compounds with UT-A inhibition activity were found in Verkman group [65,66]. These compounds have a wide range of UT-A1 *vs*. UT-B selectivity. They found that intravenous administration of an indole thiazole or α-sultambenzosulfonamide increased urine output by 3~5-fold and reduced urine osmolality by 2-fold compared to vehicle control rats, even under conditions of maximum antidiuresis produced by 1-deamino-8-D-arginine vasopressin (DDAVP). The diuresis was reversible and showed urea > salt excretion [66].

By integrated cell based high throughput screening and *in silico* methods, a class of small-molecule drug-like compounds with thienoquinolins core structure was found to have inhibition activity on both UT-A and UT-B. The structure and activity relationship analysis showed a compound PU-48, named chemically as methyl 3-amino-6-methoxythieno [2,3-b] quinoline-2-carboxylate (Figure 2A), had the best UT-A and UT-B inhibition activity. IC_{50}s of PU-48 on UT-B facilitated urea transport were micromole level in human, rat, and mouse, as determined by erythrocyte lysis assay [67]. PU-48 has stronger inhibition activity on rat UT-A (IC_{50} 0.32 μmol/l) than rat UT-B (IC_{50} 0.90 μmol/l) (Figure 2B).

To confirm the effect of PU-48 on UT-As expressed in inner medullary collecting ducts, the efficiency of urea transport in the rat terminal portion of collecting duct was measured using the tubule perfusion technique. PU-48 significantly reduced basal urea transport by 31.1% at 10 μmol/l and completely inhibited the vasopressin-stimulated component of urea transport at 1, 5, and 10 μmol/l (Figure 2C). 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 in rat kidney as determined by Western blot analysis, indicating that PU-48 is a highly selective UT inhibitor. PU-48 did not affect the hERG, NaV1.5, or CaV1.2 channels and had no cytotoxicity at effective doses, suggesting that PU-48 is not hazardous.

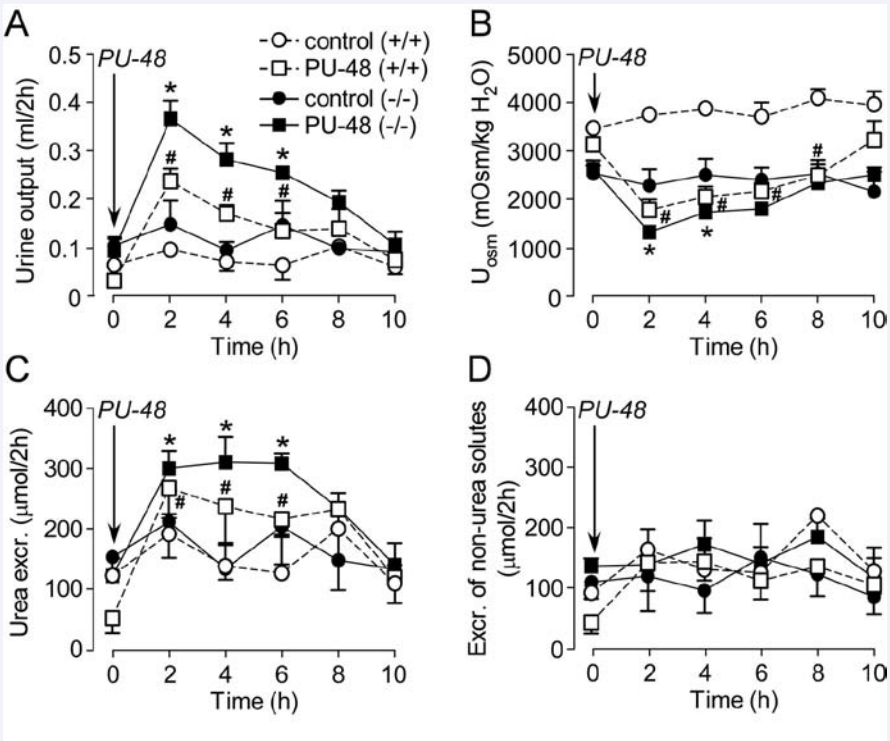

Figure 3 Effect of PU-48 on urinary concentrating activity and renal handling of solutes in wild-type and UT-B null mice**.** Mice subcutaneously injected with 100 mg/kg of PU-48 just after a 2-h urine collection (time 0). Urine samples were collected every 2 h in metabolic cages. **A.** Urine output. **B.** Urinary osmolality (U_{osm}). **C.** Urea excretion. **D.** Excretion of non-urea solutes. Mean±SEM, n=6; **P*<0.05 *vs.* control UT-B null mice, #*P*<0.05 *vs.* control wild-type mice. (reproduced from Ren et al. 2014) [13].

Diuretic activity and characteristics of UT inhibitors

In vivo activity of PU-48 on urinary concentrating function was evaluated in wild-type and UT-B null mice fed ad libitum in metabolic cages. In the 2 h before administration of PU-48, urine output was higher and urinary osmolality was lower in UT-B null mice than in wild-type mice (Figure 3A,B). Urine output significantly increased and urinary osmolality significantly decreased after subcutaneous administration of PU-48 at 100 mg/kg in both UT-B null mice and wild-type mice [68]. These data suggest that PU-48 exert sits diuretic effect by strongly inhibiting the inner medullary collecting duct UT-As. PU-48 significantly increased urea excretion in both wild-type mice and UT-B-null mice (Figure 3C). Notably, the excretion of non-urea solutes did not change (Figure 3D) in both wild-type and UT-B null mice, indicating no significant loss of Na^+, K^+, or Cl^- [13].

To confirm the diuretic activity of PU-48, rat model was assessed using metabolic cages. Urine output significantly increased in a dose-dependent manner in rats subcutaneously administered PU-48 at 3.125, 12.5, and 50 mg/kg (Figure 4A). Urinary osmolality was significantly decreased. The peak changes of urine output, urinary osmolality and urinary urea concentration occurred 2 h after PU-48 administration, with values returning to baseline by 10 h [13].

Diuretic characteristics of PU-48 for continuous use were evaluated in rats. PU-48 at 50 mg/kg was subcutaneously injected every 6 h for 6 days. As shown in Figure 4B, the 24 h urine output in PU-48 treated rats was significantly higher than that in vehicle control rats. Notably, blood urea, T-CHO, TGs, and LDL-C in PU-48-treated rats were similar with those in vehicle control rats, which are normal levels (Table 1). It was also found that total osmolalities were significantly less in inner medullary tissue of PU-48-treated rats than those in vehicle control rats, which was primarily because of a reduction in inner medulla urea concentration (Figure 4C). Na^+ and Cl^- concentration was similar with those in vehicle control rats, which indicates that the diuretic effect of PU-14 does not cause electrolyte imbalance and abnormal metabolism. These experimental results suggest that urea transporter inhibitors may be developed as a novel class of diuretics performing urea-selective diuresis without disturbing electrolyte excretion and metabolism [13].

Clinical significance of urea transporter inhibitors as diuretics

Urea transporter inhibitors used as diuretics have potential clinical significance. Urea transporter inhibitors have a different mechanism-of-action from conventional diuretics, which block salt transport across kidney tubule epithelial cells. Urea transporter inhibitors can be widely used to increase renal water excretion in conditions associated with total body fluid overload, including congestive heart failure, cirrhosis and nephrotic syndrome [69]. By disrupting countercurrent mechanisms and intrarenal urea recycling, urea transport inhibitors, alone or in combination with conventional diuretics, may induce a diuresis

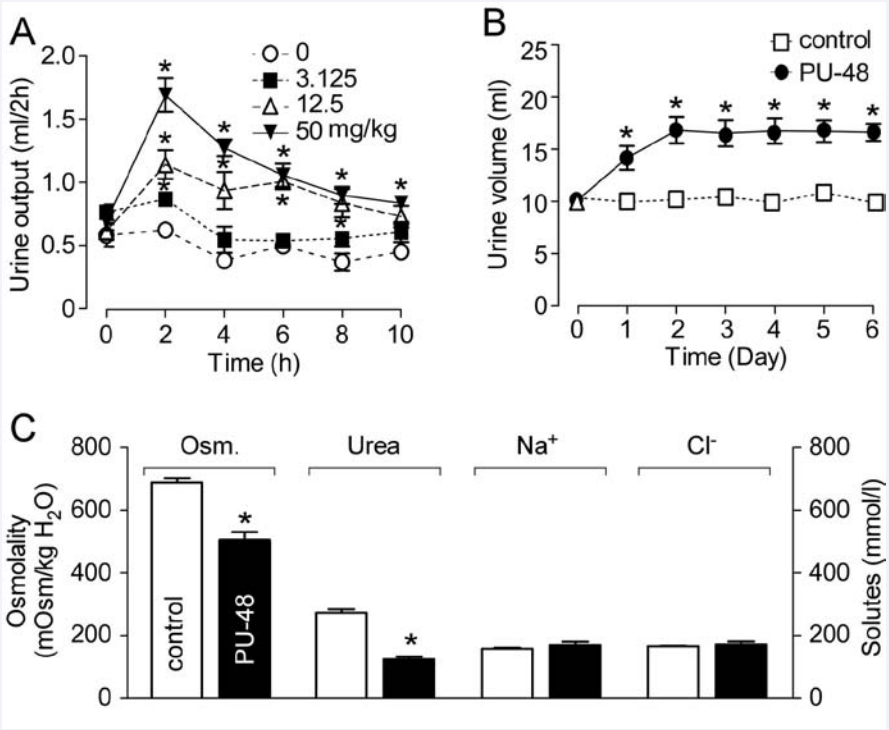

Figure 4 Diuretic effect of PU-48 in rats. **A.** Urine output of rats subcutaneously injected with 0, 3.125, 12.5, or 50 mg/kg of PU-48 just after a 2-h urine collection (time 0). Urine samples were collected every 2 h in metabolic cages. **B.** Urine output of rats that were subcutaneously treated without or with PU-48 at 50 mg/kg for 6 days. Mean ±SEM, n = 6; *P<0.05, *vs.* control rats. **C.** Concentration of osmoles, urea, Na^+ and Cl^- in renal inner medullary tissue in rats without (control) or with PU-48 treatment. Mean ± SEM; n = 6, *P<0.05 *vs.* control rats. (reproduced from Ren et al. 2014) [13].

Table 1: Blood chemistry in control or PU-48 treated rats (adapted from Ren et al. 2014) [13].

	Control (n = 6)	PU-48 (n = 6)
Serum osmolality, mOsm/kg H_2O	302±1.5	305±2.6
Serum Na, mM	150.1±10.1	145.2±6
Serum K, mM	4.4±0.8	4.2±0.8
Serum Cl, mM	107.3±9.9	104.6±4.7
Serum urea, mM	8.0±0.9	8.5±1.3
Serum TG, mM	1.0±0.8	0.8±0.5
Serum HDL-C, mM	0.6±0.2	0.7±0.2
Serum LDL-C, mM	0.2±0.1	0.3±0.1
Serum Glu, mM	9.3±0.9	8.7±1.7
Serum Chol, mM	1.4±0.3	1.7±0.4
Serum ALDO, pg/ml	75.1±12.1	78.7±5.8

in states of refractory edema where conventional diuretics are ineffective [70]. However, many challenges remain in the clinical development of urea transporter, including demonstration of efficacy in clinically relevant models of refractory edema and SIADH (syndrome of inappropriate antidiuretic hormone), and in medical chemistry in the selection of inhibitors with appropriate pharmacological properties.

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