Novel diuretic targets

Jerod S. Denton, Alan C. Pao, and Merritt Maduke

1Department of Anesthesiology, Vanderbilt University School of Medicine, Nashville, Tennessee; 2Department of Pharmacology, Vanderbilt University School of Medicine, Nashville, Tennessee; 3Institute of Chemical Biology, Vanderbilt University School of Medicine, Nashville, Tennessee; 4Institute for Global Health, Vanderbilt University School of Medicine, Nashville, Tennessee; 5Department of Medicine, Stanford University, Stanford, California; and 6Department of Molecular and Cellular Physiology, Stanford University, Stanford, California; and 7Veterans Affairs Palo Alto Health Care System, Palo Alto, California

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Approximately 67 million Americans have hypertension, a chronic disease of excessive vascular tone and fluid volume that increases the risk of stroke, myocardial infarction, and chronic kidney disease. Hypertension costs the United States roughly $50 billion annually in direct medical expenses and lost productivity, and the incidence of hypertension is projected to rise considerably as rates of obesity and life expectancy increase. Diuretics continue to be a mainstay of antihypertensive therapy and work by reducing sodium chloride (NaCl) and osmotically obliged water reabsorption in the nephron, thereby lowering blood volume and pressure. Combination therapy, in which more than one drug is prescribed to reach a target blood pressure of <140/90, is required in ~75% of hypertensive patients. Resistant hypertension, defined as blood pressure above 140/90, despite adherence to at least three antihypertensive drugs, is observed in about 20–30% of the population (16). Rotation between diuretics of different classes (e.g., loop, thiazide, potassium-sparing) or even within a class can overcome refractoriness in some diuretic-resistant patients; however, differences in drug bioavailability, metabolism, compatibility with other medications, as well as existing comorbidities like chronic kidney disease, can complicate establishing the correct combination to achieve the blood pressure goal. The development of new classes of drugs targeting diverse mechanisms would provide clinicians with greater flexibility in establishing effective treatment regimens for an increasingly diverse and aging patient population. An emerging body of physiological, genetic, and pharmacological evidence has implicated several renal ion-transport proteins, or regulators thereof, as novel, yet clinically unexploited, diuretic targets. These include the renal outer medullary potassium channel, ROMK (Kir1.1), Kir4.1/5.1 potassium channels, CIC-Ka/b chloride channels, UTA/B urea transporters, the chloride/bicarbonate exchanger pendrin, and the STE20/SPS1-related proline/alanine-rich kinase (SPAK). The molecular pharmacology of these putative targets is poorly developed or lacking altogether; however, recent efforts by a few academic and pharmaceutical laboratories have begun to lessen this critical barrier. Here, we review the evidence in support of the aforementioned proteins as novel diuretic targets and highlight examples where progress toward developing small-molecule pharmacology has been made. CIC-K; Kir4.1/5.1; pendrin; ROMK; urea transporter


Jerod.S.Denton@Vanderbilt.edu.

Address for reprint requests and other correspondence: J. S. Denton, T4208 Medical Center North, 1161 21st Ave. South, Nashville, TN 37232 (e-mail: Jerod.S.Denton@Vanderbilt.edu).

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the thick ascending limb of Henle (TALH), distal convoluted tubule (DCT), and collecting duct (CD) (56). In the TALH (Fig. 1C), ROMK forms a ~35-pS K⁺-secretory pathway that is functionally coupled to the Na⁺-K⁺-2Cl⁻ cotransporter and loop diuretic target NKCC2, which catalyzes the first step in the transepithelial NaCl reabsorption in this nephron segment (49). Approximately 30% of the total filtered NaCl is reabsorbed in the TALH, and this contributes to a hypertonic medullary interstitium that promotes distal osmotic water reabsorption and generation of concentrated urine (9, 15). NKCC2 mediates the electroneutral symport of Na⁺, K⁺, and 2 Cl⁻ ions across the luminal membrane (Fig. 1), after which Na⁺ and Cl⁻ are transported across the basolateral membrane via the Na⁺-K⁺-ATPase and ClC-Kb, respectively. Since the concentration of K⁺ in the tubular fluid is much lower than that of Na⁺ or Cl⁻, the continual replenishment of tubular fluid K⁺ by ROMK is necessary to maintain NKCC2 activity and NaCl reabsorption. A 70-pS K⁺ channel is also expressed in the TALH and can account for up to 70% of the luminal K⁺ conductance (149). The molecular identity of the 70-pS channel has not yet been determined, but the 30- and 70-pS channels are both absent in the TALH of KCNJ1-null mice. This observation has led to the proposal that ROMK is a necessary subunit of the 70-pS channel (90). Inhibition of luminal K⁺ conductance by removal of K⁺ or with the nonspecific blocker barium inhibits transepithelial NaCl reabsorption in isolated TALH segments (50, 55).

ROMK constitutes a major pathway for regulated K⁺ excretion in the mineralocorticoid-sensitive distal nephron (45, 148, 152). Transepithelial K⁺ secretion in principal cells of the CD is a two-step process, whereby K⁺ is initially pumped across the basolateral membrane by the Na⁺-K⁺-ATPase and subsequently conducted across the luminal membrane through ROMK (Fig. 1E). K⁺ secretion shifts the luminal membrane potential toward the Nernst potential for K⁺, thus increasing the electrochemical driving force favoring Na⁺ influx through ENaC, the rate-limiting step in Na⁺ reabsorption in the CD (Fig. 1E). ROMK inhibition with barium depolarizes the luminal membrane potential difference and reduces the driving force for ENaC-mediated Na⁺ reabsorption (71).

The discovery of autosomal recessive loss-of-function (LOF) mutations in KCNJ1 in patients with type II Bartter syndrome (129) and studies in KCNJ1-deletion mice (87, 91) confirmed the importance of ROMK in renal function. The clinical presentation of type II Bartter’s syndrome includes hypernatremia, hypokalemic alkalosis, polyuria, and low blood pressure (8). More recently, Lifton and colleagues (66) found that heterozygous carriers of inactivating KCNJ1 mutations have lower blood pressure and are protected from development of hypertension. These observations provide strong genetic validation for ROMK as a diuretic target and support the notion that pharmacological inhibitors of ROMK may lower blood volume and pressure without causing derangements in serum electrolytes. Specifically, by inhibiting Na⁺ reabsorption in the TALH and CD and blocking K⁺ secretion in the distal nephron, ROMK inhibitors may induce natriuresis and limit the urinary loss of K⁺ observed with conventional loop and thiazide diuretics. Furthermore, considering that ROMK participates in NaCl reabsorption in multiple nephron segments, it is conceivable that ROMK inhibitors may induce natriuresis and diuresis more effectively than conventional loop, thiazide, or K⁺-sparring diuretics, which target transport pathways present in only one nephron segment.

Clark and colleagues (22) showed that administration of the sulfonylurea K<sub>ATP</sub> channel inhibitor glyburide to rats led to a rapid and dose-dependent increase in renal Na⁺ excretion without affecting K⁺ excretion. Wang et al. (146, 147) later showed in patch-clamp experiments and isolated, perfused renal tubules that glyburide, as well as a nonsulfonylurea K<sub>ATP</sub> channel antagonist, inhibit the ~35-pS channel ROMK channel and reduce transepithelial Na⁺ and K⁺ transport in the TALH and CD. Although these observations are consistent with ROMK-mediated effects, the high doses used and potential for off-target effects complicate interpretation of the data. Antagonists with higher affinity and greater selectivity for ROMK over other channels expressed in the nephron are required to verify ROMK as a diuretic target. With few exceptions, however, the molecular pharmacology of ROMK and most other Kir channels has remained largely undeveloped (11), consequently stalling efforts to assess ROMK’s therapeutic potential. However, the wait may soon be over, as targeted drug-discovery campaigns are leading to the development of promising pharmacological tools.

Denton and colleagues (78) employed high-throughput screening (HTS) to interrogate a small-molecule library of ~225,000 compounds from the National Institutes of Health (NIH) Molecular Libraries Small-Molecule Repository (MLSMR) for pharmacological modulators of ROMK. This screen led to the discovery of VU590 (Fig. 2A), which inhibits ROMK currents by 50% at a concentration (IC<sub>50</sub>) of ~290 nM and exhibits at least 20-fold selectivity over Kir7.1 (IC<sub>50</sub> ~8 μM), Kir2.1 (IC<sub>50</sub> >10 μM), and Kir4.1 (IC<sub>50</sub> >10 μM). The chemical structure of VU590 is shown in Fig. 2A. Lead optimization efforts to improve its potency were unsuccessful but revealed that the two nitro groups flanking the cryptan linker region of the compound are indispensable for VU590 potency. VU590 inhibits K⁺ secretion in isolated, perfused rat collecting ducts (Liu W and Satlin L, unpublished observations), consistent with inhibition of ROMK. However, given the limited selectivity of VU590 for ROMK over Kir7.1, both of which are expressed in the collecting duct (102), these effects could be mediated by inhibition ROMK, Kir7.1, or both channels.

In an effort to develop a more selective ROMK antagonist, the investigators turned to another inhibitor identified in the screen, termed BNBI (Fig. 2B) (10). Unlike VU590, BNBI is a relatively weak (i.e., IC<sub>50</sub> ~8 μM) ROMK channel inhibitor. Importantly, however, BNBI has no effect on Kir7.1 at doses up to 100 μM. As shown in Fig. 2, A and B, both VU590 and BNBI contain nitro groups flanking a variable seven-atom linker region containing two nitrogen atoms. A notable difference between the two compounds is the absence of a central oxygen atom in the linker of BNBI (Fig. 2B). Remarkably, addition of the oxygen increased the potency of the BNBI-derivative by 30-fold (IC<sub>50</sub> = 240 nM). The new compound was termed VU591 (Fig. 2C). VU591 is highly selective for ROMK over other members of the Kir channel family and >65 other ion channels and receptors. As predicted, VU591 inhibits K⁺ excretion in the isolated collecting duct (10). Thus VU591 may afford the potency and selectivity to probe ROMK as a diuretic target in vivo.

Merck Research Laboratories recently disclosed their program to develop ROMK-directed diuretics with the publication...
Fig. 1. Putative diuretic targets. A: schematic of the nephron and vasa recta spanning the cortex, outer medulla, and inner medulla. AQP, aquaporin. B: thin ascending limb. CLC-Ka mediates Cl⁻/H⁺ flux across both the apical and basolateral membranes. This Cl⁻ flux helps to generate the steep solute gradient in the inner medulla that drives water reabsorption from the tubular fluid to the blood. C: thick ascending limb. Na⁺/K⁺-2Cl⁻ cotransporter (NKCC2) mediates entry of Na⁺, K⁺, and Cl⁻ across the apical membrane. Since luminal K⁺ concentration is low compared with Na⁺ and Cl⁻ concentrations, K⁺ must recycle across the apical membrane through the renal outer medullary potassium channel (ROMK) to maintain NKCC2 activity and NaCl reabsorption. Kinases such as STE20/SPS1-related proline/alanine-rich kinase (SPAK) and OSR1 independently regulate NKCC2 phosphorylation and activity. ClC-Kb mediates basolateral Cl⁻ efflux. D: distal convoluted tubule. NCC mediates NaCl entry across the apical membrane. SPAK stimulates NCC phosphorylation and activity. ClC-Kb mediates Cl⁻ exit across the basolateral membrane. Since extracellular K⁺ concentration is low compared with Na⁺ concentration, K⁺ must recycle across the basolateral membrane through Kir4.1/5.1 to maintain Na⁺/K⁺-ATPase activity. E: top: principal cell of the collecting duct. The epithelial sodium channel (ENaC) mediates apical Na⁺ entry. The resulting lumen negative potential drives K⁺ secretion via ROMK. E: middle: intercalated cell of the collecting duct. Pendrin mediates Cl⁻/HCO₃⁻ exchange across the apical membrane of type B and non-A, non-B intercalated cells. ClC-Kb mediates Cl⁻ exit across the basolateral membrane. Alkalization of the tubular fluid in the collecting duct lumen also stimulates apical Na⁺ entry via ENaC across principal cells. E: bottom: principal cell of the collecting duct. Arginine vasopressin (AVP) activates vasopressin-2 receptors in the basolateral membrane and stimulates apical insertion of AQP2 water channels to initiate water transport across the collecting duct. AVP also stimulates apical insertion of UTA-1/3 urea transporters to initiate rapid urea transport into the inner medullary interstitium. UT-B urea transporters in the vasa recta operate to recycle, trap, and maintain a high level of urea concentration in the inner medulla to drive water absorption in the collecting duct.

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of several nanomolar-affinity small-molecule ROMK antagonists (135). A high-throughput screen of ~1.5 million compounds revealed a hit containing a 4-nitrophenyl group identical to that found in VU590 (Fig. 2A). Although it was only moderately potent (IC$_{50}$ = 5 μM), the compound was highly selective for ROMK over Kir2.1 and Kir2.3. Purification of the molecule by HPLC led to a complete loss of ROMK inhibitory activity, suggesting the active component was a minor contaminant in the sample. Indeed, synthesis of a suspected impurity yielded a potent ROMK inhibitor (IC$_{50}$ = 50 nM) that maintained selectivity for ROMK over other Kir channels and >65 other ion channels and receptors (see Ref. 11 for details). The central linkers of VU590, BNBI, and VU591 are shown in bold to emphasize the common nitrogen- and oxygen-containing structures, which drove the medicinal chemistry efforts to optimize VU591 (see text for details).

Foster et al. (41a) recently proposed that ROMK is a component of the mitochondrial K$_{\text{ATP}}$ (MitoK$_{\text{ATP}}$) channel complex, which is involved in cardiomyocyte and neuronal ischemic preconditioning. A proteomic analysis of mitochondrial inner membrane protein fractions and RT-PCR analysis led to the identification of several ROMK splice variants in the heart, brain, and liver. The ROMK2 splice variant, which lacks 19 N-terminal amino acids present in the ROMK1 variant (160), is targeted to mitochondria of Chinese hamster ovary cells and H9C2 heart-derived immortalized cells by a mitochondrial localization sequence in the N terminus of the channel. The ROMK peptide inhibitor tertiapin-Q dose dependently inhibited flux of the K$^+$ surrogate thallium in rat heart mitochondria, albeit with an IC$_{50}$ three orders of magnitude lower than that observed for heterologously expressed ROMK. Consistent with a role in mitoK$_{\text{ATP}}$ function, ROMK2 overexpression is cytoprotective against oxidative stress, whereas knockdown of ROMK enhances cell death. While this study points to a potentially critical and previously unappreciated role of ROMK in ischemic preconditioning, it also raises concerns that ROMK antagonists developed for use as diuretics may have untoward consequences on mitochondrial function. The development and use of small-molecule ROMK inhibitors should address this important and timely question.

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**Fig. 2. Development of small-molecule ROMK inhibitors.**

**A:** chemical structure of VU590, a small-molecule inhibitor of ROMK and Kir7.1 discovered in an HTS at Vanderbilt University. Concentrations that inhibit channel activity by 50% (IC$_{50}$) are shown. **B:** BNBI, a weak but selective ROMK inhibitor identified in the HTS. **C:** VU591, an improved BNBI derivative that exhibits ~30-fold higher potency toward ROMK. The asterisk (*) indicates VU591 is selective for ROMK over other Kir channels and >65 other ion channels and receptors (see Ref. 11 for details). The central linkers of VU590, BNBI, and VU591 are shown in bold to emphasize the common nitrogen- and oxygen-containing structures, which drove the medicinal chemistry efforts to optimize VU591 (see text for details). **D:** chemical structure of compound 5, a ROMK inhibitor discovered from an HTS at Merck. **E:** compound 30, a derivative of compound 5 with improved pharmacokinetic properties to enable in vivo pharmacology.
**Kir4.1/5.1**

Recent genetic evidence suggests that heteromeric Kir4.1/Kir5.1 channels could represent novel drug targets for hypertension. Inactivating mutations in the gene encoding Kir4.1, KCNJ10, give rise to SeSAME (or EAST) syndrome, a complex disorder presenting with seizures, sensorineural deafness, ataxia, intellectual disability, and electrolyte imbalance (13, 122). The neurological deficits are presumably due in part to loss of extracellular “K+ buffering” mediated by homo- and heteromeric Kir4.1 channels expressed in astrocytes and glial cells (27). The renal consequences, which include polyuria, hypokalemia, metabolic alkalosis, and elevated renin and aldosterone, are consistent with impaired NaCl reabsorption in the DCT. In the DCT, apical NaCl absorption is mediated by NCC, the thiazide-sensitive NaCl cotransporter (Fig. 1D). Na+ is pumped out of the cell, across the basolateral membrane, in exchange for K+ by the Na-K-ATPase. Cl− moves down its electrochemical gradient and out of the cell through ClC-Kb. Kir4.1/5.1 channels are expressed in the basolateral membrane of the DCT (Fig. 1D), where they perform at least two important functions. First, they recycle K+ back out across the basolateral membrane to help maintain the activity of the Na+-K+-ATPase. Second, Kir4.1/5.1 hyperpolarizes the basolateral and luminal membrane potentials and thus facilitates the electrogenic exit of Cl− ions and entry of Na+ ions, respectively.

Deletion of KCNJ10 in mice recapitulates the salt-wasting phenotype of subjects with SeSAME/EAST syndrome (13), whereas inactivation of the Kir5.1-encoding gene KCNJ16 paradoxically increases renal NaCl reabsorption (103). Heteromeric Kir4.1/5.1 channels are critically regulated by intracellular pH (pH), and unlike homomeric Kir4.1, Kir4.1/5.1 is partially inhibited at physiological pH. Patch-clamp analysis revealed that as a consequence of KCNJ16 deletion, and loss of this negative pH-dependent regulation, there is an increase in the basolateral homomeric Kir4.1 activity. The ensuing increased K+ conductance is proposed to stimulate transepithelial Na+ and Cl− reabsorption in the DCT by increasing the electrochemical driving forces for these ions (Fig. 1D) (122).

Kir4.1/5.1 antagonists would be expected to mimic the actions of thiazide diuretics, by indirectly inhibiting NCC-mediated NaCl reabsorption in the DCT. Importantly, however, inhibitors of Kir4.1/5.1 may offer some advantages over conventional diuretics due to the localization of the channel on the basolateral membrane (60, 76, 89). Loop and thiazide diuretics are first secreted into the renal tubular fluid by organic anion transporters and multidrug resistance proteins present in renal proximal tubule cells before reaching their sites of action on the luminal membrane (53, 54, 141, 142; for a review see Ref. 155). Competitive interactions between diuretics and other substrates, including β-lactam antibiotics, NSAIDs, antivirals, and organic acid in the setting of renal failure, can limit their secretion and natriuretic effects. Kir4.1/5.1 inhibitors acting directly on the basolateral membrane would presumably avoid this limitation of secreted diuretics.

Currently, the molecular pharmacology of Kir4.1 is limited to neurological drugs exhibiting weak off-target effects on the channel. This include the selective serotonin reuptake inhibitors fluoxetine and sertraline, as well as the tricyclic antidepressants amitriptyline, desipramine, imipramine, nortriptyline (42, 101, 134; for a review, see Ref. 11). There are no known inhibitors that discriminate between homomeric and heteromeric Kir4.1-containing channels. Identifying subtype-selective modulators will be important moving forward, because of the broad tissue distribution of Kir4.1 and potential for pleiotropic effects of general Kir4.1 inhibitors. Kir4.1 is broadly expressed in the periphery (i.e., kidney, stomach) and central nervous system (brain, spinal cord, retina, coochlea). In the kidney, Kir4.1 appears to exist predominantly as a heterotrimer with Kir5.1 (60, 76, 89). Developing small-molecule inhibitors of heteromeric Kir4.1/5.1 channels that are excluded from the central nervous system by the blood-brain barrier may enable the selective inhibition of the channel in renal DCT cells.

**ClC-Ka/b**

The chloride channel (CLC) family, with nine human homologs, is an eclectic group of anion-selective channels and transporters. Members share a similar molecular scaffold and selectivity for anions (20, 28, 29, 64, 94, 162), but vary significantly in their physiological distribution, function, and fundamental mechanisms. About half of all CLCs are secondary active transporters, catalyzing the stoichiometric exchange of two Cl− ions for one proton, while the other half are passive channels, catalyzing rapid downhill movement of Cl− (1, 109, 120). Both types play important roles in kidney tubular function (23, 26, 30, 86, 88, 110, 151). In the channel branch of the family, the two kidney-specific homologs, ClC-Ka and ClC-Kb, have central roles in tubular Cl− reabsorption that make them important targets for novel therapeutic interventions (36, 41, 61, 63, 65, 75).

ClC-Kb is localized to the basolateral membrane in the Talh (Fig. 1C), DCT (Fig. 1D), and CD (Fig. 1E) (139). Mutations in ClC-Kb cause Bartter’s syndrome type III, which is characterized by hypokalemic alkalosis with salt wasting and low blood pressure (127). The low blood pressure associated with defective ClC-Kb function suggests ClC-Kb is a target for antihypertensive drugs (41, 81). This suggestion is further supported by the observation of an association between a common ClC-Kb polymorphism that increases channel activity and a predisposition to hypertension (7, 37, 62, 73, 126). However, it should be noted that such a predisposition has not been detected in all studies (17, 37, 73, 132). It has been suggested that salt-loading conditions as well as sex and ethnic segregation need to be considered, and that larger studies are necessary to resolve the issue (73, 126, 150).

ClC-Ka is expressed in both the apical and basolateral membranes of the thin ascending limb (92, 140) (Fig. 1B). It catalyzes the Cl− flux necessary for maintaining the steep solute gradient in the kidney medulla and provides the driving force for water absorption from the urine to the blood (72). In mice, genetic deletion of ClC-K1 (the mouse ortholog of human ClC-Ka) dissipates this solute gradient in the inner medulla and leads to an increase in urine water excretion (92). Importantly, deletion of ClC-K1 does not increase urine NaCl excretion or decrease extracellular fluid volume because renal NaCl transport in the more distal segments of the kidney tubule remain intact (2). This finding suggests ClC-Ka inhibitors may be useful as therapeutics to treat hyponatremia which develops in diseases that impair free water excretion, such as in decompensa-
lated heart failure, decompensated cirrhosis, renal failure, and syndrome of inappropriate anti-diuretic hormone secretion (41).

Traditional diuretics are not ideal therapies for hyponatremia because they can actually exacerbate hyponatremia by inducing excretion of Na⁺ in excess of free water. Thus a new class of pharmacological agents called aquarretics that selectively enhance renal water excretion has been developed. To date, antagonists that inhibit vasopressin-2 receptors (V2R) are the only class of aquarretics that have been approved by the FDA for the treatment of hyponatremia (24). Although these inhibitors are effective for treatment of hyponatremia (74), some drawbacks of their clinical use have emerged. For example, not all patients respond optimally to V2R inhibitors (52), and their use with CYP3A inhibitors (which includes numerous and diverse drugs) is contraindicated (4). Response failure to V2R inhibitors has been attributed to increased thirst (52), increased responsiveness of the receptor to its ligand (163), and the presence of activating mutations in the receptor gene in the general population (25). In addition, the FDA recently issued a safety announcement that tolvaptan should not be used for longer than 30 days and is contraindicated in patients with liver disease. Risk of liver failure was observed in an open-label extension study of a recent clinical trial using tolvaptan to treat autosomal dominant polycystic kidney disease (138). This safety announcement seriously limits the utility of tolvaptan, and possibly the entire class of V2R antagonists. Thus, if prevention or treatment of hyponatremia is clinically desirable, aquarretics with different mechanisms of action will need to be developed. Inhibiting Cl⁻ transport in the thin ascending limb with a CLC-Ka inhibitor would dissipate the medullary concentration gradient, and hence the driving force for water movement; this would represent a novel mechanism for enhancing water excretion by the kidney.

In addition to occurring in the diseases mentioned above, hyponatremia can be a serious side effect of thiazide diuretics. Thiazide-induced hyponatremia can lead to cognitive impairment, unsteady gait, falls, and hip fractures (6), which are particularly problematic in the elderly patient population (124, 133), as well as lethargy, vomiting, confusion, and even seizures (21). A combined regimen of a CLC-Ka inhibitor and thiazide diuretics would result in an increase in urine NaCl and water excretion so that hypertension can be controlled without causing hyponatremia. This strategy could lead to wider use of thiazide diuretics as a first-line therapy for hypertension.

CLC-Kb and CLC-Ka both are localized almost exclusively to the kidney and inner ear (125). In the inner ear, they are expressed in the basolateral membrane of the stria vascularis, where they are required for maintaining the K⁺ secretion performed by these cells (by allowing the parallel movement of Cl⁻) (33). The redundant role of the CLC-K channels in the inner ear is indicated by the fact that loss of either CLC-Ka or CLC-Kb function has no effect on hearing, whereas loss of both results in deafness; simultaneous loss of both CLC-Ka and CLC-Kb function can occur either via direct effects on both CLC-Ka and CLC-Kb (100, 121), or via loss of function of the required accessory subunit Barttin (12, 33). In either case, the resulting disease (Bartter’s syndrome type IV) involves severe renal salt wasting and bilateral sensorineural deafness. To preclude effects on hearing, it is crucial to develop drugs that are specific to CLC-Kb over CLC-Ka or vice versa.

In general, the pharmacology of CLC proteins is poorly developed. The so-called classic “chloride-channel inhibitors” have no selectivity among completely unrelated chloride channels, and are even known to inhibit cation channels (3, 18, 34, 67, 79). Nevertheless, recent progress demonstrates the potential for development of novel CLC-specific compounds. Compounds specific to the CLC-2 channel and to the CLC-ec1 antiporter have recently been described (59, 137). Of relevance to the kidney, Pusch and colleagues (48) have systematically characterized and developed small-molecule inhibitors and activators of the CLC-K channels. In a series of elegant studies, they evolved the low-affinity (mM) CLC-1 inhibitor p-chlorophenoxy-propionic acid (CPP) (5, 112) into a low-micromolar inhibitor of CLC-Ka, MT-189 (80, 83, 84, 108) (Fig. 3; see below). Administration of this drug to rats induced water excretion, as expected for a CLC-Ka inhibitor (81). The lack of decrease in solute accumulation in the inner medulla in this study suggests that CLC-Kb (discussed above) or other targets may be additional mediators of the effects. The excellent bioavailability of MT-189 motivates further development of this scaffold to improve specificity. The in vitro threefold selectivity of MT-189 for CLC-Ka over CLC-Kb (which is 90% identical to CLC-Ka) (83) arouses hope that it may be feasible to engineer more stringent specificity through molecular docking and rational design. Toward this end, a residue near the extracellular vestibule of the channel has been identified as a major determinant of inhibitor selectivity (83, 93).

![ClC-Ka vs CLC-Kb](image)

**Fig. 3.** Evolution of a CLC-K channel inhibitor. Evolution of CLC-K channel inhibitors is shown, as described in the text. CPP (a) was tested against a chimera of CLC-Kb and mouse CLC-Ka (CLC-K1) (79).
In parallel studies, Pusch and colleagues (82, 83, 107) also characterized the interactions of another class of compounds, the fenamates, with the CIC-K channels. The fenamates are NSAIDs that are also known to inhibit and/or activate a wide variety of ion channels (14, 19, 44, 77, 117, 153). A CIC-K channel activator could be of use in amplifying residual CIC-Kb activity in patients with Bartter’s syndrome (48, 99, 136). Extensive mutagenesis studies of CIC-Ka and CIC-Kb identified a set of three residues important for the activating effect (161). These residues form a cluster in three-dimensional space (as mapped onto a CIC-K homology model; see Ref. 47) in a region distinct from the site implicated in inhibition by MT-189. While it is not yet known whether these three residues constitute a binding site, these results will facilitate studies to design more specific drugs and thus further develop CIC pharmacology.

**Pendrin**

Pendrin is encoded by the Slc26a4 gene and mediates Na\(^+\)-independent Cl\(^-\)/HCO\(_3\)\(^-\) exchange. Inactivating mutations of Slc26a4 results in Pendred’s syndrome (123), which is characterized by hearing loss and goiter (106). In the kidney, pendrin is expressed in type B and non-A, non-B intercalated cells of the aldosterone-sensitive distal nephron (68, 116, 144), defined as the late DCT, connecting segment, and cortical CD. Pendred’s syndrome is not associated with electrolyte abnormalities in either humans or mouse models (35, 116); however, under dietary Cl\(^-\) restriction, LOF mice reveal a requirement for pendrin in maintaining acid-base balance, extracellular fluid volume, and blood pressure (145). Interestingly, although pendrin mediates Na\(^+\)-independent ion exchange, deletion of pendrin leads to urinary Na\(^+\) wasting, indicating that pendrin interacts with Na\(^+\) transport pathways (69). Indeed, expression of the epithelial sodium channel (ENaC) is reduced in the kidneys of pendrin knockout mice under conditions of NaCl restriction or mineralocorticoid administration (69). In a series of elegant experiments, Wall and colleagues (105) demonstrated that raising the luminal HCO\(_3\)\(^-\) concentration in kidney tubules reversed the decrease in ENaC expression and function in the cortical collecting ducts of pendrin knockout mice. These findings suggest that pendrin, by setting the HCO\(_3\)\(^-\) in the cortical collecting ducts of pendrin knockout mice, tubules reversed the decrease in ENaC expression and function.

Inactivation of pendrin leads to urinary Na\(^+\) wasting, indicating that pendrin interacts with Na\(^+\) transport pathways. Indeed, expression of the epithelial sodium channel (ENaC) is reduced in the kidneys of pendrin knockout mice under conditions of NaCl restriction or mineralocorticoid administration (69). In a series of elegant experiments, Wall and colleagues (105) demonstrated that raising the luminal HCO\(_3\)\(^-\) concentration in kidney tubules reversed the decrease in ENaC expression and function in the cortical collecting ducts of pendrin knockout mice. These findings suggest that pendrin, by setting the HCO\(_3\)\(^-\) concentration in the collecting duct, regulates ENaC activity and urine Na\(^+\) excretion.

Currently, no inhibitors against pendrin have been developed, but a recent study suggests that dual inhibition of pendrin and the NCC could represent a potent K\(^+\)-sparing diuretic regimen. Soleimani and colleagues (131) have demonstrated that pendrin/NCC double knockout mice exhibit severe urinary NaCl wasting, profound extracellular fluid volume depletion, and renal failure. In addition, these mice develop metabolic alkalosis and nephrogenic diabetes insipidus, but they do not develop hypokalemia. The absence of hypokalemia in pendrin/NCC double knockout mice indicates that dual inhibition of pendrin and NCC inhibits electroneutral NaCl reabsorption without stimulating K\(^+\) secretion. Thus, a potential advantage for developing pendrin inhibitors is that they could be used in combination with thiazide diuretics (inhibitors of NCC) to induce a more potent diuresis without causing hypokalemia.

**Urea Transporters**

Urea plays a critical role in the urine concentrating process because it contributes to the steep solute concentration gradient in the medullary interstitium, which drives water reabsorption. Studies in gene knockout mice demonstrate that UT-A1/3 and UT-B urea transporters are critical for urea accumulation in the inner medulla and water conservation. UT-A1/3 urea transporters are expressed in the apical membrane of the inner medullary collecting duct (40, 98) and respond to arginine vasopressin (AVP), which enhances urea permeability (70, 119) and enables urea to diffuse down a favorable concentration gradient from the collecting duct to the medullary interstitium (Fig. 1A). In UT-A1/3 double-knockout mice, isolated inner medulla CD lack urea permeability, and the urea concentration in the inner medulla of knockout mice is one-third of that in wild-type mice (39). UT-A1/3 knockout mice also demonstrate polyuria regardless of whether they are given free access to water (39). When urea excretion exceeds the ability of the inner medullary CD to reabsorb urea, urea becomes an osmotic diuretic in affected mice (38).

UT-B knockout mice also demonstrate defects in urine concentration. UT-B urea transporters are expressed in the vasa recta (Fig. 1A) and operate to ensure that urea is recycled, trapped, and maintained at a high level in the inner medulla (156). UT-B knockout mice with free access to water exhibit a higher plasma urea concentration and lower urine urea concentration than those in wild-type mice, suggesting that deletion of UT-B in vasa recta leads to washout of urea from the inner medulla. Indeed, the urea concentration of the inner medulla of knockout mice is more than two times lower compared with that in wild-type mice. Moreover, UT-B knockout mice with free access to water consume and excrete ~50% more fluid compared with their wild-type counterparts; in response to water deprivation, knockout mice cannot appropriately increase urine osmolality to conserve water (157). Interestingly, humans lacking UT-B urea transporters also exhibit modest defects in urine concentration (118).

Since urea transporters play an integral role in urine concentration, small-molecule inhibitors directed against urea transporters have the potential to disrupt renal NaCl and water handling. Using an HTS approach, Verkman and colleagues (157) identified a novel class of triazolothiopheneopyrimidine UT-B inhibitors that selectively and reversibly inhibit urea transport with nanomolar potency. Docking studies between one compound called UTB(inh)-14 and a UT-B homology model suggest that this inhibitor binds to a site in a centrally located groove of the transporter. Intraperitoneal administration of UTB(inh)-14 in mice with free access to water increases urine output and decreases urine osmolality. Additionally, administration of UTB(inh)-14 blunts DDAVP-stimulated increases in urine osmolality (159). The diuretic effect of UTB(inh)-14 is not observed in UT-B knockout mice, suggesting that effects of UTB(inh)-14 on urine concentration are specific to UT-B (159). These data illustrate that urea transport inhibitors represent a novel class of diuretics that have a different mechanism of action from conventional diuretics or aquaretics. Inhibition of urine accumulation in the medullary interstitium, either by inhibiting urea transport in the inner medullary collecting duct or urea recycling in the vasa recta, renders urea itself an osmotic diuretic.
In 2001, Lifton and colleagues (154) identified mutations in WNK1 and WNK4 that cause familial hyperkalemic hypertension (also referred to as pseudohypoaldosteronism type II or Gordon syndrome), a Mendelian form of hypertension that is also characterized by hyperkalemia and metabolic acidosis (46, 104). Mutations in WNK1 and WNK4 increase the activity of NCC in the DCT, but neither kinase directly phosphorylates the transporter. Instead, WNK1 and WNK4 signal through the STE20 kinases SPAK and OSR1. In cell-based studies, SPAK and OSR1 respond to similar signaling pathways (43) and phosphorylate and activate similar substrates such as NCC (97, 114, 143) and NKCC2 (111, 115). Moreover, both kinases are in the position to regulate NCC and NKCC2 in vivo (Fig. 1): SPAK is expressed specifically in the TALH (Fig. 1C) and DCT (Fig. 1D) (113); OSR1 is expressed in all nephron segments (96), including the TALH and DCT (158).

Transgenic knockout models, however, paint a more nuanced picture of how SPAK and OSR1 regulate NCC and NKCC2 in vivo. Kidney-specific deletion of OSR1 in mice leads to hypokalemia and hypercalciuria (85). When fed a low-Na+ diet, these knockout mice develop hypotension due to impaired renal conservation of sodium. This phenotype is reminiscent of Bartter’s syndrome type I, which arises from defects in NKCC2 (128). Indeed, expression of the activated (phosphorylated) form of NKCC2 is decreased in these knockout mice, indicating that OSR1 is a key kinase responsible for activating NKCC2 (85). On the other hand, expression of activated NCC is increased in kidney-specific OSR1 knockout mice, suggesting that NCC is not an in vivo target of OSR1 regulation but instead compensates for loss of NKCC2 function when OSR1 expression is disrupted. Consistent with this notion, the natriuretic and chloruretic effect of furosemide (an NKCC2 inhibitor), but not hydrochlorothiazide (an NCC inhibitor), is blunted in these knockout mice (85).

In contrast to the Barrter’s-like phenotype of the kidney-specific OSR1 knockout mouse model, SPAK LOF mouse models exhibit phenotypes resembling Gitelman’s syndrome, an autosomal recessive disease caused by LOF mutations in NCC (130). Similar to individuals with Gitelman’s syndrome, SPAK LOF mice have low blood pressure accompanied by hypokalemia (158), hypomagnesemia, and hypocalciuria (113, 158). Furthermore, expression of total and activated (phosphorylated) forms of NCC is decreased in SPAK LOF mouse models (113, 158), a finding that is consistent with a defect in NCC activity. Finally, the natriuretic and chloruretic effect of hydrochlorothiazide, but not furosemide, is blunted in one SPAK LOF mouse model (158), confirming that NCC is indeed an in vivo target of SPAK.

Although it is clear that SPAK regulates NCC in vivo, it is not yet clear whether NKCC2 is also an in vivo target because the mouse models differ with respect to the status of NKCC2 phosphorylation, which represents a biochemical surrogate for activity. These discrepancies may relate to how SPAK function was disrupted in the different mouse models. SPAK knockin mice, in which SPAK catalytic activity is inactivated, exhibit a decrease in NKCC2 expression and phosphorylation (113), whereas SPAK-deletion mice exhibit an increase in NKCC2 expression and phosphorylation (158). Recent studies have indicated that deletion of SPAK eliminates expression of not only full-length SPAK but also other inhibitory SPAK isoforms in the TALH, which allows other kinases such as OSR1 or AMPK to compensate and increase NKCC2 phosphorylation and activity (51, 95). Inactivation of SPAK catalytic activity, on the other hand, specifically eliminates SPAK activity, but it does not alter the inhibitory activity of other SPAK isoforms in the TALH; as a result, NKCC2 phosphorylation remains suppressed (95).

If OSR1 and SPAK LOF mouse models accurately recapitulate human renal physiology, small-molecule inhibitors of SPAK or OSR1 would represent a novel, alternative strategy for inhibiting NaCl reabsorption in the TALH (Fig. 1C) and DCT (Fig. 1D). An OSR1-specific inhibitor would preferentially inhibit NKCC2 in the TALH, whereas a SPAK-specific inhibitor would inhibit NKCC2 in the TALH and NCC in the DCT. Inhibiting NaCl reabsorption in two successive nephron segments with a SPAK inhibitor would have a similar effect to that of combination therapy with loop and thiazide diuretics, a strategy that is currently used in clinical practice to induce a potent diuresis in individuals with diuretic resistance (31, 32). Another potential advantage for developing an OSR1 or SPAK inhibitor is that the side effect profile of either inhibitor may be distinct from that of loop and thiazide diuretics. Examples of side effects of thiazides include hypernatremia, hyperglycemia, hyperlipidemia, and hyperuricemia, whereas side effects of loop diuretics include severe hypokalemia and ototoxicity. An OSR1 or SPAK inhibitor without these side effects may lead to wider use of diuretic therapy for the treatment of hypertension as well as diseases associated with elevated extracellular fluid volume.

Concluding Remarks

The molecular revolution that occurred in the late 20th century and continues today has led to an explosion in our understanding of disease pathways and potential therapeutic targets. With more than a century of elegant physiological investigations behind us, one of the grand challenges facing renal physiologists in the coming decades will be the translation of these discoveries into novel therapies to improve human health. Approximately one in three American adults have hypertension, and only about half of these are managing their blood pressure correctly due in part to inadequate therapy. A review of the physiological, genetic, and pharmacological data pointing to specific ion channels (i.e., Kir1.1, Kir4.1/5.1, ClC-Ka, ClC-Kb), transporters (i.e., UTA/B, pendrin), and regulatory proteins (i.e., SPAK) participating in the regulation of fluid-volume homeostasis by the kidney highlights the tremendous opportunities renal pharmacologists have to develop more diverse and efficacious antihypertensive drugs. Without exception, the molecular pharmacology of the putative diuretic targets described in this review is limited at best. The continued development of small-molecule ligands will provide critically needed tools to explore the integrative physiology and druggability of these pathways and accelerate efforts to bridge the bench and bedside.

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