Salt Sensitivity

Chronic Inhibition of Renal Outer Medullary Potassium Channel Not Only Prevented but Also Reversed Development of Hypertension and End-Organ Damage in Dahl Salt-Sensitive Rats


Abstract—The renal outer medullary potassium (ROMK) channel mediates potassium recycling and facilitates sodium reabsorption through the Na+/K+/2Cl− cotransporter in the loop of Henle and potassium secretion at the cortical collecting duct. Evidence from the phenotype of humans and rodents with functional ROMK deficiency supports the contention that selective ROMK inhibitors (ROMKi) will represent a novel diuretic with potential of therapeutic benefit for hypertension. ROMKi have recently been synthesized by Merck & Co, Inc. The present studies were designed to examine the effects of ROMKi B on systemic hemodynamics, renal function and structure, and vascular function in Dahl salt-sensitive rats. Four experimental groups—control, high-salt diet alone; ROMKi B 3 mg·kg−1·d−1; ROMKi B 10 mg·kg−1·d−1; and hydrochlorothiazide 25 mg·kg−1·d−1—were included in prophylactic (from week 1 to week 9 on high-salt diet) and therapeutic studies (from week 5 to week 9 on high-salt diet), respectively. ROMKi B produced sustained blood pressure reduction and improved renal and vascular function and histological alterations induced by a high-salt diet. ROMKi B was superior to hydrochlorothiazide at reducing blood pressure. Furthermore, ROMKi B provided beneficial effects on both the plasma lipid profile and bone mineral density. Chronic ROMK inhibition not only prevented but also reversed the development of hypertension and end-organ damage in Dahl salt-sensitive rats. Our findings suggest a potential utility of ROMKi B as a novel antihypertensive agent, particularly for the treatment of the salt-sensitive hypertension patient population. (Hypertension. 2017;69:332-338. DOI: 10.1161/HYPERTENSIONAHA.116.08358.)

Key Words: Dahl salt-sensitive rats ■ end-organ protection ■ hydrochlorothiazide ■ hypertension ■ ROMK inhibitor

Renal outer medullary potassium channel (ROMK) is encoded by the KCNJ1 (potassium inwardly-rectifying channel, subfamily J, member 1) gene and expressed in the apical membranes of thick ascending limb of Henle and cortical collecting duct cells; ROMK mediates potassium recycling and facilitates sodium reabsorption through Na+/K+/2Cl− cotransporter in the thick ascending limb of Henle and potassium secretion in cortical collecting duct.1−3 Thus, ROMK plays a critical role in the regulation of renal sodium reabsorption and the body’s potassium homeostasis. Human genetic studies indicated that loss-of-function mutations in ROMK cause type II Bartter syndrome,4−6 featuring polyuria, polydipsia, salt wasting, hypokalemia, alkalosis, hypercalciuria, low blood pressures, elevated plasma renin and aldosterone, and excess production of renal prostaglandins. ROMK heterozygous mutations in humans protect from the development of hypertension.7 ROMK-deficient mice exhibit a Bartter syndrome type II–like phenotype.8,9 Heterozygous disruption of ROMK in rats is associated with reduced blood pressure and less severe renal injury.10 Furthermore, acute pharmacological intervention with a small molecule ROMK inhibitor (ROMKi) compound A evoked diuresis and natriuresis in rats and dogs,11 which establishes the concept that ROMK inhibition represents a novel diuretic mechanism. We, therefore, hypothesize that chronic ROMK inhibition by selective small molecules would induce natriuresis and diuresis and, thereby, lower blood pressure and protect from end-organ damage in hypertensive subjects. To this end, we examined the effects of a selective and potent ROMKi B, recently synthesized by Merck & Co, Inc,12 on systemic hemodynamics, renal function and structure, vascular function, and cardiac structure and compared the effects with those of hydrochlorothiazide (HCTZ) in 2 separate patient populations.

The online-only Data Supplement is available with this article at http://hyper.ahajournals.org/lookup/suppl/doi:10.1161/HYPERTENSIONAHA.116.08358/-/DC1.

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Correspondence to Xiaoyan Zhou, Department of Cardiometabolic Diseases, Merck & Co, Inc, 2000 Galloping Hill Rd, Kenilworth, NJ 07033. E-mail xiaoyan_zhou@merck.com

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studies (prophylactic and therapeutic dosing regimens) in Dahl salt-sensitive (Dahl SS) rats, a rodent model of salt-sensitive hypertension.13–15

Materials and Methods
Four experimental groups—control, high-salt diet alone; ROMKi B 3 mg·kg⁻¹·d⁻¹; ROMKi B 10 mg·kg⁻¹·d⁻¹; and HCTZ 25 mg·kg⁻¹·d⁻¹—were included in prophylactic (from week 1 to week 9 on high-salt diet) and therapeutic studies (from week 5 to week 9 on high-salt diet), respectively. The compounds were administrated in feed. Blood pressure was measured by radiotelemetry. Renal function was assessed in metabolic cage studies. Vascular function was assessed using a vascular relaxation assay.

A detailed Materials and Methods section is given in the online-only Data Supplement.

Statistical Analyses
All data are presented as means±standard error of the mean. A repeated measure analysis of variance was used for time course data analysis. For end point comparisons among all groups, 1-way analysis of variance followed by Newman–Keuls post hoc test was used. P values of <0.05 were considered to be of statistical significance.

Results
Effects of ROMKi B and HCTZ on Blood Pressure and Heart Rate
In the prophylactic study, systolic and diastolic blood pressures were 148±0.9 and 100±1.5 mm Hg, respectively, while the Dahl SS rats were on a control (0.3% NaCl) diet; systolic and diastolic blood pressures increased to 161±0.5 and 110±1.0 mm Hg, respectively, after switching to a high-salt (4% NaCl) diet for 1 week. The systolic and diastolic blood pressures progressively increased to 217±5.7 and 156±4.3 mm Hg, respectively, over the subsequent 8-week period of high salt feeding in the vehicle group, which reflects the salt-sensitive characteristic of this rat strain. The systolic and diastolic blood pressures were significantly reduced in those animals that received ROMKi B at either 3 or 10 mg·kg⁻¹·d⁻¹ dose and HCTZ at 25 mg·kg⁻¹·d⁻¹ treatment; the magnitude of systolic blood pressure reduction was significantly but transiently increased heart rate (≈10% increase for the first 3 days). Heart rate in the control group was significantly increased after 5 weeks of high-salt challenge, which may reflect cardiac dysfunction in the control group (Figure 1).

In the therapeutic study, systolic and diastolic blood pressures were increased to 198±2.7 and 140±4.6 mm Hg after 5 weeks of 4% NaCl diet in all rats. Four weeks of treatment with ROMKi B at 3 or 10 mg·kg⁻¹·d⁻¹ and HCTZ at 25 mg·kg⁻¹·d⁻¹ lowered systolic blood pressure by 33, 51, and 23 mm Hg and diastolic blood pressure by 22, 35, and 14 mm Hg, respectively. Heart rate changes at the initiation of the treatment were similar to those in the prophylactic study, namely, a transient increase in the higher dose of ROMKi B treatment group (Figure 1).

Effects of ROMKi B and HCTZ on Renal Excretory Function and Kidney Injury Biomarkers
Food intake or body weight was not different among all groups during the studies. Water intake and urine output were in balance. Neither ROMKi B nor HCTZ had a significant effect on urinary excretion of Na⁺, K⁺, Cl⁻, Mg²⁺, and PO₄³⁻ (data not shown). However, ROMKi B at 10 mg·kg⁻¹·d⁻¹ caused a significant urinary loss of Ca²⁺, while animals that received HCTZ demonstrated a retention of Ca²⁺ in both the prophylactic and the therapeutic studies (Figure S2 in the online-only Data Supplement).

Proteinuria is a biomarker of glomerular and tubular injury. Urinary protein excretion was markedly and progressively increased in Dahl SS rats on the 4% NaCl diet, which was significantly attenuated in animals that received either ROMKi B or HCTZ, with a greater beneficial effect in the high dose of ROMKi B group in both prophylactic and therapeutic studies (Figure 2). Lipocalin-2 is a biomarker of renal tubular epithelial injury, and kidney injury molecule-1 is a protein that is specifically expressed in proximal tubules and is a sensitive biomarker for proximal tubular injury. Both ROMKi B and HCTZ significantly decreased urinary excretion of lipocalin-2 and kidney injury molecule-1 in both prophylactic and therapeutic studies (Figure S3). Other kidney injury biomarkers, such as osteopontin and renal papillary antigen 1, had similar changes as lipocalin-2 and kidney injury molecule-1 (data not shown).

Plasma Electrolytes, Creatinine, Cystatin C, Lipids, and Compound Concentrations
Plasma electrolytes (Na⁺, K⁺, Cl⁻, Mg²⁺, and Ca²⁺) were not different among all groups at the end of each study (data not shown). Both ROMKi B and HCTZ significantly decreased plasma creatinine and serum cystatin C levels in the prophylactic but not the therapeutic study (Figure S4). Interestingly, prophylactic and therapeutic treatment with ROMKi B or HCTZ also decreased plasma cholesterol and low-density lipoprotein levels (Figure S5). Plasma concentrations of ROMKi B at 3 or 10 mg·kg⁻¹·d⁻¹ and HCTZ at 25 mg·kg⁻¹·d⁻¹ groups were ≈0.23, 0.66, and 1.39 μmol/L, respectively. Of note, the IC₅₀ of ROMKi B is ≈20 nmol/L (Figure S5); the plasma concentrations achieved in the present study provide a >90% ROMK inhibition. Concentrations of ROMKi B and HCTZ were also monitored in feed at weeks 0, 4, and 8 and confirmed that both compounds were stable in the diet.

Bone Mineral Density
Because the high dose of ROMKi B induced increased urinary calcium loss, we evaluated whether this would affect bone mineral density (BMD). The bone dual-energy X-ray absorptiometry results demonstrated that prophylactic treatment with either ROMKi B or HCTZ significantly increased femur and lumbar spine BMD, and ROMKi B had no effect on femur and lumbar spine BMD in the therapeutic study (Figure S6).
The vasorelaxant response of thoracic aorta from all groups to acetylcholine or sodium nitroprusside (SNP) is summarized in Figure 3. Our data show that both ROMKi B and HCTZ shift the acetylcholine and SNP dose–response curve to the left, which indicates increased acetylcholine-induced nitric oxide–mediated vasorelaxation and increased sensitivity to nitric oxide donation by SNP in aorta, demonstrating preserved endothelium-dependent and -independent responses.

Organ Weights and Histopathologic Findings
Total kidney weight/body weight and heart weight/body weight ratios are summarized in Table. Representative light microscopic findings in kidneys and hearts from the prophylactic study and renal and cardiac histopathologic scores from the therapeutic study are shown in Figures 4 and 5 and Figures S7 and S8. In brief, treatment with either ROMKi B or HCTZ significantly decreased kidney and heart weight, while there were no body weight changes. Microscopic findings in the kidney exhibited severe focal-segmental or global glomerulosclerosis, tubular dilation and protein cast formation, inflammatory cell infiltration, and perivascular fibrosis. Findings in the heart showed myocardial degeneration, interstitial inflammatory cell infiltration, and arteriolar hyalinization in the vehicle group. Both ROMKi B and HCTZ significantly improved the above-described histopathological changes and
the quantitative injury scores for each component of the kidney and heart.

**Discussion**

Our results demonstrate that chronic ROMK inhibition completely blocked the development of hypertension in a rodent model of salt-sensitive hypertension, with no evidence of tolerance developing during the 8-week chronic study. The blood pressure lowering effect of HCTZ was blunted over time. Hence, ROMKi B is superior to that of HCTZ in blood pressure reduction. Chronic ROMK inhibition and HCTZ treatment also prevented end organs from high salt–induced damage as demonstrated by preserved glomerular (plasma creatinine and cystatin C levels were used as estimators of glomerular filtration rate) and renal tubular function, vascular function, renal and cardiac histopathology in the compound treatment groups. Moreover, chronic ROMK inhibition lowers blood pressure in established hypertension, ameliorates glomerular and renal tubular injury, slows down the progression of proteinuria, and improves vascular endothelial and smooth muscular function, as well as renal and cardiac histopathologic changes. Thus, chronic ROMK inhibition not only prevents but also reverses the development of hypertension and end-organ damage in Dahl SS rats.

We have previously performed a pilot study by orally dosing ROMKi B or HCTZ for 3 days to assess natriuresis/diuresis and blood pressure–dose response relationship in Dahl SS rats. We found that both compounds evoked an acute (0–4 hour) but not prolonged (0–24 hour) natriuretic/diuretic response in a dose-dependent manner; ROMKi B at 3 mg·kg⁻¹·d⁻¹ and HCTZ at 25 mg·kg⁻¹·d⁻¹ had an equivalent blood pressure–lowering efficacy (data not shown). Based on this information, we chose the above tested doses plus a high dose (10 mg·kg⁻¹·d⁻¹) of ROMKi B in our present studies. No 24-hour natriuretic/diuretic response to ROMKi was detected by weekly monitoring of renal excretory function in the present chronic studies, which is consistent with the findings from the previous pilot oral dosing study. Because compounds were administrated via medicated diet, it is difficult to determine postdosing time period, and no 0–4 hour urine collection was taken in the present studies. Nevertheless, our data clearly demonstrated that ROMKi B significantly lowers blood pressure in the absence of 24-hour natriuresis/diuresis. The underlying mechanism responsible for blood pressure reduction by ROMK inhibition is not clear, but it could be associated with pressure–natriuresis response alteration. It has been shown that Na⁺/K⁺/2Cl⁻ cotransporter and ROMK expression is elevated, and chloride and water reabsorption in the loop of Henle is enhanced in Dahl SS rats. This intrinsic inability to excrete salt and water results in abnormal

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**Table. Total KW/BW and HW/BW Ratios in all Groups**

<table>
<thead>
<tr>
<th>Groups</th>
<th>Total KW/BW, g/kg</th>
<th>HW/BW, g/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Prophylactic study</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>10.3±0.2</td>
<td>4.8±0.1</td>
</tr>
<tr>
<td>ROMKi B 3 mg·kg⁻¹·d⁻¹</td>
<td>8.7±0.2*</td>
<td>3.9±0.1*</td>
</tr>
<tr>
<td>ROMKi B 10 mg·kg⁻¹·d⁻¹</td>
<td>8.6±0.1*</td>
<td>3.7±0.1*</td>
</tr>
<tr>
<td>HCTZ 25 mg·kg⁻¹·d⁻¹</td>
<td>9.1±0.2*</td>
<td>4.0±0.1*</td>
</tr>
<tr>
<td><strong>Therapeutic study</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control diet</td>
<td>6.8±0.1</td>
<td>3.5±0.1</td>
</tr>
<tr>
<td>Baseline</td>
<td>9.6±0.3</td>
<td>4.2±0.1</td>
</tr>
<tr>
<td>Vehicle</td>
<td>10.3±0.3</td>
<td>4.7±0.1</td>
</tr>
<tr>
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<td>4.0±0.1*</td>
</tr>
</tbody>
</table>

Data are mean±SEM. BW indicates body weight; HCTZ, hydrochlorothiazide; HW, heart weight; KW, kidney weight; and ROMKi, renal outer medullary potassium inhibitor.

*P<0.05 vs vehicle group.
renal hemodynamics, abnormal pressure–natriuresis relationship, and development of hypertension. Blood pressure is increased to compensate for this intrinsic renal excretory inability to maintain sodium and water balance. With ROMK inhibition, less salt and water are reabsorbed from the loop of Henle, facilitating an increased amount of salt and water being excreted from the kidney. Blood pressure is consequently decreased, and the pressure–natriuresis curve is shifted to the left. That is to say, ROMK inhibition evoked sodium excretion, which may offset this intrinsic inability of sodium excretion from the kidney in Dahl SS rats, ultimately leading to blood pressure reduction.

ROMK plays a critical role in the regulation of renal sodium reabsorption and the body’s potassium homeostasis by facilitating sodium reabsorption through Na+/K+/2Cl− cotransporter in thick ascending limb of Henle and mediating potassium secretion in cortical collecting duct. Therefore, ROMK inhibition could be potassium sparing while causing natriuresis/diuresis, which is a favorable feature for a novel diuretic and would differentiate ROMKi from conventional loop diuretics such as furosemide that cause hypokalemia. Indeed, in our present studies, ROMKi B did not cause increased urinary K+ excretion and had no effect on plasma K+ concentration. However, it is of note that ROMK-mediated K recycling in thick ascending limb of Henle also generates positive potential in the lumen side, driving Ca2+ and Mg2+ reabsorption through a paracellular pathway. Thus, ROMK inhibition could lead to increased urinary Ca2+ and Mg2+ loss.

In our studies, the high dose of ROMKi B caused significant urinary loss of Ca2+; in contrast, HCTZ induces hypocalciuria that is suggested to result from enhancement of passive Ca2+ reabsorption in proximal tubules or stimulation of active

Figure 4. Representative light microscopic findings (10×) in renal histopathology from the prophylactic study. Vehicle group (A) exhibited severe focal-segmental or global glomerulosclerosis, tubular dilation and protein cast formation, inflammatory cell infiltration, and perivascular fibrosis. These lesions were significantly improved by either renal outer medullary potassium inhibitor (ROMKi) B (B and C) or hydrochlorothiazide (HCTZ; D) treatment.

Figure 5. Renal histopathologic scores from the therapeutic study. Data are mean±SEM (n=8 for each group, except for n=6 in the control group and 4% NaCl baseline group). Both renal outer medullary potassium inhibitor (ROMKi) B and hydrochlorothiazide (HCTZ) significantly decreased glomerular (A), tubular (B), interstitial (C), and vascular (D) scores in kidneys. Vehicle group had a remarkably greater score in each of the above components than the control group. *P<0.05 vs vehicle; †P<0.05 vs HCTZ.
Ca$^{2+}$ reabsorption in distal convoluted tubules. Because of the concern for the potential effect of increased Ca$^{2+}$ loss on bones, we measured femur and lumbar spine BMD by bone dual-energy X-ray absorptiometry, and interestingly, BMD was increased in the prophylactic study and was unaffected in the therapeutic study in ROMKi B 3 mg·kg$^{-1}$·d$^{-1}$ and 10 mg·kg$^{-1}$·d$^{-1}$-treated groups. We hypothesized that ROMK inhibition could improve regional hemodynamics and organ function by decreasing systemic blood pressure; thereby, Ca$^{2+}$ reabsorption from the digestive system could be enhanced, and ultimately BMD could be unaffected or even improved. Obviously, this presumption needs to be tested in a separate study. It is also intriguing that decreased plasma cholesterol and low-density lipoprotein levels were observed with both prophylactic and therapeutic treatment with ROMKi B or HCTZ, which again might be associated with blood pressure reduction and subsequent improved organ function and lipid metabolism.

ROMKi B at 3 mg·kg$^{-1}$·d$^{-1}$ and HCTZ at 25 mg·kg$^{-1}$·d$^{-1}$ had similar beneficial effects on organ protection. Although the blood pressure-lowering effect of HCTZ was blunted over time, the blood pressure in this group remained significantly lower than that in the vehicle group. It is of note that ROMKi B at the high dose of 10 mg·kg$^{-1}$·d$^{-1}$ had a greater effect on mitigating proteinuria in the prophylactic study and a superior effect in improving myocardial scores in the therapeutic study compared with that of HCTZ at 25 mg·kg$^{-1}$·d$^{-1}$. The beneficial organ protective effects of ROMKi B or HCTZ are most likely attributed to an improvement of systemic hemodynamics. ROMKi B at the high dose of 10 mg·kg$^{-1}$·d$^{-1}$ almost normalized blood pressure, and it provided the greatest benefits in renal and vascular function and renal and cardiac histopathology.

This is the first report to demonstrate that ROMKi lowers blood pressure and provides end-organ protection in a hypertensive rat model. Compared with the phenotype of genetically engineered ROMK-deficient mice, pharmacological inhibition of ROMK did not adversely affect the animal’s survival rate, electrolytes and acid–base balance, kidney function, and structure. In contrast, ROMKi delivered favorable outcomes.

In conclusion, chronic ROMK inhibition not only prevents but also reverses the development of hypertension and end-organ damage in Dahl SS rats. Our findings suggest a potential utility of ROMKi as novel antihypertensive agents, particularly in treating the salt-sensitive hypertension patient population.

**Perspectives**

We previously reported that heterozygous disruption of ROMK in Dahl SS rats exhibited reduced blood pressure and protection from renal injury, which underscores a critical role of ROMK in blood pressure regulation. We further demonstrated in the present study that pharmacological inhibition of ROMK using a small molecule not only prevented but also reversed development of hypertension and end-organ damage in Dahl SS rats. Our findings suggest a potential utility of ROMKi as novel antihypertensive agents, particularly in treating the salt-sensitive hypertension patient population.

**Acknowledgments**

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**Disclosures**

All authors are or were employees at Merck & Co, Inc, and may hold stock or stock options of Merck & Co.

**Reference**


What Is New?

- This is the first report to demonstrate that pharmacological inhibition of renal outer medullary potassium using a small molecule lowers blood pressure and provides end-organ protection in a hypertensive rat model.
- Renal outer medullary potassium inhibitor B was tested in prophylactic as well as therapeutic studies in chronic settings.

What Is Relevant?

- Our findings suggest a potential utility of renal outer medullary potassium inhibitor as novel antihypertensive agents, particularly in treating the salt-sensitive hypertension patient population.

Summary

We demonstrated that pharmacological inhibition of renal outer medullary potassium using a small molecule not only prevented but also reversed development of hypertension and end-organ damage in Dahl salt-sensitive rats.
Chronic Inhibition of Renal Outer Medullary Potassium Channel Not Only Prevented but Also Reversed Development of Hypertension and End-Organ Damage in Dahl Salt-Sensitive Rats


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Chronic Inhibition of Renal Outer Medullary Potassium Channel Not Only Prevented but Also Reversed Development of Hypertension and End Organ Damage in Dahl Salt Sensitive Rats

Xiaoyan Zhou¹, Michael J Forrest², Wanda Sharif-Rodriguez¹, Gail Forrest², Daphne Szeto², Olga Urosevic-Price², Yonghua Zhu¹, Andra S. Stevenson¹, Yuchen Zhou², Sloan Stribling², Maya Dajee¹, Shawn P Walsh³, Alexander Pasternak³, Kathleen A Sullivan¹

Department of ¹Cardiometabolic Diseases, ²In Vivo Pharmacology, ³Chemistry, Merck & Co., Inc., 2000 Galloping Hill Road, Kenilworth, NJ 07033 USA

Running Title: Blood Pressure and End Organ Effects of ROMK inhibition

Correspondence:

Xiaoyan Zhou

Department of Cardiometabolic Diseases

Merck & Co.,

2000 Galloping Hill Road,

Kenilworth, NJ 07033, United States

Tel: 908-740-4405

Fax: 908-740-4020

Email: xiaoyan_zhou@merck.com
Expanded Materials and Methods

**Animals.** Dahl SS rats were purchased from Harlan Labs. Inc. (Indianapolis, Indiana). All rats were housed in a temperature- and humidity-controlled room with a 12:12-hour dark-light cycle with food and water provided ad libitum. Rodent diets (#7034, control diet, containing 0.3% NaCl; TD.92034, high salt diet, containing 4% NaCl) were purchased from Harlan Teklad (Madison, Wisconsin). ROMKi B was synthesized by Merck & Co., HCTZ was purchased from MP Biomedicals, LLC (Solon, Ohio). The medicated diets (either ROMKi B or HCTZ mixed in TD.92034) were prepared by Research Diets, Inc. (New Brunswick, New Jersey). The concentration of tested compound in the diet was calculated to give a daily dose of 3 or 10 mg•kg⁻¹•d⁻¹ of ROMKi B and 25 mg•kg⁻¹•d⁻¹ of HCTZ based on food intake relative to body weight.

All procedures utilizing experimental animals were conducted in accordance with the Guide for the Care and Use of Laboratory Animals, and experimental protocols were approved in advance by the Institutional Animal Care and Use Committee at Merck Research Laboratories, Rahway, NJ. All studies were conducted in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.

**Radiotelemetry implant surgery.** Male Dahl SS rats (8 weeks old) were anesthetized with isoflurane and pre-medicated with buprenorphine (0.03 mg/kg, s.c.: Reckitt Benckiser healthcare Ltd., Hull, England) prior to surgery. Telemetry devices (TA11PA-C40, Data Sciences International, DSI, St. Paul, Minnesota) were aseptically placed in a subcutaneous pocket on one side of the body with the catheter inserted into the descending aorta via the femoral artery. Penicillin G (150,000 U/kg, s.c. Bimed Inc., Irwindale, California) was administered at the end of surgery. Rats were allowed to recover for 3 weeks prior to experimentation.

**Experimental design.** Male Dahl SS rats with implanted radiotelemetry devices were housed individually in Nalgene metabolism cages (Braintree Scientific, INC., Braintree, Massachusetts) and used for the study. All animals were maintained on a control diet (0.3% NaCl) prior to experimentation. In the prophylactic study, all rats were switched to a high salt (4% NaCl) diet for one week of diet acclimation prior to compounds administration. The rats were then randomly divided into four groups (n=8 in each group): Group 1, high salt diet alone (4% NaCl); Group 2, ROMKi B 3 mg•kg⁻¹•d⁻¹; Group 3, ROMKi B 10 mg•kg⁻¹•d⁻¹; Group 4, HCTZ 25 mg•kg⁻¹•d⁻¹. The compounds were mixed in the 4% NaCl diet in each treatment group and the treatment lasted 8 weeks. In the therapeutic study, all rats were fed the 4% NaCl diet for 5 weeks (including one week of acclimation) before being randomly assigned to one of the above-described groups. The compound treatment continued for 4 weeks. The schematic experimental protocols for prophylactic and therapeutic studies are shown in Figure 1. In addition, two separate groups of rats (without radiotelemetry devices): control diet alone for 9 weeks (n=6) and 4% NaCl diet for 5 weeks (n=6), were included for a complete comparison in the therapeutic study.

**Systemic hemodynamics, renal function and kidney injury biomarkers assessment.** Radiotelemetry signals were collected and analyzed using DSI Dataquest system version 4.1 (Data Sciences International, DSI, St. Paul, Minnesota). Systolic, diastolic, mean, and pulse arterial blood pressures and heart rate were determined on a beat-by-beat basis. Data was collected for 10 minutes every hour and was reported as average values for each animal over a
24-hour period. Twenty-four hour food and water intake, and urine output were monitored once a week during the study. Urinary electrolytes (including Na\(^+\), K\(^+\), Cl\(^-\), Mg\(^{2+}\), Ca\(^{2+}\), and PO\(_4^{3-}\)) and protein concentration were assessed by a Roche Modular Chemistry System (Roche Diagnostics, Indianapolis, IN). Kidney injury biomarkers including lipocalin-2 (LPN), osteopontin (OPN), kidney injury molecule-1 (KIM-1), and renal papillary antigen 1 (RPA-1) were detected using Kidney Injury Panel 1 (rat) kit and Argutus AKI (rat) kit from Meso Scale Discovery, LLC. (Gaithersburg, Massachusetts). All these biomarkers have previously been validated in animal studies [1].

**Plasma electrolytes, creatinine, cystatin C, lipids, and compound concentration measurement.** Upon study termination, animals were euthanized by CO2 inhalation and cardiac puncture was performed to collect blood for determination of plasma electrolytes, creatinine, cystatin C, cholesterol and LDL level (Roche Diagnostics, Indianapolis, IN) as well as ROMKIB and HCTZ concentrations (determined by LC/MS/MS following protein precipitation with acetonitrile). Serum cystatin C, used as an estimator of glomerular filtration rate (GFR), has previously been validated in animal studies [1].

**Bone mineral density measurement.** Femur and lumbar spine were harvested at the termination of study. All specimens were shipped to Numira Bioscience (Bothell, Washington) to perform bone Dual-energy X-ray absorptiometry (DEXA) analysis blindly, including bone mineral content (BMC, g), area of bone (cm\(^2\)), and bone mineral density (BMD/area, g/cm\(^2\)). Femur regions (whole, distal, distal femoral metaphysis, central, and proximal femur) and lumbar spine regions (L1-L4, L1-L2, and L3-L4) were scanned and individual data was captured and analyzed.

**Evaluation of vascular function.** The thoracic aorta was harvested for evaluation of ex-vivo vasoreactivity using a DMT Myograph system (Danish Myo Technology, DMT, Aarhus N, Denmark) at the study termination. In brief, aortic rings were mounted between two stirrups in organ chambers circulated with oxygenated modified Krebs-Ringer bicarbonate solution ([in mmol/L] NaCl 118.6, KCl 4.8, CaCl\(_2\) 2.5, MgSO\(_4\) 1.2, K\(_2\)PO\(_4\) 1.2, NaHCO\(_3\) 25.1, glucose 10.1, and EDTA 0.026) at 37°C. The aortic rings were precontracted with phenylephrine (1µmmol/L) and baseline force was recorded, then an ascending concentration (10\(^{-11}\) to 10\(^{-4}\) mmol/L) of either acetylcholine (ACH) or sodium nitroprusside (SNP) was added to the bathing solution, respective force was recorded at each concentration of either ACH or SNP in each individual ring to assess the endothelium-dependent and endothelium-independent relaxations [2], respectively.

**Histopathology.** Kidney and heart tissues were harvested from all animals, fixed in Prefer (Anatech LTD, Battle Creek, Michigan) for at least 24 hours, and then paraffin embedded. Tissue sections were stained with hematoxylin and eosin. The severity of histopathological changes in tubules, interstitium, vasculature, and glomeruli of the kidney were graded on a 0 to 5 scale corresponding to normal, minimal, mild, moderate, marked, and severe [3, 4]. Similarly, the severity of histopathological changes of vasculature, myocardium, and interstitium in the heart were also graded on a 1 to 5 scale corresponding to minimal, mild, moderate, marked, and severe [4]. Sections from both kidneys and the heart were examined and the final scores composite that from all sections of each slice. Histology scoring was performed blinded.

Reference (for Expanded Materials and Methods)


Figure S1. Schematic experimental protocols for the prophylactic (A) and therapeutic (B) studies. The experimental protocols began when rats were approximately 11 weeks old and ended at around 20 weeks of age. Control diet, 0.3% NaCl; high salt diet, 4% NaCl. In the prophylactic study, all rats were switched to 4% NaCl diet for one week of diet acclimation prior to compounds administration. In the therapeutic study, all rats were fed 4% NaCl diet for 5 weeks (including one week of acclimation) before initiation of compound treatment. Four major study groups are included: Group 1, high salt diet alone; Group 2, ROMKi B 3 mg·kg\(^{-1}\)·d\(^{-1}\); Group 3, ROMKi B 10 mg·kg\(^{-1}\)·d\(^{-1}\); Group 4, HCTZ 25 mg·kg\(^{-1}\)·d\(^{-1}\).
Figure S2. Time course of 24-hour urinary Ca^{2+} excretion in all groups. Data are mean ± SEM (n=8 for each group). ROMKi B 10 mg.kg^{-1}d^{-1} significantly increased but HCTZ 25 mg.kg^{-1}d^{-1} decreased urinary Ca^{2+} excretion in both prophylactic (A) and therapeutic (B) studies. * p<0.05 vs. vehicle.
Figure S3. Time course of 24-hour urinary kidney injury biomarkers in all groups. Data are mean ± SEM (n=8 for each group). KIM-1, kidney injury molecule-1. Urinary kidney injury biomarkers were significantly decreased by either ROMKi B or HCTZ treatment (Changes in osteopontin and renal papillary antigen 1 are similar to Lipocalin-2 and KIM-1, data are not shown here). * p<0.05 vs. vehicle.
Figure S4. Chronic effects of ROMKi B and HCTZ on plasma creatinine and serum cystatin C levels in Dahl SS rats. Data are mean ± SEM (n=8 for each group, except for n=6 in the control group and 4% NaCl baseline group). Both ROMKi B and HCTZ significantly decreased plasma creatinine and serum cystatin C levels in the prophylactic (A & B) but not therapeutic (C & D) study. * p<0.05 vs. vehicle; † <0.05 vs. HCTZ.
Figure S5. Chronic effects of ROMKi B and HCTZ on plasma lipid levels in Dahl SS rats. Data are mean ± SEM (n=8 for each group, except for n=6 in the control group and 4% NaCl baseline group). Plasma cholesterol (A & C) and LDL (B & D) levels were significantly decreased by treatment with ROMKi B or HCTZ. Vehicle group had a greater plasma lipid level than the control group. * p<0.05 vs. vehicle; † p<0.05 vs. HCTZ.
Figure S6. Chronic effects of ROMKi B and HCTZ on bone mineral density (BMD) in Dahl SS rats. Data are mean ± SEM (n=8 for each group, except for n=6 in the control group and 4% NaCl baseline group). Prophylactic (A & B) treatment with either ROMKi B or HCTZ significantly increased femur (A & C) and lumbar (B & D) spine BMD. ROMKi B had no effect on BMD, but HCTZ increased femur BMD in the therapeutic (C & D) study. * p<0.05 vs. vehicle.
Figure S7. Prophylactic study. Vehicle group exhibited significant cardiac fibrosis (Panel A), which was significantly improved by either ROMKi B (Panel B & C) or HCTZ (Panel D) treatment.
Figure S8. Cardiac histopathological scores from the therapeutic study. Data are mean ± SEM (n=8 for each group, except for n=6 in the control group and 4% NaCl baseline group). Both ROMKi B and HCTZ significantly decreased vascular (A), myocardial (B), and interstitial (C) scores in hearts. Vehicle group had a remarkably greater score in each of the above components than the control group. * p<0.05 vs. vehicle; † p<0.05 vs. HCTZ.