PHARMACOLOGICAL INHIBITION OF THE RENAL OUTER MEDULLARY POTASSIUM CHANNEL CAUSES DIURESIS AND NATRIURESIS IN THE ABSENCE OF KALIURESIS

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ROMK inhibitors cause diuresis and natriuresis in the absence of kaliuresis

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Abbreviations: ROMK, renal outer medullary potassium channel; TALH, thick ascending loop of Henle; DCT, distal convoluted tubule; CCD, cortical collecting duct;
Kir, inwardly rectifying potassium channel; DMSO, dimethyl sulfoxide; HCTZ, hydrochlorothiazide; MEM, minimum essential medium; FBS, fetal bovine serum; HBSS, Hank’s buffered saline solution; SD, Sprague-Dawley; PE, polyethylene; BW, body weight; PEG, polyethylene glycol; PAH, para-aminohippurate, LC-MS/MS, liquid chromatography-tandem mass spectrometry.

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Abstract

The renal outer medullary potassium (ROMK) channel is located at the apical membrane of epithelial cells lining the thick ascending loop of Henle and cortical collecting duct, and plays an important role in kidney physiology by regulating salt re-absorption. Loss-of-function mutations in the human ROMK channel are associated with antenatal type II Bartter’s syndrome, an autosomal recessive life-threatening salt wasting disorder with mild hypokalemia. Similar observations have been reported from studies with ROMK knock out mice and rats. Importantly, heterozygous carriers of Kir1.1 mutations associated with antenatal Bartter’s syndrome have reduced blood pressure, and a decreased risk of developing hypertension by age 60. Although selective ROMK inhibitors would be expected to represent a new class of diuretics, this hypothesis has not been pharmacologically tested. Compound A (5-(2-(4-(2-(4-(1H-tetrazol-1-yl)phenyl)acetyl)piperazin-1-yl)ethyl)isobenzofuran-1(3H)-one), a potent ROMK inhibitor with appropriate selectivity and characteristics for in vivo testing, has been identified. Compound A accesses the channel through the cytoplasmic side and binds to residues lining the pore within the transmembrane region below the selectivity filter. In normotensive rats and dogs, acute oral administration of A caused concentration-dependent diuresis and natriuresis that were comparable to hydrochlorothiazide. Unlike hydrochlorothiazide, however, compound A did not cause any significant urinary potassium losses or changes in plasma electrolyte levels. These data indicate that pharmacological inhibition of ROMK has the potential for affording diuretic/natriuretic efficacy similar to that of clinically used diuretics, but without the dose-limiting hypokalemia associated with the use of loop and thiazide-like diuretics.
Introduction

The kidneys play a critical role in the long-term regulation of blood pressure. In humans, all identified mutations in genes that cause Mendelian forms of hypertension or hypotension act in the kidney to alter net renal salt and water reabsorption (Lifton et al., 2001). Several mechanisms involving ion channels, exchangers, and transporters, act in an integrated manner along the nephron to regulate salt and water reabsorption. At the thick ascending limb of Henle (TALH), ~30% of salt reabsorption occurs through the luminal Na⁺/K⁺/2Cl⁻ co-transporter, which is the target of furosemide, a loop diuretic used clinically in the treatment of congestive heart failure. In the distal convoluted tubule (DCT), the Na⁺/Cl⁻ co-transporter is responsible for ~7% of salt reabsorption. This co-transporter represents the clinical target of the thiazide-class of diuretics, used in the treatment of hypertension. The final step in the regulation of salt reabsorption takes place in the cortical collecting duct (CCD) through the amiloride-sensitive epithelial sodium channel. Because of the tight coupling between sodium reabsorption and potassium secretion at the CCD, the use of loop or thiazide diuretics is clinically associated with hypokalemia, while amiloride intervention causes hyperkalemia.

Bartter’s syndrome, characterized by renal salt wasting and polyuria-associated low blood pressure and hypokalemic alkalosis, is caused by recessive loss-of-function mutations in any one of the four genes involved in salt reabsorption at the TALH: the apical Na⁺/K⁺/2Cl⁻ co-transporter, the basolateral Cl⁻ (α or β subunits) channel, and the renal outer medullary potassium (ROMK) channel, the product of the KCNJ1 gene, present at the apical membrane (Palmer et al., 1997; Xu et al., 1997; Lu et al., 2002).
ROMK channel has attracted significant interest because of the finding that heterozygous carriers of channel mutations associated with type II Bartter’s syndrome have reduced blood pressure and a decreased risk of developing hypertension by age 60 (Ji et al., 2008). Moreover, it is important to note that ROMK knock out mice and rats recapitulate the phenotype of type II Bartter’s syndrome (Lu et al., 2002; Lorenz et al., 2002; Zhou et al., 2013). These data suggest that ROMK represents a target for the development of novel diuretics for the treatment of hypertension and/or heart failure.

ROMK is a member of the inwardly rectifying family of potassium (Kir) channels (Nichols and Lopatin, 1997). The expression of ROMK (Kir1.1) appears to be almost exclusively restricted to the apical membrane of the epithelial cells lining the TALH and the CCD (Xu et al., 1997; Palmer et al., 1997; Lu et al., 2002). At the TALH, ROMK participates in potassium recycling across the apical membrane that is critical for the proper function of the furosemide-sensitive Na+/K+/2Cl− co-transporter because the K+ concentration in the luminal fluid is much lower than that of Na+ and Cl−. At the CCD, ROMK provides a pathway for potassium secretion that is tightly coupled to sodium reabsorption through the amiloride-sensitive epithelial sodium channel. Because of the presence of ROMK channels at both TALH and CCD, selective inhibitors of this channel would be predicted to provide equivalent or superior diuretic/natriuretic efficacy to loop diuretics, such as furosemide, with the added potential benefit of attenuating the hypokalemia associated with the use of loop diuretics or thiazides. Despite all of the evidence supporting ROMK as a novel therapeutic target, the development of selective channel inhibitors has only recently been attempted. In addition to the peptide tertiapin, which blocks with high affinity the rat but not the human channel (Jin and Lu, 1998;
Felix et al., 2006), two independent groups have reported the identification of small molecule ROMK inhibitors (Lewis et al., 2009; Bhave et al., 2011; Tang et al., 2012).

In the present study, compound A, the discovery of which is described in a separate study (Tang et al., 2013), 5-(2-(4-(2-(4-(1H-tetrazol-1-yl)phenyl)acetyl)piperazin-1-yl)ethyl)isobenzofuran-1(3H)-one, was characterized. This agent inhibits the rat and human Kir1.1 channels with high affinity, and displays good selectivity across other ion channel super-families, as well as pharmacokinetic properties suitable for in vivo testing. Acute oral dosing of A was shown to produce dose-dependent diuresis and natriuresis in normotensive rats and dogs of similar magnitude to that of hydrochlorothiazide, but with no significant kaliuresis, and no changes in plasma electrolyte levels. Taken together, these data indicate that pharmacological inhibition of ROMK provides diuretic/natriuretic efficacy similar to that of clinically used diuretics, but with the potential benefit of reducing the hypokalemia associated with the use of loop and thiazide class diuretics.
Materials and Methods

Materials. Compound A, 5-(2-(4-(2-(4-(1H-tetrazol-1-yl)phenyl)acetyl)piperazin-1-yl)ethyl)isobenzofuran-1(3H)-one, was synthesized at Merck Research Laboratories, Rahway, NJ, as described (Tang et al., 2013). FluxOR™ thallium detection kit was obtained from Life Technologies, Carlsbad, CA. Probenecid, anhydrous dimethyl sulfoxide (DMSO), and ouabain were obtained from Sigma-Aldrich, St. Louis, MO. ${}^{86}\text{RbCl}$ was obtained from Perkin Elmer, Inc., Waltham, MA. QuickChange II site-directed mutagenesis kit was from Stratagene, La Jolla, CA. The pCI-neo vector and the transfection reagents FUGENE® 6 and FUGENE® HD were from Promega, Madison, WI. Hydrochlorothiazide (HCTZ) was purchased from MP Biomedicals, LLC, Solon, OH. All other reagents were obtained from commercial sources and were of the highest purity commercially available.

Cells. All tissue culture reagents were obtained from Life Technologies, Carlsbad, CA. All Kir1.1 constructs represent the ROMK1 splice form of Kir1.1. HEK293 cell lines stably transfected with human Kir1.1 (hKir1.1) or rat Kir1.1 (rKir1.1), CHO cell line stably expressing hKir1.1 (CHO-hKir1.1), and MDCKII-Flp cell line stably expressing rKir1.1 (MDCK-rKir1.1) were obtained as previously described (Felix et al., 2006; Felix et al., 2012). A CHO cell line stably expressing hKir2.3 (CHO-hKir2.3) was constructed by transfecting cells with hKir2.3-pCIneo expression plasmid using FUGENE6. Stable pools of CHO cells expressing hKir2.3 were prepared by geneticin selection (1000 μg/ml), and analyzed for functional expression of hKir2.3 using a membrane potential, fluorescence resonance energy transfer-based assay as described (Solly et al., 2008).
Individual stable cell lines were generated by limiting dilution under continuous selection with geneticin. HEK293 cells stably expressing either human Kir4.1 (HEK-hKir4.1) or human Kir7.1 (HEK-hKir7.1) were prepared by transfecting cells with hKir4.1-phCMV1 or hKir7.1-phCMV1 expression plasmids using FUGENE HD. Stables pools of cells were selected using 1000 μg/ml geneticin. Individual stable cell lines were generated by limiting dilution under continuous selection with geneticin, and analyzed for functional expression of Kir4.1 or Kir7.1 using a fluorescence-based thallium flux assay. HEK293 cells stably transfected with human Kir2.1 were obtained from EMD Millipore Corporation, Billerica, MA. HEK293 cells were grown in minimum essential media (MEM) Alpha medium, 10% fetal bovine serum (FBS), 500 μg/ml geneticin, 1x penicillin/streptomycin/glutamine and 1x MEM non-essential amino acids. CHO cells were grown in Iscove's Modified Dulbecco's Medium supplemented with HT Supplement Solution with 10% heat inactivated FBS, 500 mg/mL geneticin, and 1% penicillin/streptomycin. The MDCK-rKir1.1 cell line was grown in DMEM+Glutamax supplemented with penicillin/streptomycin, 200 μg/ml hygromicin B, and 10% FBS. All lines were maintained at 37°C in a 10% CO2 atmosphere.

Kir1.1 Thallium Flux Assay- Permeation of thallium through open hKir1.1 channels was determined as previously described (Felix et al., 2012). Briefly, HEK293 cells stably transfected with hKir1.1 were plated using a Thermo Scientific Matrix WellMate® (Thermo Scientific Inc., Waltham, MA) at approximately 20,000 cells/well on black-wall, clear bottom, 384-well poly-D-lysine-coated plates (Becton Dickinson, Franklin Lakes, NJ) in 50 μl growth medium, and incubated overnight (16 – 20 hours) at 37°C in a
10% CO₂ atmosphere. All liquid handling was done on a Thermo Scientific Matrix PlateMate® 2X3. Cell growth medium was removed and cells were then incubated with 0.025 ml of a solution containing FluxOR dye loading reagent, prepared according to the manufacturer instructions in Hank’s Buffered Saline Solution (HBSS) containing 1.26 mM CaCl₂ and 0.49 mM MgCl₂ (Life Technologies), pH adjusted to 7.4 by addition of NaOH. After incubation in the dark for 90 min at ambient temperature (22-24°C), cells were washed once with 0.04 ml of HBSS buffer solution, and incubated in the dark for 30 min at ambient temperature (22-24°C) with 0.025 ml of FluxOR assay solution containing 2.5 mM probenecid, and 300 μM ouabain, in the absence or presence of test compound. At the end of the 30 min incubation period, the plate was placed in a FLIPR<TETRA instrument (Molecular Devices, Sunnyvale, CA), illuminated at 490 nm, and fluorescence emission was recorded at 525 nm. After an 80 s baseline reading, 0.00625 ml of a 5X solution containing 7.5 mM thallium sulfate, 0.75 mM K₂SO₄, prepared in the FluxOR chloride-free buffer was added, and fluorescence emission was recorded for an additional 8-9 minutes, with an exposure time of 0.4 s and a read interval of 10 s. The change in fluorescence emission (F/F₀) was calculated by averaging the three readings just prior to the signal reaching a plateau level, usually from 330-360 s (F), and the baseline calculated by averaging the initial four readings usually from 1-40 s.

Kir1.1 ⁸⁶Rb⁺ Flux Assays- The ability of ⁸⁶Rb⁺ to permeate through Kir1.1 channels was evaluated as previously described (Felix et al., 2012). Briefly, CHO cells stably expressing hKir1.1 or HEK293 cells stably expressing rKir1.1 were seeded at 120,000 cells/well in either 96-well white, opaque bottom tissue culture plates (PerkinElmer, Inc.,
Waltham, MA) or clear bottom poly-D-lysine coated plates (BioCoat™, Becton Dickinson, Franklin Lakes, NJ) in complete growth medium containing 1.5 μCi/ml $^{86}$Rb$^+$, and incubated in 10% CO$_2$ at 37°C overnight. On the day of the assay, the $^{86}$Rb$^+$-containing medium was removed, and the cells were washed once with Low K assay buffer containing (in mM): 126.9 NaCl, 4.6 KCl, 2 CaCl$_2$, 1 MgCl$_2$, 10 Hepes/NaOH, pH 7.4. High K assay buffer (100 μl) containing (in mM) 121.5 NaCl, 10 KCl, 2 CaCl$_2$, 1 MgCl$_2$, 10 Hepes/NaOH, pH 7.4, with or without test compound was added and cells were incubated at ambient temperature (22-24 °C) for 30 min. An aliquot (30 μl) of the assay buffer was removed and added to 170 μl of MicroScint 20 scintillation cocktail (Perkin Elmer, Inc., Waltham, MA) in 96-well plates (Packard OptiPlate-96, EMD Millipore, Billerica, MA), and the remaining assay buffer was discarded. Cells were solubilized in the presence of 1% sodium dodecyl sulfate, and 170 μl MicroScint 20 was added to each well. Radioactivity associated with the assay buffer and cells was determined on a TopCount counter (Packard, GMI, Ramsey, MN). The amount of radioactivity in the assay buffer (% efflux) was normalized to the total radioactivity content of the assay buffer and cells. For experiments in which serum was included, compounds were prepared in high K assay buffer, in the absence or presence of 10, 30, or 100% human (CHO-Kir1.1) or rat (HEK-rKir1.1) serum. All other steps were carried out as described above. For Transwell assays, 80,000 MDCK-rKir1.1 cells in 0.5 ml were seeded in BD Falcon™ cell culture inserts, 0.4 μm pore, transparent PET membrane (Becton Dickinson, Franklin Lakes, NJ), and 2 ml of growth media was added to the lower chamber (Felix et al., 2012). Cells were allowed to adhere and grow at 37°C in 10% CO$_2$ for 3 days. The Transwell media (apical compartment) was replaced with 0.5
ml of fresh media, and the growth media was removed from the lower chamber (basolateral compartment), and replaced with 2 ml of growth media containing 1.5 μCi/ml of $^{86}$Rb$^+$, followed by overnight incubation at 37°C in 10% CO$_2$. The Transwell medium was replaced with 0.4 ml of Low K assay buffer, and the Transwell was transferred to a chamber containing 2 ml of Low K assay buffer to remove excess of $^{86}$Rb$^+$. Transwell medium was replaced with 0.4 ml of High K assay buffer, with or without 10 μM compound A, and the Transwell was transferred to a chamber containing 2 ml of High K assay buffer, with or without 10 μM compound A. After 30 minutes incubation at ambient temperature, the Transwell filter was removed and radioactivity associated with cells and the apical and basolateral media was determined on a TopCount counter after addition of MicroScint 20.

**Electrophysiological Assay** - Block of wild-type and mutant hKir1.1 channels by compound A was examined by whole cell voltage clamp as previously described (Felix et al., 2006). Briefly, experiments were performed at room temperature, using an EPC-9 amplifier and Pulse software (HEKA Electronics, Lamprecht, Germany). Data were acquired at 10 kHz and filtered at 2.9 kHz. The internal (pipet) solution contained 130 mM KCl, 5 mM NaCl, 2 mM MgCl$_2$, 5 mM EGTA, 0.2 mM MgATP, 5 mM Na-HEPES, pH 7.4. Bath solutions containing two different concentrations of K$^+$, each composed of (140-x) mM NaCl, x mM KCl, 2.7 mM CaCl$_2$, 0.5 mM MgCl$_2$, 5 mM Na-HEPES, pH 7.4, were used in every experiment to assess the quality of the recording. Only cells with a shift in the reversal potential within 5 mV of the calculated shift for a K$^+$ selective current were used. Kir1.1 currents were recorded using voltage ramps over at least 100
mV and, unless noted otherwise, the current corresponding to a membrane potential of -100 mV was used to determine fractional inhibition by compound A.

**Site-directed mutagenesis** – Site-directed mutagenesis of Kir1.1, cloned into the pCIneo expression plasmid, was performed using the QuickChange II kit according to the manufacturer’s protocol. For all mutants, the entire open reading frame was sequenced to exclude secondary amino acid changes. For characterization by whole-cell voltage clamp, mutant constructs were transiently transfected into TsA-201 cells using FuGENE® 6, as previously described (Felix et al., 2006).

**Other Ion Channel Assays** - The functional activities of Kir2.1 (HEK-hKir2.1), Kir4.1 (HEK-hKir4.1), and Kir7.1 (HEK-Kir7.1) channels were determined in thallium flux assays using identical conditions as those described for hKir1.1 (Felix et al., 2012). The activity of Kir2.3 (CHO-hKir2.3) was evaluated by $^{86}$Rb$^+$ flux using identical conditions as those described for Kir1.1 (Felix et al., 2012). All procedures for evaluation of human *ether-a go-go* related gene (hERG) (CHO-hERG) by either QPatch™ automated electrophysiology or by $^{35}$S-MK-499 binding (Schmalhofer et al., 2010), the human voltage-gated sodium channel Nav1.5 (HEK-hNav1.5) using a FRET-based membrane potential assay (Felix et al., 2004), and the L-type calcium channel Cav1.2 (HEK-hCav1.2) in a fluorescence calcium influx assay (Abbadie et al., 2010) have been previously described.
Ancillary Target Binding Assays- In vitro binding assay screening for a panel of 166 ancillary targets was performed by MDS Pharma Services (King of Prussia, PA).

Animal Studies- All protocols for animal experiments were approved by the Institutional Animal Care and Use Committee of Merck Research Laboratories (Rahway, NJ and West Point, PA) and adhere to the guidelines of the Committee for Research and Ethical Issues.

Renal Excretory Function Studies in Anesthetized Rats- Twelve-week old male Sprague Dawley (SD) rats (body weight 300~350g) were anesthetized with thiobutabarbitral sodium (Inactin, 100-110 mg/kg ip; Sigma-Aldrich, St. Louis, MO) and then placed on a heating pad to maintain rectal temperature at 37°C throughout the study. A tracheostomy was performed and a polyethylene (PE) tube (PE-250) was inserted to facilitate spontaneous breathing. A PE-50 catheter was inserted into the left femoral artery to allow for intermittent blood sampling and continuous monitoring of arterial blood pressure using a digital data acquisition system (EMKA Technologies Inc., Falls Church, VA). The left femoral vein was cannulated with a PE-50 catheter for infusion of a solution of 6% albumin at a rate of 0.4 ml/100 g body weight per hour (BW/h) initially, followed by infusion of a maintenance solution of 1% albumin at a rate of 0.35 ml/100 g BW/h. The right jugular vein was also cannulated with a PE-50 catheter for infusion of vehicle (10% ethanol/40% polyethylene glycol (PEG) 400 /50% water) and either compound A or HCTZ (both compounds were dissolved in the above mentioned vehicle) at a rate of 0.05 ml/100 g BW/h. This vehicle, when tested in a separate study, was shown to have no effect on renal excretory function (data not shown). The bladder was
catheterized for urine collection with PE-100 tubing. After 60-min stabilization, urine was collected over two consecutive 30-min periods, with blood samples being withdrawn at their midpoint in order to assess control values of renal excretory function and blood electrolytes during vehicle administration. Subsequently, compound A at 1.55 mg/kg/h or HCTZ at 5.0 mg/kg/h were administered by constant intravenous infusion for 1 h, and two successive 30-min sample collections were carried out, as described for the control vehicle period. Blood and urine electrolytes (Na⁺, Cl⁻, and K⁺) were measured with an i-STAT Portable Clinical Analyzer (HESKA Corporation, Loveland, CO) and a Roche Modular Chemistry System (Roche Diagnostics, Indianapolis, IN), respectively.

Rat Diuresis Assay- Adult male SD rats (275-350 g body weight) were acclimated to single housing in metabolism cages with free access to food and water for at least three days before the experiments. On the day of the study, animals were transferred from metabolism cages to shoebox cages, and access to food and water was restricted for the entire duration of the study. Vehicle (Imwitor 742:Tween 80 (1:1, v:v)) or compound was administered at a dose volume of 1 ml/kg by oral gavage. After 30 min, voiding was induced by giving each animal a saline load (18 ml/kg by oral gavage). Animals were then transferred back to metabolism cages for urine collection over the next four hours at room temperature. Volume of urine voided was recorded for each rat; urine samples were then centrifuged, aliquoted, and frozen at -20°C until analyzed. HCTZ was used as a positive control. When needed, blood samples were obtained by jugular vein puncture to determine compound plasma exposure levels.
Dog Diuresis Assay- Female mongrel dogs were trained to lie quietly on their back. A sterile Foley catheter with lubricant on its tip was inserted into the urinary bladder after a local topical anesthetic, such as cetacaine spray or lidocaine gel, was applied to the urethra and surrounding tissue for the comfort of the dog. Once inserted into the bladder a balloon was gently inflated to retain the catheter within the bladder, which remained in place for the duration of the urine collection period (total 3 hours). The animals were then placed in a padded sling (size appropriate, manufactured by Alice King Chatham) during the experiment. Sterile percutaneous catheters were inserted into saphenous and cephalic veins for blood collection for chemistry, hematology and compound level analysis. Immediately following collection of control blood and urine samples (two 30 minutes collection periods), vehicle or compound was administered orally by gavage (feeding tube). Six additional blood and urine collections (30 min for each period) were obtained. Urine volume was recorded for each collection period. Urine and blood samples were then centrifuged, aliquoted, and frozen at -20°C until analyzed. Dogs were continually observed while in sling restraint. Upon completion of the study, the Foley catheter was gently removed and dogs were returned to their home cages.

Plasma Urine, and Kidney Level Analysis- Plasma, urine, and homogenized kidney tissue concentrations of compound A were determined by liquid chromatography-tandem mass spectrometry (LC-MS/MS) using an Applied Biosystems/MDS Sciex API 5000 LC/MS/MS mass spectrometer (Applied Biosystems/MDS Sciex, Foster City, CA) operated in positive ion atmospheric pressure chemical ionization mode with multiple-reaction monitoring. Plasma was prepared for analysis by addition of 300 μl of
acetonitrile to a 50 μl sample of plasma. The mixture was then vortexed and centrifuged. The clear liquid at the top was pipetted away from the pellet that was formed at the bottom of the tube, and injected directly on to the LC/MS/MS. The kidney homogenate was prepared by adding 3 parts of water for every 1 part tissue (v:w), and then shaking vigorously with grinding beads. At the end of the process, the homogenate becomes an opaque liquid that can be readily pipetted. To an aliquot of the homogenate a six-fold volume of acetonitrile was added, and the sample was vortexed and centrifuged. The liquid at the top (supernatant) was injected directly on to the LC/MS/MS. Extracts were chromatographed using a Phenomenex Kinetex 1.7 μm PFP 50 x 2.1 mm column (Phenomenex, Torrance, CA), and eluted at 0.75 ml/min using a linear gradient of acetonitrile.

**Statistics** - IC₅₀ values for inhibition were determined according to the Hill equation from concentration-response curves by a non-linear regression analysis, where all parameters were left unconstrained. Data are presented as either mean ±SD or mean ± SEM of n experiments. Statistical analysis was conducted using either ANOVA followed by Dunnett’s post hoc test using Prism (Version 4.0.3, Graphpad, La Jolla, CA) or Student’s t-test, as appropriate. Statistical significance was defined as two-tailed \( P < 0.05 \).
**Results**

**Compound A Inhibits Kir1.1 Channels** The search for potent and selective Kir1.1 inhibitors has led to the identification of compound A (Figure 1). In HEK cells stably expressing hKir1.1 channels, A inhibits thallium flux through these channels with an IC$_{50}$ value (mean ± SD) of 24 ± 7 nM (n = 3) (Figure 2A). In functional cell-based assays that measure the ability of $^{86}$Rb$^+$ to permeate through human or rat Kir1.1 channels, A inhibits these channels with IC$_{50}$ values (mean ± SD) of 89 ± 6 (n = 6) (Figure 2B) and 135 ± 15 (n = 2) nM (Figure 2C), respectively, in the absence of serum, and 506 ± 23 (n = 2) (Figure 2B) and 576 ± 164 (n = 2) nM (Figure 2C), respectively, in the presence of 100% human or rat serum.

The selectivity of A was assessed in functional assays of other related Kir channels, such as cardiac Kir2.1, and renal Kir2.3, Kir4.1, and Kir7.1. In the absence of serum and at concentrations of up to 100 μM, A had no significant effect on either thallium flux through Kir2.1 (Figure 2A) or $^{86}$Rb$^+$ flux through Kir2.3 channels (Figure 2B). Thallium flux through either Kir4.1 or Kir7.1 channels were not significantly inhibited by A at concentrations of up to 100 μM, in the absence of serum (Figure 1, Supplemental Data). In electrophysiological recordings of hERG channels, A inhibits with an IC$_{50}$ (average ± SEM) of 5.6 ± 1.3 μM (n=8), whereas in a binding assay that measures the interaction of $^{35}$S-MK-499 with membranes derived from HEK cells expressing the hERG channel, A displays an IC$_{50}$ value of 5.9 ± 0.4 μM (n= 5), a value that is similar to that determined by electrophysiology. In functional assays, A inhibited the human voltage-gated sodium channel Nav1.5 by 42% at 30 μM, and the human voltage-gated calcium channel Cav1.2 by 19% at 100 μM. In a panel of 166 enzyme and
radioligand binding assays run by MSD Pharma Services, A, when tested at 10 μM, only inhibited the serotonin transporter with an IC₅₀ of 9.1 μM.

The above data suggest that A is a potent and selective Kir1.1 inhibitor. The pharmacokinetic properties of A in rats and dogs (Table 1) indicate that the compound has moderate clearance rates (rat 40 ml/min/kg, dog 36 ml/min/kg) and good oral bioavailability (rat 33%, dog 80%), making it suitable for in vivo evaluation.

**Mechanism of Inhibition of Kir1.1 Channels by Compound A**

Inhibition of hKir1.1 channels by compound A was examined in more detail using standard whole cell voltage clamp protocols and HEK cells stably expressing hKir1.1 channels. Voltage ramps from -80 mV to +20 mV were applied at regular intervals to monitor hKir1.1 currents under control conditions and in the presence of increasing concentrations of compound A (Figure 3). The amplitude of the inward current at -80 mV was inhibited by compound A with an IC₅₀ (mean ± SD) of 92 ± 51 nM (n=3). Similarly, the outward current at +20 mV was inhibited with an IC₅₀ (mean ± SD) of 42 ± 20 nM (n=3) (Figure 3C). The potency observed in the electrophysiology study was consistent with data generated in the thallium and 86Rb⁺ flux assays (29 nM and 106 nM, respectively, see above). The data further suggests that inhibition of hKir1.1 by compound A is not dependent on the direction of current flow (P = 0.16 for comparing IC₅₀ values at -80 mV and +20 mV).

To gain information about the binding site of compound A in the hKir1.1 channel, several site-directed mutants were generated. Mutagenesis focused mainly on amino acids that differed between hKir1.1 and hKir2.1, and that were predicted to face the ion conduction path, based on comparisons with the chicken Kir2.2 channel (Tao et al., 2009)
Amino acids in hKir1.1 were mutated individually to the corresponding amino acids found in hKir2.1. All mutant channels were transiently expressed in TsA-201 cells, and examined by whole-cell voltage clamp. Results from these mutagenesis studies are summarized in Table 2, and representative current traces are shown in Figure 4. Two of the mutant constructs (S130A and L166V) did not generate measurable currents. With the exception of mutations at position 171, all mutant channels were inhibited potently by compound A. N171 in hKir1.1 was initially mutated to aspartate, found in the homologous position in hKir2.1. As expected, based on the work by MacKinnon and others (Lu and MacKinnon, 1994), currents generated by hKir1.1-N171D were strongly inwardly rectifying. Bath application of 100 nM compound A had no discernible effect on these currents, whereas 10 μM compound A inhibited the current at -100 mV by 59% (Figure 4A). Based on the prominent outcome of introducing a negative charge at position 171, a charge-neutral substitution to glutamine was examined. Like wild-type, hKir1.1-N171Q currents were weakly inwardly rectifying; however block by compound A, while more potent than for N171D, was still shifted to weaker potency by almost an order of magnitude (Figure 4B, Table 2).

MDCK-rKir1.1 cells grown on permeable Transwell supports provide a polarized system where the apical and basolateral membranes are physically separated by an impermeable barrier due to the formation of tight junctions (Simons and Virta, 2006). Addition of $^{86}$Rb$^+$ to the basolateral compartment allows the accumulation of the isotope inside the cell through activity of the ouabain-sensitive Na$^+$/K$^+$-ATPase pump. Following removal of $^{86}$Rb$^+$ from the basolateral compartment, efflux of the isotope into the apical side can be inhibited by addition of the peptide blocker tertiapin-K12/Q13 to
the apical, but not the basolateral compartment, suggesting that Kir1.1 is exclusively expressed at the apical surface of the MDCK-rKir1.1 cells, and that the two membrane compartments are indeed separated by tight junctions formed by the monolayer of cells. When the same experiment is carried out with 10 μM compound A, inhibition of 86Rb+ flux into the apical compartment occurs regardless of whether A was added to the apical or basolateral compartments (Figure 5). These data are consistent with the idea that channel inhibition results from A accessing the channel from the cytoplasmic side.

**Renal Excretory Function Studies in Anesthetized SD Rats.** The diuretic activity of compound A was assessed in anesthetized euvoolemic rats. Following intravenous infusion of either compound A or HCTZ for 1 h, significant and comparable increases in urine flow and urinary Na+ and Cl− excretion rates were observed with both agents (Table 3). Urinary K+ excretion, however, was only significantly increased by HCTZ, but not by compound A. HCTZ also caused a significant decrease in blood K+ levels. Plasma levels for compound A during the infusion period averaged 1.36 ± 0.13 μM, approximately three times above the IC50 for inhibition of rat Kir1.1 channels in the presence of 100% serum. Neither compound A nor HCTZ caused significant changes in blood pressure or heart rate during the study (Table 3). Vehicle had no effect on renal excretory function (data not shown). These data indicate that even at a greater diuretic and natriuretic dose, compound A induces less urinary K+ losses compared to HCTZ. Despite significant diuresis observed with both agents, blood K+ levels did not significantly change with compound A, but significantly decreased with HCTZ.
Compound A Elicits Diuresis and Natriuresis in Conscious, Volume-loaded Rats.

Oral administration of compound A evoked dose-dependent increases in urine output in conscious, volume-loaded, SD rats (Figure 6, panel A). Four hours after oral dosing, compound A significantly increased urine flow starting at a dose of 3 mg/kg (2.6-fold vs. vehicle) while maximal increases were observed at 50 mg/kg (4.3-fold vs. vehicle). Diuretic efficacy of compound A appeared to reach a plateau at doses between 10 and 50 mg/kg under these experimental conditions. In similar experiments, HCTZ significantly and dose-dependently increased urine flow starting at 10 mg/kg (2.5-fold vs. vehicle) while maximal increases were observed at 100 mg/kg (4.0-fold vs. vehicle). Thus, the diuretic efficacy of compound A and HCTZ appear to be similar at the highest doses tested. A similar trend was observed when comparing the natriuretic efficacy of compound A and HCTZ (Figure 6, panel B). Four hours post-dosing, compound A and HCTZ significantly, and dose-dependently, increased urinary Na+ excretion starting at doses of 10 mg/kg. Maximal natriuretic effects were observed at the highest doses tested, with compound A increasing urinary Na+ excretion by 3.8-fold and HCTZ by 3.4-fold compared to vehicle-treated rats. Importantly, despite robust diuresis and natriuresis associated with compound A administration, urinary K+ excretion did not significantly change with any of the doses tested, but was significantly increased by the highest dose of HCTZ (100 mg/kg, 1.4-fold vs. vehicle) (Figure 6, panel C). These data indicate that compound A, at maximally efficacious diuretic and natriuretic doses, does not lead to significant urinary K+ losses, an effect that is different from the well-known, and documented here, HCTZ-induced kaliuresis. After 4.5 h following oral dosing, concentrations of compound A in plasma were below 0.2 μM in rats given the 1 and 3
mg/kg doses, but 0.39 and 0.92 μM, respectively, in rats dosed with 10 and 50 mg/kg. Compound A levels were higher in urine samples, ranging from 0.74 μM in rats dosed with 3 mg/kg up to 4.8 μM in rats dosed with the 50 mg/kg dose. In a parallel PK study, concentrations of compound A were determined in plasma and kidney samples from rats dosed with 10 mg/kg compound A (Table 4). Kidney levels of compound A were approximately 3.9 to 6.7-fold larger than those found in plasma.

**Compound A causes Diuresis and Natriuresis in Conscious Dogs.** Oral administration of compound A increased urine output in conscious, euvolemic dogs (Figure 7, panel A). Compound A dosed at either 10 or 50 mg/kg led to significant dose- and time-dependent increases in urine output. Maximal diuretic effect of compound A was obtained with the 50 mg/kg dose, three hours post-dosing, with urine output increasing from baseline values of 0.43 ml/min up to 2.68 ml/min (8-fold change vs. baseline), but values appeared to plateau after 1.5 h post-dosing. Oral HCTZ dosed at 10 mg/kg also increased urine flow in a time-dependent fashion, and maximal diuretic effect of HCTZ was seen at 1 h post-dosing. Urine flow values increased from 0.35 ml/min at baseline up to a maximum of 1.62 ml/min at 1h, but values remained constant for the remainder of the study (~3.5 to 4.8-fold change vs. baseline). Similarly, compound A led to dose- and time-dependent increases in urinary Na⁺ excretion in dogs (Figure 7, panel B). Maximal natriuretic effect was seen with 50 mg/kg compound A, three hours post-dosing. Urinary Na⁺ excretion increased from baseline values of 55 μEq/min up to 435 μEq/min (16-fold change vs. baseline), and values appeared to plateau after 1.5 h post-dosing. Oral HCTZ also increased urinary Na⁺ excretion in a time-dependent fashion; and maximal natriuresis...
was seen at 1 h post-dosing. Urinary Na\(^+\) excretion increased from 23 μEq/min at baseline up to a maximum of 289 μEq/min at 1 h (16-fold change vs. baseline), and these values slowly decreased to 167 μEq/min after 3 h (9.1-fold change vs. baseline). Thus, the natriuretic efficacy of the largest dose of compound A and HCTZ were comparable whereas the extent of diuresis was larger with the 50 mg/kg dose of compound A. Kaliuresis, on the other hand, was only significantly enhanced by HCTZ (Figure 7, panel C), particularly during the first hour post-dosing. The two doses of compound A tested did not significantly change urinary K\(^+\) excretion rates compared to baseline values, however, HCTZ significantly increased urinary K\(^+\) excretion values from 36 μEq/min at baseline to 63 μEq/min after 1 h post-dosing (1.7-fold change vs. baseline). Glomerular filtration rate, determined by calculating creatinine clearance, and effective renal plasma flow, determined by PAH clearance, were significantly decreased by HCTZ by approximately 20% but not by either dose of compound A (data not shown). Plasma sodium and potassium levels, as well as hematocrit values, did not change following oral dosing of compound A at 10 or 50 mg/kg, however, plasma potassium levels in HCTZ-treated dogs tended to decrease toward the end of the study, but the decrease did not reach statistical significance. Several cardiovascular parameters evaluated in these conscious dogs, such as heart rate, PR interval, QRS duration, QTc interval, and blood pressure were not significantly changed by either compound A or HCTZ during the course of the experiment (data not shown). Plasma and urine levels of compound A were determined and shown to rise with time (Table 5). Similarly to the findings in SD rats, levels of compound A were found to be significantly higher in urine than in plasma. Taken together, these data demonstrate that in two different animal species, single oral
doses of compound A evokes a diuretic and natriuretic response that is comparable to HCTZ, but with minimal changes in urinary K⁺ excretion rate and without any significant cardiovascular liabilities.
Discussion

The results of this study illustrate the characterization of the first small molecule Kir1.1 inhibitor with appropriate selectivity profile and pharmacokinetic properties for in vivo evaluation. Compound A is a potent inhibitor of Kir1.1 channels stably expressed in heterologous systems, and accesses the channel through the cytoplasmic side. Residues lining the pore within the transmembrane region of the channel below the selectivity filter appear to contribute to high affinity inhibition of Kir1.1 by A. Pharmacological inhibition of ROMK upon oral dosing of A leads to diuresis and natriuresis in two different animal species, SD rats and dogs. The magnitude of these events is similar to those of the clinically used diuretic HCTZ, but A appears to have a more favorable urinary potassium/sodium ratio than relevant doses of HCTZ in these acute studies. Taken together, these data suggest that selective inhibitors of ROMK represent a novel mechanism for developing diuretic/natriuretic agents with the potential for enhanced efficacy, and assuming that acute effects predict chronic pharmacology, a more favorable potassium balance than the diuretics that are currently used in the treatment of hypertension and/or congestive heart failure.

Within the diuretic class, thiazides, such as HCTZ, are the most widely used as first line therapy to treat uncomplicated hypertension, or as add-on therapy to other mechanism of action drugs, such as angiotensin-converting enzyme inhibitors, and angiotensin II receptor blockers (Sood et al., 2010). Even when HCTZ is used as part of combination therapy with renin-angiotensin-aldosterone system blockade, patients with resistant hypertension often fail to achieve adequate blood pressure control; ROMK
Inhibitors may offer potential added efficacy benefit in these populations. Hypokalemia (serum potassium concentration < 3.5 mEq/L) and elevations in fasting blood glucose are the major liabilities associated with thiazides (Palmer and Naderi, 2007). Loop diuretics, such as furosemide, are mostly used to treat acute episodes of pulmonary and peripheral edema in congestive heart failure patients, but their chronic use in the disease is not recommended because the drugs lose efficacy with time and cause hypokalemia at higher doses. No new diuretics have been developed within the past four decades, but an ideal novel diuretic should be potassium neutral, provide equal or greater efficacy to clinically used diuretics, and be suitable for combination therapy. In this sense, ROMK has emerged as an attractive novel target for the development of such agents (Ji et al., 2008).

ROMK is present in two different regions of the nephron (Xu et al., 1997). Inhibition of ROMK at the TALH should mimic the effect of furosemide in providing natriuresis/diuresis. In addition, inhibition of ROMK at the CCD, where it participates in potassium secretion, may ameliorate the hypokalemia caused by loop and thiazide diuretics. The results presented in this study with the selective, small molecule A seem to support these expectations of a ROMK inhibitor. Thus, A provides diuresis/natriuresis effects comparable to those of clinically used diuretics but with a more favourable urinary K/Na ratio in two different species, and under different experimental paradigms. It is interesting to note that inhibition of ROMK at the CCD does not appear to decrease urinary potassium excretion, which could lead to hyperkalemia, despite the fact that ROMK contributes to potassium secretion in that part of the nephron. However, we can speculate that other mechanism(s) present at the CCD, such as high-conductance, calcium-activated potassium channels, are likely to contribute to potassium secretion, in
particular under conditions of high luminal flow rates that result from inhibition of salt reuptake at the TALH. Indeed, compensatory mechanisms between ROMK and high-conductance, calcium-activated potassium channels in the distal part of the nephron have been observed in studies with mice lacking either channel (Rieg et al., 2007). Like furosemide and thiazide diuretics, ROMK inhibitors are expected to activate the renin-angiotensin system, which could attenuate the extent of natriuresis/diuresis upon chronic dosing. Chronic treatment with A will be needed to determine if diuretic/natriuretic resistance develops with time which may limit the utility of ROMK inhibitors as monotherapy agents. If resistance develops, it would be important to evaluate whether ROMK inhibitors could be administered in combination with an angiotensin converting enzyme or an angiotensin II receptor blocker to enhance their efficacy. In addition, chronic treatment will also provide insight into the effects of ROMK inhibitors on plasma potassium levels over time.

The search for Kir1.1 inhibitors has provided a limited number of compounds with appropriate potency, selectivity, physico-chemical, and pharmacokinetic properties to be used in proof of concept studies (Lewis et al., 2009; Bhave et al., 2011; Tang et al., 2012). Compound A represents the first Kir1.1 inhibitor that fulfils the above criteria. The selectivity of A for Kir1.1 versus other Kir channels, such as Kir2.1 and Kir2.3, is especially noteworthy. Part of this selectivity appears to arise from the nature of specific residues that line the channel’s pore within the transmembrane domain below the selectivity filter, although other region(s) of the channel may also contribute to the high affinity interaction of A with Kir1.1 channels. Although more studies need to be done to determine the in vivo efficacy of A after chronic dosing, and the possibility of
combination with other mechanism of action drugs, the results of the present study support the idea that ROMK represents a target of interest for the development of novel diuretics with the potential of having an improved plasma potassium profile.
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Authorship Contributions

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Contributed new reagents or analytic tools: Tang, de Jesus, Pasternak


Wrote or contributed to the writing of the manuscript: Garcia, Priest, Alonso-Galicia, Zhou, Kaczorowski, Pasternak
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Footnotes

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RMB: Novartis, Cambridge, Massachusetts
TB: ImClone Systems, New York, New York
AS: AbbVie, North Chicago, Illinois
Figure Legends

Figure 1. Structure of compound A, 5-(2-(4-(2-(4-(1H-tetrazol-1-yl)phenyl)acetyl)piperazin-1-yl)ethyl)isobenzofuran-1(3H)-one.

Figure 2. Compound A inhibits Kir1.1 channels. (A) HEK293 cells stably expressing hKir1.1 (●) or hKir2.1 (▲) channels were preloaded with the FluxOR reagent, and incubated in the absence or presence of increasing concentrations of A, as described under Materials and Methods. Upon recording the emission of the dye for 90 sec, a thallium sulfate/potassium sulfate solution was added, and fluorescence was monitored for an additional 510 sec. Data were analyzed by the Hill equation where all parameters were left unconstrained. Compound A inhibits hKir1.1 with an IC50 value of 30 nM and nH of 1. In marked contrast, A does not have any significant effect on hKir2.1 at concentrations of up to 100 μM. Data shown are mean ± SD (n= 4). (B and C) CHO-hKir1.1 (●, △, •, □) (B), CHO-hKir2.3 (▲) (B) or HEK-rKir1.1 (C) cells were incubated with 86Rb+ overnight as indicated under Materials and Methods. On the day of the experiment the medium was removed and cells were placed into assay medium containing 0 (●) (▲), 10 (△), 30 (•), or 100% (□) human (B) or rat (C) serum, in the absence or presence of increasing concentrations of compound A, and incubated at room temperature for 30 min. The amount of 86Rb+ efflux was calculated as indicated under Materials and Methods. Data were analyzed by the Hill equation where all parameters were left unconstrained. Data shown are mean ± SD (n=4). IC50 values
Figure 3. Inhibition of hKir1.1 channels by compound A. Human Kir1.1 channels stably expressed in HEK293 cells were examined by whole cell voltage-clamp as described under Materials and Methods. (A) Kir1.1 currents were recorded during 200 ms voltage ramps from -80 mV to +20 mV in control (black) and in the presence of 100 nM compound A (grey). External K⁺ concentration was 40 mM. (B) Currents measured at a membrane potential of -80 mV were plotted as a function of time for a representative cell. Bath application of rising concentrations of compound A is indicated by the arrows. (C) Currents corresponding to membrane potentials of -80 mV and +20 mV were measured, and the observed inhibition was plotted as a function of compound A concentration for a representative cell. The solid lines represent the results of fitting the Hill equation to the data points, yielding an IC₅₀ of 53 nM and Hill coefficient of 1.4 for currents recorded at -80 mV, and an IC₅₀ of 47 nM and Hill coefficient of 1.5 for currents recorded at +20 mV.

Figure 4. Inhibition of hKir1.1 mutant channels by compound A. Mutated hKir1.1 channels were transiently expressed in TsA-201 cells as described under Materials and Methods. Currents were recorded in response to voltage ramps, using 140 mM external K⁺, and are shown in the absence (black) and in the presence (grey) of the indicated concentration of compound A. Channel constructs and compound A concentrations were as follows: (A) hKir1.1-N171D; 10 μM compound A, (B) hKir1.1-N171Q; 2 μM
compound A, (C) hKir1.1-L166V; no K⁺ selective, Ba²⁺ sensitive inward current was detected. At positive voltages, a small delayed rectifier current endogenous to TsA-201 cells was seen. (D) hKir1.1-M81L; 100 nM compound A.

Figure 5. Inhibition by compound A of rKir1.1 channels stably expressed in MDCK Cells. (A) Schematic representation of MDCK-rKir1.1 cells grown in permeable Transwell supports. The apical (A) and basolateral (B) compartments of the cell monolayer are physically separated by an impermeable barrier due to the formation of tight junctions. Kir1.1 channels are exclusively expressed at the apical surface. The Na⁺/K⁺-ATPase pump allows the accumulation of ⁸⁶Rb⁺ inside the cell when the isotope is added to the basolateral compartment. Upon removal of ⁸⁶Rb⁺ from the medium, unidirectional efflux of the isotope occurs into the apical compartment through rKir1.1 channels. (B) MDCK-rKir1.1 cells grown in Transwell supports were loaded with ⁸⁶Rb⁺ through the basolateral compartment. The radioisotope was removed and the supports were placed in fresh medium with or without 10 μM compound A. After 30 min incubation at room temperature, radioactivity in the apical compartment was determined. Apical, or basolateral, application of compound A inhibited rROMK-mediated ⁸⁶Rb⁺ efflux. Data represent the mean ± SD (n= 4) and are presented relative to an untreated control. ***P <0.001, **** P <0.0001 vs. control.

Figure 6. Effect of compound A and HCTZ on urinary output and sodium and potassium excretion rates in SD rats. Adult male SD rats (n = 5-6 per treatment) were dosed by oral gavage with indicated doses of either compound A or HCTZ in a vehicle consisting of
Imwitor 742:Tween 80 (1:1, v:v). After 30 min, voiding was induced by giving each animal a saline load as indicated under Materials and Methods. Urine collection took place over the next four hours at room temperature, and analyzed for volume (A), sodium (B) and potassium (C) content. Data are presented as fold change relative to values obtained in vehicle-treated animals. Baseline values for vehicle group were: 3.29 ± 0.42 mL/4 h (urinary output), 0.33 ± 0.06 mmol/4 h (urinary sodium excretion), and 0.72 ± 0.13 mmol/4 h (urinary potassium excretion). *P < 0.05 vs. vehicle.

Figure 7. Effect of compound A and HCTZ on urinary output and sodium and potassium excretory rates in conscious dogs. Female dogs (n = 3 per treatment) were dosed by oral gavage with either vehicle (Imwitor 742:Tween 80 (1:1, v:v)), compound A or HCTZ. Urine samples were collected before and after oral dosing at 30-min intervals up to 3 hours post-dosing, and analyzed for volume (A), sodium (B) and potassium (C) content. The zero time point (BL) represents the average of two 30-min baseline clearance periods collected prior to administration of vehicle or test compound. Data (mean ± SEM) are presented as fold change relative to baseline values obtained prior to test article dosing to each animal.
Tables

Table 1: Pharmacokinetic Properties of Compound A in Rats and Dogs

<table>
<thead>
<tr>
<th></th>
<th>Rat</th>
<th>Dog</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cl (ml/min/kg)</td>
<td>40</td>
<td>36</td>
</tr>
<tr>
<td>T₁/₂ (h)</td>
<td>0.62</td>
<td>1.4</td>
</tr>
<tr>
<td>Vdss (L/kg)</td>
<td>0.66</td>
<td>1.9</td>
</tr>
<tr>
<td>nAUC (μM•hr/(mg/kg))</td>
<td>0.34</td>
<td>0.88</td>
</tr>
<tr>
<td>Tmax (h)</td>
<td>0.67</td>
<td></td>
</tr>
<tr>
<td>F (%)</td>
<td>33</td>
<td>80</td>
</tr>
</tbody>
</table>

Pharmacokinetic parameters were obtained following intravenous and oral administration of compound A to SD rats or dogs. Compound A was dissolved in a PEG 200:water (70:30, v:v) solution and dosed to rats or dogs either intravenously at 1 mg/kg or orally at 2 mg/kg. Plasma concentrations were determined by LC-MS/MS following protein precipitation with acetonitrile. Cl, plasma clearance; T₁/₂, terminal half-life; Vdss, volume of distribution at the steady state; F, oral bioavailability; nAUC, dose-normalized area under the plasma concentration vs. time curve following oral dosing.
Table 2: Block of Kir1.1 site-directed mutants by compound A.

<table>
<thead>
<tr>
<th>hKir1.1 construct</th>
<th>n</th>
<th>IC$_{50}$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td></td>
<td>81</td>
</tr>
<tr>
<td>M81L</td>
<td>1</td>
<td>&lt;100</td>
</tr>
<tr>
<td>L128F</td>
<td>4</td>
<td>&lt;100</td>
</tr>
<tr>
<td>S130A</td>
<td>4</td>
<td>ND</td>
</tr>
<tr>
<td>Q152E</td>
<td>3</td>
<td>&lt;100</td>
</tr>
<tr>
<td>L166V</td>
<td>5</td>
<td>ND</td>
</tr>
<tr>
<td>V168C</td>
<td>3</td>
<td>&lt;100</td>
</tr>
<tr>
<td>N171D</td>
<td>4</td>
<td>~7100</td>
</tr>
<tr>
<td>N171Q</td>
<td>2</td>
<td>~720</td>
</tr>
</tbody>
</table>

Mutant constructs were expressed in TsA-201 cells, and inhibition by compound A was examined by whole-cell voltage clamp as indicated under Experimental Procedures. ND denotes constructs that did not generate detectable currents.
Table 3: Effect of intravenous infusion of Compound A or HCTZ on mean arterial pressure, heart rate, renal excretory function, and blood electrolytes in anesthetized rats.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Compound A</th>
<th></th>
<th>HCTZ</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Treatment</td>
<td>Control</td>
<td>Treatment</td>
</tr>
<tr>
<td>Systolic Blood Pressure (mm Hg)</td>
<td>132 ± 6</td>
<td>122 ± 5</td>
<td>131 ± 4</td>
<td>127 ± 4</td>
</tr>
<tr>
<td>Diastolic Blood Pressure (mm Hg)</td>
<td>93 ± 5</td>
<td>88 ± 6</td>
<td>89 ± 5</td>
<td>85 ± 5</td>
</tr>
<tr>
<td>Heart Rate (beats/min)</td>
<td>388 ± 18</td>
<td>383 ± 22</td>
<td>366 ± 15</td>
<td>379 ± 17</td>
</tr>
<tr>
<td>Urine Flow (μl/min/KW(g))</td>
<td>8 ± 0.9</td>
<td>30 ± 6.6*</td>
<td>6 ± 0.5</td>
<td>23 ± 2.4**</td>
</tr>
<tr>
<td>Urinary Na⁺ Excretion (μmol/min/KW(g))</td>
<td>0.5 ± 0.1</td>
<td>4.9 ± 1.1*</td>
<td>0.2 ± 0.1</td>
<td>3.5 ± 0.5**</td>
</tr>
<tr>
<td>Urinary Cl⁻ Excretion (μmol/min/KW(g))</td>
<td>0.5 ± 0.1</td>
<td>5.2 ± 1.1*</td>
<td>0.2 ± 0.1</td>
<td>3.8 ± 0.4**</td>
</tr>
<tr>
<td>Urinary K⁺ Excretion (μmol/min/KW(g))</td>
<td>1.5 ± 0.1</td>
<td>1.7 ± 0.1</td>
<td>1.3 ± 0.1</td>
<td>1.9 ± 0.2*</td>
</tr>
<tr>
<td>Blood Na⁺ Concentration (mmol/L)</td>
<td>139 ± 0.5</td>
<td>138 ± 0.5*</td>
<td>139 ± 0.2</td>
<td>138 ± 0.3</td>
</tr>
<tr>
<td>Blood Cl⁻ Concentration (mmol/L)</td>
<td>102 ± 0.5</td>
<td>100 ± 0.9*</td>
<td>102 ± 0.4</td>
<td>100 ± 0.4**</td>
</tr>
<tr>
<td>Blood K⁺ Concentration (mmol/L)</td>
<td>3.5 ± 0.1</td>
<td>3.4 ± 0.04</td>
<td>3.6 ± 0.1</td>
<td>3.2 ± 0.1**</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>43.6 ± 0.8</td>
<td>46.6 ± 1.5</td>
<td>41.2 ± 0.3</td>
<td>43.0 ± 2.0</td>
</tr>
</tbody>
</table>

Compound A or HCTZ were given as a continuous intravenous infusion at 1.55 mg/kg/h, or 5 mg/kg/h, respectively. Data are mean ± SEM (n=5 and 6 for compound A and
HCTZ studies, respectively). * P<0.05, ** P <0.01 vs. control. Urine flow and urinary electrolyte (Na⁺, Cl⁻, and K⁺) excretion rates were corrected for kidney weight (KW).
Table 4: Plasma and kidney concentrations of compound A after oral administration to SD rats.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Plasma (μM)</th>
<th>Kidney (μM)</th>
<th>Kidney/Plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25</td>
<td>0.37</td>
<td>1.4</td>
<td>3.9</td>
</tr>
<tr>
<td>1</td>
<td>0.61</td>
<td>4.1</td>
<td>6.7</td>
</tr>
<tr>
<td>4.5</td>
<td>0.65</td>
<td>3.3</td>
<td>5.1</td>
</tr>
</tbody>
</table>

SD rats (n =3 per each time point) were orally dosed with 10 mg/kg compound A formulated as a 10 mg/ml suspension in Imwitor 742: Tween 80 (1:1, v:v). Plasma and kidney concentrations were determined by LC-MS/MS.
Table 5. Plasma and kidney concentrations of compound A after oral administration to dogs.

<table>
<thead>
<tr>
<th>Collection Time (min)</th>
<th>Plasma Concentration (μM)</th>
<th>Urine Concentration (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10 mg/kg</td>
<td>50 mg/kg</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0-30</td>
<td>0.1</td>
<td>1.5</td>
</tr>
<tr>
<td>30-60</td>
<td>0.1</td>
<td>3.2</td>
</tr>
<tr>
<td>60-90</td>
<td>0.1</td>
<td>2.9</td>
</tr>
<tr>
<td>90-120</td>
<td>0.2</td>
<td>3.9</td>
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<td>120-150</td>
<td>0.2</td>
<td>8.5</td>
</tr>
<tr>
<td>150-180</td>
<td>0.2</td>
<td>9.9</td>
</tr>
</tbody>
</table>

Female dogs (n=3) were orally dosed with either 10 or 50 mg/kg compound A at a dose volume of 0.5 ml/kg in Imwitor 742: Tween 80 (1:1, v:v). Plasma and urine samples were collected before and after oral administration of compound A at 30-min interval periods for up to 3 hours post-dosing. Concentrations of compound A were determined by LC-MS/MS.
Figure 2

(A) Thallium flux (% control) vs. log [Compound A] (M).
(B) $^{86}$Rb$^+$ flux (% control) vs. log [Compound A] (M).
(C) $^{86}$Rb$^+$ flux (% control) vs. log [Compound A] (M).
Figure 3

A

Voltage (mV)

-80   -60   40   20

Current (nA)

-2   2

B

Current (nA)

-3

Time (s)

0   300   600   900   1200   1500   1800

1000 nM

300 nM

100 nM

30 nM

C

Percent Inhibition

0   20   40   60   80   100

Log [Compound A] (M)

-8.0   -7.5   -7.0   -6.5   -6.0   -5.5