VU6036720: The first potent and selective *in vitro* inhibitor of heteromeric Kir4.1/5.1 inward rectifier potassium channels

Samantha J. McClenahan*, Caitlin N. Kent*, Sujay V. Kharade, Elena Isaeva, Jade C. Williams, Changho Han, Andrew Terker, Robert Gresham III, Roman M. Lazarenko, Emily L. Days, Ian M. Romaine, Joshua A. Bauer, Olivier Bouteau, Gary A. Sulikowski, Raymond Harris, C. David Weaver, Alexander Staruschenko, Craig W. Lindsley, and Jerod S. Denton

Department of Anesthesiology, Vanderbilt University Medical Center, Nashville, TN (SJM, SVK, RG, RL, JSD)

Department of Cell Biology, Neurobiology and Anatomy, Medical College of Wisconsin, Milwaukee, WI (EI)

Department of Molecular Pharmacology and Physiology, University of South Florida, Tampa, FL (AS)

Department of Chemistry, Vanderbilt University, Nashville, TN (CNK, JCW, IMR, CDW, GAS, CWL)

Department of Pharmacology, Vanderbilt University, Nashville, TN (ED, CDW, CWL, CH, OB, JSD)

Department of Biochemistry, Vanderbilt University, Nashville, TN (JAB)

Vanderbilt Institute of Chemical Biology, Vanderbilt University, Nashville, TN 37232 (JAB, GS, CDW, CWL, JSD)

*SJM and CNK contributed equally

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Corresponding author:

Jerod S. Denton, Ph.D.
1161 21st Avenue South
T4208 Medical Center North
Nashville, TN 37232
jerod.s.denton@vumc.org

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Nonstandard abbreviations: AUC, area under the curve; CCD, cortical collecting duct; cDNA, complimentary DNA; CLp, plasma clearance; CMV, cytomegalovirus; C_{max}, maximal concentration; DCT, distal convoluted tubule; DMEM, Dulbecco's modified Eagle's medium; DMPK, Drug metabolism pharmacokinetics; EAST, epilepsy, ataxia, sensorineural deafness, tubulopathy; EF-1 α , Elongation factor-1 α ; EWGs, electron withdrawing group; EDGs, electron donating groups; F, oral bioavailability; f_u , fraction unbound; HBAs, hydrogen bond acceptors; HBDs, hydrogen bond donors; HCTZ, hydrochlorothiazide; IC₅₀, half-maximal inhibitor concentration; hERG, human EAG-related gene; K⁺, potassium ion; Kir, inward rectifier potassium; PO, per os (oral administration); SeSAME, seizures, sensorineural deafness, ataxia, mental disability, tubulopathy; PBL, plasma brain levels; SAR, structure-activity relationships; T_{1/2}, half-life; Tl⁺, thallium ion; T_{max}, time of maximal concentration; V_{ss}, volume at steady state

ABSTRACT

Heteromeric Kir4.1/Kir5.1 (*KCNJ10/KCNJ16*) inward rectifier potassium (Kir) channels play key roles in the brain and kidney, but pharmacological tools for probing their physiology and therapeutic potential have not been developed. Here we report the discovery, in a high-throughput screen of 80,475 compounds, of the moderately potent and selective inhibitor VU0493690, which we selected for characterization and chemical optimization. VU0493690 concentration-dependently inhibits Kir4.1/5.1 with an IC_{50} of 0.96 μ M and exhibits at least 10-fold selectivity over Kir4.1 and ten other Kir channels. Multi-dimensional chemical optimization of VU0493690 led to the development of VU6036720, the most potent (IC_{50} = 0.24 μ M) and selective (>40-fold over Kir4.1) Kir4.1/5.1 inhibitor reported to date. Cell-attached patch single channel recordings revealed that VU6036720 inhibits Kir4.1/5.1 activity through a reduction of channel open-state probability and single-channel current amplitude. Elevating extracellular K^+ by 20 mM shifted the IC_{50} 6.8-fold, suggesting VU6036720 is a pore blocker that binds in the ion-conduction pathway. Mutation of the “rectification controller” asparagine 161 to glutamate (N161E), which is equivalent to small-molecule binding sites in other Kir channels, led to a strong reduction of inhibition by VU6036720. Renal clearance studies in mice failed to show a diuretic response that would be consistent with inhibition of Kir4.1/5.1 in the renal tubule. Drug metabolism and pharmacokinetics profiling revealed that high VU6036720 clearance and plasma protein binding may prevent target engagement *in vivo*. In conclusion, VU6036720 represents the current state-of-the-art Kir4.1/5.1 inhibitor that should be useful for probing the functions of Kir4.1/5.1 *in vitro* and *ex vivo*.

SIGNIFICANCE STATEMENT:

Heteromeric inward rectifier potassium (Kir) channels comprising Kir4.1 and Kir5.1 subunits play important roles in renal and neural physiology and may represent inhibitory drug targets for hypertension and edema. Herein, we employ high-throughput compound library screening, patch clamp electrophysiology, and medicinal chemistry to develop and characterize the first potent and specific *in vitro* inhibitor of Kir4.1/5.1, VU6036720, which provides proof-of-concept that drug-like inhibitors of this channel may be developed.

INTRODUCTION

Inward rectifier potassium (Kir) channels are tetrameric, membrane-spanning proteins that conduct potassium (K^+) ions down their electrochemical gradient to regulate cell excitability, hormone secretion, and transepithelial ion transport (Hibino et al., 2010; Welling, 2016). There are sixteen genes in the Kir channel family and gain- or loss-of-function mutations in several of them are associated with human diseases. Sulfonylurea receptor (SUR) ligands that inhibit Kir6.2/SUR1 (K_{ATP}) channels in pancreatic beta cells stimulate insulin secretion and promote blood glucose homeostasis in patients with type II diabetes (Nichols, 2006). While other Kir channels are postulated to be drug targets for treating other diseases, a dearth of pharmacological tool compounds that are potent and selective enough to precisely modulate specific subtypes of Kir channels has been a critical barrier to rigorously validating these putative drug targets (Weaver and Denton, 2021).

Homotetrameric Kir4.1 and heterotetrameric Kir4.1/5.1 (Kir4.1 and Kir5.1 are encoded by *KCNJ10* and *KCNJ16*, respectively) channels are two subtypes of Kir channels expressed in the brain and kidney that may hold therapeutic potential for treating neurological and cardiovascular diseases (Bockenhauer et al., 2009; Denton et al., 2013; Reichold et al., 2010; Schlingmann et al., 2021; Scholl et al., 2009). Kir4.1 forms functional K^+ channels on the plasma membrane when expressed alone, whereas Kir5.1 does not function on its own but can form heteromeric channels with Kir4.1 (Pessia et al., 1996).

Homomeric Kir4.1 is expressed primarily in brain astrocytes where they help remove extracellular K⁺ and glutamate released from actively firing neurons, in a process called spatial K⁺ buffering (Djukic et al., 2007; Kofuji et al., 2002; Nwaobi et al., 2016). Changes in the expression levels of Kir4.1 and associated extracellular K⁺ levels have been found in models of clinical depression (Cui et al., 2018; Frizzo and Ohno, 2021), ischemic stroke (Milton and Smith, 2018), and Huntington's disease (Jiang et al., 2016; Tong et al., 2014), suggesting a mechanistic link between Kir4.1 and these diseases. Kir4.1 channels have also been implicated in the behavioral and respiratory disturbances in Rett syndrome (Kahanovitch et al., 2018; Zhang et al., 2011), although their specific contributions in this polygenic disease are unclear.

Heteromeric Kir4.1/5.1 channels are the dominant channel subtype expressed in the kidney tubule (Manis et al., 2020; Weaver and Denton, 2021; Welling, 2016). Although they appear to be expressed throughout most of the nephron, Kir4.1/5.1 channels play especially important roles in regulating sodium, chloride and K⁺ balance by the distal convoluted tubule (DCT) and cortical collecting duct (CCD) (Cuevas et al., 2017; Su et al., 2019; Su and Wang, 2016; Terker et al., 2015; Zhang et al., 2014). The importance of Kir4.1 and Kir4.1/5.1 channels in humans was definitively established with the discovery that loss-of-function mutations in *KCNJ10* cause EAST/SeSAME syndrome, an autosomal recessive disorder characterized by epilepsy, ataxia, sensorineural deafness, intellectual disability, and renal salt wasting (Bockenhauer et al., 2009; Reichold et al., 2010; Scholl et al., 2009). Similarly, recent studies revealed that mutations in *KCNJ16* are associated with hypokalemia, salt wasting, disturbed

acid-base homeostasis, and sensorineural deafness (Schlingmann et al., 2021; Webb et al., 2021)

Kir4.1 and Kir4.1/5.1 channels differ significantly in their functional and regulatory properties, as well as their sensitivity to small-molecule inhibitors. For example, heteromeric Kir4.1/5.1 channels have a larger unitary conductance, lower open-state probability, and greater sensitivity to changes in intracellular pH than homomeric Kir4.1 (Lagrutta et al., 1996; Pessia et al., 2001; Tucker et al., 2000). The molecular pharmacology of the two channels is still very rudimentary, however, homomeric Kir4.1 is known to be inhibited by the serotonin reuptake inhibitors fluoxetine and nortriptyline, and the tricyclic antidepressant amitriptyline (Furutani et al., 2009; Ohno et al., 2007). Unfortunately, these compounds are relatively weak inhibitors (i.e. IC_{50} s in the tens of micromolar) and exhibit broad inhibitory activity toward several other members of the Kir channel family. Chloroquine (Marmolejo-Murillo et al., 2017b) and pentamidine (Arechiga-Figueroa et al., 2017) also inhibit Kir4.1, but their usefulness as *in vivo* tool compounds is limited. We recently reported the discovery of a moderately potent ($IC_{50}=1\ \mu M$), *in vivo-active* Kir4.1 inhibitor that is 9-fold selective over Kir4.1/5.1 and greater than 30-fold selective over Kir1.1, Kir2.1, and Kir2.2, demonstrating that the development of tool compounds capable of discriminating between homomeric Kir4.1 and heteromeric Kir4.1/5.1 channels and other members of the Kir family is feasible (Kharade et al., 2018). Here, we report the successful development and detailed characterization of the first potent and selective Kir4.1/5.1 channel inhibitor: VU6036720.

METHODS

Molecular biology and HEK-293-Kir4.1/5.1 cell line generation

Expression plasmids carrying the complementary DNA (cDNA) sequence of human Kir4.1 or Kir5.1 were purchased from Origene Technologies. To ensure that both channel subunits are expressed in transfected cells, the cDNAs were subcloned into different multiple cloning sites of the bicistronic vector pBudCE4.1 (Invitrogen). The hKir4.1 cDNA was subcloned downstream of the CMV promoter, whereas the hKir5.1 cDNA was subcloned downstream of the EF-1 α promoter using gene synthesis methods by GenScript. The Kir5.1-asparagine 151-to-glutamate mutation was created using the QuickChange mutagenesis kit (Agilent Technologies) according to manufacturer's instructions. The correct cDNA sequences were verified with DNA sequencing.

HEK-293T cells were transfected using Lipofectamine LTX reagent according to the manufacturer's protocol and then placed under antibiotic selection using 700 ug/ml zeocin. Single clones were isolated from stably transfected polyclonal cells using limiting dilution methods, expanded, and then screened for robust Kir4.1/5.1-mediated thallium (Tl⁺) flux using the methods described below. Stably transfected monoclonal T-Rex-human embryonic kidney 293 (HEK-293) cell lines expressing Kir1.1, Kir2.2, Kir2.2, Kir2.3, Kir4.1, Kir4.2, Kir6.1/sulfonylurea receptor 2B (SUR2B), Kir6.2/sulfonylurea receptor 1 (SUR1), Kir7.1-M125R, or Kv11.1 (hERG) were generated and cultured as described previously (Bhave et al., 2011; Lewis et al., 2009; Raphemot et al., 2013; Raphemot et al., 2011; Swale et al., 2016). HEK-293 cells expressing hERG were constructed as described (Kaufmann et al., 2013).

Quantitative Ti^+ flux assay of Kir4.1/5.1 activity

High-throughput Ti^+ flux fluorescence-based assay was performed in the Vanderbilt High-Throughput Screening Center essentially as described previously (Kharade et al., 2018). Briefly, HEK-293-Kir4.1/5.1 cells were culture overnight in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS at 37 °C and 5% CO_2 in 384-well plates. The following day, the cells were incubated with dye-loading assay buffer (Hank's balanced salt solutions, 20 mM HEPES, pH 7.3) containing 0.01% (w/v) Pluronic F-127 (Life Technologies) and 1.2 μM Ti^+ -reporting dye Thallos Gold (Dutter et al., 2018) (or "Pluronic") or Brilliant Thallium (Ion biosciences, Austin, TX) in ambient conditions for 1 hour with 20 μL /well assay buffer, washing before and after dye using Hanks' balanced salt solution/20 mM HEPES (assay buffer). Media and buffer exchange were performed on a ELx405 TS plate washer (BioTek, Winooski, VT). Dye-loaded cells were then transferred to a Panoptic Kinetic Imaging Plate Reader (Wavefront Bioscience, Franklin, TN) to collect live measurements at 1 Hz (482/35 nm excitation and 536/40 nm emission Brilliant Thallium; 517/20 nm excitation and 580/60 nm emissions Thallos Gold) during simultaneous 384-well pipetting of 10 μM small molecules (0.1% DMSO) or control (100 μM fluoxetine). Compounds were incubated for 4 minutes before the addition of Ti^+ stimulus buffer (125 mM NaHCO_3 , 1.8 mM CaSO_4 , 1 mM MgSO_4 , 5 mM glucose, 1.2 mM Ti_2SO_4 , and 10 mM HEPES, pH 7.4 titrated with NaOH). Live measurements were collected 10 seconds before the addition of compounds to 2 minutes after Ti^+ stimulus addition.

For identified hits in the primary screen and structure-activity relationship libraries, compounds were tested at concentrations to obtain 10-point, 3-fold dilution

concentration-response curves. Data acquisition and analysis were performed using Waveguide (VU-HTS center) and Microsoft Excel. IC₅₀ values were determined by fitting the Hill equation using variable-slope nonlinear regression analyses performed with GraphPad Prism version 5.01 (GraphPad Software, San Diego, CA).

Whole-cell patch clamp electrophysiology

HEK-293T cells were transfected with wild-type (WT) pBudCE4.1-Kir4.1/5.1 or combinations of pcDNA5-Kir4.1 and pcDNA5-Kir5.1 with pcDNA3.1-EGFP (transfection marker) using Lipofectamine LTX reagent according to the manufacturer's instructions. The cells were dissociated the following day and plated on poly-L-lysine-coated coverslips and allowed to recover for at least 1 hour in a 37 °C 5% CO₂/95% air incubator before beginning experiments. Patch electrodes (2–3 mega Ohm) were filled with an intracellular solution containing 135 mM KCl, 2 mM MgCl₂, 1 mM EGTA, 10 mM HEPES-free acid, and 2 mM Na₂ATP (Roche Diagnostics, Risch-Rotkreuz, Switzerland), pH 7.3 titrated with KOH, 275 mOsmol/kg water. The standard bath solution contained 135 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 5 mM glucose, and 10 mM HEPES free acid, pH 7.4 titrated with NaOH. Macroscopic currents were recorded under whole-cell voltage-clamp conditions using an Axopatch 200B Amplifier (Molecular Devices, Sunnyvale, CA). Cells were voltage clamped at a holding potential of -75 mV and stepped every 5 seconds to -120 mV for 200 milliseconds before ramping to +120 mV at a rate of 1.2 mV/ms. Data were collected at 5 kHz and filtered at 1 kHz. Data acquisition and analysis were performed using the pClamp 9.2 software suite (Molecular Devices). Pharmacology experiments were terminated by applying 2 mM barium (Ba²⁺) chloride to block the heterologously expressed Kir currents and measure

residual leak current. Cells exhibiting <90% block by Ba^{2+} were excluded from the analysis. The mean current amplitude recorded over five successive steps to -120 mV in cells at a single concentration was expressed as the mean $\pm$ S.D. IC_{50} values were determined by fitting the Hill equation to CRCs using variable-slope nonlinear regression analyses. All the analyses were performed with GraphPad Prism version 5.01 (GraphPad Software).

Single-channel electrophysiology recordings

Chinese Hamster Ovary (CHO) cells were used for single-channel recordings because they express very little endogenous K^+ channel activity that could interfere with these recordings. Cells were maintained under standard culture conditions (F12K, 10% fetal bovine serum, 100 U/ml penicillin-streptomycin, 37°C, 5% CO_2), and transfected with pIRES-Kir4.1, pBudCE4.1-Kir4.1/Kir5.1, or pBudCE4.1-Kir4.1/5.1-N161E with eGFP plasmid DNA using the Polyfect reagent (Qiagen, Valencia, CA) according to manufacturer's protocol. Patch-clamp recordings of inwardly rectifying K^+ currents were carried out 24-48 hrs following transfection. Single-channel Kir4.1 and Kir4.1/Kir5.1 activity were performed in cell-attach voltage-clamp configuration using an Axopatch 200B amplifier (Molecular Devices) and Digidata 1440A analog-to-digital converter (Molecular Devices). Recordings were made using extracellular solution (in mM): 150 NaCl, 5 KCl, 1 CaCl_2 , 2 MgCl_2 , 5 glucose, and 10 HEPES, pH 7.35 titrated with NaOH. Patch electrodes were filled with a solution of the following composition (in mM): 100 NaCl, 50 KCl, 2 MgCl_2 , and 10 HEPES, pH 7.35 titrated with NaOH and had resistances from 8 to 10 M Ω . Currents were low-pass filtered at 0.3 kHz with an eight-pole Bessel filter (Warner Instruments, Hamden, CT). Channel activity was assessed using Clampfit

10.6 software (Molecular Devices). Statistical analysis was performed using two-sided paired t-test or one-way ANOVA with Bonferroni multiple comparison test, as appropriate, with statistical significance defined at $P < 0.0167$ and 0.05, respectively.

Renal clearance studies

10-week-old male C57Bl/6 mice were used for urine collection studies. Five mice were orally gavaged with vehicle (10% Tween 80 in PBS) and five with VU6036720 at a dose of 30 or 100 mg/kg prior to a four-hour urine collection in metabolic cages. One week later, the vehicle group was gavaged with VU6036720 at 30 mg/kg and the group that received VU6036720 was gavaged with vehicle prior to another four-hour urine collection. Seven of the mice underwent a third gavage treatment with VU6036720 at 100 mg/kg two weeks later prior to another four-hour urine collection. Three mice that had previously received either vehicle or VU6036720 received the diuretic hydrochlorothiazide (HCTZ) as a positive control.

General chemistry methods

All NMR spectra were recorded on a 400 MHz AMX Bruker NMR spectrometer. ^1H and ^{13}C chemical shifts are reported in δ values in ppm downfield with the deuterated solvent as the internal standard. Data are reported as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, b = broad, m = multiplet), Coupling constant (Hz) and integration. Low resolution mass spectra were obtained on an Agilent 6120 or 6150 with ESI source. Method A: MS parameters were as follows: fragmentor: 70, capillary voltage: 3000 V, nebulizer pressure: 30 psig, drying gas flow: 13 L/min, drying gas temperature: 350 °C. Samples were introduced via an Agilent 1290 UHPLC comprised of a G4220A binary pump, G4226A ALS, G1316C TCC, and

G4212A DAD with ULD flow cell. UV absorption was generally observed at 215 nm and 254 nm with a 4 nm bandwidth. Column: Waters Acquity BEH C18, 1.0 x 50 mm, 1.7 μ m. Gradient conditions: 5% to 95% CH₃CN in H₂O (0.1% TFA) over 1.4 min, hold at 95% CH₃CN for 0.1 min, 0.5 mL/min, 55 °C. Method B: MS parameters were as follows: fragmentor: 100, capillary voltage: 3000 V, nebulizer pressure: 40 psig, drying gas flow: 11 L/min, drying gas temperature: 350 °C. Samples were introduced via an Agilent 1200 HPLC comprised of a degasser, G1312A binary pump, G1367B HP-ALS, G1316A TCC, G1315D DAD, and a Varian 380 ELSD (if applicable). UV absorption was generally observed at 215 nm and 254 nm with a 4 nm bandwidth. Column: Thermo Accucore C18, 2.1 x 30 mm, 2.6 μ m. Gradient conditions: 7% to 95% CH₃CN in H₂O (0.1% TFA) over 1.6 min, hold at 95% CH₃CN for 0.35 min, 1.5 mL/min, 45 °C. High resolution mass spectra were obtained on an Agilent 6540 UHD Q-TOF with ESI source. MS parameters were as follows: fragmentor: 150, capillary voltage: 3500 V, nebulizer pressure: 60 psig, drying gas flow: 13 L/min, drying gas temperature: 275 °C. Samples were introduced via an Agilent 1200 UHPLC comprised of a G4220A binary pump, G4226A ALS, G1316C TCC, and G4212A DAD with ULD flow cell. UV absorption was observed at 215 nm and 254 nm with a 4 nm bandwidth. Column: Agilent Zorbax Extend C18, 1.8 μ m, 2.1 x 50 mm. Gradient conditions: 5% to 95% CH₃CN in H₂O (0.1% formic acid) over 1 min, hold at 95% CH₃CN for 0.1 min, 0.5 mL/min, 40 °C. Optical specific rotations were obtained using JASCO P-2000 Digital Polarimeter equipped with Tungsten-Halogen lamp (WI), 589 nm wavelength, photomultiplier tube (1P28-01) detector and CG2-100 Cylindrical glass cell, 2.5 ϕ x 100 mm. For compounds that were purified on a Gilson preparative reversed-phase HPLC, the system comprised of a 333 aqueous pump with solvent-

selection valve, 334 organic pump, GX271 or GX-281 liquid handler, two column switching valves, and a 155 UV detector. UV wavelength for fraction collection was user-defined, with absorbance at 254 nm always monitored. Method 1: Phenomenex Axia-packed Luna C18, 30 x 50 mm, 5 μ m column. Mobile phase: CH₃CN in H₂O (0.1% TFA). Gradient conditions: 0.75 min equilibration, followed by user defined gradient (starting organic percentage, ending organic percentage, duration), hold at 95% CH₃CN in H₂O (0.1% TFA) for 1 min, 50 mL/min, 23 °C. Method 2: Phenomenex Axiapacked Gemini C18, 50 x 250 mm, 10 μ m column. Mobile phase: CH₃CN in H₂O (0.1% TFA). Gradient conditions: 7 min equilibration, followed by user defined gradient (starting organic percentage, ending organic percentage, duration), hold at 95% CH₃CN in H₂O (0.1% TFA) for 7 min, 120 mL/min, 23 °C. Chiral separation was performed on a Thar (Waters) Investigator SFC Column: Chiral Technologies CHIRALPAK IF, 4.6 x 250 mm, 5 μ m column. Gradient conditions: 20% to 50% IPA in CO₂ over 7 min, hold at 50% CO₂ for 1 min. Flow rate: 3.5 mL/min. Column temperature: 40 °C. System backpressure: 100 bar. Solvents for extraction, washing and chromatography were HPLC grade. All reagents were purchased from Aldrich Chemical Co. and were used without purification. All compounds described in the SAR tables were >95% pure by LCMS (214 nm, 254 nM and ELSD) as well as ¹H NMR.

Initially, small quantities of VU0493690 were purchased from Life Chemicals (Niagara-on-the-Lake ON), and larger quantities were prepared following a variation of the experimental procedure below for the synthesis of VU6036720.

Chemical synthesis

See Supplemental Methods

Hepatic microsomal intrinsic clearance

Human, rat, and mouse hepatic microsomes (0.5 mg/mL) and 1 μ M test compound were incubated in 100 mM K⁺ phosphate pH 7.4 buffer with 3 mM MgCl₂ at 37 °C with constant shaking. After a 5 min preincubation, the reaction was initiated by addition of NADPH (1 mM). At selected time intervals (0, 3, 7, 15, 25, and 45 min), aliquots were taken and subsequently placed into a 96-well plate containing cold acetonitrile with internal standard (50 nM carbamazepine). Plates were then centrifuged at 3000 RCF (4° C) for 10 min, and the supernatant was transferred to a separate 96-well plate and diluted 1:1 with water for LC/MS/MS analysis. The *in vitro* half-life ($t_{1/2}$, min), intrinsic clearance (CL_{int} , mL/min/kg) and subsequent predicted hepatic clearance (CL_{hep} , mL/min/kg) were determined using Equations 1 – 3:

$$T_{1/2} = \frac{\ln(2)}{k} \quad [1]$$

Equation 1. Determination of half-life. k represents the slope from linear regression analysis of the natural log percent remaining of test compound as a function of incubation time.

$$CL_{int} = \frac{0.693}{in\ vitro\ T_{1/2}} \times \frac{1\ mL\ incubation}{0.5\ mg\ microsomes} \times \frac{45\ mg\ microsomes}{1\ gram\ liver} \times \frac{20^a\ gram\ liver}{kg\ body\ wt} \quad [2]$$

Equation 2. Determination of intrinsic clearance. ^ascale-up factors (gm liver/kg body weight) of 20 (human), 45 (rat), and 87.5 (mouse) were used in this calculation (scaling factors were derived from (Lin et al., 1996)).

$$CL_{hep} = \frac{Q_h * CL_{int}}{Q_h + CL_{int}} \quad [3]$$

Equation 3. Determination of predicted hepatic clearance. Q_h represents hepatic blood flow (ml/min/kg): 21 for human, 70 for rat, and 90 for mouse.

Plasma-protein and brain-homogenate binding

The protein binding of each compound was determined in plasma via equilibrium dialysis employing rapid equilibrium dialysis (RED) plates (ThermoFisher Scientific, Rochester, NY). Plasma was added to the 96 well plate containing test compound and mixed thoroughly for a final concentration of 5 μ M. Subsequently, an aliquot of the plasma-compound mixture was transferred to the *cis* chamber (red) of the RED plate, with a phosphate buffer (25 mM, pH 7.4) in the *trans* chamber. The RED plate was sealed and incubated for 6 hours at 37 °C with shaking (120 rpm). At completion, aliquots from each chamber were transferred to a new 96-well plate and were diluted 1:1 with either plasma (*trans*) or buffer (*cis*) and transferred to a new 96 well plate, at which time ice-cold acetonitrile containing internal standard (50 nM carbamazepine) (3 volumes) was added to extract the matrices. The plate was centrifuged (3000 RCF, 10 min) and supernatants transferred and diluted 1:1 (supernatant: water) into a new 96 well plate, which was then sealed in preparation for LC/MS/MS analysis. Each compound was assayed in triplicate within the same 96-well plate.

A similar approach was used to determine the degree of brain homogenate binding, which employed the same methodology and procedure with the following modifications: 1) a final compound concentration of 1 μ M was used, 2) naïve rat brains were homogenized in DPBS (1:3 composition of brain: DPBS, w/w) using a Mini-Bead Beater™ machine in order to obtain brain homogenate, which was then treated in the same manner as the plasma samples in the previously described plasma protein

binding assay. Fraction unbound for both plasma and brain samples was determined using Equation 4.

$$f_u = \frac{Conc_{buffer}}{Conc_{plasma}} \quad [4]$$

Equation 4 Determination of fraction unbound in plasma. The diluted fraction unbound (f_{u2}) in brain was calculated in the same manner by using brain homogenate rather than plasma. Undiluted fraction unbound for the brain was calculated using Equation 5

$$f_u = \frac{1/4}{\left\{ \left(\frac{1}{f_{u2}} \right) - 1 \right\} + 1/4}$$

Equation 5 Determination of fraction unbound in brain. F_{u2} represents the diluted fraction unbound.

Rat IV PBL cassette

Male Sprague-Dawley rats (n=2) weighing around 300g were purchased from Harlon laboratories (Indianapolis, IN) and implanted with catheters in the carotid artery and jugular vein. The cannulated animals were acclimated to their surroundings for approximately one week before dosing and provided food and water *ad libitum*. IV cassette PK experiments in rats were carried out according to methods described previously (Bridges et al., 2014). Briefly, a cassette of compounds (n = 4–5 compounds/cassette) was formulated from 10 mM solutions of compounds in DMSO. In order to reduce the absolute volume of DMSO that was administered, the compounds were combined and diluted with ethanol and PEG 400 to achieve a final concentration of 0.4–0.5 mg/mL for each compound (2 mg/mL total) administered in each cassette. The final dosing solutions consisted of approximately 10% ethanol, 40% PEG400, and

50% DMSO (v/v). Each cassette dose was administered IV via the jugular vein to two dual-cannulated (carotid artery and jugular vein) adult male Sprague–Dawley rats, each weighing between 250 and 350 g (Harlan, Indianapolis, IN) for a final dose of 0.2–0.25 mg/kg per compound. Whole blood collections via the carotid artery were performed at 0.033, 0.117, 0.25, 0.5, 1, 2, 4, 7, and 24 hours post dose and plasma samples prepared for bioanalysis. For tissue distribution studies in cassette format, brain dissection and blood collections via the carotid artery were performed at 0.25 hr post dose. Blood samples were collected into chilled, EDTA-fortified tubes, centrifuged for 10 minutes at 3000 rpm (4°C), and resulting plasma aliquoted into 96-well plates for LC/MS/MS analysis. The brain samples were rinsed in PBS, snap frozen and stored at -80 °C. Prior to LC/MS/MS analysis, whole brain samples were thawed to room temperature and subjected to mechanical homogenation in 3 ml of 70:30 IPA:water employing a Mini-Beadbeater™ and 1.0 mm Zirconia/Silica Beads (BioSpec Products) for 3 min, and centrifuged at 3,500 g for 5 min. 5 µL of the supernatant was diluted in 15 µL of blank plasma for quantification of the analytes. Discrete IV PK experiments in rats (n=2) were carried out analogously at a dose of 1.0 mg/kg in 10% EtOH, 50% PEG 400, 40% saline, while discrete PO PK experiments in rats (n=2) were carried out using a 3 mg/kg dose of compounds in a fine microsuspension in 30% Captisol in H₂O via oral gavage to fasted animals. Whole blood collections via the carotid artery were performed at 0.117, 0.25, 0.5, 1, 2, 4, 7, and 24 hours post dose. Plasma samples were centrifuged at 3,500 g for 5 min. A standard curve was generated by diluting the analytes DMSO stocks with blank plasma to obtain a final concentration of 10,000 ng/ml followed by a serial dilution down to 0.5 ng/ml. Quality controls were generated by a

serial dilution of the 5,000 ng/ml standard curve solution in blank plasma to obtain 3 concentrations of 500, 50 and 5 ng/ml. 20 uL of brain diluted in plasma, plasma, blank plasma, standard curve and QC samples were loaded in a V-bottom 96-well plate. 120 uL of acetonitrile containing 50 nM carbamazepine (internal standard) was added in each well and the plate was centrifuged at 3,500 g for 5 min. 60 uL of the supernatant of each well (protein free) was transferred to a new 96-well plate containing 60 uL of water. The plates were sealed for analysis by LC-MS/MS.

LC/MS/MS bioanalysis of samples

Plasma and brain tissue samples originating from *in vivo* studies were analyzed by electrospray ionization using an AB Sciex Q-TRAP 5500 (Foster City, CA) that was coupled to a Shimadzu LC-20AD pump (Columbia, MD) and a Leap Technologies CTC PAL auto-sampler (Carrboro, NC). Analytes were separated by gradient elution using a C18 column (3 x 50 mm, 3 mm; Fortis Technologies Ltd, Cheshire, UK) that was thermostated at 40 °C. HPLC mobile phase A was 0.1% formic acid in water (pH unadjusted); mobile phase B was 0.1% formic acid in acetonitrile (pH unadjusted). A 10% B gradient was held for 0.2 min and was linearly increased to 90% B over 0.8 min, with an isocratic hold for 0.5 min, before transitioning to 10% B over 0.05 minute. The column was re-equilibrated (1 minute) before the next sample injection. The total run time was 2.55 min, and the HPLC flow rate was 0.5 ml/min. The source temperature was set at 500 °C, and mass spectral analyses were performed using a Turbo-Ion spray source in positive ionization mode (5.0-kV spray voltage) and using multiple-reaction monitoring of transitions specific for each analyte. All data were analyzed using AB

Sciex Analyst 1.5.1 software. The lower limits of quantitation were determined at 1.0 ng/ml in plasma and in brain homogenates.

Mouse IV and PO PK studies

10-week-old male C57Bl/6 mice were used for the pharmacokinetic studies performed at Frontage Laboratories, Inc. (Exton, PA). Three mice were administered intra-venously with VU6036720 in ethanol: PEG400: saline (10:70:20 v/v/v) at 1 mg/kg. Three mice were orally gavaged with VU6036720 in 10% Tween 80 in water at 10 or 100 mg/kg. Blood samples were collected from all mice at 8 different time points post-dose. The concentration of VU6036720 in blood was quantified by LC/MS/MS as described above.

The IV PK experiment was used to determine clearance, Volume of distribution and half-life. The PO experiments were used to determine exposure and oral bioavailability. PK parameters of VU6036720 were calculated using Phoenix WinNonlin software (version 8.1).

RESULTS

Electrophysiological characterization of HEK-293-Kir4.1/5.1 cells

Kir4.1/5.1 channels were expressed from the bicistronic vector, pBudCE4.1, to ensure that both subunits are expressed at approximately equal levels in stably transfected cells. In comparison to non-transfected HEK-293, which exhibited only small-amplitude, outwardly rectifying endogenous currents (Fig. 1A), stably transfected cells exhibit robust, inwardly rectifying whole-cell currents that are characteristic of

Kir4.1/5.1 currents (Fig. 1B)(Tucker et al., 2000). As expected, the currents were fully inhibited by Ba^{2+} (Fig. 1C-D) and reversed near the Nernst equilibrium potential for K^+ , indicating the dominant whole-cell current is carried by heterologously expressed Kir4.1/5.1.

Development and validation of the Kir4.1/5.1 Ti^+ flux assay for HTS

Heteromeric Kir4.1/5.1 channels exhibit functional, regulatory, and pharmacological properties that are distinct from homomeric Kir4.1 (Pessia et al., 2001; Tucker et al., 2000). To confirm that our HEK-293-Kir4.1/5.1 cell line expresses primarily heteromeric channels, we compared the pharmacological properties of the HEK-293-Kir4.1/5.1 cell line to those of a HEK-293 cell line that stably expresses homomeric Kir4.1 (Kharade et al., 2018).

The experiments were performed using quantitative Ti^+ flux assays that utilize the intracellular fluorescent dye, Thallo-AM, to report the inward movement of the K^+ congener Ti^+ through Kir4.1/5.1 or Kir4.1 channels overexpressed in the HEK-293 cell membrane. The assays were performed in 384-well plates so that experiments on Kir4.1/5.1 and Kir4.1 could be performed in parallel. As shown in Fig. 2A, Ti^+ addition to a well containing HEK-293-Kir4.1/5.1 cells caused a robust increase in fluorescence emission as Ti^+ moved inwardly through the expressed channels and excited the dye. Pre-addition of the control inhibitor fluoxetine (50 μM) led to a strong reduction in the Ti^+ -induced fluorescence increase. Parallel experiments performed on stably transfected HEK-293-Kir4.1/5.1 and HEK-293-Kir4.1 cells showed that the channels are pharmacologically distinct. We constructed concentration-response curves (CRCs) for both cell lines using the known Kir4.1 blockers fluoxetine (Ohno et al., 2007),

amitriptyline (Su et al., 2007), and VU0134992 (Kharade et al., 2018). Of the three compounds tested, fluoxetine and amitriptyline were the most potent inhibitors of Kir4.1/5.1, exhibiting IC_{50} values of 20.1 μ M (CI: 18.6-21.3 μ M) and 20.8 μ M (CI: 19.2-22.5 μ M), respectively (Fig. 2B, D). Fluoxetine and amitriptyline inhibited homomeric Kir4.1 channels with IC_{50} values of 48.6 μ M (CI: 37.2-102.6 μ M) and 81.7 μ M (CI: very wide). As we reported previously (Kharade et al., 2018), VU0134992 exhibits approximately 9-fold selectivity for Kir4.1 (IC_{50} =6.1 μ M; CI: 5.5-6.6 μ M) over Kir4.1/5.1 (IC_{50} =51.7 μ M; CI: 42.0-77.0 μ M) (Fig. 2C). It is notable that our previous studies were performed using a concatemeric channel construct in which Kir4.1 and Kir5.1 were physically connected by a linker and thereby forced to assemble into heteromeric channels. The excellent agreement between the two datasets strongly suggests the channels expressed in the HEK-293-Kir4.1/5.1 cell line are likely heteromeric Kir4.1/5.1 channels.

We validated the suitability of the Kir4.1/5.1 TI^+ flux assay for library screening by evaluating its tolerance to the small-molecule solvent DMSO, as well as its well-to-well, plate-to-plate, and day-to-day uniformity. DMSO tolerance was tested by incubating Kir4.1/5.1 cells in increasing concentrations of DMSO and then subjected to TI^+ flux analysis. As shown in Fig. 2E, Kir4.1/5.1-mediated TI^+ flux was insensitive to DMSO at concentrations up to 1.25% v/v, above which TI^+ flux dropped off dose-dependently. This tolerance range is important because screening is done at a DMSO concentration of 0.1% v/v, which does not directly interfere with Kir4.1/5.1 channel activity. The uniformity of the assay was assessed by performing checkerboard assays in which alternative wells of a 384-well plate are treated with either DMSO (0.1% v/v) or

fluoxetine (control inhibitor). The scatter plot in Fig. 2F shows normalized fluorescence (see Methods) data recorded from wells of a single 384-well plate treated with either DMSO or 50 μ M fluoxetine. The experiment was repeated using three different plates on three separate days. The mean $\pm$ SD Z' calculated from these experiments was 0.64 ± 0.08 , indicating the assay is sufficiently uniform and reproducible to enable high-confidence hit picking in a primary screen.

Discovery and characterization of VU0493690

We screened 80,475 compounds from the Vanderbilt Discovery Collection (Life Chemicals, Woodbridge, CT, USA) for novel inhibitors and activators of Kir4.1/5.1. The mean $\pm$ SD Z' plate statistic for the 253-plate screen was 0.714 ± 0.04 , indicating robust assay performance. A summary of the number of inhibitors (427), weak inhibitors (719), activators (145), weak activators (946), and negative compounds (the remainder of compounds) is illustrated in Fig. 3A. Hits were re-tested in duplicate and counter-screened against parental (non-transfected) HEK-293 cells resulting in 157 activators and 291 Inhibitors that were retest positive and dependent on Kir4.1/5.1 expression. Concentration-response experiments were performed on the top 90 most potent inhibitor showing the largest decrease in TI^+ flux at 10 μ M by evaluating their potency in 10-point CRCs. VU0493690 (Fig. 3B) was one of the first moderately potent inhibitors discovered early in the screen and was therefore re-synthesized to confirm its structure and activity. In TI^+ flux assays, VU0493690 concentration-dependently inhibited Kir4.1/5.1 activity with an IC_{50} of 3.2 μ M (CI: 2.7-3.8 μ M), which is approximately 6-fold more potent than fluoxetine (IC_{50} =19.5 μ M; CI: 12.1-31.3 μ M) (Fig. 3C). In patch clamp experiments, VU0493690 inhibited Kir4.1/5.1 currents completely at 10 μ M (Fig. 3D)

and concentration-dependently at -120 mV with an IC_{50} of 0.96 μ M (CI: 0.25-1.6 μ M) (Fig. 3E). At a concentration of 10 μ M, VU0493690 inhibited homomeric Kir4.1 current at -120 mV by only $7.8 \pm 1.9\%$ ($n=5$; data not shown), affording greater than 10-fold selectivity for Kir4.1/5.1 over Kir4.1.

The selectivity of VU0493690 for Kir4.1/5.1 versus eleven other members of the Kir channel family was evaluated in concentration-response experiments using established Tl^+ flux assays (Kharade et al., 2018; Raphemot et al., 2011; Raphemot et al., 2014; Swale et al., 2016). At concentrations ranging between 0.3 nM to 30 μ M, VU0493690 is greater than 30-fold selective for Kir4.1/5.1 over homomeric Kir4.1 channels, Kir1.1, Kir2.1, Kir2.2, Kir2.3, Kir4.2, Kir6.2/SUR1, Kir6.1/SUR2b and Kir7.1 (Table 1). However, weak activity toward Kir3.1/3.2 (13% inhibition at 30 μ M) and Kir3.1/3.4 (18 % inhibition at 30 μ M), and Kir6.1/SUR2b (19% inhibition at 30 μ M) were observed. We also evaluated the activity of VU0493690 against the cardiac K^+ channel, hERG (Kv11.1), in which VU0493690 had greater than 10-fold selectivity (Table 1).

VU0493690 SAR and development of VU6036720

While VU0493690 was the most potent and selective Kir4.1/5.1 inhibitor reported to date, a more potent tool was required for proof of concept and target validation studies in pre-clinical animal models. A multi-dimensional optimization campaign, as depicted in Fig. 4, sought to identify productive regions of SAR to improve target potency and DMPK profile. Piperazine replacements, such as [3.3.0] and other spirocyclic congeners proved inactive. A wide range of alternatively substituted aryl and heteroaryl sulfonamides were prepared and evaluated, and only a 3-chloro-4-fluoro congener **3** proved as potent (Kir4.1/5.1 Tl^+ IC_{50} = 3.5 μ M) as VU0493690. Maintaining

this moiety and surveying alternative aryl groups on the distal piperazine led to the discovery of a 2-cyanophenyl derivative **4** with a slight improvement in potency (Kir4.1/5.1 TI⁺ IC₅₀ = 2.6 μM). With these SAR findings in hand, we then evaluated the addition of chiral methyl groups to the piperazine core of **4**, in an effort to further improve on target potency. The chemistry to arrive at these analogs is shown in Supplemental Figure 1, with the route to VU6036720 exemplified. Starting from commercial (S)-**5**, a Buchwald coupling affords N-Ar piperazine **6** in 89% yield. Boc-deprotection proceeds in quantitative yield delivering **7**, which is then alkylated to provide **8**. Another deprotection gives **9**, and a reaction with a sulfonyl chloride delivers the final compound **10** (VU6036720). Interestingly, the SAR proved to be highly regio- and enantiospecific. As highlighted in Fig. 5, 2-methyl substituted piperazines **10** and **11** showed clear enantiospecific activity, with the (S)-enantiomer **10** more potent (Kir4.1/5.1 TI⁺ IC₅₀ = 0.73 μM) than the HTS hit, while the (R)-enantiomer **11** was inactive. Installation of chiral methyl groups in the 3-position, as with **12** and **13** was unproductive, leading to weak or inactive analogs. At concentrations ranging between 0.3 nM to 30 μM, VU6036720 maintained greater than 30-fold selective for Kir4.1/5.1 over homomeric Kir4.1 channels, Kir1.1, Kir2.1, Kir2.2, Kir2.3, Kir4.2, Kir6.2/SUR1, Kir6.1/SUR2b and Kir7.1, similar to that of VU0493690 (Table 1). A detailed SAR optimization description is outside of the scope of this manuscript and will be reported in due course.

Effect of VU6036720 on Kir4.1/5.1 single channel properties

Cell-attached patch recordings were performed to determine how VU6036720 inhibits Kir4.1/5.1 at the single-channel level. Fig. 6A shows a representative recording

before (a) and after (b) bath application of 10 μ M VU6036720. The same recordings at an expanded time scale are shown in Fig. 6B. As shown in the summary data (Fig. 6C) from 8 independent recordings, 10 μ M VU6036720 led to a significant ($P < 0.05$) decrease in N , P_o , and single-channel amplitude.

VU6036720 blocks the Kir4.1/5.1 channel pore near Asparagine 161

Several structurally diverse small-molecule Kir channel inhibitors block the pore through interactions with residues that comprise the so-called “rectification controller” located below the selectivity filter (reviewed in (Swale et al., 2014) and (Weaver and Denton, 2021)). A common feature of these pore blockers is their tendency to exhibit K^+ -dependent “knockoff”, whereby elevation of extracellular K^+ weakens binding in the pore. As a first step toward testing if VU6036720 is a Kir4.1/5.1 pore blocker, we constructed a CRC for VU6036720 in the presence of 25 mM bath K^+ . As shown in Fig. 7A, the elevation of extracellular K^+ right-shifted the CRC and increased the VU6036720 IC_{50} of VU6036720 to 1.6 μ M (25K, gray curve), which represents 6.8-fold shift from that recorded in 5 mM bath K^+ (black curve).

The rectification controller of Kir4.1 is glutamate 158 (E158), and mutation of this residue reduces channel block by fluoxetine, nortriptyline, pentamidine, quinacrine, and VU0134992 (Arechiga-Figueroa et al., 2017; Furutani et al., 2009; Kharade et al., 2018; Marmolejo-Murillo et al., 2017a). Alignment of the Kir4.1 and Kir5.1 (Fig. 7B, bottom panel) amino acid sequences reveals that the equivalent residue in Kir5.1 is asparagine 161 (N161). The location of N161 is mapped onto the Kir2.2 model in Fig. 7B (top panels). Given the importance of this pore region in small-molecule block, we tested if mutation of Kir5.1-N161 to glutamate (Kir5.1-N161E) reduces the sensitivity of

Kir4.1/5.1 channels to VU6036720. A potential caveat of this experiment is that the Kir5.1-N161E mutation could prevent the formation of heteromeric channels, leading to the expression of homomeric Kir4.1 channels, which are markedly less sensitive to VU6036720 (Table 1). We, therefore, compared the single-channel properties of Kir4.1/5.1-N161E with those of wild type Kir4.1/5.1 and Kir4.1. Representative current recordings for the three channel subtypes are shown in Fig. 7C, whereas the mean $\pm$ SD IV plots and single-channel conductance data are summarized in Fig. 7D and 7E, respectively. As expected from published literature, the single channel conductance of Kir4.1 is significantly ($P < 0.001$) smaller than that of wild type Kir4.1/5.1. Importantly, the single-channel conductance of Kir4.1/5.1-N161E is not different from its wild type counterpart, indicating that the N161E mutation does not prevent heteromultimerization with Kir4.1. We, therefore, used this construct to test the role of N161 in the mechanism of VU6036720 action. As shown in Fig. 7F, mutation of Kir5.1-N161E led to a striking reduction in VU6036720 block (filled triangles), which was similar to that of homomeric Kir4.1 (filled circles). In these experiments, the IC_{50} for Kir4.1 was much greater than 10 μ M, affording greater than 40-fold selectivity of VU6036720 for Kir4.1/5.1 over Kir4.1.

VU6036720 does not induce diuresis in vivo

Having developed the most potent and selective Kir4.1/5.1 inhibitor reported to date, we tested if VU6036720 could induce diuresis by inhibiting renal Kir4.1/5.1 channel activity in mice. The animals were gavaged with either vehicle control, 30 mg/kg VU6036720, 100 mg/kg VU6036720, or the diuretic hydrochlorothiazide (HCTZ; 25 mg/kg) and placed in metabolic cages for 4 hrs to collect urine. In contrast to HCTZ,

which induced a significant ($P < 0.05$) increase in urine output, neither 30 mg/kg nor 100 mg/kg of VU6036720 stimulated urine production (Supplemental Fig. 2).

DMPK properties of VU6036720

We profiled VU6036720 in a battery of *in vitro* and *in vivo* DMPK assays in an effort to understand its inability to induce urine production *in vivo* (Table 2). VU6036720 was compliant with Lipinski rules and had a favorable xLogP of 2.78. In addition, VU6036720 had modest fraction unbound across species (f_u human = 0.013; f_u rat = 0.009 and f_u mouse = 0.019) and similar brain homogenate binding (f_u brain rat = 0.007 and f_u brain mouse = 0.020). Unfortunately, the compound displayed high predicted hepatic clearance across species (CL_{hep} human – 20.3 mL/min/kg; CL_{hep} rat – 68.9 mL/min/kg; CL_{hep} mouse – 89.4 mL/min/kg). *In vivo* IV/PO PK experiment confirmed that VU6036720 was highly metabolized in mouse (CL_p mouse – 124 mL/min/kg; 137.7% QH) and had very low oral bioavailability (1.3%) (Table 2). Based on these data, metabolite identification studies are underway to identify metabolic hot spots to improve the pharmacokinetic profile.

DISCUSSION

Here we describe the development of a small-molecule inhibitor, VU6036720, with unprecedented potency and selectivity for heteromeric Kir4.1/5.1 over homomeric Kir4.1. With an IC_{50} in TI^+ flux assays of approximately 3 μ M (Table 1), VU6036720 is approximately 7-times more potent at inhibiting Kir4.1/5.1 than fluoxetine (IC_{50} = 20 μ M; Fig. 2B) and amitriptyline (IC_{50} = 20 μ M; Fig. 2D). The actual IC_{50} for VU6036720 from

gold-standard whole-cell voltage clamp experiments is approximately 240 nM, making it greater than 40-fold selective over Kir4.1 ($IC_{50} > 10 \mu M$). Thus, VU6036720 represents the current state-of-the-art in Kir4.1/5.1 channel inhibitors.

The screening assay was developed using a bicistronic vector that enables approximately equal expression of both Kir4.1 and Kir5.1 subunits in transfected cells, which should promote the formation of predominantly heterotetrameric channels for study. Several lines of evidence suggest that this is the case. TI^+ flux experiments clearly showed that channels in HEK-293-Kir4.1/5.1 cells are pharmacologically distinct from those expressed in HEK-293-Kir4.1 cells. For example, concentration-response-experiments using the Kir4.1-preferring inhibitor VU0134992 revealed a 9-fold selectivity for Kir4.1 over Kir4.1/5.1, which is the same selectivity window we reported from patch clamp recordings comparing homomeric Kir4.1 channels and a Kir4.1/5.1 concatemer where the two subunits are physically coupled together to ensure heteromultimerization (Kharade et al., 2018). Pharmacological separation between the two cell lines was also observed for fluoxetine and amitriptyline. Electrophysiological recordings showed the characteristically larger unitary conductance of Kir4.1/5.1 compared to Kir4.1. Thus, most of the channels expressed in HEK-293-Kir4.1/5.1 cells are heterotetramers.

VU6036720 is a pore blocker that likely accesses its binding site by first crossing the plasma membrane and then entering the cytoplasmic pore of the channel. Three lines of evidence support this model. First, the potency of block of inward current is reduced by increasing extracellular K^+ concentration, a phenomenon known as “knockoff” and reflects the displacement of VU6036720 from its binding site by inwardly directed K^+ ions. This behavior has been observed for small-molecule inhibitors of

Kir1.1 (Bhave et al., 2011; Lewis et al., 2009), Kir2.1 (Wang et al., 2011), Kir4.1 (Kharade et al., 2018), and Kir7.1 (Swale et al., 2016). Second, bath applied VU6036720 blocks the channel in the cell-attached configuration of the patch clamp technique, making it unlikely that the compound accesses the extracellular pore of the channels being recorded. Single-channel measurements revealed that VU6036720 reduces the number of active channels, channel open probability, and unitary current amplitude. The latter effect is probably due to membrane depolarization associated with Kir4.1/5.1 channel inhibition and a reduction in the electrochemical driving force acting on K^+ ions. The third piece of evidence is that mutation of Kir5.1-N161E almost completely abrogates block of the heteromeric channel by VU6036720. This residue is located just below (intracellularly) the narrow selectivity filter of the channel, which likely creates a physical barrier between extracellular VU6036720 and its deeper binding site in the pore. The most likely explanation is that VU6036720 enters the cytoplasmic pore of the channel to reach its binding site near N161.

The development of an *in vivo*-active probe of Kir4.1/5.1 will be required to rigorously explore the channel's therapeutic potential as a diuretic target. Both genetic and physiological studies support Kir4.1/5.1 as a viable diuretic target. In the distal convoluted tubule (DCT), Kir4.1/5.1 channels critically regulate sodium chloride reabsorption mediated by the electroneutral transporter, NCC, whose activity is modulated by its phosphorylation state. Several elegant genetic, biochemical, and physiological studies have established that the phosphorylation state of NCC is controlled by a Kir4.1/5.1-WNK-SPAK-NCC regulatory pathway by which Kir4.1/5.1 channel activity promotes the phosphorylation and activity of NCC (Cuevas et al., 2017;

Su et al., 2019; Su et al., 2020; Terker et al., 2015). Genetic disruption of *KCNJ10* or depolarization of the DCT membrane potential with high K^+ decreases NCC phosphorylation/activity and reduces sodium chloride reabsorption in the DCT. Sodium that is not reabsorbed in the DCT is delivered to the CCD where it can be reabsorbed by epithelial sodium channels, ENaC, in exchange for K^+ secretion via Kir1.1 (ROMK). Thus, loss of Kir4.1/5.1 in the DCT accounts for most of the sodium and K^+ wasting observed in patients with SeSAME/EAST syndrome. This human genetic data offers strong validation for Kir4.1/5.1 as a novel diuretic target. The therapeutic value of Kir4.1/5.1 might be especially high in the setting of loop diuretic resistance, where the efficacy of loop diuretics is lost due in part to the expansion of the DCT and sodium chloride reabsorption capacity of this nephron segment.

We recently reported that the Kir4.1-preferring inhibitor VU0134992 induces urine volume, sodium, and K^+ wasting in orally dosed, volume-loaded rats (Kharade et al., 2018). While we intended to specifically target homomeric Kir4.1 channels expressed in the nephron, the observed diuretic effect could potentially be mediated at least in part through inhibition of Kir4.1/5.1 channels in the DCT. VU0134992 is only 9-fold selective for Kir4.1 over Kir4.1/5.1 channels and diuretic effects were only observed at relatively high doses of 50 and 100 mg/kg, raising the possibility that off-target inhibition of Kir4.1/5.1 occurred. The development of an inhibitor that is highly selective for Kir4.1/5.1 over Kir4.1 is needed to test if selective inhibition of Kir4.1/5.1 is enough to induce salt and water wasting. VU6036720 is greater than 40-fold selective for Kir4.1/5.1 showing proof-of-concept that the development of highly selective probes is feasible. Unfortunately, VU6036720 showed no diuretic effects in mice, likely due to

high clearance and poor bioavailability after parenteral administration. Additional chemistry is underway to identify VU6036720 analogs that maintain potency at Kir4.1/5.1 but exhibit more favorable *in vivo* DMPK properties.

The 4-hour dosing regimen used here should be long enough to induce a measurable diuretic response following inhibition of Kir4.1/5.1. The control diuretic used in the present study was the NCC inhibitor, hydrochlorothiazide (HCTZ), which mediates sodium chloride reabsorption in the DCT, the same tubule segment we are targeting with a Kir4.1/5.1 inhibitor. We observed a statistically significant increase in urine volume in mice treated with HCTZ over 4 h, but not in animals treated with VU6036720. A potentially important difference in determining the kinetics of diuresis caused by inhibiting NCC directly with HCTZ versus indirectly with an inhibitor of Kir4.1/5.1 is that the latter is mediated by the WNK-SPAK-NCC pathway, which may take longer. However, several lines of evidence suggest that this should take on the order of minutes as opposed to hours or days. For example, Loffing and colleagues (Sorensen et al., 2013) showed that an oral K⁺ load leads to rapid NCC phosphorylation and natriuresis within 30 mins of dosing in mice. Similarly, NCC dephosphorylation is observed in kidney slices treated with high K⁺ or a high dose (5 mM) of the non-specific Kir channel blocker barium (Penton et al., 2016). Taken together, these studies suggest that inhibition of Kir4.1/5.1 should trigger rapid NCC dephosphorylation and inhibition and natriuresis *in vivo*. The development of a potent and selective VU6036720 analog with improved DMPK properties that can effectively engage Kir4.1/5.1 *in vivo* will help resolve this issue.

In conclusion, we developed a robust HTS assay that enables the interrogation of large compound libraries for small-molecule modulators of heterotetrameric Kir4.1/5.1 K⁺ channels. Hundreds of novel inhibitors were discovered from a screen of 80,475 chemically diverse compounds, some of which are more potent than VU6036720 even before chemical optimization (data not shown). Although VU6036720 did not exhibit *in vivo* activity in the metabolic cage studies reported here, it should still be useful as a selective probe of Kir4.1/5.1 function in heterologous expression experiments, isolated tubule studies, and kidney slice preparations. A surprising outcome of the primary screen is the discovery of the first-in-class potentiators of Kir4.1/5.1, which could be useful for determining how activation of the channel affects the physiology of the kidney tubule and brain astrocytes and glial cells. We anticipate that the diverse collection of chemically tractable inhibitors and activators discovered in the screen will be instrumental in transforming the molecular pharmacology of Kir4.1/5.1 by enabling the development of potent, specific, and *in vivo*-active channel probes.

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AUTHOR CONTRIBUTIONS:

Participated in research design: McClenahan, S., Kent, C., Kharade, S., Isaeva, E., Williams, J., Han, C., Terker, A., Gresham, R., Lazarenko, R., Days, E., Romain, I.,

Boutaud, O., Sulikowski, G., Harris, R., Weaver, D., Staruschenko, A., Lindsley, C., Denton, J.

Conducted experiments: McClenahan, S., Kent, C., Kharade, S., Isaeva, E., Williams, J., Han, C., Terker, A., Gresham, R., Lazarenko, R., Days, E., Romain, I., Boutaud, O.

Contributed new reagents or analytical tools: McClenahan, S., Kent, C., Kharade, S., Williams, J., Han, C., Romain, I., Boutaud, O., Weaver, D., Lindsley, C.,

Performed data analysis: McClenahan, S., Kent, C., Kharade, S., Isaeva, E., Williams, J., Han, C., Terker, A., Gresham, R., Lazarenko, R., Days, E., Romain, I., Boutaud, O.,

Wrote or contributed to the writing of the manuscript: McClenahan, S., Kent, C., Kharade, S., Isaeva, E., Williams, J., Han, C., Terker, A., Gresham, R., Lazarenko, R., Days, E., Romain, I., Boutaud, O., Sulikowski, Bauer, J., G., Harris, R., Weaver, D., Staruschenko, A., Lindsley, C., Denton, J.

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FOOTNOTES

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LEGENDS FOR FIGURES

Figure 1. Electrophysiological properties of HEK-293-Kir4.1/5.1 cells. A) Exemplar whole-cell currents recorded from non-transfected HEK-293 cells evoked with 200 msec steps between -120 mV and 120 mV from a holding potential of -75 mV. B) Currents evoked using the same voltage clamp protocol but from stably transfected monoclonal Kir4.1/5.1 cells. C) Residual currents following bath application of 2 mM barium (Ba²⁺).

D) Currents evoked using a step-ramp protocol used for concentration-response experiments before and after block with Ba^{2+} . See Methods for details.

Figure 2. Development and validation of Kir4.1/5.1 Ti^+ flux assay for HTS.

A) Representative Ti^+ flux experiment in which Thallo fluorescence is recorded from HEK-293-Kir4.1/5.1 cells plated a 384-well plate, treated with either 0.1% v/v DMSO (solvent control) or 50 μM fluoxetine (control blocker) before adding Ti^+ stimulus buffer to wells (arrow). The fluorescence increase is inhibited by the Kir4.1/5.1 blocker fluoxetine. CRCs against HEK-293-Kir4.1/5.1 cells (grey, square symbols) or HEK-293-Kir4.1 cells (black, circle symbols) using B) fluoxetine (Kir4.1/5.1 IC_{50} = 20.1 μM ; Kir4.1 IC_{50} = 48.6 μM), C) VU992 (Kir4.1/5.1 IC_{50} = 51.7 μM ; Kir4.1 IC_{50} = 6.1 μM), or D) amitriptyline (Kir4.1/5.1 IC_{50} = 20.8 μM ; Kir4.1 IC_{50} = 81.7 μM). Data are means $\pm$ SEM from triplicate experiments performed on two separate days. E) DMSO tolerance curve. HEK-293-Kir4.1/5.1 cells were incubated with the indicated concentrations of DMSO for 10 min before initiating Ti^+ flux experiments. Note that Ti^+ flux is unaffected at concentrations up to 1.25% v/v, indicating that screening and other experiments where DMSO is <0.3% v/v are unaffected by the solvent. Data are means $\pm$ SEM from triplicate experiments performed on two separate days. F) Representative scatter plot of “checkerboard” experiments in which alternative wells of a 384-well plate are treated with either DMSO (solvent control) or 50 μM fluoxetine (control Kir4.1/5.1 blocker) for 10 min before initiating Ti^+ flux. Note the clear separation between the two cell/well populations. This experiment was performed in triplicate on three separate days, yielding a mean Z’ value of 0.64.

Figure 3. Discovery and characterization of Kir4.1/5.1 inhibitor VU0493690. A) HEK-293-Kir4.1/5.1 cells plated in 384-well plates were treated with 0.1% v/v DMSO (solvent control), 100 μ M fluoxetine (control blocker), or test compounds at a nominal concentration of 10 μ M for 10 min before initiating Ti^+ flux. Test compounds were assigned either inactive (black), potentiators (dark blue), weak hits (light blue), or inhibitors (green) based on their effect on Ti^+ flux relative to vehicle controls. Hit categories were defined per 384-well plate as exceeding 3 standard deviations of the mean Ti^+ response in both slope and amplitude. These values are plotted as a percentage of maximal inhibition defined by fluoxetine as the control. Weaker hits only exceeded in either slope or amplitude (weak potentiators not shown). B) Scatter plot showing the discovery of VU043690 (purple circle) among several thousand other test compounds. The chemical structure of VU043690 is shown in the inset. C) Comparison of VU043690 (black) and fluoxetine (grey) CRCs in Kir4.1/5.1 in Ti^+ flux assays. Data are means $\pm$ SEM from triplicate experiments performed on two separate days. IC_{50} values derived from logistical fits to the data are 3.2 μ M and 19.5 μ M for VU043690 and fluoxetine, respectively. D) Confirmation of VU0493690 activity against Kir4.1/5.1 in whole-cell patch-clamp orthogonal assays. Data are means $\pm$ SEM (n=4-5) where SEM is indicated in shaded area. 2 mM Ba^{2+} was used as a control blocker. E) VU043690 CRC in whole-cell patch clamp experiments. Data are means $\pm$ SD (n=4-5).

Figure 4. Multi-dimensional optimization plan for 1, VU0493690, and initial productive SAR affording 3 and 4. HBAs: hydrogen bond acceptors, HBDs: hydrogen bond donors, EWGs: electron withdrawing groups, EDGs: electron donating groups.

Figure 5. SAR of regio-and enantiopure methyl piperazine analogs 10-13.

Figure 6. Effects of VU6036720 on Kir4.1/5.1 single-channel properties. A) Cell-attached patch single-channel recordings of Kir4.1/5.1 channel activity before (a) and after (b) bath application of 10 μ M VU6036720. (B) Channel activity from (a) and (b) shown at higher temporal resolution. C) Summary of single channel data showing significant ($*P<0.0167$) effects of VU6036720 on N , P_o , and amplitude.

Figure 7. VU6036720 is a Kir4.1/5.1 channel pore blocker. A) VU6036720 CRC generated in the presence of 5 mM K^+ ($IC_{50} = 240$ nM; black symbols and line) and 25 mM K^+ ($IC_{50}=1.6$ μ M; grey symbols and line) illustrating the rightward shift associated with weakened channel block due to knockoff of VU6036720 from the intracellular pore. B) Location of N161 in a structural model of heteromeric Kir4.1/5.1 channels based on Kir2.2 (PDB 3JYC) crystal structure. The model is shown from the side (left) and top-down (right) views in a Kir4.1-Kir5.1-Kir4.1-Kir5.1 arrangement. Kir4.1 and Kir5.1 subunits are colored grey and cyan, respectively. Alignment of Kir4.1 and Kir5.1 are shown below with a red dot indicating the location of Kir4.1-E158 and Kir5.1-N161. C) Representative single-channel recordings of wild type Kir4.1/5.1, Kir4.1/5.1-N161E, and Kir4.1. D) Mean $\pm$ SD IV relationships for type Kir4.1/5.1, Kir4.1/5.1-N161, and Kir4.1. (n=6-11). E) Scatter plot summary showing the unitary conductances of wild type Kir4.1/5.1 and Kir4.1/5.1-N161E are not statistically different from each other but are significantly ($***P<0.001$) larger than that of homomeric Kir4.1. F) Mean $\pm$ SD (n=4-8) VU6036720 CRC for wild type Kir4.1/Kir5.1, Kir4.1/5.1-N161E, and Kir4.1 showing dramatic effect of Kir5.1-N161E mutation on VU6036720 and essentially no activity toward Kir4.1.

Table 1. Selectivity of VU6036720 over members of the Kir channel family and

Data are mean IC₅₀ values (in μ M) derived from concentration response curves performed in triplicate. Inactive compounds are those that inhibited the indicated

channel by less than 50% at 30 μ M and exhibited no trend in curve-fitting analyses. Numbers in parentheses indicate the maximal percent inhibition at 30 μ M. Fluoxetine or the indicated compounds were used as control inhibitors.

Channel	VU0493690	VU6036720	Fluoxetine	Special Controls
Kir4.1/5.1	3.2 (100%)	2.2 (100%)	11.2	18.9 (VU0134992)
Kir4.1	Inactive	Inactive	32.5 (32%)	8.02 (VU0134992)
Kir4.2	Inactive	Inactive	39.5	3.4 (VU0134992)
Kir1.1	Inactive	Inactive	35.5 (18)	0.243 (VU591)
Kir7.1	Inactive	>30 (33%)	NA	6.4 (ML 418)
Kir2.1	Inactive	Inactive	>30 (5%)	7.3 (ML133)
Kir2.2	Inactive	Inactive	>30 (1%)	21.5 (ML133)
Kir2.3	Inactive	>30 (17%)	26.6	9.6 (ML133)
Kir3.1/3.2	>30 (13%)	>30 (9%)	4.32	8.24 (SCH23390)
Kir3.1/3.4	>30 (18%)	>30 (8%)	7.8	6 (SCH23390)
Kir6.2/SUR1	Inactive	Inactive	>30	0.008 (Glibenclamide)
Kir6.1/SUR2b	>30 (19%)	>30 (54%)	8.01	0.024 (Glibenclamide)
Kv11.1	11.4 (61%)	6.4 (92%)	5.1	3.9 (Quinidine)

Table 2. Pharmacokinetic parameters for VU6036720

Dose	Route	CLp (mL/min/kg)	t_{1/2} (hr)	Vss (L/kg)	AUC (hr*ng/ml)
1 mg/kg	IV	124	0.68	6.00	129

Dose	Route	C_{max} (ng/ml)	t_{max} (h)	AUC (hr*ng/ml)	F (%)
10 mg/kg	PO	3.25	0.75	BLQ	NA
100 mg/kg	PO	142	0.42	207	1.30

CLp: plasma clearance; t_{1/2}: half-life; Vss: Volume of distribution at steady state; AUC: area under the curve; C_{max}: maximal concentration; T_{max}: time of maximal concentration; F: oral bioavailability

Figure 1

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Figure 2

Figure 3

Figure 4

Figure 5

10, VU6036720
Kir4.1/5.1 TI+ IC₅₀ = 0.73 μ M

11, VU6036721
Kir4.1/5.1 TI+ IC₅₀ >10 μ M

12, VU6036722
Kir4.1/5.1 TI+ IC₅₀ >10 μ M

13, VU6036724
Kir4.1/5.1 TI+ IC₅₀ = 6.1 μ M

Figure 6

Figure 7